PREFACE

The International Conference on Agriculture and Agro-Industry 2010 (ICAAI2010) under the theme of “Food, Health and Trade” is the first international conference organized by School of Agro-Industry. It was held at Mae Fah Luang University, Chiang Rai, Thailand on November 19-20, 2010. ICAAI2010 is aimed to create connections amongst scientists in the areas of agriculture and agro-industry, hence the sustainable agriculture and agro-industry for the world.

Approximately 350 delegates from overseas and Thailand have attended the conference with totally 184 abstracts submitted to be presented as oral and poster presentations. With those fruitful discussions during the presentation, 84 full papers were selected by the conference scientific reviewers who come from various academic institutes from overseas and Thailand to be published in the supplement issue of Thai Journal of Agricultural Science. Those papers cover three categories (1) Food Science and Technology (2) Agricultural Science and Technology and (3) Agribusiness Management regarding to the sessions in the ICAAI2010.

All in all, ICAAI2010 was very successful in engaging scientists from different fields to share their ideas, to develop bridge between institute-to-institute from various parts and regions in the world, and consequently fulfill the original purposes of the conference.

Lastly, we would like to thank those people who have supported to make this ICAAI2010 achieved. We also would like to extend my thanks to Professor Dr. Irb Kheoruenromne who provided generous support allowing this proceeding to be published in the supplement issue of Thai Journal of Agricultural Science. This will be benefit to the ones who may interest those findings presented in the ICAAI2010 for further applied in their research or practical works.

ICAAI 2010 Organizer
# Thai Journal of Agricultural Science

## Content

<table>
<thead>
<tr>
<th>Volme</th>
<th>Number</th>
<th>Special Issue</th>
<th>2011</th>
</tr>
</thead>
<tbody>
<tr>
<td>44</td>
<td>5</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

### Agricultural Science and Technology

1. The Influence of the Interaction between Jasmonates, Ethylene, and Polyamines on Fruit Quality  
   *S. Kondo and M. Kittikorn*

2. Physiological and Phytochemical Changes in Cayenne Pepper  
   *S. Srilaong and N. Kaewkhum*

3. Application of Chitosan for Reducing Chemical Fertilizer Uses in Waxy Corn Growing  
   *S. Boonlertnirun, R. Suvannasara, P. Promsomboon and K. Boonlertnirun*

4. Photo-stability of Mango Seed Kernel Extract and Its Encapsulated Product  
   *P. Maisuthisakul*

5. Development of Artificial Neural Network on Transparent Soap Base Containing Sonneratia caseolaris Extract  
   *S. Piriyaprasarth, G. Chansiri, T. Phaechamud, and S. Puttipipatkhachorn*

6. Indented Longan Detection with Computer Vision-based Software in Consideration of Roundness Value  
   *P. Poonnoy*

7. Antimicrobial Resistance Profile of Escherichia coli Isolates From Fattening Pigs in Khon Kaen Province, Thailand  
   *P. Sornplang, N. Na-ngam, and S. Angkititrakul*

8. Improvement of Rheological and Functional Properties of Defatted Rice Bran Protein Bioplastic by Kraft Lignin Addition  
   *P. Rattanatham, T. Kunanopparat, and S. Siriwattanayotin*

9. Mangiferin and Antioxidant Capacity from Mango (*Mangifera indica* L.) Leaves Extracts  
   *P. Kitbumrungsart, O. Suntornwat, and K. Rayanil*

10. Preliminary Investigation of Biodiesel Wastes Utilization in Bacterial Fermentation  
    *C. Sinprasertchok, A. Thanapimmetha, M. Saisriyoot and P. Srinophakun*

11. Identification of Sugarcane Somaclones Derived from Callus Culture by SSR and RAPD Markers Analysis  
    *S. Thumjamras, S. Iamtham, R. Lersrutaiyotin and S. Prammanee*

12. Potential of Six Plant Species for Food Processing Wastewater Treatment in Wetland  
    *P. Sohsalam*

13. Phosphorus Accumulation in Wetland for Food Processing Wastewater Treatment  
    *P. Sohsalam and S. Klangkongsup*

14. Identifying Parameters Influencing Growth and Astaxanthin Production by Xanthophyllomyces dendrorhous Cultivated in Pineapple Juice Concentrate Base Low Cost Medium  
    *S. Abdullah, K. Poomputsa, P. Mekvichitsaeng, V. Ruanglek and S. Akeprathumchai*
15. Carotenoids Production from Red Yeasts Using Waste Glycerol as a Sole Carbon Source  
   A. Manowattana, C. Techapun, P. Seesuriyachan and T. Chaiyaso

16. Studying the Genomic Function of Rice β-glucosidase via RNA Interference   
   D.T.T. Tam and M. Ketudat-Cairns

17. Bioactivities of Carica papaya Latex Extract  
   C. Bandasak, S. Rawdkuen, P. Pintathong and P. Chaiwut

18. Extraction of Phenolic Antioxidants From Peels and Seeds of the Royal Project’s Fruits  
   T. Sroimori, S. Srisunton, S. Rawdkuen, P. Pintathong and P. Chaiwut

19. Antioxidant Capacity and Total Phenolic Content of Moringa oleifera Grown in Chiang Mai, Thailand  
   W. Wangcharoen, and S. Gomolmanee

20. Expression Analysis of Na+/ H+ Exchanger and Monosaccharide Transporter Genes in Rice Suspension Cells Under Salt Stress  
   K. Mahasal, A. Chaopaknam and B. Ngampanya

21. Determination of Relationships and Genetic Variation Among Amorphophallus sp. From Northern Part of Thailand  
   O. Mekkerdchoo, P. Holford, G. Srzednicki, C. Prakitchaiwattana, C. Borompichailchartkul and S. Wattananon

22. Process Optimization of Anhydrous Ethanol Production Using Vapor Permeation (VP) and Pressure Swing Adsorption (PSA) Techniques  
   S. Pimkaew, S. Kanchanatawee, and A. Boontawan

23. Tangerine Quality Monitoring by Ethanol Concentration Measurement  
   S. Wongsila, W. Kumpoun, A. Gardchareon, D. Wongratanaphisan and S. Choopun

24. Screening for Physical Stability of Nanoemulsions Containing Plai Oil by Box-Behnken Desig  
   S. Manchun, S. Piriyparasarth, and P. Srijornnsak

25. Effect of Ozone on Oxidative Stress Defense Enzymes and Quality Changes in Tangerine (Citrus reticulata Blanco cv. Sai Nam Pung) Fruit  
   P. Boonkorn, H. Gemma, S. Sugaya, S. Setha, J. Uthaiibutra, and K. Whangchai

26. Effects of Prebiotics on Growth Performance and Pathogenic Inhibition in Sex-Reversed Red Tilapia (Oreochromis niloticus × Oreochromis mossambicus)  
   V. Plongbunjong, W. Phromkuntong, N. Suanyuk, B. Viriyapongsutee and S. Wichienchot

27. Validation of Modified QuEChERS Method for Simultaneous Determination of Organophosphates and Carbamates in Mangosteens by LC-MS/MS  
   W. Meeccharoenv, N. Tayapatuch, P. Pityont and N. Leepipatpiboon

28. Effect of Seed Development on Seed Quality of Physic Nut (Jatropha curcas Linn.)  
   S. Ngamprasitthi, S. Juntakool, I. Sookasatan, S. Sukprakarn and S. Techapinyawat

29. Reduction of Residual Chlorpyrifos on Harvested Bird Chillies (Capsicum frutescens Linn.) Using Ultrasonication and Ozonation  
   S. Pengphol, J. Uthaiibutra, O. A. Arquero, N. Nomura and K. Whangchai
30. Antioxidant Activities of Curcumin-metal Complexes
   A. Thakam and N. Saewan
   188

31. Lactic Acid Bacteria from Thai Fermented Meat Products as Biological Control Agents against Anthracnose Disease
   Bussaman P., Sa-uth C., Tonsao A., Sawangeaw A., Rattanasena P.
   194

32. Antimicrobial Activity of Agricultural By-products Extracts Against Vibrio spp.
   S. Charoenrak, S. Boonprasop, P. Sutthirak, and N. Wongmongkol
   200

33. Quality Attribute and Antioxidant Activity Changes of Jerusalem Artichoke Tubers (Helianthus tuberosus L.) During Storage at Different Temperatures
   T. Plangklang and R. Tangwongchai
   204

34. Seed Soaking with Three Essential Oils from Herbal Plants for Controlling Sclerotium rolfsii Sac. Causing Damping – off Disease in Tomato
   R. Duamkhanmanee
   213

35. Effect of Ozone and Vapor Phase Hydrogen Peroxide Fumigation on the Control of Postharvest Diseases of Longan Fruit (Dimocarpus longan Lour.)
   K. Whangchai, N. Nuanaon and J. Uthaibutra
   219

36. Development of Shellac From Source Available in Thailand as an Alternative Polymer for Postharvest Treatment
   P. Sriamornsak, S. Puttipipatkhachorn and S. Limmatvapirat
   224

37. Nanoemulsions Containing Volatile Oils as Novel Antimicrobial for Oral Health Care Products
   S. Pengon, C. Limmatvapirat, S. Limsririchaikul and S. Limmatvapirat
   230

38. Effect of Bio-extract as Microbial Inoculum on Composting of Cassava Leaves and Stems
   P. Feunganksorn, S. Akeprathumchaisit and S. Tripetchkul
   236

39. Anti-aging Cosmetics from Schizophyllum commune Fries
   P. A. Pirshahid, C. Phromtong, S. Laovitthayanggoon, Y. Khampan,
   T. Hemthanon, P. Chueboonmee, J. Eiamwat and V. Arunpairojana
   242

40. Preparation and Characterization of Shellac/PVP Iodine Blend as Antimicrobial Film Patch
   T. Thammachat, C. Limmatvapirat, S. Limsririchaikul and S. Limmatvapirat
   247

41. Medium Optimization for Antimicrobial Compound Production by an Endophytic Fungus of Stemona burkiiilii for Plant Pathogenic Control
   T. Pairaj, N. Ratnarathorn, J. Anu-aun and T. Vichitsoonthonkul
   252

42. Varietal Cross Heterosis of Thein Waxy Corn
   K. Boonlertnirun, R. Suvannasara, and S. Boonlertnirun
   256

43. Factors Affecting on the Enhancement of Mechanical Properties of Composite Edible Film based on Shellac and Gelatin
   S. Soradech, J. Nunthanid, P. Sriamornsak, S. Limmatvapirat
   and M. Luangtana-anan
   263
44. Off-flavor in Tilapia (Oreochromis niloticus) Reared in Cages and Earthen Ponds in Northern Thailand
   N. Whangchai, S. Wiraiboon, K. Shimizu, N. Iwami and T. Itayama

45. Effect of Germination on Antioxidative Property of Pigmented and Non-Pigmented Rice
   S. Jiapong, R. Singanusong, and S. Jiamyangyuen

46. Antioxidant and Anti-inflammatory Activities of Freshwater Macroalga, Cladophora glomerata Kützing
   D. Amornlerdpison, K. Mengumphun, S. Thumvijit and Y. Peerapornpisal

47. Packaging Development to Support Export Supply Chain of Mangosteen Fruit
   S. Sugiyono and I.M Edris

Food Science and Technology

48. Effect of Drying Conditions on Isoflavones and α-Glucosidase Inhibitory Activity of Soybean [Glycine max (L.) Merrill]
   C. Niamnuy, M. Nachaisin and S. Devahastin

49. Stability and Rheological Properties of Fat-Reduced Mayonnaises Containing Modified Starches as Fat Replacer
   K. Khantarat and S. Thaitudom

50. Mathematical Models for Electrical Conductivities of Fresh Juices, Concentrated Juices and Purees undergoing Ohmic Heating
   T. Tumpanuvatr and W. Jittanit

51. Contamination of Acrylamide in Thai-conventional Foods From Nong Mon Market, Chonburi
   P. Komthong, O. Suriyaphan, and J. Charoenpanich

52. Antimicrobial Activities of the Edible Bird’s Nest Extracts Against Food-borne Pathogens
   W. Saengkrajang, N. Matan, and N. Matan

53. Evaluation of Oxidative Stability and Some Quality Characteristics of Chinese-Style Sausage as Affected by the Addition of Roselle Extract and Different Sweeteners
   T. Parinyapatthanaboot and P. Pinsirod

54. Rapid and Highly Sensitive Analysis of Ethoxyquin Residues in Shrimp Using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry
   S. Chikakul and N. Leepipatpiboon

55. Changes in Cooking Behavior of Organic and Inorganic Phatthalung Sungyod Rice During Ageing
   I. Keawpeng and M. Meenune

56. Extraction of Collagen from Hen Eggshell Membrane by Using Organic Acids
   W. Ponkham, K. Limroongreungrat and A. Sangnark

57. Farinograph and Extensograph Properties of Frozen Dough Added With Psyllium Husk Powder or Locust Bean Gums
   S.Y Sim, A.A.N Aziah, T.T Teng, L.H Cheng
58. Extraction and Characterization of Acid-soluble Collagen from Skin of Striped Catfish (Pangasianodon hypophthalmus)  
   W. Wongwien, N. Srirach, S. Rawdkuen and N. Thitipramote  

59. Antioxidant Activity of Plant by-Products (Pink Guava Leaves and Seeds) and Their Application in Cookies  

60. Infrared and Hot Air Drying of Mullet Fish: Drying Kinetics, Qualities and Energy Consumption  
   Y. Tirawanichakul, S. Kaseng and S. Tirawanichakul  

61. One and Two-Stage Drying of Shrimp using Hot Air and Infrared: Quality Aspect and Energy Consumption  
   S. Tirawanichakul and Y. Tirawanichakul  

62. Development of a Composite Tubular Membrane for Separation of Acetone-Butanol-Ethanol (ABE) from Fermentation Broth by using Pervaporation Technique  
   W. Inthavee, S. Kanchanatawee and A. Boontawan  

63. Effect of High-Pressure Microfluidization on the Structure and Properties of Waxy Rice Starch  
   K. Kasemwong, K. Meejaiyen, S. Srisiri and T. Itthisoponkul  

64. Determination of Multiclass Pesticides in Onion Using Gas Chromatography with Tandem Mass Spectrometry (GC-MS/MS)  
   T. Semathong and N. Leepipatpiboon  

65. Effect of Nutrients in Trypticase Soy Agar on Growth Kinetics of Salmonella spp. under Micro-Cultivation  
   W. Sangadkit, W. Saeung, A. Boonyaprapasorn and A. Thipayarat  

66. Assessing Awareness on Food Quality and Safety among Food Small and Medium-Size Enterprises in Thailand  
   V. Suwanpidokkul and C. Waisarayutt  

67. Use of Viscozyme L for Pre-treatment of Coconut Prior to Extraction by Screw Press  
   N. Krasaechol, S. Chinnasarn, T. Itthisoponkul and W. Yuenyongputtakal  

68. Spoilage Bacteria Changes During Storage of Oyster (Crassostrea belcheri) in Ice-bath  
   S. Manatawee, N. Boonprasop, S. Boonprasop and P. Sutthirak  

69. Water Sorption Isotherm and Thermo-Physical Properties for the Analysis of Natural Rubber Drying  
   J. Tasara, S. Tirawanichakul and Y. Tirawanichakul  

70. Fast and Less Thermal Degradation Protocol for Chromocult® Coliform Agar (CCA) Preparation to Detect E. coli  
   P. Supanivatin, J. Khueankhancharoen, W. Saeung and A. Thipayarat  

71. Microbiological Quality of Fresh Cockle (Anadara granosa) During Storage at Room Temperature  
   P. Sutthirak and S. Boonprasop
72. Cloning of Beta-Galactosidase Gene from Lactobacillus delbrueckii subsp. bulgaricus TISTR 892 and Expression in Escherichia coli
   W. Srila, B. Ngampanya and P. Jaturapiree

73. Chemical Compositions of Eggs from Chicken, Quail and Snail-Eating Turtle
   T. Tunsaringkarn, W. Siriwong and W. Tungjaroenchai

74. Crude Malva Nut Gum Affects Pasting and Textural Properties of Wheat Flour in the Presence or Absence of Sodium Chloride
   Y. Phimolsriripol, U. Siripatrawan and C. J. K. Henry

75. Purification and Characterization of Microbial Transglutaminase from Enterobacter sp. C2361
   C. Bourneow, S. Benjakul and A. H-Kittikun

76. Effect of Adding Ling-zhi (Ganoderma lucidum) on Oxidative Stability, Textural and Sensory Properties of Smoked Fish Sausage
   W. Wannasupchue, S. Siriamornpun, K. Huaisan, J. Huaisan, and N. Meeso

77. Study on Preparation and Quality of Tomato Crispy Crackers
   N. Panyoyai, S. Sanjai and P. Mungkan

78. Effect of the Physical Properties on Consumer Preference of Nuggets
   P. Nantapatavee, A. Jangchud, K. Jangchud, J. Lin and T. Harnsilawat

79. Simple Fed–Batch Technique for the Production of Recombinant Enterokinase Light Chain By Pichia pastoris
   N. T. T. Dung, M. Ketudat-Cairns, and A. Boontawan

80. Structure Characterization and Molecular Docking Studies of α-Amylase Family-13 Glycosyl Hydrolases from Lactobacillus plantarum Complexed with Maltoheptaose: a Novel Feature of α-Amylase Catalytic Mechanism
   W. Bomrungnok, N. Khunajakr, A. Wongwichan, T. Dussadee, R. Saiprajong and S. Pinitglang

81. Origin of Proteolytic Enzymes Involved in Production of Malaysian Fish Sauce, Budu

82. Simple Determination of Ochratoxin A in Rice by Ultra Performance Liquid Chromatography Coupled with Mass-Spectrometry
   K. Sanguankaew and N. Leepipatpiboon

Agribusiness and Management

83. A Comparative Study of Rice Production and Trade Dynamics between Thailand and Vietnam
   N. L. Bach and N. Hempattarasuwan

84. Thai Consumer Willingness to Pay for Genetically Modified Rice
   W. Udomroekchai and Y. Chiaravutthi

List of ICAAI2010 Committee
Agricultural Science and Technology
The Influence of the Interaction between Jasmonates, Ethylene, and Polyamines on Fruit Quality

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Abstract

Jasmonates (jasmonic acid and methyl jasmonate) could regulate ethylene biosynthesis. The expression of the ACC synthase (ACS) 1 and ACC oxidase (ACO) 1 genes increased in pears (Pyrus communis L.) treated by n-propyl dihydrojasmonate (PDJ) at the preclimacteric stage. However, the accumulations of ACS1 mRNA decreased in the fruit treated by PDJ at the climacteric stage. Jasmonate treatment also influenced aroma volatiles (alcohols and esters) and anthocyanin formation as well as ethylene in apples (Malus domestica Borkh.). Jasmonates stimulated anthocyanin accumulation in the skin related to ethylene action. The PDJ or polyamine treatment decreased low-temperature damages such as splitting in apple fruit. The EC50 values of DPPH radial-scavenging activity in PDJ-treated or polyamine-treated fruit after a low-temperature treatment were lower than in the untreated control.

Keywords: aroma volatile, ethylene, environmental stress, jasmonic acid, polyamine

Introduction

Phytohormones have a correlation with each other. For example, 2, 4-DP application before harvest increased ethylene production in fruit and promoted fruit ripening (Kondo and Hayata, 1995; Kondo et al., 2006). Auxin influences 1-Aminocyclopropane-1-carboxylate (ACC) synthase in the ethylene pathway (Ishiki et al., 2000). In tomatoes, ACC synthase cDNAs for ACS1, 2, 3, 4, 5, 6, 7, and 8 have been isolated (Sato and Mizuno, 2003). The levels of the expression of their mRNAs differed with factors such as wounding and flooding. In pears, PcACS1 and ACC oxidase PcACO1 mRNA accumulations were observed in rewarmed fruit after low temperature treatment (Lelieuvre et al., 1997). These facts imply that MdACS1 and MdACO1 may be related to the fruit ripening. But the ACS genes of messenger RNA (mRNA) increased by the auxin application differed among fruits (Ishiki et al., 2000). In this report, the interaction between jasmonates, ethylene, and polyamine on fruit quality is discussed.

The Effects of Jasmonates and Ethylene on Aroma Volatile Compounds and Antioxidant Activity in Fruit

Aroma volatiles are primarily synthesized in the skin of fruit (Knee and Hatfield, 1981). The volatile compound production of apples is affected by various other substances. For instance, 1-MCP, which blocks ethylene receptors and inhibits ethylene action, delays apple fruit ripening (Blankenship and Dole, 2003). The levels of volatile compounds such as...
alcohols, esters, and ketones increase gradually toward ripening; however, their concentrations were the lowest in 1-MCP-treated fruit (Kondo et al., 2005). Furthermore, volatile compounds in 1-MCP-treated fruit did not increase greatly. This was true even at ripening. These results suggest that 1-MCP inhibits the production of volatile compounds. Volatile compounds in apples, produced by lipid and amino acid catabolism, are primarily synthesized in the skin (Rudell et al., 2002). Palmitic acid, stearic acid, oleic acid, linoleic acid, and triacontane were the predominant lipids detected in apple skin at harvest, but the levels of melissic acid, montanic acid, and heptacosan were greater in immature fruit skin (Noro et al., 1985). Thus, the late-forming lipids may be associated with aroma volatile synthesis during fruit ripening. Ranjan and Lewak (1995) showed that the lipid catabolism enzyme lipase is associated with aroma volatile production.

In addition, 1-MCP’s influence on the enzyme activity in the lipid catabolism pathway is well-known. This effect may be difficult to recover from due to its ethylene inhibition properties, through suppression of enzyme activity. Aroma volatiles in mangoes increased with the application of jasmonates (Lalel et al., 2003). However, Kondo et al. (2005) demonstrated that the effect of jasmonates on aroma volatile production was dependent on the developmental stage of the fruit. Jasmonates may decrease volatile compound production when applied at the climacteric stage. In contrast, jasmonate application at the pre-climacteric stage may stimulate aroma volatile production, as well as the relationship between ethylene and aroma volatiles in alcohols and esters (Fig. 1).

Furthermore, jasmonate application at the pre-climacteric stage could increase anthocyanin formation in apple skin (Kondo et al., 2001). It is known that the anthocyanin, which is a kind of polyphenolics, is an effective antioxidant. For instance, the hemolysis of red blood cells was delayed in the buffer including solution extracted from the skin of apples compared to the only buffer (Fig. 2). Ethylene is associated with anthocyanin formation in apple skin (Kondo and Hayata, 1995). In addition, jasmonates could stimulate anthocyanin accumulation in the skin related to ethylene action because a combination of jasmonates and AVG (an inhibitor of ACC synthase) increased anthocyanin concentrations,
compared to the untreated control (Kondo et al., 2001).

![Image of fruit skin extract buffer and Buffer](image)

**Figure 2** Effect of fruit skin extract on the hemolysis of red blood cells induced by a peroxyl radical generator (AAPH).

### The Effect of Jasmonates on Ethylene Production in Fruit

The changes of jasmonates differed between climacteric and non-climacteric fruit (Kondo et al., 2000; Kondo and Fukuda, 2001). Jasmonate concentrations increased at the ripening stage in climacteric fruit, but did not in non-climacteric fruit. In addition, the interactions between ethylene and jasmonates have been also reported in fruit. MeJA application at the pre-climacteric stage increased ethylene production in apples (Saniewski et al., 1988), but production decreased when MeJA was applied at the climacteric stage (Miszczyk et al., 1995).

PDJ application also influenced the ACS activity, ACC concentration, and the ACO activity at the pre-climacteric stage (Kondo et al., 2007). In PDJ-treated fruit at the pre-climacteric stage, an expression of the ACS1 and ACO1 mRNA was increased (Fig. 3). However, at the climacteric stage in pears, the expression of ACS1 and ethylene production was decreased in PDJ-treated fruit (Fig. 4; Kondo et al., 2007). These results suggest that jasmonate application may regulate ethylene synthesis of system 2 through the action of ACS1.

![Image of Northern blots](image)

**Figure 3** Northern blots from ‘La France’ pear skin at the pre-climacteric stage.

Chilling injury is generally caused by membrane damages based on cellular dehydration (Thomashow, 1999). Furthermore, membrane damage is caused by the freeze-induced production of reactive oxygen (McKersie and Bowley 1998). The production of reactive oxygen is induced by environmental factor such as low temperature (Matsui and Li, 2003). The treatments of PDJ or spermine decreased low-temperature injuries such as splitting and spotting in apple fruit (Yoshikawa et al., 2007). The EC50 of DPPH-radical scavenging activities in PDJ-treated fruit at 5 days after the low-temperature treatment was lower than in the untreated control at 20 °C and -2 °C (Fig. 5). It has been shown that phytohormone influenced low-temperature tolerance in rice cultivars.
That is, an increase of ABA or putrescine concentrations was observed in low temperature-tolerant cultivars but not in low-temperature sensitive cultivars when they were put at -5 °C (Lee at al., 1995). Low temperatures below -2 °C can induce frost damage such as splitting of the fruit. By applying PDJ or spermine, the rate of fruit damage caused by low temperatures was reduced from 14% to 10% (Yoshikawa et al., 2007). This coincided with an increase of endogenous ABA concentrations. The result that ABA increased in PDJ- or spermine-treated fruit suggests that these treatments may be effective for increasing low-temperature tolerance. When stored at -2 °C, the fruit’s endogenous JA concentrations declined more slowly. However, in Spm-treated fruit, the concentration either showed significant difference or decreased when compared to untreated fruit at -2 °C (Yoshikawa et al., 2007). This result shows that polyamine may reduce the increase of JA by increasing the tolerance of the fruit to low temperature. Therefore, the low-temperature tolerance induced by the jasmonate application may occur through polyamine.

**Conclusions**

The interactions between jasmonates and ethylene could influence fruit quality, although the effect differed with the stage of fruit ripening. The changes in physiological active substances including jasmonates, ethylene, and polyamine correlate with environmental stress and changes in genes. These genes were also affected by both environmental factors and phytohormones. Although environmental conditions significantly influence plant response, in many cases reactions are caused by changes in phytohormones. Plant reactions can be regulated by exogenous treatments of phytohormones, as well as regulation of environmental conditions. During the cultivation process of agricultural crops, environmental stresses such as drought and low temperatures induce plant dormancy. This is a kind of self-defense reaction of the plant to environmental stress and simultaneously jasmonate or ethylene levels increase dramatically.

**References**


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Physiological and Phytochemical Changes in Cayenne Pepper

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Abstract

Cayenne pepper (\textit{Capsicum annuum} Linn. Var acuminatum Fingerh) is widely consume in Thailand, however information about its phytochemical components is still limited. Thus, this research aimed to study the changes in physiological and phytochemical components in two cultivars of cayenne pepper during the postharvest period. Green and red cayenne peppers were harvested from a commercial orchard in the central part of Thailand. The peppers were kept at 4°C for 30 days and samples were withdrawn for analysis every 5 days. It was found that red cayenne pepper had a higher antioxidant activity (DPPH radical scavenging activity) than green cayenne pepper. This was concomitant with a higher abundance of total phenolic compounds, ascorbic acid and \(\beta\)-carotene contents in green cayenne pepper compared with the red one. Respiration and ethylene production rates of green cayenne pepper were higher than that of the red cultivar. The content of total phenolic compounds and \(\beta\)-carotene in both green and red cayenne peppers decreased after day 20 of storage, while ascorbic acid content slightly increased. Based on the antioxidant contents, a consumption of red cayenne pepper would appear to provide a greater health benefit than consumption of the green cultivar.

Keywords: antioxidant activity, ascorbic acid, \(\beta\)-carotene, Cayenne pepper

Introduction

Nowadays, consumers tend to eat more fresh fruit and vegetables than previously, and this is linked with a belief that fresh fruit and vegetables are enriched with antioxidative compounds which can eliminate or scavenge the free radical in our bodies. Many fruit and vegetables produced in tropical regions are rich in plant pigments which have free radical scavenging properties and act as electron donors to unpaired electrons of reactive oxygen species. Chili is widely consumed around the world. They are many common species of chili peppers such as \textit{Capsicum annuum}, \textit{Capsicum frutescens}, \textit{Capsicum chinense}, \textit{Capsicum pubescens} and \textit{Capsicum baccatum}. However, chilis are commonly classified into three groups; bell peppers, sweet peppers, and hot peppers. There are only a few commonly used species, most especially \textit{Capsicum annuum} which includes bell peppers, cayenne, jalapeños, and the chiltepin, and \textit{Capsicum frutescens} which includes the chiles de árbol, malagueta, tabasco and Thai.
peppers. Previous reports found that chili contains high amounts of vitamin C and carotene (provitamin A). In addition, it is a good source of vitamin B6 and is very high in potassium, magnesium, and iron. Moreover, chilis are rich in phenolic compounds which accumulate in the form of pigments such as anthocyanin and flavonoids. The bioactive compounds in chili are believed to promote a healthy human body by dilating the blood vessels, cleansing the mucus lining and lowering blood cholesterol. Research in humans found that, with the intake of capsaicin (a hot compound in chili), the LDL or bad cholesterol actually resisted oxidation for a longer period. This reduced the risks of heart attacks and strokes.

In Thailand, Cayenne pepper is a widely used species of hot chili. Most of the research in the past has focused on the advantages of red hot chili. However, a few studies have dealt with the nutritional and postharvest research of Cayenne pepper. Thus the objective of this research was to study changes in the physiology and phytochemicals of Cayenne pepper during the postharvest period.

**Materials and Methods**

**Plant Material Preparation**

Red and green Cayenne peppers (*Capsicum annuum* Linn. var acuminatum Fingerh) were harvested at commercial maturity from an orchard in Nontaburi Province, Thailand. The fruit were transported to the Postharvest Technology laboratory at King Mongkut’s University of Technology Thonburi within 3 hours after harvesting and then were selected for uniformity of maturity, color and size, and also freedom from any defects and diseases. The selected red and green Cayenne peppers were cleaned with running tap water and left to dry under ambient condition for 30 minutes after which the fruit were stored separately (red and green) in plastic basket covered with a polyethylene bag at 4°C (90-95% RH). Samples of red and green Cayenne peppers were taken every 5 days for physiological and phytochemical analysis during the storage period of 30 days. Respiration and ethylene production rates were monitored and total phenol content, total ascorbic acid content, β-carotene content and DPPH radical scavenging activities were measured.

**Respiration and Ethylene Production**

The rate of ethylene production was measured by gas chromatography, using a flame ionization detector (FID) equipped with an 80/100-mesh Pora pack-Q column with nitrogen as the carrier gas. The Cayenne peppers were kept in plastic chambers and incubated at 4°C for 3 h after which a gas sample (1 mL) was taken with a syringe. Respiration rates were also determined by gas chromatography using a 80/100-mesh Pora pack-Q column and a thermal conductivity detector (CHROMA-TOPAC C-R 8A, SHIMADSU Co., Kyoto, Japan).
Total Phenol Content

Total soluble phenolic compounds were measured using the method of Singleton and Rossi (1965). Extracts were separately prepared from the top (around the calyx), the middle, and the bottom part of the fruit with 3 replications. Two grams of fruit sample were homogenized with 20mL of 80% ethanol for 1 min. The extract was then filtered and centrifuged at 10,000×g for 15 min. One millilitre of the supernatant was mixed with 1mL of Folin Ciocalteu reagent (Sigma–Aldrich, Buchs, Switzerland) and 10mL of 7% sodium carbonate. The volume was increased to 25 mL with distilled water and left to settle for 1 h. The total phenolic content was then read at 750nm using a spectrophotometer (UV-1601; Shimadsu Co., Kyoto, Japan). A standard curve of gallic acid was used to quantify the total phenolic content.

Total Ascorbic Acid Content

The total ascorbic acid content was measured according to the method of Hashimoto and Yamafuji (2001). Five millilitres of fruit juice were mixed with 20mL of cold 5% metaphosphoric acid, and filtered through Whatman No. 1 paper. A 0.4mL aliquot of the filtrate was mixed with 0.2 mL of 2% di-indophenol. The mixture was then added to 0.4mL of 2% thiourea and 0.2mL of 1% dinitrophenol hydrazine, and incubated at 37° C for 3 h. After incubation, 1 mL of 85% sulphuric acid was added, and the resultant solution was incubated again at room temperature for 30 min. Total ascorbic acid was determined by measuring absorbance at 540 nm using a spectrophotometer (UV-1601; Shimadsu Co., Kyoto, Japan). The concentration of total ascorbic acid was expressed in mg/100g on a fresh weight basis.

β-Carotene Content

Each 2g sample of tissue was placed in 50 ml of solution containing hexane:acetone:ethanol (2:1:1) and then homogenized and mixed on a magnetic stirrer for 10 min. To this homogenate was added 7.5 ml of distilled water and the solution again mixed on a magnetic stirrer for 5 min. The sample was allowed to form two phases with the upper phase being the hexane phase (25 ml) and the lower being a mixture of acetone and ethanol. The upper phase was collected and its absorbance was determined at 450 nm according to the method of Scott (2005).

DPPH Radical Scavenging Activity

The DPPH assay was carried out according to the method of Brand-Williams et al. (1995) with some modifications. The stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and this stock solution was stored at 20 oC until needed. A working solution was obtained by mixing 10 mL of the stock solution with 45 mL methanol to obtain an absorbance of 1.1±0.02 units at 515 nm using the spectrophotometer. Fruit extracts (150 µL) were allowed to react with 2850 µL of the DPPH solution for 24 h in the dark. The absorbance was then taken at 515 nm. The standard curve was linear between 25 and 800 µM Trolox. Results are expressed in µM TE/g fresh mass. Additional dilution was needed if the DPPH value measured was over the linear range of the standard curve.
Results and Discussion

The respiration rates of both the red and green cayenne peppers showed the same pattern throughout the storage time of 30 days (Figure 1A). A peak of respiration was observed on day 15 in both pepper cultivars. Green cayenne pepper had a significantly higher rate of respiration than the red cultivar. The ethylene production rates of red and green cayenne peppers showed the same trend of change during storage. The maximum peak of ethylene evolution in green pepper was on day 10. In the red pepper, the ethylene production rate was lower and the peak was shifted to day 15 (Figure 1B). These results indicate that ethylene production rose after the initial day of storage whereas the respiration rate was changed little during the first ten days of storage. The later increase in respiration may have been induced by the ethylene production. Green cayenne pepper had higher respiration and ethylene production rates than the red cultivar. This may imply that green cultivar was an immature fruit while the red cultivar was a more mature fruit. Normally, the immature fruit have higher respiration and ethylene production rates than the mature fruit (Tadesse et al., 1998).

Total phenolic content in both red and green cayenne peppers was slightly decreased during 20 days of storage and then sharply declined through to the end of storage (Figure 2). The total phenolic content in the red pepper was higher than in the green cultivar. This may be due to the red pepper containing anthocyanin as its major pigment and anthocyanin is one class of flavonoid compounds, which are widely distributed plant polyphenols thus the total phenolic content in red pepper was greater compared to the green one. This result was in contrast with the finding of Zhang and Hamauzu (2003) who determined that the phenolic content in green bell pepper was higher than in the red and yellow cultivars. The differences between the levels of phenolic compounds in the peppers in our study compared with the levels found by Zhang and Hamauzu (2003) may be due to the different cultivars of pepper examined and may also be influenced by differences in the environments in which peppers were grown, with the bell peppers being grown in a cooler climate than that of cayenne pepper.

The β-carotene content in the green pepper was in the range of 7-11 mg gFW-1 while in the red pepper, the content was some two-fold higher, in the range of 15-30 mg gFW-1 (Figure 3). The content in the red pepper increased after day 10 to a peak at about day 20 then decreased to the end of storage. In contrast the content in green cayenne pepper did not change significantly across the period of storage. A similar observation was reported by Zhang and Hamauzu (2003) who reported that the carotenoid content in red and yellow bell peppers were greater than in green cultivar due to the carotenoid pigments.

Total ascorbic acid contents in both the red and green cayenne peppers changed little over the first 20 days of storage (Figure 4) but increased sharply to a peak at day 25 in the red pepper and remained higher than in the green pepper until the end of storage. In contrast, the content in the green pepper declined slowly from day 20 until the end of the storage period. As mentioned above, the green and red cayenne pepper were at different maturity stages, thus the red mature fruit had higher ascorbic acid contents than the less mature green
pepper. Zhang and Hamauzu (2003) also found that red bell pepper contained higher ascorbic acid than the green cultivar.

Antioxidant activity in this research was determined as the DPPH radical scavenging activity (Figure 5). There was little difference in DPPH radical scavenging activity between the two cultivars during the first 20 days of storage. However, after day 20 the DPPH radical scavenging activity in the red pepper increased to a higher level than in the green cultivar and was about 2-fold higher than in the green pepper by the end of study. The higher antioxidative activity in red pepper was related with a higher content of ascorbic acid, total phenolic compound and β-carotene.

**Figure 1** Respiration (A) and ethylene production rate (B) of red and green cayenne peppers during storage at 4°C. The vertical bars indicate standard errors (n=3).

**Figure 2** Changes in total phenolic contents in red and green cayenne peppers during storage at 4°C. The vertical bars indicate standard errors (n=3).

**Figure 3** Changes in β-carotene contents in red and green cayenne peppers during storage at 4°C. The vertical bars indicate standard errors (n=3).

**Figure 4** Total ascorbic acid content in red and green cayenne peppers during storage at 4°C. The vertical bars indicate standard errors (n=3).

**Figure 5** DPPH radical scavenging activity in red and green cayenne peppers during storage at 4°C. The vertical bars indicate standard errors (n=3).

**Conclusions**

Red cayenne pepper has higher antioxidant activity (DPPH radical scavenging activity) than green cayenne pepper due to its higher levels of total phenolic compounds, ascorbic acid and β-carotene. This higher antioxidant
activity would suggest that consumption of the red cayenne pepper might gain more beneficial to human health than consumption of the green pepper.

References


Application of Chitosan for Reducing Chemical Fertilizer Uses in Waxy Corn Growing

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Abstract

Chitosan is abundant biopolymer found in nature. It has been used to stimulate plant growth and enhance crop yields. The objectives were to reduce chemical fertilizer uses in waxy corn growing and also conserve soil physical properties and environment. This experiment was conducted using a split plot in Randomized Complete Block Design with two main plots and four subplots and replicated four times. Main plot was chitosan application and control (no chitosan) and subplot was four rates (50+50, 50+25, 25+50 and 25+25 kg/rai) of chemical fertilizer mixed between formula 16-20-0 and 46-0-0. Field experiment was carried out at field crop plot of Plant Science section, Rajamangala University of Technology Suvarnabhumi, Pranakhon Sri Ayuttaya province during February to April 2010. The results showed that chitosan application significantly increased (p<0.05) leaf area, ear size, waxy corn yield and fiber percentage. It also significantly retained (p<0.05) leaf greenness at harvesting time but no significant effects at V8 (8 true leaves) and R1 (silking) growth stage. However, chitosan did not affect leaf nitrogen content. In regard to chemical fertilizer rate effects, it was found that no significant difference was found in terms of waxy corn yield and agronomic characteristics, except nitrogen content and fiber percentage. This finding suggested that chitosan application can be used to enhance waxy corn yield and also to reduce chemical fertilizer uses in waxy corn growing.

Keywords: chemical fertilizer, chitosan application, waxy corn

Introduction

Waxy corn is a type of fresh corns popularly consumed in Thailand due to being gentle and lightly sweet, furthermore ear size is also fitting and harvesting time is rather short (55-70 days). It can be grown through the year as cash crops in irrigated areas. Corn takes up nitrogen higher than the other crops (35 kg ha⁻¹ throughout cropping season). Nitrogen absorption from soil is increased depending on growth stages with the actual rates depending upon soil type and previous cropping history (Weir et al., 1996). Nitrogen application in excess of crop requirements contributes to the increased levels of NO₃⁻ in the soil profile (Roth and Fox, 1990). Reducing N application rate by 5% less than that of the required rate to achieve maximum corn yield reduces NO₃⁻ leaching by 40 to 45% (Sexton et al., 1996). Chitosan, a natural biopolymer, can enhance germination index and shoot and root dry weight (Guan et al., 2009). It also promotes seedling growth, accelerates the first flowering date of several ornamental plants (Ohta et al., 2004), stimulates plant growth and enhances yield of crop species such as Oryza sativa L. and Glycine max L. (Boonlertnirun et al., 2008; Zheng et al.,...
Furthermore, it has been shown to trigger immune system to resist plant disease in many plant species (Nandeeshkumar et al., 2008). Chitosan can be absorbed to the root after being decomposed by bacteria in the soil. Application of chitosan in agriculture, even without chemical fertilizer, can increase the microbial population by large numbers, and transforms organic nutrient into inorganic nutrient, which is easily absorbed by the plant roots (Somashkar and Richard, 1996; Brian et al., 2004). Chitosan is useful as the nutrient for the soybean cultivation when the nitrogen is insufficient (Zheng et al., 2005). Oligosaccharides (oligochitosan) act on plants as phytohormone-like compounds in regulation processes of morphogenesis, growth and development (Thama et al., 2001). The objectives were to reduce chemical fertilizer uses in waxy corn growing and also conserve soil physical properties and environment.

**Materials and Methods**

**Experimental Design**

The experimental design was a split-plot in Randomized Complete Block with 2 main plots and 4 subplots and replicated 4 times. The treatment details are as follows:

- **Main plot (M)** was 2 types of application
  1. Chitosan application
  2. No chitosan application (control)
- **Subplot (S)** was 4 chemical fertilizer rates
  1. Basal application with 16-20-0 (50 kg/rai) and top dressing with 46-0-0 (50 kg/rai)
  2. Basal application with 16-20-0 (50 kg/rai) and top dressing with 46-0-0 (25 kg/rai)
  3. Basal application with 16-20-0 (25 kg/rai) and top dressing with 46-0-0 (50 kg/rai)
  4. Basal application with 16-20-0 (25 kg/rai) and top dressing with 46-0-0 (25 kg/rai)

**Planting Method and Cultural Practice**

Plot size was 3x4 m², each comprising of 4 rows, the spacing between rows and plants were 75 and 20 cm (75x20cm) respectively. Two central rows (about 40 plants) were utilized to record growth and yield data. Three corn seeds cv. Big white 852 were sown per hole, 2 weeks after sowing, corn seedlings were thinned to be one healthy plant per hole. All recommended cultural practices such as irrigation, weed control, except pesticide application were uniformly done according to standard crop requirement. Oligomer chitosan from shrimp with 90% degree of deacetylation at the concentration of 80 ppm was sprayed 4 times at 14, 21, 28 and 35 days after planting. This experiment was conducted at field crop plot of Rajamangala University of Technology Suvarnabhumi, Phranakhon Sri Ayutthaya province, during January to February 2010.

**Data Collection**

1. Leaf greenness, 10 leaf samples per plot were measured on both sides of the midrib, midway between leaf base and tip of subtending leaf (ear leaf) by using chlorophyll meter (SPAD 502) at harvesting time
2. Leaf area, all leaves of each corn plant were recorded at harvesting time by measuring leaf width (W) and length (L) of 10 plants per plot and averaged to be leaf area per plant by the following equation:

\[
\text{Leaf area} = 3.3 + 0.642 (L \times W) + 0.001 (L \times W)^2, \quad R^2 = 0.995
\]

3. Fiber percentage (% fiber) was performed at silking stage (R1) by Approximate analysis method.
4. Nitrogen content was detected at silking stage (R1) by Kjeldahl method (1883)
5. Yield and yield components (ear width and length and ear number) were recorded after harvesting.

Results and Discussion

Waxy Corn Yield
Yield of waxy corn was significantly affected by chitosan application. The average yields of chitosan treatment and control (no chitosan) were 1.37 and 1.19 ton/rai respectively. In regard to chemical fertilizer effects, it showed no significant difference, however basal application with 16-20-0 at 50 kg/rai and topdressing with 46-0-0 at 50 kg/rai (recommended rate) after 21 days after planting tended to produce the maximum yield. Reduction of chemical fertilizer application without or the least yield reduction can be performed by decreasing basal application to be 25 kg/rai whereas top dressing was still the same rate (50 kg/rai) as recommendation. This result was closely to the work of Zheng et al. (2005) who found that chitosan was useful as the nutrient for the soybean cultivation when the nitrogen was insufficient. There was not an interaction effect between chitosan application and chemical fertilizer rates on waxy corn yield (Figure 1).

Marketable Ear Numbers
Chitosan application significantly influenced marketable ear numbers. A large number of marketable ears were achieved with chitosan treatment of 4,391 ears/rai whereas those of the control (no chitosan) were only 3484 ears/rai. Abdel-Mawgoud et al. (2010) found that chitosan application at 2cc/l improved yield components (number and weight) of strawberry plants. Application of various rates of chemical fertilizer did not affect number of marketable ears. However, application following the recommended rate (50 kg/rai of basal+50 kg/rai of topdressing) tended to gain more marketable ear numbers than the others. Interaction between chitosan application and chemical fertilizer rates was not significantly found (Figure 2). Lu et al. (2002) found that panicle numbers of rice were increased with spraying chitosan at the rate of 0.4 g/50 cc of water.

Ear Size
Chitosan showed positive effects on ear size of waxy corns. In term of husk ear, both ear width and length were increased by chitosan application. No significant difference was detected in terms of various chemical fertilizer rates and interaction.
effect. With regard to unhusk yield, chitosan affected ear width but no effect on ear length (Figure 3,4). Uthairatanakij et al. (2007) reported that application of chitosan to Dendrobium orchid plants tended to increase the size of open florets and inflorescence length.

![Figure 3](image1)

**Figure 3** Effects of chitosan with various chemical fertilizer rates on ear width of husk and unhusk ear.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Husk ear</th>
<th>Unhusk ear</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD.05 (M)</td>
<td>0.1</td>
<td>0.04</td>
</tr>
<tr>
<td>LSD.05 (S)</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LSD.05 (MxS)</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CV(%)</td>
<td>1.72</td>
<td>1.64</td>
</tr>
</tbody>
</table>

![Figure 4](image2)

**Figure 4** Effects of chitosan with various chemical fertilizer rates on ear length of husk and unhusk ear.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Husk ear</th>
<th>Unhusk ear</th>
</tr>
</thead>
<tbody>
<tr>
<td>LSD.05 (M)</td>
<td>0.215</td>
<td>ns</td>
</tr>
<tr>
<td>LSD.05 (S)</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>LSD.05 (MxS)</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>CV(%)</td>
<td>3.58</td>
<td>5.44</td>
</tr>
</tbody>
</table>

Leaf Greenness

Application of chitosan obviously promoted leaf greenness of waxy corn and significantly differed from the control. Leaf greenness value of chitosan treatment and the control was 54.45 and 49.72 spad unit respectively. The similar result reported by Go'nik et al. (2008); Dzung et al. (2010) revealed that chitosan significantly increased chlorophyll content in grapevine and coffee seedlings. Furthermore, leaf greenness was also influenced by chemical fertilizer rates. The recommended rate (50+50 kg/rai) significantly contributed to leaf greenness increasing. Boonlertnirun et al. (2010) reported that leaf greenness value of waxy corn was 2335.24 cm²/plant. This finding was supported by the result of Abdel-Mawgoud et al (2010) who reported that number of strawberry leaves were positively improved after application of chitosan at 2 cc/l. Dzung et al. (2010) indicated that leaf area of coffee seedlings was increased up to 60.53% after spraying chitosan oligomer with the concentration of 60 ppm. Leaf areas of waxy corn were not significantly differed with various chemical fertilizer rates. Interaction effect between chitosan application and chemical fertilizer rates on leaf areas was not detected (Figure 5).

![Figure 5](image3)

**Figure 5** Effects of chitosan with various chemical fertilizer rates on leaf areas.

Leaf Area

There was significant difference between chitosan application and the control (no chitosan). Leaf areas of waxy corn were well developed of 2472.54 cm²/plant after chitosan application while those of the control were 2335.24 cm²/plant. This finding was supported by the result of Abdel-Mawgoud et al (2010) who reported that number of strawberry leaves were positively improved after application of chitosan at 2 cc/l. Dzung et al. (2010) indicated that leaf area of coffee seedlings was increased up to 60.53% after spraying chitosan oligomer with the concentration of 60 ppm. Leaf areas of waxy corn were not significantly differed with various chemical fertilizer rates. Interaction effect between chitosan application and chemical fertilizer rates on leaf areas was not detected (Figure 5).
increased with increasing of nitrogen fertilizer rates. No interaction effect (chitosan x chemical fertilizer rates) was found (Figure 6).

![Figure 6: Effects of chitosan with various chemical fertilizer rates on leaf greenness.](image)

LSD.05 (M) = 4.67  
LSD.05 (S) = 2.88  
LSD.05 (MxS) = ns  
CV(%) = 4.40

Figure 6 Effects of chitosan with various chemical fertilizer rates on leaf greenness.

**Nitrogen Content**

Chitosan did not contribute to nitrogen content increases in waxy corn leaves. On the contrary, Abdel-Mawgoud et al (2010) reported that leaf nitrogen content of strawberry was increased with increasing chitosan concentration. Application of various chemical fertilizer rates significantly affected nitrogen content. Boonlertnirun et al. (2010); Kulsum et al., (2007) revealed that nitrogen content (concentration) in plant leaves was increased with increasing nitrogen level. Top dressing (46-0-0) at high rate (50 kg/rai) tended to accumulate nitrogen greater than did with low rate (25 kg/rai), particularly when applied with chitosan, however interaction effect (chitosan x chemical fertilizer rates) was not found (Figure 7).

![Figure 7: Effects of chitosan with various chemical fertilizer rates on nitrogen content.](image)

LSD.05 (M) = ns  
LSD.05 (S) = 0.0017  
LSD.05 (MxS) = ns  
CV(%) = 10.42

Figure 7 Effects of chitosan with various chemical fertilizer rates on nitrogen content.

**Fiber Percentage**

Both chitosan and chemical fertilizer application significantly affected fiber percentage in waxy corn leaves. Chitosan contributed to fiber (cellulose, hemicelluloses and lignin) increases because chitosan molecule comprised many nitrogen atoms which was components of fiber. This was the good point to increase plant cell vigour. Fiber percentage of chitosan treatment and the control was 27.36 and 21.77 % respectively. Considering chemical fertilizer effect, top dressing (46-0-0) at high rate (50 kg/rai) tended to increase fiber percentage. Application of chitosan with chemical fertilizer at 25 kg/rai for basal application and 50 kg/rai for top dressing obviously increased fiber percentage. Srisiripan et al. (2008) reported that lignin (one of fiber components) of two bell pepper cv. Torcal and Gold Frame was increased after coating with chitosan at the concentration of 1.0 and 1.5% and stored at 25 °C , 82% RH for 15 days. Application of chitosan by seed soaking before planting and four times of soil application throughout cropping season tended to increase fiber percentage in rice plants (Boonlertnirun et al., 2008).
LSD.05 (M) = 1.112
LSD.05 (S) = 0.699
LSD.05 (MxS) = 0.988
CV (%) = 2.26

Figure 8 Effects of chitosan with various chemical fertilizer rates on fiber percentage.

Conclusions

The present study revealed that chitosan had strongly positive effects on growth and yield as well as some characteristics relating to yield potential of waxy corn. According to this result, application of chitosan at the concentration of 80 ppm with mixed chemical fertilizer between 16-0-0 and 46-0-0 at the rate of 25 and 50 kg/rai respectively can be suggested to apply for reducing chemical fertilizer uses in waxy corn growing without yield losses.

Acknowledgement

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Photostability of Mango Seed Kernel Extract and Its Encapsulated Product

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Abstract

The effect of light on the oxidative stability of phenolic compound from mango (Mangifera indica cultivar Chok-Anan) seed kernel extract and its encapsulated product was determined by absorptivity in the Ultraviolet (UV) spectrum and by total phenolic content induced with white fluorescent lamp (28 watt) intensity over a 1, 3, 6, 24 and 48 h period. Maximum UV absorptive at 276 nm of mango seed extract highly increased during light exposure more than the others. While, encapsulation of the extract showed the shoulder at 252 and 321 nm, the strongest UV absorption change was obtained at 276 nm from 0.625 to 1.054 after 48 hours of photo induction. Total phenolic content of samples showed a significant difference in the initial stage of photoinduced (1–6 hours), but after this time, the total phenolic content slowly decreased. However, the phenolic content of extract was more highly decreased than that of encapsulated product. The correlation of maximum UV absorption change values and total phenolics decreasing was highly related (r = 0.71). These results showed that UV absorption is a simply tool for photo-oxidation monitoring in quality control of mango seed kernel extract and its encapsulation.

Keywords: encapsulation, mango seed, phenolic, photostability, UV absorption

Introduction

Extracts from kernels of eleven cultivars of Thai mango (Mangifera indica Linn.) seed showed antioxidant activity comparable to that of α-tocopherol. Most of the phenolic compounds of mango seed kernel were phenolic acids and flavonoids (Maisuthisakul, 2010). The extracts from the mango seed kernel of the cultivar Chok-Anan also possessed tyrosinase inhibitory activity, which was greater than that of arbutin and they did not cause acute irritation of rabbit skins (Maisuthisakul and Gordon, 2009). 1,2,3,4,6-penta-O-galloyl-beta-D-glucopyranose, methyl gallate and gallic acid have been identified as components of an ethanolic extract of Thai mango seed kernel cultivar Fahlun (Nithitanakool et al., 2009).

During the process of food development, stability has been identified as an essential element to assure the safety and efficacy of food products because of the possibility of toxic degradants and loss of active ingredient (Yasueda et al., 2004). The double bonds conjugated with some functional groups such as hydroxyl group present in the phenolic structure is responsible for electron delocation which affect antioxidant activity, but also for the poor stability of the molecule under processing and storage condition such as high temperature, oxygen and light (Barbosa et al., 2005).

One of the alternatives used to improve phenolics stability is the microencapsulation technique, which entraps a sensitive ingredient inside a coating material. The structure formed around the microencapsulated substance (core) is called the wall. Of the techniques available for microencapsulation, spray-drying, a well-known technology is the most commonly used. The wall materials most frequently used include gums and maltodextrin. The
objectives of the study were to evaluate the photostability of encapsulation with two polysaccharides on mango seed kernel (MSK) phenolics and to evaluate the relation between phenolic degradation and UV-spectrum of MSK.

Materials and Methods

Materials

Sun dried seeds from ripened mango (Mangifera indica cultivar Chok-Anan) were donated from a mango processing manufacturer in Thailand from March to June in 2008 as by-products. Moisture content on a dry weight basis according to AOAC (1990) of dried mango seed kernel equaled to 9.81±0.34%. The dried material was kept in freezer at -20°C no longer than two months.

Maltodextrin (DE = 16-20, 5% moisture, bulk density = 6000 kg m-3 was purchased from natural Starch and Chemical (Sydney, Australia). Arabic gum (food grade) was provided by Colloides Naturels International Co. (Rouen, France). Polyglycerol polyricinoleate (PGPR, 4150, HLB ≈ 3) was obtained from Palsgaard (New Jersey, USA). The emulsifier used was Tween 80 (Fisher Scientific, New Jersey, USA). The other chemicals and solvents used in this experiment were analytical grade purchased from Sigma-Aldrich Co., Ltd (Steinheim, Germany).

Extraction of Mango Seed Kernel Antioxidant

The freezing kernel (80 g) was blended for 1 min with 95% ethanol and refluxed with 1.2 M hydrochloric acid in ethanol for 3 h. The supernatant, after filtration through cheesecloth and Whatman No 4 filter paper, was evaporated under vacuum. Sample was dried in a freeze dryer and stored in aluminum foil after flushing with nitrogen at -20°C until usage.

Preparation of W1/O/W2 Emulsions

W1/O/W2 emulsions were prepared using the modified two-step emulsification method describes by Fechner et al. (2007). The inner aqueous phase (W1) was prepared by hydrating gelatin (1%, W/W), NaCl (0.6%, W/W) and 200 mg kg⁻¹ of crude mango seed kernel extract (MSKE) in water at 60°C for 5 min using moderate magnetic stirring. Gelatin was added to solidify the inner aqueous phase as it has been previously reported to enhance the encapsulation efficiency and the oil droplet stability of double emulsion (Fechner et al., 2007). The oil phase was refined soybean oil containing required antioxidants and the hydrophobic emulsifier PGPR (8%, W/W) and heated to 60°C for 10 min. The outer aqueous phase (W2) was prepared by mixing distilled water at pH 6.6 containing Tween 80 (0.5 %, W/W), arabic gum (10 %, W/W) and maltodextrin (20 %, W/W) under moderate magnetic stirring conditions.

Primary water-in-oil (W1/O) emulsion (300 g, 20.0% aqueous phase, W/W) was prepared by initially mixing the inner aqueous phase with the oil phase. The mixtures were homogenized with a high-pressure homogenizer (Armfield model FT9, UK) twice at 3000 psi. Subsequently, the W1/O emulsion (10%, W/W) was gradually added to the outer phase (W2, 90%, W/W), mixed hand-held laboratory homogenizer at 10,000 rpm for 2 min (PRO200 Homogenizer, Pro Scientific, CT, USA) to produce the final double emulsion. The droplet size distribution of W1/O/W2 emulsions was measured.

Preparation of Spray Dried Encapsulated Powders

The dispersions were spray dried using Nitro Minor Dryer (Gea Nitro A/S, Denmark) pilot scale spray dryer. The dispersion was fed by a peristaltic pump at a fixed rate of 30-35 ml/min. Drying was carried out in the concurrent mode. Inlet and outlet air temperatures were 180 °C and 90 °C, respectively. The microcapsule powder was collected at the dryer’s cyclone. The microcapsules were analyzed for characterization and used for photostability study.
Characterization of Microcapsules

The characterization of the encapsulated samples was water activity, moisture content (AOAC, 1990), hygroscopicity and bioactive compounds microencapsulated yields.

Water activity was measured using an aqualab analyzer (Decagon Devices, USA) at 25°C after stabilization of the samples at this temperature for 1 h.

For hygroscopicity, samples (about 2 g) of each powder were placed in Petri dishes at 25°C in an airtight plastic container filled with Na2SO4 saturated solution (81%RH). After 1 week the samples were weighed and hygroscopicity was expressed as g of water absorbed/100 g of dry solids (Cai and Corke, 2000).

The determination of bioactive compounds microencapsulated yield (BMY) was determined according to Saénz et al. (2009). The 100 mg of microcapsules were treated with 10 and 1 ml of a mix of ethanol and methanol (1:1), respectively. These dispersions were agitated in a Vortex at room temperature for 1 min and then filtered (0.45 µm Millipore filter). The amount of phenolic compound was quantified by the Folin-Ciocalteu method. The surface bioactive compounds percentage (SB) and bioactive compounds microencapsulated yield (BMY) were calculated using the equation SB% = [(measurable phenolic content/theoretical phenolic content) × 100], BMY% = 100 – SB%. All tests were carried out in triplicate.

Statistical Analysis

Each experiment, from sample preparation to analysis, was repeated in triplicate, and the data were analyzed by SPSS software program (SPSS Inc., Chicago, IL, USA). The general linear model procedure was applied and Duncan’s multiple range tests was used to compare the mean values at p<0.05. Mean values and pooled standard error of the mean (SEM) were then estimated.

Results and Discussion

Characterization of Microcapsules

The characterization of the encapsulated samples is shown in Table 1. Microcapsule with mango seed kernel extract (MSKE) showed the similar value of water activity to microcapsule with no antioxidant (control) which contrast to moisture content. The moisture content of encapsulated MSKE was higher than control. The MSKE encapsulation yields (BMY) reached value above 98%. Typically, phenolic compounds are amphiphilic structure. Adding phenolic substances in the encapsulated system cause higher hygroscopicity. These results suggest that the hydrophobic groups were less

Photostability of Spray Dried Encapsulated powders

Unencapsulated mango seed kernel extract and microcapsules were exposed in glass tubes to white fluorescent lamp (28 watt) intensity inside a light chamber. Glass tubes were 1 cm in diameter and contained a volume of 8 ml. Tubes were completely filled to eliminate any interference from air. Light chambers (aquariums painted with flat black paint) were fitted with overhead fluorescent lights (General Electric Cool White No. F15T8-CW), and a rheostat was used to adjust light intensity as measured by a type 214 General Electric light meter. Sample tubes, closed with Teflon-lined screw caps, were laid horizontally, without agitation. Light chambers and samples were maintained at 30°C. Samples were removed at 1, 3, 6, 24 and 48 h of exposure and analyzed for UV spectrum and phenolic content.

The absorbance of triplicate samples of treated unencapsulation of mango seed kernel extract and microcapsules was measured after different exposure times between 200 to 400 nm and the λmax for aqueous samples.

The total phenolic content of extracts was determined using the Folin-Ciocalteu’s phenol reagent (modified from Maisuthisakul et al., 2008). The concentration of total phenolic compounds in all samples was expressed as mg of tannic acid equivalent per g dry weight of MSK using a linear equation. All determinations were performed in triplicate.
exposed, probably due to hydrophobic interaction with emulsifier. These interactions caused conformational changes with increased the amount of superficial hydrophilic groups increasing the water activity, the moisture and the hygroscopicity of the encapsulated MSKE compared to control.

Table 1 Aw, surface bioactive compounds percentage (SB), bioactive compounds microencapsulated yield (BMY) and hygroscopicity of studied samples †

<table>
<thead>
<tr>
<th>Microcapsule with no antioxidant</th>
<th>MSKE microcapsule</th>
</tr>
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<tbody>
<tr>
<td>Aw (g/100g)</td>
<td>0.189 ± 0.006</td>
</tr>
<tr>
<td>Moisture content</td>
<td>0.74 ± 0.13</td>
</tr>
<tr>
<td>SB (%)</td>
<td>ND</td>
</tr>
<tr>
<td>BMY (%)</td>
<td>ND</td>
</tr>
<tr>
<td>Hygroscopicity (g of water/100 g, db)</td>
<td>109.33 ± 0.08</td>
</tr>
</tbody>
</table>

†dry weight basis; Means of three replications ± SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each row.

Photostability of Spray Dried Encapsulated Powders

Photoinduced changes of the spectra when exposed to white fluorescent lamp at different time are examined (Figure 1). The spectrum of each unencapsulated mango seed kernel extract and microcapsules were different. It is found that spectra of control (encapsulated without MSKE) did not show any shoulder or peak between 200 to 400 nm (Figure 2). While spectrum of encapsulated MSKE showed maxima peak at 276 nm and shoulder at 252 and 321 nm (Figure 1b). The exposure of samples to light brings about a significant increase of initially more intense absorption band of all samples in the region of 200 to 400 nm at a time. In MSKE, photoinduced changes are less effect to absorption tail (321 to 400 nm). The spectrum of MSKE increased in the spectral interval of 243 to 285 nm, especially at 276 nm (Figure 1a).

Figure 1 UV spectrum of treated (a) unencapsulation of mango seed kernel extract and (b) microcapsules.

Typically, different phenolic compound showed different UV spectral characteristics. It has been also seen in the present work that effect of light leads to results in terms of enhanced degradation, particularly at UV maxima wavelength. While at this stage, the observed limiting values cannot be convincingly explained, it may possibly be due to a degradation of many flavonoid molecules to be many molecules of smaller phenolics. Therefore, it is shown a higher value of absorbance when the samples were exposed to light. This is consistent to phenolic degradation data (Figure 3).
from the absorbance decreasing data/measurement time (h). Table 2 showed a comparison of phenolic degradation under varying conditions. The encapsulated mango seed kernel extract photolysis reveals a much slower reaction rate. The MSKE has a maximum UV absorbance at approximately 276 nm (Figure 1a), hence the comparable values were used absorbance value at 276 nm. Moreover, the absorbance at this wavelength showed the highest relationship between phenolic degradation and absorbance reading values (Figure 4).

Table 2 The absorbance decreasing values and degradation rate at different time exposure to light†

<table>
<thead>
<tr>
<th>Exposure time (h)</th>
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<th>6</th>
<th>24</th>
<th>48</th>
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<tbody>
<tr>
<td>Absorbance decreasing data</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unencapsulation</td>
<td>0.049&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.176&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.201&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.434&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>±0.006</td>
<td>±0.002</td>
<td>±0.001</td>
<td>±0.003</td>
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<tr>
<td>Encapsulation</td>
<td>0.024&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.079&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.084&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.084&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>±0.004</td>
<td>±0.005</td>
<td>±0.006</td>
<td>±0.001</td>
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<tr>
<td>Degradation rate (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unencapsulation</td>
<td>0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.029&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.008&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.009&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>±0.002</td>
<td>±0.004</td>
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<tr>
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<td>0.004&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.002&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>±0.002</td>
<td>±0.004</td>
<td>±0.005</td>
<td>±0.006</td>
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</table>

†Means of three replications ± SD (standard deviation); Different superscript letters mean significant differences (P<0.05) between conditions in each column.

Encapsulation resulted in enhanced extents of phenolic degradation as compared to unencapsulated one (Figure 3). The similar rates of phenol photodegradation after 6 hour exposure to light were obtained from encapsulated MSKE. The coating material can protect phenolic active ingredient from light surrounding. Typically, the higher of phenolic content in plant extract, the higher antioxidant capacity is obtained (Maisuthisakul et al., 2008). The result implied that antioxidant capacity from encapsulation of MSKE was stable more than the extract. Thus, encapsulation technique should be promising method for prolong shelf life of phenolic mango seed kernel extract.
Conclusions

By coupling the results of different assays for evaluating phenolic degradation of unencapsulated and encapsulated mango seed kernel extract exposure to light, we can conclude that absorbance value at 276 nm can be used to measure phenolic degradation during storage under illuminated condition. Overall outcome of the present investigation depicted that encapsulated method can stabilize phenolic extract from irradiation. Discussed study offer some information about antioxidants potential during storage in this source, moreover, more work is required to further establish particular phenolic composition of components involved during storage for application.

Acknowledgments

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References


Development of Artificial Neural Network on Transparent Soap Base Containing *Sonneratia caseolaris* Extract

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**Abstract**

The objective of this study was to use artificial neural network in development of transparent soap. The different eighteen transparent soap formulations were prepared and the physical properties of them such as clearness, hardness, foam ability and surface tension were investigated. Moreover, the correlation between each formulation and response parameters was examined using feed-forward back-propagation neural networks. The results showed that the amounts of SLES-N70, glycerine, sodium stearate and PVP-K30 were the important parameters on foam ability, clearness, hardness and surface tension, respectively. The proposed models were able to predict the properties of transparent soap with a reasonable degree of accuracy. The predictive ability of these models was validated by an external set of 6 formulations which were not included in the training set. The predictions were in good agreement with the observed and the predicted values. Moreover, the 5% of *Sonneratia caseolaris* extract was successfully incorporated into the soap. These results could be applicable for development of transparent soap containing *S. caseolaris* extract.

**Keywords**: glycerine, transparent soap, artificial neural network, *Sonneratia caseolaris*

**Introduction**

Soap is the result from saponification reaction between fat and caustic agent such as sodium hydroxide and potassium hydroxide (Umbach, 1991). Various kinds of fat could be used to produce soap: animal fat such as tallow, lard, chicken fat and vegetable oil. Soap can be classified into many categories. However, if the classification criterion is based on the clearness, soap will be divided into transparent soap and opaque soap. Presently, both transparent and opaque soap containing natural plant extracts or aromatic oil are very popular in the body shop and especially spa business.

*Sonneratia caseolaris* Linn. (family Sonneratiaceae; local name: cork tree), is found in along deep muddy river banks, mangroves forest and river mouths. It has cone-shaped pneumatophores. Its stem and branch are used for firewood, building boats, posts of bridges and houses. The half-ripe fruits are traditionally used as medicine for cough and the ripe fruits are used for expelling parasites in intestine. Fermented fruit juice is useful for arresting haemorrhage. *S. caseolaris* composed of polyphenols, flavonoids, triterpenoids, and sterols (Wu et al., 2009). The extract of this plant showed antioxidant, anti-inflammatory, and hepatoprotective activities (Sadhu et al., 2006).

Natural transparent soap is considerably milder than opaque soap since it rinses off the skin much more easily (Ahmad et al., 2008). Natural transparent soap can be easily prepared by melting the hard glycerin and mix with herbal extracts.
However, the cost of hard glycerin is quite expensive and imported from outside Thailand. To find the optimal soap formulation is cost and time consuming. Recent advances in the area of computer science, neuroscience, and applied mathematics have resulted in the development of artificial neural network (ANN). The ANN is computer program that is designed to simulate some functions of the human brain using different learning algorithms that can learn from experience (Bourquin et al., 1997; Sun et al., 2003). The ANN has the remarkable information processing features of the human brain, such as nonlinearity, high parallelism, robustness, fault and failure tolerance, learning, ability to handle imprecise and fuzzy information, and the capability to generalize (Basheer & Hajmeer, 2000]. Hence, ANN has been successfully applied to various pharmaceutical fields such as preformulation studies (Murtoniemi et al., 1994) pharmaceutical process development (Wu et al., 2000), formulation optimization (Ebube et al., 2000; Vaithiyalingam & Khan , 2002; Piriyaprasarth et al., 2009), in-vitro-invivo correlation (Dowell et al., 1999), and pharmacokinetic parameters prediction (Opara et al., 1999).

Therefore, the purposes of this research were to use ANN in development of transparent glycerin soap base. The effect of formulation parameters on physical properties of transparent glycerin soap was investigated and the transparent glycerin soap containing *S. caseolaris* extracts was developed.

### Materials and Methods

#### Materials

The leaves of Cork tree, (*Sonneratia caseolaris* Linn.), Sonneratiaaceae were collected from Tachin river, Sampran district, Nakhon-Pathom province by researcher of Faculty of Pharmacy, Silpakorn University in September-October, 2009. The dry leaves were deposited in the Department of Pharmacognosy, Silpakorn University in Nakhon-Pathom, Thailand.

#### Preparation of Soap Bar

The eighteen soap formulation were prepared by varying the concentration of sodium stearate, sodium lauryl ether sulfate (SLES)-N70, glycerin and polyvinyl-pyrrolidone (PVP)-K30 as shown in Table 1. Briefly, sodium stearate was dissolved in the mixture of water, glycerin, SLES-N 70 and PVP-K30 using the heat from water bath. The resulted soap solution was stirred and poured into molds.

#### Evaluation of Physical Properties of Soap Bar

The clearness of soap bar was visually observed (n=3) and grading according to the following criteria: 1 means the soap bar is clear and 0 means the soap bar is turbidity. A 10%w/w of soap in distilled water was prepared and measured for pH using pH meter (Denver UltraBasic Benchtop Meters, New York, USA) at room temperature (n=3). The penetrometer with a cone type of 102.745 gram (Precision. Scientific Co., Chicago,Illinois) was used to evaluate hardness of soap bar. The distance (mm) the cone penetrated through soap bar in 10 second was measured (n=3) to represent hardness of soap. In addition, the surface tension of the soap was measured using tensiometer (FTA 1000, Portsmouth, VA). The measurement was repeated three times.

#### Measurement of Foaming Ability of Soap Bar

A 1%w/w solution of soap in distilled water was prepared. Forty ml of soap solution was filled in burette and another 10 ml in cylinder. The soap solution in the burette was arranged to flow into the cylinder. The distance between the tip of burette and the surface of soap solution in cylinder was 25 cm. The volume of foam was measured at 0, 10, 20 and 30 minutes (n=3).
Table 1 Compositions of eighteen soap formulations.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sodium stearate (%)</th>
<th>SLES N 70 (%)</th>
<th>glycerine (%)</th>
<th>PVP K30 (%)</th>
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</table>

Development of Artificial Neural Network Model

The concentrations of formulation variables in this study were 5-25% for SLES-N70, 5-60% for glycerine, 5-30% for sodium stearate and 0-30% for PVP-K30. The physical properties of transparent soaps such as hardness, surface tension, foam ability and clearness were used as response variables. The correlation between the formulation composition variables and physical properties of transparent soap was investigated using feed-forward back-propagation neural networks. Trajan Software, (The Old Rectory, Low Toytont, Horncastle, Lincs., LN9 6JU, UK) was used throughout the study. The Input layer consisted of four formulation variables whereas the output layer contained the response variable. The error between the output of the network and the desired output was calculated. The changes for the connection weights was computed by feeding the summed squared errors from the output layer back through the hidden layers to the input layer. In order to validate the ANN model, the model was trained again using 18 trial formulations and withholding one formulation. Once the ANN model was trained, the model predicted the output parameters for the withhold formulation. This process was repeated 18 times, each time withholding a different trial formulation from the training set. This is called the “leave-one-out method.” Then, a regression plot was constructed for the observed output parameters and predicted output parameters. The goodness-of-fit was evaluated by the predictive root mean square error (predictive RMSE) and the predictive $r^2$, defined as follows:

$$\text{Predictive RMSE} = \left( \frac{\sum (y_{obs} - y_{pred})^2}{N} \right)^{1/2} \quad (1)$$

$$\text{Predictive } r^2 = 1 - \frac{\sum (y_{obs} - y_{mean})^2}{\sum (y_{obs} - y_{pred})^2} \quad (2)$$

The determination of the final optimized model was based on the predictive RMSE and predictive $r^2$ values for all 18 formulations. The predictive ability of these models was validated by an external set of 6 formulations that were not included in the training set.

Preparation of Transparent Soap bar Containing Cork Tree Extract

The 100 g of leaves of Cork tree was macerated in 400 mL methanol. The maceration was shaked at room temperature for 24 hrs, filtered and evaporated until dry under vacuum. The crude extract was kept in temperature controlled chamber at 4°C. The transparent soap bar containing S. caseolaris extracts was prepared by melting the transparent soap bar and incorporated with S. caseolaris extracts.
Results and Discussion

Physical Properties and Foam Ability of Soap Bar

The eighteen transparent soaps were prepared and characterized for the physical properties. The hardness of the prepared soap bars was represented by the penetration distance in the unit of mm. It was in the range of 2.37-18.13 mm. The pH values of the prepared soaps were 10.26-11.17. The surface tension of the prepared soaps was in the range of 25.54-38.20 mN/m and their foam volumes were in the range of 4.67-25.67 mL (Table 2).

<table>
<thead>
<tr>
<th>No.</th>
<th>penetration distance (mm)</th>
<th>Surface tension (mN/m)</th>
<th>Foam volume (ml)</th>
<th>Clearness</th>
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<td>1</td>
<td>14.85</td>
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</table>

Development of Artificial Neural Network Model

The concentration of SLES-N 70, glycerine, sodium stearate and PVP K30 was used as formulation parameters (Table 1). The physical properties of transparent soaps such as clearness, hardness, foam ability and surface tension were used as response parameters (Table 2). The correlation between the formulation parameters and response parameters of the eighteen prepared soaps was examined using feed-forward back-propagation neural networks. The best ANN model of hardness with a configuration of 4–3–1 for input, hidden, and output layers for hardness gave the $r^2$ of 0.966 and the root mean square (RMS) errors of 0.88 as shown in Figure 1.1A. For surface tension and foam ability, the best ANN model with a configuration of 4–4–1 gave $r^2$ of 0.9699 and 0.996 and RMS errors of 0.05 and 0.36, respectively (Figure 1.2A and 1.3A).
The “leave-one-out” cross-validation revealed that the neural network models could predict the physical properties of the transparent soap with a reasonable accuracy. The predictive $r^2$ were 0.891, 0.973 and 0.758 and the predictive RMSE were 1.57, 0.80 and 0.91 for hardness, surface tension and foam ability, respectively (Figure 1B). Table 3 shows the leave-one-out predictability of the ANN model for clearness of the soap with a configuration of 4–3–1 which provided the predictive error of less than 0.0090. This indicated that the ANN model could predict the clearness of the transparent soap with a reasonable degree of accuracy. The results indicated that the concentration of SLES-N70, glycerine, sodium stearate and PVP K30 were the important parameter for foam ability, clearness, hardness and surface tension, respectively.

**Table 3** The Leave one out predictability of the ANN model for clearness of the transparent soap with a configuration of 4–3–1.

<table>
<thead>
<tr>
<th>No.</th>
<th>Experimental property</th>
<th>Predicted property</th>
<th>Error</th>
<th>Error cleanness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Clear</td>
<td>Clear</td>
<td>Right</td>
<td>0.0090</td>
</tr>
<tr>
<td>2</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>3</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>4</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>5</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>6</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>7</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>0.0003</td>
</tr>
<tr>
<td>8</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>9</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>10</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>11</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>12</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>0.0096</td>
</tr>
<tr>
<td>13</td>
<td>Clear</td>
<td>Clear</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>14</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>15</td>
<td>Clear</td>
<td>Clear</td>
<td>Right</td>
<td>0.0002</td>
</tr>
<tr>
<td>16</td>
<td>Clear</td>
<td>Clear</td>
<td>Right</td>
<td>0.0002</td>
</tr>
<tr>
<td>17</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>0.0024</td>
</tr>
<tr>
<td>18</td>
<td>Turbidity</td>
<td>Turbidity</td>
<td>Right</td>
<td>0.0024</td>
</tr>
</tbody>
</table>

In addition, the predictive ability of the models was examined by a set of six formulations that were not included in the training set (Table 4). Table 5 shows the values of response parameters such as hardness, surface tension foam and clearness of the six soap formulations which were used as the external data for testing the ANN model. The predictions were in good agreement with the observed values for hardness, surface tension and foam ability (Figure 2). The predictive RMSE were 1.92 for hardness and 3.05 for surface tension and foam volume.

**Table 4** The physical property parameters of the six prepared soap bars for external testing of the ANN model.

<table>
<thead>
<tr>
<th>No.</th>
<th>Sodium stearate (%)</th>
<th>SLES N 70 (%)</th>
<th>glycerine (%)</th>
<th>PVP K30 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>10</td>
<td>20</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>25</td>
<td>35</td>
<td>0</td>
</tr>
<tr>
<td>21</td>
<td>25</td>
<td>15</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>22</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>23</td>
<td>5</td>
<td>20</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>20</td>
<td>30</td>
<td>20</td>
</tr>
</tbody>
</table>

**Table 5** The response parameters of the external data for testing the ANN model.

<table>
<thead>
<tr>
<th>No.</th>
<th>penetration distance (mm)</th>
<th>Surface tension (mN/m)</th>
<th>Foam volume (ml)</th>
<th>Clearness</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>10.97</td>
<td>26.19</td>
<td>23.33</td>
<td>clear</td>
</tr>
<tr>
<td>20</td>
<td>2.62</td>
<td>25.74</td>
<td>19.67</td>
<td>turbidity</td>
</tr>
<tr>
<td>21</td>
<td>5.2</td>
<td>25.76</td>
<td>19.00</td>
<td>turbidity</td>
</tr>
<tr>
<td>22</td>
<td>4.97</td>
<td>35.82</td>
<td>23.67</td>
<td>clear</td>
</tr>
<tr>
<td>23</td>
<td>15.00</td>
<td>38.48</td>
<td>27.00</td>
<td>turbidity</td>
</tr>
<tr>
<td>24</td>
<td>18.00</td>
<td>38.70</td>
<td>24.50</td>
<td>turbidity</td>
</tr>
</tbody>
</table>

Table 6 shows the predictability of the ANN model for clearness of the transparent soap using external validation. Thus, the results demonstrated that the ANN models of physical properties of transparent soap base in terms of type and concentration of formulation factors was successfully developed with a reasonable accuracy of prediction.
When *S. caseolaris* extracts were incorporated into the transparent soap in the concentration of 1-10% and investigated for their physical properties, it was found that the soap containing up to 5% of *S. caseolaris* extracts exhibited good performance. The foam ability was in the range of 24.5-28.8 mL, the surface tension was in the range of 36.28-38.83 mN/m and the penetration distance was in the range of 0.314-0.591 mm. However, the clearness of the soap decreased with increase in the extract concentration. This might be due to the interaction between methanol in the extract and the soap formulation that could be solved by further varying the formulation parameters. These results could be useful and applicable for development of transparent soap containing *S. caseolaris* extracts.

**Conclusions**

The proposed ANN model, where the formulation parameters were used as the independent parameters, was able to predict the physical properties such as hardness, surface tension foam ability and clearness of transparent soap with a reasonable degree of accuracy. The concentration of SLES-N70, glycerine, sodium stearate and PVP-K30 were the important parameters on foam ability, clearness, hardness and surface tension, respectively. The incorporation of *S. caseolaris* extract up to 5% into the soap was successfully developed.

**Acknowledgments**

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Indented Longan Detection with Computer Vision-based Software in Consideration of Roundness Value

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Abstract

Sorting indented-longan is time-consuming when done by humans. The performance of the process depends upon individual experience and reduces due to fatigue after a long working period. Image analysis approach has been applied for indented-longan detection. A digital image representing a shape of a longan was analyzed by a computer vision-based software developed with MATLAB. The software determined the roundness of the longan by dividing the area of a longan fruit with the area of the minimum circumscribed circle. A sensitivity analysis was carried out to find the optimum threshold roundness for normal and indented longan detection. The optimum threshold was 0.9052. The software identified any longan of which the roundness was lower than the threshold as “indented”, otherwise “normal”. The detection accuracy of the software for normal and indented longans was evaluated and found to be 68% and 62% respectively. The computational time was 0.33 ± 0.09 second. Fault detection for normal longan occurred with oblate and prolate spheroid longans due to large deviation between the fruit boundary and perimeter of the minimum circumscribed circle. The deviation was comparable to that created by indents. For longans with small indents, the deviation is relatively small and comparable to the deviation for normal longans leading to misclassification. Application of this software is, therefore, limited to round shape longan with large indents. The computer vision-based software should take into account the information on curvature of the fruit boundary to overcome the limitation.

Keywords: computer vision, image analysis, indented longan, roundness, sorting

Introduction

Longan (Dimocarpus longan Lour) is one of the most important fruit products of Thailand. It is mainly planted in the northern part of the county especially in Chiangmai, Lamphun and Chiangrai provinces. The total production of fresh longan in the year 2009 was 623,027 tons which is 30% higher than the total production in the year 2008 of 476,930 tons (Anonymous, 2010b). The major importer of Thai longan is China, where the whole-dried longan is the most preferable. This is because the whole-dried longan has a round black and shiny seed with a circular white spot at the base, giving the aspect of a dragon eyes (Rangkadilok et al., 2005). Chinese people believe that eating longan or ‘dragon eye’ is good for health (Lapsongphol et al., 2007). The exported values for the whole-dried longan were 1,832.6 and 2,589.6 million Baht in 2008 and 2009, respectively (Anonymous, 2010a). Therefore Thailand has been considered as the biggest longan exporter in the world (Jiang et al., 2002).

A commercial fixed-bed hot-air dryer has been widely used to dry whole-fresh longans. This equipment is capable of drying fresh longans of 800-2,000 kg per batch. The drying time of 60-72 hours at hot-air temperature of 60-75 °C is required to reduce the moisture from 80% (w.b.) to 14% (w.b.) (Varith et al., 2008). During the drying process, moisture inside the cells migrates out of the cellular structure, which
causes shrinkage. Consequently, space underneath the brittle and thin brownish skin shrinkage is created. Finally, this area becomes less resistant to the external forces causing indentation (Varith et al., 2008).

According to the Thai Agricultural Commodity and Food Standard for Whole Dried-longan, the longan with indented surface should not be included in the package (Attapanyo et al., 2006). In order to separate the indented longan from others, whole dried-longan fruits are put on a sorting table and inspected by humans standing around the table. This sorting process is time-consuming and it requires intensive human labors whose sorting performance decreases with time. This can result in inconsistent product quality.

Computer vision system is an advanced technology which is able to describe characteristics of products with consistency, accuracy, and high repeatability (Chen, 2002). It is also capable of performing continuous inspection within a reasonably short time. Computer vision system has been widely applied for industrial products inspection including agricultural and food products (Brosnan and Sun, 2002) such as quality assessment of citrus fruits (Blasco et al., 2007), determination of pizza topping distribution (Sun and Brosnan, 2003), recognition of fish species (Strachan, 1993), estimation of mass and volume of citrus fruits (Omid et al., 2010), classification of mangoes (Poonnoy, 2003), and determination of longan size (Poonnoy et al., 2008).

An achievement of the computer vision system in classification of food products is critically dependent upon an algorithm used to interpret the data embedded in a digital image. Classification of different food product requires a unique algorithm for data interpretation. For example, the computer vision system identifies straight and curved tamarind pods using the shape index derived from the coordinates of stem and tail of the tamarind pod searched by analyzing the curvature of the pod (Jarimopas and Jaisin, 2008). For mangoes, on the other hand, the computer vision system detected abnormality of shape by considering the ratio of the major and minor axis lengths of a mango with the index value generated from normal shape mango (Poonnoy, 2003). In a different way, the sharing line method was adopted for strawberry gradation (Liming and Yanchao, 2010). However, the morphology of longan fruit is different from those products; the algorithm for detection of indented longan using computer vision has not been reported and it is needed to be developed. The objective of this research is, therefore, to develop the computer vision-based software and evaluate the software efficiency of indented longan detection in consideration of roundness value.

Materials and Methods

Image Acquisition

A digital camera (FujiPix: S9, Japan) was used to capture the longan images with a resolution of 640×480 pixels. A longan sample was placed on a turntable under the light from the high frequency fluorescent lamps. The digital camera took an image of a normal longan sample in a random manner at every 45 degree of rotation. Figure 1(a) illustrates a typical shape of a normal longan. For an indented longan sample, on the other hand, each sample was manually rotated until the borders of the indented area were parallel to the line of sight. At this point, the flat surface caused by the indent definitely revealed as indicated in Figure 1(b). The camera took an image of the indented sample. For some indented longans with multiple indents on the surface, an image was taken by focusing on one indent at a time. Two-hundred images of normal longan samples and fifty images representing indented longan samples were acquired from this step. The images obtained were stored in a memory card of the camera and then transferred to the computer for analysis.

Image Processing

The computer vision-based software for indented longan detection was developed on a personal computer (CPU: Core2Duo
2.80GHz, RAM: 4.00 GB) with MATLAB® version 2008b (Mathworks: Natick, USA). The image processing functions of Image Processing Toolbox of MATLAB® (Mathworks: Natick, USA) were utilized for features extraction.

At first, the RGB-image of longan (Figure 2 a) was converted to a gray-scaled image (Figure 2 b). The ‘graythresh’ command was applied to obtain the optimum threshold value required for image binalization. Using ‘im2bw’ function together with the determined threshold from the previous step yielded binary image of the longan (Figure 2 c). The longan’s profile was displayed in white against a black background. These steps allowed the computer to determine the projected area and the maximum radius of the longan which were necessary for roundness determination.

**Estimation of Roundness Value Using Image Analysis Approach**

In order to obtain the projected area of the longan ($A_p$), the computer counted the number of white pixels in the image and reported the obtained projected area in terms of number of pixels.

The boundary of the sample was searched by comparing the contrast among pixels in the image with ‘bwtraceboundary’ function. The best-fitted circle along with the boundary was found using least square method and its center was then located. The distances between the center and the coordinates on the searched boundary were calculated and compared to obtain the maximum radius. The maximum radius was used to calculate the area of the minimum circumscribed circle as follow:

$$A_c = \pi d^2$$

where:
- $A_c$ = Area of the minimum circumscribed circle (pixels)
- $\pi$ = Constant equals to 3.14
- $d$ = Maximum radius (pixels)

As the $A_p$ and $A_c$ were obtained, the roundness ($R$) of the longan was then determined by the following equation (Mohsenin, 1970):

$$R = \frac{A_p}{A_c}$$

where:
- $R$ = Roundness (Dimensionless)
- $A_p$ = Projected area of a longan (pixels)

The computer software stored the estimated roundness value in the memory and displayed it on the screen.

**Determination of Threshold Roundness for Indented Longan Detection**

Based on the definition of roundness which refers to the ratio of the projected area of the object and the area of the minimum circumscribed circle, threshold roundness values were set for the longan detection. The estimated roundness value of each longan was then compared with these threshold values for classification.
circumscribed circle, the indented longan tends to have lower roundness value comparing to the normal longan.

A sensitivity analysis was carried out to find the optimum value for indented longan detection by assigning various threshold values. The computer software analyzed 50 images of normal longan and 50 images of indented longan in accordance with the assigned value. The initial threshold value was determined by calculating the average roundness value of 100 images of normal longan.

Once the threshold was given, the software identified any longans as ‘indented’ if their roundness values were found to be lower than the threshold. Otherwise, the software identified a longan as ‘normal’.

The detection accuracy for normal and indented longan was evaluated by comparing the detection results from the computer software with human. It was evaluated by dividing a number of longans identified by the computer software with a number of longans identified by human. Assuming human identification is 100% correct; therefore, the accuracy of the computer software was calculated by the following equation:

\[ A = \frac{N_{\text{computer}}}{N_{\text{human}}} \times 100 \]

where:  
A = Accuracy of the computer vision-based software (%)  
\( N_{\text{computer}} \) = Number of longan image correctly classified by computer  
\( N_{\text{human}} \) = Number of longan image classified by human

The detection accuracy was plotted against the threshold roundness value. The threshold value that yielded the maximum accuracy in both normal and indented longan was considered as the optimum roundness value for indented longan detection. Two linear regression models were developed to express the relationship between the threshold values and the detection accuracy for normal and indented longans. The intercept between those equations represented the optimum threshold value. It is noted that the normal longan images used in this step were not used for determination of the initial threshold value.

Computational Time

The computational time for each image was programatically determined with a stop watch function of MATLAB. Average processing time and standard deviation were calculated and reported.

Results and Discussion

Characteristics and Interpretation of Analyzed Images

The typical characteristics of normal and indented longan images are different. As seen in figure 3a, the boundary for the normal-shape longan is adjacent to the perimeter of the circumscribed circle. Small spaces between the longan fruit boundary and the perimeter were observed as expected because the normal longan fruit has no perfect round shape. For the indented longan (figure 3b), the indent on the longan surface created a larger space between the longan boundary and the perimeter compared to the normal longan. Hence, a normal longan tends to have roundness converging to one as its appearance is similar to a circle. On the other hand, the roundness for the indented longan approaches zero because the indent on an indented longan created a large space between the searched boundary and the perimeter of the circumscribed circle. The decrease in roundness value correlates with the degree of indentation: the higher degree of indentation, the lower roundness value.

Determination of The Optimum Threshold Roundness Value for Indented Longan Detection

The developed computer software processed and analyzed numerous sample images of normal and indented longan
samples. A roundness value for normal longans ranged from 0.8653 to 0.9615 with an average of 0.9146 ± 0.0233. On the other hand, the roundness value for indented longan was in a range of 0.8921 to 0.9258 with an average of 0.921±0.0198. The overlap of the roundness values for normal and indented longan was observed. The optimum threshold value for roundness value was, therefore, searched by a sensitivity analysis.

The typical roundness value obtained from 100 images of normal longan was 0.9229 ± 0.0203. The initial threshold for roundness used in the sensitivity analysis was then set to 0.9200 with an increment and decrement of 0.0100. The maximum and minimum of the threshold values were correspondent to the maximum and maximum accuracy of normal and indented longans detection.

In Figure 3, the detection accuracy was plotted against the various threshold roundness values. It can be seen that the accuracy for normal and indented longan varies with the given threshold values. The increase in the threshold value improved the accuracy of the software for indented longan detection since the software identified any longan as “indented” if the determined roundness value was lower than a specified threshold value. The highest accuracy of indented longan detection reached 100% when the threshold was set to 0.9700. However the accuracy of the normal longan detection was at 0% because all normal longans having roundness value lower than 0.9700 were misclassified as indented longans. 0.9700 were misclassified as indented longans.

By contrast, a reduction in the threshold value increased the software accuracy in normal longan detection because the software identified any longan as “normal” if the roundness value was higher than a given threshold value. The maximum performance of normal shape longan identification was 100% when the threshold was set to 0.8500. On this condition, the lowest accuracy of 0% for indented longan was reached.

The maximum accuracy for both normal and indented longans detection is at the intercept lying between the threshold values of 0.900 and 0.9100. In order to find the optimum threshold value yielding maximum accuracy, two linear regression models representing the relationship between the threshold values and the detection accuracy for normal and indented longan detection were developed. The linear relationships between threshold values and the detection accuracy for normal and indented longan were:

\[ A_n = -2000TR + 1880 \]  
\[ A_i = 3000TR - 2646 \]

where:  
\[ A_n \] = Accuracy for normal longans detection  
\[ A_i \] = Accuracy for indented longans detection  
\[ TR \] = Threshold for roundness

Figure 3 Typical characteristics of analyzed images: (a) normal longan and (b) indented longan
Equation (4) represents the linear relationship between the threshold values and the detection accuracy for normal longan detection. On the other hand, equation (5) represents the linear relationship between the threshold values and the detection accuracy for indented longan detection. The developed equations were equilibrated and solved for the optimum threshold value. The result of TR was 0.9052. This value was assigned to the software.

The images of normal and indented longans were presented to the software again. The accuracy and computational time of the software were evaluated.

**Evaluation of Software Efficiency**

The developed computer vision-based software determined the roundness values of the longan images and identified the longan types with accuracy of 68% and 62% for normal and indented longan, respectively. The computational time was 0.32±0.03 second.

The fault detection for normal longans was, however, found in some oblate and prolate spheroid shape longans. For the oblate spheroid shape longan, the flats at stem and apex of a longan create large spaces between the fruit boundary and the circumscribed circle perimeter around the stem and the apex areas (Figure a). Similar to the prolate longan, the large spaces are created beside the fruit (Figure b). These spaces increase the difference between the projected area and the area of the circle; as a result, a roundness value decreases below the threshold and leads to misclassification.

For indented longan detection, the error was observed for longans with small dents (Figure ). In this experiment, the threshold value for the roundness was specified at 0.9052. The software identified any longan image as “indented” if the space between the longan boundary and the perimeter of the minimum circumscribed circle was higher than 9.48% of the circle area. In Figure 8a, the longan has a flattened stem and a small...
indent around the apex area. The area of the spaces was 9.27% of the circle area. Such a small space was comparable to the existing space in normal longan. Although the longan had dent on a surface, it was identified as normal. Similar result was found in a round shape longan with a small indent as shown in Figure b.

From the results, it was implied that the computer vision-based software was able to distinguish normal-shape longans from indented longans as long as the indent created sufficient large space between the fruit boundary and the circumscribed circle perimeter. The use of this method for indented longan detection is limited to some oblate and prolate spheroid shape longans. This is because the boundary of such longans deviates from the perimeter of the minimum circumscribed circle. The increase in the degree of deviation lowers the roundness value, even though those longans have no indent. Fault detection for oblate and prolate longans is unavoidable.

In order to improve the detection efficiency, further study on algorithms for the detection of the indent on longan surface should be developed based on the curvature of the boundary. This is because the boundary of the longan at the indented surface appears to be a straight line whereas the boundary at normal surface is curved. Analysis on the curvature by modifying the algorithm for locating stem and tail of tamarind (Jarimopas and Jaisin, 2008) may overcome the limitations of indented longan detection by using only the roundness value.

In reality, since the computer vision-based software needs to process and analyze numerous images for each longan, the computational time increase proportionally to the amount of images. Consequently, the

Figure 5 Characteristics of normal longans with fault detection: (a) Oblate spheroid and (b) Prolate spheroid

Figure 6 Characteristics of indented longans with fault detection: (a) Oblate spheroid with dent and (b) Round longan with dent
sorting speed is reduced. The computational time for image analysis may be reduced by modifying the simple algorithm for roundness evaluation (Gadelmawla, 2010).

It is noted that the detection accuracy significantly correlates with the setting of the threshold value that may vary according to varieties of longans. The threshold used in this paper was specific to the sample groups. Using this method for identifying other longans may require different optimum threshold value.

Conclusions

The computer vision-based software was developed under MATLAB® environment to analyze longan images. Analyzing longan image yielded roundness value of longan which was determined by dividing the projected area with the minimum circumscribed circle area. The software compared such a roundness value with the specified threshold. It identified longans as “indented” if the determined roundness value was below the specified threshold, otherwise, “normal”. Fault detection was observed for some oblate and prolate spheroid shape normal longans because of the relatively large spaces existing beside the stem and the apex for oblate spheroid longan and on both sides of longan for prolate spheroid longan. Further research on detection of indent based on the curvature of longan fruit boundary should be conducted to improve the efficiency of the computer vision-based software.

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Antimicrobial Resistance Profile of *Escherichia coli* Isolates From Fattening Pigs in Khon Kaen Province, Thailand

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**Abstract**

*Escherichia coli* is a bacteria with many strains. It can be found in the gastrointestinal tract in humans and animals. Some strains do not cause disease, but some strains of bacteria cause disease in humans and animals. The pathogenic strains of *E. coli* are a high risk of transfer of an antibiotic resistance gene from animals to humans via contaminated food, due to an overuse of antibiotic therapy in humans and animals. The aim of this study was to evaluate antimicrobial resistance in porcine *E. coli*, which was isolated from 50 fattening pigs. Sixteen *E. coli* strains were screened for antimicrobial resistance profile against 8 antibiotics using the disk diffusion method. All of the *E. coli* strains resisted to penicillin, vancomycin and metronidazole. Resistance rates against ampicillin, tetracycline, gentamicin and sulfamethoxazole-trimethoprim of 16 *E. coli* strains were 56.25, 81.25, 12.5 and 43.75 %, respectively. These *E. coli* strains showed 8 multi-resistance patterns, which were resistance to 3 or more antibiotics. These results indicated that multi-resistance of *E. coli* from fattening pigs was high and antimicrobial resistance surveillance program is important to detect bacterial resistance.

**Keywords**: antimicrobial resistance, *Escherichia coli*, fattening pigs

**Introduction**

An overuse of antibiotics for bacterial infections in livestock increases a resistance in pathogenic bacteria and in microflora, which some strains of the microflora can be acted as pathogens such as *Escherichia coli* in gastrointestinal tract in animals. This situation presents a high risk of transfer of this antibiotic resistance from animals to humans via contaminated food. *E. coli* has been described to be indicator to evaluate the development of antimicrobial resistance (Von Baum and Marre, 2005). This is due to the high frequency of mutation exhibited by *E. coli* leading to the development of antimicrobial resistance, as compared to other microorganisms frequently found in animals and food (Kijima-Tanaka et al., 2003). In different parts of the world, multi-drug resistant strains of *E. coli* are ubiquitous in both human and animal isolates (Amara et al., 1995) and multiple drug resistant, nonpathogenic or pathogenic *E. coli* found in the intestine of animal origin is probably an important reservoir of resistance genes transferring to other bacteria and to humans via contaminated food. In Thailand, researcher has studied antibiotic resistance of pathogenic bacteria such as *Salmonella* in native chickens and broilers (Chalermchaikit et al., 2005) that failed to treat in animals. Conversely, there is little information about the antibiotic susceptibility in commensal or pathogenic bacteria such as *E. coli* isolated from gastrointestinal tract in pigs’ origin. Therefore, the present study was to isolate and screen antimicrobial resistance profile of *E. coli* strains isolated from fattening pigs in Khon Kaen province, Thailand.
Materials and Methods

Sample Collection and Isolation of Escherichia coli

A total of 50 fecal samples were taken from each fattening pig at slaughterhouses in Amphur Muang, Khon Kaen province during July to August 2010. The samples were collected using rectal swab, which transported at 5°C in transport media (Cary Blair transport medium, Oxoid, England) and tested for laboratory microbiology within 24 hours.

Each sample was cultured in EC broth with Durham tube added and incubated at 44°C for 24 hours. Gas positive samples were further streaked onto Eosin Methylene Blue (EMB, Oxoid, England) agar and incubated at 37°C for 24 hours. One to three typical E. coli colonies that showed metallic green sheen appearance to dark colonies were picked, transferred onto nutrient agar slant, incubated at 37°C for 24 hours and kept at 4°C for further tested. E. coli pure cultures were confirmed by biochemical tests, which included indole, methyl red and Simmons citrate tests according to guidelines of the Manual of Systematic Bacteriology (Krieg, 1984).

Antimicrobial Susceptibility Test

A total of 16 representative E. coli isolates were further tested for antimicrobial susceptibility by agar disk diffusion on Mueller-Hinton agar plates (Oxoid) according to Clinical and Laboratory Standards Institute (CLSI formerly NCCLS) guideline (NCCLS, 2002). Eight antibiotics commonly used in treating human or animal infections (European Food Safety Authority, 2008) and provided different antibiotics classes were chosen for the test. They were penicillin G (10µg), ampicillin (10µg), erythromycin (15µg), tetracycline (30µg), vancomycin (30µg), gentamicin (10µg), sulfamethoxazole-trimethoprim (25µg) and metronidazole (50µg). All antibiotic disks (diameter = 6 mm) were obtained from Oxoid (Oxoid, England).

Each E. coli isolate was inoculated with $10^8$ CFU (turbidity of 0.5 Mac Farland standard) at 44°C in EC broth and incubated for 18 hours. Culture solution was dipped using sterile cotton swap and swabbed in three directions on Mueller-Hinton agar plates. All antibiotic disks were seeded in the plates and incubated at 37°C for 48 hours. The diameters of antibiotic inhibition zones were measured using a ruler under a colony counter apparatus (Gallenkamp, England) and expressed in millimeters which included diameter of antibiotic disk. Antimicrobial susceptibility interpreted according to the cut-off levels proposed by CLSI for veterinary pathogens (NCCLS, 2002). E. coli ATCC 25922 was used as a reference strain for this study. Isolates exhibiting resistance to at least two of the antimicrobial agents tested were considered to be multi-resistant strains. All antibiotics were tested in duplicate.

Results and Discussion

Bacterial isolation and further biochemical tested from the 50 fecal samples were identified as 16 strains of E. coli. Antibiotic susceptibility pattern of these E. coli isolates from fecal samples of fattening pigs are shown in Table 1. Among these E. coli strains, penicillin, vancomycin and metronidazole were resisted at 100% while ampicillin, tetracycline, gentamicin and sulfamethoxazole-trimethoprim showed 56.25, 81.25, 12.5 and 43.75 % resistance, respectively. All of E. coli strains were sensitive to erythromycin. A high percentage resistance to penicillin in this study showed similar results of Akond et al. (2009). Vancomycin resistance in E. coli in this study showed similar result of Arthi et al. (2003) who reported that the most of gram negative bacteria were resistant to vancomycin. A large percentage resistance to metronidazole in this study may due to the increasing of antibiotic inactivation gene of these E. coli strains (Land and Johnson, 1999). The strains of E. coli in present study showed resistance to antibiotics commonly used in treating of bacterial infection in pig farms,
Table 1 Interpretative zone diameter (mm) with eight antibiotics of 16 representatives *E. coli* strains isolated from fattening pigs

<table>
<thead>
<tr>
<th>Bacteria strains</th>
<th>Antibiotics&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P (10µg)</td>
</tr>
<tr>
<td>G5</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N1</td>
<td>8 (R)</td>
</tr>
<tr>
<td>N2</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N3</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N4</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N5</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N6</td>
<td>9 (R)</td>
</tr>
<tr>
<td>N7</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N8</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N9</td>
<td>0 (R)</td>
</tr>
<tr>
<td>N10</td>
<td>0 (R)</td>
</tr>
<tr>
<td>T1</td>
<td>0 (R)</td>
</tr>
<tr>
<td>T2</td>
<td>0 (R)</td>
</tr>
<tr>
<td>T3</td>
<td>0 (R)</td>
</tr>
<tr>
<td>T8</td>
<td>0 (R)</td>
</tr>
<tr>
<td>T11</td>
<td>9 (R)</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0 (R)</td>
</tr>
</tbody>
</table>

<sup>1</sup>P=penicillin; AMP= ampicillin; E=erythromycin; TE=tetracycline; VA=vancomycin; CN= gentamicin; SXT= sulfamethoxazole-trimethoprim; MTZ= metronidazole

<sup>2</sup>Susceptibility expressed as (R), resistant; (MS), moderately susceptible; (S), sensitive; susceptible

<sup>3</sup>ATCC= American Type Culture Collection

<sup>4</sup>showed pinpoint colonies within the inhibition zone

the levels of antibiotic resistance descending order included tetracycline, ampicillin, sulfamethoxazole-trimethoprim and gentamicin. In this study we demonstrated the widespread occurrence of antimicrobial resistance to tetracycline. Similar information has been reported by several researchers such as Akond et al. (2009) and Minas et al. (2008). Ampicillin and sulfamethoxazole-trimethoprim were increased resistance of strains of *E. coli*. These results indicated that a long-time use of these antibiotics for veterinary therapy. In previous studies reported only 2% resistance of *E. coli* to gentamicin (Minas et al., 2008) but in our study gentamicin resistance was 12.5 %. The previous study of *E. coli* strains isolated from chickens was found 100% resistance to erythromycin (Akond et al., 2009) but in this study, no isolates of *E. coli* was found resistant to erythromycin. However, there were 2 out of 16 *E. coli* isolates (N6 and T2) showed pinpoint colonies within the inhibition zone with erythromycin. This observation for these bacterial strains indicated the mutation that leads to antimicrobial resistance (Danielsen and Wind 2003). The most of *E. coli* strains were susceptible to erythromycin may be due to a low use of this antibiotic in this area.

*E. coli* strains isolated from fattening pigs, demonstrated 8 antibiotic resistance patterns as shown in Table 2. The strains of *E. coli* were resistant at least to three antibiotics used in the study at 12.5%. According to our results, all strains of *E. coli* (100%) were resistant to more than one antimicrobial agent. The highest pattern of multi-drug resistance was penicillin/ampicillin/tetracycline/vanco mycin/metronidazole at 31.25%. This result suggests that an antibiotic used in pig farms in this area is the critical multi-resistance problem.
Table 2 Patterns of multi-resistance in 16 *Escherichia coli* strains isolated from fattening pigs in Khon Kaen province.

<table>
<thead>
<tr>
<th>Multi-resistance patterns in various antimicrobial agents</th>
<th>Percentage of resistant strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AMP-VA-MTZ</td>
<td>6.25</td>
</tr>
<tr>
<td>P-AMP-TE-VA-MTZ</td>
<td>31.25</td>
</tr>
<tr>
<td>P-AMP-TE-VA-SXT-MTZ</td>
<td>12.5</td>
</tr>
<tr>
<td>P-AMP-TE-VA-CN-SXT-MTZ</td>
<td>6.25</td>
</tr>
<tr>
<td>P-TE-VA-MTZ</td>
<td>12.5</td>
</tr>
<tr>
<td>P-TE-VA-SXT-MTZ</td>
<td>12.5</td>
</tr>
<tr>
<td>P-TE-VA-CN-SXT-MTZ</td>
<td>6.25</td>
</tr>
<tr>
<td>P-VA-MTZ</td>
<td>12.5</td>
</tr>
</tbody>
</table>

1/ P=penicillin; AMP= ampicillin; E=erythromycin; TE=tetracycline; VA=vancomycin; CN= gentamicin; SXT= sulfamethoxazole-trimethoprim; MTZ= metronidazole

Conclusions

The results of the present study demonstrate that *E. coli* strains as being nonpathogenic or pathogenic bacteria isolated from fattening pigs in Khon Kaen are high resistant to penicillin (100%) and tetracycline (81.25%) and increase resistant to ampicillin and sulfamethoxazole-trimethoprim. They are also high multi-drug resistance indicating a long-time use of these antibiotics for veterinary therapy in this area and the antibiotics use should be recommended by veterinarians and utilizing control measures to decrease resistance on pig farms. Because of an antibiotic resistance of this enteric bacteria can be transferred from animals to humans via the food chain.

Acknowledgements

The authors would like to thank the Department of Veterinary Public Health, Faculty of Veterinary Medicine, Khon Kaen University, Thailand for bacterial laboratory facility. This work was partially supported by a grant (2010 research project grant) of the National Research Council of Thailand.

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Antimicrobial resistance profile of *Escherichia coli*

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Improvement of Rheological and Functional Properties of Defatted Rice Bran Protein Bioplastic by Kraft Lignin Addition

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Abstract

The aim of this research was to study the effect of Kraft lignin (KL) on rheological and functional properties of defatted rice bran protein (DRBP) blend. DRBP was extracted from defatted rice bran by alkaline extraction. DRBP plasticized with 30% glycerol was blended with 0-70% KL and then hot-molded using compression molding. Viscosity, storage modulus, mechanical properties and water absorption of plasticized KL/DRBP blend were respectively determined by capillary rheometer, dynamic mechanical thermal analysis, texture analyzer and water immersion method. Concerning the rheological properties, KL addition resulted in a decrease in viscosity and storage modulus in rubbery state of DRBP blends at processing temperature. In addition, an introduction of KL in DRBP blend improved functional properties of materials in terms of mechanical properties and water absorption. The KL/DRBP bioplastics exhibited higher Young’s modulus and tensile strength and lower water absorption compared to DRBP-based materials. Therefore, the addition of KL can be considered as a good way to improve processability due to a decrease in viscosity and also improve properties of DRBP-based bioplastic.

Keywords: bioplastic, defatted rice bran protein, kraft lignin, polymer blend

Introduction

Plastic waste is now regarded as a worldwide environmental problem. Biodegradable materials from renewable agricultural resources such as carbohydrates, starches, and proteins have attracted much attention as possible replacements. Interest in these materials is mainly because of their sustainable supply and biodegradability (Verbeek et al., 2010). Thailand is the world’s largest exporter of rice, sending over 8.5 million metric tons of milled rice around the globe in 2008/2009, while also ranking first in global production (Silpradit et al., 2010). One of the major by-products in rice mill process is rice bran which is accounted for 10% of milled rice (Sereeawathanawut et al., 2008). The minimum protein content of bran is about 13%. Defatted rice bran protein (DRBP) can be produced by alkaline extraction defatted rice bran and then by isoelectric precipitation (Agboola et al., 2005).

Protein-based materials can be defined as a stable three-dimensional macro-molecular network stabilized by low-energy interactions and strengthened by covalent bonds. Although protein is an interesting resource to produce bioplastic, a main drawback of protein-based plastics is a narrow window processing on common synthetic plastic process such as extrusion and injection molding due to its high viscosity (Verbeek et al., 2010). During processing protein blend has high viscosity due to protein aggregation via an exchange of thiol/disulfide bond. In addition, in terms of functional properties, protein-based bioplastic has poor water resistance and
moderate mechanical properties (Pommet et al., 2003).

To improve the processability of protein-based materials, a number of researches (Jane et al., 1996) (Ullsten et al., 2006) proposed to use chemical additives such as salicylic acid (Ullsten et al., 2006) in order to decrease the viscosity of protein during processing. Salicylic acid has free radical scavenging properties, so it can act as a viscosity reducer and interfered protein aggregation. Although salicylic acid can improve processability and enlarge the extrusion window of protein-based materials, the disadvantage of using chemical additive is a decrease in the mechanical properties of materials.

Blending of polymer is known to be an effective way to improve polymer properties such as flow ability, mechanical performance and water resistance (Torres-Giner et al., 2009). Kraft lignin (KL) is by-product from alkaline pulping process. KL is a polyphenolic compound which is known for its radical scavenging properties (Thielemans et al., 2002). KL may interfere protein aggregation and may decrease viscosity of protein due to its free radical scavenging properties. In addition, it is relatively hydrophobic and poorly soluble in water. Therefore, the objective of this research was to study the effect of KL on the processability of protein in terms of viscosity of protein blend and on functional properties of protein-based materials consisting of mechanical properties and water resistance.

Materials and Methods

Materials

Commercial defatted rice bran was provided by Thai Edible Oil Co., Ltd. (Bangkok, Thailand). Its protein and moisture content were respectively 18% and 10% (wet basis) according to the manufacturer.

Kraft lignin (KL) was obtained from Meadwastvaco corp (Virginia). Glycerol and Mg(NO$_3$)$_2$ were purchased from Ajax Finechem Ltd. (Auckland, New Zealand). NaN$_3$ was purchased from Merck and P$_2$O$_5$ was from Carlo Erba Ltd. All solvents and reagents were analytical grade.

Preparation of Defatted Rice Bran Protein

Defatted rice bran was ground by hammer to pass through a 70 mesh sieve. Then, defatted rice bran was suspended in 10 times distilled water and the pH adjusted to 9.0 with 2 M NaOH (Pincirolib et al., 2009). The suspension was stirred for 60 min and then centrifuged for 15 min at 9,000g. The supernatant was adjusted to pH 4 with 2 M HCl and then centrifuged at 9,000g for 15 min. The pellet was suspended in water, neutralized with 2 M NaOH, and freeze-dried.

Preparation of Defatted Rice Bran Protein/Kraft lignin materials

Materials in this study contain a mixture of defatted rice bran protein (DRBP): KL: glycerol in a weight ratio ranging from 70:0:30 to 0:70:30.

Mixing process

Forty-five gram of KL/DRBP/glycerol was blended in a mixer (Brabender, Plastograph®, Germany) for 15 min using 100 rpm of mixing speed. Temperature of mixing chamber was 80 °C using silicone oil (Brabender T300 B, electronic, Germany).

Compression molding process

Twenty-five gram or nine gram of the mixed blend was placed in a squared mould (9 × 9 cm) with 2 or 1 mm of thickness of spacer to avoid the direct pressure on the sample. Then, sample was thermo molded at 100 °C for 15 min.

Characterization of Defatted Rice Bran Protein/Kraft Lignin Materials

Viscosity

Sixty gram of mixed blend was used to determine the shear viscosity of blend (ASTM D3835-08) by using a high-pressure capillary rheometer (Rosand Rh2200, Malvern Instruments, UK) with a 1-mm capillary die, using two capillaries with the same radius but different length/radius.
ratios. Sample was pre-heated at 140°C for 2 min. Measurements were carried out 140°C under a shear rate ranging from 10 to 3000 s⁻¹. Viscosity is plotted against shear rate. The power law model was used to describe relationship between viscosity and shear rate as described in equation 1.

\[ \eta = K\gamma^n \]  

(eq.1)

where the consistency (K) corresponds to the viscosity value for a shear rate \(\gamma \) of 1s⁻¹ and the power-law index (n) characterizes the deviation from the Newtonian behavior.

Thermomechanical properties

Rectangular samples (10×3×1 mm³) were analyzed with a dynamic mechanical thermal analyzer (NETZSCH DMA 242, Piscataway, USA) equipped with a cryogenic system fed with liquid nitrogen. A tensile test was performed with a temperature ramp from –100 to 200°C at a heating rate of 3°C.min⁻¹. A variable sinusoidal mechanical stress was applied to the sample (frequency = 1 Hz) to produce a sinusoidal strain amplitude of 0.05% which ensure measurements in the linear domain of viscoelasticity. During analysis, the storage modulus (E’), the loss modulus (E’’), and tan δ (E’’/E’) were recorded and plotted against temperature. \(T_g\) was identified as the temperature of the tan δ maximum.

Mechanical properties

Tensile tests were performed on a Texture Analyzer (Stable Micro System, TA-XT.plus, Surrey, UK). Samples were cut into dumb-bell-shaped specimens of 70 mm overall length and 4 mm width for the elongating part and preconditioned at 25 °C and 53% relative humidity over a saturated salt solution of Mg(NO₃)₂. Sample thickness was measured with a micro meter. The elongation speed was 1 mm/s. Stress values (MPa) were calculated by dividing the measured force values (N) by the initial cross-sectional area of the specimen (mm²). Strain values were expressed in percentage of the initial length of the elongating part of the specimen (\(L_0 = 20\) mm). Young’s modulus was determined as the slope of the linear regression of the stress–strain curve. Given tensile strengths and elongations at break values, as well as Young moduli, are the means of at least four replicates.

Water absorption

The samples (20 mm in diameter) were dried at 50°C for 24 h in an oven. Subsequently, they were cooled in a desiccators for a few minutes, weighed (\(W_i\)), and then submerged in distilled water containing 0.05% NaN₃ (to avoid the microbial growth) for 1 week. The extra water on the surface of the specimen after soaking was removed with a paper towel, and the specimen was weighed again (\(W_f\)). The container without the soaking specimen was placed in an air oven at 50°C for 72 h until weigh constant then weighing specimen (\(W_i\)), and the water-soluble content in the specimen was determined. Water absorption was calculated by (Huang et al., 2003).

\[ \text{Water absorption (％)} = \left( \frac{W_f - W_i}{W_i} \right) \times 100\% \]  

(eq. 2)

Statistical Analysis

The experimental data were analyzed and presented as mean values with standard deviations. The one-way ANOVA, LSD test was used to compare means at 95% confidence (\(p \leq 0.05\)). The statistical program SPSS (version 11) was used to perform all statistical calculations.

Results and Discussion

Rheological Properties of KL/DRBP Blends

The apparent viscosity of plasticized DRBP blend containing 0-70% KL after mixing in an internal mixer were measured by capillary rheometer at 140 °C as shown in Figure 1. A decrease in the apparent viscosity can be observed when KL content increased. The higher the KL content, the lower the viscosity. However, it can be noticed that at high KL content (60-70%KL) it seems to be two slopes. At the shear rates of 10-100 s⁻¹, it showed steep decline slope and at shear rates of 100-1000 s⁻¹ it showed gentle decline slope.
The power-law index (n) and consistency (K) present in Table 1. All samples demonstrated the shear thinning. The plasticized DRBP mixed blend showed the highest apparent viscosity as the same trend as the apparent viscosity of sunflower protein (Orliac et al., 2003) and soy protein based material (Li et al., 2009). Material with 70%KL corresponded to material without KL shows the lowest viscosity. This may be attributed to the low molecular weight of KL (Kunanopparat et al., 2009). The addition of KL reduced the consistency (K) and power law index (n) of blends. This may be associated with the low molecular weight of KL compared to DRBP.

<table>
<thead>
<tr>
<th>KL (%)</th>
<th>K consistency (Pa·s)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>2938 ± 1058</td>
<td>0.59 ± 0.18</td>
</tr>
<tr>
<td>20%</td>
<td>1226 ± 345</td>
<td>0.72 ± 0.11</td>
</tr>
<tr>
<td>40%</td>
<td>1918 ± 922</td>
<td>0.52 ± 0.07</td>
</tr>
<tr>
<td>60%</td>
<td>1277 ± 93</td>
<td>0.29 ± 0.06</td>
</tr>
<tr>
<td>70%</td>
<td>478 ± 203</td>
<td>0.65 ± 0.07</td>
</tr>
</tbody>
</table>

ns indicated that values in the same column are not significant difference (p > 0.05).

**Thermomechanical Properties of KL/DRBP Materials**

Table 2 shows the values of $E'$ in rubbery regions as determined at 140°C, of $T_g$ as determined by the maximum of tan δ and of tan δ peak height. The addition of KL resulted in a decrease in $E'$ in rubbery state. In addition, tan δ peak height increased with increasing KL content until 60%KL, then slightly decreased with 70%KL. This may be associated with a low molecular weight of KL. In addition, KL has a number of hydroxyl groups which are well known for its plasticization properties (Pommet et al., 2005b).

<table>
<thead>
<tr>
<th>KL (%)</th>
<th>$E'$ at 140°C (MPa)</th>
<th>$T_g$ (°C)</th>
<th>Tan δ peak height</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>9.4±1.5$^a$</td>
<td>103±7$^a$</td>
<td>0.47±0.40$^b$</td>
</tr>
<tr>
<td>20%</td>
<td>7.6±0.3$^a$</td>
<td>112±0.2$^a$</td>
<td>0.56±0.08$^b$</td>
</tr>
<tr>
<td>40%</td>
<td>7.1±0.8$^b$</td>
<td>118±7$^a$</td>
<td>0.67±0.13$^{ab}$</td>
</tr>
<tr>
<td>60%</td>
<td>4.4±0.2$^b$</td>
<td>113±2$^a$</td>
<td>0.78±0.04$^a$</td>
</tr>
<tr>
<td>70%</td>
<td>3.3±1.3$^b$</td>
<td>110±7$^a$</td>
<td>0.68±0.04$^{ab}$</td>
</tr>
</tbody>
</table>

Values with different superscript letters in the same column are significant difference (p ≤ 0.05).

**Mechanical Properties of KL/DRBP Materials**

Figure 2 shows the mechanical properties of DRBP-based materials with 0-50%KL content. However, the mechanical properties of DRBP material with 60-70%KL cannot be determined because the samples are brittle and fragile.
which exhibited hydrophobic nature of KL (Whetten 1998). KL appeared to be an interesting filler to reduce the water absorption of DRBP-based materials. This observation confirmed the evolution previously study on soy protein plastics with increasing KL content (Huang et al., 2003).

**Figure 3** Water absorption of DRBP-based materials with 0-70%KL content

**Conclusions**

The addition of KL decreased the apparent viscosity of plasticized DRBP blend. In addition, storage modulus ($E'$) in rubbery state of DRBP materials decreased when KL was added. This may be associated a low viscosity of plasticized KL due to its low molecular weight. In addition, the addition of KL increased the mechanical properties of DRBP-based material. However, when KL was the major component in materials (50-70% KL) Young’s modulus and tensile strength of materials decreased due to the hard brittle property of KL. Moreover, the addition of KL decreased the water absorption of DRBP materials because of hydrophobic nature of KL. From this study, it may be concluded that KL is an alternative additive to enlarge the protein thermal processing window. KL not only improved the processability of protein by decreasing the viscosity at high processing temperature, but also improved functional properties in terms of mechanical properties and water absorption of DRBP-based materials.
Acknowledgments

The authors are very grateful to Thai Edible Oil Co., Ltd. Bangkok, Thailand for providing commercial defatted rice bran.

References


Mangiferin and Antioxidant Capacity from Mango (*Mangifera indica* L.) Leaves Extracts

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Abstract

Mangiferin, a naturally occurring xanthone glucoside with potent antioxidant and anti-inflammatory properties, is particularly abundant in the bark of *Mangifera indica* L. (mango tree) and can also be found in other parts of this plant. In this study, we looked at its abundance in the mango leaves. An ethanolic extract of leaves was prepared. The extract was then subjected to sequential extractions with hexane, ethyl acetate, n-butanol and water. Various fractions were investigated for antioxidant capacity and total phenolic content. Antioxidant capacities and phenolic content were detected in all fractions tested with the highest in the ethyl acetate fraction, 2.82 g trolox/g extract and 0.44 mg gallic acid/g extract, respectively. Mangiferin was found in both the butanol and water fractions. Mangiferin was isolated from the butanol fraction as a major component. The identity of mangiferin was confirmed by NMR spectroscopy.

Keywords: antioxidant capacity, mangiferin, mango leaves, phenolic content

Introduction

Mango (*Mangifera indica* L.) is one of the most common garden trees throughout the tropics including Thailand. Besides its juicy and highly nutritious fruit, the mango plant has been the focus of attention by many scientists searching for potent natural antioxidants. Various parts of the mango, such as stem bark, leaves, seeds and pulp have been used for biomedical purposes, including antioxidative, free radical scavenging (Ajila et al., 2007, Rocha Ribeiro et al., 2007), anti-inflammatory (Hernandez et al., 2007), and anticancer (Percival et al., 2006) activities. Garrido et al. (2001) and Delgado et al. (2001) reported the potent *in vivo* and *in vitro* anti-inflammatory activity of the standardized aqueous extract from the stem bark of selected species of *Mangifera indica* L., (VIMANG®) while the immunomodulatory and strong antioxidative effects were also shown (Sanchez et al., 2000, Martinez et al., 2000). This aqueous extract is composed of a variety of phenolic acids, phenolic esters, flavonols and mangiferin as the major components (Rodríguez et al., 2006). Mangiferin, a naturally occurring xanthone glucoside (2-C-β-D-glucopyranosyl-1,3,6,7-tetrahydroxyxanthone) (Figure 1), was found in the leaves, stem bark, fruit peel and roots of many higher plants including mango (Nong et al., 2005). Pharmacological studies of mangiferin reveals that this compound possesses antitumor (Leiro et al., 2003), antiviral (Guha et al., 1996), antidiabetic (Garcia et al., 2003), and immunomodulatory (Makare et al., 2001) activities.

In leaf material of *Mangifera indica*, var. ‘taimour’, quercetin 3-glucoside and kaemferol 3-glucoside were found, along with protocatechuic acid, gallic acid, mangiferin, quercitin and kaemferol (El Sissi and Saleh, 1970). Saleh and El Sissi (1975) reported the co-occurrence of mangiferin, isomangiferin and homomangiferin from the polyphenol
screening of 20 mango varieties and that mangiferin was the main compound of leaves and twigs. In a recent study, aqueous and ethanolic extracts of mango leaves were reported to be an ideal antioxidant (Ling et al., 2009). Mango leaves are not commercially used and are available year round so they could be another promising source of useful phytochemicals such as polyphenols, carotenoids, vitamins and mangiferin.

The aim of the present study was to evaluate the antioxidant capacity and phenolic content of an ethanolic extract of mango leaves. Three different assays were used to determine the antioxidant capacity of the ethanolic extract. These assays included free radical scavenging activity (DPPH. and ABTS .+), and reducing power (FRAP). The Folin-Ciocalteau method was applied to all samples in order to monitor the total phenolic content. The purification and structural characterization of mangiferin in the leaf extract was also performed.

**Materials and Methods**

**Plant Collection**

Fresh mature green leaves of *Mangifera indica* L. (cv Oklong) were collected during September and October from trees cultivated on the Sanamchan Palace campus, Silpakorn University in central Thailand.

**Preparation of Plant Extracts**

The leaves were washed with distilled water and allowed to air dry at room temperature. Then the leaves were placed in an oven at 40°C until completely dried. They were then ground and extracted exhaustively with ethanol (room temperature, 5 L of ethanol/kg leave) for twenty four hours and filtered. The residues were re-extracted with ethanol. The filtrates from each round of extraction were pooled and the solvent was removed under reduced pressure and referred to as crude extract. The crude extract was then subjected to sequential extractions with hexane, ethyl acetate, *n*-butanol and water.

**Antioxidant Assays**

The antioxidant capacities of the crude extract and the subfractions from sequential extractions were investigated using three different assays. The ability of the extracts to scavenge DPPH radical and ABTS cation radical were determined by previously described methods (Brand-William et al., 1995 and Re et al., 1999). The reducing power of the extracts was determined by the ferric reducing ability of plasma or FRAP methods (Benzi and Strain, 1996).

**Determination of Phenolic Content**

The total phenolic content of the extracts was determined by the Folin method with the modifications of Singleton and Rossi (1965) and expressed as gallic acid equivalents which reflected the phenolic content as mg of gallic acid/g extract.

**Purification of Mangiferin by Chromatographic Methods**

The crude extract and the subfractions were further analyzed by thin layer chromatography (silica gel 60 F254 on an aluminium backing, Merck, Darmstadt, Germany) using toluene: ethyl acetate: formic acid (4:5:1, v/v) as the mobile phase. Antioxidants in the crude extract and subfractions were detected by staining the silica gel chromatogram with 0.4 M DPPH solution in methanol (Takao et al., 1994). All subtractions were investigated for antioxidation capacity and total phenolic content using the same methods as for the crude extract. The *n*-butanol and water subfractions were analyzed by thin layer chromatography (RP-18 F254s, on an aluminium backing, Merck, Darmstadt, Germany) along with standard mangiferin from *Mangifera indica* bark (Sigma-Aldrich) using methanol: water (10:1, v/v) as the mobile phase. The *n*-butanol fraction was recrystallized in methanol yielding mangiferin as a pale yellow amorphous solid.
Results and Discussion

Antioxidant Capacities and Total Phenolic Content

The crude ethanolic extract from the mango leaves represented approximately 3.3% of the dry weight of the leaves. The chemical components in the crude extract were further resolved by sequential extraction with hexane, ethyl acetate, n-butanol and water. The highest yield of extractable material was with hexane (33.9% of the ethanol extract), n-butanol (26.6%), ethyl acetate (20.7%) and the lowest with water (11.4%).

The results obtained from the various antioxidant assays with reference to trolox confirmed the potential free radical and reducing power of the crude extract and subfractions of mango leaf (Table 1). The crude extract from mango leaf showed a concentration-dependent DPPH scavenging activity, with an EC$_{50}$ of 0.63 mg/mL or 603.47 mg trolox/g extract. The ABTS cation radical scavenging activity of the crude extract was parallel to that observed with the DPPH assay with EC$_{50}$ of 0.49 mg/mL or 468.22 mg trolox/g extract. The reducing property of the extract obtained from FRAP assay or FRAP value was 4266.95 µmol FeSO$_4$.7H$_2$O/g extract. The total phenolic content of the crude extract was found to be 248.22 (mg gallic acid/g extract). Compared to the ethanolic extracts from unripe and ripe flesh and peel of mango as reported earlier (Kim et al., 2010) the phenolic content in leaf is much higher and also exhibited much greater antioxidant capacity.

The evaluation of the antioxidant capacity and phenolic content of all the fractions were performed in parallel with the crude extract. The antioxidant capacity, determined by DPPH and ABTS assay, and total phenolic contents was found in all fractions, the highest in the ethyl acetate fraction followed by the n-butanol, hexane and water fractions respectively (Table1).

Isolation and Characterization of Mangiferin

The chemical components in the subfractions of the crude extract were analyzed by silica gel thin layer chromatography and compared to the standard phenolic compounds, quercetin, catechin and gallic acid. The DPPH scavenging activity was found in all fractions with the highest activity in the ethyl acetate and n-butanol fractions. Mangiferin was detected in the n-butanol and water fractions. Recrystallization of n-butanol fraction by methanol afforded mangiferin as a major product. Identification of magiferin was confirmed by comparison of the $^1$H and $^{13}$C NMR spectra (Table 2) with literature data (Martin et al., 2008).

Conclusions

Results of the present study indicate that the ethanolic extract of mango leaves, especially the ethyl acetate and n-butanol subfractions, exhibit good antioxidant capacity by effectively scavenging DPPH and ABTS radicals and reducing Fe$^{3+}$ (FRAP assay). The total phenolic contents of the extract correlate well with the antioxidant capacity. Mangiferin was found to be the main component of the n-butanol subfractions as confirmed by NMR spectrum. Mango leaves are a promising source of mangiferin, an antioxidant with promising pharmacological properties.
Table 1  Antioxidant capacity and total phenolic content of crude extract and the subfractions from mango (Mangifera indica L.) leaves. Each value is expressed as mean ±standard deviation (n=3)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>DPPH method mg trolox/g extract</th>
<th>ABTS method mg trolox/g extract</th>
<th>FRAP method μmol FeSO₄.7H₂O/g extract</th>
<th>Total phenolic content mg gallic acid/g extract</th>
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<tr>
<td>Crude extract</td>
<td>603.47 ± 16.33</td>
<td>468.22 ± 36.62</td>
<td>4266.95 ± 237.34</td>
<td>248.22 ± 6.58</td>
</tr>
<tr>
<td>Hexane</td>
<td>202.27 ± 6.63</td>
<td>197.05 ± 31.23</td>
<td>1474.41 ± 73.57</td>
<td>99.53 ± 3.29</td>
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<tr>
<td>Ethyl acetate</td>
<td>1226.66 ± 39.59</td>
<td>2817.99 ± 230.40</td>
<td>10172.59 ± 808.46</td>
<td>431.56 ± 25.13</td>
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<tr>
<td>n-butanol</td>
<td>942.28 ± 13.38</td>
<td>1974.75 ± 100.60</td>
<td>11589.54 ± 325.02</td>
<td>381.92 ± 7.19</td>
</tr>
<tr>
<td>Water</td>
<td>68.27 ± 0.50</td>
<td>174.30 ± 19.44</td>
<td>1114.97 ± 15.79</td>
<td>60.47 ± 4.81</td>
</tr>
</tbody>
</table>

Figure 1  Chemical structure of mangiferin (2-C-β-D-gluco-pyranosyl-1,3,6,7-tetrahydroxyxanthone)

Table 2  ¹H (300 MHz) and ¹³C (75 MHz) NMR data of mangiferin (1) in DMSO-d6

<table>
<thead>
<tr>
<th>Position</th>
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<td>3</td>
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Acknowledgments

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References


Preliminary Investigation of Biodiesel Wastes Utilization in Bacterial Fermentation

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Abstract

A surplus of crude glycerol from a biodiesel manufacture can be utilized into many potential ways of an application. Adding the crude glycerol as a nutrient in fermentation process is one of the interesting options. In this study crude glycerol was served as a solo carbon source for a cell cultivation of Rhodococcus opacus PD 630. In comparison of pure glycerol, this waste from biodiesel process did not have a similar effect to support the growth rate of R. opacus PD 630 during 0-36 hour. A possible reason is that an inhibit mechanism of microorganism might have been occurred from many impurities in crude glycerol. Moreover, an employment of pure glycerol representing a higher purity of glycerol at 1-10 % (w/v) concentration for feeding this strain revealed that R. opacus PD 630 could exploit glycerol as a carbon source. The highest biomass, 0.043 g l⁻¹, was found in 10 % (w/v) pure glycerol at 72 hour. According to results from this work, crude glycerol can possibly be a carbon source for Rhodococcus opacus PD 630 but it is necessary to abate impurities’ effect by increasing a treatment step before and/ or blending with other carbon source. In addition to exploiting crude or pure glycerol as a carbon source for this bacterium efficiently, adding other nutrients in fermentation medium is recommend.

Keywords: fermentation, crude glycerol, Rhodococcus opacus PD630

Introduction

The world energy crisis is developed from an imbalance between endless demand in energy and limit of supply. Even though actions in less energy consumption can abate this trouble, an increase in a new source and development of a new technology in power is a necessarily parallel key. Biodiesel is one of the renewable technologies that almost everyone pays attention to. Since there are many pros, for instance, diesel-like properties (Srivastava and Prasad, 2000), less modification of present engine technology and less pollution from exhaustion (Fangrui and Hanna, 1999), a manufacture of this sustainable energy is widespread in many countries (Sarin et al., 2007, Lim and Teong, 2010).

The transesterification reaction not only produces biodiesel but also glycerol, so-called crude glycerol, as a product. 1 kilogram of crude glycerol is approximately generated from the reaction with 3 gallons of biodiesel (Cavalheiro et al., 2009). A thriving of a biodiesel business causes a rising huge quantity of this byproduct at present; however, a usage of crude glycerol into many downstream fields is not high. Many complicated steps in purification and lacking of attraction in economic way are some problems making crude glycerol less attractive.
Nitayavardhana and Khanal, 2011). A bottleneck in utilization of crude glycerol should be managed by applying its implication into many areas. Serving biodiesel waste as a microbial nutrient is an appealing way from various alternatives. There are many industrial processes or studies exploiting crude glycerol to nourish microbes for producing a designed product. Crude glycerol was added in a packed-bed reactor with Enterobacter aerogenes HU-101 to produce ethanol and hydrogen (Ito et al., 2005). Klebsiella pneumonia, Pantoea agglomerans and several bacterial strains could produce 1,3-propanediol from using glycerol as a substrate (Mu et al, 2008). Furthermore, phytase enzyme (Tang et al., 2009) and docosahexaenoic acid (Chi et al., 2007, Ethier, 2011) were some products from taking advantage of crude glycerol as a substrate for microbial growth. A utilization of triacylglycerol’s microbe is an engaging approach for an industry of renewable energy. Because of less area exploitation for plantation and market competition, growing of the microorganism especially bacteria which is capable of lipid formation can serve as a new practical way to exert influence on waste from biodiesel plant (Xin et al., 2009). Even though many genus, e.g. Acinetobacto, Mycobacterium, Nocardia, (Marc et al., 2005), have capabilities to accumulate lipid, Rhodococcus opacus PD630 has also proven to produce lipid during its growth. Alvarez et al. (1996) found that this genus can accumulate lipid up to 76% cell dry mass (CDM) if gluconate was used in cultivation. This work will investigate the possibility to use the crude glycerol as a solo carbon source for a cell cultivation of R. opacus PD630. A potential in an employment of crude glycerol will be introductory evidence that can lead to a further study in lipid production of this bacterium.

Materials and Methods

Media and Culture

Rhodococcus opacus PD 630 was received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM number 44193). For preparing a seed flask, R. opacus PD 630 was taken into the nutrient broth as reported by Pfennig (1974). Crude glycerol, 76% of purity, from a palm biodiesel plant in Thailand and a 97% purity of glycerol, so-called “pure glycerol”, were used as carbon sources in this study. The fermentation medium was the mineral salts medium (MSM) according to Schlegel et al. (1961). At desired concentration, carbon source was added into MSM and sterilized for 15 minutes in an autoclave (121°C, 15 lb/in²). 250-mL Erlenmeyer flasks of medium were inoculated with 5% (w/v) of seed culture. After that, all flasks were incubated at 30 °C and 200 rounds per minute (rpm) of shaking speed for specified time.

Measurement of Biomass

Culture medium was taken and centrifuged at 6,000 × g, 4°C for 15 minutes. Cell pellet separated from supernatant was rinsed with distilled water and then dried in an oven until a constant weight. The dry weight cell was measured and record.

Results and Discussion

A Culture Response to a Purity of Glycerol

Demethanolized crude glycerol was brought to study a characteristic of Rhodococcus opacus PD 630 growth. The mineral salt medium was added with this untreated crude glycerol at 1% (w/v) and cultivated for 0-36 hours in comparison to a same concentration of pure glycerol. According to figure 1, pure glycerol can promote cell growth better than crude glycerol at every cultivation time. These results can be implied that impurities in
crude glycerol influence negatively cell functions of *R. opacus* PD 630. There are many suspicious substances negatively influencing this bacterium growth such as sodium hydroxide, di- and/or monoacylglycerols, methyl esters, free fatty acids and soap. It is essential, therefore, to have a treatment step for this byproduct before serving as a carbon source like a study of Liu and coworkers (2011). Moreover a concentration limiting in crude glycerol not over than 1% (w/v) without a pretreatment step could alleviate a detrimental effect in *R. opacus* PD 630 cells from those intoxicants and help more uptake of crude glycerol into cells.

**Concentration Effect on Bacterial Growth**

Even though *R. opacus* barely consumes crude glycerol as a carbon source, it is compelling to evaluate capability of this strain to utilize pure glycerol at higher concentration. MSM mixed with each 3 concentrations, 1, 5 and 10% (w/v) was inoculated with *Rhodococcus opacus* PD 630 and monitored cell growth during 0-72 hours. A correlation between biomass and time can be depicted as figure 2. At every concentration, cell weight increases obviously with cultivation time. This bacterium can tolerate and take advantage of having pure glycerol at higher concentration in medium. The highest biomass, 0.043 g l⁻¹, can be found at 10% (w/v) of pure glycerol. This consequence exhibited a probability of glycerol in being a nutrient for *Rhodococcus opacus*. Nevertheless, pure glycerol should not be suitably used as a solo carbon source. An additional other nutrients, such as peptone used in a study of Trchounian (2011), is suggested in pure glycerol fermentation to boost a growth of this bacterium. Besides, results of experiment varying pure glycerol concentration showed that crude glycerol at over 1% (w/v) has a propensity to be a carbon source for this strain of *Rhodococcus* genus when there is an addition step for eliminating or reducing toxicants in crude glycerol. Moreover, a mixing with other carbon source as a co-substrate like a study of Xiu et al. (2007) is a possible option for utilizing this biodiesel waste.

**Conclusions**

A hasty blooming of the biodiesel manufacture introduces us to know its waste, crude glycerol. It’s crucial to have a contingency plan to manage it. One of the interesting ways is serving as a microbial nutrition for producing desired products. In this work, crude glycerol was brought to cultivate a gram positive bacterium, *Rhodococcus opacus* PD 630. This biodiesel byproduct was aimed at supplying as a solo carbon source in cell density cultivation of this strain. A result showed that a purity of glycerol play a major role in microorganism growth. Pure glycerol,
at 1% (w/v), is a nutrient for supporting a growth of *R. opacus* PD 630 better than crude glycerol at every cultivation time. Since there are many substances in this biodiesel waste, those could possibly suppress or inhibit cell functions. Moreover, a higher level of pure glycerol concentration, up to 10% (w/v), promote a cell mass without inhibition. According to this results, a usage of high crude glycerol concentration tend to be suitable for microbial cultivation. However, a direct use of this biodiesel waste as a solo carbon source is not recommended. The pretreatment steps, adding other nutrients and/ or blending with other carbon source are still required.

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References


Identification of Sugarcane Somaclones Derived from Callus Culture by SSR and RAPD Markers Analysis

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Abstract

Somaclonal variation in sugarcane variety K84-200 derived from callus culture, was evaluated. For callus induction, leaf and shoot explants were subjected to in vitro culture on MS medium supplemented with 3 mg L⁻¹ 2,4-D for 5 times of subculture (15 weeks). All explants were regenerated in MS medium without growth regulators. Three different DNA-based techniques, random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR) and SSR combine with RAPD markers were used for detecting genetic variation and the phylogenetic relationship in the chosen explants. The result showed that the similarity coefficient were in range of 0.80-1.00 for SSR, 0.65-1.00 for RAPD and 0.72-1.00 for SSR combine with RAPD markers, respectively. The phylogenetic tree of SSR and RAPD can distinguish genetic diversity of 58 samples of sugarcane somaclones into 6 groups, which group 5 and 6 showed distinctly different from mother plant (K84-200). Interestingly, all somaclones of group 5 and 6 could be survived in medium containing 1.5% NaCl (w/v).

Keywords: somaclonal variation, SSR, RAPD, sugarcane

Introduction

Sugarcane is a major economic crop which was used as material for sugar production and sustainable fuel as bioethanol. Genetic variation is an essential component of any conventional crop breeding program. The adoption of new technologies as plant tissue culture and molecular markers may help in achieving some of the goals to produce the better plants. Plant tissue culture is a method for rapid propagation of somaclones in short time but some of regenerated plants are different from mother plant. Heinz and Mee (1969) were observed that the new plantlets of sugarcane are varied via callus culture on MS medium (Murashige and Skoog, 1962) supplemented with 3 mg L⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D). Plant tissue culture induced genetic variation in regenerated plants, also called somaclonal variation (Larkin and Scowcroft, 1981). Matheka et al. (2008) selected drought tolerance maize clones. Some clones in N6 medium (Chu, 1978) with mannitol and polyethylene glycol (PEG-600) could survive and were also found the genetic variation. Biswas et al. (2009) showed the three group of strawberry varieties which was suitable for economic trade. The somaclonal variation started from callus culture of strawberry and tested on natural field. Molecular marker as SSR and RAPD can be used for examination of the variation of DNA, gene and protein during somaclonal variation. Linacero et al. (2000) examined the DNA variation in rye regenerated from seedling callus and flower...
callus. The result showed that rye physiology was varied for 40 percent. Somaclonal variation could produce the different clones from mother plant and the good quality could also be found in clones (Rajeswari et al. 2009). Cordeiro et al. (2003) studied on the genetic variation of sugarcane by using 6 SSR marker primers; SMC334BS [(TG)_{36}], SMC336BS [(TG)_{23}(AG)_{19}], MSSCIR5 [(GGC)_9], MCSA068G08 [(CAG)_9], MCSA042E08 [(GAT)_{7}…(GAT)_{6}] และ MCSA053C10 [(CAG)_5] for identification of 66 variaties of sugarcane. This method could identified into Erianthus, Saccharum, Sorghum, Slerostachya and Miscanthus with the clearly result. RAPD marker was easily and rapidly way for identification of somaclonal variation of sugarcane (Tawar et al., 2008, Da Silva et al., 2008) This study was identified sugarcane by using SSR compare with RAPD and collaborate result of both methods.

Materials and Methods

Plant Materials

The K84-200 sugarcane variety was obtained from Suphan Buri Field Crops Research Center, Thailand.

Callus Induction and Plant Regeneration

The explants were derived from young shoots and young leaf. Surface sterile by Clorox 5-10% for 15-30 minutes and cultured the sterile explant on MS medium supplemented with 2,4-D 3 mg.L^{-1}. Subculture was done every 3 weeks for 15 weeks for somaclonal variation induction. Plant regeneration was conducted by MS medium without plant growth regulator.

In Vitro Salt Treatment

The total regenerated somaclones was planted in MS medium with 1 % NaCl w.v^{-1} for 4 weeks.

DNA Extraction & PCR Amplification

DNA extraction from sugarcane somaclones were randomly selected for 58 samples (modified method of Dellaporta et al., 1983). Eight primers as MCSA068G08, MCSA175A08, MCSA108E02, MCSA205C07, SMC226CG, SMC319CG, SMC477CG and SMC863CG were used for SSR marker. Polymerase Chain reaction (PCR) was performed in a 25 µL reaction volume containing 75 ng of template DNA, 50 pmole of each primer, 0.2 mM of the each dNTP, PCR buffer 1X, 1.5 mM MgCl_{2} and 1 U Taq DNA polymerase. All PCR reactions were performed on a Gene Amp PCR system (Perkin Elmer GeneAmp PCR system 2400) with the following profile: 2 min at 94°C, followed by 30 cycles of 2 min at 94°C, 0.5 min at 55°C, 1 min at 72°C, and a final elongation for 5 min at 72°C. Amplified products were electrophoresed on 4% polyacrylamide gel. φx-174/HindIII was used for standard DNA marker.

Ten primers as universal primer 2 (pharmacia kit), universal primer 3 (pharmacia kit), universal primer 6 (pharmacia kit), OPA-01, OPA-02, OPA-04, OPA-08, OPA-10, OPA-16 and OPA-18 were used for RAPD marker. PCR was performed in 25 µL reaction volume containing 80 ng of template DNA, 25 pmole of each, 0.2 mM of the each dNTP, PCR buffer 1X, 2 mM MgCl_{2} and 1 U Taq DNA polymerase. All PCR reactions were performed on a Gene Amp PCR system (Perkin Elmer GeneAmp PCR system 2400) with the following profile: 5 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 36°C, 2 min at 72°C, and a final elongation for 5 min at 72°C. Amplified products were electrophoresed on 4% agarose gel. Lamda DNA/HindIII ladder was used as the standard DNA marker.

Data Analysis

DNA fingerprint analysis for SSR and RAPD marker were scored “1” when band was occurred while scored “0” for absence band. The result of scored band was analyzed for similarity index by using Numerical Taxonomy and Multivariate Analysis System
Grouping was done following unweighted pair group method using arithmetic average (UPGMA) (Sneath and Sokal, 1973) and shown in phylogenetic trees.

**Results and Discussion**

MS medium supplemented with 3 mg L⁻¹ 2,4-D could induce callus formation in 6 weeks (Fig 1.) 16 of survived somaclones were found from 80 initial somaclones (data not shown).

According to using 8-primers for SSR marker, the result showed that 6 alleles in primer SMC863CG was mostly found but only 2 alleles in primers MCSA205C07, SMC319CG and SMC477CG were found. Using 10-primers for RAPD marker, 13 alleles could be found in primer universal primer6 but only 5 alleles could be found in primer OPA-08. Some of DNA bands were showed in Fig.2 for SSR and Fig.3 for RAPD.

**Figure 1** Callus induction., A Callus from young leaf tissue and B Callus from young shoot tissue.

**Figure 2** Some of SSR profile tested with primer SMC226CG on some somaclones (M=Φx-174/HindI).

**Figure 3** Some of RAPD profile tested with primer OPA-10 on some somaclones

**Data Analysis for SSR and RAPD Marker**

The above results of SSR were analyzed by NTSYSpc 2.2 program to calculate the similarity of 58 somaclones of sugarcane. Similarity coefficient was in range of 0.79-1.00 and similarity matrix was in range of 0.6785-1.0000. Phylogenetic tree could identified these samples into 6 groups (Fig.4).

The above results of RAPD were analyzed by the same program. Similarity coefficient was in range of 0.65-1.00 and similarity matrix was in range of 0.4404-1.0000. Phylogenetic tree could identified these samples into 2 groups and 6 subgroups (Fig.5).

Data analysis by using collaborate techniques of SSR and RAPD. Similarity coefficient was in range of 0.72-1.00 and similarity matrix was in range of 0.5625-1.0000. Phylogenetic tree could identified these samples into 2 groups and 6 subgroups (Fig.6).

SSR could identify sugarcane somaclones variation into 6 groups but there were not genetic diversity in many somaclones. On contrary, RAPD could identify into 6 groups which every somaclones were difference. This could ensure that RAPD was suitable for sugarcane identification. The used primers were not the specific for any position on genome. Then, there were many possible position that primers could detect which could give a lot of analytical data in short time. RAPD is widely used to study the variation at DNA level among the variants (Orapeza et al.,1995; Geisteira et al.,2002; Bennici et al., 2003). In this study, SSR gave less analytical data than RAPD. Phylogenetic tree grouping by using data from SSR and RAPD gave the similar result while only 8-
primers were used in SSR marker method. This could be confirmed by collaborate method of SSR and RAPD which could clearly identified the genetic variation. However, Selvi et al. (2003) were used SSR detection for genetic variation in sugarcane. The result showed that SSR marker could be clearly identification. Somaclonal variation could be found in callus culture. The phylogenic tree which was developed from RAPD data could identified into 2 groups and 6 subgroups (Fig.5,6). The 1st group was a callus culture from apical shoot and the 2nd group was a callus culture from young leave. This indicated that genetic variation can occur in different direction. The collaborate method of SSR and RAPD showed the similar result to RAPD but different in some somaclones (33LN(T)). Also different explants on callus

Figure 4 Phylogenetic tree of 58 somaclones sugarcane which developed from SSR marker method

Figure 5 Phylogenetic tree of 58 somaclones sugarcane which developed from RAPD marker method
induction in tissue culture technique were showed the variation in different ways (Ogura, 1990).

In this analysis, SSR, RAPD and SSR+RAPD method could not show the different between salt tolerance somaclone and other somaclones which were planted in salted medium. One of salt tolerance somaclone could be clearly identified in 5th and 6th groups in phylogenic tree which was developed from SSR and RAPD method. The important factor in characteristic under saline conditions, is determined by at least three genes (Akbar et al., 1972; Akbar and Yabuno, 1977) and salt tolerance is a multigenic trait (Flowers, 2004). This experiment showed some data which related with salt tolerance expression but could not indicate the relationship between each phenotype.

Conclusions

Somaclonal variation could be found in callus culture. SSR and RAPD marker methods were suitable for genetic variation study. More primers should be used to give the higher performance of somaclone identification.

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Potential of Six Plant Species for Food Processing Wastewater Treatment in Wetland

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Abstract

Six emergent plant species; Cyperus involucratus, Canna siamensis, Heliconia spp., Hymenocallis littoralis, Typha augustifolia and Thalia dealbata J.Fraser) were compared in small wetland. They were fed with seafood wastewater, cassava starch wastewater and molasses wastewater. These high strength wastewaters were studied in wetland system at different organic loading rates (OLR) of 612, 696, 806, 929 and 1,213 kg BOD₅.ha⁻¹.day⁻¹. The high performance plant species as Cyperus involucratus, Typha augustifolia and Thalia dealbata J.Fraser were studied. An increase of OLR affected pollutants removal efficiency and plant growth. Tested species may not be able to bear extensive under the high organic loading rate. Final plant dry weight was lower than the initial while ORL was increased up to 806 kg BOD₅.ha⁻¹.day⁻¹. The acceptable pollutant removal efficiency was found at lowest OLR of 612 kg BOD₅.ha⁻¹.day⁻¹ except for COD removal because input COD was very high. Pollutants reduction were 89-92 percent for SS, 74-89 % for BOD₅, 15-68 % for COD, 10-81 % for ammonium and 95-99 % for nitrate, respectively.

Keywords: cassava starch wastewater, molasses, constructed wetland, emergent plant, seafood wastewater

Introduction

Wastewater problem is seem more effective than other issues due to short term of affected period and its apparently impact. The effective industry on wastewater problems are pulp paper, palm oil, starch and seafood processing. These kinds of industry discharge large quantity and high strength of wastewater. The wastewater of palm oil, flour mill and seafood processing contain 30,000, 6,800 and 2,300 mg/l of BOD₅ (Bureau of Industrial Environmental Technology, 1998). The number of food processing factory in Thailand was increased year by year and flour mill showed the highest number. Treatment of food processing wastewater before discharge has been proposed. Constructed wetlands have been widely used in treating different types of contaminant found in domestic sewage, storm water, various industrial wastewaters, agricultural runoff, acid mine drainage and landfill leachate (Reed et al., 1988; Vrhovsek et al., 1996; Higgins et al., 1993; Bernard and Lauve, 1995). They are also economical and simple to construct and operate and are ideally suited for application in tropical climate when land is available and low cost. Traditionally, the constructed wetland is employed as a terminal treatment component, where it is designed and operated at rather low loads for the purposes of producing effluent that could meet discharged criteria.

Typical upper ranges of organic loading rates (OLR) as BOD₅ for design are 67.25...
kg.ha\(^{-1}\).d\(^{-1}\) and 44.84 kg.ha\(^{-1}\).d\(^{-1}\) for subsurface flow wetland and surface flow wetlands, respectively (Tchobanoglous et al., 1991). This requires more hydraulic detention times and land. In Thailand, the most frequently used of wetland emergent plants are cattail (\textit{Typha latifolia}), bulrush (\textit{Scirpus lacustris}) and reed (\textit{Phragmites australis}) (Brix, 1993) for domestic, but the other species may be more efficient under site specific conditions and wastewater type.

In this investigation, a microcosm surface flow constructed wetland (SFCW) was tested for its capability treatment of seafood wastewater, cassava starch wastewater and molasses wastewater under various loading conditions. The specific objectives of this study are: (i) to examine the wetland performance during operations at various OLR; and, (ii) to compare the performance of six emergent plant species for treating seafood wastewater.

**Materials and Methods**

**Surface Flow Constructed Wetland (SFCW) Operation**

The size of each wetland microcosm cell was 0.6 m deep, 2.0 m long and 0.5 m wide and bed slope of 1\%. The empty-bed volume of each cell was approximately 0.6 m\(^3\). The inner wall of the concrete cell was coated with waterproofing paint to prevent leakage. The wetland cell was filled with gravel, with a diameter of 1.34-1.55 cm. A 0.5 Hp water-pump was used to transfer the raw wastewater (influent) from a storage tank to the wetland cell and the excess flow was recirculated back to the storage tank for homogeneous mixing (Fig.1). Upon entering the microcosm, the wastewater flew down and the percolated through brick into the treatment zone. A transparent roof covered the system to prevent rainfall and other plant litter which can be interfered the experimental result. Six emergent plant; \textit{Cyperus involucratus}, \textit{Canna siamensis}, \textit{Heliconia spp.}, \textit{Hymenocallis littoralis}, \textit{Typha augustifolia} and \textit{Thalia dealbata} J.Fraser were investigated. Rhizomatous cuttings of each species from the field were collected. Each microcosm was planted with 22 cutting (approximately 20 cm in length) of each species in monocultured microcosm at the same age. The microcosms were monitored three times per week, and invasive seedlings detected were immediately removed. The water level was kept constant at 10 above the level of gravel surface. The OLR had five levels; 612, 696, 806, 929 and 1,213 kg BOD\(_5\).ha\(^{-1}\).day\(^{-1}\). These OLRs were conducted over a period of 90 days. Wastewater addition began at which the time the plants were well established.

![Figure 1 Wastewater flow direction into each wetland microcosm](image)

**Analysis Method**

**Water**

100-ml water samples were collected twice a week from influent and at the outlet of the wetland cell. Sampling was usually performed at around 10 A.M. on each sampling date. The samples were analyzed for Suspended solids (SS), biochemical oxygen demand (BOD\(_5\)), chemical oxygen demand (COD), ammonia nitrogen (AN) and nitrate nitrogen (NO\(_3\)\(^{-}\)-N) were analyzed according to Standard Methods for Water and Wastewater Examination (APHA, 1995).

**Plant Dry Weight Analysis**

All plants were observed throughout the experimental period for general appearance and health. Plant tissues were sampled...
following completion of each experiment (each OLR operation). Whole plant tissue in wetland was harvested and oven dried for approximately 48 hours at 80°C and weighted.

**Statistical Analysis**

All statistical analyzed were performed using SPSS 14.0 by SPSS Inc.. In all cases, significance was defined by P < 0.05. Test for significant difference in water quality between hydraulic retention times and plant species of the treatment wetland were tested using a completely randomized design (CRD) analysis of variance (ANOVA) with a posteriori LSD.

**Results and Discussion**

The average measurements in influent contaminant concentrations over the five values of OLR were BOD₅ 155 -307 mg.L⁻¹, COD 2,734-5,637 mg.L⁻¹, SS 30-58 mg.L⁻¹, ammonia 30-59 mg.L⁻¹ and nitrate 0.696-1.366 mg.L⁻¹.

**Suspended Solid Removal Efficiency**

SS loading rate in this experiment was not high and most of SS was removed during settlement in the influent zone before passing through the treatment zone. Increase of OLR affected SS removal performance. All plant species showed similar performance at each OLR except at OLR of 1,213 kg BOD₅.ha⁻¹day⁻¹ which was characterized by lowest performance. The averaged effluent concentration met discharge limitations for the wastewater. The range of SS reduction was found to be 89-92 percent for all OLRs. Performance reduction was observed at high loading rates (P<0.05). SS concentration percent removal attained 93.6-90.1 approximately 50 cm from the inlet point at all OLRs. Throughout this study, no clogging problem was observed. This may due to the low input SS.

**Plant Dry Weight COD and BOD₅ Removal Efficiency**

Increase of OLR affected plant dry weight for all plant species (Fig.2). The highest plant dry weight could be obtained at low OLR but the plant dry weight was decrease and began to die at OLR of 929 and 1,213 kg BOD₅.ha⁻¹day⁻¹. The average effluent concentration attained effluent limitations imposed by the government of Thailand, BOD₅<20 mg.L⁻¹ and COD<120 mg.L⁻¹. The initial BOD₅ and COD concentration of food processing wastewater were in range of 149-315 mg.L⁻¹ and 2,588-5,372 mg.L⁻¹ at five studied OLRs. Increase of OLR affected BOD₅ and COD removal performance significantly. All species removed BOD₅ efficiently at OLR 61 and 696 kg BOD₅.ha⁻¹day⁻¹ but they could not achieve the limitation at OLR over 806 kg BOD₅.ha⁻¹day⁻¹. Contrary, this wetland system could not achieve effluent limitation of COD even at the lowest OLR. BOD₅ and COD removal efficiencies were observed in range of 74-89 percent and 15-68 percent. BOD₅ removal efficiency was related with plant dry weight.
Ammonia and Nitrate Removal Efficiency

The most constituent of influent total nitrogen (TN) concentration was 47 percent of ammonium (NH$_4^+$). All plant species demonstrated the similar removal performance (Fig.3). Increase of OLR affected ammonium removal performance but nitrate removal was found while increases OLRs. Nitrogen removals were 10-81 percent for ammonium and 95-99 percent for nitrate. Presence of ammonia and nitrate were correlated with oxidation reduction potential (ORP) as shown in Fig.4. High nitrate could be found at positive value of ORP while ammonia was low.

Molasses Decolorization

All species showed the accepted decolorization performance only at OLR of 612 kg BOD$_5$/ha.day (Fig.5). The range of color reduction was found to be 21-74 percent for all OLRs. Performance reduction Color limitation of effluent was stated that “the treated wastewater which the acceptable color is not obtained can be discharged” but a nominal value is not stated.

All tested species could grow in high strength wastewater as food processing wastewater. These species are halophyte which possess specific characteristics of growth physiology that guarantee their survival even under extreme rhizosphere condition (Stottmeisters et al., 2003). Molasses is a high organic concentration wastewater and it affected growth of many
organisms as bacteria, fungi and plant (Satayawali and Balakrisnan, 2008). This may cause of plant biomass reduction when OLR was increased. The nutrient content was constant for all species at OLR of 612, 696, 806, 929 and 1,213 kg BOD$_5$.ha$^{-1}$.day$^{-1}$. However the nutrient uptake rate was affected by increase OLR. Polprasert (1997) reported a greater nitrogen uptake rate of 0.287 g.m$^{-2}$.day in treatment wetlands receiving a lesser nitrogen loading rate than this study; clearly indicate that high organic loading rates resulted in reduction in nitrogen uptake rate. The wetland evaluated in this study received an organic loading rate almost 2.5-12.3 times higher than that reported in the recent research (Ronald, 1994), acceptable effluent BOD$_5$ levels were achieved for all plant species even as the organic loading rate was increased up to 696 kg BOD$_5$.ha$^{-1}$.day$^{-1}$. The suspended solid was removed entirely by physical processes, involving sedimentation, filtration and adsorption (USEPA, 2000).

The settling zone modification enhanced all pollutants removal efficiency since solids removal was also accompanied with a considerable reduction in SS, BOD$_5$ and total nitrogen. Dissolved and small particle passed through the permeable brick into wetland treatment zone. The various forms of nitrogen were involved in chemical transformations from inorganic to organic compounds and back from organic to inorganic. Some of these processes require energy (typically derived from an organic carbon source) to proceed, and others release energy, which is used by organisms for growth and survival (Kadlac and Knight, 1996). The microorganisms, which grow on submerged portions of plant, on litter and other detritus, were a major contributor to wastewater treatment efficiency in the attached growth system. As the microorganisms attach on different parts of plants and media, they form a biofilm layer, which played an important role in nitrogen removal from wastewater (Polprasert, 1997). These processes are influenced by temperature, pH, alkalinity of the water, inorganic carbon source, microbial population and concentration of dissolved oxygen (Vymazal et al., 1998). Dissolved oxygen in this system was quite low at 0.03-0.16 mg L$^{-1}$ for all treatment (data not shown). Increase OLR reduced the amount of oxygen transferred into the wetland and lowered the ORP (Reddy and D’Angelo, 1994). Most TN was oxidized within the system using oxygen. Particulate TN was trapped in settling zone and converted into its soluble forms; ammonia, nitrite nitrate. Only the inorganic forms of nitrogen passed into gravel zone where it was removed. The removal mechanism of BOD$_5$ was similar to TN as state above. Molasses decolorization mechanisms were adsorption, enzymatic reaction, assimilation, chemical degradation of melanoidin at low pH. Wetland gave the acceptable treatment results for BOD$_5$, SS, ammonium, nitrate and color at lowest OLR of 612 kg BOD$_5$.ha$^{-1}$.day$^{-1}$ except COD. This OLR is the same dilution rate as recent study of molasses wastewater decolorization. According to result, pollutants removal performance can achieve at long hydraulic retention time and the tested emergent plant species can survive under organic loading rate less than 327 kg BOD$_5$/m$^2$.day for no harvest during operation. Plant harvesting during operation can increase wetland performance (Satayawali and Balakrisnan, 2008). The high biomass production plant species gives high wastewater treatment performance. Plantation with mixing plant species should be studied for enhance wetland performance.

Conclusions

Organic loading rate (OLR) effected the pollutant removal efficiency and plant growth performance. All tested plant species (Cyperus involucratus, Canna siamensis, Heliconia spp., Hymenocallis littoralis, Typha augustifolia and Thalia dealbata J.Fraser) could not grow under high OLR of 1,213 kg BOD$_5$.ha$^{-1}$.day$^{-1}$. The system showed the highest removal efficiency under the lowest OLR of 612 kg BOD$_5$.ha$^{-1}$.day$^{-1}$.
SS, BOD₅, COD, ammonia and nitrate removal efficiencies were 90-93%, 88-89%, 67%, 77-82% and 94-95%, respectively. However, the effluent BOD₅ still higher than the standard permission of Department of Industrial Work of Thailand at OLR of 806, 929 and 1,213 kg BOD₅ ha⁻¹ day⁻¹.

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Phosphorus Accumulation in Wetland for Food Processing Wastewater Treatment

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Abstract

A gaseous form of phosphorus, phosphine (PH₃) has not frequently found in environment. Phosphine is soluble in water, but has a high vapor pressure. It may be emitted from regions of extremely low redox potential as in a high water level in constructed wetland. The food processing wastewater was fed in wetland at various organic loading rate (OLR). The phase of phosphorus accumulation in wetland was studied by using six plant species, *Cyperus involucratus*, *Canna siamensis*, *Heliconia* spp., *Hymenocallis littoralis*, *Typha augustifolia* and *Thalia dealbata* J.Fraser, under 3 OLR. Accumulation in each part of wetland (plant tissue, sediment, treat wastewater, media) was observed. Increasing of OLR can increase the absent of phosphorus as phosphine. Reduction of OLR increased phosphorus accumulation in plant tissue.

Keywords: constructed wetland, food processing wastewater, phosphine, phosphorus accumulation

Introduction

Wetlands frequently are used to remove nitrogen and phosphorus from treated wastewater prior to release into receiving ground and surface waters (Kadlec, 1989). Several studies (Boyt et al., 1977; Fetter et al., 1978) suggest that freshwater wetlands, swamps, marshes and flooded soil systems can also reduce P levels of nutrient laden waters. Richardson (1985) cautioned, however, that wetland soils can function as either a source or sink for phosphorus to the overlying floodwater moving through the wetland. Physical, chemical and biological functioning in overlying water and underlying sediment dynamically regulate phosphorus in wetlands. A significant portion of floodwater and pore water phosphorus can be removed through uptake by macrophytes and algae (Toth, 1972; Syers et al., 1973). Kadlec (1989) states, associated litter and sediments are the key components of wetland systems in the regulation of nutrient cycling.

Although wetlands are in wide use, the nutrient uptake by plants and nutrient distribution in wetland compartments are less investigated, especially in tropical climate. The purposes of this research are to determine the relative effect of organic loading rates (OLR) and specific plant species on nutrient distribution for each wetland compartment. Three organic loading rates (OLR) were studied. Six plant species including umbrella sedge (*Cyperus involucratus*), canna (*Canna siamensis*), heliconia (*Heliconia* spp.), spider lily (*Hymenocallis littoralis*), cattail (*Typha augustifolia*) and water canna (*Thalia dealbata* J.Fraser) were selected as the representative species in tropical regions.
such as Thailand. A high organic concentration wastewater from the food processing industry was used as influent to investigate nutrient removal and plant uptake performance.

**Materials and Methods**

**Wetland Operation**

The surface flow constructed wetland (SFCW) system employed in this study was located Bangkok, Thailand. The wetland cell constructed using concrete was 0.6 m deep, 2.0 m long and 0.5 m wide (Fig.1). The empty-bed volume of the wetland cell was approximately 0.6 m³. The inner wall of the concrete cell was coated with waterproofing paint to prevent leakage. The wetland cell was filled with gravel, with a diameter of 1.34-1.55 cm. An electrical pump was used to transfer the influent wastewater from a storage tank to the wetland cell and the excess flow was recirculated back to the storage tank for homogeneous mixing. Upon entering the microcosm, the wastewater flew down and the percolated through brick into the treatment zone. A transparent roof covered the system to prevent rainfall and other plant litter which can interfere the experimental result. The plant treatment had seven levels; one unvegetated and six monocultures; *Cyperus involucratus*, *Canna siamensis*, *Heliconia* spp., *Hymenocallis littoralis*, *Typha augustifolia* and *Thalia dealbata* J.Fraser. Rhizomatous cuttings of each species from the field were collected. Each microcosm was planted with 22 cutting (approximately 20 cm in length) of each species in monocultured microcosm at the same age. The microcosms were monitored three times per week, and invasive seedlings detected were immediately removed. The microcosms were watered few times weekly. The water level was kept constant at 10 above the level of gravel surface. The OLR had three levels; 283, 473 and 1,412 kg BOD ha⁻¹·day⁻¹. These OLRs were conducted over a period of 90 days. Wastewater addition began at which the time the plants were well established. Prior to discharge into the wetland, raw seafood wastewater was 50 percent diluted with the treated wastewater from the existing three-stage facility at food factory including solid separation, facultative pond (with hydraulic retention time, HRT, of 14 days), and aerated lagoon (HRT of 6 hours).

![Figure 1 Dimension of constructed wetland of side view and water flow direction](image)

**Analysis Method**

**Water**

100-ml water samples were collected twice a week from influent and at the outlet of the wetland cell. Sampling was usually performed at around 10 A.M. on each sampling date. The samples were analyzed for dissolved oxygen (DO), Oxidation Reduction Potential (ORP), both was determined in the field. Other measurements were performed from field samples which were immediately transferred to the laboratory. Orthophosphate (PO₄³⁻-P) and total phosphate (TP), were analyzed according to Standard Methods for Water and Wastewater Examination (APHA, 1995).

**Plant Tissue Analysis**

All plants were observed throughout the experimental period for general appearance and health. Plant tissues were sampled following completion of each experiment.
(each OLR operation). Whole plant tissue in wetland was harvested and then chopped. 100 g sub-samples were sampled randomly and oven dried for approximately 48 hours at 80°C and weighted. To estimate nitrogen and phosphorus in each species, 50 g of dried material were crushed by using a grinder with 0.5 mm trapezoidal-perforation sieve. Total Nitrogen in plant tissue was determined using an induction furnace and a thermal conductivity detector. Samples were ignited in an induction furnace at approximately 900°C, in a helium and oxygen environment in a quartz combustion tube. An aliquot of the combustion gases was then passed through a copper catalyst to remove oxygen and convert nitrous oxides to N₂, scrubbed of moisture and carbon dioxide, and the nitrogen content determined by thermal conductivity. Total phosphorus in plant tissue was determined by using a nitric acid/hydrogen peroxide microwave digestion followed by atomic absorption spectrometry (AAS) and Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES) (AOAC, 1997)  

Gravel Media and Sediment Analysis  
Gravel media was weighted and 100-ml of deionized water was then added. This sample was then shake (120 rpm) horizontally for 30 minutes for biofilm separation. The suspension was next analyzed for total nitrogen and total phosphorus, using the same method as that employed for plant tissue analysis.  

Statistical Analysis  
All statistical analyzed were performed using SPSS 14.0 by SPSS Inc.. In all cases, significance was defined by P < 0.05. Test for significant difference in water quality between hydraulic retention times and plant species of the treatment wetland were tested using a completely randomized design (CRD) analysis of variance (ANOVA) with a posteriori LSD.  

Results and Discussion  
Phosphorus Distribution in Each Wetland Compartment  
Most input phosphorus was organic phosphorus. About 72-78 percent of total phosphorus (TP) was removed from influent. Increase of OLR did not affect phosphorus removal for all plant species. Nutrient removal efficiency in each study was depended on influent concentration. The increasing of OLR also effect phosphorus distribution in wetland compartments (Fig. 2). The proportion of phosphorus in the water body increased when OLR was increased. Most phosphorus was stored in the sediment. This indicates that important nutrients removal mechanisms were sedimentation process, plant uptake and microbial assimilation. At the highest OLR (1,412 kg BOD ha⁻¹.day⁻¹), nutrient accumulation in plant tissue decreased when the OLRs were increased. Different species showed similar results for nutrient distribution in sediment, wastewater and media. However differences were observed among species regarding nutrient accumulation in plant tissue. The unvegetated system showed higher nitrogen content in water body (Data not shown). However, phosphorus cumulative was not different. Phosphorus distribution can be accounted for in ranges of 0.79-17.01 percent, 20.35-28.37 percent, 40.96-56.27 percent and 9.09-20.47 percent in plant tissue, treated wastewater, sediment and media, respectively.  

Phosphorus Mass Balance  
As included an increase of OLR (473 and 1,412 kgBOD. ha⁻¹.day⁻¹) affected phosphorus accumulation but this increased phosphorus loss from wetland. A gaseous form of phosphorus, phosphine (PH₃) has been identified as a potential compound of significance in wetland environment (Gassman and Glindemann, 1993).
Phosphine is soluble in water, but has a high vapor pressure. It may be emitted from regions of extremely low redox potential, together with methane. Devai et al., 1988, measured PH$_3$ emission from a constructed wetland (1.0 ha, Phragmite and bulrushes) in Hungary and estimated that 1.7 g.m$^{-2}$.year$^{-1}$ of phosphorus was being lost by this chemical route. In this study, phosphorus may have been lost as plant litter which could have left the system due to wind. It may also have been lost as PH$_3$. According to plant biomass production, the higher biomass production species were characterized by more positive ORP value (Cyperus involucratus, Typha augustifolia and Thalia dealbata J.Frazer).

Phosphate was rapidly depleted in the unvegetated system. This indicates that the unvegetated presented a more reductive environment. This indicates that the unvegetated presented a more reductive environment. Because emergent plant species have the ability to adsorb oxygen and other needed gasses from the atmosphere through their leaves and above-water stems, and they have large gas vessels, which conducted these gasses to the root. Therefore, their roots are sustained aerobically in an otherwise anaerobic environment. It has been estimated that these plants can transfer between 5-45 g of oxygen.day$^{-1}$.m$^{-2}$ of wetland surface area, depending on plant density and oxygen stress levels in the root zone (Sherwood, 1996). Below ground biomass in the form of root material facilitates nutrient uptake and oxygenation of the rhizosphere. However 90% of the below ground material for all species was rhizomatous. This reduces the effectiveness of harvesting as it represents a relatively inaccessible store of biomass and nutrients. The large above ground biomass is
desirable in surface flow systems as it provides a substrate for epiphytic growth. Gerberg et al. (1986) stated that microbial transformations of nutrients are the main treatment process in wetlands. The nutrient uptake observed in above ground tissue was found higher than for below ground tissue.

This is associated with biomass production. However, the percentage of nitrogen and phosphorus content of the biomass were similar at all HRTs. Influent nutrient content was higher as compared to the effluent which produced a nutrient gradient through the length of the systems. Higher nutrient availability promotes growth (Radojevic’and Bashkin, 1999). Aerts and de Caluwe, 1994 have shown that the total productivity of three species of Carex can be increased by adding nutrients to the environment. Nutrient uptake for Cyperus involucratus, Typha augustifolia and Thalia dealbata J.Frazer was observed at 102-194 kg P/ha. This is higher than a recent study by Ennabili et al, 1998, which reported uptake of 105 kg P/ha due to high nutrient feeding to the system. The higher concentration of influent nutrients yielded higher removal efficiencies (Kadlec and Knight, 1996). The various forms of nitrogen and phosphorus were continually involved in chemical transformations of inorganic to organic compounds and back from the organic to inorganic. Some of these processes require energy (typically derived from an organic carbon source) to proceed, and other release energy, which is used by organisms for growth and survival (Kadlec and Knight, 1996).

Conclusions

Increasing of the organic loading rate affected nutrient distribution in plant tissue, media, sediment and water body. The tropical plant species studied were Cyperus involucratus, Canna siamensis, Heliconia spp., Hymenocallis littoralis, Typha augustifolia and Thalia dealbata J.Fraser. All plant species had an even distribution between above ground (shoot) and below ground (root) biomass. The high biomass production species (Cyperus involucratus, Typha augustifolia, and Thalia dealbata J. Fraser) exhibited a higher oxidative environment in the wetland system. This may be due to oxygen transferred through their leaves and above-water stems to rhizosphere. Increase of organic loading rate affected plant biomass production and also affected nutrient accumulation in plant tissue. Phosphorus accumulation in plant tissue was associated with plant biomass production. Increase of organic loading rate may result in an increase of nutrients in the water body as well as sediment and media (gravel). Therefore the optimal organic loading rate and hydraulic retention time were significant parameters in determining nutrient distribution in the wetland system. Tropical plant species which provided high biomass production are also characterized by high nutrient accumulation. Loss of phosphorus in gaseous form was found in anoxic and anaerobic condition as observed by oxidation and reduction potential value in wetland environment. The wetland close system that can prevent lost of N₂, NH₃ and PH₃ should be set. Operation under various organic loading rates and C:N ratio should be observed the exactly mass balance of wetland system, and wetland mass balance model for tropical region to be developed.

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References


Identifying Parameters Influencing Growth and Astaxanthin Production by *Xanthophyllomyces dendrorhous* Cultivated in Pineapple Juice Concentrate Base Low Cost Medium

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Abstract

Astaxanthin, a potent antioxidant, has gained considerable attention in several applications such as food, feed, and pharmaceutical industries. However, synthetic astaxanthin is expensive and poses safety concern when applying as feed additive. Therefore, biologically synthesized astaxanthin represents a safer alternative. *Xanthophyllomyces dendrorhous* is the red yeast capable of synthesizing astaxanthin as its major carotenoid pigment. As wild types in general produce low pigment concentration, objective of this study was to identify parameters significantly influencing growth as well as astaxanthin production by *X. dendrorhous* cultivated on pineapple juice concentrate based growth medium, a sugar as well as amino acids rich agricultural waste derived from canned pineapple manufacturing process. Screening conducted on six culturing factors, i.e., sucrose, glucose, (NH₄)₂SO₄, KNO₃, n-hexadecane, and pH was accomplished using fractional factorial design (FFD). Results showed that sucrose, KNO₃, and n-hexadecane were identified as the significant factors affecting cell growth (p<0.05) while sucrose, glucose, (NH₄)₂SO₄, KNO₃, and n-hexadecane were considered statistically significant for astaxanthin production. Results further suggested that increasing cell growth would increase astaxanthin production since astaxanthin production is directly proportional to the concentration of biomass. Therefore, the most important factors, namely, sucrose, KNO₃, and n-hexadecane, were adopted for further optimization study by Doehlert design.

**Keywords**: astaxanthin, FFD, pineapple juice concentrates, screening, *X. dendrorhous*

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione) is a carotenoid pigment classified as xanthophyll capable of providing a characteristic coloration to salmons, trouts, and crustaceans. It has been used as a feed in the aquaculture industry, and has also been considered a potential functional food and pharmaceutical supplement because of its excellent antioxidant activity (Johnson, 2003; Guerin *et al.*, 2003). *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) is a superior astaxanthin-producing yeast because it can be grown in fermentor to very high cell densities, more than 50 g L⁻¹ dry weight (Johnson, 2003). Generally, yeast extract was employed as supplement in cultivating medium; however, high price hinders its use in large quantities. Attempts have been made to
seek inexpensive cultivation growth medium for *X. dendrorhous* cultivation. Several inexpensive carbon sources have been proposed for production of astaxanthin by *X. dendrorhous* including cane molasses (Haard, 1988), sugar cane juice (Fontana *et al*., 1996), grape juice (Meyer and du Preez, 1994), hydrolyzed peat (Martin *et al*., 1993), and raw coconut milk (Domínguez-Bocanegra and Torres-Muñoz, 2004).

Thailand is one of the largest pineapple exporter producer in the world (Department of Agricultural Extension, 1998) providing that approximately 2.47 million tons was produced in 2008 (Bureau of Merchandise Trade Administration, 2008). Approximately 70–80% of pineapple produced was supplied to factories for processing into several products such as canned pineapple, frozen pineapple and pineapple juice concentrate. Pineapple juice concentrate contains high amount of sugar, especially, sucrose, as well as citric acid and malic acid (Elkins *et al*., 1997). Moreover, pineapple juice concentrate is rich in amino acids and several trace elements such as iron, potassium and phosphorous. Therefore, pineapple juice may potentially be employed to cultivate *X. dendrorhous* and, at the same time, yielding higher level of astaxanthin production.

Since growth and astaxanthin production by *X. dendrorhous* are dependent on several factors, statistical experiment design is an effective and reliable tool for identifying significant variables. Fractional factorial designs (FFD) is most widely used for screening the few main important factors from many less important ones (Nair *et al*., 2008). This statistical design method has also been applied previously to screen out the significant factors for astaxanthin production by *X. dendrorhous* in flask cultures.

This study aimed at identifying factors greatly affecting both growth and astaxanthin production by *X. dendrorhous* cultivated in pineapple juice concentrate based growth medium.

**Materials and Methods**

**Strain and Inoculation preparation**

The red yeast *X. dendrorhous* TISTR 5730 was purchased from Thailand Institute of Scientific and Technology Research. Inoculum was prepared by inoculating two loopful of yeast from an agar plate into a 250 ml flask containing 50 mL Yeast Malt medium, and incubated at 22°C under 200 rpm agitation for 24 h.

**Medium Preparation**

Pineapple juice concentrate was obtained from the Pilot Plant Development and Training Institute (PDTI) King Mongkut’s University of Technology Thonburi. The juice was diluted with distilled water to appropriate final sugar concentration and then filtered through double-fold-cotton cloth. Particulate matter was further removed by centrifugation at 4,500 rpm for 30 min at 4°C. The High Performance Liquid Chromatography (HPLC) method was used for quantitative analysis of sugars with Sugarpak column (Waters, USA). Deionized water containing 5 ppm CaEDTA served as mobile phase at flow rate 0.5 mL min⁻¹ whose temperature was maintained at 80°C while 20 µL sample size was employed. Stock solution containing sucrose, glucose, and fructose at 23.14, 37.72, and 35.51 g L⁻¹, respectively, was then stored at -20°C until use.

**Determination of Growth and Astaxanthin**

Yeast cells collected at a specified time were separated from the liquid medium by centrifugation at 4500 rpm for 5 minutes and rinsed twice with double distilled water. Cell growth was spectrophotometrically determined at 660 nm. Carotenoid content in the yeast cells was determined according to the method recommended by Sedmak *et al.* (1900). Briefly, yeast cells were disrupted with hot dimethyl sulfoxide (DMSO) together with glass bead, extracted with hexane:ethyl acetate at 50:50 (v v⁻¹) and separated by centrifugation.
at 4500 rpm for 5 minutes. Absorbance at 480 nm was determined.

**Statistical Experimental Design and Data Analysis**

Experiments were set up according to a $2^{6-2}$ fractional factorial design to investigate effects of six variables, i.e., sucrose, glucose, (NH$_4$)$_2$SO$_4$, KNO$_3$, n-hexadecane, and pH (Table 1) on growth and astaxanthin production by *X. dendrorhous*. 16 trials together with 3 runs at center point (Table 2) were necessary. According to Table 2, concentrations of each variable were coded as +1, 0, and -1 designating, respectively, as high, intermediate (central) and low level. Statistical analysis was conducted using Minitab 14 (Minitab Inc, USA).

<table>
<thead>
<tr>
<th>Variables</th>
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<th>(0)</th>
<th>(+1)</th>
</tr>
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<tbody>
<tr>
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<td>30</td>
<td>40</td>
</tr>
<tr>
<td>B : Glucose (g L$^{-1}$)</td>
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<td>20</td>
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<tr>
<td>C : (NH$_4$)$_2$SO$_4$ (g L$^{-1}$)</td>
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<td>3</td>
</tr>
<tr>
<td>D : KNO$_3$ (g L$^{-1}$)</td>
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<tr>
<td>F : pH</td>
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<td>5.5</td>
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</table>

**Results and Discussion**

As shown in Table 2, the highest biomass and astaxanthin production was observed 12.35 g L$^{-1}$ and 1,351.49 µg g$^{-1}$ yeast at Run 14 and 5, respectively. The coefficient of determination ($R^2$) for dry weight and astaxanthin production were 99.77 and 99.96%, respectively. These values showed good agreement between experimental observations and predicted values. The $R^2$ value also indicated that only 0.23 and 0.04 % of the variation was not explained by the model. Analysis of Variance (ANOVA) showed that all terms were considered significant statistically at the confidence interval of 95% for both dry weight and astaxanthin production.

As can be seen from pareto plot (Figure 1A), sucrose, KNO$_3$, and n-hexadecane were regarded statistically significant and influenced biomass production positively. Moreover, the two way interactions, sucrose×KNO$_3$ or its alias, n-hexadecane×pH, had a large positive effect on biomass production whose significance was confirmed by ANOVA analysis with corresponding p-value of 0.005. Further, interaction between glucose×KNO$_3$ and its alias, (NH$_4$)$_2$SO$_4$×pH and sucrose×pH, together with its alias, KNO$_3$×n-hexadecane showed small positive effect on biomass production given that these interactive effects were regarded statistically significant with the p-value of 0.037 and 0.045, respectively (Table 3). For astaxanthin production, glucose was considered statistically significant and influenced astaxanthin production negatively (Figure 1B). Moreover, sucrose as well as KNO$_3$ was considered significant statistically posing simultaneously small negative effect on astaxanthin production. It could be observed further that all variables (main effects) chosen affected astaxanthin production negatively except n-hexadecane (p≈0.016) whose presence appeared to favor astaxanthin production by *X. dendrorhous*. Additionally, sucrose×n-hexadecane, and its alias, glucose×(NH$_4$)$_2$SO$_4$ and glucose×pH, as well as its alias, (NH$_4$)$_2$SO$_4$×KNO$_3$ showed a small negative effect on astaxanthin production whose significance was indicated by the p-values of 0.003 and 0.008, respectively (Figure 1B). Further, interactions between glucose×KNO$_3$, and its alias, (NH$_4$)$_2$SO$_4$×pH, and sucrose×KNO$_3$, and its alias, n-hexadecane×pH showed small positive effect on astaxanthin production (Figure 1B) providing that these interactive effects were considered statistically significant with the p-value of 0.004 and 0.038, respectively.
Figure 1 Pareto charts depicting the influence of factors on biomass production (A) and Astaxanthin production (B) at 8-day post inoculation. Open and shaded bars signify positive and negative effect, respectively, while the dot line indicates significance at 95%.

Table 2 Experimental Design and Responses of 2^{6-2} Fractional Factorial Design

<table>
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<tr>
<th>Runs</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Dry Weight (g L(^{-1}))</th>
<th>Astaxanthin Content (µg g(^{-1}) yeast)</th>
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<td>-1</td>
<td>-1</td>
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Table 3 Analysis of variance for dry weight and astaxanthin production at 8-day post inoculation

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<th>DF</th>
<th>Adj[MS]</th>
<th>F</th>
<th>p</th>
<th>Adj[MS]</th>
<th>F</th>
<th>p</th>
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<td>69.75</td>
<td>0.014*</td>
<td>26490</td>
<td>590.52</td>
<td>0.002*</td>
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<tr>
<td>Astaxanthin</td>
<td></td>
<td>2.93922</td>
<td>41.69</td>
<td>0.024*</td>
<td>45480</td>
<td>101.66</td>
<td>0.010*</td>
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<tr>
<td>R(^2) = 99.77%</td>
<td></td>
<td>4.30821</td>
<td>61.11</td>
<td>0.016*</td>
<td>10993</td>
<td>24.57</td>
<td>0.039*</td>
</tr>
<tr>
<td>Curvature</td>
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<td>25.61</td>
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<td>12268</td>
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<td>0.07050</td>
<td>447</td>
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<td>Total</td>
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<td></td>
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</table>

* indicates significance at 95%
and its alias, n-hexadecane, pH showed small positive effect on astaxanthin production (Figure 1B) providing that these interactive effects were considered statistically significant with the p-value of 0.004 and 0.038, respectively.

This is not surprising that sucrose was identified as significant variable since the basidiomycetous yeast *X. dendrorhous* is known to produce an invertase (Killian *et al.*, 1996), an enzyme responsible for hydrolyzing sucrose into glucose and fructose, which could be utilized for either growth or astaxanthin production. Moreover, transfructosylation activity of invertase involves in transferring a fructose moiety to sucrose molecule resulting in neokestose, a prebiotic, which, in turn, could promote growth of *X. dendrorhous* (Killian *et al.*, 1996; Kritzinger *et al.*, 2003).

Therefore, high biomass as well as astaxanthin production may be anticipated since pineapple juice concentrate contained high sucrose and glucose as well as fructose concentration.

Nitrogen source, particularly potassium nitrate, was found to significantly affect growth and carotenoid production of the yeast *X. dendrorhous* (Figure 1). This is in good agreement with several reports available in literature (Fang and Cheng, 1993; Parajó *et al.*, 1998; Ni *et al.*, 2007). Parajó *et al.* (1998) found that when potassium nitrate was utilized at low concentration, approximately 0.1-0.2 g L\(^{-1}\) as sole nitrogen source for *X. dendrorhous* cultivation high astaxanthin concentration could be achieved.

Even though glucose has not been found to be statistically significant on growth, however, high glucose concentration largely affected pigment biosynthesis in a negative fashion. *X. dendrorhous* has been demonstrated to suffer glucose or catabolite repression, (Ramírez *et al.*, 2001) when glucose concentration presence in the growth medium exceeded 19 g L\(^{-1}\). Further, when glucose is present at high concentration, fermentation sets in leading to a marked decrease in the astaxanthin biosynthesis and an increase in the accumulation of ethanol and organic acids (Walker, 1998; Meyer and Du Preez, 1994).

N-hexadecane was found to affect growth and astaxanthin production by *X. dendrorhous* significantly. Liu and Wu (2006) found that supplementation of n-hexadecane into growth medium led to an increase in carotenoid yield due to increasing oxygen transfer rate. It has been reported that oxygen supply was advantageous in increasing astaxanthin biosynthesis and biomass production (Wang and Yu, 2009; Liu and Wu, 2006).

pH was considered insignificant on both growth and astaxanthin production which may be due to the fact that ranges of pH tested coincided with the optima in literature (Johnson and Lewis, 1979; Fang and Cheng, 1993; Meyer and du Preez, 1994; An *et al.*, 1989).

**Conclusion**

Sucrose, potassium nitrate, and n-hexadecane were identified as parameters significantly influencing both growth and astaxanthin production by *X. dendrorhous*. Therefore, their corresponding optimal concentrations will be investigated using Doehlert design.

**References**


Carotenoids Production from Red Yeasts Using Waste Glycerol as a Sole Carbon Source

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Abstract

Fourteen strains of red yeasts from a culture collection Thailand Institute Scientific and Technological Research (TISTR) were screened for carotenoids production using yeast malt-extract medium (YM), basal medium supplemented with either pure glycerol (BMP) or waste glycerol (BMW) as a sole carbon source. The results revealed that eleven strains of the red yeasts could grow and produced carotenoids in YM and BMP but only nine strains were observed in BMW. Sporobolomyces pararoseus TISTR5213 showed the maximum total carotenoids content of 912.74, 845.52 and 948.34 µg (g DCW)-¹ in YM, BMP and BMW, respectively. The optimal conditions for carotenoids production in BMW by S. pararoseus TISTR5213 were; 20 g L⁻¹ waste glycerol, 1.0 g L⁻¹ yeast extract, 5.3 g L⁻¹ (NH₄)₂SO₄, 5.5 g L⁻¹ KH₂PO₄, 3.7 g L⁻¹ K₂HPO₄, 0.5 g L⁻¹ MgSO₄.7H₂O, 0.2 g L⁻¹ MnSO₄.H₂O and 0.25 g L⁻¹ NaCl with an initial pH of 6.0 and agitation speed of 200 rpm at 25 °C for 5 days. Under the optimized conditions, the maximum total carotenoids content of 1,058.42 µg (g DCW)-¹ was achieved. In comparison, the cost of carotenoids production in YM, BMG and BMW were 0.177, 0.043 and 0.025 Baht (µg carotenoids)-¹ (g medium)-¹, respectively. These results indicated that waste glycerol showed high potential as a carbon source for carotenoids production.

Keywords: Sporobolomyces pararoseus, red yeasts, carotenoids, waste glycerol

Introduction

Carotenoids are the yellow to orange-red pigments that are ubiquitous in nature (Waites et al., 2001). They are a group of 600 molecules which can be found in many plants and microorganisms, including bacteria, algae, molds and yeasts (Frengova and Beshkova, 2009) but are not synthesized in animals (Rock, 1997). Carotenoids are used in pharmaceuticals, nutraceuticals, chemicals and animal feed additives industries (Frengova and Beshkova, 2009), as well as colorants in cosmetics and foods (Das et al., 2007). They are usually C₄₀ tetraterpenoids built from eight C₃ isoprenoid units joined, so that the sequence is reversed at the center. The basic linear and symmetrical skeleton, which can be cyclized at one or both ends, has lateral methyl groups separated by six C atoms at the center and five C atoms elsewhere. Carotenoids consist of two classes of molecule, the carotene which, are strictly hydrocarbons and xanthophylls which contain oxygen (Rodriguez-Amaya, 2001; Johnson, 2007).

Various species of red yeasts in genus Cryptococcus, Rhodotorula, Sporidiobolus, Sporobolomyces and Xanthophyllomyces can produce and accumulate carotenoids in their cells (Simson et al., 1971). The commercial production of carotenoids using microorganisms is highly efficient because they are easily manipulated in the bioprocessing schemes (Tinoi et al., 2005) in particularly in red yeasts because they have
high growth rate and they are convenient for large-scale fermentation (Frengova and Beshkova, 2009).

Waste glycerol or crude glycerol is a by-product from biodiesel production process. As the biodiesel production is increasing exponentially, waste glycerol is generated by the transesterification of vegetable oils has also been generated in a large quantity. Glycerol is present in the form of its esters (triacylglycerol and derivatives) in all fats and oils (Pachauri and He, 2006).

For every 9.0 kg of biodiesel produced, about 1.0 kg of waste glycerol by-product is obtained (Dasari et al., 2005). The usage of low-grade quality of waste glycerol obtained from biodiesel production is a big challenge as this glycerol cannot be used for foods and cosmetic applications (Pachauri and He, 2006). The aim of this study is to find an application of waste glycerol obtained from biodiesel production process as a sole carbon source for the growth of red yeasts and also produced carotenoids as a high value added products.

Materials and Methods

Microorganisms

Fourteen strains of red yeasts were obtained from the culture collection Thailand Institute Scientific and Technological Research (TISTR) namely Sporobolomyces pararoseus TISTR5213, S. shibatanus TISTR5563, S. nylandii TISTR5581, S. vermiculatus TISTR5589, S. salmonicolor TISTR5600, S. poonsookiae TISTR5722, Sporobolomyces sp. TISTR5899, Rhodosporidium toruloides TISTR5123 and TISTR5158, Rhodotorula rubra TISTR5134, and TISTR5158, Bullera crocea TISTR5565, Dioszegia sp. TISTR5792 and Xanthophyllomyces dendrorhous (formerly Phaffia rhodozyma) TISTR5730. All of red yeasts were maintained in glycerol stock at -80°C (Kusdiyantini et al., 1998).

Screening of Carotenoids Producing Yeast

The red yeasts were screened for carotenoids production in three conditions. The first condition was grown in yeast malt-extract medium (YM) contained 4.0 g L⁻¹ yeast malt extract, 10.0 g L⁻¹ malt extract and 4.0 g L⁻¹ glucose. The second condition was grown in basal medium supplemented with pure glycerol (BMP) contained 20.0 g L⁻¹ glycerol, 1.0 g L⁻¹ yeast extract, 5.3 g L⁻¹ (NH₄)₂SO₄, 5.5 g L⁻¹ KH₂PO₄, 3.7 g L⁻¹ K₂HPO₄, 0.5 g L⁻¹ MgSO₄·7H₂O, 0.2 g L⁻¹ MnSO₄·H₂O and 0.25 g L⁻¹ NaCl. The third condition was grown in basal medium supplemented with waste glycerol (BMW) which was similarly to BMP but 30.0 g L⁻¹ waste glycerol was added to replace pure glycerol. The initial pH was adjusted to be 6.8 and the cultivation conditions were incubation in the incubator shaker (Kühner, Switzerland) with shaking speed 200 rpm at 25°C for 5 days (Maldonade et al., 2008).

Optimization Conditions

The physicochemical effects of BMW and cultivation conditions for carotenoids production in red yeasts that produced the highest carotenoids was also studied step by step as described below.

Effect of Waste Glycerol Concentration

The waste glycerol concentration in BNW was varied at 10, 20, 30 and 40 g L⁻¹. Each flask was adjusted initial pH to be 6.8 and incubated with shaking speed 200 rpm at 25°C for 5 days.

Effect of Initial pH

The initial pH of BMW containing optimal waste glycerol concentration was adjusted to be 4.0, 5.0, 6.8, 7.0 and 8.0. The cultivation conditions were incubated with shaking speed 200 rpm at 25°C for 5 days.

Effect of Temperature

The cultivation temperature of BMW with optimal initial pH was varied at 20, 25 and 30°C. Each flask was incubated in the incubator shaker at 200 rpm for 5 days.

Carotenoids Analysis

Ten milliliters of 5 days cultivation broth was taken from each flask and centrifuged at 6,000 rpm at 4°C for 10 min. Cell pellets
were washed twice with n-hexane and once with distilled water. The carotenoids contents of cell pellets was extracted by breaking of (DCW)-1 in YM, BMP and BMW, respectively. yeast cell method which carried out in 30 mL test tube containing 10 mL acetone mixed with glass beads (size 3.0 mm, 30% w v⁻¹) then vigorously shake with vortex mixer for 15 min under the presence of 100 ppm ascorbic acid. The acetone phase was determined by measuring the absorbance at 480 nm for total carotenoids with the UV-VIS spectrophotometer using astaxanthin (Sigma, USA) as the standard (Kusdiyantini et al., 1998; Liu et al., 2006; Moriel et al., 2005). Dried cell weight (DCW) of each flask was collected from 5 days cultivation broth, centrifuged and washed as described above before drying at 80°C overnight.

Results and Discussion

Screening of Carotenoids Producing Yeast

The results revealed that among fourteen strains, only eleven strains of red yeasts could grow and produce carotenoids in YM and BMP but only nine strains were observed in BMW. Table 1 and Figure 1 show that S. pararoseus TISTR5213 gave the highest production yield or total carotenoids content (Yₚ/ₓ) of 912.74, 845.52 and 948.34 µg (gDCW)-¹ in YM, BMP and BMW, respectively.

![Figure 1](image)

**Figure 1** The production yield or total carotenoids content (Yₚ/ₓ) of red yeasts cultivated in YM ( ), BMP ( ) and BMW ( ).

### Table 1

<table>
<thead>
<tr>
<th>TISTR strains</th>
<th>YM</th>
<th>BMP</th>
<th>BMW</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Xₘ [g L⁻¹]</td>
<td>Pₘ [µg L⁻¹]</td>
<td>Yₚ/ₓ [µg (g DCW⁻¹)]</td>
</tr>
<tr>
<td>5123</td>
<td>6.86</td>
<td>1,637</td>
<td>238.63</td>
</tr>
<tr>
<td>5134</td>
<td>6.67</td>
<td>1,443</td>
<td>216.34</td>
</tr>
<tr>
<td>5158</td>
<td>7.10</td>
<td>1,331</td>
<td>187.46</td>
</tr>
<tr>
<td>5213</td>
<td>5.22</td>
<td>4,765</td>
<td>912.74</td>
</tr>
<tr>
<td>5563</td>
<td>4.20</td>
<td>3,604</td>
<td>857.98</td>
</tr>
<tr>
<td>5565</td>
<td>5.10</td>
<td>956</td>
<td>187.45</td>
</tr>
<tr>
<td>5581</td>
<td>5.44</td>
<td>1,222</td>
<td>224.63</td>
</tr>
<tr>
<td>5600</td>
<td>3.60</td>
<td>550</td>
<td>152.78</td>
</tr>
<tr>
<td>5730</td>
<td>5.87</td>
<td>1,080</td>
<td>183.99</td>
</tr>
<tr>
<td>5792</td>
<td>6.19</td>
<td>1,464</td>
<td>236.51</td>
</tr>
<tr>
<td>5899</td>
<td>6.38</td>
<td>1,516</td>
<td>237.62</td>
</tr>
</tbody>
</table>

Effect of Waste Glycerol Concentration

As evident in Table 2 shows that the production yield or total carotenoids content (Yₚ/ₓ) of the strain S. pararoseus TISTR5213 in BMW containing different waste glycerol concentrations. The results revealed that waste glycerol concentration affected to DCW (Xₘ) and carotenoids production (Pₘ) of S. pararoseus TISTR5213. As shown in Table 2, waste glycerol concentration of 20.0
g L\(^{-1}\) gave the highest production yield or total carotenoids contents of 1,026.77 µg (g DCW\(^{-1}\) and further increasing of waste glycerol concentration, decreasing of the production yield was observed. Waste glycerol can enhanced the biomass and carotenoids production in red yeasts because it contained many types of macro elements such as calcium, potassium, magnesium, sodium, phosphorus and sulfur which were necessary for biomass synthesis (Thompson and He, 2006). The effect of these macro elements on the biosynthesis of carotenoids in red yeasts could be explained by hypothesizing a possible activation or inhibition mechanism by metal ions on the specificity of carotenogenic enzymes, in particularly, desaturase which involved in carotenoids biosynthesis (Frengova and Beshkova, 2009).

Table 2 Effect of waste glycerol concentration on production yield or total carotenoids content (\(Y_{p/x}\)) of \(S.\) pararoseus TISTR5213 in BMW after 5 days cultivation.

<table>
<thead>
<tr>
<th>Waste glycerol concentration (g L(^{-1}))</th>
<th>(X_m) [g L(^{-1})]</th>
<th>(P_m) [µg L(^{-1})]</th>
<th>(Y_{p/x}) [µg (g DCW(^{-1}))]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>3.06</td>
<td>2,436</td>
<td>796.05</td>
</tr>
<tr>
<td>20</td>
<td>3.16</td>
<td>3,245</td>
<td>1,026.77</td>
</tr>
<tr>
<td>30</td>
<td>3.18</td>
<td>2,935</td>
<td>922.96</td>
</tr>
<tr>
<td>40</td>
<td>3.25</td>
<td>2,485</td>
<td>764.46</td>
</tr>
</tbody>
</table>

Effect of Initial pH
The pH is one of most important environment parameters affecting cell growth and carotenoids production. However, these effects vary with the different microorganisms, medium compositions and operation conditions (Saenge et al., 2011). The initial pH of BMW affected on the biosynthesis activity and growth rate of \(S.\) pararoseus TISTR5213 as well as waste glycerol concentration. As evident from Figure 2, raising the initial pH, the biomass and carotenoids production rate increased and reached to the maximum level at pH 6.0. The highest production yield of 1,058.42 µg (g DCW\(^{-1}\)) was obtained at pH 6.0. However, the maximum biomass of 3.60 g L\(^{-1}\) was obtained at pH 7.0. The similarly result was reported by Hu et al. (2006) that the optimal pH for cell growth of \(X.\) dendrorhous was pH 6.0, and the optimal pH for astaxanthin production was pH 4.0.

![Figure 2 The effect of initial pH of BMW on the production yield or total carotenoids content (\(Y_{p/x}\)) (■) and dried cell weight (♦) of \(S.\) pararoseus TISTR5213 after 5 days cultivation.](image)

Effect of Temperature
The cultivation temperature is another important factor affecting the performance of yeast cells and carotenoids formation (Liu et al., 2006). The maximum production yield of 1,057.75 µg (g DCW\(^{-1}\) and DCW of 3.24 g L\(^{-1}\) were obtained at 25°C (Figure 3). Further increasing of temperature, the production yield and biomass were dramatically decreased. This might be that the high temperature caused the denaturation of the enzyme systems involved in carotenoids biosynthesis (Aksu and Eren, 2007). Moreover, most of red yeasts are the psychrotrophic yeasts with a temperature range of growth from 15 to 30°C (Miller et al., 1976).

Time Course of Carotenoids Production by \(S.\) pararoseus TISTR5213 in BMW Under Optimal Conditions
Figure 4 shows the growth and carotenoids production behaviors of \(S.\) pararoseus TISTR5213 under the optimal conditions. This strain needed cultivation time up to 5 days for the highest carotenoids accumulation in its cell. As evident from
Figure 4, carotenoids production was strongly growth associated and increased during the log phase of the yeasts growth. The maximum production yield of 1,058.42 µg (g DCW)^{-1} and DCW of 3.40 g L^{-1} were obtained at days 5 of cultivation and further increasing of incubation time, decreasing of the production yield and DCW were observed. As a result, it could be showed that carotenoids were formed almost parallel with the cell growth. The similarly result was reported by Aksu and Eren (2005) that the carotenoids production of *R. mucilaginosa* was also growth associated product and the highest carotenoids content of 69.8 mg L^{-1} and DCW of 5.0 g L^{-1} were obtained at the end of log phase (5 days).

### Table 3 Carotenoids production cost in various media.

<table>
<thead>
<tr>
<th>Media</th>
<th>Cost of medium [Baht g^{-1}]</th>
<th>Production yield [µg (g DCW)^{-1}]</th>
<th>Production cost [Baht µg^{-1}g^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>YM</td>
<td>162</td>
<td>912.74</td>
<td>0.177</td>
</tr>
<tr>
<td>BMP</td>
<td>38</td>
<td>845.52</td>
<td>0.045</td>
</tr>
<tr>
<td>BMW</td>
<td>26</td>
<td>948.34</td>
<td>0.025</td>
</tr>
</tbody>
</table>

Figure 3 Effect of cultivation temperature on the production yield or total carotenoids content (Y_{p/x}) (■) and dried cell weight (♦) of *S. pararoseus* TISTR5213 after 5 days cultivation.

**The Carotenoids Production Cost**

In table 3, the estimated cost of carotenoids production by *S. pararoseus* TISTR5213 in YM, BMP and BMW medium were calculated. The waste glycerol showed the lowest production cost of 0.025 Baht per µg carotenoids per g medium. This result indicated that waste glycerol had a high potential as an alternative carbon source for carotenoids production in red yeasts.

Figure 4 Time course of the production yield or total carotenoids content (Y_{p/x}) (■) and dried cell weight (♦) of *S. pararoseus* TISTR5213 cultivated in BMW under optimal conditions.

**Conclusions**

*S. pararoseus* TISTR5213 could produce high carotenoids in BMW with the production yield of 948.34 µg (g DCW)^{-1}. The optimal conditions for carotenoids production were; waste glycerol concentration of 20.0 g L^{-1}, initial pH 6.0 and 25°C for 5 days. Under these optimal conditions the maximal of total carotenoids contents of 1,058.42 µg (g DCW)^{-1} was obtained.

**Acknowledgments**

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References


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Studying the Genomic Function of Rice β-glucosidase via RNA Interference

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Abstract

In this research, a group of five rice β-glucosidase genes were knocked down via a high throughput inducible RNAi vector (pOpOff2) using dexamethasone (dex) as an inducer. The coding region of Os3bglu7 was used as the target genes to produce the construct. The transformation was done with Agrobacterium strain EHA105. The dex was added in the transformation processes. The results showed that the control treatment (no dex added) had lower number of brown calli (higher transformation efficiency) on selection medium and lower Agrobacterium population on the calli surface than the treatment that dex was added. This indicated that when the five β-glucosidase genes were knocked down, perhaps Agrobacterium developed better than usual. The structure of calli were broken and damaged, so lower transformation efficiency was obtained. This result reflects the effect of knocking down β-glucosidase genes on the efficiency of transformation of rice callus. This information supports the knowledge that β-glucosidases could play a role in callus defense against Agrobacterium infection.

Keywords: agrobacterium transformation, β-glucosidase, gene silencing, RNAi

Introduction

Beta-glucosidases are enzymes mainly belonging to GH1 family (http://www.cazy.org/fam/GH11.html). They catalyze the hydrolysis of the β-glucosidic linkages between two carbohydrate moieties or a carbohydrate and an aglycone moiety. These enzymes have been found in all living organisms and contribute to variety of physiology mechanisms including lignification (Dharmawardhana et al., 1995), cell wall degradation (Leah et al., 1995; Akiyama et al., 1998), chemical defense, activations of phytohormones. (Brzobohaty et al., 1993; Kristoffersen et al., 2000), responses to biotic or abiotic stress as well as plant secondary metabolisms (Forslund et al., 2004; Sue et al., 2000; Morant et al., 2008). However, the understanding about rice β-glucosidases is still not complete. In rice, 40 β-glucosidase genes have been found and 34 genes are expressed in a range of organs and stages of development, based on the cDNA and EST sequences in public databases (Opassiri et al., 2006). To date, only a few rice β-glucosidases have been characterized for their expression, physiology, biochemical properties, and structure (Akiyama et al., 1998; Opassiri et al., 2003, 2004, 2006, 2010; Chuenchor et al., 2008, 2010; Seshadri et al., 2009; Kuntothom et al., 2009, 2010; Wakuta et al., 2010).

The physiological functions of specific β-glucosidase in defense mechanism as well as in the development of rice are still controversial. To date, the largest number of studies of rice β-glucosidase has been conducted on Os3bglu7 (bglu1). Opassiri et al. (2003) suggested that the highly expressed Os3Bglu7 protein plays a role in cell wall expansion, cell division, in activation of phytohormones and in defense of young tissue. The Os3Bglu7 is grouped in the same phylogenetic cluster with four other rice β-glucosidase isoenzymes (Os1Bglu1,
Os3BGlu8, Os7BGlu26 and Os12BGlu38) (Opasiri et al., 2006, Kuntothom et al., 2009). Our previous studies suggested that β-glucosidases may protect rice from the infection of Agrobacterium. To investigate the functions of these genes, the RNA interference (RNAi) method was used; since RNAi has been routinely used to characterize gene function (Fu et al., 2007).

**Materials and Methods**

**Plant Material**

The *Japonica* rice cultivar, Koshihikari used in this study was obtained from the Chiang-mai Rice Research Center, Thailand.

**Target Sequence for RNAi Vector**

Five rice β-glucosidase genes (*Os1bglu1*, *Os3bglu7*, *Os3bglu8*, *Os7bglu26* & *Os12bglu38*) were chosen. To knock down these genes, a 399 bp conserved region (Kn5) of the *Os3bglu7* coding region was used as the target sequence. The Kn5 sequence shows high identity with the mRNA sequences of the 4 β-glucosidase genes (Table 1).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Description</th>
<th>Max identity</th>
<th>Query coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>OSU28047</td>
<td><em>Os3bglu7</em></td>
<td>399/399</td>
<td>100%</td>
</tr>
<tr>
<td></td>
<td>1909 bp</td>
<td></td>
<td>(100%)</td>
</tr>
<tr>
<td>AK120790</td>
<td><em>Os3bglu8</em></td>
<td>330/393</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td>2082 bp</td>
<td></td>
<td>(84%)</td>
</tr>
<tr>
<td>AK068499</td>
<td><em>Os7bglu26</em></td>
<td>270/392</td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td>1897 bp</td>
<td></td>
<td>(69%)</td>
</tr>
<tr>
<td>AK069177</td>
<td><em>Os1bglu1</em></td>
<td>249/345</td>
<td>85%</td>
</tr>
<tr>
<td></td>
<td>1833 bp</td>
<td></td>
<td>(72%)</td>
</tr>
<tr>
<td>AK071058</td>
<td><em>Os12bglu3</em></td>
<td>76/102</td>
<td>25%</td>
</tr>
<tr>
<td></td>
<td>8 1796 bp</td>
<td></td>
<td>(74%)</td>
</tr>
</tbody>
</table>

**RNAi Vector Construction**

The primers used to amplify Kn5 fragment were as follows forward 5' CACCCCTCGAGGTTCCCATAGCGG TTCGTTG (CACC for directional cloning into pENTR/D TOPO vector) and reverse 5' GGGAATTCCATTCACCAGCCTCG.

*Os3bglu7* cDNA was used as template to amplify the Kn5 fragment. The Kn5 was then cloned into pENTR/D TOPO vector (Invitrogen) and sequenced. The correct recombinant pENTR/D Kn5 was then used to transfer the Kn5 fragment into the pOpOff2 vector by LR clonase enzyme (Invitrogen). After that, the recombinant pOpOff2 that contains the inverted repeats of the Kn5 sequence (pOpOff/Kn5) were checked by restriction enzyme analysis. The correct recombinant pOpOff/Kn5 plasmid was then transformed into Agrobacterium strain EHA105.

**Agrobacterium-mediated Transformation**

Mature sterilized rice seeds (*Oryza sativa* L. cv. Koshihikari) were cultured on callus induction Chu N6 medium (pH 5.8) that contains 30 g/L sucrose, 3.98 g/L CHU basal salt mixture (Phytotechnology), 300 mg/L casaminoacid, 2.878 g/L L-proline, 5 ml/L 100x N6 vitamin, 2 mg/L 2,4D and 4 g/L gellengum (Phytotechnology) in the dark at 28°C. After 4-6 weeks, the secondary calli were subcultured on new callus induction medium for 3 days. After sub-cultured, the secondary calli were co-cultivated with Agrobacterium harboring pOpOff/Kn5 for 3 days at 25°C in the dark. After 3 days, the infected calli were washed to get rid of the Agrobacterium and the fresh healthy calli were transferred to selection medium (N6D calluss induction medium containing 300 mg/L timentin and 50 mg/L hygromycin). Dex dissolved in ethanol was spread on the surface of solid co-cultured medium and selection medium. The percent of brown calli were collected at week 1 and week 2 of selection. All of the calli on selection medium after 4 weeks were transferred to the regeneration medium.

**Agrobacterium Population on the Calli After Co-cultivation**

The Agrobacterium transformations were done with 128 µg of dex or with out dex. After 3 days of co-cultivation, 30 pieces of calli (2-3 mm) were selected and rinsed in 10 ml of sterile water, and then 1 ml was used to measure OD$_{500}$ to quantify the
number of *Agrobacterium* population. The experiments were done in triplicates for each treatment (each experiment) and 3 transformation experiments were done.

**Results and Discussion**

**Effect of the Treatments on Agrobacterium Population**

The transformations were done with *Agrobacterium* carrying the RNAi construct to knock-out 5 β-glucosidase genes (pOpOff2/Kn5) in Koshihikari. The *Agrobacterium* populations on the calli after co-cultivation were determined by measuring the OD₆₀₀ value of *Agrobacterium* suspension. The results showed that the treatment supplemented with dex to activate the production of dsRNA (Kn5) had higher level of *Agrobacterium* population than the control (Table 2).

**Table 2** The relative level of *Agrobacterium* population.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Agrobacterium population (OD₆₀₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R1</td>
</tr>
<tr>
<td>No dex</td>
<td>0.025 (+)</td>
</tr>
<tr>
<td>With dex</td>
<td>0.037 (+)</td>
</tr>
</tbody>
</table>

R: replication; (+) means the same *Agrobacterium* population as control; (+++) means the *Agrobacterium* population higher than control.

These data indicated that adding dex to knock down the expression of β-glucosidase genes could effect the *Agrobacterium* population in the transformation process. Since adding dex on the co-culture medium for 10 days, did not affect the un-infected calli (data not shown). Dex also did not show any effect on the growth of *Agrobacterium*. It means that dex treatment does not harm the uninfected calli or affect *Agrobacterium* population. Numerous studies have shown that the suppression of the target gene expression by dbRNA can happen within 24 hours in many organisms (Akashi et al., 2001; Makimura et al., 2002; Wang et al., 2005; Rothermel et al., 2006). In this research, the calli were co-cultured with *Agrobacterium* for 3 days. The T-DNA to knock down the β-glucosidase genes could be integrated into the rice genome and dbRNA expressed and induce the knocking down of β-glucosidase genes via the RNAi mechanism. The pOpOff2/Kn5 transformed calli (4 weeks of selection) after 24 hours dex treatment and GUS staining (Figure 1) indicated that the dex did activate the *Gus* gene expression which should also activate the Kn5 dbRNA expression. All of the transformed calli showed expression of *Gus* gene when treated with dex. These data suggested that adding dex can induce the Kn5 RNAi and GUS production.

**Effect of the Treatments on Efficiency of Transformation**

In this experiment, the treatment was done by adding dex on the surface of the co-culture medium and compare with the control (no dex added). The calli were co-cultured with *Agrobacterium* for 3 days in the dark at 25°C. After extensive washing, the calli were transferred to selection medium. Theoretically, on selection medium non-transformed calli will turned brown with time. The percentage of brown calli on selection medium was evaluated to reflect the efficiency of transformation in different treatments.

![Figure 2 GUS staining of non-transformed calli (A) and 4 weeks of transformed calli (B) after treating with dex.](image)
The results showed that the control treatment had lower number of brown calli after 2 weeks on selection medium than the treatment. When adding chemical inducer, dex in order to induce the RNAi to knock down 5 β-glucosidase genes, the results showed high number of Agrobacterium population (Table 2) and also higher percent of brown calli (Table 3). These results indicated that when five β-glucosidase genes were knocked down more Agrobacterium development were observed. This caused over growth of Agrobacterium. This may be due to the defense system of calli became inactive by the RNAi knock down of the b-glucosidases so that the Agrobacterium could easily break down the calli defense mechanism. Consequently, the calli became more sensitive and damaged. The structures of calli in the dex treatment were broken and damaged. Although the calli after 3 days of co-culture were washed extensively to get rid of the Agrobacterium, the calli were not healthy on the selection medium. After 2 weeks, these calli became weak, turned brown and died.

Conclusions

Although we cannot give the exact answers of how rice β-glucosidases contribute to the Agrobacterium transformation process, we have shown the evidence that rice β-glucosidases could play an important role in Agrobacterium transformation process. On the one hand, adding dex to induce the RNAi to knock down the β-glucosidase genes, the treatment reduced the efficiency of transformation when compare to control. On the other hand, knocking down gene expression also increased the growth of Agrobacterium on the surface of calli. In conclusion, our results supported that the five β-glucosidase genes could play an important role in defense of callus to Agrobacterium infection. However, to give the exact answer for the functions of β-glucosidase genes in Agrobacterium transformation process more investigation is needed.

Acknowledgements

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References


Bioactivities of *Carica papaya* Latex Extract

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²Food Technology Program, School of Agro-Industry, Mae Fah Luang University, Muang, Chiang Rai, 57100, Thailand,
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Abstract

Bioactivities of latex tapping from newly and repeated wounded papaya fruit were investigated. Four types of papaya latex including the first time tapped liquid latex (FL), the first time clotting latex (FC), repeated tapped liquid latex (RL) and repeated tapped clotting latex (RC) were used. All papaya latex samples were fractionated with ultracentrifugation employing 3, 10 and 30 kDa MW cut-off membranes. The highest protease activity of 10,789 units was found in the crude latex of RL, while the highest protein content of 300.6 mg was observed in that of FC. After fractionation, the protease activities (more than 80% of crude latex) and proteins were found in the fractions of MW >30 kDa and almost absent in the fractions of MW lower 10 kDa. On the other hand, the potential antioxidant activities with IC₅₀ 1.13-1.64 µg/mL (assayed by ABTS method) were obtained in the fractions of low MW, especially those of ≤ 3 kDa. The crude latex and >30 kDa fractions showed high absorbance readings at UVB range and could be comparable to that of oxybenzone at around 282 nm. This study showed potential bioactivities of papaya latex extract for food and cosmetic applications.

Keywords: antioxidant, *carica papaya* latex, phenolic, protease activity, protein

Introduction

Cosmetics and food industries often concentrate on natural products with high levels of bioactivity, especially antioxidants. It is believed that these natural compounds are safe, easily absorbed and utilized by human body. Consequently, a vast number of researches were documented for the discovery of new substances from plants and other living organisms for those requirements.

Papaya (*Carica papaya* Linn.), a soft-stemmed and unbranched tree, is widely cultivated in tropical and subtropical regions around the world for its edible fruit (Azarkan *et al.*, 2003). Papaya latex abrupt release is a mechanical wounding response of the plant. It rapidly coagulates and seals the injured area to prevent further entry of pathogens into the phloem. Papaya latex is a thixotropic fluid with a milky appearance that contains about 85% water. Approximately 15% of dry matter consists of both soluble and insoluble parts (Azarkan *et al.*, 2003). The insoluble particulate fraction is tightly associated with lipase and other practically unknown compounds. On the other hand, the soluble fraction contains both the usual ingredients such as carbohydrate (~10%), salts (~10%) and lipids (~5%), and representative biomolecules for instance, cysteine proteases (~30%), several other proteins (~10%) such as superoxide dismutase (SOD) and glutathione.
Altogether, papaya cysteine proteases make up to 40% of the dry matter (Azarkan et al., 2003) and account for more than 80% of the whole enzyme fraction (Oberg et al., 1998). Presumably, they circulate freely within the laticiferous cell with their concentrations higher than 1 mM (Oberg et al., 1998). Glutathione possesses high efficiency as an antioxidant and tyrosinase inhibitor, while superoxide dismutase (SOD) has the ability to eliminate oxygen free radical.

After injury the papaya tree will release the latex and then the bleeding process goes on for a few minutes until a latex clot forms on the affected area for further repair and wound healing. This defense process strongly resembles blood coagulation and clot formation during wounding in mammals. Therefore, papaya latex is expected to contain a key substance stimulating new cell formation like collagen and elastin (Banchhor and Saraf, 2008). The finding on this attractive topic will be useful for cosmetics and other applications in the future.

Therefore, in this research papaya latex will be fractionated into 4 ranges of molecular weight fractions. Biological activity such as proteolytic activity, protein content, total phenolic content, and antioxidant activity will be determined. Results obtained from this research will be beneficial for cosmetic application offering attractive alternative ingredient with synergistic activity.

Materials and Methods

Chemicals and Plant Materials

Folin–Ciocalteu phenol reagent, bovine serum albumin (BSA), casein and cysteine were obtained from Fluka. ABTS (2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and Coomassie Brilliant Blue G-250 were purchased from Sigma. Potassium phosphate (K2HPO4), dipotassium phosphate (KH2PO4), trichloroacetic acid (TCA) and other chemicals with analytical grade were obtained from Merck.

Papaya Latex Collection

Latex from unripe papaya fruits (average 700 g weight and 4 months age) was collected in the early morning at 5.00–7.30 AM during June 2010 from locally grown papaya tree in Tasud District, Muang, Chiang Rai. Papaya latex samples obtained in this research were from only one tree. The liquid latex obtained from newly wound of papaya fruit was collected as the 1st liquid tapping latex (FL). After the liquid latex exuded for 2-3 min, the milky latex becomes clotting. The clotting latex obtained from newly incision of papaya fruit was collected as the 1st clotting tapping latex (FC). Other samples of papaya latexes were collected in the same manner after 7 days. The milky latex collected from the already made incision fruit was designed as repeated liquid tapping latex (RL). After the milky latex expelled from this already wounded fruit, it becomes clotting. This form of latex was collected and named as repeated clotting latex (RC).

Crude Latex Preparation

Papaya latex was mixed with DI water at the ratio of 1:1 (w/v) with agitation for 10 min and then centrifuged at 5,000 g, 4°C for 45 min. The obtained supernatant was collected and referred to crude latex. The crude papaya latex was then fractionated by ultrafiltration using ultracentrifuge tube. Molecular weight (MW) cut-off the tubes were 30, 10 and 3 kDa. Therefore, the fractions obtained from the crude would be > 30 kDa, > 10 - 30 kDa, > 3 - 10 kDa and ≤ 3 kDa. The papaya latex fractions were stored at –20°C for future experiment.

Proteolytic Activity Determination

Due to feasibility for application of papaya latex extract in cosmetic is the main purpose, pH value of 8.0 was used in the proteolytic activity assay for achievability of enzyme using in cleansing product which possess the pH around 8-9. An enzyme sample of 0.10 ml was mixed with 1.10 ml of 1% (w/v) casein in 0.10 M Tris-HCl, pH 8.0 containing 12 mM cysteine. The reaction was started by incubating the mixture at 37°C for
20 min. The reaction was stopped by adding 1.8 ml of 5% TCA. After centrifugation at 3,000xg for 15 min, the UV absorption of the soluble peptide in supernatant was measured at 280 nm. One unit of proteolytic activity was defined as the amount of enzyme releasing the product equivalent to 1 mmole tyrosine min⁻¹ at assayed condition (Chaiwut et al., 2007).

**Protein Determination**

The protein concentration in the sample was measured using the Bradford method (Bradford, 1976). The sample at appropriate concentration was mixed with distilled water and 5.0 mL of Bradford reagent. The mixture was kept at room temperature for 15 min and then the absorbance was measured at 595 nm by using UV-VIS spectrophotometer. The amount of protein in the extracted sample was calculated by using a BSA (1-10 µg/mL) standard curve.

**Determination of Phenolic Content**

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method, which was modified from Soong and Barlow (Soong and Barlow, 2006). Each extract (0.1 mL) was mixed with 0.75 mL of Folin–Ciocalteu reagent and 0.50 mL of 2% (w/v) Na₂CO₃. The volume of the reaction mixture was adjusted to 2.50 mL with deionized water. The mixture was then thoroughly mixed using a vortex and allowed to stand for 30 min. The absorbance at 760 nm of a mixture was measured by using a UV-VIS spectrophotometer. The concentration of EPC was expressed as mg per mL of sample using a linear equation of gallic acid as a standard. All the studies were carried out at least in triplicate.

**ABTS Radical Scavenging Assay**

The ABTS assay was performed followed the method of Re et al., (1999) with a slight modification. ABTS was dissolved in distilled water to give a 7 mM concentration. ABTS radical cation (ABTS⁺) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate. The mixture was stored at room temperature in the dark for 16 h. For the study, ABTS⁺ was diluted with phosphate buffer saline, pH 7.4 to an absorbance 0.70-0.80 at 734 nm. Sample (50 µL) was mixed with 1450 µL of ABTS solution and the mixture was left at room temperature for 20 min in dark. The absorbance was then measured at 734 nm using the spectrophotometer. The antioxidant activity was expressed as IC₅₀ which was obtained from plotting between % ABTS scavenging and concentration of the sample.

The percentage ABTS scavenging was calculated as:

\[
\text{% Radical inhibition} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

where; \(A_{\text{control}}\) is the absorption at 734 nm of ABTS solution without sample.
\(A_{\text{sample}}\) is the absorption at 734 nm of ABTS solution with sample.

**UV Absorption Scanning**

Papaya latex Fractions of >30 kDa was diluted to proper concentration. Oxybenzone was used as standard UV absorber. All samples were scanned the absorption in the wavelength ranged 200-800 nm using UV-spectrophotometer (Biochrom/Libra S22, England).

**Statistical Analysis**

All chemical analyses were performed in triplicate. Data were subjected to analysis of variance (ANOVA). Analysis was performed by using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

**Results and Discussion**

**Crude Latex Preparation**

There were 4 types of crude papaya latex samples; liquid latex obtained from the first time tapping of new fruit called 1st liquid (FL), clotted latex obtained from the first time tapping of new fruit called 1st clotting (FC), liquid latex obtained from repeated tapping of
the fruit called repeated liquid (RL) and clotted latex obtained from repeated tapping of the fruit called repeated clotting (RC). All of these 4 crude latexes were fractionated into 4 fractions distinguished by molecular weight (MW) range; > 30 kDa, >10–30 kDa, >3-10 kDa and ≤3 kDa as shown in Table 1.

Protease Activity and Protein Content

Enzyme activities of all sample fractions were assayed by casein digestion method. Table 1. revealed that, apart from crude extract, the fractions >30 kDa possessed the highest protein content and total protease activity. This was due to the papaya cysteine proteases as well as major proteins consisting in papaya latex were mainly retained above the filter with 30 kDa MW cut-off. There were some protease activities present in the fraction of MW >10–30 kDa. This was possibly from some papaya cysteine protease contained in this fraction. A vast number of works have been documented that these proteases exhibit the relative molecular mass in the range of 23–26 kDa (Moutim et al., 1999; Azarkan et al., 2004).

However, though the papaya protease have their MW of 23–26 kDa, the results shown in Table 1. displaying the proteolytic activities were mostly found at >30 kDa fractions. This may be due to the initial presence of the enzymes immature forms. This agrees with the previous reports (Thomas et al., 2009; Chaiwut et al., 2010; Mousaoui et al., 2001). Generally, the 4 papaya protease are all synthesized as pro-enzymes in which the mature forms usually contain from 212 to 218 amino acids and are preceded by long pro-sequenced (Mousaoui et al., 2001). The presence of pro-regions seems to be required for proper folding of the mature enzymes. From this regards the pro-proteases exhibit MW of around 31.8 to 32.6 kDa. There has been reported that papain is synthesized as a 40 kDa inactive precursor with a 170 amino acid N-terminal pro-region (Mousaoui et al., 2001).

Bradford method was used to determine the protein content due to its very fast and convenient assay. Similarly to protease activity, protein concentrations of fractions >30 kDa were higher than others including the crude latexes. From the results shown in Table 1., the most of protein contents were pooled in the fraction of MW >30 kDa.

Table 1. Protease activity and protein concentration of papaya latex fractions obtaining from 7 g fresh papaya latex.

<table>
<thead>
<tr>
<th>Fraction Sample</th>
<th>Total activity (Unit)</th>
<th>Total Protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude FL</td>
<td>2,305.17 ± 113.72a/c/</td>
<td>296.46 ± 2.86a/c/</td>
</tr>
<tr>
<td>FC</td>
<td>3,288.06 ± 124.56b/a</td>
<td>300.60 ± 1.25b/a</td>
</tr>
<tr>
<td>RL</td>
<td>10,788.93 ± 92.49d/a</td>
<td>293.85 ± 3.73d/a</td>
</tr>
<tr>
<td>RC</td>
<td>9,717.75 ± 105.64d/a</td>
<td>286.11 ± 2.57d/a</td>
</tr>
<tr>
<td>&gt; 30 kDa FL</td>
<td>1,976.64 ± 11.32b/a</td>
<td>282.50 ± 2.58b/a</td>
</tr>
<tr>
<td>FC</td>
<td>745.34 ± 19.22a/b</td>
<td>289.64 ± 3.41a/b</td>
</tr>
<tr>
<td>RL</td>
<td>8,669.30 ± 22.56c/a</td>
<td>281.38 ± 3.83a/b</td>
</tr>
<tr>
<td>RC</td>
<td>8,049.44 ± 16.01c/a</td>
<td>279.92 ± 1.43c/a</td>
</tr>
<tr>
<td>&gt;10–30 kDa FL</td>
<td>12.62 ± 0.61a/b</td>
<td>0.94 ± 0.16a/b</td>
</tr>
<tr>
<td>FC</td>
<td>17.38 ± 0.07b/a</td>
<td>2.74 ± 0.28b/a</td>
</tr>
<tr>
<td>RL</td>
<td>12.20 ± 0.13a/b</td>
<td>2.06 ± 0.17b/a</td>
</tr>
<tr>
<td>RC</td>
<td>12.64 ± 0.97a/b</td>
<td>2.06 ± 0.20b/a</td>
</tr>
<tr>
<td>&gt;3-10 kDa FL</td>
<td>3.40 ± 0.01c/a</td>
<td>0.44 ± 0.06c/a</td>
</tr>
<tr>
<td>FC</td>
<td>1.32 ± 0.00c/a</td>
<td>0.20 ± 0.00c/a</td>
</tr>
<tr>
<td>RL</td>
<td>2.24 ± 0.00b/a</td>
<td>0.20 ± 0.00b/a</td>
</tr>
<tr>
<td>RC</td>
<td>2.82 ± 0.04b/a</td>
<td>0.62 ± 0.01c/a</td>
</tr>
<tr>
<td>≤ 3 kDa FL</td>
<td>0.46 ± 0.00a/b</td>
<td>0.16 ± 0.04a/b</td>
</tr>
<tr>
<td>FC</td>
<td>0.90 ± 0.05b/a</td>
<td>0.20 ± 0.02b/a</td>
</tr>
<tr>
<td>RL</td>
<td>1.92 ± 0.01c/a</td>
<td>0.20 ± 0.03b/a</td>
</tr>
<tr>
<td>RC</td>
<td>0.36 ± 0.00a/b</td>
<td>0.16 ± 0.03a/b</td>
</tr>
</tbody>
</table>

Values followed by different letters compared in each papaya latex fraction sample within the same column are significantly different (P<0.05).
acid. The chitinase and the trypsin inhibitor are members of the so called pro-region protein. Their induction after wounding can thus, be explained by the role they can fulfill in the plant defense mechanism (Azarkan et al., 2006).

Among the crude fractions, RL showed the highest activity of 10,789 units, while the highest protein of 300.6 mg was belonged to the FC. According to the newly tapped latex (FL and FC), protease activity of FC was lower than FL. This agrees with previous report that the activity of papaya protease gradually decreased 60% at 3 min after injured (Moutim et al., 1999). The fraction of MW >3–10 kDa and ≤3 kDa exhibited rather low protein content and protease activity. This might be due to the limit movement of major proteins and papaya protease through the membrane of 10 kDa pore size.

**Phenolic Content and Antioxidant Capacity**

The total phenolic content of papaya latexes are shown in Fig. 2. It can be observed that total phenolic contents were highly increased in the fraction of MW 30 kDa. However in the lower MW fractions (>10–30, >3–10 and ≤3 kDa), the phenolics were dramatically reduced and their values were not significant different \((P > 0.05)\). However, high concentrations of phenolics found in the fraction of MW >30 kDa and the crude extract might be representative of the protein content which probably reacted with Folin reagent.

The antioxidant capacity by using ABTS radical scavenging assay of papaya latex extract is present in Fig. 3. Although the fractions of MW ≥30 kDa exhibited high protein content, high protease activity and phenolic content, they contained much lower capacity of radical scavenging capacity. The IC\(_{50}\) value represents the concentration of extract which inhibited 50% of the ABTS free radical. The low IC\(_{50}\) value suggested the high antioxidant capacity.

When comparing with standard BHT (IC\(_{50}\) 350 µg/ml), all fractions of papaya latex samples showed very much higher antioxidant activity. From the result, papaya latex fractions of MW lower than 30 kDa displayed their very high radical scavenging IC\(_{50}\) 1.13-1.64 µg/mL.

The antioxidant capacity observed in the papaya latex fraction could possibly be due to the presence of some other phytochemicals such as peptides and other compounds which also contribute to the total antioxidant capacity. There have been reports on peptide showing excellent antioxidant activity (Tironi and Anon, 2010; Liu et al., 2010) and the presence of carenolides (Oloyede, 2005), glutathione and other reducing agent in papaya latex possibly with good antioxidation activity (Moussaoui et al., 2001).

**UV Absorption Scanning**

Ability of papaya latex fraction to absorb UV was studied. This would be useful in application of papaya latex fraction as a sunscreen agent. The UV absorption of papaya latex fraction MW >30 kDa is shown in Fig. 4. All samples mostly absorbed the UV range of 200 – 280 nm which belonging to UVC and UVB ranges (190–240, 240-320 nm, respectively).
Antioxidant capacity of papaya latex fraction (FL; liquid latex obtained from first time tapping, FC; clotting latex obtained from first time tapping, RL; liquid latex obtained from repeated tapping and RC; clotting latex obtained from repeated tapping) comparing to IC50 350 µg/mL of standard BHT. Different letters indicate significant difference among the samples (P<0.05).

The standard oxybenzone showed good absorption in wide ranges of UV. It is notable that the amount of oxybenzone used (5 µg/ml) was 170 times higher than those of papaya latex fractions. The result showed a good indication that papaya latex fractions could be used as UVB absorbent.

Conclusions

Papaya latex fraction of MW lower than 30 kDa displayed much higher antioxidant capacity with IC50 values ranged from 1.13-1.64 ng/mL than those of higher MW fractions. These antioxidant capacities were comparable to the efficacy of BHT. Papaya latex fraction also exhibited good UV absorption property. The result revealed the papaya latex might be good source for other bioactive compound besides the proteolytic enzymes.

Acknowledgments

The authors would like to thank Mae Fah Luang University for financial support.

References


Soong, Y. Y. and Barlow, P. J. 2006. Quantification of gallic acid and ellagic acid from longan (Dimocarpus longan Lour.) seed and mango (Mangifera indica L.) kernal and their effects on antioxidant activity. Food Chem. 97 : 524–530.

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Extraction of Phenolic Antioxidants From Peels and Seeds of the Royal Project’s Fruits

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Abstract
The values added of peels and seeds of Royal Project’s fruits, i.e. papaya (Carica papaya L.), cantaloupe (Cucumis melo var. cantalupensis), muskmelon (Cucumis melo var. reticulates) and Japanese pumpkin (Cucurbita maxima) were carried by using them as a source for phenolic antioxidants extraction. In this study, effects of different ratios of ethanol and water (80:20, 50:50 and 20:80) and extraction times (0, 2, 4 and 8 h) on phenolic content and antioxidant activity were investigated using Folin-Ciocalteu method and 2,2-diphenyl-1-picrylhydraxyl (DPPH) radical-scavenging assay, respectively. The results showed that ethanol and water of 80:20 was the optimum ratio for phenolic compounds extraction from the most extracts, while the optimum for extraction of antioxidant activity was varied depending on the type of the samples. The time of 2 h was the optimum condition to extract antioxidants from Japanese pumpkin peels (JP), papaya seeds (PS), Japanese pumpkin seeds (JS), cantaloupe peels (CP), cantaloupe seeds (CS) and muskmelon seeds (MS), whereas optimum extraction time of 8 h was obtained from papaya peels (PP) and muskmelon peels (MP). Among the fruit extract tested, papaya and muskmelon peel extracts had high antioxidant potential with IC₅₀ value of 0.75±0.01 and 1.20±0.03 mg mL⁻¹, respectively. They could be used as an alternative source for antioxidant extraction.

Keywords: antioxidant, peel, phenolic, Royal project’s fruit, seed

Introduction
Antioxidants are both natural and artificial (synthetic) compounds which are capable to scavenge free radicals and to inhibit oxidation processes. However, artificial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) that have been widely used for preventing lipid peroxidation in several food products are gradually limited and controlled in the food industry because they are suspected to be toxic and carcinogenic (Namiki, 1990). Therefore, the development and extraction of natural antioxidants from natural plants are more attractive and have been highly studied for new antioxidants. Many plants such as fruits and vegetables contain various phenolic compounds, which can possess antioxidant activity (Grigonis et al., 2005; Maisuthisakul et al., 2007). Several isolated plant constituents as well as crude extracts of fruits and vegetables have been well recognized as natural antioxidants that
possess beneficial effects against free radicals in biological systems (Prasad et al., 2005). These extracts can be employed in cosmetic, pharmaceutical and food applications.

Consumption of the Royal Project fruits have been increasing continuously in recent years due to their favors, qualities, and bioactivities. However, high consuming of these fruit also produce high amount of their wastes such as peels and seeds. Besides the flesh fruits, these wastes have also been found the natural antioxidant, mainly phenolic compounds such as flavonoid, catechins, tannins, as well as terpenoids and other bioactive compounds. Therefore, the aim of this study was targeted to investigate phenolic antioxidants activities from the seeds and peels of four Royal project’s fruits, i.e., papaya (Carica papaya L.), cantaloupe (Cucumis melo var. cantalupensis), muskmelon (Cucumis melo var. reticulates.) and Japanese pumpkin (Cucurbita maxima) that are generally considered as wastes.

Materials and Methods

Chemicals and Raw Materials

Ethanol was purchased from Merck, Germany. Folin–Ciocalteu phenol reagent was obtained from Fluka (Steinheim, Germany). 2,2′-diphenyl-picrylhydrazyl (DPPH), gallic acid was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Papaya (Carica papaya L.), cantaloupe (Cucumis melo var. cantalupensis), muskmelon (Cucumis melo var. reticulates.) and Japanese pumpkin (Cucurbita maxima) were purchased from The Royal Project Foundation shop, Chiang Rai and Chiang Mai Province, Thailand.

Preparation of Samples

After separation of the pulp of samples, all peels and seeds were dried by hot air oven at 55°C until their weights were constant. The peels and seeds were stored at -20°C until used. Study of optimum ethanol:water (EtOH: H₂O) ratio for extraction of phenolic antioxidants

The ratio between sample to solvent was fixed at 1:6 (g mL⁻¹). Various ratios of EtOH:H₂O (80:20, 50:50, 20:80, v/v) affecting the extraction efficiency were studied. The mixture was shaken for 24 h and then filtered through Whatman filter paper No.1. The filtrate was centrifuged at 10,000 rpm for 15 min. The obtained volume of supernatant was recorded and kept at 4 °C until used. Study of optimum extraction time for extraction of phenolic antioxidants

Various extraction times affecting to the extraction efficiency were also determined. The ratio of sample to solvent was still fixed at 1:6 (g mL⁻¹). The mixture was shaken for 0, 2, 4 and 8 h then filtered through the Whatman filter paper No.1. The filtrate was centrifuged at 10,000 rpm for 15 min. The obtained volume of supernatant was recorded and kept at 4 °C until used.

Determination of Extractable Phenolic Content (EPC)

Extractable phenolic content (EPC) was determined by the Folin–Ciocalteu method, which was adapted from Swain and Hillis (1959). Each extract (0.1 mL) was mixed with 0.75 mL of Folin–Ciocalteu reagent and 0.50 mL of 2% (w/v) Na₂CO₃. The volume of the reaction mixture was adjusted to 2.50 mL with deionized water. The mixture was then thoroughly mixed using a vortex and stand for 30 min. The absorbance at 760 nm of a mixture was measured by using a UV-spectrophotometer (Biochrom/Libra S22, England). The concentration of EPC was expressed as mg per dry weight of samples.

Determinations of Antioxidant Activity

DPPH radical scavenging activity of fruit...
peel and seed extracts were determined as described by Brand-Williams et al. (1995) with a slight modification. The DPPH radical (0.004%) was prepared by dissolving DPPH with 95% ethanol. The extracts with various amounts were added to 1 mL of the DPPH radical solution and the final volume of 2.50 mL was adjusted with 95% ethanol. After 30 min, the absorbance was measured at 517 nm. The inhibitory effect of the extract was expressed as extract concentration causing 50% loss of enzyme activity (IC50).

Statistical Analysis
The all experiments were performed in triplicate. One way analysis of variance (ANOVA) and Duncan’s New Multiple-range test were determined for the differences among the means using SPSS package version 11.5 for Windows (SPSS Inc., Chicago, USA). P-values less than 0.05 (<0.05) were regarded as significant.

Results and Discussion

Preparation of Peel and Seed Samples
Waste seeds and peels of each sample were dried by hot air oven at 55°C until their weights were constant. The weights after drying were aligned in Table 1. The dried peels were more loss their moistures obtaining the yields in a range of 7.60 to 20.28%. In contrast, the fruit seeds were more retained their moisture after drying, especially CS, which most water was still retained in the seed. This is probably due to seed coat of CS is quite strengthen when compared to those of PS, JS and MS.

Effect of Ethanol:Water (EtOH:H2O) Ratio
Peels and seeds extracts were obtained with various solvent ratios of EtOH:H2O. The extractable phenolic contents (EPC) of extracted peel and seed samples were measured using the Folin-Ciocalteu method. The results shown in Figure 1 indicate that the highest EPC of 2.014 mg GAE g⁻¹ was found in the extract of papaya peels (PP) extract whereas the lowest EPC of 0.339 mg GAE g⁻¹ was found in the extract from Japanese pumpkin seed (JS).

The total free radical-scavenging capacity of sample was determined by using the DPPH method and evaluated in term of IC50 value. The lowest IC50 represents the highest antioxidant capacity. The results showed that the IC50 values derived from both seeds and peels for all fruits sample were present in the range of 0.682±0.088 to 23.610±2.289 mg mL⁻¹. From the Figure 1, it can be summarized that ethanol and water of 80:20 was the optimum ratio for phenolic compounds extraction from the most extracts, while the optimum ratio of solvent for extraction of antioxidant activity was varied depending on the type of the samples. Water and ethanol are commonly used to extract phytochemicals from plant due to absence of toxicity (Maisuthisakul et al., 2009). These extractants have been extensively used to extract the bioactive compounds from fruit wastes such as mango (Arogba, 2000; Guo et al., 2003; Soong and Barlow, 2004, 2006; Maisuthisakul et al., 2009).

Effect of Extraction Time
The optimum ratio of EtOH:H2O (50:50 v/v⁻¹) was used as a medium for determination of extraction time affecting the phenolic antioxidant separation from the peel and seed of the Royal Project fruit samples. The optimum time of extraction was estimated from the IC50 value in which the lowest value was the highest antioxidant capacity. The time of 2 h was the optimum condition to extract antioxidants from Japanese pumpkin peels (JP), papaya seeds (PS), Japanese pumpkin seeds (JS), cantaloupe peels (CP), cantaloupe seeds (CS) and muskmelon seeds (MS), whereas optimum extraction time of 8 h...
Table 1 Percent yield of seed and peel of the Royal Project’s fruits.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fresh weight (g)</th>
<th>Dried weight (g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP</td>
<td>1,689.50</td>
<td>213.73</td>
<td>12.65</td>
</tr>
<tr>
<td>JP</td>
<td>1,173.00</td>
<td>237.87</td>
<td>20.28</td>
</tr>
<tr>
<td>CP</td>
<td>3,215.50</td>
<td>244.27</td>
<td>7.60</td>
</tr>
<tr>
<td>MP</td>
<td>1,613.20</td>
<td>171.69</td>
<td>10.64</td>
</tr>
<tr>
<td>PS</td>
<td>234.00</td>
<td>68.79</td>
<td>29.40</td>
</tr>
<tr>
<td>JS</td>
<td>236.10</td>
<td>66.32</td>
<td>28.09</td>
</tr>
<tr>
<td>CS</td>
<td>164.20</td>
<td>62.56</td>
<td>38.10</td>
</tr>
<tr>
<td>MS</td>
<td>84.33</td>
<td>25.48</td>
<td>30.21</td>
</tr>
</tbody>
</table>

was obtained from papaya peels (PP) and muskmelon peels (MP) (Table 2).

Comparison of Antioxidants Activity in Peel and Seed Extracts From the 4 Royal Project’s Fruits

The antioxidant capacities (IC$_{50}$ values) in the seed and peel from the 4 Royal Project’s fruits extracted under each optimum extraction time were compared. As result shown in Table 2, papaya and muskmelon peel extracts had significantly highest antioxidant potential in which the IC$_{50}$ value was equivalent to 0.75±0.01 mg mL$^{-1}$ and 1.20±0.03 mg mL$^{-1}$, respectively. These 2 values were significantly higher about 10 times than BHT. As previous report, Leong and Shui, 2002 found that papaya have higher primary antioxidant potential, as measured by scavenging DPPH and iron (III) reducing assays. In our experiment, it can be summarized that papaya peels and muskmelon peel could be an alternative source for efficient antioxidant extraction.

Figure 1 EPC (A and B) and antioxidant activity, IC$_{50}$ (C and D) in fruit peels and seeds extracted with different ratios of EtOH:H$_2$O (20:80, 50:50 and 80:20) : Papaya peel, PP; Papaya seed, JS; Japanese pumpkin peel, JP; Japanese Pumpkin seed, JS; Cantaloupe peel, CP; Cantaloupe seed CS; and Muskmelon peel; and Muskmelon seed MS. Bars show SD values from triplicate analysis.
Table 2 Comparison of antioxidant activity (IC$_{50}$) in Royal Project fruit seed and peel extracts under optimum time of extraction.

<table>
<thead>
<tr>
<th>Fruit</th>
<th>Part</th>
<th>Extraction time (h)</th>
<th>IC$_{50}$ (mg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Papaya</td>
<td>Peel</td>
<td>8</td>
<td>0.75±0.01$^{a2}$</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>2</td>
<td>1.67±0.13$^{b}$</td>
</tr>
<tr>
<td>Japanese pumpkin</td>
<td>Peel</td>
<td>2</td>
<td>12.57±2.25$^{d}$</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>2</td>
<td>15.72±0.65$^{e}$</td>
</tr>
<tr>
<td>Cantaloupe</td>
<td>Peel</td>
<td>2</td>
<td>16.65±1.13$^{f}$</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>2</td>
<td>12.50±0.88$^{d}$</td>
</tr>
<tr>
<td>Muskmelon</td>
<td>Peel</td>
<td>8</td>
<td>1.20±0.03$^{a}$</td>
</tr>
<tr>
<td></td>
<td>Seed</td>
<td>2</td>
<td>4.56±0.11$^{c}$</td>
</tr>
<tr>
<td>BHT</td>
<td>-</td>
<td>-</td>
<td>11.97±0.55$^{d}$</td>
</tr>
</tbody>
</table>

$^{1}$Values are given as mean ± S.D from triplicate determinations.

$^{2}$Different letters in the same column indicate significant differences (p<0.05).

Conclusions

In this study, papaya and muskmelon extracts seem to have high antioxidant potential, especially papaya peels (PS) and muskmelon peels with IC$_{50}$ value of 0.75±0.01 and 1.20±0.01 mg mL$^{-1}$, respectively. This indicates that two of them are possibly applied as a strong antioxidant for replacing the use of synthetic ingredient on cosmetics. Moreover, the use of wastes from Royal Project’s fruits will be an alternative way for value-add the wastes and subsequently increase the income of Thai’s agriculturist.

Acknowledgments

We gratefully acknowledge Mae Fah Luang University for partially financial support.

References


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Antioxidant Capacity and Total Phenolic Content of Moringa oleifera Grown in Chiang Mai, Thailand

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Abstract

Leaves, pods and seeds of Moringa oleifera (variety: Num Phare, Ang Thong, and PKM1) grown in Chiang Mai, Thailand, were extracted with water or 95% ethanol and their antioxidant capacities were estimated using a Ferric Reducing Antioxidant Power (FRAP), an improved ABTS radical cation decolorization assay, and a DPPH free radical scavenging activity assay; together with determination of their total phenolic contents by the Folin-ciocalteau micro method. The results of aqueous extracts (0.80 – 12.25 mg standard equivalent per gram of dry weight) were greater than those of ethanolic extracts (0.40 – 8.85 mg standard equivalent per gram of dry weight). Leaf extracts (1.80 – 12.25 mg standard equivalent per gram of dry weight) showed the highest antioxidant capacity and total phenolic content, followed by those of pods (1.80 – 6.54 mg standard equivalent per gram of dry weight) and seeds (0.40 – 1.79 mg standard equivalent per gram of dry weight), respectively. Leaf extracts of Ang Thong and PKM1 varieties (2.08 – 12.25 mg standard equivalent per gram of dry weight) were composed of higher antioxidant capacities and total phenolic contents than those of Num Phare variety (1.80 – 10.10 mg standard equivalent per gram of dry weight). Pod extracts of Ang Thong and PKM1 varieties (3.53 – 6.54 mg standard equivalent per gram of dry weight) consisted of higher reducing power (FRAP values) and total phenolic contents than those of Num Phare variety (2.59 – 3.61 mg standard equivalent per gram of dry weight). The FRAP value and total phenolic content of Ang Thong seed extracts (0.95 – 1.65 mg standard equivalent per gram of dry weight) were larger than those of PKM1 and Num Phare seed extracts (0.73 – 1.30 mg standard equivalent per gram of dry weight) while the ability to quench both ABTS and DPPH radicals of PKM1 seed extracts (0.48 – 1.79 mg standard equivalent per gram of dry weight) were better than those of Ang Thong and Num Phare seed extracts (0.40 – 1.13 mg standard equivalent per gram of dry weight).

Keywords: Moringa oleifera, antioxidant, phenolic content

Introduction

Oxidative damage in the human body is a crucial etiological factor contributing to the aging process and several chronic diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, neurodegenerative diseases (Gutteridge, 1995; Pong, 2003). This can occur if antioxidant defenses in the body are inadequate. Therefore, it is desirable to increase the intake of antioxidants from dietary sources (Soong and Barlow, 2004). Safety concerns about the use of synthetic antioxidants, e.g., BHA (butylated hydroxy anisole) and BHT (butylated hydroxyl toluene), have been expressed in recent reports (Sun and Ho, 2005; Hossain et al., 2008), while edible plants rich in antioxidants, especially spices and herbs, have become the focus of current interest (Nakatani, 1997; Rice-Evans et al., 1997).
Moringa oleifera Lamarak commonly known as drumstick tree or horseradish tree, belongs to the Moringaceae family. It is indigenous to many countries in Africa, Arabia, South East Asia, the Pacific and Caribbean Island, and South America (Nadkami, 1976). This plant has been used for several purposes such as foods, medicines (Anwar et al., 2007), animal feeds (Amaglo et al., 2010), and industrial uses (Bhuptawat et al., 2007; Rashid et al., 2008). Ayurvedic medicine has declared that *M. oleifera* can provide the nutrients and therapeutic ingredients to prevent, mitigate, or treat many diseases. Its leaves, fruits, seeds, and roots are used for treating abdominal tumors, hysteria, scurvy, paralytic attacks, helminitic, bladder, prostate troubles, sores and skin infections (Fugie, 1999).

In Thailand, the tender pods, fruits, and leaves of *M. oleifera*, a local vegetable has become more popular recently, especially as a dried leave powder, because Thais believe in its potential therapeutic values against several diseases. The work of Chumark et al. (2008) indicated that leaves of *M. oleifera* possess antioxidant, hypolipidaemic and antiatherosclerotic activities and have therapeutic potential for the prevention of cardiovascular diseases.

Various nutrients and phytochemicals which have been identified and quantified in different tissues of *M. oleifera* show that those tissues could be good sources of nutrients and potential sources of dietary antioxidants (Singh et al., 2009; Amaglo et al., 2010). However, there are many factors which can affect the composition of *M. oleifera* tissues regarding their antioxidant activities. For examples, season and production location (Iqbal and Bhanger, 2006), and stages of maturity (Sreelatha and Padma, 2009) have been proven to influence antioxidant activity of *M. oleifera* leaves.

The objective of this present work was to compare antioxidant capacities (FRAP, DPPH and ABTS) and total phenolic contents of aqueous and ethanolic extracts from leaves, pods, and seeds of *M. oleifera* (variety: Num Phare, Ang Thong, and PKM1) grown in Chiang Mai, Thailand.

**Materials and Methods**

**Chemicals**

- DPPH (2,2-diphenyl-1-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine)[Sigma], ABTS (2,2’-azinobis(3-ethylbenzothiazoline -6-sulfonic acid)), ferric chloride, Folin-Ciocalteu phenol reagent, gallic acid, glacial acetic acid, hydrochloric acid, potassium persulphate, sodium acetate, sodium carbonate, vitamin C [Fluka] were of analytical grade.

**Sample Extraction**

Fresh leaves and pods of *M. oleifera* (variety: Num Phare, Ang Thong, and PKM1) grown in Chiang Mai, Thailand, were harvested in April–June. Leaves and pods were homogenised using a blender, and seeds were ground in mortar. Two grams of blended or ground sample were transferred into a 25 cm x 150 cm tube, and 10 mL of deionized water or 95% ethanol were added. The extraction was carried out using a vortex mixer for 60 s (Modified from Leong and Shui, 2002). The mixture was filtered through Whatman No. 1 paper and the filtrate volume was adjusted to 10 mL for using as extract examples for FRAP, ABTS, DPPH, and total phenolic content assays. A Spectronic 20D+ spectrophotometer of Milton Roy was used for all assays.

**Ferric Reducing Antioxidant Power (FRAP) Assay**

The FRAP assay, a method for measuring total reducing power of electron donating substances, was carried out according to the procedure of Benzie and Strain (1999). Briefly, 6 mL of working FRAP reagent (0.1 M acetate buffer:0.02 M FeCl₃:0.01 M TPTZ = 10:1:1) prepared daily was mixed with 20-50 μL of extract sample. The absorbance at 593 nm was recorded after a 30-min incubation at 37 °C. FRAP values were obtained by comparison with standard curves created using vitamin C (0-15 μg), and are reported as mg vitamin C equivalent per gram of dry weight.
DPPH Free Radical Scavenging Activity
The DPPH method of Brand-Williams et al. (1995), based on the reduction of DPPH radical solution in the presence of hydrogen donating antioxidants, was used with some modifications. 0.8 mM DPPH radical solution in 95% ethanol was prepared. Twenty to fifty µL of the extract sample was diluted to 5.4 mL using deionized water and 95 % ethanol (1:1) before 0.6 mL DPPH solution was added, and shaken vigorously. The decrease of absorbance was recorded at 1 min after mixing. Vitamin C (0-15 µg) was used as the standard, and results were reported as mg vitamin C equivalent per gram of dry weight.

ABTS Radical Cation Decolorization Assay
The ABTS method of Re et al. (1999), based on the ability of antioxidant molecules to quench the long-lived ABTS radical cation (ABTS•+), was used with some modification. The ABTS•+ was produced by reacting 7 mM ABTS stock solution with 2.45 mM postassium persulphate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 hours before use. The ABTS•+ solution was diluted with deionized water and 95 % ethanol (1:1) to an absorbance of 0.70 (+0.02) at 734 nm. Twenty to fifty µL of the extract sample was mixed with 6 mL of diluted ABTS•+ solution. The decrease of absorbance was recorded at 1 min after mixing. Vitamin C (0-15 µg) was used as standard, and results were reported as mg vitamin C equivalent per gram of dry weight.

Total Phenolic Content (TPC)
The Folin-ciocalteau micro method of Waterhouse (n.d.) was used. Twenty to fifty µL of the extract sample was diluted with deionized water to 4.8 mL, and 300 µL Folin-ciocalteau reagent was added and shaken, after which 900 µL of 20% sodium carbonate was added with mixing. The solution was incubated at 40 °C for 30 min before measuring the absorbance at 765 nm. Gallic acid (0-30 µg) was used as standard, and results were reported as mg gallic acid equivalent per gram of dry weight.

Values of FRAP, DPPH, ABTS and TPC (mg standard per gram of dry weight)

\[
= \frac{[(SA - BA)/Slope]x[10/U]}{[2x[1 - MC]x[1,000]]}
\]

When: SA = Sample absorbance for FRAP and TPC values or absorbance decrease of sample for ABTS and DPPH values
BA = Blank (no extract) absorbance for FRAP and TPC values or absorbance decrease of blank for ABTS and DPPH values (extract was substituted by deionized water for blank)
Slope = Slope of standard curve
[10/U] = Total volume of extract (10 mL) divided by used volume of extract (mL)
[2] = Weight of used sample (g)
MC = Moisture content of sample/100 (Table 1)
[1,000] = Factor for changing µg to mg.

Table 1 Moisture content of samples (%w/w)

<table>
<thead>
<tr>
<th></th>
<th>Num Phare</th>
<th>Ang Thong</th>
<th>PKM1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>70.4-77.7</td>
<td>75.4-78.8</td>
<td>73.3-78.1</td>
</tr>
<tr>
<td>Pods</td>
<td>83.4-84.0</td>
<td>83.5-84.9</td>
<td>86.1-88.5</td>
</tr>
<tr>
<td>Seeds</td>
<td>53.3-55.5</td>
<td>49.9-53.3</td>
<td>72.9-73.3</td>
</tr>
</tbody>
</table>

Statistical Analysis
Each determination was repeated three times and was conducted on separate lots of materials. A randomized complete block design (RCBD) was used and the separate lots served as the blocking variable. Mean comparisons were performed by Duncan’s new multiple range test (DMRT) at P <0.05.
Results and Discussion

Antioxidant capacities including FRAP, DPPH and ABTS, and total phenolic contents of almost all of aqueous extracts were higher than those of ethanolic extracts (Table 2). These results agreed with the work of Maksab and Wichairam (2009), and indicated that antioxidant compounds in *M. oleifera* leaves, pods and seeds were water-soluble rather than ethanol-soluble. Leaf extracts showed the highest antioxidant capacity and total phenolic content, followed by extracts from pods and seeds, respectively (Table 2). These results concurred with the findings of Singh et al. (2009) and Amaglo et al. (2010) who reported that leaves had higher contents of phenolic compounds (including gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin) and flavonoids (including glucosides, rutinosides, malonylglycosides and acetylglycosides of kaempferol, quercetin and isorhamnethin) than pods and seeds.

The different values of each antioxidant capacity assay result from the different mechanism of each method. The FRAP assay is a method for measuring total reducing power of electron donating substances, while ABTS and DPPH assays are methods for measuring the ability of antioxidant molecules to quench ABTS and DPPH free radicals, respectively. In addition, Wang et al. (1998) showed that some compounds which have ABTS$^+$ scavenging activity may not show DPPH scavenging activity, and Arts et al. (2004) found that some products of ABTS$^+$ scavenging reaction may have a higher antioxidant capacity and can continually react with ABTS$^{2-}$.

Aqueous extracts from leaves of the Ang Thong and PKM1 varieties had larger antioxidant capacities and total phenolic contents than those of the Num Phare variety (Table 2), suggesting that Ang Thong and PKM1 leaves contained more water-soluble antioxidants and phenolic compounds than Num Phare leaves. With the ethanolic extracts, values of antioxidant capacities and total phenolic contents of extracts from Ang Thong leaves were higher than those from Num Phare and PKM1 leaves (Table 1), implying that Ang Thong leaves contained more ethanol-soluble antioxidants and phenolic compounds than Num Phare and PKM1 leaves.

In addition, ABTS values were higher than FRAP and DPPH values. The leaves for these experiments were harvest in the summer time when the environmental temperatures were highest and, according to the work of Iqbal and Bhanger (2006) this may have resulted in the assay values being lower than they would have been if harvested during a cooler period.

FRAP values and total phenolic contents of aqueous extracts from pods of Ang Thong and PKM1 were better than those of Num Phare (Table 2). This implies that pods of the Ang Thong and PKM1 varieties contained more water-soluble reducing power and phenolic substances. The antioxidant capacities of ethanolic extracts from the Ang Thong and PKM1 pods were higher than those from the Num Phare pods, but the total phenolic content of only the Ang Thong pods had a higher value than the Num Phare pods (Table 2). This implied that the pods of Ang Thong and PKM1 varieties contained more ethanol-soluble antioxidants, but only pods of Ang Thong variety had a higher content of ethanol-soluble phenolic compounds. FRAP and ABTS values of aqueous extracts were higher than DPPH values, while FRAP values of ethanolic extracts were higher than DPPH and ABTS values.

The FRAP value and total phenolic content of aqueous extracts from Ang Thong seeds were larger than those from PKM1 and Num Phare seeds, while ABTS and DPPH values of aqueous extracts from PKM1 variety’s seeds were higher than those from Ang Thong and Num Phare varieties (Table 2). This implies that Ang Thong seeds
have more water-soluble reducing power and phenolic compounds, while PKM1 seeds contain more water-soluble substances with ability to quench ABTS and DPPH radicals. The FRAP and DPPH values as well as the total phenolic contents of ethanolic extracts from Ang Thong seeds were higher (Table 3), indicating their greater content of ethanol-soluble compounds with reducing power and the ability to quench DPPH radicals, and of phenolic substances. DPPH and ABTS values of aqueous extracts of PKM1 seeds were higher than their FRAP values, but FRAP values of all ethanolic

Table 2 Antioxidant capacities and total phenolic contents of Moringa oleifera leaves, pods and seeds (mg standard equivalent per gram of dry weight)

<table>
<thead>
<tr>
<th>Varieties (Mean ± S.D.)</th>
<th>Num Phare</th>
<th>Ang Thong</th>
<th>PKM1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaves</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqueous extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>5.44 b ± 1.14</td>
<td>5.62 b ± 1.71</td>
<td>6.74 a ± 2.23</td>
</tr>
<tr>
<td>DPPH</td>
<td>4.43 b ± 0.84</td>
<td>6.07 a ± 0.96</td>
<td>6.75 a ± 2.49</td>
</tr>
<tr>
<td>ABTS</td>
<td>10.10 b ± 1.78</td>
<td>12.25 a ± 2.85</td>
<td>11.81 a ± 3.39</td>
</tr>
<tr>
<td>TPC</td>
<td>8.43 b ± 1.32</td>
<td>9.63 b ± 1.53</td>
<td>9.69 a ± 2.56</td>
</tr>
<tr>
<td>Ethanol extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>2.93 b ± 0.78</td>
<td>3.49 a ± 0.85</td>
<td>3.15 b ± 0.99</td>
</tr>
<tr>
<td>DPPH</td>
<td>1.80 b ± 0.87</td>
<td>3.17 a ± 1.69</td>
<td>2.08 b ± 1.68</td>
</tr>
<tr>
<td>ABTS</td>
<td>7.34 a ± 2.75</td>
<td>8.85 a ± 2.60</td>
<td>6.48 b ± 2.08</td>
</tr>
<tr>
<td>TPC</td>
<td>5.26 b ± 2.56</td>
<td>6.70 a ± 2.71</td>
<td>4.59 b ± 1.76</td>
</tr>
<tr>
<td><strong>Pods</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqueous extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>3.32 b ± 0.23</td>
<td>3.98 a ± 0.41</td>
<td>3.88 a ± 0.30</td>
</tr>
<tr>
<td>DPPH</td>
<td>2.06 ± 0.12</td>
<td>2.32 ± 0.20</td>
<td>2.31 ± 0.28</td>
</tr>
<tr>
<td>ABTS</td>
<td>3.41 ± 1.78</td>
<td>3.84 ± 0.49</td>
<td>3.67 ± 0.34</td>
</tr>
<tr>
<td>TPC</td>
<td>3.61 ± 0.46</td>
<td>6.54 ± 0.72</td>
<td>6.36 ± 0.68</td>
</tr>
<tr>
<td>Ethanol extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>2.59 b ± 0.56</td>
<td>4.05 a ± 0.48</td>
<td>3.81 a ± 0.26</td>
</tr>
<tr>
<td>DPPH</td>
<td>1.80 ± 0.62</td>
<td>2.42 ± 0.33</td>
<td>2.06 ± 0.41</td>
</tr>
<tr>
<td>ABTS</td>
<td>2.12 ± 0.26</td>
<td>2.96 ± 0.33</td>
<td>2.90 ± 0.28</td>
</tr>
<tr>
<td>TPC</td>
<td>3.17 ± 0.49</td>
<td>4.09 ± 0.31</td>
<td>3.54 b ± 0.38</td>
</tr>
<tr>
<td><strong>Seeds</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aqueous extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>0.80 b ± 0.08</td>
<td>1.03 ± 0.08</td>
<td>0.85 ± 0.04</td>
</tr>
<tr>
<td>DPPH</td>
<td>0.95 b ± 0.12</td>
<td>1.13 b ± 0.20</td>
<td>1.79 ± 0.21</td>
</tr>
<tr>
<td>ABTS</td>
<td>0.99 b ± 0.09</td>
<td>0.93 b ± 0.04</td>
<td>1.43 ± 0.05</td>
</tr>
<tr>
<td>TPC</td>
<td>1.30 b ± 0.13</td>
<td>1.65 ± 0.15</td>
<td>1.20 b ± 0.11</td>
</tr>
<tr>
<td>Ethanol extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FRAP</td>
<td>0.73 b ± 0.05</td>
<td>0.95 a ± 0.16</td>
<td>0.74 b ± 0.07</td>
</tr>
<tr>
<td>DPPH</td>
<td>0.51 b ± 0.04</td>
<td>0.40 ± 0.06</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>ABTS</td>
<td>0.56 b ± 0.04</td>
<td>0.64 a ± 0.42</td>
<td>0.50 ± 0.04</td>
</tr>
<tr>
<td>TPC</td>
<td>0.95 b ± 0.10</td>
<td>1.27 a ± 0.17</td>
<td>0.79 b ± 0.01</td>
</tr>
</tbody>
</table>

FRAP = Ferric reducing / antioxidative power assay; DPPH = DPPH free radical scavenging activity; ABTS = Improved ABTS radical cation decolorization assay; TPC = Total phenolic content by Folin-ciocalteau micro method.

The standard for FRAP, DPPH, and ABTS is vitamin C, while the standard for TPC is Gallic acid.

Means with different letters in the same row are significantly different (P<0.05)

Means in the same row are insignificantly different (P>0.05)
extracts were higher than DPPH and ABTS values.

Conclusions

Leaves, pods and seeds of *M. oleifera* grown in Chiang Mai, Thailand, contained antioxidant and phenolic substances which were water-soluble rather than ethanol-soluble. Those of the Ang Thong and PKM1 varieties were better sources of antioxidants and phenolic compounds than those of Num Phare variety. Leaves contained more antioxidant and phenolic substances than pods and seeds, respectively. Antioxidants of the leaves had the ability to quench ABTS\(^{+}\) rather than reducing power and ability to quench DPPH radicals, while antioxidants of the pods and seeds possessed reducing power equal to or higher than the ability to quench DPPH and ABTS radicals.

Acknowledgments

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Expression Analysis of Na\(^+\)/ H\(^+\) Exchanger and Monosaccharide Transporter Genes in Rice Suspension Cells Under Salt Stress

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Abstract

The expression analysis of genes, which are probably related to salt tolerance mechanisms in rice was conducted in this study. Rice suspension cells treated with 100 mM NaCl were harvested at 0, 1, 3, 6, 10 and 14 days for subsequent gene expression analysis. The *OsNHX1* (*Oryza sativa* L. Na\(^+\)/ H\(^+\) exchanger1) had higher expression at the early period of NaCl treatment and gradually declined which suggested the response of rice suspension cells to cope the harmful of salt stress by compartmentalisation of sodium ion in cytoplasm. In addition, the increased expression level of *OsMST3* (*Oryza sativa* L. monosaccharide transporter3) and high glucose concentration at the early period of NaCl treatment also indicated the higher requirement of reserve substance to repair the cells damage by toxicity of sodium ion.

Keywords: rice, salt stress, *OsNHX1*, *OsMST3*

Introduction

Salinity is a major factor restricts plant growth and productivity. Many mechanisms were generated by plant in both the cellular and whole-plant levels to overcome salt stress. The maintenance of K\(^+\)/ Na\(^+\) ratio by sodium transporter protein to reduce the toxicity of excess Na\(^+\) ion concentration is fundamental response found in plants (Blumwald et al., 2000). In rice, the increased expression of Na\(^+\)/ H\(^+\) exchanger gene (*OsNHX1*) that catalyzes the exchange of Na\(^+\) for H\(^+\) across membranes, contributes to regulation of internal pH, cell volume, and sodium level in the cytoplasm was observed in roots and shoots of 7- day-old seedling after 24 h of treatment with 100 mM NaCl (Fukuda et al., 1999). Additionally, the over expression of *OsNHX1* in perennial rye grass improved the salt tolerance which suggests the importance roles of this gene in salt stress (Wu et al., 2005). Beside the specific mechanism to adjust internal osmotic status of plant cells by accumulation of potassium, sodium and chloride ions, the increased amount of compatible solutes (e.g., sugar, sugar alcohols, low complexity of carbohydrates, tertiary amines, sulfonium compounds and amino acids) which is a part of normal metabolisms was also reported (Bohnert and Sheveleva, 1998). To our knowledge, the interrelationship among the expression of sugar transporter gene, sodium exchanger gene and the determination of monosaccharide accumulation in rice at the cellular level under salt stress has not yet been reported. There is only one experiment involved in the expression of sugar transporter gene (*AgSUT1*) in the whole-plant of celery (*Apium graveolen* L.) during salt stress (Noiraud et al., 2000). When celery plants were subjected to 300 mM NaCl for 30 days, the expression of *AgSUT1* was decreased in all organs, especially in root which is representative of strong sink organ. In celery plant which favored to maintain a high level of mannitol as osmoprotectant, mannitol dehydrogenase is repressed by high level of sucrose which indicated that sucrose transport has to be maintained. It is consistent with the low expression of *AgSUT1*. However, the occurrence in celery which is dicotyledonous...
plant might not be the same as in rice which is monocotyledonous plant. Moreover, the higher expression of monosaccharide transporter under biotic stress condition which suggested the increased carbohydrate demand of injured cells was documented. Under stress condition, carbohydrate in form of monosaccharide such as glucose as a major energy source might be required and imported to plant cells (William et al., 2000). Therefore, this research aims to study the expression of sodium exchanger and monosaccharide transporter genes (Toyofuku et al., 2000) in rice at the cellular level. In addition, the glucose content was also determined. This will lead to understand carbon metabolism when rice at cellular level is in unfavorable condition.

Genes Expression Analysis
To determine the expression of OsNHX1 and OsMST3, semi-quantitative RT-PCR was performed. Total RNA was isolated from rice cell suspensions. The 1 µg total RNA was reverse-transcribed to generate the first stranded cDNAs. The cDNAs are then subjected to PCR amplification (28 cycles) with specific primers for OsNHX1 and OsMST3. In addition, specific transcripts of each gene were confirmed based on technique of Southern blotting by using Dig-labeled cDNA of OsNHX1 and OsMST3 as probes. To help validate the results obtained, control PCR using primers for housekeeping gene, rice actin whose expression level are constant in all tissue types, was performed alongside the experimental samples.

Determination of Glucose Content
The sugar contents were extracted from 0.2 g rice cell suspension samples which used 3 ml 80% ethanol as solvent. The extraction was done under water boiling temperature for 1 min and then incubated the extracts at 65°C for additional 1 hr. The extracts were centrifuged to remove cell debris. The extract solutions were used to determine glucose content by glucose oxidase-peroxidase (GOD/POD) method.

Results and Discussion
As shown in figure 1 and 2, treatment with 100 mM NaCl stimulated the transcript of OsNHX1 and OsMST3 at the early period of treating and gradually declined. The earlier expression of OsNHX1 than OsMST3 suggested the response of rice suspension cells to cope the harmful of salt stress by compartmentalisation of sodium ion in cytoplasm. To reduce toxicity of sodium ion, maintaining of the K+/Na+ ratio inside cells is required. Therefore, rice cells which direct contact to salt solution has to transport sodium ion across membrane into vacuole via the expression of OsNHX1. This situation in the cell level of rice is the same as the respond of whole-plant level under salt stress as previously documented. The increased expression of OsNHX1 was observed in roots.
and shoots of 7-day-old rice seedling after 24 h of treatment with 100 mM NaCl, especially in root (Fukuda et al., 1999). In addition, the higher expression of OsMST3 which is rice monosaccharide transporter and the high content of glucose at the early period of treatment as shown in figure 2 and table 1 in respectively indicated the increased carbohydrate demand of NaCl-treated cells to generate the defense mechanisms or repair damaged cells. Under stress condition, carbohydrate in form of monosaccharide such as glucose as a major energy source might be required and imported to plant cells (William et al., 2000). Moreover, the decreased expression of OsSUT1 which is rice sucrose transporter gene at 1, 3 and 6 days followed by the increased expression at 10 and 14 days after NaCl treating in rice cell suspensions (data not shown) may suggest the similar mechanism as observed in celery plant when subjected to 300 mM NaCl (Noiraud et al., 2000). To keep osmotic potential in cells, mannitodehydrogenase is repressed by sucrose and glucose is also needed as substrate for sugar alcohol synthesis. Once glucose is depleted, sucrose should be hydrolyzed into glucose and fructose in order to compensate the used glucose. The higher expression of OsSUT1 at the end of NaCl treating well supported this explanation.

![Figure 1](image1.png) **Figure 1** The expression of OsNHX1, OsMST3 and actin in 100 mM NaCl-treated rice cell suspensions.

Lane 1: 0 day NaCl treatment
Lane 2: 1 day NaCl treatment
Lane 3: 3 days NaCl treatment
Lane 4: 6 days NaCl treatment
Lane 5: 10 days NaCl treatment
Lane 6: 14 days NaCl treatment

![Figure 2](image2.png) **Figure 2** The Southern blot analysis of OsNHX1 and OsMST3 expression in 100 mM NaCl-treated rice cell suspensions.

Lane 1: 0 day NaCl treatment
Lane 2: 1 day NaCl treatment
Lane 3: 3 days NaCl treatment
Lane 4: 6 days NaCl treatment
Lane 5: 10 days NaCl treatment
Lane 6: 14 days NaCl treatment

![Table 1](image3.png) **Table 1** Glucose content in 100 mM NaCl-treated rice cell suspensions.

<table>
<thead>
<tr>
<th>NaCl-treated Time (day)</th>
<th>Glucose content (mM/g fresh weight) ± SD, n= 3</th>
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<tr>
<td>0</td>
<td>0.166 ± 0.024</td>
</tr>
<tr>
<td>1</td>
<td>0.248 ± 0.038</td>
</tr>
<tr>
<td>3</td>
<td>0.785 ± 0.152</td>
</tr>
<tr>
<td>6</td>
<td>0.559 ± 0.030</td>
</tr>
<tr>
<td>10</td>
<td>0.213 ± 0.020</td>
</tr>
<tr>
<td>14</td>
<td>0.246 ± 0.026</td>
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</table>

Conclusions

Under salt stress, the early response mechanism to cope the harmful of rice cells from toxicity of sodium ion was done via the expression of OsNHX1. In addition, the abundant transcript of OsMST3 and high glucose content at the early period of salt stress also suggested that glucose is a major carbohydrate required for stabilizing osmotic potential inside cells and generating defense mechanisms to protect cellular structures and activities of rice cells.

Acknowledgments

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Determination of Relationships and Genetic Variation Among
Amorphophallus sp. From Northern Part of Thailand

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Abstract

Amorphophallus sp. are known under the common name of konjac or elephant foot yam. Some of the species have the potential to become highly profitable crops for South East Asia as raw material for various industries. However, considerable variation in morphological features of many species of Amorphophallus makes it difficult to identify them in the vegetative form. Randomly Amplified Polymorphic DNA (RAPD) and DNA Sequencing are often used to determine the genetic relationship of plants. The aim of this study was to determine the relationship and genetic variation of various accessions of Amorphophallus sp. collected in northern regions of Thailand. Fifty samples were characterised by RAPD with four primers (ERIC1R, ERIC 2, BOXA1R, RPO1) the data from which were used to calculate genetic distances which were then visualized using multidimensional scaling. In addition, the psbM-trnD region of their chloroplast genome was also sequenced from which phylogenetic relationships were determined using parsimony analysis. The results from the RAPD analysis placed the accessions into 35 different groups with distance values between 0.075 and 0.949. The DNA sequence data found the accessions into 30 different groups. Further work will be carried out to more closely determine the relationships between the accessions and to relate them to each other.

Keywords: Amorphophallus sp., genetic distance, RAPD, DNA sequencing

Introduction

Amorphophallus sp. belongs to the family Araceae. It is a perennial herbaceous plant, known in English under the names of konjac or elephant foot yam. There are around 125 species found in mountainous or hilly areas of subtropical regions especially in South East Asia mainly in Thailand and Indonesia (Gandawijaja et al., 1983; Hetterscheid and Ittenbach, 1996). There are 46 species of Amorphophallus found in Thailand (Sukumalanand, 2005), mostly in the northern part of the country. Some of the species have the potential to become highly profitable crops for South East Asia as their main component is glucomannan (KGM) (Zhang et al., 2005; Chua et al., 2010). Glucomannan is one of the most important and economically useful hydrocolloids and is used in many industries such as those associated with the manufacture of foods, pharmaceuticals, textiles and chemicals. Because of the potential of Amorphophallus sp., various studies about this
genus including its morphology (Hetterscheid and Ittenbach, 1996), palynology (Van der Ham et al., 1998; Punekar and Kumaran, 2010) and odor biochemistry (Kite and Hetterscheid, 1997) were studied. However, the morphology and palynology characters are highly variable, so it is difficult to identify them in the vegetative form (Grob et al., 2002).

Currently, a number of DNA techniques are used to determine relationships and genetic variation in plants. Among those techniques, there are two different techniques that are based on DNA pattern namely Randomly Amplified Polymorphic DNA (RAPD) and nucleotide sequence (DNA Sequencing) that are being used to determine the genetic relationship of a particular plant. For RAPD technique, it can be used in a wide range of applications because of its sensitivity, simplicity, cost-effectiveness and no requirement for DNA sequence information (Bardakci, 2001). RAPD can be used in population genetic studies to estimate affinities between closely related plants (Na et al., 2009; Verma et al., 2009) and has been used for studies of *A. albus* (Hu et al., 2008), *A. titanum* (Poerba and Yuzammi, 2008) and *A. muelleri* (Poerba and Martanti, 2008). DNA sequencing technique is the most direct way to determine nucleotide sequence of defined regions. It can provide highly robust, reproducible, informative data sets and can be adapted to different levels of discriminatory potential by choosing appropriate genomic target regions (Weising et al., 2005). In *Amorphophallus* sp., there is number of DNA regions that have been used in sequencing such as the gene FLO/LFY (*FLint2*) and the chloroplast regions *rbcL*, *matK*, and *trnL* gene (Grob et al., 2004). However, those regions have some limitations as *matK*, *trnL* and *FLint2* can not provide enough non-conflicting informative characters to produce highly informative cladograms. Recently, the non-coding cpDNA is rapidly increasing for research on closely related species and one such region is *psbM-trnD* (Shaw et al., 2005). The study presented in this paper was aiming at determining relationship and genetic variation of *Amorphophallus* sp. from northern part of Thailand based on RAPD and *psbM-trnD* region sequences.

**Material and Methods**

**Plant Materials Collection**

Wild species of *Amorphophallus* were collected from 5 provinces in northern part of Thailand including Tak (14 samples), Lampang (13 samples), Lampoon (6 samples), Chiangmai (12 samples) and Mae Hongson (5 samples). All leaf material was taken from living plants and subjected to freeze drying at -47 °C under vacuum pressure of 0.048 mbar for 24 hours (Model DTF-530-090U, UK). The dried leaf samples were then ground and kept in transparent plastic cup with lid at room temperature for further experiments.

**DNA Extraction**

Genomic DNA was extracted from 1 mg dried sample by using REDExtract-N-Amp Plant PCR kit (Sigma, USA).

**Random Amplified Polymorphic DNA (RAPD) analysis**

RAPD-PCR was used to generate banding patterns for the different *Amorphophallus* accessions using 4 different RAPD primers: ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′), ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic et al., 1991), BOXA1R (5′-CTACGGCAAGGCGACGCTGACG-3′) (Versalovic et al., 1994) and RPO1 (5′-AATTTTCAAGCGTCGTGCACA-3′) (Watson and Schofield 1985). Amplification of genomic DNA was performed in a 25 µl reaction mixture containing 18.5 µl PCR grade distilled water (Promega, USA), 2.5 µl of 10× Thermopol buffer (2 mM MgCl₂) (Bio Lab Inc., USA), 0.5 µl bovine serum albumin (BSA, 10 mg/ml) (Promega, USA), 1 µl of 10x dNTPs (10 mM) (Fisher, USA), 1 µl of primer and 0.5 µl of Taq DNA polymerase (Bio Lab Inc., USA) and 1 µl of template DNA. PCR application conditions were an initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 50°C for 30 s, and 65°C for 8 min, followed by 65°C for 5 min. PCR products were separated by
electrophoresis in a 1% (w/v) agarose gel in tris-borate-EDTA (TBE) buffer at 100 V with DNA ladder (1 kbp and 50 bp) (Promega, USA) used for size estimation.

Sequencing Analysis

The two primers used for both amplification and sequencing of the psbM-trnD region were TRND (5’-ACCAATTGAACTACAATCCC-3’) and psbMF (5’AGCAATAAATGCRAGAATATTTACTTCCAT-3’) (Shaw et al., 2005). The PCR amplification conditions were initial denaturing at 94°C for 5 min, followed by 34 cycles of 94°C for 60 s, 55°C for 60 s, and 72°C extension for 3 min 30 s, followed by 72°C extension for 5 min. PCR products were then separated on 1% agarose gels to indicate the quantity of the fragments. PCR products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and DNA concentration was estimated by spectrophotometry (Nanodrop 1000, Thermo Scientific, USA). Sequencing was performed using the forward and reverse primers by Macrogen (Korea) using standard dye-chemistry.

Data Analyses

The RAPD technique produces multiple bands and various lengths of PCR products. Each amplified product was scored according to the presence of DNA band. Score 1 for presence and 0 for absence of a DNA band across samples. The data were used to calculate genetic distances according to the method of Nei and Li (1979) which were then visualized using multidimensional scaling by using STATISTICA (Version 9.1. StatSoft, Inc., USA). The data were also subjected to parsimony analysis using PAUP (Phylogenetic Analysis Using Parsimony, Swafford, 1992). For DNA sequence data, raw sequences were examined and contigs of forward and reverse sequences made using DNA Baser (Version 2.11.0, Heracle BioSoft, Germany) and aligned using ClustalW as enabled by Bioedit Sequencer Alignment Editor (Version 5.0.1, Hall,1999) and manually corrected. Phylogenetic trees were reconstructed using PAUP as detailed above.

Results and Discussion

The genetic relationships and variation among the 50 accessions of Amorphophallus species (A. muelleri, A. paeonifolius, A. corrugatus, A. macrorhizus and unknowns) collected from five provinces in northern part of Thailand were characterized using two different methods namely RAPD technique and sequencing of psbM-trnD region.

Table 1 Amorphophallus accessions collected from different provinces of northern Thailand.

<table>
<thead>
<tr>
<th>No.</th>
<th>source</th>
<th>species</th>
<th>No.</th>
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</table>

Random Amplified Polymorphic DNA (RAPD) Analysis

Random amplified polymorphic DNA (RAPD) technique based on the polymerase chain reaction (PCR) is one of the most common molecular techniques used to develop DNA markers by amplification of anonymous DNA sequences. RAPD employs single, short and arbitrary oligonucleotide primers and does not require prior knowledge of DNA sequence. In addition,
This technique is capable of developing large numbers of DNA markers in a short time, a low cost and less requirement for special equipments. The number of DNA bands that were generated after PCR amplification depended on primary homology of DNA template which was used as well as on sites of attachment of the primers (Tingey et al., 1994). In this study, long RAPD primers (LP-RAPD) were used because they are sensitive to intraspecific and interspecific genetic variation, reproducible and more stable than shorter primer (Gillings and Holley, 1997). The four primers used were ERIC1R, ERIC2, BOXA1R and RPO1 were used that resulted in a total of 60 bands with 20 bands coming from ERIC1R, 15 from ERIC2, 12 from BOXA1R and 13 from RPO1. Only clear DNA bands were used for scoring and DNA fragments obtained ranged in size from 50 bp to 2000 bp; all bands were polymorphic. The highest number of polymorphic bands was found in the primer ERIC1R while the lowest number of bands was found in the BOXA1R primer. PCR results from one primer (ERIC1R) are shown in Fig. 1.

![Figure 1](https://example.com/figure1.png)

**Figure 1** PCR results of 50 *Amorphophallus* sp. samples with primers ERIC1R. Description: M = DNA marker (1 Kb ladder, Promega).

However, results of DNA amplification by PCR using the four primers showed 5 accessions (2, 14, 33-35) of 50 accessions could not produce DNA fragments from some primer such as sample no. 2, 14 (BOXA1R primer) and sample 33-35 (RPO1 primer). This results from random binding when genomic priming site is large and may exceed the capacity of PCR. Hence it can cause fragment loss (Bardakci, 2001).

Genetic distances were calculated according to the method of Nei and Li (1979) using only those accessions that produced DNA bands from all four primers. Genetic distances for the 45 accessions ranged from 0.075 to 0.949. The largest genetic distance was between sample no. 1 (*A. muelleri* from Tak) and no. 28 (*A. macrorhizus* from Lampang); the large distance value is probably due to the plants being of different species and originating from different regions. The smallest genetic distance (0.075) was found between sample no. 12 (Unknown from Lampang) and no. 15 (*A. corrugatus* from Lampang). It means that the two samples have genetic properties close to each other. Both accessions were collected from the same location that is Wang Kaew Waterfall National Park in Lampang. Estimates of genetic distances of *Amorphophallus* species using RAPDs are as follows: *A. konjac* (0.36 to 0.52), *A. albus* (0.34 to 0.38), *A. pingbianensis* (0.36 to 0.46) (Wenbing et al., 2008); *A. titanum* (0.14 to 0.59) (Poerba and Yuzammi, 2008); *A. muelleri* (0.02 to 0.36) (Poerba and Martanti, 2008).

The estimates of genetic diversity in this study are high when compared with these other studies. The cause of the high variation should be investigated further and may be due to differences in methodology, differences in relatedness of populations of plants in Indonesia, China and Thailand or geographic distribution (distant collection areas) and a possible recombination of genetic material from sexual reproduction allowing genotype development in new environment that made it maintain genetic diversity (Poerba and Yuzammi, 2008). Moreover, the authors suggested that population groups which have the highest levels of genetic variation, need more attention for its conservation in future.
The genetic distances were visualized using multidimensional scaling (MDS, Figure 2). MDS showed that *A. paeoniifolius* population had closest genetic distances values than other species. It can be seen that accessions of *A. muelleri* clearly group separately from accessions of *A. paeoniifolius*. The grouping of *A. macrorhizus* and *A. corrugatus* is uncertain, and they appear to group with *A. paeoniifolius*. This may be a function of the few accessions of *A. macrorhizus* and *A. corrugatus* assessed (no. 3 and no. 1).

However, the results of the RAPD analysis suggest that the genetic distances calculated using this technique seem to be related to both the origin of the accessions and/or their taxonomic relatedness. To further investigate any relationships in the RAPD data, they were subjected to parsimony analysis using PAUP (Figure 3). However, this analysis revealed little structure within the data. Within each analysis of phylogenetic tree on 45 accessions of *Amorphophallus* species shows separation of accessions into three distinct groups that seem to be distinct from one another. This phylogenetic tree is composed of 35 groups with 7 main clades. The main clades are partly clustered based on species. Some samples that are based on species and locations namely no. 9 and no. 17 (*A. paeoniifolius* from Lampang). Other single clusters are at random even within the same species (Fig. 3).

**Nucleotide Sequence Analysis**

DNA sequencing reveals DNA sequence variation in unparallel data. Therefore it provides accurate molecular information (Weising et al., 2005). Fifty accessions of *Amorphophallus* sp. at psbM-trnD region in the noncoding chloroplast genome were sequenced. The psbM-trnD region sequences of those species were approximately 597-645 bp. In the alignment of these sequences, it was found that medium conservative block showed that psbM-trnD region is not saturated in *Amorphophallus*. Phylogenetic tree was created by using parsimony analysis of psbM-trnD region sequences (Fig. 4). It showed that this strict consensus tree is relatively well resolved with basal polytomy as shown by a Bootstrap (BS) higher than 60%.

**Figure 2** The genetic distances of 45 accessions of *Amorphophallus* sp. visualized after multidimensional scaling.

**Figure 3** Cladogram derived from parsimony analysis of the RAPD profile data of 45 accessions of *Amorphophallus* species from Thailand.
Figure 4 Cladogram derived from parsimony analysis of 50 accessions based on psbM-trnD region sequences, numbers indicate bootstrap support, taken from bootstrap analysis.

The result of phylogenetic relationships between *Amorphophallus* sp. shows that main 5 clades are partly clustered based on species except first clade which includes samples 1-3, 7 and 19 based on species and location, all identified as *A. muelleri* from Tak, had 15-16 bp insertions compared to all other accessions, indicating that these species are related closely within 91% Bootstrap. Sample No. 7 (unknown from Tak) grouped with the accessions containing the 15-16 bp insertion; this grouping is also reflected in the RAPD analysis. However, this insertion was missing in accession 10 also from Tak and identified as *A. muelleri* in second clade. Moreover, the large third group of clade including samples 23, 25, 26, 32, 35 and 38 had a 8 bp insertion compared to other sequences, could be *A. paeoniifolius* because they are closely related to each other in same group of Chiang Mai province except samples 23 and 32 which are in Lampoon and Lampang respectively. This result is again reflected in the RAPD analysis. Finally, two unknown samples (no.34 and no.41) formed a fifth clade, and sample 41 is outside of the groups formed by *A. muelleri* and *A. paeoniifolius* in the RAPD analysis. Therefore, this study found a reasonable level of congruence between the RAPD and sequence analysis. Therefore, these results indicate high genetic variation in different 30 clusters from 50 accessions that is probably caused by geography or highly heterogeneous morphology (Grob et al., 2002).

From this study, *psbM-trnD* region may be useful as noncoding chloroplast phylogenetic marker and provides a good hypothesis on intrageneric relationships of *Amorphophallus* sp. *Psbm-trnD* region sequences are short (<700 bp) thus it is suitable for sequencing and the alignment is relatively less complicated (Baker et al., 2000). The results show that *psbM-trnD* region is highly variable and not to be saturated with respect to phylogenetic utility of this region. However, *psbM-trnD* region alone did not provide enough information of DNA characters to produce highly informative phylogenetic tree of *Amorphophallus* sp. like some of the previous studies. Grob et al. (2002) analyzed nucleotide sequences of the chloroplast gene *matK* and the *trnL* intron gene alone was insufficient to define all intrageneric relationships. Similarly, the results from sequencing the *trnL* gene resulted in poorly resolved trees with low bootstrap values. Further, Grob et al. (2004) investigated second intron of *FLO/LFY* (*FLI2*) in phylogeny of 46 *Amorphophallus* sp. found only a low percentage of informative sites. However, when this region was used in combination with chloroplast regions (*trnL, rbcL, matK* gene) a number of well-supported clades were produced. Therefore, in a further study, it should be considered to use another DNA region sequencing to combine with *psbM-trnD* region in order to obtain more...
informative data on intrageneric relationship in this species. There were some successful studies to identify Amorphophallus sp. such as study of noncoding spacer between the atpB and rbcL genes in two populations of A. henryi (Chiang et al., 1998), mannoselinding lectin gene (aka) in A. konjac found this species related with monocot species (Fei et al., 2003), chloroplast trnL-trnF intergenic spacer in A. glossophyllus and A. variabilis found that they are sister group which are closely related (Rothwell et al., 2004) However, all of those successful studies, dealt only with few samples and species of Amorphophallus.

Conclusions

Fifty accessions of Amorphophallus species (A. muelleri, A. paeoniifolius, A. corrugatus, A. macrorhizus and unknown accessions) were collected from five different provinces in northern part of Thailand. Relationship and genetic variation among accessions based on RAPD and psbM-trnD region sequences were investigated. Forty-five accessions were detected using four RAPD primers resulting in the production of 60 DNA fragment sizes from 50 bp - 2000 bp. Estimates of genetic distance values ranged from 0.075 to 0.949. These values are high compared to other studies. Multidimensional scaling showed three species that are clearly separated from each others. Phylogenetic tree showed separation of 35 groups with 7 main clades. All of the results indicated high genetic variation and genetic diversity based on species and geography as compared with other studies. All fifty accessions were sequenced at psbM-trnD region in cpDNA with a final alignment of 597-645 bp. Phylogenetic relationships were determined using parsimony analysis showing 30 different groups with 5 main clades and there was a reasonable degree of congruence between the RAPD and sequence data. Both of RAPD analysis and DNA sequence data showed that Amorphophallus sp. in northern Thailand have high genetic variation and high genetic diversity among accessions. Therefore, molecular characters have potential to determine various accessions of Amorphophallus sp. on the basis of its relationship and genetic variation. Moreover, the molecular information has potential to help in identifying unknown samples. Further works on determination of the relationship between accessions will be carried out to relate them to each other.

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Introduction

Bioethanol is considered as a renewable and sustainable fuel for automotives. Ethanol is the most promising biofuel in the future due to its high energy value, and ease of production. The ethanol-blended gasoline is marketed under the fuel name, gasohol. It is not only naturally renewable, but gasohol also has lower emissions of carbon monoxide, carbon dioxide and hydrocarbons (Chang et al., 1998). Moreover, anhydrous ethanol is widely used in industries such as organic syntheses, painting, medicine, cosmetics, and perfume, etc. Ethanol can be easily produced from agricultural substrates by the means of fermentation. Primary recovery of ethanol from fermentation broth is normally accomplished by mean of distillation, but it is known that ethanol forms azeotrope with water at 89.4 mole% (95.6 wt% ethanol), 78 °C and at atmospheric pressure. Hence, an additional dehydration process must be added in order to produce anhydrous ethanol. A major challenge in the dehydration of ethanol is the high energy cost associated with the separation of water from the large excess of ethanol. Different techniques have been introduced to dehydrate azeotropic mixture of ethanol/water namely azeotropic distillation, membrane separation, and adsorption technique. The first is no longer economically attractive because of high energy input. In addition, there is a major health concern of the toxic additive used in the process. During the past few decades, membrane separation as an alternative dehydration process has been developed to replace this energy consuming process. Vapor permeation (VP) is a membrane separation process where the feed side is in...
In this work, a commercial composite membrane prepared from modified Poly(vinyl alcohol) (PVA) as a selective layer and polyacrylonitrile (PAN) as a supportive layer was employed. PVA is often used in the dehydration of water-ethanol mixtures because it exhibits a good hydrophilic property (Boontawan et al., 2007). A vapor permeation (VP) system was investigated for its dehydration performances in order to produce anhydrous ethanol.

However, the relationship between required membrane area and desired purity of ethanol is not linear. Figure 1 shows that membrane area increases exponentially with required purity. Ranging from 90 to 99% purity of ethanol, the required membrane area gradually increases with the increasing purity of ethanol in the retentate stream until the value reaches approximately about 12 m² when the purity of ethanol is about 99%.

![Graph showing the relationship between membrane area and ethanol purity](image)

**Figure 1** Mathematical simulation of required purity on membrane area (Pettersen et al., 1995)

The adsorptive option is particularly attractive because of its low energy consumption, which takes up 50–80% of the overall energy required by the fermentative plan (Banat et al., 2000; Hills et al., 1990). The pressure swing adsorption (PSA) process is attractive for the final separation since it requires little energy input, and is capable of producing a very pure product (Simo et al., 2008). The pressure swing adsorption column is filled with 3 Å type molecular sieve beads. It is an adequate adsorbent for the removal of small amounts of water from organic solvents. In virtue of their small diameter (0.28 nm), the water molecules can easily penetrate the structural zeolite canals, while many organic molecules, such as ethanol (0.44 nm), are simultaneously excluded (Carmo et al., 1997). For this work, pressurized beds packed with molecular sieve 3Å (individual bed volume of 0.50 L) were used as the final step to dehydrate ethanol vapor with the objective of the highest purity for up to 99.99%.

**Materials and Methods**

**Vapor Permeation**

The schematic diagram for experimental setup of VP system was shown in Figure 2. A composite PVA/PAN membrane supplied by Sulzer Chemtech, Switzerland with a surface area of 0.0288 m² (0.16 m × 0.18 m) was placed in a module, and temperature was controlled by re-circulating silicone oil through the jacket surrounding it. The liquid feed was heated in a pressurized vessel, and the vapor feed then entered the membrane module. The permeate vapor was collected using a condenser set at -20 °C, and permeate pressure was kept low using a vacuum pump. Permeate was sampled periodically to determine the flux and water composition. The permeation rate was measured gravimetrically by weighing the permeate samples collected over a period of time whilst determination of water concentrations in both feed and permeate were carried out using an
automatic Karl Fischer's titrator (Schott, Germany). The membrane performance can be described by permeate flux \( J \) (kg.m\(^{-2}\).h\(^{-1}\)), and separation factor \( \alpha \) which can be defined as follow:

\[
J = \frac{W(\text{kg})}{A(\text{m}^2) \cdot t(\text{h})}
\]

Where; \( W \) is the weight of permeate, \( A \) is the membrane area, and \( t \) is the time, respectively.

\[
\alpha = \frac{w_{\text{P,H,O}}}{w_{\text{F,H,O}}} / \frac{w_{\text{P,EtOH}}}{w_{\text{F,EtOH}}}
\]

Where; \( w_F \) and \( w_P \) are the weight fraction of water and ethanol in the feed and permeate side, respectively.

![Figure 2](image)

**Figure 2** Schematic diagram for hybrid vapor permeation and pressure swing adsorption experiment.

**Pressure Swing Adsorption (PSA)**

The PSA experiment was also conducted as shown in Figure 2. Each column was made from a stain steel shell with an oil jacket surrounded it. The internal volume of the bed was 500 mL which accommodated 386.24 g of molecular sieve beads. The height of the column was 30 cm. Ethanol-water mixture was stored in a pressurized vessel. The vapor pressure in the column was controlled by the heater at pressurized vessel. The temperature of the vapor was well above the dew point in order to avoid condensation. The vapor feed subsequently went through the column, and the ethanolf-rich product was collected at the end of the tube after being condensed. Product samples were collected at every 50 ml and water content was analyzed. When the adsorption reached equilibrium, the feed direction was switched to another column where the saturated bed entered regeneration step by applying vacuum. The desorbed vapor was collected in the same permeate glass reservoir as it was used for the VP system. When combining the two processes together, the vapor feed firstly entered the VP module followed by PSA system.

**Results and Discussion**

**Vapor Permeation**

Application of the composite PVA/PAN membrane from Sulzer Chemtech, Switzerland for dehydration of ethanol was investigated using vapor permeation technique at operating pressure between 1.2-1.8 bars, and module temperature between 80-120 °C. Higher module temperature was not investigated because this value was the maximum temperature recommended by the manufacturer. The retentate (product) flow was controlled by a back pressure valve with the flow rates between 0.875-3.025 mL.min\(^{-1}\). Separation of water and ethanol is governed by preferential absorption into the cross-linked polymer, and mass transfer characteristic is also affected by operating conditions. In this work, separation performances were investigated including the effect retentate flow rate, feed pressures, and operating temperatures, respectively.

**Effect of feed pressure on permeate flux, separation factor and ethanol concentration in retentate.**

The influence of feed pressure on ethanol concentration retentate is illustrated in Figure 3. It is shown that the ethanol concentration depended on the feed pressure, indicating that the higher quality ethanol concentration in retentate can be gained at higher feed pressure.
The ethanol concentration increased from 96.82 to 98.86 wt% as the feed gage pressure increased from 1.2 to 1.8 bars.

**Figure 3** The compositional evolution in ethanol retentate with proceeding of dehydration at different feed pressures; flow rate 1.85 ml/min., feed concentration of EtOH 95 wt% and T_cell 120 ºC.

**Figure 4** The influence of operating feed gauge pressure on fluxes and separation factor with 95 wt% of ethanol in the feed side, vacuum 6 mbar, retentate flow rate 1.85 mL/min, and cell temperature 120 ºC, respectively.

Figure 4 shows the effect of operating feed gauge pressure on fluxes and separation performances. Permeation experiments were carried out at different feed gauge pressure, and membrane performances were also investigated in terms of fluxes and separation factor. For separation factor, it was clearly seen that separation factor slightly increased with increasing feed pressure. The separation factor increased from 4.58 to 6.04 as the feed pressure increased from 1.2 to 1.8 bar. Total, ethanol, and water fluxes, there was slightly increased with increasing feed pressure.

**Effect of cell temperature on permeate flux, separation factor and ethanol concentration in retentate.**

The effect of operating cell temperature on ethanol concentration retentate is shown in Figure 5. The experimental results show that the ethanol concentration depended on the cell temperature, indicating that the higher quality ethanol concentration in retentate can be gained at higher cell temperature.

**Figure 5** The compositional evolution in ethanol retentate with proceeding of dehydration at different cell temperature; feed pressure gage 1.4 bar, feed concentration of EtOH 95 wt% and flow rate 0.95mL/min

**Figure 6** The influence of operating cell temperature on fluxes and separation factor with 95 wt% of ethanol in the feed side, vacuum 6 mbar, feed pressure gage 1.4 bar and retentate flow rate 0.95 mL/min.

Figure 6 shows a membrane performance as a function of cell temperature. Usually, when the cell temperature is higher, the diffusivity of permeant molecules will be increased but the activity of feed vapor is reduced, resulting in a significant decline in the
solubility of the vapor into membrane (C.K. Yeom et al., 1997). Thus, the overall permeation rate shows a decrease with cell temperature as shown in this figure. On the other hand, separation factor was observed to change little with cell temperature increasing from 80 to 100 °C, and change dramatically with increasing of cell temperature from 100 to 120 °C. It might be explained that higher cell temperature could produce a higher saturated vapor pressure which would increase the solubility of feed vapor to the membrane and thus the difference in densities of the saturated vapor or the interfacial resistance could be lowered to increase a net flux across the interface between feed and membrane. In addition, high cell temperature could also be positive factor for the vapor permeation of ethanol-water mixtures (C.K. Yeom et al., 1997).

Figure 7 The compositional evolution in ethanol retentate with proceeding of dehydrating at different retentate flow rate; feed pressure gage 1.6 bar, feed concentration of EtOH 95 wt% and T_cel 120 °C.

Effect of retentate flow rate on permeate flux, separation factor and ethanol concentration in retentate.

The effect of operating retentate flow rate on ethanol concentration in retentate is shown in Figure 7. The experimental results show that the ethanol concentration depended on the retentate flow rate, indicating that the higher quality retentate can be gained at lower flow rate.

The influence of retentate flow rate on fluxes and separation factors are illustrated in Figure 7. It is shown that total, ethanol, and water fluxes increase with increasing retentate flow rate. The total fluxes increase from 1.26 to 1.57 kg.m⁻².h⁻¹ as the retentate flow rate increases from 0.89 to 3.03 mL/min. For ethanol and water, it is clearly seen that water fluxes and ethanol fluxes slightly increase with increasing retentate flow rate. For separation factor, it is clearly seen that separation factor increase dramatically with increasing of retentate flow rate.

All experiments were carried out at the vapor feed ethanol concentration 95 wt% whilst the vacuum pressures was kept constant at 6 mbar. In general, the lower the downstream pressure applied to the membrane, the higher the water flux can be obtained. This is simply due to the lower boiling point of water caused by low vacuum pressure. Vacuum pressure plays an important role on water permeation through the selective layer. The most influential region is in the range for up to 100 mbar. This is because the boiling point of water significantly reduces at pressure lower than this value [Boontawan A et al., 2007]. However, application of very low vacuum requires a large amount of energy input, and also needs a large vacuum pump resulting in higher investment cost. Therefore, optimization between a high
water flux and energy consumption is essential for engineer to design the dehydration plant. In addition, high energy for cooling is required for a very low vacuum applied.

From this experiment, the highest purity ethanol concentration of 99.34 wt% was obtained with the following operating conditions: temperature of cell 120 °C, flow rate 0.875 mL/min and feed pressure of 1.6 bar, respectively. However, separation becomes more difficult at very high ethanol concentration due to low driving force.

**Pressure Swing Adsorption**

For adsorption experiment, adsorption beds packed with molecular sieve 3Å were used as the final step to dehydrate ethanol vapor with the objective of the highest purity for up to 99.95 wt%. Various concentrations of the feed ethanol increased from 95 to 99.0 wt% and feed pressure gage from 1 to 3 bars. In this work, operating parameters on adsorption capability and on productivity were investigated including the effect of feed ethanol concentrations and feed pressures.

**Effect of Feed Pressure on Adsorption Capability**

Figure 9 shows the effect of feed pressure on adsorption capability. Experimental result shows that the adsorbed masses of water increased with increasing feed pressure. It can be seen that, at higher feed pressure gage, the adsorbed quantity for water went down faster than lower feed pressure gage. From the experimental results, adsorption works much better at higher feed pressure.

![Figure 9](image1.png)

**Figure 9** The relationship between wt% of H₂O after adsorption and volume (mL); Feed 95 wt% EtOH and T_column 145 °C.

![Figure 10](image2.png)

**Figure 10** The relationship between wt% of H₂O after adsorption and volume (mL); feed pressure 3.2 bar, and T_column 145 °C.

**Effect of feed ethanol concentration on adsorption capability.**

The effect of feed ethanol concentration on adsorption capability and on productivity is shown in Figures 10. It can be seen that when the feed concentration of ethanol was increased from 95.0 to 99.0 wt%, the adsorption capability increased. It was noted that, at the feed ethanol concentration of 99.0 wt %, the adsorption capability effect is the largest. From the experimental results, adsorption works much better at lower water content, coincidently at 1 wt% water content of the feed. Residual water content goes down to ppm level before gradually increases after 300 mL accumulation.
Conclusions

A major challenge in the dehydration of ethanol is the high energy cost associated with the separation of ethanol from the large excess of water. Vapor permeation and pressure swing adsorption have been proposed as alternative technologies for azeotropic separation problems. For vapor permeation technique, the performance of water across the selective layer depends on many operating parameters including partial feed pressure, module temperature, retentate flow rate, and down stream pressure. In this work, a composite PVA/PAN membrane can be expected to be utilized in motor fuel grade ethanol (MFGE) production. The mathematical simulation suggested that membrane area increased exponentially with the required purity of ethanol. The membrane area increases for nearly 4 times when the purity of ethanol in the retentate stream increases from 99 to 99.99%. Therefore, it is reasonable to employ vapor permeation system to obtain water content in retentate at 99% since separation becomes much less effective. For adsorption system, pressurized beds packed with molecular sieve 3Å (individual bed volume of 0.5 L) used as the final step to dehydrate ethanol vapor for up to 99.99%. The adsorption capacity of 3Å molecular sieves for ethanol dehydration was investigated on lab scale apparatus. The masses of water and ethanol adsorbed were measured for various feed ethanol concentrations and operating feed pressure. From the experimental results, it can be concluded that adsorption works much better at higher feed pressure gage and at lower water content, incidently at 1 wt% water content of the feed.

Acknowledgements

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References


Tangerine Quality Monitoring by Ethanol Concentration Measurement

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Abstract
The objective of this work is attributed to postharvest quality of ‘Sai Nam Pung’ tangerine. The ethanol concentration of the tangerine quality was monitored during keeping. Commercially, a tangerine is coated with wax in order to increase the shelf-life. However, the waxed tangerine can generate an anaerobic respiration known as “fermentation” which has an effect on the tangerine quality. As a result of the fermentation, ethanol is produced. In this work we have tested the tangerine quality with our home-made alcohol meter based on a semiconductor oxide gas sensor. The alcohol meter is designed to measure four tangerines at the same time. The ethanol, diffused from the tangerine surface, was measured by the alcohol meter in a close system at a constant temperature of 35°C. In order to check the capability of the home made system, the alcohol meter was calibrated with standard ethanol solutions. With these standard solutions, the tangerine quality was classified to 3 levels which are 25 ppm, 50 ppm and 75 ppm. The ethanol level results were compared with the human taste. For human score, the tangerine was orally tasted by trained specialist then a score was given. From the experimental results, a correlation between the human scores and the ethanol levels was determined and can be used for indicating the tangerine quality. From the correlation results, the home-made alcohol meter can be used for continuously monitoring the tangerine quality in a non-destructive way.

Keywords: alcohol meter, postharvest, quality monitoring, tangerine

Introduction
‘Sai Nam Peung’ Tangerine fruit is a hybrid cultivar grown commercially in northern part of Thailand. The fruit is in oblate shape, orange peel and firm texture. The fresh tangerine fruit is juicy and sweet/sour taste, which has a rich flavor. Normally harvested fruits are mostly waxed thus, after a few days at room temperature they will develop off-flavored due to increasing of ethanol content inside the tangerine. The wax coating is needed for high gloss skin, for preventing water loss and also for decreasing of shrinkage. Therefore, the wax coating will prolong the marketing life of the fresh fruit. However, many reports showed that coating in various citrus fruit cultivars has effect in respiration and composition of the internal fruit leading to the development of distinct off-flavor. Although the external look of the tangerine is in a good shape, the off-flavor tangerine also has an impact on the buying decision of the consumer. An off-flavor indication is an increase of ethanol content. (Cohen et al., 1990; Ke and Kader, 1990). The off-flavor tangerines reported so far have an ethanol for 50-225 ppm at the skin of the tangerine (Mangkornthong et al., 2007) and the good quality tangerines will have an ethanol lower than 50 ppm (Wongsila et al., 2005). In this study we developed the method and equipment for monitoring the quality of the tangerines during storage time by non-destructive way. The quality monitoring equipment was used to measure an evaporated ethanol from the skin of the waxed ‘Sai Nam
Peung’ tangerines and the measured value of the ethanol was then checked with the testing score of the trained volunteer.

Materials and Methods

Preparation of Tangerine

‘Sai Nam Peung’ Tangerine fruits were harvested at mature green state on July from Fang district, 200 kilometers far from Chiang Mai city, and transported to laboratory at Chiang Mai University. The tangerine fruits were washed with dish washing detergent, rinsed with tap water and then selected for uniform size and free from bruise. The fruits were divided into 3 groups. One was used as “control”, no coating material; the other two were coated with 2 types of coating materials. Type A called “rosy” was composed of oxidized polyethylene, shellac and wood resin and type B called “wax” was composed of bee wax and resin. Then, each group was kept at 25 ºC, 70% RH. Every two day during storage the alcohol measurement was performed.

Hardware

In this research, non-destructive technique was used to measure the evaporated ethanol from the skin of the tangerine fruits. In this tangerine measuring system, a tangerine fruit was put inside the plastic box (39 cm x 71 cm x 25 cm ) called chamber. As shown in Figure 1, thermal insulator was covered inside the chamber. The internal temperature of the chamber was heated up to 35 ºC using nichrome heater and was checked using the thermocouple type-T together with temperature controller (Maxthermo, MC-2438).

Calibrating Methods

The tangerine monitoring system was composed of four ethanol sensors (based on a semiconductor oxide gas sensor) controlling by the data acquisition system (agilent 34970a), supplied of the electrical current by the programmable power supply (TTi TSX1820P). The whole systems were controlled by the microcomputer as shown in Figure 2.

The alcohol vapor was blown to the four ethanol sensors of the system for one minute. The data from the four sensors were recorded. It is also worth to mention that when the prepared concentration was changed the re-programming of the system was needed for displaying the right value as the standard alcohol solution.

Figure 1 Heating chamber covered by thermal insulator for heating up to 35 ºC.

Figure 2 Home-made alcohol measuring system for tangerine quality monitoring.
Quality testing for tangerine

Each prepared tangerine was put in a glass bottle and all glass bottles were placed inside the temperature controlled chamber. When the chamber was filled by the glass bottles that have the tangerines inside, the temperature was set at 35 ºC for 5 minutes. Next, the evaporated ethanol from the skin of the tangerines was determined by the alcohol sensor system, then the system will record the measured value in the computer. In this study the quality of the tangerines was monitored until the tangerine was in fermented state. The tangerine was orally tasted by four trained volunteer, and then a given score was compared with the measured value. One replication of tangerine sample was used in the panelist test.

Results and Discussion

Figure 3 showed plots of ethanol concentration measured by our home-made alcohol meter as a function of time for 30 tangerines with coating type (a) control, (b) rosy and (c) wax. It can be seen that large amount of tangerines with rosy had high ethanol concentration but smaller amount of tangerines with wax had high ethanol concentration. This indicated that the tangerines with rosy were the most fermented tangerines and the tangerines with no wax (control) were the least fermented tangerines. Thus, wax was more suitable for coating tangerines than rosy due to better prevent fermentation process and can be used to increase the shelf-life of tangerines.

The ethanol concentration results were changed to ethanol level and compared with the human taste. Figure 4 showed plots of ethanol level as a function of human score for 30 tangerines with coating type (a) control, (b) rosy and (c) wax.
It can be seen that a linear relation was observed indicating good agreement between ethanol level measured by our home-made alcohol meter and human score gave by volunteers.

The consistence results were summarized in Table I. It was found that there was a small number of inconsistence. There were only 2, 1 and 4 inconsistence for coating type of control, rosy, and wax, respectively. Moreover, the consistence of our home-made alcohol meter was high with percentage of more than 86.7% and can be as high as 96.7% for rosy tangerines. It should be noted that the measurement time for each tangerine measurement by our home-made alcohol meter is about 3 min. Thus, our home-made alcohol meter can be used for continuously monitoring the tangerine quality in a non-destructive way.

**Table I** Summary of consistence results between ethanol level measured by our home-made alcohol meter and human score gave by volunteers.

<table>
<thead>
<tr>
<th>Coating type</th>
<th>No. of test</th>
<th>Consistence</th>
<th>Inconsistence</th>
<th>Percentage of consistence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>30</td>
<td>28</td>
<td>2</td>
<td>93.3</td>
</tr>
<tr>
<td>rosy</td>
<td>30</td>
<td>29</td>
<td>1</td>
<td>96.7</td>
</tr>
<tr>
<td>wax</td>
<td>30</td>
<td>26</td>
<td>4</td>
<td>86.7</td>
</tr>
</tbody>
</table>

**Conclusions**

Tangerine quality monitoring by ethanol concentration measurement was successfully performed by using our home-made alcohol meter. The results of ethanol concentration measurement were changed to ethanol level and compared with the human taste. The good agreement between ethanol level measured by our home-made alcohol meter and human score gave by volunteers was observed with consistence percentage of more than 86.7% and can be as high as 96.7% for rosy tangerines. Thus, our home-made alcohol meter can be used for continuously monitoring the tangerine quality in a non-destructive way.

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**Screening for Physical Stability of Nanoemulsions Containing Plai Oil by Box-Behnken Design**

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**Abstract**

The aim of this work was to screen for physical stability of nanoemulsions containing plai (Zingiber cassumunar Roxb.) oil using 3-factor, 3-level Box-Behnken experimental design. Oil-in-water nanoemulsions containing plai oil were prepared by using pectin, which is a biopolymer, combined with Tween® 80 as emulsifiers and homogenized at 9500 rpm for 20 minutes. For Box-Behnken experimental design, the three independent variables investigated were the concentration of pectin, plai oil and Tween® 80. The droplet size and zeta potential of the prepared formulations were investigated using a laser scattering particle size distribution analyzer and zeta potential analyzer, respectively. All nanoemulsions were subjected to temperature cycling for physical stability test by storage at 4°C for 24 h and 45°C for 24 h for 5 cycles. Percentage of creaming was calculated and used as response variable. The results showed that droplet size of plai oil-loaded nanoemulsions ranged from 98 nm to 19.7 µm and the zeta potential was in the range of -20.27 to -1.37 mV. The physical stability of nanoemulsions increased with an increased concentration of pectin and Tween® 80. In addition, the increase in plai oil concentration resulted in a decrease in the physical stability. The application of Box-Behnken experimental design facilitated the prediction and identified important formulation variables on physical stability of nanoemulsions containing plai oil.

**Keywords:** nanoemulsions, plai oil, Box-Behnken experimental design, physical stability

**Introduction**

Zingiber cassumunar Roxb., commonly known as “plai”, is a Thai herbal plant that has been exploited for medicinal purposes in Thailand for centuries. Plai is composed of curcuminoinds, essential oil and other compounds. Essential oil of plai is steam-distilled from the rhizome and a pale amber color. The scent is a cool, green peppery one with a touch of a bite. The active chemicals, which are major constituents, are terpinene-4-ol (25-42%), sabinene (25%-45%), α-terpinene(5-10%), γ-terpinene and (E)-1-(3’,4’-dimethoxyphenyl) butadiene (DMPBD) (1-10%) (Wanauppathamkul, 2003). These active ingredients were used in pharmaceutical applications for its anti-inflammatory, antioxidant and antimicrobial activities. However, plai oil itself is volatile and insoluble in water. Development of plai oil formulations as nanoemulsions may provide a solution to these problems.

Nanoemulsions are a class of emulsions with very small and uniform droplet size, typically in the range of 20–500 nm. In general, the size of emulsion droplets formed is controlled by the interplay between droplet break up and droplet coalescence (McClements, 1999; Dickinson, 2003). Droplet break up is controlled by the type and amount of shear applied to the droplets as well as the droplets resistance to deformation which is determined by the surfactant. The rate of droplet coalescence is determined by the ability of the surfactant to
adsorb on the surface of newly formed droplets (Dickinson, 2003). Emulsifiers are surface-active molecules with adsorb to the surface of freshly formed droplets during homogenization, forming a protective membrane which prevent the droplets from coming close enough together to aggregate (Dickinson, 2003). The chemical structure of the emulsifier molecules at the interface of emulsion droplets has an important impact on the stability and physical properties of a number of foods. Emulsifiers that formed thicker and more viscoelastic films at the oil-water interface are more resistant to penetration by fat crystals and so provide greater stability to partial coalescence (Dickinson and McClements, 1995).

Pectin, a naturally occurring biopolymer, is a complex mixture of polysaccharides that makes up about one third of the cell wall dry substance of higher plants. Basically, it is a polymer of α-D-galacturonic acid with 1-4 linkages. The presence of surface-active molecules in pectin provides the emulsification properties. In addition, pectin is capable of reducing the interfacial tension between an oil phase and a water phase and can be effective in the preparation of emulsions (Sriamornsak et al., 2004). Pectin from citrus fruits and apples can also exhibit good surface activity and emulsion stabilizing characteristics if the average molecular weight is reduced to <80 kDa (Mazoyer et al., 1999). Low methoxyl pectin stabilizes oil in water (O/W) emulsion when it interacts with an emulsifier (mainly protein) in the system (Einhorn-Stoll, 1998).

Experimental design has been frequently applied for nanoemulsions optimization considering the advantages such as reduction in the number of experiments that need to be performed, development of mathematical models to assess the relevance and statistical significance of the factor effects, and evaluation of interaction effect between studied factors (Bouchemal et al., 2004; Pey et al., 2006; Gutiérrez et al., 2008). The Box-Behnken design is one of the most efficient experimental designs (Ferreira et al., 2007). An advantage of the Box-Behnken design is that it does not contain combinations for which all factors are simultaneously at their highest or lowest levels. So these designs are useful in avoiding experiments performed under extreme conditions, which unsatisfactory results are often obtained.

This work describes experimental design to reveal interactions among three factors (concentration of pectin, Tween® 80 and plai oil) with respect to their influences on stability properties of nanoemulsions. Box-Benhken methodology was used to design a set of 15 experimental conditions that are capable of elucidating the influence of these factors on the stability responses including second-order and interaction effects. The measured responses were fit to polynomial model functions, and an analysis of the polynomial coefficients and their standard errors was used to identify the factors and interaction terms that are statistically significant for each model.

Materials and Methods

Materials

Plai oil extracted from *Zingiber cassumunar* Roxb. was obtained from Thai-China Flavors and Fragrances Industry (TCFF) Co., Ltd. (Thailand). High methoxyl pectins with degree of esterification of 70% were a gift from Herbstreith & Fox KG (Germany). Tween® 80 was purchased from Fluka (Switzerland).

Experimental Design

A three-level, three-factorial Box–Behnken experimental design was used for conducting the experiments. The nonlinear quadratic model generated by regression of the variables is as follows:

\[
Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{23}X_2X_3 + b_{13}X_1X_3 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2
\]

(1)

where \( Y \) represents the response associated with each factor combination, \( b_0 \) is an intercept and \( b_1–b_{33} \) are the regression coefficients of the factors. The formulation factors studied and the levels of factors are presented in Table 1. A total of 15
experimental runs, which the dependent variables was physical stability ($Y$) and the independent variables were concentration of pectin ($X_1$), plai oil ($X_2$), and Tween® 80($X_3$), was generated and analyzed.

Table 1 Formulation factors and levels used in Box-Behnken experimental design

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>$X_1$: Pectin (%w/w)</td>
<td>-1 3 5</td>
</tr>
<tr>
<td>$X_2$: Plai oil (%w/w)</td>
<td>3 5 7</td>
</tr>
<tr>
<td>$X_3$: Tween®80 (%w/w)</td>
<td>1 3 5</td>
</tr>
<tr>
<td>$Y$: Physical stability of nanoemulsions</td>
<td></td>
</tr>
</tbody>
</table>

Preparation of Nanoemulsions Containing Plai Oil

Oil-in-water nanoemulsions were prepared by using various concentrations of plai oil, pectin and Tween® 80 and mixed by homogenizer (Ultra-Turrax® T50 Basic, IKA, Germany) at a speed of 9500 rpm for 20 minutes in an ice bath to avoid overheating.

Evaluation of Morphology, Particle Size and Zeta Potential

The morphology of nanoemulsions was evaluated by a light microscope (CX41RF, Olympus, Japan). The particle size and the zeta potential of the nanoemulsions were measured by a laser scattering particle size distribution analyzer (Horiba LA-950, Japan) and zeta potential analyzer (Zeta Plus, Brookhaven, USA). The measurement of particle size and zeta potential was performed in triplicates.

Stability Test

Stability test was performed using the method reported by Aoki et al. (2005) with some modifications. Briefly, the prepared nanoemulsions were transferred into a cylindrical glass (internal diameter 15 mm, height 180 mm) until an emulsion height of 135 mm and stored at 45 °C for 24 hours and then at 4 °C for 24 hours. This cycle was repeated five times. The percentage of creaming that represents the cream layer formed at the top of the tube was calculated and expressed as a percentage (of the total volume of emulsion in the tube) using the following equation:

$$C = 100 \times \left( V_t - V_s \right) / V_t$$  (2)

where $C$ is the creaming (volume) percentage, $V_t$ (mL) is the total volume of the sample, and $V_s$ (mL) is the volume of the lower phase layer. According to this equation, it is worth noticing that a greater value of the $C$ is an indication of a more stable emulsion.

Statistical Analysis

Responses observed were fitted to linear, second order and quadratic models, and were evaluated in terms of statistically significant coefficients, $p$-values and $R^2$ values. Polynomial equations involving the main effect and interaction factors were determined based on estimation of statistical parameters such as multiple correlation coefficient and the predicted root mean squares error (RMSE) generated by Statistica™ software (Statsoft Inc., Tulsa, OK, USA). The statistical analysis of the polynomial equations was established by ANOVA provision available in the software.

Results and Discussion

Characterization of Nanoemulsions

The microscopic images of nanoemulsions containing various concentrations of plai oil can be observed in Figure 1. The nanoemulsions obtained were spherical in shape and showed a polydisperse in the droplet size (0.090-18.784 µm). The smaller size of droplets was obtained when the plai oil concentration was decreased from 7% to 3%. The formulation with Tween® 80 (3%) revealed very small droplets (Figure 1d); the size was lesser than 900 nm, comparing to the size of the formulations without Tween® 80 which were more than 10 µm. This indicated that the addition of Tween® 80 to the emulsion formulation was very important to obtain the nano-sized emulsions. The reason for this behavior could be the influence of Tween® 80 in the molecular association with pectin.
Figure 1 Microscopic images of nanoemulsions containing (a) 3% pectin + 7% plai oil, (b) 3% pectin + 5% plai oil, (c) 3% pectin + 3% plai oil, and (d) 3% Tween® 80 + 3% plai oil.

The droplet size of the formulations containing both Tween® 80 and pectin was even smaller. The results from a laser scattering particle size analyzer (Figure 2a) showed a decrease of the droplet size with a decrease in the concentration of plai oil and an increase in the concentration of pectin (the formulations containing 3% Tween® 80). All the zeta potential values obtained are negative (-21.31 to -1.16 mV). Increasing of pectin concentration tended to decrease the zeta potential of nanoemulsions (Figure 2b). This is probably due to the increase of a negatively charged polysaccharide, e.g., pectin, which contains higher amount of carboxyl groups, could dissociate to negative charge (e.g., COO-) (Sriamornsak, 2003) in the emulsion formulation.

Stability of Nanoemulsions

In this study, the stability of nanoemulsions were evaluated by temperature cycling testing that is an environmental stress test used in evaluating reliability nanoemulsions to catch early-term, latent defects by inducing failure through thermal fatigue. The results showed that, after 2-cycle storage, most formulations of nanoemulsions separated into a cream layer at the top, a turbid layer in the middle, and a transparent layer at the bottom. However, the formulations containing 5%w/w pectin showed the highest percent creaming (100% cream) within five cycles of temperature cycling test. It could be observed that decreasing of plai oil and increasing of pectin concentration in the formulations tended to increase the stability of nanoemulsions (Figure 3). Stability is reflected by the value of the creaming percentage that provided indirect information about the extent of droplet aggregation in the nanoemulsions. The lower the percentage of creaming, the greater the aggregation. The results indicated that higher concentrations of pectin produced more stable nanoemulsions in which pectin was tightly bound onto the oil droplets, then the extent of droplet aggregation were reduced. It is thought that the increase of pectin, negatively charged polysaccharide, in the nanoemulsion formulations resulted in an
increase in negative repulsive forces (electrostatic and steric) between oil droplets. Therefore, the presence of pectin can reduce the interfacial tension and form a cohesive interfacial film around the nanoemulsion droplets thereby retarding nanoemulsion instability (Funami et al., 2007).

**Figure 3** Percent creaming of nanoemulsions containing 3% Tween® 80 and various concentrations of plai oil and pectin.

### Statistical Analysis

Box-Behnken experimental design was executed to determine the relationship (X₂) and Tween® 80 (X₃) against physical stability of nanoemulsions. The formulation composition factors and response data for Box-Behnken experimental design were presented in Table 2. In this study, physical stability of nanoemulsions was presented as score of percentage creaming, i.e., 100% creaming were expressed as 1 and less than 100% creaming was expressed as 0. A statistical method is used based on analysis of the variance (ANOVA) which determines the statistical signification of effects and interactions comparing the mean square with an estimation of experimental error. Once the variables and their experimental field were selected, the influence of variables and their interactions on physical stability was checked. Table 3 shows ANOVA table for the effect of formulation factors on the physical stability using Box-Behnken experimental design with a confident level of 95%. The concentration of pectin had highest impact (F ratio = 237.53, ***P<0.001).

#### Table 2 Formulation compositions (X) and the physical stability expressed as score (Y) in Box-Behnken design.

<table>
<thead>
<tr>
<th>No.</th>
<th>%Pectin (X₁)</th>
<th>%Plai oil (X₂)</th>
<th>%Tween® 80 (X₃)</th>
<th>Score (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
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<tr>
<td>2</td>
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<td>15</td>
<td>3</td>
<td>5</td>
<td>3</td>
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</table>

<table>
<thead>
<tr>
<th>Factors</th>
<th>Sum of squares</th>
<th>d.f.</th>
<th>Mean square</th>
<th>F-ratio</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>X₁ (L+Q)</td>
<td>6.2875</td>
<td>2</td>
<td>3.14375</td>
<td>237.53</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>X₂ (L)</td>
<td>0.1875</td>
<td>1</td>
<td>0.1875</td>
<td>14.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>X₃ (L)</td>
<td>0.1875</td>
<td>1</td>
<td>0.1875</td>
<td>14.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>X₁*X₂</td>
<td>0.525</td>
<td>3</td>
<td>0.175</td>
<td>13.22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>X₁*X₃</td>
<td>0.45</td>
<td>2</td>
<td>0.225</td>
<td>17.00</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>X₂*X₃</td>
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<td>1</td>
<td>0.375</td>
<td>28.33</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Error</td>
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<td>34</td>
<td>0.013235</td>
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<td></td>
</tr>
<tr>
<td>Total SS</td>
<td>8.8</td>
<td>44</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

L= linear, Q =quadratic

The responses observed for 15 prepared formulations were fitted to first order, two-factor interaction and quadratic models. For the estimation of the significance of the model, the analysis of variance was applied. The quadratic model was established for physical stability with the R² of 0.9511 and RMSE of 0.098. The equation which describes physical stability in terms of the significant variables (***P-values<0.001) was as follows:

\[
\text{Physical stability (Y)} = 0.140744 \times X₁^2 - 0.75443 \times X₁ - 0.140625 \times X₂ - 0.109375 \times X₃ + \text{No.} \]
Response surface analysis was presented in three-dimensional graphs to explain the factor affecting physical stability of nanoemulsions containing plai oil. The response surface plots were used for interpretation of the interaction effects of two independent variables on the responses or dependent variables when a third factor was kept at constant level zero. The effects of pectin \((X_1)\) and plai oil \((X_2)\) concentration when Tween® 80 was kept constant \((3\%\text{w/w})\) and their interaction on physical stability \((Y)\) (Figure 4). It was estimated that plai oil could be successfully incorporated into the nanoemulsions in the concentration of 3-7%w/w using of 5%w/w pectin and 3%w/w Tween® 80. However, low concentration of plai oil in nanoemulsions tended to be more stable and the physical stability increased with the increased concentration of both pectin and Tween® 80. In these results, Tween® 80 acted as O/W emulsifier and pectin acted as co-emulsifier. The possible explanation for this is that pectin having amphiphilic character and has been used as a natural emulsifier in this study. An effective emulsifier is therefore one that (i) rapidly reduces the interfacial tension at the freshly formed oil–water interface, (ii) binds strongly to the interface once adsorbed, and (iii) protects the newly formed droplets against flocculation or coalescence (Dickinson and McClements, 1995). Once an emulsion has been formed, the main factor determining stability is the strength/range of the repulsive interactions between pairs of closely approaching droplet surfaces. As a hydrocolloid, pectin contains hydrophobic groups that are numerous enough and sufficiently accessible on a short timescale to enable the adsorbing molecules to adhere to and spread out at the interface, thereby generating a greater short-range steric repulsion between the droplets that may prevent them from coming close enough together to coalesce (Dickinson, 2003). Moreover, the presence of pectin can reduce the interfacial tension and form a cohesive interfacial film around the nanoemulsion droplets thereby retarding nanoemulsion instability (Funami et al., 2007).

\[
0.141614 \ X_1X_2 - 0.0079 \ X_1X_2^2 - 0.015625 \ X_1^2X_2 + 0.015625 \ X_1^2X_3 + 0.03125 \ X_2X_3 +
0.909968
\] (3)

Figure 4 (a) Response surface plot and (b) contour plot showing effect of pectin \((X_1)\) and plai oil \((X_2)\) concentrations, at constant Tween® 80 \((3\%\text{w/w})\) on physical stability \((Y)\).

Consequently, higher concentrations of pectin produced more stable nanoemulsions in which pectin was tightly bound onto the oil droplets (Burapapadh et al., 2010).

Conclusions

Experimental designs allowed evaluating the most important factors on observed responses, and investigating the relationship between factors by the response surface methodology. Box-Behnken design was successfully used to statistically evaluate the
main interaction and quadratic effects of independent variables on the physical stability of nanoemulsions. In this study, the physical stability increased with the increased concentration of both pectin and Tween® 80 and the decreased plai oil concentration. Pectin, which was used as a natural polymeric emulsifier, was the most important factors on physical stability of nanoemulsions containing plai oil.

Acknowledgments

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References


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Effect of Ozone on Oxidative Stress Defense Enzymes and Quality Changes in Tangerine (Citrus reticulata Blanco cv. Sai Nam Pung) Fruit

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Abstract
The effect of ozone on oxidative stress defense enzymes and quality changes in tangerine (Citrus reticulata Blanco cv. Sai Nam Pung) fruit were studied. The fruits were either not wounded and non-inoculated or wounded and inoculated with Penicillium digitatum (1.04×10⁵ spores ml⁻¹) and then fumigated with ozone (200 ppm) for 0, 0.5, 1, 2, 4 or 6 hours and stored at 25±2 °C for 7 days. Samples were taken everyday for investigation on oxidative stress defense enzymes activity (superoxide dismutase, peroxidase and catalase), some physical qualities (fruit firmness, weight loss and peel color) and chemical qualities (total soluble solids, titratable acidity and ascorbic acid content) changes. The results showed that exposing fruit to ozone for 4 and 6 hours delayed the disease incidence in inoculated fruit. One day after ozone fumigation, the activities of superoxide dismutase, peroxidase and catalase in the fruit peel were increased, especially in the 4 and 6 hour treatments. Throughout the 7 days of the experiment, the physical and chemical qualities of tangerine fruit in all treatments were not affected by ozone exposure. The results suggest that exposing fruit to ozone was effective in controlling postharvest disease and can increase superoxide dismutase, peroxidase and catalase activities.

Keywords: Citrus reticulata Blanco cv. Sai Nam Pung, oxidative stress defense enzymes, ozone, Penicillium digitatum

Introduction
Tangerine (Citrus reticulata Blanco cv. Sai Nam Pung) is an economically important fruit in Thailand. Fungal decay is the main cause of citrus postharvest loss. The high incidence of postharvest disease in citrus is primarily due to green mold, Penicillium digitatum and blue mold, Penicillium italicum. Although rotting may be reduced with chemical disinfection, recently many people have been concerned about food safety. So the alternative methods to chemical disease control techniques are needed. Among a number of new strategies being investigated, ozone may constitute a desirable and effective residue-free alternative to traditional postharvest fungicide practices (Tzortzakis et al., 2007). Ozone is a powerful oxidizing agent and is widely used as an antimicrobial agent to inactive bacteria, fungi, viruses and protozoa for disinfecting water, in the food and beverage industries, for reusing the wastewater, and controlling the alkalinity and pH of shrimp pond water (Kim et al., 1999; Whangchai, 2001). Ozone has been used during postharvest treatments in order to reduce...
growth, sporulation and mycotoxin production of pathogenic microorganisms, and to extend the shelf-life of various products. Palou et al. (2003) reported that ozone gas continuously generated in a cold storage room at the rates ranging from 0.5-1.0 ppm effectively control the sporulation of *P. digitatum* and *P. italicum* on orange (*Citrus sinensis* L.). Tzortzakis et al. (2007) reported that ozone fumigation (0.1 µmol mol⁻¹) of tomatoes, strawberries, table grapes, and plums kept at 13 °C resulted in a decline in spore production of gray mould (*Botrytis cinerea*). Whangchai et al. (2005) found that ozone exposure of longan at a concentration of 200 ppm for 60 minutes significantly reduced growth of *Lasiodiplodia* sp. and *Cladosporium* sp. Ozone treatment also results to some biochemical changes. When ozone comes in contact with plant tissue, it may induce oxidative stress that will produce peroxides and free radicals which cause oxidative damage in plants (Mittler, 2002). Plants have evolved a defense system to prevent the accumulation of potentially damaging reactive oxygen species (ROS). Willekens et al. (1994) reported that ozone fumigation caused an increase in transcription levels of some enzymes such as catalase, superoxide dismutase and ascorbate peroxidase in *Nicotiana plumbaginifolia* L. Magbanua et al. (2007) found a relationship between resistance to *Aspergillus flavus* infection of maize and a high level of catalase activity.

However, less attention has been paid to the impacts of ozone on the quality of treated fruits and vegetables. Low-level atmospheric ozone-enrichment during cold storage is capable of controlling not only disease development and proliferation, but also maintains fruit quality in terms of taste and firmness. Aguayo et al. (2006) found an increase in fructose, glucose, ascorbate and fumarate contents in tomato after exposure to ozone at a rate of 4 µl l⁻¹ for 30 minutes every 3 hours. This study was conducted to verify the effect of ozone on some biochemical and quality changes in tangerine fruit cv. Sai Nam Pung during storage.

**Materials and Methods**

**Plant Materials and Ozone Treatment**

Tangerine fruits cv. ‘Sai Nam Pung’ that grown under standard cultural practices were obtained from a commercial orchard in Amphoe Fang, Chiang Mai province, Thailand. Fruits were harvested in February, 2009, when commercially mature (in season with yellow peel). Before any commercial postharvest treatment was applied, the fruits were selected by hand and transported to the Postharvest Physiology Research Laboratory at Chiang Mai University within 3 hours. The surface of the fruits were washed with tap water and dried for 3 hours at 25±2 °C. Tangerine fruits were separated into 2 groups; inoculated and non-inoculated with *P. digitatum*. For the inoculated group, the fruits were wounded with a sterile needle (about 1 mm deep), inoculated with the spore suspension of *P. digitatum* and kept at room temperature for 3 hours before ozone treatment. The fruits were fumigated in a closed chamber at room temperature with ozone gas produced by ozone generator (Ozonizer, Sky zone S50AE) at a concentration of 200 ppm for 0, 0.5, 1, 2, 4 or 6 hours and then kept at 25±2 °C. Samples were collected daily for 7 days and subjected to examination.

**Estimation of Fruit Disease Incidence**

The disease incidence was visually estimated everyday during the experiment for 7 days. Tangerine fruits showing surface mycelia development were considered infected. Fruit disease incidence was expressed as percentage of infected fruits.

**Quality Attributes**

The firmness was measured equatorial by an Effigi pressure tester with a 0.79 cm diameter puncture head. The puncture head was inserted to a depth of 0.5 cm on opposite side of each fruit. Peel color changes fruits were determined using CIE color space coordinates L*, a*, b* obtained with colorimeter (Minolta CR-200). Measurements were conducted on four sides in the middle of each fruit.
For total soluble solids (TSS) and organic acids analysis, a juice sample was taken from each fruit. TSS was measured by digital refractometer (ATAGO PAL-1) and expressed as %Brix. Titratable acidity (TA) was determined by the titrimetric method, involving the titration of fruit juice with 0.1 \( N \) sodium hydroxide, and the formation of pink precipitate monitored using phenolphthalein. The results were calculated as equivalents of citric acid, which is the main organic acid in tangerine fruit. Ascorbic acid content was determined by the 2,6-dichloroindolephenol titrimetric method. Ascorbic acid reduced indicator dye, 2,6-dichloroindophenol, to a colorless solution. At the end point, excess unreduced dye was rose pink in acid solution.

**Enzymatic Assays**

Freeze-dried samples of tangerine peel (0.5 g) were homogenized with 50 mM potassium phosphate buffer (pH 7.0) containing 3 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 g of polyvinyl pyrrolidone (PVP). The homogenate was centrifuged at 15,000 \( \times g \) for 20 min at 4 °C. The supernatant were analyzed for activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT), immediately.

SOD activity was determined spectrophotometrically at 560 nm according to the method of Ukeda et al. (1997). One unit of SOD was defined as the amount of enzyme that caused a 50% decrease of the SOD-inhibitable NBT reduction. POD activity was determined spectrophotometrically at 470 nm according to the method of Nagle and Haard (1975). CAT activity was measured following the decrease in absorbance at 240 nm due to the reduction of \( \text{H}_2\text{O}_2 \) according to the method of Aebi (1984). The specific activity of the enzymes was expressed as U mg\(^{-1}\) protein. Soluble protein content was determined according to the modified method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

**Statistical Analysis**

The Statistical Package for the Social Science (SPSS) software for windows was used for Analysis of Variance (ANOVA) and Least-Significant Difference (LSD) at 95% confidence level of each variable value under Completely Randomized Design (CRD).

**Results and Discussion**

**Effect of Ozone on Disease Incidence**

Ozone fumigation for 4 and 6 hours delayed the disease incidence in inoculated tangerine fruit stored at 25±2 °C. The 0, 0.5, 1, or 2 hours of ozone fumigation had a storage life of 4 days while the 4 and 6 hours ozone fumigation had a storage life of 5 days. The non-inoculated fruit did not show any disease incidence for more than 7 days of storage (Figure 1). This result agreed with the previous studied by Palou et al. (2003) whom found that ozone gas continuously generated in a cold room (0.5-1.0 ppm) could control the sporulation of *Penicillium digitatum* and *P. italicum* on orange.

**Figure 1** Percent of disease incidence of tangerine fruit fumigated with ozone at 0 (◊), 0.5 (□), 1 (△), 2 (○), 4 (–) or 6 (×) hours of exposure and storage at 25±2 °C (Note that no disease incidence for the non-inoculated groups through 7 days of the storage).
Effects of Ozone on Quality Attribute

Weight loss in fruits treated by ozone for 4 and 6 hours were not statistically different among treatments. The weight loss in all treatments was less than 3% which did not affect the total fruit quality. Firmness of the inoculated fruit decreased rapidly after the *P. digitatum* infestation. This mold attacks injured areas of the peel and appeared as a soft, watery, decolorized spot on the rind causing the fruit to collapse eventually. While the non-inoculated fruits maintained their firmness throughout the storage time at 25±2 °C.

After ozone fumigation for 2, 4 or 6 hours, the lightness (*L*), redness (*a*) and yellowness (*b*) index were not statistically different among all treatments (Table 1). The chemical qualities such as the content of total soluble solids (TSS), titratable acidity (TA), and ascorbic acid were not statistically different among all treatments (Table 2). Similar to the report of Tzortzakis *et al.* (2007) whom found that ozone treatment at 1.0 µmol mol⁻¹ at 13 °C did not affect the changes of organic acid and vitamin C content. However, after storage for longer periods, it was found that the content of citric acid and ascorbic acid decreased (data not shown). The observed decrease may be due to the fact that these acids are used as substrate for respiration (Nagar, 1994) and as an antioxidant to scavenge free radicals that occurred from various activities in the cells (Dalton, 1995).

### Table 1 Physical qualities parameters of tangerine fruit after inoculated or non inoculated with *P. digitatum* and fumigation with ozone for various times and then stored at 25±2 °C for 4 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ozone fumigation (hr)</th>
<th>Weight loss (%)</th>
<th>Firmness (kg cm⁻²)</th>
<th>Peel color measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated with <em>P. digitatum</em></td>
<td>0.5</td>
<td>0.94⁺</td>
<td>0.18⁻</td>
<td>65.17⁺</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.84⁺</td>
<td>0.21⁻</td>
<td>65.56⁺</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>0.97⁺</td>
<td>0.23⁻</td>
<td>65.66⁺</td>
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<tr>
<td></td>
<td>4.0</td>
<td>0.80⁻</td>
<td>1.90⁺</td>
<td>65.96⁺</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0.86⁻</td>
<td>1.92⁺</td>
<td>65.00⁺</td>
</tr>
<tr>
<td>Non-Inoculate</td>
<td>0</td>
<td>0.51⁺</td>
<td>3.08⁺</td>
<td>64.65⁺</td>
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<tr>
<td></td>
<td>0.5</td>
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<td>66.36⁺</td>
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<tr>
<td></td>
<td>6.0</td>
<td>0.84⁺</td>
<td>2.85⁺</td>
<td>66.01⁺</td>
</tr>
</tbody>
</table>

Means with difference letters within the same column represent significant differences at *P*<0.05

### Table 2 Chemical qualities parameters of tangerine fruit after inoculated or non inoculated with *P. digitatum* and fumigation with ozone for various times and then stored at 25±2 °C for 4 days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ozone fumigation (hr)</th>
<th>Total soluble solids (%)</th>
<th>Titratable acidity (% citric acid)</th>
<th>Ascorbic acid (mg 100 ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inoculated with <em>P. digitatum</em></td>
<td>0</td>
<td>11.9⁺</td>
<td>0.59⁺</td>
<td>22.0⁺</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>11.9⁺</td>
<td>0.60⁺</td>
<td>23.4⁺</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>12.3⁺</td>
<td>0.67⁺</td>
<td>20.3⁺</td>
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<tr>
<td></td>
<td>2.0</td>
<td>12.0⁺</td>
<td>0.60⁺</td>
<td>21.8⁺</td>
</tr>
<tr>
<td></td>
<td>4.0</td>
<td>11.8⁺</td>
<td>0.68⁺</td>
<td>23.2⁺</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>12.1⁺</td>
<td>0.69⁺</td>
<td>23.4⁺</td>
</tr>
<tr>
<td>Non-Inoculate</td>
<td>0</td>
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<td>0.79⁺</td>
<td>24.4⁺</td>
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<td>0.67⁺</td>
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<tr>
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<td>2.0</td>
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<td>0.64⁺</td>
<td>25.6⁺</td>
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<tr>
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<td></td>
<td>6.0</td>
<td>10.9⁺</td>
<td>0.64⁺</td>
<td>23.6⁺</td>
</tr>
</tbody>
</table>

Means with difference letters within the same column represent significant differences at *P*<0.05

Effect of Ozone on Oxidative Stress Defense Enzymes

Induced resistance in plants relied on the activation of many different defense mechanisms within the plant. One of biochemical defenses includes the activation of oxidative stress defense enzymes such as SOD, POD or CAT. In this experiment, SOD activity increased rapidly during the first two days after ozone fumigation, and the increase was sharper at 6, 4 and 2 hours than at 1, 0.5 and 0 hours of ozone treatment (Figure 2). SOD is a primary scavenger for superoxide free radicals; the induction of SOD activity after ozone exposure may inhibit radical accumulation in fruit peel resulting in an increased dismutation of the radicals to...
hydrogen peroxide which then will be change to non-toxic molecules by another enzyme such as POD or CAT (Yoshida et al., 1994). SOD activity in inoculated fruits was slightly lower than that of the non-inoculated fruits. Ballester et al. (2006) found that the activity of this enzyme in the area around the inoculation point was lower than that found in the healthy area in citrus fruit inoculated with P. digitatum.

Figure 2  Effect of ozone fumigation on superoxide dismutase (SOD) activity of tangerine fruit inoculated with P. digitatum (A) or non-inoculated with P. digitatum (B) and then fumigated with ozone at 0 (◊), 0.5 (□), 1 (∆), 2 (○), 4 (–) or 6 (×) hours of exposure and storage at 25±2 °C.

POD activity in ozone fumigated fruits, especially in the 4 and 6 hours of the fumigation, showed a marked increase at the beginning of the storage, reaching the highest level after 2 days and then decreased there on rapidly. The activity increased again at the end of storage as shown in Figure 3. The observed increase may have caused by the role of POD in detoxifying the H₂O₂ generated as a consequence of ozone exposure. Ranieri et al. (2000) reported that POD activity was enhanced by ozone fumigation (150 ppb for 4 h repeated for 4 days) in sunflower plants. The decrease of the activity after two days of storage may be due to the transient effect of ozone in activated enzyme activity. Peroxidase activity increases in plants in response to a great variety of stresses, so the increased activity at the end of the storage may be due to stress from pathogenic attack because the level of enzyme activity was closely related to the severity of fungal decay.

Figure 3  Effect of ozone fumigation on peroxidase (POD) activity of tangerine fruit inoculated with P. digitatum (A) or non-inoculated with P. digitatum (B) and then fumigated with ozone at 0 (◊), 0.5 (□), 1 (∆), 2 (○), 4 (–) or 6 (×) hours of exposure and storage at 25±2 °C.

CAT plays a key role in maintaining H₂O₂ homeostasis in plant cells and Magbanua et al. (2007) found a correlation between resistance of maize to the Aspergillus flavus infection and the level of catalase activity. In this experiment, CAT activity increases with ozone fumigation time, the higher values were found in the fruits subjected to 2, 4 and 6 hours fumigation time. The activity still
increased until the third day of the storage and then steadily decreases, but observed to be higher than that of 1, 0.5 or 0 hours fumigation time (Figure 4). The results showed that the increase in CAT activity is principally related to the ozone exposure time and had an important role in inducing the fruit protection against ozone stress. The ability of fruits to maintain high CAT activity may be an important role in the defense mechanism of tangerine fruit against pathogenic attack. The severity of fungal attack or fruit senescence in tangerines after

stored for a longer time at 25±2 °C might be associated with a reduction in the activity of CAT at the end of storage. The results supported the view of Sala and Lafuente (2000), who stated that heat-conditioning treatments increasing chilling tolerance of citrus fruits may be related to induction of CAT activity.

Figure 4 Effect of ozone fumigation on catalase (CAT) activity of tangerine fruit inoculated with P. digitatum (A) or non-inoculated with P. digitatum (B) and then fumigated with ozone at 0 (○), 0.5 (□), 1 (△), 2 (◇), 4 (○) or 6 (×) hours of exposure and storage at 25±2 °C.

Conclusion

Our study showed that exposing fruit to ozone influenced the SOD, POD and CAT activity. The effectiveness of enzyme activation was closely related with the time of ozone exposure; the longer exposure time, the higher enzymes activity. The enzyme activities induced by ozone increased the tolerance of citrus fruit to the fungal attack. Throughout the 7 days of the experiment, the physical and chemical qualities of tangerine fruit in all treatments were not significantly affected by ozone exposure.

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References


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Effects of Prebiotics on Growth Performance and Pathogenic Inhibition in Sex-Reversed Red Tilapia 
(Oreochromis niloticus × Oreochromis mossambicus)

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Abstract
Effect of three types of prebiotics, inulin, galactooligosaccharide and soybean oligosaccharide were tested in vitro and in vivo. In vitro study, 1% and 5% prebiotics were evaluated on their antipathogenic bacterial (Pseudomonas putrefaciens, Vibrio parahaemolyticus or Streptococcus iniae) properties and their ability on growth promotion to a useful bacteria in fish (Bacillus subtilis). Among prebiotics tested, galactooligosaccharide gave the highest inhibition to all tested pathogenic bacteria while inulin and soybean oligosaccharide showed lower inhibition (p<0.05). For growth promotion property, galactooligosaccharide gave the highest growth values while soybean oligosaccharide gave the lowest. All tested prebiotics in vitro at 5% showed antipathogenic bacterial property in fish and growth stimulation on B. subtilis. In vivo test (20 fish with 6 replications) on sex-reversed red tilapia (Oreochromis niloticus × Oreochromis mossambicus) fed with diet containing prebiotics showed that galacto-oligosaccharide (5%) gave the highest specific growth rate (5.01%) and weight gain (720.45%). Feed conversion ratio of diet containing prebiotics was not significantly different from that of the basal diet, which was 1.08. The fish had the lowest mortality rate (37.78%) after receiving diet containing 5% galactooligosaccharide, whereas inulin and soybean oligosaccharide gave the mortality rates of 40.00 and 48.89%, respectively.

Keywords: prebiotic, nile tilapia, growth performance, pathogenic inhibition

Introduction
Prebiotic research is one of the newest fields of nutritional science, offering promising benefits for aquaculture. A prebiotic is a type of functional food defined as a non-digestible ingredient that beneficially affects the host by selectively stimulating the growth and/or the activity of one or a limited number of beneficial microorganisms (Gibson and Roberfroid, 1995) enabling colonization of the gastrointestinal (GI) tract and improving host health at the enteric level (Brunser et al., 2006).

During the last two decades, traditional use of antibiotics in aquaculture has been criticized because of the potential development of antibiotic-resistant bacteria, the presence of antibiotic residues in seafood, the destruction of microbial populations in the aquacultural environment and the suppression of the aquatic animal’s immune system (Smith et al., 2003; Cabello, 2004; Sørum, 2006; Sapkota et al., 2008). As an alternative strategy to antibiotics, probiotics have recently attracted extensive attention in aquaculture. Many reports have been published regarding applications of probiotics in the
aquatic environment (Ringø and Gatesoupe, 1998; Verschuere et al., 2000; Irianto and Austin, 2002; Ringo, 2004; Balcazar et al., 2006; Wang et al., 2008). However, because of high cost, potential impact on the environment, regulatory issues, and food safety and challenges regarding incorporation into modern extruded feeds, large-scale applications of probiotics in water has been limited. Alternatively, it appears more practical to manipulate the GI tract microbiota in aquatic animals through the use of prebiotics that alter the conditions to favour certain bacterial species which may enhance fish growth efficiency and reduce disease susceptibility of the host organism (Gatlin, 2002; Burr et al., 2005; Nicolas et al., 2007).

The improved economy and social concerns by decreasing the use of antibiotics in fish farming have encouraged more environmentally friendly approaches to increase fish growth (Verschuere et al., 2000). Therefore, alternative techniques such as the use of prebiotics (GOS, SOS, inulin) and other related carbohydrates have received considerable attention (Mussatto and Mancilha, 2007; Cerezuela et al., 2008). Currently, the most widely studied prebiotics are inulin and oligofructose, derived from chicory roots, and secondly, GOS derived from enzymatic synthesis of lactose (Roberfroid, 2005; Bosscher et al., 2006). Prebiotics are defined as “non-digestible food ingredient(s) that beneficially affect host health by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon” (Gibson and Roberfroid, 1995). Many functional food products containing prebiotic substances have been successful in human health promotion. However, the evidence for health benefits in animals, especially fish is limited. Administration of specific prebiotics typically results in enhancement of the components of the immune system that also become activated in fish under Amyloodinium epizootics (Noga et al., 1991; Smith et al., 1994; Cobb et al., 1998) and prebiotic supplementation is effective against a variety of bacteria, e.g., Clostridium sp., Listeria sp., Salmonella sp. and Escherichia coli (Holt et al., 2005), including fish pathogenic agents such as Streptococcus iniae (Li and Gatlin, 2004). Thus, the use of prebiotic oligosaccharides as a food or feed ingredient, especially in aquaculture, is gaining popularity.

Materials and Methods

Diets and Experimental Design

Four treatments were carried out with sex-reversed red tilapia (Oreochromis niloticus × Oreochromis mossambicus). The experiment was carried out in twenty-four aquaria with six replicates of three test diets and one control diet. The ingredients and chemical composition of the basal diets used in this study were according to Tudkaew et al. (2007). Three prebiotics (inulin, GOS and SOS) at 5% (w/w) of oligosaccharide content were included in basal diet to replace the filler (milled cassava). The control group was fish receiving the basal diet. The basal diet and each prebiotic were mixed following the pre-calculated formula then extruded and air-dried at room temperature. The diets were kept at -20 ºC until used.

Fish and Experimental Management

Healthy juveniles of the sex-reversed red tilapia were acclimated in the concrete tanks and were fed with basal feed twice daily for 2 weeks. Then healthy juveniles were distributed into 24 aquaria with an initial stocking density of 20 sex-reversed red tilapia per aquarium for 60 days of culture. All fish had similar initial weights (3.05-3.09 g). The experiment was conducted as a completely randomized design with four treatments (tested diets 1-3 and control). The 1st group (control) was given basal diet without prebiotics. The 2nd group was fed the basal diet supplemented with inulin. The 3rd group was fed the basal diet supplemented with GOS and the 4th...
group was fed the basal diet supplemented with SOS.

Fish were fed two times daily at 9 A.M. and 5 P.M. Everyday the feed remaining in the aquaria was cleaned by siphoning.

**Sampling and Analytical Methods**

Feed raw materials, feed, and fish meat were analyzed for crude protein, fat, ash, energy and moisture content. Crude protein was determined using Kjeldahl analyzer and crude fat was determined by Soxhlet extraction method. Energy was determined by bomb calorimeter. Weights of all sex-reversed red tilapia were measured at the beginning (initial weight) and at the end (final weight) of the experiment.

**Growth Parameters and Calculations**

Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated according to the following formulae:

\[
\text{SGR} = 100 \times \left[ \ln(\text{final body mean weight}) - \ln(\text{initial body mean weight}) \right] / \text{duration of feeding (day)};
\]

\[
\text{FCR} = \text{dry feed intake (g)} / \text{wet weight gain (g)}.
\]

**Survival Rate**

The fish were counted after 4 and 8 weeks from the beginning of the experiment to determine the survival percentage, which was as follows:

\[
\text{survival rate} \% = \frac{\text{final fish number}}{\text{initial fish number}} \times 100.
\]

**Bacterial Enumeration**

For bacteriological studies, samples of fish gut homogenate randomly selected from the upper, middle and lower gut, were weighed then added 1:9 (w/w) sterile sodium chloride to dilute up to \(10^4\) dilution. A 0.1 ml sample of each dilution was spread onto duplicate plates of Tryptone soy agar (TSA), Rimler shott agar, Columbia CNA agar and MYP agar. Plate counts were performed and colony forming units (CFU/ml) were determined for cultured bacteria. Representative colonies of dominant bacteria, i.e. those bacteria of similar morphology present in the highest numbers, were randomly picked and subcultured onto TSA, from which subsequent identification tests were performed and the isolates were initially grouped on the basis of their morphology, Gram staining, motility, catalase and oxidase tests and glucose fermentation.

**Immune Response**

After feeding fish with each diet for forty-two days, fish were divided into two groups, i.e. infected group (B) and non-infected group (A). Infected fish were challenged with 0.1 ml of \(S.\ iniae\) containing \(1 \times 10^8\) CFU/ml by injection at peritoneal. After injection, fish were returned to their respective aquaria where they were fed with their respective assigned experimental diets twice daily for 14 days. Following routine observation, fish behavior and mortality were recorded twice daily for 14 days.

Twenty fish were randomly collected from each treatment including the control. The fish were anesthetized by immersion in water containing clove oil. Whole blood was collected from the caudal vein of each fish using syringes (1 ml) and 27-gauge needles that were rinsed in heparin, to determine the hematocrit values. A blood sample was further centrifuged at 1000\(g\) for 5 min at 4\(\degree\)C in order to separate the plasma.

Hematocrit capillary tubes were two-third filled with the whole blood and centrifuged in a hematocrit centrifuge for 5 min and the percentage of the packed cell-volume was determined by the hematocrit tube reader. Data were analyzed by ANOVA using SPSS software version 13.0.

**Results and Discussion**

**Growths Performance**

Feed diets containing 5\% of each prebiotic had a significant effect on growth performance. Feed conversion ratio (FCR) was highest in the control group (1.08±0.04) while diet added with galactooligosaccharide gave the highest specific growth rate (SGR) and % weight
gain of 5.01±0.17 and 720.45±47.48, respectively (Table 1).

Table 1: Feed conversion ratio, specific growth rate and weight gain of sex-reversed red Nile tilapia fed diets containing 5% of each prebiotic.

<table>
<thead>
<tr>
<th>Diets</th>
<th>FCR</th>
<th>SGR</th>
<th>Weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.08±0.04ab</td>
<td>4.57±0.17ab</td>
<td>582.75±46.67ab</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.94±0.11a</td>
<td>4.76±0.36ab</td>
<td>643.36±79.29ab</td>
</tr>
<tr>
<td>GOS</td>
<td>0.88±0.07ab</td>
<td>5.01±0.14ab</td>
<td>720.45±47.48ab</td>
</tr>
<tr>
<td>SOS</td>
<td>0.93±0.12ab</td>
<td>4.72±0.25ab</td>
<td>629.75±76.12ab</td>
</tr>
</tbody>
</table>

Means±S.D. with different superscript letter(s) in the same column are significantly different (p<0.05).

Survival Rate

Table 2: Percent mortality and percent relative survival (RPS) after 14 days post-challenged with *S. iniae* of fish fed diets containing each prebiotic for 5 weeks.

<table>
<thead>
<tr>
<th>Diets</th>
<th>Mortality (%)</th>
<th>RPS (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.11±7.69a</td>
<td>0</td>
</tr>
<tr>
<td>Inulin</td>
<td>49±13.33a</td>
<td>21.74</td>
</tr>
<tr>
<td>GOS</td>
<td>37.78±3.85a</td>
<td>25.09</td>
</tr>
<tr>
<td>SOS</td>
<td>48.89±13.82a</td>
<td>4.36</td>
</tr>
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</table>

Means±S.D. with different superscript letter(s) in the same column are significantly different (p<0.05).

The average percent mortality and relative percent survival (RPS) after 14 days among the fish fed with diets containing various prebiotics and challenged with *S. iniae* were not significantly different (Table 2). However, galactooligosaccharide gave the lowest mortality (37.78±3.85), or the highest survival rate.

Bacterial Stimulation and Inhibition

Total bacteria were counted by aga plate method. Galactooligosaccharide showed the highest stimulation on the growth of *Bacillus subtilis* (Fig 1). Moreover, galactooligosaccharide showed the highest growth inhibition of *Pseudomonas putrefaciens*, *Vibrio parahaemolyticus* and *Streptococcus iniae* (Fig 2, 3 and 4, respectively).

Immune Response

The immune response of fish fed diets containing 5% of each prebiotic is shown in Table 3. Type of prebiotics tested did not significantly affect hematocrit and white blood cell count, whereas red blood cell
count was significantly affected ($p<0.05$). Fish fed diet containing 5% SOS had the highest red blood cell count ($17.5\pm47.78$).

In this study, we tested whether the addition of prebiotics (inulin, GOS and SOS) would improve the growth performance and immune response of sex-reversed red tilapia. Prebiotics are a source of carbohydrate which may influence the increase in the availability of nutrient(s) and also maximize the metabolic pathway for energy and protein conversion efficiency. Feed efficiency was improved in tilapia when fed with diet containing GOS compared to inulin or SOS. These results are in agreement with those for hybrid striped bass fed a diet supplemented with the commercial prebiotic Grobiotic™AE (Li and Gatlin, 2004).

Olsen et al. (2001) observed that a diet supplemented with 15% inulin caused harmful effects in Arctic charr (Salvelinus alpinus L.). Previous studies by Ringo et al., (1998) and Ringo and Olsen (1999) showed that dietary fatty acids and carbohydrates altered the bacterial flora of gastrointestinal tract of fish.

Survival of sex-reversed red tilapia after being challenged with S. iniae was not significantly different. Other studies have reported an increase in disease resistance in fish fed diets supplemented with prebiotics. McNulty et al. (2003) reported a reduction in Hb and RBC concentration in nile tilapia infected with S. iniae. The authors hypothesize that the breakdown of Hb increases circulating iron levels and possibly overloads the “iron withholding system” in S. iniae infected tilapia.

Grisdale-Helland et al. (2008) fed Atlantic salmon with 1% GOS for 16 weeks and reported that GOS was unable to affect growth parameters relative to the control group. Apparent nutrient digestibility and body composition also remained unaffected. However, body gross energy content of GOS-fed fish was reduced. More research is needed to fully elucidate the physiological mechanisms by which prebiotics enhance the selected parameters and to provide a complete characterization of their effects on several other defense systems. Also, more evidence is required on the structure-function relationship of the various prebiotics pointed out in this study to gain a more complete understanding of the potential applications of prebiotic therapy in aquaculture. Nevertheless, it is possible to foresee prebiotic utilization in reducing aquaculture’s reliance on expensive chemotherapeutic treatments by promoting

![Figure 4](image_url)
the controlled colonization of the fish’s GI tract to improve their overall production efficiency and disease resistance.

Conclusions

The results of this study indicate that supplementation of diets with inulin, SOS, and especially GOS, seems to be most positive for sex-reversed red tilapia production. However, the prebiotics tested did not appear beneficial in improving the immune response of sex-reversed red tilapia against *S. iniae* infection.

Acknowledgments

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References


Validation of Modified QuEChERS Method for Simultaneous Determination of Organophosphates and Carbamates in Mangosteens by LC-MS/MS

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Abstract

Thailand is the world’s major producer and exporter of mangosteens, known as the “queen of fruits,” earning 1,879 million baht from trade in 2009. Chemical residue analysis of whole mangosteen, to ensure compliance with export standards, is challenging due to interference from the dark-colored phenolic components in the fruit peel. QuEChERS (quick, easy, cheap, effective, rugged and safe) technique has been developed for sample preparation to test pesticide residues in mangosteens by liquid chromatography tandem quadrupole mass spectrometry (LC-MS/MS) analysis. Extraction parameters, such as types of organic solvents and buffer agents, were optimized. Sorbent types and composition of dispersive solid-phase extraction (d-SPE) were evaluated for optimum interference removal. The final QuEChERS parameters for the extraction of 10 g homogenized whole mangosteen employed 10 mL acetonitrile/acetate buffer mixture followed by a d-SPE (1:1 PSA:alumina N) cleanup step. The final analysis was performed by LC-MS/MS. Mass spectral acquisition was done by multiple reaction monitoring (MRM) with two mass transitions per compound to provide quantification and confirmation. In-house validation parameters included: matrix-matched linearity, method accuracy, method precision, limits of detection (LODs) and limits of quantitation (LOQs). LODs and LOQs for organophosphates were 0.005 mg kg⁻¹ and 0.01 mg kg⁻¹, for carbamates were 0.005 mg kg⁻¹ and 0.01 mg kg⁻¹, respectively. Extraction recoveries were: 71–116%, relative standard deviation (RSD) 8–19%, for carbamate; and 75–97%, RSD 6–20%, for organophosphate. The validation data met the requirements of regulation EC 396/2005, proving that the developed method is fit for routine analysis of organophosphates and carbamates in mangosteens.

Keywords: carbamates, LC-MS/MS, mangosteen, organophosphates, QuEChERS

Introduction

Mangosteen is one of the major fruits exported from Thailand to foreign markets, especially the European Union (EU). Since September 1, 2008, the European Union has implemented regulation EC 396/2005, by which the 27 member states are mandated to have one unified limit for any pesticide residue whose MRL (maximum residue limit) was not listed as a default MRL of 0.01 mg kg⁻¹ (European Commission, 2005). Testing of pesticide residues in mangosteen, including pulp and peel, is required to prove that the product is safe, conforming to “Good Agricultural Practices” (GAP). However, the
trace analysis of pesticide residues in whole mangosteen is quite complicated due to its intense coloration and the number of high-molecular-weight components in the thick peel, such as polyphenols and wax; this can cause serious interference in pesticide residue analysis. Insecticides such as organophosphate and carbamate are mostly used to control insects in food plants, and become a serious concern in terms of health certification for export. Whole fruit of exported mangosteen, including pulp and peel, must be analyzed by homogenization and extraction of a wide range of pesticide residues, using a multi-residue method (MRM); these are then analyzed using a chromatographic technique. The first multi-residue method (MRM) was developed by a chemist at the U.S. Food and Drug Administration (FDA) (Mills et al., 1963).

Multi-residue methods for pesticides are most often used for monitoring of food, risk assessment studies, and routine pesticide residue analysis. Therefore, additional cleanup is required in order to obtain a reliable analysis result. Currently, the QuEChERS (quick, easy, cheap, effective, rugged and safe) method has been demonstrated to be a very effective sample cleanup procedure for simultaneous analysis of pesticides in a variety of fruit and vegetable matrices, it’s known as the “original version” (Anastassiades et al., 2003).

The method was developed by added citrate buffering agents to induce liquid separation and stabilize acidic and basic labile pesticides and has been used in collaborative studies of a large number of commodity/pesticide combinations. It has now become the European Committee for Standardization (CEN) standard method EN 15662:2008 (European Committee for Standardization, 2008). Later, a additional technique was developed to improve the results for problematic pesticides by adding 1% acetic acid in extraction solvents and acetate buffer during the extraction/partitioning step (Lehotay et al., 2005). This new method was used in a collaborative study of fortified pesticides in grapes, lettuce and oranges (Lehotay, 2007). The method has been approved by the AOAC for the analysis of grapes (representative matrix for berries and small fruit); lettuce (representative matrix for leaf vegetables and fresh herbs) and oranges (representative matrix for citrus fruit) (AOAC International, 2007). However, no representative matrix has yet been determined for mangosteen.

In 2006 the European Parliament and the Council established Annex I (amending regulation EC No. 396/2005), listing the food and feed products to which maximum levels for pesticide residues apply, as well as selected representative matrices of vegetables and fruits for validation (European Commission, 2006). Mangosteen was not specifically categorized, and so is classified as “miscellaneous fruit,” representing a unique commodity. Hence, for validation of MRL data, whole mangosteen after removal of stem is needed for full validation (European Food Safety Authority, 2009).

In this study we used a modified QuEChERS method for simultaneous determination of carbamate and organophosphate residues in mangosteen, using liquid chromatography–tandem mass spectrometry technique. The validation method of optimized conditions is based on SANCO/10684 (European Food Safety Authority, 2009).

Materials and Methods

Chemicals

Acetonitrile (HPLC grade) of sufficient quality for pesticide residue analysis was purchased from RCI Labscan (Bangkok, Thailand). Sodium chloride, anhydrous sodium acetate and trisodium citrate dihydrate of analytical reagent grade were purchased from Merck. Disodium hydrogen citrate sesquihydrate was obtained from Fluka (Buchs, Switzerland). Anhydrous magnesium sulfate (MgSO₄), in powder and granular
forms, were purchased from UCT (Bristol, PA, USA) and Fluka, respectively.

**Sorbents**

Florisil®, silica, alumina N, alumina B, C-18, NH2, Oasis® MAX, Oasis® MCX, Oasis® HLB, Silica based trimethylaminopropyl (SAX), and Accell™ Plus CM were purchased from Waters Corp. (Milford, MA, USA). Graphitized carbon black (GCB) was obtained from Supelco, Inc. (Bellefonte, PA, USA). Primary secondary amine sorbent (PSA) was supplied by UCT.

**Sample Preparation**

The stems of fresh mangosteen fruits were removed and the whole fruits, including pulp and peel, were homogenized in a high-speed blender for 3–5 min.

**Sample Extraction**

Carbamate and organophosphate were extracted from 10 g of homogenized mangosteen with 10 mL of acetonitrile in a 50 mL centrifuge tube. Four g of anhydrous MgSO4, 1 g of NaCl and 1 g of anhydrous sodium acetate were added, and the tube was capped tightly. The mixture was immediately vortexed for 1 min, then centrifuged for 10 min at 3,500 rpm. The acetonitrile layer was transferred into another tube for cleanup by dispersive solid-phase extraction using mixed sorbents of 25 mg PSA and 25 mg alumina N. The tube was capped tightly, shaken, vortexed for 30 s, and then centrifuged for 5 min at 10,000 rpm. The clear extract was transferred into a 2 mL amber vial for LC-MS/MS analysis.

**Standards**

Standard calibration curves were prepared by matrix-matched calibration standard to compensate for matrix effects. Organophosphate and carbamate standards were added to blank mangosteen extracts at concentrations of 5, 25, 50, 100, 125 µg L⁻¹. Quantitative determination was analyzed by bracketing calibration of external standard.

**Fortification**

In recovery and repeatability studies, the mixtures of each pesticide standards in acetonitrile were spiked to 10 g homogenized mangosteen blank sample at spiking levels such as 0.005, 0.01, 0.02, 0.05 and 0.10 mg kg⁻¹. The tubes containing spiked samples were vortexed for 30 s and left standing for 10 min to allow time for pesticides to interact with the matrix.

**Determination by LC-MS/MS**

Residue analyses were performed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). A high performance liquid chromatograph was connected to a Micromass® Quattro Premier™ XE benchtop tandem quadrupole mass spectrometer (Waters). Electrospray ionization (ESI) was used as an ionizing source in positive mode. LC was performed by injecting 10 µL via an autosampler on a Luna C-18 (2.0 mm x 150 mm x 2.1 µm) column (Phenomenex, Torrance, CA, USA) connected with guard column, maintained at 40 °C, 0.2 mL min⁻¹ flow rate. The mobile phase, solution A (0.01 M ammonium acetate) and solution B (methanol), was set at linear gradient from 0% B to 95% B for 14 min and held for 6 min. Chromatographic separations of the 21 compounds were achieved within 20 min.

The tandem mass spectrometer parameters were ion spray voltage at 4000 V, cone gas flow at 0–55 L h⁻¹, desolvation gas flow at 600–650 L h⁻¹ desolvation at 350 °C, and ion source temperature at 120 °C. Estimation of the residues was performed by multiple reaction monitoring (MRM), with two mass transitions for each pesticide: one for quantification and the other for confirmation. The details of MRM transitions of all analytes are shown in Table 1. Instrument control, data acquisition and evaluation were performed using a MassLynx 4.0 software package (Waters Corp, Milford, MA, USA).

**Results and Discussion**

The method successfully analyzed carbamate and organophosphate residues in mangosteen. The extraction and cleanup steps were optimized. After the modified sample preparation steps, the clear solution was subjected to LC-MS/MS analysis.

**Extraction Solvents and Buffers**

The extraction solvents, types of salts and amounts were evaluated for their extraction
efficiency. The comparison between using acetonitrile and 1% acetic acid in acetonitrile as extractant is shown in Figure 1A. Figure 1B shows the difference of % recovery when using: (a) 4 g MgSO₄ + 1 g NaCl; (b) 4 g MgSO₄ + 1 g NaCl + 1 g trisodium citrate dehydrate + 0.5 g disodium hydrogen citrate sesquihydrate; and (c) 4 g MgSO₄ + 1 g NaCl + 1 g anhydrous sodium acetate. The best recovery was obtained when using 100% acetonitrile and a mixture of 4 g magnesium sulfate, 1 g sodium chloride and 1 g anhydrous sodium acetate as the extraction solution.

Sorbent Cleanup and Dispersive-SPE

Mangosteen extracts were cleaned up with 13 different sorbents: GCB, PSA, Florisil®, silica, alumina N, alumina B, C-18, NH₂, Oasis® MAX, Oasis® MCX, SAX, Oasis® HLB and Accell™ Plus CM. The extractions after cleanup were evaluated to determine the efficiency of sorbents in removing interference from mangosteen matrix in terms of: weight of residues after cleanup; color of extract after cleanup (by studying its ability to absorb UV-VIS light); interference peak effect (by gas chromatography); and the effect of temperature on removal of matrix interference (by freezing out and centrifuging at low temperature). Five out of the 13 sorbents were found to be suitable for removal polar interferences and large molecules of mangosteen matrix.

Polar interferences and large molecules such as fatty acids and wax can be further removed by adding dispersive-SPE sorbent. Five different dispersive-sorbent types were tested: PSA, alumina N, Florisil®, Oasis® MCX, and SAX. Primary secondary amine sorbent, alumina N and Florisil® worked very well for removing interferences of fatty acids and wax in the matrix, as shown in Figure 2.

A mixture of PSA and alumina N was found to be very good for cleanup by removing the polar interferences and fatty acids in mangosteen matrix. Ratios of mixed sorbents were evaluated to determine the optimum quantities showed in Figure 3. Mixtures of PSA:alumina N and PSA:Florisil® sorbents were evaluated in term of precision in 10 replicates at 0.01 mg kg⁻¹ spiked level. PSA:alumina N mixed sorbents had greater precision: RSD 5.98-25.52% for PSA:alumina N (25:25 mg), and RSD 4.74-39.08% for PSA:Florisil® (25:25 mg).

**Figure 1** Effect of extraction solvent and buffer, shown as mean recovery data (n = 3).

(A) Carbamate and organophosphate residues spiked at 0.1 mg kg⁻¹ extracted with acetonitrile and 1% acetic acid in acetonitrile.

(B) Carbamate and organophosphate residues spiked at 0.1 mg kg⁻¹ extracted with acetonitrile plus added salts.
Figure 2 Effect of sorbent type. Mean recovery (n = 3) of 21 pesticides spiked at 0.1 mg kg\(^{-1}\) and cleaned up with PSA, alumina N, Florisil, MCX, and SAX.

Figure 3 Extraction efficiency of mixed sorbents and amount employed. Mean recovery (n = 3) of 21 pesticides spiked at 0.1 mg kg\(^{-1}\), cleaned up with mixtures of (A) PSA:alumina N, and (B) PSA:Florisil, at three mixture ratios.

Method Validation

Method validation was performed to provide evidence that the method was fit for the purpose. The method, tested to assess linearity sensitivity, covered mean recovery, precision, limits of detection (LODs) and limits of quantitation (LOQs).

Linearity of method was obtained from a matrix-matched standard calibration curve of range 5–125 µg L\(^{-1}\) with three replicates. The lowest calibration level (LCL) was 5 µg L\(^{-1}\). The calibration curves exhibited the intercepts, slopes and coefficients of determination (R\(^2\)), representing the linearity of the method, R\(^2\) showed in Table 1.

Limits of Detection (LODs)

In trace analysis, it is important to know the lowest concentration of analyte or property value that can be confidently detected by the method. The limits of detection is the lowest concentration of analytes applied to the optimized method. The method employed was determined by analyzing the lowest spiked sample of all analytes at a concentration of 0.005 mg L\(^{-1}\) in 10 replicates. The LOD results are shown in Table 1.

Limits of Quantitation (LOQs)

The limit of quantitation is the minimum concentration of analyte that can be quantified with acceptable accuracy and precision. It should be applied to the complete analytical method compliance with MRL at 0.01 mg kg\(^{-1}\). The LOQ must not be lower than the corresponding lowest calibration level (LCL). LOQs were determined by analyzing spiked samples at concentration 0.01 mg kg\(^{-1}\) under the optimized condition in 10 replicates. The concentration level obtained was evaluated in terms of accuracy and precision by calculating the percentage recovery and RSD. The limits of quantitation (LOQs) obtained are shown in Table 1.

Accuracy and Precision

The accuracy and precision was obtained by analyzing the spiked recovery samples to determine their accuracy at different concentrations, with a minimum of five replicates (European Food Safety Authority, 2009). The LOQ is defined as the lowest spiked level that meets the method performance acceptability criteria. Ten replicates (for accuracy and precision of data) at spiked levels of 0.01, 0.02, 0.05 and 0.10 mg kg\(^{-1}\) were analyzed under the optimized
Table 1 MRM transition and performance of method in recovery data (% mean recovery, \( n \geq 7 \)) obtained for 20 pesticides in the mangosteen matrix.

<table>
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<tr>
<th>Pesticide</th>
<th>MRM transition</th>
<th>Collimation energy (V)</th>
<th>Rec1 %RSD</th>
<th>Rec 1 %RSD</th>
<th>Rec 1 %RSD</th>
<th>Rec 1 %RSD</th>
<th>LOD (mg kg(^{-1}))</th>
<th>LOQ (mg kg(^{-1}))</th>
<th>R(^2)</th>
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<td>0.01</td>
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</table>

1 Mean recovery, \( n \geq 7 \)
condition. The pesticides gave good recoveries in the range of 70-122% with RSDs 5.01-22.72% for carbamate and recoveries in the range of 63-104% with RSDs 3.54-19.58% for organophosphate. The obtained values were in agreement of the EU requirement (European Food Safety Authority, 2009), indicating the reliability of the proposed method. The accuracy and precision data are shown in Table 1.

Conclusions

A modified QuEChERS procedure was proved to be satisfactory for the extraction of 12 carbamate and 8 organophosphate residues in whole mangosteen. The optimum method employed acetonitrile and an added combination of salts (magnesium sulfate and sodium chloride, and sodium acetate buffering agent) to induce liquid phase separation as well as stabilize acid and base labile pesticides. PSA and alumina N mixed sorbents at a ratio of 25:25 mg (1:1) were used as dispersive mixed sorbent to cleanup the polar interferences and fatty acids in the mangosteen matrix. The validation data demonstrated good method performance with a satisfactory recovery range: 70-122% and RSD 5-23% for carbamate; and 63-104% and RSD 4-20% for organophosphate. The LODs were 0.005 mg kg\(^{-1}\) and the LOQs were 0.01 mg kg\(^{-1}\). Range of methods was 0.01-0.10 mg kg\(^{-1}\). This method determined pesticide residues in mangosteen, such as carbamate and organophosphate, by LC-MS/MS. Tandem mass spectrometry (MS/MS) was operated in multiple reaction monitoring mode, with the two most sensitive transitions used for both quantification and confirmation. This method was able to analyze residues at a low concentration level of 0.01 mg kg\(^{-1}\), which is in compliance with the benchmark parameters of regulation EC 396/2005.

Acknowledgments

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References


Effect of Seed Development on Seed Quality of Physic Nut

*(Jatropha curcas Linn.)*

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Abstract

Physic nut (*Jatropha curcas* Linn.), a multipurpose herbaceous plant (hedge, soap, pesticide, medicinal uses, and energy source), belonging to Euphorbiaceae family is gaining lot of importance for the production of biodiesel. Study of seed development of physic nut accession KUBP 74 was determined the appropriate time of harvesting for high seed quality. The experiments were carried out in early and late rainy season of 2007-2008 at National Corn and Sorghum Research Center. The results showed that seed approached the physiological maturity at 70 DAA. At this stage the seed contained maximum dry weight (0.80 g/seed) and attained the highest germination potential (98%) but seed moisture content was 34.44%. The main objective of seed production was highly seed quality which can be achieved by harvesting at 90-120 DAA (SMC. 9-12%). Seed oil content of accession KUBP 74 was measured at 50, 70, 90 and 110 DAA. It was found that the highest percentage of oil content at 90 DAA was 56.7% from seed kernels. The main fatty acids of physic nut oil from KUBP 74 was palmitic 13.96%, stearic 7.26%, oleic 46.15%, linoleic 31.38%, ash 5.7% and acid value 1.9%. The highest oil content could be extracted at 90 DAA.

Keywords: seed development, seed quality, oil seed, physic nut

Introduction

The scientific name of physic nut is *Jatropha curcas* Linn. comprise in Euphorbiaceae family (Linnaeus, 1753). It is a drought resistant shrub with 2-5 meters tall. Every part of the trunk has white-grey latex. The original native is in Mexico and Central America (Heller, 1996). Physic nut is well adapted plant and can grow in many soil types even with poor soils and low rainfall (Dehgan and Schutzman, 1994). Among the oily seed plants physic nut is one of the best potential for future biodiesel production in tropic and sub-tropic region. The cost of seed production is low and has appropriated size to use for industry because seed has high oil content (Jones and Miller, 1991; Francis, 2005). The properties of physic nut oil is similar to diesel but has higher viscosity (Pasboot and Suttipiboon, 1992), so physic nut is interested plant because they can be extracted as a biodiesel fuel and other parts of the plant are valuable for many other uses. Because of the plant shows indeterminate growth with a morphological discontinuity at both flowering and maturity (Chinawong, 2006). These were affected to find out the appropriate time of harvesting while the period at maximum dry seed weight is indicated for seed physiological maturity (Delouche, 1976: Harrington, 1972; Robert,
(1972). Bewley and Black (1978) reported that castor commercially growth varieties would have maximum dry seed weight at 60 days after flowering. Ratanaubon and Juntakool (1988) reported that soybean was harvested at 30 days after physiological maturity showed lower quality than harvested at 10 and 20 days after physiological maturity. Kaushik et al. (2001) reported that during the development of physic nut seed and fruit (17-57 days after flowering) which grown in Bawal Haryana India, there were the increment of fresh weight, dry weight, size of seed and fruit. The color of fruit at 47 days after flowering was changed from green to brown at 57 days after flowering but seed color was changed from white at 17-27 DAA to brown at 37-47 DAA and black at 57 DAA respectively. Moisture content of seed and fruit has been reduced from 26-67 DAA. Germination percentage increased during 17-57 DAA and had maximum to 85 percent at 67 DAA. Optimum temperature for seed germination was 30°C (Kaushik et al., 2003). For high quality seed production of physic nut at optimum harvesting time, the development of seed maturity is necessary to study. On this experiment has provided to study effect of seed development on the quality of Jatropha seeds in order to use for seed multiplication.

Materials and Methods

Study on effect of seed development on high seed quality of physic nut accession KUBP 74 was conducted at Suwanwajokkasikit Field Crop Research Station, Pakchong, Nakorn-Ratchasima during March 2007–May 2008. Experimental field was divided into 4 plots, 140 square meters per plot. Applied basal fertilizer with 18-46-0 rate 312.5 kg/ha. Rows and plant spaces were 2x2 meters. Chemical fertilizer was applied as top dressing with 46-0-0 rate 156.25 kg/ha. Chlorpyriphos was applied for pest control at the rate 40 ml/20 l (water) by spraying leaves and plant at 30 and 60 days after planting. Harvesting times were 13 stages at 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 and 120 days after flowering (DAA). Color of fruit for shell characteristic, separately between epicarp, mesocarp and endocarp; color of seed and fruit size were recorded. Each stage was randomly collected of 10 fruits and measured width, length and thickness of seed and fruit. Moisture content was determined by hot air oven methods, weighed 10 random seeds, ground before drying at temperature 103±2°C for 17± 1 hours. Moisture content percentage was calculated following the formula of ISTA (2003).

Fresh weight and dry weight of seed and fruit were weighed from random 10 fruits and seeds per sample. Germination test and seed vigor of fresh seed were determined in every stages by using 50 random fresh seeds/replication by sand test and counted number of seedling at 10 days after sowing (ISTA, 2003). Germination test and seed vigor of dry seed were provided from random fresh seed in every stages and dried in open room temperature for 1 month. After drying seed samples were brought 50 seeds/replication germinated in the sand and counted number of seedling at 10 days after sowing.

Germination data were analyzed on the basis of seedling number (ISTA, 2003). Seed vigor was assessed from mean germination time (MGT) (Ellis and Roberts, 1980). Random seed were brought to culture, counted number of seedlings everyday and calculated for seed vigor by the formula; MGT=$\Sigma$Ti.Ni/$\Sigma$Ni where Ni = number of seeds germinating on the Ti day of germination testing. The oil content of jatropha accessions KUBP 74 was analyzed at stage of before physiological maturity, physiological maturity and after physiological maturity stages (50, 70, 90 and 110 DAA.) and was extracted by hexane (AOAC, 2000).
Statistical Analysis

Statistical analysis was carried out using analysis of variance followed by LSD method. Mean differences with P<0.05 were considered statistically significant.

Results and Discussion

Development on Color, Weight, Size and Characteristic of Fruit

Color of Fruit

After fertilization, young fruit color was green. The obvious change when fruit color was changed from green to yellow at 50 DAA. (Fig.1, Fig 2 and Table 1) and outer shell of fruit changed to dark brown, mesocarp changed from white to brown, endocarp changed from white to brown and finally black at 70 DAA. Change of color could be used as the index to harvest physic nut by collecting only yellow and brown color of fruit (Kaushik et al, 2001; Chinawong, 2006).

Fruit Size

Fruit size increased as same as seed development. Moisture content was 86.50% when fruit had maximum size after that fruit size reduced as moisture decreased. At physiological maturity stage, fruit size had 2.72 cm wide, 3.42 cm long and 2.80 cm thick (Fig.1, Fig 2 and Table 1).

Fresh Weight of Fruit

Fresh weight of physic nut accession KUBP 74 increased after fertilization and reduced when moisture content decreased. Fresh weight of fruit was 2.0 g/fruit at 120 DAA. (Table 1).

Dry Weight of Fruit

After fertilization dry weight changes in fruit of physic nut accession KUBP 74 during seed development so dry weight was increasing until the mature stage of physiological maturity, fruit dry weight reached maximum of 0.76 g/fruit at 70 DAA. (Table 1). After that dry weight reduced because of moisture content reducing. The dry weight was stable because main sources of food stopped translocations (Tekrony et al., 1979).

Moisture Content of Pericarp

For moisture content of pericarp determination, moisture content had statistically significant difference (Table 1). The initial stage had high moisture content at 87.32%, after fertilization moisture content increased to 89-90% and decreased to 86.90% at physiological maturity. Moisture content was declined to 11-14% during harvest at 100 DAA.

Statistical analysis was carried out using analysis of variance followed by LSD method. Mean differences with P<0.05 were considered statistically significant.

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Development on Color, Weight, Size and Characteristic of Fruit

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Fresh Weight of Fruit

Fresh weight of physic nut accession KUBP 74 increased after fertilization and reduced when moisture content decreased. Fresh weight of fruit was 2.0 g/fruit at 120 DAA. (Table 1).

Dry Weight of Fruit

After fertilization dry weight changes in fruit of physic nut accession KUBP 74 during seed development so dry weight was increasing until the mature stage of physiological maturity, fruit dry weight reached maximum of 0.76 g/fruit at 70 DAA. (Table 1). After that dry weight reduced because of moisture content reducing. The dry weight was stable because main sources of food stopped translocations (Tekrony et al., 1979).

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Table 1 Changes in characteristics of fruit and mean germination time (MGT) during seed development of physic nut accession KUBP 74

<table>
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<tr>
<th>Age of seed (day)</th>
<th>Fresh fruit Width (cm)</th>
<th>Long (cm)</th>
<th>Thick (cm)</th>
<th>Fresh fruit Weight (g/fruit)</th>
<th>Dry fruit Weight (g/fruit)</th>
<th>Thick of fruit (cm)</th>
<th>Fruit MC (%)</th>
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<td>2.90</td>
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<td>2.08</td>
<td>7.93</td>
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<td>0.38</td>
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<td>5.44</td>
</tr>
<tr>
<td>C.V. (%)</td>
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<td>2.21</td>
<td>3.78</td>
<td>13.29</td>
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F-test ** ** ** ** ** ** ** **

** Significant at 99% probability level 0.01
1/ Physiological maturity

Seed Quality Changes During Development

Seed Fresh Weight
Fresh weight of physic nut accession KUBP 74 was increased after fertilization and decreased when moisture content decreased. Seed fresh weight was 1.38 g/seed at physiological maturity and 0.80 g/seed at 120 DAA. (Fig 3) as well as Adams and Rinne (1980) reported that the percentage moisture content of seed was rapidly reduced, seed had maximum size and highest vigor. At the third stage seed weight had been stabled or slightly reduced due to seed still used stored food for biochemical activity in the seed. Moisture content percentage was reduced to 10-12% depend on weather conditions.

Seed Dry Weight
The seed accumulated dry weight after fertilization. At physiological maturity, the ratio of food consumption and production had balanced and gave the maximum dry weight. (Delouch, 1976). At 10-20 days after pollination of physic nut accession KUBP 74, dry weight had slightly increased and reached maximum at 70 DAA (Physiological maturity). Maximum seed dry weight was 0.80 g/seed (Fig 3) and decreased due to seed moisture decreased until its stable because seeds did not receive nutrients transferred from the main tree (Tekrony et al., 1979). After physiological maturity seed dry weight would be stable or slightly changed. Globerson (1981) and Sukprakarn (1985) who studied on lettuce was reported that physiological maturity of lettuce was not different among varieties at the same environmental conditions. Whereas, Physiological maturity of physic nut accessions KUBP 74 showed different result from the report of Kaushik (2003) which found that physic nut when grown in Haryana India had physiological maturity at 57 days after pollination depending on weather and varieties.

Seed Moisture Content
The development and maturation of seeds was associated with seed moisture content loosed (Adams and Rinne, 1980). In the early stage of physic nut development, it was found that young seeds had moisture content
about 88.45%, after that moisture content reduced while seed dry weight accumulation increased. At physiological maturity seeds moisture content was 34.44% (Fig 3) as same as in many crops such as para-rubber (Mohamad, 1981), lablab bean (Ngamprasitthi, 2001), sesame (Jankam, 1989) and mung bean (Wajasatya, 1990), then the moisture content reduced to 9.27% at harvesting. Hocking (1982) found that seeds of caster bean giant types at harvesting stage had moisture content between 11-15%. At physiological maturity, physic nut seeds had high moisture content and decreased within 10-20 days after physiological maturity that was optimum harvesting.

**Color of Seed**

After pollination the color of outer shell of physic nut seeds was white at 10 DAA after that seed color had changed. At 50 DAA, seed color was mixing white and black and changed to black at 70 DAA. or physiological maturity (Fig. 2). For seed color change Kaushik et al. (2001) reported that during development of physic nut (17-57 DAA) which grown in Bawal Haryana, India, at 47 DAA fruit was green and changed to yellow at 57 DAA and at 17-27 DAA seed was white then changed to brown at 37-47 DAA. and black at 57 DAA.

**Seed Size.**

After fertilization the size of physic nut seeds was increased to reach maximum size at physiological maturity while moisture content was 34.44%. After that, seed size had fully developed as moisture content decreased. At physiological maturity physic nut accession KUBP 74 had 1.34 cm wide, 2.14 cm long and 1.68 cm thick and had 0.90 cm wide, and 0.66 cm thick at harvesting stage 120 DAA. (Fig. 2). This result was similar to Poapun (2005) reported that average size of seeds had length of 1.7 – 1.9 cm and thickness of 0.8-0.9 cm.

**Seed Vigor**

Although seeds can germinate before it had maximum dry weight but seeds had the highest vigor at maximum dry weight stage. In this experiment, seed vigor was measured from mean germination time (MGT). It was found that physic nut accession KUBP 74 seeds had statistically significant different between different ages. Seeds at 5-40 DAA could not germinated after that seed vigor increased and seeds had the highest vigor at physiological maturity, seed germination for 6 days after planted at 70 DAA while seeds color was black and fruit color was yellow. After physiological maturity seeds vigor had reduced (Table 1). Kaushik (2003) reported that seedling which germinated from seeds harvested at the age of 57 and 67 DAA showed maximum root and shoot length at 30°C while minimum seed vigor was found in seeds from green fruits, this consistent with studied in soybean (Andrews, 1966) and maize (Knittile and Burries, 1976).

**Seed oil Component**

Seed oil components were significantly different during seed development of physic nut accession KUBP 74 at 50, 70, 90 and 110 DAA. The highest percentage of oil content at 90 DAA was 56.7% from seed kernels. The fatty acid compositions of physic nut accession KUBP 74 consisted of palmitic 13.96%, stearic 7.26%, oleic 46.15%, linoleic 31.38%, ash 5.7% and acid value 1.9%. (Table 2).
Table 2 The composition of physic nut seed accession KUBP 74 at different stages during seed development.

<table>
<thead>
<tr>
<th>Days after anthesis (DAA)</th>
<th>SMC (%)</th>
<th>Acid (w/w) (%)</th>
<th>Ash (w/w) (%)</th>
<th>Oil (w/w) (%)</th>
<th>Fatty Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Palmitic</td>
</tr>
<tr>
<td>50 DAA</td>
<td>3.32</td>
<td>4.47</td>
<td>6.45</td>
<td>16.96</td>
<td>14.45</td>
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<tr>
<td>70 DAA  (1)</td>
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<td>5.41</td>
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<td>90 DAA</td>
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<td>1.89</td>
<td>5.68</td>
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<td>110 DAA</td>
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<tr>
<td>C.V. (%)</td>
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</table>

F-test

** Significant at 99% probability level 0.01
(1) Physiological maturity

Conclusions

It can be concluded that physic nut seed had physiological maturity at 70 DAA when fruit and seeds became black and gave maximum dry weight of 0.80 g/seed, moisture content of 34.44%, germination for fresh seeds and dry seeds of 98.50% and 97.00%, respectively. Seed vigor at physiological maturity was 6 days (MGT). For high seed quality production, physic nut seed should be harvested when seed moisture content decreased. Therefore, the appropriated time for harvest was 100-120 DAA as moisture content decreased to 9-11%.

The oil composition of physic nut accession KUBP 74 consisted of palmitic 13.96%, stearic 7.26%, oleic 46.15%, linoleic 31.38%, ash 5.7% and acid value 1.9%. The highest oil content at 90 DAA was suitable time for oil extraction.

Acknowledgments

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Reduction of Residual Chlorpyrifos on Harvested Bird Chillies 
(\textit{Capsicum frutescens} Linn.) Using Ultrasonication and Ozonation 

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\textbf{Abstract} 

Reduction of chlorpyrifos using ultrasonication, ozonation and a combination of ultrasonication and ozonation was investigated. A standard chlorpyrifos at the concentration of 1 mg l\textsuperscript{-1} was subjected to different frequencies at 108, 400, 700 and 1000 kHz, as well as ozone at the concentration of 25 ml min\textsuperscript{-1} with various exposure times (0, 10, 20, 30, 40, 50 and 60 min). It was found that ultrasonication at 1000 kHz for 60 min was the most effective treatment for reducing chlorpyrifos. In addition, ozone could reduce the concentration of standard chlorpyrifos after increased exposure time to 60 min. Ultrasonication in combination with ozone had a synergistic effect in reducing standard chlorpyrifos concentration with the highest rate of reduction occurring within the first 10 min. The standard chlorpyrifos was reduced 75.18\% after ultrasonication at 1000 kHz and exposure to ozone for 60 min. The effectiveness of ultrasonic and ozone washing for reduction of chlorpyrifos residues on Bird chillies (\textit{Capsicum frutescens} Linn.) were also determined by GC-FPD. The chlorpyrifos residue on fresh chillies after ultrasonic and ozone washing was reduced 73.03\% after 60 min. The present study demonstrates that ultrasonication and ozonation are effective processes for the reduction of chlorpyrifos residues from aqueous solution and chlorpyrifos treated Bird chillies.

\textbf{Keywords:} bird chillies, chlorpyrifos, ozonation, ultrasonication

\textbf{Introduction} 

Chlorpyrifos has been used in agriculture for reducing insect pest populations. While the inherent toxicological properties of pesticides allow them to control pests in agriculture, there is significant concern about the potential risks posed by chlorpyrifos to the environment and humans. The Thai Agricultural Commodity and Food Standard (2006) reported that imported countries detected chlorpyrifos in chillies over the MRL. Chlorpyrifos is generally used in a formula as a broad spectrum insecticide. Chlorpyrifos use for crop protection is extensive covering a wide variety of horticultural and field crops. The mode of chlorpyrifos action is non-systemic, and exposure of insects to active chlorpyrifos (via contact, ingestion and/or inhalation) affects the nervous system by inhibiting the activity of acetyl cholinesterase (National
Registration Authority for Agricultural and Veterinary Chemicals, 2000).

Ultrasonication technology has been reported to be effective in reducing a variety of organic and inorganic contaminants in water (Weavers et al., 1998) and its acoustic cavitations generate many hot spots with locally high temperatures and pressures that cause the sonolysis of H₂O molecules. Consequently, radical species (H, *OH, *OOH) are formed which lead to a direct destruction of chemicals in aqueous solution. Hoffmann et al. (1996) reported that ultrasonication at the frequency of 20 to 500 kHz was effective in rapid destruction of organic contaminants in water. In addition, Wang et al. (2006) indicated that methyl parathion could be degraded using the ultrasonication at a frequency of 40 kHz. Moreover, Song and O'Shea (2007) reported that ultrasonication at 640 kHz effectively and rapidly degrade 2-methylisoborneol (MIB) and geosmin (GSM). Their study revealed that hydroxyl radical scavengers play a major role in the degradation.

Ozonation was a promising method for the reduction of sulfite residues in the pericarp and aril of fresh longan (Whangchai et al., 2004). Furthermore, the effect on aflatoxin reduction was reported by Inan et al. (2007) who found that after 60 min of ozone exposure, the aflatoxin B₁ content was reduced in flaked and chopped red peppers (Capsicum annuum) by 80 and 93% at the rate of 33 and 66 mg l⁻¹, respectively. Wu et al. (2007) demonstrated that using dissolved ozone (1.4 mg l⁻¹) for 60 min was effective in the reduction (60-99%) of methylparathion, cypermethrin, parathion and diazinon in aqueous solution. This research was studied the effectiveness of ultrasonication and ozonation on the reduction of standard chlorpyrifos and residual chlorpyrifos on Bird chillies.

Materials and Methods

Reference chlorpyrifos standard was purchased from Sigma-Aldrich Laborchemikalien GmbH (Stienheim, Germany) with a 99.9% purity. Chlorpyrifos stock solution (1000 mg l⁻¹) was prepared in acetone for the pesticide residue analysis, high-performance liquid chromatography (HPLC) grade 99.9%, having been obtained from RCI Labscan Ltd. (Bangkok, Thailand). The solution was diluted to the appropriate concentrations.

Ultrasonic devices, with an input power of 3 watts and 4 varying frequencies; 108, 400, 700 and 1000 kHz, and made by Honda Electronics Company (Toyohashi, Aichi, Japan), were utilized for three replications. A polyethylene cylinder reactor 10 cm in diameter, equipped with a transducer at the lower part was used. Chlorpyrifos (25 ml) in a flask was sonicated in an ultrasonic reactor. Ozone gas produced by an ozone generator (Ozonizer, Sky zone model S05AE) was bubbled into chlorpyrifos solution in a flask. The ozone gas was bubbled with a flow rate of 25 ml min⁻¹. Dissolved ozone was determined by the indigo colorimetric method (Eaton et al. 2005).

Study of Optimum Frequency and Time for Reducing Standard Chlorpyrifos Solution by Ultrasonication and Ozonation

A standard chlorpyrifos concentration of 1 mg l⁻¹ was prepared in a flask and then placed inside the ultrasonic reactor mentioned earlier and subjected to different frequencies at 108, 400, 700 and 1000 kHz. A 200 mg l⁻¹ ozone gas was bubbled through the chlorpyrifos solution. In this experiment the same procedure was done as in experiment using ultrasonic and ozone treatments except that the standard chlorpyrifos solution was exposed to combined ultrasonication and ozone treatment (Figure 1). All samples were taken out every 10 min for 1 hour. Finally, three replicates of chlorpyrifos samples were analyzed. Concentration was determined using gas a chromatograph equipped with a flame photometric detector (GC-FPD) and percent removal of the chlorpyrifos was calculated at each collection time.

Reduction of Chlorpyrifos on Harvested Bird Chillies Using Ultrasonication and Ozonation

Bird chilli samples were treated with chlorpyrifos one day before harvest. Ten
grams of chillies were immersed in 50 ml distilled water in an ultrasonic reactor (108, 400, 700 and 1000 kHz) and ozonated. Chlorpyrifos residues of the Bird chillies as extracted following Fenoll et al. (2007), the chopped chillies (5 grams) was extracted with acetone and methylene chloride homogenized at 13,000 rpm for 1 min and dried using rotary vacuum evaporation at 340 mbar. The final solvent was acetone (HPLC grade). The samples were analyzed chlorpyrifos residue using GC-FPD.

Figure 1 Schematic diagram of experimental set-up.

**GC-FPD Instrument and Conditions**

The GC-FPD analysis was performed with an Agilent Technologies (Wilmington, DE) model 6890 gas chromatograph equipped with a flame photometric detector. The GC was carried out using a fused silica capillary column HP-5, 5% Phenyl Methyl Siloxane, with dimensions of 30 m × 0.32 mm i.d. and a 0.25 µm film thickness (Agilent Technologies). The temperature regime was as follows: initial temperature at 100 °C, increasing 10 °C min⁻¹ to 200 °C and then 4 °C min⁻¹ to the final temperature of 220 °C. A purified helium carrier gas was used at a flow rate of 3.6 ml min⁻¹. The detector temperature was 250 °C. Sample solution (1.0 µl) was injected in splitless mode, and the quantification of chlorpyrifos was performed using chlorpyrifos standard.

**Statistical analysis**

All experiments were replicated three times and evaluated with a regression procedure using the SPSS version 17. Differences were considered significant at *P < 0.05.

**Results and Discussion**

**Study of Optimum Frequency and Time for Reducing Standard Chlorpyrifos Solution by Ultrasonication and Ozonation**

The effectiveness of ultrasonication on reducing standard chlorpyrifos of 1 mg l⁻¹ was investigated. The effect of chlorpyrifos oxidation in standard solution using different frequencies of ultrasonication and different exposure times was determined by GC-FPD. It was found that the removal of chlorpyrifos residue increased with extending ultrasonic exposure time. Moreover, ultrasonication at 1000 kHz for 60 min was the most effective treatment for reducing chlorpyrifos (64.87%) (Figure 2). It was observed that the beneficial effect of high ultrasonic frequency may be due to increased cavitational activity which lead to a higher number of collapsing cavities that the ultrasonic frequency increasing positively affecting the removal rate. Similarly, Yao et al. (2010) reported that the optimal frequency for parathion degradation was 600 kHz which is attributed to the much higher ●OH yield compared to treatments at 200 kHz.

Results from the ozone treatment of the standard solution of chlorpyrifos varied with exposure time. It was found that the removal of chlorpyrifos residue was directly proportional to increase in ozone exposure time. At exposure time at 60 min, the chlorpyrifos removal was 41.31% (Figure 3). The ozone is a strong oxidant and possibly can degrade the chlorpyrifos. As reported by Ku et al. (1998) the diazinon degradation occurred within 1 hour of ozone treatment. These results are in agreement with many studies on the reduction of pesticide in water solution after ozone treatment with the same range of contact times (Faust and Gomma, 1972).

The chlorpyrifos solution that was treated with ultrasonication in combination with ozone had an improved rate of chlorpyrifos degradation, with the peak degradation rate occurring within the first 10 min 2-4 times faster when compared with using ultrasonication or ozonation alone. This may be due to the synergistic effect of ultrasonic-
ozone treatment in degraded or oxidized the chlorpyrifos structure. Moreover, the combination of ultrasonic and ozone treatments that achieved the highest chlorpyrifos removal occurred at 1000 kHz for 60 min, these was a significant (*P<0.05) increment of chlorpyrifos removal (75.18%), compared with other treatments (Figure 4). Therefore, ultrasonication and ozonation appeared to be a promising treatment for residual chlorpyrifos removal. These results showed that the chlorpyrifos degradation achieved in conjunction with ultrasonication was twice as fast as that accomplished by ozone alone. So, there was a synergistic effect of using ultrasonic and ozone treatments together. This is similar to the findings of Weavers et al. (1998) who reported the importance of the rapid degradation of organic contaminants using ozonation and ultrasonication (20 kHz) resulting in an increase of hydroxyl radicals.

![Figure 2](image2.png)

**Figure 2** Percent removal of chlorpyrifos by ultrasonication.

**Reduction of Chlorpyrifos on Harvested Bird Chillies Using Ultrasonication and Ozonation**

The Bird chillies were immersed in an ultrasonic reactor with frequencies at 108, 400, 700 and 1000 kHz in order to reduce residual chlorpyrifos. It was found that the chlorpyrifos removal on fresh chillies of all ultrasonication frequencies increased with increasing contact time (Figure 5).

![Figure 3](image3.png)

**Figure 3** Percent removal of chlorpyrifos by ozonation.

![Figure 4](image4.png)

**Figure 4** Percent removal of chlorpyrifos by ultrasonic combined with ozone treatments.

Reduction of residual chlorpyrifos increased with the combination of ultrasonication and ozonation. The results showed that the highest percentage of chlorpyrifos removal on chillies was 73.03% when the ultrasonic frequency was 1000 kHz combination with ozone treatment for 60 min. In addition, the first 10 min of all treatments significantly (*P<0.05) increased chlorpyrifos degradation on chillies when compared with the control, which was immersed in distilled water (Figure 6). This tendency can be explained by the reaction of ultrasonic frequencies with ozonation producing the •OH radical, which effectively decomposed chlorpyrifos residue on the chillies surface. Similarly, Wenrong and Haiyan (2000) reported that the
decomposition rate of arsenazo treated by ozone or ultrasonic is more rapid than that treated by ozone alone and the structure of arsenazo is decomposed more completely. In addition, Wu et al. (2007) also indicated that ozone water treatment could effectively reduce pesticides (methyl-parathion, parathion, diazinon and cypermethrin) residue on Pak Choi (Brassica rapa) surface.

However, washing chillies fruit with ultrasonication and ozonation were not significantly different in the quality changes of fruits (Data not shown). Moreover, chlorpyrifos reduction by ultrasonication and ozonation is a very promising technique that can be applied in commercial scale of fresh chillies or agricultural products for exportation.

Conclusions

The removal of chlorpyrifos after ultrasonic or ozone treatment increased with increasing contact time. The higher the ultrasonic frequencies, the greater the percent removal. The combined ultrasonic and ozone treatments induced a synergistic effect on chlorpyrifos degradation. Moreover, these results also show that chlorpyrifos residue removal using ultrasonication and ozonation on Bird chillies was positively correlated to contact time. Therefore, further studies to increase the reduction of chlorpyrifos residues in commercial facilities lowering the toxicity of treated chlorpyrifos products are also required.

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Antioxidant Activities of Curcumin-metal Complexes

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Abstract
Curcumin is a potent antioxidant agent. This compound has an intense yellow color and is insoluble in water, degraded in alkaline solution and unstable to light and heat which usually limits its applications. The stability and antioxidant activity of curcumin is believed to increase when it is complexed with transition metals. The aim of this study was to investigate the best metal to enhance the antioxidant activity of curcumin. Seven divalent transition metals (Zn²⁺, Se²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺, and B²⁺) were selected to complex with curcumin by a reflux method. The complexes were characterized by spectroscopic techniques including UV and IR analyses. The DPPH radical scavenging activity and ferrous reducing power of the complexes were investigated. The result proved that curcumin–metal complexes have better antioxidant activity than free curcumin at the same dose level. The best transition metal enhancing antioxidant ability of curcumin was Zn²⁺.

Keyword: antioxidant activity, curcumin, curcumin-metal complexes, reducing power

Introduction
Curcumin is a polyphenol found in curcuma species which is popularly used in food supplements, cosmetics and pharmaceuticals due to multiple therapeutic activities including anti-cancer (Hatcher et al., 2008; Yang, 2005), anti-inflammatory (Ammon et al., 1992), the inhibition of bacterial and fungal growth (Srinivasan et al., 2001; Roth et al., 1998) high antioxidant activity (Jayaprakash et al., 2006), an inhibition of lipid peroxidation which is 8 folds higher than provided by vitamin E, and an inhibition of tyrosinase enzyme activity which is equal to that of kojic acid (Shirota et al., 1986; Khunlad et al., 2008). With these features curcumin was used as an active ingredient in many products such as anti-wrinkles, anti-acne and whitening products. However, there are restiction on the used of curcumin due to its intense yellow color, its insolubility in water, its degradation in alkaline solution and its instability to light and heat (Tonnesen et al., 2002; Sowbhagya et al., 2005) that affect the appearance of the products.

The efficacy and stability of the substance can be enhanced by complexing it with transition metal ions (Hesham et al., 2004). The objectives of this study were to complex curcumin with a number of different transition metals and to compare the stability, DPPH radical scavenging and ferrous reducing power of the complexes.

Materials and Methods

Synthesis of Metal-curcumin Complex
The curcumin complexes were prepared by refluxing the mixture of curcumin and transition metals (ratio 1:1 mol) at 60 °C in absolute ethanol for 3 hours. The precipitates were washed with cold ethanol-water mixture and the curcumin complex powders were obtained.

Recording the UV Absorption Spectrum
A UV-visible spectrophotometer was used to record spectra of curcumin and its
complexes in DMSO. The spectra were scanned from 350–600 nm.

**Recording the FT-IR Spectrum**

FT-IR spectrum of curcumin and its complexes were performed using FT-IR spectroscopy with fixed resolution to 4 cm⁻¹. The KBr pallets of samples were scanned on the wave number range of 4000–400 cm⁻¹.

**Kinetic Degradation Study**

A UV-visible spectrophotometer was used to record spectra and absorbance of curcumin and its complexes in varies pH buffer solution (pH 3, 7 and 12). Kinetic degradation reactions of the complexes were followed between 0-50 hours. The percent residual of curcumin and its complexes were calculated by comparing the absorbance with standard curcumin at different concentrations.

**Determination the DPPH Radical Scavenging activity**

The analytical procedure was modified from Rangkadilok (Rangkadilok et al., 2005). This assay detects scavenging of free radicals of the complexes through the scavenging ability of the stable DPPH free radical. Each 5 µl aliquot of each sample was reacted with 195 µl of DPPH 100 µM in a 96-well microplate. The plate was incubated at 37 ºC for 30 minutes in dark. After that the absorbance was measured at 515 nm using a microplate reader. The percentage of radical scavenging was calculated by following equation

\[
\text{Radical scavenging (\%)} = \frac{(\text{Abs control} - \text{Abs sample})}{\text{Abs control}} \times 100
\]

**Determination of Ferrous Reducing Power Activity**

The reducing power of the curcumin and its complexes was determined according to the ferrous reducing power method from Takashi (Takashi et al., 2009) with some modifications. Each 25 µl of sample or ascorbic acid (as a positive control), together with 25 µl of 0.1 M phosphate buffer (pH 7.2) and 50 µl of 1% potassium ferricyanide were added to a 96-well microplate. After incubation at 37 ºC for 60 minutes, 25 µl of 10% trichloroacetic acid and 100 µl of distilled water were added. The absorbance was then measured at 700 nm (Abs1). After 25 µl of 0.1% ferric chloride was added to the mixture, the absorbance was measured again (Abs2). Ferrous reducing power was calculated by following equation

\[
\text{Reducing Power} = \frac{\text{Abs}2\text{sample} - \text{Abs}1\text{sample}}{\text{Abs}2\text{control} - \text{Abs}1\text{control}}
\]

**Results and Discussion**

**Synthesis of Metal-curcumin Complex**

Curcumin-metal complexes were successfully prepared by refluxing mixtures of curcumin and transition metals (ratio 1:1 mol) in ethanol for 3 hours. The complexes were obtained as difference color powders. The yield percent was calculated with curcumin-B being the highest as showed in Table 3.1.

**UV Absorption Spectrum**

The UV absorption spectra of curcumin and its complexes were recorded between 200-600 nm. The maximum absorption of curcumin was 434 nm while the maximum absorption of the complexes was shifted (Figure 3.1). The maximum absorption of curcumin-Zn, curcumin-Se, curcumin-Cu, curcumin-Fe, curcumin-Mn, curcumin-Mg, and curcumin-B were 425.5, 424, 425.5, 423, 424, 424, and 424.5 nm respectively.
The FT-IR spectrum of the complexes at 3560 cm\(^{-1}\) is attributed to vibrations of free hydroxyl-group of phenol (Ar–OH). At 1628, 1603 and 1509 cm\(^{-1}\) is attributed to vibrations of the carbonyl bond (C=O). Compared with the reference spectrum of curcumin, all complexes shows a decrease in the intensity of (C=O) carbonyl band, by a shift wave values (\(\Delta \nu =3-19\) cm\(^{-1}\)) (Table 3.2).

### Table 3.1 The physical appearance of curcumin complexes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% yield</th>
<th>Physical appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>-</td>
<td>Yellow powder</td>
</tr>
<tr>
<td>Curcumin-Zn</td>
<td>68.4</td>
<td>Orange powder</td>
</tr>
<tr>
<td>Curcumin-Se</td>
<td>66.1</td>
<td>Black powder</td>
</tr>
<tr>
<td>Curcumin-Cu</td>
<td>79.2</td>
<td>Dark Gold powder</td>
</tr>
<tr>
<td>Curcumin-Fe</td>
<td>87.3</td>
<td>Black powder</td>
</tr>
<tr>
<td>Curcumin-Mn</td>
<td>75.7</td>
<td>Chocolate color powder</td>
</tr>
<tr>
<td>Curcumin-Mg</td>
<td>67.4</td>
<td>Dark gold powder</td>
</tr>
<tr>
<td>Curcumin-B</td>
<td>89.7</td>
<td>Orange-red powder</td>
</tr>
</tbody>
</table>

### FT-IR Spectrum

The FT-IR spectra of curcumin and its complexes are shown in Figure 3.2. The IR spectra of curcumin complexes are compared with the reference spectrum of curcumin (Figure 3.2). The IR data of curcumin and its complexes are shown in Table 3.2.

### Table 3.2 Wave length changes from infrared (KBr pellets) spectral data of curcumin and curcumin-metal complexes.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IR (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ketone (C=O)</td>
</tr>
<tr>
<td>Curcumin-Zn</td>
<td>1625 ((\Delta \nu = 3))</td>
</tr>
<tr>
<td>Curcumin-Se</td>
<td>1626 ((\Delta \nu = 2))</td>
</tr>
<tr>
<td>Curcumin-Cu</td>
<td>1618 ((\Delta \nu = 10))</td>
</tr>
<tr>
<td>Curcumin-Fe</td>
<td>1618 ((\Delta \nu = 10))</td>
</tr>
<tr>
<td>Curcumin-Mn</td>
<td>1627 ((\Delta \nu = 2))</td>
</tr>
<tr>
<td>Curcumin-Mg</td>
<td>1630 ((\Delta \nu = 2))</td>
</tr>
<tr>
<td>Curcumin-B</td>
<td>1627</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1628</td>
</tr>
</tbody>
</table>
These results indicate that some interaction has occurred at these sites between the metal and curcumin. Below 500 cm\(^{-1}\) showed some weak bands that attribute to metal-oxygen vibration (Bich et al., 2009). The FT-IR spectrum of curcumin-Zn, curcumin-Se, curcumin-Fe, curcumin-Cu, and curcumin-B showed more significant change than those of the curcumin-Mn and curcumin-Mg due to the stronger metal-oxygen bond.

Kinetic Degradation

The kinetics of hydrolytic degradation reactions of curcumin has been proposed by Tonnesen and Karlsen. When the pH is less than 1, curcumin has a red color, which indicates the protonated form (H\(_4\)A\(^{+}\)) (Figure 3.3). Curcumin in solution at pH 1 to 7 has yellow color. The majority of the curcumin molecules are in the neutral form (H\(_3\)A) (Figure 3.3). At pH more than 7.5, the color is changed to orange red. The curcumin molecule was extremely unstable due to it is postulated to be in between tree form; H\(_3\)A, H\(_2\)A\(^{-}\), and HA\(^{2-}\) (Figure 3.3) (Tonnesen & Karlsen, 1985).

To compare the stability of curcumin and its complexes the chemical degradation was investigated in “in vitro” over a 50 hours period.

At acids condition (pH 3), the complexes were more stable than free curcumin with curcumin-Fe and curcumin-Cu being the most stable. The stability of the complexes was decrease in the order of: curcumin-Fe > curcumin-Cu > curcumin-Zn > curcumin-Mn > curcumin-B > curcumin-Mg > curcumin-Se > curcumin (Figure 3.4).

At neutral condition (pH 7), curcumin was totally degraded after 10 hours, while less than 10% of the curcumin-Fe complex was degraded. After 50 hours all of complexes remained relatively stable except the curcumin-Mg that was completely degraded. The stability of complexes was decrease in order: curcumin-Fe ≅ curcumin-Cu > curcumin-Se > curcumin-Zn > curcumin-B > curcumin-Mg > curcumin-Mn > curcumin (figure 3.5).

At alkaline condition (pH 12), for the same time interval, the stability of complexes was much higher than curcumin on its own. Curcumin-Fe was more stable than other complexes. The stability of the complexes was decrease in order: curcumin-Fe ≅ curcumin-Cu > curcumin-Zn > curcumin-Se ≅ curcumin-B ≅ curcumin-Mn > curcumin-Mg > curcumin (figure 3.6).

The results indicated that the complexes was more stable than free curcumin with curcumin-Fe, curcumin-Cu, and curcumin-Zn being the most stable in acids, neutrals, and alkaline conditions.

---

**Figure 3.3** Curcumin form in aqueous solution

**Figure 3.4** Kinetic degradation in buffer solution pH3
**Ferrous Reducing Power Activity**

To measure the ferrous reducing power activity, the reduction of $[\text{Fe(CN)}_6]^{3-}$ to $[\text{Fe(CN)}_6]^{4-}$ that was formed in blue complex from excess Fe$^{3+}$ ions was determined. The absorbance of the reaction was measured at 700 nm. Increasing absorbance of the reaction mixture indicate higher reduction ability. The reducing power of complexes decreased in the following order: curcumin-Zn > curcumin-Fe > curcumin-Se > curcumin > ascorbic acid > curcumin-B > curcumin-Mn > curcumin-Mg > curcumin-Cu. The curcumin-Zn complex showed the highest reducing ferrous ions and was 3 fold higher than that of the free curcumin (Table 3.3).

**Table 3.3 Show the bioactivity of curcumin-metal complexes**

<table>
<thead>
<tr>
<th>Sample</th>
<th>DPPH radical scavenging (IC$_{50}$ mM)</th>
<th>Reducing power at OD 0.5 (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic acid</td>
<td>1.88</td>
<td>0.48</td>
</tr>
<tr>
<td>Curcumin</td>
<td>1.15</td>
<td>0.32</td>
</tr>
<tr>
<td>Curcumin-Zn</td>
<td>0.41</td>
<td>0.12</td>
</tr>
<tr>
<td>Curcumin-Se</td>
<td>4.10</td>
<td>0.50</td>
</tr>
<tr>
<td>Curcumin-Cu</td>
<td>1.57</td>
<td>3.12</td>
</tr>
<tr>
<td>Curcumin-Fe</td>
<td>1.47</td>
<td>0.28</td>
</tr>
<tr>
<td>Curcumin-Mn</td>
<td>1.40</td>
<td>1.22</td>
</tr>
<tr>
<td>Curcumin-Mg</td>
<td>0.95</td>
<td>1.47</td>
</tr>
<tr>
<td>Curcumin-B</td>
<td>1.60</td>
<td>0.53</td>
</tr>
</tbody>
</table>

**Conclusions**

Curcumin-metal complexes were obtained by refluxing curcumin and a number of different transitions metal in ethanol. The complexes that were formed had different colors developed by the interaction between the metal ions and the curcumin. The curcumin-B complex provided the highest yield percent. The complexes were characterized by IR and UV...
spectroscopic techniques. The reaction between curcumin and transition metals was established by comparing the spectra of the complexes to that of the free curcumin. The chemical degradation of the curcumin and the complexes was determined over 50 hours. All the complexes showed higher stability than the free curcumin with the curcumin-Fe showing the highest stability under all condition, followed by curcumin-Cu, curcumin-Zn, curcumin-Se, curcumin-B, curcumin-Mn and the curcumin-Mg complexes. The DPPH radical scavenging and ferrous reducing power activities of the complexes indicated that the curcumin–metal complexes had higher antioxidant activity than the free curcumin. The curcumin-Zn showed highest enhanced antioxidant ability.

Acknowledgments

The authors express thanks to Mae Fah Luang University for support of the scientific equipment for this work.

References


**Lactic Acid Bacteria from Thai Fermented Meat Products as Biological Control Agents against Anthracnose Disease**

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**Abstract**

Lactic acid-producing bacteria (LAB) and their metabolites have been used as biological control (biocontrol) agents against other microorganisms. Herein, the efficacy of the metabolites of 23 isolates of LAB derived from Thai fermented meat products, including sour rice sausages (Sai Krog Isan), fermented pork sausage (Nham) and fermented liver sausages (Mham), has been evaluated against fungal anthracnose disease. The supernatants of LAB culture were tested for efficacy to inhibit the growth of *Colletotrichum gloeosporioides* (Penz) Penz & Sacc in Penz, a fungal causative agent of anthracnose disease in mango. Among these, the supernatants derived from three isolates of LAB (3ST1, 4MT8, 5NBM1) showed 100% inhibition against *C. gloeosporioides* mycelial growth for up to 3 days. The lowest concentration of these LAB supernatants capable of 100% inhibition was $10^5$ cfu ml⁻¹. These supernatant could also inhibit the growth of both vegetative cells and spores of *C. gloeosporioides* after treatment up to 7 days. These results revealed that the metabolites from LAB may potentially be used as natural preservatives and biological control agents for inhibition of *C. gloeosporioides* growth on post harvest products, especially fresh fruit or fresh cut products.

**Keywords**: anthracnose diseases, biocontrol, *Colletotrichum gloeosporioides*, lactic acid-producing bacteria, Thai fermented meat products

**Introduction**

Anthracnose is one of the major diseases that cause devastating loss to the post-harvest fruit industry in tropical, subtropical and temperate regions. The common etiological agent of anthracnose is fungal *Colletotrichum gloeosporioides* (Penz) Penz & Sacc in Penz (Koomen et al., 1993). Infection by *C. gloeosporioides* in tropical fruits, for instance, mango (*Mangifera indica* L) (Alahakoon et al., 1992), avocado (*Persea americana* Mill) (Coates et al., 1993) and papaya (*Carica papaya* L) (Gamagae et al., 2003), is extremely difficult to notice because the symptoms are not shown until the fruits become ripen. *C. gloeosporioides* can remain on fruit surface by producing appressoria and infection peg in the fruit cuticle and eventually appear to cause anthracnose disease during transportation, storage or market shelf distribution (Estrada et al., 2000). The common method for prevention of *C. gloeosporioides* infection is hot water dipping (50-55°C for 10 minutes) which often incorporates the use of benomyl (500-1000 ppm). However, this method requires time precision because overexposure to heat may result in damages of fruit quality. Also, the development of resistant strains of *C. gloeosporioides*, the ban of benomyl and other fungicide uses in post-harvest fruit industry in several countries and the environmental concerns have resulted in several attempts for the search of safe and effective biological agents for controlling of *C. gloeosporioides* (Kefialew et al., 2008; Koomen et al., 1993).
The use of biological control agents for controlling of *C. gloeosporioides* infection has been shown to be successful in several experimental settings. These include the use of microorganisms for controlling of *C. gloeosporioides* infection of mango fruits. Such microorganisms include *Bacillus licheniformis* (Govender et al., 2006), *Brevundimonas diminuta*, *Stenotrophomonas maltophilia*, *Enterobacteriaceae* sp., *Candida membranifaciens* (Kefialew et al., 2008), *Pseudomonas fluorescens*, *Bacillus subtilis* and *Saccharomyces cerevisiae* (as pre-harvest application) (Vivekananthan et al., 2004). The use of *Cryptococcus magnus*, *Candida oleophila*, and *Pseudomonas putida* for controlling of *C. gloeosporioides* infection of papaya fruits has also been reported (de Capdeville et al., 2007; Gamagae et al., 2003; Gamagae et al., 2004; Shi et al., 2010). The major issues of using these biocontrol agents were their safety warrant and effectiveness (Sharma et al., 2009). From previous reports, there are several lactic acid bacteria (LAB) isolates, especially *Lactobacillus* spp. and *Pediococcus* spp., that have been used as effective anti-fungal agents (Dalié et al., 2010; Schnürer et al., 2005) against *Aspergillus niger*, *Penicillium* sp., *Fusarium graminearum* (Gerez et al., 2009), *A. fumigatus*, *A. nidulans*, *P. commune*, *F. sporotrichioides* (Magnusson et al., 2003), *P. roqueforti*, *Endomyces fibuliger* (Valerio et al., 2009), *P. candidum* (Voulgarì et al., 2010), *P. nordicum* (Schilling et al., 2010), *A. flavus* and *A. parasiticus* (Roy et al., 1996). However, the use of LAB as anti-fungal agent against *C. gloeosporioides* has never been reported.

The purpose of this study is to use the naturally isolated LAB as the beneficial and safe approach for post-harvest controlling of anthracnose disease in tropical fruits. In this study, the supernatants of LAB isolated from Thai fermented meat products sour rice sausages (Sai Krog Isan), fermented pork sausage (Nham) and fermented liver sausages (Mham) were in vitro tested against *C. gloeosporioides* to determine the efficacy of these bacteria as biological control agents for anthracnose disease.

### Materials and Methods

#### Microorganisms and Culture Conditions

From previous experiments, twenty three isolates of lactic acid bacteria (LAB) were derived from Thai fermented meat products, including sour rice sausages (Sai Krog Isan), fermented pork sausage (Nham) and fermented liver sausages (Mham), by using lactobacilli MRS agar (Criterion, USA), and they were then identified using biochemical tests. These isolates of LAB were cultured using MRS broth at 37 °C for 24 hours until the bacterial concentration reached $10^{11}$ cfu ml\(^{-1}\). The bacterial cultures were then centrifuged at 9000 rpm at 4 °C for 10 minutes. Their supernatants were filtered using 0.22 µm filter kit and collected for further experiments.

The fungus, *C. gloeosporioides*, was isolated from mango fruits, which have been shown to have anthracnose disease, and was identified by morphological characteristics under microscope. *C. gloeosporioides* was grown using potato dextrose agar (PDA) at 28±2 °C for 5-7 days and used for further experiments.

#### Inhibition of *C. gloeosporioides* Mycelial Growth

The modified food-poisoned technique (Grover et al., 1962) was used to determine the effect of LAB supernatants against the growth of *C. gloeosporioides* mycelia. The supernatants of LAB cultures (0.5 ml) grown at the concentrations of $10^2$, $10^4$, $10^6$, or $10^8$ cfu ml\(^{-1}\) were individually mixed with PDA, poured into sterile Petri dish plates and left in laminar flow for 5-10 minutes to have dry surfaces. The agar discs (with diameter of 0.7 cm) containing young mycelia of 7-day old *C. gloeosporioides* were obtained using sterile cork borer and were placed onto plates of PDA mixed with LAB supernatants. The agar discs of *C. gloeosporioides* were also
placed onto PDA plates without LAB supernatants and used as control group (R1). The plates were incubated at 28±2 ºC for 3 days. The experiments were performed in three replicates. The diameters of the C. gloeosporioides colonies grown on control group (R1) and those of C. gloeosporioides colonies grown on PDA mixed with LAB supernatants (R2) were used for calculation of percent inhibition of radial growth (PIRG) using the following formula:

\[ \text{PIRG} = \frac{(R1-R2)}{R1} \times 100 \]

**Inhibition of C. gloeosporioides Spore Germination**

The glass slide technique was used to determine the effect of LAB supernatants against the germination of C. gloeosporioides spores. Glass slides were sterilized at 121 ºC for 30 minutes and were then placed in sterile Petri dish. The agar discs (with diameter of 0.7 cm) of PDA mixed with LAB supernatants (according to experiments described above) were placed onto the glass slides. A sterile needle was used to pick up the spores of C. gloeosporioides and the spores were inoculated onto the PDA agar discs and the plates were incubated at 28±2 ºC for 7 days. The glass slides were then examined for germination of C. gloeosporioides spores. The experiments were performed in three replicates.

**Statistical Analysis**

The data were analyzed for variance using the general linear models procedure (SAS Institute, Cary, NC). The significant differences between treatment means were determined using the LSD test at \( P \leq 0.05 \).

**Results and Discussion**

Herein, this is the first report of using LAB as biocontrol agents against C. gloeosporioides. The initial screening showed that supernatants derived from three LAB isolates, including 3ST1, 4MT8, and 5NMB1, at \( 10^6 \) cfu ml\(^{-1} \) showed 100% inhibition of C. gloeosporioides mycelia growth on day 2 (Table 1). Therefore, these three isolates were then used to further determine the effective concentration of LAB supernatants (Table 2). On day 3, at the LAB concentration of \( 10^2 \) cfu ml\(^{-1} \), 5NBM1 supernatant was shown to have levels of inhibition (40.00±0.00%) significantly higher than those of 4MT8 (35.40±0.50%) and 3ST1 (18.84±0.76%). In addition, at the LAB concentrations of \( 10^4 \) cfu ml\(^{-1} \) and higher, all three isolates produced supernatants with 100% inhibition against C. gloeosporioides mycelia growth, which were not significantly different from Cabedazim (50 ppm.), a commercial fungicide. These three isolates were then used for evaluation against C. gloeosporioides spore germination on day 7 (Table 3). At the LAB concentration of \( 10^4 \) cfu ml\(^{-1} \), 4MT8 was found to produce supernatant with the highest efficacy to suppress spore germination (100%), followed by 5NBM1 (95.00±2.00%) and 3ST1 (78.80±1.15). The efficacy to inhibit spore germination of 5NBM1 and 3ST1 supernatants rose up to 100% when using at \( 10^5 \) and \( 10^6 \) cfu ml\(^{-1} \), respectively. In addition, on day 7, C. gloeosporioides mycelial growth was slowly presented and in a form of short germ tubes, suggesting the low capability of this fungus to recover.

This study showed that LAB isolates (3ST1, 4MT8, and 5NMB1) could produce and secrete their metabolites to the culture supernatants, which have been found to have antifungal activities against C. gloeosporioides. There are a number of reports suggesting that LAB produce a number of antifungal compounds, such as proteinaceous compounds, phenyllactic acid and cyclic dipeptides, hydroxylated fatty acids, hydrogen peroxide, phenolic compounds, reuterin, and bacteriocin-like substances, which resulting in the damage of fungal mycelial growth and spore germination; however this also may simply due to the fungal sensitivity to lactic and acetic acids produced by LAB (Dalié et al., 2010; Roy et al., 1996; Schnürer et al., 2005; Sharma et al., 2009; Valerio et al., 2009; Voulgari et al., 2010).
The abilities of three LAB isolates, 3ST1, 4MT8, and 5NMB1, to produce supernatants that inhibit mycelial growth and spore germination of *C. gloeosporioides* may suggest their application as biocontrol agents for controlling of fungal anthracnose disease in post-harvest products.

<table>
<thead>
<tr>
<th>LAB isolate</th>
<th>PIRG of <em>C. gloeosporioides</em> mycelia</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1NBT4*</td>
<td>0</td>
<td>50.54±1.48&lt;sup&gt;de&lt;/sup&gt;</td>
<td>65.33±0.67&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>1NBT6*</td>
<td>0</td>
<td>52.59±3.48&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>70.00±1.20&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2NPT1*</td>
<td>0</td>
<td>3.19±1.80&lt;sup&gt;km&lt;/sup&gt;</td>
<td>5.33±2.91&lt;sup&gt;ih&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2NPT2*</td>
<td>0</td>
<td>10.99±2.19&lt;sup&gt;kl&lt;/sup&gt;</td>
<td>35.33±5.30&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>2NPT3*</td>
<td>0</td>
<td>39.70±4.45&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>45.33±10.73&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>3ST1***</td>
<td>0</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4MT2**</td>
<td>0</td>
<td>1.04±1.04&lt;sup&gt;mn&lt;/sup&gt;</td>
<td>11.33±4.67&lt;sup&gt;eh&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4MT5**</td>
<td>0</td>
<td>50.67±4.84&lt;sup&gt;de&lt;/sup&gt;</td>
<td>70.00±4.16&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4MT7**</td>
<td>0</td>
<td>63.54±4.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>72.66±4.67&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4MT8**</td>
<td>0</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>4MT9**</td>
<td>0</td>
<td>7.43±4.52&lt;sup&gt;km&lt;/sup&gt;</td>
<td>21.33±2.90&lt;sup&gt;ef&lt;/sup&gt;</td>
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<tr>
<td>4MT11**</td>
<td>0</td>
<td>3.23±1.80&lt;sup&gt;mm&lt;/sup&gt;</td>
<td>7.33±1.34&lt;sup&gt;hi&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5NBMI*</td>
<td>0</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5NBM2*</td>
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<td>8.79±4.02&lt;sup&gt;klm&lt;/sup&gt;</td>
<td>15.33±2.91&lt;sup&gt;eh&lt;/sup&gt;</td>
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<td>5NBMS*</td>
<td>0</td>
<td>51.30±6.12&lt;sup&gt;de&lt;/sup&gt;</td>
<td>66.66±2.91&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6NPM1*</td>
<td>0</td>
<td>27.45±4.77&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>53.33±1.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6NPM5*</td>
<td>0</td>
<td>1.04±1.04&lt;sup&gt;nm&lt;/sup&gt;</td>
<td>20.00±3.50&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>7SM2***</td>
<td>0</td>
<td>41.83±4.08&lt;sup&gt;ef&lt;/sup&gt;</td>
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<td></td>
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<tr>
<td>10NPJJ*</td>
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<td>60.42±1.88&lt;sup&gt;be&lt;/sup&gt;</td>
<td>76.00±1.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>10NPJ3*</td>
<td>0</td>
<td>14.32±1.23&lt;sup&gt;kl&lt;/sup&gt;</td>
<td>18.66±3.53&lt;sup&gt;f&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>11ST4***</td>
<td>0</td>
<td>31.66±3.95&lt;sup&gt;ef&lt;/sup&gt;</td>
<td>18.66±1.34&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>11ST7***</td>
<td>0</td>
<td>17.36±0.70&lt;sup&gt;k&lt;/sup&gt;</td>
<td>44.00±1.20&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>12MT2**</td>
<td>0</td>
<td>20.19±4.01&lt;sup&gt;ij&lt;/sup&gt;</td>
<td>37.33±1.34&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>MRS broth</td>
<td>0</td>
<td>0.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Carbendazim</td>
<td>0</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Percent inhibition of *C. gloeosporioides* spore germination by LAB supernatants at various concentrations (cfu ml<sup>-1</sup>) on day 7

<table>
<thead>
<tr>
<th>LAB isolate</th>
<th>Percent inhibition of <em>C. gloeosporioides</em> spore germination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10&lt;sup&gt;7&lt;/sup&gt;</td>
</tr>
<tr>
<td>3ST1***</td>
<td>78.80±1.15&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>4MTS**</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>5NBMI*</td>
<td>95.00±2.00&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>MRS broth</td>
<td>0.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carbendazim</td>
<td>100.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Also, their effective suppression of spore germination on day 7 indicated their stability and perhaps their application for extending the period of transportation, storage, and shelf life of tropical fruits. Furthermore, LAB have GRAS status (generally recognized as safe) and therefore are considered safe for the consumers (Dalié et al., 2010; Schnürer et al., 2005). Hence, the use of these supernatants of LAB isolated from meat products could warrant their safety as biocontrol agents.
Conclusions

In the present study, the supernatants derived from three LAB strains (3ST1, 4MT8, and 5NMB1) that were isolated from Thai fermented meat products were shown to have antifungal activities against C. gloeosporioides. These results may suggest their application as safe natural biological control agents against C. gloeosporioides in post-harvest and food industries.

Acknowledgments

We thank for the Biotechnology department, Mahasarakham University, for providing the laboratory facilities. We also thank Assist Prof. Dr. Pariyaporn Itsaranuwat and Khun Surat Vangpikul for providing the isolates of lactic acid bacteria.

References


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Antimicrobial Activity of Agricultural By-products Extracts Against *Vibrio* spp.

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Abstract

Agricultural by-products are residues from the utilization of agriculture materials, i.e., generic peels and seeds. The extractions of the by-products could inhibit foodborne pathogens. This work aimed to study the inhibitory effect of six agricultural by-products extracts (pomegranate peels, mango seeds, rambutan peels, rambutan seeds, mangosteen peels and longkong peels) against *Vibrio parahaemolyticus* and *V. vulnificus*. The by-products were extracted with either water or 95% ethanol. It was found that the pomegranate peels extracted by soaking in 95% ethanol for 6 hours was the best condition to inhibit *V. parahaemolyticus* and *V. vulnificus* and the MIC (minimum inhibitory concentration) was 2.5 and 2.3 mg/mL, respectively. Meanwhile, the mango seeds extracted by soaking in 95% ethanol for 12 hours showed the best condition to inhibit *V. vulnificus* with the MIC at 4.1 mg/mL. Hence, the agricultural by-product extracts could be used to replace the antibiotics, resulting in the reduction of antibiotic resistance and safety for consumers. Moreover, this would be another way to make the highest utilization of agricultural by-products.

Keywords: agricultural by-products, antimicrobial activity, *V. parahaemolyticus*, *V. vulnificus*

Introduction

Thailand, being an agricultural country, acquires vast agricultural products for both international exportations and domestic consumptions. The products include rice, corn, cassava, and other local fruits especially those growing in the south of the country where different types of fruits are planted and become very popular among consumers. These local fruits include mangosteen, rambutan, durian, longkong, etc. The situation of post consumption or after various forms of fruit processing is the by-products of the productions or consumptions which become agricultural waste in the form of fruit skins or seeds.

Today, all waste is used for fertilizer. Moreover, fruit skins i.e. rambutan, mangosteen, pomegranate, banana, dragon fruit, passion fruit, coconut and longkong are used to make antioxidant and applied to be a mixture of various beauty products which further reduce the amount of chemical importation for cosmetic supplement. There were some studies on natural extracts against bacterial and it was found that they have remarkable antimicrobial activities (kil, *et al*., 2009; Yaltirak, *et al*., 2009; Kossah, *et al*., 2010).

*Vibrio* is in the species of *Vibrionaceae* which is gram negative in a shape of a rod or curve-rod. It can be found in fresh water, seawater, and human or animal’s intestines.
In addition, this bacterial can cause diseases in human beings with a symptom of either mild diarrheas or severe ones. The significant diseases include *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* (Jay, 2000). Antimicrobial of *V. parahaemolyticus* (VP) and *V. vulnificus* (VV) by using antibiotic substances such as tetracycline, chloramphanical, penicillin, and amphicelin can cause leftover toxic or can lead to antibiotic resistant in a patient. In our work, we conducted the antimicrobial experiment by using agricultural by-products i.e. skins and seeds of various fruits and extracting them against VP and VV that are the cause of diarrheas for those who are in favour of raw seafood.

**Materials and Methods**

**Agricultural By-product Samples**

The agricultural by-products using in this experiment were either the by-products of consumption or the processed agricultural products i.e. *Garcinia mangostana*, *L. Lansium domesticum*, Corr., *Punica granatum*, *L.*, *Nephelium lappaccum*, *L.*, *Mangifera indica*, *L.*

**Test Pathogenic Bacteria**

VP and VV (ATCC 27562) were obtained from the Department of Medical Science, Ministry of Public Health.

**Preparation of Fruit Peels and Seeds Powder**

Fruit skins or seeds, either fresh or dry, were cleaned in fresh water and cut into small pieces before drying in a hot air oven at 65°C for 3 days or until the skins/seeds totally dry. Then, they were ground into powder.

**Preparation of Fruit Peels and Seeds Extracts**

The extracts were prepared according to the method of Dupont et. al (2004). Ethanol (95%) and distilled water were used as an extraction solvent. Briefly, two types of extracts (distilled water and ethanol) of each of the five agricultural by-products were prepared. Aqueous extracts were prepared by adding 1 g of agricultural by-products to 19 ml of extract and stirring. The mixtures were filtered through Whatman No. 4 filter paper, centrifuged at 10,000g for 15 min. The ethanol filtrates were evaporated to dryness under vacuum at 40°C. The water extracts were freeze-dried to dry powder. After that, they were redissolved in 1 ml distilled water and stored at -20°C until use. The extracts were filtered again through a 0.45 µm filter before antimicrobial testing.

**Antimicrobial Screening of Agricultural By-product Extracts**

The antimicrobial activity of agricultural by-products against VP and VV was conducted using the Disc diffusion method. VP and VV were grown in tryptic soy broth plus 1% sodium chloride at 35°C, shaking for 24 hrs prior to being used in experiments. The testing bacterial with the concentration of $10^8$ cfu/ml (the turbidity of 0.5 Mc Farland Standard) was then spread on tryptic soy agar plates plus 1% sodium chloride to make bacterial lawn. Agar diffusion assays were done aseptically using sterile 6-mm-diameter paper disks to which 20 µl of test extracts was added. Extraction saturated disks were placed onto the surface of seeded agar plates. Each experiment was repeated three times. Controls consisted of disks with and without distilled water only. After 24 hrs, the diameter of inhibition zone surrounding each disk was measured. The best experiment was selected for the next step of testing.

**Effect of Extraction Time on Antimicrobial Activities**

The extraction time for agricultural by-products was conducted at 6, 12, 24, and 48 hours. The antimicrobial assay was done according to above method. The suitable experiment was selected for the next step of testing.

**Determination of MIC (Minimum Inhibitory Concentration)**

MIC value of the extracts was evaluated for the bacterial strains which were determined using the disc diffusion assay.
The extract was 2-folds diluted prior to test using sterile 6-mm-diameter paper disks to which 20 µl of test extracts was added. Extraction saturated disks were placed onto the surface of seeded agar plates. After incubated for 24 hrs, the diameter of inhibition zone surrounding each disk was measured. The MIC value was evaluated as the lowest concentration of the extract that demonstrated no inhibition zone.

Data Analysis

Data were analyzed using SPSS ANOVA and Tukey’s was used to describe the significance of the effect of treatment.

Results

Inhibitory Effect of Agricultural by-product Extracts on VP and VV

The inhibitory effect of 6 agricultural by-products extracts against VP and VV was investigated (Table 1 and 2). The results showed that the extraction of mango seeds, pomegranate peels and rambutan peels, using distilled water and 95% ethanol for 24 hrs, could inhibit both VP and VV growth. Pomegranate peels extracted with 95% ethanol showed the best condition to inhibit VP while mango seeds and pomegranate peels extracted with 95% ethanol worked best against VV.

Effect of extraction time on antimicrobial activities

Pomegranate peels and mango seeds extracted with 95% ethanol were conducted at 6, 12, 24, and 48 hours. It was found that pomegranate peels extracts could inhibit VP and VV at all extraction periods. They showed the clear zone of similar sizes ($p \geq 0.05$). However, in order to select the most suitable time in extracting substances from pomegranate peels, it was found that the best choice was at 6 hours as it was the most adequate length of time. For mango seeds extract, the results showed that in all extraction periods of mango seeds were able to inhibit VV. Extraction for 12 to 48 hrs showed wider clear zone in comparison with 6 hrs ($p>0.05$). However, there was no significant difference of inhibitory effect during 12 to 48 hrs of extraction time ($p \geq 0.05$) (Table 3). This meant that the extract of mango seeds at 12 hrs was the suitable condition for MIC determination. Determination of MIC

<table>
<thead>
<tr>
<th>By product</th>
<th>Solvent</th>
<th>Clear zone* (mm)</th>
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<tbody>
<tr>
<td>rambutan seeds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>longkong peels</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mangosteen peels</td>
<td>Distilled water</td>
<td>9.60±0.52&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>pomegranate peels</td>
<td>water</td>
<td>9.13±0.69&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>mango seeds</td>
<td>8.34±0.93&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>rambutan seeds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>longkong peels</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mangosteen peels</td>
<td>95%</td>
<td>-</td>
</tr>
<tr>
<td>pomegranate peels</td>
<td>ethanol</td>
<td>13.10±1.03&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>rambutan peels</td>
<td>11.14±0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>mango seeds</td>
<td>10.35±0.57&lt;sup&gt;bc&lt;/sup&gt;</td>
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</tbody>
</table>

* Numbers with the same letters are not significant difference ($p \geq 0.05$)

<table>
<thead>
<tr>
<th>By product</th>
<th>Solvent</th>
<th>Clear zone* (mm)</th>
</tr>
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<tbody>
<tr>
<td>rambutan seeds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>longkong peels</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>mangosteen peels</td>
<td>Distilled water</td>
<td>9.96±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>pomegranate peels</td>
<td>water</td>
<td>10.63±0.81&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>mango seeds</td>
<td>11.30±0.51&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td>rambutan seeds</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>longkong peels</td>
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<tr>
<td>mangosteen peels</td>
<td>95%</td>
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<td>13.49±0.94&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>rambutan peels</td>
<td>12.94±1.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>mango seeds</td>
<td>10.60±0.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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</table>

* Numbers with the same letters are not significant difference ($p \geq 0.05$)

<table>
<thead>
<tr>
<th>Clear zone (mm)*</th>
<th>mango seeds</th>
</tr>
</thead>
<tbody>
<tr>
<td>VV</td>
<td>VP</td>
</tr>
<tr>
<td>pomegranate peels</td>
<td>14.77±1.65&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>14.94±1.30&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>24</td>
<td>13.75±1.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>48</td>
<td>15.44±0.87&lt;sup&gt;b&lt;/sup&gt;</td>
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</tbody>
</table>

* Numbers with the same letters are not significant difference ($p \geq 0.05$)
The study on the MIC from the extracts of pomegranate peels and mango seeds soaking in 95% ethanol were studied. As a result, the extracts of pomegranate peels with 95% ethanol for 6 hrs were able to inhibit VP and VV with the MIC of 2.5 and 2.3 mg/ml, respectively. On the other hand, the extracts of mango seeds with 95% ethanol at 12 hours showed the MIC of 4.1 mg/ml.

**Discussion**

The result of 6 agricultural by-products extracted with both water and 95% ethanol to inhibit VP and VV revealed that the extract of pomegranate peels was able to inhibit these two pathogenic bacterial while the one of mango seeds worked well to inhibit VV. Palakawonge et al. (2010) reported that the antimicrobial activity of mangosteen extract against some Gram-positive bacteria (*L. monocytogenes* and *S. aureus*) and Gram-negative bacteria (*E. coli* and *Salmonella* sp.). They showed the MIC values of peel, leaves, and bark extracted against Gram-positive bacteria were ranged from 0.025-0.78 mg/ml. Snyder (1997) reported that the oregano extracts were able to inhibit pathogenic bacteria.

The inhibitory effect of agricultural by product extract might be due to the primary component of antibacterial substances within the peels and seeds. These substances are in the group of tannins, sterol glycosides as well as phenolic compounds and they were able to perform antibacterial activities.

**Conclusions**

Six agricultural by-products extracted with distilled water and 95% ethanol were tested for antibacterial activities of VP and VV using agar disc diffusion method. Pomegranate peels and mango seeds extracted with 95% ethanol could perform antibacterial activities against both VP and VV with the MIC of 2.5 and 2.3 mg/ml, respectively. While the mango seeds extracted with 95% ethanol for 12 hrs could inhibit VV with the MIC of 4.1 mg/ml. Although, this study on the antibacterial of agricultural by-products extracts is considered a primary one, it can be adopted and developed to effectively perform inhibitory activities against the other pathogenic microorganism in order to reduce antibiotic usage as well as drug resistance. Moreover, it is to maximize the use of agricultural by-products.

**Acknowledgments**

We would like to thank Prince of Songkla University for providing the fund for this research.

**References**


Quality Attribute and Antioxidant Activity Changes of Jerusalem Artichoke Tubers (*Helianthus tuberosus* L.) During Storage at Different Temperatures

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Abstract

Jerusalem artichoke (*Helianthus tuberosus* L.) tuber contains high inulin. Qualities of the tubers are governed by storage temperature and time. This study was aimed to investigate the changes in quality attribute and antioxidant activity of peeled fresh Jerusalem artichoke tubers (HEL65 and JA89 varieties) during storage at 5 and 10 °C for 5 weeks. The statistical data analysis showed that storage temperature influenced on firmness, total phenolic compound and DPPH-scavenging activity in JA89 and affected only *a* in HEL65. The decrease in *L* and the increase in *a* were observed in HEL65 stored at 5°C (*p*≤0.05), however both storage temperature and time did not affect *b* (*p*>0.05). Firmness was determined by a puncture test using TA XT Plus, the firmness of HEL65 did not change during storage (*p*>0.05) but that of JA89 significantly decreased in two and three weeks time at 5°C and 10°C (*p*≤0.05), respectively. Exudate was observed in the JA89 but not for the HEL65. There were no significant changes in moisture and fructan content during storage. TSS in HEL65 slightly increased (*p*≤0.05) but rapidly decreased in JA89 after two weeks of storage (*p*≤0.05). Total phenolic compound, ABTS- and DPPH-scavenging activity initially increased up to three weeks of storage (*p*≤0.05), then remained constant through the storage at 5 and 10°C for HEL65 and at 5°C for JA89 (*p*≤0.05). However, TPC decreased in JA89 stored at 10°C (*p*≤0.05). Therefore, storage temperature was recommended at 5°C for JA89 and at 10°C for HEL65.

Keywords: antioxidant activity, Jerusalem artichoke (*Helianthus tuberosus* L.), quality attribute, storage temperature

Introduction

Quality parameters of fresh-cut fruit and vegetable products include appearance, texture, flavor, and nutritive value which depend upon the cultivar, pre-harvest practices, climatic conditions, maturity, and harvesting method. Handling and operation conditions during fresh-cut processing also have major impacts on quality of intact fruits and vegetables and consequently to quality of the fresh-cut products (Kader, 2002). Three basic problems confront the extension of shelf life of fresh cut fruit and vegetable products. Firstly, the oxidation of phenolics catalyzed by polyphenol oxidase due to peeling and cutting which undergo enzymatic browning to produce an undesirable brown color. Secondly, tissue wounding induces high respiration rate which triggers tissue deterioration and, thirdly the growth of microorganisms (Shah and Nath, 2008).

Jerusalem artichoke (*Helianthus tuberosus* L.) is a fructan plant, composing of inulin and fructo-oligosaccharides. Functional properties of inulin rely on degree of polymerisation (DP) which was influenced by storage temperature and duration. The longer chain, the more benefit to health as dietary fiber, fat replacement, prebiotics, intestinal mineral absorption enhancer *etc.* (Kays and Nottingham, 2008). Thompson (2003) reported that Jerusalem...
Artichoke tubers could be kept at room temperature and 60% RH for 1-2 week. The tubers lost moisture and shriveled at temperature over 40°C while a mouldy growth was observed at 16°C during 2-3 weeks time. Saengthongpinit and Sajjaanantakul (2005) found that the tubers stored at 2 and 5°C increased in sucrose and DP 3-10 while DP>10 fractions decreased after 4-6 weeks storage. They also reported that the inulin composition did not changed under frozen storage (-18°C) for 3 months.

The aim of present study was to investigate the effect of different storage temperature and time on quality attributes and antioxidant activities of peeled fresh Jerusalem artichoke tubers.

Materials and Methods

The Sample Preparation

Two varieties of Jerusalem artichoke tubers (HEL65 and JA89 of the July-2010 harvesting crop) were obtained from the Plant Breeding Research Center for Sustainable Agriculture, Khon Kaen University. The tubers were washed several times with tap water to get rid of contaminants and final washed with 200 ppm sodium hyperchloride. The tubers were trimmed out the defects and peeled. The peeled tubers were maintained at low temperature using ice water, then soaked in 50 ppm sodium hyperchloride (ambient temperature) for 15 s to reduce microorganism load, then packed in zipper polyethylene bags and kept at 5 and 10°C. The peeled tubers were sampled every week to analyze for color, texture, total soluble solid (TSS), ABTS- and DPPH-scavenging activity, moisture, fructan and total phenol content (TPC).

Determination of Quality Attribute

Surface color of the tubers was determined for L*, a* and b* value using Hunter Lab (Ultrascan XE, Hunter Associates Laboratory; USA). Firmness was measured by puncture test with P/3 probe, test speed of 1.5mm s^-1 and test distance of 5mm using texture analyzer (TAXT2 Plus, Stable Micro System; UK). The peak force (g) was recorded as the firmness or hardness of the tubers.

Moisture content was determined by hot-air oven drying method (AOAC, 1999), TSS (°brix) was measured by hand refractometer (Atago, Japan). Fructan content was analysed with the enzymatic/spectrophotometric (AOAC method 999.03) using the enzyme assay kit K-FRUC (Megazyme International, Ireland).

Total phenolics were extracted in methanolic solution and antioxidant activity was measured. Ten grams of grounded sample was extracted in 50 ml of 80% (v/v) methanol, and then mixture was vigorously in a shaker for 1 h. The sample suspensions were centrifuged at 10,000×g for 15 min. The supernatant were filtered through Whatman No.1 and filtrates were stored at -20°C till analysis. TPC was measured by colorimetric method with Folin’s reagent (Queiroz et al., 2009). Results are expressed as milligrams of gallic acid equivalent (GAE) per 100g of fresh weight (fw).

Determination of Antioxidant Activity

ABTS- and DPPH-radical scavenging activity were determined according to Kornkasem et al., (2006). Briefly, ABTS-radical scavenging activity, stock solution of ABTS radical was prepared by reacting ABTS (7 mM in water) solution with potassium per sulfate (2.45 mM final concentration) and allowing this mixture to stand in the dark at room temperature for 12–16 h before use. The stock solution of ABTS was diluted just before use to obtain an absorbance of 0.70 (+0.02) at 734 nm. To 10 µl of the sample extract or standard and 1 ml of diluted ABTS was added, mixed well and absorbance was read immediately. For DPPH- radical scavenging activity, 77 µl of sample extract was added to 3 ml of DPPH reagent (0.06 mM in methanol) and mixed vigorously. The reaction mixture was...
allowed to stand in the dark for 15 min at room temperature and the discoloration of DPPH was measured against blank at 515 nm. Trolox solutions of 0-100 and 0-200 µg Trolox ml⁻¹ were drawn for a standard curve of DPPH and ABTS, respectively. The antioxidant activity was expressed as micrograms of trolox equivalent (TE) per 1g of fresh weight.

**Statistical Analysis**

All data were presented as means± standard deviation of at least 2 replicate and 2 determinations for each replicates. Differences between variables were tested for significance by using ANOVA. Differences between means were considered by Duncan’s multiple rang test at $p<0.05$ (SPSS for Windows v 17).

**Results and Discussion**

The yield of HEL65 peeled pulp was 71.02%. The initial moisture (% fresh wet; fw), TSS (° brix), fructan (% dry weight), TPC (mg GAE 100 g⁻¹fw), ABTS- and DPPH-radical scavenging (µgTE g⁻¹fw) were 79.17±0.15, 17.50±0.78, 63.20±0.80, 32.72±1.08, 89.38±0.59 and 62.48±0.23, respectively. The peeled tuber was lighter yellow color having $L^*$, $a^*$ and $b^*$ value of 74.93±2.35, -1.12±0.30 and 15.99±2.16, respectively. The firmness was 2261.20± 74.90 g .

The JA89 had edible pulp of 67.97% with initial moisture, TSS, fructan, TPC, ABTS- and DPPH-radical scavenging of 78.58± 0.13, 21.38±1.52, 64.20±0.57, 46.05±0.57, 116.87± 5.85 and 73.71±2.57, respectively. The pulp lightness was lower than that of HEL65 but $a^*$ and $b^*$ value was not significant different from those of HEL65. The JA89 had $L^*$, $a^*$ and $b^*$ value of 66.31±1.48, -1.26±0.60 and 14.18±2.29, respectively. However, the firmness was 1991.79±2.93, lower than that of HEL65.

Changes in appearances and chemical compositions of peeled Jerusalem artichoke tubers stored at 5 and 10°C were observed. Storage temperature influenced on firmness, TPC and DPPH-scavenging activity in JA89 but affected only $a^*$ in HEL65 (Table 1), while storage time affected on TSS, firmness, TPC, ABTS- and DPPH-radical scavenging in JA89 and also influenced on TSS, $L^*$ and $a^*$ value, TPC, ABTS- and DPPH-radical scavenging in HEL65.

**Physical Attribute Change**

Table 2 summarizes changes in color and firmness over the storage period. Color changes on the surface of the HEL65 tubers were observed during the 4-5 weeks of storage at 5°C. Lightness ($L^*$) decreased

| Table 1 Analysis of variance of the storage temperature and time |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | HEL65           | JA89            |
| % Moisture (fw)                 | ns              | ns              |
| % Fructan (dry weight)          | ns              | ns              |
| TSS (°Brix)                     | ns              | ns              |
| Color $L^*$                     | ns              | ns              |
| $a^*$                           | ns              | ns              |
| $b^*$                           | ns              | ns              |
| Firmness (g)                    | ns              | ns              |
| TPC (mg GAE 100 g⁻¹fw)          | ns              | ns              |
| ABTS (µg TE g⁻¹ fw)             | ns              | ns              |
| DPPH (µg TE g⁻¹ fw)             | ns              | ns              |

* Significant at $p \leq 0.05$

** Significant at $p \leq 0.01$

ns No significance
from 74.24 to 67.29. The red spots, probably due to chilling injury, were observed resulting in an increase in \( a^* \) from -0.96 to 0.29 in the week-4 of storage but not found in the other temperature. The degree of yellowness on the surface, described by \( b^* \) values, remained unchanged over the storage period. Storage temperature and time influenced the surface color of JA89. Color change of fresh-cut fruits and vegetables mainly occurs from the oxidation of phenolic compounds and the primary enzyme responsible for the browning reaction is polyphenol oxidase (PPO) (Ding, et al., 1998; Mayer and Harel, 1979). The PPO activity of Jerusalem artichoke was found mainly in the skins. Thus, the browning reactions could be considerably reduced by removing the skins. (Tchoné et al., 2005).

A decrease in firmness of JA89 was observed at 5 and 10°C storage (\( p \leq 0.05 \)). It decreased from 1783.82 to 7801.6 g and 1975.27 to 1780.06 g during the last week of storage. The HEL65 stored at 5 and 10°C for 5 weeks remained firm and crisp. However, the reduction of firmness in JA89 might probably resulted from microbial decay and enzyme activity, such as cellulase, pectinmethylsterase (PME) and polygalacturonase (PG), which played an important role in remodeling, ripening and softening of cell wall. (Lamikanra, 2002)

### Chemical Composition Changes

The table 3 shows that the tubers had constant moisture content and no significant weight loss during 5 week storage. The temperature and duration of storage did not affect moisture content in both varieties (\( p>0.05 \)). This was possibly due to the resistance of vapor transmittance of the zipper polyethylene bag used in this study. Duration of storage affected on the TSS, depending on cultivar (Table 3). TSS of the HEL65 variety tubers increased significantly from 17.50 to 19.20 °Brix in 4 weeks and upto 20.05 °Brix in 5 weeks at 5°C. At 10°C, TSS increased to 18.85 °Brix in 3 week and 20.05-20.88 °Brix after 4 week storage. The increase in TSS could be due to biochemical reactions, such as starch conversion, degradation of starch and hydrolysis of cellulose by cellulase resulting in glucose units. On the other hand, the JA89 lost TSS during 3 week storage at both temperatures, from 21.38 to 17.85 at 5°C and to16.13 at 10°C. The reduction in TSS during storage was perhaps due to a microbial growth which consumed sugars (Tassou and Boziaris, 2002; Shah and Nath, 2008) Furthermore, there some exudate found in bags of JA89, indicating cell decays and the loss of TSS.

Fructan content of tubers was in the range of 61-65% (Table 3). The temperature and time did not affect on fructan contents of both two cultivars during storage. Ernst et al. (1996) reported that the DP of fructan in Jerusalem artichoke (DP of 2-70) could alter slowly during storage. The change of DP distribution profile of fructan during storage was depending on the variety, maturity and storage conditions (Modler et al., 1993; Saengthongpinit & Sajjaanantakul, 2005). However, the DP change was not monitored in this study.

### Total Phenolic Content and Antioxidant Activity

Table 4 shows the changes in TPC, ABTS- and DPPH-radical scavenging activity of Jerusalem artichoke tuber during storage at 5 and 10°C. The HEL65 increased in TPC during 2 week storage (from 32.72 to 38.93) and then remained constant from week 3-5. While an increase in TPC of the JA89 was observed at 5°C during the week-2 and the TPC decreased after 3 weeks of storage. The tubers at 10°C increased in TPC from 46.05 to 51.04 in first week; then decreased gradually during the 2-5 weeks storage. Phenylalanine ammonia lyase (PAL) activity, the key enzyme in biosynthesis of phenolic compounds and accumulation of phenolic constituent, could be a reason for the rise of total phenolic content in tissues (Hwang et al., 1994; Ritenour et al., 1995; Benkeblia, 1999). Whereas, the decrease in phenolics content during a longer storage
Table 2  The physical changes of Jerusalem artichoke during storage at 5 and 10°C.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time</th>
<th>HEL65**</th>
<th>JA89</th>
<th>HEL65*</th>
<th>JA89</th>
<th>HEL65</th>
<th>JA89</th>
<th>HEL65</th>
<th>JA89**</th>
<th>Firmness (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>74.93b ±2.35</td>
<td>66.31 ±1.48</td>
<td>-1.12a ±0.30</td>
<td>-1.26 ±0.60</td>
<td>15.99 ±2.16</td>
<td>14.18 ±2.29</td>
<td>2261.20 ±74.90</td>
<td>1991.79c ±2.93</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>72.71b ±2.95</td>
<td>66.87 ±1.34</td>
<td>-1.12a ±0.06</td>
<td>-1.05 ±0.11</td>
<td>15.08 ±1.15</td>
<td>14.67 ±1.03</td>
<td>2190.28 ±10.70</td>
<td>1958.67c ±48.59</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>74.35b ±1.27</td>
<td>67.16 ±2.46</td>
<td>-0.56a ±0.61</td>
<td>-1.17 ±0.25</td>
<td>17.28 ±1.70</td>
<td>15.18 ±0.20</td>
<td>2195.33 ±90.45</td>
<td>1805.77ab ±35.31</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>74.75b ±2.96</td>
<td>67.43 ±1.21</td>
<td>-0.96a ±0.11</td>
<td>-0.73 ±0.13</td>
<td>16.04 ±2.38</td>
<td>15.59 ±0.57</td>
<td>2413.72 ±76.80</td>
<td>1783.82b ±15.73</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>74.24b ±0.33</td>
<td>63.04 ±1.81</td>
<td>0.29b ±0.07</td>
<td>-0.33 ±0.31</td>
<td>17.46 ±1.29</td>
<td>16.52 ±0.06</td>
<td>2177.24 ±119.96</td>
<td>1658.58a ±78.87</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>67.29a ±0.21</td>
<td>0.31b ±0.04</td>
<td>17 ±0.26</td>
<td>13 ±35.05</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>0</td>
<td>74.93±2.35</td>
<td>66.31 ±1.48</td>
<td>-1.12a ±0.30</td>
<td>-1.26 ±0.60</td>
<td>15.99 ±2.16</td>
<td>14.18 ±2.29</td>
<td>2261.20 ±74.90</td>
<td>1991.79b ±2.93</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>72.02±0.73</td>
<td>65.63 ±2.03</td>
<td>-0.96a ±0.11</td>
<td>-0.72 ±0.13</td>
<td>16.1 ±2.60</td>
<td>14.91 ±0.16</td>
<td>2174.94 ±93.51</td>
<td>1991.32b ±69.97</td>
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</tr>
<tr>
<td>2</td>
<td>0</td>
<td>73.76±0.77</td>
<td>66.13 ±2.68</td>
<td>-0.90a ±0.51</td>
<td>-0.83 ±0.38</td>
<td>15.55 ±1.56</td>
<td>15.91 ±0.22</td>
<td>2272.27 ±72.47</td>
<td>1975.27ab ±37.42</td>
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<tr>
<td>3</td>
<td>0</td>
<td>74.27±0.78</td>
<td>61.69 ±3.4</td>
<td>-0.86a ±0.16</td>
<td>-0.71 ±0.05</td>
<td>14.38 ±0.03</td>
<td>16.02 ±0.45</td>
<td>2364.8 ±173.91</td>
<td>1780.06b ±22.40</td>
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</tr>
<tr>
<td>4</td>
<td>0</td>
<td>74.51±2.88</td>
<td>-0.93a ±0.07</td>
<td>17.12 ±0.82</td>
<td>13 ±59.14</td>
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<td></td>
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</tr>
<tr>
<td>5</td>
<td>0</td>
<td>68.62±1.29</td>
<td>-0.99a ±0.06</td>
<td>13.26 ±1.56</td>
<td>2262.48 ±93.73</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Means follows by different letters were significantly different (p < 0.05) within a column.
** Means follows by different upper case or lower case letters were significantly different within storage temperature and time, respectively.
Table 3  The chemical composition changes of Jerusalem artichoke during storage at 5 and 10°C.

<table>
<thead>
<tr>
<th>Storage Temp</th>
<th>Time</th>
<th>HEL65 Moisture (%fw)</th>
<th>JA89 Moisture (%fw)</th>
<th>TSS (°Brix) HEL65**</th>
<th>TSS (°Brix) JA89**</th>
<th>HEL6 Fructan (%dw)</th>
<th>JA89 Fructan (%dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>79.20 ± 0.10</td>
<td>78.60 ± 0.10</td>
<td>17.50a ± 0.78</td>
<td>21.38cd ± 1.52</td>
<td>63.20 ± 0.80</td>
<td>64.20 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78.85 ± 0.21</td>
<td>78.59 ± 1.19</td>
<td>17.28a ± 0.32</td>
<td>21.68d ± 0.60</td>
<td>64.06 ± 1.28</td>
<td>63.84 ± 1.68</td>
</tr>
<tr>
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<td>2</td>
<td>78.07 ± 0.28</td>
<td>78.37 ± 0.21</td>
<td>17.90bc ± 0.99</td>
<td>19.30bc ± 0.92</td>
<td>63.44 ± 1.04</td>
<td>62.64 ± 0.87</td>
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<tr>
<td></td>
<td>3</td>
<td>78.68 ± 0.15</td>
<td>79.36 ± 1.10</td>
<td>18.03ab ± 0.18</td>
<td>17.85ab ± 0.21</td>
<td>61.55 ± 0.61</td>
<td>62.87 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>78.96 ± 1.64</td>
<td>79.73 ± 0.57</td>
<td>19.20bc ± 0.64</td>
<td>16.13a ± 0.39</td>
<td>61.22 ± 0.11</td>
<td>61.55 ± 0.87</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>78.71 ± 0.63</td>
<td>20.05c ± 0.49</td>
<td>61.15 ± 1.56</td>
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</tr>
<tr>
<td>10</td>
<td>0</td>
<td>79.20 ± 0.10</td>
<td>78.60 ± 0.1</td>
<td>17.50a ± 0.78</td>
<td>21.38cd ± 1.52</td>
<td>63.20 ± 0.80</td>
<td>64.20 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>78.85 ± 0.96</td>
<td>79.26 ± 0.29</td>
<td>17.75a ± 0.42</td>
<td>20.45b ± 0.99</td>
<td>64.15 ± 2.05</td>
<td>63.91 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>78.01 ± 0.25</td>
<td>77.82 ± 0.75</td>
<td>17.18a ± 0.46</td>
<td>20.15b ± 0.78</td>
<td>61.80 ± 0.97</td>
<td>61.06 ± 1.18</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>78.77 ± 0.25</td>
<td>79.06 ± 0.24</td>
<td>18.85ab ± 0.35</td>
<td>16.13a ± 0.11</td>
<td>62.05 ± 1.99</td>
<td>60.48 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>78.15 ± 0.96</td>
<td>20.05c ± 0.35</td>
<td>63.10 ± 1.04</td>
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</tr>
<tr>
<td></td>
<td>5</td>
<td>78.53 ± 0.37</td>
<td>20.88c ± 0.25</td>
<td>62.81 ± 1.32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Means follow by different letters were significantly different (p ≤ 0.05) within a column.
** Means follow by different upper case or lower case letters were significantly different (p ≤ 0.05) within storage temperature and time, respectively.
Table 4 The total phenolic compound and antioxidant activity by ABTS and DPPH assay of Jerusalem artichoke during storage at 5 and 10°C

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time</th>
<th>Storage</th>
<th>TPC (mgGAE·100g⁻¹ fw)</th>
<th>ABTS (µgTE·g⁻¹ fw)</th>
<th>DPPH (µgTE·g⁻¹ fw)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HEL65**</td>
<td>JAS9*</td>
<td>HEL65**</td>
<td>JAS9**</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>32.72 a ±1.08</td>
<td>46.05bc ± 0.57</td>
<td>89.38a ± 0.59</td>
<td>116.87a ± 5.85</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>36.31b ±0.49</td>
<td>50.97d ± 0.90</td>
<td>92.01bc ± 2.56</td>
<td>145.91b ±10.82</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>38.93c ±0.89</td>
<td>48.65cd ± 2.27</td>
<td>95.99b ± 1.46</td>
<td>157.75b ±5.78</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>38.97c ±0.48</td>
<td>46.40bc ± 0.16</td>
<td>106.22c ± 4.38</td>
<td>150.93b ±3.29</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>38.96c ±1.32</td>
<td>39.60a ± 1.41</td>
<td>102.55c ± 1.68</td>
<td>142.29b±4.38</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>39.31c ±0.30</td>
<td>103.48c ± 1.68</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Means follow by different letters were significantly different (p ≤ 0.05) within a column.
** Means follow by different upper case or lower case letters were significantly different (p ≤ 0.05) within storage temperature and time, respectively.
was probably due to a degradation of phenols under low temperature conditions (Shah and Nath, 2008). The presence of exudates, indicating a deterioration of cell structure, might be a reason for a decrease TPC.

Antioxidant activity of the methanol extract from the tubers was measured by ABTS and DPPH assays (Table 4). It was found that antioxidant activity by the DPPH assay was lower than that of ABTS assay. Antioxidant activity of the HEL65 stored at 5 and 10°C increased from 89.38 to 106.22 for ABTS assay and 62.48 to 77.64 for DPPH assay during 3 week period and then kept constant. The antioxidant activity of the JA89 stored at 5°C increased during the first week of storage compared to the fresh tuber ($p \leq 0.05$) and then remained constant ($p > 0.05$). At 10°C, the JA89 tubers reduced in antioxidant capacity at the last week of storage.

Conclusions

Storage temperature at 5 and 10 °C had small effect on the physical and chemical attributes changes. The storage time and variety played an important role to quality attributes and antioxidant activity changes of Jerusalem artichoke tubers. It is well-known that phenolics are effective antioxidants in fruits and vegetable. Therefore, the increase in total phenolic content might contribute antioxidant capacity in the tubers during storage. The storage temperature of 5°C and 10 °C was recommended for the JA89 and HEL65 tubers respectively.

Acknowledgments

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References


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Seed Soaking with Three Essential Oils from Herbal Plants for Controlling Sclerotium rolfsii Sacc. Causing Damping-off Disease in Tomato

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Abstract

Essential oils possess the antimicrobial properties against a wide range of plant fungi. This study evaluated the efficacy of three essential oils and their combinations at different ratio from lemon grass (Cymbopogon citratus), holy basil (Ocimum sanctum) and clove (Eugenia caryophyllus) to control Sclerotium rolfsii causing seedling damping-off disease in tomato by means of seed soaking at 1000 and 2000 ppm under two experiments, using completely randomized design of eighteen treatments. It was found that at the 1000 ppm concentration, the highest germination percentage (84.5%) was obtained in seeds soaked in a mixture of lemon grass and holy basil oil at the ratio 1:1 whereas seeds soaked in a mixture of holy basil and clove oil (2:1) at 2000 ppm revealed the highest germination percentage (90%). The results indicated highly significant difference from those of seed planted in S. rolfsii infested soil. This recommends an alternative way to control seed damping-off as a substitute for chemical treatments.

Keywords: Sclerotium rolfsii, bio-fungicide, essential oil

Introduction

Damping off in tomato (Lycopersicon esculentum Mill.) is caused by Sclerotium rolfsii, a devastating soil borne fungal pathogen, which attacks on a wide host range of crop plants throughout the world including in Thailand. Seedlings are very susceptible and die quickly when it was infected. Essential oils are, as secondary metabolites, made up of many different volatile compounds which are composed almost entirely of two classes of compound, terpines and phenylpropenes (Bowles, 2003; Pichersky, 2006). Lemongrass (Cymbopogon citratus) oil is characterized by a high content of citral which composed of neral and geranial isomers. (Paviani et al., 2006, Combrinck, 2011). The main chemical component of holy basil (Ocimum sanctum) oil and clove (Eugenia caryophyllus) oil is eugenol (Kumar et al, 2010, Lv et al., 2011).

Those active constituents provide an important defense strategy to the plant, against disease fungi.

It is generally believed that essential oils principally achieved against the cell cytoplasmic membrane of microorganism. The hydrophobicity is an important properties of essential oils (Sikkema et al., 1995). The occurrence of leakage of intracellular constituents, impairment of microbial enzyme systems (Carson et al. 2002) and extensive loss of the cell contents will cause the death of cell. Omidbeygi et al. (2007) have suggested that antimicrobial components of the essential oils cross the cell membrane, interacting with the enzymes and proteins of the membrane, then producing a flux of protons towards the cell exterior which induces changes in the cells and, eventually, causing the cell death. Many researches showed that essential oils from different herbal plants exhibit antifungal
activity against a wide range of fungal pathogen. (Deans, 1991; Isman, 2000; Duamkhanmanee, 1999, 2005, 20008 a, 2008 b, 2009; Locke, 2010.). Duamkhanmanee (2005) reported that the usage of single essential oil from lemon grass, holy basil, clove and phlai by seed soaking and soil drench treatment tended to decrease seedling damping-off disease in tomato, particularly, soil drenched with holy basil oil. In a former day, there are a few research controlling soil borne disease by using essential oil application. At present - , the works of those are gradually increasing. Locked (2010) reported that formulated clove oil could effectively reduce the population of various soilborne fungal pathogens and control diseases caused by them. Other plant extracts are also being evaluated for their potential as biopesticides to control diseases caused by soilborne pathogens. Due to side effects of chemicals used to protect the plant and eradicate the disease, it has caused such problematic results as serious hazards to human and environment, induction of non target pests and fungicide resistance (Fry, 1982). This research intends to find a potential non chemical means to solve the problem. Therefore, the studies were conducted to determine the efficacy of three essential oils from lemon grass (Cymbopogon citratus), holy basil (Ocimum sanctum) and clove (Eugenia caryophyllus) to control Sclerotium rolfsii causing seedling damping-off disease of tomato by means of seed soaking.

Materials and Methods

Experimental Design

Two experiments were conducted using Completely randomized design (CRD) and having 18 treatments with four replications. The treatment details are as follows:

1. sterilized soil + sterilz. dist. water (control-no pathogens)
2. S. rolfsii infested soil+ sterilz. dist.water (control-pathogens)
3. S. rolfsii infested soil+ lemon grass oil
4. S. rolfsii infested soil+ holy basil oil
5. S. rolfsii infested soil+ clove oil
6. S. rolfsii infested soil+ lemon grass oil: holy basil oil: 1:1
7. S. rolfsii infested soil+ lemon grass oil: holy basil oil : 1:2
8. S. rolfsii infested soil+ lemon grass oil: holy basil oil : 2:1
9. S. rolfsii infested soil+ lemon grass oil: clove oil : 1:1
10. S. rolfsii infested soil+ lemon grass oil: clove oil : 1:2
11. S. rolfsii infested soil+ lemon grass oil: clove oil : 2:1
12. S. rolfsii infested soil+ holy basil oil: clove oil : 1:1
13. S. rolfsii infested soil+ holy basil oil: clove oil : 1:2
14. S. rolfsii infested soil+ holy basil oil: clove oil : 2:1
15. S. rolfsii infested soil+ lemongrass oil : holy basil oil: clove oil : 1:1:1
16. S. rolfsii infested soil+ lemongrass oil : holy basil oil: clove oil : 1:2:1
17. S. rolfsii infested soil+ lemongrass oil : holy basil oil: clove oil : 2:1:1
18. S. rolfsii infested soil+ lemongrass oil : holy basil oil: clove oil : 1:1:2

Three essential oils from lemon grass, holy basil and clove were evaluated to control Sclerotium rolfsii causing seedling damping-off disease of tomato by means of seed soaking at 1000 and 2000 ppm under two experiments. Both experiments included seed planted in disinfested soil (control-no pathogens), seed planted in S. rolfsii infested soil (control-pathogens), seed soaked with lemon grass, holy basil and clove oil (tr3-tr5) together with the other thirteen treatments in which seeds were soaked in the above ratios of these essential oils prior to planting in S. rolfsii infested soil. Tween 80 was added in all treatments for oil dispersion.

Oil Extraction

Three essential oils from lemon grass, holy basil and clove (Figure 1) were extracted by water distillation (Figure 2). The method started with 300 grams of clove
flower bud, and fresh leaves of the other plants, cut into small pieces and then put in 700 ml. of water in a 2 litre-round flask placed on electrical mantel. The steam and extracted essential oil passes through a water condenser allowing the volatile oil fraction to float on top of the water. The oil was collected by drawing out the water.

Isolation of Fungi

Sclerotium of Sclerotium rolfsii was put on Potato Dextrose Agar to obtain the isolate of fungi (Figure 3). The subculture was done to attain the even inoculum.

Mass Culture of Pathogen (S. rolfsii)

Inoculum of the isolated pathogens was prepared using soil : powdered corn (9:1 v/v) medium. The ingredients were mixed with water and adjusted to the appropriate moisture (60%). The mixture was put into the 5x8 inch² plastic bag for sterilization. The sterilized medium was inoculated with mycelium discs obtained from the margin of actively growing cultures of the 2-days old colony using a cork borer (5 mm diameter) and incubated at room temperature for 10 days (Figure 4) to gain the entirely infested media which were mixed thoroughly with the sterilized soil (1:9 v/v), then, watered and covered with plastic for 2 days (Figure 5).

Efficacy of Essential Oils on Damping-off Disease

Hundred tomato seeds soaked with three essential oils for six hours in different ratios at 1000 and 2000 ppm were sown in tray containing S. rolfsii infested soil. Besides, untreated seeds grown in sterilized and infested soil served as two controls. The data were recorded on a number of germinated plants and the survival seedling after 20 days of sowing. Percentage of seed germination and seedling survival was calculated and developed to the Arc Sine Transformation (Snedecor and Cochran, 1973) prior to analysis of variance (ANOVA) followed by Duncan’s multiple range test to compare treatment means.

Results and Discussion

Effects of Essential Oils in the Different Ratios on Damping-off Disease: Seed Germination

According to the experiment I (1000 ppm), it was found that the untreated seeds sown in sterilized soil (control-no pathogens) achieved the highest germination (89.75%) as usual. Among the treated seeds planted in S. rolfsii soil, (tr3-tr15), the highest germination percentage (84.5%) was obtained from seeds soaked in a mixture of lemon grass and holy basil oil at the ratio 1:1 (tr6) which was higher than the others (Figure 6). Besides, the percentage of seed germination of untreated seeds (control-pathogens) sown in S. rolfsii was only 60%.
The application of single pure oil by seed soaking seemingly brought high percentage of seed germination. It was 79.75, 78.50 and 76.75 % from oil of lemon grass, holy basil and clove respectively. The three combinations of three oils exhibited the lower percentage of seed germination than the single pure oil and some of two oil combinations (tr6-8). Moreover, they still were not different from the control-pathogens except the eighteenth treatment.

Effects of Essential Oils in the Different Ratios on Damping-off Disease: Survival Seedling

In the experiment II (2000 ppm), Among the treated treatment (tr3-18), except the control-no pathogens, the highest percentage of seed germination (90%) was achieved with seed soaking in a mixture of holy basil and clove oils at the ratio 2:1 (Figure 7), whereas the treatment with the single pure oil from lemon grass and holy basil also resulted in a high percentage of seed germination of 85.25 % and 83.75 % respectively. The result showed a great accomplishment when compared to the untreated seed in infested soil, which the germination percentage was only 48.50 %.

Effects of Essential Oils by Seed Soaking in the Different Ratios at 1000 ppm on Seed Germination.


According to the result, essential oil showed positive effects on controlling S. rolfsii damping-off disease by seed soaking. As known, the incidence of damping-off
disease occurred both in pre- and post-emergence of seedling. In this study the percentage of seedling survival was high. It seems that essential oil could both inhibit damping-off disease and protect tomato seedlings. This recommends an alternative way to control seed damping-off as a substitute for chemical treatments.

In the present day, biofungicides based on natural product are effective against plant diseases. There is increasing interest in essential oil as biofungicides because of their environmental safety. Further research should involve the formulation and the method for application.


**Conclusions**

It was found that at the 1000 ppm concentration, the highest germination percentage (84.5%) was obtained from seeds soaked in a mixture of lemon grass and holy basil oil at the ratio 1:1 whereas seeds soaked in a mixture of holy basil and clove oil (2:1) at 2000 ppm revealed the highest germination percentage (90%). The three combinations of three oils exhibited the lower percentage of seed germination than the single pure oil and some of two oil combinations (tr6-8). The result indicated highly significant difference between seed soaking with essential oil and untreated seed in terms of seed germination and survival seedling under both experiments. Thus, it can be recommend that the application by seed soaked in a mixture of lemon grass and holy basil oil at the ratio 1:1 at the 1000 ppm
concentration and seeds soaked in a mixture of holy basil and clove oil (2:1) at 2000 ppm tended to reduce the damping-off in tomato. This study provides evidence that seed soaking with essential oils from lemon grass, holy basil and clove have the potential as biofungicide for controlling Sclerotium rolfsii damping-off disease.

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Effect of Ozone and Vapor Phase Hydrogen Peroxide Fumigation on the Control of Postharvest Diseases of Longan Fruit (\textit{Dimocarpus longan} Lour.)

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Abstract

This study evaluated the effect of vapour phase hydrogen peroxide (VPHP) at different fumigation times and temperatures. The combination of VPHP and 200 ppm ozone for 60 minutes to control the decay of fresh longan fruit during storage was also investigated. The results showed that fumigation with VPHP for 60 minutes at 60ºC was the most effective condition to reduce disease incidence in longan fruit during storage at 5ºC for 21 days. Exposure of longan fruit to VPHP combined with ozone or VPHP alone significantly reduced microbial population compared to control. However, the combination of ozone and VPHP treatment had better results in controlling browning and reduction of polyphenol oxidase (PPO) activity.

Keywords: ozone, longan fruit, postharvest diseases, vapor phase hydrogen peroxide

Introduction

Longan (\textit{Dimocarpus longan} Lour.) is one of the most economically important fruits in the north of Thailand. In 2010, fresh longan fruit of 219,395 tonnes were exported to China, HongKong and Indonesia (Custom Facilitation, 2011). The quantity of domestic and exported longan has been limited by its highly perishable nature, short storage life and susceptibility to postharvest diseases, as a result of bacterial, yeast and fungal infections. Rapid pericarp browning during storage is the main problem resulting in restricting the export of longan to long-distance markets (Sardsud \textit{et al.}, 1994). For many years, the recommended method to control postharvest decay and prevent pericarp browning in longan has been the sulfur dioxide (SO\textsubscript{2}) treatment. Recently, importing countries such as China and Singapore have restricted the import of longan products and other fruits and the maximum permitted residual level of SO\textsubscript{2} has been reduced. Longan consumers are becoming cautious regarding SO\textsubscript{2} residues, due to its allergenic property. There is a need to develop effective methods to replace SO\textsubscript{2} treatment, with something less harmful to humans and the environment. An alternative method is the use of ozone. Ozone is triatomic oxygen (O\textsubscript{3}) and is widely used as an antimicrobial agent to inactivate bacteria, fungi, viruses and protozoa for disinfecting water, in the food industry, and for reuse of waste water, as well as for control of alkalinity and pH of shrimp pond water (Kim \textit{et al.}, 1999).

Recent work indicates that ozone is an effective treatment for increasing shelf-life and decreasing fungal deterioration in the postharvest treatment of fresh fruit such as table grapes (Sarig \textit{et al.}, 1996) stone fruit (Palou \textit{et al.}, 2002) and longan (Whangchai \textit{et al.}, 2006) because of the lack of residues on the product. The effects of ozone on the reduction of growth of microorganisms have been also reported with regards to
sporulation and mycotoxin production by *Aspergillus flavus* and *Fusarium moniliforme* (Mason *et al*., 1997). In addition, populations of *Escherichia coli* are reduced by ozone treatment (Zhao *et al*., 1993).

Hydrogen peroxide (H$_2$O$_2$) is a highly reactive oxidizing agent that is widely used as a disinfectant (Chapman, 1998). The vapor phase of H$_2$O$_2$ has been used to sterilize packaging and medical supplies and equipment (Block, 1991). H$_2$O$_2$ is considered as a safe chemical and the USFDA has approved its use for the treatment of milk, cheese and carcasses of red meat. Forney and Rij (1991) reported that vapor phase H$_2$O$_2$ had a positive effect at preventing the postharvest spoilage of grapes. Peng *et al*., (2008) found that H$_2$O$_2$ can be used as a potential anti-browning treatment for fresh-cut Chinese water chestnut.

Pericarp browning has been attributed to oxidation of phenolics by polyphenol oxidase (PPO), producing brown-coloured by-products (Ferrar and Walker, 1996). PPO has been widely studied in various fruits such as apple (Harel *et al*., 1964), grape (Harel and Mayer, 1971), litchi (Tan and Li, 1984) and plum (Lin *et al*., 1994), but little is known about longan fruit. In this study, the effects of ozone in combination vapor phase H$_2$O$_2$ with on the postharvest decay of longan fruit were investigated.

**Materials and Methods**

**Effect of Temperature and Fumigation Time of Vapor Phase Hydrogen Peroxide on Postharvest Decay of Longan**

Longan fruits were selected for uniformity and size, and any bruised or diseased fruits were discarded from the harvested longan. H$_2$O$_2$ was vaporized at 40 and 60°C for 20, 40 and 60 min. (Figure 1). The treated fruit were put into plastic boxes and stored at 5°C for 21 days. At weekly intervals, skin samples were taken by swabbing the skin surface of each fruit with two sterile swabs, placing the swabs in 10 ml of sterile distilled water (SDW) and vortexing for 1 minute. Samples (1 ml) of the suspension were spread over Potato Dextrose Agar (PDA) medium. The PDA plates were incubated at 28°C for 3-5 days and the survival of microorganisms was expressed as the mean number of colony forming units (cfu/ml) while the percentage of disease incidence was estimated by a lesion of area of fungal infection on the fruit surface.

![Figure 1](image-url) Experimental set up for vapour hydrogen peroxide application.

**Effect of Ozone and Vapor Phase Hydrogen Peroxide on Fruit Quality of Longan**

The fruits were fumigated with VPHP at 60°C for 60 min and then exposed to ozone gas at a concentration of 200 ppm for 60 min, compared to ozone or VPHP treatment alone, and then packed into plastic boxes and over-wrapped with plastic film at 5°C for 3 weeks. The number of colony forming unit and the percentage of disease incidence were determined as described above. Fruit eating quality was assessed by a trained panel of six researchers. At each interval, 30 fruits per replication were randomly selected and rated for quality on the scale of 1 = poor, 5 = acceptable and 9 = excellent.

Pericarp browning was estimated by measuring the extent of total browned area on each fruit surface on the following scale: 1 = no browning, 2 = slight browning (<20%), 3 = moderate browning (20-40%), 4 = moderated-serious browning (40-60%) and 5 = serious browning (>60%). A browning index was calculated, using the
following formula: \( \Sigma \) (browning scale / proportion of corresponding fruits within each class).

Polyphenol oxidase (PPO) was extracted by the method of Huang et al. (1990). Longan pericarp (10 g) was homogenized in 40 ml of 0.05 M potassium phosphate buffer (pH 6.2) containing 1 M KCl and 2% polyvinylpyrrolidone K15 and then centrifuged for 5 min. at 13,500 rpm (Hermel model Z383K) at 4°C. The supernatant was collected as the enzyme extract. PPO activity was assayed by a modification based on the method of Jiang and Fu (1998) using the reaction mixture of 0.05 M potassium phosphate buffer (pH 7.5) containing 0.2 M catechol (0.2 ml) and crude enzyme (0.5 ml). Tubes were incubated for 5 min. at 30°C, the absorbance was measured at 420 nm by visible spectrophotometer (model Thermo Spectronic). The unit of enzyme activity was defined as the amount of enzyme that caused a change of 0.01 in absorbance per minute.

Results and Discussion

Effect of Temperature and Fumigation Time of Vapor Phase Hydrogen Peroxide on Postharvest Decay of Longan

Postharvest decay of longan fruits was affected by VPHP at higher temperature (60°C) and directly related to the contact time during the 3-week storage. A longer exposure time was more effective in reducing the population of microorganism (Figure 2a) and in controlling percentage of disease incidence (Figure 2b), when treated at 60°C. Forney and Rij (1991) reported the same positive results of decreased microorganism population after \( \text{H}_2\text{O}_2 \) application in grapes. \( \text{H}_2\text{O}_2 \) is a highly reactive molecule that performs multiple functions to against pathogens.

Effect of Ozone and Vapor Phase Hydrogen Peroxide on Fruit Quality of Longan

The best conditions using VPHP to control the decay of longan from the first experiment was considered in this study. Based on the prior preliminary screening test, 60 minutes was the optimal duration of ozone exposure to control postharvest diseases of longan fruit in vitro of Lasiodiplodia sp. and Cladosporium sp. (Whangchai et al., 2005)

Microorganism population on the fruit surface inducing fungi, yeasts and bacteria were reduced to 0.27, 0.16 and 0.03 cfu/ml when exposed to VPHP, ozone, and the combination, respectively as compared with control (3 cfu/ml) during the 3-week storage. This suggests that VPHP or ozone alone was not effective in controlling microorganism growth on longan peel. It is possible that pathogens are deeply embedded in rough surface of (Figure 3) longan pericarp, and fungal hyphae penetrate the cuticle through micro cracks and lenticels.

Therefore ozone and VPHP alone dose not completely prevent conidia germination under the front skin when the infection occurred. Victorin (1992) found that ozone destroy microorganisms by oxidation of the cellular components such as sulfohydryl groups of amino acids in enzymes and oxidation of the cell membrane.

The eating quality of all treated fruits decreased gradually with storage. A comparison among all treatments showed that the treatments did not adversely affect eating quality (Figure 4a).

In this study, treatment of ozone in combination with VPHP markedly reduced the browning of longan pericarp (Figure 4b) and also inhibited the increase in PPO activity. At week 3, the PPO activity of longan fruit treated with VPHP, ozone and the combination were 1.85, 1.75, 1.68 unit/mg protein when compared with control (2.6 unit/mg protein) (Figure 5). Low PPO activity correlated with discoloration as indicated by the low browning index.

Similar results have been reported in inhibition of PPO activity for fresh-cut Chinese water chestnut (Peng et al., 2008) and fresh-cut apple, pears and jicama (Buta and Abbott, 2000; Aquino-Bolaños and
Mercado-Silva, 2004), which suggested that dysfunction of phenolic-related enzyme by the oxidation could directly inhibit browning.

Figure 2 Effect of VPHP at various times and temperatures on populations of microorganisms on fruit surface.

Figure 3 Effect of ozone and VPHP on microorganism population during storage at 5°C for 3 weeks.

Figure 4 Effect of ozone and VPHP on eating quality (a) and Browning index (b) during storage at 5°C for 3 weeks.

Figure 5 Changes in activity of polyphenol oxidase (PPO) in longan pericarp of ozone and VPHP treated fruits stored at 5°C for 3 weeks.

Conclusions
The application of VPHP alone or combined with ozone had synergistic effect to decrease decay, suppress PPO enzymatic activity and delay surface discoloration, thereby, maintaining eating quality. The
treatments are potentially useful to replace SO₂ fumigation for extending the shelf-life of fresh longan fruit. Future investigation to increase the efficiency of controlling pericarp browning and decay for commercial application should be conducted.

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Development of Shellac From Source Available in Thailand as an Alternative Polymer for Postharvest Treatment

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Abstract

Shellac, which is abundant in Thailand, had been thought as an obsolete polymer for a few decades because of its poor solubility and instability. The purpose of study was to solve the problems by structure modification at carboxyl groups and hydroxyl groups of shellac molecules. The modification of carboxyl groups was conducted by salt formation. Shellac salts were prepared by dissolving shellac with ammonia or meglumine. The solid-state reaction was applied for esterification of shellac molecules at hydroxyl groups. Shellac was co-ground with cyclic anhydrides and then annealed at various temperatures to obtain shellac esters. The results demonstrated that percent insoluble solid of shellac salts (less than 12% w/w) was significantly decreased as compare to native shellac (40% w/w), suggesting the stability enhancement after salt formation. The conversion of carboxylic acid to carboxylate, after salt formation, could protect the polymerization which was the major cause of instability. However, the solubility was still a problem. The problem was further clarified by esterification shellac with succinic and phthalic anhydrides. The succinate and phthalate esters of shellac demonstrated better aqueous solubility as compared to native shellac which made them more ready for using by dissolving in water. In addition, the stability enhancement was also observed for the shellac esters because of protection of hydroxyl groups. In conclusion, the findings could give the solution for problems of shellac and made it more potential as a coating polymer for postharvest treatment of agricultural products in the future.

Keywords: esterification, polymer, post harvest, salt formation, shellac

Introduction

During the past 50 years, edible coating technology has been applied on the fruit surface for controlling decay and prolonging shelf-life during postharvest storage and handing (Embascado, 2009). One of the oldest coating polymers is shellac. Shellac is a mixture of polyester and single ester obtained from secretion of lac insect (Laccifera lacca), which live on trees called lac host tree in India, Thailand, and to a minor extent other Southeast Asian countries. Shellac has been wildly used in food and agriculture industry, to some extent still in a variety of fields because of its FDA approval (Cole et al., 2002). Shellac is used to coat apple (Bai et al., 2003), grapefruit (McGuire and Hagenmaier, 1996), citrus fruit (McGuire and Hagenmaier, 1996), mango (Hoa et al., 2002) to maintain their quality. Furthermore, the numerous
advantages of shellac based coating on agricultural products are glossy appearance, extended shelf-life, reduced moisture loss and delayed onset of ripening.

However, the utilization of shellac as a coating polymer has fallen into disfavor for several reasons. One of the problems is that shellac undergoes an “ageing effect” upon storage. The reduced solubility of shellac on storage is attributed to polymerization resulting from trans-esterification of the hydroxyl group of one shelloic or aleuritic acid molecule with the carboxyl group of another of the hydroxyl containing carboxylic acids (Wruble, 1930). Another problem of shellac is that it has poor solubility. Shellac comprises mainly of hydroxyl and carboxyl groups (Fig. 1). The low amount of carboxylic acid group per shellac molecule and the high $pK_a$ lead to the low solubility. Several works attempted to solve these problems; the hydrolysis treatment was used for improving the solubility of shellac. The solubility of shellac increased with increasing hydrolysis time. The partial hydrolyzed shellac showed better solubility property (Limmatvapirat et al, 2005), however the stability problem was not solved. The addition of organic acid or hydrophilic polymer was a simplified approach to improve the solubility of shellac. Organic acids and hydrophilic polymer performed as plasticizer, reducing glass transition temperature, that deceased disintegration time. On the other hand, the polymerization effect could occur during storage (Pearnchob et al, 2004). Furthermore, the improved enteric properties of shellac by 2-amino-2-methyl-1-propanol (AMP) and ammonium salt were investigated. The ammonium salt demonstrated increased solubility and stability; however the stability problem was decreased by losing ammonium ion from carboxylic group during storage time. The AMP salt presented better solubility and stability than ammonium salts, whereas the AMP salt film was showed decreasing moisture protection and film strength (Limmatvapirat et al, 2007). So another base (salt forming agent), which strongly bound to carboxylic acid, should be studied. In addition, the increasing carboxylic groups affect the more polarity and can be improve solubility of shellac. As shellac consists of a large amount of hydroxy group (Fig. 1), esterification with cyclic anhydride, may be an alternative way for increasing a number of carboxylic acid.

The objectives of this study were to improve the stability of shellac by salt formation and to enhance the solubility by esterification with cyclic anhydride. Ammonium hydroxide (AMN) and D(-)-N-methylglucamine (MGM) were chosen as the salt forming agent. Succinic anhydride (SUC) and phthalic anhydride (PHT) were selected for reaction with shellac. The shellac salt form and its ester form were prepared and comparatively evaluation with native shellac.

![Figure 1 Chemical structure of shellac, polyester (a), single ester (b).](image)

**Materials and Methods**

**Materials**

Shellac (SHL) was obtained form Thananchai Part., Ltd. (Bangkok, Thailand). Ammonia solution (AMN) and D(-)-N-methylglucamine (MGM) were purchased from Merck (Darmstadt, Germany). Succinic anhydride (SUC), phthalic anhydride (PHT) were obtained from Merck (Hohenbrunn, Germany). All the reagents were of analytical grade.
Preparation of Native Shellac Films
Shellac was dissolved in 95% ethyl alcohol overnight and then the final concentration was adjusted to 6% w/w. The solution was poured onto a coated glass plate and was allowed to dry at 50 °C for 2–3 h. The film was peeled off and stabilized at 25 °C, 75% RH prior to testing.

Preparation of Shellac Films in Various Salts Forms
Films were prepared to examine the effect of two salt forming agents. A 6% w/w aqueous solution of shellac was prepared by dissolving shellac with AMN or MGM. The amounts of added bases were calculated on the basis of acid value of shellac. The mixture was stirred until the shellac sample was completely dissolved and kept stirring overnight. The solution was poured onto a glass plate, and allowed to evaporate at 50 °C for 4–5 h. The film was peeled off and kept at room temperature, 75% RH prior to testing.

Characterization of Shellac and Shellac Salts
Fourier Transformed Infrared (FTIR) Spectroscopy
FTIR spectra of shellac samples were recorded with a FTIR spectrophotometer (Nicolet 4700, Thermo Electron Corporation, USA) using the KBr disc method. Each sample was dried over silica gel and pulverized. The ground sample was blended with KBr powder and then compressed with pressure of 5 tons. The KBr disc was scanned from 4000 to 400 cm⁻¹ at a resolution of 4 cm⁻¹.

Acid Value and Insoluble Solid
Acid value (AV) was measured by the acid–base titration according to the method described in the USP XXXIII (USP 33, 2010). The shellac sample was dissolved in 95% w/w ethanol, centrifuged and filtered through filter paper. The filtrate was titrated with 0.1 N sodium hydroxide VS. The equivalent point was determined by potentiometric titration. The insoluble solid on filter paper was washed with excess 95 %w/w ethanol and dried at 70 °C until the weight was constant, and then the percentage of insoluble solid was calculated.

Thermal Degradation Test
Film of shellac and shellac salts was heated at 80 °C 24 h. The heated samples were then dissolved in 95% ethanol overnight. The acid value and insoluble solid of all samples were determined by the previously described method (Limmatvapirat et al., 2007).

Preparation of Shellac Esters and Their Films
The SHL and cyclic anhydrides (succinic anhydride or phthalic anhydride) were transferred into a 500 ml stainless steel grinding bowl together with 5 stainless steel grinding balls having a radius of 30 mm and then ground by planetary ball mill (PM 100, Retsch, Germany) for 90 min. The ground mixture was then annealed at 60 °C for 24 h (succinate formation) and 80 °C for 12 h (phthalate formation). After annealing, the mixture was withdrawn and immediately washed with purified water to remove excess cyclic anhydride. Then the cast films of shellac esters were prepared by the same method as described above.

Solubility of Shellac Esters
The solubility was determined by measuring the weight loss of shellac and shellac ester film. The film was cut in 1 cm × 1 cm and placed in the USP disintegration apparatus. The obtained film was tested in distilled water for 3 h. The resulted film was dried at 70 °C for 24 h, and weighed to determine percent dissolved.

Statistical Analysis
The statistical data were expressed as mean ± standard deviation (SD). The statistical analysis was carried out using
analysis of variance (ANOVA) at the significant level of 0.01.

Results and Discussion

Acid Value of Shellac and Shellac Salt Films

The effect of bases (AMN and MGM) on acid value, insoluble solid, FTIR spectra and other physicochemical properties was investigated.

The amount of carboxylic acid in shellac was indicated by acid value. Figure 2 indicated the acid value of shellac (SHL) and shellac salts (SHL-AMN or SHL-MGM). Acid value of SHL, SHL-AMN, and SHL-MGM were 76 ± 1, 85 ± 18, and 70 ± 0, respectively. The acid values of all salt form were not significantly different as compared to SHL (p>0.01). Results suggested the quantity of carboxylic acid was unchanged during the salt formations with both the salt forming agents. The unchanged acid value after salt formation was in agreement with previous report (Luangtana-Anan et al., 2007).

FTIR Spectroscopy

FTIR spectroscopy was utilized for indicating the change of acid to salt form. Figure 3 demonstrated the FTIR spectra of SHL, SHL-AMN, and SHL-MGM. SHL showed carbonyl stretching (C=O) vibration band at 1716 cm⁻¹. The SHL-AMN and SHL-MGM salts showed the decrement of relative absorbance of carbonyl stretching band around 1716 cm⁻¹ while the clearly new peaks at 1560 and 1385 cm⁻¹, assigned to the asymmetric and symmetric carbonyl stretching of carboxylate salt, were observed. The results indicated the conversion of carboxylic acid to carboxylate by salt formation. Furthermore, SHL-MGM demonstrated the higher intensity of carbonyl stretching band of carboxylate, as compared to SHL-AMN. The relative absorbance ratios of the FTIR peaks, that demonstrated carbonyl stretching band of carboxylate and carboxylic acid (ABS₁₅₅₆/ABS₁₇₁₆), are shown in Figure 4. The ABS₁₅₅₆/ABS₁₇₁₆ ratio of SHL-MGM was higher than SHL-AMN. The results suggested that the carboxylate conversion of SHL-MGM salt was more pronounced, as compared to SHL-AMN salt.

Percent Insoluble Solid

Shellac consists of a number of hydroxyl and carboxylic acid groups as shown in Figure 1. Instability can occur by the esterification among the functional group (polymerization) (Wruble, 1930). Insoluble solid is a parameter for monitoring the ageing of shellac.
The percent insoluble solid of shellac samples before and after heating at 80 °C for 24 h is illustrated in Figure 5. Before heating, the insoluble solid of all samples was less than 2.5%. The significant increment of percent insoluble solid of SHL (from 2.5% to 40%) was observed after heating (p<0.01). However, percent insoluble solid of SHL-AMN and SHL-MGM was less increased after heating (from 2.3% to 12% and 1.0% to 1.6%, respectively). The results showed that the polymerization of shellac could be protected by the salt formation. SHL-MGM salt could provide better ageing protection, as compared with shellac in acid and ammonium salt forms.

As indicated by FTIR spectroscopy the more carboxylate conversion of SHL-MGM was observed. MGM should be more strongly interact with carboxylate, resulting in more stability than AMN. The results were in good agreement with the previous report (Limmatvapirat et al., 2007).

Solubility of Shellac and Shellac Ester Film

Shellac is recognized as a coating material that possesses good resistance to water and desirable gloss. However, one of the limitations is poor solubility of shellac. So, our study was designed to investigate the solubility improvement of shellac by esterification with cyclic anhydrides.

Figure 6 shows percent dissolved of SHL, SHL-SUC and SHL-PHT in distilled water. The percent dissolved of SHL, SHL-SUC, and SHL-PHT was 11.6%, 89.2%, 100%, respectively. The results suggested the aqueous solubility of SHL-PHT was better as compared to SHL-SUC and SHL. The result suggested that shellac esters were more ready for coating by dissolving in water.

Conclusions

The resolved stability and solubility problems were succeeded by salt formation and esterification, respectively. The salts forms, especially MGM salt demonstrated the improved stability. The solubility of shellac was successfully improved by added succinate or phthalate moieties. The increased amount of
carboxylic acid to shellac molecule significantly affected the high solubility in water. The study may give the easier approach for improving the properties of shellac that should be an alternative polymer for postharvest treatment in the near future.

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Nanoemulsions Containing Volatile Oils as Novel Antimicrobial for Oral Health Care Products

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Abstract

Dental caries is one of the most common diseases in children. *Streptococcus mutans* has been identified as a plaque forming bacterium and able to produce dental caries. This work demonstrated the guideline for development of oral health care products for eradication of *S. mutans* through nanoemulsification with volatile oils. Nanoemulsions were prepared by a simple emulsification method. Formulation factors, including type, amount of surfactants and oils were evaluated by monitoring of particle size, particle charge of oil droplets as well as other physical stability. The emulsions containing Cremophor® RH40, as a surfactant, showed relatively smaller oil droplets comparing to those containing Tween-60 or sodium lauryl sulfate. The result suggested that physical stability of nanoemulsions could be formed by specific selection of type and amount of surfactants. Nanoemulsions containing spearmint, peppermint, and tea tree oils, individually, demonstrated good physical stability as compared to that containing clove oil. The optimized formula was tested on antimicrobial properties against *S. mutans*. The nanoemulsions containing spearmint, peppermint and tea tree oils indicated 100% killing at the level of 10-fold dilution while that containing eucalyptus oil showed 95% killing. The knowledge gain from the study should provide the guideline for formulation of stable and effective oral health care products.

Keywords: oral health care products, volatile oils, nanoemulsion, antimicrobial

Introduction

Dental caries is one of the most worldwide public health problems especially in children and the elderly. The main etiology of the problem is related with the infection by *Streptococcus mutans* (Loesche, 1986). Generally, the bacterial infection can be prevented by utilization of suitable antiseptics, e.g. phenolic, chlorinated compounds, alcohols, and chlohexidine (Hibbard, 2005; Khan and Naqvi, 2006; Vernon et al., 2006). However, most substances cause irritation and allergic reactions. Thus the finding of more safe and efficient antiseptics is still the matter of interest for eradicating of *S. mutans*. Some herbal extracts, especially volatile oils from plants, demonstrated interesting activities, including anti-allergic, enzyme inhibitory, anti-inflammatory, anti-mutagenic, anti-carcinogenic, antiviral, and antimicrobial properties (Nakatsu et al., 2000 and Bakkali et al., 2008). Recently, volatile oils is widely use in oral therapeutics and oral cosmetics e.g. mouth wash,
toothpaste. Mouthrinse (Listerine®) containing essential oil combined with antiseptic could reduce the *Streptococcus mutans* counts in plaques and saliva (Fine et al., 2000). However, volatile oils have a limited aqueous solubility. They have also been difficult to prepare aqueous solutions for oral administration. Therefore, the development of alternative dosage forms should be further investigated.

Nanoemulsion is the dosage form that consists of 2 phases, *i.e.* water and oil phases. The popular system is oil in water emulsion which is safe, compatible with water, easy removing and can be used for drug delivery (Gutiérrez et al., 2008; Hamouda et al., 2001; Myc A et al., 2002).

As mentioned above, the volatile oils have the limited application due to their water immiscibility therefore formulation into emulsion should be a possible troubleshooting. However, the factors affecting emulsions properties, *e.g.* stability, antibacterial properties were not clearly indicated. The purpose of study was to evaluate the formulation factors affecting the physical stability of the emulsion containing volatile oils. Formulation factors, including types and amount of oil, surfactants were studied.

**Materials and Methods**

**Materials**

Soybean oil was purchased from Thai Vegetable Oil Public Company Limited, Thailand. Phytosterols (Batch No. U18B040005) was obtained from Cognis Thai Ltd., Thailand). Glycerol monooleate (Lot No. 1-6297) was purchased from Imperial Industrial Chemicals Co., Ltd., Thailand. Cremophor® RH40 (Lot No. 98-2394) was obtained from BASF, Germany. Tween®60 (Lot No.59630) was obtained from P.C. Drug Center Co., Ltd. Sodium lauryl sulfate (Lot No. 000148) was obtained from Vidhyasom Co., Ltd., Thailand. Clove oil (Batch No. 6168) was purchased from H.E. Daniel Ltd., England. Spearmint oil (Batch No. R00249), peppermint oil (Batch No. R00068), eucalyptus oil (Batch No. R00245) and tea tree oil (Batch No. R00364) were obtained from Greater Pharma Co., Ltd., Thailand. Brain Heart Infusion broth (BHI broth) (Batch No. VM008293) and Brain Heart Infusion agar (BHI agar) (Batch No. VM006625) were purchased from Merck, Germany. The culture media were mixed with water according to the prescription of the supplier and were sterilized at 121°C for 15 min before use. *Streptococcus mutans* (104B) used for the microbiological assay were obtained from Department of Microbiology, Faculty of Science, Silpakom University.

**Oil-in-Water Emulsions Preparation**

Oil-in-water (O/W) emulsions are the system consisting of 2 phases, *i.e.* oil and aqueous phases. Oil phase was prepared by mixing of phytosterol, glycerol monooleate with soybean oil and then heated upto 60 °C with magnetic stirrer equipped with hot plate. For some formulations, volatiles oils (clove oil spearmint oil, peppermint oil, eucalyptus oil or tea tree oil) were added after complete dissolution of oil phase. Aqueous phase was prepared by dissolving of surfactants (Tween®60, Cremophor® RH40 or sodium lauryl sulfate) in distilled water at 65°C. The oil phase was added into water phase and kept stirring until the emulsion formed. The resulted emulsion was then homogenized with a homogenizer (X1020, Ystral GmbH, Germany) for 5 min at 2350 rpm and stored at room temperature prior evaluation.

**Evaluation of Emulsion Stability**

Emulsion samples (50 ml) containing various type of surfactants (Tween®60, Cremophor® RH40 or sodium lauryl sulfate) and various type of volatile oils (clove oil, spearmint oil, peppermint oil, eucalyptus oil or tea tree oil) were transferred into glass bottle with plastic cap (n=3). The bottles were then placed in a 25°C for 7 days or accelerated condition (4°C and 45°C for 6
cycles) and then evaluated for physical stability (percent creaming and cracking). The percent creaming was the ratio between the height of cream layer and the total height of emulsion according to Eq. 1.

\[
\text{%Creaming} = \frac{\text{Total height of cream layer}}{\text{Total height of emulsion}} \times 100
\]  

Evaluation of Droplet Size of Oil

A 300 µl of emulsions were diluted in 2700 µl distilled water. An aliquot of sample (100 µl) were then measured for droplet size of oil by particle sizer (Horiba, LA-950, Japan).

Microscopic Observation

The morphology of emulsions was observed using a light microscope (CX41RF, OLYMPUS, Japan). Prior to the microscopic observation, emulsion samples were shaken gently and diluted with distilled water in 1:1 ratio. Subsequently, a drop of the emulsion was placed on a microscope slide and covered with a cover slip. Photomicrography images of the emulsion were captured using digital image processing software (Dinolite, AM423X, Taiwan).

Antimicrobial Properties of Nanoemulsions

The optimized formula was tested on antimicrobial properties against *S. mutans*. Stock culture concentration 10^5 CFU/ml was prepared by broth dilution method (Wright, 1996). Mixed emulsions of various concentration (nondiluted and diluted 10x) and stock culture in 9:1 ratio were incubated in shaking incubator (SI4-2, SL SHELF LAB, USA) at 37°C, 200 rpm for 10 min. These suspensions were diluted with BHI broth (50x). These diluted suspension 100 µl were spread on BHI agar and incubated in bacterial incubator (Contherm Digital Series, New Zealand) at 37°C for 18-24 hr. Data were collected by colony count and calculated by follow equation.

\[
\%\text{inhibition} = \frac{\text{Total colony} - \text{Survival colony}}{\text{Total colony}} \times 100
\]  

Results and Discussion

Effect of Surfactant on Physical Stability of Emulsions

The effect of types of surfactants, e.g. Tween® 60, Cremophor® RH40 and sodium lauryl sulfate on physical stability of nanoemulsion were investigated. The physical stability of emulsions was characterized by particle size measurement and visual observation of creaming and cracking. Generally, the stable emulsion did not show phase separation as observed by the distinct layer of oil phase and water phase (cracking). In addition, the homogeneous emulsion should be observed (100% creaming). In the case of instability, the emulsion would separate into cream layer. The more height of cream layer indicated the more stability of emulsion. For the present study, all emulsions showed no evidence of cracking whereas different percent creaming among formula was observed (Figure 1). Emulsion containing Tween® 60 and sodium lauryl sulfate demonstrated low stability as indicated by low percentage of creaming while the emulsion containing Cremophor® RH40 showed 100% creaming even storage up to 7 days and accelerated condition. The result suggested that the Cremophor® RH40 should be the most suitable surfactant for the present study.

Effect of Surfactant on Droplet Size of Oil

Since droplet size of emulsion could affect the antimicrobial properties, the effect of types of surfactant on droplet size was also investigated. As illustrated in Figure 2, the droplet size was in nanometer range for the emulsion containing Cremophor® RH40. In addition, the droplet size did not change even storage up to 7 days and at the accelerated condition. However, the emulsion containing Tween® 60 and sodium lauryl sulfate demonstrated droplet size about 2.58 and 1.24 micron respectively. The results indicated that droplet size of oil should relate with the physical stability of emulsion. Droplet size of oil in nanometer range could provide better physical stability.
Effect of Types of Oil on Physical Stability of Emulsions

This research was also investigate the effect of types of volatile oils such as clove oil, spearmint oil, peppermint oil, eucalyptus oil and tea tree oil on physical stability of nanoemulsions. The physical stability of emulsions was characterized by visual observation of creaming and cracking. The results showed that physical stability of emulsions containing clove oil was rapidly decreased while emulsions prepared from other volatile oils were stable after storage (Figure 3). Emulsion containing clove oil showed cracking whereas emulsions containing spearmint oil, peppermint oil, eucalyptus oil and tea tree oil showed good physical stability after kept in accelerated condition (Figure 4). As the density of clove oil was relatively higher as compared to that of other volatile oils, the poor physical stability should be related with the different density between oil and aqueous phases of emulsion systems.

Effect of Types of Oil on Droplet Size of Oil

This part was to investigate the effect of types of volatile oils such as clove oil, spearmint oil, peppermint oil, eucalyptus oil and tea tree oil on droplet size of oil. The results showed that emulsion containing clove oil demonstrated the largest particle size (Figure 5) whereas emulsion containing other volatile oils (spearmint oil, peppermint oil, eucalyptus oil and tea tree oil) demonstrated particle size in the range of 169-262 nanometers after storage for 7 days and at accelerated condition. In addition, photographs from light microscope were used to confirm the results. Photomicrographs revealed the large crystals that precipitated from the emulsion containing clove oil while the emulsion containing spearmint oil, peppermint oil, eucalyptus oil and tea tree oil showed small spherical droplet of oils (Figure 6). The result confirmed the poor physical stability of clove oil emulsion.
The optimized formula, i.e., the emulsion containing spearmint oil, peppermint oil, eucalyptus oil and tea tree oil was further investigated for antimicrobial properties against S. mutans. The results showed that emulsion containing spearmint oil, peppermint oil and tea tree oil indicated 100% killing at the level of 10-fold dilution while that containing eucalyptus oil showed 95% killing (Figure 7).

Conclusions

The emulsions containing Cremophor RH40, as a surfactant, showed relatively good stability and smaller oil droplets comparing to those containing Tween 60 or sodium lauryl sulfate. The droplet size of emulsion was decreased when Cremophor RH40 was increased. The result suggested that physical stability of nanoemulsions could be achieved by specific selection of type and amount of surfactants and volatile oils. Nanoemulsions containing spearmint, peppermint, and tea tree oils demonstrated good physical stability as compared to that containing clove oil. The nanoemulsions containing spearmint, peppermint and tea tree oils indicated 100% killing at the level of 10-fold dilution while that containing eucalyptus oil showed 95% killing. The possible mechanism might be involved with the disruption of bacterial cell wall. The knowledge gain from the study should provide the guideline for formulation of stable and effective oral health care products.

Acknowledgments

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Effect of Bio-extract as Microbial Inoculum on Composting of Cassava Leaves and Stems

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Abstract

Utilization of bio-extract (BioEx) as microbial inoculum on composting process are widespread in Thailand, however, the information about bio-extract as microbial inoculum is sparingly available in literature. In addition, wastes from cassava plantation, particularly, leaves and stems, are recalcitrant for biodegradation due to their high lignocelluloses content. This study interested in the use of microbial inoculum from cassava leaves and stems bio-extract on composting of cassava leaves and stems in the combination with cow manure and molasses. Inoculum concentrations employed were in the range of 0-15 % by weight (0 % (experiment 1), 5 % (experiment 2), 10 % (experiment 3) and 15 % (experiment 4)). The results showed that inoculums concentration had no effect on the increasing temperature as well as the pH change within the pile during composting. Inoculum addition affected total carbon loss (TC loss) and total nitrogen loss (TN loss) significantly during composting process of 203 days. Additionally, TC loss of approximately 64.8 % w/w in experiment 3 was significantly higher than those of the others (p<0.05). Moreover, the highest TN (3.41) and TN loss (23.62 %) were accomplished using 10 % BioEx inoculum; however, insignificantly different from that of experiment 4 (BioEx 15 %) (p>0.05).

Keywords: bio-extract, composting, cassava leaves and stems, inoculum

Introduction

In cassava plantation, wastes such as cassava stems, rhizomes and leaves were usually left in the field after harvest. In Thailand, such wastes amounted to approximately 4.6 million tons annually (Office of national research council of Thailand, 2002 – 2006). In 2008, Laptrakoon proposed to add value to these agricultural wastes by producing compost using cassava leaves and stems as principal raw materials. However, both cassava leaves and stems contain high lignocelluloses content, longer composting time is necessary. In general, composting could be enhanced by appropriately adjusting physical and biological factors involved such as aeration system design (Cayuela et al., 2006), C/N ratio (Noohom, 2001) and microbial inoculation (Barrena, 2006). Previous studies on application of microbial inoculation to improve degradation of raw material containing high lignocelluloses content revealed that degradation efficacy depended on several factors such as type, source and concentrations of inoculums used (Vargas-Gracia et al., 2006, Barrena 2006). Vargas-Gracia (2006) employed Bacillus shackletonni, Steptmyces thermovulgaris and Ureibacillus thermosphaericus isolated from pepper plant compost as inocula for composting of wastes from olive-oil mill, pruning waste, rice straw and almond shell. The authors found that U. thermosphaericus was the most effective in degrading
lignocellulose materials in the bio-oxidative phase during composting was accelerated. Besides the pure culture inocula, the commercially available microbial inocula have been employed in composting. Barrena (2006) investigated effectiveness of MicroGest 10X, the commercially available microbial inoculum, on composting of municipal wastes and reported that microbial concentration of the inoculum directly affected degradation activity and that inoculums containing $10^6$ CFU/g was optimal capable of reducing composting time by half of that generally required. It has also been reported that commercially available culture was ineffective since these microorganisms were more susceptible to harsh environmental conditions than innate microbial consortium. Therefore, attempts have been made to utilize microorganisms isolated from natural sources, such as spoiled leaves, rice straw left in paddy field and bamboo stem, as inoculum. (Tancho, 2006). Ruangcharus (2008) found that composting of palm fruit bunch supplemented with microbial inoculum isolated from palm fruit bunch compost led to the lower C/N ratio than those obtained with inoculum isolated from both bamboo leave litter and longkong leave litter indicating that inoculum isolated from the same sources as that of composting materials yielded better composting efficacy than that isolated from different sources.

Moreover, bio-extract has also been employed as microbial inoculum for compost production since bio-extract was considered a good source of microorganisms (Department of agriculture, 2001; Sassanakij et al., 2004). The microorganisms typically found in the bio-extract are Bacilli, Lactobacilli and Streptococci. Additionally, fungi such as Aspergillus niger, Penicillium, Rhizopus and yeast, i.e., Candida sp., could be commonly observed. These microorganisms may play an important role in composting. Further, bio-extract usually contains high nitrogen content, which could then be employed as the C/N ratio adjuster. Information regarding the role of bio-extract as an inoculum for composting is rarely available in literature. However, Kusuwanvichid (2006) reported that fish bio-extract positively enhanced microbial activities in a concentration dependent manner leading to high total carbon loss.

The objective of this study was to investigate effectiveness of bio-extract prepared from cassava leaves and stems as an inoculum for composting of cassava stems and leaves. Insight gained from this study may serve as basis for producing compost from other high lignocelluloses materials in order to reduce composting time rendering the process to be more cost effective.

**Materials and Methods**

**Inoculum Preparation (Bio-extract)**

Bio-extract was prepared by combining cassava leaves and stems, molasses and water, at the ratio of 1:1:1:1 by weight in 60L vessel and left at room temperature. The mixture was stirred every 7 days. At day 14 post fermentation, bio-extract could then be utilized as inoculum (Narphahsin, 2009).

**Compost Preparation**

Compost materials, leaves and stems of cassava, cow manure and molasses at ratios 0.8: 6.4: 4.7: 0.03 (w/w) (Laptrakoon, 2008), were mixed in 0.5 m$^3$ vessel, adjusted moisture content to about 50-60% (w/w) with water. The compost was turned once a week during bio-oxidative phase. The compost pile was inoculated with 0, 5, 10 and 15% by weight of bio-extract previously prepared and subsequently designated as control, BioEx 5%, BioEx 10% and BioEx 15%, respectively. Experiments were done in duplicate.

**Analytical**

During the first 2 months, temperature of the compost was measured every day. Small samples were taken once a week for physical and chemical analysis, such as pH, total nitrogen (AOAC, 1995), carbon (Navarro et
Total carbon (TC) and total nitrogen (TN) loss were quantified according to the method proposed by Peredes et al. (2002). Rate constant of reaction (k) was estimated using second-order kinetic as shown below;

$$\frac{1}{C} - \frac{1}{C_0} = kt$$

where $C$, $C_0$, $k$, and $t$ were total carbon concentration at any time (%TC), initial total carbon concentration (%TC), rate constant of reaction ( (%TC.day)$^{-1}$) and time (day), respectively.

Statistical analysis was accomplished using Minitab 14 (Minitab, Inc., USA)

**Results and Discussion**

**Temperature Profile**

Temperature is a parameter directly representing microbial activities taking place during composting (Epstein, 1997). Figure 1 shows that the temperature in all piles increased and reached the highest value of approximately 56.3 – 59.7°C at day 3 post fermentation. It should be noted that the temperature within all compost piles remained higher than 40°C, dubbed thermophilic phase (Bernal et al., 1998), for 8 – 10 days. This prolong thermophilic phase helped killing plant pathogens, parasites’ eggs and worms (Polprasert, 1996).

It was found that thermophilic phase of compost piles inoculated with bio-extract 10 and 15% were longer, approximately 9 and 10 days, respectively (Table 1), than those of control and BioEx 5% indicating that the inoculum supplementation positively affected interval of thermophilic phase. Results found were in good keeping with that of Bolta et al. (2003) who reported that when the compost of one week old was employed as an inoculum for preparation of compost of the same composting materials, the temperature within the pile increased faster and longer thermophilic phase in comparison with those without inoculum.

![Temperature profile during the composting of cassava leaves and stems at different inoculum concentrations: control (●), BioEx 5% (○), BioEx 10% (▼) and BioEx 15% (△) and ambient temperature (■).](image)

**Table 1 Temperature during composting of composting cassava leaves and stems**

| Exp | $T_{max}$ (°C) | Bio-oxidative phase (days) | MR phase
<table>
<thead>
<tr>
<th>(°C)</th>
<th>TP phase (T ≥ 40°C)</th>
<th>MP phase (40°C&gt;T&gt;RT)</th>
<th>(T=RT) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57.5 (0.5)</td>
<td>1-8</td>
<td>9-48</td>
</tr>
<tr>
<td>2</td>
<td>56.3 (0.6)</td>
<td>1-8</td>
<td>9-48</td>
</tr>
<tr>
<td>3</td>
<td>57.7 (0.6)</td>
<td>1-9</td>
<td>10-48</td>
</tr>
<tr>
<td>4</td>
<td>59.7 (0.6)</td>
<td>1-10</td>
<td>11-48</td>
</tr>
</tbody>
</table>

Note: Exp: Experiment, $T_{max}$: Maximum temperature at day 3, RT: Room temperature, TP phase: Thermophilic phase, MP phase: Mesophilic phase, MR phase: Maturation phase 1, 2, 3 and 4 in the first column represent Control, BioEx5%, BioEx10% and BioEx15%, respectively. * indicates significant difference from the control. (n = 3) The value in parenthesis is the standard deviation.

**pH**

Figure 2 provides time profile of pH during composting of cassava leaves and stems. It was found that only at day 1 post inoculation, the pH of compost prepared with 15% inoculum (BioEx 15%) was significantly different from others, which were prepared with lower inoculum concentration. From then onwards, the pHs of all piles were insignificantly different for the entire composting period of 203 days, approximately 7.38 – 7.65. The initial pH of compost pile containing 15% bio-extract was
rather acidic which may be due to the fact that larger quantity of bio-extract with the pH of 3.64 was employed. The pH of all composts tended to decline during thermophilic phase, in other words, the first 7 day of composting, due possibly to degradation of carbohydrate leading to organic acid production (Epstein, 1997). Subsequently, towards the end of composting, the pH within the compost piles turned to be alkali because of the mineralization of protein, amino acid, and peptide to ammonia (Parades et al., 2002). The final pH of all compost piles conformed well to that of standard specified by the Department of agriculture (2005), approximately 5.5 – 8.5.

Biodegradation

Total Carbon Loss (TC Loss)

Since carbon is a source of energy and microbial proliferation, therefore, total carbon (TC) reduction during composting may be employed as indicator of effectiveness of composting process. As can be observed from Figure 3, the TC of all composts prepared was reduced from 44.55 – 45.64% to 34.16 – 37.25% at day 203 of fermentation. Table 2 shows that inoculum supplementation during composting of cassava leaves and stems could enhance microbial activities to degrade the composting materials at the beginning of composting process during thermophilic phase in particular. Total carbon loss, 5.9 – 22.1%, of compost piles prepared with 5, 10 and 15% bio-extract addition was significantly higher than that of control (p<0.05). Moreover, the degradation rate constant (k) (Table 3) of compost pile supplemented with microbial inoculum (0.00003 %TC day⁻¹) was also significantly higher than that of control (0.00002 %TC*day⁻¹) (p<0.05). Results found indicated that the inoculum supplementation in form of BioEx could accelerate the TC degradation, especially at the beginning of composting process.

When considering the effect of inoculum concentration on TC loss (Table 2), it was found that at 10% supplementation the highest TC loss (65%) was attained while too high inoculum (15%) led to lower TC loss. Similar result was reported by Kusuwanvichid (2006) who found that the supplementation of fish bio-extract at concentration higher than 22% resulted in a decline in TC degradation. The adverse effect observed when bio-extract rich in trace elements was supplemented at high concentration may perturb trace element balance which subsequently inappropriate to microbial growth (Kusuwanvichid, 2006). Moreover, bio-extract may also contain some inhibitory substances capable of inhibiting the growth of microorganisms, thus, lowering degradation rate.

Total Nitrogen Loss (TN Loss)

For all composts prepared, the TN tended to increase from 1.98 – 2.25 % (w/w) initially to 3.37 – 3.59% (w/w) at day 203 post inoculation (Figure 4). When the TN loss was considered as indicator of composting process efficacy, TN loss of control during bio-oxidative and maturation phase were approximately 4.09 and 11.54%, respective, which were significantly lower than those obtained with 5-15% inoculum supplemented (p<0.05), approximately 15.21 – 19.85% and 15.91 – 23.96%, respectively.
Lower TN loss observed for control may be resulted from the fact that thermophilic phase was shorter than those prepared with inoculum supplementation (except BioEx 5%) leading to lower rate of ammonia evaporation which is usually high during thermophilic phase (T>45°C) (Beck-Friis et al., 2000; Peredes et al., 2000). Further, high temperature kept the nitrification process in check causing high ammonia evaporation. Nevertheless, it has been reported that the compost with high organic matter degradation also suffered high TN loss.

Even though the control suffered less TN loss for the entire composting process, however, at day 203 post inoculation, nitrogen content of compost piles supplemented with 10 and 15% inoculum was still significantly higher approximately 3.41-3.59% than that of control (p<0.05) which may be because high nitrogen present in bio-extract was proportionally added to the composting materials. Therefore, even with higher TN loss during composting, larger fraction added through bio-extract remained at the end of composting.

**Conclusions**

Supplementation of inoculum in the form of bio-extract prepared from cassava leaves and stems enhanced degradation activity during composting of cassava stems and leaves, particularly during thermophilic phase. Compost supplemented with inoculum resulted in higher TC and TN loss than that prepared without inoculum added.
Optimal bio-extract supplementation was approximately 10%, which led to the highest TC loss while TN content remained was also high even with higher loss during composting.

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Anti-aging Cosmetics from *Schizophyllum commune* Fries

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**Abstract**

*Schizophyllum commune* Fries. is an edible mushroom, common name, “Krang”. It is available in the southern part of Thailand, i.e. Phuket and Krabi. The fresh harvest can be prepared to many kinds of food. It is high in nutrients sufficient to human body and antioxidants which are claimed to be protective for oxidative damage in living cells that will cause aging. The study of anti-oxidant activity of *Schizophyllum commune* Fries. extract by DPPH radical scavenging activity showed that the extract had a potent anti-oxidative capacity at 1,408TE when compared with Trolox as a reference standard. The anti-aging product was formulated with the active extract of *Schizophyllum commune* Fries. Quality control of the raw material and product are performed by HPLC fingerprints. The stability test by Heating-Cooling method for 6 cycles revealed that the product was in the good condition of cream (viscosity, pH). Physical properties (odor, color) had been evaluated and shown to have the same appearance. The product exhibited no sign of skin irritation and acute dermal toxicity. All processes had been patented.

**Keywords:** *Schizophyllum commune* fries., DPPH, anti-oxidant, anti-aging cosmeceutical

**Introduction**

Currently, the Thai cosmetics market has increasing revenue each year. Skin care products (lotion and cream) have a market share of over 4,000 million Baht. They are mostly developed for skin firming, nourishing and anti-aging.

Mushrooms are a good source of nutrients compared to meats and some soy. About 650 known species possess both preventive and therapeutic benefits (Wasser *et al.*, 2000). Several mushrooms have been used as ingredients in cosmetics (Lemann, 2007).

*Schizophyllum commune* Fries., commonly known as split gill, is one of the common edible mushrooms of widespread distribution. It is popular and has been consumed widely in Southern Thailand. Recent studies reported that *Schizophyllum commune* Fries produce the polysaccharide schizophyllan which is useful as skin anti-aging, depigmenting and healing agent (Kim *et al.*, 1999, 2000).

In the present study, *Schizophyllum commune* Fries was selected as a potential source for further development of cosmetics product.

**Materials and Methods**

**Plant Materials**

Fresh Krang mushrooms were purchased from local mushroom farmers in Phuket. They were washed, cut and dried at 50 °C
for 24 h. Then they were ground to coarse powder for extraction purpose.

**Biological Materials**

New Zealand white rabbits were purchased from Department of Animal Science, Faculty of Agriculture, Kasetsart University. White Wistar rats, both male and female, were purchased from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhon Pathom province.

**Extraction of Plant Materials**

One kilogram of air-dried ground coarse powder of *Schizophyllum commune* Fries. was macerated with 2 L of 95% ethanol by stirring at room temperature for 30 min. The solid was separated from the mother liquor by filtration, followed by re-extraction for 5 times. The filtered solution was combined and then evaporated under vacuum at 40 °C to yield the crude extract for subsequent tests.

**Quality Control of Crude Extract**

The crude extract of 1 g was dissolved with 25 mL of methanol. The solution was sonicated for 5 min, filtered and then adjusted to a final volume of 100 mL with methanol. Five concentrations of the solution (200, 400, 600, 800 and 1000 ppm) were prepared for the standard calibration curve. For each concentration, the solution was filtered through a 0.45-µm filter before injecting into the HPLC system.

**Determination of Anti-oxidant Activity Using DPPH Radical Scavenging Activity Assay**

The anti-oxidant activity of samples was evaluated according to Brand-Williams *et al.* (1995) with some modifications. Trolox was used as a standard anti-oxidant. An appropriate amount of sample was mixed with 4.5 mL of methanol to obtain final concentrations at 0.2, 0.4, 0.8, 1.2 and 1.6 mg/mL. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) in methanol was used as control. The absorbance of each sample solution was measured at 517 nm. The anti-oxidant capacity was expressed in µmole equivalent in activity of Trolox.

**Preparation of Product**

The oil phase which includes emollient, emulsifier, moisturizer and thickener were mixed together and heated at 70 °C. A blend of humectants and thickener as the water phase was heated at 75 °C. The water phase was added to the oil phase, and then mixed uniformly using a homogenizer. *Schizophyllum commune* Fries. extract was dissolved in a solubilizer. After that, the extract in solubilizer, preservative were added and the mixture was homogenized.

**Quality Control of Product**

Product was weighed for 0.5 g and then dissolved with 4.5 mL of methanol. The solution was mixed by a rotating shaker for 4 h at room temperature, followed by sonication for 30 min and filtration through a 0.45-µm filter prior to HPLC analysis.

**Safety Evaluation of Product**

The product was evaluated for the safety by primary skin irritation test and acute dermal toxicity in accordance with the OECD 402 (1987) and OECD 404 (2002), respectively.

**Preservative Efficacy Test of Product (Challenge Test)**

The effectiveness of the preservative was evaluated by measuring microbial contamination to the product. Samples were inoculated with microorganisms to obtain $10^7$ cfu/mL of *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 10231, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538. The samples were tested for viable microorganisms at 28 days after inoculation using a standard plating method.

**Stability Test of Product**

The product was evaluated for the stability by 6 cycles of Heating-Cooling method (4 °C for 48 h and 45 °C for 48 h).
After completing each cycle, physical properties of the samples were determined by measuring color, odor, viscosity, pH and texture.

**Results**

**Quality Analysis Using HPLC Technique**

HPLC fingerprints of the crude extract and anti-aging cream from *Schizophyllum commune* Fries. containing marker at $R_t = 9.5$ min are shown in Figure 1. The linear calibration curve of the extract ($Y=0.5461X-3.6069$, $R^2=0.9997$) is given in Figure 2.

![Figure 1. HPLC fingerprints of (a) crude extract and (b) anti-aging cream from *Schizophyllum commune* Fries. showing marker at $R_t = 9.5$ min](image)

![Figure 2. Calibration curve of *Schizophyllum commune* Fries. extract](image)

**DPPH Radical Scavenging Assay**

The DPPH radical scavenging activity of *Schizophyllum commune* Fries. extract at various concentrations (0.2, 0.4, 0.8, 1.2 and 1.6 mg/ml) increased in a dose-dependent manner. The extract had the anti-oxidant capacity equivalent to Trolox at 1,408 µmole.

**Skin Irritation Test**

At 24 h, it was found that in three rabbits, erythema with score 1 was observed on the skin sites applied with the anti-aging cream from *Schizophyllum commune* Fries. given in Table 1. After 48 h, all erythemas reduced in severity and disappeared. There was non-occurrence of edema on the sites applied with the cream.

<table>
<thead>
<tr>
<th>Rabbit No.</th>
<th>Reaction</th>
<th>Score for skin reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Erythema</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Edema</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>Erythema</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Erythema</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Erythema</td>
<td>0</td>
</tr>
</tbody>
</table>

**Acute Dermal Toxicity**

Results showed that, in ten rats (5 males and 5 females), no reaction was observed on the skin sites applied with the anti-aging cream from *Schizophyllum commune* Fries. at the dose of 2,000 mg/kg body weight. Gross pathology examination conducted on the test rats at the end of a 14-day observation period indicated that there were no detectable abnormalities of internal organs in both male and female rats.

**Antimicrobial Preservative Efficacy Test**

The antimicrobial preservative efficacy of the anti-aging cream from *Schizophyllum commune* Fries. challenged with *A. niger*, *C. albicans*, *P. aeruginosa* and *S. aureus* is shown in Table 2. After 28 days, the growth of the test microorganisms was not observed.
Table 2. Antimicrobial preservative efficacy of the anti-aging cream from *Schizophyllum commune* Fries. challenged with *A. niger*, *C. albicans*, *P. aeruginosa* and *S. aureus*

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Sampling time/Viable counts (cfu/mL)</th>
<th>0</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em> ATCC 6404</td>
<td>2.0 × 10⁷</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td><em>C. albicans</em> ATCC 10231</td>
<td>2.0 × 10⁷</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td><em>P. aeruginosa</em> ATCC 9027</td>
<td>4.0 × 10⁷</td>
<td>&lt; 10</td>
<td></td>
</tr>
<tr>
<td><em>S. aureus</em> ATCC 6538</td>
<td>1.2 × 10⁷</td>
<td>&lt; 10</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Stability test of the anti-aging cream from *Schizophyllum commune* Fries. using six cycles of heating-cooling

<table>
<thead>
<tr>
<th>Property</th>
<th>Before 6 cycles</th>
<th>After 6 cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>White</td>
<td>White</td>
</tr>
<tr>
<td>Odor</td>
<td>Soft scent</td>
<td>Soft scent</td>
</tr>
<tr>
<td>Viscosity</td>
<td>2741 cP at 27 °C</td>
<td>2720 cP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2780 cP</td>
</tr>
<tr>
<td>pH</td>
<td>6.71</td>
<td>6.70</td>
</tr>
<tr>
<td>Texture</td>
<td>Smooth homogenous cream</td>
<td>Smooth homogenous cream</td>
</tr>
</tbody>
</table>

Stability Test of the Anti-aging Product
The quality evaluation of the anti-aging cream from *Schizophyllum commune* Fries. was accessed using six cycles of heating-cooling (4 °C for 48 h and 45 °C for 48 h) as shown in Table 3. No marked changes in color, odor, texture and viscosity were observed.

Discussion

Quality Analysis Using HPLC
The quality analysis was conducted by using HPLC technique. The active compound as marker at Rₜ = 9.5 min was proved in the anti-aging cream from *Schizophyllum commune* Fries. at concentration of 0.37% w/w.

Anti-oxidant Activity
The anti-oxidant activity of the *Schizophyllum commune* Fries. extract was conducted by using DPPH radical scavenging assay. The results have shown that the extract have the anti-oxidant capacity of 1,408 μmole equivalent in activity of Trolox.

Safety Evaluation of the Anti-aging Product
The safety evaluation of the anti-aging cream from *Schizophyllum commune* Fries. was examined by skin irritation test and acute dermal toxicity. Based on the results of this *in vivo* investigation, the cream produced no signs of irritation. In addition, visible signs of toxicity had not been observed. This finding indicates that the product can be considered as non-irritant and non-toxic.

Antimicrobial Preservative Efficacy Test
The anti-aging cream from *Schizophyllum commune* Fries. was investigated for its efficacy of antimicrobial preservation against *A. niger*, *C. albicans*, *P. aeruginosa* and *S. aureus*. It was shown that the product was generally effective to control the growth of the reference microorganisms over a 28-day period.

Stability Test of the anti-aging product
The stability test was conducted by six cycles of a heating-cooling method. It was shown that there was no change of the properties of the anti-aging cream from *Schizophyllum commune* Fries. The product had a smooth, white homogenous texture and soft scent with a medium viscosity and neutral pH (approximately 7).
Conclusions

The anti-aging cream from *Schizophyllum commune* Fries. has been scientifically proven to have anti-oxidant capacity and tested that is safe for usage.

Acknowledgements

The authors are thankful to Food Technology Department for providing necessary equipments. Acknowledgement is extended to Thailand Institute of Scientific and Technological Research for financial support.

References


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Preparation and Characterization of Shellac/PVP Iodine Blend as Antimicrobial Film Patch

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Abstract

Shellac is a purified resinous secretion of lac insects which are mostly cultivated in host trees in Thailand. As of nature origin and some attractive advantages such as low water permeability and good film forming property, shellac could be used for various pharmaceutical and agricultural applications. In the present study, shellac was applied as a matrix forming agent for antimicrobial film patch. It was blended with polyvinyl pyrrolidone iodine (PVPI) and casted into the films. The blend films were then characterized for physicochemical properties and antimicrobial property against \textit{Staphylococcus aureus}. The result demonstrated that all films were easily prepared. However, the mechanical properties, melting and solubility were depended on the composition of blended films. The percent elongation was increased as increasing the fraction of shellac in blend films, suggesting the more flexibility of shellac. The solubility of blend films was decreased after addition of PVPI. The result was agreed well with the increment of melting temperature. For antimicrobial property, the films containing 30\% w/w or more of PVPI clearly demonstrated inhibition zone of \textit{S. aureus}. The inhibition zone was increased from 7.4 to 13.0 mm after increasing the percentage of PVP iodine from 30\% to 50 \% w/w. In conclusion, shellac could be a promising polymer for fabrication of antimicrobial film patch.

Keywords: antimicrobial, film, PVP iodine, shellac

Introduction

In the view of biodegradable and eco-friendly, natural polymers have been extensively studied. Shellac (SHL) is a natural polymer derived from the secretion of lac (\textit{Kerria Lacca}) insects which are mostly cultivated in host trees in China, India and Thailand. Thailand is the second of shellac exporter in the world and SHL have many advantages, including low water vapor permeability and excellence film forming. Therefore, it is widely used in the food, agriculture, wood and pharmaceutical industry. In the pharmaceutical industry, shellac has been used for moisture protection and glossing, and enteric coating of pharmaceutical products (Limmatvapirat et al., 2004; 2007, Luangtana-anan et al., 2007; Sushanta et al., 2010).

As illustrated in Fig. 2, polyvinyl pyrrolidone iodine (PVPI) is a complex of polyvinylpyrrolidone (PVP) and elemental iodine. It demonstrates a broad range of antiseptic activity against bacteria, fungi, protozoa, and viruses (Yanai et al., 2006; Panzova and Bogdanov, B 1990). Generally, it is used in the form of solution. However,
the application of PVPI in the film form was not investigated.

Recently, there has been a growing of interest for developing materials with film-forming capacity and having antimicrobial properties (Vladimir et al., 2010; Maolin et al., 2004; Yu-Bey et al., 2004). As mentioned earlier, SHL showed very good film properties. However, it did not provide an antimicrobial activity which was the importance characteristic for wound dressing or wound healing. Thus, a combination of shellac with PVPI should be a possible mean for improving film properties and antimicrobial activity of SHL.

The aim of the present study was to investigate the physicochemical properties and antimicrobial activity of SHL-PVPI blend film patch.

**Preparation of SHL/PVPI Antimicrobial Films Patch**

The blend films of SHL/PVPI, at the weight ratios of 10:90 to 50:50, were prepared by casting method as previous described (Limmavapirat et al., 2004). Briefly, SHL and PVPI was dissolved in 95% ethanol for 6 hr, poured onto Teflon plate and then allowed to dry by hot air oven at 50 °C about 6 h.

**Characterization of SHL/PVPI Blend Film**

**Hot Stage Microscopy**

The thermal analysis of film was characterized by hot stage microscope (Mettler Toledo, Switzerland). All HSM photographs were recorded using a light microscope equipped with digital camera (Olympus, BX50, Japan). Finely ground films were observed under the microscope at a heating rate of 10 °C/min. Changes in the morphology of each samples were comparatively evaluated as a function of temperature.

**Mechanical Properties**

The mechanical properties of film were performed by texture analyzer (TA.XT plus Texture Analyzer, Stable Micro Systems, UK). The film was placed in a holder with a cylindrical hole (r = 1.0 cm). The probe was driven through the film with a speed of 0.1 mm/s and force–displacement curves were recorded through a 50 N load cell. The maximum load and the maximum displacement of films were measured, and then converted to puncture strength, elongation at puncture and modulus at puncture. The maximum load and the maximum displacement of films were

---

**Materials**

SHL was kindly supplied from Union Shellac Part., Ltd. (Bangkok, Thailand). Ethanol absolute anhydrous (Lot No. 00336) was purchased from Liquor distillery organization excise department. (Chachoengsao, Thailand). PVPI was purchased from Merck, Germany. Tryptic soy broth (TSB) (Batch No.809199) and Tryptic soy agar (TSA) (Batch No. 814987) were purchased from Merck, Germany. The culture media were mixed with water according to the prescription of the supplier and were sterilized at 121°C for 15 min before use. *Staphylococcus aureus* (ATCC 25923) was obtained from the Department of Microbiology, Faculty of Science, Silpakorn University. All other reagents were of analytical grades.
measured. The parameters were calculated as equation.

\[
\text{Puncture strength} = \frac{F_{\text{max}}}{A_{\text{CS}}}
\]

Where \(F_{\text{max}}\) is the maximum applied force, \(A_{\text{CS}}\) the cross-sectional area of the edge of the film located in the path of the cylindrical hole of the film holder, with \(A_{\text{CS}} = 2\pi r \delta\), \(r\) is the radius of the hole and \(\delta\) is the thickness of the film.

\[
\text{Elongation (\%)} = \frac{\sqrt{r^2 + d^2} - r}{r} \times 100
\]

d represents the displacement of the probe from the point of contact to point of puncture (mm).

Antimicrobial Test of SHL/PVPI Film Patch

*Staphylococcus aureus* (ATCC 25923) was selected as the model gram positive bacteria for testing. The antibacterial activity of the SHL/PVPI films was evaluated by zone of inhibition test. A standard inoculum of the test organism containing 100 µl of \(10^8\) colony forming units (CFU)/ml was swabbed on the surface of a tryptic soy agar plate (TSA), and then strips of 6.0 mm diameter of SHL/PVPI film were placed on the agar plates. The plates were incubated overnight at 37 °C for 24 h, and the inhibition zones surrounding the sample were measured.

Results and Discussion

Thermal Analysis

Figure 3 shows the HSM pictures of SHL and SHL/PVPI blend film after heating at various temperatures. SHL showed the clear melting after heating up to 60 °C which was agreed well with the endothermic peak of DSC (data not shown). However, SHL/PVPI blend films, did not clearly demonstrated melting even heat up to 200 °C. All ratios of SHL/PVPI films were charred (without melting) at the high temperature. The result suggested that PVPI might strongly interact with SHL and changed the melting characteristic of SHL. Several studies reported the interaction of PVP with some materials through hydrogen bonding with pyrrolidone moiety (Tantishaiyakul et al., 1999). Since SHL composed of many hydroxyl and carboxyl groups, the possible interaction between these functional groups with alicyclic nitrogen or carbonyl groups of PVPI might be occurred.
**Mechanical Properties**

The puncture strength and percentage of elongation of shellac/PVPI blend film are shown in Table 1. PVPI could not form film since it was too fragile while shellac can easily be prepared. SHL/PVPI blend films demonstrated that the increased percentage of elongation as increasing the fraction of SHL from 10-50% w/w. The film containing more than 50% w/w of PVPI was too fragile. The result suggested that SHL could promote the film forming properties of PVPI and the SHL/PVPI blend film could load PVPI up to 50% w/w.

**Table 1** Mechanical properties of SHL/PVPI blend films

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Mechanical properties</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Puncture strength (MPa)</td>
</tr>
<tr>
<td>SHL</td>
<td>1.34±0.47</td>
</tr>
<tr>
<td>SHL/PVPI 10:90</td>
<td>2.27±0.58</td>
</tr>
<tr>
<td>SHL/PVPI 20:80</td>
<td>2.54±0.18</td>
</tr>
<tr>
<td>SHL/PVPI 30:70</td>
<td>3.13±1.12</td>
</tr>
<tr>
<td>SHL/PVPI 40:60</td>
<td>2.54±0.72</td>
</tr>
<tr>
<td>SHL/PVPI 50:50</td>
<td>1.07±0.14</td>
</tr>
</tbody>
</table>

**Antimicrobial Test of SHL/PVPI Film Patch**

Films containing PVPI were tested for antimicrobial activity against *S. aureus*. The results of growth-inhibition tests were given in Table 2 and Figure 4. As indicated by the clear zone in Figure 4, SHL film did not show clear zone while pure PVPI demonstrated large inhibition zone, suggesting the inability of pure SHL for eradication of *S. aureus*. However, SHL/PVPI films indicated clear zone at the concentration of PVPI 30% and more. The diameter of inhibition zone was increase as increasing percentage of PVPI (Table 2). The result suggested that antimicrobial film patch of SHL could be achieved.

**Table 2** Diameter of inhibition zone of SHL, PVPI and SHL/PVPI blend films

<table>
<thead>
<tr>
<th>Test samples</th>
<th>Diameter of inhibition zone (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHL</td>
<td>-</td>
</tr>
<tr>
<td>SHL/PVPI 10:90</td>
<td>-</td>
</tr>
<tr>
<td>SHL/PVPI 20:80</td>
<td>-</td>
</tr>
<tr>
<td>SHL/PVPI 30:70</td>
<td>7.4 ± 0.4</td>
</tr>
<tr>
<td>SHL/PVPI 40:60</td>
<td>8.4 ± 0.4</td>
</tr>
<tr>
<td>SHL/PVPI 50:50</td>
<td>10.8 ± 0.2</td>
</tr>
<tr>
<td>PVPI</td>
<td>13.0 ± 0.0</td>
</tr>
</tbody>
</table>

**Figure 4** Inhibition zone of (a) pure SHL film (b) PVPI film and (C) SHL/PVP iodine film patch with 30% w/w PVPI.
Conclusions

In this present study, The SHL-based PVPI films were successfully prepared. Factors affecting properties of films were characterized. The result obtained for the study could give a more alternative application of shellac as an antimicrobial film patch.

Acknowledgements

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References


Povidone Iodine antiseptic agent available online at http://online1.ispcorp.com/Brochures/Pharma/pvpiodine.pdf (3 Sep. 2010)


This paper was originally presented at the International Conference on Agriculture and Agro-Industry 2010 (ICAA2010), November 19-20, 2010 Mae Fah Luang University, Chiang Rai, Thailand
Medium Optimization for Antimicrobial Compound Production by an Endophytic Fungus of *Stemona burkillii* for Plant Pathogenic Control

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\(^2\)Division of Biotechnology, School of Bioresources and Technology, King Mongkut’s University of Technology Thonburi, Bangkok 10150, Thailand  
\(^3\)Pilot Plant Development and Training Institute, King Mongkut’s University of Technology Thonburi, Bangkok 10150, Thailand

*Corresponding author. E-mail: taweerat.vic@kmutt.ac.th

Abstract

In the present work, the optimum composition and concentration of a liquid medium for the production of bioactive compounds against phytopathogenic microorganisms by NHL-L 6/6, an endophytic fungus isolated from *Stemona burkillii*, were studied. Antimicrobial activities against *Erwinia caratovora* and *Penicillium* sp. were determined in cultures of five different media used. Sucrose yeast extract (SY) medium (2% sucrose and 0.5% yeast extract) gave the best activities against both test microorganisms. Yeast extract and sucrose concentrations in SY medium were optimized for secondary metabolite biosynthesis. The liquid medium containing 2% sucrose and 0.3% yeast extract presented maximum antimicrobial agent production by NHL-L 6/6.

**Keywords**: antiphytopathogenic compounds, endophytic fungi, culture medium, sucrose yeast extract (SY) medium

Introduction

Agrochemical treatment of phytopathogenic diseases could lead to environmental impact and poses threat in humans and animals. Therefore, research on potential biocontrol agents has been increasing in order to reduce use of chemicals in the treatment of phytopathogens.

By the end of 2002, more than 2,000 bioactive compounds from microorganisms were reported (Berdy, 2005). Endophytes are symbionts living in plant tissue without apparent disease symptoms. Endophytic fungi are important microorganisms that are being explored for novel products for agrochemical industries because the secondary metabolites that a fungus produces may correspond with its respective ecological niche, e.g. the mycotoxins of plant pathogen (Gloer, 1997). In addition, fungi have versatile synthetic capability (Suryanarayanan et al., 2009).

In a search for alternative phytopathogenic control, an endophytic fungus, NHL-L 6/6, isolated from *Stemona burkillii* leaves, produces antimicrobial compounds against some fungal phytopathogens (Rat-narathorn et al., 2008) and bacterial pathogens (unpublished data). Antimicrobial compounds are secondary metabolites and their production in microorganisms requires substrates from primary metabolism. Secondary metabolism is regulated by culture conditions and medium composition (Demain, 2000) such as carbon sources, nitrogen sources, phosphate and trace elements (Betina, 1994). Carbon and nitrogen sources which are favorable for growth may not be beneficial to secondary metabolite production (Demain, 1992). Study on secondary metabolite biosynthesis involved an optimization of culture medium in achieving high productivity and low cost. Furthermore, fermentation medium design is critical important for developing biolotechnological-
based industrial process. In the present work, preliminary study on optimization of medium constituents was carried out to increase yield of antimicrobial compound produced by NHL-L 6/6.

**Materials and Methods**

**Fungal Strain**

NHL-L 6/6 stock culture was maintained in PDA (Potato Dextrose Agar) slants and stored at 4°C. The culture was transferred to freshly prepared PDA every two months.

**Medium Optimization**

Five different media were tested: MID (Stroble et al., 2002), PDB (Potato Dextrose Broth) + 2% sucrose, SY (2% sucrose, 0.5% yeast extract), Sucrose ammonium chloride (2% sucrose, 0.025% yeast extract, 2.9 g L⁻¹ NH₄Cl), Sucrose ammonium sulfate (2% sucrose, 0.025% yeast extract, 3.58 g L⁻¹ (NH₄)₂SO₄).

The antimicrobial activity determination revealed that SY culture medium gave best activity. The effect of sucrose and yeast extract concentrations in SY medium on antimicrobial agent production in NHL-L 6/6 was further investigated. Sucrose (1-3%) was individually added to SY medium while 0.5% yeast extract was used as a sole nitrogen source. By keeping sucrose concentration at optimum level (2%), yeast extract concentrations, ranging from 0.3 to 1%, were added into the medium. pH of all media was adjusted to 6.5 before sterilization at 121°C.

**Fungal Cultivation**

Since NHL-L 6/6 does not produce spores. It was grown on PDA at 28°C for 7 days and used as inoculum. A set of 3 replicates was carried out through out the experiments. Five mycelial disks of NHL-L 6/6 were inoculated into 250 mL flasks containing 100 mL of one of the mentioned medium. After inoculation, the cultures were incubated at 28°C, 150 rpm for 7 days. The culture broth was collected and centrifuged at 10000xg, 4°C for 10 min to remove fungal pellets.

**Antimicrobial Activity Assay**

To evaluate the antimicrobial activity in culture broth, *Erwinia caratovora* and *Penicillium* sp. were used as test microorganisms. The agar well diffusion method as adopted earlier (Boonmagard, et al., 2007; Ratnarathorn et al., 2008) was used. Since diffusion assays in this study were relative, a standard was required to calibrate the system. Mercuric chloride solutions (500 to 5000 mg mL⁻¹) were used as standard solutions in the assay. The formula of neutral particle diffusion gas recommended by Cooper and Woodman (1946) can be applied to the diffusion of antibiotic through agar gels. The formula is written as follows:

\[x^2 = 4DT (\ln m_o - \ln m')\]

\[x^2 = \text{square of the distance between the reservoir and the edge of the zone of inhibition}\]

\[D = \text{the diffusion coefficient for the antibiotic under study in the test medium and at the temperature of the test system}\]

\[T = \text{the time taken for the zone to be fixed}\]

\[m_o = \text{the concentration of antibiotic at the origin}\]

\[m' = \text{the critical concentration of antibiotic which inhibits the test organism under the conditions of diffusion}\]

One unit activity was equivalent to one natural logarithm of mercuric chloride concentration at the corresponding diameters of clear zone.

**Results and Discussion**

Five different media tested were favorable to antimicrobial compound production in NHL-L 6/6, in the condition (temperature and agitation rate) used in this study, although to different extents (Table 1). The results showed that culture broth from sucrose yeast extract (SY) medium gave the best antimicrobial activities against both test microorganisms. This could be a result of a negative effect exerted by some media components. A rapidly utilizable carbon source, such as glucose in PDB amended with 2% sucrose, could lead to carbon catabolite regulation which is widely
distributed among microbial systems (Sanchez and Demain, 2002). Similarly, some nitrogen sources support microbial growth but are negatively affected to microbial secondary metabolism. Many studies reported that ammonium is a rapidly assimilated nitrogen source and can cause nitrogen catabolite repression that interfere antibiotic production (Sanchez and Demain, 2002). Ammonium in Sucrose ammonium chloride and Sucrose ammonium sulfate media could also give negative results due to nitrogen catabolite regulation to antibiotic biosynthesis in NHL-L 6/6.

Table 1 Antimicrobial activities of culture broth of NHL-L 6/6 in five different media.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Antimicrobial Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. caratovora</td>
</tr>
<tr>
<td>MID</td>
<td>5.64</td>
</tr>
<tr>
<td>PDB+2% sucrose</td>
<td>5.53</td>
</tr>
<tr>
<td>SY</td>
<td>6.8</td>
</tr>
<tr>
<td>Sucrose-NH$_4$Cl</td>
<td>5.98</td>
</tr>
<tr>
<td>Sucrose-(NH$_4$)$_2$SO$_4$</td>
<td>6.39</td>
</tr>
</tbody>
</table>

Table 2 Effect of sucrose concentration on antimicrobial compounds produced by NHL-L 6/6 when 0.5% yeast extract was used as a sole nitrogen source.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Antimicrobial Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. caratovora</td>
</tr>
<tr>
<td>SY (1% Sucrose)</td>
<td>6.47</td>
</tr>
<tr>
<td>SY (2% Sucrose)</td>
<td>6.8</td>
</tr>
<tr>
<td>SY (3% Sucrose)</td>
<td>6.95</td>
</tr>
</tbody>
</table>

Table 3 Effect of yeast extract (YE) concentrations on antimicrobial activity when 2% sucrose was used as a sole carbon source.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Antimicrobial Activity (U/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E. caratovora</td>
</tr>
<tr>
<td>SY(0.3% YE)</td>
<td>8.64</td>
</tr>
<tr>
<td>SY(0.5% YE)</td>
<td>6.8</td>
</tr>
<tr>
<td>SY(0.7% YE)</td>
<td>NA</td>
</tr>
<tr>
<td>SY(1% YE)</td>
<td>NA</td>
</tr>
</tbody>
</table>

* NA = No Activity

Yeast extract concentrations ranging from 0.3 to 1% were added to SY medium containing 2% sucrose as a sole carbon source. The results were shown in Table 3. Antimicrobial activities increased with low concentration (0.3%) of yeast extract. The antibacterial and antifungal activities increased 1.3 and 1.6 folds, respectively, when compared to medium with 0.5% yeast extract. Yeast extract is commonly used as a common complex raw material in microbial fermentation because it provides proteins, free amino nitrogen, vitamins and minerals. The results suggested that yeast extract could provide substrates for primary and secondary
metabolism in NHL-L 6/6. Yeast extract was found to be very important components of the medium for the production of squalostatin (Para et al., 2005). However, it should be noted that high yeast extract concentrations inhibited antimicrobial compound formation in NHL-L 6/6.

High concentrations of sucrose and yeast extract inhibited the antibiotic production in NHL-L 6/6. Generally, secondary metabolism seems to be activated after growth phase ends as a result of nutrient depletion (Sanchez and Demain, 2002). High concentrations of some nutrients, e.g. carbon and nitrogen sources, perhaps delay the fungal growth and antibiotic production in NHL-L 6/6 resulting in non detectable antibiotic level on day 7 of cultivation. In addition, different C/N ratios in SY medium as a consequence of varying either C or N source concentration probably affect antibiotic production. Therefore, the effect of C/N ratios on fungal growth and production phase should be evaluated for the future work.

**Conclusions**

Carbon and nitrogen sources in culture medium influenced antimicrobial agent formation in NHL-L 6/6. The liquid medium containing 2% sucrose and 0.3% yeast extract was the best medium for antimicrobial agent production in this fungus.

**References**


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Varietal Cross Heterosis of Thein Waxy Corn

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Abstract

Waxy corn breeding program in Thailand is in the beginning stage with high potential in commercializing to new varieties. Thein corn is one of waxy corn with small ear and popularly consumed as fresh corn. Plant breeders are currently interested in improving varieties for desired characteristics and high yield. Study on heterosis in 10 Thein corn varieties aimed to classify varieties to heterotic groups for reciprocal recurrent selection. The results found that the studied Thein corn varieties showed significant differences on yield and ear size. Varietal cross between TNW and TSW gave the highest green yield and marketable dehusked yield of 14.5 and 8.2 Mg ha\(^{-1}\) respectively, and 19 crosses from 45 crosses gave green yield over than 12.5 ton/ha. For yield heterosis, the values were between -21 % (TNW/TMP) and 69 % (TLO/TSW) for green yield and between -25% (TNW/TMP) and 115% (TLO/TSW) for marketable dehusked yield. The SCA for GY and MDY ranged from -4.9 to 3.2 Mg ha\(^{-1}\) and -2.4 to 1.5 Mg ha\(^{-1}\), respectively. TLO and TSW also had significantly positive GCA effects of 0.6* and 0.9 ** Mg ha\(^{-1}\) for marketable dehusked yield. TKKU1 and TPR gave positive significant SCA effect for dehusk yield, they are recommended for reciprocal recurrent selection program for yield improvement. For ear size (ear width, ear length and seed row number), its heterosis was -14 to 30%.

Keywords: heterosis, combining ability, waxy corn

Introduction

Thein corn is one of waxy corns with small ear and also popularly consumed in Thailand. Most of farmers prefer to use local variety selected by themselves due to its good adaptation to local practices, furthermore, there are diverse characteristics depending on consumer desirable. Presently, consumer demand is to especially grow in urban and city. The standard size of thein corn is 10-15 cm length, 2-3 cm diameter and 8-12 ear rows which size is determined by Department of Agriculture. Thein corn breeding program in Thailand is in the beginning stage with high potential in commercializing to new varieties. Local varieties are valuable basis populations for improving synthetic or composite variety and developing inbred line. Understanding on genetic diversity in corn helps breeders to efficiently plan and use the existing germplasm for improving new varieties (Hallaure and Miranda, 1988).

Differences in genetic background, origin, and level of heterozygosity within and among populations, are the basis of that diversity (Melani and Carena, 2005). In general, genetic diversity and heterosis are positive association (Troyer et. al., 1988) but heterosis increases with increasing genetic distance are only up to an optimum level (Moll et. al., 1965). Diallel analysis of crosses among varieties proposed by Gardner and Eberhart (1966) is suitable and has been widely used for evaluation of open pollinated populations to identify heterotic pattern (Mungoma and Pollak, 1988; Melani and Carena, 2005; Jampatong et.al., 2010) and to determine the relative potential of populations as breeding populations in hybrid breeding.
program (Hallaure and Miranda, 1988). The objectives of this study were to evaluate performance combining ability and heterosis for yield and ear size of 10 thein corn varieties collected from Thailand.

Materials and Methods

Plant Materials

Ten thein corn varieties from different sources: TheinBanKhoa (TBK), TheinNamOm (TNO), TheinNamWung (TNW), TheinSaWan (TSW), and TheinSaiNamPung (TSN) from farmers and local market in Phranakhon Si Ayutthaya province, TheinLeungOn (TLO) from Chai-Nat province, Thein MunPoo (TMP) from Pichit province, PadTaewRachaburi (PTR) from Rachaburi province, Thein LeungKhonkaen (TLK1), and TheinLai Khonkaen (TLK2), from Khon Kaen University, were obtained. They were grown and crossed during December 2009 to February 2010 in a half diallel mating manner to produce forty-five varietal crosses.

Ten parents and forty-five varietal crosses were used as the entries. Yield testing was conducted at Agronomy Farm, Faculty of Agricultural Technology and Agro-industry, Rajamangala University of Technology Suvarnabhumi, Phranakhon Si Ayutthaya province, in early rainy season (May-June, 2010). The experimental design was a Randomized Complete Block with two replications. The experimental plots were grown with two rows of 5 m long and 0.25x0.75 m² of plant row spacing. Weeds were controlled by spraying atrazine at 2.3 L ha⁻¹ and hand weeding. Fertilizer was applied as basal application with 46.8 kg ha⁻¹ each of N, P₂O₅ and K₂O and top dressing with 143.8 kg ha⁻¹ of N at 20 days after emergence.

The recorded traits were green yield (GY), marketable dehusk yield (MDY), marketable ear numbers (MEN) ear length (EL), ear width (EW) and number of seed rows per ear (NSR). Analysis of variance was performed for all traits. The analysis III of Gardner and Eberhart (1966) was used to estimate genetic information on varietal parents. The analysis III was based on fitting only the variety cross means: \( Y_{ij} = \mu + g_i + g_j + s_{ij} \) where \( Y_{ij} \) is the mean of the cross of variety i and j; \( \mu \) is the mean of all crosses; and \( g_i \), \( g_j \) are the general combining ability effects and \( s_{ij} \) is specific combining ability effects of varieties i and j, respectively. Mid-parent heterosis (MPH) were determined from MPH= (VC – MP)/MP. Where VC is the trait of varietal crosses, MP = (P₁ + P₂)/2 in which P₁ and P₂ are the trait average of the varietal parents.

The t-test of significance was used to test the null hypothesis that mid-parent heterosis, GCA and SCA effects value were equal to zero.

Results and Discussion

Analysis of variance indicated that differences among entries were significantly different (P<0.01) for all traits. Base on Analysis III of Gardner and Eberhart (1966), the data from varieties and crosses showed significant differences in trait means and general combining ability (GCA) effects while specific combining ability (SCA) effects were not different in number of marketable ears and number of seed rows per ear (Table 1).

The Performances of Varietal Parent and Their General Combining Ability.

The performances of local varieties in this study were significantly different. TSN, TLK2 and TNW gave high green yield and marketable dehusk yield (Table 2 and Table 3), especially TLK2 showed the highest marketable dehusk yield of 6.4 Mg ha⁻¹. Green yield and marketable dehusk yield average over all varieties were 9.4 and 4.6 Mg ha⁻¹, respectively.
### Table 1 Mean square from Gardner and Eberhart (1966) analysis III of yield and ear size for 10 Thein corn varieties and their 45 crosses.

<table>
<thead>
<tr>
<th>SOV</th>
<th>df</th>
<th>Mean Squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GY(^1)</td>
</tr>
<tr>
<td>Entries</td>
<td>54</td>
<td>7.75   **</td>
</tr>
<tr>
<td>Varieties</td>
<td>9</td>
<td>11.88 **</td>
</tr>
<tr>
<td>Varieties vs Crosses</td>
<td>1</td>
<td>45.74 **</td>
</tr>
<tr>
<td>Crosses</td>
<td>44</td>
<td>6.05   **</td>
</tr>
<tr>
<td>GCA(gi)</td>
<td>9</td>
<td>16.46 **</td>
</tr>
<tr>
<td>SCA(sij)</td>
<td>35</td>
<td>3.37 *</td>
</tr>
<tr>
<td>Error</td>
<td>54</td>
<td>1.99</td>
</tr>
<tr>
<td>Total</td>
<td>109</td>
<td></td>
</tr>
<tr>
<td>C.V. (%)</td>
<td></td>
<td>14.79</td>
</tr>
</tbody>
</table>

\(^1\)GY = green yield, MDY = marketable dehusk yield, MEN = marketable ear numbers, EL = ear length, EW = ear width, and NSR = number of seed rows per ear.

* Significant at P < 0.05. ** Significant at P < 0.01.

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### Table 2 Green yield (Mg ha\(^{-1}\)) mean for 10 Thein corn varieties (diagonal) and their 45 varietal crosses (above diagonal) specific combining ability (below diagonal) and general combining ability.

<table>
<thead>
<tr>
<th>variety</th>
<th>THT</th>
<th>TNO</th>
<th>TLK1</th>
<th>TLO</th>
<th>TNW</th>
<th>TSN</th>
<th>TSW</th>
<th>PTR</th>
<th>TMP</th>
<th>TLK2</th>
</tr>
</thead>
<tbody>
<tr>
<td>THT</td>
<td>5.9</td>
<td>7.4</td>
<td>8.9</td>
<td>10.0</td>
<td>12.8</td>
<td>11.6</td>
<td>9.6</td>
<td>7.5</td>
<td>7.9</td>
<td>10.6</td>
</tr>
<tr>
<td>TNO</td>
<td>1.4</td>
<td>7.1</td>
<td>12.6</td>
<td>11.1</td>
<td>11.5</td>
<td>12.5</td>
<td>11.8</td>
<td>9.3</td>
<td>9.6</td>
<td>11.9</td>
</tr>
<tr>
<td>TLK1</td>
<td>0.5</td>
<td>1.8</td>
<td>8.6</td>
<td>13.1</td>
<td>14.0</td>
<td>12.1</td>
<td>6.9</td>
<td>14.0</td>
<td>9.7</td>
<td>11.4</td>
</tr>
<tr>
<td>TLO</td>
<td>0.4</td>
<td>0.7</td>
<td>0.6</td>
<td>9.2</td>
<td>13.2</td>
<td>12.2</td>
<td>13.8</td>
<td>11.5</td>
<td>12.8</td>
<td>13.0</td>
</tr>
<tr>
<td>TNW</td>
<td>2.2</td>
<td>0.5</td>
<td>1.4</td>
<td>0.4</td>
<td>12.1</td>
<td>13.0</td>
<td>14.5</td>
<td>12.1</td>
<td>7.6</td>
<td>13.3</td>
</tr>
<tr>
<td>TSN</td>
<td>1.0</td>
<td>0.5</td>
<td>-0.6</td>
<td>-1.5</td>
<td>-0.8</td>
<td>14.3</td>
<td>13.8</td>
<td>11.9</td>
<td>11.1</td>
<td>13.9</td>
</tr>
<tr>
<td>TSW</td>
<td>-0.2</td>
<td>0.6</td>
<td>-4.9*</td>
<td>1.0</td>
<td>1.4</td>
<td>0.8</td>
<td>7.2</td>
<td>11.6</td>
<td>10.3</td>
<td>13.4</td>
</tr>
<tr>
<td>PTR</td>
<td>-1.2</td>
<td>-0.8</td>
<td>3.2*</td>
<td>-0.3</td>
<td>0.1</td>
<td>0.0</td>
<td>0.4</td>
<td>9.7</td>
<td>9.0</td>
<td>10.3</td>
</tr>
<tr>
<td>TMP</td>
<td>0.2</td>
<td>0.4</td>
<td>-0.1</td>
<td>2.0</td>
<td>-3.3*</td>
<td>0.2</td>
<td>0.1</td>
<td>-0.1</td>
<td>7.3</td>
<td>11.2</td>
</tr>
<tr>
<td>TLK2</td>
<td>0.4</td>
<td>0.3</td>
<td>-0.8</td>
<td>-0.3</td>
<td>-0.2</td>
<td>0.5</td>
<td>0.8</td>
<td>-1.3</td>
<td>0.6</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Vi effect: -3.5 -2.3 -0.9 -0.3 2.6 4.9 -2.2 0.2 -2.1 3.5

GCA\(_i\) effect: -2.0** -0.6 0.1 1.1* 1.2* 1.2* 0.4 -0.6 -1.6** 0.8

* Significant at P < 0.05. ** Significant at P < 0.01.

Test statistics: LSD\(_{0.05}\) (varietal crosses mean difference) = 3.7 Mg ha\(^{-1}\), gi – gj differences = 1.5 Mg ha\(^{-1}\)
The significantly positive values of variety effect ($V_i$) in green yield and marketable dehusk yield revealed by TSN, TLK2 and TNW indicated that their yields were over than the variety average. Jampatong et al. (2010) recommended that they were promising breeding materials for selecting desirable genotypes by recurrent selection. The largest GCA effect of 0.9 Mg ha$^{-1}$ for marketable dehusk yield was contributed by TSW but variety effect showed negative value. TLO and TNW also had significantly positive GCA effects of 0.6 Mg ha$^{-1}$ for marketable dehusk yield (Table 3). It could be explained that TSW, TLO and TNW were good combiners for marketable dehusk yield. TLO showed positive effects on both variety and GCA effect, indicating that it was consisted of favorable genes with additive effects (Medici et al., 2004).

Marketable ear numbers of 82700 and 62700 ears ha$^{-1}$ of TLK2 and TLK1 respectively, were greater than the other varieties, but TSW gave the lowest ear numbers of 51300 ears ha$^{-1}$. TLK1, TSW and TLK2 showed high GCA effects for marketable ear numbers of 10300, 6470 and 6300 ears ha$^{-1}$, respectively (Table 4). TLK2 and TLK1 were prolific varieties and good combiners for marketable ear number, indicating that it was consisted of favorable genes with additive effects for yield in terms of marketable ear number. Large GCA effects of the parents were mainly due to additive and additive x additive gene actions (Griffing, 1956). SCA effect for marketable ear number was not significant (Table 1). Therefore, for improving ear number, the germplasm can be chosen based on variety effect and GCA. TLK2 was a better choice to be a parent for improving marketable ear number.

Ear length, ear width and numbers of seed row of the varietal parents were 12.7, 3.5 cm and 11.2 rows, respectively (Table 4). PTR showed the longest ear of 16.0 cm and the best general combiner for ear length but the worst GCA effect for

| Table 3 | Marketable dehusk yield (Mg ha$^{-1}$) mean for 10 thein corn varieties (diagonal) and their 45 varietal crosses (above diagonal) specific combining ability (below diagonal) and general combining ability. |
|---------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
|         | THT    | TNO    | TLK1   | TLO    | TNW    | TSN    | TSW    | PTR    | TMP    | TLK2   | Vi effect | GCA effect |
| THT     | 3.1    | 3.7    | 4.8    | 6.1    | 7.0    | 5.1    | 5.2    | 4.0    | 4.2    | 5.5    | -1.4**    | -1.5**    |
| TNO     | -0.6   | 3.6    | 5.8    | 6.3    | 5.9    | 6.0    | 6.5    | 4.9    | 6.1    | 6.5    | -0.6*     | 0.3**     |
| TLK1    | -0.3   | -0.1   | 3.9    | 6.5    | 8.3    | 6.8    | 7.3    | 7.6    | 5.4    | 5.8    | -1.0      | -0.6**    |
| TLO     | 0.5    | 0.0    | -0.6   | 4.3    | 7.5    | 6.7    | 7.9    | 6.2    | 7.0    | 7.2    | 0.2       | 0.6**     |
| TNW     | 1.5*   | -0.3   | 1.2    | 0.1    | 5.4    | 7.0    | 8.2    | 6.2    | 3.7    | 7.3    | 0.8       | 0.9**     |
| TSN     | -0.1   | 0.0    | 0.0    | -0.5   | -0.2   | 6.1    | 7.8    | 7.2    | 5.8    | 6.5    | 1.2       | 0.1       |
| TSW     | -0.6   | -0.1   | -0.1   | 0.1    | 0.4    | 0.3    | 3.0    | 6.8    | 6.3    | 7.7    | 1.3       | 0.4       |
| PTR     | -0.6   | -0.5   | 1.3    | -0.4   | -0.4   | 0.9    | -0.1   | 5.0    | 5.4    | 5.9    | -2.4*     | -0.1      |
| TMP     | 0.0    | 1.2    | -0.4   | 0.9    | -2.4*  | -0.1   | -0.2   | 0.1    | 4.6    | 6.8    | 0.9       | 0.9       |
| TLK2    | 0.3    | 0.4    | -1.0   | 0.0    | 0.1    | -0.4   | 0.2    | -0.4   | 0.9    | 6.4    | 0.8       | 0.3       |

* Significant at P<0.05  ** Significant at P<0.01
Test statistics; LSD$_{0.05}$ (varietal crosses mean difference) = 1.9 Mg ha$^{-1}$, $g_i – g_j$ differences = 0.8 Mg ha$^{-1}$
numbers of seed row. TNW, TSN and TMP produced the same ear width of 4.0 cm and also showed positive GCA effects for ear width. Large GCA effects of the parents were mainly due to additive and additive x additive gene actions (Griffing, 1956). TNW and TSN, high green yield varieties and high GCA effect, showed more ear width than that of standard ear size which determined by Department of Agriculture and may produce larger ear size progeny. Breeder should be careful to use TNW and TSN as germplasm and consider both ear size and green yield. THT, TNO, TLK1 and TLK2 showed negative GCA effect for ear width, which was suitable to be parents for reducing ear width in hybrid varieties.

Heterosis

The variance of variety and cross comparison showing significant differences indicated that average heterosis was important, the average of varietal cross performances were higher than that of parental mean in all traits. The average heterosis for yield performances was 4, 6, 8, 11, 20 and 38% for number of seed row, ear width, ear length, marketable ear number, green yield and marketable dehusk yield, respectively (Table 5). The top three high yield varietal crosses, TNW/TSW, TLK1/TNW and TLK1/PTP, gave green yield of 14.5, 14.0 and 14.0 Mg ha⁻¹ respectively (Table 2). TLK1/TSW, TNW/TSW and TLO/TSW gave marketable dehusk yield of 8.3, 8.2 and 7.9 Mg ha⁻¹ respectively (Table 3). For yield heterosis, the values ranged from -21% (TNW/TMP) to 69% (TLO/TSW) for green yield and -25% (TNW/TMP) to 115% (TLO/TSW) for marketable dehusk yield. While heterosis values for ear size ranged from -5% to 30% for ear length, -9% to 19% for ear width and -14% to 22% for numbers of seed row (Table 5).

### Table 4

Variety means (VMᵢ) and general combining ability effects (gᵢ) of marketable ear numbers and ear size performance for 10 local thein corn varieties.

<table>
<thead>
<tr>
<th>variety</th>
<th>MEN¹</th>
<th>EL</th>
<th>EW</th>
<th>NSR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VMᵢ</td>
<td>Gᵢ</td>
<td>VMᵢ</td>
<td>Gᵢ</td>
</tr>
<tr>
<td></td>
<td>x₁₀⁻³</td>
<td>ears ha⁻¹</td>
<td>cm</td>
<td>----------------------</td>
</tr>
<tr>
<td>THT</td>
<td>51.3</td>
<td>-4.70</td>
<td>12.6</td>
<td>-0.19</td>
</tr>
<tr>
<td>TNO</td>
<td>58.0</td>
<td>0.80</td>
<td>13.4</td>
<td>0.29</td>
</tr>
<tr>
<td>TLK1</td>
<td>62.7</td>
<td>10.30 **</td>
<td>12.2</td>
<td>-0.18</td>
</tr>
<tr>
<td>TLO</td>
<td>54.0</td>
<td>0.97</td>
<td>10.8</td>
<td>-0.61 *</td>
</tr>
<tr>
<td>TNW</td>
<td>52.0</td>
<td>1.47</td>
<td>13.1</td>
<td>-0.25</td>
</tr>
<tr>
<td>TSN</td>
<td>54.0</td>
<td>-1.62</td>
<td>13.7</td>
<td>-0.05</td>
</tr>
<tr>
<td>TSW</td>
<td>51.3</td>
<td>6.47 *</td>
<td>11.1</td>
<td>-0.07</td>
</tr>
<tr>
<td>PTR</td>
<td>57.3</td>
<td>-7.70 **</td>
<td>16.0</td>
<td>1.36 **</td>
</tr>
<tr>
<td>TMP</td>
<td>56.0</td>
<td>-12.28 **</td>
<td>10.4</td>
<td>-0.52 *</td>
</tr>
<tr>
<td>TLK2</td>
<td>82.7</td>
<td>6.30 *</td>
<td>13.7</td>
<td>0.22</td>
</tr>
</tbody>
</table>

|          | Mean  | 57.9 | 12.7 | 3.5  | 11.2 |
|          | LSD₀.₀₅ (vᵢ-vᵣ) ‡ | 4.9 | 1.4 | 0.2  | 1.5 |
|          | LSD₀.₀₅ (gᵢ-gᵣ) ‡ | 2.46 | 0.72 | 0.10 | 0.76 |

¹ MEN= marketable ear numbers, EL= ear length, EW= ear width and NSR= number of seed rows per ear.

* Significant at P≤0.05  ** Significant at P≤0.01

‡ LSD₀.₀₅ (VMᵢ-VMⱼ) denotes differences between variety mean

‡ (gᵢ – gⱼ ) denotes differences between general combining ability effects.
Table 5 Mean values of varietal crosses, varietal parents and estimates of average heterosis based on mid-parent values for green yield (GY) marketable dehusk yield (MDY) marketable ear number (MEN) ear length (EL) ear width (EW) and number of seed row (NSR).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Varietal crosses</th>
<th>Varietal parents</th>
<th>Average heterosis</th>
<th>Range of heterosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td></td>
<td></td>
<td>Min</td>
</tr>
<tr>
<td>GY (Mg ha⁻¹)</td>
<td>11.4 ± 1.6¹</td>
<td>9.4 ± 2.3</td>
<td>20</td>
<td>-21 (TNW/TMP)</td>
</tr>
<tr>
<td>MDY (Mg ha⁻¹)</td>
<td>6.3 ± 0.9</td>
<td>4.6 ± 1.0</td>
<td>38</td>
<td>-25 (TNW/TMP)</td>
</tr>
<tr>
<td>MEN (10³ ears ha⁻¹)</td>
<td>64.5 ± 8.2</td>
<td>57.9 ± 5.9</td>
<td>11</td>
<td>-28 (TNW/TMP)</td>
</tr>
<tr>
<td>EL (cm)</td>
<td>13.6 ± 0.7</td>
<td>12.7 ± 1.7</td>
<td>8</td>
<td>-5 (TSN/TLK2)</td>
</tr>
<tr>
<td>EW (cm)</td>
<td>3.7 ± 0.2</td>
<td>3.5 ± 0.5</td>
<td>6</td>
<td>-9 (TNW/TMP)</td>
</tr>
<tr>
<td>NSR (no.)</td>
<td>11.6 ± 1.1</td>
<td>11.2 ± 2.2</td>
<td>4</td>
<td>-14 (TSN/TMP)</td>
</tr>
</tbody>
</table>

¹ Mean ± S.D.

SCA or specific heterosis was different among varietal crosses for green yield, marketable dehusk yield, ear length and ear width but no significant difference was found for ear numbers and numbers of seed row (Table 1). These parameters were the results of dominance gene action and difference in gene frequencies between parental varieties (Murray et al., 2003). The SCA for GY and MDY ranged from -4.9 to 3.2 Mg ha⁻¹ (Table 2) and -2.4 to 1.5 Mg ha⁻¹ (Table 3), respectively. Heterosis in varietal crosses was probably due to the difference between genotypes of the parental varieties. The expression of heterosis depended on the difference in allele frequency of the parents and dominant effect at various loci (Falconer and Mackay, 1996). TLK1/PTR gave high green yield and exhibited significant SCA of 3.2 Mg ha⁻¹. While THT/TNW showed positive significant SCA effect of 1.2, 0.6 Mg ha⁻¹, 0.22 cm. and 0.85 seed row for green yield, marketable dehusk yield, ear width and number of seed row respectively. Recommendation for TNW, it is high population potential for marketable dehusk yield improvement; however, it should be carefully selected because large ears are usually out of standard. THT/TNW showed the highest SCA effect. These varieties were assigned to different heterotic group. GCA effect for ear width and number of seed row of THT was negative effect. THT and TNW may be coupled for reciprocal recurrent selection program and

Conclusions

TLK1 and TLK2, prolific varieties having high GCA for marketable ear number, are recommended as parents with high potential to be selected as breeding program for increasing marketable ear number.

TNW gave positive significant GCA effect of 1.2, 0.6 Mg ha⁻¹, 0.22 cm. and 0.85 seed row for green yield, marketable dehusk yield, ear width and number of seed row respectively. Recommendation for TNW, it is high population potential for marketable dehusk yield improvement; however, it should be carefully selected because large ears are usually out of standard. THT/TNW showed the highest SCA effect. These varieties were assigned to different heterotic group. GCA effect for ear width and number of seed row of THT was negative effect. THT and TNW may be coupled for reciprocal recurrent selection program and
will be improved to be source of inbred for hybrid breeding program

TLK1 and PTR gave positive significant SCA effect for green yield and marketable dehusk yield. These varieties were assigned to different heterotic group. TLK1 was good combiner for ear number while PTR was good combiner for ear length. Therefore, they are recommended to be utilized in reciprocal recurrent selection program for developing hybrid variety parental lines.

Acknowledgments

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References


Factors Affecting on the Enhancement of Mechanical Properties of Composite Edible Film based on Shellac and Gelatin

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Abstract

The purpose of this study was to investigate the factors affecting on the enhancement of mechanical properties of composite film based on shellac and gelatin. The various factors including gelatin content, temperature, pH, ionic strength and mixing equipment were investigated. The composite film solutions were prepared by adding different concentrations of gelatin into the 6% w/w shellac solution and were prepared in a free film by casting method. The mechanical properties of films were measured by using a texture analyzer. The result demonstrated that the higher gelatin contents gave the higher puncture strength and the higher percentage of elongation, resulting in the enhancement of strength and flexibility of shellac film. The composite film solution at the concentration of 40% w/w gelatin was selected for further investigation for the influence of heat, pH, ionic strength and mixing equipment during the preparation process. Thus, the changes in temperature and pH affected the change in mechanical properties of film, causing the denatured or hydrolyzed secondary and tertiary structure of gelatin during the film formation. The addition of NaCl attributed to the increased elongation of film, resulting from the increased solubility of gelatin solution. The study indicated that the mixing equipment, e.g. mechanical stirrer, ultrasonic generator and homogenizer, had an influence on the mechanical properties. The ultrasonic generator was a suitable technique for the preparation of the composite film solution. Hence, the mechanical properties of composite film based on shellac and gelatin could be modified by the various factors such as pH, heat, ionic strength and mixing condition.

Keywords: shellac, gelatin, composite film, mechanical properties, denatured protein

Introduction

Shellac has been widely studied for applying as an edible film coating. It is a thermosetting resin secretion of lac insects, Laccifer Laca which is mostly cultivated on host trees from India and Thailand. It is considered as a complex of polyhydroxy polybasic acid. Various hydroxyl acids such as aleuritic acid, shelloic acid, kerrolic acid and jalaric acid have been isolated by chemical degradation of the hard resin portion of shellac (Qussi and Suess, 2006). The shellac is widely used in the food industry and agro industry for water, gas, lipid and microbial spoilage protection and hence prolonging the shelf-life of product. It has been used for moisture protection of drugs, controlled drug delivery and enteric coating in the pharmaceutical industry due to its excellent film forming, low water vapor permeability, alcohol and alkaline solubility (Limmatavapirat et al., 2004; Limmatavapirat et al., 2007; Luangtana-anan et al., 2007). Edible films from the shellac alone show weak mechanical strength and low flexibility. The stress value of shellac film was 1-2 N/mm² and the strain value was 1-2 % lower than other natural polymers (Luangtana-anan et al., 2007). An
approach to improve the weak mechanical strength and low flexibility of shellac was prepared by the formation of composite films based on shellac and hydrophilic polymer (Qussi and Suess, 2006). In this study, gelatin was chosen as a second film forming, due to a naturally complex polypeptide obtained by hydrolysis of collagen from skin, bones and connective tissue. It is a protein used in food, pharmaceutical, and cosmetic industries and has been widely used in edible films and coating due to the abundance of raw material, biodegradability, biocompatibility, low cost, excellent film forming and good mechanical properties (Gomez-Guillen et al., 2009).

However, there were several researches reported the change in the mechanical properties during the film preparation process due to the changes in concentration of gelatin, temperature, pH and ionic strength (Cao, Fua, and He, 2007, Stuchell and Krochta, 1994, Rhim et al.2000). This results from the amino acid structure of gelatin giving the sensitivity to the film preparation condition. The higher gelatin content attributed to the higher tensile strength and elongation at break of composite films based on soy protein isolate and gelatin (Cao, Fua and He, 2007). The increase in temperature during the protein preparation has an effect on the structure of protein, causing the denaturation of the secondary and tertiary structures of proteins and disulfide interchange among protein molecules (Stuchell and Krochta, 1994). Rhim et al. (2000) observed that the heat induced cross-linking contributed to the increased strength and the reduction in extensibility of soy protein films. Furthermore, the mechanical property of composite film solution was dependent on the change in pH of protein preparation. Generally, the dispersion of protein was made at pH 4-7 to unfold the protein (Swain et al., 2004). The change in structure of protein was dependent on the pH which was associated with the high content of ionized polar amino acids. The higher pH in protein solution led to the hydrolyzed and denatured protein indicated by the enhancement of soluble and gel free formation in water solution. The denaturation of protein affected on the change in the shape of the protein from globular to extended chain (Brandenburg, Weller, and Testin, 1993). Furthermore, the concentration of electrolyte in protein film forming solution affected on the solubility and hydrophilicity of protein due to the effect of salting in at the lower concentration of electrolyte whereas the higher concentration showed the salting out effect causing lower solubility and protein precipitation, leading to the change in mechanical properties of film. Therefore, the addition of electrolyte in protein film shows a similar manner to that of plasticizer. The higher concentration of NaCl exhibited the higher flexibility of film due to the increase in adsorption of water content (Lee, Shim and Lee, 2004; Sarabia, Gomez-Guillen; Montero, 2000). The various types of mixer to prepare the composite film solutions had an influence on the compatibility and homogenous of polymer and polymer interaction. It was expected that the dispersion of polymer into a polymer matrix could act as excellent supporting filler, enhancing the mechanical properties of the composite film (Bae et al.,2009).

In the present study was to investigate the factors affecting on the enhancement of mechanical properties of composite film based on shellac and gelatin. The various factors including gelatin content, thermal treatment temperature, pH, ionic strength and mixing equipment were investigated. The conditions of these studies were described as the following statements:

**Materials and Methods**

**Materials**

Shellac was supplied from Union Shellac Part., Ltd. (Bangkok, Thailand). Gelatin type A, Porcine skin was purchased from Sigma Aldrich, (USA). The solvents used were ammonium solution obtained from Merck, (Germany)
Preparation of Shellac and Gelatin Composite Films

Effect of Gelatin Content
The shellac and gelatin composite film was prepared by the film casting method. The 6% w/w shellac was dissolved in ammonium solution to prepare shellac in ammonium salt form. The amount of ammonium salt solutions (NH₃) were calculated on the basis of acid value of shellac, that is, the amount of salt which is used to interact with the acid groups of shellac. These solutions were centrifuged for 10 min at 6,000 rpm and insoluble solid were removed by filtration, while the 6% w/w gelatin was prepared by hydration at room temperature for 30 min and then dissolved in water at 50 °C with stirring for about 1 hr. The 6% w/w composite solutions were prepared by mixing shellac solution with gelatin solution to give different ratio of shellac and gelatin, i.e., 100:0, 90:10, 80:20, 70:30, 60:40 50:50 and 0:100. All composite solutions were then poured on a glass plate and were allowed to dry at 50 °C for 8 h. The composite films were kept in desiccators and were evaluated for the mechanical properties by texture analyzer measurement (TA.XT.plus Texture Analyzer, Stable Micro Systems, UK). In this study, the composite films based on shellac and gelatin at the ratio of 60:40 was selected for further investigation for the effect of thermal treatment temperature, pH ionic strength and mixing equipment on the mechanical properties of composite film. The details of studied conditions for the preparation of gelatin solution were described as the following statements:

Effect of Thermal Treatment Temperature of Gelatin Solution
The 6% w/w gelatin solution was heated on a hot plate- magnetic stirrer until its temperature reached at 40, 50, 60, 70, 80 and 90 °C and the gelatin solution was stirred for 1 hr. The chosen temperature for further study for the effect of pH, ionic strength and mixing equipment was at 50 °C.

Effect of pH of Gelatin Solution
The 6% w/w gelatin solution was prepared by dissolving at 50°C and adjusted the pH to be 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 with 0.1 N NaOH and stirring for 30 min. The chosen pH for further study for the effect of ionic strength and mixing equipment was at pH 6.0.

Effect of Ionic Strength of Gelatin Solution
The 6% w/w gelatin solution at pH 6.0 was prepared by dissolving gelatin in water at 50 °C and was then added with 1-4% w/w NaCl and stirring for 30 min. The chosen concentration of electrolyte for the study of the effect of mixing equipment was 1% w/w NaCl.

Effect of Mixing Equipment to Prepare Shellac and Gelatin Composite Solution
The 6% w/w gelatin solution with the addition of 1% w/w NaCl was prepared at 50°C, and pH 6.0. The solution was then stirred by three types of mixers; they were mechanical stirrer; ultrasonic generator and homogenizer. The condition of mixers was 650 rpm, 30 min for mechanical stirrer, 11,000 RPM, 5 min for homogenizer and 20 kHz, 5 minute for ultrasonic generator.

Film Characterization

Mechanical Properties
The mechanical properties of the films were evaluated by puncture test as described in Limmatvapirat et al., (2007). A texture analyzer (TA.XT.plus Texture Analyzer, Stable Micro Systems, UK) equipped with a spherical puncturing probe (diameter 5 mm) was employed. The film sample was cut to a square piece of 7 x 7 cm². The film was placed in a holder with a cylindrical hole (r = 1.0 cm). The probe was driven through the film with a speed of 0.1 mm/s and force displacement curves were recorded through a 50 N load cell. The maximum load and the maximum displacement of films were measured. The average of ten measurements was performed (n=10). The puncture strength and percentage
of elongation were calculated using the following equations

\[
Puncture \text{ strength} = \frac{F_{\text{max}}}{A_{CS}}
\]

where \(F_{\text{max}}\) is the maximum applied force, \(A_{CS}\) the cross-sectional area of the edge of the film located in the path of the cylindrical hole of the film holder, with \(A_{CS} = 2\pi \delta\), where \(r\) is the radius of the hole and \(\delta\) is the thickness of the film.

\[
\text{Elongation (\%)} = \frac{\sqrt{r^2 + d^2} - r}{r} \times 100
\]

where \(r\) is the radius of the film exposed in the cylindrical hole of the film holder and \(d\) represents the displacement of the probe from the point of contact to the point of puncture.

**Water Content**

The film samples were accurately weighed (\(W_0\)) and was dried by loss on drying measurement (\(W_1\)). The temperature of drying is 100 °C. The weight of films before and after drying was calculated for the water content. All samples were performed in triplicate. Water content (WC) was determined as the percentage of water in films as the following equation: \(WC (\%) = 100\left(\frac{w_0 - w_1}{w_0}\right)\) where \(w_0\) and \(w_1\) were the weights before and after drying by loss on drying measurement.

**Scanning Electron Microscope (SEM)**

The morphology of the film surface was characterized by scanning electron microscope (SEM, model MX2000, Cam Scan, Cambridge, United Kingdom). The samples were coated with a fine gold layer before obtaining the micrographs. The accelerating voltage was used at 10 kV.

**Statistical Analysis**

The data of research were analyzed as means and standard deviation (Means ± SD). The statistical analysis was carried out using analysis of variance (ANOVA). Significant difference (\(P < 0.01\)) were performed using the LSD test.

**Results and Discussion**

**Effect of Gelatin Content on the Mechanical Properties of Shellac and Gelatin composite Film**

The effect of gelatin content on the mechanical properties of shellac and gelatin composite films is displayed in Table 1. The result demonstrated that the higher gelatin content contributed to the higher puncture strength and the higher percentage of elongation, resulting in the enhancement of strength and flexibility of shellac film at the ratio of 6% w/w gelatin was increased to 50% w/w (50:50 shellac and gelatin composite film). The significant (\(p < 0.01\)) change was also reported. The incorporation of gelatin into shellac network resulted in the hydrogen bonding formation between carboxyl and hydroxyl groups of shellac molecule and amino and carboxyl groups of gelatin molecule, attributing to the increase in the puncture strength. The higher elongation might be the result of the increase in the hydrophilicity with the increase in gelatin content. Apart from the concentration of gelatin, the mechanical properties of composite film with gelatin were dependent on the temperature, pH and ionic strength during gelatin preparation. The highest concentration of gelatin that could be incorporated in the composite film was chosen for further study. However, the sensitivity to water is another important parameter requirement for determining the proper gelatin content for the food and the pharmaceutical application, predicting by water vapor permeability coefficient (WVPC). The WVPC of composite film based on 10-40% gelatin did not change in the WVPC in comparison to single shellac film while at 50% w/w gelatin (50:50 shellac and gelatin composite film) caused the significant increase in the higher WVPC (Data not shown in this study) Therefore, the ratio of 60:40 of composite film based on shellac and gelatin was chosen in this study for further study for the effects of various factors such as temperature, pH, ionic strength and mixing equipment on the mechanical properties of...
composite film as shown in Tables 2-4 and Figures 1-2.

**Table 1 Effect of gelatin content on the mechanical properties of shellac and gelatin composite film**

<table>
<thead>
<tr>
<th>Shellac:gelatin</th>
<th>Puncture strength (MPa)</th>
<th>Elongation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:0</td>
<td>3.61 ± 0.90</td>
<td>3.80 ± 1.35</td>
</tr>
<tr>
<td>90:10</td>
<td>5.01 ± 1.35</td>
<td>6.51 ± 2.30</td>
</tr>
<tr>
<td>80:20</td>
<td>8.51 ± 2.44*</td>
<td>12.89 ± 3.01*</td>
</tr>
<tr>
<td>70:30</td>
<td>11.83 ± 0.73*</td>
<td>19.65 ± 4.45*</td>
</tr>
<tr>
<td>60:40</td>
<td>14.06 ± 3.20*</td>
<td>26.66 ± 4.77*</td>
</tr>
<tr>
<td>50:50</td>
<td>15.58±3.40*</td>
<td>32.47 ± 8.42*</td>
</tr>
<tr>
<td>0:100</td>
<td>23.18 ± 2.34*</td>
<td>38.23 ± 3.40*</td>
</tr>
</tbody>
</table>

* The data was significant differences (p<0.01) between average obtained by LSD test.

**Figure 1 Effect of thermal treatment temperature of gelatin solution on the mechanical properties of shellac and gelatin composite film**

**Effect of Thermal Treatment Temperature of Gelatin Solution on the Mechanical Properties of Shellac and Gelatin Composite Film**

The influence of thermal treatment temperature of gelatin solution on the mechanical properties of composite film is shown in Figure 1. The puncture strength value decreased significantly (p < 0.01) from 17.33 to 9.83 MPa whereas the percent elongation increased (p < 0.01) from 22.65 to 61.35 % when the gelatin solution was heated at 90 °C. The change in mechanical properties resulted from the denatured and hydrolyzed the secondary and tertiary structure of protein when the protein was heated during the preparation process. The result was in agreement with another study (Stuchell and Krochta, 1994). The higher temperature, the denaturation of protein was obtained, resulting in the change in the shape of the protein from globular to extended chain of protein, giving the change in the mechanical properties. The temperature of gelatin solution at 50 °C was chosen for further study as a result of less denatured protein.

**Table 2 Effect of pH value of gelatin solution on the mechanical properties of shellac and gelatin composite film**

<table>
<thead>
<tr>
<th>pH</th>
<th>Puncture strength (MPa)</th>
<th>Elongation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>14.06 ± 3.20</td>
<td>26.66 ± 4.77</td>
</tr>
<tr>
<td>6.0</td>
<td>15.30 ± 2.84</td>
<td>27.13 ± 4.92</td>
</tr>
<tr>
<td>7.0</td>
<td>14.81 ± 1.63</td>
<td>49.41 ± 14.06*</td>
</tr>
<tr>
<td>8.0</td>
<td>12.47 ± 2.56*</td>
<td>56.88 ± 15.55*</td>
</tr>
<tr>
<td>9.0</td>
<td>11.53 ± 3.39*</td>
<td>61.35 ± 8.44*</td>
</tr>
<tr>
<td>10.0</td>
<td>8.84 ± 1.84*</td>
<td>100.56 ± 35.40*</td>
</tr>
<tr>
<td>11.0</td>
<td>8.14 ± 3.30*</td>
<td>132.56 ± 35.71*</td>
</tr>
<tr>
<td>12.0</td>
<td>7.35 ± 1.08*</td>
<td>160.56 ± 27.92*</td>
</tr>
</tbody>
</table>

* The data was significant differences (p<0.01) between average obtained by LSD test.

**Effect of pH value of gelatin solution on mechanical properties of shellac and gelatin composite film**

Table 2 shows the effect of pH on the mechanical properties of composite film based on shellac and gelatin. The puncture strength decreased (p < 0.01) from 14.06 to 7.35 MPa while the percentage elongation of film increased (p < 0.01) from 26.66 to 160.56 % with the increase in pH of gelatin solution. The result was due to the denaturation and hydrolyzation of protein and the change in the shape from globular to extended chain of protein giving the reduction in strength and the increase in the flexibility of composite film. This result was in accordance with other reports (Swain et al., 2004 and Brandenburg et al., 1993). Swain et al. (2004) reported that the sensitivity of proteins to the change in pH was generally related to a high content of ionized polar amino acids. Brandenburg et al. (1993) found that the denaturation of protein at the higher pH resulted in the change in the shape of the protein from globular to extended chain of protein leading to the change in the mechanical properties. The too high pH led to the extreme hydrolyzation and denaturation of protein, resulting in the lower strength and
higher percentage elongation of composite film. The pH 6.0 was then chosen for further study as the composite film showed the highest puncture strength.

**Table 3** Effect of ionic strength of gelatin solution on the mechanical properties of shellac and gelatin composite film

<table>
<thead>
<tr>
<th>Content of NaCl (%)</th>
<th>Puncture strength (MPa)</th>
<th>Elongation (%)</th>
<th>Water Content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>15.30±2.84</td>
<td>27.13 ± 4.92</td>
<td>12.46</td>
</tr>
<tr>
<td>1</td>
<td>17.25±2.88</td>
<td>37.65 ± 3.89</td>
<td>12.73</td>
</tr>
<tr>
<td>2</td>
<td>14.07±3.15</td>
<td>58.94 ±14.20*</td>
<td>13.88</td>
</tr>
<tr>
<td>3</td>
<td>12.78±1.52</td>
<td>76.98 ±18.21*</td>
<td>14.20</td>
</tr>
<tr>
<td>4</td>
<td>9.35±2.24*</td>
<td>89.38 ±14.39*</td>
<td>18.78</td>
</tr>
</tbody>
</table>

* The data was significant differences (*p*<0.01) between average obtained by LSD test.

**Effect of Ionic Strength of Gelatin Solution on the Mechanical Properties of Shellac and Gelatin Composite Film**

Table 3 shows the effect of ionic strength by the addition of NaCl at 1-4 % w/w on the mechanical properties of composite films. The puncture strength of composite film decreased from 15.30 to 9.35 MPa while the elongation of composite film increased from 27.13 to 89.38 when the NaCl was increased to 4% w/w. It was due to the higher amount of NaCl causing the enhancement of the solubility of gelatin in shellac solution, resulting in the formation of homogeneous composite solution. The high content of electrolyte could interfere in the intermolecular force of gelatin causing the reduction in the puncture strength while the higher water content was obtained giving the increase in the elongation. The water content of film increased from 12.46 to 18.78 % when the NaCl was added up to 4% w/w. The result showed the similar manner with the addition of plasticizers (Porter, 1990).

**Effect of Mixing Equipment on the Mechanical Properties of Shellac and Gelatin Composite Film**

The condition used to study for the effect of mixing equipment such as mechanical stirrer, ultrasonic generator and homogenizer to prepare the shellac and gelatin composite solution on the mechanical properties of composite film was 1%w/w NaCl, pH 6.0. The ultrasonic technique gave the suitable technique for the preparation of the composite film solution, indicated by the higher percentage of elongation of composite film, attributing to the improved flexibility of the film as shown in Table 4.

This was the result of the higher mixing efficiency of ultrasonic technique giving the homogeneous solution confirmed by the SEM micrograph as shown in Figure 2.

**Table 4** Effect of mixing equipment on the mechanical properties of shellac and gelatin composite films

<table>
<thead>
<tr>
<th>Technique</th>
<th>Puncture strength (MPa)</th>
<th>Elongation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirrer</td>
<td>17.25 ± 2.88</td>
<td>37.65 ± 3.89</td>
</tr>
<tr>
<td>Ultrasonic</td>
<td>14.50 ± 2.65</td>
<td>43.39 ± 11.75</td>
</tr>
<tr>
<td>Homogenizer</td>
<td>11.84 ± 2.25*</td>
<td>39.90 ± 7.54</td>
</tr>
</tbody>
</table>

* The data was significant differences (*p*<0.01) between average obtained by LSD test.

![Figure 2 SEM micrograph of shellac and gelatin composite films when prepare by stirrer (2a), ultrasonic technique (2b) and homogenizer (2c)](image-url)
Conclusions

The mechanical properties of composite film based on shellac and gelatin could enhance by various factors such as the content of gelatin, temperature, pH and mixing equipment. The high content of gelatin could enhance the mechanical properties while the high pH and temperature caused the destruction of protein structure, leading to the significant decrease in the strength and increase in the elongation of shellac and gelatin composite film. The addition of NaCl and the mixing by ultrasonicator could enhance the solubility of gelatin film, resulting in the homogeneous of composite film, causing the change in the mechanical properties of composite film. Therefore, the understanding of the process of preparation of composite film could enhance the mechanical properties of composite films and contribute to the benefit of edible film coating for the food and pharmaceutical industries.

Acknowledgments

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Off-flavor in Tilapia (*Oreochromis niloticus*) Reared in Cages and Earthen Ponds in Northern Thailand

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Abstract

Tilapia, the highest produced fish in Thailand, is facing problems in the fish product quality, especially the contamination of off-flavor (geosmin and 2-methylisoborneol, MIB) in fish flesh. The aim of this study was to compare off-flavor levels in fish from two different cultures, cage and earthen pond. The samples of fish flesh from the two types of culture were analyzed for off-flavor by solid phase microextraction (SPME) and by gas chromatography mass spectrometry (GC-MS). The results revealed that geosmin and MIB levels in pond water samples were higher than those obtained from cages (*P*<0.05). Both geosmin and MIB levels from the fish samples cultured in the cages were 4 and 1.9 times lower than those cultured in the earthen ponds (*P*<0.05), respectively. The levels of geosmin in cages and earthen ponds were 0.66±0.11 and 2.61±0.51 µg kg⁻¹, respectively, while the levels of MIB in cages and earthen ponds were 2.45±0.50 and 4.55±0.59 µg kg⁻¹, respectively. In addition, it was found that 87.5% of tilapia from cage culture contained geosmin that were lower than the threshold level, which is set at 0.9 µg kg⁻¹. Cyanobacteria causing off-flavor, *Oscillatoria tenuis* Gardiner, *Anabaena circinalis* Kütz and *Pseudanabaena catenata* Lauterborn detected from the water samples from the cage culture were less than those from the earthen ponds. In terms of odor contamination, tilapia flesh derived from cage culture provided a lower possibility of off-flavor contamination than those from earthen ponds.

Keywords: off-flavor, geosmin, MIB, tilapia, cage, earthen pond

Introduction

A common problem of growing concern with freshwater fish production in earthen ponds is unpleasant muddy-odors and off-flavor leading to low quality of fish flesh for export (Smith et al., 2008) Tilapia is among those fish susceptible to such problems because they are normally raised in earthen ponds. Substances causing unpleasant odors and off-flavors include geosmin and 2-methyl-isoborneol (MIB) (Izaguirre et al., 1982) which are found in aquaculture systems integrated with livestock farming, such as chicken and pig as well as in water source with high concentrations of nutrients.

Fish can absorb geosmin and MIB directly by osmosis through their skin and/or by ingesting food contaminated with the substances which can then accumulate in their flesh (Tanchotikul, 1990). This contamination causes low-quality fish flesh leading to lower prices and affects the fisheries industry as a whole. Geosmin and MIB in aquaculture are...
produced by blue-green algae, such as Anabena sp., Symploca sp. and Oscillatoria sp. and some actinomycetes bacteria. Klausen et al. (2006) reported the accumulation of actinobacteria, producing 2-9 ng L\(^{-1}\) geosmin, in fish-culture ponds, whereas the levels of geosmin and MIB found in the water source were 55% and 100% higher than those found in the pond water, respectively. At present, the acceptable threshold level of geosmin is 0.9 µg L\(^{-1}\) (Robertson et al., 2006) while that of MIB is 0.6 µg L\(^{-1}\) (Persson, 1980).

In Thailand, most tilapia are raised in earthen ponds, especially in the “green culture system ” where fertilizer is added into the culture water to induce natural growth of algae as feeds for the fish, hence reducing capital investment. This study compared the unpleasant muddy-odor level in culture-water, as well as off-flavor levels in tilapia flesh from 2 different culture methods, cages and earthen ponds.

**Materials and Methods**

**Study Design and Site**

The experiment was carried out in a private fishery from (month) to (month) (year), in Phan District of Chiang Rai Province, Thailand where 9 experimental-cages were hung in a 21,600 m\(^2\) earthen pond with 6 m depth. The dimension of the experimental-cage was 3.0x4.0x1.5 m in size. Tilapia were raised with the density of 60 fish per m\(^3\) in average. The earthen ponds experiment was carried out in 9 ponds where each pond was about 5,600 m\(^2\) in area with 1.5 m depth. The stocking density in ponds was 3 fish per m\(^2\). Both culture methods used commercial pellets. Throughout the study, 10-20% culture-water of both culture methods was replaced once a month.

**Sampling and Analysis**

After 4 months before harvest, 27 tilapias were obtained from the cage-culture where 3 fish were sampled from each of 9 cages. Similarly, twenty-seven 5-month old tilapias were obtained from the earthen pond-culture where 3 fish were sampled from each of 9 ponds. The average weight of tilapia samples from the cage-culture was 497.0±40.7 g whereas that of the fish sampled from the earthen pond-culture was 502.0±22.1 g. All sampled tilapias were then filleted and the flesh was kept at 20°C.

Off-flavor contamination in the water and fish flesh was analyzed by SPME and GC-MS (Grimm et al., 2004). Five grams of ground fish flesh was put into 20-ml vials before 10 ml of methanol was added followed with 1.9 g Sodium Chloride. For water samples, 10 ml of water sample was put into 20-ml vials and 1.9 g sodium chloride was added. All vials were closed with high-temperature tolerant rubber stoppers and aluminum caps. Each experimental-vial was placed in an electromagnetic stirrer on a hot-plate at 70°C for 5 minutes before injection of SPME fiber into the vial. The fiber was exposed for 12 minutes to allow the fiber to capture geosmin and MIB in the sample. The SPME fiber was then removed from the vial and injected into GC-MS Agilent Technologies 6890 N Network GC System. The splitless-mode was used through capillary column (DB-DURABOND) HP-5 (30m. × 0.32mm. × 0.25µm film-thickness), using Helium gas as the carrier with a flow rate of 2.5 ml min\(^{-1}\). The process was run for 5 minutes then the SPME fiber was removed from the device. The standard geosmin and MIB samples from Sigma Company were used for calibration in this study.

**Water Quality Analysis**

Physico-chemical and biological parameters for quality of water sampled from both experimental cage and earthen pond cultures were determined. The physico-chemical parameters included; dissolved oxygen (DO); water temperature; turbidity—using TOA multimeter version WQC-22A;
alkalinity-following titration method Boyd and Tucker (1992); chlorophyll-a adapting methods of Wintermans and De Mots (1965) and Saijo (1975); NO\textsubscript{3}-N-following phenate method Boyd and Tucker (1992); NO\textsubscript{2}-N following diazotizing colorimetric method Boyd and Tucker (1992); NO\textsubscript{3}-N following cadmium reduction method Boyd and Tucker (1992); and PO\textsubscript{4}-P -following stannous chloride method Boyd and Tucker (1992). The biological parameters include phytoplankton, species and abundance; and total cyanobacteria cell number following method of Wongrat and Boonyapiwat (2003).

**Data Analysis**

Data obtained from the analysis, especially the levels of geosmin and MIB found in sampled tilapia flesh and culture-water, as well as, chlorophyll-\(a\) and total cyanobacteria cell number were treated with paired t-test analysis.

**Results**

**Levels of Geosmin and MIB Found in Culture-water From Cage and Earthen Pond Cultures, Chlorophyll-\(a\) and Cyanobacteria Count**

The levels of both geosmin and MIB found in the water sampled from the cage-culture were significantly lower than those sampled from the earthen pond-culture (\(\ast P<0.05\)). The levels of geosmin and MIB found in the fish flesh sampled from the cage-culture were 0.66±0.11 \(\mu g\) kg\(^{-1}\) and 2.45±0.50 \(\mu g\) kg\(^{-1}\), while those sampled from the earthen pond-culture were 2.61±0.51 \(\mu g\) kg\(^{-1}\) and 4.55±0.59 \(\mu g\) kg\(^{-1}\), respectively (Table 1).

Geosmin and MIB levels in fish flesh from the cage-culture were 4.0 and 1.9 times less than those from earthen pond-culture. In addition, 87.5\% of tilapias from the cage-culture contained lower geosmin and MIB level than the acceptable threshold which is 0.9 \(\mu g\) kg\(^{-1}\) (Table 2).

**Table 1** Geosmin and MIB levels in water samples and in the fish flesh from two culture systems (n=9)

<table>
<thead>
<tr>
<th>Culture system</th>
<th>Water sample (mean±se)</th>
<th>Fish flesh sample (mean±se)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geosmin ((\mu g) L(^{-1}))</td>
<td>MIB ((\mu g) L(^{-1}))</td>
</tr>
<tr>
<td>Cage</td>
<td>0.92±0.08(^a)</td>
<td>3.10±0.15(^a)</td>
</tr>
<tr>
<td>Earthen pond</td>
<td>24.56±7.11(^b)</td>
<td>26.26±6.56(^b)</td>
</tr>
</tbody>
</table>

*Values in the same column, not sharing superscript letters are significantly different (\(^\ast P<0.05\))

**Table 2** Geosmin and MIB levels, percentage of samples containing geosmin and MIB lower than threshold level (0.9 \(\mu g\) kg\(^{-1}\) of geosmin and 0.6 \(\mu g\) kg\(^{-1}\) of MIB) in tilapia cultured in cage and earthen pond cultures

<table>
<thead>
<tr>
<th>Culture system</th>
<th>Geosmin</th>
<th>MIB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Samples containing geosmin lower than threshold level</td>
<td>T2/T1</td>
</tr>
<tr>
<td>Cage</td>
<td>0.66±0.11(^a)</td>
<td>87.5%</td>
</tr>
<tr>
<td>Earthen pond</td>
<td>2.61±0.51(^b)</td>
<td>0.0%</td>
</tr>
</tbody>
</table>

*Values in the same column, not sharing superscript letters are significantly different (\(^\ast P<0.05\))
Both chlorophyll-a and total cyanobacteria number found in the water sampled from the cage-culture were significantly lower than those sampled from the earthen pond-culture (*P<0.05) (Table 3 and Figure 1).

Cyanobacteria species causing off-flavor found in the water sampled from the cage-culture included *Anabaena circinalis* Kütz, *Oscillatoria tenuis* Gardiner and *Pseudanabaena catenata* Lauterborn, whereas those found in water sampled from the earthen pond-culture included *A. circinalis* Kütz, *A. macrospora* Klebahn, *O. limosa* C. Aghardh and *O. tenuis* Gardiner, *P. catenata* Lauterborn and *P. limnetica* (Lemmermann) (Table 4).

**Table 3** Chlorophyll-a content and total cyanobacteria number of cells in water from the two culture systems

<table>
<thead>
<tr>
<th>Culture system</th>
<th>Water samples (mean±se)</th>
<th>Water samples (mean±se)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chlorophyll-a (µg L⁻¹)</td>
<td>Total cyanobacteria numbers of cells (x10³ cells m L⁻¹)</td>
</tr>
<tr>
<td>Cage</td>
<td>401.35±21.11ᵃ</td>
<td>26.98±2.72ᵃ</td>
</tr>
<tr>
<td>Earthen pond</td>
<td>922.08±143.74ᵇ</td>
<td>63.56±13.12ᵇ</td>
</tr>
</tbody>
</table>

*Values in the same column, not sharing superscript letters are significantly different (*P<0.05).*

**Figure 1** Numbers of cells of *Oscillatoria* sp., *Anabaena* sp. And *Pseudanabaena* sp. (mean±se) in the water samples from cage and earthen pond cultures.

**Table 4** Off-flavor causing cyanobacteria found in both cultures

<table>
<thead>
<tr>
<th>Cage-culture</th>
<th>Earthen pond-culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Anabaena circinalis</em> Kütz</td>
<td><em>A. circinalis</em> Kütz</td>
</tr>
<tr>
<td><em>Oscillatoria tenuis</em> Gardiner</td>
<td><em>A. macrospora</em> Klebahn</td>
</tr>
<tr>
<td><em>Pseudanabaena catenata</em> Lauterborn</td>
<td><em>O. limosa</em> C. Aghardh</td>
</tr>
<tr>
<td><em>Pseudanabaena catenata</em> Lauterborn</td>
<td><em>P. catenata</em> Lauterborn</td>
</tr>
<tr>
<td><em>P. limnetica</em> (Lemmermann)</td>
<td><em>P. limnetica</em> (Lemmermann)</td>
</tr>
</tbody>
</table>

**Water quality during the experiment**

Water quality of both treatment cultures throughout the duration of the experiment met the required aquaculture standard (Boyd and Tucker, 1992). The values of studied parameters for the water sampled from the cage-culture and the earthen pond culture were presented in Table 5.

**Table 5** Water qualities of the culture systems

<table>
<thead>
<tr>
<th>parameters</th>
<th>Cages (mean±se)</th>
<th>Earthen ponds (mean±se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5±0.0ᵃ</td>
<td>8.1±0.2ᵇ</td>
</tr>
<tr>
<td>DO (mg L⁻¹)</td>
<td>4.6±0.1ᵃ</td>
<td>9.4±0.6ᵇ</td>
</tr>
<tr>
<td>Alkalinity (mg L⁻¹)</td>
<td>43.7±1.5ᵃ</td>
<td>92.0±7.8ᵇ</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>24.7±1.9ᵃ</td>
<td>215.5±47.6ᵇ</td>
</tr>
<tr>
<td>NH₄-N (mg L⁻¹)</td>
<td>0.40±0.02ᵃ</td>
<td>0.63±0.10ᵇ</td>
</tr>
<tr>
<td>NO₂-N (mg L⁻¹)</td>
<td>0.12±0.00ᵃ</td>
<td>0.24±0.09ᵇ</td>
</tr>
<tr>
<td>NO₃-N (mg L⁻¹)</td>
<td>0.16±0.02ᵃ</td>
<td>0.12±0.02ᵇ</td>
</tr>
<tr>
<td>PO₄-P (mg L⁻¹)</td>
<td>0.15±0.01ᵃ</td>
<td>0.45±0.08ᵇ</td>
</tr>
</tbody>
</table>

*Values in the same row, not sharing superscript letters are significantly different (*P<0.05).*

**Discussion**

Tilapias are usually raised in ponds with high densities of phytoplankton which served as natural food source for fish. Such
phytoplankton could lead to higher possibility of getting more off-flavor caused by cyanobacteria in the water. The present study showed a significantly higher amount of chlorophyll-a in ponds compared to that of the cage-culture.

Results showed that off-flavor causing substances; geosmin and MIB, were detected in higher levels in the earthen pond culture relative to the cages. Accordingly, off-flavor causing cyanobacteria were also detected more abundantly in the ponds. The result was similar to a study conducted by Van Der Ploeg and Boyd (1991) where cyanobacteria found more abundant include Oscillatoria sp. and Anabaena sp. Other off-flavor producing blue-green algae such as Aphanizomenon sp., Lyngbya sp., Planktothrix sp. and Phormidium sp. were also detected.

Some actinomycetes bacteria, such as Streptomyces tendae, S. avermitilis and Penicillium expansum can cause the off-flavor in fisheries (Jüttner and Watson, 2007). Such bacteria were also reported in the sediment at the bottom of the fish ponds. Tilapia raised in ponds, can be contaminated with off-flavor causing substances from the sediment at the pond bottom, whereas the fish raised in the cage hung in the culture-water have less possibility to be contaminated from the sediment. Moreover, the off-flavor causing substances (geosmin and MIB) can also exist as colloid-particles in the culture-water. Very high levels were detected in the pond-water reflecting a high turbidity.

Table 6 shows the geosmin and MIB levels found in different fin fishes (Johnsen and Lloyd, 1992) and shrimp (Lovell and Broce, 1985) including this study. Geosmin and MIB levels in tilapias from the present study were comparatively higher than other studies which could be due to different pond management, sediment structure and different amount of off-flavor sources.

Cyanobacteria such as Oscillatoria spendida, O. brevis, O. tenuis, Lyngbia subtilis and L. allogei which are off-flavor causing cyanobacteria can grow in benthic form living on the surface of side-net and in the bottom part of the cage (Jüttner and Watson, 2007). In addition, some benthic cyanobacteria can produce off-flavor substances that cause offflavor taints at extremely low concentration in fish (Sugiura et al., 1998).

The acceptable threshold level of geosmin is 0.9 µg kg⁻¹ (Robertson et al., 2006), while the threshold level of MIB is 0.6 µg kg⁻¹ (Persson, 1980). In the present study 87.5% of tilapias sampled from the cage-culture have lower geosmin level than the threshold level. The study by Yamprayoon and Noomhorm (2000) shows that geosmin and MIB can get into the fish within a short period of time but require a much longer period, can be in weeks, in order to get rid of them.

Interestingly, geosmin has been found in this study to be less prevalent than MIB in both pond and cage cultures (Table 1). In water, geosmin and MIB are degraded by microbial activities (Izaguirre, 1992). However, this degradation is slow with geosmin being biodegraded in approximately 3 days and with MIB appearing more resistant with a degradation time ranging from 5 to 14 days (Izaguirre et al., 1988). This difference between geosmin and MIB degradation could speculatively explain the latter’s prevalence in both cage and earthen pond cultures and why all values obtained for MIB were all above its threshold level.

Therefore, prior to harvest and marketing, fish should be carefully checked for muddy odors and off-flavors. Changing culture-water regularly and/or storing fish in a tank with clean running water and aeration after harvesting, to give the fish time to purge their gills and guts of any off-flavor can help reduce the contamination.
Conclusions

This study found the levels of off-flavor substances in water and tilapia from cage cultures were significantly lower than those from earthen ponds (\(P<0.05\)). Cyanobacteria were also found more abundantly in the earthen pond culture than in the cage. In addition, 87.5% of tilapia samples from the cage-culture have lower geosmin level than the threshold level of 0.9 \(\mu g \, kg^{-1}\). Therefore, it can be concluded that tilapia raised in the cages have a lower possibility of being contaminated with off flavor than those raised in earthen ponds.

Table 6 Geosmin and MIB in various kinds of fin fish and shrimp

<table>
<thead>
<tr>
<th>Fin fish / shrimp</th>
<th>Geosmin ((\mu g , kg^{-1}))</th>
<th>MIB ((\mu g , kg^{-1}))</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td>–</td>
<td>0.055</td>
<td>Persson, 1980</td>
</tr>
<tr>
<td>Channel catfish</td>
<td>0.25-0.50</td>
<td>0.10-0.20</td>
<td>Grimm et al., 2004</td>
</tr>
<tr>
<td>Channel catfish</td>
<td>–</td>
<td>20.1-20.8</td>
<td>Johnsen and Lloyd, 1992</td>
</tr>
<tr>
<td>Shrimp</td>
<td>78</td>
<td>–</td>
<td>Lovell and Broce, 1985</td>
</tr>
<tr>
<td>Channel catfish</td>
<td>0.7</td>
<td>–</td>
<td>Dionigi et al., 2000</td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cage</td>
<td>0.66±0.11</td>
<td>2.45±0.50</td>
<td>Present study</td>
</tr>
<tr>
<td>Earthen ponds</td>
<td>2.61±0.51</td>
<td>4.55±0.59</td>
<td></td>
</tr>
</tbody>
</table>

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References


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Effect of Germination on Antioxidative Property of Pigmented and Non-Pigmented Rice

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Abstract

The objective of this study was to determine effect of germination time on total phenolic content (TPC) and % DPPH radical inhibition as determine by DPPH scavenging method of rice (Oryza sativa L). The non-pigmented rice (non-waxy rice variety Phitsanulok 2) and 2 pigmented rice (black glutinous; local name Niew Dam, and black non-waxy rice; local name Hom Nil) were used in this study. Rice grains with husk or without husk were steeped in water for 12 hrs or 6 hrs, respectively, before further left to germinate for 0, 6, 12, 18, and 24 hr. Rice grains without husk showed higher germination rate. After germination, the results showed that average TPC of pigmented rice (Niew Dam 1.14 and Hom Nil 0.87 mg gallic acid equivalent/g rice) were higher than that of non-pigmented rice (0.22 mg gallic acid equivalent GAE/g rice). The same trend was found for average percent inhibition (54.61%, 41.90%, and 14.05% for Niew Dam, Hom Nil, and Phitsanulok 2, respectively). Pigmented-germinated rice prepared from rice grains without husk showed lower TPC and % DPPH radical inhibition compared to those of control (ungerminated) and thoseone prepared from grains with husk. Compared to pigmented rice, the non-pigment rice (Phitsanulok 2) was the least affected by germination process and germination time as far as the TPC and %DPPH radical inhibition were concerned. Since pigmented rice contain more bioactive compounds, thus rice with husk intact should be employed for the preparation of pigmented - germinated rice to protect TPC loss during germination.

Keywords: antioxidant, germination, pigmented rice

Introduction

In Thailand, pigmented rice varieties are differently called depending on the color of grain including black, purple, or red. In pigmented rice, there are natural color substances that belong to the flavonoid group called anthocyanins. Positive health effects of the pigments present in the bran layer of rice have been reported. A commonly found anthocyanin in red and black rice is acetylated procyanidins, which is reported to possess a free radical scavenging activity (Oki et al., 2002). Recently, germinated rice receives more attention from consumers, especially those who are health conscious due to its health benefit. Germination of seeds commences with the uptake of water by the dry seed via a process called imbibition and is completed when a part of the embryo, usually the radicle, extends to penetrate the structures that surround it (Bewley, 1997). The brown rice is usually available as a raw material for making germinated rice. However, pigmented rice becomes an alternative choice for increasing benefit from germinated rice intake. Nevertheless, there are not much existing scientific data to support the combined effect of germination and roles of pigment in rice. The objective of this study was therefore to determine effect of germination on total phenolic content and antioxidative property of pigmented and non-pigmented rice.
Materials and Methods

Chemicals
Gallic acid and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO). Folin–Ciocalteu reagent was purchased from Merck (Germany). All other reagents and solvents used were of analytical grade.

Rice Samples
The rice samples compose of 3 varieties of rice including a non-pigmented rice (Phitsanulok 2 variety) and 2 pigmented rice (black waxy rice or Niew Dam and black non-waxy rice or Hom Nil variety). All rice samples were harvested from Pichit province, Thailand.

Germination of Rice Samples
Two forms of rice were prepared; (1) grains with husk intact and (2) grains with the husk removed. Rice grains, both with and without husk (100 g) of all three cultivars were steeped in water at room temperature for 12 hrs. and 6 hrs for grains with husk and without husk, respectively. Water was drained off and grains were washed again. Grains were then left to germinate in the dark at room temperature for 0, 6, 12, 18, and 24 hrs. After germination, the rice grains were dried at 50 degree celsius for 3 hour (moisture content was less than 13%).

Extraction of Antioxidants
The husks of grains were removed before analysis. The extraction for each sample was performed in duplicate. The method of Jang and Xu (2009) with slight modification was used for extraction step. Briefly, one gram of each sample was transferred into a test tube (25 x 150 mm.) to which methanol (3 mL) was added, and the mixture was vortex mixed for 30 sec. The test tubes were capped and placed in a 60 °C water bath for 20 min. These test tubes were vortex mixed twice during the incubation. Then, the methanol layer in each tube was separated by centrifugation at 10,000 rpm for 10 min. The solvent supernatant was transferred to a 10 mL volumetric flask. The residue was again mixed with 3 mL of methanol. The supernatant was separated as previously described and combined with the previous supernatant. The tube containing supernatant was adjusted to 10 ml. The extracted solution was kept in 0 °C until analysis.

Determination of Total Phenolic Content (TPC)
The Folin-Ciocalteau reagent method was employed to determine the TPC content in the rice bran extracts according to Jang and Xu (2009). The Folin-Ciocalteau reagent was diluted 10 times with deionized water. The rice extract 0.1 mL was placed in the test tube, which contained 0.75 mL of diluted Folic-Ciocalteau reagent. The reaction was carried out at 25 °C for 5 min in a dark room. Then 0.75 mL of sodiumbicarbonate solution (60 g L-1) was added. The mixture was incubated at 25 °C for 90 min and filtered through a 0.45 µm syringe filter. The absorbance of the filtered solution was determined at 750 nm using a UV-visible spectrophotometer (Thermo Spectronic Genesis 20). Gallic acid was used to prepare a standard curve. The TPC of the extract was calculated and expressed as mg gallic acid equivalent per g of rice (mg GE g⁻¹).

Determination of Antioxidant Activity Using the DPPH Free Radical Scavenging Method
The DPPH free radical scavenging capability in the extract was determined using the method of Yue and Xu, 2008. The DPPH reagent (0.025 g) was dissolved in 1000 mL of methanol for preparing the DPPH reagent solution. The bran extract solution which was reconstituted with 6.0 mL of methanol for being employed to measure TPC was used for the DPPH free radical scavenging test. Two milliliters of DPPH solution was mixed with 50, 100, and 150 µL of the extract/methanol solution and transferred...
to a spectrophotometer cuvette. The reaction solution was carried out at 25 °C for 30 min in a dark room. Then the absorbance of the reaction mixture was monitored at 515 nm using a UV-visible spectrophotometer. The inhibition percentage of the absorbance of the DPPH solution was calculated using the following equations:

\[
\text{Inhibition (\%)} = \left(\frac{\text{Abs}_{t_{0 \text{ min}}} - \text{Abs}_{t_{30 \text{ min}}}}{\text{Abs}_{t_{0 \text{ min}}}}\right) \times 100
\]

Where \(\text{Abs}_{t_{0 \text{ min}}}\) was the absorbance of DPPH at zero time and \(\text{Abs}_{t_{30 \text{ min}}}\) the absorbance of DPPH after 30 min of incubation for the reaction.

**Results and Discussion**

**Total Phenolic Contents**

The total phenolic contents in the methanolic extracts of rice grains, both germinated from grains with and without husk, were expressed as mg gallic acid equivalents/g rice and shown in Fig. 1. Ungerminated rice was used as a control for each rice variety. In general, pigmented rice shows higher TPC than that of non-pigmented rice. For non-pigmented rice (Phitsanulok 2), it was found that TPC of germinated rice prepared from with and without husk was not significantly different from that of control.

For pigmented rice, TPC of germinated rice prepared from grains with husk was not significantly different from control, but showed more TPC than those prepared from rice grain without husk. The average TPC of germinated Phitsanulok 2, Niew Dam, and Hom Nil rice was 0.22, 1.14, 0.87 mg GAE/g rice, respectively. This means that the average TPC of pigmented rice was higher than that of non-pigmented rice. Upon germinating of grains without husk, pigmented rice lost some phenolic content. Since this study focused on total phenolic content in rice, it was inconclusive of which part that have been lost during germination. However, it was noticed that the water used for steeping rice grain turned to black color at the end of soaking step. This water might contain some water soluble phenolic compounds and could be a partial reason for lost of phenolic content in pigmented rice. However, in-depth cause of this phenomenon requires verification through further experiments. Three major phenolic compounds found in different fraction of Khao Dawk Mali 105 have been reported as ferulic, vanillic, and p-coumaric acids (Butsat and Sirimornpun, 2010). Data regarding different types and contents of antioxidant in whole grain cereals have been collected and reported by Fardet et al. (2008) that polyphenol content in rice (brown and black) ranges from 0.54-3.13 mg/g. In addition, Choi et al (2007) reported that polyphenol content of methanolic extracts from some grains consumed in Korea, including white rice, black rice, and brown rice were 0.18, 3.13, and 0.54 mg GAE/g rice, respectively. The wide range in those reported value is due to differences in many factors such as variety, growth site, and antioxidant components. However, the TPC of rice varieties used in this study fall within those reported range, in which black waxy rice (Niew Dam) exhibited the highest TPC compared to the other samples.
The DPPH radical inhibition percentage of rice is shown in Fig. 2 and it was found that inhibition percentage of germinated Phitsanulok 2 rice prepared from grains with husk was not significantly different from that of control but was higher than that of prepared from grains without husk. In the pigmented rice, inhibition percentage of Niew Dam rice prepared from grains with husk was not significantly different from the control. However, black non-waxy rice (Hom Nil rice) showed a lower DPPH radical inhibition than that of control even though it was prepared from grains without husk. This may be due to the fact that the husk of Hom Nil rice was thinner than the husk of Niew Dam rice. As a result, some antioxidants in Hom Nil rice grain might be lost or bleached out during the germination step. The average inhibition percentages showed a similar trend as found for TPC (14.05, 54.61 and 41.90% for Phitsanulok 2, Niew Dam, and Hom Nil, respectively). This implies that the inhibition percentage of pigmented rice was higher than that of non-pigmented rice. It is known that colored rice (black, red, or purple) contains pigments that play important role in antioxidative property. The anthocyanins in pigmented rice have been identified as cyanidin 3,5-di-glucoside, cyanin 3-glucoside and peonidin 3-glucoside (Abdel-Aal et al., 2006; Ryu et al., 1998). Amongst the group of pigmented rice, Niew Dam exhibited the highest TPC and strongest antioxidant abilities in terms of DPPH radical inhibition percentage, followed by Hom Nil.

The correlation between TPC and the DPPH radical inhibition percentage of pigmented and non-pigmented rice is shown in Figure 3-5. In general for all three rice varieties, it was found that when TPC increased, the DPPH radical inhibition percentage also increased. This indicated that the germinated rice, both prepared from grains with and without husk, showed a positive correlation between TPC and inhibition percentage.
Figure 2  DPPH radical inhibition percentage of germinated rice. In parenthesis, the first and second numbers indicate soaking times and germination times (hrs.), respectively Different small letters (a,b,c) indicate statistically different TPC within the same variety (p ≤ 0.05) by DMRT Different capital letters (A,B,C) indicate statistically different TPC between varieties of rice germinated in the same condition (p ≤ 0.05) by DMRT

Figure 3 Correlation between TPC and the DPPH radical inhibition percentage of non-pigmented rice (Phitsanulok 2 variety)

Figure 4 Correlation between TPC and the DPPH radical inhibition percentage of black waxy rice (Niew Dam variety)
Conclusions

The pigmented rice contained more TPC and DPPH radical inhibition percentage than that of non-pigmented rice. The rice prepared from grains with husk retained higher levels of TPC and DPPH radical inhibition percentage than rice prepared from grains without husk. Study of germination effect on rice grains revealed that there are many cellular and metabolic changes undergone after imbibition of grains and during germination. Husk of rice grains play important role in protection of some bioactive compound loss of pigmented rice during a soaking step. Therefore, pigmented rice in the form of grains with husk intact is recommended as a raw material for preparation of germinated rice. Finally, all rice samples, both prepared from rice grains with and without husk, show the positive correlation between TPC and the DPPH radical inhibition percentage.

Acknowledgment

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References

Antioxidant and Anti-inflammatory Activities of Freshwater Macroalga, *Cladophora glomerata* Kützing

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Abstract

*Cladophora glomerata* Kützing is an edible filamentous alga. Macroalga has traditionally been consumed as part of an indigenous diet in northern Thailand. The belief of the local people is that the alga could serve to protect against various diseases. Therefore, the aqueous extract of *C. glomerata* (Aq. C) was examined for antioxidant and anti-inflammatory activities. The Aq. C showed antioxidant activity when tested in the 1-diphenyl-2-picrylhydrazyl (DPPH), superoxide and lipid peroxidation assays at 71, 73, and 66%, respectively. Furthermore, the Aq. C exhibited anti-inflammatory activity, as was shown by evidence obtained from the ethyl phenyl propiolate (EPP)-induced ear edema and carrageenin-induced paw edema experiments in rats. The Aq. C possessed inhibition of the ear and paw thickness at the levels of 58 and 52%, respectively. The mechanism of anti-inflammatory activity of Aq. C might be due to its inhibitory effect on the release of some inflammatory mediators. The Aq. C contained phenolic compounds, which the substances associated with antioxidant and anti-inflammatory activities. According to the acute toxicity tests in rats, no deaths or other signs of toxicity were observed. Therefore, the findings could support the premise that *C. glomerata* is safe for general consumption. From these results, the evidence substantiates the potential of *C. glomerata* as a food substance or as a nutraceutical product.

Keywords: acute toxicity tests, anti-inflammatory activity, antioxidant activity, *Cladophora glomerata*, nutraceutical

Introduction

*Cladophora glomerata* Kützing occur in abundance especially in the Nan River, Nan Province, and the Mekong River in Chiang Khong District, Chiang Rai Province. The Thai common name of the alga is “Kai”. According to the traditional wisdom of the local people, it is believed that consumption of the alga could protect against various diseases. Moreover, there has been research to process various foods from Kai, such as crisps, baked goods, paste, and noodles, in order to obtain more market products for the people in these areas (Peerapornpisal et al., 2005).

*C. glomerata* is a freshwater macroalga in Division Chlorophyta, Order Cladophorales, Family Cladophoraceae. It is green or light green, filamentous in form (Fig.1a), attached on rocks or cobble in the bed of shallow rivers. Microscopically, thalli are composed of joined cylindrical cells, with a length of 6 - 20 µm and a width of 4 -10 µm and with dichotomously branching filaments. Branches are narrowed towards the tip, cell walls are thick and usually lamellate (Fig.1b) (Peerapornpisal et al., 2006).
The ethanolic extract of *C. glomerata* was evaluated for pharmacological screening in rats. The results showed anti-gastric ulcer activity, as well as anti-inflammatory, analgesic, anti-oxidant, and hypotensive activities and suggested its value as a therapeutic agent (Peerapornpisal *et al*., 2006).

Free radical oxidative stress has been implicated in the pathogenesis of a variety of human diseases. Natural antioxidants have been found to be effective in the treatment of many diseases. In recent years it has become increasingly apparent that, free radicals play a role in a variety of functions of the normal regulatory system, the deregulation of which may play an important role in inflammation (Winrow *et al*., 1993). Therefore, the objectives of this research were to examine both the antioxidant and anti-inflammatory activities of the aqueous extract of *C. glomerata* for the potential development of these algae to be used in nutraceutical products.

### Materials and Methods

#### Preparation of the Aqueous Extract of *C. glomerata*

Fresh *C. glomerata* was collected from the Nan River, Tha Wang Pha District, Nan Province, Thailand. The alga was washed in tap water and rinsed with deionized water before being dried in an oven at 60 °C for 48 h. A 200 g sample of the dried alga was pulverized into a fine powder and boiled with 1 L of distilled water at 90 -100°C for 2 h and then filtered through Whatman’s No.4 filter paper. The filtrate was evaporated *in vacuo* and lyophilized to obtain a dry extract. The yield of aqueous extract was 37.83% from the dried alga. Figure 2 shows the extraction of the aqueous extract of *C. glomerata* (Aq. C) to be used in testing for its biological activities. The extract was dissolved in distilled water to the required concentrations before being used.

![Figure 1 Cladophora glomerata Kützing](image1)

![Figure 2 Extraction protocol and work plan for biological activities of C. glomerata](image2)
Phytochemical Testings

**Determination of Phenolic Compounds**

Total phenolic content was determined according to Folin-Ciocalteu Method (Hammerschmidt and Prat, 1978). Briefly, 0.2 ml of the sample (Aq. C) solution was mixed with 1.0 ml of 10% Folin-Ciocalteu solution and 0.8 ml of 7.5% sodium carbonate solution. The mixture was incubated for 1 hr at room temperature. The absorbance at 760 nm was measured and converted to phenolic content according to the calibration curve of gallic acid.

**Gas Chromatography - Mass Spectrometry (GC-MS) Analysis**

Aq. C was used to determine the quantitative chemical analysis by GC-MS, a service provided by the Science and Technology Service Center, Chiang Mai University. The Aq. C was analyzed by GC 6890 (Agilent Technologies Co., Ltd. USA) using a HP-5MS column (30m x 0.25mm i.d. x 0.25 µm film thickness), the focusing of which was coupled to a mass spectrometer (Hewlett-Packard 5973(EI)). The analysis was carried out on the basis of MS spectra and the results were compared with the database (Wiley 7n.l). The components of the extract were identified by their TIC (Total Ion Chromatogram), retention time, area percent report and library search report. The percentage of composition of the identified compounds was computed from the GC peak area.

**Biological Activities**

**Antioxidant activities**

**Scavenging Activity of DPPH (1-diphenyl-2-picrylhydrazyl) Radicals**

The Aq. C was measured for DPPH radical-scavenging activity according to the method of Mensor *et al.* (2001). Briefly, each 0.3 ml of the solution of the extract was added to 0.1 ml buffer (pH 7.9), and then mixed with 0.6 ml of 100 mM DPPH in methanol for 20 min, under light protection. The concentrations of the Aq C. were in the range of 0.1-20 mg.ml⁻¹. The absorbance at 517 nm was then determined. Deionized water was used as a blank. The DPPH radical-scavenging activity was calculated according to the following equation:

\[ \text{Scavenging activity (\%) } = \frac{(A_{517} \text{blank} - A_{517} \text{sample})}{A_{517} \text{blank}} \times 100\% \]

where, \(A_{517}\) is the absorbance at 517 nm.

\(L\)-ascorbic acid was used as a positive control. All determinations were carried out in triplicate. The IC50, which stands for the concentration required for 50% scavenging activity, was calculated from the dose-response curve.

**Scavenging Activity of Superoxide Radicals (O₂⁻)**

The superoxide anion scavenging activity of the extract was measured by the method of Nikishimi *et al.* (1972). To a 0.5 ml sample, 0.5 ml nitroblue tetrazolium or NBT (2.52 mM), 0.5 ml Nicotinamide adenine dinucleotide or NADH (624 µM) and 0.5 ml Phenazine methosulphate or PMS (120 µM) were added. The NBT, NADH and PMS solutions were prepared in 0.1 M phosphate buffer at pH 7.4. After 14 min of incubation at room temperature, the absorbance was measured at 560 nm and the scavenging activity on the superoxide anion was calculated by the following equation:

\[ \text{Scavenging activity (\%) } = \frac{(A_{560} \text{blank} - A_{560} \text{sample})}{A_{560} \text{blank}} \times 100\% \]

where, \(A_{560}\) is the absorbance at 560 nm.

All determinations were carried out in triplicate. The IC50 was calculated from the dose-response curve.

**Lipid Peroxidation Assay**

Lipid peroxide formation was measured by the method of Masao *et al.* (1993). The reaction mixture was composed of 0.2 ml of rat liver homogenate in phosphate buffer,
30 mM KCl, 0.5 mM ferrous iron, 0.06 mM ascorbic acid, and various concentrations of the Aq. C in a final volume of 1.0 ml. After the mixture was incubated at 37 °C for 1 hr, 0.4 ml of the reaction mixture was treated with 0.2 ml of 8% sodium dodecyl sulfate (SDS), 0.2 ml of 0.8% thiobarbituric acid (TBA), 1.5 ml of 20% acetic acid and 0.4 ml of distilled water. Then, the mixture was incubated in a water bath at 100 °C for 1 hr. After the mixture was cooled at (25 °C), 1 ml of distilled water and 5 ml of n-butanol were added, followed by vigorous shaking for 1 min. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The inhibition of lipid peroxide formation was calculated by the following equation:

Inhibition (%) = \( \frac{A_{532}\text{blank} - A_{532}\text{sample}}{A_{532}\text{blank}} \times 100 \)

where, \( A_{532} \) is the absorbance at 532 nm.

The inhibition was expressed as trolox equivalent antioxidant capacity (TEAC). TEAC expressed as mM Trolox per gram of sample. All determinations were carried out in triplicate. The IC50 was calculated from the dose-response curve.

**Anti-inflammatory Activity**

**Ethyl Phenylpropiolate (EPP)-induced Ear Edema in Rats**

Ear edema was induced in rats by locally applying EPP at a dose of 1 mg per 20µl per ear to the inner and outer surfaces of both ears of each rat (Brattsand et al., 1982). Male rats weighing 50-60 g were used. The Aq. C or the reference drug (phenylbutazone) was applied in the same manner in a volume of 20 µl before the application of EPP. The thickness of each ear was measured using automatic vernier calipers at 15, 30, 60 and 120 min. The edema volume of the ear and the percent edema inhibition was calculated by the following calculation:

\[
\begin{align*}
ED_x &= ET_x - ET_o \\
% ED_x &= \frac{ED_c - ED_t}{ED_c} \times 100 \\
\end{align*}
\]

where,

- \( ED_x \) = edema thickness at time \( x \)
- \( ET_x \) = ear thickness (µm) at time \( x \)
- \( ET_o \) = ear thickness (µm) before application of EPP
- \( ED_c \) = edema thickness (µm) of control group at time \( x \)
- \( ED_t \) = edema thickness (µm) of test group at time \( x \)
- % \( ED_x \) = percent edema inhibition of test group at time \( x \)

**Carrageenin-induced Hind Paw Edema in Rats**

This method was used for the investigation of the inhibitory effect of anti-inflammatory drugs on the edema formation of the rat paw induced by carrageenin (Winter et al., 1962). Male rats weighing 100-120 g were used. The Aq. C was orally given 1 hr prior to the carrageenin injection. A 1% solution of lamda carrageenin was injected intradermally into the plantar side of the right hind paw. The foot volume of each animal was determined by means of a volume displacement technique using a plethysmometer. Each paw volume was measured at 1, 3 and 5 hrs after carrageenin injection. The edema volume of the paw and the percent edema inhibition were obtained by the following calculation:

\[
\begin{align*}
EV_x &= PV_x - PV_o \\
% EI_x &= \frac{EV_x^{\text{control}} - EV_x^{\text{test}}}{EV_x^{\text{control}}} \times 100 \\
\end{align*}
\]

where,

- \( EV_x \) = edema volume at time \( x \)
- \( PV_x \) = paw volume (ml) at time \( x \)
- \( PV_o \) = paw volume (ml) measured before carrageenin injection
- %EI_x = percent edema inhibition of test group at time \( x \)

**Acute Toxicity**

The acute oral toxicity study was conducted using the limit dose test of up and down procedure according to OECD Test Guideline 425 (OECD, 2001). Female Wistar albino rats were used. The rats were fasted overnight prior to dosing. The Aq. C at the dose of 5000 mg.kg\(^{-1}\) was administered by way of a gastric feeding tube in five rats. Distilled water was used as the vehicle. Behavioural manifestations of acute oral toxicity were also noted.
Observations include changes in skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous systems, and somatomotor activity, as well as behaviour patterns. Attention should be directed to observations of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma. Each rat was observed for signs of toxicity every 15 min in the first 4 hrs, after which monitoring was continued for a period of 14 days after dosing.

**Laboratory Animals**

Male Sprague-Dawley rats were obtained from the National Laboratory Animal Center, Salaya Mahidol University, Thailand. All animals received humane care in compliance with the principles of ethics in the use of animals and the experimental procedures were approved by the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Thailand.

**Statistical Analysis**

The data were expressed as mean ± S.E. Statistical comparison between groups and was analyzed using one-way analysis of variance (ANOVA) and post hoc least-significant difference (LSD) test. All \( p \) values of less than 0.05 were considered significant.

**Results and Discussion**

**Phytochemical Testings**

**Determination of phenolic compounds**

It was found that total phenolic content of the Aq. \( C \) was \( 184 ± 2 \) mg GAE (GAE expressed as mg gallic acid per gram of extract). These compounds show a wide range of antioxidant activities in vitro and are thought to exert protective effects against major diseases, such as cancer and many cardiovascular diseases (Rice-Evans et al., 1995).

**Gas Chromatography - Mass Spectrometry (GC-MS) analysis**

The GC-MS spectra and screening analysis of the Aq. \( C \) are shown in Figure 3. It was found that the phenolic compounds, such as phynyl ester (peak 2) and methoxyl phenyl (peak 4), were shown at the retention times of 6.669 and 7.784 minutes, respectively.
Biological Activities

Antioxidant Activities

Scavenging activity of DPPH (1-diphenyl-2-picrylhydrazyl) radicals

The Aq. C. at the doses of 0.1 - 20.0 mg.ml\(^{-1}\) exhibited scavenging activity of 1 - 71%. Additionally, the Aq. C showed the 50% inhibition concentration (IC50) for a DPPH radical scavenging activity of 11.76 mg.ml\(^{-1}\), with the correlation coefficient value (r) of 0.9565.

The DPPH free radical is a stable free radical, which has been widely accepted as a tool for estimating the free radical-scavenging activities of antioxidants (Hu et al., 2004).

Scavenging activity of superoxide radicals (O\(^{2-}\))

The Aq. C was found to have the ability to scavenge O\(^{2-}\) in a concentration-dependent manner (Figure 5), with the correlation coefficient value (r) of 0.9011. The Aq. C exhibited the maximum scavenging activity of 73%, whereas that of IC50 was found to be 6.41 mg.ml\(^{-1}\).

Superoxide anion is an initial free radical formed from mitochondrial electron transport systems. It plays an important role in the formation of other reactive oxygen species, such as hydrogen peroxide, hydroxyl radicals, or singlet oxygen in living systems (Lee et al., 2004).

Lipid peroxidation assay

The percentages of inhibition of lipid peroxide formation (MDA) of the Aq. C are shown in Figure 6. The Aq. C exhibited the maximum scavenging activity of 66%, whereas that of IC50 was 21.79 mg/ml. The correlation (r) value of the extract was 0.9949. The TEAC of the Aq. C showed 0.92 mM trolox/gram of the extract.

Lipid peroxidation is a very important process in free radical pathology as it is so damaging to cells (Winrow et al., 1993). The livers of the rats were used as a source of polyunsaturated fatty acids to determine the extent of lipid peroxidation. Malondialdehyde (MDA), a lipid peroxidation product, as an indicator of...
reactive oxygen species (ROS) generation in the tissue (Rice-Evans et al., 1996).

It has been reported that phenolic substances are associated with antioxidative activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993). Interestingly, the phenolic compounds of the Aq. C were demonstrated in this study and caused the inhibition of lipid peroxide formation.

To compare the effect of antioxidant activity in different assays, the IC50 values of the Aq. C and standard substances are shown in Table 1. The positive control, L-ascorbic acid and trolox, indicated more effective antioxidant activities than the Aq. C. in three assays. However, The Aq. C showed the potential antioxidant activity when tested in DPPH, superoxide and lipid peroxidation assays at 71, 73, and 66%, respectively. The Aq.C showed a concentration-dependent antioxidant activity in all assays.

According to phytochemical testings, the Aq C showed the presence of phenolic compounds, such as phenyl ester and methoxy phenyl, substances known to possess antioxidant activities. It is therefore likely that the antioxidant activity is due to the presence of phenolic substances in the Aq. C. Phenolic substances are known to possess the ability to reduce oxidative damage and act as antioxidants (Halliwell. et al., 1987). From the results, it is suggested that the phenolic compounds in the Aq. C are the substances associated with the antioxidant activity.

Table 1 Comparison of IC50 of the aqueous extract of C. glomerata (Aq. C) and standard substances in DPPH, superoxide (O^{-2}) and Lipid peroxidation (LPO) assays

<table>
<thead>
<tr>
<th>Group</th>
<th>IC50 (mg.ml^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DPPH</td>
</tr>
<tr>
<td>Aq. C</td>
<td>11.76 ± 0.41</td>
</tr>
<tr>
<td>L-ascorbic acid</td>
<td>0.05 ± 0.002</td>
</tr>
<tr>
<td>trolox</td>
<td>-</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E. of triplicate measurements.

Anti-inflammatory Activity

Ethyl phenylpropiolate (EPP)-induced ear edema in rats

The inhibitory effects of the Aq. C on EPP-induced ear edema in rats are shown in Table 2. The Aq. C at the dose of 2.5 mg per ear inhibited marked edema formations at 1 and 2 hours. Phenylbutazone (PBZ), a nonsteroidal anti-inflammatory drug exhibited significant inhibitory effects at 30 minutes, and 1 and 2 hours. Moreover, edema thickness of the Aq. C at 1 and 2 hours was not significantly different from that of PBZ.

The inflammatory process is the response to an injurious stimulus. It can be evoked by a wide variety of noxious agents (e.g., infections, antibodies, or physical injuries). The inflammatory response includes warmth, pain, redness and swelling. The EPP-induced ear edema in rats is a useful screening model to investigate the anti-inflammatory activity of the test substance on the acute phase of inflammation (Brattsand et al., 1982). The inflammatory response provoked by EPP, is associated with a transient increase in prostaglandin (PG) production. PGs and other inflammatory mediators are capable of promoting vasodilatation and increasing vascular permeability, as well as synergistically producing edema (Carlson et al., 1985).

From the results, it is suggested that Aq. C possesses anti-edematogenic activity by inhibition of inflammatory mediators of the acute phase of inflammation.
Table 2 Effect of the aqueous extract of *C. glomerata* (Aq. C) on EPP-induced ear edema in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema thickness (µm)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15 min</td>
<td>30 min</td>
</tr>
<tr>
<td>Control</td>
<td>64 ± 10</td>
<td>126 ± 14</td>
</tr>
<tr>
<td>Phenylbutazone 1 mg per ear</td>
<td>40 ± 10</td>
<td>67 ± 12*</td>
</tr>
<tr>
<td>Aq. C 2.5 mg per ear</td>
<td>46 ± 18</td>
<td>84 ± 22</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E. (n = 6)
Significantly different from control group: *p < 0.05

Table 3 Effect of the aqueous extract of *C. glomerata* (Aq. C) on carrageenin-induced paw edema in rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Edema volume (mm$^3$)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h</td>
<td>3 h</td>
</tr>
<tr>
<td>Control</td>
<td>0.11 ± 0.02</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td>aspirin 250 mg.kg$^{-1}$</td>
<td>0.05 ± 0.03*</td>
<td>0.22 ± 0.04*</td>
</tr>
<tr>
<td>Aq. C 250 mg.kg$^{-1}$</td>
<td>0.16 ± 0.04</td>
<td>0.25 ± 0.10*</td>
</tr>
</tbody>
</table>

Data expressed as mean ± S.E. (n = 6)
Significantly different from control group: *p < 0.05

Carrageenin-induced hind paw edema in rats

The inhibitory activities of the Aq. C at the dose of 250 mg.kg$^{-1}$ showed a significant inhibitory effect at 3 hours. Aspirin, a COX-inhibitor, at the dose of 250 mg.kg$^{-1}$ exhibited significant inhibitory effects at 1 and 3 hours.

Carrageenin-induced paw edema in rats is known to be sensitive to cyclooxygenase (COX) inhibitors and has been used to evaluate the effects of the non-steroidal anti-inflammatory agents which primarily inhibit the cyclooxygenase synthesis (Winter *et al.*, 1962).

The acute inflammation has two phases: the first phase begins immediately after injection and lasts for one hour and the second phase begins after one hour and lasts for three hours (Garcia-Pastor *et al.*, 1999). Histamine and serotonin are usually responsible for eliciting the immediate response of inflammation in rats (first phase), whereas the kinins and prostaglandins mediate the more prolonged delayed onset responses (second phase) (Vane and Botting, 1987). The anti-inflammatory activity of Aq. C was depicted in EPP-induced ear edema and carrageenin-induced paw edema in rats. The mechanism of the anti-inflammatory activity of Aq. C might be due to its inhibitory effect on the biosynthesis and/or the release of some inflammatory mediators. Phenolic compounds have been reported to have biological effects including antioxidant, anti-inflammatory, anti-aging and anti-carcinogen activities (Han *et al.*, 2007). Interestingly, the Aq. C was found to possess both antioxidant and anti-inflammatory activities. It has been suggested that these activities are due to its phenolic contents in Aq.C.

Acute toxicity

There were no deaths or other signs of toxicity, including in rats that were administered with 5000 mg.kg$^{-1}$ of the Aq. C during the 14 days of observation. No gross pathologic lesions of organs, including the liver, kidney, heart, spleen, ovaries, and testes, were observed in any of the animals at necropsy. The toxicity test of the ethanolic extract of *C. glomerata* has been studied in rats. The results showed that the extract caused neither mortality, nor abnormalities in subchronic toxicity, when the ethanolic extract of *C. glomerata* was
administered orally for 60 days with at the doses of 0.5 and 1.0 mg.kg⁻¹ (Prannapus et al., 2006). Therefore, these results support the supposition that C. glomerata is safe for general use and human consumption.

Conclusions

The Aq. C was found to show both antioxidant and anti-inflammatory activities. Interestingly, the safety of the Aq. C was demonstrated according to the acute toxicity tests in rats. No signs of acute toxicity were observed. The findings provide the evidence to substantiate the nutraceutical potential of C. glomerata.

Acknowledgements

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References


Packaging Development to Support Export Supply Chain of Mangosteen Fruit

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Abstract

The changes of the quality of mangosteen fruits for export market occur during the distribution handling from production center to the shipment port and then to the destination market countries. This research was carried out to develop optimum packaging design of mangosteen fruit for export market using corrugated carton material which enable to be stacked in maximum load. The result showed that compressive strength of C-flute packaging type with inner package was 474.5 kgf, while the BC-flute packaging type was 663.2 kgf. The maximum stacking was determined based on the capacity, compressive strength of packaging and safety/environment factor in ASTM D4269 standards. The designated packaging of C-flute and BC-flute type could be maximally stacked up to 29 units and 35 units of packaged fruits, respectively. Compressive strength decreased higher under storage at 13°C (RH approximately at 90-95%) than 8°C with 60-75% RH i.e. 40.04-41.64% and 6.75-30.31%, respectively. Mechanical damage symptom occurred in C-flute type with 2x2 and 2x3 inner model during transportation was broken calyx at 1.39 and 1.67%, respectively. Packaging model under different capacity did not significantly influence firmness and TSS but significantly influence weight loss.

Keywords: compressive strength, mangosteen, mechanical damage, packaging design

Introduction

Mangosteen, The Queen of Fruit, has become one of the most popular tropical fruit in the world because of its exotic shape, taste and color. The largest import markets of mangosteen from Indonesia are China (44.4%), Hongkong (35.47%), Singapore (8.54%), expand to Middle Eastern (6.14%) and Europe (0.39%). For Indonesia, mangosteen has become the main export fruit commodity. Meanwhile, it is reported that a big shrinkage occurred in exported fruit of which only 35%-40% was accepted by importing countries (Sutrisno et al., 2009).

Packaging is conducted to maintain the safety of a product during transportation and to protect it from pollution and quality degradation as well as to make it easy in handling. The advantages of using appropriate packaging are efficiency in handling, ease in storage and distribution as well as reducing transportation and marketing cost (Hardenberg, 1986).

Box which is made of corrugated carton is a type of packaging which mostly used for transportation of goods including fruits, vegetables and other industrial goods. Corrugated carton box is very functional because of its practical for retail sale, needs little stock room and is made from material which is environmentally friendly. This type of carton box can also be designed for application which have various strengths and forms needed. However, the compressive strength of packaging box depends on various design factors. The factors affecting
the design are board component, dimension, design, dispatch condition and storage environment. Jinkarn et al. (2006) stated that corrugated board lost its compressive strength when subjected to distribution hazards such as high relative humidity, excessive stacking load, long term storage and uneven stacking pattern.

Transportation is an important link in the mangosteen agribusiness activities. In the process of transportation, fresh mangosteen damage can be caused by environmental and physical risks. Mechanical damage that occurs in agricultural products during transport can reach 32%-47%. Products experiencing mechanical damage will be more vulnerable to physiological and biological damage (Satuhu, 2004).

The objective of this research was to develop a design of mangosteen packaging for export purposes and determine the change in compressive strength and quality changes during cold storage as well as mechanical damage during transportation simulation.

**Materials and Methods**

**Materials**

Fruits used were mangosteen which was classified as export quality (major size at 61 mm - 63 mm and 100-120 gram individual weight) and 6.53 kgf bio-yields with 5.2 mm deformation. The material used for packaging was corrugated carton of BC and C-flute types for the construction of prototypes of outer packaging and B flute type for inner packaging with grammature 150.

**Methods**

Design of packaging was carried out based on packaging requirement with some conditions, i.e., number of fruits per package, dimension and weight, number of stacks, characteristics and type of carton material. Design testing was conducted on the compressive strength as indicated in Figure 1. Eight prototypes were designed in this research, conforming to the criteria previously mentioned and listed as follow:

A1B1C1, A1B1C2, A1B2C1, A1B2C2, A2B1C1, A2B1C2, A2B2C1 and A2B2C2 (A1: 2x2 inner model; A2 2x3 inner model; B1: C-flute type; B2: BC-flute type; C1 without inner; C2: with inner). The samples of design are presented in Figure 2.

Compressive strength test was carried out using two wooden plates with smooth surfaces which were attached to the upper and the lower compressive jaws of the machine to evenly distribute the compressive load on the corrugated board panel. Each wooden plate weighed 4.6 kg and sized at 48 cm x 40 cm x 4 cm. The crosshead speed was set at 10 mm/min.

The value of compressive strength in the test was used to calculate the number of stacks using the following equations (Twede et al., 2005).

\[ C_s = \frac{P}{f} \]  
\[ S = \frac{C_s}{W_b} \]

where: \( C_s \) is compressive strength; \( P \) is compressive load, \( S \) is number of stacks and \( f \) is a coefficient.

Transportation simulation was carried out by converting time needed for transporting mangosteen from production center to the collector which was held at 2.23 hours of truck with speed of 60 km/hour. The result was approximately at 3.295 Hz and 3.88 cm for 2 hours which equal with 133.878 km of road in outer city.

Observation on the mechanical damage was visually conducted by calculating the occurrence of broken calyx, fruits cracking and bruising. Fruit was considered to be dented if its skin surface was not smooth. This condition was known by means of touching and examining the skin surface. Measurement of the fruit damage was calculated by the following equation.
\[ Y_b = \frac{S_r}{S_b} \times 100 \quad (3) \]

whereas: \( Y_b \) is percentage of fruit damage; \( S_r \) is number of damage fruits (broken calyx, fruits cracking and bruising); and \( S_b \) is total number of fruits.

The observation of quality change i.e. weight loss, firmness and total soluble solids (TSS) was conducted after 10 days storage at 8 and 13°C and transportation simulation. Control treatment which was conditioned to be un-packaged and un-transported was also developed for comparison.

\[ Y_b = \frac{S_r}{S_b} \times 100 \]

**Results and Discussion**

**Model of Packaging Design**

Supply chain of mangosteen export in Indonesia involved farmers, small middle man, packing house and exporters. In this supply chain the product distribution was conducted by the use of packaging of plastic basket or wooden crate. The mechanical damage of mangosteen during the transportation simulation for wooden crate with Styrofoam partition was 5.2 %, which was higher compared to the plastic basket packaging with 3.7 % damage. The difference was because the size of the wooden crate was bigger than that of the plastic basket, so the stacking load among the fruits became bigger (Sutrisno et al., 2009). To reduce the mechanical damage during transportation, a development in packaging of mangosteen fruit was carried out using corrugated carton material. In this research, C-flute and BC-flute type with average capacity at 4.8 kg and 6.0 kg were used. Figure 3 shows the dimension of the packaging design developed.

**Compressive Strength and Maximum Stack of the Designed Package**

Compressive strength test under number of stacks was carried out during distribution using container or in warehouse during storage. Stacking caused top to bottom compression. Based on the magnitude of compressive strength, the maximum stack could be calculated. Peleg (1985) stated that there are two factors affecting the compressive strength, i.e., material of the box, like flute type carton used and type of packaging.

Compressive strength test of the packaging design with A2B2C2 experienced the highest compressive strength of 790.89 kgf (Figure 4). It also revealed that packaging using BC-flute type and 2x3 inner model had higher
compressive strength than C-flute type and 2x2 inner model, respectively. Based on the Equation 3, the number of maximum stack could be calculated, i.e., 29 stacks for 2x2 inner model with C-flute material and 35 stacks for BC-flute material.

The compressive strength of the BC-flute material was higher than that of the C-flute so that the estimated number of stacks was also higher (Figure 5).

**Change in Compressive Strength During Cold Storage**

After 10 days storage, test showed that storage at 13\(^\circ\)C with high RH (approximately at 90-95\%) experienced higher rate in reducing compressive strength than storage at 8\(^\circ\)C with 60-75\% RH i.e. 40.04-41.64\% and 6.75-30.31\%, respectively in each packaging (Table 1). In order to explain the effect of cold storage condition towards the compressive strength, the hygro-instability properties of corrugated board was best could define the phenomenon since there was no control in RH in storage room and investigated in different temperature. Hygro-instability of material is defined as the capability of material to absorb water from the ambient air which caused the deflectance of the material. The material which in this case was represented by corrugated carton would continue to absorb water from the ambient air until reach equilibrium where it was not capable to absorb water anymore. In this research, high RH caused higher decreasing rate which could be described that higher RH had higher water content so the possibility to water absorbed was also relatively high.

![Figure 3 Dimension of the packaging design](image)

![Figure 4 Compressive strength of packaging design](image)

![Figure 5 Relationship of compressive strength and number of maximum stacking](image)

**Mechanical Damage and Change in Quality of Mangosteen**

The main symptom of mechanical damage in C-flute type with 2x2 and 2x3 inner model was broken calyx at 1.39 and 1.67\% respectively which occurred because unfitted inner space of fruits which led to free movement and impact between fruits and the wall area. Position of fruits inside the package experienced 45\(^\circ\) and 90\(^\circ\) sliding which accounted at 2.77 and 1.67\%, respectively. While the mechanical damage such as breaking, cracking and bruising were not found.Darmawati et al. (2009) used 8 kg capacity packaging design with fcc pattern resulting in 3.1\% and 2.3\% damage with jumble pattern. While 15 kg packaging
Table 1 Change in compressive strength during cold storage

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>474.50</td>
<td>474.50</td>
<td>663.23</td>
<td>663.23</td>
<td>590.19</td>
<td>590.19</td>
<td>790.89</td>
<td>790.89</td>
</tr>
<tr>
<td>1</td>
<td>372.89</td>
<td>292.25</td>
<td>619.26</td>
<td>427.11</td>
<td>558.12</td>
<td>367.24</td>
<td>605.85</td>
<td>481.50</td>
</tr>
<tr>
<td>5</td>
<td>337.31</td>
<td>285.90</td>
<td>622.65</td>
<td>402.75</td>
<td>549.41</td>
<td>338.36</td>
<td>630.46</td>
<td>469.35</td>
</tr>
<tr>
<td>10</td>
<td>330.66</td>
<td>280.64</td>
<td>618.45</td>
<td>397.67</td>
<td>548.75</td>
<td>344.46</td>
<td>625.88</td>
<td>474.08</td>
</tr>
</tbody>
</table>

Design for each pattern were 7.5% and 2.5%, respectively. So that packaging design using corrugated carton of C-flute type and partition with a capacity of 4.8 kg and 6.0 kg could reduce the damage of mangosteen fruit during transportation.

Lower weight loss was encountered in packaged fruits than control i.e. 24.14% (A1B1C2), 25.04% (A2B1C2) and 27.68% (control). Statistically, weight loss in A1B1C2 was not significantly different with control, while A2B1C2 was significantly different with A1B1C2 and control. Different result was found for two others quality parameters. Three treatments did not significantly influence firmness and TSS (Table 2).

Table 2 Quality changes of mangosteen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Weight loss</th>
<th>Firmness</th>
<th>TSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1B1C2</td>
<td>106.5573 b</td>
<td>0.7983 a</td>
<td>17.9503 a</td>
</tr>
<tr>
<td>A2B1C2</td>
<td>99.8307 a</td>
<td>0.8053 a</td>
<td>17.9507 a</td>
</tr>
<tr>
<td>Control</td>
<td>108.0493 b</td>
<td>0.7841 a</td>
<td>18.0433 a</td>
</tr>
</tbody>
</table>

Means followed by the same letter within a column is not significantly different as determined by Duncan test P< 0.05.

Conclusions

In this research, two types of packaging material were used and then were compared by applying B-flute as inner packaging to determine the quality of mangosteen under transportation and cold storage for export purpose. BC-flute type had higher compressive strength than C-flute which resulted in higher number of stacks. Mechanical damage symptom occurred during transportation simulation was broken calyx. Packaging model under different capacity was not significantly influence firmness and TSS but significantly influence weight loss in which A2B1C2 was significantly different with A1B1C2 and control.

Acknowledgments

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Food Science and Technology
Effect of Drying Conditions on Isoflavones and \( \alpha \)-Glucosidase Inhibitory Activity of Soybean \([Glycine \textit{max} (L.) \textit{Merrill}]\)

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Abstract

The health benefits of soybean have been well known. The main bioactive compound in soybean is isoflavones (\(\beta\)-glucoside, malonyl glucoside, acetyl glucoside and aglycone forms). Isoflavones possess \(\alpha\)-Glucosidase inhibitory activity that related to the treatment of Type 2 diabetes mellitus. Drying is the important process for shelf-life extension of soybean before soy food or pharmaceutical processing. The objectives of present study, therefore, were to investigate effects of drying method and drying temperature on isoflavones distribution, extraction yield and \(\alpha\)-Glucosidase inhibitory activity of dried soybean at the moisture content of 10% (d.b.). Hot air fluidized bed drying (HAFBD), superheated steam fluidized bed drying (SSFBD) and gas-fired infrared combined with vibrating drying (GFIR-HAVD) were carried out at various drying temperature (50, 70, 130, 150°C). The results showed that the higher drying temperature led to lower content of total isoflavones. At the same drying temperature, it was found that GFIR-HAVD resulted in highest content of total, \(\beta\)-glucoside and aglycone isoflavones as well as extraction yield and \(\alpha\)-Glucosidase inhibitory activity of dried soybean, followed by SSFBD and HAFBD, respectively. In addition, the drying temperature of 130°C gave the highest \(\alpha\)-Glucosidase inhibitory activity in all drying techniques.

Keywords: \(\alpha\)-Glucosidase inhibitory activity, gas-fired infrared drying, isoflavones, soybean, superheated steam drying

Introduction

Soybean has been appreciated by consumer as healthy food. There are various important nutrients in soybean viz. protein, oil and several bioactive compounds such as isoflavones, phytosterols, saponins and oligosaccharides (Shao and Oszmianski, 2009). Isoflavones, the main phenolic compounds found much in soybean, have received high attention because of their weak estrogenic property and other beneficial functions (Klein et al., 1995). A large number of researches reported the positive aspects of isoflavones on human health such as reducing the risk of cardiovascular, atherosclerotic, hemolytic and carcinogenic diseases; improvement of bone health, osteoporosis, menopausal symptoms and blood cholesterol levels; inhibition of the growth of hormone related human breast cancer and prostate cancer cell lines in culture; and increased antioxidant effect in human subjects (Lee et al., 2008). Isoflavones in soybean are found as \(\beta\)-glucoside, malonyl glucoside, acetyl glucoside and aglycone forms. Malonyl glucoside is the predominant form in raw soybean. Recently, there were several works reported that aglycone is able to play the role of \(\alpha\)-glucosidase inhibitor that can be...
used for type 2 diabetes mellitus treatment through the lowering of blood glucose levels (Choi et al., 2008). Isoflavones distribution of soybean can be changed during various processing such as fermentation, cooking, frying, roasting and drying (Lee and Lee, 2009). Drying is an important process to extend the shelf life of soybean before consumption or isoﬂavones extraction for pharmaceutical production. Nowadays, new drying techniques, such as superheated steam and gas-fired infrared drying, are used to improve the quality of products, especially, agricultural products. Therefore, the objectives of this study were to investigate the effect of drying method and drying temperature on the isoﬂavones distribution, extraction yield and \( \alpha \)-glucosidase inhibitory activity of soybean.

**Materials and Methods**

**Materials**

Fresh soybean \([\text{Glycine max (L.) Merill}]\) samples of Chiangmai 60 variety, harvested at Loei province, were used for this experiment. After harvesting, the samples were first thoroughly cleansed of foreign materials and immature, undersized and oversized grains. Fresh soybean was rewetted to the uniform moisture content of 20% (d.b.), mixed and kept in cold storage at temperatures of 4°C for 7 days. Before the drying experiment, the soybean was kept in ambient air until grain temperature was close to the ambient temperature.

**Experimental Set-up**

The drying experiments were conducted using 200±0.5 grams of rewetted soybean samples with three replications. Prepared soybean was dried at drying temperatures of 50, 70, 130 and 150°C for a hot air fluidized bed dryer (HAFBD) and a gas-fired infrared combined with hot air vibrating dryer (GFIR-HAVD). Experiments were also conducted in a superheated steam fluidized bed dryer (SSFBD) at the drying temperature of 130 and 150°C. During the process soybean was continuously sampled for moisture measurement. Soybean was dried until it reached the final moisture content of around 10% (d.b.) then taken for various qualities analysis. All experiments were performed in duplicate.

The qualities of dried soybean, namely, moisture content, isoﬂavones content (\( \beta \)-glucoside, malonyl glucoside, acetyl glucoside and aglycone forms), extraction yield and \( \alpha \)-glucosidase inhibitory activity were determined. Moisture content of soybean was determined using an AOAC method (1995). The content of various isoﬂavones forms of soybean were performed using HPLC according to the methods of Wardhani et al. (2008). The methanolic extraction of soybean, according to the method of Huang and Chou (2009) with some modifications, was carried out for extraction yield determination and \( \alpha \)-glucosidase inhibitory activity analysis according to the method of Lebovitz (1998) with some modifications.

**Results and Discussion**

Figure 1 showed that the different drying conditions gave the different isoﬂavones distribution of soybean. Typically, the chemical structure of various form of isoﬂavones can be transformed during food processing, especially, heating. Malonyl glucoside can be decarboxylated to produce the acetyl glucoside form. The malonyl and acetyl glucoside can be transformed to \( \beta \)-glucoside via de-esterification reaction. The malonyl, acetyl and \( \beta \)-glucoside can be hydrolyzed to produce aglycone (Riedl et al., 2005; Vaidya et al., 2007). All forms of isoﬂavones can be lost due to thermal degradation and oxidation (Chien et al., 2005). It can be seen that higher drying temperature led to lower content of total isoﬂavones. However, drying at low temperature (50 and 70°C) gave low content of \( \beta \)-glucoside and aglycone but high content of malonyl and acetyl glucoside. It was indicated that at low
drying temperature, the conversion of various forms of isoflavones occurred at low rate. Soybean dried using GFIR-HAVD has the highest content of total isoflavones, β-glucoside and aglycone isoflavones, followed by SSFBD and HAFBD, respectively, at the same drying temperature. It was shown that high heat transfer rate for a short drying time in case of GFIR-HAVD, compared to HAFBD and SSFBD, gave high transformation but low loss of various isoflavones. Drying without oxygen by SSFBD can prevent isoflavones loss from oxidation reaction. Soybean dried at 150°C gave lower aglycone content than that was dried at 130°C. It was due to degradation of aglycone increase with an increase of heating temperature (Chien et al., 2005).

**Figure 1** Distribution of isoflavones in soybean dried undergoing different drying conditions

**Table 1** Extraction yield and α-glucosidase inhibitory activity of soybean dried undergoing different drying conditions.

<table>
<thead>
<tr>
<th>Drying condition</th>
<th>Moisture content (% dry basis)</th>
<th>Extraction yield (mg/g dry sample)</th>
<th>α-Glucosidase inhibitory activity (mg acarbose/g dry sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raw soybean</td>
<td>20.38 ± 0.11b</td>
<td>18.75 ± 0.82</td>
<td>-</td>
</tr>
<tr>
<td>HAFBD 50°C</td>
<td>10.06 ± 0.12a</td>
<td>12.66 ± 1.05b</td>
<td>-</td>
</tr>
<tr>
<td>HAFBD 70°C</td>
<td>9.96 ± 0.10d</td>
<td>10.18 ± 0.69b</td>
<td>-</td>
</tr>
<tr>
<td>HAFBD 130°C</td>
<td>9.82 ± 0.07e</td>
<td>9.04 ± 0.80b</td>
<td>4.94 ± 0.50c</td>
</tr>
<tr>
<td>HAFBD 150°C</td>
<td>10.04 ± 0.14f</td>
<td>7.50 ± 0.99e</td>
<td>1.71 ± 0.35d</td>
</tr>
<tr>
<td>GFIR-HAVD 50°C</td>
<td>9.87 ± 0.12a</td>
<td>17.96 ± 0.93b</td>
<td>-</td>
</tr>
<tr>
<td>GFIR-HAVD 70°C</td>
<td>9.85 ± 0.05e</td>
<td>16.75 ± 1.02d</td>
<td>-</td>
</tr>
<tr>
<td>GFIR-HAVD 130°C</td>
<td>9.82 ± 0.10c</td>
<td>14.90 ± 0.86c</td>
<td>11.87 ± 0.79g</td>
</tr>
<tr>
<td>GFIR-HAVD 150°C</td>
<td>9.97 ± 0.10c</td>
<td>13.54 ± 0.94c</td>
<td>8.59 ± 0.64d</td>
</tr>
<tr>
<td>SSFBD 130°C</td>
<td>9.83 ± 0.11d</td>
<td>9.39 ± 0.57b</td>
<td>7.73 ± 0.71d</td>
</tr>
<tr>
<td>SSFBD 150°C</td>
<td>9.71 ± 0.15d</td>
<td>8.95 ± 0.70b</td>
<td>3.68 ± 0.43b</td>
</tr>
</tbody>
</table>

Values in the same column with different superscripts are significantly different (p<0.05).
were corresponding to total isoflavones content and aglycone content, respectively. Soybean dried at 130°C using GFIR-HAVD had the highest α-glucosidase inhibitory activity. However, α-glucosidase inhibitor behavior was not detected in case of raw soybean as well as soybean dried at low temperature drying (50 and 70°C) for both GFIR-HAVD and HAFBD according to their low aglycone content.

Conclusions

The results demonstrated that drying method and drying temperature significantly influenced on isoflavones distribution, extraction yield and α-glucosidase inhibitory activity of soybean. The isoflavones losses in all drying methods were increased with an increase of drying temperature. The GFIR-HAVD gave high conversion of various isoflavones but low isoflavones loss. Soybean dried at 130°C with GFIR-HAVD gave the highest α-glucosidase inhibitory activity.

Acknowledgments

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References


Stability and Rheological Properties of Fat-Reduced Mayonnaises Containing Modified Starches as Fat Replacer

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Abstract

The effects of modified starch (hydroxypropyl distarch phosphate: HDP; starch sodium octenyl succinate: SSO or maltodextrin: MD) and 50% fat substitution by these starches on stability (mean diameter of oil droplet: D_{43} and thiobarbituric acid: TBA) value) and rheological properties (storage modulus: G’; loss modulus: G’’; consistency index: K; and flow behavior index: n) of fat-reduced mayonnaise (FR) were studied throughout 12 weeks of storage time. These properties were compared to those of full fat mayonnaise (FF). Oil droplet size, detected by optical microscope, of all FR was smaller than that of FF. However, D_{43} of FR, prepared from HDP was significantly greater than that of FF (p<0.05). This might be due to the presence of starch granules of HDP that dominated the oil droplet effect. The rheological properties of all mayonnaises exhibited thixotropic behavior and weak gel-like characteristic. G’ of FF and FR, prepared by SSO or HDP tended to decrease when the storage time increased whereas G’ of FR, prepared from MD tended to increase due to MD retrogradation which enhanced solid-like characteristic. D_{43} of FF and FR, prepared by SSO or HDP did not significantly change throughout the storage time (p>0.05). However, D_{43} of FR, prepared from MD dramatically increased at week 4. TBA value of all samples except FR which was prepared by SSO tended to increase during a period of storage time. Thus, SSO could prevent oxidative reaction better than HDP. However, the use of SSO to substitute oil in FR was limited by the SSO substitution level (in this study is not higher than 50%).

Keywords: emulsions, hydroxypropyl distarch phosphate, maltodextrin, octenyl succinate starch,

Introduction

The relationship between dietary fat and the development of cardiovascular disease and hypertension has prompted consumers to be more aware of the amount of fat in their diet. Therefore, food manufacturers have responded to consumer demands, resulting in a rapid market growth of products with a healthy image. However, as a major food component, fat contributes to some key sensory and physicochemical properties in the products. These properties could be related to flavor, mouthfeel, texture, and stability of fat-based product such as emulsion. Therefore, modification of this product is often viewed as the effects of using fat replacers or fat mimetics to maintain the physicochemical of fat-reduced mayonnaise to be close to the traditional one (Worrasinchai, et al., 2006; Liu et al., 2007; Mun et al., 2009).

Mayonnaise is an O/W emulsion, typically containing high oil content (70-80%), egg yolk, vinegar and additives such as spice, salt, sugar and thickening agent (Depree and Savage, 2001). To produce fat-reduced mayonnaise, it is necessary to decrease the dispersed phase and to increase the water content. Some fat replacers such as modified starch, pectin, β-glucan, carrageenan, and proteins have been incorporated into fat-
reduced products to provide and improve the quality of functional attributes of emulsion (Cheug et al., 2002; Worrasinchai et al., 2006; Liu et al., 2007; Mun et al., 2009).

Modified starches are commonly used as a fat replacer because of their low cost, tastelessness, and uniqueness, providing a creamy texture (Cheung et al., 2002; Mun et al., 2009). Modified starches are also used as a thickening agent to prevent oil separation during the storage of mayonnaise. Some of modified starches have been found highly suitable for obtaining desirable organoleptic (texture, fat-like mouthfeel, etc.) as well as physical properties (water holding, gelling, crystallization prevention, freezing control, retrogradation retard, acid tolerant, shear tolerant, etc.) of various food products. Hydroxypropyl distarch phosphate (HDP), starch sodium octenyl succinate (SSO) and maltodextrin (MD) are such modified starches, often used in food products. HDP is a dual modification of substituent hydroxyl group in the polysaccharide by hydroxypropyl function group and cross-linked with the cross-linked agent. This makes such starch resistant to acid, thermal and high shear process, and delay retrogradation during storage (Singh et al., 2007). SSO is a product of the substitution of hydroxyl group in the polysaccharide by octenyl succinic acid (Tesch et al., 2002; Nilsson and Bergenståhl, 2006). This polysaccharide exhibits amphiphilic character which enhances its emulsifying property. MD is an enzyme/acid hydrolysis product which provides soft gel, resulting in a neutral taste and a smooth creamy consistency (Lenchin et al., 1985; Sajilata and Singhal, 2005). However, so far, no information has been available about the effects of HDP, SSO and MD on functional properties of fat-reduced mayonnaises as a function of storage time. Thus, the objectives of this research were to study the rheological properties and stability of such mayonnaise including mean fat particle size diameter, microstructure and lipid oxidation during storage at 25°C for 12 weeks.

**Materials and Methods**

**Materials**

Cool water soluble starches: HDP, MD (Dextrose equivalent: DE 3-5), and SSO were purchased from Siam Modified Starch Co., Ltd. Eggs, soybean oil, vinegar, salt and sugar were purchased from supermarket in Nakhon Ratchasima, Thailand.

**Mayonnaise Preparation**

The mayonnaise formulations are demonstrated in table 1. The recipe and method of mayonnaise preparation were modified from Worrasinchai et al. (2006) and Liu et al. (2007). However, the concentration of modified starch paste used in this study was different in each starch as follows; HDP, MD and SSO were fixed at 8, 28 and 10% (w/w), respectively. This depended on their optimum water soluble capability and viscosity that would be suitable for mayonnaise production with different modified starches. The substitution of fat by modified starch was fixed at 50% of total oil content in the formulation, resulting in the codes of HFR, MFR and SFR for HDP, MD and SSO, respectively.

**Table 1** Mayonnaise formulations prepared at 1.5 kg

<table>
<thead>
<tr>
<th>Ingredients (g)</th>
<th>FF</th>
<th>HF</th>
<th>SFR</th>
<th>MFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil</td>
<td>120</td>
<td>600</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td>Vinegar</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Egg yolk</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Sugar</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Salt</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Water</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Starch/water</td>
<td>0/0</td>
<td>48/5</td>
<td>60/5</td>
<td>168/4</td>
</tr>
</tbody>
</table>

The mayonnaise samples were prepared using a food mixer (Moulinex, Easy Max...
Perfect model). The water, sugar, salts, starch paste, egg yolk and 0.5 % (w/w) benzoic acid were mixed together at the mixer speed 1 for 5 min. Then, oil and vinegar were gradually added into the mixer at the mixer speed 4. This process was controlled to be completed in 5 min.

**Rheology**

Rheological properties of mayonnaise were carried out using a control stress Rheometer (GR-2 TA instruments, New Castle, DE, US) with 40 mm diameter parallel plates. The measurements were conducted at a 1 mm. gap distance at 25 °C.

The linear viscoelastic range (LVR) was determined under mode of strain sweep (0.1-100%) at a fixed frequency of 5 Hz. The viscoelastic properties were studied through a dynamic frequency sweep (0.1 - 100 Hz) by applying a constant strain of 0.5% which was within LVR.

The steady flow behavior of mayonnaise was carried out following the method of Worrasinchai et al. (2006). To characterize the flow behavior, the experimental data were fitted to Power’s law model: \( \sigma = K(\dot{\gamma})^n \), where \( \sigma \) is the shear stress (Pa), \( \dot{\gamma} \) is the shear rate (1/s), \( K \) is the consistency index (Pa.s^n), and \( n \) is the flow behavior index. All rheological data were calculated using TA instrument software program (TA Instruments Advantage Software, 5.0.1, US).

**Optical Microscopy**

The mayonnaise microstructure was observed using Nikon 641463 camera (Nikon corp., Japan). A drop of each mayonnaise sample which was mixed with a drop of 2% (w/v) iodine solution was placed on a microscope slide. The samples were covered with a cover slip and were observed at a magnification of 100X at room temperature.

**Particle Size Measurement**

Mayonnaise samples (0.05g) were then the sample was homogenized using a Moulinex portable homogenizer (speed 4) for 10 min. The mayonnaise samples were kept in a glass container with hermitical seal and wrapped with aluminum foil to protect it from light. Then, they were stored at 25°C overnight before analysis. Diluted with 100 ml of 0.1% (w/v) sodium dodecyl sulfate solution. The mixes were gently stirred before analysis using a particle size analyzer (Mastersizer, Malvern Instrument Ltd, Worestershire, UK). The sample solution was dispersed in distilled water until an obscuration rate of 20-30% was obtained. Optical properties of the sample were defined as follows; refractive index of particle, dilatants and absorption were 1.5295, 1.330 and 0.100, respectively. Droplet size measurements were reported as the volume-weighted mean diameter: \( d_{43} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \), where \( n_i \) is the number of droplets of diameter \( d_i \). Each sample was measured in triplicate.

**Thiobarbituric Acid Analysis (TBA)**

The extraction of oil from samples for TBA analysis was performed following the method of Lagunes-Galvez et al. (2002). Twenty grams of samples were kept at -35 °C for 16 hr. then they were thawed at 5°C for 2 hr. Two milliliters of water were added to those samples and centrifuged at 7,500xg for 10 min. The oil, separated from centrifugal technique was kept in vial at -35 °C before analysis. The TBA reactivity was determined following the method of Luotola and Luotola (1985). The TBA chromagens in the aqueous layer were measured at 532 nm and compared with blanks. Each sample was measured in triplicate.

**Statistical Analysis**

One-way analysis of variance (ANOVA) and Duncan's New Multiple Range Test were used to establish the significance of differences of all property data. Differences were considered to be significant at \( p \leq 0.05 \). The analysis was
performed using the SPSS version 14.0 for windows program (SPSS Inc).

Results and Discussion

Microstructure
The images of mayonnaise microstructure after day 1 storage, observed under microscope, are shown in Fig. 1. There were red-blue regions in continuous phase in all FR samples that represented the amylose rich region which could be dyed with iodine solution. However, the oil droplets of FF sample showed a bigger size and broader distributions than those of all FR samples. In addition, many droplets in these samples had nonspherical shapes (Langton et al., 1999). The larger the droplets, the greater the nonspherical shapes. This may be due to their lower Laplace pressure which resulted in an easier deformation of oil droplets (McClements, 2005). However, HFR obviously showed oil entrapment in the clumps of starch granules (red-blue region) when compared to the others (Fig.1). This might protect both small droplets in the clumps and free oil droplets in dispersed phase from their deformation and might enhance the emulsion stability, consequently. The MFR image exhibited the oil droplets dispersed in void spaces surrounded by the MD gel network (red-blue region) while the oil droplets of SFR were close and attached together, resulting in less void spaces in continuous phase (Fig.1). Moreover, SFR gave the smallest emulsion size among the others. This might be due to the amphiphilic character of SSO which could enhance its emulsifying property.

The particle size distribution of mayonnaises was shown in Fig.2. All size distributions showed a monomodal curve except HFR which had binominal distributions, attributed to the oil droplet (range 0.5-10 µm) and starch granule (10-100 µm) distribution. FF sample provided the widest distribution and a bigger particle size than the others. On the contrary, SFR exhibited the narrowest particle size distribution and had the smallest size when compared to the others. This was confirmed by Fig.1

Rheological Properties
The results showed that all mayonnaises exhibited thixotropic and shear-thinning behavior throughout the study range of shear rates (0.1-300 s⁻¹) that was agreed with Worrasinchai et al. (2006) and Mun et al. (2009). Flow behavior index (n) of all samples were less than 1 (Table 2) and the flow characteristics depended on shear rate and time. This might be because shear forces during measurement could deform the aggregated particle networks of mayonnaise. Consequently, there was a reduction of its apparent viscosity (McClements, 2005). Compared to the other starches and samples, it was found that SFR had the highest K and viscosity (Table 2) even though the SSO paste had the lowest viscosity. This might be because SSO is a well-known associative thickener and made the system more viscous by forming networks with other polymers in aqueous solution via hydrophobic interaction (Ortega-Ojeda, et al., 2005), resulting in an increase of viscosity which might stabilize the dispersed phase of mayonnaise effectively. In addition, these results might be due to the smallest particle size of oil that could enhance friction force among oil droplets (McClements, 2005; Liu et al., 2007).

In contrast, K and viscosity of MFR were lower than those of FF. This might be because MFR had less oil content than FF and the polysaccharide chains of MD were easily destroyed during the homogenization process (Nilsson et al., 2006). This phenomenon was not found in HPD because of its cross-linkage which contributed such starch to resist to acid, thermal and high shear process (Singh et al., 2007).
Table 2. Flow parameters of the upward curve using Power’s law

<table>
<thead>
<tr>
<th>sample</th>
<th>Consistency index (Pa.s)</th>
<th>Flow behavior index</th>
<th>Thixotropic index (Pa/s)</th>
<th>Viscosity (Pa.s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF</td>
<td>43.54 ±3.85b</td>
<td>0.289± 0.021b</td>
<td>6802 ± 112d</td>
<td>2.663± 0.002b</td>
</tr>
<tr>
<td>HFR</td>
<td>48.93±14.2ab</td>
<td>0.251±0.053c</td>
<td>14,835±2662a</td>
<td>2.542± 0.217a</td>
</tr>
<tr>
<td>SFR</td>
<td>51.24±2.21a</td>
<td>0.256±0.009c</td>
<td>12,193±603b</td>
<td>2.790±0.121b</td>
</tr>
<tr>
<td>MFR</td>
<td>13.69±2.31c</td>
<td>0.471±0.024a</td>
<td>7,499±584c</td>
<td>1.815±0.163b</td>
</tr>
</tbody>
</table>

Remark: Mean ± SD in the same column followed by different letter are significantly different (p<0.05). 1Viscosity at shear rate 50s⁻¹ and 25°C

In this study, the storage modulus (G’) of all samples was higher than the loss modulus (G'”) (Fig. 3). This was classified as a weak gel-like characteristic (Clark and Ross-Murphy, 1987) which was a good agreement with the previous reports (Worrasinchai et al., 2006; Liu et al., 2007; Santiphanichwong and Suphantarika, 2007; Mun et al., 2009). The order of G’ at frequency 1 Hz. from the highest to the lowest was ranked as follows: HFR>SFR>FF>MFR. This might be due to the starch granules of HFR and a tight compact of smaller oil droplets in SFR as mentioned previously. Similar results were also found by Langton et al. (1999) and Worasinchai et al. (2006) who reported that the G’ was found to be more solid-like when the mayonnaise was formed by the smaller oil droplets.

Stability of Mayonnaise
Change of Oil Droplet Size and Storage Moduli

The viscoelastic properties in terms of G’ of HFR and SFR were higher than that of
MFR (Fig. 4). It could be explained that MFR which was prepared with MD which had low DE could form hydrogen bonds between amylopectin and amylose (Chronakis, 1998), resulting in a more gel-like structure. The structure could be stronger and showed a more solid-like characteristic when the storage time increased. This might be because the hydrogen bonds in this structure could bind and interact with the other parts of amylose and amylopectin in MD molecules itself and between the other MD molecules, resulting in closed and tight structure in gel networks, leading to MD retrogradation. Consequently, depletion flocculation, water and oil syneresis and higher G’ during storage time (Fig. 4) could be observed. This was agreed with Biliaderis (1992), who reported that starch gel was an unstable system, which could change continuously during storage, and the viscoelasticity of amylose gel changed little, but amylopectin could make gel viscoelasticity increase during storage.

However, the retrogradation was not found in HDP or SSO due to the substitutions of hydroxypropyl group or octenyl succinic acid in such polysaccharides, respectively. The substitution molecules in these polysaccharides might stabilize the mayonnaise system by steric hindrance which prevented the closed association of chains and restricted the formation of interchain hydrogen bonds (Singh, et al., 2007). This made HFR and SFR have a small change of G’ throughout the storage time.

Oil droplet sizes of FF, HFR and SFR did not change so much throughout the storage time while those of MFR increased rapidly after 2 weeks (Fig. 5). This meant that HDP or SSO provided more stable emulsion system than MD. The rapid change of oil droplet size in MFR might be because of the coalescence of oil droplets which was attributed to retrogradation of MD as mentioned previously (Dokic-Baucal et al., 2004)

### Lipid Oxidation

The TBA reactivity, referred to the amount of malonaldehyde which is a major secondary by-product of lipid oxidation, was investigated during storage time. The TBA reactivity of FF, MFR and HFR samples was continuously increased as storage time increased (Fig. 6). It might be concluded that MD and HDP could not prevent the lipid oxidation (Matsumura et al., 2003).

![Figure 4](image)

**Figure 4** Storage moduli of mayonnaise at frequency 1 Hz and 0.5% strain at 25 °C. ¹ The study was ended because of phase separation.

However, the TBA reactivity of SFR increased as a function of time in the early storage time but it was stable after week 4 (Fig. 6). This might be because the free fatty acid and phospholipids found in continuous phase could oxidize until they were reacted with oxygen completely in the early storage time. Subsequently, there were no more free fatty acids and phospholipids existing to oxidize with oxygen in continuous phase. Meanwhile, SSO might cover the interfaces of dispersed phase and prevented oxygen diffusion to the surface by steric hindrance (Matsumura et al., 2003), resulting in a protection of dispersed phase from oxidation reaction.
Conclusions

Types of modified starches used as fat replacers in this study could affect the rheological properties and stability of fat-reduced mayonnaise because of the different functionality of each starch. MD could retrograde when storage time increased, resulting in instability of oil droplet size and rheological properties in MFR. On the contrary, there was no retrogradation in HFR and SFR, resulting in a higher stability than MFR throughout storage time. However, SSO could prevent oxidative reaction better than HDP. Thus, using SSO as fat-replacer in FR was the most suitable but the oil substitution level of SSO was limited at 50% in this study.

Acknowledgments

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References


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Mathematical Models for Electrical Conductivities of Fresh Juices, Concentrated Juices and Purees undergoing Ohmic Heating

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Abstract

Electrical conductivity is the most important parameter for applying ohmic heating in food processing. In this study, 10 kinds of juices were heated in a static ohmic cell to the temperature of 80 °C applying voltage gradients in the range between 10 and 32 V cm⁻¹. Furthermore, the concentrated orange and pineapple juices and the purees were heated at various concentration levels. The data of sample temperature, electrical current, and voltage gradient were recorded at each time step in order to determine the ohmic heating characteristics and calculate the electrical conductivities of samples. It appeared that the electrical conductivities of 10 juices were in the range from 0.08 to 1.75 S m⁻¹ while those of concentrated juices and purees were between 0.34 and 1.51 S m⁻¹. The electrical conductivities of samples were significantly affected by their concentrations and temperatures. As a result, a number of empirical models were created for estimating the electrical conductivities as a function of these factors with the good fitting result (R² ≥ 0.954 and RMSE ≤ 0.048 S m⁻¹ for 10 juice samples and R² ≥ 0.969 and RMSE ≤ 0.069 S m⁻¹ for concentrated juices and purees). In addition to electrical conductivity, pH, total soluble solid, density and specific heat of juices and purees were investigated in order to be background information for the researchers and food industry that intent to apply ohmic heating technology for these products.

Keywords: concentration, electrical property, empirical model, orange, pineapple

Introduction

Ohmic heating is an alternative heating technique using an electrical current passing through the food product. Conventional heating requires long time to sterilize food product due to low thermal conductivities of foods especially for particulate food. This results in destruction of flavor, texture and nutrient to the product. These heat transfer obstacles have now been overcome with the development of ohmic heating (Parrott, 1992). The potential applications of this technique in food industry are varied including blanching, evaporating, dehydration, fermentation and pasteurization (Sastry et al., 2002).

Many factors affect the heating rate of foods undergoing ohmic heating such as electrical conductivities, particle size, shape and concentration (Kim et al., 1996). However, ohmic heating rate is directly proportional to the electrical conductivity of product and the square of electric field strength (Sastry and Palaniappan, 1992).

Until now, there have been some studies on the electrical conductivities of juices and puree (Palaniappan and Sastry, 1991; Castro et al., 2003; Icier and Ilicali, 2004 and 2005). Palaniappan and Sastry (1991) stated that electric current can pass through food and generate heat rapidly if food contains ionic species such as salts and acids. Also, they investigated the electrical
conductivities of tomato and orange juices and found that the electrical conductivities of these samples increased along the rising temperature and decreased with the larger particle size. Furthermore, Castro et al. (2003) studied the electrical conductivities of strawberry products and discovered that they were influenced by sugar or solid contents and applied field strength. The result indicated that the electrical conductivity decreased when the solid content and the size of particles increased. The high solid content (> 20% w/w) and sugar content over 40 °Brix resulted in the low electrical conductivity of product. Icier and Ilicali (2004) investigated the electrical conductivities of apple and sourcherry. It appeared that at the same temperature the sourcherry had higher electrical conductivity than apple juice at all concentration and for all voltage gradients applied. In addition, Icier and Ilicali (2005) found that the electrical conductivities of fruit puree increased with the higher temperature, ionic concentration and pulp content.

Although there have been a number of studies on electrical conductivities of some juices, the information about electrical conductivities and physical properties of some juices of Thailand are still limited. Thus, the aim of this research was to investigate the data of electrical conductivities and some attributes for 10 kinds of juices, the concentrated juices and purees of orange and pineapple. The outcome of this work would be useful for the food industry that intents to apply ohmic heating technology for their processing.

Materials and Methods

Raw Material Preparation

Ten kinds of juices

There were 10 sorts of local juices applied in this study consisting of tamarind, tomato, germinated brown rice, mulberry, pomelo, Thai blueberry, sugarcane, passion fruit, coconut and guava juices. For the tamarind, tomato, sugarcane, coconut and guava juices, they were newly prepared from the fresh fruits prior to the experiment. However the juices of germinated brown rice, mulberry, Thai blueberry and passion fruit from “Doikham” brand and the pomelo juice from “Chaba” brand were purchased at the local market for experiment. These juices were thermally-processed products in the aseptic packaging.

Orange and pineapple concentrated juices

The fresh orange “Tangerine” variety and pineapple “Sriracha” variety were bought from the local market. They were washed, cut and separated juice by applying the hydraulic press machine (Sakaya, Bangkok, Thailand). Then, the juices were concentrated using a vacuum evaporator (Hisaka, model REV-T, Japan) at temperature of 60 °C and 71 cmHg of vacuum pressure until reaching concentration of 32 °Brix. The specimens at concentrations of 12, 17, 22 and 27 °Brix were prepared by diluting the 32 °Brix juice with distilled water.

Orange and pineapple concentrated purees

Similarly to the concentrated juices, the concentrated purees were prepared from the fresh orange “Tangerine” variety and pineapple “Sriracha” variety. They were washed, cut and then putting into the pulper and finisher machine (Stroter, USA). After that, the purees were concentrated using rotary vacuum evaporator (Buchi, model R152, Flawil, Switzerland) at temperature of 60 °C and 71 mbar of vacuum pressure until the concentration of puree was 12, 17, 22 and 27 °Brix were prepared by diluting the 32 °Brix puree with distilled water.
Electrical Conductivity Measurement

The samples were measured their electrical conductivities using a static ohmic heating device that was built at the department of Food Science and Technology, Kasetsart University. A schematic diagram of the electrical circuit is shown in Figure 1. The cylindrical ohmic cell was made from acrylic pipe while the electrodes were stainless steel grade 316. The diameter of electrodes was 0.043 m. The distance between electrodes was 0.036 m. The electric field strength applied in this measurement was in the range between 10-32 V cm⁻¹. The sample temperature was measured using type-T thermocouple located at the center of the ohmic cell and recorded by a data logger (Yokogawa, model DX 1012, Japan). The electrical voltage and current were measured using digital multimeter (Fluke, model 8808A, USA). The experiments were conducted in triplicate for each type of sample. Electrical conductivity of sample was calculated by applying the Eq. (1)

\[ \sigma = \frac{IL}{AV} \quad (1) \]

where
\( \sigma \) = Electrical conductivity (S m⁻¹)
\( A \) = Cross sectional area of electrode (m²)
\( I \) = Electrical current (Ampere)
\( L \) = Distance between the electrodes (m)
\( V \) = Applied voltage (Volt)

Mathematical Models for Electrical Conductivity

The relationship between electrical conductivity of food and temperature is commonly linear (Jittanit, 2001). Hence, the electrical conductivity data of 10 local juices collected from the 3 replications of experiments were fitted into the Eq. (2).

\[ \sigma = aT + b \quad (2) \]

where
\( a, b \) = Empirical constants
\( T \) = Temperature (°C)

Electrical conductivities of concentrated juices and purees are generally correlated to both temperature and concentration; thus, the Eq. (3) that was proposed by Icier and Ilicali (2004) was fitted by the measured electrical conductivities of concentrated juices and purees of orange and pineapple.

\[ \sigma = E_1 \cdot (\text{Conc.})^{N_2} + B_2 \cdot T + C_2 \quad (3) \]

where
\( B_2, C_2, E_1, N_2 \) = Empirical constants
\( \text{Conc.} \) = Concentration (°Brix)

The experimental data were fitted into the mathematical models by regression method using the software Statistica 5.5 (StatSoft, Inc. Tulsa, OK 74104 USA).
**Total Soluble Solid and pH**

The total soluble solid (TSS) values of samples were determined by digital refractometer (HANNA, model HI 96801, USA) whereas the pH values were measured at 25 °C using the digital pH meter. Both TSS and pH were measured in triplicate.

**Density**

The densities of juices were determined using the specific gravity bottle method applying a hydrometer as a tool. The specific gravity (SG) of sample was indicated by the hydrometer (Nikkei Nihon, Japan). Then, the density of sample was calculated by the Eq. (4).

\[
\text{Density of sample} = \text{SG} \times \text{density of water (4)}
\]

**Specific Heat**

The specific heat values of samples were determined applying the method of Manohar et al. (1991). The differential scanning calorimetry (Mettler Toledo, model DSC 1, USA) was used by setting the rate of temperature rise at 20 °C min⁻¹. The initial and final temperatures of samples were 20 and 80 °C respectively.

\[
\text{Density of sample} = \text{SG} \times \text{density of water (4)}
\]

**Results and Discussion**

The measured electrical conductivities of 10 kinds of juices at temperatures of 30, 40, 50, 60, 70 and 80 °C from three replications were averaged and illustrated in Figure 2. Electrical conductivities appeared to range between 0.08 and 1.75 S m⁻¹. Furthermore, they significantly increased along the rising temperature. This result is similar to the previous studies of some researchers such as Palaniappan and Sastry (1991), Jittanit (2001) and Icier and Ilicali (2005). According to the Figure 2, it was apparent that the coconut juice had the maximum electrical conductivities among 10 samples. It should be due to the high ion contents in coconut juice such as potassium, iron, sodium, calcium and magnesium (Campbell et al., 2000). Palaniappan and Sastry (1991) pointed out that the electrical conductivity of food directly depends on the amount of ionic species such as salts and acids. On the other hand, the germinated brown rice juice had the lowest electrical conductivity according to its pH. The other 9 juices had lower pH (< 7) while the germinated brown rice juice had neutral pH (≈7.4). The juices that have higher electrical conductivities will be ohmically heated more rapid than those with lower conductivities (Sastry and Palaniappan, 1992).

![Figure 2 Electrical conductivities of 10 juices (Thai blueberry, sugarcane, passion fruit, coconut, guava, tamarind, tomato, germinated brown rice, mulberry, pomelo).](image)

Electrical conductivities of orange juices, pineapple juices, orange purees and pineapple purees at concentrations of 12, 17, 22, 27, and 32 °Brix were presented in Figure 3 (a) to (d). Similarly to 10 previous juices, electrical conductivities of all juices and purees in Figure 3 increased when temperature was raised. In general, at the same concentration and temperature the electric conductivities of orange specimens appeared to be significantly higher than those of pineapple. It should be due to the higher ion contents in orange samples. Additionally, it was found
that the puree samples had less electrical conductivities than juice samples especially at high temperatures and concentration levels. This phenomenon could be explained by the larger solid content and particle size of solids in purees that obstructed the electrical current movement leading to the lower electrical conductivities (Castro et al., 2003). The negative influence of the solid content and particle size on the electrical conductivities of purees was obvious at high temperature and concentration levels.

The experimental results in Figure 3 also indicated that the electrical conductivities of juices and purees would be amplified if the concentrations of samples were raised especially from 12 °Brix to 17 °Brix. However, the amplification of electrical conductivities would be less for the further raising in concentration. Moreover, the opposite effect would take place if the concentration of samples was increased from 27 °Brix to 32 °Brix. This occurred from too high sugar and other soluble solid contents that cause the resistance for ionic movement inside the samples. This result is similar to the finding of Castro et al. (2003) who stated that if the solid content in strawberry product was increased to higher than 20% w/w or the sugar content was raised to over 40 °Brix, the decrease in electrical conductivities of product would occur.

According to the results, the electrical conductivities of concentrated juices and purees of orange and pineapple were rather high; therefore, these products can be quickly heated by ohmic heating. It is feasible to replace the conventional pasteurizers of concentrated juices and purees that apply heat conduction and convection by the ohmic heater that directly generates heat inside the product by passing the electrical current through them.
The linear models developed by fitting the experimental data of electrical conductivities and temperatures of 10 local juices during ohmic heating into the Eq. (2) are shown in Table 1. Moreover, the outcome of fitting the electrical conductivities of concentrated juice and puree samples into the Eq. (3) is presented in Table 2. The results in Table 1 and 2 indicate the goodness of fit due to their high coefficients of determination ($R^2$) and low root mean square error (RMSE). These empirical equations are useful for estimating the electrical conductivities of these products at any temperatures in the range between 30 to 80 °C.

Apart from the electrical conductivity, the properties of samples in aspects of TSS, pH, density and specific heat ($C_p$) were determined in this study. The results are provided in Table 3. These properties are useful for the researchers and food industries that intend to apply ohmic heating technology for these kinds of products.

**Table 1** The empirical models expressing the relationship between electrical conductivities and temperatures of 10 local juices.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Model</th>
<th>$R^2$</th>
<th>RMSE (S m$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thai blueberry</td>
<td>$\sigma = 0.0051T+0.076$</td>
<td>0.995</td>
<td>0.007</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>$\sigma = 0.0098T+0.265$</td>
<td>0.980</td>
<td>0.025</td>
</tr>
<tr>
<td>Passion fruit</td>
<td>$\sigma = 0.0120T+0.353$</td>
<td>0.985</td>
<td>0.048</td>
</tr>
<tr>
<td>Coconut</td>
<td>$\sigma = 0.0156T+0.502$</td>
<td>0.970</td>
<td>0.036</td>
</tr>
<tr>
<td>Guava</td>
<td>$\sigma = 0.0058T+0.188$</td>
<td>0.995</td>
<td>0.007</td>
</tr>
<tr>
<td>Tamarind</td>
<td>$\sigma = 0.0082T+0.229$</td>
<td>0.979</td>
<td>0.024</td>
</tr>
<tr>
<td>Tomato</td>
<td>$\sigma = 0.0064T+0.325$</td>
<td>0.954</td>
<td>0.028</td>
</tr>
<tr>
<td>Germinated brown rice Mulberry</td>
<td>$\sigma = 0.0018T+0.028$</td>
<td>0.988</td>
<td>0.004</td>
</tr>
<tr>
<td>Pomelo</td>
<td>$\sigma = 0.0036T+0.035$</td>
<td>0.996</td>
<td>0.004</td>
</tr>
</tbody>
</table>

**Table 2** Estimated empirical constants of the Eq. 3 for the juice and puree samples and the statistical parameters.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Orange juices</th>
<th>Pineapple juices</th>
<th>Orange purees</th>
<th>Pineapple purees</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td>1.805</td>
<td>0.415</td>
<td>0.587</td>
<td>0.058</td>
</tr>
<tr>
<td>N2</td>
<td>0.125</td>
<td>0.286</td>
<td>0.198</td>
<td>0.409</td>
</tr>
<tr>
<td>B2</td>
<td>0.016</td>
<td>0.011</td>
<td>0.01</td>
<td>0.009</td>
</tr>
<tr>
<td>C2</td>
<td>-2.585</td>
<td>-0.925</td>
<td>-0.816</td>
<td>-0.027</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.973</td>
<td>0.969</td>
<td>0.999</td>
<td>0.999</td>
</tr>
<tr>
<td>RMSE (S m$^{-1}$)</td>
<td>0.069</td>
<td>0.056</td>
<td>0.040</td>
<td>0.031</td>
</tr>
</tbody>
</table>

**Acknowledgments**

This research was financially supported by the Faculty of Agro-Industry, Kasetsart University, Thailand.
Table 3 Mean values of some properties of fruit juices and purees.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TSS (°Brix)</th>
<th>pH</th>
<th>Density (g cm⁻³)</th>
<th>Cₚ (J g⁻¹ °C⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamarind</td>
<td>10.5</td>
<td>2.14</td>
<td>1.08</td>
<td>3.02</td>
</tr>
<tr>
<td>Tomato</td>
<td>4.4</td>
<td>4.11</td>
<td>1.07</td>
<td>3.28</td>
</tr>
<tr>
<td>Germinated brown rice</td>
<td>2.8</td>
<td>7.43</td>
<td>1.02</td>
<td>3.57</td>
</tr>
<tr>
<td>Mulberry</td>
<td>14.9</td>
<td>3.01</td>
<td>1.06</td>
<td>3.40</td>
</tr>
<tr>
<td>Tomato</td>
<td>4.4</td>
<td>4.11</td>
<td>1.07</td>
<td>3.28</td>
</tr>
<tr>
<td>Germinated brown rice</td>
<td>2.8</td>
<td>7.43</td>
<td>1.02</td>
<td>3.57</td>
</tr>
<tr>
<td>Mulberry</td>
<td>14.9</td>
<td>3.01</td>
<td>1.06</td>
<td>3.40</td>
</tr>
<tr>
<td>Thai blueberry</td>
<td>16.2</td>
<td>2.75</td>
<td>1.06</td>
<td>3.56</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>16.1</td>
<td>5.63</td>
<td>1.05</td>
<td>3.37</td>
</tr>
<tr>
<td>Thai blueberry</td>
<td>16.2</td>
<td>2.75</td>
<td>1.06</td>
<td>3.56</td>
</tr>
<tr>
<td>Sugarcane</td>
<td>16.1</td>
<td>5.63</td>
<td>1.05</td>
<td>3.37</td>
</tr>
<tr>
<td>Passion fruit</td>
<td>14.4</td>
<td>2.91</td>
<td>1.07</td>
<td>3.34</td>
</tr>
<tr>
<td>Coconut</td>
<td>6.1</td>
<td>5.03</td>
<td>1.03</td>
<td>3.55</td>
</tr>
<tr>
<td>Guava</td>
<td>9.6</td>
<td>4.25</td>
<td>1.03</td>
<td>3.40</td>
</tr>
<tr>
<td>Orange juices</td>
<td>12.32</td>
<td>1.04</td>
<td>3.29</td>
<td></td>
</tr>
<tr>
<td>Orange juices</td>
<td>17</td>
<td>3.26</td>
<td>1.07</td>
<td>3.21</td>
</tr>
<tr>
<td>Orange juices</td>
<td>22</td>
<td>3.22</td>
<td>1.09</td>
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<td>1.12</td>
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<tr>
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References


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Contamination of Acrylamide in Thai-conventional Foods From Nong Mon Market, Chonburi

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Abstract

This study reports the acrylamide contamination in some Thai-conventional foods available in Nong Mon market, Chonburi. Analysis was done by GC-MS system using ¹³C₃-acrylamide as an internal standard. The highest levels of acrylamide (> 1 mg kg⁻¹) were found in a few sweet potato crisps, Khanom Jak and Khanom Kai Hong. Moderate levels (200-500 µg kg⁻¹) were detected in whole sweet-based fried samples (sweet potato crisps, sweet taro crisps and banana fritters). Low contamination of acrylamide (<200 µg kg⁻¹) were found in Khao Larm, durian chips and a few of Khanom Jak but the content in Pa Tong Koo was below the detectable level (50 µg kg⁻¹) of acrylamide. These strongly suggest that the formation of acrylamide is influenced by sugar additive and processing conditions. Consumption habits indicated that the acrylamide levels in the studied traditional foods could lead to a daily intake of a few micrograms.

Keywords: Bangsaen, GC-MS analysis, Maillard reaction, starchy food

Introduction

Acrylamide is a well-known difunctional monomer used as a conjugated reactive molecule in various industrial applications (Prasad, 1982; Wampler and Ensign, 2005; Prabu and Thatheyus, 2007). It is thought also to be a neurotoxicant, carcinogen and terratogen in animals (Cherry et al., 1956; Tilson and Cabe, 1979; IARC, 1994; Segerbäck et al., 1995; Prabu and Thatheyus, 2007). Neurotoxic effects in humans have been observed at high levels of exposure in occupational settings. Acrylamide is oxidized to the epoxide glycidamide via an enzymatic reaction involving cytochrome P450 2E1 (Besaratinia and Pfeifer, 2004). Both acrylamide and glycidamide can form hemoglobin adducts and induce abnormalities in the daughter cells of animals and plants (Shairashi, 1978; Shanker et al., 1987; Bergmark et al., 1991).

After the first announcement made by the Swedish National Food Administration and a research group at the University of Stockholm that acrylamide could form in foods during the cooking process (Tareke et al., 2000; Swedish National Food Administration, 2002; Tareke et al., 2002). Many researchers have confirmed the presence of acrylamide in different processed foods, and it was shown that its concentration might reach levels as high as several mg kg⁻¹ depending on the composition and the way of processing (Svensson et al., 2003; Hoenicke and Gatermann, 2005; Zhang et al., 2006; Zhang et al., 2007). Numerous paths of formation have been discussed, predominantly the thermal degradation of the free amino acid asparagines via a Maillard reaction, which involves the reaction with reducing sugars,
such as fructose and glucose in the presence of heat (Mottram et al., 2002; Stadler et al., 2002). In addition, some reports suggested that acrylamide could form in lipid-rich foods by the reaction of acrylic acid from acrolein and ammonia from the amino acid upon heating (Yasuhara et al., 2003; Zhang et al., 2006; Zhang et al., 2007). From these alarming informa"ons, the consumption of foods with this contaminant has become a public health concern and a list of acrylamide-contaminated foods is subject to be monitored for this problem. However, no intensive information is available for acrylamide content in Thai-conventional foods. Since Nong Mon Market is the largest Thai-conventional products and the most visited local market of Chonburi. Famous products of this market are Khao Larm (sweet rice in bamboo pipe) and Khanom Jak (sweet coconut wrapped in palm leaf) as well as dried and sweetening preserved fruits. For reasons of consumer health protection, the present study aims to survey the exposure of acrylamide in the foods samplings from this market and evaluate a daily intake of this compound in consumers based on the consumption habits analysis.

Materials and Methods

Chemicals

Acrylamide (99%) and $^{13}$C$_3$-labeled acrylamide (isotopic purity 99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively. All of other solvents and chemicals used for the extraction and analysis of acrylamide were of analytical grade. Water was purified with a Milli-Q system (Millipore, Bedford, USA).

Acrylamide and $^{13}$C$_3$-labeled acrylamide are hazardous and must be handled carefully. Sample pretreatment procedures referring to organic reagent operations should be carried out in a fume cupboard.

Samples

Thai-conventional food samples (24 samples covering 8 product types) were randomly purchased from the food suppliers in Nong Mon market, Thailand during April - August 2010. The analytical survey comprised a sequence of signature products such as Khao Larm, Khanom Jak, Khanom Kai Hong (sweet and fried flour ball), Pa Tong Koo (fried flour stick), banana fritters, durian chips, sweet potato crisps and sweet taro crisps.

Sample Preparation, Extraction, Clean-up and Bromination Methods

The food samples were freeze-dried at -80°C for 24 h, pulverized and homogenized by variable speed waring blender prior to sampling. Then, 10 g of the above-mentioned samples was weighed in 250 mL beaker. To make a defatting process, 100 mL of isohexane/t-butyl methyl ether (95:5) was added, and each beaker was placed for 10 min for the passable mixing of the solvent with sample and then slightly shaken by hand for 5 min. The supernatant fluids were removed by filtration with filter paper, and defatting step was performed again as described above. The filter paper (sample) was dried in air for 1 h, cut in small pieces (1 x 1 cm) and put into the 250 ml beaker. The sample matrixes were additionally spiked with 10 µL of $^{13}$C$_3$-labeled acrylamide (1 µg µL$^{-1}$) as the internal standard and the beaker was placed for 15 min at 4°C in order that labeled acrylamide could adequately mix with sample matrix via osmotic effect. To extract the analyte, one hundred milliliters of water were added into the residue of each beaker and shaken in an ultrasonic shaker at 40°C for 15 min. After that, 1 ml of Carrez solution I (0.35 M K$_4$[Fe(CN)$_6$]) and Carrez solution II (1M ZnSO$_4$) were promptly added into the solution with continuously shaking. The samples were subsequently centrifuged at 5,000 rpm for 15 min and filtrated with filter paper to obtain the clarified aqueous layer. Solid-phase extraction (SPE) clean-up was further performed via Chromabond ABC18 cartridges (500 mg polypropylene, 6 mL) purchased from Macherey-Nagel (Düren,
The cartridges were conditioned with 10 mL of methanol followed by 10 mL of water; the methanol and water portions were discarded. Then, air was aspirated through the column for 30 sec. The 2 mL of extract was allowed to pass through the sorbent material and discarded. Then, the cartridge was eluted with another 18 mL of sample solution and collected. For bromination, the sample solutions were cooled on ice for 15 min and 1200 µL of derivatization reactants (KBr 15.2 g, hydrogen bromide 0.8 mL, bromine water 5 mL, water 60 mL) (Castle et al., 1991) was added to the precooled solution. The reaction mixture was allowed to stand on ice for 3 h in dark and subsequently terminated by adding 1 mM sodium thiosulfate until excess bromine was reduced to colourless (typically 100 µL). Then 20 mL of redistilled ethyl acetate were added for extraction and the combined extracts were dried over anhydrous sodium sulfate. Finally, 1 mL of the final test solution was used for GC-MS analysis.

**Determination of Acrylamide by GC-MS**

The final test solution was introduced into the GC-MS system by splitless injection method with a purge activation time of 1.0 min and an injection temperature of 280°C. The GC-MS system used in this study was an Agilent 6890N gas chromatography coupled with a Agilent 5973N quadruopole mass selective detector (MSD) operated in selected ion monitoring (SIM) mode with positive electron impact (EI) ionization (70 eV). The analytical separation was performed on a HP5-MS capillary column, (polysiloxane polymers, 30 m × 0.25 mm, 0.25 µm, J&W Scientific, Agilent, CA, USA) and helium was chosen as the carrier gas at a flow rate of 1.0 mL min⁻¹. After injection, the column was held at 60°C for 2 min, then programmed at 10°C min⁻¹ to 200°C, and held for 5 min at 200°C (total run time: 21 min). The GC-MS interface transfer line was held at 280°C. Under such conditions, the retention time of acrylamide and ¹³C₃-acrylamide derivatives was 6.9 min. Ions monitored were m/z 151 for 2-bromopropenamide and m/z 154 for 2-bromo (¹³C₃)-propenamide (Figure 1). Calculation of acrylamide content in the food samples was estimated from the ratio of peak area of 2-bromopropenamide to 2-bromo (¹³C₃)-propenamide.

**Consumption Habits Analysis and Estimated Daily intake**

The consumption habits data were obtained based on a food frequency questionnaire with questions on 25 food items. The questionnaire was carefully designed to minimize its effect on consumer responses and to collect information that reflected the consumer’s own attitude. Questions related to frequency of visiting and consumption behaviors were asked. Also, the questions relating to consumer knowledge about food safety issues and socio-economic characteristics of participants were asked. All subjects who did not consume the food in the market were designated as a group of nonconsumers and all consumers were divided by the frequency of consumption. For the acrylamide intake estimation, we focused on foods that are likely to contain considerable acrylamide concentrations according to the mechanism of formation (Mottram et al., 2002; Stadler et al., 2002). Thus, the following list of food products was established: Khao Larm, Khanom Jak, Khanom Kai Hong, Pa Tong Koo, durian chips, sweet potato crisps, sweet taro crisps, and banana fritters.

Estimated daily intake (EDI) values of acrylamide by consumer were calculated according to the average contents of acrylamide individually found in food type and consumption habits data. The EDI (µg kg body weight⁻¹) was estimated from the concentration of acrylamide (µg kg⁻¹) multiplied by the amount of food consumed day g⁻¹ and divided by the average weight of an individual (50 kg) (Dybing and Sanner, 2003; Daniali et al., 2010).
Statistical Analysis

Statistical analysis was carried out using analysis of variance by SPSS program. P value of less than 0.05 was considered statistically significant.

Results and Discussion

Nowadays, consumers are more interested in the health benefits of foods in order to promote health and/or reduce the risk of certain diseases. Acrylamide has been known as a neurotoxicant and probably carcinogen in human, which capable to form in heated foodstuffs. Thai-conventional food is one of the most demand foods for consumers due to its good taste and flavor and reasonable price. However, the preparation might require either Maillard reaction or lipid peroxidation to obtain the specific tastes. With respect to food safety, data on acrylamide contamination in these foods are needed. In this study, we randomly selected eight Thai-conventional foods available in Nong Mon market for analysis. Over 1 mg kg\(^{-1}\) of acrylamide was found in a few sweet potato crisps, Khanom Jak and Khanom Kai Hong (Table 1). Moderate levels (200-500 µg kg\(^{-1}\)) were detected in most sweet potato crisps, except one sample contained the highest acrylamide concentration (1.46 mg kg\(^{-1}\)) and whole sweet taro crisps and banana fritters. Low concentrations (<200 µg kg\(^{-1}\)) were found in Khao Larm, durian crisps and a few of Khanom Jak (Figure 2). For Pa Tong Koo, the detected level of acrylamide was not found to be as significant as expected. These results are in agreement with the facts that fried products of plant origin seem to give high concentration of acrylamide (Lingnert et al., 2002). Moreover, acrylamide formation is increased by increased concentration of (reducing) sugar in the raw materials or ingredients (De Wilde et al., 2006). This could explain in the case of Khanom Kai Hong with the sugar covering surface. The detected values of acrylamide in sweet potato crisps and Khanom Jak were wide-ranging as 365-1465 µg kg\(^{-1}\) and 118-1571 µg kg\(^{-1}\), respectively. These differences might likely due to the different processing methods, temperatures, raw materials and repeated usage of frying oil and maturity stage of potato.
Table 1 Acrylamide contents in Thai-conventional foods available in Nong Mon market, Chonburi.

<table>
<thead>
<tr>
<th>Food types</th>
<th>Number of tests</th>
<th>Acrylamide contents (µg kg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Minimum contents</td>
<td>Maximum contents</td>
</tr>
<tr>
<td>Khao Larm</td>
<td>3</td>
<td>106.67</td>
</tr>
<tr>
<td>Khanom Jak</td>
<td>4</td>
<td>117.90</td>
</tr>
<tr>
<td>Khanom Kai Hong</td>
<td>2</td>
<td>1241.42</td>
</tr>
<tr>
<td>Pa Tong Koo</td>
<td>2</td>
<td>&lt; 50</td>
</tr>
<tr>
<td>Durian chips</td>
<td>3</td>
<td>104.23</td>
</tr>
<tr>
<td>Sweet potato crisps</td>
<td>4</td>
<td>365.11</td>
</tr>
<tr>
<td>Banana fritters</td>
<td>3</td>
<td>264.33</td>
</tr>
<tr>
<td>Sweet taro crisps</td>
<td>3</td>
<td>219.06</td>
</tr>
</tbody>
</table>

Previous reports revealed that the Maillard reaction, a reaction between reducing sugar and amino acid, being an important reaction route for acrylamide formation (Lingnert et al., 2002; Mottram et al, 2002; Friedman, 2003). However, lipid degradation pathways to the formation of acrolein should also be considered (Lingnert et al., 2002). The variation among the samples can be explained by different chemical composition of the raw materials (Fiselier et al., 2006). Moreover, the duration of time and temperature for processing might affect the stability of acrylamide in food (Hoenicke and Gatermann, 2005). Duration of frying time in Khanom Kai Hong is higher than the other samples; moreover repeated usage of cooking oils can also enhance the formation of acrylamide. Since a small number of samples were analyzed for each food, the detected values are not representative of each food. Further work is in progress to examine the amount of acrylamide in various foods and the effect of manufacturing processes on acrylamide formation.

Food consumption habits were studied in 100 tourists visiting the market. The distribution of the consumers was equally, female (51%) and male (49%). The mean age of the consumers was close to 30 years and about 47% of the consumers had at least some college education. Three-quarter (70%) of the consumers had an annual household income of less than 240,000 baths and more than 40% of the consumers had visiting the market once a month for shopping about 500 baths. Five foods which big demands by the consumers are Khao Larm, Khanom Jak, durian chips, sweet potato crisps and sweet taro crisps. The estimated daily intake of acrylamide in the five studied foods is presented in Table 2. The highest estimated exposure of acrylamide was found from the consumption of Khanom Jak that is the biggest demand product. This study illustrates that consumption habits have a real impact on acrylamide dietary exposure in terms of daily intake levels.

The relationship between eight types of food safety concerns and the corresponding change in food consumption habits of 100 consumers in Nong Mon market, Chonburi was also evaluated. Results showed a close relationship between food safety concerns and food consumption habits. For example, 100% of consumers were extremely concerned about food contaminant and all actually took extreme precaution in buying items considering this perceived threat. The study indicated that educating consumers about preventive methods to reduce food safety threats will lead to reduced concerns and changes in food consumption habits.
Figure 2 Acrylamide concentrations of different food samples determined in triplicate. Average levels and standard deviations are given.

Table 2 Estimated daily intake (EDI) of five Thai-conventional foods from Nong Mon market, Chonburi

<table>
<thead>
<tr>
<th>Food types</th>
<th>Consumption (g day⁻¹)</th>
<th>Acrylamide (µg kg⁻¹)</th>
<th>EDI (µg kg bodyweight⁻¹)</th>
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</thead>
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<tr>
<td>Khao Larm</td>
<td>17.2</td>
<td>109</td>
<td>0.37</td>
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<td>Khanom Jak</td>
<td>18.6</td>
<td>532</td>
<td>1.98</td>
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<td>Durian chips</td>
<td>11.17</td>
<td>109</td>
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<tr>
<td>Sweet potato crisps</td>
<td>7.5</td>
<td>681</td>
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<td>Sweet taro crisps</td>
<td>6.83</td>
<td>223</td>
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</table>

Conclusions

The present study surveys the contamination of acrylamide in some Thai-conventional foods available in Nong Mon market, Chonburi. High levels of acrylamide up to 1.5 mg kg⁻¹ were found in sweet potato crisps, banana fritters, sweet taro crisps, durian chips, Khao Larm, Khanom Jak and Kanom Kai Hong but the contents in Pa Tong Koo was below the detectable level (50 µg kg⁻¹) of acrylamide. Study on the tourist consumption habits indicated the risk of consumer intake of a few micrograms acrylamide daily.

Acknowledgments

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Antimicrobial Activities of the Edible Bird’s Nest Extracts Against Food-borne Pathogens

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Abstract

Antimicrobial activities of extracts from the edible bird’s nest (EBN) against four food borne pathogens (Staphylococcus aureus, Escherichia coli, Candida albican, and Aspergillus niger) were investigated. The EBN extracts were prepared using two methods: a soaking extraction method in ethyl acetate or methanol at 100-3,000 mg/L and a solvent extraction method in ethyl acetate or methanol at 20-100 mg/L. The agar well diffusion method was employed to examine antimicrobial activity of each EBN extract at various concentrations. For the soaking extraction method using methanol, the EBN extract showed stronger antimicrobial activity for S. aureus (100 mg/L) and C. albican (100 mg/L) than for E. coli (1,000 mg/L) and A. niger (>3,000 mg/L) while the EBN extract derived by the soaking extraction in ethyl acetate showed no inhibitory effects. Results of the solvent extraction method showed that the EBN extracts using ethyl acetate were more effective than using methanol with C. albican (20 mg/L) and A. niger (20 mg/L). However, using methanol showed some activity with S. aureus at 20-100 mg/L. Using the solvent extraction method with methanol showed slight effects with E. coli (>100 mg/L). This study has demonstrated a potential of the EBN extract as an antimicrobial agent against food borne pathogens.

Keywords: antimicrobial activity; edible bird’s nest; food borne pathogens

Introduction

The edible bird’s nest (EBN) or, in Chinese, Yan Wo and Yanchoo is the natural saliva nest produced by swiftlets. EBN farming is produced in many countries in Southeast Asia such as Thailand, Malaysia, Vietnam, and Indonesia. Recently, the price of EBN in Thailand is sold by swiftlet farmers at about ฿65,000 per kilogram, depending on the quality. The export value from swiftlet farming has reached around ฿126 million in 2007 (Jory and Saengthong, 2007).

It has been used as traditional Chinese medicine (Chan, 2009; Oda et al., 1998). Consumers believe that consummation of EBN could be good for their health such as inhibition of influenza viral infection (Guo et al., 2006). This Chinese delicacy uses rare EBN in soup which makes it amongst one of the world’s most expensive animal products consumed by humans. The main nutritional contents of the EBN are carbohydrates and protein with trace elements of sodium, calcium, potassium, magnesium, phosphorus, and iron (Huda et al., 2008; Marcone, 2005).

Although the nutritional aspects of the EBN have been examined, medical aspects such as antimicrobial activity have not yet been confirmed and this is the main objective of this research. Emphasis was placed on an evaluation of antimicrobial
properties of the EBN extracts against various food borne pathogens such as Staphylococcus aureus (gram positive bacteria), Escherichia coli (gram negative bacteria), Candida albican (yeast) and Aspergillus niger (mold). These microorganisms were selected because of their harm to human health. S. aureus is a pathogen associated with both human and animal diseases including mastitis, toxic shock syndrome, and staphylococcal food-poisoning. Symptoms can cause vomiting, abdominal cramps and diarrhea (Jørgensen et al., 2005; Hrstka et al., 2006). To date, outbreaks caused by enterohaemorrhagic E. coli have been attributed to strains found in foods; the vegetable, for example. Most strains of E.coli are harmless, but produce toxins that could cause diarrhea (Hales et al., 1991). C. albicans is a form of yeast that could release chemicals into the bloodstream and cause various symptoms like lethargy and chronic diarrhea (Reagan et al., 1995). A. niger could produce Ochratoxin A as a mycotoxin and contaminate food. It has been implicated in immunotoxicity in both animals and humans (Esteban et al., 2006).

The objective of this work is to evaluate the effectiveness of the EBN extracts to reduce four food borne pathogens (Staphylococcus aureus, Escherichia coli, Candida albican, and Aspergillus niger) using both a soaking method and a solvent method.

Materials and Methods

Materials

Bird nests were collected from a local swiftlet farm in the Nakhon Si Thammarat province in Southern Thailand from June to October 2010. Feathers and dirt were removed from the nests using sterile forceps and scissors. Then, the nests were ground in a mill to produce a fine powder and later packed into vacuum packaging and kept dry in the desiccators at 25 °C.

Soaking Extraction Method

Milligrams (100, 500, 1,000, 2,000, and 3,000) of dried EBN were extracted and soaked in 1 L of methanol or ethyl acetate for 12 hours at 25 °C in a shaker to give a concentration of 100, 500, 1,000, 2,000, and 3,000 mg/L. The extract was then filtered through a buchner funnel with Whatman No. 1 filter paper three times. Amounts of EBN’s extract after filtration was between 400-500 ml. It was then preserved in a sealed vial at 4 °C until further analysis. Methanol and ethyl acetate were the control.

Solvent Extraction Method

One hundred grams of dried EBN was soaked in 1 L of methanol or ethyl acetate, for 12 hours at 25 °C and stirred every hour using a sterilized glass rod. At the end of the extraction, it was passed through Whatman No. 1 filter paper. This filtrate was concentrated under vacuum on a rotary evaporator at 40 °C and then stored at 4 °C for further use. The crude extract was prepared by dissolving to have a stock solution of 100 mg/L concentration. Then, methanol or ethyl acetate was added onto the crude extracts to meet concentrations of 0, 20, 50, 70, and 100 mg/L.

Microbial Strains and Culture Media

The pathogenic bacteria (Staphylococcus aureus, Escherichia coli), yeast (Candida albicans) and mold (Aspergillus niger) were obtained from the Center for Scientific and
Technology Equipment at Walailak University in Southern Thailand. Suspension of the test bacteria, yeast and mold were prepared from fully grown bacteria on Nutrient Agar, NA (Merck, Thailand) at 37°C. Fully grown yeast and mold were made on Malt Extract Agar, MEA at 25°C. Microbial strains were collected by flooding the surface of the plates with ~5 ml sterile saline solution (NaCl, 8.5 g/l water) containing Tween 80 (0.1% v/v). The viability of all strains was checked using quantitative colony counts at 10^7 CFU ml^-1.

**Antimicrobial Activity of the EBN Extract**

Antimicrobial activity of the EBN extract against *S.aureus*, *E.coli*, *C.albican* and *A. niger* were determined by the disc diffusion assay. Sterile plastic plates of 90 mm diameter (P. Intertrade Equipments, Thailand) containing NA for bacteria and MEA for yeast and mold were spread with 0.1 ml of each appropriate suspension. A cork borer was used to make a 6 mm hole on the agar. Fifty microliters of EBN extracts obtained by soaking extraction (100, 500, 1,000, 2,000, and 3,000 mg/L) and solvent extraction (20, 50, 70 and 100 mg/L) were impregnated into the hole. Fifty microliters of dilution solvent (methanol or ethyl acetate) were added to the hole on the control plates. The diameter of the zone of inhibition (mm) around the disc was measured after cultivation at 37°C for 24 hours for bacteria and at 25°C for 72 hours for yeast and mold. The clear zone from the control plate was used to minus different clear zone from test plate.

Minimum inhibitory concentration (MIC) was tested using the broth dilution method. One ml of EBN extracts obtained by the soaking extraction (100, 500, 1,000, 2,000, and 3,000 mg/L) and the solvent extraction (20, 50, 70 and 100 mg/L) were adding 0.1 ml of in each *E.coli*, *S.aureus*, *C.albican* and *A. niger* into the sterile screw-cap tubes. Methanol and ethyl acetate were the control. The tubes were shaken using a platform shaker at 150 rpm, 24 hours for bacteria and 72 hours for yeast and mold. The viable count of the *E.coli* and *S.aureus* in each sample was determined by plating 0.1 ml portions directly or after serially diluted in sterile 0.1% peptone water on Compact Dry "Nissui" EC (for *E. coli*), and Compact Dry "Nissui" SA (for *S. aureus*). All of Compact Drys were purchased from Oskon Ltd, Thailand. *E. coli* and *S.aureus* incubated at 37°C for 24 hours before counting. *C. albicans* and *A. niger* were counted using MEA after incubating at 25°C for 72 hours. The lowest concentration showing no visible growth was regarded as the MIC.

**Results and Discussion**

**Effectiveness of EBN Using the Soaking Extraction Method**

Antimicrobial properties of the EBN extracts obtained by the soaking extraction method represented as a zone of inhibition is shown in Table 1. For the methanol soaking extraction, the EBN extract at 100 mg/L exhibited a clear inhibitory zone by the absence of gram positive bacterium (*S. aureus*) and yeast (*C.albican*) growth around the hole. At concentration of 1,000 mg/L, the clear zone of inhibition was observed with gram negative bacterium (*E.coli*). Growth of the test mold (*A. niger*) was, however, not affected by the presence of the EBN extract up to the concentration of 3,000 mg/L. For the ethyl acetate, no clear zone was found for all bacterial, yeast and mold test. According to the results given in Table 1, MIC of the EBN extracts weren’t found at the concentration between 100 to 3,000 mg/L. These results show that soaking the EBN with methanol has a higher level of activity than ethyl acetate. Marcone (2005) reported that protein was a main composition of the EBN. In addition, it was also found that the EBN share a common 77 KDa protein that has properties similar to those of the ovotransferrin protein in eggs.
Table 1 Effect of the EBN extract obtained by the soaking extraction on the growth of the four food-borne pathogens.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Con. (mg/L)</th>
<th>Inhibition zone diameter of strains (mm.)</th>
<th>S.aureus</th>
<th>E.coli</th>
<th>C.albican</th>
<th>A.niger</th>
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<td>11±3</td>
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<tr>
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<td>2,000</td>
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<td>&gt;3,000</td>
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<td>&gt;3,000</td>
<td>&gt;3,000</td>
</tr>
</tbody>
</table>

1/ Mean values ± standard deviations (n = 3)
2/ Minimum inhibitory concentration

Nevertheless, some protein could be soluble in alcohol (Mathew and Juang, 2007). These activities might depend on the compounds being extracted by methanol, the polarity of the solvents, and their intrinsic bioactivity.

Effectiveness of EBN Using the Solvent Extraction Method

Antimicrobial properties of the EBN extracts obtained by the solvent extraction method is shown in Table 2. The EBN extract with ethyl acetate at concentrations ≥20 mg/L was capable of inhibiting the growth of yeast (C.albicans) and mold (A. niger) strains with zone inhibition of 11 to 20 mm. Although, active EBN extract from ethyl acetate showed against S.aureus at 70 mg/L, it could not inhibit E.coli in this test at concentration of 100 mg/L. The methanol extract of the EBN also showed effect on S.aureus at 20 mg/L and slightly effect on E.coli at 100 mg/L, but did not show any anti yeast and mold. It may be due to low concentration of EBN. Therefore, our next study will focus on different higher concentration of the EBN.

Table 2 Effect of the EBN extract obtained by solvent extraction on the growth of the four food-borne pathogens.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Con. (mg/L)</th>
<th>Inhibition zone diameter of strains (mm.)</th>
<th>S.aureus</th>
<th>E.coli</th>
<th>C.albican</th>
<th>A.niger</th>
</tr>
</thead>
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<tr>
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<tr>
<td>Ethyl acetate</td>
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<tr>
<td>MIC2</td>
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<td>&gt;100</td>
</tr>
</tbody>
</table>

1/ Mean values ± standard deviations (n = 3)
2/ Minimum inhibitory concentration

The crude extracts with MIC of the EBN in all samples were more than 100 mg/L. The relationship between the zone of inhibition and MIC value may not be related. On the other hand, these test strains may have different level of intrinsic tolerance to antimicrobials and thus the MIC values differ from isolate to isolate. There are limited reports on the antimicrobial activity of the EBN extract in the literature, even though inhibition of some components in the EBN extract have been reported against viruses (Guo et al., 2006), Staphylococcus aureus, Streptococcus sp., Escherichia coli, Salmonella sp., Klebsiella pneumonia, and Pasteurella multocida (Suriya, et al., 2004). However, in depth exploration on antibacterial activity of the bird's nest needs to be carried out. The future work should be focused on determination of major active components in the EBN extract responsible for its antimicrobial activity against food borne pathogens.

Conclusions

The EBN extract obtained by soaking the extraction in methanol showed good inhibition against growth of gram positive bacteria and yeast. The EBN extract obtained by solvent extraction using ethyl
acetate at concentration ≥ 20 mg/L inhibited growth of yeast and mold (C. albicans and A. niger). The minimal inhibitory concentration of the EBN were not found for all samples in this test. The EBN extract showed good potential as a healthy antimicrobial agent against food borne pathogens in the alimentary canal.

Acknowledgments

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References


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Evaluation of Oxidative Stability and Some Quality Characteristics of Chinese-Style Sausage as Affected by the Addition of Roselle Extract and Different Sweeteners

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Abstract

The purpose of this study was to evaluate the oxidative stability and qualities during storage of Chinese-style sausages with addition of roselle extract and different sweeteners. The Chinese-style sausage with 0.3% (w/w) roselle extract and 16.6 % (w/w) sucrose or sugar alcohols (lactitol, maltitol and xylitol) were stored at room temperature (29±1 °C) for four weeks in a vacuum plastic bag. The results showed that Chinese-style sausage with xylitol addition had lower moisture content and water activity (p<0.05). Chinese-style sausages with sugar alcohols addition exhibited the lower TBARS values (p<0.05) compared to those of sucrose added sausage or control (no roselle extract). According to color parameters of Chinese-style sausage, lactitol added sample was higher in lightness (CIE L*) and yellowness (CIE b*). However, no significant differences (p≥0.05) on chroma and browning index of all Chinese-style sausage samples were observed. Considering texture profile analysis (TPA), xylitol added sausage showed better values in hardness, adhesiveness, springiness and gumminess than those of lactitol or maltitol added samples. Adding roselle extract with xylitol in Chinese-style sausage not only improves quality and delays lipid oxidation, but also retains the similar texture qualities compared to the original Chinese-style sausages.

Keywords: antioxidant, chinese-style sausage, roselle, sugar alcohols, sweetener

Introduction

Rancidity in meat products results in a loss of nutritional and sensory quality such as color, texture, and flavor, thereby limiting their shelf life. The addition of antioxidants has been used to retard lipid oxidation in meat products. The synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tert-butylhydroquinone (TBHQ) and propylgallate (PG) have been used to control or retard the development of rancidity in meat products (Decker et al., 2000). However, there is increasing concern about their safety because of their potential as mutagens and carcinogens (Williams et al., 1999; Sebranek and Bacus, 2007). As a result, the use of natural antioxidants is preferred in the production of foods. Most natural antioxidants are of plant origin and their antioxidant properties have been proved and reported extensively (Maisuthisakul et al., 2008; Patthamakanokporn et al., 2008; Kevers et al., 2007). These compounds are primarily phenolic or polyphenolic, flavonoids (anthocyanins, flavonols, flavones, flavanones and catechins), and related compounds (phenolic acids, chalcones, and isoflavones). They act as reducing agents, free radical scavengers, complexers of pro-oxidant metals or quenchers of the formation of singlet oxygen.

Antioxidative activity of anthocyanins has been extensively reviewed by Kong et al. (2003) and Castañeda-Ovando et al. (2009). Anthocyanins extracted from fruits and vegetables have been evaluated for its antioxidative effect in meat products and have been reported to improve the oxidative...
stability of cooked chicken (Sáyago-Ayerdi et al., 2009), dry-fermented sausage (Karabacak and Bozkurt, 2008) and cooked beef (Ismaiil and Yee, 2006).

Recently, anthocyanins extracted from roselle (Hibiscus sabdariffa L.) and commercial anthocyanin powder such as black carrot (Daucus carota, L.) and grape skin (Vitis vinifera L.) have been reported to be an effective antioxidant (in terms of antilipoperoxidant activity) in ethnic meat products including Chinese-style sausage (Parinyapatthanaboot and Pinsirodom, 2010) and Pork chips (Parinyapatthanaboot et al., 2010). However, high concentrations of sucrose (>10%) has been shown to negatively affect the antilipoperoxidant capacity of roselle anthocyanins when incorporated at 0.3% (w/w) in Chinese-style sausage (Parinyapatthanaboot et al., 2010). In addition, when the xylitol was used to replace sucrose in Chinese-style sausage, the roselle anthocyanins efficiently exhibited antilipoperoxidant capacity (Pinsirodom, 2008). It is; therefore, of interest to further investigate whether different types of sugar alcohols will affect the antioxidant activity of roselle anthocyanins in the same manner.

The use of sugar alcohols as an alternative sweetener in meat products has not yet been reported elsewhere. The interest in application of sugar alcohols in food products is not only due to their low calorie and ability to improve blood sugar control, but also their excellent hydroxyl radical (•HO') scavenging capacity and in vitro inhibition of diazocompound-induced erythrocyte damage (den Hartog et al., 2010). Thus, sugar alcohols would be a promising sweetener for meat products especially when roselle anthocyanins will be incorporated as anti-lipid oxidation agent.

The objectives of this study were to evaluate the effect of different sweeteners (sucrose, lactitol, maltitol and xylitol) on oxidative stability and some qualities of Chinese-style sausage with addition of roselle extract during storage.

### Materials and Methods

#### Materials and Reagents

Lactitol-monohydrate crystalline (Danisco, Denmark) and Xylitol-crystalline (Danisco, Denmark) were kindly supported by Rama production Co., Ltd., Thailand. Maltitol syrup powder MU90G (Ueno, Japan) was kindly supported by UENO Fine Chemicals Industry (Thailand). Ltd. Sucrose (Mitrphol) was purchased from Mitr Phol Sugar Corp., Ltd. Thailand. All chemicals used in this study were analytical grade.

#### Preparation of Roselle Anthocyanin Extract

Roselle anthocyanin extract (RAE) was prepared as described by Parinyapatthanaboot et al. (2010). The dried calyces of roselle were purchased from local herbal marketed brand (Dr. Green; Thailand) and were cleaned and powdered. The extract was prepared by soaking dried roselle calyces powder in 95% ethanol (1:10 w/v) in the dark for 24 hr with an occasional shaking to increase the extraction capacity. The extract was rapidly filtered through a Buchner funnel with Whatman no 1 filter paper and the filtrate was evaporated under reduced pressure in rotary evaporator (Büchi model R210, Switzerland) at 30 °C. The crude extracts were kept in amble glass vial with screw cap at -18 °C until use.

The RAE obtained was dark red, viscous oleoresin with pH 1.0-1.1. The antioxidant properties of the RAE had been previously reported by (Parinyapatthanaboot et al., 2010).

#### Preparation of Chinese-style Sausage

Fresh lean pork and back fat (Lot no. 10183E1380507) purchased from a CP Fresh Mart, Bangkok, Thailand. The lean tissue was trimmed off heavy connective tissue and external fat and then ground through a 4 mm grinder plate (SevenFive, Thailand). Chinese-style sausages were prepared according to previous study (Parinyapatthanaboot et al., 2010). Briefly, minced back fat (16 %) was mixed with 0.3 % RAE (by total weight of the mixture) in a
bowl using an electrical mixer (Moulinex Mixer BM8, USA) at medium speed for 1 min. The minced lean pork (65 %) was added and mixed well at low speed for 2 min. The other ingredients were added in equal amounts to all samples of Chinese-style sausage: 0.2 % potassium nitrite (prague powder), 0.1 % Chinese five-spice blend and 0.3 % monosodium glutamate. To compare the effect of sucrose and sugar alcohols (lactitol, moltitol and xylitol) on qualities of Chinese-style sausage, the concentration of each sweetener were assigned as 16.6 % (w/w).

The sausage mixtures were stuffed into collagen casing with 15 mm diameter (Nippi casing, Japan), linked into 10 cm length and then dried at 60 °C for 24 hr (0 day storage). Sausages were packed under vacuum in nylon/PE vacuum bag (PA/LLDPE/LDPE, thickness: 80 micron) stored at room temperature (29 ± 1 °C) and analyzed every week for 4 weeks (7, 14, 21 and 28 day). Three replicates with batches were conducted in this study.

Determination of Moisture Content
The moisture content of sausages was determined using the hot air oven method at 105±1 °C (AOAC, 1990)

Determination of pH
The pH of the Chinese-style sausage was measured after homogenisation (Ultra-Turrax® T25Bbasic, Germany) with distilled water at a ratio of 1:10 using pH meter Level1 couple with pH Electrode SenTix81 probe (inoLab, Germany)

Determination of Water Activity (aw)
Water activity of the ground Chinese-style sausage was measured by a water activity meter (AquaLab TMSeries3TE, USA).

Determination of Color Parameters
Color parameters (CIE L*, a*, b*) were analyzed by a colormeter (Minolta CR-400, Japan) standardized with a white plate (Y = 93.74; X = 0.3133; y = 0.3194). The color measurements of ground Chinese-style sausage were performed at room temperature (29±1 °C) in triplicate. Total color difference (TCD; Equation 1), hue angle (Equation 2), chroma (saturation index; Equation 3), and browning index (BI; Equation 4) was calculated using CIE L*, a*, and b* values (Maskan, 2001) as:

\[
TCD = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2}
\]

\[
\text{Hue angle} = \arctan \left( \frac{b}{a} \right)
\]

\[
\text{Chroma} = \sqrt{a^2 + b^2}
\]

\[
\text{BI} = \frac{[100 \times (X - 0.31)]}{0.17}
\]

Determination of Texture Profile Analysis (TPA)
Texture profile analysis (TPA) was applied as described by Bourne (2002) using a TAXT2i plus texture analyzer (Stable Micro Systems, Surrey, UK) equipped with a cylindrical probe P/25 to determine at ambient temperature. The cubic samples with approximately 2.5 cm long and 1.5 cm wide were compressed twice to 50 % of their original height with a compression platen of 75 mm in diameter. Force–time deformation curves were recorded at a crosshead speed of 5 mm/s and recording speed was also 5 mm/s. The following parameters were determined: hardness (H) = maximum force required to compress the sample; springiness (S) = ability of sample to recover its original form after a deforming force was removed; cohesiveness (Coh) = extent to which sample could be deformed prior to rupture (A2/A1, A1 being the total energy required for the first compression and A2 the total energy required for the second compression); gumminess (G) = force necessary for disintegrate a semisolid sample for swallowing (H × cohesiveness); chewiness (C) = work to masticate the sample for swallowing (S × gumminess).

Determination of 2-Thiobarbituric Acid Reactive Substances (TBARS)
Chinese-style sausage was analyzed for TBARS value, as a lipid oxidation index, according to the method of Min et al. (2009)
with slight modifications. Briefly, 500 mg of ground sausage was homogenized with 5 mL distilled water using a homogenizer (Ultra-Turrax® T25Bbasic, Germany) for 30 s at the highest speed. One milliliter of homogenate was transferred to a test tube (16x150 mm) and TBA/trichloroacetic acid solution (5 ml) was added. The mixture was vortexed and then incubated for 15 min in a boiling water bath to develop color. After cooling, the mixture was centrifuged at 14000g (Hettich Universal 16, Germany) for 5 min. The absorbance of the supernatant was determined at 531 nm against a reagent blank. The amount of TBARS was expressed as milligrams malondialdehyde per kilogram sausage. A standard curve was prepared using 1,1,3,3 tetraethoxypropane (TEP).

Statistical Analysis

A completely randomized experimental design (CRD) was used. The results were analyzed using General Linear Models (GLM) procedure. When significant (P<0.05), mean values was determined by Duncan’s New Multiple Range Test. The statistical analysis was conducted with SPSS software.

Results and Discussion

Changes of Moisture Content, Water Activity and pH of Chinese-style Sausages

The effect of sweeteners on moisture content, water activity and pH of Chinese-style sausage containing RAE are shown in Table 1 and changes of those parameters during storage are presented in Figure 1.

No significant difference (p≥0.05) of moisture content was observed for all sausage samples, except for xylitol added sample. The moisture content of all samples ranged from 23 to 25% and tended to decrease (p<0.05) during storage for 28 days (Figure 1a). The moisture content of the sausages in this experiment agrees with the Thai Industrial Standard (TISI, 2003) which mentioned that moisture content of Chinese-style sausage should not be higher than 29%.

The water activity of all samples was between 0.71 to 0.78, and was not significantly different over storage time (data not shown). The results showed that all Chinese-style sausage samples corresponded to a typical characteristic of intermediate moisture food. In this study, the Chinese-style sausage with xylitol addition tended to have a lowest water activity. This was probably due to the fact that xylitol has greater influence in lowering the water activity of food, and its high osmotic pressure could lead to an increased preservative effect (Comesaña et al., 2001).

The pH values were significantly lower (P<0.05) for the Chinese-style sausages with sucrose or sugar alcohols addition when the RAE was incorporated (Table 1). This slightly lower pH of samples with RAE addition was probably due to the acidic pH of roselle extract. The slightly reduced pH of meat products as affected by roselle extract addition have been observed in pork chip (Pinsirodom, 2008), Turkish dry-fermented sausage “sucuk” (Karabacak and Bozkurt, 2008), cooked meat “kavurma” (Bozkurt and Belibaği, 2009) and Chinese-style sausage (Parinyapatthanaboot and Pinsirodom, 2010). In addition, the pH of all sausage samples slightly increased during storage. The values were approximately 5.9 to 6.1 (Figure 1b).

Changes of Color Parameters of Chinese-style Sausages

The addition of sugar alcohols to the formulation showed significant effect on the internal color values of Chinese-style sausages (Table 2). The data showed that the sausage with lactitol addition tended to have higher lightness value (CIE L*). However, the lightness of xylitol and sucrose added samples was not significantly different (p≥0.05) compared to that of the control (sucrose added without RAE).
Table 1 Effect of sweeteners on moisture content, water activity ($a_w$) and pH of Chinese-style sausage containing RAE.

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>MC (%)</th>
<th>$a_w$</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.86±0.53$^a$</td>
<td>0.777±0.00$^a$</td>
<td>6.11±0.01$^a$</td>
</tr>
<tr>
<td>Sucrose</td>
<td>24.93±0.50$^b$</td>
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<tr>
<td>Lactitol</td>
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<tr>
<td>Maltitol</td>
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<tr>
<td>Xylitol</td>
<td>23.10±0.41$^b$</td>
<td>0.709±0.00$^d$</td>
<td>5.97±0.01$^b$</td>
</tr>
</tbody>
</table>

*Control (sucrose added without RAE), sucrose (sucrose added with RAE), Lactitol (lactitol added with RAE), Maltitol (maltitol added with RAE) and Xylitol (xylitol added with RAE).

abc Means values in the same column with different letters indicate significant differences (p<0.05).

Figure 1 Changes of a. moisture content (%) and b. pH of Chinese-style sausages containing RAE and different sweeteners during storage. All results are from 3 experiments with average coefficient of variation (CV) = 3.10 %.

Comparing to the control, all sausage samples with sweeteners and RAE addition, showed significantly lower redness values except for lactitol added sausage which exhibited no significant difference. Results also indicated that red color of RAE did not affect the redness value of all sausage samples. This could be explained by the fact that the anthocyanins undergo a variety of molecular transformations as the pH of the medium changes. The pH of the sausage in this study was about 5.9 to 6.1, which was beyond the acidic pH that the red color species of anthocyanins (flavylium cation) could be formed. Moreover, pH values between 5 and 6, only two colorless species (carbinol pseudobase and chalcone) can be existed (Castañeda-Ovando et al., 2009). Therefore, addition of RAE at the concentration of 0.3% (w/w) in this study would not affect the redness of Chinese-style sausage.

As seen in Figure 2a, the redness of all samples tended to increase during storage. This was probably due to the formation of nitrosomyoglobin (Suman et al., 2006) and moisture loss (Pérez-Alvarez et al., 1999) leading to the increase in redness.

In terms of yellowness, xylitol added sausage showed no difference to the sucrose added and control samples, while the lactitol and maltitol added sausages had significantly higher values (p<0.05). However, a slight increase of yellowness was observed during storage for all samples (data not shown).

The total color difference (TCD), which is a combination of CIE $L^*$, $a^*$ and $b^*$ values, is a parameter commonly used to characterize the variation of colors in foods. A decrease in TCD for all Chinese-style sausage was observed throughout storage time (data not shown). The decrease of TCD in this study agreed with previous reports in relation to the discoloration of bologna (Fernández-Ginés et al., 2003) and pork sausage (Jo et al., 2000) during storage. These authors suggested that color deterioration during storage of sausages can be explained by the degradation of certain nitrosopigments caused by oxidative processes.

Hue angle, Chroma and Browning index (BI) (Figure 2b, 2c and 2d) of all samples...
showed a slightly increase during storage period. However, sucrose and xylitol added samples did not show significant difference for these values (p≥0.05). Hue angle of control and sweeteners added samples increased from 24 to 32 and 29 to 40 during storage, respectively. This indicates that the color of all sausages located in shades of an orange-red (Hue<90) (Maskan, 2001). The Chroma and browning index (BI) values of all sausage samples were significant difference (p<0.05) between beginning and the end of storage. This indicated that the development of color intensity might affected by non-enzymatic browning reaction during storage.

**Texture Profile Analysis (TPA) of Chinese-style Sausages**

A comparative study of the effect of different sweeteners on texture quality of the Chinese-style sausage in the present of RAE was carried out and the results are shown in Table 3. When RAE was used as natural antioxidant, addition of all sweeteners studied, in hardness and gumminess values compared to the control. except for xylitol resulted in Chinese-style sausages with significantly higher (p<0.05) In addition, xylitol added sausage showed significantly lower chewiness and springiness compared to the control. However, cohesiveness of all sausage samples was not affected (p≥0.05) by sweeteners and/or RAE addition. The results also indicated that Chinese-style sausage with xylitol addition yielded more tender texture compared to that with other sweeteners.

**Table 2** Effect of sweeteners on lightness (CIE L*), redness (CIE a*), yellowness (CIE b*), total color difference (TCD), Hue angle (Hue), Chroma and browning index (BI) of Chinese-style sausage containing RAE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CIE L*</th>
<th>CIE a*</th>
<th>CIE b*</th>
<th>TCD</th>
<th>Hue</th>
<th>Chroma</th>
<th>BI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.64±2.22c</td>
<td>11.41±0.78a</td>
<td>5.05±0.65b</td>
<td>52.79±2.17a</td>
<td>23.90±3.37a</td>
<td>12.49±0.71b</td>
<td>29.21±2.24b</td>
</tr>
<tr>
<td>Sucrose</td>
<td>46.18±1.47a</td>
<td>9.69±0.44c</td>
<td>5.97±0.84b</td>
<td>52.24±1.54a</td>
<td>31.51±3.06bc</td>
<td>11.41±0.73c</td>
<td>28.68±3.13bc</td>
</tr>
<tr>
<td>Lactitol</td>
<td>50.46±1.31a</td>
<td>11.53±0.48b</td>
<td>7.64±0.13a</td>
<td>48.60±1.22c</td>
<td>33.56±0.95db</td>
<td>13.84±0.45a</td>
<td>32.49±0.76a</td>
</tr>
<tr>
<td>Maltitol</td>
<td>48.62±1.24b</td>
<td>10.75±0.29b</td>
<td>7.65±0.36a</td>
<td>50.29±1.18bc</td>
<td>35.43±1.21a</td>
<td>13.20±0.37a</td>
<td>32.69±1.20a</td>
</tr>
<tr>
<td>Xylitol</td>
<td>46.22±1.14c</td>
<td>9.37±0.40c</td>
<td>5.37±0.34b</td>
<td>52.06±1.15ab</td>
<td>29.82±1.57c</td>
<td>10.80±0.43c</td>
<td>26.59±1.48c</td>
</tr>
</tbody>
</table>

*Control (sucrose added without RAE), sucrose (sucrose added with RAE), Lactitol (lactitol added with RAE), Maltitol (maltitol added with RAE) and Xylitol (xylitol added with RAE).

abc Means values in the same column with different letters indicate significant differences (p<0.05).

**Figure 2** Changes of color parameters, a. redness (CIE a*), b. Hue angle, c. Chroma and d. Browning index (BI) in Chinese-style sausages containing RAE and different sweeteners during storage. All results are from 3 experiments with with average coefficient of variation (CV) = 9.4%.
Regardless of the type of sweeteners, hardness, springiness, gumminess and chewiness of the Chinese-style sausage tended to increase during storage (Figure 3), while cohesiveness remained the same for all samples (data not shown). The increase in hardness values are in agreement with the study of Fernández-López et al. (2004), who reported the increased hardness of ostrich pâté, a French sausage or spread made from ground liver, meat and fat, during 28-day storage. It has been suggested that the increased hardness of meat products during storage may be due to the polymerization of lipids and/or proteins and the decrease of moisture content (Fernández-Ginés et al., 2005). Many factors can affect protein/fat stability such as minced degree and water-holding capacity, but pH is most likely to be the most important factors impacting protein/fat stability in meat products (Fernández-López et al., 2004). It is possible that the lower pH of RAE might impact on protein/fat stability, leading to the aggregation and complex formation due to cross link formation. Results from the present study suggest that RAE might cause the increased hardness and gumminess of Chinese-style sausage by reducing protein/fat stability.

Increasing in gumminess and chewiness values indicated that the sausage became more gummy and tougher during storage (Szczeniak, 2002). Considering the springiness values which related to elastic properties of the sausages, control and xylitol added samples showed a slightly increase in springiness during storage. However, no significant difference (p≥0.05) was found in springiness of all samples at the latest period of storage.

Oxidative Stability of Chinese-style Sausages

In Figure 4, the data obtained for milligrams of MDA per kilograms of Chinese-style sausage treated with 16.6% (w/w) of different sweeteners and 0.3% (w/w) RAE along with the control sample (sucrose added without RAE) are shown.

The result indicated that the formation of MDA increased for all samples with storage time meaning that lipid oxidation increased with the time during storage. There was a significant difference (p<0.05) in the TBARS values between sausages with different sweeteners (lactitol, maltitol and xylitol) and the control sample. Sugar alcohols added samples accounted for a reduction of approximately 1 mg MDA/kg sausage compared to the sucrose added or control sample at the end of storage. However, there was no significant deference in TBARS value (p≥0.05) among sugar alcohols treatments during storage.

### Table 3 Effect of sweeteners on texture profile analysis of Chinese-style sausage containing RAE

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Hardness (g)</th>
<th>Springiness (cm)</th>
<th>Cohesiveness (ratio)</th>
<th>Gumminess (N)</th>
<th>Chewiness (N cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1564.46±147.07c</td>
<td>0.42±0.06c</td>
<td>0.89±0.02c</td>
<td>1386.38±121.08d</td>
<td>584.57±101.61c</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2087.60±201.93a</td>
<td>0.53±0.05a</td>
<td>0.90±0.04a</td>
<td>1879.97±205.45a</td>
<td>993.84±143.68a</td>
</tr>
<tr>
<td>Lactitol</td>
<td>1908.48±98.45b</td>
<td>0.36±0.01c</td>
<td>0.86±0.02a</td>
<td>1630.00±79.60c</td>
<td>594.88±47.65a</td>
</tr>
<tr>
<td>Maltitol</td>
<td>2051.53±136.80b</td>
<td>0.45±0.05b</td>
<td>0.86±0.03b</td>
<td>1765.65±133.85b</td>
<td>798.31±121.59b</td>
</tr>
<tr>
<td>Xylitol</td>
<td>1551.88±50.57c</td>
<td>0.34±0.05c</td>
<td>0.91±0.03a</td>
<td>1416.13±47.00d</td>
<td>478.41±67.64d</td>
</tr>
</tbody>
</table>

*Control (sucrose added without RAE), sucrose (sucrose added with RAE), Lactitol (lactitol added with RAE), Maltitol (maltitol added with RAE) and Xylitol (xylitol added with RAE).

abcMeans values in the same column with different letters indicate significant differences (p<0.05)
Figure 3 Changes of texture parameters, a. hardness (g), b. springiness (cm), c. gumminess (N) and d. chewiness (N cm) in Chinese-style sausages containing RAE and different sweeteners during storage. All results are from 3 experiments with with average coefficient of variation (CV) = 10.8 %.

Figure 4 Development of TBARS (mg MDA/kg sausage) in Chinese-style sausages containing RAE and different sweeteners during storage. All results are from 3 experiments with with average coefficient of variation (CV) = 2.4 %.

abcdMeans (type of sweeteners) with different letters indicate significant differences (p<0.05).

ABCDEMeans (storage time; days) with different letters indicate significant difference (p<0.05).

TBARS values were found to be highest in control sample (0.35-1.27 mg MDA/kg sausage) and lowest in xylitol added samples (0.06-0.13 mg MDA/kg sausage). The results clearly showed that sugar alcohols added Chinese-style sausages exhibited higher oxidative stability compared to sucrose added sausage when RAE was used as natural antioxidant. This agreed with the previous studies which reported that changes of TBARS values during storage of Chinese-style sausages and pork chips treated with 0.3% (w/w) RAE and sucrose at the concentration of 7-20% (w/w) were higher than those for the control (0% sucrose with RAE added) (Parinyapatthanaboot et al., 2010; Pinsirodom, 2008). Results suggested that xylitol or other sugar alcohols could enhance reducing potential of RAE for lipid oxidation in Chinese-style sausage.

Conclusions

When RAE was used as natural antioxidant, addition of different sweeteners affected the physicochemical properties and oxidative stability of Chinese-style sausage. The sausages with sugar alcohols addition, especially xylitol, showed lower moisture content and water activity compared to the sucrose added sample. Results also showed that xylitol added Chinese-style sausage exhibited all color and texture parameters more closely to the sucrose added sausage. All sugar alcohols in the present of RAE were
efficiently improves the oxidative stability of Chinese-style sausages during storage. The findings in this study demonstrate a potential use of sugar alcohols as an alternative sweetener and RAE as a natural antioxidant in the development of novel healthy meat products with improved shelf-life. This would be the first introduction of using sugar alcohols as sweetener in processed meats.

Acknowledgments

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References


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Rapid and Highly Sensitive Analysis of Ethoxyquin Residues in Shrimp Using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

A rapid, simple and sensitive method for quantitation and confirmation was developed for the analysis of ethoxyquin in shrimp. The method involved a new sample preparation process based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) methodology. Extraction and cleanup procedures were optimized with confirmed positive and spiked samples. Ethoxyquin was extracted from homogenized samples using 2% acetic acid in acetonitrile, followed by a cleanup step of dispersive solid-phase extraction using 50 mg of primary secondary amine and 10 mg of graphitized carbon black. The detection of ethoxyquin was achieved by ultra high performance liquid chromatography–tandem mass spectrometry with 2 min retention using positive electrospray ionization mode. The performance characteristics of the method were established by in-house validation procedures employing tests with standard solutions, sample blanks, spiked samples and positive samples. Linearity, matrix effect, trueness, precision, selectivity, limit of detection and limit of quantitation were determined. The mean recoveries of ethoxyquin from the shrimp samples spiked at 5.0–50.0 µg kg\(^{-1}\) were 91–97% with relative standard deviation between 4.09–6.61%. Linear calibration curve was obtained in the range of 0.5–8.0 µg kg\(^{-1}\) with good coefficient of determination above 0.995. The limit of detection was 1.5 µg kg\(^{-1}\), which is significantly lower than the maximum residue limits for ethoxyquin in food products (10 µg kg\(^{-1}\)). This method has been successfully applied in routine analysis with high sample throughput.

Keywords: ethoxyquin, residues, shrimp, tandem mass spectrometry, ultra high performance liquid chromatography

Introduction

Ethoxyquin is a synthetic antioxidant widely used as a pesticide and a food preservative in animal feeds. The applications of ethoxyquin are based on its ability to interrupt the oxidation reaction in the propagation process initiated by air, light, or transition metals in unsaturated lipids of feeds. Despite its importance as a preservative, there is a concern about ethoxyquin utilization in feeds because it may leave harmful residues in shrimp tissue, inducing food safety problems for the consumer. Recently it has been reported that ethoxyquin residue was found in frozen chicken at 130 µg kg\(^{-1}\) imported to Japan from Brazil in 2008. The Japan Ministry of Health, Labour and Welfare (MHLW) became aware of this problem and introduced a positive list system for agricultural chemicals remaining in food (MHLW, Notifications 497 and 499, 2005) to protect human health from potentially harmful ethoxyquin residues. This mandates that foods shall not be produced, imported, processed, used, cooked or stored for sale that exceed an ethoxyquin level of 10 µg kg\(^{-1}\). It is therefore important to develop a rapid and sensitive
method for analysis of trace levels of this compound in shrimp samples.

Several analytical methods for the determination of ethoxyquin in feeds and foodstuffs, such as Gas chromatography-Mass Spectrometry (GC-MS) and high performance liquid chromatography (HPLC) with UV fluorescence, have been reported (Aoki et al., 2010; Berdikova Bohne et al., 2007; Mezcua et al., 2009). However, these methods are time-consuming and not sensitive enough to utilize as a standard confirmatory method. Currently HPLC with tandem mass spectrometry (HPLC-MS/MS) is the most efficient technique, which is suitable for the determination of residues such as pesticides and veterinary drugs because of its high selectivity and sensitivity (Gentili et al., 2005; Romero-González et al., 2008). In this study, the use of ultra performance liquid chromatography (UPLC) provides high efficiency together with short analysis time. The objective of this work is to develop a rapid and sensitive method for the determination of ethoxyquin in shrimp. Performance parameters were evaluated in terms of selectivity, recovery, precision, linearity, the limit of detection (LOD) and limit of quantitation (LOQ).

![Figure 1 Chemical structure of ethoxyquin.](image)

### Materials and Methods

#### Chemicals and Reagents

Ethoxyquin standard (96.50%) was purchased from Dr. Ehrenstofer (Augsburg, Germany). Acetonitrile LC-MS grade was obtained from J.T. Baker (Phillipsburg, NJ, USA). Acetonitrile HPLC grade was purchased from Kanto Chemical (Tokyo, Japan), and glacial acetic acid of analytical grade from J.T. Baker (Phillipsburg, NJ, USA). Ultra-pure water was obtained using a Milli-Q Ultrapure system (Millipore, Bedford, MA, USA). Anhydrous magnesium sulfate was obtained from Panreac (Barcelona, Spain). Primary secondary amine (PSA, 40 µm particle size) was supplied by Varian (Harbor City, CA, USA). Graphitized carbon black (GCB, 120/140 mesh, 100 m² g⁻¹) was obtained from Supelco (Bellefonte, PA, USA).

#### UPLC-MS/MS System and Conditions

Sample analysis was performed on an Acquity™ UPLC system coupled with a Micromass Quattro Premier XE triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA).

#### UPLC Condition

The UPLC consisted of a binary pump system, degasser, autosample and column chamber. The Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm i.d.) stainless steel analytical column (Waters), maintained at 40 °C, was used for the analysis. The mobile phase flow rate was 0.50 mL min⁻¹. The injection volume was 5 µL using the partial loop with needle overfill loop mode. The optimized mobile phase composition was 10 mM ammonium acetate containing 0.3% acetic acid in water (mobile phase A), and acetonitrile containing 0.3% acetic acid (mobile phase B). A mobile phase gradient program was started at 80% B to 100% B at 1.1 min (held for 0.3 min). The column was re-equilibrated at the initial mobile phase condition (80% B) for 0.5 min before the next injection.

#### MS Condition

The MS instrument performed electrospray ionization in positive mode (ESI⁺). MS conditions used in this study were as show in Table 1. MassLynx Version 4.1 software (Waters) was used for instrument control and data acquisition /processing.
Table 1 MS optimization

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polarity</td>
<td>ESI+</td>
</tr>
<tr>
<td>Capillary voltage (kv)</td>
<td>1</td>
</tr>
<tr>
<td>Extractor voltage (v)</td>
<td>3</td>
</tr>
<tr>
<td>Source temperature (ºC)</td>
<td>120</td>
</tr>
<tr>
<td>Desolvation temperature (ºC)</td>
<td>350</td>
</tr>
<tr>
<td>Cone gas N2 flow (Lh⁻¹)</td>
<td>50</td>
</tr>
<tr>
<td>Desolvation gas N2 flow (Lh⁻¹)</td>
<td>1000</td>
</tr>
<tr>
<td>Low MS 1 resolution</td>
<td>14</td>
</tr>
<tr>
<td>High MS 1 resolution</td>
<td>14</td>
</tr>
<tr>
<td>Ion energy 1</td>
<td>0.5</td>
</tr>
<tr>
<td>Low MS 2 resolution</td>
<td>14</td>
</tr>
<tr>
<td>High MS 2 resolution</td>
<td>14</td>
</tr>
<tr>
<td>Ion energy 2</td>
<td>0.5</td>
</tr>
<tr>
<td>Collision gas flow Ar (mL.min⁻¹)</td>
<td>0.21</td>
</tr>
<tr>
<td>Multiplier</td>
<td>675</td>
</tr>
</tbody>
</table>

Sample Preparation Method

For validation experiments, shrimp samples were purchased from a local market. The shrimp samples were homogenized, and also checked using UPLC-MS/MS to ensure that samples were free from analytes. Samples were then stored at -20 ºC prior to analysis.

After thawing, 5 g samples of homogenized shrimp were weighed into 50 mL polypropylene centrifuge tubes (Corning, Lowell, MA, USA); then 2 mL of water and 20 mL of acetonitrile containing 2% acetic acid was used as extraction solvent in each tube. The mixtures were vortexed briefly for 45 s and then mechanically shaken for 10 min. Then 5 g of anhydrous magnesium sulfate (MgSO₄) was added to each tube, which was then vigorously shaken by hand for 45 s and subsequently centrifuged at 3400 rpm for 5 min. An aliquot of 1 mL of each extract was transferred to a 2 mL microcentrifuge tube containing 50 mg of PSA and 10 mg of GCB sorbent, this was followed by vortexed mixing for 30 s and centrifuging for 3 min at 14000 rpm. Finally, 0.5 mL of the supernatant was diluted with 0.5 mL of an acetonitrile:water (80:20, v/v) solution. The extract solution was filtered through a 0.2 μm nylon filter membrane (Whatman, Florham Park, NJ, USA) prior to UPLC-MS/MS analysis.

Results and Discussion

The chromatographic conditions of UPLC were optimized on the BEH C18 UPLC column in order to achieve the best separation and retention for the analyte. The main advantage of UPLC is the column efficiency, providing a narrow peak, higher sensitivity and shorter analysis time. The MS/MS parameters were evaluated to determine the most sensitive ionization condition. The most abundant ESI-MS/MS transition for ethoxyquin was monitored in the multiple reaction monitoring (MRM) mode to obtain the highest quantitative sensitivity. The MS/MS system was performed by infusing a 10 mg L⁻¹ solution of ethoxyquin in an acetonitrile: water solution (50:50, v/v) in positive mode. The compound produced a precursor ion [M+H⁺] at m/z 218.15 and two product ions at m/z 160.10 and 148.10 with highly selective, sensitive and stable forms. The retention time and optimized MS/MS conditions obtained for ethoxyquin are shown in Table 2.

Under these optimization conditions, this method was successfully developed for ethoxyquin analysis with efficient separation and a suitable detection limit. The good peak shape of the analyte was achieved, and separation performed, using an Acquity™ BEH C18 column (100 mm × 2.1 mm, 1.7μm i.d.) with a gradient elution mixture of 10 mM ammonium acetate containing 0.3% acetic acid in water and 0.3% acetic acid in acetonitrile. The selectivity was assessed by comparing the chromatograms of five different batches of blank shrimp samples. MRM chromatograms of blank shrimp and spiked shrimp samples of ethoxyquin at 50 μg kg⁻¹ are shown in Figure 2. The retention time of ethoxyquin was about 0.86 min. Endogenous peaks at the retention time of the analyte were not
observed for any of the blank shrimp, indicating no significant interference in the MRM mode during detection of the analyte.

Table 2 Retention time and optimized MS/MS conditions obtained for ethoxyquin.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Ethoxyquin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>271.31</td>
</tr>
<tr>
<td>tR (min)</td>
<td>0.86±0.01</td>
</tr>
<tr>
<td>Cone voltage (V)</td>
<td>40</td>
</tr>
<tr>
<td>1st transition quantification (m/z)</td>
<td>218.15&gt;160.10</td>
</tr>
<tr>
<td>Collision energy (eV)</td>
<td>32</td>
</tr>
<tr>
<td>2nd transition confirmation (m/z)</td>
<td>218.15&gt;148.10</td>
</tr>
<tr>
<td>Collision energy (eV)</td>
<td>30</td>
</tr>
<tr>
<td>Ion ratio (2nd/1st)</td>
<td>0.49±0.12</td>
</tr>
</tbody>
</table>

In sample preparation optimization, the first step was the evaluation of a suitable extraction solvent. Acidified acetonitrile was selected as the extraction solvent because it offers satisfactory yield of residues with low levels of matrix co-extractives and effective denaturing proteins. The acidified acetonitrile was employed for extraction in the presence of anhydrous magnesium sulfate. The addition of salt induces phase separation of the solvent from the aqueous phase. The residues of interest and matrix co-extractives are separated into the relevant liquid phase based on their polarity. The residues are partitioned into the organic phase and the matrix co-extractives into the aqueous phase. Five different concentrations of acetic acid were tested to evaluate which of the extraction solvents offered good yield recovery of the analyte. Figure 3 shows the recovery values for ethoxyquin. Increasing recovery was directly proportional to the concentration of acetic acid up to 2%, then the recovery decreased up to 3%. It can be assumed that degradation of analytes may occur in a highly acidic condition. Therefore, 2% acetic acid was selected to extract ethoxyquin and enhance the effectiveness of protein precipitation by eliminating turbidity of the solution.

Figure 2 Representative multiple reaction monitoring (MRM) chromatograms of ethoxyquin in shrimp: (A) blank shrimp sample, and (B) spiked shrimp sample at 50 µg kg⁻¹.

Figure 3 Effect of acetic acid concentration in acetonitrile (%) on the recovery of ethoxyquin for the spiked shrimp sample at 50 µg kg⁻¹ (n = 5).

The second step in the method development was the evaluation of a suitable sorbent to provide selective and complete isolation of the co-extracted compounds from acetonitrile extracts. Different commercially available sorbents were compared. Figure 4 shows the extracted solution after cleanup with different types of sorbent, including 50 mg of C18, NH₂, GCB and PSA. The best recovery for ethoxyquin was obtained using PSA sorbent. However, the result shown in Figure 5 demonstrates that only C18 and GCB sorbent can obviously remove the pigment from shrimp sample extract. Only GCB provided satisfactory recovery. Therefore, GCB was selected for the further optimization procedure.
Figure 4 Effect of different sorbent types for d-SPE cleanup on the recovery (%) of ethoxyquin for the spiked shrimp sample at 50 µg kg⁻¹ (n = 5).

In the third step, different amounts of GCB (5–50 mg) were applied. The PSA could remove polar matrixes from the sample extract and GCB has a strong affinity for planar molecules, and thus effectively removes pigment. The results of the experiment showed that the use of a combination of 50 mg PSA and 10 mg GCB provided excellent recovery of ethoxyquin, then the recoveries were decreased and remain constant when the amount of GCB higher than 20 mg. The results are shown in Figure 6.

Figure 5 Comparison of solution extract colors after cleanup with different sorbents using UV-visible spectrophotometer.

Finally, the properties of the extracted solution before injection into UPLC-MS/MS should be considered, because the direct injection of acetonitrile sample extract produces low sensitivity for ethoxyquin determination. In general, the dilution of samples can sufficiently reduce the signal suppression from the matrix effect. Therefore, the dilutions studied were 0, 20, 40, 60 and 80% solutions of acetonitrile in water. The results indicated that 80% acetonitrile in water is the most suitable ratio for sample dilution, providing the highest sensitivity and best peak shape, because the selected dilution solution is similar to the initial composition of the mobile phase.

Method performance was investigated by assessing if linearity was demonstrated for the ethoxyquin analyte by preparing a nine point matrix-matched calibration curve at concentration levels in the range of 0.5–8.0 µg L⁻¹. The calibration curve showed a coefficient of determination ($R^2$) representing good linearity in the range of 0.5–8.0 µg L⁻¹, with $R^2 = 0.9959$. LOD and LOQ were evaluated on the basis of signal to noise ratios of 3 and 10, respectively. The LOD value was 1.5 µg kg⁻¹ and the LOQ value was 5.0 µg kg⁻¹ for ethoxyquin, representing excellent sensitivity of the proposed method as summarized in Table 3.
Table 3: Coefficient of determination ($R^2$), linear range, LOD and LOQ.

<table>
<thead>
<tr>
<th>Performance Parameter</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Coefficient of determination ($R^2$)</td>
<td>0.9959</td>
</tr>
<tr>
<td>Linear range ($\mu$g L$^{-1}$)</td>
<td>0.5-8.0</td>
</tr>
<tr>
<td>LOD ($\mu$g kg$^{-1}$)</td>
<td>1.5</td>
</tr>
<tr>
<td>LOQ ($\mu$g kg$^{-1}$)</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The average recovery percentages ($n = 10$) of EQ were 97, 91 and 92% at LOQ, 5LOQ and 10LOQ spike levels, respectively. The repeatability was expressed as relative standard deviation (%RSD), which was lower than 6.61% ($n = 10$) for all levels. Obtained RSD values at stated concentration levels did not exceed the acceptable values calculated from the Horwitz equation (Horwitz and Albert, 2006), indicating the reliability of the developed method (Table 4).

Table 4: Percent recoveries and RSD of ethoxyquin analysis

<table>
<thead>
<tr>
<th>Spike level</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Acceptable % RSD value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOQ</td>
<td>97</td>
<td>4.49</td>
<td></td>
</tr>
<tr>
<td>5LOQ</td>
<td>91</td>
<td>6.61</td>
<td>23.44</td>
</tr>
<tr>
<td>10LOQ</td>
<td>92</td>
<td>4.09</td>
<td></td>
</tr>
</tbody>
</table>

Application to Real Samples

To prove the validity of the proposed method when applied to real samples, more than 50 shrimp samples were obtained (raised for export) from a shrimp farm in Thailand. The quality control for each batch of shrimp samples consisted of a matrix-matched calibration with a reagent blank, and a spike blank shrimp sample at 5 $\mu$g kg$^{-1}$ for EQ. Among the shrimp samples analyzed, 20 samples presented traces of ethoxyquin (<LOQ), and in 5 samples ethoxyquin was detected at concentrations in excess of their LOQ (5 $\mu$g kg$^{-1}$).

The positive finding was confirmed by performing two transitions in MS/MS for quantification and identification in compliance with recent EU guidelines. The ion ratio of analytes in incurred samples was confirmed to be within the range of permitted tolerance when compared to ions in the matrix-matched standard. The retention time of the analyte in positive sample was identical, within instrumental variation, when compared to the retention time of the analyte in the matrix-matched standard, according to the data in Table 5 according to SANCO/10684/2009 (European Food Safety Authority, 2009). Chromatograms of the determination of EQ in shrimp samples are shown in Figure 7.

Table 5: Confirmatory UPLC-MS/MS analysis of incurred shrimp sample.

<table>
<thead>
<tr>
<th>Product</th>
<th>Incurred</th>
<th>Matrix-</th>
<th>Incurred</th>
<th>Matrix-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ion</td>
<td>sample</td>
<td>ion</td>
<td>standard</td>
</tr>
<tr>
<td>EQ</td>
<td>Ion</td>
<td>$t_R$</td>
<td>Ion</td>
<td>$t_R$</td>
</tr>
<tr>
<td></td>
<td>ratio (min)</td>
<td></td>
<td>ratio (min)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>148.0/160.0</td>
<td>0.51</td>
<td>0.86</td>
<td>0.50</td>
</tr>
</tbody>
</table>

Figure 7: UPLC-MS/MS chromatograms of EQ obtained from real sample analysis: (A) positive sample (8.1 $\mu$g kg$^{-1}$), and (B) negative sample.
Acknowledgments

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Changes in Cooking Behavior of Organic and Inorganic Phatthalung Sungyod Rice During Ageing

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Abstract

Organic and inorganic Phatthalung Sungyod rice were grown and harvested in Phatthalung province, southern Thailand. They were processed to obtain brown rice and stored in a vacuum Nylon/LLDPE plastic bag, at room temperature for 6 months. The objective of this study was to monitor the changes in cooking properties of rice (Elongation ratio, colour, water uptake, hardness and percent of crystallinity) during ageing. Brown rice were collected at every 2 months and cooked with the ratio of water and rice 2:1 (w/w). Cooked inorganic rice showed significantly darker than cooked organic rice (P<0.05), and the lightness (L*) of cooked rice decreased as ageing time. The lightness of cooked organic and inorganic rice at after harvested was 41.15 and 38.47, respectively. After stored for 6 months the lightness of cooked rice was changed to 40.45 and 36.74, respectively. Elongation ration of cooked organic rice was significantly different from inorganic rice (P<0.05). Cooked organic rice showed higher in water uptake capacity than inorganic rice. These was significantly decreased with increased storage time (P<0.05). The water uptake of cooked organic and inorganic rice was decreased during storage. Cooked inorganic rice showed higher hardness than organic rice at after harvested until 2 months of storage (P>0.05). However, the sharply increase in hardness value was observed after 2 months of storage, but, no difference was shown after 4 months of storage.

Keywords: ageing, cooking quality, organic rice, Phatthalung Sungyod rice

Introduction

Rice has long been a staple food in Southeast Asia. It is consumed principally as a whole grain and, therefore the texture of the whole grain is a matter of primary importance. Nowadays, organic rice becomes more interest for human who concern the healthy foods. The organic rice is rice that is produced without the use of chemical fertilizers and chemicals in the control of pests. Its benefit to the consumers is the safety from chemical residues. In addition, it reduces pollution in the environment and preserves the balance of nature. The organic fertility management also has an effect on physicochemical properties and sensory quality of diverse rice cultivars. Champagne et al. (2007) have reported the difference in properties of organic and inorganic rice. The perception of differences in flavour and texture between organically- and conventionally-cooked rice was reported. These may affect from protein content of rice. The protein content of rice grown with the typically-used 100% nitrogen fertilizer was higher than that of the cultivar grown organically. Moreover, the stickiness which correlates negatively with protein content was observed to be higher in the organically-grown rice than in the same cultivars grown conventionally using the 100% nitrogen fertilizer.

There are much more than 4,000 varieties of the traditional rice in southern Thailand. Phatthalung Sungyod rice is a rice variety which most produce in Songkhla Lake Basin,
covering three provinces, Nakhon Si Thammarat, Songkhla and Phatthalung. Phatthalung Sungyod rice is a pigmented rice, which it contain a natural colourant, called anthocyanin. A commonly found anthocyanin in red rice is acetylated procyanidins, which is reported to possess a free radical scavenging activity. The contents of GABA (γ-aminobutyric acid), γ-Oryzanol and Ferulic acid were found in Phatthalung Sungyod rice, as reported by Banchuen et al. (2010).

The different cultivars of rice are usually classified according to the grain dimension, amylose content, pasting properties, thermal properties of the extracted starch from rice and the texture, from sensory or hardness measurement, of cooked rice. The physicochemical properties of freshly harvested rice changed during ageing. From freshly harvested, rice is generally sticker than that aged rice (Sodhi et al., 2003). The textural properties of cooked rice are depending on many characteristics, such as, variety, storage conditions, amylose content, cooking method and degree of cooking (Indudhara Swamy et al., 1978; Chrastil, 1990a, Vronique et al., 2007).

The aim of this work was to investigate the changes in cooking behaviors of rice from different cultivated system, organic and inorganic, and the effects of rice ageing on cooking properties.

**Materials and Methods**

Rice from two different cultivation systems, organic and inorganic rice, were grown and harvested in Phatthalung province, southern Thailand, in the 2008/2009 growing season. After harvesting, paddy rice was dehydrated by sunlight for approximately 7 days and was processed to obtain brown rice. The brown rice was stored in a vacuum Nylon/LLDPE plastic bag, 0.5 kg per bag, under room temperature (~30°C). Samples were stored for 6 months; this was considered as the maximum aging period known to be used in the commercial production of brown rice.

**Cooking Method**

Rice samples were collected and analyzed for cooking quality at every 0, 2, 4 and 6 months. Rice grain were combined with distilled water by varied the ratio of water and rice grain at 2:1 in quick-fit conical flask fitted with a glass stopper. The flask was immersed in a water bath at constantly temperature (100°C) for 20 min. The cooking experiment was done in 3 replicates.

**Elongation Ratio**

Ten cooked rice kernels were collected randomly and measured for the length. The Elongation ratio was calculated by cumulative length of 10 cooked rice kernels divided by the length of 10 uncooked kernels (Singh et al., 2005).

**Texture**

Hardness of the cooked rice grain was determined using Stable Microsystems TA-XT2i Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) fitted with a 50 kg load cell. Samples were prepared and were packed into a Kramer shear cell. The Kramer probe (5 blades, 3 mm thick, 64 mm high, 82 mm wide, 11 mm apart) was used in this study. Test speed was 2.0 mm/s. Parameters recorded from test curves as maximum force (hardness). The texture of cooked rice grain was measured for 10 times (Juliano, 1985).

**Colour**

The colour of cooked rice grains were measured by colourimeter (Hunter Lab Reston, VA, USA). The instrument was calibrated with a white and black calibration tile. Samples were placed on a clear petri dish and colours were measured. The colour was measured in CIE L*, a*, b* colour spaces. L* is a measure of the brightness from black (0) to white (100); a* describes red-green colour with positive a* values indicating redness and negative a* values indicating greenness; b* describes yellow-blue colour with positive b* values indicating yellowness and negative b* values indicating blueness.
Amylose Content
The amylose content was determined by an iodine colourimeter at 620 nm using amylose from potato starch for preparing standard mixture (Juliano et al., 1985).

Water Uptake Capacity
The weight of cooked rice sample was measured. The water uptake of cooked rice was calculated by the ratio of cooked rice grain divided by uncooked rice grain weight (Zhou et al., 2007).

Crystallinity by XRD
The crystallinity of cooked rice was measured by the X-ray diffractometer (X’Pert MPD, PHILIPS). The operating conditions were CuKα radiation (λCu = 1.5406), voltage 40 kV and current 30 mA. The scanning angular (2θ) ranged between 4.0-40.0° at a scan rate of 0.5°/min (Kim et al., 2001).

Statistical Analysis
Data were subjected to analysis of variance (ANOVA). A comparison of means was carried out by Duncan’s multiple-range test. The analysis was undertaken using an SPSS package (SPSS 11.0 for windows, SPSS Inc, Chicago, IL).

Results and Discussion
Elongation Ratio
The relationship between rice cultivation system and storage time on elongation ratio of cooked organic and inorganic rice is presented in Table 1. Inorganic rice showed significantly higher expansion when compared with organic rice (P<0.05). The higher elongation ratio of cooked organic rice grain was due to the absorption of more water in organic rice compared with inorganic rice. The higher amylose content has positive effect on the volume elongation ratio, as it improves the capacity of starch granule to absorb water and expand in volume without collapsing (Juliano, 1985; Singh et al., 2005; Mohapatra and Bal, 2006). This is because of the greater capacity of amylose to hydrogen bond or retrograde. The elongation ratio of both cooked rice grain was not changed during ageing for 6 months.

Colour
Phatthalung Sungyod rice is a pigmented rice variety. It contains pigment compounds, such as, anthocyanin, which responds for red colour. The colour of organic and inorganic rice grain after cooked is showed in Table 1. Cooked inorganic rice grain showed significantly darker compared with cooked inorganic rice (P<0.05), indicated with the lower in lightness (L*) and the redness (a*) value of inorganic rice. This may attributed from higher in anthocyanin content of inorganic rice (the data is not shown here). During ageing, the changes in lightness and redness of both cooked rice were not observed (P>0.05). The L* of cooked organic rice after harvested was 41.15 and it was changed to 40.45 at the 6 months of storage. While, L* of cooked inorganic rice was changed from 38.47 to 36.74. However, the difference in colour of rice grains during ageing cannot be distinguished by sight.

Water Uptake
Water uptake capacity of cooked organic and inorganic rice is shown in Figure 1. Both of rice showed different water uptake capacity. The higher water uptake capacity in cooked organic rice was observed (P<0.05). Water uptake capacity of cooked organic rice after harvest was 4.77% and decreased to 4.52% at the 6 months of storage. This trend of decreasing can be observed in the cooked inorganic rice, which it decreased from 4.57 to 4.47% during storage. The difference in water uptake capacity of cooked rice may attribute from the protein content at outer layer of rice grain. The main protein in rice is oryzenin (Martin and Fitzgerald, 2002). It is composed of subunits that are linked by both intra- and inter-molecular disulphide bridges. During storage, the molecular weight of oryzenin increases significantly, which correlates with an increase in disulphide bonding (Sugimoto et al., 1986; Chrastil,
From this reason, the strongly bonding of disulphide bridges may affect penetration of water into rice grains. 

![Figure 1](image1.png) Water uptake of cooked organic and inorganic Phatthalung Sunygod rice during storage for 6 months.

![Figure 2](image2.png) Hardness of cooked organic and inorganic Phatthalung Sunygod rice during storage for 6 months.

**Texture**

The textural property of cooked rice was determined by texture analyzer. The important parameter for the evaluation of the texture of the cooked rice grain is hardness. Hardness is defined as the maximum force that occurs at the first compression cycle (Smewing, 1999). Changes in hardness of both cooked rice are shown in Figure 2. Cooked rice become harder during ageing. In each rice cultivars, there is no significant different in hardness of cooked rice during storage for 4 months (P>0.05), but a significant difference was observed after storage for 6 months (P<0.05). Hardness of cooked organic and inorganic rice after harvest was 530.03 and 533.03 N, respectively. The highest compression force of cooked organic and inorganic rice was found during 6 months of storage. The values were 541.40 and 540.73 N, respectively. An increase in hardness is likely associated with the lower hydration process of starch granules in aged rice grains. After aged, rice grains showed higher resistance to the first compression of the probe. This increase is related to an increase in the resistance to the hydrothermal disruption of starch granules and the increase in insoluble material contents that are very tough and difficult to break up in the mouth (Tsugita et al., 1983 and Meullenet et al., 2000).

Inorganic rice showed slightly higher amylose content compared with organic rice (P<0.05). Amylose content of inorganic rice and organic rice was 16.27 and 15.32 % (db), respectively. However, no changes in amylose content during storage time for 6 months were observed. The high value of hardness in rice cultivars may be attributed to differences in their textural properties and granular structure (Singh et al., 2005). The positive correlation between amylose content and firmness of cooked rice grains was reported by Sowbhagya et al. (1987) and Juliano et al. (1987). Rice with higher amylose content and long chain amylopectin tended to have a hard texture, while rice with a lower amylose content and short chain amylopectin tended to have a softer texture (Juliano et al., 1987).

**Crystallinity**

Starch is partially crystalline, as a typical cereal starches. Rice starch has the A-type of X-ray diffraction pattern (Boa, 2004). In native starch, both of rice types showed a diffractogram of type-A pattern. Their diffractogram showed the peaks at 15.2, 17.0, 17.9 and 22.9Å. The crystallinity of organic and inorganic rice was 38.75 and 38.72%, respectively.
Table 1 Cooking properties of organic and inorganic Phatthalung Sungyod rice during storage for 6 months.

<table>
<thead>
<tr>
<th>Rice cultivars</th>
<th>Storage month</th>
<th>Elongation ratio</th>
<th>Cooking properties</th>
<th>Crystallinity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L*</td>
<td>a*</td>
</tr>
<tr>
<td>Organic rice</td>
<td>0</td>
<td>1.07±0.01c</td>
<td>41.15±1.35a</td>
<td>8.07±0.57b</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.08±0.01c</td>
<td>40.93±1.09a</td>
<td>8.42±0.46cd</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.07±0.01c</td>
<td>40.81±2.05ab</td>
<td>8.91±0.18bcd</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.06±0.01d</td>
<td>40.45±0.98abc</td>
<td>8.97±0.43bcd</td>
</tr>
<tr>
<td>Inorganic rice</td>
<td>0</td>
<td>1.11±0.01b</td>
<td>38.47±0.83bcd</td>
<td>9.44±1.32abc</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.12±0.01a</td>
<td>38.17±1.46cd</td>
<td>9.51±0.35abcd</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.11±0.01b</td>
<td>37.12±0.52d</td>
<td>9.85±0.27ab</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.10±0.01b</td>
<td>36.74±0.77d</td>
<td>10.48±0.96a</td>
</tr>
</tbody>
</table>

Means with the same letter in the same column are not significantly different (P<0.05). Data are reported as means ± SD (standard deviation) for 3 determinations.

But significantly difference was not observed (P>0.05). After cooking, the crystallinity of cooked rice organic and inorganic rice cultivars was decreased. The changes in crystallinity of both cooked rice during ageing are shown in Table 1. They showed a small difference in percent of crystallinity. After cooked, rice starch was fully gelatinized and the structure of starch became amorphous. The percent of crystallinity of both cooked rice increased significantly during ageing (P<0.05). An increase in crystallinity of cooked rice may be due to the formation of amylose and lipid complex (Hibi et al., 1990).

Conclusions

Cooked rice from different cultivation systems, organic and inorganic cultivation systems, showed difference in cooking behaviors. Cooked inorganic rice was darker, harder and more expanded compared with cooked organic rice. Moreover, the ageing of brown rice caused the decrease in water uptake capacity and the increase in hard texture of cooked rice grains, but, ageing was not affected to the changes of colour and the expansion of cooked rice grains in both of rice cultivars. The crystallinity of both cooked organic and inorganic rice was increased during ageing.

Acknowledgments

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References


Cooking Behavior of organic and inorganic rice

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Extraction of Collagen from Hen Eggshell Membrane by Using Organic Acids

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Abstract

The procedure of membrane separation and collagen extraction, presented in the membrane, were investigated. Membranes were separated from the shell wall in a tank (15 L) of water fitted with a mechanical stirrer. Membrane separation was enhanced by addition of the elutant, EDTA at 5% w v⁻¹. Optimum weight of eggshells was 500 g 15 L⁻¹ aqueous solution, with maximum yielding of the membrane about 8% of eggshells. Collagen was extracted from the separated membranes with the addition of either of two organic acids, 0.5 M acetic or 0.5 M citric acid. Highest collagen yields of 507 and 495 mg 100g⁻¹ dry sample were obtained when acetic and citric acids relative to membrane weight were added at a ratio of 1:8. Thermal solubility of collagen at 40 ºC was about 14.7 and 18.0 mg 100 g⁻¹ dry sample for water and 0.45 M NaCl solution, respectively. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the collagen from eggshell membranes yielded type 1 collagen with α1 and α2 fibrils.

Keywords: collagen, hen eggshell membrane, organic acid

Introduction

Raw collagen is a biochemical product now increasing demand for the production of skin graft and tissue replacement products, dental implants, and cornea repair. Purified collagen is now selling for up to $1,000 per gram (Abdullah, 2003). The skins of cattle and pig, as well as their bone, are the main sources of collagen used in food, pharmaceutical, cosmetic and leather industries (Bailey and Light 1989; Cavallaro, et al., 1994; Ogawa et al., 2004). The outbreak of mad cow diseases has resulted in anxiety among users of cattle gelatin, due to the not fully confirmed hypothesis, that the infective agent can be transferred from animals into human beings. Additionally, the collagen obtained from pig’s bones cannot be used as a component of some foods for religious reasons. Therefore, there is a strong need to develop alternative collagen sources such as fish offal, bone, scales, fins as well as skins (Sadowska et al., 2003; Nalinanon et al., 2007; Sadowiska and Skierka, 2007).

Hen eggs provide a useful amount of complete protein at the cheapest price when compare with meat, poultry and fish; thus eggs are one of the important food that are consumed throughout the world. Besides, eggs have some which are very useful for food modification, such as gel production and emulsifying agent. Therefore, eggs are widely used as a raw material for making many different types of food such as ice-cream, deep-fat fried food, bakery products and mayonnaise. In Thailand, hen-eggs were produced 9,424 million units or 471,200 tons of each day, generating 47,120 tons of waste eggshells (Office of Agricultural Economic of Thailand, 2009). The shells from industrial companies were used as a source of calcium in animal feed. However, the shells were still over supplied for this case. Companies, in USA, are paying up to $ 100,000 per year to dispose of this waste in landfills that are quickly
reaching capacity (Abdullah, 2003). Mac Neil (2001) reported that eggshell had 8% (w w⁻¹) shell membrane and the membrane contained 10% (w w⁻¹) collagen. Collagens from hen-eggshell membrane are type I, V and X (Candish and Scougall, 1969; Wong, et al., 1984; Arias et al., 1991).

There are three methods for collagen extraction. Saito et al (2002) extracted collagen from sea cucumber by using enzyme pepsin at 4 °C for 2 days. The result indicated that collagen was extracted up to 70%, however, the enzyme was too expensive for commercial production. Davison (1991) extracted collagen from rat-tail tendons by using organic amine, 0.1 M methylenediamine hydrochloride, at 4 °C for overnight. During the reaction, thiol reagent (mercaptoethanol) was added to increase collagen yield and periodically homogenized. The result indicated that collagen was extracted marked type I, III and V. Nevertheless, the chemicals were expensive and the procedure of extraction was too complicate. Moreover, almost of proteolytic enzyme, such as pepsin trypsin and papain, and ethylenediamine were non-specific for collagen extraction. These enzymes removed only the telopeptide (non-helical ends) of collagen resulted in changed collagen properties and solubilized non-collagen protein (Cliche, et al; 2003; Sadowska and Skierka, 2007). Subsequently Sadowska and Skierka (2007) indicated that the best solvents for solubilized collagen fibrils were acetic and lactic acid. However, only α₁, α₂, and β chains were observed in the electrophoretogram. Sadowska et al. (2003) extracted collagen from cod skin by using organic acid as acetic and citric acids. The extractability of collagen depends both on the concentration of acids and the ratio of skin to acid. The largest percentage of total content of collagen about 85% could be extracted by either 0.5 M acetic or 1.5 M citric acid, at 4 °C for 24 h; using the ratio of skin to acetic and citric acids at 1:40 and 1:20 (w v⁻¹), respectively.

Mac Neil (2001) investigated the method to separate the shell membrane from hen eggshell by using their physical properties. The shell particles, being heavier than the membrane particles, settle down at the bottom of the tank while the membrane particles are relatively light and therefore to maintain suspended in the water of the tank. So the suspend membrane was removed from the shell. However, Arias et al (1991) indicated that after soaking the shell in 0.5M EDTA (tetrasodium salt) could improve the separating of membrane from shell. So it should have way to research how the shell membrane to be able to separate from the eggshell and study for the appropriate condition of collagen extraction from eggshell membrane, as the economic value added and the use of eggshell.

Materials and Methods

Egg shell waste was obtained from the process of pasteurized liquid egg of Bangkok Produced Agroindustry Co. Ltd., Thailand. The eggs were washed by using 50 °C water before passing through the egg breaking machine to separate egg white, egg yolk and egg shell. Liquid egg white and egg yolk was pasteurized while egg shell was ground by centrifugal mill, resulted 2-5 mm diameter particle size.

Separation of Egg Shell Membrane

Egg shell membrane was separated from egg shell by using 20 liters separating equipment as in Figure 1. The equipment composed of a stirring container and a separating container. The egg shell (300, 500, 700, 900 and 1,100 g) was mixed with 5 L of 5% EDTA solution and stirred at the speed of 50 rpm for 30 min by using stirrer paddles of the stirring tank. The heavier egg shell would be precipitated while the lighter membrane would be float and collected on a sieve of separating container. The membrane was then rinsed with distilled water.

Proximate Analysis

Total protein, fat and ash of the separated collagen were determined by the methods of AOAC (1990).
Collagen Extraction From Egg Shell Membrane

Pretreatment of egg shell membrane

Pretreatment process was followed the method of Sadowska et al. (2003) to remove the impurity such as soluble non-collagen compound, lipid, pigment and off-flavor. In the first step, soluble non-collagen compounds were removed by cold water and salt solution. Egg shell membrane was blended with cold water (1:6 w/v) by using a blender at 4 °C for 3 min and filtered. The membrane was then mixed with 0.45 M NaCl (1:6 w/v) by using magnetic stirrer for 3 min. The retentate was homogenized with 0.45 M NaCl (1:6 w/v) at 6,000 rpm for 4 min. Subsequently, the retentated membrane was washed with distilled water (1:6 w/v) and centrifuged at 2,000 g, 4 °C for 30 min. The supernatant was analyzed hydroxyproline amino acid by using HPLC and total nitrogen by Kjeldahl method to evaluate the pretreatment loss. Finally, the precipitate was collected to remove lipid, pigment and off-flavor by stirring in the following chemicals: 0.2% (w/v) NaOH, 0.2% (w/v) H₂SO₄ and 0.7% (w/v) citric acid, respectively, at the ratio of precipitate to each solution 1:7 (w/v), for 4 min, washed with water to pH 7 and filtered. After that, the retentate was soaked in 10% (w/v) NaCl for 24 h at room temperature, filtered, bleached with alkaline hydrogen peroxide (1% v/v) H₂O₂ in 0.01 M NaOH in the ratio of 1 to 6 (w/v) for 24 h at room temperature, neutralized and washed with distilled water.

Collagen extraction

Collagen was extracted from membranes, obtained from the pretreatment process, with either 0.5 M acetic acid or 0.5 M citric acid. Membranes were thoroughly mixed with either acid in one of four ratios (1:4, 1:6, 1:8 and 1:10 w/v), in a shaker water bath at 4 °C for 2 h. Mixtures were then removed, centrifuged for 4 min at 6,000 rpm, again mixed in the shaker bath (4 °C) for 24 h, homogenized for 2 min (6000 rpm, 4 °C) and finally centrifuged at 10,000g 10 °C for 20 min. The precipitate was extracted three times. The volume of each supernatant (soluble collagen) was determined the hydroxyproline amino acid following the method of Dunphy at al. (1987) and the type of collagen was determined following the method of Goncalves-Note et al. (2002). The supernatant of samples extracted with citric acid was also dialyzed in distilled water at 4 °C for 24 h before analysis.

Analysis of Hydroxyproline and Collagen

The extracted collagen compound (1 ml) was hydrolyzed with 5 ml 6 N HCl at 116 °C for 16 hr and then flushed with nitrogen gas to evaporate acid. The dried sample was prepared to phenylthiocarbamyl (PTC) derivatives following the method of Koop et al. (1982). The sample was added with 3 μL 50% (v/v) ethanol and 7 μL of PTC mixture, (contained 90% (v/v) ethanol, triethylamine phenylisothiocyanate, 7:2:1 v/v/v), hold for 10 min at room temperature, and diluted with 1 ml mobile phase (contained acetonitrile:pure water:140 mM acetate buffer pH 6.3 at ratio 0.6:0.4:9.0 v/v/v, respectively).

The 10 μL diluted sample was injected into auto sample to determine hydroxyproline content by using HPLC (Model 717 Plus, Water Associate, MA, U.S.A.), following the method of Dunphy et al (1987). Analysis was performed by using 250 mm length x 4.6 mm diameter of
C18 Nova-Pak Column (Supelco, Bellefonte, PA, USA). A system comprised of the mobile phase, 1900 psi pressure, 1.5 ml min⁻¹ flow rate and UV detector at 254 nm. The peak area of chromatogram was calculated as amino acid content, compared with hydroxyproline standard, and converted to collagen content, µg (µg of hydroxyproline content × 14.7, Dunphy at al., 1987).

Thermal Solubility of Collagen
Thermal solubility of collagen was investigated in the temperature range from 0 to 40 °C as the method of Montero and Borderias (1991) with some modification. The egg shell sample (2 mg) was suspended in water or 0.45 M NaCl solution (1:6, w v⁻¹) and incubated in a water bath for 24 h at 0, 5, 10, 15, 20, 25, 30, 35 and 40 °C. The samples were then centrifuged at 10,000 g for 30 min at 15 °C. Supernatant (1 ml) was analyzed as hydroxyproline content by HPLC and the result was then converted to collagen to determine the collagen solubility at various temperatures.

Type of Collagen Determination by SDS-PAGE
Standard collagen type I, from bovine achilles tendon, Sigma Chemical Co., St. Louis, Mo, USA, and extracted collagen samples were treated according to SDS page method.

Preparation of standard
The standard collagen type I was dissolved in 0.5 M acetic acid (3 mg ml⁻¹), dialyzed with buffer A (50 mM Tris-HCL, contained 0.2 M NaCl, 1 mM CaCl₂ and 0.02% v v⁻¹NaN₃, pH 7.4) and vacuum dried at 200 mbar 50°C for 5 h. The sample was dissolved with 100 µL 0.01M acetic acid, added with 50 µL 2% (v v⁻¹) β- mercaptoethanol and the dried at 110 °C for 1 min. Finally, the collected sample was stored at –20 °C for analysis.

Preparation of collagen sample
One milliliter of the extracted collagen was vacuum dried at 200 mbar 50 °C for 5 h. The dried sample was dissolved in 100 µL 0.01 M acetic acid and 50 µL 2% (v v⁻¹) β- mercaptoethanol, dried at 110 °C for 1 min and collected the sample at –20 °C for analysis.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)
SDS-PAGE were performed according to the method of Laemmli (1970). The stacking gel contained 5% polyacrylamide and the separating gel contained 12.5% polyacrylamide. The prepared sample was loaded per well and the migration was induced at a constant current 24 mA. for 90 min, or until the migration reached the end of the resolving gel. The gel were stained with Comassie blue R-250 for 30 min and destained with a solution of 10% glacial acetic, 40% methanol and 50% water. The type I collagen was used as a standard.

Statistical Analysis
The experimental data were analyzed statistically by analysis of variance, for statistical significance (p = 0.05) using Duncan’s Multiple Range Test, SPSS for Window Version 17.0 (SPSS ID 5066789, permanent) and inferences were reported at the appropriate place.

Results and discussion
Amount of Membrane Separation and Chemical Components of the Membrane
By a preliminary experiment, the 5% EDTA solution assisted membrane separation from eggshell. As a result, the membrane separated about 6.5 g 100 g⁻¹ eggshell after soaking in 5% EDTA solution for 30 min while the yield was only 5.0 g 100 g⁻¹ eggshell by water soaking at the same time. Tung and Richad (1972) explained that disodium salt of EDTA solution could remove calcium, main component of eggshell, thus the eggshell membrane was separated from the shell. EDTA, prominence as a chelating agent, has ability to sequester di and tri-cationic ions such as Ca²⁺ and Fe³⁺. After being bound by
EDTA, the Ca ions remained in the solution but exhibit diminished reactivity (Lewis, 1993). Thus, membrane was removed from the shell which contained 95% calcium carbonate (Fennema, 1985).

The amount of eggshell was significantly effect on percent yield of separated membrane (p<0.05). The 500 g eggshell weight gave the most enable membrane, with 7.5% yield (Table 1). While the increasing of eggshell from 700 to 1,100 g caused decrease membrane separation from 6.3 to 5.5%, respectively. This might be due to the exceeding of eggshell weight.

The chemical components of eggshell membrane were composed of 88.2% protein, 10.5% ash, 1.04% carbohydrate and 0.35% fat. As in the report of Candish and Scougall (1969) indicated that eggshell membrane mostly composed of protein and less in fat and carbohydrate.

<table>
<thead>
<tr>
<th>Egg shell (g)</th>
<th>% Yield*</th>
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<tr>
<td>300</td>
<td>6.70 ± 0.01 b</td>
</tr>
<tr>
<td>500</td>
<td>7.53 ± 0.50 a</td>
</tr>
<tr>
<td>700</td>
<td>6.29 ± 0.25 b</td>
</tr>
<tr>
<td>900</td>
<td>5.56 ± 0.01 c</td>
</tr>
<tr>
<td>1100</td>
<td>5.45 ± 0.01 c</td>
</tr>
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</table>

*Means average of three replications
b, c: Means with different letters are significantly different (p<0.05)

**Acetic Acid and Citric Acid Extraction**

Protein was loss only 0.11% from pretreatment step and hydroxyproline was not detected in the pretreatment solution. The extractability of collagen depends on the ratio of membrane to acid. Increasing of acetic and citric acid ratio could be able to increase in collagen extraction. With citric acid extraction, yield of collagen decreased with cycle of extraction (Figure 2). The extracted collagen was increased 4 times when the ratio of membrane to acetic acid increased from 1:4 to 1:8 (p<0.05, Figure 3). Compared with citric acid, extracted collagen increased 2 times at the same range ratio of membrane to acid. The largest percentage of the extracted collagen content was about 507 and 495 mg 100 g⁻¹ dried sample by acetic and citric acid extraction, respectively. Yi et al. (2003) indicated that collagen protein was insoluble in water but the protein could soluble in common non toxic organic solvents, such as 3-mercaptopropionic acid in the presence of 10% acetic acid. This mainly caused by the presence of crosslinks of disulfide bonds. Sadowska et al. (2003) also reported that the increasing the ratio of fish (Baltic cod) skin to 0.5 M acetic acid from 1:10 to 1:40 could increase extractability of collagen in range 20 to 90%, respectively. However, collagen of the skin could limitedly dissolve in citric acid. Analysis type of extracted collagen from eggshell membrane by SDS-PAGE, demonstrated band pattern typical of type I collagen (Figure 4) which composed of α 1 and α 2 bands as reported in Wong et.al. (1984) and Yi et al. (2004).

**Thermal Solubility of Collagen**

The thermal solubility of hen-eggshell membrane collagen depended on the medium solution (Figure 5). As a result, the membrane was heated in 0.45 M NaCl and water the solubility of collagen increased significantly at temperature above 20 ºC and 30 ºC, respectively. At 40 ºC, collagen solubilities were about 14.7 and 18.0 mg 100 g⁻¹ dry sample in 0.45 M NaCl and water, respectively. Sadowska et al. (2003) reported that the suitable temperature for collagen extraction was less than 20 ºC to protect protein denaturation. In addition, Montero et al. (1995) investigated the water solubility of plaice skin collagen was increased from 4 to 90% at the temperature in range from 10 ºC to 40 ºC, respectively.

**Conclusions**

The maximum yielding of the membrane was 8% of eggshells by using the eggshells at 500 g per 15 L of 5% EDTA solution. The optimum ratio of the membrane to 0.5 M acetic acid for collagen extraction was 1:8 wt v⁻¹, with the highest collagen yields of 507 mg 100g⁻¹ dry
sample and the extracted collagen was type I collagen.

Figure 2 Extracted collagen by (a) 0.5 M acetic acid and (b) 0.5 M citric acid at various ratio of acid to membrane in each extraction

Figure 3 The extracted collagen from eggshell membrane by 0.5 M acetic acid (—) and 0.5 M. citric acid (—–)

Figure 4 Protein bands from SDS-PAGE of eggshell membrane collagen. Lane 1 Standard collagen type I by acetic acid. Lane 2 Extracted collagen from eggshell membrane by acetic acid. Lane 3 Extracted collagen from eggshell membrane by citric acid.

Figure 5 Solubility of eggshell membrane collagen at various temperature in 0.45 M NaCl (—) and water (—–)

Acknowledgements

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vol. 44, no. 5, special issue 2011 Extraction of collagen from hen eggshell membrane


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Farinograph and Extensograph Properties of Frozen Dough Added With Psyllium Husk Powder or Locust Bean Gums

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Abstract

Mixing properties and extensional properties of frozen dough upon addition of psyllium husk powder (PSY) and locust bean gums (LBG) were studied. Food gums were incorporated into wheat flour at addition levels of 0.2, 0.4, 0.6 and 0.8% w/w (wheat flour basis). Mixing properties and extensional behaviours of dough were assessed by farinograph and extensograph, respectively. Based on farinograms obtained, water absorption of dough increased progressively with the addition levels of food gums (P<0.05). PSY added dough had higher water absorption as compared to dough added with similar amount of LBG. Dough mixing stability was reduced with increasing levels of both PSY and LBG. In general, extensograph results revealed that dough resistance for all the samples was increased slightly at initial frozen storage period. However, it exhibited a significant decrease with extended frozen storage. A reverse trend was observed in dough extensibility. PSY added dough was found to show higher ratio of resistance to extensibility when compared to LBG added dough and an anomaly trend was demonstrated by dough added with 0.2% PSY. In general, mixing stability of dough was decreased with food gums addition. Weakening of dough was found to occur during freezing and frozen storage. In addition, dough added with higher amount of PSY was reported to be rigid as compared to LBG added counterparts.

Keywords: dough, extensograph, farinograph, locust bean gums, psyllium husk

Introduction

Frozen dough is gaining popularity in bakery industry. It serves as a good alternative because the shelf life of fresh bakery products is usually short (Asghar et al., 2009). The use of frozen dough allows fresh bread to be easily available to the customers besides facilitate the production of bread and also baking or steaming of end products in retail outlets (Rouillé et al., 2000). In addition, industrial bakers are able to save on the use of equipments and labour costs (Kenny et al., 1999).

However, there are several difficulties associated with frozen dough which include long proofing time, low specific volume, unacceptable crumb texture, and loss of dough performances (Kenny et al., 1999). Freezing and frozen storage caused decreased number of viable yeast cells and hence a decline in gassing power. Furthermore, the variable capability of the gluten network to retain carbon dioxide as a result of the gradual loss of dough strength. This resulted in deterioration of frozen dough quality and poor crumb structure (Räsänen et al., 1997; Ribotta et al., 2004).

Characterization of rheological behaviours of wheat flour dough is the key step to determine the dough mechanical handling properties, dough stability during processing such as proofing and baking or steaming as well as quality of the end products (Stojceska et al., 2007). Empirical rheological tests such as the farinograph and the extensograph tests have been widely used to evaluate the quality of wheat flour wherein farinograph determines the mixing properties of flour while extensograph
provides information about dough extensibility and resistance towards stretching (Stojceska et al., 2007).

Food gums which differ in chemical structures and functionalities have been widely used for purposes such as thickening and gelling, retarding starch retrogradation, improving moisture retention and textural properties, as stabilizers, emulsifiers or foaming agents as well as to control the water migration of a food system (Rosell et al., 2007). Several research works have been carried out to study the effects of food gums incorporation on dough and bakery products quality. Sharadanant and Khan (2003a) reported that bread staling was retarded as a consequence of addition of food gums and higher loaf volume was observed for bread prepared from frozen dough incorporated with gum additives as compared to frozen wheat dough without gums addition. Guarda et al. (2004) studied the effect of hydrocolloids from different sources and chemical structures on the quality of fresh bread and concluded that hydrocolloids showed potential application in retarding bread staling.

Changes in water distribution and moisture loss might take place in food matrix during extended frozen storage and freeze-thaw cycles (Xu et al., 2009). As a result, deterioration of dough structures may occur. Food gums is believed to be able to help in solving this problem due to its high hygroscopicity as well as ability to develop a better gluten network. The aim of this study was to determine the effects of psyllium husk powder and locust bean gums addition on mixing properties of wheat flour and extensographic properties of wheat dough upon frozen storage.

**Materials and Methods**

**Materials**

Wheat flour (10% protein, 0.47% ash, and 13.3% moisture) was obtained from United Malayan Flour Mill (Butterworth, Malaysia). Psyllium husk powder (*Plantago ovata*) (99.29% purity) and locust bean gums from *Ceratonia siliqua* seeds (Sigma-Aldrich brand, product of Morocco) were procured from Country Farms Sdn. Bhd. (Selangor, Malaysia) and Sigma-Aldrich Sdn. Bhd. (Selangor, Malaysia), respectively. Sodium chloride (SYSTERM® brand) was purchased from Classic Chemicals Sdn. Bhd. (Selangor, Malaysia).

**Farinograph Analysis**

Wheat flour was dry-blended with psyllium husk powder or locust bean gums at addition levels of 0.2, 0.4, 0.6 and 0.8% w/w (wheat flour basis). Wheat flour without any food gums added was designated as control. The farinograph analysis was performed following AACC method 54-21 (AACC, 2000). 300 g of wheat flour blend (corrected to 14% moisture basis) was mixed in a 300-g mixing bowl of a Brabender Farinograph®-E (Brabender OHG, Duisburg, Germany). Three replicates measurements were done.

**Extensograph Analysis**

This test was carried out according to AACC method 54-10 (AACC, 2000). Dough was prepared in a Brabender Farinograph®-E from 300 g wheat flour blend (14% moisture basis) in which 6 g of sodium chloride dissolved in part of the water added and the amount of water added was fixed by subtracting 2% of the water absorption determined by the farinograph. The dough was initially mixed for 1 min, rested for 5 min and mixing was resumed until 500 Farinograph unit (FU) consistency was obtained. The dough is then rounded, moulded and stored in a freezer at temperature of -18 °C until further analysis. Extensograph test was performed for fresh dough, dough after frozen storage for 1 month and 3 months. For dough after frozen dough, thawing was needed before analysis. The dough is then placed in a humidity cabinet (< 90% relative humidity) at 30 °C for 50 min until the dough reached temperature of 28 ± 2 °C. After resting time of 45 min, the dough was stretched until rupture. The same dough piece was then moulded and stretched again after rest for another 45 min. This step was repeated once more. In this study, the result was reported as
average of three replicates measurements for dough after 90 min and 135 min resting time.

**Statistical Analysis**
Where necessary, differences between treatment means were determined using the Duncan test (P<0.05) by SPSS software for Windows Release 15.0 (SPSS Inc., Chicago, Illinois, USA).

**Results and Discussion**

**Dough Mixing Properties Upon Addition of Psyllium Husks Powder or Locust Bean Gums**
Mixing of dough is crucial in breadmaking as it homogenizes the bakery ingredients and causes the development of gluten network until optimum rheological properties was achieved for later gas retention and stabilization of gas cells. It also helps in dough aeration and contributes to proper bread crumb structure (Martin et al., 2004). A well developed dough has strong dough network and optimum resistance to extension (Abang Zaidel et al., 2008).

As shown in Table 1, water absorption of wheat flour was increased with increasing addition level of food gums (P<0.05). However, wheat flour with addition of PSY had higher water absorption than those added with LBG. This could be attributed to the high water holding capacity or hygroscopicity shown by PSY as compared to LBG. When there is relatively higher amount of food gums present, more water is required in order to achieve the 500 Farinograph Unit (FU) consistency. This could be due to the high hydroxyl groups content presents in food gums that binds more water (Guarda et al., 2004). Similar observation was obtained from the study of Rosell et al. (2007) and Guarda et al. (2004).

For dough development time, all the samples including the control are insignificant different (P>0.05). Stability of dough showed a decline with increasing amount of food gums added. This suggests that low addition level of food gums may interact well with other flour components. However, excessive food gums might interrupt the inter- and intra-bonding of

<table>
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<th>Table 1 Farinograph parameters of wheat flour added with psyllium husk powder or locust bean gums</th>
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<td>Sample</td>
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<tr>
<td>Control</td>
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<tr>
<td>0.2% PSY</td>
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<td>0.4% PSY</td>
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<td>0.6% PSY</td>
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<td>0.8% PSY</td>
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<th>Addition of locust bean gum (LBG)</th>
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<tbody>
<tr>
<td>Sample</td>
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<tr>
<td>Control</td>
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<tr>
<td>0.2% LBG</td>
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<td>0.4% LBG</td>
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<td>0.6% LBG</td>
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<td>0.8% LBG</td>
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Values followed by the same letter and with the same capital letter in the same column are not significant different at 95 % confidence interval in the comparison between samples.
wheat dough that formed through the disulphide-sulphhydryl interchange which happened during the mixing and hence lower dough stability was observed. Dough added with PSY possessed higher dough stability as compared to their respective counterparts of LBG added samples at any given addition level. Mixing tolerance index (MTI) for all the samples are insignificant different (P>0.05) with the exception of sample added with 0.2% PSY. As shown in Table 1, inconsistent result was observed in time to breakdown of PSY added samples. However, as for LBG added samples, time to breakdown of dough showed a decrease with increasing addition level of LBG (P<0.05).

Dough Extensograph Properties Upon Addition of Psyllium Husks Powder or Locust Bean Gums

Dough expands during proofing as a consequence of carbon dioxide produced by the viable yeast cells. Dough resistance to extension and dough extensibility are essential for gas cells stabilization as well as gas retention in order to achieve good crumb structure and high loaf volume (Matuda et al., 2008). Different requirements of dough resistance to extension and extensibility are needed for different wheat-based products. For instance, pasta making needs dough with high resistance whereas dough with lower resistance but high extensibility is required to make biscuits in order to avoid dough pieces from elastic shrinkage after formation. Dough with moderate dough resistance and high extensibility are able to offer optimum performance to breadmaking (Bangur et al., 1997; Larroque et al., 1999).

In the making of bread, dough with high extensibility is preferred as this property is needed for the gas cells expansion during proofing and also early stages of baking. Besides high extensibility, bread dough is favoured to possess high resistance to extension so that the gas cells will not easily collapse under the dough weight (Larroque et al., 1999).

Extensograph analysis appears as the most appropriate method to evaluate the quality of frozen dough as the dough extensional properties affect oven spring and loaf volume of the end product (Kulp, 1995; Bhattacharya et al., 2003). The deformation observed in this test very much resembles practical conditions whereby the cell walls keep expanding during proofing stage (Dobraszczyk and Salmanowicz, 2008). Based on the results shown in Figure 1, all dough samples showed slightly higher dough resistance at constant deformation (R50) than their respective fresh samples after 1 month of frozen storage. However, extended frozen storage reduced the dough resistance. Dough resistance for PSY added samples was higher than that of LBG added samples, with the exception shown in sample added with PSY at 0.2% addition level. For PSY added samples, addition at 0.2% level exhibited the lowest resistance among all the other samples. In general, dough resistance increased in the order of 0.2% PSY <Control< 0.4% PSY < 0.6% PSY < 0.8% PSY, especially for dough after 1 month.

![Figure 1](image-url) Resistance at constant deformation after 50 mm of stretching for dough after resting for 90 min. (a) PSY added samples, (b) LBG added samples. Typical coefficient of variation did not exceed 10%.
frozen storage. LBG added samples had lower resistance as compared to the control with 0.6% LBG showed relatively low resistance.

Similar trend was true for dough after 135 min resting time (Figure 2). However, dough after resting for 135 min displayed slight increase in dough resistance. The decrease in dough resistance after prolonged frozen storage signifies the weakening of gluten network as a consequence of physical damage caused by ice crystals (Kulp, 1995).

Similar result was obtained by Inoue and Bushuk (1992) which showed maximum resistance of dough decreased gradually with frozen storage time. Dough with higher addition level of PSY had higher dough resistance than the LBG added dough and might not be suitable for dough rising due to higher dough toughness. Tough dough is not able to expand to an optimum height during proofing (Sharadanant and Khan, 2003b).

A reverse trend was observed for dough extensibility (Ext) as compared to dough resistance. As can be seen in Figure 3, after 90 min of resting time, extensibility of all dough samples reduced at initial frozen storage period. An increase in dough extensibility was shown by dough stored for 3 months at frozen condition. For PSY added samples, all the doughs had lower extensibility values than the control except for 0.2% addition level which showed the highest extensibility. Dough extensibility values decreased in the order of 0.2% PSY > Control > 0.4% PSY > 0.6% PSY > 0.8% PSY. Dough with LBG addition generally possessed higher extensibility than the
control with sample added with 0.6% LBG displayed relatively high value. Dough after resting for 135 min exhibited similar observation with dough after 90 min resting time (Figure 4). Dough after resting for 90 min showed higher extensibility as compared to dough after 135 min resting time. One plausible explanation for the increase in dough extensibility after prolonged frozen storage might be due to the protein depolymerisation during frozen storage and causes changes in distribution of molecular weight of glutenin subunits. Larroque et al. (1999) stated that dough extensibility increased with increasing amount of low molecular weight glutenins. In the study of Inoue and Bushuk (1992), they reported that dough extensibility increased progressively with extended frozen storage.

Dough requires an optimum resistance to extension as a prerequisite to have better gas retention properties and higher loaf volume. Extremely high resistance to extension causes lower loaf volume as tough dough not capable to proof to an optimum height, whereas too low resistance to extension also not good for dough performance because the dough will not be able to retain gas well and thus resulting in lower loaf volume (Sharadanant and Khan, 2003b). Hence, ratio storage might be due to the protein depolymerisation during frozen storage and causes changes in distribution of molecular weight of glutenin subunits. Larroque et al. (1999) stated that dough extensibility increased with increasing amount of low molecular weight glutenins. In the study of Inoue and Bushuk (1992), they reported that dough extensibility increased progressively with extended frozen storage.

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Figure 4 Extensibility for dough after resting for 135 min. (a) PSY added samples, (b) LBG added samples. Typical coefficient of variation did not exceed 10%.

Figure 5 Ratio of resistance to extensibility for dough after resting for 90 min. (a) PSY added samples, (b) LBG added samples. Typical coefficient of variation did not exceed 10%.
of resistance to extensibility ($R_{50}^{\text{Ext-1}}$) of dough is important and is closely related to quality of yeast-leavened wheat products. In this study, similar trend was shown by $R_{50}^{\text{Ext-1}}$ with $R_{50}$ of dough for both resting time of 90 min and 135 min (Figure 5 and Figure 6). As a general observation, all the dough samples increased $R_{50}^{\text{Ext-1}}$ after 1 month frozen storage followed by a decline in this parameter with prolonged frozen storage. PSY added samples possessed higher value that that of LBG added samples with exception shown in dough added with 0.2% PSY. Ratio of resistance to extensibility of dough after 135 min resting time was higher than that of dough after 90 min resting time. Kenny et al. (1999) also reported a decrease in ratio of resistance to extension due to freezing storage of frozen dough. Similar trend was observed in this study which could be caused by the deterioration of gluten network.

Conclusions

Ratio of resistance to extension of dough decreased as a function of frozen storage time. This indicates that dough became weaken after frozen storage. The resistance to extension increased with higher amount of PSY. However, exception was shown when 0.2% PSY added which showed lower resistance to extension as compared to the control. Opposite trend was shown by dough added with LBG. Resistance to extension showed slight decrease with higher addition level of LBG. In general, dough added with PSY had higher resistance to extension than dough added with LBG.

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Extraction and Characterization of Acid-soluble Collagen from Skin of Striped Catfish (Pangasianodon hypophthalmus)

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Abstract
Collagen was extracted from skin of striped catfish (SC: Pangasianodon hypophthalmus) using acid-solubilization process at 4°C for 48 hours. Some characteristics of extracted collagen were also determined. Before collagen extraction, proximate composition of SC fish skin was analyzed. SDS-PAGE patterns of the collagen and the skin of SC were monitored as well as the viscosity and UV-visible spectra absorption of collagen solution. The extraction yield of acid-soluble collagen from SC skin was 10.2. Protein patterns of collagen, and fish skin showed three major bands, representing α₁-, α₂- and β-components. However, the high intensity of these major bands was clearly observed in the collagen rather than those of fish skin. The temperature at which the change in collagen viscosity (half completed) was about 30°C. UV-visible spectra of the collagen showed the highest absorption peak at 230 nm. These finding suggested that the striped catfish skin could be used as an alternative source for collagen extraction.

Keywords: acid-soluble collagen, fish skin, striped catfish

Introduction
Collagen is the most abundant protein polymer (~30% of total protein of body) that is the main structural element of bones, cartilage, tendons and skin (Muyonga et al., 2004). It is widely and diversely used in food, medicine and cosmetic industries. Collagens used in commercial products are mainly obtained from cattle and swine. However, the use of mammalian collagen has limited by the spread the animal disease to human such as the bovine spongiform encephalopathy and the foot/mouth disease. Thus, the finding of new materials for collagen extraction has been investigated, especially in fishery industries. About 30% of the wastes consist of skin and bone, which are very rich in collagen (Gomez-Guillen et al., 2002). The collagen extraction from the skin, fin and bone of marine fish has been broadly studied (Jongiareonarak et al., 2005; Kittiphattanabawon et al., 2005), but not in case of the freshwater fish. In Thailand, the freshwater fish that are widely commercially cultured has little been studied for collagen extraction, especially in thick skin species. The striped catfish (SC) is one of importantly economic fish mostly cultured in Chiang Rai province (Department of Fisheries of Thailand, 2010). As mentioned before, during fish processing, large amount of by-products are generated. Utilization of freshwater fish industry by-products as a material for value-added products preparation from those wastes would be useful. Therefore, the objective of this study was to extract and characterize the collagen from striped catfish skin.
Materials and Methods

Materials

Acetic acid and sodium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Farmed striped catfish were obtained from Charan Farm in Chiang Rai, Thailand. Giant catfish collagen was obtained from our previous study (Thitipramote and Rawdkuen, 2010). Commercial collagen from bovine achilles tendon (C9879-5G) was obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Methods

Fish Skin Preparation

Fish skin of striped catfish was removed manually and cleaned samples were washed with tap water and drained. The skin was then cut into small pieces (0.5x0.5 cm) and placed in a polyethylene bag. The prepared skin was stored at -20°C until used.

Collagen Extraction

The collagens were extracted followed the method of Kittiphattanabawon et al. (2005) with a slight modification. All preparation procedures were performed at 4°C.

Preparation of skin for collagen extraction

To remove non-collagenous proteins, the skin was mixed with 0.1 N NaOH at a sample/alkaline solution ratio of 1:10 (w/v). The mixture was stirred for 6 h with changing of the alkaline solution every 2 h. Then, the alkali-treated skins were washed with cold water until neutral pH of wash water was obtained. The treated skins were then defatted with 10% butyl alcohol with a solid/solvent ratio of 1:10 (w/v) for 18 h. Defatted skins were washed with 10 volumes of cold water for three times.

Acid-solubilization process

Pretreated skin was soaked in 0.5 M acetic acid with a solid to solvent ratio of 1:20 (w/v) for 48 h with continuous stirring. The mixtures were filtered with two layers of cheesecloth and the residue skins were re-extracted with four proteases. The collagen in the filtrate was precipitated by adding NaCl to a final concentration of 2.6 M in the presence of 0.05 M Tris-HCl, pH 7.5. The resultant precipitate was collected by centrifugation at 20,000g at 4°C for 60 min. The pellet was dissolved in a minimum volume of 0.5 M acetic acid, dialyzed against 25 volumes of 0.1 M acetic acid for 12 h. Thereafter, it was dialyzed against 25 volumes of distilled water for 48 h. The resulting dialysate was freeze dried and referred to as “acid soluble collagen; ASC”.

Characterization of Collagen

Proximate analysis

Fish skin was subjected to proximate analyses, including moisture, ash, fat and protein contents, according to the AOAC (2000) method Nos. 950.46, 928.08, 960.39 and 920.153, respectively.

Yield

The yield of collagen (Yc) was calculated by:

\[ Y_c = \frac{V_c \times C_c}{W_t} \]

where \( V_c \) is the volume of extracted collagen solution in milliliter, \( C_c \) is the protein concentration of the same solution (g/mL), and \( W_t \) is the weight of the fish skin in gram. Each set of the test was done in duplicate.

SDS-PAGE

SDS–PAGE was performed by the method of Laemmli (1970). The collagens were dissolved in 0.02 M sodium phosphate containing 1% SDS and 3.5 M urea (pH 7.2) with continuous stirring at room temperature. Fish skin was minced and dissolved in 5% SDS and heated at 85°C for 1 hour. Solubilized samples were mixed at 1:1 (v/v) ratio with the sample buffer (0.5 M Tris–HCl, pH 6.8, containing 4% SDS, 20% glycerol) in the presence or absence of reducing agent (10% β-ME). Samples were loaded onto the polyacrylamide gel (7.5%
gel) and subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Molecular weight markers were used to estimate the molecular weight of proteins. Commercial collagens and giant catfish (GC) collagens were used as a reference.

Viscosity of collagen solution
Five hundred milliliter of 0.03% each collagen in 0.1 M acetic acid were subjected to viscosity measurements using a Brookfield viscometer with spindle No. 1 at a speed of 100 rpm. The solution was heated from 4°C to 50°C with a heating rate of 4°C/min. At the designated temperature, the solution was held for 30 min prior to viscosity determination. Measurement was carried out in triplicate. The relative viscosity was calculated in comparison to that obtained at 4°C. Td was defined as the temperature causing 50% decrease in the relative viscosity of the collagen solution.

Spectra absorption
Collagen was dissolved in 0.5 M acetic acid to obtain a concentration of 1 mg/ml. The solution was then subjected to UV-Vis measurement using a double-beam spectrophotometer (UV-1700, Shimadza Kyoto, Japan). Prior to measurement, the base line was set with 0.5 M acetic acid. The spectrum was obtained by scanning the wavelength in the range of 220-600 nm with a scan speed of 50 nm/min.

Results and Discussion

Proximate Compositions and Collagen Recovery
The proximate compositions of SC skin and the extraction yield of collagen were shown in Table 1. The SC skin contained the lower protein content (27%) with the high lipid content (20%) compared with GC skin (34%) (Thitipramote and Rawdkuen, 2010). The extraction yield of acid-soluble collagen from SC skin was 10.2% (wet basis) that was higher than the ASC (wet basis) from previous study in same species (5.1%) (Singh et al., 2011) and other catfish skin e.g. giant catfish (8.48%) (Thitipramote and Rawdkuen, 2010) as well as other fish skin e.g. the brownbanded bamboo shark (9.38%) (Kittiphattanabawon et al., 2010) and brownstripe red snapper (9%) (Jongjareonrak et al., 2005). The variation of the extraction yield of collagen from fish skins probably due to the different structure of skins used.

<table>
<thead>
<tr>
<th>Composition (%)</th>
<th>Striped Catfish skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction yield of collagen</td>
<td>10.2</td>
</tr>
<tr>
<td>Protein</td>
<td>27.26±0.28</td>
</tr>
<tr>
<td>Moisture</td>
<td>51.85±0.40</td>
</tr>
<tr>
<td>Lipid</td>
<td>20.24±0.59</td>
</tr>
<tr>
<td>Ash</td>
<td>0.23±0.03</td>
</tr>
</tbody>
</table>

Molecular Weight Distribution of Collagens
The protein patterns of the collagen and fish skin of SC under reducing and non-reducing conditions were presented in Figure 1. Three major bands were found in all samples, representing β-, α1, α2. However, the different band intensity of each source was observed that the high intensity of β, α1 and α2 band was clearly observed in SC and GC collagen rather than commercial collagen (STD) and fish skins. Comparing between collagens under reducing and non-reducing conditions, the patterns of the samples were similar; indicating the absence of disulfide bonds in those collagens, although the intensity of these bands is slightly different. Characteristic for type I collagen β-, α1, α2 components were also observed in skin
Collagen of Nile perch (Muyonga et al., 2004), Nile tilapia (Thitipramote and Rawdkuen, 2010), yellowfin tuna (Woo et al., 2008) and brownstripe red snapper (Jongjareonrak et al., 2005), and skin and bone collagens of bigeye snapper (Kittiphattanabawan et al., 2005).

**Figure 1** SDS-PAGE patterns of the fish skin collagen from giant catfish (1) and striped catfish (2) and the SC fish skin (3). M=molecular weight marker. STD= commercial collagen.

**Viscosity of Collagen Solution**

The change of viscosity of SC collagen solutions with increasing temperature was shown in Figure 2. The temperature at which the change in viscosity was half completed in SC skin collagen solutions was about 30°C. The viscosity of SC collagen gradually decreased at constant rate until 20°C, after which it continued to decrease more rapidly in range of 25-35°C. Denaturation of collagen structure caused by heat-treatment in associated with the change in viscosity (Nagai and Suzuki, 2002; Kittiphattanabawan et al., 2005). The denaturation temperature of SC collagen was higher than the closely species, giant catfish (17°C) (Thitipramote and Rawdkuen, 2010) but its high denaturation temperature was similar to the values reported for those other fish species e.g. Nile tilapia (30°C) (Thitipramote and Rawdkuen, 2010) and Nile perch (36.5°C) (Muyonga et al., 2004).

**Figure 2** Denaturation curve of fish skin collagen of striped catfish.

**UV-visible Spectra**

The UV absorption spectrum of SC collagen was shown in Figure 3. SC collagen (1 mg/ml) had the highest absorption peak at 230 nm. It indicates that the SC collagen had very low tyrosine and phenylalanine contents with no absorption peak at 280 nm (Lin and Liu, 2006). The result was accordance with results from skin collagen of Nile tilapia, giant catfish (Thitipramote and Rawdkuen, 2010), channel catfish (Liu et al., 2007), walleye pollock (Yan et al., 2008), carp (Duan et al., 2009) and shark (Kittiphattanabawon et al., 2010) that a same absorption peak at around 230 nm is characteristic for triple-helical collagen (Duan et al., 2009).

**Conclusions**

Collagen could be successfully extracted from striped catfish skin by cid collagen was characterized as type I without disulfide bond. Based on the results, the striped
catfish skin could be used as an alternative source for collagen extraction.

Acknowledgements

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Antioxidant Activity of Plant by-Products (Pink Guava Leaves and Seeds) and Their Application in Cookies


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Abstract

The study was conducted with the aim to investigate the potential of agriculture by-products such as leaves and seeds from pink guava as an antioxidant in food product. There are two phases of the study being conducted whereby the first phase involved the determination of antioxidative potential of the leaves and seeds extracts of pink guava while the second phase emphasized on the effects of the extracts on the oxidative stability of cookies. Antioxidant potential of pink guava leaves and seeds were evaluated by using Total Phenolic Content (TPC), Total Flavonoid Content (TFC), scavenging of 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assays. The natural antioxidants were then incorporated into cookies prepared from seven formulations (F1 to F7) and were kept at room temperature for 12 weeks of storage. The effects on the oxidative stability of cookies were evaluated by measuring the Peroxide Values (PV) and Thiobarbituric Acid (TBA) Values. Results showed that guava leaves possessed higher TPC and TFC values compared to the seeds. The leaves exhibited high scavenging effect (90%) as determined by DPPH while the seeds had 36% scavenging effect. The results also demonstrated that the FRAP value of guava leaves was higher than guava seeds. For the oxidative stability analysis, PV and TBA values in all formulations of cookies increased with the storage time but at a significantly lower rate than the control. All cookies added with antioxidants delayed the rancidity during 12 weeks of storage period compared to the control sample.

Keywords: antioxidant, plant by-products, pink guava, oxidative stability

Introduction

Psidium guajava Linn. (fam. Myrtaceae), referred to as guava, grows as a large spreading shrub or a small tree up to 15 m high. India is the major producer of guava in the world. In Malaysia, Perak state is the largest area for guava plantation (Kwee & Chong, 1990). Guava is one of the local fruits with superior nutritive value. As a fresh table fruit, guava is delicious and has one of the most nutritive values. Guava is rich in tannins, phenols, triterpenes, flavonoids, essential oils, saponins, carotenoids, lectins, vitamins, fiber and fatty acids. Guava fruit is higher in vitamin C than citrus (80 mg of vitamin C in 100 g of fruit) and contains appreciable amounts of vitamin A as well. Guava fruits are also a good source of pectin - a dietary fiber. The leaves of guava are rich in flavonoids, in particular, quercetin. Most of guava's therapeutic activity is attributed to these flavonoids. The flavonoids have demonstrated antibacterial activity. Quercetin is thought to contribute to the anti-diarrhea effect of guava; it is able to relax intestinal smooth muscle and inhibit bowel contractions. In addition, other flavonoids and triterpenes in
guava leaves showed antisplasmodic activity. Guava also has antioxidant properties which is attributed to the phenolic compound. Phenolic compounds such as myricetin and apigenin (Miean & Mohamed, 2001), ellagic acid, and anthocyanins (Misra & Seshadri, 1968) are at high levels in guava fruits. Therefore, producing guava specially bred for higher levels of antioxidant compound is a realistic approach to increase dietary antioxidant intake.

Antioxidants are substances that can prevent or delay oxidative damage of lipids, proteins and nucleic acids by reactive oxygen species, which include reactive free radicals such as superoxide, hydroxyl, peroxy, alkoxyl and non-radicals such as hydrogen peroxide, hypochlorous, etc. They scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen (Shi et al., 2001). The most abundant antioxidants in fruits are polyphenols and Vitamin C. Vitamins A, B and E and carotenoids are present to a lesser extent in some fruits. These polyphenols, most of which are flavonoids, are present mainly in ester and glycoside forms (Fleuriet & Macheix, 2003).

Up to date, there are lack of findings on the antioxidant and antimicrobial activities from pink guava by-products such as seeds and leaves grown in Malaysia. Hence, this study was conducted with the aim to evaluate the potential of leaves and seeds of pink guava as a natural antioxidant agent.

### Materials and Methods

Guava seeds and leaves (pink variety) were obtained from Golden Hope Plantation, Sitiawan Perak, Malaysia. Matured leaves and seeds were used for the extraction process.

#### Preparation of Extracts

Extraction using water was determined according to the method of Duh & Yen (1997). 20 g of the fresh sample (extracted with boiling water (600 ml) for 10 minutes. After boiling, sample was blended and filtrated with filter paper (Whatman No. 4). Water was removed by using Rotary Evaporator (Model: BUCHI Rotavapor R-200) at the temperature of 70°C. Concentrated extracts were frozen in blast freezer (SANYO VIP Series -86°C, Japan) at -70°C for 2 days and freeze-dried in freeze dryer (CHRIST Alpha 2-4,United Kingdom) at -16°C for 3 days. The extracts were stored in the amber bottles at 0°C prior to analysis.

#### Total Phenolic Content (TPC)

The Total Phenolic Content (TPC) was determined using Folin-Ciocalteu reagent following the method of Waterman and Mole (1994) using gallic acid as a standard. Briefly, 0.1 ml diluted extract (1 mg of extract/1 ml of distilled water) solution was added in a 10 ml volumetric flask. Then, 0.5 ml of Folin-Ciocalteu reagent was added and the content of the flask thoroughly mixed. After 1-8 minutes, 1.5 ml of Na₂CO₃ (20%) was added and the volume was made up to 10 ml using distilled water. The mixture was allowed to stand for 2 hours with intermittent shaking. The absorbance was measured at 760 nm using a UV-
Vis Spectrophotometer (Cole Parmer 1100, Canada). Total phenolic content was expressed as mg of gallic acid equivalent (GAE) per milligrams of crude extract using the equation obtained from the standard gallic acid calibration graph.

**Total Flavonoid Content**

Determination of Total Flavonoid Content (TFC) was determined according to the method by Zhishen et al. (1999) with some modification. One ml of aliquots was mixed with 4 ml of distilled water. At zero time, 0.3 ml of (5% w/v) NaNO₂ was added. After 5 min, 0.3 ml of (10% w/v) AlCl₃ was added. At 6 min, 2 ml of 1 M solution of NaOH were added. The volume then was made up to 10 ml, by the addition of 2.4 ml of distilled water. The mixture was shaken vigorously and the absorbance of the mixture was read at 510 nm. A calibration curve was prepared using a standard solution of quercetin (200, 400, 600, 800 and 1000 ppm, r² = 0.991). The results were also expressed on a fresh weight basis as mg quercetin equivalents (mg QAE) /g of sample.

**DPPH Radical Scavenging Activity**

The effect of plant extracts on DPPH radical is estimated according to the method of Yamaguchi et al. (1998) with some modifications. 600 µl of the extracts (0.2 mg /1ml distilled water = 200 ppm) with increasing concentration was added to 4.5 ml of DPPH (1 mM in ethanolic solution) in a 10 ml bottle with screw cap. The mixture was shaken and left to stand at room temperature for 20 minutes in dark place. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The radical scavenging activity was measured as a decreasing in the absorbance of DPPH and was calculated by:

\[
\text{Scavenging affect} (\%) = 1 - \frac{A_{\text{sample(517nm)}}}{A_{\text{control(517nm)}}} \times 100
\]

**Ferric Reducing Power Assay (FRAP)**

Ferric reducing/antioxidant power assay (FRAP assay) FRAP assay was performed according to a modified method described by Benzie and Strain (1996) with some modifications. Briefly, 0.1 ml of extract was mixed with 2.9 ml of FRAP reagent to mark up 3 ml. The reaction mixture was incubated in dark place for 1 hour to make sure all the samples were reacted with the solvent. Then, the absorbance was determined at 593 nm against a blank that was prepared using distilled water. FRAP reagent should be pre-warmed at 37°C and should always be freshly prepared by mixing 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with 20 mM FeCl₃. 6H₂O and 0.3 M acetate buffer, pH 3.6. A calibration curve was prepared, by using trolox as a standard (200, 400, 600, 800 and 1000 µM, r² = 0.991). FRAP values were expressed on a fresh weight basis as micromoles of trolox equivalent per gram of sample (µM trolox/g fresh weight).

**Preparation of Cookies**

The margarine (without antioxidant) which was prepared at Malaysian Palm Oil Board (MPOB) and castor sugar were beaten in a mixing bowl on medium speed until light and fluffy (creamy) for a few minutes. One whole egg (white and yolk) were added one by one. Addition of eggs had to be careful
ensuring that each addition is completely incorporated. The batter was beaten for five minutes until it is smooth and fluffy. The antioxidant (natural or synthetic) was then added into the batter for 1 minute at low speed. The sifted flour were fold in carefully without over mixing into the batter for 2 minutes. All of these mixtures were put in the refrigerator for one hour to make sure all the ingredients are well mixed and crunchy when it is baked. After an hour the dough was cut in a small size before baked in an oven. The cookies were baked in an oven at an approximate temperature of 160°C for 30 minutes. Once baked, the cookies were allowed to cool for 30 minutes and then stored in plastic containers at ambient temperature for 12 weeks for further analysis.

Cookies were prepared to provide seven variations. Seven formulations consists of control sample (cookies without addition of antioxidant) (F1), cookies added with BHA/BHT at 200ppm (F2), cookies with 100ppm leaves extract (F3), cookies added with 200ppm leaves extract (F4), cookies incorporated with 900ppm seeds extract (F5), cookies added with 1300ppm seeds (F6) and cookies added with combination of 100ppm leaves and 100ppm seeds (F7).

**Peroxide Value (PV)**

The peroxide value of cookies for each formulation was determined at 0, 2, 4, 6, 8, 10, 12 weeks of the storage at room temperature. Triplicates samples were taken for each formulation. According to the method of AOAC (1984), 5 g of fat sample was weighed in 250 ml conical flask and 30 ml mixture of acetic acid: chloroform (3:2) was added. The mixture was stirred until dissolved. 0.5 ml of saturated potassium iodide was added and left for 1 minute. Then, 30 ml of distilled water was added and stirred. The mixture was slowly titrated with 0.01 N sodium thiosulphate until the yellow colour almost disappeared. 1.0 ml of starch indicator (1%) was added and titration continued until the blue colour disappeared. A blank sample was determined simultaneously. The peroxide value was expressed as milliequivalent of peroxide oxygen per kilogram of sample (mEq/kg).

\[
\text{Peroxide value} = \frac{(V_f - V_b) \times N \times 1000}{W}
\]

Where:
- \(V_f\) = Volume of titration in ml of sodium thiosulphate used for the sample
- \(V_b\) = Volume of titration in ml of sodium thiosulphate used for the blank
- \(N\) = Normality of sodium thiosulphate used
- \(W\) = Weight in grams of extracted lipid

**Thiobarbituric Acid (TBA) Value**

The 2-thiobarbituric acid (TBA) distillation method of Tarladgis et al. (1960) was used to determine lipid oxidation. 10 g of samples were blended with distilled water (50 ml) for 2 minutes at high speed by using a homogenizer. The sample was then transferred to a 500 ml distillation flask with 47.5 ml of distilled water and the pH was adjusted to 1.5 with 2.5 ml of 4 N HCl. 4 bumping agents was added to avoid any explosive. The flask was connected to a distillation model BüCHI Distillation Unit B-324 apparatus consisting of a Y-type connector, dropping funnel, splash head and condenser. The mixture was boiled until 50 ml of distillate was collected. In a screw capped test tube, 5 ml of the distillate was reacted with 5 ml of TBA reagent (0.02 M 2-Thiobarbituric acid in 90% glacial acetic acid) and placed in a
boiling water bath for 35 minutes. A control made up of 5 ml distilled water and 5 ml of TBA reagent was also boiled for 35 minutes. The tubes were cooled to room temperature and the absorbance was read at 535 nm with spectrophotometer model Cole-Parmer 6100 UV-VIS. The TBA were calculated by multiplying the absorbance readings by a factor of 7.8 (Tarladgis et al., 1960) and expressed as mg malonaldehyde (MDA)kg⁻¹ of sample.

Statistical Analysis
Experimental data were analysed by the analysis of variance (ANOVA) and the significant differences among means were determined by Duncan’s multiple range test using the Statistical Analysis System (SAS, 2008) computing program.

Results and Discussion
Total Phenolic and Total Flavonoid Content
The results for Total Phenolic and Total Flavonoid Content are presented in Table 1. The data showed that guava leaves contain more phenolic and flavonoid content than guava seeds. Our results complies with the previous work by Ojan and Nihorimbere (2004) where the total phenolic content in dried guava leaves determined spectrophotometrically according to Folin-Ciocalteu’s phenol method and calculated as gallic acid equivalent (GAE) remarkably showed high total phenolic content (575.3 ± 15.5mg/100g GAE). Those antioxidant properties are associated with its phenolic compounds such as protocatechuic acid, ferulic acid, quercetin and guavin B (Thaipong et al., 2005), quercetin, ascorbic acid, gallic acid and caffeic acid (Jimenez et al., 2001). Studies from Wojdylo et al. (2007) also revealed that the total phenolic content varied widely in herb materials are ranged from 0.00 to 15.2 mg GAE/100g dry weight. Guava leaves extracts are a potential source of natural antioxidants (Ojan and Nihorimbere, 2004). For total flavonoid, the result complies with the study by Marinova et al. (2005) whereby the Total Flavonoid Content in fruits was in the range of 15.0 - 190.0 mg CAE/100g.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total Phenolic Content (mg GAE/100 g dry extracts)</th>
<th>Total Flavonoid Content (mg QAE/g dry extracts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>368.61±25.85a</td>
<td>162.92±19.73a</td>
</tr>
<tr>
<td>Seeds</td>
<td>79.03±3.48b</td>
<td>83.97±6.32b</td>
</tr>
</tbody>
</table>

Note: Analysis data were obtained from triplicate samples. Means with the different letter (a-b) within the column are statistically different at p< 0.05.

Plant derived antioxidants especially polyphenols and flavonoids have been ascribed to previous properties like anticancer, antidiabetic, antiaging and prevention of cardiovascular diseases (Dixon et al., 2005; Rice-Evans, 2004). The action of polyphenol is believed to be mainly due to redox properties, which play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen or decomposing peroxides (Birt et al., 2001). They contain conjugated ring structures and hydroxyl groups that have the potential to function as antioxidants in vitro or cell free systems by scavenging superoxide anion, singlet oxygen, lipid peroxyradicals and stabilizing free radicals involved in oxidative processes.
leaves contain more phenolic and flavonoid content than guava seeds. Our results complies with the previous work in herb materials are ranged from 0.00 to 190.0 mg CAE/100g. Those antioxidant compounds such as protocatechuic acid, caffeic acid, quercetin, ferulic acid, quercetin and guavin B properties are associated with its phenolic structures and hydroxyl groups that have the total phenolic content varied widely in herb materials are ranged from 0.00 to 190.0 mg CAE/100g. Studies from (Jimenez et al., 2001) showed that guava leaves extract showed high total phenolic content (575.3 ± 16.1 mg GAE). Those antioxidant compounds such as protocatechuic acid, caffeic acid, quercetin, ferulic acid, quercetin and guavin B properties are associated with its phenolic structures and hydroxyl groups that have the total phenolic content varied widely in herb materials are ranged from 0.00 to 190.0 mg CAE/100g. Studies from (Jimenez et al., 2001). They contain conjugated ring and single oxygen or singlet oxygen or triplet oxygen or neutralizing free radicals, quenching radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species (Birt et al., 2001).

2, 2-diphenyl-1-picrylhydrazyl (DPPH)

The abilities of extracts from guava seeds and leaves, assayed to scavenge the DPPH radical in comparison with synthetic antioxidant (BHA/BHT) are shown in Figure 1. Results are expressed as a percentage of the ratio of the decrease in absorbance at 517 nm. The scavenging activity of the guava leaves and seeds extracts on inhibition of the DPPH radical was related to the amounts of the extracts added. The scavenging effect of BHA/BHT was observed to be higher than the plant extracts. The scavenging effect of extracts and standards with the DPPH radical is in the following order: BHA/BHT (96 %) > guava leaves extract (90%)> guava seeds extract (36 %) at the dose of 200 µg/ml (200 ppm). The results showed high scavenging effect of the leaves as compared to seeds extract.

![Figure 1 Scavenging power of pink guava leaves and seeds extract at different concentration.](image)

According to Naik et al. (2003), on interaction with DPPH, antioxidants itself can either transfer an electron or hydrogen atom to DPPH, thus neutralizing its free radical character. The color changes from purple to yellow and its absorbance at wavelength 517 nm decreases. Generally, the antioxidant activity depends on the extract concentration. The increasing of antioxidant activity was found with increasing extract concentration. From the present results, the scavenging activity was increased as the concentration of BHA/BHT and guava leaves increased until it reached 200 µg/ml. Meanwhile, the scavenging effect of guava seeds also increased with the increasing concentration but at a lower rate.

Ferric Reducing Antioxidant Power (FRAP)

FRAP assay treats the antioxidants in samples as reductants in a redox-linked calorimetric reaction. FRAP is relatively simple and easy to be standardized. The FRAP value was determined from regression equation of calibration curve (y = 0.2904x - 0.1905, R² = 0.9991) and expressed in mM trolox equivalents, in each of the selected samples extracts and shown in Table 2.

![Table 2 Antioxidant capacity (ferric reducing antioxidant power, FRAP) of guava leaves and seeds extract](image)

<table>
<thead>
<tr>
<th>Samples</th>
<th>FRAP value (mM trolox equivalent/g dry extracts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guava leaves extract</td>
<td>116.0±11.42a</td>
</tr>
<tr>
<td>Guava seeds extract</td>
<td>13.3±1.96b</td>
</tr>
</tbody>
</table>

Note: Analysis data were obtained from triplicate samples. Means with the different letter (a-b) within the column are statistically different at p< 0.05.

From the results in Table 2, guava leaves extract showed significantly high FRAP value as compared to guava seeds.
extract. FRAP assay was selected to evaluate the antioxidant activities of guava leaves and seeds in this study because of the following reason. First, FRAP assay treats the antioxidants in the samples as reductants in a redox-linked colorimetric reaction. Second, the procedure of FRAP assay is relatively simple and easy to standardized. One possible disadvantage with FRAP assay is the fact that this assay is still suitable for assessment of antioxidant activity of fruit samples because only limited amounts of plant glutathione are absorbed by humans.

Plant based natural antioxidants can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc. that is any part of the plant may contain active components. FRAP is a sensible and practicable indicator of total antioxidant capacity (Vassalle et al., 2004). Results showed that FRAP method is sensitive in the measurement of total antioxidant power of fresh biological fluids, such as plant homogenates and pharmacological plant products (Szollosi & Varga, 2002). FRAP assays is based on ferric reducing ability (Vassalle et al., 2004). Ferric to ferrous reduction at low pH causes a blue ferrous tripyridyl triazine complex to form (Benzie and Strain, 1996; Olabinri, 2006). The FRAP assay offers a putative index of antioxidant or reducing potential of biological fluid within the reach of every laboratory and researcher interested in oxidative stress and its effects (Benzie and Strain, 1996). The FRAP assay uses antioxidants as reductants in a redox-linked colorimetric reaction (Benzie and Strain, 1996).

**Peroxide Value (PV)**

The PV was estimated in the products as an indication of the degree of oxidation. Changes occurring in the PV values of cookies were given in Figure 2. In general, all the cookies which were added with antioxidants slow down the rate of peroxide formation, since PV of all samples which contained synthetic (BHA/BHT) or natural antioxidants were lower than the control sample, during storage period. All samples were able to maintain PV less than 10mEq/kg until 12 weeks of storage. In the present study, all samples were considered not rancid and still acceptable. Initially at week 0, all the cookies including control indicated similar values of PV (0.4 mEq/kg). But from the second weeks onwards, all the treatment had significantly (p< 0.05) lower PV compared to the control. Control sample exhibited the highest PV throughout the storage period, showing a high oxidation process. A slow rise in PV was observed for all the treated samples, revealing the effectiveness of natural plant extracts and synthetic antioxidants (standards) in the stabilization of cookies during 12 weeks of storage under room temperatures. The PV of the control sample (F1) rapidly increased from 0.40 to 2.6 mEq/kg fat during 2 weeks of storage and then increased slowly up to 5.65 mEq/kg fat at weeks 12. The PV of the cookies incorporated with synthetic antioxidants (BHA/BHT) F2, slowly increased from 0.4 to 3.7
mEq/kg fat during 12 weeks of storage. Meanwhile, the cookies incorporated with natural antioxidants also showed slow increment from week 0 until the end of storage. Among all samples, the cookies treated with BHA/BHT showed the lowest PV throughout storage period followed by F4<F6<F3<F7<F5. All cookies samples treated with natural antioxidant showed no significant different on week 12 compared to sample with synthetic antioxidant. This indicated that plants extract possessed good antioxidant activities which are comparable to BHA/BHT at 12 weeks of storage. These results suggested that guava leaves and seeds were effective in suppressing the oxidation of cookies comparable to that of BHA/BHT.

**Thiobarbituric Acid (TBA) Value**

TBA is defined as the quantity of malondialdehyde (in mg) present in 1 kg of sample. TBA values for seven formulations of cookies which include control sample were observed until 12 weeks of storage. Malonaldehyde (MDA) reacting specifically with thiobarbituric acid causes an increase in TBA value at 12 weeks of storage in all samples. The results showed that TBA value and red colour intensity for control sample was higher than other samples. Incorporation of natural antioxidants (guava leaves and seeds extracts) and synthetic antioxidants significantly (p < 0.05) reduced TBA values compared to the control sample throughout the storage. Figure 3 showed an increased in TBA values for all samples throughout the storage at room temperature for 12 weeks.

According to Lim & Suhaila (1999), TBA values less than 8 µmol (0.576 mg) kg⁻¹ sample are considered not rancid. Whereas values greater than 9-20 µmol (0.65-1.44mg)kg⁻¹ sample are reported as rancid but still acceptable and values greater than 21 µmol (1.5mg) kg⁻¹ sample are said to be rancid and unacceptable. Figure 3 shows an increase in TBA values after 12 weeks of storage in all formulations of cookies. Based on the results, all cookies were not rancid at the initial stage (0 week). From week 2 onwards, TBA values of control and cookies containing natural antioxidants started to increase. At the week 8, control and cookies containing seeds reached TBA values which are considered become rancid but still acceptable. However, by the week 12, all the samples were rancid but still acceptable. Within acceptable level where control sample had significantly highest TBA values among all samples.

**Conclusions**

In the present study, the extracts obtained from pink guava leaves extract were found to possess strong antioxidant activity compared to pink guava seeds extract. This could be due to the high phenolic compounds presence in the leaves extract of the guava. Cookies formulation with the
incorporation of guava leaves extract showed powerful oxidative stability effect compared to the formulation with guava seeds extracts and control sample (without antioxidant). However, cookies with BHA/BHT showed the strongest oxidative stability throughout the storage period. Hence, pink guava leaves extract has the potential to be used as a functional food ingredient or as a bioactive ingredient in the food and pharmaceutical industry.

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Infrared and Hot Air Drying of Mullet Fish: Drying Kinetics, Qualities and Energy Consumption

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Abstract

The main purposes of this work were to study drying kinetic of mullet fish using three heating technique and to evaluate physical and sensory analysis of dried mullet fish. hot air convection (HA), infrared radiation (IR) and combined hot air convection and infrared radiation (HA+IR). Effect of drying conditions on quality and specific energy consumption was evaluated and compared to the conventional solar drying. Initial moisture content of fresh mullet fish was in ranges between 280 and 360% dry-basis and was thin-layer dried by three different heat sources until the final average moisture content was reached to 15±0.1% dry-basis. The inlet drying temperature for all batches was varied between 70 and 90°C with a constant air flow rate of 1.0-1.2 m s⁻¹ (HA and HA-IR drying). The experimental data was evaluated and used for fitting the drying kinetic models. The results showed that the evolution of moisture transfer using the empirical drying model has a good relationship with the experimental results. Average drying rate with IR irradiation was faster than those drying rate of combined HA–IR sources and solar energy, respectively. To determine the physical qualities of dried mullet fish samples, the results showed that the redness value of dried mullet fish using only IR and HA heat source was more uniformity than the other drying conditions. Finally, sensory evaluation of dried samples was carried out. The total acceptability values for all dried mullet fish products were not different significantly. Moreover, the specific energy consumption and drying time of mullet fish drying with IR source was relatively low compared to the other heat sources (7.6–11.8 MJ kg of water evaporated⁻¹).

Keywords: batch drying, dried seafood, infrared radiation, quality, sensory evaluation

Introduction

Mullet fish (Mugilidae) is normally purchased as fresh and dried fish. The mullet fish is valuable sea-food product which can be harvested in the southern part of Thailand, especially in 4 provinces nearby Malaysia. This fish is not only product for domestic consumption but also for export. Its price depends on quality for examples, dryness, color and size. Dried mullet fish can be kept for long period of shelf life and there are a few research works on mullet fish drying (Jain and Pathare, 2007). Commonly, mullet fish is dried traditionally under open sun for 3-5 days. To maintain high quality of dried mullet fish, the good practice for drying is necessary. The soaked mullet fish has high moisture content which is preferred for microorganism growth. However, the ambient surrounding conditions and water activity affecting some properties of samples were reported (Shepherd and Bhardwaj, 1986; Dutta et al., 1988; Joshi et al., 1993;
A decrease in bulk density with increase in moisture content was also determined for soybeans, gram seed, sunflower seed, pigeon pea, neem nut and lentil seeds (Desphande et al., 1993; Dutta et al., 1988; Gupta and Das, 1997; Shepherd and Bhardwaj, 1986; Viswanathan et al., 1996; Amin et al., 2004). The review papers indicated there are a few works focused on physical quality of fish, especially on mullet fish. This may because the fresh mullet fish has major good in the market whilst the dried mullet fish products in small or medium enterprises just have been started. In addition, the suitable drying method needs to have some physical property of sample for improvement the good practice drying process.

Then the objectives of this work was to investigate novel drying method for good quality of mullet fish and evaluate the quality and energy consumption from mullet fish drying using HA, IR and combined IR-HA sources comparing to the conventional solar drying. The sensory evaluation by untrained panelists was presented in term of smell, salty taste, and appearance.

Materials and Methods

Materials

Raw mullet fish with 20-25 fish kg\(^{-1}\) was provided by Tarosamelae fresh market, Pattani province, Thailand. The fresh mullet fish were washed and cut along length of samples and taken their organs off such as stomach, blood etc. To be deactivated and disinfected the fish samples by microorganisms, raw mullet fish was washed and soaked in 3% (w/v) of salt water solution for 3 min (Namsanguan et al., 2004; Tirawanichakul, et al., 2007). Then the soaked mullet fish samples were fermented with weight ratio of 1 kg of fish per 100 g of salt for 12-15 hours. The moisture content of all samples was determined according to the AOAC method (AOAC, 1995). The initial moisture content of fish samples was in ranges between 282.2 and 361.8 % dry-basis. For the drying strategies, the mullet fish was dried by various drying conditions until the final safe moisture content was about 18-20% dry-basis for long period of shelf life. The fresh mullet fish sample was illustrated in Fig.1.

A hybrid cabinet dryer can be operated using 3 heat resources (Tirawanichakul et al., 2008) consisting of electric heating air convection, infrared radiation and solar energy as illustrated in Figure 2. The drying cabinet dimension was 60×80×158.5 cm. The drying cabinet was made of stainless steel and the wall inside was insulated by microfiber sheet of 5 cm thickness. Electric heater was made of fin electric heating rod (1,000×10 W) and electric infrared rod (500×3 W) while solar collector was used for solar heating. The dimension of solar collector was of 32.5×133×240 cm and the top of collector was covered by perspex glass with 0.5 cm thickness. The solar collector was placed at 10° from horizontal level for taking direct solar radiation. Forward-blade blower driven by AC motor of 1 hp was used for force convection. Hot air after drying could be released to surrounding using air outlet pipe and could be fed back into the drying cabinet by using recycle pipe.

The drying temperature, surrounding air temperature, sample temperature and outlet air temperature were measured by K-typed thermocouples parallel connected to a data logger with an accuracy of 0.5°C (SUPCON, China). During drying period, the samples were weighed by an electronic balance with an accuracy of 0.1 g (A&D model GF 3000, Taiwan). The air inlet flow rate was fixed at 1.0±0.5 m s\(^{-1}\) and was measured by hot wire anemometer with an accuracy of 0.01 m s\(^{-1}\) (DIGICON model DA-45, USA).
In this experiment, 1.0-2.0 kg of mullet fish sample was placed on three stainless steel trays with dimension of 30\times40 \text{ cm} and dried by using 2 drying heat sources and 3 drying strategies (HA, IR and combined IR+HA heating). For quality analysis, the color measurement, sensory evaluation and energy consumption were determined as follows in the next subsection.

Establishment of Thin-layer Drying Model

To study the drying kinetic of mullet fish, the soaked mullet fish with initial moisture content ranging of 282.2-361.8 \% dry-basis was thin-layer dried by inlet air temperature ranging from 60 to 90\degree \text{ C} at increment intervals of 10\degree \text{ C}/step. The inlet drying air temperature was controlled by a PID controller with an accuracy of \pm 1\degree \text{ C}.

According to evaluate evolution of moisture transfer, the mathematical model of thin layer drying equations was formulated using the different empirical drying models. The experimental data were mathematical analyzed by non-linear regression analysis. The four empirical drying models were used for predicting the experimental results and all drying equations were listed as follows:

Newton model

\[ MR = \exp(-k_1t) \]  

Page model (1949)

\[ MR = \exp(-k_3t') \]  

Logarithmic model

\[ MR = A \exp(-k_3t) + (1-A)\exp(-k_3At) \]  

Henderson and Pabis model (1956)

\[ MR = C \exp(-k_4t) \]  

Determination of Color (CIE-Lab unit)

The color measurement of the fish samples was determined in CIE a*-value by using Juki color meter (JP7100p Tokyo Japan). The color values (L*-a*-b*) of dried mullet fish were measured by mean in five replications. In addition, the positive CIE L*-a*-b* values indicate brightness, redness and yellowness whilst negative CIE L*-a*-b* values indicate darkness, greenness and blueness, respectively (Tirawanichakul et al., 2008). Additionally, the total color difference (\Delta E*) was determined as follow in (6)

\[ \Delta E^* = \pm \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \]  

where \( L_0^* \) and \( L^* \) is the initial and final brightness value of sample, respectively. The \( a_0^* \) and \( a^* \) value is the initial and final redness value of sample, respectively. The \( b_0^* \) and \( b^* \) value is the initial and final yellowness value of sample, respectively.

Sensory Evaluation

The sensory evaluation of dried mullet fish samples was determined on the basis of its palatability. Twenty-five untrained panelists evaluated for the Aroma (flavor), tenderness and cohesiveness (texture), whiteness (color), glossiness (moisture) and overall acceptability of dried mullet fish using hedonic scale of 1-9 with the following scales: 1 = dislike extremely, 2 = dislike very much, 3 = dislike moderately, 4 = dislike slightly, 5 = like nor dislike, 6 = like slightly, 7 = like moderately, 8 = like very much and 9 = like extremely.

Determination of Energy Consumption

Specific energy consumption was calculated by total energy consumption dividing by water evaporated during drying period. The formula for calculating of energy consumption was written as follows:
\[ SEC = \frac{3.6P}{(m_i - m_o)} \]  \hspace{1cm} (7)

where \( P \) is energy consumption, kWh; \( m_i \) and \( m_o \) is weight of mullet fish before and after drying, kg. The value of 3.6 is conversion factor of power unit to energy unit.

**Drying rate**

Drying rate (DR) is defined as ratio of mass of water evaporated from material and time interval during drying period for driving evaporated water as follows:

\[ DR = \frac{m_w}{\Delta T} \]  \hspace{1cm} (8)

where \( m_w \) is the mass of water evaporated from mullet fish (kg of water) and \( \Delta T \) is the drying time (min).

**Results and Discussion**

**Drying Kinetic and Empirical Models**

The evolution of moisture transfer in three drying techniques with average temperatures of 70-90°C was carried on. The initial moisture content of salty mullet fish was in range of 280-360% dry-basis. The evolution of moisture transfer curve showing in terms of moisture ratio (MR) during drying time of HA, IR and combined IR+HA heat sources was illustrated in Figure 3, 4 and 5, respectively. The results showed that for all drying technique, the drying rate of mullet fish was relatively dependent on the drying temperature. Moreover, the drying with combined heat sources of infrared and hot air was faster than those of drying with infrared and hot air. This is because of the rapid transfer of electromagnetic energy directly to the water molecules in the material in order to bypass the surface-to-centre conduction stage. This is corresponded to Chua and Chou (2005).

To predict the drying kinetics of mullet fish with three different heat sources, the experimental data were curved fitted using 4 empirical thin-layer drying model which was defined in (2)-(5). By using non-linear regression analysis, the arbitrary constants were shown in Table 1.
Table 1 Illustration of arbitrary constants of empirical drying models for hot air, infrared and combined infrared and hot air heat sources

<table>
<thead>
<tr>
<th>Mathematical Model</th>
<th>Constant of Model</th>
<th>R²</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newton</td>
<td>( MR = \exp(-kt) )</td>
<td>k = 5.77exp(-2624.045/RT)</td>
<td>0.997</td>
</tr>
<tr>
<td>Page</td>
<td>( MR = \exp(-kt*+n) )</td>
<td>k = 6.31exp(-2355.802/RT), n = 1.037</td>
<td>0.997</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>( MR = \exp(-kt*+c) )</td>
<td>k = 5.62exp(-2394.309/RT), c = 1.0509</td>
<td>0.988</td>
</tr>
<tr>
<td>Two term exponential</td>
<td>( MR = \exp(-kt*) )</td>
<td>k = 1.61exp(-0.358/RT)</td>
<td>0.983</td>
</tr>
<tr>
<td>Henderson and Pabis</td>
<td>( MR = \exp(-kt*) )</td>
<td>a = 1.0506</td>
<td>0.977</td>
</tr>
<tr>
<td>Infrared radiation of 1,000 W</td>
<td>( MR = \exp(-kt) )</td>
<td>k = 35.19exp(-2762.144/RT)</td>
<td>0.938</td>
</tr>
<tr>
<td>Page</td>
<td>( MR = \exp(-kt*+c) )</td>
<td>k = 21.07exp(-2222.205/RT)</td>
<td>0.973</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>( MR = \exp(-kt*+c) )</td>
<td>k = 1.32exp(-2106.331/RT), c = 0.789</td>
<td>0.968</td>
</tr>
<tr>
<td>Two term exponential</td>
<td>( MR = \exp(-kt*) )</td>
<td>k = 240.77exp(-2847.008/RT)</td>
<td>0.969</td>
</tr>
<tr>
<td>Henderson and Pabis;</td>
<td>( MR = \exp(-kt*) )</td>
<td>a = 0.921</td>
<td>0.940</td>
</tr>
<tr>
<td>Combined Hot Air and IR of 1,000 W</td>
<td>( MR = \exp(-kt) )</td>
<td>k = 0.07exp(-767.043/RT)</td>
<td>0.994</td>
</tr>
<tr>
<td>Page</td>
<td>( MR = \exp(-kt*+c) )</td>
<td>k = 0.008exp(-0.159/RT)</td>
<td>0.988</td>
</tr>
<tr>
<td>Two term exponential</td>
<td>( MR = \exp(-kt*) )</td>
<td>k = 1.93exp(-0.085/RT)</td>
<td>0.988</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>( MR = \exp(-kt*+c) )</td>
<td>k = 0.08exp(-777.003/RT), c = 1.014</td>
<td>0.994</td>
</tr>
<tr>
<td>Henderson and Pabis;</td>
<td>( MR = \exp(-kt*) )</td>
<td>a = 1.008</td>
<td>0.994</td>
</tr>
</tbody>
</table>

Determination of Color

Table 2 showed the results of color value in terms of brightness, redness and yellowness (CIE-lab unit) which was determined by color meter (JP7100p Tokyo Japan). It stated that drying using temperature of 60-70°C gave high positive value of brightness (L*) and redness (a*) compared to drying with temperature of 80°C. This implies the dried mullet fish looks more brightness and redness. For the yellowing phenomena, the dried mullet fish with high temperature was lower than low drying temperature. This may because of non-enzymatic reaction. In addition, drying with different heat sources had no effect on color value of dried sample as illustrated in Table 2.

Table 2 Color value of dried mullet fish

<table>
<thead>
<tr>
<th>Source</th>
<th>Drying Temp. (°C)</th>
<th>Color value (CIE lab)</th>
<th>ΔE*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>L*</td>
<td>a*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48.72</td>
<td>4.20</td>
</tr>
<tr>
<td>HA</td>
<td>64.7</td>
<td>47.91</td>
<td>5.16</td>
</tr>
<tr>
<td>IR (1,000 W)</td>
<td></td>
<td>71.3</td>
<td>46.82</td>
</tr>
<tr>
<td></td>
<td>80.1</td>
<td>44.10</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>61.0</td>
<td>44.35</td>
<td>4.90</td>
</tr>
<tr>
<td></td>
<td>77.5</td>
<td>43.57</td>
<td>6.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89.5</td>
<td>41.36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>84.3</td>
<td>41.42</td>
</tr>
<tr>
<td>HA+IR</td>
<td>67.1</td>
<td>46.52</td>
<td>5.42</td>
</tr>
<tr>
<td>1,000 W</td>
<td></td>
<td>89.2</td>
<td>45.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>84.3</td>
<td>41.42</td>
</tr>
</tbody>
</table>

Note: L*, a* and b* means brightness, redness and yellowness value, respectively. The same superscript letter in the same column means data has no significantly different.

From the Table 2, total color difference (ΔE*) of dried mullet fish using three different heat sources tends to increase with drying temperature. To compare with control fish sample dried with solar energy, the mullet fish drying using three heating method. This is because an average drying temperature (45°C) of solar drying was low and there is no effect on color value such as browning reaction. This is corresponded to the previous works (Tirawanichakul et al., 2004).

Sensory Evaluation

For the sensory evaluation, the results showed that the acceptability of dried mullet fish can be acceptable for all drying conditions and specific energy consumption of infrared drying was slightly lower than those of experiment. For sensory evaluation in terms of flavor, color, moisture and texture was higher than 6.0 implying as like taste. An acceptability value for all drying conditions was higher than 7.0. The experimental results were presented in Table 3. To comparative study of each drying technique on drying temperature ranging of 60-90°C, the results showed that dried mullet fish of all heating
techniques had insignificant difference. Acceptability value was in ranges of 7.2-7.5 implying like moderately and like very much taste. Moreover, for flavor, color, moisture and texture analysis, they were clearly stated that drying temperature of 60-90°C has no effect on sensory evaluation of dried mullet fish.

Table 3 Sensory evaluation of dried mullet fish

<table>
<thead>
<tr>
<th>Drying Temp. (°C)</th>
<th>Sensory evaluation</th>
<th>Flavor</th>
<th>Color</th>
<th>Moisture</th>
<th>Texture</th>
<th>Accept -ability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot Air</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64.7</td>
<td>6.8a</td>
<td>7.6a</td>
<td>7.2a</td>
<td>7.5ab</td>
<td>7.4a</td>
<td></td>
</tr>
<tr>
<td>71.3</td>
<td>6.8a</td>
<td>7.6a</td>
<td>7.1a</td>
<td>7.5ab</td>
<td>7.4a</td>
<td></td>
</tr>
<tr>
<td>80.1</td>
<td>6.8a</td>
<td>7.6a</td>
<td>7.1a</td>
<td>7.4ab</td>
<td>7.5a</td>
<td></td>
</tr>
<tr>
<td>Infrared radiation 1,000 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61.0</td>
<td>6.6a</td>
<td>7.3a</td>
<td>6.9a</td>
<td>7.1a</td>
<td>7.2a</td>
<td></td>
</tr>
<tr>
<td>77.5</td>
<td>6.4a</td>
<td>7.4a</td>
<td>6.9a</td>
<td>7.3ab</td>
<td>7.2a</td>
<td></td>
</tr>
<tr>
<td>89.5</td>
<td>6.4a</td>
<td>7.3a</td>
<td>6.9a</td>
<td>7.3ab</td>
<td>7.3a</td>
<td></td>
</tr>
<tr>
<td>Combined Hot Air and Infrared radiation 1,000 W</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67.1</td>
<td>7.0a</td>
<td>7.6a</td>
<td>7.1a</td>
<td>7.3ab</td>
<td>7.4a</td>
<td></td>
</tr>
<tr>
<td>69.2</td>
<td>7.0a</td>
<td>7.6a</td>
<td>7.1a</td>
<td>7.5ab</td>
<td>7.4a</td>
<td></td>
</tr>
<tr>
<td>84.3</td>
<td>6.6a</td>
<td>7.3a</td>
<td>6.8a</td>
<td>7.1ab</td>
<td>7.2a</td>
<td></td>
</tr>
</tbody>
</table>

Note: The same column with different letters mean the data is significantly different.

Specific Energy Consumption

Table 4 illustrated the specific energy consumption of mullet fish drying with three heat sources and drying temperature ranging of 60-90°C.

The conclusion was that drying with high temperature had relative low energy consumption compared to drying at low temperature. This is because the high drying temperature increase drying rate correlated to low operating time. For all drying conditions as shown in Table 3, the drying rate at the lower temperature was higher than drying rate of the higher temperature. In addition, at the same drying temperature, the drying rate using combined hot air and infrared of 1,000 W was faster than those of drying method. This is because there are composed of both heat convection (hot air) and radiation (infrared) phenomena. So the infrared drying has the lowest energy consumption.

Table 4 Specific energy consumption of mullet fish drying with temperature of 60-90°C

<table>
<thead>
<tr>
<th>Drying Temp. (°C)</th>
<th>Drying Time (min)</th>
<th>Drying Rate (kg/h)</th>
<th>Specific Energy Consumption (MJ/kg H2O evap.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy source: Hot Air</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>64.7</td>
<td>300</td>
<td>0.99</td>
<td>46.9</td>
</tr>
<tr>
<td>71.3</td>
<td>240</td>
<td>1.23</td>
<td>49.4</td>
</tr>
<tr>
<td>80.1</td>
<td>220</td>
<td>1.47</td>
<td>39.8</td>
</tr>
<tr>
<td>Energy source: Infrared radiation 1,000 W</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61.0</td>
<td>200</td>
<td>0.81</td>
<td>10.4</td>
</tr>
<tr>
<td>77.5</td>
<td>200</td>
<td>1.40</td>
<td>8.6</td>
</tr>
<tr>
<td>89.5</td>
<td>120</td>
<td>1.67</td>
<td>9.1</td>
</tr>
<tr>
<td>Energy source: Combine Hot Air and Infrared radiation 1000 W</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>67.1</td>
<td>160</td>
<td>1.12</td>
<td>22.0</td>
</tr>
<tr>
<td>69.2</td>
<td>160</td>
<td>1.16</td>
<td>21.2</td>
</tr>
<tr>
<td>84.3</td>
<td>120</td>
<td>1.78</td>
<td>21.9</td>
</tr>
</tbody>
</table>

Conclusions

Hybrid drying using combined hot air and infrared heat sources was one of an appropriate drying technique for producing high quality of dried mullet fish. The specific energy consumption with high temperature for mullet fish give high efficient evaporation of moisture content correlated to short period of operation. Determination of specific energy consumption shows that specific energy consumption of mullet fish drying with high temperature tends to be decrease. However, total color difference (ΔE*) is also affected by high temperature over 70°C especially on yellowing. Finally, total acceptability of dried mullet fish with all drying conditions can be acceptable in like moderately and like very much level (>7.0 of hedonic scale).

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References


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One and Two-Stage Drying of Shrimp using Hot Air and Infrared: Quality Aspect and Energy Consumption

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Abstract

The 1-stage and 2-stage drying with electric heating convection (HA) and infrared radiation (IR) for boiled shrimp were studied and presented. The initial moisture content of boiled fresh shrimp was in range of 250-350\% dry-basis and desired final moisture content was about 20-25\% dry-basis. The 1-stage drying of shrimp was operated using constant drying temperature ranged from 40 to 90\°C while the 2-stage drying was carried on with the 1\textsuperscript{st} stage drying temperature between 90 and 109.4\°C for 30 min and was then dried continuously using the drying temperature of 70\°C until the desired final moisture was 20-25\% dry-basis. The result showed that drying rate of the 2-stage drying using combined between HA and IR was faster than using the 1-stage IR drying, 1 stage HA drying and solar drying, respectively. Effective diffusion coefficients of shrimps determined by a diffusion model using a finite cylindrical shape was relatively dependent on the drying temperature and the value was in order of $10^{-6} \text{ m}^2 \text{ s}^{-1}$ with good fitting to the experimental results. Finally, the effect of drying strategies on qualities and energy consumption were determined. The redness value of dried shrimp using hybrid IR and HA heating delivered the highest color uniformity. The 1-stage drying had relatively high shear force compared to the 2-stage drying while the percentage of shrinkage and rehydration of dried shrimp were insignificantly different for all drying strategies (p<0.05). The specific energy consumption of 2-stage drying using combined IR and HA was relatively low compared to 1-stage drying with HA. It was in the range of 16.9-34.1 MJ kg.$\text{H}_2\text{O}$ evaporated\textsuperscript{1}.

Keywords: drying kinetic, quality, shrimp drying, specific energy consumption, texture evaluation

Introduction

Dried shrimp is an important fishery product both for domestic consumption and export to many countries. Its price depends on quality of dried shrimp, for examples, dryness, color and size (Namsanguan et al., 2004; Devahastin et al., 2006). Normally, process for dried shrimp production can be divided to 3 stages [boiling, drying and packaging]. The main process of three stages is the drying which can reduce high moisture content to safe value for long shelf life. Moreover, the good drying process should maintain quality of dried shrimp. Commonly, shrimp is dried traditionally under open sun for 3-5 days (Jain and Pathare, 2007). The suitable management for producing the high quality of dried shrimp is necessary because the boiled shrimp has high moisture content which is suitable for microorganism growth. However, the ambient surrounding conditions and water activity affecting on some properties of samples were reported (Shepherd and Bhardwaj, 1986; Dutta et al., 1988; Kukelko et al., 1988; Joshi et al.,
1993; Suthar and Das, 1996; Viswanathan et al., 1996). Some previous works reported the effect of drying temperature on physico-chemical properties of fish (Sobral, and Habitante, 2001; Sablani et al., 2002). Moreover, physical, chemical and rheological characteristics of water surrounding could affect to quality of sea food (Marek and Tadeusz, 2000). Since 1985 the use of energy source utilization for drying process and the drying with various techniques have been widely reported and carried out (Soponronnarit, 1997). As a result, the suitable drying process not only maintain quality but also it can improve some quality of food inventory or the products such as superheated steam combined with heat pump (Namsangua et al., 2004), fluidized-bed drying enhanced physicochemical quality of rice (Tirawanichakul et al., 2006).

Tirawanichakul et al. (2008) reported the change of color values and percentage of shrinkage of dried shrimp was significantly dependent on drying temperature. These properties play an important role in the design and prediction of heat transfer operations during the handling, processing, canning, storing, and distribution of foods. Thus the understanding of thermo-physical properties and drying process by mathematical modeling can be used to predict the change of qualities and evolution of moisture transfer for shrimps, especially on moisture content and its thermo-physical parameters.

Thus the aims of this work were to feasible study of shrimp drying with one and two-stage drying using electric heat convection and infrared radiation. In addition, effect of drying temperatures on physical qualities of dried shrimp (color, the percentage of shrinkage, the percentage of rehydration and texture), specific energy consumption and effective diffusion coefficient derived from experimental data were evaluated.

Materials and Methods

Materials
Raw white shrimp (300-400 shrimps kg$^{-1}$) of the genus *Penaeus spp.* was provided by Songkhla fresh market, Thailand. To deactivate and disinfect the shrimp from micro-organisms, raw shrimps were washed and boiled in 3% (w/v) of salt solution for 3 min (Namsanguan et al., 2004; Tirawanichakul et al., 2008). The initial moisture content of shrimp samples was in ranges between 270 and 384% dry-basis and the shrimps were dried by various drying conditions until the final moisture content was about 20-25% dry-basis and then the dried sample were air ventilated until the final moisture content reached to 14-16% dry-basis which was safe for long shelf life or storage.

Experimental Procedure
A hybrid cabinet dryer can be operated using 3 heating resources (Tirawanichakul et al., 2008) such as electric heating air convection, infrared radiation and solar energy as illustrated in Figure 1. Drying chamber dimension was 60×80×158.5 cm. The chamber was made of stainless steel and the wall inside was insulated by microfiber sheet of 5 cm thickness. Electric heater was made of fin electric heating rod (1,000×10 W) and infrared rod (500×3 W) while solar collector was used for solar heating. The dimension of solar collector was of 32.5×133×240 cm and the top of collector was covered by perspex glass with 0.5 cm thickness. The solar collector was placed at 14° from horizontal level for taking direct solar radiation. Forward-blade blower driven by AC motor of 1 hp was used for force hot air convection. Hot air after drying could be released to surrounding using air outlet pipe and could be fed back into the drying chamber using recycle pipe.
Drying Kinetic Study

Effective diffusion coefficient

The effective diffusion coefficient (D) [the so-called effective diffusivity] is principal parameter for predicting of moisture transfer in material and can be used to design drying system. The diffusion coefficient depends on the surrounding temperature and the relative humidity of surrounding air. From the drying experiment during the falling drying rate, drying rate highly depends on mass transfer, so the diffusion coefficient can be evaluated by Fick’s second law of diffusion as follows:

\[
\frac{\partial M}{\partial t} = D V^2 M
\]

with initial condition (IC) and boundary condition (BC) as:

IC: \( t = 0; \ -L < z < L \quad M = M_{\text{initial}} \)

BC: \( t = 0; \ z = 0 \quad \text{and} \quad z = L \quad M = M_{\text{initial}} \)

\( t > 0; \ z = 0 \quad \text{and} \quad z = L \quad M = M_{\text{equilibrium}} \)

where

\( M = \) moisture content, decimal (dry basis)

\( D = \) diffusion coefficient, \( \text{m}^2 \text{s}^{-1} \)

\( t = \) time, \( \text{s} \) and \( z = \) distance, \( \text{m} \)

The geometry of the shrimp was considered as spherical and finite cylindrical shape. Then, the mathematical model of thin layer drying equation can be solved and shown in general form as follows: (Crank, 1975)

For a spherical shape,

\[
MR = \frac{M - M_{\text{eq}}}{M_{\text{in}} - M_{\text{eq}}} = \frac{6}{\pi^2} \left[ \exp \left( -\frac{\pi^2 D t}{r_0^2} \right) + \left( \frac{1}{4} \right) \exp \left( -\frac{4\pi^2 D t}{r_0^2} \right) + \left( \frac{1}{9} \right) \exp \left( -\frac{9\pi^2 D t}{r_0^2} \right) \right]
\]

For a finite cylindrical shape,

\[
MR = \frac{M - M_{\text{eq}}}{M_{\text{in}} - M_{\text{eq}}} = \frac{8}{\pi} \left[ \exp \left( \frac{\pi^2 D t}{l^2} \right) \right] \left[ \frac{1}{\lambda_1} \exp \left( \frac{\lambda_1^2 D t}{l^2} \right) + \frac{1}{\lambda_2} \exp \left( \frac{\lambda_2^2 D t}{l^2} \right) + \frac{1}{\lambda_3} \exp \left( \frac{\lambda_3^2 D t}{l^2} \right) \right]
\]

where

\( r = \) initial radius of the cylinder, \( \text{m} \)

\( (r = 0.00755 \text{ m}) \)

\( l = \) half length of the cylinder, \( \text{m} \)

\( (l = 0.0415 \text{ m}) \)

\( \lambda_n = \) root of the Bessel function of the n\textsuperscript{th} kind of zero order.

\( (\lambda_1 = 2.4048, \lambda_2 = 5.5201, \lambda_3 = 8.653) \)
Quality Determination

Color measurement

The color value (Hunter $L$, $a$ and $b$) of dried shrimp was measured using Juki color meter (JP7100p Tokyo Japan) at three positions: P1 (the head), P2 (middle part) to P3 (the tail) as illustrated in Figure 3. The $L$ coordinate represents as lightness ($+L$), the $a$ coordinate represents as redness ($+a$) or greenness ($-a$), and the $b$ coordinate represents as yellowness ($+b$) or blueness ($-b$). Additionally, the total color difference ($\Delta E$) was determined as follows: (Devahastin et al., 2006; Namsanguan et al., 2004).

$$\Delta E = \sqrt{(L_0 - L_f)^2 + (a_0 - a_f)^2 + (b_0 - b_f)^2}$$  \hspace{1cm} (4)

where

$L_0, L_f =$ initial and final brightness value of sample, respectively

$a_0, a_f =$ initial and final redness value of sample, respectively

$b_0, b_f =$ initial and final yellowness value of sample, respectively

Figure 2 Flow chart of drying system for boiled shrimps. (300-400 shrimp/kg).

Figure 3 Measured positions for determining of dried shrimp color.
The percentage of shrinkage and rehydration

The diameter of sample before and after drying was measured in three positions as illustrated in Figure 3 by using vernier caliper with an accuracy of 0.01 mm. The average value was determined by mean of three replication and the percentage of shrinkage was defined as follows:

\[ \text{%Shrinkage} = \frac{D_{\text{initial}} - D_{\text{final}}}{D_{\text{initial}}} \times 100 \]  

where \( D_{\text{initial}} \) and \( D_{\text{final}} \) are the geometric mean diameters of the shrimp at the beginning and at the end of the drying experiment, respectively.

The diameters of sample before and after soaking at 100°C for 10 min were measured using the same method as mentioned above. The experiment data was confirmed by duplication. The percentage of rehydration (Tapaneyasin et al., 2005) was calculated as follows:

\[ \text{%Rehydration} = \frac{D'_{\text{after}} - D'_{\text{before}}}{D'_{\text{before}}} \times 100 \]  

where \( D'_{\text{after}} \) and \( D'_{\text{before}} \) are the geometric mean diameters of the shrimp before soaking and after soaking, respectively.

Texture analysis

The texture of dried shrimp sample was determined by texture analyzer (Model TA-XT2i plus, Stable Micro System, United Kingdom) using the head Warner Bratzler Meat Shear Compression type at the cross head speed of 13.5 mm s\(^{-1}\). The maximum shear force was recorded and the average value was determined from five replications.

Specific energy consumption

From the experimental set-up, the specific energy consumption can be evaluated as follows:

\[ \text{SEC} = \frac{3.6E_p}{(M_{\text{in}} - M_{\text{f}}) \times W_w} \]  

where \( E_p = \) total energy, kW-h, \( M_{\text{in}} = \) initial moisture content, decimal (dry basis), \( M_{\text{f}} = \) final moisture content, decimal (dry basis), \( W_w = \) dry weight of sample, kg

Results and Discussion

Drying Kinetics and Diffusion Coefficient

From the experiments with various drying conditions, the evolutions of moisture ratio of 1-stage and 2-stage drying during drying time were illustrated in Figure 4 and 5.

The result showed that drying with high temperature was relatively short period compared to the drying with low temperature. Additionally, the 2-stage drying with combined infrared (IR) and hot air (HA) heat source was faster than 1 and 2 stage drying with HA only. At the beginning of drying time, moisture ratio of shrimp decreased rapidly because the main part of moisture content of shrimp exists around the exterior surface, thus allowing the easier water removal without any interference of disordered void spaces inside tissue when the tissue is contacted with infrared radiation. At nearly end of drying period, heat and mass transfer didn’t occur at the surface of shrimp but it stimulated water inside the shrimp. However, moisture inside tissue of shrimp move to surface slowly than the movement from its surface to ambient environment, also drying rate will be lower. Consequently, at a higher drying air temperature, rate of moisture removal became relatively faster compared to those of a lower temperature. These drying curves are typical to ones for food stuff and grain kernel (Soponronnarit et al., 1997; Bala and Mondol, 2001; Namsanguan et al., 2004; Panagiotou et al., 2004). Thus the combined HA+IR drying caused more energy transfer for evaporating water content than drying with only HA convection.
To determine effective diffusion coefficient, the Eq.(2) and Eq.(3) were used for simulation drying kinetic of shrimp sample in form of a spherical and a finite cylindrical shape. By using non-linear regression analysis, the results showed that an effective diffusion coefficient with finite cylindrical shape assumption had a high goodness of fit compared to those value obtained using spherical shape assumption as shown in Table 1.

From the Table 1, the effective diffusion coefficient for finite cylindrical shape was in ranges between $6.4 \times 10^{-10}$ and $12.1 \times 10^{-10}$ m$^2$/s$^{-1}$ at drying temperatures of 72.0-129.8°C. These values are within the general range of $10^{-11}$–$10^{-9}$ m$^2$/s$^{-1}$ for drying of seafood matters reported works (Panagiotou et al., 2004; Krokida et al., 2004; Tirawanichakul et al., 2008).

Determination of Quality

Color measurement

From all drying conditions using 1 and 2 stage strategies as illustrate in Table 2 and 3 using HA and combined HA+IR, respectively. The total color difference of samples ($\Delta E$) tended to decrease with an increase in drying temperature. Dried shrimp with combined HA+IR significantly enhanced redness value (positive Hunter $a$-value) compared to solar drying. The initial color of shrimp before drying: $L_0=57.73\pm0.28$, $a_0=12.48\pm0.06$ and $b_0=12.44\pm0.06$. 

Table 1 The effective diffusion coefficient values of shrimp dried at two-stage temperature drying with hot air, combined hot air-solar and combined hot air-infrared heat sources.

<table>
<thead>
<tr>
<th>Drying conditions</th>
<th>Shape</th>
<th>Diffusivity constant ($D_0$), cm$^2$/S</th>
<th>Activation energy ($E_a$) kJ/kmol</th>
<th>Diffusivity ($D$), $\times 10^{-6}$ cm$^2$/S</th>
<th>$R^2$ (%)</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA (113.6-129.8°C, 83-89°C)</td>
<td>sphere</td>
<td>0.0004</td>
<td>9,999.99</td>
<td>16.94-17.86</td>
<td>97.48</td>
<td>0.0374</td>
</tr>
<tr>
<td></td>
<td>finite cylinder</td>
<td>0.0008</td>
<td>14,834.27</td>
<td>6.44-6.94</td>
<td>99.53</td>
<td>0.2602</td>
</tr>
<tr>
<td>HA - Solar (98.4-123.4°C, 74-81°C)</td>
<td>sphere</td>
<td>523.67</td>
<td>52,388.33</td>
<td>13.67-27.08</td>
<td>95.69</td>
<td>0.0592</td>
</tr>
<tr>
<td></td>
<td>finite cylinder</td>
<td>87.56</td>
<td>49,807.22</td>
<td>5.83-10.08</td>
<td>98.22</td>
<td>0.0436</td>
</tr>
<tr>
<td>HA - IR 1000 W (99.2-112.1°C, 72-75°C )</td>
<td>sphere</td>
<td>82.71</td>
<td>45,075.01</td>
<td>24.53-31.42</td>
<td>96.94</td>
<td>0.0469</td>
</tr>
<tr>
<td></td>
<td>finite cylinder</td>
<td>4,295.30</td>
<td>59,976.42</td>
<td>8.86-12.31</td>
<td>98.89</td>
<td>0.1175</td>
</tr>
</tbody>
</table>
The color values in form of brightness, redness and yellowness and total color difference of dried shrimp were illustrated in Table 2 and 3, respectively. For the results of Table 2 and 3, they showed that redness value of dried shrimp using IR heat source slightly increased compared to the other drying techniques. In addition, the total color difference of dried shrimp using 1-stage and 2-stage drying was significant different from solar drying. It implied the color value can be improved by drying technique without any chemical coloring agent, especially on IR drying technique.

**Shrinkage and rehydration analysis**

The results of percentage of shrinkage and rehydration in Table 4 and 5 revealed that the percentage of shrinkage of dried shrimp tended to increase with an increase of drying temperature for 1-stage HA. Moreover, the 1-stage and 2-stage drying with various drying strategies had significant difference on shrinkage and rehydration (p<0.05). Quality of dried shrimp with various drying temperature of 40-110°C in terms of rehydration was different significantly (p<0.05). The shrinkage phenomena were corresponded to the previous works (Namsanguan et al., 2004).

**Table 2** Color value of shrimp dried at two-stage drying with hot air.

<table>
<thead>
<tr>
<th>Drying conditions</th>
<th>L</th>
<th>a</th>
<th>b</th>
<th>ΔE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA (113.6°C, 30 min) + 83.5°C</td>
<td>50.12</td>
<td>±0.98d</td>
<td>±0.33</td>
<td>±0.38b</td>
</tr>
<tr>
<td>HA (121.4°C, 30 min) + 89.3°C</td>
<td>48.54</td>
<td>±0.60b</td>
<td>±0.52b</td>
<td>±0.38b</td>
</tr>
<tr>
<td>HA (129.8°C, 30 min) + 83.3°C</td>
<td>44.58</td>
<td>±0.54b</td>
<td>±0.34b</td>
<td>±0.23b</td>
</tr>
<tr>
<td>HA 79.7°C</td>
<td>76.08</td>
<td>22.83</td>
<td>28.34</td>
<td>26.40</td>
</tr>
<tr>
<td>HA 112.8°C</td>
<td>73.44</td>
<td>22.23</td>
<td>26.90</td>
<td>23.48</td>
</tr>
<tr>
<td>Solar 40.1°C</td>
<td>52.61</td>
<td>13.54</td>
<td>14.30</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Note: *a-e in the same column with different superscripts are significantly different (p<0.05)

**Table 3** Colors of shrimp dried at two-stage temperature drying with combined Infrared and hot air.

<table>
<thead>
<tr>
<th>Drying conditions</th>
<th>L</th>
<th>a</th>
<th>b</th>
<th>ΔE</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA+IR 1,000 W 99.2°C (30 min)+72.0°C</td>
<td>37.84</td>
<td>±0.52f</td>
<td>±0.27</td>
<td>±0.42d</td>
</tr>
<tr>
<td>HA+IR 1,000 W 109.7°C (30 min)+74.2°C</td>
<td>42.13</td>
<td>±0.95c</td>
<td>±0.74c</td>
<td>±0.56d</td>
</tr>
<tr>
<td>HA+IR 1,000 W 112.1°C (30 min)+75.0°C</td>
<td>43.17</td>
<td>±0.92d</td>
<td>±0.30b</td>
<td>±0.14c</td>
</tr>
<tr>
<td>HA+IR 1,000 W 78.1°C</td>
<td>73.84</td>
<td>25.43</td>
<td>28.77</td>
<td>28.38</td>
</tr>
<tr>
<td>HA+IR 1,000 W 94.7°C</td>
<td>±0.36b</td>
<td>±0.41d</td>
<td>±0.20b</td>
<td>±0.50c</td>
</tr>
<tr>
<td>Solar 40.1°C</td>
<td>52.61</td>
<td>13.54</td>
<td>14.30</td>
<td>5.55</td>
</tr>
</tbody>
</table>

Note: *a-e in the same column with different superscripts are significantly different (p<0.05)

**Table 4** Shrinkage, rehydration and maximum shear force of dried shrimp at using two-stage temperature drying with hot air in comparison with solar drying.

<table>
<thead>
<tr>
<th>Drying conditions</th>
<th>Shrinkage (%)</th>
<th>Rehydration (%)</th>
<th>Max. Shear (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA (113.6°C, 30 min) + 83.5°C</td>
<td>20.48</td>
<td>±0.92d</td>
<td>±0.78b</td>
</tr>
<tr>
<td>HA (121.4°C, 30 min) + 89.3°C</td>
<td>21.81</td>
<td>±0.77b</td>
<td>±0.68b</td>
</tr>
<tr>
<td>HA (129.8°C, 30 min) + 83.3°C</td>
<td>21.50</td>
<td>±0.84b</td>
<td>±0.09b</td>
</tr>
<tr>
<td>HA 79.7°C</td>
<td>21.38</td>
<td>±0.42b</td>
<td>±0.80b</td>
</tr>
<tr>
<td>HA 112.8°C</td>
<td>30.73</td>
<td>±0.45b</td>
<td>±0.47b</td>
</tr>
<tr>
<td>Solar 40.1°C</td>
<td>18.31</td>
<td>±1.18</td>
<td>±2.77</td>
</tr>
</tbody>
</table>

Note: *a-e in the same column with different superscripts are significantly different (p<0.05)

For texture analysis, the statistical results showed that maximum shear force of drying with the 1-stage and 2-stage drying with high temperatures provided low maximum shear force. This may because dried shrimp tissue had more porosity getting their low strength and crispy tissues.

**Specific energy consumption**

The specific energy consumption (SEC) of shrimp drying using the two stage hot air drying was illustrated in Figure 5. The
specific energy consumption tended to decrease when the drying temperature increases correlated to operating time. This is the same incidence as another drying with combined IR+HA heat sources. Figure 6 showed the SEC of shrimp drying with two stage using combined IR+HA heat sources.

Table 5 Shrinkage, rehydration and maximum shear force of dried shrimp at using two-stage temperature drying with combined hot air and infrared sources in comparison with solar drying

<table>
<thead>
<tr>
<th>Drying conditions</th>
<th>Shrinkage (%)</th>
<th>Rehydration (%)</th>
<th>Max. Shear (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA+IR (99.2°C, 30 min) + 72.0°C</td>
<td>21.14 ± 1.07</td>
<td>12.47 ± 0.59</td>
<td>77.62 ± 1.32</td>
</tr>
<tr>
<td>HA+IR (109.7°C, 30 min) + 74.2°C</td>
<td>21.42 ± 0.01</td>
<td>12.53 ± 0.44</td>
<td>43.56 ± 0.83</td>
</tr>
<tr>
<td>HA+IR (112.1°C, 30 min) + 75.0°C</td>
<td>22.11 ± 0.18</td>
<td>12.65 ± 0.12</td>
<td>39.40 ± 0.53</td>
</tr>
<tr>
<td>HA+IR 78.1°C</td>
<td>21.03 ± 0.58</td>
<td>7.86 ± 0.55</td>
<td>70.24 ± 0.18</td>
</tr>
<tr>
<td>HA+IR 94.7°C</td>
<td>22.03 ± 0.58</td>
<td>8.78 ± 0.55</td>
<td>65.78 ± 2.08</td>
</tr>
<tr>
<td>Solar 30.1°C</td>
<td>18.31 ± 1.18</td>
<td>8.45 ± 0.27</td>
<td>61.84 ± 1.74</td>
</tr>
</tbody>
</table>

Note: *a-e in the same column with different superscripts are significantly different (p<0.05)

Figure 5 Specific energy consumption of shrimp drying using two-stage hot air drying for initial moisture content of 306-384% dry-basis and with inlet drying air flow rate of 1.1 m s⁻¹.

Figure 6 showed the SEC of shrimp drying using combined IR+HA heating. The results showed that the specific energy consumption of shrimp drying with two-stage techniques was lower than using one and two stage drying with constant drying temperature.

![Graph showing SEC vs Drying Temperature](image)

**Conclusions**

The following conclusion can be stated as below:

1. Evaluation of an effective diffusion coefficient using the Fick’s law of diffusion show that the effective diffusion coefficient highly related to the drying temperature. An effective diffusion coefficient considered as a finite cylindrical shape had a good description for drying kinetics of shrimp compared to an effective diffusion coefficient of shrimp determined by a diffusion model using a spherical shape.

2. For quality determination in each drying condition, the dried shrimp using infrared provided high physical quality, especially on redness value and uniform moisture content. The dried shrimp with high temperature got more shrinkage than that obtained with low temperature for one stage hot air drying. On the other side, quality of dried shrimp with various drying temperature of 40-110°C in terms of rehydration was different significantly (p<0.05) whilst high temperature significantly affected on the shear force (p<0.05).

3. Two stage drying using combined heat sources was appropriate method for
high quality of shrimp with short drying time and low specific energy consumption.

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References

Development of a Composite Tubular Membrane for Separation of Acetone-Butanol-Ethanol (ABE) from Fermentation Broth by using Pervaporation Technique

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Abstract

Biobutanol has been considered as a potential alternative fuel with characteristics sufficiently similar to gasoline. However, product inhibitions, low productivities, and high recovery costs are the consequent limitations of acetone-butanol-ethanol (ABE) fermentation. A Polydimethyl siloxane/ Polyvinylidene fluoride (PDMS/PVDF) composite membrane, and Natural rubber/Ceramic (NR/CRM) composite tubular membrane were used to investigate the membrane performances by pervaporation technique. ABE solutions were prepared to study the effect of feed temperatures in the range of 35-80 °C. The effect of feed butanol concentration was also investigated using butanol/water aqueous solutions varying from 1.25 to 10.0 %v/v. The results showed that total flux, and butanol selectivity increased rapidly with the increase of temperature, while the corresponding water selectivity showed the reverse tendency. Increasing the feed butanol concentration resulted in an increase of the total flux and slightly decrease of butanol selectivity. Both membranes showed the similar trend under the same experimental condition but the PDMS/PVDF composite membrane gave significantly better results in term of flux. However, NR/CRM composite tubular membrane showed higher performance in term of butanol selectivity. ABE fermentations were also carried out with in situ product removal (ISPR) using Clostridium acetobutylicum TISTR 1462, and PDMS/PVDF composite membrane. The experimental results indicated that the total solvents, and production yield were higher (16.9 g/L and 0.43 g/g, respectively) than that of batch fermentation (13.7 g/L and 0.41 g/g, respectively). Compared to batch fermentation, 1.5 times more productivity was found in this system.

Keywords: biobutanol, extractive fermentation, hydrophobic membrane, pervaporation

Introduction

At present, the petroleum exploration has met several problems, such as increasing petroleum price which has been opposed to rapid decrease of whole petroleum stock in the world. In addition, the fuel crisis is an important problem in Thailand which will be possibly extended in the near future. Moreover, other growing concerns are the greenhouse gas emission and global warming. Therefore, these reasons force researches to find the alternative fuels.

In the past two decades, there were many researches related to the renewable fuels which can either be completely replaced or blended with the petroleum fuel without requiring specially adopted engines in vehicles (Ranjan and Moholkar, 2009). The most popular alternative fuel is ethanol, which has been recommended as a great alcohol fuel. However, there are some disadvantages of this alcohol fuel, such as limitations of low energy content (or heat of combustion) and it causes problems with corrosion including phase separation in the gasoline mixture. Another alternative alcohol fuel that has emerged in recent past is biobutanol, which overcomes most of above constraints. Biobutanol has currently
attracted considerable attention as an alternative biofuel to the petroleum-derived fuel (Ha et al., 2008) due to several advantages including high energy content, low water absorption, and easy application to the existing gasoline infrastructure.

Biobutanol can be produced through the process of acetone-butanol-ethanol (ABE) fermentation of various substrates by using solvent producing strains of Clostridium spp. However, ABE fermentation processes still have limited productivity. A common reason for this is the presence of the products that can cause alcohol inhibitory or toxic effects (making poor use of the enzyme) or promote unfavourable equilibria (giving low conversions) (Lye and Woodley, 1999). In each case, the desired product needs to be removed as soon as it is formed in order to overcome these constraints and hence increase the productivity and yield of the biocatalytic process. The usual concentration of total solvents in the fermentation broth is 18–33 g/L (using starch or glucose) of which butanol is only about 13–18 g/L (Ezeji et al., 2004). Such a low product concentration adversely affects the economics of recovery of these solvents from dilute fermentation broth by distillation, making the process unable to compete with the petroleum-based products. Recently, a variety of butanol recovery techniques have been developed to reduce the cost of butanol production. Pervaporation is a part of downstream processing which appears to be particularly promising. Furthermore, a combination of production process and downstream technology (integrated processing) offers a great potential in micro-biotechnology.

**Materials and Methods**

**Preparation of the Aqueous Solutions**

Analytical grade \( n \)-butanol, acetone, and ethanol (Sigma-Aldrich, Singapore) were used together with de-ionized water to prepare the aqueous feed solutions for the pervaporation studies on membrane performances. The membranes used in this work were two different membrane materials. A commercial Polydimethylsiloxane/Polyvinylidene fluoride (PDMS/PVDF) composite membrane was supplied by Sulzer Chemtech GmbH, Switzerland and a Natural rubber/Ceramic (NR/CRM) composite tubular membrane was kindly provided by the Prince of Songkla University (PSU), Thailand. In order to evaluate the effects of operating conditions on the separation performance, both the membranes mentioned previously were used for the separation of binary butanol/water solution with butanol concentration in the range of 1.25-10.0 \% \text{v/v} and quaternary mixture solution (acetone/butanol/ethanol/ water) containing 3.0 g/L acetone, 10 g/L \( n \)-butanol, 1 g/L ethanol. Both the binary and quaternary solutions were performed at varying temperature of 35-80 °C. These operation steps can be influenced positively by the time and cost intensive downstream processes. Also importantly, high permeation flux and high selectivity are the essential requirements for a successful product separation process by pervaporation.

In order to meet these requirements, a hydrophobic polymeric membrane plays an important role on high specific recovery and should also have an ultra thin layer, while this layer must maintain its integrity and mechanical stability under operation. Composite tubular membrane has attracted a great deal of attentions because of its many advantages in contrast to the traditional extraction process (Liu et al., 2003). In the fabrication of a composite tubular membrane, a microporous tubular support with good mechanical strength is coated with a thin layer of selective hydrophobic polymer to perform the separation. Generally, the defect free top layer should be as thin as possible while the support membrane should possess a high porosity with reasonably small pore size.

In many researches on ABE production, it was found that organic solvents had very strong action on biotransformation cells and membranes. Here, this work aimed to produce the ABE with \textit{in situ} product-removal (ISPR) or extractive fermentation, integration of production and separation process with the
development of composite membrane using pervaporation technique. The ISPR processing, in which a potentially inhibitory product is continuously removed from the fermentation broth as it is produced, has important advantages in improving yield and conversion relative to conventional processes.

**Pervaporation Experiments**

The experimental setup of the pervaporation apparatuses were performed with two different types of the membranes (flat-sheet and tubular membranes) as shown in Figure 1. The first apparatus, a PDMS/PVDF flat-sheet membrane was installed in a stainless steel module. 2 L of the feed in a 3 L stirred tank reactor was circulated at 10 L/h through the membrane module and return to reactor as retentate by using a peristaltic pump (Masterflex® Peristaltic Pump, Cole parmer, USA). For the second apparatus, a NR/CRM tubular membrane was immersed directly inside the bioreactor. For both of the experiment, the feed temperature was controlled by a circulating thermostat water bath (Julabo, Germany). The feed side was kept at atmospheric pressure, whereas the permeate pressure was maintained below 5 mbar using a vacuum pump coupled with a pressure controller. Permeates were condensed using two glass cold traps filled with liquid nitrogen to ensure that all permeates were fully collected. Both the feed and permeate samples were collected at a fixed interval (0.5-1 h for aqueous solution, and 6 h for in situ product removal, respectively). The total flux (g/m² h) and selectivity were calculated by the following equations.

\[
\text{Total flux} = \frac{W}{A \times t} \\
\text{Selectivity} = \frac{y/(1-y)}{x/(1-x)}
\]

Where W is the weight of the permeate in grams, A is the membrane area in m² and t the time in hour for the sample collection. The x and y represent the weight fraction of components in feed and permeate samples, respectively.

**Preparation of ABE Fermentation**

*Clostridium acetobutylicum* TISTR 1462 was obtained from Thailand Institute of Scientific and Technological Research (TISTR). In order to prepare the inoculum, spores were suspended in cooked meat medium, and the suspension was heat shocked for 2 min, then cooled in ice-cold water for 1 min as described by Qureshi et al. (2001). The heat shocked spores were incubated in an anaerobic jar at 30 °C for 18-24 h. Fermentations were carried out by using a medium with the following composition: glucose 50 g/L, yeast extracts 5 g/L, ammonium acetate 2 g/L, KH₂PO₄ 0.75 g/L, K₂HPO₄ 0.75 g/L, MgSO₄•7H₂O 0.40 g/L, MnSO₄•7H₂O 0.01 g/L, and FeSO₄•7H₂O 0.01 g/L. Before inoculation, the medium was autoclaved at 121 °C for 15 minutes (0.01 g/L p-aminobenzoic acid and 0.001 g/L biotin were filtered through 0.45 μm filter prior to adding to the medium after cooled down to 35°C and cooled to 35 °C.

![Figure 1](image_url)
In Situ Product Removal

ABE fermentations were performed in a manner of integrated production, and separation process at the same time with total medium of 2.3 L in a 3 L bioreactor (Sartorius, Germany) by using a PDMS/PVDF composite membrane. The bioreactor containing 2.07 L of medium was inoculated with 0.23 L of inoculum from a 18-24 hours culture. All experiments were conditioned at optimal growth temperature of 35 °C, pH at around 6.0 (adjust pH via the addition of 3M NaOH), and the agitation speed was set at 100 rpm (in order to make the broth homogeneously under an anaerobic environment). The fermentation was initially run for 24 h without separation before the ABE were continuously removed by using pervaporation process. The fermentation broth was re-circulated through a feed channel of the membrane module before returned back to the bioreactor. The vacuum pressure was supplied by a vacuum pump. This experiment was performed for 102 h with collection of retentate and permeate samples as well as changing the glass cold traps every 6 h.

Analysis

Solvent concentration in the feed and permeate samples were analyzed by using a SRI 8610C gas chromatography equipped with a Carbowax® column (Restek, USA) of 30 m x 0.32 mm x 0.25 µm and a flame ionization detector (FID). Helium, 99.99% pure, was used as carrier gas with flow rate of 20 mL/min. Samples containing cell or suspended solids were centrifuged at 14,000 rpm for 2 min in a microcentrifuge. Glucose and organic acids (acetic and butyric acid) in the fermentation broth were measured using high performance liquid chromatography (HPLC) with RI detector (Model 1200 series, Agilent technology, USA) and 4 mM sulfuric acid was used as the mobile phase. The temperature of the column was operated at ambient temperature with a flow rate of 1.0 mL/min. The cell concentration in the fermentation broth was determined by optical density at 600 nm (OD<sub>600</sub>) with a spectrophotometer.

Results and Discussion

Effect of Feed Solution Temperature on Membrane Performances

Pervaporation of the ABE solvents from aqueous solutions through the PDMS/PVDF composite membrane, and NR/CRM composite tubular membrane were firstly investigated with varying feed temperatures. The concentration of the organic solvents in the feed solution was prepared at 10 g/L butanol, 3 g/L acetone, and 1 g/L ethanol in the total solution volume of 2 L. The effect of temperature on separation performances was exposed in Figure 2. Total flux of both the PDMS/PVDF composite membrane, and NR/CRM composite tubular membranes increased from 515 to 1,665 g/m<sup>2</sup>h and 9.2 to 97.6 g/m<sup>2</sup>h, respectively with increasing the feed temperature of 35 to 80 °C. This phenomenon can be traditionally explained by the increase of solubility and diffusivity of organic solvent (particularly butanol) and water in membrane as well as the increase of sorption and desorption rate of permeant molecules in membrane matrix. As the temperature increased to 80 °C, the butanol selectivity increased to 7.2 and 11.2 for PDMS/PVDF composite membrane, and NR/CRM composite tubular membrane, respectively. In contrast, the water selectivity decreased slightly for both membranes at the same condition due to the increase of solvent diffusivity through the membrane at higher temperature.

Effect of Feed Butanol Concentration on Membrane Performances

The separation of butanol by pervaporation using two different membrane materials were investigated from butanol/water binary solution with varying feed butanol concentration from 1.25 to 10 %v/v at a set temperature of 80 °C. Figure 3 shows that the PDMS/PVDF composite membrane conferred linear increasing of butanol flux (from 140.9 to 1,211.2 g/m<sup>2</sup>h) and concentration in permeate (from 54.0 to 382.1 g/L) with the increase of feed butanol concentration. The results mentioned above
showed similar trends to NR/CRM composite tubular membrane, butanol flux and concentration in permeate increased from 9.3 to 116.2 g/m²h, and from 118.9 to 431.7 g/L, respectively. This phenomenon occurred by the interaction between the polymeric membrane and the permeate molecules. PDMS and NR active layers are hydrophobic materials, and had relatively high polarity groups resulting in strong interactions with organic solvents. With the increase of butanol content in feed, more butanol molecules can be easily absorbed and passed through the membrane. The total flux also increased with the increase of feed butanol concentration and reached 3,070 to 3,170 and 221 to 269 g/m²h at a feed concentration of 5 to 10 %v/v for both the PDMS/PVDF composite membrane and the NR/CRM composite tubular membrane, respectively. Butanol selectivity of two membranes used in this work decreased slightly with the increase of the feed butanol concentration. The similar trend was observed by Qureshi et al. (1999) to separate butanol from aqueous solution using a silicalite/silicone membrane at a retentate temperature of 78 °C. At higher feed concentrations, the denominator term in the selectivity relationship becomes larger, thus giving low selectivity.

**Comparison of PDMS/PVDF Composite Membrane and NR/CRM Composite Tubular Membrane**

The comparison of efficiencies of two different membrane materials is listed in Table 1. The PDMS/PVDF composite membrane showed significantly better results in terms of butanol flux. With active layer of 2 µm, the membrane offered higher butanol flux than that of the NR/CRM composite tubular membrane when using feed butanol concentration of 10 g/L at temperature of 35-80 °C. Moreover, at optimum temperature (35 °C) of ABE fermentation, the butanol flux of NR/CRM composite tubular membrane was almost absented. However, NR/CRM composite tubular membrane showed higher performance in terms of selectivity when compared to the PDMS/PVDF composite membrane. It could be seen that incorporating the ceramic supportive layer with the dense natural rubber active layer makes both membrane layers thicker than 2 µm PDMS active coated on PVDF supportive layer. The thinner membrane exhibited a higher permeation flux and a lower selectivity than the thicker membrane. Therefore, all experiments mentioned above indicated that the permeability of butanol across the membrane was found to follow the same relationship with membrane thickness, feed temperature and butanol concentration.
Feed butanol concentration (g/L)

0 2 4 6 8 10 12

Total flux (g/m²h)

2400 2600 2800 3000 3200 3400

Butanol concentration

Flux (g/m²h), Concentration (g/L), and Selectivity

-200 0 200 400 600 800 1000 1200 1400 1600

Total flux

Butanol flux

Butanol concentration

Butanol selectivity

Figure 3 Effect of feed butanol concentration on flux, concentration, and selectivity using: (a) PDMS/PVDF composite membrane (b) NR/CRM composite tubular membrane.

Table 1 Summary of two different membranes for butanol separation by pervaporation using 10 g/L butanol/water aqueous solution.

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Active layer thickness (µm)</th>
<th>Feed temperature (°C)</th>
<th>Butanol flux (g/m²h)</th>
<th>Butanol Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDMS/PVDF composite membrane</td>
<td>2</td>
<td>35</td>
<td>14.7</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>27.6</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>68.1</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>113.0</td>
<td>7.2</td>
</tr>
<tr>
<td>NR/CRM composite tubular membrane</td>
<td>N/A</td>
<td>35</td>
<td>0.4</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>50</td>
<td>2.4</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>70</td>
<td>5.1</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>9.9</td>
<td>11.2</td>
</tr>
</tbody>
</table>

ABE Production With in situ Product Removal

As described earlier, 2.3 L broth was fermented in a 3 L bioreactor. After 24 h of fermentation time, whole ABE fermentation broth was circulated directly through a module of PDMS/PVDF composite membrane in order to begin the separation process.

The permeate molecules were condensed by using two cold traps filled with liquid nitrogen and retentates were flown back to the reaction side. For the total fermentation time of 102 h, 16.9 g/L of total solvent concentration produced in a fermentation broth, and 0.44 g/L h of productivity were obtained in the ABE production with ISPR system (Table 2). Throughout fermentation, cell concentration in the bioreactor still kept increasing with the time while glucose was consumed rapidly until the end of fermentation as shown in Figure 4.

It can be seen that once the inhibitory products were produced, the ISPR process can simultaneously removed the products which affected the cell in the reaction side. Similarly, Chauhan and Woodley (1997) reported that ISPR method can increase the productivity or yield of a biocatalytic reaction by any of the following means: (1) overcoming inhibitory or toxic effects, (2) shifting unfavourable reaction equilibria, (3) minimizing product losses owing to degradation or uncontrolled release, and (4) reducing the total number of downstream-processing steps. These reasons gave the higher productivity and total solvent concentration as well as the production yield when compared with traditional batch carried out in our laboratory.
**Table 2** ABE fermentation with ISPR using *C. acetobutylicum* and PDMS/PVDF composite membrane.

<table>
<thead>
<tr>
<th></th>
<th>Batch with ISPR process</th>
<th>Batch process*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone (g/L)</td>
<td>3.3</td>
<td>2.7</td>
</tr>
<tr>
<td>Butanol (g/L)</td>
<td>13.3</td>
<td>10.8</td>
</tr>
<tr>
<td>Ethanol (g/L)</td>
<td>0.34</td>
<td>0.18</td>
</tr>
<tr>
<td>Total solvents (g/L)</td>
<td>16.94</td>
<td>13.68</td>
</tr>
<tr>
<td>Solvent productivity (g/L h)</td>
<td>0.44</td>
<td>0.32</td>
</tr>
<tr>
<td>Solvent yield (g/g)</td>
<td>0.43</td>
<td>0.41</td>
</tr>
<tr>
<td>Glucose utilized (g) (50g/L at beginning)</td>
<td>48.3</td>
<td>43.7</td>
</tr>
</tbody>
</table>

*ABE production in batch carried out in our laboratory with the same condition.

Pervaporation results of acetone, butanol, and ethanol produced in one fermentation broth were shown in Figure 5. In the production side, total solvent concentration increased linearly at the beginning until fermentation time of 30 h followed by stationary phase with the highest concentration of 10.2 g/L during pervaporation. Compared to the permeate side, the total solvent concentration showed similar trend to the concentration in the reaction side as well as the total flux. The result indicated that the rate of removal of solvent strongly depends upon solvent concentration, particularly on butanol. Lower concentrations in broth result in lower concentration in permeate and flux as well.

**Figure 4** Glucose, cell, and total solvent concentration in ABE fermentation with ISPR using *C. acetobutylicum* and PDMS/PVDF composite membrane.

**Figure 5** Total flux and solvent concentration in reaction side and permeate in ABE fermentation with ISPR using *C. acetobutylicum* and PDMS/PVDF composite membrane.

**Conclusions**

The separation of acetone, butanol, and ethanol (ABE) from aqueous solution by pervaporation using two different membrane materials, (PDMS/PVDF composite membrane and NR/CRM composite tubular membrane), was investigated for separation performances. The effect of feed temperature and feed concentration were studied and compared individually. The results revealed that the total flux and butanol selectivity of both the membranes investigated increased with increasing feed temperature from 35 to 80 °C. This phenomenon did not occur with water selectivity. The increase of butanol concentration in the feed solution from 1.25 to 10 %v/v resulted in the total flux increasing rapidly with inverse trend of butanol selectivity which slightly decreased at working temperature of 80 °C. This result showed the similar trend between the two different membranes, but it could be seen that the PDMS/PVDF composite membrane with thinner active layer gave the best result for permeation flux. However, the thicker NR/CRM composite tubular membrane showed the higher performance in terms of butanol selectivity but it did not work efficiently with low temperature (insignificantly low flux was found at 35 °C). The PDMS/PVDF composite membrane...
membrane was therefore chosen to perform the in situ product removal system due to its higher permeation flux. ABE production with ISPR revealed that the total solvent and production yield were higher (16.9 g/L and 0.43 g/g, respectively) than that of traditional batch fermentation (13.72 g/L and 0.41 g/g, respectively) as well as 1.5 times more productivity was found in this system.

Acknowledgments

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Effect of High-Pressure Microfluidization on the Structure and Properties of Waxy Rice Starch

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Abstract
Waxy rice starch-water suspensions (5, 15%) were subjected to high-pressure microfluidization treatment at 50 MPa, 100 MPa, and 150 MPa. The influence of microfluidizing pressure on the temperature, thermal properties, particle size and morphology of starch granules were investigated, with native waxy rice starch as a control sample. The temperatures of microfluidized starch slurries increased with the increase of the applied pressure and the rate was 0.12°C MPa⁻¹ and 0.08°C MPa⁻¹ for 5 and 15% starch concentration, respectively. However, the increasing temperatures were below the gelatinization temperatures of waxy starch. Differential scanning calorimetry analysis showed a decrease in gelatinization temperatures (Tₒ, Tₚ) and gelatinization enthalpy (ΔH₆₆) with increasing homogenizing pressure. Laser scattering measurement of particle size showed an increase in the granule size at a homogenizing pressure of 150 MPa. This was attributed to the gelatinization and aggregation of the starch granules. The morphology of waxy starch studied by scanning electron microscope showed that the waxy starch was partly gelatinized at the pressure of 100 and 150 MPa, and the gelatinized granules were tend to aggregate with each other, resulting in an increase of granule size.

Keywords: high pressure, microfluidization, starch gelatinization, waxy rice starch

Introduction
Starch is a natural substance widely used in industrial and food applications (Parker and Ring 2001; Che et al, 2007). Native starch occurs as granules which are relative dense and insoluble in cold water. When heated in the presence of excess water, gelatinization takes place. Starch gelatinization is an irreversible process which includes granular swelling, native crystalline melting, loss of birefringence and starch solubilization (Sullivan and Johnson, 1964; Hermansson and Svegmark, 1996). In addition, starch can also be gelatinized by high pressure (Douzals et al., 1996; Stute et al., 1996; Katopo et al., 2002). It has been reported that high pressure (up to 650 MPa) treatment caused an irreversible alteration of the crystalline region in starch granules prior to a reversible hydration of the amorphous phase, which in turn leads to the destruction of the starch granules (Rubens and Heremans, 2000; Blaszczyk et al., 2007).

Although most research has focused on the high hydrostatic pressure treatment of starch, the limitation of a lengthy period of process is economically undesirable in the starch industry. High-pressure microfluidization treatment could be used as an emerging technology in the starch industry. High-pressure microfluidization, where a dynamic high pressure is introduced over very short time (less than 5 s), is different from a static high-pressure system. Under high-pressure microfluidization, a rapid change in pressure induces liquid to
expose high pressure, high shear, turbulence and cavitation which contributes to the change of treated sample properties (Paquin, 1999). The use of high pressure technique has been introduced to create unique properties in starch granules which in turn can affect the gelatinization and pasting properties of starches. Some starch granules after high pressure treatment did not show any extensive swelling and kept granular structure, which resulted in a weaker gel (Stolt et al., 2001; Oh, et al., 2008a, b; Wang et al., 2008; Che et al., 2009; Vallons and Arendt, 2009). The gelatinization of starches treated with high pressure can occur at ambient temperature. However, the degree of gelatinization depended on the botanical origin, the pressure, the temperature and the duration of treatment (Stute et al., 1996; Bauer and Knorr, 2005; Oh, et al., 2008a). It was suggested that B-type starches (potato starch, high amylose maize starch) were more resistant to pressure gelatinization than A-type starches (normal maize starch, waxy maize starch, rice starch) due to the different crystalline structure (Rubens et al., 1999; Katopo et al., 2002). It was also reported that wheat and cassava starch completely lost their crystalline structure and formed a gel-like structure after treated with high pressure at 600 MPa, while potato starch under the same treatment still retained its granule structure with decreased crystallinity (Douzals et al., 1996; Bauer and Knorr, 2005; Huang et al., 2007). Regarding rice and waxy rice starch, gelatinization of those occurred when treatment at above 500 MPa was applied (Oh, et al., 2008b). Understanding pressure induced gelatinization of starch is important for application of high pressure microfluidization in starch-containing products.

In this study, the effects of different treatment pressures and starch concentrations on the structure and properties of waxy rice starch were investigated. The process was studied in the pressure range 0 – 150 MPa at ambient temperature. In order to find out the influences of microfluidization pressure, the temperatures, morphology, partial size distributions and thermal properties of treated starch were measured. Differential scanning calorimetry (DSC), microscopy and scanning electron microscopy (SEM), were used to monitor the gelatinization of starch and laser scattering analyzer was used to study the particle size distributions of starch granules after pressured treatment.

Materials and Methods

Materials

Commercial waxy rice starch was purchased from Cho Heng Rice Vermicelli Factory Co., Ltd. The moisture content of the starch was determined by drying measurement in air-oven at 105°C for 24 h, and the average value was determined to be 13% (w/w). An analytical grade alcohol (95%, w/w) was purchased from Sigma Chemical Co. (St.Louis, MO, USA).

High-pressure Microfluidization of Waxy Rice Starch Suspension

Five hundred milliliters of waxy rice starch suspensions (5, 15 wt.%) were prepared by adding waxy rice starch in de-ionized water at 25°C. The well mixed suspensions were passed through a benchtop microfluidizer (M-110P, Microfluidic Corporation, USA) for one pass at 50, 100 and 150 MPa, respectively. The temperatures of starch-water suspensions were monitored just before and after microfluidization with an immersion thermometer. The microfluidized starches were vacuum filtered and dehydrated with anhydrous alcohol. After that starches were dried in an oven at 37°C for 24 h to obtain dried sample for further characterizations. The native waxy rice starch was soaked in de-ionized water for 10 min and then treated using the method described above without microfluidization.

Microscopy of Waxy Rice Starch Granules

The treated and native waxy rice starches were observed using an optical microscope (Olympus BX51 series, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a polarized light.

Scanning Electron Microscopy

Starch samples were mounted on metal
stubs and coated with platinum using an Ion Sputter E-100 coating system (Hitachi High-Technologies, Tokyo, Japan). Samples were observed using a scanning electron microscope at 15 kV (Hitachi S-3400N SEM, Hitachi High-Technologies, Tokyo, Japan).

**Laser Scattering Measurement**

The particle size distributions of microfluidized starches and the native sample were determined using the laser scattering particle-size distribution analyzer LA-950 V2 (Horiba, Ltd., Kyoto, Japan). About 10 mg of starch was dispersed in distilled water in the dispersion tank of the instrument with sonicated circulation for 1 min. The granule size was taken to be the number-average diameter automatically calculated by the instrument software.

**Thermal Properties of Starch Samples**

The gelatinization properties of the native waxy rice starch and microfluidized waxy rice starches in the presence of excess water were studied using a differential scanning calorimeter (DSC-823, Mettler Toledo, Switzerland) equipped with a thermal analysis data station. The system was calibrated with indium and cyclohexane standards. Starch (3.5 mg) was accurately weighed into a 40 µl capacity aluminum pans (Mettler, ME-27331) and distilled water was added to obtain a starch-water suspension containing 70% water. Samples were hermetically sealed and equilibrated for 3 h at room temperature before heating in DSC. The samples were heated from 20 °C to 120 °C at 10 °C min⁻¹. Thermal transitions of starch samples including onset, peak and conclusion temperatures ($T_o$, $T_p$ and $T_c$) together with gelatinization enthalpy ($\Delta H_{gel}$) were determined.

**Statistical Analysis**

All experiments were carried out in triplicate. The results reported are means ± standard deviation of three determinations. Statistical analysis was carried out using analysis of variance followed by Duncan’s Multiple Range Test. Mean difference with P<0.05 were considered statistically significant.

**Results and Discussion**

**Effect of High-pressure Microfluidization on the Temperatures of Waxy Rice Starch Suspensions**

The temperatures of microfluidized waxy rice starch at different concentrations are shown in Figure 1. The microfluidized pressures showed a positive correlation with the temperatures of treated starch suspensions in both low and high concentration of starch suspensions. The rates of increasing temperature were 0.12°C MPa⁻¹ and 0.08°C MPa⁻¹ for 5% and 15% starch concentration, respectively. The temperature increase in this study is unlikely to be responsible for the gelatinization of waxy rice starch because the temperature of starch-water suspension after treatment was not high (45 °C when used pressure at 150 MPa).

The linear increase in temperature with high pressure of starch samples has been reported with different values. Che et al (2009) found that the rates of increasing temperature with applied pressure of potato and cassava starch (2 wt.%) were 0.177 °C MPa⁻¹ and 0.186 °C MPa⁻¹, respectively. The results are different from this study and this may be due to the factors of starch types, starch concentration, and applied pressure. However, the temperature increased as a result of pressure is due to the high velocity of fluid flow, friction between the fluid, the value and the cavitations that occurs in the microfluidization chamber.

**Microscopy of Waxy Rice Starch Granules**

Micrographs of starch granules are shown in Figure 2. The native waxy rice starch (A) exhibited birefringence pattern. For all samples, the radial orientation of crystallite starch granules exhibits a maltese cross or birefringence when observed under polarized light.
The effect of microfluidization pressure on the temperatures of starch suspensions. \( T_{5\%} \) and \( T_{15\%} \) are the temperature of 5 wt. % and 15 wt. % starch concentration, respectively; and \( P \) is the microfluidization pressure.

However, the maltese cross was not clearly observed after high pressure treatment, particularly at 150 MPa. As the loss of birefringence can be used as a measure of gelatinization (Thomas and Atwell, 1999), the high pressure microfluidization applied in this study may affect the gelatinization of starches. It was found that maize starch was significantly deformed and partially gelatinized when it was treated with high pressure homogenization at above 100 MPa (Wang et al., 2008). Observation of sorghum starch granules showed a decreasing number of birefringence when increasing pressure and temperature above 300 MPa (Vallons and Arendt, 2009). Wheat and cassava starch completely lost their crystalline structure and formed a gel-like structure after being treated with high pressure at 600 MPa, while potato starch under the same treatment still retained its granule structure with decreased crystallinity (Douzals et al., 1996; Bauer and Knorr, 2005; Huang et al., 2007). The different behavior of the starches under high pressure could be due to the botanical origin, the pressure, the temperature and the duration of treatment (Stute et al., 1996; Bauer and Knorr, 2005; Oh, et al., 2008a).

It was reported that some loss of birefringence was observed in waxy rice starch after treatment at 350 MPa and no birefringence was observed after treatment at 500 MPa (Oh et al., 2008b). In this study, the granules of waxy rice starch still retained some degree of integrity despite the loss of birefringence after pressure treatment at 100-150 MPa. This is because the pressures used in this study were not high enough to impact on the integrity of all starch granules. However, at the pressure of 100 and 150 MPa, the aggregation of starch granules was observed. This may suggest the partial gelatinization of starch granules occurred at the surface and then resulted in the aggregation of each other.

The results showed and discussed in this and all of the following sections were focused on 5 wt. % starch concentration only. The results involving the 15 wt. % starch concentration were not reported because they had the similar trend of results compared to 5 wt. % starch. Therefore, it may be concluded from this study that the concentration of waxy rice starch was not a significant factor to affect the structure and properties of high pressure treated starch.

**Scanning Electron Microscope of Waxy Rice Starch Granules**

SEM was used to study the starch granule morphology and gelatinization after microfluidization treatment (Figure 3). The images obtained from the native waxy rice starch showed irregular polygon shapes. After treatment with high pressure at 50 MPa, the granules retained some degree of integrity. The retention of the
granular structure is typical for pressure gelatinization (Douzals et al., 1998; Stolt et al., 2001). This was suggested that disintegration of the crystalline region was not completed by pressure due to the stabilization of hydrogen bonds which only rupture at very high pressures; >700 MPa (Hendrickx et al, 1998; Buckow et al., 2007). However, after being treated at 100 and 150 MPa, it can be seen that some starch granules formed a gel-like structure by aggregating with each other. This may be attributed to the partial gelatinization of starch granules at high pressure level. This is in agreement with Che et al. (2007) and Wang et al. (2008) and also confirmed the results of microscopy investigation.

### Particle Size Distributions of Waxy Rice Starch Granules

The particle size distributions of waxy rice starch granules investigated by laser light scattering are presented in Table 1 and Figure 4.

![Figure 3](image)

**Figure 3** SEM images of waxy rice starch at different pressures: native (A), microfluidized at 50 MPa (B), 100 MPa (C) and 150 MPa (D). Arrow signs in image indicated the aggregating of starch granules to each other.

The volume mean diameter of the native waxy rice starch granules used in this study was 10.52 μm (Table 1), which was similar to the result obtained by Oh et al. (2008). From Figure 4, the results showed that high pressure microfluidization at 50 MPa had a slightly effect on the particle size for \(d(0.1)\) and \(d(0.5)\), while the \(d(0.9)\) had a decrease from 16.51 to 9.63 μm. At 100 and 150 MPa, the \(d(0.1)\), \(d(0.5)\) and \(d(0.9)\) increased with the pressure level. Although, there was a significant increase in granule size at 150 MPa, the volume mean diameter of microfluidized starches (8.95 μm) is still smaller than native starch granules (10.52 μm). It could be suggested from the results that high pressure at above 100 MPa, particularly at 150 MPa contributed to the partial gelatinization of starch granules, leading to the aggregation with each other and resulting in an increasing number of big granule sizes (Table 1) as well as the observation obtained from the SEM images. During the microfluidization process, starch suspension undergoes high shear forces as well as high pressure resulting in the disruption of the semicrystalline structure followed by swelling of the granules and forming a into gel-like structure. The strength of chemical bonds such as van der Waal’s force and electrostatic force between the gelatinized granules would be sufficient enough to penetrate the boundaries of each other and forming aggregate granules (Huang et al., 2007).

### Differential Scanning Calorimetry

The gelatinization temperatures (onset, \(T_o\); peak, \(T_p\) and conclusion, \(T_c\)) and enthalpy of gelatinization (\(\Delta H_{gel}\)) are presented in Table 2. The \(T_o\), \(T_p\) and \(\Delta H_{gel}\) of high pressure microfluidized waxy rice starch decreased with increasing pressure. The \(T_c\) of high-pressure microfluidized

### Table 1 Diameters of waxy rice starch granules treated under different microfluidizing pressure (μm)

<table>
<thead>
<tr>
<th>Starch</th>
<th>(d_{4,3})</th>
<th>(d(0.1))</th>
<th>(d(0.5))</th>
<th>(d(0.9))</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>10.52 a</td>
<td>3.33 b</td>
<td>7.10 c</td>
<td>16.51 c</td>
</tr>
<tr>
<td>50 MPa</td>
<td>5.86 a</td>
<td>2.85 b</td>
<td>5.37 a</td>
<td>9.63 a</td>
</tr>
<tr>
<td>100 MPa</td>
<td>6.44 a</td>
<td>3.30 b</td>
<td>5.93 b</td>
<td>10.59 a</td>
</tr>
<tr>
<td>150 MPa</td>
<td>8.95 b</td>
<td>6.02 b</td>
<td>8.65 b</td>
<td>13.63 b</td>
</tr>
</tbody>
</table>

Values are means of duplicate.  
1/ \(d_{4,3}\) is the volume mean diameter (De Brouckere diameter)  
2/ \(d(0.1)\), \(d(0.5)\) and \(d(0.9)\) are the particle sizes at which 10%, 50% and 90% of all the particles by volume are smaller, respectively.  
Values represent the means of triplicate. Values with the same superscript in a column do not differ significantly (p<0.05)
samples was similar to native starch. It is likely that the high pressure applied in the microfluidization process enhanced the diffusion of water into starch granules, especially into the amorphous phase, then led to destroy the compact arrangements of molecules in the semicrystalline structure of waxy rice starch resulting in a decrease of onset temperature ($T_o$) of gelatinization. Previous researchers (Muhr and Blanshard, 1982; Stute et al., 1996; Stolt et al., 1999) suggested that gelatinization of starch could start at the room temperature, zero or even subzero temperature if the pressure is high enough. The peak temperatures ($T_p$) of treated waxy rice starches compared to those of native starch were much different at high pressure. These results are in accordance with Che et al. (2007) and Wang et al. (2008) who studied with cassava and maize starch, respectively. The decrease of gelatinization enthalpies of waxy rice starches after being treated with high-pressure microfluidization could be explained as the high shear stress destroyed some order structure of starch granules. Moreover, as previously discussed that increasing pressure of microfluidization treatment contributed the increase temperature of the starch suspension, therefore starch granules are prone to absorb water molecules much easier. The energy input in the gelatinization process of treated starch will be less. In summary, the gelatinization of waxy rice starch might be due to the combined effect of the temperature rise and the high shear stress during microfluidization process.

<table>
<thead>
<tr>
<th>Starch</th>
<th>$T_o$ ($^\circ$C)</th>
<th>$T_p$ ($^\circ$C)</th>
<th>$T_c$ ($^\circ$C)</th>
<th>$\Delta H_{gel}$ (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>57.3a</td>
<td>65.5a</td>
<td>72.5a</td>
<td>13.5a</td>
</tr>
<tr>
<td>50 MPa</td>
<td>56.7a</td>
<td>65.0a</td>
<td>73.8b</td>
<td>12.1b</td>
</tr>
<tr>
<td>100 MPa</td>
<td>55.2b</td>
<td>64.3b</td>
<td>73.0a</td>
<td>10.1c</td>
</tr>
<tr>
<td>150 MPa</td>
<td>54.3c</td>
<td>60.3c</td>
<td>71.9b</td>
<td>8.2d</td>
</tr>
</tbody>
</table>

$T_o$, onset temperature; $T_p$, peak temperature; $T_c$, conclusion temperature; $\Delta H_{gel}$, enthalpy of gelatinization. Values represent the means of triplicate. Values with the same superscript in a column do not differ significantly ($p<0.05$)

Conclusions

Waxy rice starch suspension at the concentration of 5 wt.% and 15 wt.% were microfluidized at pressure ranging from 0-150 MPa to study the effect of high-pressure microfluidization on the structure and properties of starch granules. The concentration of starch slurry was an insignificant factor to affect the structure and properties of starch after being subjected to microfluidization. Pressure induces gelatinization of starch, which occurs much more easily as the energy input is dissipated in the starch suspension as heat and causes the temperature of the starch suspensions to increase with the increasing of pressure. The shear force involved in the high pressure microfluidization (100-150 MPa) possibly disrupts starch granules causing further hydration of granules and partial gelatinization which can be seen from the SEM image. The laser scattering measurement results also showed that there was a significant increase in granule size when using high pressure (100-150 MPa) as a result of granule aggregation. Thermal properties of waxy rice starch after high pressure microfluidized treatment were changed. A decrease in gelatinization temperature ($T_o$, $T_p$) and gelatinization enthalpy ($\Delta H_{gel}$) with increasing pressure was exhibited. Further studies may focus on the change of crystalline structures of
waxy rice starch to explain the behavior of starch gelatinization. Moreover, the rheological properties of the starch paste should be examined in order to find new applications in industry.

References


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Determination of Multiclass Pesticides in Onion Using Gas Chromatography with Tandem Mass Spectrometry (GC-MS/MS)

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Abstract
A gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) multi-residue method was developed for the determination and identification of 170 pesticide (45 organophosphorous, 45 organochlorines, 7 pyrethroids, and 73 other pesticides) residues in onion. The modified buffered QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) approach was optimized for sample preparation. Pesticides were extracted/partitioned from the samples with acetonitrile containing 0.5% acetic acid, followed by cleanup using dispersive solid phase extraction (d-SPE) with 150 mg MgSO₄, 50 mg primary secondary amine (PSA), and 50 mg alumina neutral (Al-N) per mL of extract. By using multiple reaction monitoring (MRM) mode, we monitored 2 MRM transitions for each analyte at the optimum collision energy of each transition in order to increase selectivities and signal sensitivities (S/N) of the method and also reduced the background noises. The average recoveries ranged from 70 to 120% (n=5) with relative standard deviations (RSDs) were lower than 15% at 0.05, 0.1, and 0.2 mg kg⁻¹ spiking levels. The limit of detections (LODs) and limit of quantitation (LOQs) of all analytes were 0.001-0.003 mg kg⁻¹ and 0.003-0.009 mg kg⁻¹, respectively. These values were far below the MRLs established by the European Union (at 0.01 mg kg⁻¹), which led to be efficient method for screening, identifying, and quantitating pesticides in real samples. The developed method was further verified in the analyses of 40 onion samples; 3 pesticides were detected in 4 samples.

Keywords: multiple reaction monitoring, onion, pesticide, QuEChERS, tandem mass spectrometry

Introduction
Onion (Allium cepa Linn.) is a member of the pungent Allium genus of the lily family, including garlic, leeks, shallots, scallions, and chives. Onion is a good source of nutrients consisting of protein, carbohydrates, sugar, soluble and insoluble fiber, fatty acids, essential amino acids, flavonoids, quercetin, vitamins, and various minerals, which have been shown to be an antioxidant and anticancer, reduce the risk of heart disease, and lower blood cholesterol levels. Thus, these health-promoting effects are originated from sulfur-containing compositions in onions. For these possible reasons, onions are being used as diet ingredient in various foods. Pesticides are widely used during the production of foods to prevent plant diseases, the damage by insects and pests, or to control the growth of weeds and fungi. Pesticides are also frequently applied after harvest to prolong storage life and improve quality of onion. Due to strong concerning about pesticide residues in foods in terms of human health issue and export products, the European Union (EU) has been established the maximum residue limit (MRL) of pesticide residues in onion at 0.01 mg kg⁻¹. Not only a stringent sample preparation method, but also a high selective and sensitive determination method becomes necessary in order to meet all regulatory requirements. Gel permeation chroma-
sensitive determination method becomes necessary in order to meet all regulatory requirements. Gel permeation chromatography (GPC) (Ueno et al. 2003), microwave-assisted heating and solid phase extraction (SPE) (Zhang et al. 2008), and matrix solid phase dispersion (MSPD) (Rodrigues et al. 2010), have been used for extracting many pesticides from onion matrices. However, these methods are time and labor consuming, and require a lot of disposable materials (solvent, SPE materials, etc.), which reduce laboratory productivity. Original QuEChERS (quick, easy, cheap, effective, rugged and safe) (Anastassiades et al. 2003), and two versions of buffered-QuEChERS (Lehotay 2007 and Payá et al. 2007), sample preparation approaches have been introduced for the analysis of pesticide residues in fruits and vegetables and further extended in other foods. Theses approaches have been used and modified in many laboratories to provide high-quality of results, save time, cost, and labor, and other beneficial features.

Onion is rich of sulfur compounds and other sulfur derivatives, which are the most difficult matrix interferences. The use of GC-FPD (Ueno et al. 2003), GC-ECD and GC–MS (Zhang et al. 2008), may not selective enough for trace level of residues in this matrix. GC–MS/MS (Walorczyk. 2007), is a powerful tool for analysis of various pesticides at low level. Triple quadrupole mass spectrometry allows for operating in multiple reaction monitoring (MRM) mode, resulting in reduction/elimination of matrix interferences that limit the accuracy and detection limit of the method, especially in complicated matrices.

The aim of this study was to devise and validate a simple and efficient method for the analysis of 170 pesticides in onion extracts. These pesticides were commonly found in routine analysis and were chosen from the list of controlled pesticides. The acetate-buffered QuEChERS method was modified to efficiently remove sulfur interferences and to obtain acceptable analytical results for the majority of analytes in the method validation. GC–triple quadrupole mass spectrometry conditions were optimized to accommodate a variety of pesticides and provide reliable quantitation and identification results.

Materials and Methods

Chemicals and Reagents

All pesticide standards (45 organophosphorous, 45 organochlorines, 7 pyrethroids, and 73 other pesticides (purity ≥95%)) were obtained from Dr. Ehrenstorfer GmbH (Augsburgm, Germany). Acetonitrile (MeCN), acetone, and methanol (MeOH) were pesticide grade purchased from Kanto chemical (Tokyo, Japan). Toluene was from Fisher Scientific (Loughborrough, UK). Glacial acetic acid (HOAc) was analytical grade obtained from J.T. Baker (Philipsburg, NJ, USA). Primary secondary amine (PSA) sorbent was supplied by Varian (Harbor City, CA). Alumina neutral (Al-N) was purchased from Supelco (Bellefonte, PA, USA). Anhydrous magnesium sulphate (anh. MgSO₄) and sodium acetate (NaOAc) were obtained from Sigma-Aldrich (Madrid, Spain).

Standard Solution Preparation

Individual stock solutions of pesticides at about 1,000 mg kg⁻¹ were prepared in appropriated solvents (MeOH, MeCN, or acetone) according to their solubility. A mixture of working standard solution at about 10 mg kg⁻¹ of each pesticide was prepared by diluting the stock solutions with MeCN. All standard solutions were kept in a freezer at -20°C.

GC–MS/MS Conditions

GC–MS/MS system was operated using an Agilent 7890 GC instrument, which was equipped with a multimode inlet (MMI), in combination with a 7000B triple quadrupole mass spectrometer. Mass Hunter Workstation software was used for instrument control and data acquisition/processing.

Injection was performed by 7693 automatic liquid sampler (ALS) with a programmable temperature vaporizer
(PTV) inlet mode. Injection volume was 5 µL into sintered glass liner which was inside of the standard 4 mm i.d. liner. The PTV conditions involved solvent vent mode was initiated at 80°C for 1.1 min (vent time) at split flow 100 mL/min and then ramped to 300°C at 700°C/min (held for the entire GC run). The analytes were separated on a J&W DB 5MS 30 m × 0.25 mm i.d., 0.25µm film thickness. High purity helium was used as carrier gas at 1.0 mL/min constant column flow rate. The oven temperature was started after 1.5 min of solvent vent period and programmed as follows: 70°C (held for 2 min), ramped to 180°C at 30°C/min, ramp to 260°C at 5°C/min, ramped to 280°C at 10°C/min, and ramped to 300°C at 3°C/min (held for 3 min).

The transfer line and ion source temperature were set at 280°C and 300°C, respectively. The solvent delay was 4.5 min. The electron ionization energy was -70 eV. The triple quadrupole mass analyzer was operated in MRM mode. Two MRM transitions of each analyte were monitored with optimal collision energy ranging from 2-40 V and dwell time in the range of 10-15 ms for each transition. The MS acquisition was divided into 19 time segments.

Sample Preparation Method
A 10 g homogenized onion sample was weighed into 50 mL polypropylene centrifuge tube. A 10 mL of 0.5% HOAc in MeCN was added and vortexed for 1 min. A preweighed mixture of 4 g anh. MgSO₄ and 1 g NaOAc was added to the tube and then shaken vigorously by hand (or vortexed) for 1 min to prevent the agglomeration of powders. The extract was centrifuged for 2 min at 3,500 rpm. A 1 mL of MeCN extract (upper layer) was transferred to 2 mL minicentrifuge tube containing 150 mg anh. MgSO₄, 50 mg PSA, and 50 mg Al-N. The tube was shaken (or vortexed) for 30 s and centrifuged for 5 min at 12,000 rpm. The extract was carefully transferred to autosampler vial for GC–MS/MS analysis.

Method Validation
In this study, we performed validation experiments in accordance with the EU guidelines for method validation (SANCO/2009/10684). Onion samples were purchased from an organic food store. The onion samples were extracted and checked to ensure samples were free of analytes using GC–MS/MS method. The well homogenized onion blank sample was kept at -20°C prior to the analysis.

For standard calibrations, mixture of standard solutions was spiked at 0.03, 0.08, 0.13, 0.18, and 0.23 mg kg⁻¹ in onion blank extracts. For recovery study, blank samples were fortified with spiking standard solution at 3 concentration levels (0.05, 0.10, 0.20 mg kg⁻¹) in 5 replicates at each level. For analytical limits of the method, limit of detection (LOD) and limit of quantitation (LOQ) of each analyte were estimated from spiked blank onion extracts and measured the signal-to-noise (S/N) ratios at 3 and 10, respectively.

Results and Discussion
GC–MS/MS Method Development and Optimization
For the PTV injection part, the solvent venting conditions (initial inlet temperature, vent time, and split flow) were carefully optimized to sufficiently eliminate large amount of MeCN solvent without losing early eluting compounds and minimizing amount of MeCN entering to the column. Analyte transfer conditions (inlet temperature programming rate, final inlet temperature, and column flow) were optimized to ensure that all analytes quantitatively transferred to the column, especially the late eluting compounds. GC oven temperature was also optimized to obtain maximum signal intensities and acceptable total run time.

For the MS/MS part, many parameters were optimized in order to achieve the
best response of each analyte. After obtaining the full scan spectra ($m/z$ 50-500), the precursor ion of each analyte, which provided high selectivity and less interference, was chosen. The collision energy (CE) 2-50 V was subsequently applied and optimized to the precursor ion to generate MS/MS product ions. Precursor ions fragmenting into two product ions at specific collision energy values were defined for each pesticide in order to provide the best sensitive and less interference in the MRM method. The most abundance product ion was used for quantitation and another ion was used for identification purpose.

The instrument software was used to set up retention time and collection time segments for each transition, and automatically calculated the optimal dwell time for each transition. Two MRM transitions of each analyte (340 transitions for all analytes) were used with dwell time in the range of 10-15 ms for each transition. The MS acquisition was divided into 19 time windows to provide maximum number of data points across peaks, good chromatographic peak shape, and improve sensitivity and selectivity of analytes, as shown in Figure 1. The total run time was 33.33 min, including ≈6 min post run. The last eluting analyte was azoxystrobin at 27.31 min.

![Figure 1](image)

**Figure 1** Total ion chromatogram ($m/z$ 50-500) of 170 pesticides at 0.01 mg kg$^{-1}$ spiked in onion blank extract.

**Sample Preparation Method Development and Optimization**

Due to the different in chemical property of analytes and our attempt to develop a fast and efficient sample preparation method for a number of pesticides, the buffered AOAC QuEChERS method was used as a template in this study. The addition of HOAc helps to stabilize some difficult pesticides those are sensitive to acidic or basic condition. The modification involved optimization of %HOAc in MeCN and type and amount of additional sorbents in the d-SPE cleanup.

We varied the %HOAc in MeCN in the range of 0.1-1%, and used 150 mg anh. MgSO$_4$ + 50 mg PSA in the d-SPE step. At 0.5% HOAc in MeCN, most analytes gave acceptable recoveries up to 120%. However, some analytes still showed low recoveries and strong co-eluting interferences (mainly carbohydrate, lipids, fibers, vitamins, and various minerals) at this condition. To address this problem, we also tried to add C$_{18}$ and Al-N in the d-SPE cleanup. Al-N is non-polar sorbent and has a strong interaction with vitamins, glycosides, plant sterols, and hydrophobic co-extracted compounds in onion matrix. By varying amount of additional sorbents, the use of 50 mg Al-N in the d-SPE provided cleaner extract comparing with 50 mg C$_{18}$ as shown in Figure 2. Therefore, we chose 150 mg anh. MgSO$_4$ + 50 mg PSA + 50 mg Al-N for cleanup step in this study to obtain overall recoveries (≥70%) for the majority of the analytes with minimizing in matrix interferences.

**Method Validation**

**Linearity and Matrix Effects**

Linearity of the method was demonstrated using matrix-matched calibration standards. The calibration curves were prepared at 10-concentration points (3 replicates at each point) in the range of 0.01-0.27 mg kg$^{-1}$. By plotting the peak area of analytes against the analyte concentrations, good linearity was found with linear
regression coefficient $R^2$ greater than 0.99 for most analytes.

In GC analysis, signal enhancements are normally occurred when matrix components fill the active sites in the injection port, column, and MS source instead of analytes. Matrix effects relate to the concentration and co-extracted components, and cannot be measured precisely. To access this effect, the slopes of calibration curves obtained from solvent-based standards and matrix-matched standards were compared. All analytes showed signal enhancements ranging from 1-75%. For more accurate results, we decided to use matrix-matched calibrations for quantitation throughout the validation experiments in this study.

**Figure 2** Overlaid total ion chromatogram (m/z 50-500) of onion blank extracts using d-SPE cleanup: (1) 150 mg anh.MgSO$_4$ + 50 mg PSA + 50 mg C$_{18}$ and (2) 150 mg anh.MgSO$_4$ + 50 mg PSA + 50 mg Al-N.

**Figure 3** Linearity in the range of 0.01-0.27 mg kg$^{-1}$ and signal enhancement of flusilazole observed in spiked onion extract.

**Accuracy and Precision**

The accuracy and precision of the method were demonstrated in term of recovery and repeatability (intra-day precision) of each analyte in this study. We performed the analyses at 3 spiking levels (0.05, 0.10, and 0.20 mg kg$^{-1}$) in 5 replicates at each level. Figure 4 shows distribution of the recoveries and relative standard deviations (RSDs) for the 170 tested pesticides in onion extracts. The vast majority of pesticides gave good recoveries in the range of 70-120% for 95% of the analytes with less than 20% RSDs in all cases. The obtained values were in an agreement of the EU requirement (SANCO/2009/10684), indicating the reliability of the proposed method.

**Figure 4** Distribution of recoveries (a) and RSDs (b) obtained from the method validation for the 170 pesticides at 0.05, 0.10, and 0.20 mg kg$^{-1}$ in onion.
Limit of Detection and Limit of Quantitation

LODs and LOQs were estimated by injecting spiked blank onion extracts and calculating the concentration of each analyte which provided signal (S/N) equal to 3 and 10 times above the background noises, respectively. In this study, we chose the transition ion of each analyte which showed the greatest signal intensity and less of matrix interferences for the calculation. The calculated LODs were 0.001-0.003 mg kg\(^{-1}\) and LOQs ranged from 0.003-0.009 mg kg\(^{-1}\) for all analytes. Thus, these values were well below the MRLs regulated by the EU for pesticide residues in onion.

Application in Real Samples

In order to access the efficiency of the method, we also tested the proposed method for multiresidue analysis of 170 pesticides in 40 real onion samples obtained from the export companies in Thailand. Matrix-matched calibrations were used for quantitation. Acceptable retention time windows and specific ion ratios were also set up for identification criteria of each analytes.

From the results, 3 pesticides were detected in 4 onions from all tested samples as summarized in Table 1. These compounds met all identification criteria used in this study. Figure 5 shows the signal of flusilazole found in tested onion at 0.114 mg kg\(^{-1}\).

<table>
<thead>
<tr>
<th>Code</th>
<th>Pesticide</th>
<th>Class</th>
<th>Concentration (mg kg(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-1</td>
<td>Flusilazole</td>
<td>Fungicide</td>
<td>0.188</td>
</tr>
<tr>
<td>O-7</td>
<td>Flusilazole</td>
<td>Fungicide</td>
<td>0.114</td>
</tr>
<tr>
<td>O-21</td>
<td>Difenoconazole</td>
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</tr>
<tr>
<td>O-34</td>
<td>Cypermethrin</td>
<td>Pyrethroid</td>
<td>0.070</td>
</tr>
</tbody>
</table>

Conclusions

A simple multiresidue method has been developed and validated for the determination of 170 GC-amenable pesticides in onion. The method is based on the acetate-buffered modified QuEChERS approach and GC–MS/MS.
Acknowledgements

This work was financially supported by Thailand National Research University Project of the Office of the Higher Education Commission (FW648I), TRF Master Research Grants (TRE-MAG-WI535S001), and Center for Petroleum, Petrochemicals, and Advanced Materials at Chulalongkorn University. We express a special acknowledgement to Overseas Merchandise Inspection Co., Ltd. (OMIC) for instrumental support of this project.

References


Effect of Nutrients in Trypticase Soy Agar on Growth Kinetics of *Salmonella* spp. under Micro-Cultivation

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Abstract

Growth kinetics of *Salmonella* spp. colonies was studied to evaluate its growth on different levels of carbon and nitrogen sources in trypticase soy agar (TSA). The logistic model was proposed to describe the Sigmodial mathematic of the area of colony expansion well under limited carbon and nitrogen supplies. The images of colony expansion were digitized and monitored every hour using a reflected light microscope equipped with a 1.5 megapixel CCD camera. The key kinetic attribute (i.e., the maximum specific growth rate) was estimated from these colony images to compare *Salmonella* growth subject to different nutrient conditions. The volume of each inoculum in this micro-cultivation scheme was limited to 5 µL and the culture was incubated on TSA at 35°C. The carbon and nitrogen sources were varied at 1.0, 1.5, 2.0, 2.5 and 3.0X strength of the original TSA recipe. The original composition of the carbon and nitrogen sources in the TSA medium did not produce the highest *Salmonella* growth in the agar plate culture. The nitrogen concentrations between 2.5- and 3.0-fold strength, however, were able to accelerate colony growth rate and enlarge the colony size. Presumably, the higher concentration of nitrogen made the colony growth more susceptible to carbon source alteration. At the 3.0-fold nitrogen level, the optimal carbon concentration at 1.5-fold strength resulted in the fastest growth rate of *Salmonella* colony having the maximum specific growth rate up to 0.305 hr⁻¹.

**Keywords:** growth kinetic, *Salmonella* growth, sigmoideal growth model, specific growth rate, trypticase soy agar

Introduction

*Salmonella* spp. is widely regarded as one of the most lethal pathogens and can inflict serious heath complication (e.g., gastroenteritis), usually caused by consuming contaminated food (Tirado and Schmidt, 2001; Favrin et al., 2003; Borowsky, 2007). There have been countless product recalls and incidents of foodborne outbreaks associated with *Salmonella* contamination (Andrews and Hammack, 1998; Anon, 2009; Harris, 2009). Each incident imposed incalculable financial burdens when combining the social and economic impacts.

Many standards and guidelines (e.g., Bacteriological Analytical Manual) strictly require traditional cell culture to detect foodborne pathogens in industrial food samples (Andrews and Hammack, 1998). The routine method for detecting *Salmonella* spp. involves the use of a non-selective pre-enrichment, selective enrichment and subsequent culturing on selective media. The identification is based on biochemical tests followed by serotyping (Popoff and Le Minor, 1997). These traditional culture techniques are often found impractical, labor-intensive and time-consuming for industrial applications. It is necessary to search for an efficient way to promote growth and accelerate the detection...
process of this critical food-borne pathogen. Bacteriological Analytical Manual (BAM) frequently recommends utilizing the Trypticase Soy Agar (TSA) medium to facilitate the cell repair for better growth of the few stressed Salmonella that may contaminate food samples (Hayashi and Yamasaki, 1998). The medium contains enzymatic digests of casein and soybean meal, which provides amino acids and other nitrogenous substances making it a nutritious medium for a variety of organisms. The original recipe of TSA contains the dextrose and tryptone component, which are the carbon (C) and nitrogen (N) sources to support the growth of Salmonella. (Rall et al., 2005)

Many recent research and publications suggested that using multiple enrichment procedures for each sample can increase the isolation rate of Salmonella in foods. The experiments were relying on improving growth parameters. Razvi et al., (1999) suggested that the increase of the complex nitrogen components can significantly increase biomass concentration and yield coefficient, leading to a high cell density culture in liquid medium. Barnes (1964) reported that glucose as energy source and metabolic precursor is essential for microorganisms. It can considerably impact bacteria size, growth and cellular energy quick conversion.

In this work, Salmonella spp. was selected as a model foodborne pathogen to study the growth kinetics of colony expansion on Trypticase Soy Agar (TSA). The effect of carbon and nitrogen sources was investigated to optimize colony growth and fasten the analytical time to detect surface colonies.

**Materials and Methods**

**Micro-cultivation of Salmonella**

Micro-cultivation in 96-well micro titter plates was proposed to carry out the Salmonella colony growth using TSA medium (Figure 1). Micro colony images were monitored and captured digitally to demonstrate the concept of micro-colony culture and enumeration for industrial practice. Figure 1 shows the developed reflected light microscope equipped with a CCD camera and an X-Y linear stage. The microscope prototype was utilized to collect digitized image of colonies formed on the surface of the TSA plates. Together with an auto counting computer software, this micro-cultivation enabled the reduction of detection time to only 18 hours. The concept of reaction miniaturization was also explored to not only enhance the cultivation efficiency but also reduce the sample and medium cost per analysis (Kim and Fung, 2005; Saeaung and Thipayarat, 2010).

![Figure 1: Photographs and schematic pictures of the MIC equipments used to perform miniaturized micro-culture of Salmonella and enumerate colony detection.](image)

**Cell Culture Preparation**

Frozen stock of Salmonella spp. strain DMST 5880 (From Department of Medical Science, Thailand) was thawed at 37°C for 1 h prior to usage. 100 µl of culture was recovered in 100 ml of Trypticase Soy Broth (TSB; Difco, USA) for 6 hr at 37°C to reach exponential growth phase. Recovered culture was harvested by centrifugation at 3,600 rpm for 20 min at 25°C. Cell pellet was resuspended with 0.9% NaCl solution. Salmonella spp. suspension was serially dilutes to receive a desirable initial cell number at $10^4$ CFU/ml. Initial cell number of each lot of recovered Salmonella inoculum had been determined. Enumeration method followed the Standard Plate Count (SPC)
using spread plate technique (Juneja et al., 2009).

**Nutrient Optimization Experiment**

Each micro well contains 150 µL of Trypticase Soy Agar (TSA). To study the effect of nutrient to the growth of bacteria, different concentrations of tryptone and dextrose were added to agar as nitrogen (N) and carbon (C) sources of bacteria, respectively. C and N sources were prepared at 1.0, 1.5, 2.0, 2.5 and 3.0X strength of the original TSA recipe. The modified TSA was solidified in the microtiter plate format. Inoculum volume at 5 µL was carefully placed onto the surface in each TSA media and the cultivation were incubated at 35 °C. During the colony growth, samples were collected periodically to determine the colony area occupied on the agar surface. Duplication of each experimental treatment was performed.

**Growth Characteristic Determination Using Logistic Model**

Mathematical models have often been used as a tool to describe and predict the growth of microorganisms. The logistic models were successfully implemented on many biological systems in batch-type cultivation (Brewster, 2003; Juneja and Marks, 2006). The key growth characteristics, such as maximum specific growth rate (µ_max), can be evaluated as a function of time using curve-fitting approach (McKellar and Lu, 2004; Gerwen and Zwietering, 2007). Colony area growth data of *Salmonella* were simulated to estimate the µ_max values. Growth curves were collected and curve fitting was applied as in Equation 1.

\[
y = y_0 + \frac{s}{1-e^{-\frac{x-x_0}{b}}}
\]

where
- \( y_0 \) is the amount of initial inoculation of *Salmonella*
- \( a \) is the maximal value of *Salmonella* growth (pixels)
- \( x_0 \) is the first derivative maximum of the function (h)
- \( b \) is the slope of curve time
- \( \mu_{\text{max}} \) is the maximum specific colony growth rate, \( \frac{1}{h} \)

A 2D contour plot was used to interpret the performance of different TSA recipes to determine the optimal compositions in maximizing the specific growth rate.

**Statistical Analysis**

The designed experiment was completely randomized. The data were investigated using the analysis of variance (ANOVA). To observed difference between factor levels, Duncan’s multiple comparisons were selected. Mean values were considered at 95% confidential interval (α=0.05). All pair wise were compared at significant level P<0.05.

**Results and Discussion**

By varying the carbon concentrations at 1.0, 1.5, 2.0, 2.5 and 3.0-fold and keeping the other ingredients unaltered, the growth profiles of *Salmonella* colonies only changed slightly under different carbon availabilities (Figure 2). There appeared an optimal concentration of carbon source at 2.0-fold strength that seemed to produce the fastest colony expansion. The similar growth profiles suggested that the carbon source might not be the limiting substrate determining the rate of colony expansion. Neither was oxygen dissolusion nor incubation conditions. Since the scattering nature of colonies on micro-cultivation allowed growing colonies to spread evenly on the agar surface (Amrane et al., 1999b). During incubation, the
temperature was maintained at its optimum (approximately 35°C) as suggested by most standards and handbooks (Andrews and Hammack, 1998).

Noticeably, the colony area expansion was rather sluggish in the initial period of incubation (approximately first 10 hours) and the final colony areas were varied between 23,822.33 to 22,319.00 pixels. Once the cells were well adjusted their metabolisms to assimilate the TSA medium, the distinct exponential growth can be observed and this acceleration of colony expansion was described by the $\mu_{\text{max}}$ values from the logistic model. The steeper the growth profiles and the faster the colony expanded were reflected by the higher values of $\mu_{\text{max}}$.

Higher nitrogen concentrations together with high carbon concentration produced a profound effect on colony expansion and suppressed the final colony sizes. Perhaps, high osmotic pressure exerting on the agar medium restricted the availability of free water for Salmonella cell growth. (Payne et al., 2006; Baron et al., 2009) The improvement of Salmonella colony growth on TSA medium between 1 and 1.5-fold carbon concentrations was observed as the nitrogen concentration increased (Figure 4, 5 and 6). As shown in Figure 3, the differences of growth profiles as a result of different carbon concentrations were more pronounced at higher nitrogen concentrations in the TSA.

![Figure 3](image-url)  
**Figure 3** Growth profiles of Salmonella spp. at 1.5 – fold strength of regular nitrogen concentration and various the carbon concentrations (i.e., 1.0, 1.5, 2.0, 2.5 and 3.0X strength) applying the initial cell loading at $10^8$ CFU/ml.

Noticeably, the colony area expansion when the nitrogen concentration was increased from 1 to 1.5-fold. As opposed to the previous experiment, the growth profiles of Salmonella spp. were more spread apart showing an optimal carbon concentration at 1.5-fold resulting in the steepest slope ($\mu_{\text{max}} = 0.262 \, \text{h}^{-1}$) and highest colony area expansion (20,457.33 pixels). However, higher carbon concentration exceeding 1.5-fold produced a negative effect on the growth profiles. Not only the rates of colony growth were deteriorated but also the final colony sizes were diminished.

![Figure 4](image-url)  
**Figure 4** Growth profiles of Salmonella spp. at 2.0 – fold strength of regular nitrogen concentration and various the carbon concentration at (i.e., 1.0, 1.5, 2.0, 2.5 and 3.0X strength) applying the initial cell loading at $10^8$ CFU/ml.

The same results occurred at higher nitrogen concentrations as well. The results of the variation of carbon and nitrogen sources in the TSA suggested that the nitrogen supply in the original TSA was limited. The increase of nitrogen availability was able to greatly enhance growth kinetics and final colony area. However, the original TSA recipe already has high concentration of salt complex and substrates (especially, dextrose) such that it exerts hypertonic environment for Salmonella cells to grow.
Increase carbon and nitrogen concentration substantially decreased \( a_w \) of medium and increased bacterial plasmolysis, which is equivalent with the hypertonicity of the suspending medium leading to retard growth of *Salmonella* spp. (Doyle, 1989; Jay, 2000; Doyle, 2001). The increase of carbon source, therefore, produced a significant negative effect on both growth rate and final colony size.

In Figure 5 and 6, the largest achievable size was increased to 32,723.33 and 37,020.67 respectively. As always the case, the availability of the limiting substrate governs the overall growth kinetics of microorganism (Aldarf et al., 2002; Shuler and Kargi, 2002). The modified medium containing excess nitrogen supplies providing a wide array of amino acids and other nitrogenous substances making it highly nutritious medium for *Salmonella*. This result was corresponding to Touratierl et al., (1999) which studied the effect of initial controlled C:N ratio and concentration of organic substrate on bacterial growth. They revealed that C:N ratio of the substrate could be a major determinant of bacterial growth. Other physicochemical factors can also affect the growth of microorganism to some degree such as water, pH, temperature, etc. (Eddleman, 1999).

The logistic model (Eq. 1) was applied to extract useful kinetics information from all previous growth profiles. The kinetic parameters were summarized in Table 1. The high correlation coefficient (higher than 0.97 in all cases) reflected good agreement between the data and the model prediction. The value of \( x_0 \) indirectly describes how fast the colony grows to reach its maximum area.

In Table 1, treatments with lower carbon concentration (between 1.0 and 1.5-fold) and higher nitrogen content (between 2.0 and 3.0-fold) generated the combination that returned the highest colony expansion rate and size. As mentioned earlier, the nitrogen supply in the main compositions of TSA may be inadequate. The increase of nitrogen content can strengthen not only the \( \mu_{\text{max}} \) but also the final colony area at lower carbon concentrations (Table 1). The largest colony size at 37,020.67 pixels was obtained by modifying the original TSA to 1.5-fold carbon supplement and 3-fold additional nitrogen. It was hypothesized that high nitrogen concentration promoted the amino content and vitamin level in the medium (Yokota et al., 1994b) and led to accelerate colony growth rate and enlarge colony size.

However, the increase of both nitrogen and carbon supplies simultaneously created high tonicity and decreased \( a_w \). Doyle (1989) reported that *Salmonella* spp. cultivated at \( a_w \) lower than 0.99 increased the lag period and decreased the total cell yield. Slower growth...
kinetics and smaller colony sizes were observed in treatments with hypertonicity.

The MIC protocol was able to accelerate colony detection using a digitized microcopy. This technique is useful to observe the colony and detect the development of color attributes at early stage. The MIC technique also reduced the use of expensive medium and miniaturized the reaction to a small volume and size. This miniaturization of the MIC protocol presented both advantages and disadvantages to industrial applications.

The obvious advantages were to the costing and throughput of the miniaturization. However, the intrinsic negative side of reaction miniaturization was the smaller inoculum volume and higher restriction to detect minute levels of Salmonella contamination.

A previous study by Saeaung et al., (2010) reported the same limitation in studying growth kinetics of E. coli on Lactose Broth Agar using same technique. The authors also utilized sigmoid equation model to evaluate the specific growth rate comparing E. coli growth on single and double strength concentration of the LB agar and optimized the medium recipe. The result of colony expansion showed that at low agar concentration the treatments using double strength medium concentration achieved significantly faster colony expansion than that using normal strength.

In Figure 7 also showed the contour profile of the $\mu_{\text{max}}$ at different combinations of carbon and nitrogen sources (i.e., 1.0, 1.5, 2.0, 2.5 and 3.0X) in the modified TSA recipe. It was found that when the carbon source was higher than 2.0-fold strength, it gave no significant improvement to the value of maximum specific growth rate. On the other hand, when keeping carbon source lower than 2.0-fold strength and varying the nitrogen source, there is an optimum concentration for modified TSA medium. Actually when the carbon source was maintained at 1.5-fold strength and N source was increased to 3.0-fold strength, at this point it gave the highest maximum specific growth rate. This result leads to concept of TSA recipe modification for Salmonella spp. to accelerate its growth on the agar culture.

Table 1 Growth characteristics of Salmonella spp. under micro-cultivation on the TSA medium at various carbon and nitrogen concentration sauces and incubation temperature was at 35 °C.

<table>
<thead>
<tr>
<th>C source</th>
<th>N source</th>
<th>$\mu_{\text{max}}$ (1.0-fold)</th>
<th>$\mu_{\text{max}}$ (2.0-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.0</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>1.5</td>
<td>1.0</td>
<td>0.28</td>
<td>0.32</td>
</tr>
<tr>
<td>2.0</td>
<td>1.0</td>
<td>0.30</td>
<td>0.35</td>
</tr>
<tr>
<td>2.5</td>
<td>1.0</td>
<td>0.32</td>
<td>0.40</td>
</tr>
<tr>
<td>3.0</td>
<td>1.0</td>
<td>0.35</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Table 1. Mean value of duplication of each treatment. Different subscripts in the same column mean that the values are significantly different at 95% confidential level. Duplication of each treatment was performed.

Figure 7. 2D contour plot of specific growth rate of Salmonella spp. by varying C and N source.

The optimal carbon and nitrogen concentrations in Trypticase Soy Agar (TSA) can produce significant impact on the colony growth rate which cell metabolism required both carbon and nitrogen for energy production and for enzyme and nucleic acid synthesis (Shuler and Kargi, 2002).
Conclusions

Growth kinetic of Salmonella spp. on solid substrate cultivation (Trypsicase Soy Agar) subject to different levels of carbon and nitrogen sources was studied. The cultivation volume was reduced to 5 μL to investigate the exploitation of the micro-cultivation strategy. The use of logistic model was utilized to evaluate the specific growth rate comparing Salmonella spp. growth on normal and different strength of carbon and nitrogen concentrations of the TSA agar. The model represents the growth characteristics and the kinetic of colony expansion well. The finding help describe the effect of nutrient sources on Salmonella spp. growth for further optimization of their colony growth on solid substrate.

The original composition of the carbon and nitrogen sources in the TSA medium did not generate the highest Salmonella spp. growth in the agar plate culture. The study of C:N ratio showed that a nitrogen supply was limited in the original recipe. The nitrogen concentration between 2.5- and 3.0-fold strength were able to accelerate colony growth rate and enlarge colony size while keeping the carbon concentration low (1-1.5 fold strength). The substantial increase of both carbon and nitrogen supplies rather produced negative effect on Salmonella growth. Perhaps, hypertonic environment created was responsible for poor growth performance as a result of substrate toxicity.

Acknowledgments

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References


Aldarf, M., Amrane, A. and Y. Prigent. 2002. Carbon and on spp subject to different levels of carbon cultivation strategy. The use of logistic investigate the exploitation of the micro-Salmonella growth rate comparing TSA agar. The model represents the growth carbon and nitrogen concentrations of the spp. growth for further optimization of their Window I) and industrial RGJ grant.


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Assessing Awareness on Food Quality and Safety among Food Small and Medium-Size Enterprises in Thailand

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Abstract

Key success for food safety and quality management in food supply chain depends on the awareness of how important food safety and quality is. After such understanding is established, the managerial aspect should be introduced. This research aimed to classify Thai food SMEs using the awareness level on food safety and quality assurance implementation in their production. Based on the level of awareness, the information of business owner profile in each group was identified as well as all relevant needs for food safety and quality management improvement. All information from the survey and analysis was then crystallized for strategic plan on food SMEs training program in Thailand. From questionnaire surveys on 209 food SMEs of fruit/vegetable, seafood, meat and cereal production, the awareness level was classified by K-means clustering analysis. There were ‘High awareness’ and ‘Low awareness’ group. Such two groups had differences in profile of education, business operation model, enterprises’s size and income. Companies based on their product types had no impact on the awareness level. One interesting observation from the awareness assessing, the high awareness group still had unclear understanding on food poisoning from microbiological contamination. For capacity building requests, the high awareness group acquired the knowledge more in marketing oriented, while the low awareness group still needed training on the quality and safety management for food production. Results from this study will be conveyed to further creating a long-term strategic plan for continuously enhancing the Thai food SMEs capacity and developing for innovative training.

Keywords: capacity building, cluster analysis, food safety and quality awareness, food small and medium enterprises.

Introduction

Under concept of food security, small and medium enterprises in food processing are one of important stakeholders in Thai food supply chain. 78.20% of the total employment was employed by SMEs (Office of small and medium enterprises Promotion [OSMEP], 2010). For all food and agricultural business, SMEs accounted for 99.7% (OSMEP, 2010). It also had the influence on the rural development which sought to economic activities in the rural sector.

Nowadays consumers are become increasingly in food safety mind. Food quality and safety is one of the most concerned issues that confront food SMEs in Thailand. According to this fact, the development and improvement of SMEs products quality and safety could be the basic requirement in order to fulfill the customer satisfaction. It had been in line with the study of Tabai and Salay (2003), the consumers tend to substitute that product with another whose quality is not doubt. Similar to the state of Briz and Ward (2009), in which food safety issues have driven consumers to search for safer foods whose qualities and attributes are guaranteed.

Although food safety is of crucial important to the consumer and food industry (Jevšnik et al., 2008), there were still many studies about difficulties in adopting of food quality and
safety system in SMEs, for example, results from Iffour (2003) reported that lack of clear advantages of having the quality system would be one of the mind barriers for SMEs to obtain successful in food safety and quality management. This was in agreement with Baş (2007) who indicated that the lack of understanding was one of the main difficulties to food safety programs implementation including the feeling of potentially insurmountable. Insufficient knowledge of food safety principles and lack of awareness of food safety issues among food workers were the problems that food SMEs were facing on (Yapp and Fairman, 2006; Raspor, 2008). About the unclearly understanding in concept of good practices in food industries was also mentioned by Raspor (2008).

From previous reports, it is clearly to state that the attitude, understanding and awareness among small and medium processors should be assessed before the food quality and safety managerial aspect would be introduced if they would like to maintain their competitiveness in the processed food market.

Materials and Methods

Marketing Survey on Food Product From SMEs

To obtain information on current trend of processed food market from SMEs, marketing survey had been conducted. Retail stores in Bangkok area included supercenter, supermarket, specialty store and convenient store was chosen by purposive sampling method for proceed the marketing survey.

Questionnaire Design

The questionnaires were structured in four sections. First section, SMEs were asked about education, business profile; business operation model, enterprises’s size and income. Questions in section two and three were formatted as tables with a six-point rating scale, in which SMEs were responded to 28 statements for indicating their awareness on food safety and quality and pinpoint the factors affecting the decision of SMEs to adopt their food safety and quality system respectively. The last section is related to capacity building requested from SMEs.

Questionnaire Validation/Reliability

Content validity and wording of the statements were reviewed and revised on comment of the food quality and safety system professional.

The questionnaires were pilot tested by 29 SMEs during August 2010 to confirm clarity, identify response option. Cronbach’s alpha was used to assess the internal consistency of variables or average correlation of items in a survey instrument to gauge its reliability (Reynaldo and Santos, 1999). The results showed the alpha was 0.776, indicating an adequate scale as Gliem et al. (2003) stated the closer Chronbach’s alpha coefficient is to 1.0 the greater the internal consistency of the statements in the scale.

Data Analysis

This paper reported on a quantitative research (n=209) and SPSS version 16.0 statistical program were used for all analysis. We utilized cluster analysis as this technique classifies a large number of observations along multiple variables (Ketchen and Shook, 1996, 453 cited in Lim et al.2006) into two or more mutually groups. Members of the same group share properties in common (Stockburger, 2006). Initially, K-means clustering was conducted to cluster the observations into exactly group number. Rujasiri (2009) found the K-mean clustering analysis was the most effective algorithm although the sample size and number of variables had changed in all dataset. After that, the samples were subjected to cross tabulations, the Chi-square test, T-test and ANOVA to examine the relationship between the variables, profile of each segment.

Results and Discussion

Food Products from SMEs

As shown in Figure 1, nearly half of products from SMEs found were in the fruits and vegetables processed products, followed
by the grain products which include flour mill. Fruit, vegetable and grain were the majority parts of food SMEs in Thailand because such products had a variety of raw material available. Together with several processing, many forms of these raw materials product based available such as fruit chip, infused dried vegetables, fruits pickle.

For other type of raw material like fish, meat or milk, it turned out that many of these various products had great similarities basically from the ingredients used. Viewing from the preservation technology, besides of the traditional processing such as meat floss, fermented sausage, there were also being preserved with high in capital processing such as freezing. Therefore, relatively few number of SMEs found in such product based.

Figure 1 Products based on raw material comparison survey.

Profile of Respondents

Respondents’ characteristics were listed in Table 1. About half of respondents had less education than bachelor’s degree (53.1%). Most of them were small enterprises with less than 15 employees (40.9%).

Food SMEs Quality and Safety Awareness

Two-clusters represent two SMEs group which had different profile, labeled as ‘High awareness’ and ‘Low awareness’ group. 55.02% of respondents were assigned to high awareness group and 44.98% to low awareness group. Majority of sampling in the low awareness segment finished high school or lower. There were cooperative business and small enterprises which had monthly business income less than 30,000 baht. High awareness segment represent the large enterprises that earned more than 50,000 baht per month. Most of them had bachelor’s degree education.

Table 1 Respondents profile (n=209)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Education level</td>
<td></td>
</tr>
<tr>
<td>Less than Bachelor’s degree</td>
<td>53.1</td>
</tr>
<tr>
<td>Bachelor’s degree</td>
<td>30.1</td>
</tr>
<tr>
<td>Higher than Bachelor’s degree</td>
<td>16.7</td>
</tr>
<tr>
<td>Business Operation Model</td>
<td></td>
</tr>
<tr>
<td>Individual</td>
<td>41.5</td>
</tr>
<tr>
<td>Cooperation</td>
<td>58.5</td>
</tr>
<tr>
<td>Enterprises’ Size (Employees)</td>
<td></td>
</tr>
<tr>
<td>Less than 15</td>
<td>40.9</td>
</tr>
<tr>
<td>16-25</td>
<td>24.1</td>
</tr>
<tr>
<td>26-35</td>
<td>5.4</td>
</tr>
<tr>
<td>36-45</td>
<td>4.4</td>
</tr>
<tr>
<td>46-55</td>
<td>1.5</td>
</tr>
<tr>
<td>More than 56</td>
<td>23.6</td>
</tr>
<tr>
<td>Business income (Baht)</td>
<td></td>
</tr>
<tr>
<td>Less than 30,000</td>
<td>42.7</td>
</tr>
<tr>
<td>30,000-50,000</td>
<td>12.5</td>
</tr>
<tr>
<td>More than 50,000</td>
<td>44.8</td>
</tr>
</tbody>
</table>

Karipidis et al. (2009) suggested the characteristics of a company such as the kind of products business produces may affect determining the adoption decision, however, we found the enterprises’ characteristics in aspect of different product type’s producers had no association with the awareness level as presented in table 2.

The 28 statements for assessing awareness of food safety and quality had been categorized into four groups’ issues concerned. First issue that should be focused on was the microbiological concept as both groups obtained such low score. Followed by the understanding of the certified label, important of the process certified system and the misunderstanding on hygiene practice in raw material preparation. The mean awareness scores for each segment are present in figure 2.
**Table 2** Comparison percentage respondents of each segment in personal profile and business profile

<table>
<thead>
<tr>
<th>Profile</th>
<th>Group 1</th>
<th>Group 2</th>
<th>Significant (P-Value)</th>
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<tbody>
<tr>
<td>Education level</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>&lt;Bachelor’s degree</td>
<td>35.9</td>
<td>75.9</td>
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</tr>
<tr>
<td>Bachelor’s degree</td>
<td>38.8</td>
<td>20.7</td>
<td></td>
</tr>
<tr>
<td>&gt;Bachelor’s degree</td>
<td>25.2</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>Business Operation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td></td>
<td></td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Individual</td>
<td>57.3</td>
<td>14.9</td>
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<tr>
<td>Cooperation</td>
<td>42.7</td>
<td>85.1</td>
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<tr>
<td>Enterprises’ Size</td>
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<tr>
<td>(Employees)</td>
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<tr>
<td>Less than 15</td>
<td>31.1</td>
<td>51.7</td>
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</tr>
<tr>
<td>16-25</td>
<td>18.4</td>
<td>33.3</td>
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<td>26-35</td>
<td>5.8</td>
<td>4.6</td>
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<td>36-45</td>
<td>5.8</td>
<td>3.4</td>
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<tr>
<td>46-55</td>
<td>1.0</td>
<td>2.3</td>
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</tr>
<tr>
<td>More than 56</td>
<td>37.9</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>Business income (Baht)</td>
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<td></td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>(Baht)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Less than 30,000</td>
<td>2.9</td>
<td>87.4</td>
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</tr>
<tr>
<td>30,000-50,000</td>
<td>13.6</td>
<td>12.6</td>
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</tr>
<tr>
<td>More than 50,000</td>
<td>83.5</td>
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<tr>
<td>Business product type</td>
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</tr>
<tr>
<td>Fruit/Vegetables</td>
<td>38.0</td>
<td>42.9</td>
<td></td>
</tr>
<tr>
<td>Fish/Seafood</td>
<td>11.3</td>
<td>12.9</td>
<td></td>
</tr>
<tr>
<td>Meat/Poultry</td>
<td>18.3</td>
<td>10.0</td>
<td></td>
</tr>
<tr>
<td>Cereal Production</td>
<td>32.4</td>
<td>34.3</td>
<td></td>
</tr>
</tbody>
</table>

a High awareness group
b Low awareness group

The high awareness segment had average score of 3.87 which more than another segment in every aspect toward food quality and safety such as certified label understanding. However, for the microbiological contamination concept still had unclear understanding even in high awareness group. As both groups got average score less than 3 from 5. This is in agreement with statement from Mortimore (2001), in which the lack of microbiological knowledge was particularly a problem for SMEs.

**Factors Affecting Food Quality and Safety Appliances**

In high awareness group and low awareness group, social responsibility was the main factor for adopting food quality and safety system as mean score 4.45 and 4.21 respectively. Similar study had shown that most of food SMEs was motivated to take action to comply with food safety requirements in order to protect their reputation and their business from potential legal action (Yapp and Fairman, 2006). Market-driven, which can be implied by mean values of establishing business image factor in table 3, and regulation were more likely to be the important factors that affect food quality and safety appliances within high awareness group. It can be explained by the study of Spillan and Parnell (2006) in which it showed that market driven strategies are created when a firm becomes market-oriented. Another report from Fairman and Yapp (2005) state that the compliance in most of food SMEs was driven by legal duty.

The important of product differentiation, budget and social responsibility view point were the top three factors that face low awareness group for adopting food quality and safety system with had no statistical significant different (p>0.05). Moreover, according to the states from International institute for trade and development (2009), states that the access to the formal sector financing and difficult access to credits remains major impediment for many Thai SMEs.

From the other similar studies, Baş et al., (2007) found that the main barriers for food safety in food businesses were negative attitude and lack of knowledge toward food safety programs. Moreover, Yapp and Fairman (2006) also discussed in deeper details that money and time which were commonly cited barriers to food safety and quality compliance may conceal the deep-
rooted issues; lack of trust in food safety legislation, lack of motivation in dealing with food safety legislation and lack of knowledge and understanding.

**Table 3** Factors influencing the appliance of food quality and safety system in SMEs

<table>
<thead>
<tr>
<th>Factors</th>
<th>Enterprises Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1</td>
</tr>
<tr>
<td>Budget</td>
<td>3.82c</td>
</tr>
<tr>
<td>Customers concerned on quality certified products</td>
<td>3.00b</td>
</tr>
<tr>
<td>Sales increasing</td>
<td>2.38a</td>
</tr>
<tr>
<td>Establishing business image</td>
<td>4.20d</td>
</tr>
<tr>
<td>Law/Regulation</td>
<td>4.29d</td>
</tr>
<tr>
<td>Social Responsibility</td>
<td>4.45e</td>
</tr>
<tr>
<td>The differentiation of products</td>
<td>4.33d</td>
</tr>
</tbody>
</table>

Group 1 High awareness group
Group 2 Low awareness group

**Capacity Building Request**

Table 4 summarize the frequency on training programs acquired from respondents, which can be the information to further developing innovative training. It was important support as Karipidis et al. (2009) reported that lack of suitable training programs was one of the discouragements found in the adoption of quality assurance system in small food enterprise.

Results indicated training on market oriented (31.3%) among SMEs who had high awareness in food safety and quality should be conducted. It was difference from low awareness segment that need more on training of the quality and safety food management. This was similar to the state of Baş et al. (2007) in which it reported that training programs, both basic food safety and HACCP to support implementation of prerequisite programs should be introduced. Comparing with the similar study from institute for small and medium enterprises development (n.d.) that reported Thai SMEs need training acquired on financial management, marketing strategy, law/regulation in workshop pattern and focused on case study.

**Table 4** Percentage of respondents on training programs acquired

<table>
<thead>
<tr>
<th>Training Programs</th>
<th>Enterprises group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>1</td>
</tr>
<tr>
<td>Technology of processing</td>
<td>26.3</td>
</tr>
<tr>
<td>Quality and Safety food management</td>
<td>17.2</td>
</tr>
<tr>
<td>Computer Program for data analysis and improved decision</td>
<td>8.1</td>
</tr>
<tr>
<td>Financial management</td>
<td>5.1</td>
</tr>
<tr>
<td>Food marketing</td>
<td>31.3</td>
</tr>
<tr>
<td>Supply Chain management</td>
<td>2.0</td>
</tr>
<tr>
<td>Law/Regulation related to food products</td>
<td>10.1</td>
</tr>
</tbody>
</table>

Group 1 High awareness group
Group 2 Low awareness group

**Conclusions**

The growth in the population and improvements in living standards had resulted in changing food consumption patterns, constant changes in consumer tasted and preferences. The approach to successful food supply chain in safety and quality management would obviously vary by awareness level on food safety and quality assurance implementation in their production, subsequent to the different of business operation model and education level of each entrepreneur. For opting proper innovative training to resolve the problems properly and achieve sustainable growth in SMEs, it is essential that Thai SMEs should improve themselves by starting with having clear perception on their own fundamental problems. As the results indicated, acquirement of capacity building should be imposed differently depend on the awareness level. Initially, microorganism concept should be the focus in order to improve the fundamental concept of food quality and safety understanding for all food SMEs, followed by the knowledge in the certified label and the process certified system.

We found factor affecting low awareness group food quality and safety appliances was firm’s productive capacity, current products rather than market forces like high awareness group.
Interestingly, subsequent to identify these problems, low awareness SMEs perceive such problem as their acquisitions to capacity building in the aspects of quality and safety food management, technology of processing to improve their products and financial management. Noticeably, once the food product quality and safety including the consistency of goods had been ensured, SMEs would like knowledge on the marketing-oriented consequently.

This would be very useful information for the facilitators like government sector to clearly understand the difficulty of SMEs and to perceive which groups should be prioritized for the supporting scheme (International institute for trade and development, 2009).

Acknowledgments

This work would not have been possible without the financial support of the FAO. The authors would like to thank the Bank for Agriculture and Agricultural Cooperatives Research Center for their support and food SMEs who gave their times to participate within the research.

References


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Use of Viscozyme L for Pre-treatment of Coconut Prior to Extraction by Screw Press

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Abstract

The effect of moisture content of coconut crushes and enzyme treatment prior to extraction by screw press on the yield and qualities of virgin coconut oil was investigated in this study. Results showed that the screw pressing efficiency was higher for coconut crushes with low moisture content. The highest yield of extracted oil (14%) was obtained when using coconut crushes containing moisture content of 15%. The optimum concentration of Viscozyme L for pretreatment before oil compression was 0.4% (w/w). The lightness (L*), redness (a*), yellowness (b*), specific gravity, refractive index, moisture content, peroxide value, acid value, iodine number and saponification number of the oil compressed from grated coconut with and without enzymatic pre-treatment were not significantly different (p > 0.05). However the viscosity of oil from enzymatic pretreated coconut meat (45.66 ± 0.10 cP) was significantly lower (p ≤ 0.05) than those from non-pretreated coconut meat (47.12 ± 0.05 cP). The changes in quality of the oil kept in glass or PET bottles during storage at room temperature (30 ± 2°C) for 28 days were studied. It was found that moisture content of oil did not change (p > 0.05), while L*, peroxide value, acid value and sensory quality in terms of cleanness increased but a*, b* and sensorial quality in terms of coconut odour decreased during storage. An accelerated shelf life study of coconut oil at 55°C and 65°C indicated that the predicted shelf life of coconut oil stored at 30°C was 57 days.

Keywords: enzymatic extraction, physical and chemical characteristics, virgin coconut oil

Introduction

Coconut is one of the most important vegetable oil crops in the world. It is cultivated throughout the tropics including Thailand. Generally, coconut oil can be obtained by compressing dry coconut (Copra). During the compression process, pressure and heat create the dark brown color and high free fatty acid content of the oil, which further require purification at great cost. Various methods have been developed to extract coconut oil of high quality, for example, the cold pressed method. Fresh coconut crush dried in a short period of time at temperature of 40-50 °C is used to extract oil by the cold compressions, either through hydraulic press or screw press. The critical factors such as the coconut crush moisture content and the drying temperature before compression could be considered to obtain a natural oil flavor as well as the valuable compounds remaining in the oil. It has been reported that screw press extraction of coconut improved the amount and qualities of extracted oil (Mpagalile et al., 2007). Although, cold pressing oil extraction is a good alternative in comparison to the traditional high-temperature extraction process, it affords only low-yields. Enzymatic incorporation
could improve the oil extraction yield as well as the qualities (Che Man et al., 1996). Enzymes will degrade the oilseed cell wall components, facilitating oil release. Enzyme treatment was reported to enhance the oil extractability of raw material. Sunflower oil extraction by using enzyme assisted process resulted in oil yield improvement and meal fiber reduction (Zuniga et al., 1995). A study of coconut oil extraction based on the enzymatic action of mixed enzymes such as cellulase, α-amylase, polygalacturonase and protease obtained a yield of about 60-74% coconut oil (McGlone et al., 1986; Che Man et al., 1996). In addition, an enzyme mixture of Viscozyme L (cellulase, hemicellulase, arabanase, xylanase and β-glucanase) at 0.6% concentration and neuraste 0.3% concentration was tested by Beatriz et al. (2003) for obtaining about 92% yield of coconut oil. As coconut tissue structure consists of polysaccharides such as cellulose, hemicelloses, arabinogalactans and pectin (Rosenthal et al., 1996), the use of enzyme hydrolyzing coconut tissue to breakdown the polysaccharides is desirable.

The present study was undertaken with the primary objective to investigate the effect of coconut crush moisture content on the yield of oil extracted by screw press. A second objective was to determine the effect of Viscozyme L, a multi-enzyme complex containing cellulase, hemicellulase, arabanase, xylanase and β-glucanase, treatment on the yield of coconut oil. The final objective was to examine the quality change in coconut oils kept in different containers (glass and PET bottles) during storage at room temperature and at the accelerated temperature.

Materials and Methods

Materials

Coconuts harvested at the age of 11-12 months were purchased from a local market (Nong Mon, Chonburi, Thailand). Then, fresh coconuts were crushed and used within 24 hours. Enzyme Viscozyme L (100 FBG / g), an enzyme mixture including cellulase, hemicellulase, arabanase, xylanase and beta-glucanase were purchased from Novozymes (sub-contact with the U & V Holding (Thailand) Co., Ltd., Thailand.

Effect of Coconut Crush Moisture Content on Oil Extraction by Screw Press

The fresh coconut crushes 500 g were oven-dried at 50 °C to adjust moisture contents to 15 ± 0.5, 20 ± 0.5 and 25 ± 0.5 % (w.b.), respectively. The samples were used to extract oil by a screw press and determined the percentage of extracted oil.

\[
\text{Extracted oil (\%)} = \left( \frac{\text{wt of extracted oil} - \text{wt of water in oil}}{\text{wt of coconut}} \right) \times 100
\]

- wt of extracted oil, the total weight of oil extracted by screw pressing (the original weight).
- wt of water in oil, coconut oil was dried in a forced air oven at 105 °C for 24 hr. (to constant mass), cooled in a desiccator and then weighed to obtain the dried oil content.
- The water in oil was obtained from the difference between the original extracted oil weight and the dried oil weight
- The suitable moisture content of coconut crushes that yield the maximum amount of extracted oil was chosen for the experiment in the next step.

Effect of Viscozyme L Concentration on Coconut Oil Extraction by Screw Press

The coconut crushes at moisture content of 50 ± 2 % were mixed with Viscozyme L enzyme at the concentration 0, 0.4, 0.6 and 0.8% ww⁻¹ and incubated at 50 °C for 1 h (Sant’ Anna et al., 2003). They were then dried in a tray dryer at 50 °C to obtain the most suitable moisture content as determined in former experiment and compressed with a screw press.

Physical and Chemical Characterization of Extracted Coconut Oil

The physical and chemical properties of coconut oil obtained by screw pressing with and without enzyme pre-treatment were analysed at the time of extraction and after kept in glass and PET bottles. In each container, 250 ml of oil were filled and kept in the light, room temperature (30 ± 2 °C)
for 28 days and samples were taken to investigate the change during storage at 0, 7, 14, 21 and 28 days. The chemical and physical properties including specific gravity, refractive index, moisture content, peroxide value, acid value, iodine value and saponification number were analysed, in 9 replicate, following the analytical methods of AOAC (1990). Color and viscosity were determined according to the method proposed by Mpagalîle et al (2007), respectively.

**Sensory Evaluation of Extracted Coconut Oil**

Descriptive sensory analysis was conducted to describe the coconut oil samples. A total of 10 trained panelists participated in the tests. The samples were evaluated using paper ballots with 10 cm line scale. Panelists identified appearance and smell of coconut oils and rated the intensity of each oil attribute based on 10 cm line scales.

**Accelerated Shelf Life Evaluation**

Coconut oils obtained by screw pressing with and without enzyme pre-treatment were stored in glass and PET bottles (250 mL/bottle) and kept at 55 and 65°C for 28 days. Peroxide values of samples were determined every 7 days. (The peroxide value is used as an indicator of oil degradation. Peroxide values of fresh oils are less than 10 meq oxygen.kg⁻¹). Shelf life was calculated by the equation (1) and the accelerated shelf life at 30°C by the equation (2).

\[
Q_{10} = \frac{\text{shelf life at } T \degree C}{\text{shelf life (T+10) } \degree C} \quad (1)
\]

\[
Q_{10}^{\Delta T/10} = \frac{\text{shelf life at } T1 \degree C}{\text{shelf life at } T2 \degree C} \quad (2)
\]

By \( \Delta = T2 - T1 \)

\( Q_{10} \) = shelf life of the sample

\( T1 \) = temperature at which to know the shelf life

\( T2 \) = temperature of a known shelf life

**Statistical Analysis**

Data was analysed using SPSS (Version13). Analysis of variance was performed by the ANOVA procedure. Mean values were considered at the significance level of 95% \(( p<0.05)\).

**Results and Discussion**

**Effect of Coconut Crushes Moisture Content on Oil Extraction by Screw Press**

The oils recovered from coconut crushes extracted by screw press as affected by the moisture content are shown in Table 1. It was found that an increase in moisture content reduced the yield of extracted oil due to the water content. About 4 to 14% oil was extracted from coconut crushes depending upon moisture content. At presence of high amount of water a negative effect of the moisture content on the extraction yield was observed. The extracted oil was higher for sample at moisture content of 15%, however this was not significantly different \(( p>0.05)\) with the sample contained moisture content of 20%. The lowest yield was obtained when the coconut crushes had high moisture content (25 %). Although, the moisture content of 15 and 20% did not affect oil recovery, in practice it was easier to compress the coconut crushes at 15%MC than those of 20%MC. At 20%MC, the presence of coconut cream (viscous liquid) impeded the operation of the screw machine. Therefore, the optimum moisture level of 15% for coconut crushes yielded the maximum oil recovery and was selected for using in the next step.

<table>
<thead>
<tr>
<th>Moisture content (%)</th>
<th>Percentage of extracted oil (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 ± 0.5</td>
<td>14.43 ± 0.74</td>
</tr>
<tr>
<td>20 ± 0.5</td>
<td>12.05 ± 3.32</td>
</tr>
<tr>
<td>25 ± 0.5</td>
<td>3.90 ± 0.74</td>
</tr>
</tbody>
</table>

**Table 1** Effect of coconut crushes moisture content on the percentage of oil extracted

\( a,b \) Values with the same superscript in a column do not differ significantly \( ( p<0.05)\).
Effect of Viscozyme L Concentration on Coconut Oil Extraction by Screw Press

Effect of Viscozyme L concentrations on oil extraction are shown in Table 2. All hydrolysis were carried out at 50°C for 1 h at 50% moisture content. Results showed that the yield of extracted oil increased with enzyme concentration. The amounts of extracted oils were not significantly different when using enzyme concentration of 0.4-0.8% (ww⁻¹) (p > 0.05); however the oil recovery obtained by enzymatic pre-treatment was higher than those of non-enzymatic treatment (p ≤ 0.05).

As coconut tissue structure consists of cellulose, hemicelluloses, arabinogalactans and pectin, the carbohydrase enzymes containing in the Viscozyme L were thought to be involved in disintegration of cell wall tissue, facilitating oil extraction. When Viscozyme L at different concentrations were used, high yields were obtained, however the data were not significantly different (p>0.05). For the economic reason, the lowest enzyme concentration was preferable. Therefore, enzyme concentration at 0.4 percent by weight was selected in this study because it costs only 246 Baht per 1 kg of coconut, while concentrations of 0.6 and 0.8 percent by weight of coconut cost up to 352-457 Baht per 1 kg of coconut.

Quality Evaluation of Coconut Oils

The quality of coconut oil obtained by screw pressing with and without enzyme pre-treatment was evaluated and the results are shown in Table 3. The qualities of both coconut oils extracted by different methods were not significantly different (p>0.05) in terms of color, specific gravity, refractive index, moisture, peroxide value, acid value, iodine value and saponification number. It was found that the acid value and peroxide value of both coconut oils were within the set limit of 4 mg g⁻¹ and <10 meq oxygen kg⁻¹, respectively (Thai Community Product Standard, 670/2547). The viscosities of oils were significantly different (p ≤ 0.05). Viscosity of the oil obtained by enzymatic treatment was 45.66 ± 0.10 cP, while that of oil extracted by non-enzymatic treatment was 47.12 ± 0.05 cP. This is because the Viscozyme L is a multi-component carbohydrase and can effectively hydrolyze plant cell wall polysaccharide. Lipase which is located within cell wall then can be released and activated. Lipase activity was activated at a temperature of more than 20 °C and then completely lost their activity at 80 °C. In this work, the process was carried out at 50°C for 1 h which is suitable for lipase to hydrolyze triacylglycerol in coconut oil. Hence, the viscosity of oil decreased (Pahoja and Sethar, 2002).

The effect of storage condition on the quality of oils kept in different packaging were shown in Table 4-6. The moisture content of all coconut oil samples were not different whether they were kept in a glass bottle or a PET bottle during 3 months storage. These results are in agreement with those of Mendez and Falque (2007) which reported the moisture content of virgin olive oil stored in different packages including glass bottle, PET bottle, tin plate and Tetra-brik did not differ during the first 3 months. The viscosities of all oil samples significantly decreased after 14 days of storage (p≤ 0.05). This is because the triacylglycerol in the oil degraded into free fatty acids, diacylglycerol, monoacylglycerol. These molecules have lower molecular weight than triacylglycerol and contributed to the decrease in viscosity of the oil. The brightness color (L*) of oils kept in glass and PET bottles increased after storage for 7 days, after that the color did not change.

Table 2 Effect of Viscozyme L concentration on the percentage of coconut oil extracted

<table>
<thead>
<tr>
<th>Amount of Viscozyme L (w/w)</th>
<th>Percentage of extracted oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.43b ± 0.74</td>
</tr>
<tr>
<td>0.4</td>
<td>16.66b ± 1.19</td>
</tr>
<tr>
<td>0.6</td>
<td>17.65b ± 0.38</td>
</tr>
<tr>
<td>0.8</td>
<td>18.11b ± 0.79</td>
</tr>
</tbody>
</table>

a,b Values with the same superscript in a column do not differ significantly (p<0.05)
Table 3 Physical and chemical properties of coconut oil, treated with Viscozyme L and non-enzymatic treatment

<table>
<thead>
<tr>
<th>Properties</th>
<th>Coconut oil control (without enzyme)</th>
<th>Viscozyme L L*ns</th>
<th>Viscozyme L a*ns</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Density (g/ml)</td>
<td>0.89 ± 0.02</td>
<td>0.93 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Viscosity (cP)</td>
<td>47.12 ± 0.05²</td>
<td>45.66 ± 0.10b</td>
<td></td>
</tr>
<tr>
<td>Refractive index (%)</td>
<td>1.45 ± 0.05</td>
<td>1.45 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>0.18 ± 0.01</td>
<td>0.17 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Peroxide value (meq oxygen/kg)</td>
<td>0.67 ± 0.03</td>
<td>0.59 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Acid value (mg/g)</td>
<td>0.38 ± 0.01</td>
<td>0.38 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Iodine value (mg/g)</td>
<td>8.56 ± 0.07</td>
<td>8.93 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>Saponification number (mg/g)</td>
<td>257 ± 0.05</td>
<td>258 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

² Values with the same superscript in a row do not differ significantly (p<0.05). ns represent the means are not significantly different (p > 0.05).

It was noticed that after storing oils for a while, the sediments at the bottom of containers were observed because the suspended particles and gum in oil precipitated. This is in consistent with reports of Villarino et al. (2007), who reported that centrifugation of coconut oil provided a higher brightness than those not treated with centrifugation. The red (a*) and a yellow (b*) value decreased during the storage. This may because the glass and PET container cannot prevent alpha–tocopherol, the yellow viscous compound, from light and led to the decomposition of the compounds.

Reactions contributing to an increase in peroxide value are auto-oxidation which occurring in the absence of light and photo-oxidation which occurs in the presence of light. An increase in the peroxide value was observed in all samples after 7 days of storage. This is because the oxygen dissolved in the oil contributed the auto-oxidation of oil. Moreover, the presence of light during the storage initiated the photo-oxidation. This result is the same as Sensidoni et al. (1995) and Mendez and Falque (2007). An increase in acid values of both oils in the glass and PET bottles was observed during 28 days of storage. Mendez and Falque (2007) suggested that plastic exhibits a higher tendency for hydrolysis of triglycerides which can be justified by their higher oxygen permeability as compared to glass, while the glass container is sensitive to the action of light. Therefore, oxygen and light in this study would contribute to the oxidation of oils and decomposition of hydroperoxide leading to the formation of carboxylic acids, responsible for an increase in acidity.

The sensory evaluation data obtained from trained panelists are shown in Table 6. As expected, the appearance of the oil samples stored in glass and PET bottle was clear and the brightness increased with the storage time. The results were in accordance with data obtained from instrumental analysis mentioned above. In terms of coconut scent of the oil, it was found that the intensity of the coconut aroma tended to decrease over the time (p≤0.05). This is because oils were oxidized by light and oxygen resulting in the formation of oxidation products or free fatty acids which masked the natural aroma of coconut oil. The intensity of coconut oil aroma decreased while the peroxide value and acid value increased as reported previously.

Accelerated Shelf Life Evaluation

Accelerated shelf life of coconut oil was studied by keeping oil at the temperature of 55°C and 65°C, respectively. The peroxide value was determined every 7 days until the value obtained was more than 10 meq oxygen per kilogram, which is the indicator of oil deterioration. The predicted shelf life of oils kept in different containers is shown in Table 7. Results showed the predicted shelf life of coconut oil obtained by non-enzymatic and enzymatic treatment kept in glass and PET bottle were 57 days when stored at 30°C.
Table 4 Changes in moisture content, viscosity and color of coconut oils obtained by non-enzymatic (control) and enzymatic pre-treatment during storage time in different containers

<table>
<thead>
<tr>
<th>Value</th>
<th>Treatment</th>
<th>Duration time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Moisture content (%)</td>
<td>non-enz</td>
<td>0.18 ± 0.17</td>
</tr>
<tr>
<td>Viscosity (Cp)</td>
<td>/ glass</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>non-enz</td>
<td>0.18 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>enz</td>
<td>0.17 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>0.02 ± 0.00</td>
</tr>
</tbody>
</table>

Table 5 Changes in peroxide value and acid value of coconut oils obtained by non-enzymatic (control) and enzymatic pre-treatment during storage time in different containers.

<table>
<thead>
<tr>
<th>Value</th>
<th>Treatment</th>
<th>Duration time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Peroxide value (mg equivalent/Kg)</td>
<td>non-enz</td>
<td>0.67 ± 1.81</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>0.70 ± 1.78</td>
</tr>
<tr>
<td></td>
<td>non-enz</td>
<td>0.67 ± 1.84</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>0.60 ± 0.29</td>
</tr>
</tbody>
</table>

Table 6 Sensory evaluation of coconut oils obtained by non-enzymatic (control) and enzymatic pre-treatment during storage time in different containers.

<table>
<thead>
<tr>
<th>Value</th>
<th>Treatment</th>
<th>Duration time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Appearance intensity</td>
<td>non-enz</td>
<td>2.81 ± 4.46</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>2.58 ± 4.03</td>
</tr>
<tr>
<td></td>
<td>enz</td>
<td>1.46 ± 4.46</td>
</tr>
<tr>
<td></td>
<td>/ glass</td>
<td>2.77 ± 4.03</td>
</tr>
</tbody>
</table>

Values with the same superscript in a row do not differ significantly (p<0.05). *ns represent the means are not significantly different (p>0.05).
Table 7 The shelf life of coconut oil obtained by non-enzymatic and enzymatic extraction kept in glass and PET bottles at 30 °C for the prediction of accelerated shelf life conditions at 55 and 65°C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-enz / glass</td>
<td>57</td>
</tr>
<tr>
<td>non-enz/ PET</td>
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<td>enzc / glass</td>
<td>57</td>
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<tr>
<td>enz/ PET</td>
<td>57</td>
</tr>
</tbody>
</table>

Conclusions

The initial moisture content of coconut crushes and the enzyme concentration affected the yield of extracted oil. To obtain the maximum oil recovery, the initial moisture content of coconut crushes was 15% and the Viscozyme L concentration was 0.4 percent by weight of coconut oil. The enzymatic treatment improved the recovery of total oil and did not affect the oil quality. However, the qualities of coconut oils obtained by non-enzymatic and enzymatic treatment changed during storage at room temperature (30 ± 2 °C) for 28 days in both glass and PET container. The moisture content was not significantly different during storage, while L*, peroxide value, acid value and sensory quality in term of viscosity and color increased, but a*, b* and sensory quality in term of coconut odor decreased during storage. The predicted shelf life of coconut oil stored at 30 °C was 57 days.

References


This paper was originally presented at the International Conference on Agriculture and Agro-Industry 2010 (ICAA2010), November 19-20, 2010 Mae Fah Luang University, Chiang Rai, Thailand.
Abstract

The study on spoilage bacterial growth (mesophile, psychrophile, Enterobacteriaceae and Pseudomonas spp.) of fresh oysters (Crassostrea belcheri) during storage in ice-bath for 10 days was conducted. The fresh oysters were divided into 2 groups that are natural whole shelled oyster (WS) and open-up, internally cleaned with tap water and re-closed shelled oyster (CS). As a result, the numbers of the mesophile and psychrophile found in the WS samples were higher than those of in the CS ones. Furthermore, the cell numbers of the mesophile, the psychrophile and Pseudomonas spp. of the WS samples increased during storage times. On the contrary, the mesophile and Enterobacteriaceae counts of the CS samples decreased. The pH values of oysters were in the range of 5.85-6.46. The freshness odor score rapidly decreased in the WS as compared with that of the CS. It can be concluded thoroughly cleaning of the oysters could help controlling the numbers of spoilage bacterial growth leading to a better quality oyster.

Keywords: bacterial growth, oyster (Crassostrea belcheri), spoilage bacteria, storage

Introduction

An oyster (Crassostrea belcheri) is the large well-known mollusk that becomes very important seafood for Suratthani Province, Thailand. This is because of its taste, its high nutrition value and can be cooked into various dishes. Hence, it is valuable aquaculture animal that is economically significant. Before transportation, the oysters might be cleaned and kept in a basket at the room temperature which can easily lead to a spoilage stage resulting in the short storage period. In order to extend the freshness, some farmers develop the method of transporting by storing oysters in foam boxes containing sufficient amount of ice flakes. Most previous studies of bacterial quality toward the oyster focused on the food pathogenic bacteria including our previous work that published on 2009 (Heller et al., 1986; MMWR, 1999; Lozano-Leon et al., 2003; FDA, 2005; Sutthirak et al., 2009) while there were no reports on the spoilage bacteria including Pseudomonas spp., Acinetobacter spp. and Flavobacterium spp. during the ice storage of raw oyster. Our work aimed to determine the numbers of spoilage bacteria in oysters (WS and CS) during storage in ice-bath. The findings might be useful for a development method in reducing the bacterial changes in fresh oysters.

Materials and Methods

Samples Preparation

The oysters of a market size (6-7 animals/kg) aging around one year and six months were collected from farms within Bandon Bay area, Suratthani Province during October and December, 2009. They were externally cleaned with fresh water, and the outer shells were scraped with a knife in order to clean the shell.
Storage Conditions
The fresh oysters were divided into 2 groups. Group one was the natural whole shelled oysters (WS). Group two was the opened-up, internally cleaned with tap water and re-closed shelled oysters (CS). They were stored in ice flake with the proportion of oyster: ice flake of 1:2 according to our preliminary data. After that, they were put in ice-bathes. At days 0 (control sample), 2, 4, 6, 8 and 10, the oyster samples were randomly selected for monitoring bacterial changes, pH, and sensory evaluation (freshness odor).

Oyster Qualities Determination

Microbiological Quality Determination
The shells were aseptically removed and the oyster samples (50 g) were mixed with normal saline (450 mL) and homogenized in a stomacher. The final concentration obtained was 0.1 g/ml. Serial dilutions were done in normal saline solution. These dilutions were used to determine the spoilage bacteria. Mesophile and psychophile counts were determined based on the method as described by Hernandez et al. (2009). Briefly, the total number of mesophilic micro-organisms was determined on Plate Count Agar (PCA) following the pour plate method, and incubated at 35°C for 48 h. Psychophile counts were determined on PCA with an incubation temperature of 7-8°C for 10 days, following the pour plate method. The number of Enterobacteriaceae and Pseudomonas spp. were determined based on the method as described by Vaz-Pires et al. (2008). Enterobacteriaceae were counted by spread the samples onto MacConkey agar after incubated at 35°C for 2 days. Pseudomonas spp. was determined by added and spread onto the Pseudomonas agar following by incubated at 20°C for 3 days.

Sample Preparation for pH Analysis
The shells were removed and the fresh meat was brought out. Then, the meat was cut to small pieces and blended using homogenizer. Forty-five milliliters of distilled water were added into 5 g of the homogenized meat. The sample solution was then mixed well prior to pH measurement with a pH meter (AOAC, 1999).

Sample Preparation for Sensory Evaluation
The shells were removed and the fresh meat was brought out. The freshness odor was evaluated using 7-point scoring test by 5-7 trained testers panelist.

Results and Discussion

Enumeration of Mesophile
The mesophile count of oysters during storage period is illustrated in Fig 1. The results showed that the mesophile counts of WS increased throughout the storage period from 4.21 log cfu/g on the day 0 (control) to 5.47 log cfu/g on the day 10. The amount of total viable count was higher than the standard level (<5 log cfu/g) set by Department of Medical Science, Thailand (2010) for the ready to eat seafood products. On the contrary, the mesophile counts of CS tended to decrease from 4.40 log cfu/g on the day 0 to 3.37 log cfu/g on the day 10. This might be due to the preservation as the whole natural oysters (WS) meant being contaminated and at the same time the chilling of ice was unable to get through to the inner of the oyster meat resulting in the increasing of the bacterial count. On the other hand, for shell opening of internal cleanness (CS), the chilling of ice can get through to the meat to retard growth of the bacteria. This was consistent with the research of Chytiri et al. (2004) who stated that mesophile count in ice storage trout increased throughout the preserving duration as bacterial in the boneless fish meat was 7 log cfu/cm² after the tenth day and 6 logcfu/cm² for the whole fish meat on the eighteenth day of the storage. This was also consistent with the research of Papadopoulos et al. (2003) who reported that on the ninth day of ice storage fish, mesophile count was 5.3 log cfu/g for the outside intestines and 7.5 log cfu/cm for the inside intestines.
Enumeration of Psychrophile

It was found that psychrophile count at day 0 of oyster samples, WS and CS, were 3.14 log cfu/g and 2.43 log cfu/g, respectively (Fig. 2). On the tenth day, as the duration of the storage was prolonged, the quantity of psychrophile accordingly increased. This was because the psychrophile bacteria were able to grow well at the low temperature (subzero to 20°C). These results are agreed with the work of Rezaei et al. (2007) who explained that the psychrotroph count in ice storage trout increased according to the storage time to 6.11 log cfu/g on the eighteenth day of the preservation.

Enumeration of Enterobacteriaceae

The Enterobacteriaceae of WS samples was 3.26 log cfu/g on the first day and decreased to 2.15 log cfu/g on the sixth day (Fig. 3). However, the Enterobacteriaceae count increased to 3.41 log cfu/g on the tenth day. This might be due to the adaptation of Enterobacteriaceae group on the refrigeration temperature or psychrophile play the role after prolonged incubation period. Similarly, the Enterobacteriaceae count in CS samples decreased from 3.46 on day 10 to 0.33 log cfu/g on the eighth day before increased to 0.65 log cfu/g on the tenth day. It was discovered that the longer the storage duration the quantity of Enterobacteriaceae tended to decrease. This was because it has the ability to grow in the moderate temperature and reduced the number when the temperature was chiller. This finding was inconsistent with the research of Chytiri et al (2004) stating that Enterobacteriacea count in ice storage trout increased after the eighteenth day of storage to 5.5 log cfu/cm² for the boneless fish meat sample and to 4.2 log cfu/cm² for the whole fish meat.

Enumeration of Pseudomonas spp.

*Pseudomonas* spp. in the WS increased from 3.29 log cfu/g on the first day to 5.49 log cfu/g on the tenth day. In the CS, it decreased from 4.1 log cfu/g on the first day to 2.78 log cfu/g on the eighth day. At day 10, it was 3.55 log cfu/g. The results showed that while the storage time is longer, *Pseudomonas* spp. also increased as the temperature facilitated its growth. These bacteria are usually the main cause of spoilage food being stored at the low temperature. This was agreed with Chytiri et al. (2004). They found that the increasing of *Pseudomonas* spp. count of more than 7 log cfu/cm² in the ice storage trout for the boneless fish meat after the tenth day and 6 log cfu/cm² for the whole fish meat after the eighteenth day.
Changes of pH in the Ice Storage Oysters

The pH values of the WS increased from 6.30 to 6.53 during storage for 6-days, and decreased to 5.85 on the tenth day. On the other hand, the pH value of the CS was 6.39 on the first day. Then, it was 6.29 on the tenth day. The pH value reduced because oysters possess carbohydrate in the form of glycogen both from the autolysis and enzymes produced by bacteria. So, the spoilage was caused from the hydrolysis of carbohydrate substance whereas the pH reduction could be an indicator for a spoilage level.

Changes of Freshness for the Ice Storage Oysters

The freshness odor acceptance for both samples (WS and CS) reduced along with the storage time was prolonged. However, the CS showed odor acceptance higher than that of the WS (data not shown). The reduction of freshness odor might be due to the activity of bacteria which the count increased during prolonged incubation times, and resulted in the reduction of pH.

Conclusions

It can be concluded that the longer storage duration tended to cause the increasing of spoilage bacterial count. The pH value revealed that it reduced during the longer storage time whereas the odor acceptance was also reduced. Nevertheless, the cleaning procedure by opened-up the shell, internally cleaned with tap water and re-closed shelled oysters before the ice storage was a better one when being compared on the aspect of spoilage bacterial and sensory acceptance.

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Water Sorption Isotherm and Thermo-Physical Properties for the Analysis of Natural Rubber Drying

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Abstract

The water sorption isotherm, thermo-physical properties (apparent density, percentage of void and specific heat capacity) and effective diffusion coefficient ($D_{\text{eff}}$) of natural rubbers samples (STR 20 and Skim block rubbers) have been determined and presented in this work. Water sorption isotherms determined in term equilibrium moisture content (EMC) was evaluated by static gravimetric method using saturated salt solutions at surrounding temperatures and relative humidity varying from 45 to 60°C and 10 to 90%, respectively. Apparent density, specific heat capacity and void fraction were evaluated at initial moisture contents ranging from 1.6 to 55.3% dry-basis. With evaluating $D_{\text{eff}}$ values using Fick’s law of diffusion, experiments were carried out at drying temperatures ranging of 100 to 130°C for STR 20 block rubber drying, and drying temperatures in the range of 85 to 115°C for Skim block rubber drying with constant air flow rate of 1.7 m s\textsuperscript{-1}. Six commonly cited EMC equations have been fitted to experiment data. Modified Oswin equation provided the best fitting in describing EMC isotherms for all study cases. The apparent density and specific heat capacity of all natural rubbers were found to be linearly dependent on moisture content whereas percentage of void was found to be inversely related. For evaluation of thin-layer drying kinetic, the predicted value of Page’s model and the Two-term exponential model provided good fits to the experimental results for both STR 20 and the Skim block rubber, respectively. In addition, drying rate of both block rubber was highly related to drying temperature. By using the Fick’s law of diffusion, The $D_{\text{eff}}$ values for STR 20 and Skim block rubbers ranged between $1.00 \times 10^{-6}$ and $2.41 \times 10^{-12}$ m\textsuperscript{2} min\textsuperscript{-1} and between $1.08 \times 10^{-6}$ and $3.54 \times 10^{-13}$ m\textsuperscript{2} min\textsuperscript{-1}, respectively.

Keywords: effective moisture diffusivity, equilibrium moisture contents, natural rubber

Introduction

Thailand has been among the first rank of rubber exporting countries in the world. Approximated ninety percentages of natural rubbers produced was exported in form of air dried sheet (ADS), ribbed smoked sheets (RSS), block rubber, concentrated rubber latex and the rest was used in country. At present, consumption of RSS and block rubber in the world market has leveled on the rise. This follows the growth of rubber tire industries, especially block rubber industry from the Standard Thai Rubber (STR) because this “STR type” is classified and graded on technical properties rather than visual inspections. STR 20 has excellent processing characteristics and good physical properties. Its low viscosity and easier mixing characteristics (compared with RSS grades) reduce mastication and mixing period considerably. It is used mostly for tires, cushion gum stocks, conveyor belts, and other general products (Rubber Research Institute of Thailand, 2008). Other block rubber of interest is the
Skim block rubber that has low dirt contents, often accompanied by light color and has relatively mild odors. Skim block rubber is produced from by-products of concentrated latex. It is mainly used in the production of rubber bands, car mats, etc (Rubber Research Institute of Thailand, 2008). Several commercial processes used for production of STR and Skim block rubbers are similar. Operation step, however, the drying conditions and operating period has individual difference. Most block rubber factories require high energy consumption not only in the drying process, but also in the pretreatment process. From the report, over 80% of the total energy consumption in block rubber factory is used in the drying stage (Rubber Research Institute of Thailand, 2008).

There have been very few studies relating to natural rubbers drying and its production process, thus the design of this drying system has become an important consideration for this processing operation. In this respect, drying energy and operating period are the parameters to be minimized. However, optimization of the drying process requires understandings of the thermo-physical parameters and drying behaviors of raw materials. Properties of natural rubber involve effective moisture diffusivity, specific heat capacity, viscosity, percentage of void and equilibrium moisture content as well as preview work done on grains and cereal grains (Achaviriya and Soponronnarit, 1990; Aghbashlo et al., 2005; Bitra et al., 2010), natural rubber (Tirawanichakul et al., 2007; Tirawanichakul and Tirawanichakul, 2008) etc. Knowledge of these physical parameters and thermal properties is necessary not only to describe the drying kinetics and to interpret experimental observations but is also to be used for simulation of the drying process. Consequently, apparent density, percentage of void and specific heat capacity of foods, porous materials and agricultural materials (Kahyaoglu et al., 2010; Sereno et al., 2007; Tirawanichakul et al., 2008). Equilibrium moisture content (EMC) contributes to predict stability during storage and to select appropriate packaging materials. The EMC value is mostly dependent upon temperature and relative humidity surrounding, as well as species variety of materials (Achaviriya and Soponronnarit, 1990; Pahlevanzadeh and Yazdani, 2005; Wani et al., 2006).

During the drying process of agricultural products and porous materials, moisture diffusivity is the most crucial transport property because it provides the moisture transfer between products and surrounding. Moisture diffusivity in agricultural products and porous materials is dependent on temperature and moisture content. Temperature dependence of diffusion coefficients has been described using Arrhenius equation (Azzouz et al., 2002; Tong and Lund, 1990; Vagenas and Karathanos, 1991) in which the activation energy is independent of moisture content. However, information on the thermo-physical properties, equilibrium moisture content, and moisture diffusion coefficient of natural rubber is nonexistent in literatures. The objective of this work is to study the thermo-physical properties (apparent density, percentage of void and specific heat capacity), equilibrium moisture content, drying kinetics and diffusion coefficient of two type natural rubber (STR 20 and Skim block rubber) in order to use for natural rubber drying simulation and design.

Materials and Methods

Natural Rubber Samples

STR 20 and Skim block rubbers sample used in the experiments were provided from Southland Resource Co., Ltd., Suratthani province, and from B TECH Industry Co., Ltd., Songkhla province, Thailand, respectively. Through several processes, raw natural rubber (wet coagulates, cup
lumps, latex sheets and tree laces) have been converted into granular form, first by chopping and washing, and subsequently in processes that involve blending and granulating (crumbing). Dimensions of the natural rubber samples were measured using Vernier calipers with an accuracy of ±0.01 mm. Moisture content was determined under ASABE method (ASABE, 1988). Initial moisture content of both natural rubbers was between 1.6 and 55.3% dry-basis to determine the thermo-physical properties.

**Water Sorption Isotherm**

In this study, equilibrium moisture content (EMC) value was determined by static gravimetric method (Wang and Brennan, 1991). Five saturated salt solutions to achieve an equilibrium state were used in the experiment as KNO₃, NaCl, Mg(NO₃)₂•6H₂O, MgCl₂•6H₂O and LiCl. The natural rubbers were weighed (20–35 g) and kept in small stainless-steel basket hanging in glass bottles which contained the saturated salt solutions. The bottles were then placed in an incubator at constant temperature and relative humidity (RH) of 45–60°C and 10–90%, respectively. After a few weeks, the samples were in equilibrival state with the saturated salt solutions. This state was acknowledged when three consecutive weight measurements showed a difference less than 0.001 g. Then the natural rubbers were taken to determine moisture contents under ASABE method (ASABE, 1988). The average EMC were determined by means of triplication. Six isotherm models for predicting EMC were chosen to correlate the experimental data, the surrounding temperature and the relative humidity. Formulated functions of relative humidity, temperature and EMC conditions are shown in Table 1. Constants in these models were derived from non-linear regression analysis on the experimental data.

**Table 1** Determination of sorption isotherm employing different models. (ASABE, 2007; Pappas and Rao, 1987)

<table>
<thead>
<tr>
<th>Model name</th>
<th>Model equation</th>
<th>Equation No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Oswin</td>
<td>( M_{eq} = \frac{(A + B T) [R(1 - R)]}{[A(T + B)]} )</td>
<td>(4)</td>
</tr>
<tr>
<td>Modified Henderson</td>
<td>( M_{eq} = \frac{-\ln(R(1 - R))}{[A(T + B)]} )</td>
<td>(5)</td>
</tr>
<tr>
<td>Modified Halsey</td>
<td>( M_{eq} = \frac{-\ln(R)}{\exp(A + BT)} )</td>
<td>(6)</td>
</tr>
<tr>
<td>Oswin</td>
<td>( M_{eq} = A [R(1 - R)]^{\frac{1}{3}} )</td>
<td>(7)</td>
</tr>
<tr>
<td>Smith</td>
<td>( M_{eq} = \frac{A + B}{[R]} )</td>
<td>(8)</td>
</tr>
<tr>
<td>Iglesias and Chirife</td>
<td>( M_{eq} = \frac{A + B}{[R]} [R(1 - R)] )</td>
<td>(9)</td>
</tr>
</tbody>
</table>

where \( M_{eq} \) is equilibrium moisture content, decimal (dry-basis), \( R \) is an universal gas constant (8.314 J mole⁻¹ K⁻¹), RH is relative humidity (decimal), \( T \) is absolute temperature (K), \( A \), \( B \) and \( C \) are constant values.

**Thermo-Physical Properties**

**Apparent Density**

Apparent density is the ratio of mass to gross volume of matter. The mass of the natural rubber samples was weighed using an electronic balance with an accuracy of ±0.01 g and the volume was measured using a volumetric flask. Apparent density was then calculated using the following equation:

\[
\rho = \frac{m}{V_b}
\]

where \( \rho \) is apparent density (kg m⁻³), is weight of natural rubber (kg), and \( V_b \) is volume of natural rubber (m³).
**Percentage of Void**

Percentage of void is fraction of void in the bulk object multiplied by 100 and the following equation of percentage of void for the natural rubbers was determined as:

$$\epsilon = \left( \frac{V_{oil}}{V_b} \right) \times 100$$  \hspace{1cm} (2)

where $\epsilon$ is percentage of void ($\%$), $V_{oil}$ is volume of oil replaced in the void (m$^3$), and $V_b$ is volume of natural rubber (m$^3$).

**Specific Heat Capacity**

Specific heat capacity of the natural rubbers was determined using a calorimeter. Equilibrium temperatures measured using a K-type thermocouple was recorded by a data logger (YOGOKAWA model FX 100, Japan). By the first law of thermodynamic, specific heat capacity of the samples is calculated using the following equation:

$$c_p = \frac{m_c c_c (T_{eq} - T_{ci}) + m_w c_w (T_{eq} - T_{wi})}{m_p (T_{eq} - T_{pi})}$$  \hspace{1cm} (3)

where $c_p$, $c_c$ and $c_w$ is specific heat capacity of the natural rubber sample, calorimeter and water, respectively; $m_p$, $m_c$ and $m_w$ is mass of the natural rubber sample, calorimeter and water, respectively, $T_{pi}$, $T_{ci}$ and $T_{wi}$ is initial temperature of the natural rubber sample, calorimeter and water, respectively and $T_{eq}$ is equilibrium temperature. Units of specific heat, mass, and temperature are kJ kg$^{-1}$ °C$^{-1}$, kg, and °C, respectively.

**Drying Experiments**

To study the drying kinetics of natural rubbers, fresh samples were thin-layer dried and experimental values were simulated by Fick’s law of diffusion. Then the effective diffusion coefficients were evaluated. The thin-layer dryer (Figure 1) comprises three major components: a stainless steel cylindrical drying chamber with an inner diameter of 0.20 m and a height of 0.40 m, electric heating unit of 1000 W×2 and a backward-curved blade centrifugal fan driven by a 1.5 kW motor. Drying was performed under hot-air inlet temperatures ranging from 100 to 130°C for the STR 20 block rubber and from 84 to 115°C for the Skim block rubber with inlet air flow rate of 1.7 m s$^{-1}$ and a natural rubber bed-depth of 0.03 m. The initial moisture content [24] of natural rubber was in the range of 32.0–56.0% dry-basis and reduced to the final moisture content of 0.5% dry-basis.

The drying temperature was controlled by a Proportional, Integral, and Derivative (PID) controller with an accuracy of ±0.5°C. The inlet and outlet air temperature, the ambient air and the temperature in each rubber bed-depth were continuously measured using K-type thermocouples connected to a data logger with an accuracy of ±1°C (YOGOKAWA model FX 100, Japan).

**Figure 1** Illustration of the thin-layer drying system (PSU-TLD1).

Mathematical Modeling of The Drying Curves

Relationship between moisture ratios and drying time normally is determined by three major mathematical drying models as follows: (1) simulation including heat and mass transfer, (2) the diffusion model modified by Crank (Crank, 1975) and (3) the empirical or semi-empirical model mostly developed from experimental data. The first and second model also called as theoretical model and semi theoretical model. Both of them are solved by complicated mathematical analysis. The rest of model is quite suitable for the experimental data but it has limitation use because it is in-situ condition of each experiment. To predict the evolution of
moisture ratio in this work, the empirical equations were developed by assumption of moisture ratio (MR) as function of drying time and the simplified equation can be written as follows:

\[
MR \left[ \frac{(M_t - M_{eq})}{(M_i - M_{eq})} \right] t(t) \tag{10}
\]

where MR is the moisture ratio, dimensionless, \(M_t\) is the average moisture content at drying time (% dry-basis), \(M_i\) is the initial moisture content (% dry-basis), \(M_{eq}\) is the equilibrium moisture content (% dry-basis) and \(t\) is the drying time (h).

The drying curves were fitted by means of ten different moisture ratio models that are widely used in most food and biological materials are tabulated in Table 2. These models are generally derived by simplifying the general series solution of Fick’s second law. Values of constants in these models were estimated by non-linear regression analysis from the experimental data.

<table>
<thead>
<tr>
<th>Model name</th>
<th>Model equation</th>
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<tbody>
<tr>
<td>Newton</td>
<td>(MR = e^{-kt})</td>
<td>(11)</td>
</tr>
<tr>
<td>Page</td>
<td>(MR = e^{-k't})</td>
<td>(12)</td>
</tr>
<tr>
<td>Henderson and Pabis</td>
<td>(MR = a e^{-kt})</td>
<td>(13)</td>
</tr>
<tr>
<td>Modified Henderson and Pabis</td>
<td>(MR = a e^{-k't} + b e^{-gt} - c e^{-h't})</td>
<td>(14)</td>
</tr>
<tr>
<td>Sharma</td>
<td>(MR = a e^{-bl} + b e^{-ct})</td>
<td>(15)</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>(MR = a e^{-kl} + b)</td>
<td>(16)</td>
</tr>
<tr>
<td>Two term</td>
<td>(MR = a e^{-kl} + b e^{-k_1t})</td>
<td>(17)</td>
</tr>
<tr>
<td>Two term exponential</td>
<td>(MR = a e^{-kl} + (1-a) e^{-kt})</td>
<td>(18)</td>
</tr>
<tr>
<td>Verma et al.</td>
<td>(MR = a e^{-kl} + (1-a) e^{-gt})</td>
<td>(19)</td>
</tr>
<tr>
<td>Midilli-Kucuk</td>
<td>(MR = a e^{-kl_0} + b_0)</td>
<td>(20)</td>
</tr>
</tbody>
</table>

where MR is moisture content (% dry-basis), \(t\) is drying time (min), and \(a, b, d, g, k, k_1\) and \(n\) are constant values.

**Table 2** Different empirical models for thin-layer drying (Tirawanichakul et al., 2008).

**Determination of Effective Moisture Diffusivity**

Drying models based on liquid diffusion theory have attracted special attentions from researchers. Liquid diffusion mechanism is extremely complex due to diversity of chemical composition and physical structure of different product. Moreover, the use of different research methodology renders comparisons difficult. According to Booker et al. (1975), Fick’s second law is used in liquid diffusion theory to establish moisture diffusion as a function of concentration gradient. A partial differential equation of moisture diffusion for natural rubber, considered geometrically either as an infinite slab shape can be generally formulated as follows (Crank, 1975).

\[
(cM/\partial t) - D_{eff} \nabla^2 M \tag{21}
\]

where \(M\) is the moisture at time \(t\), \(D_{eff}\) is the effective diffusion coefficient (m² min⁻¹) and \(t\) is the drying time, (min).

In the falling rate period of drying, moisture transfer is transferred by diffusion effect. To apply analytical method with initial and boundary conditions to Eq. (21), general solution for an infinite slab shape of moisture ratio can be derived thus:

\[
MR = \frac{8}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{(2n-1)^2} \exp \left[ -\frac{\pi^2 (2a+1)^2}{4L^2} D_{eff} t \right] \tag{22}
\]

where MR is moisture ratio (decimal); \(8/\pi^2\) is shape factor for infinite slab; \(t\) the drying time (min); \(L\) is characteristic half-thickness (m); \(n\) is number of parameters and \(D_{eff}\) is effective diffusivity (m² min⁻¹).
The dependence of the effective diffusivity on temperature is generally described by the Arrhenius equation (Simal et al., 2005)

\[ D_{\text{eff}} = D_0 \exp \left( -\frac{E_a}{RT} \right) \]  

(23)

where \( E_a \) is activation energy of the moisture diffusion (kJ mol\(^{-1}\)); \( D_0 \) is Arrhenius factor (m\(^2\) min\(^{-1}\)); \( D_{\text{eff}} \) is moisture diffusivity (m\(^2\) min\(^{-1}\)); \( R \) is universal gas constant (8.314 J mol\(^{-1}\) K\(^{-1}\)) and \( T \) is absolute temperature (K).

**Statistical Analysis**

The regression analysis was performed by using the STATISTICA computer program. The determination of coefficient (\( R^2 \)) and root mean square error (RMSE) were used in this study to evaluate the goodness of fit. The higher \( R^2 \) values and the lower RMSE values are the goodness of fit. These parameters can be calculated by using the following equations:

\[ R^2 = 1 - \frac{\sum (Y_{\text{exp}} - Y_{\text{cal}})^2}{\sum (Y_{\text{exp}} - \bar{Y}_{\text{exp}})^2} \]  

(24)

\[ \text{RMSE} = \sqrt{\frac{1}{N} \sum (Y_{\text{exp}} - Y_{\text{cal}})^2} \]  

(25)

where \( Y_{\text{exp}} \) is measured in the experiment, \( Y_{\text{cal}} \) is calculated using the models, \( Y_{\text{exp}} \) is mean of the measured in the experiment data, \( N \) is number of data points and \( z \) is the number of constants.

**Table 3** Equilibrium moisture contents for STR 20 block rubber under different model of determination.

<table>
<thead>
<tr>
<th>Model</th>
<th>Arbitrary constants</th>
<th>( R^2 )</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Oswin</td>
<td>0.1021 -0.0003 0.3941</td>
<td>0.981</td>
<td>0.001</td>
</tr>
<tr>
<td>Modified Henderson</td>
<td>34.9470 -294.4345 1.6506</td>
<td>0.951</td>
<td>0.002</td>
</tr>
<tr>
<td>Modified Halsey</td>
<td>-6.0499 0.0403 0.5981</td>
<td>0.958</td>
<td>0.002</td>
</tr>
<tr>
<td>Oswin</td>
<td>0.0117 0.4271 -</td>
<td>0.856</td>
<td>0.003</td>
</tr>
<tr>
<td>Smith</td>
<td>0.0045 -0.0104 -</td>
<td>0.849</td>
<td>0.003</td>
</tr>
<tr>
<td>Iglesias and Chirife</td>
<td>0.0069 0.0034 -</td>
<td>0.876</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Table 4** Equilibrium moisture contents of Skim block rubber under different model of determination.

<table>
<thead>
<tr>
<th>Model</th>
<th>Arbitrary constants</th>
<th>( R^2 )</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Oswin</td>
<td>0.0621 -0.0002 0.6768</td>
<td>0.977</td>
<td>0.001</td>
</tr>
<tr>
<td>Modified Henderson</td>
<td>1.4480 -282.0911 0.8487</td>
<td>0.969</td>
<td>0.002</td>
</tr>
<tr>
<td>Modified Halsey</td>
<td>-2.5283 0.0195 1.2763</td>
<td>0.968</td>
<td>0.002</td>
</tr>
<tr>
<td>Oswin</td>
<td>0.0047 0.8205 -</td>
<td>0.881</td>
<td>0.003</td>
</tr>
<tr>
<td>Smith</td>
<td>-0.0012 -0.0102 -</td>
<td>0.846</td>
<td>0.006</td>
</tr>
<tr>
<td>Iglesias and Chirife</td>
<td>0.0012 0.0033 -</td>
<td>0.881</td>
<td>0.003</td>
</tr>
</tbody>
</table>

**Results and Discussion**

**Water Sorption Isotherm**

Six widely recommended models which were used to investigate natural rubbers are given in Table 1. The constant values of A, B and C were statistical analyzed for each of the models. The indices for estimating the errors associated with these six models - which are the coefficient of determination (\( R^2 \)) and the root mean square error (RMSE) - were shown in Tables 3 and 4 for STR 20 and Skim block rubbers, respectively at temperatures of 40-60°C and relative humidity of 10-90%. The modified Oswin model yielded a better correlation to the experimental values according to higher \( R^2 \) and lower RMSE compared to other models for both the STR 20 and Skim block
rubbers. For STR 20 block rubber, the $R^2$ and RMSE values in the modified Oswin model are 0.981 and 0.001, respectively, (Table 3) while the $R^2$ and RMSE values for the Skim block rubber employing this same model are 0.977 and 0.001, respectively, (Table 4).

![Figure 2](image2.png)

**Figure 2** Influence of temperature on equilibrium moisture content of STR 20 block rubbers at temperature of 45-60°C and relative humidity of 10-90% using modified Oswin model and observed data.

Figure 2 and Figure 3 illustrates the effect of temperature on the EMC value of STR 20 and Skim block rubbers at surrounding temperature of 40-60°C and relative humidity of 10-90%. It shows that EMC increases with decreasing temperature at constant RH. EMC curves for STR 20 and Skim block rubbers have similar rate, whilst the EMC value of STR 20 block rubber was higher than those Skim block rubber. Both of the EMC models have been corresponded to many plants, food and porous materials (Achaviriya and Soponronnarit, 1990; Pahlevanzadeh and Yazdani, 2005; Teanchai and Soponronnarit, 1991; Wani et al., 2006). The EMC values for all block rubbers will be used in the evaluation of empirical and semi-theoretical moisture ratios in the next section.

![Figure 3](image3.png)

**Figure 3** Influence of temperature on equilibrium moisture content of Skim block rubbers at temperature of 45-60°C and relative humidity of 10-90% using modified Oswin model and observed data.

**Apparent Density**

The apparent density of natural rubber increases with increasing moisture content from 1.6 to 55.3% dry-basis, as shown in Figure 4 were simulated equations which were evaluated by linearly regression method for STR 20 and Skim block rubber, respectively. Apparent density of STR 20 and Skim block rubber at different moisture content varied from 250.6 to 370.4 kg m$^{-3}$ and 236.6 to 352.2 kg m$^{-3}$, respectively. The results stated that apparent density increased linearly with increasing moisture content corresponding to the previous work (Tirawanichakul et al., 2007). Additionally, an apparent density of STR 20 block rubber is always higher than that of Skim block rubber. For employing least square regression analysis, the relationship between apparent density and moisture content of natural rubbers are shown in Table 5.

**Percentage of Void**

Percentages of void of STR 20 and Skim block rubbers as a function of moisture content (Figure 5 and Table 5) were found to decrease with increasing moisture content from 1.6 to 55.3% dry-basis and 3.0 to 53.1% dry-basis, respectively. Figure 3
Table 5 Thermo-physical properties of natural rubber samples.

<table>
<thead>
<tr>
<th>NR Samples</th>
<th>Parameter</th>
<th>Equation</th>
<th>R²</th>
<th>RMSE</th>
</tr>
</thead>
<tbody>
<tr>
<td>STR 20</td>
<td>Apparent density</td>
<td>ρ=237.22+2.397M</td>
<td>0.999</td>
<td>0.165</td>
</tr>
<tr>
<td></td>
<td>Percentage of void</td>
<td>ε=100.170-0.316M</td>
<td>0.998</td>
<td>0.199</td>
</tr>
<tr>
<td></td>
<td>Specific heat capacity</td>
<td>c_p=3.560+0.094M</td>
<td>0.998</td>
<td>0.081</td>
</tr>
<tr>
<td>Skim</td>
<td>Apparent density</td>
<td>ρ=225.03+2.338M</td>
<td>0.999</td>
<td>0.248</td>
</tr>
<tr>
<td></td>
<td>Percentage of void</td>
<td>ε=96.374-0.374M</td>
<td>0.999</td>
<td>0.171</td>
</tr>
<tr>
<td></td>
<td>Specific heat capacity</td>
<td>c_p=3.315+0.086M</td>
<td>0.996</td>
<td>0.092</td>
</tr>
</tbody>
</table>

M is moisture content of crumb rubbers (%dry-basis).

Figure 4 Relationship between apparent density and moisture content of STR 20 and Skim block rubbers in range of surrounding temperature of 40-60°C.

Figure 5 Relationship between percentage of void and moisture content of STR 20 and Skim block rubbers in range of surrounding temperature of 40-60°C.

shows the percentage of void of natural rubbers at various initial moisture contents. The results showed that percentage of void decreased linearly with increasing moisture content (Tirawanichakul et al., 2007). Values of the percentage of void of STR 20 and Skim block rubbers were found to vary from 82.9 to 99.4% and 76.2 to 95.4%, respectively. Comparing STR 20 and Skim block rubbers, it was found that when moisture content increased, the value of the percentage of void for all blocks rubber decreased. Additionally, percentage of void of the Skim block rubber was slightly higher than that of the STR 20 block rubber. This is correlated to apparent density of these rubbers. Employing the least square regression technique, a relationship between percentage of void and moisture content of natural rubbers are shown in Table 3.

Specific Heat Capacity

Generally, specific heat capacity is independent of ambient temperature within range for their storage or dehydration (Tirawanichakul et al., 2007). However, moisture dependence of the specific heat capacity has been well-recognized and model. The values of specific heat capacity for STR 20 and Skim block rubbers at different moisture content varied from 3.6 to 8.7 kJ kg⁻¹ °C⁻¹ and 3.5 to 8.3 kJ kg⁻¹ °C⁻¹, respectively. Figure 6 shows the relationship between specific heat capacity and moisture content of the natural rubbers. It was found that specific heat capacities of these natural rubbers were linearly proportional to moisture content. At moisture content ranging from 20-60% dry-basis, the specific heat capacity of STR 20 block rubber was slightly higher than that of other Skim block rubbers. Least square regression technique was used to determine
the relationship between specific heat capacity and moisture content of natural rubbers are shown in Table 5.

![Figure 6](image-url) Relationship between specific heat capacity and moisture content for STR 20 and Skim block rubbers in range of surrounding temperature of 40-60°C.

**Drying Characteristics**

The typical characteristic drying curve (moisture ratio versus time) of natural rubbers during thin-layer drying operation at different drying temperature of 100-130°C and 85-115°C for STR 20 and Skim block rubbers are shown in Figure 7 and 8, respectively. The moisture ratio decreased continuously with time. Drying occurrence was only in the falling rate period for all materials during this investigation.

The result in Figure 7 and 8 stated that drying temperature had a prominent influence on the drying rate and affects the value of the diffusion coefficient. The drying rate increased greatly with the increase in drying temperature level. This fact has been reported by several authors (Azzouz et al., 2002; Gunhan et al., 2005; Tirawanichakul et al., 2007). Higher drying temperature increases the effective diffusivity of mass transfer in the samples. In addition, the drying time and drying rate of STR 20 block rubber was slower than these of Skim block rubber. This is corresponded to the percentage of void. The Skim block rubber has a high percentage of void comparing to the STR 20 block rubber so the hot air could more pass through the Skim block rubber bed depth.

**Table 6** Statistical parameters for various models of STR 20 and skim block rubbers drying.

<table>
<thead>
<tr>
<th>Model name</th>
<th>STR 20 block rubber</th>
<th>Skim block rubber</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>RMSE</td>
</tr>
<tr>
<td>Newton</td>
<td>0.947</td>
<td>0.058</td>
</tr>
<tr>
<td>Page</td>
<td>0.999</td>
<td>0.008</td>
</tr>
<tr>
<td>Henderson and Pabis</td>
<td>0.963</td>
<td>0.049</td>
</tr>
<tr>
<td>Modified Henderson and Pabis</td>
<td>0.978</td>
<td>0.038</td>
</tr>
<tr>
<td>Sharma</td>
<td>0.972</td>
<td>0.042</td>
</tr>
<tr>
<td>Logarithmic</td>
<td>0.978</td>
<td>0.038</td>
</tr>
<tr>
<td>Two-term</td>
<td>0.915</td>
<td>0.074</td>
</tr>
<tr>
<td>Two-term exponential</td>
<td>0.963</td>
<td>0.049</td>
</tr>
<tr>
<td>Verma et al.</td>
<td>0.910</td>
<td>0.187</td>
</tr>
<tr>
<td>Midilli-Kucuk</td>
<td>0.953</td>
<td>0.055</td>
</tr>
</tbody>
</table>

**Modeling of Drying Curves**

The ten thin-layer drying models were compared in terms of the statistical parameters $R^2$ and RMSE. The statistical analysis values are summarized in Tables 6. In all cases, the values of $R^2$ for the models were greater than the desirable threshold of 0.90, indicating good fittings. The best empirical model, the one with the highest correlation coefficient ($R^2$) and the lowest RMSE, in describing the thin layer drying characteristics was chosen. The $R^2$ values varied between 0.910 and 0.999 and RMSE values between 0.008 and 0.187. Thus, it was selected to represent the thin-layer drying characteristics of NR samples.
This Page model with R^2 value of 0.999 and RMSE value of 0.008 thus was chosen to represent the thin layer drying behavior of STR 20 block rubber. The R^2 values for all models for Skim block rubber were all very reasonable, with a highest value of 0.999 for a few models, including the Two-term exponential model. However, the least RMSE value (RMSE=0.009) was obtained in the Two-term exponential model and hence is expected to yield better prediction than others in describing the thin-layer drying characteristics of Skim block rubber.

Figure 7 Drying kinetics of STR 20 block rubber (Page model selected) for drying temperature of 100-130°C and air flow rate of 1.7 m s^{-1}.

Figure 8 Drying kinetics of Skim block rubber (Two-term exponential model selected), for drying temperature of 85-115°C and air flow rate of 1.7 m s^{-1}.

Figure 7 and Figure 8 compare the experimental data with the predicted ones using Page model for STR 20 block rubber using Two-term exponential model for Skim block rubber. The prediction using the model showed MR values banded along a straight line, which proved the suitability of these models in describing the drying characteristics of NR samples. Similar observations have been reported by Menges and Ertekin (2006) and Hassan-Beygi et al. (2009).

Effective Moisture Diffusivity and Activation Energy

From non-linear regression of the mean effective diffusion coefficient (D_{eff}) value and the corresponding drying temperature, Eq. (26) for STR 20 block rubber and Eq. (27) for Skim block rubber were derived. R^2 and RMSE for the STR 20 block rubber were 0.979 and 0.036, respectively and 0.988 and 0.033 for the Skim block rubber. At drying temperature of 100 to 130°C the value of D_{eff} is in the range 1.51×10^{-6} to 3.24×10^{-6} m^2 min^{-1} for the STR 20 block rubber; and at drying temperature between 85 to 115°C the D_{eff} range is 1.46×10^{-6} to 2.54×10^{-6} m^2 min^{-1} for the Skim block rubber. Figure 9 shows the effective diffusivity versus drying temperature of STR 20 and Skim block rubbers. The results show that the D_{eff} value was exponentially related to the drying temperature. This indicates that Skim block rubber transfers moisture more rapidly when compared to STR 20 block rubber. Corresponding, to the drying rate and percentage of void value meantime above. Tirawanichakul and Tirawanichakul (Tirawanichakul and Tirawanichakul, 2008) have report the effective diffusivity value of natural rubber rang from 6.0×10^{-8} to 1.3×10^{-3} m^2 min^{-1} at drying temperatures between 40 and 150°C.

The activation energy (E_a) value was 3826.970 and 2601.427 J mol^{-1} for STR 20 block rubber and Skim block rubber, respectively. E_a value is an indication of the required energy to remove moisture from material. Higher E_a value indicated greatest temperature sensitivity of diffusion.
coefficient. The reduction in the $E_a$ value of a process results from an increase in the average energy of the molecules, which take part in process. The $E_a$ value decreased with decreasing density; this indicated that at lower densities, water molecules could move easier than it could at higher densities. The experimental results found in this study were similar to those reported by Thuwapanichayanan et al. (Thuwapanichayanan et al., 2008), and Prakotmak et al. (Prakotmak et al., 2010), besides found that $E_a$ value dependences on sample thickness (Lee and Hsieh, 2008), air velocity (Amin-Nayyeri et al.; Mirzaee et al., 2009). This value from the present study is comparable with those of other similar food grains in comparison of experimental $D_{eff}$ values at different drying temperature and at constant moisture content with those predicted from Arrhenius equation from the study by (Tirawanichakul et al., 2007; Tirawanichakul and Tirawanichakul, 2008).

Conclusions

In this study, equilibrium moisture content (EMC), apparent density, percentage of void, specific heat capacity, drying kinetics and effective moisture diffusivities ($D_{eff}$) for both STR 20 block rubber and Skim block rubber can be presented in form of mathematical model and they are useful for predicting the natural rubber behavior during drying process. For determination of thermo-physical properties, the conclusions stated that apparent density and specific heat capacity of both natural rubbers relatively increase with moisture content. In the other hand site, percentage of void was inversely proportional to moisture content corresponding to the other previous researches in bio-materials. From another results, EMC values of both natural rubbers were mathematical evaluated by non-linear regression employing the six preferred models. Comparisons between experimental results and calculated EMC values show that the predicted results of modified Oswin model appears to be most suitable in describing the experimental results for both the STR 20 and the Skim block rubbers. Drying kinetics and modeling of natural rubber drying was investigated. Simulated results showed that the Page model and the Two-term exponential model are the best fittings to experimental data for the STR 20 and the Skim block rubbers, respectively. The $D_{eff}$ can be expressed as a function of drying temperature and the drying rate relatively depends on drying air temperature. All thermo-physical properties, drying kinetic equation and effective diffusion coefficients from this work are useful in simulation drying modeling of heat and mass transfer during drying processes including to energy analysis.

Acknowledgements

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Office of the Higher Education Commission for financial support for their combined financial support in this research.

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Fast and Less Thermal Degradation Protocol for Chromocult® Coliform Agar (CCA) Preparation to Detect E. coli

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Introduction

E. coli is a bacterium commonly found in the enteric tract of humans and warm-blooded animals and occasionally in contaminated food products, including raw milk, meat, fresh produce and unchlorinated water (Kaspar et al., 1987; Finneya et al., 2003 and Hamilton et al., 2005). The detection of E. coli and total coliform (TC) in foods usually indicates poor hygienic practice in handling and production operations, inadequate storage and post-process contamination (McMeekin et al., 2006 and Gormley et al., 2010). The presence of E. coli O157:H7 bacteria, in particular, can cause a lethal illness called E. coli enteritis and a wide range of symptoms (e.g., diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome). Hence, major food industry and regulatory bodies, including the US Food and Drug Administration (FDA), the US Department of Agriculture (USDA), the ISO detection and enumeration methods, recommend using E. coli and TC detection and enumeration as a key food-quality parameter to determine hygienicity of food manufacturing (Muller et al., 2001; Fang et al., 2003 and Koohmaraie et al., 2007).

Abstract

Effects of Chromocult® Coliform Agar (CCA) preparation methods on E. coli colony size and chromatic development were evaluated by comparing different mechanical and heat treatments. The colony growth kinetics obtained from the conventional preparation protocol (without autoclave) were assessed against that from two alternative approaches including the adjusted protocols applying autoclave sterilization and the high mechanical shear blending using fast microwave pasteurization. In this research, micro inoculation culture (MIC) technique was applied to detect E. coli using 5 µL inoculum and the incubation temperature was fixed at 35±2°C. The size expansion and chromatic changes of E. coli colonies were detected using digital image analysis. The CCA prepared using the alternative applying high shear and fast pasteurization protocol promoted more effective colony expansion of E. coli than the other two methods. The red and green color attributes were less affected by fast heating; however, the blue property of the colony showed substantial deterioration in color intensity. The overall chromatic characteristic of E. coli colonies was significantly affected by heat and preparation variation. High mechanical force helped dissolving agar media very rapidly and mitigated thermal degradation of heat sensitive components, resulting in the better nutrient assimilation of E. coli colonies. All these CCA preparation can be used interchangeably. The application of fast preparation of the CCA was of great benefit to industries where E.coli and coliforms detection are compulsory. The proposed fast CCA preparation can greatly enhance throughput and reduce complexity of the overall detection protocol.

Keywords: chromatic, chromocult® coliform Agar, Escherichia coli, micro inoculation culture
Most classical detection and enumeration of *E. coli* / coliforms in foods are laborious, expensive and time-consuming using conventional culture media (Loessner et al., 1988 and Beumer and Curtis, 2003). The main obstacle is to recover and detect low numbers of the injured pathogenic targets present in foods and environmental samples. The conventional protocols, hence, require the use of enrichment cultures followed by selective plating and a pre-enrichment step. Currently, several commercial test kits based on alternative rapid methods, for example, nucleic acid, fluorescent antibody or immuno-chromatography techniques, are emerging technologies to replace the decade-old conventional culture media. These modern rapid protocols, however, are not practical and affordable for many food manufacturers, especially for developing world applications. These methods require additional sophisticated equipment and expensive devices (Seo et al., 1998 and Blackburn and McCarthy, 2000).

For industrial food sampling, the enrichment steps are still compulsory to amplify the numbers of the target pathogens (Reissbrodt, 2004). To many food industries, chromogenic media as a result are still very popular allowing the differentiation results of food-borne pathogen within a reasonable time frame by developing different colonies appearance and color. These protocols are still based on agar culture technique with the presence of various chromogenic substrates in the media. The expression of specific enzymes and the availability of chromogenic substrates are the key success factors to differentiate the unique and characteristic colony color of the target pathogen. Optimization and simplification of the chromogenic protocol can effectively facilitate this simple recognition. Fast cell recovery and less thermal degradation of chromogenic substrate were hypothesized to have a positive effect on the overall detection of target pathogens (Scharlau Chemie S.A.).

In this research, the chromogenic culture media detecting *E. coli* served as a model selective plating protocol was used to demonstrate the concept of protocol optimization. The Chromocult® agar preparation step was simplified and compared to study the effect of thermal degradation on the colony expansion kinetics and chromatic development of *E. coli* colonies.

### Materials and Methods

#### Microorganisms

*E. coli* culture was prepared in shake tubes using Tryptic Soy Broth (TSB) and incubated to reach the final cell density at $10^9$ CFU/ml. Serial dilution was done to achieve the initial cell density approximately at $10^4$ CFU/ml. One hundred microliters of *E. coli* cultures was transferred into a microtube containing 900 µl of 0.86% normal saline and mixed well to successively reach desirable dilutions (usually, 5-fold dilution). The number of viable cells was detected using plate count technique (Juneja et al., 2009). The stain of *E. coli* was confirmed and enumerated in Chromocult® Coliform Agar (CCA) using the micro-inoculation technique (MIT) as described elsewhere (Khueankhancharoen et al., 2010; Saeaung and Boonyaprapasorn, 2010; Sangadkit et al., 2010 and Supanivatin et al., 2010). Different preparation strategies (i.e. Autoclave, Normal and Blender treatments) were then investigated and the *E. coli* growth kinetics was captured using a sigmoidal mathematical model.

#### Chromocult® Coliform Agar Preparation

##### The Effect of Moderate Temperature and Long Heating Time Treatment (Un-sterilization treatment)

The CCA powder was gently stirred with a glass rod and dissolved in a 500 ml beaker using distilled water. Heating was supplied via a boiling water bath and the mixture was kept stirred to assist dissolution for approximately 35 min (Merck, Darmstadt, Germany).
The Effect of High Temperature and Long Heating Time Treatment (Sterilization treatment)

The CCA solution was prepared as described in the normal preparation procedure above. The CCA was transferred into a 1 l media bottle and autoclaved at 121°C (15 lbs pressure) for 15 min. The autoclave cycle lasted 1 hr 30 min including the heating up, autoclaving and cooling time.

The Effect of High Temperature and Short Heating Time Treatment (Blender treatment)

As opposed to the previous two preparation procedures, the CCA powders in this treatment was mixed vigorously with distilled water in a blender. The mixing was very brief and lasted 30 sec. The CCA mixture was transferred into a 1 l media bottle and then heated using a full-power microwave setting for 2 min. Boiling during fast microwave (MW) pasteurization allowed the mixture to dissolve completely and achieve homogeneous consistency.

The CCA solution prepared from different preparation procedures was set to solidify in a self-fabricated plate allowing multiple samples to be monitored the number and colony size using a developed microscope prototype (Saeaug et al., 2010 and Sangadkit et al., 2010).

Micro Inoculation Culture

The cultivation volumes on the CCA plate were fixed at 5 µL per sample. The CCA plates were incubated at 35±2°C. The image of colony growth and color changed was digitized using a reflected light microscope equipped with a 1.5 megapixel CCD camera. A constructed prototype of digital image analysis protocol was implemented to evaluate the area that each colony occupied on the agar surface. Colony count in the colony forming unit per milliliter of sample (CFU/ml) was done manually. The experimental assumption was that the colony only expanded horizontally and the area of expansion was highly correlated with the growth of pathogens on the solid medium. The areas of colony growth were monitored every 1 hour (Melzoch et al., 2004).

Determining the Maximum Specific Growth Rate Using Logistic Model

The logistic mathematical representation was selected to model the *E. coli* batch growth curve. Several authors suggested utilizing this logistic function (Eq. 1) simulated sigmoidal-type growth profiles as shown below (Mitchell et al., 2004 and Saeaung and Boonyaprapasorn, 2010).

\[
y = y_0 + \frac{a}{1 + e^{\frac{t-t_0}{b}}}
\]

where

- \(y_0\) is the amount of initial inoculation of *Salmonella*
- \(a\) is colony areamax - colony areamin (pixels)
- \(t\) is cultivation time (h)
- \(t_0\) is the time (h) that colony area equals to

\[
\frac{colony\ area\ max + colony\ area\ min}{2}
\]

\(\frac{1}{b}\) is maximum specific colony growth rate (\(\mu_{max}\) (h\(^{-1}\))

Statistical Analysis

All data were analyzed at p<0.05 for significant values by ANOVA and Duncan’s multiple range tests (Statistical Analysis System).

Results and Discussion

The overall impact of different preparation protocols on the total number of colony count was first evaluated. Table 1 showed each preparation treatment returned statistically the same number of total colony reading. The different degrees of thermal degradation did not compromise the effectiveness of Chromocult® Coliform Agar (CCA) to supply adequate nutrients for colonies to grow. Either excessive temperature at 121°C with long treatment time (~90 min) or minimal heat exposure (100°C for ~ 2 min) did not affect the numbers of the colony that generated from the MIC plates.
Table 1 Comparison of agar preparation treatments for the enumeration of *E. coli* when performed on pure culture

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Autoclave</th>
<th>Normal</th>
<th>Blender</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> count (log cfu/ ml)</td>
<td>5.02±0.03</td>
<td>5.05±0.03</td>
<td>5.07±0.04</td>
</tr>
</tbody>
</table>

*Values in a row with different superscripts are significantly different at P<0.05.*

Despite the same number reading, the expansion kinetics of colony area from these different treatments did, however, significantly differ as a result of different degrees of heat exposure (Figure 1). The 104,712±0.03 colonies from the autoclave treatment not only had slower area expansion rate but also grew substantially smaller in term of sizes. The thermal degradation may play an important role in deteriorating the nutrient supply and suppressing the colony growth.

Generic CCA (Merck, Darmstadt, Germany) contains 11.42% peptone, 0.03% sorbitol, 0.03% sodium pyruvate, 0.005% inhibitory agent, indicators, etc (Rall et al., 2005). Most of the ingredients are heat-stable except for sodium pyruvate as a carbon supply for quick colony growth and recovery. Generally, sodium pyruvate is heat-sensitive and most chemical manufacturers recommend to keep this substance in cold storage for longer shelf-life (Mediatech, Inc.). The high degree of thermal degradation of pyruvate was likely in the intense heat treatment (i.e., autoclave and normal treatments). The evidence of some degree of thermal degradation can be observed by the μ<sub>max</sub> values estimated using the logistic model description (Eq 1). A statistical analysis showed the μ<sub>max</sub> values of the autoclave and normal treatments was significantly less than the blender treatment (Table 2). The consequence of pyruvate destruction was also observable by comparing the final achievable colony area. The blender treatment will lesser and shorter heat exposure was able to produce the largest colony area 82,625 pixels (more than twice as many pixels obtained from the autoclave treatment).

The role of pyruvate as a carbohydrate source cannot be over emphasized. Cells can easily access this easily-absorbed substrate for energy production. It is also involved in amino acid metabolism and aerobic respiration. Some publication suggested pyruvate was able to increases the number of *E. coli* 0157:H7 cells recovered after freezing and heating (Czechowicz et al., 1996 and Mizunoe et al., 1999). In addition to the carbon source, *E. coli* also requires nitrogen sources and other trace elements. The use of peptone, tryptone and other amio supplements in CCA media was to provide building blocks and synthesize essential proteins and nucleic acids for colony growth (Harold et al., 1999). Nitrogen source was widely-known to foster biomass production and improve yield coefficient in cell culture (Razvi et al., 1999). As the end product of glycolysis, pyruvate readily is assimilated into oxidative decarboxylation to form acetyl CoA, the metabolite that initiates the Kreb’s cycle and generates cell growth. Many enzymes are involved in the oxidative respiratory pathway. The pyruvate dehydrogenase complex (PDHC), for instance, facilitates the oxidation of pyruvate to acetyl-CoA by *E. coli* during aerobic respiratory growth (Abdel-Hamid et al., 2001).

Figure 1 Profiles of colony growth in three treatments (blue line: Autoclave, green line: Normal and red line: Blender)
Table 2 Comparison of key kinetic parameters in term of colonies size expansion

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Treatments</th>
<th>µ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>x&lt;sub&gt;max&lt;/sub&gt;</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Autoclave</td>
<td>0.148±0.002</td>
<td>40044±484</td>
<td>0.993±0.002</td>
</tr>
<tr>
<td></td>
<td>Normal</td>
<td>0.149±0.002</td>
<td>66016±749</td>
<td>0.985±0.004</td>
</tr>
<tr>
<td></td>
<td>Blender</td>
<td>0.155±0.002</td>
<td>82625±1233</td>
<td>0.986±0.001</td>
</tr>
</tbody>
</table>

a,b,c values in a column with different superscripts are significantly different at P<0.05.

Detection of a specific enzyme or enzymes can be used to differentiate and identify genus, species, or groups of microorganisms. The growth of *E. coli* colonies on chromogenic mixture involves the production of unique enzymes producing distinct dark blue to violet color to *E. coli* colonies (Gonzalez et al., 2003 and Finney et al., 2003). The β-D glucuronidase cleaves both substrates Salmon-Gal and X-glucuronide in CCA developing more intense dark blue color as the incubation time progresses (Figure 2). As early as 12 hr, small violet colonies were detected. The colonies grew larger and the color was more imbrued in violet chroma. The growth of other competing bacteria was discouraged by the presence of Tergitols. This selective agent was able to effectively inhibit the growth of Gram-positive flora. The development of color kinetics was captured by blue color increment from background blue color at the initial time (Figure 3). As opposed to the colony area expansion in Figure 2, the color kinetic reached its asymptotic value rather quick (by approximately 14 hours after incubation).

The effect of thermal degradation on the color precursors and derivatives was also apparent (Figure 3). As seen earlier in the colony growth experiment, intense and long heat treatment produced negative consequence on the color intensity as well. The blue color saturation in the Autoclave treatment was less developed than that of the Normal and Blender treatments. The short exposure with intense heat from MW pasteurization was able to retain not only the quality of the pyruvate but also integrity of the chromogenic substrates. The intact Salmon-Gal and X-glucuronide were effectively converted to dark blue derivatives as reflected by high value of the maximum specific color development rate (µ<sub>max</sub>) and significant improvement of blue color intensity (x<sub>max</sub>) in Table 3.

![Figure 2](image1.png)

**Figure 2** Photographs of colony formed on plate count agar using different treatments (a) Autoclave, (b) Normal and (c) Blender

![Figure 3](image2.png)

**Figure 3** Profiles of color change (delta blue) in three treatments (blue line: Autoclave, green line: Normal and red line: Blender)
Table 3 Comparison of key kinetic parameters in term of color development (delta blue)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>$\mu_{\text{max}}$</th>
<th>$X_{\text{max}}$</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclave</td>
<td>0.632±0.046</td>
<td>57.95±0.02</td>
<td>0.995±0.004</td>
</tr>
<tr>
<td>Normal</td>
<td>0.710±0.032</td>
<td>75.94±0.03</td>
<td>0.987±0.001</td>
</tr>
<tr>
<td>Blender</td>
<td>0.703±0.048</td>
<td>86.00±0.00</td>
<td>0.985±0.002</td>
</tr>
</tbody>
</table>

a,b,c values in a column with different superscripts are significantly difference at P<0.05.

Conclusions

High and long heat exposure to the preparation of CCA was detrimental to the effectiveness of chromogenic analysis of *E. coli*. Both colony growth and color development were significantly affected by the degree of heat applied to the CCA. It was proposed that the thermal degradation of pyruvate and chromogenic substrate (i.e., Salmon-Gal and X-glucuronide) was responsible for poor colony growth rate and inferior blue color development, respectively. The routine preparation (stirring CCA in hot plate or 100°C water bath for extended period of time) was also less effective when comparing to the proposed intense mixing and fast MW pasteurization. This new method was not only improve the effectiveness of CCA in detecting *E. coli* in food samples but facilitate the preparation throughput for industrial application as well.

Acknowledgments

The authors are grateful to have strong industrial partnership with Buono (Thailand) Co., Ltd. The authors would like to express sincere appreciation to Mrs. Achara Jiamthaworn and Mr. Chethta Supparasuwat for their kind collaboration through the course of our research.

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Microbiological Quality of Fresh Cockle (*Anadara granosa*) During Storage at Room Temperature

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Abstract

The microbiological quality of fresh cockle (*Anadara granosa*) was studied during storage at room temperature for 72 hrs. Two separated trials were conducted (cleaned and uncleaned fresh cockle shell before storage). The fresh meat was examined for the microbiological quality changes during storage. The results showed that the total viable count (TVC) increased during storage. TVC increased from 2.89 to 7.23 and 3.45 to 5.97 log CFU g⁻¹ in the cleaned and uncleaned shell treatments, respectively. During the entire storage period, TVC was lower than the standard set by the National Bureau of Agricultural Commodity and Food Standard (ACFS) except in the last storage period of the cleaned fresh cockle shell sample. For coliforms group, coliforms and fecal coliforms increased during the first six hrs of storage. After that, they decreased to only 3 and 1 MPN g⁻¹ in the cleaned and uncleaned shell samples, respectively. *Escherichia coli* count was low and absent within 3 days. *Vibrio parahaemolyticus* decreased from 747 to 20 MPN g⁻¹ in the cleaned shell. This was similar to the uncleaned shell. *V. cholerae*, *Staphylococcus aureus* and *Salmonella* spp. were not detected in any of the samples, indicating that the cockle meat may not pose a risk to the consumer for acquisition of these pathogens.

Keywords: *Anadara granosa*, cockle, microbiological quality, storage

Introduction

The cockle (*Anadara granosa*) is bivalve mollusk that is economically important species in Thailand. Suratthani Province is important of culture cockle source in the southern part of Thailand and the distribution was sent to neighboring provinces and other regions across the country. Problems of transportation and storage of cockle is easily spoilage. Transportation from culture farm to the remote region such as north or northeast which the cockle sent to the state is not fresh and may be contaminated with the microorganisms. Natural cockle could accumulate pathogens and toxins from the environment, especially pathogenic and spoilage microorganisms cause by water quality in the areas of culture or contamination during the storage and transport.

The fresh cockle produced in two product forms, live cockle and shell-off cockle. The quality of cockle changed during post-harvest cultivating from cockle farm (period 7 to 8 hrs), assemblers (period of not less than 14 to 18 hrs), wholesalers (period 4 to 6 hrs) and retailers (from 1 to 36 hrs). Cockle harvesting may be live after the third day (Department of Fisheries, 1993). During transportation period, the cockles might dead or even alive, this causes the loss of quality and freshness for consumers. Usually the transport of cockle from farm to wholesalers are not clearing or grading. They may be keeping in low temperature by using big pack ice.
In this work, the microbiological quality of fresh cockle was examined during storage at room temperature after harvesting. This could be used as guidelines for quality control and standardization of storage and transport cockle to safety from cockle farm to consumer.

**Materials and Methods**

**Sample Collection and Preparation**

The cockles were sampling from cockle farms at Ban Don Bay, Amphur Karnchanadit, Surat Thani Province, Thailand. The size of sample was marketable size (60 to 70 pcs kg⁻¹). Samples were collected in plastic bag brought to the laboratory immediately. Samples were storage at room temperature (∼28°C) and analyze microbiological for 0, 6, 12, 24, 48 and 72 hrs. Samples were tested at the beginning within two hours received in the laboratory. Two separated trials were conducted. Trial 1; the fresh cockles were cleaned shell by rinse with clean water and then storage. Trial 2; the fresh cockles were kept without cleaning the shell.

**Microbiological Quality Analysis**

The fresh meat cockles were examined for the microbiological quality changes during storage. The samples were analyzed by standard methods (BAM online, 2001). Microbiological determined were total viable count (TVC), coliforms, fecal coliforms, *Escherichia coli*, *Vibrio parahaemolyticus*, *V. cholerae*, *Staphylococcus aureus* and *Salmonella* spp. *S. aureus* and *Salmonella* spp. were analyzed only the initial and the end of storage period of the experiment.

**Results and Discussion**

**Enumeration of Total Viable Count (TVC)**

The results showed that the total viable bacteria count increased during storage in both cleaned and uncleaned fresh cockles shell. During storage for 72 hrs, TVC of fresh cockles increased from 2.89 to 7.24 and 3.45 to 5.79 in cleaned and uncleaned fresh cockles shell treatments, respectively (Fig. 1). However, the numbers of bacteria counts in the cleaned shell were less than uncleaned shell. This is in agreement with results obtained later (Iddhibongsa et al., 2008). In this study, the total viable count of fresh cockle during storage at room temperature for 72 hrs was lower than the standard set by the National Bureau of Agricultural Commodity and Food Standard (ACFS) and in acceptable condition according to the microbiological quality standard of Department of Medical Science, Ministry of Public Health (DMSC, 1993), i.e. not exceeding 1x10⁶ CFU g⁻¹ except in the last storage period of the cleaned fresh cockle shell sample.

**Enumerate of Coliforms, Fecal Coliforms and *E. coli***

The number of coliforms and fecal coliforms increased during the first six hrs of storage. The counts were 44 and 39 MPN g⁻¹ (coli), 82 and 81 MPN g⁻¹ (fecal coliforms) in the cleaned and uncleaned shell samples, respectively. After that, they decreased during storage period. The coliforms count in cleaned and uncleaned fresh cockles shell treatments decreased to 3 and 1 MPN g⁻¹ at the end of storage, respectively. The results were similar to the fecal coliforms count which decreased to 3
and 0 MPN g\(^{-1}\) in cleaned and uncleaned fresh cockles shell treatments, respectively (Fig. 2).

**Figure 2** The number of Coliforms and Fecal coliforms in fresh cockles during storage at room temperature for 72 hrs.

- ■ = Coliforms-cleaned fresh cockles shell
- ◼ = Coliforms-uncleaned fresh cockles shell
- □ = Fecal coliforms-cleaned fresh cockles shell
- ○ = Fecal coliforms-uncleaned fresh cockles shell

The number of *E. coli* was a few detected during storage for 72 hrs except in first six hrs storage period of the cleaned fresh cockle shell sample. *E.coli* count tended to decreased from 8 and 9 MPN g\(^{-1}\) to not detected level in the cleaned and uncleaned fresh cockles shell, respectively (Fig. 3).

Coliforms, fecal coliforms and *E.coli* are the commonly used bacterial indicator of sanitary quality of foods. Coliforms and fecal coliforms increased during the first six hrs of storage. After that, they decreased to only 3 and 1 MPN g\(^{-1}\) in the cleaned and uncleaned shell samples, respectively. The fresh cockles were in acceptable condition according to the microbiological quality standard of Department of Medical Science, Ministry of Public Health (DMSC, 1993), i.e. not exceeding 20 MPN g\(^{-1}\). *E. coli* count was low and absent within 3 days. However, the presence of fecal coliforms bacteria is indicative possible of fecal contamination of the samples.

The number of coliforms group was different according to various environmental factors. Cefas (2006) reported that the initial experiments undertaken in the UK in support of definition of protocols for the sampling, sample transport and testing of bivalve mollusk harvesting areas for *Escherichia coli* showed no significant effect of sample storage at 4°C, 10°C or 13°C for up to 72 hours. The effect of storage at 19 – 22°C differed between three experiments. One (mussels) showed no change, one (mussels) showed significant growth by 48 hours and one (Pacific oysters) showed some decline.

**Figure 3** The number of *E. coli* in fresh cockles during storage at room temperature for 72 hrs.
- • = *E. coli*-cleaned fresh cockles shell
- ◼ = *E. coli*-uncleaned fresh cockles shell

**Enumeration of *V. parahaemolyticus***

The number of *V. parahaemolyticus* in fresh cockles during storage at room temperature for 72 hrs decreased from 747 to 20 and 743 to 19 in cleaned and uncleaned fresh cockles shell treatments, respectively (Fig. 4). *V. parahaemolyticus* is commonly found in fisheries products and seawater. It was found that *V. parahaemolyticus* count was high at the initial storage but it decreased during storage for 72 hrs. The amount of contamination was in acceptable level according to the microbiological requirement of frozen and chilled fishery products (ready to eat) export to the United States (FIQD, 2004), i.e. not exceeding \(1 \times 10^4\) MPN g\(^{-1}\).
Figure 4 The number of V. parahaemolyticus in fresh cockles during storage at room temperature for 72 hrs. • = V. parahaemolyticus-cleaned fresh cockles shell
ο = V. parahaemolyticus-uncleaned fresh cockles shell

Enumeration of V. cholerae, Staphylococcus aureus and Salmonella spp.

V. cholerae, S. aureus and Salmonella spp. were not detected in any of the samples. This is in agreement with results obtained later (Sutthirak et al., 2009).

The results of the above studies showed that the microbiological quality of fresh cockle during storage at room temperature for 72 hrs was still within the standard. However, it is important considered to ensure that the cockle is suitable for human consumption. The practice follow requirement of Thai Agricultural Commodity and Food Standard about the Code of Practice for Fish and Fishery Products (ACFS, 2005) which the purpose of protecting the health of consumers and ensuring fair practices in the food trade. This code is intended for all those engaged in the handling, production, storage, transportation, distribution and sale to consumers. The practical following appropriate procedures are the harvesting equipment and container should be clean. On removal from water or during handling and transportation, the cockle should not be subjected to extremes of heat or cold or sudden variations in temperature. Before storage, they should be washed to remove mud by washing with clean seawater or portable water. During storage, the cockle should be laid out at a density and under such conditions that will permit them to open and function normally. Storage periods should be kept as short as possible. Temperature control is important in handling and storage the cockle. Since Degner et al. (2005) has shown that alternative harvesting, handling and storage techniques, such as tempering, use to increase survival of hard clams (Mercenaria mercenaria) in refrigerated storage when harvest water temperatures exceed 80°F should also be considered for the Blood ark clam (Anadara ovalis).

Conclusions

This study was to investigate the microbiological quality of fresh cockle (cleaned and uncleaned shell) during storage at room temperature for 72 hrs. The total viable count (TVC) increased during storage. At the initial storage the number of coliforms group including E.coli was considerable. After that, they decreased to low and E.coli absent within 3 days. V. parahaemolyticus decreased during storage. V. cholerae, S. aureus and Salmonella spp. were not detected in any of the samples.

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Cloning of Beta-Galactosidase Gene from *Lactobacillus delbrueckii* subsp. *bulgaricus* TISTR 892 and Expression in *Escherichia coli*

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**Abstract**

β-Galactosidase enzyme catalyzes not only the hydrolysis reaction but also the transgalactosylation reaction of β-D-galactopyranosidic linkage. Hence, β-galactosidase is among the most common industrial enzymes due to its various applications in dairy and food industries. More recently, thermophilic bacteria have been of interests for the commercial production of β-galactosidase. This research focused on cloning and expression of the β-galactosidase gene from acidotolerant and thermophilic *Lactobacillus delbrueckii* subsp. *bulgaricus* TISTR 892. The gene encoding for β-galactosidase was cloned by PCR technique. About 3 kb amplified product was then ligated to p10HisFLAG expression vector. The recombinant plasmid with β-galactosidase gene was screened and transformed into *Escherichia coli* TOP10. The obtained recombinant enzyme showed higher activity when compared with native enzyme. In addition, recombinant enzyme was purified to apparent homogeneity and characterized. The recombinant β-galactosidase displayed high efficiency in lactose hydrolysis and galactosyl-oligosaccharides production.

**Keywords**: β-galactosidase, *Lactobacillus*, recombinant enzyme

**Introduction**

The enzyme β-galactosidase (β-D-galactohydrolase, lactase; EC 3.2.1.23) hydrolyzes lactose into two moieties–glucose and galactose. β-Galactosidase is among the most common industrial enzymes due to its various applications in dairy and food industries. Another advantage of the enzymatic process is that, during the hydrolysis of lactose by β-galactosidase, some galactosyl-oligosaccharides (GOS) are formed. These compounds are indigestible, acting as dietary fibre, and promote the growth of bifidobacteria in the intestine, with the subsequent healthy effects in the intestine and the liver (Mahoney, 1998). More recently, thermophilic bacteria have become an object of interest for the commercial production of β-galactosidase. The main advantage of thermophilic enzymes is higher reaction rates due to decreasing the viscosity of the reaction mixture and thus increasing the diffusion coefficient of substrate. A higher production yield was observed when the solubility of substrate and product increasing and product inhibition decreasing (Zeikus et al., 1998). Among these, lactic acid bacteria are interesting, because of their generally regarded as safe (GRAS) status and they can produce GOS. Several reports have described lactose hydrolysis by crude *Lactobacillus delbrueckii* subsp. *bulgaricus* β-galactosidase (Vasiljevic and Jelen, 2001, 2002, 2003). The β-galactosidase of *L. delbrueckii* subsp. *bulgaricus* has been characterized showing high activity and stability at temperatures above 50 °C. These conditions can enhance the rate of lactose hydrolysis and preventing the growth of microbial contamination (Itoh et al., 1980; Greenberg and Mahoney, 1982). However, most native thermostable enzymes are synthesized at very low levels.
by the thermophilic bacteria. Expression of the gene coding the thermostable β-galactosidase in E. coli could help to solve this problem. So, it was interesting to improve the production of β-galactosidase by cloning β-galactosidase gene.

**Materials and Methods**

**Bacterial Strains and Culture Conditions**

*L. delbrueckii* subsp. *bulgaricus* TISTR 892 was obtained from Microbiological resources centre (Thailand Institute of Scientific and Technological Research, Thailand). The strain was grown on MRS broth (Difco Laboratories. Detroit, MI) and incubated in anaerobic jar at 37 °C for 16 h. *Escherichia coli* TOP10 was an expression host. The *E. coli* was grown in Luria Bertani (LB) medium supplementation with ampicillin (100 µg mL⁻¹). The expression vector, p10HisFLAG, was obtained from Biotechnology, Suranaree University of Technology.

**Cloning and Expression of the β-Galactosidase Gene**

The β-galactosidase gene from *L. delbrueckii* subsp. *bulgaricus* TISTR 892 was cloned by PCR method. The oligonucleotide primers (LblacZ-F2: GGAA TTCTCTGAGGTATTCAAGTAAGGAAG and LblacZ-R2.2: CCACGAAGAT CTITTTAGTAAAGGCTGAATCAC) were used for PCR amplification of *lacZ*. These primers were designed using the published sequence from the genomic database of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 (NCBI accession number CR954253) and were compatible with the *XhoI* and *BglII* cloning sites of p10HisFLAG expression vector. The amplified products of about 3 kb were cut with *XhoI* and *BglII* and inserted into the respective sites of the expression vector p10HisFLAG resulting in the overexpression plasmid pHFgal24 (Figure 1) encodes 10 x His tagged recombinant enzymes for further purification. The DNA sequence of β-galactosidase gene was determined with automated DNA sequencer (BioDesign, Thailand)

*E. coli* TOP10 harbouring the pHFgal24 plasmid was inoculated in 5 mL of LB broth containing 100 µg mL⁻¹ of ampicillin, and incubated at 37 °C for overnight. Then, 1 mL of the overnight culture was inoculated in LB medium (100 mL) containing 100 µg mL⁻¹ ampicillin, and incubated at 37 °C in a rotary shaker (200 rpm). After the optical density (OD₆₆₀) of the culture reached 0.5-0.6, Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1.0 mM. The culture was incubated further at 30 °C for 12 h. after that, the induced cells were harvested. Enzyme in the periplasmic content was extracted according to a previously published protocol (Yamabhai et al., 2007). To enzyme extraction in the cytoplasm, the pellets from the previous step were suspended in buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and disrupted by using a French press (AMINCO,USA). The cell debris was removed by centrifugation at 8000 x g for 20 min at 4 °C.

**Figure 1** Scheme for construction of the expression plasmid pHFgal24. The gene was inserted between the *XhoI* and *BglII* sites of plasmid p10HisFLAG. The plasmid is based on the pFLAG-CTS vector (Sigma, USA)
Enzyme Purification
The crude extract was applied onto a Histrap column (Ni Sepharose 6 Fast Flow, GE Healthcare, Sweden) equilibrated with buffer A. The enzyme was eluted at a rate of 1 mL min\(^{-1}\) with a 15 column volumes linear gradient from 0 to 100% buffer B (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4). The active fractions were pooled and concentrated by ultrafiltration with a membrane with a 10 kDa cutoff (Millipor, Ireland). The purity of the enzyme was examined by 12% SDS-PAGE and protein concentration was determined by Bradford (1976) method using bovine serum albumin as a standard.

Enzyme Activity Assay
β-Galactosidase activity assay was performed according to a previously described (Nakkharat and Haltrich, 2005). Twenty microliters of suitably diluted enzyme was added into 480 µL of 22 mM o-nitrophenyl-β-D-galactopyranoside (oNPG) in 20 mM sodium phosphate buffer (pH 7.4) and incubated at 40 °C for 15 min. The reaction was stopped by adding 750 µL of 0.4 M sodium carbonate (\(\text{Na}_2\text{CO}_3\)) and the o-nitrophenol (oNP) releasing amount was determined by reading the increase in absorbance at 420 nm.

One unit of β-galactosidase activity (U) was defined as the amount of enzyme that releases 1 µmol of oNP from oNPG per minute under experimental conditions described above.

Characterization of Recombinant β-Galactosidase Effect of pH
The optimum pH of the enzyme activity was determined by using suitably diluted enzyme. The activity was investigated under standard assay conditions, 22 mM oNPG in different buffers (pH 4.0-10.0). The buffers used were 50 mM of acetate buffer (pH 4.0-6.0), phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 7.0-9.0) and glycine-NaOH buffer (pH 9.0-10.0). The stability of β-galactosidase at various pH was also studied by incubating the enzyme in the above buffers at 40 °C for 24 h. Subsequently, the residual activity was determined under standard assay conditions.

Effect of Temperature
Temperature dependence of β-galactosidase was studied by monitoring the enzyme activity on oNPG in 20 mM sodium phosphate buffer (pH 7.4), at various temperatures (25-80 °C). Stability studies were carried out by incubating the enzyme in 20 mM sodium phosphate buffer (pH 7.4), at different temperatures (35-70 °C) for 24 h, and the residual activity was determined by standard assay conditions.

Effect of Various Cations
The effect of various cations on β-galactosidase activity were assayed at 40 °C with 22 mM oNPG in 20 mM sodium phosphate buffer (pH 7.4) as a substrate in the presence of various cations added in final concentrations of 1 mM. The measured activities were compared with the activity control of the enzyme solution determined under same conditions but without added cations.

Hydrolysis of Lactose and GOS Production
The hydrolysis of lactose was performed by incubating 250 g L\(^{-1}\) initial lactose concentration with 200 U mL\(^{-1}\) final concentration of β-galactosidase in sodium phosphate buffer (pH 6.5) at 40 °C for 48 h. Samples were withdrawn at specific time intervals and immediately incubated at 100 °C for 10 min to inactivate the enzyme. The samples were stored at -20 °C for subsequent analysis. Lactose and GOS were analyzed by using high performance liquid chromatography (HPLC).

Analysis of Lactose Hydrolysis and GOS Production
The carbohydrate composition of the reaction mixtures was determined by HPLC under the following conditions:
refractive index detector (Waters model 410, USA); column oven (Waters column Heater Module); carbohydrate column (Phenomenex, Rezek RNM Carbohydrate Column 7.8 x 300 mm); pump (waters model M510); mobile phase, deionized distilled water; flow rate, 0.4 mL/min and column temperature, 80 °C. The yield of GOS was expressed as percentage of carbohydrate weight formed per weight of lactose initially present.

Results and Discussion

Cloning and Expression of β-Galactosidase Gene

Several transformants harboring the lacZ gene were analyzed by direct colony method. A plasmid pHFgal24 carrying the lacZ gene (Figure 2) under the control of tac promoter and could be induced for high expression using IPTG. At 12 h after induction, more β-galactosidases activities could be found in the cytoplasm than in the periplasmic space and culture medium, respectively. Comparison of the yield of recombinant β-galactosidase in different cell compartments is shown in Figure 3. To prepare the enzyme for purification and analysis in the next step, we preferred to use the cytoplasmic and periplasmic space, facilitating the subsequent affinity purification step. A purified β-galactosidase (6.11 mg) was obtained with a yield of 67.9%, and had a specific activity of 3153 U/mg protein, with a final purification factor of 5.4-fold. The subunit molecular weight was determined as being approximately 115 kDa by SDS-PAGE (Figure 4 (A)) whereas the molecular mass of the active form of the enzyme was estimated at 230 kDa by native-PAGE (Figure 4 (B)). One complete open reading frame (ORF) of 3027 bp encoding 1008 amino acid residues with a calculated molecular mass of 114,160 Da was obtained and belongs to glycoside hydrolase family GH2, according to the CAZy (CArbohydrate-Active EnZymes) databank (Cantarel, 2009). It showed highly significant identities with published β-galactosidase genes of L. delbrueckii ssp. bulgaricus ATCC 11842 (100%), ATCC BAA-365 (100%) and L3 (99%).

Characterization of Recombinant β-Galactosidase Effect of pH

The enzyme activity of the L. delbrueckii subsp. bulgaricus TISTR 892 recombinant β-galactosidase was examined at pH ranging from 4.0 to 10.0 (Figure 5). The optimum pH for enzyme activity was determined to be 7.5. Similar results have been reported for several β-galactosidases from Bifidobacterium infantis (Hung and Lee 2002), Arthrobacter sp. C2-2 (Karasova-Lipovova et al, 2003) and Flavobacterium sp. (Sorensen et al., 2006) which have optimum of 7.5. At pH 6.0-6.5
and 7.0, the enzyme activities showed more than 70% of the maximum activity. The enzyme retention of about 50% of maximal activity was at pH value of 6.0.

Enzyme stability studies at different pH values indicates that the enzyme exhibited more than 70% of residual activity at pH 5.0 and 5.5 while pH 7.0 and 7.0, the enzyme retained 60% and 49% of residual activity, respectively. It may be suitable for hydrolysis of lactose in milk and in sweet whey. At pH 4.0, the enzyme activity sharply declined due to enzyme denaturation.

**Effect of Temperature**

The effect of temperature on the enzyme activity is shown in the Figure 6. The enzyme was the most active at 55 °C and its similar to β-galactosidases from _L. acidophilus_ R22 (Nguyen et al., 2007). However, at the temperature below 55 °C, the enzyme activity decreased. The enzyme activity sharply declined due to enzyme denaturation at the temperature higher than 60 °C.

![Figure 5](image_url) **Figure 5** Effect of pH on recombinant β-galactosidase activity at 40 °C: (•) citrate phosphate buffer; (■) phosphate buffer; (♦) Tris-HCl buffer and (▲) glycine-NaOH buffer.

![Figure 6](image_url) **Figure 6** Effect of temperature on recombinant β-galactosidase activity: Enzyme activity was determined at various temperatures as described in material and methods.

The temperature dependence of stability was tested by incubation of the enzyme at 25-80 °C and determination of the remaining activity under standard conditions. The half-life of the enzyme (0.5) for β-galactosidase activity at 35, 40, 50 and 55 °C were found to be 82, 53, 41 and 4.3 h, respectively.
Effect of Various Cations

The influences of different metal ions were investigated. Metal ions that are Mg²⁺, Ca²⁺, Mn²⁺, Co²⁺, Fe²⁺, Ni²⁺, Li²⁺ increased enzymatic activity, whereas Zn²⁺ did not affect enzymatic activity. The activity was inhibited by Na⁺, K⁺ and Pb²⁺. The activity was completely inhibited by Cu²⁺ and Ag²⁺ (Table 1). Previously reported that Mn²⁺ plays an activator role for *Thermotoga maritime* A4 (Kim et al., 2004) and *Thermus* sp. (Ohtsu et al., 1998) while it inactivates *L. acidophilus* R22 (Nguyen et al., 2007). And it is know which Mg²⁺ is needed for activity in most β-galactosidases (Kim et al., 2004; Wanarska et al., 2005) where as Cu²⁺ deactivated the activity of β-galactosidases (Wanarska et al., 2005; Shipkowski and Brenchley, 2006)

### Table 1 Influence of metal ions on β-galactosidases activity

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>%Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>Na⁺</td>
<td>81</td>
</tr>
<tr>
<td>K⁺</td>
<td>85</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>0</td>
</tr>
<tr>
<td>Li⁺</td>
<td>123</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>213</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>124</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>101</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>287</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>0</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>282</td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>262</td>
</tr>
<tr>
<td>Pb²⁺</td>
<td>42</td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>244</td>
</tr>
</tbody>
</table>

Hydrolysis of Lactose and GOS Production

A time course of GOS synthesis is shown in Figure 7. At the beginning of the reaction, lactose decreased rapidly and the amount of glucose increased above the amount of galactose, as galactose was used to form the GOS. GOS formation reached approximately 33.03% from 66.24% lactose hydrolysis at 24 h and then leveled off. As the reactions continued, glucose and galactose continued to increase. Yield of GOS obtained from recombinant enzyme of *L. delbrueckii* subsp. *bulgaricus* TISTR 892 higher than those of enzyme of *Bifidobacterium pseudolongum* (Rabiu et al., 2001), *Thermotoga maritime* (Kim et al., 2004) and *Lactobacillus reuteri* (Maischberger et al., 2008). This result indicates that the enzyme exhibited higher transgalactosylation activity.

![Figure 7](image)

Conclusions

*E. coli* expression system is an efficient enzyme production. This enzyme is able to catalyze lactose hydrolysis and galactosyl-oligosaccharides production. In addition, it was interesting to overproduction of β-galactosidase in food-grade expression system for food the food and dairy industries.

Acknowledgments

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References


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Chemical Compositions of Eggs from Chicken, Quail and Snail-Eating Turtle

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Abstract

This study was aimed to evaluate chemical compositions, profiles of amino acids and fatty acids, vitamin and sex hormone contents of eggs from chicken, quail and snail-eating turtle, respectively. Whole quail egg had higher levels of fat (9.89 g 100g⁻¹) and energy (156.50 kcal 100g⁻¹) than the others. Snail-eating turtle egg contained the highest levels of total solids (5.36%) and ash (1.45 g 100g⁻¹). Total of amino acids content of either quail or chicken egg white (10.78 or 9.33 g 100g⁻¹) was higher than that of snail-eating turtle. Ratio of unsaturated and saturated fatty acids content found the highest in snail-eating turtle eggs. Quail egg yolks contained the highest content of vitamin A (retinol) and D (717.00 and 1.14 µg 100g⁻¹), while the chicken egg contained the highest level of vitamin E (6.37 mg 100g⁻¹). Most of sex hormones content; Luteinizing Hormone (LH), Follicle Stimulating Hormone (FSH), Prolactin (PRL), Progesterone (P), Estradiol (E2) and Testosterone (T), were high in egg yolks. Whole eggs of chicken egg contained the highest content of hormone P (807.06 ng g⁻¹) which was higher than that of quail or snail-eating turtle (314.83 or 157.36 ng g⁻¹). This study indicated that quail egg contained higher contents of amino acid, such as aspartic acid and leucine, vitamin A and D, than the others. While, the chicken egg contained higher levels of vitamin E and sex hormones P than the others. Total minerals were the most abundant in snail-eating turtle egg.

Keywords: chemical compositions, chicken, quail, snail-eating turtle, egg

Introduction

Eggs have been consumed by mankind for millennia. There are many different species, including birds, reptiles, amphibians, and fishes. Eggs are the one's diet and have the various nutrients which supply proteins, all essential amino acids for humans and provide several vitamins and minerals (Messier, 1991; Seuss-Baum, 2007). All of which are easily absorbed by the body. The nutritional compositions, such as proteins affect growth, protection and development of embryo. In addition, lipids are also important nutritional components of the many living organisms. They are the major energy sources and provide a range of essential nutrients for tissue developments and functions (Neuringer et al., 1988; Noble and Cocchi, 1990; Maldjian et al., 1995). Eggs are also an inexpensive single-food source of proteins. Chicken egg is the most commonly consumed eggs. The recent study of older adults consumed one egg a day for 5 weeks showed significant increasing serum lutein and zeaxanthin concentrations without elevating serum lipids and lipoprotein cholesterol concentrations (Goodrow et al., 2006). At present, quail egg has become a vital ingredient around the world which people believed that there is an abundant of useful minerals and vitamins (Iwasawa et al., 2009; Sahin et al., 2010). Snail-eating turtle egg can be found in all lowland areas of Chao Phraya River Basin (Brophy, 2006) and most people in this area consume for 2 eggs a day (Siriwong, 2010) Some people
believed that snail-eating turtle egg is as a supplement of male sex hormone. Thus, this study was aimed to determine chemical compositions of chicken, quail and snail-eating turtle eggs.

**Material and Methods**

**Samples Collection**

Chicken (*Gallus gallus*) and quail (*Coturnix coturnix japonica*) egg samples were purchased from local market Ayutthaya, and snail-eating turtle (*Malayemys macrocephala*) egg samples were purchased from provider in Bangban district, Ayutthaya province during November 2009 to March 2010. Eggs of snail-eating turtle and quail were similar, and they were about 5 times smaller than that of chicken egg. Triplicate samplings of eggs were conducted, and samples of 100 eggs of quail or snail-eating turtle, and 50 eggs of chicken were collected for each sampling. Eggs samples were 3-days after laying and were carefully handled in an ice-box, and transported to the laboratory. They were kept in the refrigerator (@ 7°C) until used for the experiment.

**Samples Preparation and Analyses for Chemical Compositions, Amino Acids, Fatty Acids and Vitamins**

Egg samples were weighed for whole, egg white, and egg yolk. Samples were homogenized by a laboratory blender (Moulinex A327, France), and subjected to proximate analyses (AOAC, 2005; Shapiro, 1995a; Shapiro, 1995b), profiles of amino acids by acid hydrolysis and derivertization with GC-FID detection (Robert and Sarwar, 2005), fatty acids profile by acid hydrolysis and methylation with Capillary GC detection (Ratnayake et al., 2006). Vitamin analysis was conducted by saponification and liquid extraction of organic solvents with HPLC-Fluorescence detection (DeVries, 2005). All analyses were performed by a certified laboratory (ALS Laboratory Group Co., Ltd., Bangkok).

**Samples Preparation for Sex Hormones Analyses**

Egg samples were separated for egg whites and egg yolks, and they were homogenized. Each sample was weighed for 5.0 g of each and 50.0 ml of hexane was added for a 3-minute extraction in a mixer. Solvent of the extract was evaporated in nitrogen gas. Sample extracts were soluble in phosphate buffer (pH 7.0): dimethylsulfoxide (DMSO) (1:1, vol/vol) before determinations of sex hormones of LH (Luteinizing Hormone), FSH (Follicle Stimulating Hormone), P (Progesterone), E2 (Estradiol), T (Testosterone) by ECLIA (Electrochemiluminescence immunoassay, method (Xin et al., 2010) using Roche Diagnostics kits and PRL (Prolactin) by IFMA (Time-resolved fluoroimmunoassay) method (Diamandis, 1988) using PerkinElmer Life and Analytical Sciences kit. The hormonal analyses were performed by reference laboratory of King Chulalongkorn Memorial Hospital, Bangkok.

**Statistical Analyses**

Statistical analyses were carried out using descriptive statistical analysis and compared with One-Way ANOVA: Post Hoc Multiple Comparision (LSD). Mean differences with \( p<0.05 \), \( p<0.01 \) and \( p<0.001 \) were considered statistical significant. All statistical analyses performed by SPSS for window version 17.0.

**Results and Discussion**

Average weight of a chicken egg was 60.80 g egg\(^{-1}\) which was higher than that of the snail-eating turtle and the quail eggs (\( p<0.001 \) and \( p<0.01 \)) (Table 1). Contents of protein, fat and calories in the whole quail egg were the highest among three different types of eggs. Quail egg had higher contents of fat and calories than those of the chicken egg (\( p<0.01 \) and \( p<0.001 \)) (Table 1). Higher content of ash in the snail-eating turtle compared to those of the quail and chicken eggs (\( p<0.05 \) and \( p<0.01 \)), might suggest the higher content of total mineral constituents in the snail-eating turtle egg. While total
carbohydrates and moisture contents were found the highest in the chicken egg. Carbohydrates are macronutrients which human needs to uptake in reasonably large quantities to be converted to energy for body needs (Noor et al., 2010; Owen et al., 2010). Dietary fiber has such a complicated chemical structure that the human body cannot metabolize it at all. So, fiber remains very important for both health and weight control (Babio et al., 2010; Pal et al., 2010). Moreover, it also helps to protect us against some serious diseases, including various cancers (Slavin, 2008).

Table 1 Proximate analyses of chicken, quail and snail-eating turtle whole eggs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chicken Mean ± S.D.</th>
<th>Quail Mean ± S.D.</th>
<th>Snail-eating turtle Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g egg⁻¹)</td>
<td>60.80 ± 1.46 e,i</td>
<td>10.67 ± 0.89 e,g</td>
<td>11.55 ± 2.39 d,i</td>
</tr>
<tr>
<td>Ash (g 100g⁻¹)</td>
<td>0.88 ± 0.01 e</td>
<td>1.06 ± 0.05 d</td>
<td>1.45 ± 0.11 d,e</td>
</tr>
<tr>
<td>Dry matter (%)</td>
<td>3.73 ± 0.28 f</td>
<td>3.82 ± 0.21 d</td>
<td>5.36 ± 0.28 f,g</td>
</tr>
<tr>
<td>Carbohydrate (Include Dietary Fiber) (g 100g⁻¹)</td>
<td>5.96 ± 0.18 b</td>
<td>4.01 ± 0.34 b</td>
<td>4.64 ± 0.89</td>
</tr>
<tr>
<td>Calories (kcal 100g⁻¹)</td>
<td>112.50 ± 0.71 c,e,e</td>
<td>156.50 ± 0.71 c</td>
<td>145.17 ± 14.29 c</td>
</tr>
<tr>
<td>Fat (g 100g⁻¹)</td>
<td>4.37 ± 0.17 b</td>
<td>9.89 ± 0.08 b</td>
<td>8.56 ± 2.40</td>
</tr>
<tr>
<td>Moisture (g 100g⁻¹)</td>
<td>76.40 ± 0.00 c,f,e</td>
<td>72.25 ± 0.21 c</td>
<td>72.93 ± 0.64 f</td>
</tr>
<tr>
<td>Protein (N * 6.25) (g 100g⁻¹)</td>
<td>12.40 ± 0.00</td>
<td>12.70 ± 0.14</td>
<td>12.42 ± 1.63</td>
</tr>
</tbody>
</table>

ab, c,e significant difference between types of egg at p<0.05
d,e,f significant difference between types of eggs at p<0.01
g,h,i significant difference between types of eggs at p<0.001

The essential amino acid profiles (EAAs, Young, 1994) of all egg whites were mostly found in chicken and quail eggs which total EAAs were significantly higher than snail-eating turtle egg (p<0.001) as shown in Table 2. The most contents of EAAs were lysine, leucine and phenylalanine which found in chicken egg at 1,410.00, 1,195.00 and 1,140.00 mg 100g⁻¹ respectively. Quail egg had highest of total EAAs content at 5,486.50 mg 100g⁻¹. In addition, total non-essential amino acids (NEAAs) was highest in quail egg whites at 5,297.99 mg100g⁻¹ which was significantly higher than chicken and snail-eating turtle eggs (p<0.001). Most of NEAAs of quail egg whites were aspartic acid, alanine and tyrosine. The EAAs are health benefits for solving malnutrition problem and improve the quality of life worldwide (Wenefrida et al., 2009). Total amino acid of quail eggs and chicken were significantly higher than snail-eating turtle eggs (p<0.001).

The essential fatty acids (EFAs) contents of all egg yolks were lower than non-essential fatty acids (NEFAs) and total fatty acids of quail and chicken were significantly higher than snail-eating turtle (p<0.001) (Table 3). Most NEFAs of all eggs were oleic acid, palmitoleic acid, stearic acid while most EFAs was linoleic acid. Both saturated and unsaturated fatty acids (SFA and UFA) were highest content in quail egg at 7.41 and 13.30 g 100g⁻¹. They were significant difference with snail-eating turtle egg (p<0.001). Unsaturated to saturated fatty acid ratios of all types eggs were in range from 1.79 to 2.33 which was highest ratio in snail-eating turtle egg. EFAs are omega-3 (ω-3, linolenic acid) and omega-6 (ω-6, linoleic acids) which humans cannot synthesize, and must be obtained through diet. Linolenic acid (ALA) will convert into eicosapentaenoic acid (EPA), and later into docosahexaenoic acid (DHA). EPA and the GLA synthesized from linoleic acid are later converted into hormone-like compounds known as eicosanoids, which aid in many bodily functions including vital organ function and intracellular activity. The level of ω-3 in eggs can be naturally high.
advantage, particularly through their DHA content may be similar to those produced following the fish oils consumption and their ability can decrease risk factors for heart disease (Oh et al., 1991). The previous conducted that one egg consumption per day is unlikely to have impact on cardiovascular disease risk in non diabetes and egg consumption has been promoted satiety (Vander et al., 2005). The quail egg had the most content of EFAs. They can support the cardiovascular, reproductive, immune, and nervous systems (Santos and Santos, 1979; Mahesh and Brann, 2005; Cobbold et al., 2009). The snail-eating turtle egg is the highest of $\omega$-3 to $\omega$-6 ratio at 0.23, which is the better value for human nutritional need (Simopoulos, 1998). Moreover, quail egg had highest of UFA and SFA content but snail eating-turtle egg was the highest of UFA to SFA ratio at 2.33.

Table 2 Amino Acids in egg whites of of chicken, quail and snail-eating turtle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chicken (mg 100g$^{-1}$)</th>
<th>Quail (mg 100g$^{-1}$)</th>
<th>Snail-eating turtle (mg 100g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Non-Essential Amino Acid (NEAA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>337.00 ± 5.66$^{h,i}$</td>
<td>739.00 ± 33.94$^{g,h}$</td>
<td>16.65 ± 4.99$^{g,i}$</td>
</tr>
<tr>
<td>Arginine</td>
<td>459.50 ± 9.19$^{h,i}$</td>
<td>311.00 ± 4.24$^{g,h}$</td>
<td>13.92 ± 24.10$^{g,i}$</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>482.00 ± 26.87$^{h,i}$</td>
<td>1488.00 ± 91.92$^{g,h}$</td>
<td>16.35 ± 6.51$^{g,i}$</td>
</tr>
<tr>
<td>Cystine</td>
<td>39.20 ± 1.13$^{d,e}$</td>
<td>107.00 ± 7.07$^{g,h}$</td>
<td>16.10 ± 27.89$^{d}$</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>738.50 ± 19.09$^{h,i}$</td>
<td>170.50 ± 14.85$^{g,h}$</td>
<td>46.07 ± 8.33$^{g,i}$</td>
</tr>
<tr>
<td>Glycine</td>
<td>197.50 ± 7.78$^{h,i}$</td>
<td>473.50 ± 36.06$^{g,h}$</td>
<td>16.04 ± 5.35$^{g,i}$</td>
</tr>
<tr>
<td>Histidine</td>
<td>523.00 ± 26.87$^{h,i}$</td>
<td>293.5 ± 19.09$^{g,h}$</td>
<td>7.22 ± 6.25$^{g,i}$</td>
</tr>
<tr>
<td>Proline</td>
<td>342.50 ± 0.71$^{h,i}$</td>
<td>488.50 ± 20.51$^{g,h}$</td>
<td>31.27 ± 9.35$^{g,i}$</td>
</tr>
<tr>
<td>Serine</td>
<td>720.00 ± 1.13$^{h,i}$</td>
<td>646.50 ± 22.63$^{g,h}$</td>
<td>54.37 ± 37.37$^{g,i}$</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>49.00 ± 0.99$^{h,i}$</td>
<td>664.50 ± 0.64$^{g,h}$</td>
<td>0.00 ± 0.00$^{g,i}$</td>
</tr>
<tr>
<td>Total NEAAs</td>
<td>3960.50 ± 68.17$^{h,i}$</td>
<td>5297.00 ± 9.83$^{g,h}$</td>
<td>216.16 ± 87.37$^{g,i}$</td>
</tr>
<tr>
<td>Essential Amino Acid (EAA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>530.50 ± 12.02$^{b,i}$</td>
<td>597.5 ± 43.13$^{b,g}$</td>
<td>22.5 ± 10.53$^{g,i}$</td>
</tr>
<tr>
<td>Leucine</td>
<td>1,195.00 ± 205.06$^{b,i}$</td>
<td>1,139.00 ± 73.54$^{b,g}$</td>
<td>53.83 ± 21.57$^{g,i}$</td>
</tr>
<tr>
<td>Lysine</td>
<td>1,410.00 ± 42.43$^{h,i}$</td>
<td>790.00 ± 24.04$^{g,h}$</td>
<td>68.90 ± 23.56$^{g,i}$</td>
</tr>
<tr>
<td>Methionine</td>
<td>481.50 ± 3.54$^{i}$</td>
<td>484.00 ± 5.66$^{g}$</td>
<td>4.49 ± 4.05$^{g,i}$</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1,140.00 ± 28.28$^{h,i}$</td>
<td>727.50 ± 27.58$^{g,h}$</td>
<td>32.37 ± 16.88$^{g,i}$</td>
</tr>
<tr>
<td>Threonine</td>
<td>42.10 ± 4.81$^{f,h}$</td>
<td>740.00 ± 0.00$^{g,h}$</td>
<td>5.50 ± 9.53$^{g,f}$</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>85.50 ± 5.09$^{e,f}$</td>
<td>146.00 ± 0.00$^{g,f}$</td>
<td>14.16 ± 17.90$^{e,g}$</td>
</tr>
<tr>
<td>Valine</td>
<td>488.00 ± 12.73$^{h,i}$</td>
<td>862.5 ± 65.76$^{g,h}$</td>
<td>25.8 ± 6.89$^{e,g}$</td>
</tr>
<tr>
<td>Total EAAs</td>
<td>5372.60 ± 5166.60$^{i}$</td>
<td>5486.50 ± 2908.2$^{g}$</td>
<td>227.55 ± 22.81$^{g,i}$</td>
</tr>
<tr>
<td>Total AAs</td>
<td>9333.10 ± 104.36$^{i}$</td>
<td>10783.00 ± 58.62$^{g}$</td>
<td>443.70 ± 160.70$^{g,i}$</td>
</tr>
</tbody>
</table>

$^{a,b,c}$ significant difference between types of egg at $p<0.05$
$^{d,e,f}$ significant difference between type of eggs at $p<0.01$
$^{g,h,i}$ significant difference between type of eggs at $p<0.001$

Trans fatty acids were not significant difference among three types of eggs. They were in low range between 0.03 to 0.04 g 100g$^{-1}$. Trans fat is the UFAs with trans-isomer fatty acids which may be MUFA or PUFA. The primary health risk identified for trans fat consumption is an elevated risk of coronary heart disease (Mazaffarain, et al., 2006).

Moreover, egg yolks of quail had the highest contents of vitamin A and vitamin D, whilst the chicken egg had highest level
of vitamin E but had no vitamin D (Table 4). It was inconsistent with previous report that egg yolk is one of the few foods naturally containing vitamin D (NRC, 1976). The retinol levels of quail, chicken and turtle in this study were similar to recent study (4.97, 8.96 and 0.04 to 0.23 µg g⁻¹ yolk) in Japan (Irie et al., 2010). Vitamin D is a fat-soluble vitamin that is naturally present in very few foods and essential for promoting calcium absorption in the gut and maintaining adequate serum calcium and phosphate concentrations to enable normal mineralization of bone and prevent hypocalcemic tetany, also helps protect older adults from osteoporosis. Vitamin D has other roles in human health, including modulation of neuromuscular and immune function and reduction of inflammation (Hayes, et al., 2003; DeLuca, 2004). Vitamin E of chicken, quail and turtle eggs were 6.37, 5.92 and 3.93 mg 100g⁻¹ respectively whose all eggs were higher than their vitamin A and vitamin D. There are many reports of health benefits from vitamin E diets (Cordero et al., 2010; Usoro and Mousa, 2010).

Lastly, sex hormones (LH, FSH, PRL, P, E2 and T) were high in egg yolks but all types of eggs were highest of P (Table 5). The hormone P was highest in chicken egg yolks (1149.40 ng g⁻¹) which was significantly higher than quail (307.79 ng g⁻¹, p<0.05) and snail-eating turtle eggs (121.88 ng g⁻¹, p<0.05). Whilst the other hormone was not statistical different among three types of eggs. Over all of whole eggs, all hormones were similar profiles to egg yolks which P was highest in chicken (807.06 ng g⁻¹) which its concentration was significantly higher than quail (314.83 ng g⁻¹, p<0.05) and snail-eating turtle (157.30 ng g⁻¹, p<0.05) as shown in Table 6. Health benefits of natural P are antidepressant, balancing blood sugar level, decreasing PMS and menopause symptoms and weight loss (Daniel B). The PRL of all eggs were low which human PRL functions during pregnancy and lactation (Tucker, 1979; Gregg, 2009). A poor life can be caused by hormonal problems.

The previous studies reported that dietary sources of maternal diet affected on fatty acid compositions (Rizzo et al., 1999; Huang et al., 2003) and lipid compositions in egg yolk are important for viability, growth and/or embryo development (Delaunay et al., 1993; Noble and McCartney, 1993; Noble et al., 1996). This study showed that most of fatty acid profiles of chicken and quail egg yolks were similar cause of their same species as well as EAAs but they were significant different from snail eating-turtle. Snail eating turtle had highest of UFA to SFA and ω-3 to ω-6 ratio but low of vitamin A, D and E contents. The fact that, in case of chicken and quail eggs contained much higher levels of vitamin E which may be relevant to the successful development of embryo (Hossain et al., 1998). Vitamin E is very effective natural antioxidant and may have important role in preventing peroxidative damage to developing tissues (Speake et al., 1999). Thus, snail-eating turtle may resist the embryo development. Moreover, sex steroid of maternal origin could be found in egg yolks of many species (Adkins-Regan et al., 1995; Janzen et al., 1998) and correlated with maternal circulating plasma (Schwabl, 1996). Steroid hormones varied with the species reproductive strategy and social requirement which may affect male and female offspring differently (Petrie et al., 2001).

Conclusions

Snail-eating turtle egg was relatively poor in nutritional value compared to those of chicken or quail eggs. Essential fatty acid of ω-3 fatty acid, linolenic acid, was found more in snail-eating turtle egg, and chicken was an alternative source of vitamin E and progesterone (P) sex hormone.
Table 3  Fatty acid profiles of egg yolks of chicken, quail and snail-eating turtle

<table>
<thead>
<tr>
<th>Parameters (mg 100g⁻¹)</th>
<th>C number</th>
<th>Chicken Mean ± S.D.</th>
<th>Quail Mean ± S.D.</th>
<th>Snail-eating turtle Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-Essential Fatty Acid (NEFA)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caprylic acid</td>
<td>C8:0</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Capric acid</td>
<td>C10:0</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Lauric acid</td>
<td>C12:0</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Tridecanoic acid</td>
<td>C13:0</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>C14:0</td>
<td>0.06 ± 0.00^c</td>
<td>0.14 ± 0.06</td>
<td>0.21 ± 0.06^c</td>
</tr>
<tr>
<td>Tetradecenoic acid</td>
<td>C14:1</td>
<td>0.01 ± 0.00^c</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01^c</td>
</tr>
<tr>
<td>Pentadecanoic acid</td>
<td>C15:0</td>
<td>0.01 ± 0.00^c</td>
<td>0.02 ± 0.02</td>
<td>0.05 ± 0.02^c</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C16:0</td>
<td>4.38 ± 0.10^b,i</td>
<td>5.13 ± 0.08^b,g</td>
<td>1.24 ± 0.34^g,i</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>C16:1</td>
<td>0.41 ± 0.01^c</td>
<td>0.76 ± 0.01</td>
<td>0.98 ± 0.22^c</td>
</tr>
<tr>
<td>Margaric acid</td>
<td>C17:0</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.00</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C18:0</td>
<td>1.44 ± 0.03^c,j</td>
<td>2.03 ± 0.05^c,g</td>
<td>0.45 ± 0.13^g,i</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1</td>
<td>8.07 ± 0.25^i</td>
<td>8.84 ± 0.18^g</td>
<td>2.65 ± 0.62^g,i</td>
</tr>
<tr>
<td>trans-Elaidic acid</td>
<td>C18:1trans</td>
<td>0.03 ± 0.00</td>
<td>0.04 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Eicosenoic acid</td>
<td>C20:1, ω-9</td>
<td>0.05 ± 0.00</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td><strong>Total NEFAs</strong></td>
<td></td>
<td>14.58 ± 0.40^i</td>
<td>17.09 ± 0.33^g</td>
<td>5.83 ± 1.43^g,i</td>
</tr>
<tr>
<td><strong>Essential Fatty Acid (EFA)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>C18:2, ω-6</td>
<td>2.98 ± 0.08</td>
<td>2.58 ± 0.05</td>
<td>0.32 ± 0.09</td>
</tr>
<tr>
<td>g-Linolenic acid</td>
<td>C18:3, ω-6</td>
<td>0.02 ± 0.00</td>
<td>0.05 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>C18:3, ALA, ω-3</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>Eicosadienoic acid</td>
<td>C20:2, ω-6</td>
<td>0.04 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>g-Eicosatrienoic acid</td>
<td>C20:3, ω-3</td>
<td>0.00 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>Eicosatrienoic acid</td>
<td>C20:3, ω-6</td>
<td>0.05 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>C20:4, ω-6</td>
<td>0.42 ± 0.00</td>
<td>0.44 ± 0.01</td>
<td>0.47 ± 0.13</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>C20:5, EPA, ω-3</td>
<td>0.00 ± 0.00</td>
<td>0.02 ± 0.00</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>C22:6, DHA, ω-3</td>
<td>0.15 ± 0.00</td>
<td>0.50 ± 0.00</td>
<td>0.04 ± 0.02</td>
</tr>
<tr>
<td>ω-3/ω-6</td>
<td></td>
<td>0.06</td>
<td>0.19</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>Total EFAs</strong></td>
<td></td>
<td>3.71 ± 0.08^i</td>
<td>3.70 ± 0.03^g</td>
<td>1.33 ± 0.20^g,i</td>
</tr>
<tr>
<td><strong>Total FAs</strong></td>
<td></td>
<td>18.28 ± 0.48^i</td>
<td>20.79 ± 0.35^i</td>
<td>7.87 ± 1.24^g,i</td>
</tr>
</tbody>
</table>

^a,b,c,d: Significant difference between types of egg at p<0.05
^de,f: Significant difference between type of eggs at p<0.01
^g,h,i: Significant difference between type of eggs at p<0.001
Table 4 Vitamin A, D and E in egg yolks of chicken, quail and snail-eating turtle

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chicken Mean ± S.D.</th>
<th>Quail Mean ± S.D.</th>
<th>Snail-eating turtle Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A (Retinol) (µg 100g⁻¹)</td>
<td>482.5 ± 23.33 b,c</td>
<td>717.00 ± 0.00 g,h</td>
<td>19.47 ± 4.40 g,i</td>
</tr>
<tr>
<td>Vitamin D (µg 100g⁻¹)</td>
<td>0.00 ± 0.00 h</td>
<td>1.14 ± 0.00 g,h</td>
<td>0.12 ± 0.12 g</td>
</tr>
<tr>
<td>Vitamin E (mg 100g⁻¹)</td>
<td>6.37 ± 0.16 f</td>
<td>5.92 ± 0.09 d</td>
<td>3.93 ± 0.65 d,f</td>
</tr>
</tbody>
</table>

a,b,c significant difference between types of egg at p<0.05  
d,e,f significant difference between type of eggs at p<0.01  
g,h,i significant difference between type of eggs at p<0.001

Table 5 Sex hormones in egg yolks of snail-eating turtle, quail and chicken

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chicken Mean ± S.D.</th>
<th>Quail Mean ± S.D.</th>
<th>Snail-eating turtle Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (IU g⁻¹)</td>
<td>0.23 ± 0.07</td>
<td>0.19 ± 0.15</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>FSH (IU g⁻¹)</td>
<td>0.38 ± 0.03</td>
<td>0.33 ± 0.18</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>PRL (ng g⁻¹)</td>
<td>&lt;0.25 ± 0.00</td>
<td>&lt;0.25 ± 0.00</td>
<td>&lt;0.25 ± 0.00</td>
</tr>
<tr>
<td>P (ng g⁻¹)</td>
<td>1149.40 ± 571.13 b,c</td>
<td>307.79 ± 231.11 b</td>
<td>121.88 ± 39.37 c</td>
</tr>
<tr>
<td>E2 (ng g⁻¹)</td>
<td>0.27 ± 0.04</td>
<td>0.22 ± 0.08</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>T (ng g⁻¹)</td>
<td>2.78 ± 3.53</td>
<td>4.28 ± 5.31</td>
<td>4.23 ± 1.00</td>
</tr>
</tbody>
</table>

a,b,c significant difference between types of egg at p<0.05  
d,e,f significant difference between type of eggs at p<0.01  
g,h,i significant difference between type of eggs at p<0.001

Table 6 Sex hormones in whole eggs of snail-eating turtle, quail and chicken

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Chicken Mean ± S.D.</th>
<th>Quail Mean ± S.D.</th>
<th>Snail-eating turtle Mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>LH (IU g⁻¹)</td>
<td>0.18 ± 0.06</td>
<td>0.11 ± 0.08</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>FSH (IU g⁻¹)</td>
<td>0.26 ± 0.12</td>
<td>0.19 ± 0.14</td>
<td>0.28 ± 0.14</td>
</tr>
<tr>
<td>PRL (ng g⁻¹)</td>
<td>&lt;0.19 ± 0.00</td>
<td>&lt;0.19 ± 0.00</td>
<td>&lt;0.19 ± 0.00</td>
</tr>
<tr>
<td>P (ng g⁻¹)</td>
<td>807.06 ± 342.34 b,c</td>
<td>314.83 ± 7.04 b</td>
<td>157.30 ± 48.92 c</td>
</tr>
<tr>
<td>E2 (ng g⁻¹)</td>
<td>0.23 ± 0.04</td>
<td>0.11 ± 0.11</td>
<td>0.22 ± 0.09</td>
</tr>
<tr>
<td>T (ng g⁻¹)</td>
<td>3.57 ± 0.79</td>
<td>3.08 ± 1.20</td>
<td>3.23 ± 1.00</td>
</tr>
</tbody>
</table>

a,b,c significant difference between types of egg at p<0.05  
d,e,f significant difference between type of eggs at p<0.01  
g,h,i significant difference between type of eggs at p<0.001

Acknowledgments

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Centenary Academic Development Project, Chulalongkorn University.

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Brophy, T.R. 2006. A llometry and sexual dimorphism in the snail-eating turtle


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Crude Malva Nut Gum Affects Pasting and Textural Properties of Wheat Flour in the Presence or Absence of Sodium Chloride

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² Department of Food Technology, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand
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Abstract

This research aims to investigate the effect of crude malva nut gum (CMG) addition on pasting and textural properties of wheat flour in the presence or absence of sodium chloride (NaCl). The mixtures were prepared by adding 0, 2.5, 5, 7.5 and 10% of CMG into wheat flour. Pasting results revealed that addition of CMG significantly elevated (P≤0.05) the peak viscosity (126-676 RVU), hot paste viscosity (75-333 RVU), breakdown (50-342 RVU) and final viscosity (153-552 RVU). Pasting temperature (61-83°C) of the pastes decreased with increasing CMG content. The textural parameters including hardness, springiness, cohesiveness, gumminess and chewiness of the mix gels decreased with addition of CMG. The presence of 0.75% NaCl in the mixtures had higher viscosity than the presence of 0 and 1.5% NaCl in the mixtures. The hardness of gel mixed with 0-2.5% CMG increased with increasing NaCl levels, while no significant effect (P>0.05) at higher level of CMG (7.5-10%). Higher paste viscosity and softer gel texture of wheat flour could be altered using CMG. The presence of NaCl affects both pasting and textural properties in different ways.

Keywords: crude malva nut gum, pasting property, sodium chloride, textural property, wheat flour,

Introduction

Wheat flour is commonly used in many food industries. Starch gelatinization and gelling behavior quantified by pasting and textural properties are considered as basic processes in the making of food containing starch such as noodle, bread, etc (Rojas et al., 1999). Pasting properties of starch depend on several factors including hydrocolloids, sucrose or salt incorporation. Salt, especially sodium chloride (NaCl), is a basic ingredient used in food products and has an influence on the gelatinization and retrogradation of starch (Funami et al., 2008). In the presence of salt, the hydration and swelling of starch granules decreases due to the reduction of available water for gelatinization (Baker and Rayas-Duarte, 1998). The most general effect of salt is to raise the gelatinization temperature when compared to gelatinization in excess of pure water (Jang et al., 2001). During storage, starch gels undergo structural transformation in terms of recrystallization and retrogradation (Biliaderis and Zawistowski, 1990). Addition of salts retards the retrogradation of the starch due to an improvement of the starch granule integrity and an increase of paste consistency (Chang and Liu, 1991). Thirathumthavorn and Trisuth (2008) found that NaCl increased the gelatinization temperatures and decreased retrogradation of both native and hydroxypropylated crosslinked tapioca starches.

Hydrocolloids or non-starch polysaccharides are usually applied to starch-based foods due to their desirable effects with improvement of food texture by
the retardation of starch retrogradation (Pongsawatmanit and Srijunthongsiri, 2008) and the increase of moisture retention with the reduction of syneresis. These advantages result in extending the overall quality of the product during the storage time, and improving texture stability of gel (Williams et al., 2009). Funami et al. (2008) found that the addition of iota-carrageenan decreased the pasting temperature of the composite system in the absence of salts and in the presence of monovalent cations, while the temperature increased in the presence of divalent cations.

Malva nut (Scaphium scaphigerum) seed is found in South-East Asia and China, and used in traditional medicine. The seed contains a large amount of mucilaginous substance which can be extracted from the seed by soaking in water and collected as jelly. The malva nut jelly is commonly consumed as a dessert when mixed with sugars. Purification of this jelly can be further done by ethanol and sodium hydroxide (NaOH) to obtain gum or hydrocolloid. The major carbohydrate compositions of the gum are the monosaccharide arabinose, galactose and rhamnose. The molecular weight of malva nut gum was very high, which was significantly reduced by the purification processing (dialysis and decoloring). FT-IR spectroscopy and methylation analysis showed that malva nut gum had similar structure to gum arabic (Somboonpanyakul et al., 2006). Srichamroen and Chavasit (2011) extracted the malva nut gum by different concentrations of NaOH to discard the protein content in the gum. They found that rheological properties of the pure gel of malva nut gum influenced by different conditions of solvent (pH, ionic strength, and co-solutes addition). NaCl has a strong effect on swelling of gum solution. Due to high water absorption capacity of crude malva nut gum (CMG), the beneficial aspect of the CMG is observed in the reduction of cooking loss and improved textural properties (hardness and springiness) of frankfurters (Somboonpanyakul et al., 2007).

Although some researchers have been reporting the applications of this gum in starch-based food systems, the information of the influence of NaCl in wheat flour-CMG mixtures is not known. Therefore, the objective of this research was to investigate the effects of CMG addition in wheat flour and the presence or absence of NaCl on the pasting and textural properties of wheat flour systems.

**Materials and Methods**

**Crude Malva Nut Gum Preparation**

Malva nut seeds were purchased from Chantaburi province, Thailand. Crude malva nut gum (CMG) was extracted following the method of Somboonpanyakul et al. (2007) with a slight modification. The malva nut seeds were soaked in water (1:80 w/v) at 30°C for 2 h to completely hydrate and swell the fruit. The excessive water was removed by filtering through a 60-mesh silk screen. The crude mucilage was precipitated with 3 volumes of 95% absolute ethanol and dried at 40°C for 12 h. Dried CMG was milled using an ultra centrifugal mill (ZM 200, Retsch, Germany) and then sieved through a 80-mesh screen. Yield of dried CMG was about 19.4%. The CMG samples were vacuum packed in laminated foil bags until further analyzed.

**Color and Proximate Analyses of CMG**

Colorimetric measurement of CMG was determined in triplicate using a Chromameter (CR-410, Konica-Minolta, Japan). Chemical composition of CMG sample including moisture, protein, fat, fiber, and ash content was determined in triplicate using procedures of AOAC (2000). Total starch content of CMG was determined using a method derived from Megazyme (Megazyme International Ireland Ltd., Wicklow, Ireland).

**Pasting Properties of Wheat-CMG Mixtures**

Wheat flour-CMG dry mixtures were prepared by incorporating CMG at the concentrations of 2.5%, 5%, 7.5% or 10% (w/w) of the flour weight. To prepare the samples with the presence or absence of
NaCl, NaCl was incorporated in the wheat flour content to the wheat flour-CMG mixtures regarded to the presence (0.75 and 1.5%) or absence (0%) of NaCl. No CMG and NaCl sample (100% wheat flour) was used as control sample. Pasting properties of wheat flour-CMG mixtures were examined using a Rapid Visco Analyzer (RVA-4D, Newport Scientific, Warriewood, Australia). A suspension of 10% wheat/CMG powder in deionized water (db, w/v) was heated from 50 to 95°C at a uniform rate of 12°C/min with constant stirring at 160 rpm. The sample was held at 95°C for 2.5 min, then cooled to 50°C at a rate of 13°C/min, and held for 2 min. Total cycle time was 13 min using the standard profile (Jangchud et al., 2003). Pasting temperature (PT), peak viscosity (PV), breakdown (BD), hot paste viscosity (HPV), final viscosity (FV) and setback (SB) were recorded. The viscosity values were reported in term of RVA units (RVU). All measurements were performed in triplicate.

Gel Textural Properties of Wheat Flour-CMG

Samples of wheat-CMG gels were prepared according to the methods of Brennan et al. (2008) with a slight modification. Warm pastes obtained from the RVA determination above were used for gel texture analysis. The pastes were poured into cylindrical stainless moulds (20 mm inner diameter by 10 mm height). The mixed gels wrapped with plastic film to avoid moisture loss were held at 25°C for 24 h to equilibrate before measurement. Three batches of each mixture were prepared. Texture profile analysis (TPA) tests were performed with a Texture Analyzer (TA-XTplus, Stable Micro Systems, Surrey, UK) fitted with a P35 probe (35 mm probe). The samples were compressed for 50% strain. The textural parameters were calculated: hardness, springiness, cohesiveness, gumminess and chewiness. Ten replicate samples were measured for each batch.

Statistical Analysis

Analysis of variance (ANOVA) was performed. Duncan’s multiple range test (DMRT) was performed for multiple comparisons at 95% confidence (P≤0.05).

Results and Discussion

Color and Chemical Composition of CMG

Color of the CMG was dark brown with the CIE L*, a* and b* values were 47.0, 6.6 and 12.2, respectively. The moisture content of the CMG sample was about 12.5%. The CMG sample had 6.7% protein and 0.36% fat content as shown in Table 1. Total starch in CMG was not detected, representing the non-starch polysaccharides of CMG. Protein and fat content in material could affect pasting properties of starchy materials (Jangchud et al., 2003). The purification of malva nut gum can be obtained by alkaline extraction. The higher alkaline concentration decreased protein content in the gum but it also led to reduction of carboxylic bonds with galacturonic acids (Srichamroen and Chavasit, 2011).

Table 1 Chemical composition of CMG.

<table>
<thead>
<tr>
<th>Chemical composition</th>
<th>Content (% wb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>12.50</td>
</tr>
<tr>
<td>Protein</td>
<td>6.72</td>
</tr>
<tr>
<td>Fat</td>
<td>0.36</td>
</tr>
<tr>
<td>Fiber</td>
<td>3.83</td>
</tr>
<tr>
<td>Ash</td>
<td>4.99</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>71.53</td>
</tr>
</tbody>
</table>

Effect of CMG and NaCl on Pasting Behavior

The pasting properties measured using a RVA for wheat flour pasted with 0, 2.5, 5, 7.5 and 10% of CMG are presented in Figure 1. The viscosities of wheat pastes were strongly affected by CMG concentrations. The temperature at the onset of this rise in viscosity can be considered as the starting point of gelatinization and is defined as the pasting temperature in RVA test. The PT of pure wheat flour sample (non-CMG) was about 71.8°C. The PT significantly decreased (P≤0.05) with 2.5-10% of CMG (61-68°C) as shown in Table 2. The PT of wheat flour
depended on the types of flour and flour mixture (Ragaee and Abdel-Aal, 2006). A reduction about 3°C of the PT was also found when 1% alginate was added implying an earlier beginning of starch gelatinization (Rojas et al., 1999). Funami et al. (2008) also found that the addition of hydrocolloids as iota-carrageenan decreased the PT of the composite system in the absence of salts. For non-CMG sample, the PT increased from 71.8°C to 83.1°C as the salt concentration increased from 0 to 1.5%. When the wheat flour incorporated with CMG, the presence of NaCl resulted in the PT of mixtures in different ways. Increase of NaCl from 0 to 1.5% of most wheat-CMG mixtures increased the PT, except for the mixtures incorporated of 5% CMG which reduction of the PT was observed (Table 2). These results were also consistent with other published studies of other starches on pasting properties in the presence of salts (Baik et al., 2010). As the concentration of NaCl increased, the gelatinization temperature increased to a certain level (ca 5-10°C), and then decreased as the concentration increased. However, as the increase in gelatinization temperature caused by sodium ion was limited, the influence of the anion (Cl⁻) became dominant when the concentration of NaCl was raised beyond 2% (Maaurf et al., 2001). Furthermore, repulsion between the electronegative OH groups of starch and the negatively charged Cl⁻ ions increases the resistance of starch to gelatinization (Pukkahuta et al., 2008).

Degree of swelling of granule during heating and the starch with higher swelling capacity causes the greater peak viscosity (Ragaee and Abdel-Aal, 2006). The PV is considered to represent the equilibrium point between swelling and the rupture of starch granules. Addition of CMG resulted in significantly increased (P≤0.05) in the PV (Table 2). The increase in viscosity is probably due to higher water absorption of CMG. CMG absorbs high amount of water and reduces the free water in the systems, resulting in increased viscosity. The PV of 10% CMG addition increased about four folds when compared to the non-CMG sample. Either a thickening effect from CMG or the interactions between CMG and swollen starch particles might be responsible for this result (Rojas et al., 1999). Charoenrein et al. (2011) also found that addition of konjac glucomannan resulted in increased viscosity of rice starch.

Swelling of granules accompanied by leaching of amylose increases the viscosity while granules may rupture during further shearing which results in viscosity decrease. The hot paste viscosity of wheat flour was about 78.5 RVU (Table 2). In addition, the HPV increased significantly (P≤0.05) with an increase in all levels of CMG concentrations. Leached amylose is more or less aligned in the direction of flow that contributes to the breakdown. The BD is correlated with the stability of starch granule under high shear condition (Ragaee and Abdel-Aal, 2006). It is shown that increasing of CMG addition gave greater BD. The 10% of CMG had the highest BD value (342.5 RVU). Due to high viscosity of wheat flour-CMG mixture, the SB from peak (calculated from FV-PV) is useful for indicating retrogradation. Increasing of CMG had no significant (P>0.05) effect on the SB from peak, except for wheat flour mixed with 10% CMG. It is revealed that addition of CMG into the wheat flour could retard the rate of starch retrogradation (Figure 1A).

The presence of NaCl in the wheat flour-CMG mixture resulted in a significant change (P≤0.05) in the PV, HPV, BD, FV and SB from peak (Table 2). When small amount of NaCl (0.75%) was incorporated into the wheat-CMG mixtures, the PV of the mixtures increased, except for 10% CMG incorporated sample. This indicates that synergistic effect of salt and CMG on the viscosity can be pronounced. Ionic strength from salt increased the molecular bonding of the CMG, resulting in increasing viscosity of the pastes (Pukkahuta et al., 2008). In contradictory, the viscosity may retard
diffusion of salt into the starch granules when 10% CMG was incorporated, leading to lower the PV. When 1.5% NaCl was added into the mixtures, the PV of wheat-CMG mixtures significantly decreased ($P \leq 0.05$) when compared to no NaCl sample for all levels of CMG incorporation. In the presence of higher NaCl, CMG is more difficult to penetrate into the starch granules in ordered molecular structures, building up the osmotic pressure in the continuous phase and inhibiting water migration for starch swelling, resulting in reduction of viscosity (Funami et al., 2008). The lower value of BD indicates the better stability of the starch paste. Addition of NaCl improved the stability of starch paste incorporating CMG by lowering BD value. This is consistent with the results of Zhou et al. (2011) which found that addition of 3% NaCl in Angelica dahurica starch resulted in decreased BD.

**Effect of CMG and NaCl on Gel Textural Properties**

Response surface curve demonstrated the effect of both CMG and NaCl on textural properties of gel mixtures including hardness, springiness, cohesiveness, gumminess, and chewiness (Figure 2-6). CMG resulted in significantly reduced ($P \leq 0.05$) the hardness, springiness, gumminess and chewiness of gel. Hardness of gel mixed with CMG varied from 107-207 g-force (Figure 2). Lower gel hardness is probably due to incompletely dissolved in water and only swells to a mucilaginous dispersion of CMG as non-gelling polysaccharide (Sricharoen and Chavasit, 2011). CMG is not capable of forming an undivided network structure, leading to lower hardness of wheat flour gel. This is consistent with the results of Charoenrien et al. (2011) which found that konjac glucomannan reduced the hardness of rice starch. However, the gel hardness depended on types and concentration of hydrocolloids. Techawipharat et al. (2008) found that the hardness of rice starch pastes increased by addition of carrageenan, but were unaffected by the other hydrocolloids such as cellulose derivatives. In the presence of NaCl, higher concentration of NaCl increased the electrostatic repulsion of polymer to form the solid like gel. Sricharoen and Chavasit (2011) stated that 1% of NaCl improved the gel formation of CMG by increased storage modulus ($G''$). However, there was no gel formation if 0.5% NaCl was used. This is probably due to intramolecular electrostatic repulsion between charged carboxyl groups and polymer-water interactions acting in competition with polymer-polymer interactions (Evageliou et al., 2000).

**Figure 1** Pasting behavior of wheat flour mixed with 0-10% of crude malva nut gum in the presence or absence of NaCl. A indicates no NaCl, B indicates 0.75% NaCl and C indicates 1.5% NaCl.
Table 2 Pasting profile of wheat flour pasted with different concentrations of CMG and NaCl.

<table>
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<tr>
<th>RVA parameters</th>
<th>CMG (%)</th>
<th>0</th>
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<td></td>
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<td>83.1±3.4</td>
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<td>68.6±1.2</td>
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</tr>
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<td>61.2±9.2</td>
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<td>B</td>
</tr>
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<td></td>
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<tr>
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<td>SB from peak (RVU)</td>
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<td>52.2±21.2</td>
<td>22.5±49.6</td>
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Mean values with different lower case letters are significantly different (P<0.05) for interaction effects.
Mean values with different upper case letters are significantly different (P<0.05) for main effects.
<sup>ns</sup> indicates no significantly different (P>0.05).

Springiness represents the elasticity of the materials which is measured from the extent of gel height recovery (Choi and Kerr, 2003). When the sample returns to its original height after pressing, this indicates high springiness value (100% elasticity) of materials (Hoseney and Smewing, 1999). High springiness appears when the gel structure is broken into few large pieces during the first TPA compression, whereas low springiness results from the gel breaking into many small pieces. Less springy gels will break down more
easily during mastication than a firm and spring gel. Results showed that addition of CMG significantly decreased (P≤0.05) springiness which ranged from 0.83-0.89. Addition of 10% CMG reduced the springiness of wheat gel about 3.4% when compared to non-CMG gel sample but the different concentrations of NaCl had no significant effect on springiness (Figure 3).

Cohesiveness is the extent to which a material can be deformed before rupturing (Szczesniak, 2002). Highest cohesiveness (0.66) of gel mixtures was observed when 10% of CMG was used (Figure 4). In addition, higher level of NaCl increased cohesiveness of gel mixtures as shown in Figure 4. Cohesiveness is how well the product withstands a second deformation relative to how it behaved under the first deformation. Higher values of cohesiveness indicate that the product will be tough and difficult to break up in the mouth in relation to sensory perception (Hoseney and Smewing, 1999; Rosenthal, 2010).

Chewiness is the quantity to simulate the energy required for masticating a semi-solid sample to a steady state of swallowing. Chewiness and gumminess values were parallel in their variations with hardness value due to the high relative weight of hardness in the calculation of chewiness. The chewiness and gumminess of wheat flour-CMG gels showed that both values significantly decreased (P≤0.05) with higher CMG addition (5-10%). The textural properties of the wheat flour-CMG mixed gels were dependent on the concentration of CMG. Addition of NaCl (0.75-1.5%) could help to increase chewiness and gumminess of gel mixtures by about 9.7-12.0% and 10.3-13.3% as demonstrated in Figure 5 and 6, respectively.
Conclusions

Addition of crude malva nut gum (CMG) increased paste viscosity. Wheat flour gel could be softer when higher CMG concentrations were used. The presence of NaCl in the mixtures resulted in different effect on both paste viscosity and gel texture. However, the mixtures incorporated with 1.5% NaCl increased setback from peak when 5-10% CMG was applied. The finding of this study demonstrated that use of CMG and incorporated with NaCl altered paste viscosity and gel texture of wheat flour. Therefore, the application of CMG has potential to be applied to starch-based food systems for improving gel viscosity and retarding retrogradation. Further research is required to investigate its application in a starch-based food system containing both starch and NaCl, for example, bakery and dessert products.

Acknowledgments

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References


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Purification and Characterization of Microbial Transglutaminase from *Enterobacter* sp. C2361

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Abstract

Microbial transglutaminase (MTGase) is a family of transferase that catalyzes the acyl-transfer reaction. MTGase has been applied to many proteins containing foods to improve their texture, taste and appearance. In this work the MTGase from *Enterobacter* sp. C2361 was studied after cultivation in fish protein hydrolysate (FPH) medium (3.0 % soluble starch, 2.0% FPH, 0.2% yeast extract, 0.2% K₂HPO₄, 0.2% KH₂PO₄, pH 7.0) at 37°C and 150 rpm for 18 h. The extracellular, cell-bound and cytoplasmic MTGase specific activities of *Enterobacter* sp. C2361 were 0.11, 0.05 and 0.05 U mg⁻¹ protein, respectively. The MTGase activity in the supernatant of *Enterobacter* sp. C2361 was 1.18 U mL⁻¹. The MTGase from *Enterobacter* sp. C2361 was purified to 19.1 folds with the yield of 15.3% by using ammonium sulphate precipitation (50–80 % saturation), ion-exchange chromatography (SP-Sepharose) and size exclusion chromatography (Sephadex G-100). The purified MTGase of *Enterobacter* sp. C2361 had the molecular weight 29.84 KDa by sodium dodecyl sulphate gel electrophoresis. The enzyme exhibited the optimum activity in a range of pH 5.0 to 7.0 and at 37 to 45°C by the hydroxamate assay. The MTGase of *Enterobacter* sp. C2361 was independent to Ca²⁺ and stable in a rage of pH 5.0-7.0 and temperature at 4°C. The obtained MTGase was moderately inhibited by Cu²⁺, Mn²⁺, Hg²⁺ and Zn²⁺. MTGase of *Enterobacter* sp. C2361 possessed not inhibited by ethylene diamine tetraacetate, but was inhibited by iodoacetic acid, N-ethylmaleimide, p-chloromercuri benzoate and phenylmethyl sulfonylfluoride.

Keywords: *Enterobacter* sp., microbial transglutaminase, purification, transferase

Introduction

Transglutaminases (TGases, 2.3.2.13) are known as the amine transferase or protein glutamine γ-glutamyl transferase (Motoki and Seguro, 1998). TGases catalyze the formation of isopeptide by the exchange of an acyl group from γ-carboxyamide (–C(O)NH₂) group of peptides/proteins containing glutamine residue, an acyl donor, to a variety of acyl acceptor including primary amines or ε-amino group of lysine. It results in polymerization, inter- and intra-molecular cross-linking of protein via formation of ε-(γ-glutamyl) lysine linkages (Motoki and Seguro, 1998). TGase could be found in several organisms with various activities (Aeschliman and Paulsson, 1994). Recently, TGase has potential application in food industries (Zhu et al., 1996; Motoki and Seguro, 1998) and textile industries (Cortez et al., 2004).

MTGase is independent to Ca²⁺ ion in catalytic activity. MTGase was reported to produce highly by *Streptoverticillium mobaraense*, Sv. ladakanum, *Sv. cinnamoneum*, *Streptomyces* and *S. lividans* (Duran et al., 1998; Ashie and Lanier, 2000; Lin et al., 2006). MTGase has been purified from cultural filtrate of *Sv. mobaraense* and *Streptoverticillium* sp.
Intracellular MTGase were found in Bacillus subtilis and in the spherules of Physarum polycephalum (Ramanujam and Hageman, 1990; Klein et al., 1992). However, the finding of novel MTGase-producing microorganism with new profile activity is also necessary for further application. To achieve commercialization of MTGase, studies have been continually performed in many laboratories including the screening of microorganisms, scale-up cultivation, downstream process optimization and its application (Zheng et al., 2002b; Lu et al., 2003; Yan et al., 2005; Cui et al., 2007). Recently, a new bacterial strain producing a high MTGase activity were isolated from soil and classified as Enterobacter sp. C2361. There are no report on purification and characterization of MTGase of Enterobacter sp. The objectives of this study were to purify and characterize MTGase produced by these newly isolated strains.

**Materials and Methods**

**Chemicals and Media**

Carbobenzoxy glutamine glycine (CBZ-Gln-Gly), hydroxylamine, glutathione (reduced form), tri-chloroacetic acid, and hydroxamic acid were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Iron (III) chloride, magnesium sulphate, potassium dihydrogen phosphate, and di-potassium hydrogen phosphate were purchased from Fluka Chemical Co. Ltd. (Buchs, Switzerland). Nutrient broth, Bactopeptone, and yeast extract were purchased from Difco™ (Becton Dickinson and Company, USA).

**Strain and Culture Conditions**

Enterobacter sp. C2361 was isolated from wastewater pond of seafood factory in Songkhla, Thailand, and preserved in our laboratory. The strain from agar slant was cultured FPH medium (3.0% starch, 2.0% fish protein hydrolysate, 0.2% yeast extract, 0.2% Mg2SO4, 0.2% KH2PO4 and 0.2% K2HPO4, pH 7.0 in 250 mL conical flask and incubated at 37˚C, 150 rpm for 18 h. The cell pellet was removed by centrifugation at 7,500×g for 20 min at 4˚C. The MTGase activity and protein content were analyzed using hydroxamate assay (Folk and Cole, 1965) and Lowry assay method (Lowry et al., 1951).

**Preparation of Cell-free Extracts**

The cells of Enterobacter sp. C2361 were collected by centrifugation at 7,500×g for 20 min at 4˚C. The supernatant was saved as extracellular proteins and the cell pellets were used for extraction of cytoplasmic and periplasmic proteins. Firstly, the cell pellets were washed once in cold 20 mM Tris-HCl (pH 7.3) buffer containing 30 mM NaCl and resuspended in 1.5 mL of the same buffer (Urban and Celis, 1990). An aliquot of 750 µl was transferred to an Eppendorf tube and recollected by centrifugation at 7,500×g for 20 min at 4˚C. The packed cells were resuspended in 750 µL of 33 mM Tris-HCl containing 10 mM EDTA (pH 8.0) and ruptured by disruption twice for 15 sec using an ultrasonic homogenizer (Cole Parmer Instrument Co., Chicago IL, USA). After centrifugation at 10,000×g for 15 min at 4˚C, the supernatant was saved as cytoplasmic extract. The cell pellets were dried with iced-cold acetone and blended using mortar to be used as cell bound protein powder. The obtained fractions were analyzed in the hydroxamate assay (Folk and Cole, 1965) and Lowry assay method (Lowry et al., 1951).

**Precipitation of MTGase**

The culture broth was precipitated by three different methods, using solvents or (NH4)2SO4. In the solvent methods, the culture broth was added by chilled ethanol or acetone to give a final concentration of 70% (v/v) at 4˚C. A latter method, the culture broth was precipitated at 50% (NH4)2SO4 and centrifuged at 10,000×g for
20 min at 4˚C to discard undigested starch and some proteins. After that, the supernatant was further precipitated to 60, 70 or 80 % saturation of (NH₄)₂SO₄. The whole mixtures were left at 4˚C for 12 h. The precipitates were collected by centrifugation at 10,000×g for 20 min at 4˚C. The obtained precipitates were dissolved in 50 mM citrate-phosphate buffer (pH 6.5) and dialyzed overnight against 20 mM citrate-phosphate buffer, pH 6.5. The obtained samples were analyzed for the hydroxamate assay (Folk and Cole, 1965) and Lowry assay method (Lowry et al., 1951). The method gave the highest MTGase specificity was chosen for further processes.

**Purification of MTGase**

The enzyme was applied to a SP-Sepharose chromatography column (2.6×20 cm) previously equilibrated with 50 mM citrate-phosphate buffer, pH 6.5. The column was washed extensively with the same buffer and eluted with a linear gradient of 0.0-0.5 M NaCl in the same buffer at a flow rate of 0.5 mL min⁻¹. The fractions with MTGase activity were pooled and re-precipitated using 80 % saturation of (NH₄)₂SO₄, dissolved in the same buffer and dialyzed overnight against the same buffer. The dialyzed enzyme solution was freeze-dried and dissolved in the same buffer to the final concentration of 1 mL min⁻¹. The MTGase was further purified by gel filtration on a column of Sephadex G-100 (2.6×90 cm) which was pre-equilibrated with the same buffer. The sample was loaded onto the column with a flow rate of 0.25 mL min⁻¹. The MTGase active fractions were analyzed for activity by hydroxamate assay (Folk and Cole, 1965), protein content by Lowry assay method (Lowry et al., 1951), and protein profiles by SDS-PAGE (Laemmli, 1970).

**Effect of pH on Activity and Stability of MTGase**

The purified MTGase activity was assayed over the range of pH 2-10 (50 mM citrate buffer for pH 2.0-4.0, 50 mM citrate-phosphate buffer for pH 4.0-7.0, 50 mM Tris-HCl buffer for pH 7.0-8.0 and 50 mM glycine–NaOH buffer or pH 9.0-10.0) at 37°C for 30 min. The stability of MTGase at various pHs were studied using the same buffers. The MTGase solutions were pre-incubated at 37°C for 30 min and then enzyme activity was assayed at the optimum pH, 37°C.

**Effect of Temperature on Activity and Stability of MTGase**

The MTGase activity was determined at different temperatures (4, 8, 25, 37, 45, 55 and 60°C) for 30 min at the optimum pH. MTGase stability was evaluated by measuring the residual activity of MTGase after incubation at different temperatures (4, 25, 37, 45, and 60°C) for 30, 60, 90 and 120 min in a temperature controlled water bath (Memmert, Germany). Thereafter, treated sample was suddenly cooled in iced water. The residual MTGase activity of the incubated samples was determined at the optimal pH and temperature.

**Effect of Various additives on MTGase Activity**

The MTGase (1.0 U·mL⁻¹) was pre-incubated in the presence of 1 and 10 mM BaSO₄, CaCl₂, CuSO₄, KCl, HgCl₂, MnSO₄, NaCl, CoSO₄ and ZnCl₂ at the optimum conditions for 30 min. After that, the substrate mixture was added and incubated at the optimum condition to monitor the residual activity.

**Effect of Some Inhibitors on MTGase Activity**

The inhibitors including, ethylenediamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), phenyl methyl sulfonylfluoride (PMSF), monooiido acetic acid (IAA), N'-ethylmaleimide (NEM) and p-chloro mercuribenzoate (pCMB) were dissolved in citrate-phosphate buffer, pH 6.5 at various concentration. The mixtures were incubated at 45°C for 30 min to allow coupling of the inhibitor to the MTGase, thereafter the residual MTGase activity
was monitored by hydroxamate assay (Folk and Cole, 1965).

**Determination of MTGase Activity**

The sample (100 µL) was mixed with 200 µL of 200 mM citrate-phosphate buffer (pH 6.0), 25 µL of 125 mM hydroxylamine, 25 µL of 12.5 mM glutathione and 75 µL of 37.5 mM CBZ-Gln-Gly. The reaction mixture was incubated at 45°C for 1 h. Thereafter, an equal volume of 5% ferric chloride in 15% TCA solution was added to the reaction mixture. After 5 min, the mixture was centrifuged at 10,000 × g for 10 min and measured the absorbance at 525 nm. The calibration curve was performed using L-glutamic acid γ-mono hydroxamate as a standard. One unit of MTGase activity was defined as the amount of enzyme used to produce 1 µmole of hydroxamic acid per min at the assay conditions.

**Protein Determination**

The amount of protein was determined by the Lowry method (Lowry et al., 1951) with bovine serum albumin as the standard. During separation by chromatography the protein of each fraction was monitored by measuring the absorbance at 280 nm.

**Statistical Analysis**

All the tests were done at least triplicate, and data means and standard deviations were analyzed. The analysis of variance of the data was performed, and the Duncan’s multiple range tests at P<0.05 was used to compare the means using SPSS version 10.0.

**Results and Discussion**

**Localization of MTGase of Enterobacter sp. C2361**

The MTGase of Enterobacter sp. C2361 had different activity among three locations including at periplasmic membrane (cell bound) (0.05 U mg⁻¹), cytoplasm (0.05 U mg⁻¹) and extracellular (0.11 U mg⁻¹) (Table 1). The MTGase mainly excreted to the extracellular medium that is an advantage due to the variety of protein matrices was lower than using the cytoplasmic proteins for downstream harvesting process (Rashad and Nooman, 2009). MTGase from bacteria usually was excreted to the extracellular media (Ando et al., 1989; Bech et al., 2002). MTGase activity varies expression with responsible to their environment. In some case, MTGase was excreted to the extracellular to form coating material such as capsule, spore, and slime. In motile bacteria, MTGase would be involved during the formation of cilia or flagella in which (Klein et al., 1992). Enterobacter sp. C2361 had the peritrichous flagella (Sanders and Sanders, 1997). Therefore, MTGase might be an important catalyst to enhance the cross-linking during the contribution of the flagella.

**Table 1 MTGase activity at various cellular compartments of Enterobacter sp. C2361**

<table>
<thead>
<tr>
<th>Location</th>
<th>Total MTGase activity (U)</th>
<th>Total Protein content (g)</th>
<th>Specific Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular</td>
<td>153</td>
<td>1.93</td>
<td>100</td>
</tr>
<tr>
<td>Cell bound</td>
<td>113±5</td>
<td>1.06±0.2</td>
<td>89.77</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>31±12</td>
<td>0.67±0.01</td>
<td>21.31</td>
</tr>
<tr>
<td>Overall</td>
<td>185</td>
<td>1.93</td>
<td>78.78</td>
</tr>
</tbody>
</table>

L¹¹²² -Total MTGase activity and protein content were calculated from the culture 100 mL.

²² -Specific activity (U mg⁻¹) = total MTGase activity (U)/total protein content (mg).

²² Relative distribution (%) = 100×(total MTGase activity/overall MTGase activity).

Means ± SD from triplicate determinations. Different superscripts in the same column indicate significant differences (p<0.05).

**Precipitation of MTGase of Enterobacter sp. C2361**

The precipitation of crude MTGase was an important step to concentrate the enzyme of interest. Initially, the culture of Enterobacter sp. C2361 had the total MTGase activity at 118 U and total protein content at 1.07 g in 100 mL of the culture. The MTGase was precipitated from the culture broth by acetone, ethanol and (NH₄)₂SO₄ to compare % recovery of MTGase. Precipitation of MTGase of
Enterobacter sp. C2361 was conducted by using stepwise from 0-50% of (NH₄)₂SO₄ to discard soluble starch and some junks. After that the supernatant was further precipitated by addition of (NH₄)₂SO₄ from 50 to 80% saturation which gained the MTGase activity higher than precipitation by using other methods (Table 2). MTGase was strongly affected by high ethanol concentration, especially under the higher temperature during precipitation (Cui et al., 2006). Crude MTGase obtained by stepwise precipitation from 50 to 80% gained higher activity compared to those treatments. Therefore, (NH₄)₂SO₄, a neutral salt, was chosen for precipitation of MTGase in this study.

### Purification of MTGase of *Enterobacter* sp. C2361

The crude MTGase of *Enterobacter* sp. C2361 was increased 2.5 times with a yield of 76.5% by stepwise precipitation of the supernatant by 50 to 80% (NH₄)₂SO₄ (Table 3). Subsequently, the dialyzed precipitate was purified by an ion-exchange chromatography using SP-sepharose at pH 6.5. The MTGase active fractions was eluted from 0.20 to 0.25 M NaCl with a yield of 67.1%, a specific activity of 1.33 U mg⁻¹ and 12.4 fold purification (Figure 1a and Table 3). The obtained fractions were pooled, concentrated, and loaded on Sephadex G-100 column (Figure 1b), which resulted in a 19.1 fold purification with a

---

**Table 2** Comparison of MTGase activity from *Enterobacter* sp. C2361 by various precipitating agents

<table>
<thead>
<tr>
<th>Precipitation</th>
<th>Total MTGase activity² (U)</th>
<th>Total Protein content² (g)</th>
<th>Specific activity² (U mg⁻¹)</th>
<th>MTGase recovery² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% Acetone</td>
<td>74±7b</td>
<td>0.76±0.01a</td>
<td>0.10</td>
<td>62.35</td>
</tr>
<tr>
<td>70% Ethanol</td>
<td>72±1b</td>
<td>0.80±0.01b</td>
<td>0.09</td>
<td>60.76</td>
</tr>
<tr>
<td>50-60% (NH₄)₂SO₄</td>
<td>37±4c</td>
<td>0.87±0.01c</td>
<td>0.04</td>
<td>31.55</td>
</tr>
<tr>
<td>50-70% (NH₄)₂SO₄</td>
<td>91±3c</td>
<td>0.88±0.02c</td>
<td>0.10</td>
<td>75.93</td>
</tr>
<tr>
<td>50-80% (NH₄)₂SO₄</td>
<td>101±2d</td>
<td>0.95±0.01d</td>
<td>0.11</td>
<td>85.76</td>
</tr>
<tr>
<td>Initial</td>
<td>118±5e</td>
<td>1.07±0.01e</td>
<td>0.11</td>
<td>100</td>
</tr>
</tbody>
</table>

² Total MTGase activity and protein content were calculated from the culture 100 mL.
³ Specific activity (U mg⁻¹) = total MTGase activity (U)/total protein content (mg).
⁴ MTGase recovery (%) = 100 × (total MTGase activity / overall MTGase activity).

Means ± SD from triplicate determinations.

Different superscripts in the same column indicate significant differences (p<0.05).
yield of 15.3% and a specific activity of 2.05 U mg⁻¹. The obtained MTGase showed
the mass approximately 29.84 KDa by
SDS-PAGE (Figure 2).

Effect of pH on MTGase Activity and
Stability
The purified MTGase from Enterobacter
sp. C2361 showed broad spectrum for
catalytic activity in a range of pH 5.0-7.5,
which was more than 80% relative activity
(Figure 3a). The optimum pH for MTGase
activity was in a range of 5.0-6.5 The
MTGase from Enterobacter sp. C2361 was
more stable in a range of pH 5.0-7.0 than
other pH ranges (Figure 3b). MTGase activity
of Enterobacter sp. C2361 was gradually
decreased in acidic pH and decreased rapidly
at the alkaline pH. It was similar to the
MTGase of B. substilis, but it was in contrast
to S. hygroscopicus (Cui et al., 2007). The
optimal temperature for MTGase activity of
Enterobacter sp. C12361 was similar to the
optimum pH for MTGase from Sv. mobaranes, Sv. ladakanum, Sv. cinnamoneum and S. hygroscopicus (Ando et
al., 1989; Duranetal., 1998; Cui et al., 2007;
Ho et al., 2008). But it was different from
MTGase of Bacillus spp. and yeasts which
was around pH 8.0-9.0 (Bech et al., 2002).

Effect of Temperature on MTGase
Activity and Stability
The effect of temperature on the
MTGase activity was studied by
determining the activity between 4-60˚C at
pH 6.0 for 30 min. The optimal temperature
for MTGase activity of Enterobacter sp.
C2361 was at 37-50˚C (Figure 4a) which
was similar to MTGase from Sv. ladakanum, Sv. mobaranes and B. substilis
that had the optimal temperature at 30-50˚C
(Ando et al., 1989; Suzuki et al., 2000; Ho
et al., 2008). The activity of MTGase was
decayed sharply at temperature over 45˚C.
Thermal stability of the purified MTGase of
Enterobacter sp. C2361 and was
investigated between 4-60˚C (Figure 4b).
The MTGase of Enterobacter sp. C2361
was stable at 4˚C but was slowly decreased
at the room temperature. The MTGase was
rapidly inactivated at higher temperature
and preserved lower than 20% of initial
activity when exposed at 60˚C for 30 min.

Table 3 Purification steps for MTGase from Enterobacter sp. C2361

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Total MTGase activity (U)</th>
<th>Total protein content (g)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme</td>
<td>100</td>
<td>102</td>
<td>0.95</td>
<td>0.11</td>
<td>1.0</td>
</tr>
<tr>
<td>Precipitation</td>
<td>25</td>
<td>78</td>
<td>0.29</td>
<td>0.27</td>
<td>2.5</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>15</td>
<td>68</td>
<td>0.05</td>
<td>1.33</td>
<td>12.4</td>
</tr>
<tr>
<td>Sephadaex G-100</td>
<td>5</td>
<td>16</td>
<td>0.01</td>
<td>2.05</td>
<td>19.1</td>
</tr>
</tbody>
</table>

1 Total MTGase activity and protein content were calculated from the culture 100 mL.
2 Specific activity (U mg⁻¹) = total MTGase activity (U)/total protein content (mg).

Figure 2 SDS-PAGE of MTGase on acrylamide gel for crude enzyme. M, standard markers; lane A, broth; lane B, MTGase after SP-Sepharose and lane C, MTGase after sephadex G-100.
Effect of Some Metal Ions on MTGase Activity

The effect of metal ions on MTGase activity of *Enterobacter* sp. C2361 was studied in the presence of metal ions at 1 and 10 mM (Table 4). The purified MTGase from *Enterobacter* sp. C2361 was moderately inhibited by Ba$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ at 10 mM. Co$^{2+}$ and Na$^+$ possessed less effect on lowering of MTGase activity. The sensitivity of MTGase towards cations has been reported in *Bacillus circulans*, *Sv. mobaraense*, *Sv. lividans*, *Sv. cinnamoneum*, *Sv. ladakanum* and *Streptomyces hygroscopicus* (Ando et al., 1989; Seguro et al., 1995; Duran et al., 1998; Barros et al., 2003; Lin et al., 2006; Cui et al., 2007; Ho et al., 2008). Cations including Cu$^{2+}$, Pb$^{2+}$, Li$^+$ and Zn$^{2+}$ were significantly inhibited MTGase functioning (Seguro et al., 1995). Because heavy metals such as Cu$^{2+}$, Hg$^{2+}$, Pb$^{2+}$ and Zn$^{2+}$ can bind to the thiol group of the cysteine residue at the active site of MTGase (Seguro et al., 1995; Cui et al., 2007).

Effect of Some Inhibitors on MTGase Activity

The effect of inhibitors including, EDTA, EGTA, IAA, pCMB, PMSF and NEM on MTGase of *Enterobacter* sp. C2361 was studied at 1 and 5 mM in citrate-phosphate buffer, pH 6.0. EDTA and EGTA had no inhibitory effect on MTGase from both strains at 1 mM but possessed a slightly inhibition at 5 mM (Table 5). The result showed that MTGase from *Enterobacter* sp. C2361 was function without metal activation. The result was concomitance with the previous study the effect of metal ions on MTGase activity. The MTGase activity did not increase with the addition of various divalent metal ions. The MTGase was a Ca$^{2+}$ independent enzyme which was confirmed by the MTGase with the addition of EDTA and EGTA, an inhibitor used to block the specific site for binding of Ca$^{2+}$ ion.
Table 4 Effect of some metal ions on residual activity (%) of MTGase of Enterobacter sp. C2361

<table>
<thead>
<tr>
<th>Metal ion</th>
<th>Concentration</th>
<th>1 mM</th>
<th>10 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>121.95±0.94&lt;sup&gt;b,E&lt;/sup&gt;</td>
<td>89.02±3.21&lt;sup&gt;C,D&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>113.41±2.49&lt;sup&gt;b,D&lt;/sup&gt;</td>
<td>106.34±2.32&lt;sup&gt;F&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Co&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>103.66±1.17&lt;sup&gt;b,C&lt;/sup&gt;</td>
<td>93.90±3.57&lt;sup&gt;D&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cu&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>114.63±2.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.37±4.84&lt;sup&gt;BC&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Hg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>93.90±5.05&lt;sup&gt;b,A&lt;/sup&gt;</td>
<td>80.49±5.65&lt;sup&gt;AB&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>101.20±0.88&lt;sup&gt;a,BC&lt;/sup&gt;</td>
<td>107.14±1.02&lt;sup&gt;F&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>98.78±2.4&lt;sup&gt;b,ABC&lt;/sup&gt;</td>
<td>84.15±0.30&lt;sup&gt;BC&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>100.08±1.24&lt;sup&gt;a,BC&lt;/sup&gt;</td>
<td>97.14±3.13&lt;sup&gt;E&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Zn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>96.45±5.48&lt;sup&gt;b,AB&lt;/sup&gt;</td>
<td>74.82±3.26&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Means ± SD from triplicate determinations.
The different letters in the same row indicate the significant differences (P<0.05).
The different capital letters within the same column indicate the significant differences (P<0.05).

Table 5 Effect of some inhibitors on residual activity (%) of MTGase of Enterobacter sp. C2361

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>1 mM</th>
<th>5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>100.00±0.20&lt;sup&gt;b,CD&lt;/sup&gt;</td>
<td>96.34±1.86&lt;sup&gt;D&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>99.04±2.65&lt;sup&gt;a,JD&lt;/sup&gt;</td>
<td>97.72±3.46&lt;sup&gt;D&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IAA</td>
<td>71.47±3.87&lt;sup&gt;b,BC&lt;/sup&gt;</td>
<td>35.48±4.21&lt;sup&gt;B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>pCMB</td>
<td>68.08±3.78&lt;sup&gt;b,BC&lt;/sup&gt;</td>
<td>28.70±1.92&lt;sup&gt;a,B&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>75.22±2.54&lt;sup&gt;b,BC&lt;/sup&gt;</td>
<td>36.95±3.91&lt;sup&gt;B,BC&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>NEM</td>
<td>53.61±5.46&lt;sup&gt;a,BC&lt;/sup&gt;</td>
<td>21.60±3.52&lt;sup&gt;A&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

Means ± SD from triplicate determinations.
The different letters in the same row indicate the significant differences (P<0.05).
The different capital letters within the same column indicate the significant differences (P<0.05).

The MTGase was strongly inhibited by NEM, pCMB and IAA which affected to cysteine residues at the active site of MTGase and also inhibited by PMSF. The inhibition of MTGase by thiol effective reagents strongly showed that the enzyme functioned by thiol (-SH) groups. In addition, many synthetic inhibitors including, IAA, NEM, PMSF, pCMB could inhibit both TGase and MTGase activity (Kang et al., 1998; Leblanc et al., 2001; Lu et al., 2003; Worratao and Yongsawatdigul, 2005; Lin et al., 2006). MTGase comprised of cysteine at the active site, therefore, MTGase could be inactivated if the sulphydryl group is destroyed by some environmental factors. The addition of pCMB, NEM and IAA could destroy the sulphydryl group of MTGase, resulting in inhibition of MTGase activity by oxidation of the sulphydryl group (Ando et al., 1989; Duran et al., 1998; Lin et al., 2009).

Conclusions

The purified MTGase from a newly isolated Enterobacter sp. C2361 was obtained after two purification steps had molecular weight at 29.84 kDa by SDS-PAGE. The enzyme exhibited optimum activity in a range of pH 5 to 7.0 and at 37 to 50°C. The MTGase of Enterobacter sp. C2361 was independent to Ca<sup>2+</sup> and stable in a range of pH 5-7 and temperature at 4°C. The obtained MTGase from both sources was moderately inhibited by Cu<sup>2+</sup>, Hg<sup>2+</sup>, Mn<sup>2+</sup> and Zn<sup>2+</sup>. MTGase of Enterobacter sp. C2361 possessed no inhibitory effect from EDTA and EGTA but it was inhibited IAA, NEM, pCMB and PMSF.

Acknowledgements

The authors are grateful to express their sincere thank to Graduate School, Prince of Songkla University for their financial support. This work was also supported by the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission.

References


Effect of Adding Ling-zhi (Ganoderma lucidum) on Oxidative Stability, Textural and Sensory Properties of Smoked Fish Sausage

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Abstract
Ling-zhi (Ganoderma lucidum) has long been regarded as a food and medicinal mushroom. We investigated the oxidative stability, textural and sensory properties of added Ling-zhi in smoked fish sausage at levels of 1% (crushed Ling-zhi and Ling-zhi water extract) and 0.25% w/w (Ling-zhi spore). The DPPH radical scavenging activity was used for evaluating their antioxidant activity. Lipid oxidation was evaluated through measurement of peroxide value (PV) and thiobarbituric acid (TBA) value. The values of PV and TBA remained not significantly different (P>0.05) among all samples at the first 3 months of storage at -18°C. However after that those parameters of the control (no added Ling-zhi) was sharply increased at 4 months and steadily at 5 and 6 months with the final PV of 3.8 (mg peroxide equivalent/kg sample) and TBA of 1.56 (mg malonaldehyde equivalent/ kg sample) which were significantly higher than those of all added Ling-zhi sausages. This indicates that addition of any forms of Ling-zhi significantly delayed lipid oxidation when compared with the control. Addition of Ling-zhi water extract and spore did not affect the textural properties of smoked fish sausages while addition of crushed Ling-zhi resulted in undesirable texture. The sensory evaluations indicated that the greatest overall acceptability score was obtained at the 1% Ling-zhi water extract added sausages. This study has demonstrated that addition of Ling-zhi in the smoked fish sausages not only increased the nutritional value but also retarded the lipid oxidation.

Keywords: Ling-zhi, smoked fish sausage, antioxidant, storage, sensory evaluation

Introduction
Ling-zhi (Ganoderma lucidum), which belongs to the family of Ganodermataceae of Polyporales, is regarded as a panacea, because it is reported to have a broad spectrum of medicinal properties for both health maintenance and treatment of disease. Ling-zhi is also called “marvelous herb” or “mushroom of immortality”, emphasizing its function in enhancing longevity. Evidence from both in vitro experiments and in vivo animal and human, support Ling-zhi extract as a potential anticancer agent (Gao et al., 2004; Yuen and Gohel, 2005).

Recently, phytochemicals in food materials and their effects on health, especially the suppression of active oxygen species by natural antioxidants from teas, spices and herbs, have been extensively studied (Ho et al., 1994). Xiaoping et al. (2009) reported that Ling-zhi (Ganoderma lucidum) has been used as traditional edible and medicinal materials in China. G. lucidum polysaccharides exhibited the higher DPPH radicals scavenging activities.
This result suggested that the Ling-zhi polysaccharides is a good scavenger for DPPH radical (Xiaoping et al., 2009). However, antioxidant supplements, or foods containing antioxidants, may be used to help the human body reduce oxidative damage (Yang et al., 2002).

Smoked fish sausages are popular in Thailand. Fish is known to be a source of protein rich in essential amino acids (lysine, methionine, cystine, threonine, and tryptophan) (Sikorski, 1994), micro- and macroelements (calcium, phosphorus, fluorine, iodine), fats that are valuable sources of energy, fat-soluble vitamins, and unsaturated fatty acids that, amongst other benefits, have a hypocholesterolemic effect (antiarteriosclerosis) (Fernandez and Venkatrammann, 1993; Ismail, 2005). Moreover, the consumer interest in the development of meat analogs using alternative protein sources is gaining in popularity. Several recent studies have examined the use of various functional ingredients or adjuncts in sausage formulations. Among such ingredients are mushroom (Murphy et al., 2004). Thus, our then get added mushroom was Ling-zhi species *Ganoderma lucidum* in smoked fish sausage using alternative for consumer take an interest the health. With the present of radical scavenging activity, consumption of food is contain Ling-zhi which antioxidant properties might be beneficial to protect human body against oxidative damage, which can be further developed into health related degenerative illnesses.

Our objective was to study the effect of adding Ling-zhi (*Ganoderma lucidum*) on antioxidant activity, textural and sensory properties of smoked fish sausage. We selected three forms namely crushed Ling-zhi, Ling-zhi water extract and Ling-zhi spore. The overall acceptance of consumer was also evaluated.

**Materials and Methods**

**Sample**

Three forms of Ling-zhi namely crushed Ling-zhi, Ling-zhi water extract and spore were obtained from mushroom farm, Kalasin, Thailand. For each of samples, 100 g. were randomly selected and prepared for analyses. Ling-zhi was air-dried in an oven at 40 °C for 2-3 days after a crushed before sample preparation. Ling-zhi was extracted by hot water (Mau et al., 2005) ratio 1:10 (w/v) for 5 hour and then filtering through Whatman No. 4 filter paper (Whatman International Ltd., Maidstone, England) and used for analyzing and added to smoked fish sausage. All analyses were performed in three replicates.

**Sample Extract**

Three forms of Ling-zhi namely crushed Ling-zhi, Ling-zhi water extract and spore were extracted with 80% ethanol at the ratio of 1:10 and shaken for 2 h at room temperature. The extracts were then separated from the sample residue by filtration through Whatman No.4 filter paper. The residue was then extracted with two additional ratio 1:10 portions of ethanol as described above. The combined ethanolic extracts were then rotary-evaporated at 40 °C to dryness. The dried extract was used directly for analyses of antioxidant properties or redissolved in ethanol to a concentration of 50 mg/ml and stored at 4 °C for further use. The smoked fish sausages with Ling-zhi were extracted using the same method with that of Ling-zhi. Each extract was analyzed in three replicates for each antioxidant test, and the results were reported as mean values ± SD.

**Smoked Fish Sausage Preparation**

Smoked fish sausages were prepared according to the procedure described by National center for genetic engineering and biotechnology (2004), Thailand. Crushed Ling-zhi, Ling-zhi water extract and spore were added in the sausages at levels of 1%, 1% and 0.25% w/w, respectively. Control sausages were also prepared without the addition of Ling-zhi. Smoked fish sausage was produced using catfish (*Clarias gariepinus*), 61.67 % catfish meat, 6.88 % striped catfish fat, 20.65 % ice water, 3.44
% soy protein, 1.38 % salt, 1.38 % sugar, 0.68 % ground coriander seed, 0.18 % ground nutmeg, 0.18 % ground nutmeg flower, 0.68 % ground pepper, 0.17 % ground cinnamon, 1.72 % ground garlic, 0.34 % phosphate and 0.34 % monosodium glutamate. For each batch of sausages, fish, Ling-zhi and other ingredients were mixed thoroughly using a mixer for 3 min at 4–6 °C. After mixing, the mixtures were stuffed into synthetic cellulose casings (approximate diameter of 30 mm) using a stuffer.

Sensory Evaluations
Thirty panelists included students and staff members in the Department of Food Technology and Nutrition, Faculty of Technology, Mahasarakham University. Sausage samples were cooked in the oven as previously described, then sliced into pieces, and served warm to the panelists. The panelists evaluated each characteristic of the sample using a 9-point hedonic scale for 6 attributes (color, flavor, taste, texture, appearance and overall acceptability) where one (1) was “dislike extremely” and nine (9) was “like extremely”.

DPPH Radical-Scavenging Activity
The 1,1–diphenyl-2-picrylhydrazyl (DPPH) free radical, was determined by the method described by Braca et al. (2001). Aqueous extract (0.1 ml) was added to 3 ml of a 0.001 M DPPH in methanol. Absorbance at 517 nm was determined after 30 min, and the percent inhibition of activity was calculated as [(Ao - Ae)/Ao] × 100 (Ao = absorbance without extract; Ae = absorbance with extract).

Determination of Total Phenol Content
The total phenolic content of each extract was determined using the Folin–Ciocalteu reagent (Zhou and Yu., 2006). The reaction mixture contained 0.3 ml of sample extract, 2.25 ml of the Folin–Ciocalteu reagent (1:10 v/v) and 2.25 ml of 6% sodium carbonate. After 2 h of reaction at ambient temperature, the absorbance at 725 nm was measured and used to calculate the phenolic contents, using gallic acid as a standard. The total phenolic contents were then expressed as gallic acid equivalents (GAE), in mg/g dry sample.

Proximate Aomposition
Proximate composition analysis of the sausages was performed according to AOAC (AOAC, 2000b). Moisture, protein, fat, fiber and ash parameters were determined in triplicate from product of sausage mixtures.

Texture Profile Analysis (TPA)
Texture profile analysis (TPA) testing was performed at about 22 °C using a TA.XT2i Stable Micro Systems Texture Analyser (Stable Micro systems Ltd., Surrey, England) with the Texture Expert program (Bourne, 1978). Both textural procedures involved discarding the external case of the smoked fish sausages.
In general, this procedure involved the preparation of four cylinders of 1.5 cm high and 2 cm wide from each smoked fish sausage. When samples of mini-fuet (diameter < 2 cm) were analyzed a width of 1 cm was employed. A double compression cycle test was performed up to 50% compression of the original portion height with an aluminium cylinder probe P/25. A time of 5 s was allowed to elapse between the two compression cycles. Force-time deformation curves were obtained with a 25 kg load cell applied at a cross-head speed of 2 mm/s.

Measurement of Peroxide Value
Peroxide value (PV) was determined according to the AOAC International (1999a). The sample (5 g) was weighed in a 250-ml glass stoppered Erlenmeyer flask and heated in a water bath at 60 °C for 3 min to melt the fat, then thoroughly agitated for 3 min with 30 ml acetic acid–chloroform solution (3:2 v/v) to dissolve the fat. The sample was filtered under vacuum through Whatman filter paper. Saturated potassium iodide solution (0.5 ml) was added to the filtrate, which was transferred into the burette. The titration was allowed to run
against standard solution of sodium thiosulfate (25 g/l). PV was calculated and expressed as milliequivalent peroxide per kg of sample: 

\[
PV \text{(meq/kg)} = \left( \frac{S \times N}{W} \right) \times 1000,
\]

where \( S \) is the volume of titration (ml), \( N \) the normality of sodium thiosulfate solution (\( N = 0.01 \)), and \( W \) the sample weight (kg).

**Measurement of TBA Value**

The 2-thiobarbituric acid (TBA) assay was carried out according to the procedure of Schmedes and Holmer (1989). Sausage sample (0.15 g) was mixed with 25 ml of trichloroacetic acid solution (0.2 g/l of TCA in 100 ml/l n-Butanol solution) and homogenized in a blender for 30 s. After filtration, 5 ml of the filtrate were added to 5 ml TBA solution in a test tube. The test tubes were incubated at water bath 95 °C in the dark for 2 h; then the absorbance was measured at 530 nm by using UV–VIS spectrophotometer (model UV-1200, Shimadzu, Japan). TBA value was expressed as mg malonaldehyde per kg of sausage. Bank was no sample.

**Statistical Analysis**

The data were analyzed by analysis of variance (ANOVA) using statistical analysis systems (SAS). Duncan’s multiple range test was used to determine the statistical significance among the means (SAS, 1997) at 95% significant level.

**Results and Discussion**

**Sensory Evaluations**

The sensory panels were convened to assess the effects of the addition of Ling-zhi and quality on the color, flavor, taste, texture, appearance and overall acceptability in a smoked fish sausage (Figure 1). All sensory attributes were significantly different (\( P > 0.05 \)) except for flavor and appearance. The scores for color were ranged from 4.20 to 6.75 being highest in smoked fish with Ling-zhi water extract and the control. Smoked fish with crushed Ling-zhi have lowest score color. Flavor and appearance scores were not significantly different among the sausages, ranging from 4.30-5.45, 5.85-6.45, respectively. The smoked fish sausage with Ling-zhi water extract (6.65±1.32) gave the highest score for the taste, followed by the control and the smoked fish sausage with spore whereas smoked fish sausage with crushed Ling-zhi has the least score (3.85). It was observed that panelists dislike texture of smoked fish sausage with crushed Ling-zhi. The overall acceptability scores ranged from 4.35 to 7.05. Maximum acceptability obtained at 1% Ling-zhi water extract added smoked fish sausage followed by control, spore added and crushed Ling-zhi added sausages, respectively.

**DPPH Radical-Scavenging Activity**

The antioxidant properties assayed herein were in Figure 2 and the results were normalized and expressed as IC\(_{50}\) values (mg/ml) for comparison. A synthetic antioxidant; BHT showed excellent scavenging abilities of IC\(_{50}\) 0.12 mg/ml (\( P > 0.05 \)), followed crushed Ling-zhi (IC\(_{50}\) =1.12), spore (IC\(_{50}\) =2.67) and Ling-zhi water extract (IC\(_{50}\) =4.35 mg/ml). Scavenging effects of each sample extracts from Ling-zhi on DPPH radicals increased with the increased concentrations (Yang et al., 2002).
We hence evaluated the effect of adding three forms of Ling-zhi as an ingredient on antioxidant properties of smoked fish sausage. The results showed that the scavenging activity was increased in all sausages added Ling-zhi compared with the control (no added Ling-zhi). The results are expressed as IC\textsubscript{50} value in Figure 2. The highest scavenging activity was found in smoked fish sausage with crushed Ling-zhi 1% (3.36±0.15) followed by smoked fish sausage with spore 0.25%, smoked fish sausage with Ling-zhi water extract 1% and control. A decrease of scavenging activity might be affected by several factors involved in the cooking process such as heat, pH, moisture etc (Turkmen et al., 2005).

**Figure 2** IC\textsubscript{50} of Ling-zhi (■) and smoked fish sausage with/without Ling-zhi (■). (n = 3; mean ± SD)

Determination of Total Phenol Content

Phenolic compounds are widely distributed in plants (Li et al., 2006), which have gained much attention, due to their antioxidant activities, which potentially have beneficial implications for human health (Govindarajan et al., 2007; Imeh and Khokhar, 2002; Li et al., 2006; Ross and Kasum, 2002). Total phenol content (TPC) was determined in comparison with standard gallic acid and the results expressed in terms of mg GAE/g dry sample. The TPC values in the part of Ling-zhi in descending order: crushed Ling-zhi > spore > Ling-zhi water extract, all as mg GAE/100g dry sample (Figure 3).

**Figure 3** Total phenol content of Ling-zhi (■) and smoked fish sausage with/without Ling-zhi (■). (n = 3; mean ± SD)

This study aimed to evaluate the effect of adding Ling-zhi as an ingredient on total phenolic content (Figure 3). The highest phenolic content was smoked fish sausage with spore followed by smoked fish sausage with crushed Ling-zhi and smoked fish sausage with Ling-zhi water extract 269.25±0.71, 254.75±0.55, 183.75±0.81 mg GAE/100g dry sample, respectively whereas, the control had the least phenolic content. Natural antioxidant can be used to replace the synthetic antioxidant in the food industry such as BHT, BHA and TBHQ, which may possess mutagenic activity (Skrinjar et al., 2007; Namiki, 1990).

**Proximate Composition**

Proximate compositions of the smoked fish sausages with/without Ling-zhi are shown in Table 1. The addition of Ling-zhi did not significantly affect (P>0.05) the moisture contents, ash content and protein content of all samples ranged from 80.74%-83.17%, 1.33-1.62% and 11.10-12.89 g protein/100 g, respectively. Smoked fish sausage with spore has the highest fat content followed by smoked fish sausage with crushed Ling-zhi and smoked fish sausage with Ling-zhi water extract 269.25±0.71, 254.75±0.55, 183.75±0.81 mg GAE/100g dry sample, respectively whereas, the control had the least phenolic content. Natural antioxidant can be used to replace the synthetic antioxidant in the food industry such as BHT, BHA and TBHQ, which may possess mutagenic activity (Skrinjar et al., 2007; Namiki, 1990).
Texture Profile Analysis (TPA)

The texture attributes (TPA) of the smoked fish sausages with/without Ling-zhi are shown in Table 2. Hardness (N) was the maximum force required to compress the sample. To observe, smoked fish sausages with Ling-zhi water extract have the highest hardness (5.53±0.02 N) followed by control smoked fish sausage with spore and smoked fish sausage with crushed Ling-zhi have the least hardness were 1.45±0.04 N.

Cohesiveness was extent to which the sample could be deformed prior to rupture. The addition of Ling-zhi did not significantly affect (P>0.05) the cohesiveness of all samples, ranged from 0.19 to 0.32. Adhesiveness (N×s) was area under the abscissa after the first compression. Control and smoked fish sausages with Ling-zhi water extract have the highest adhesiveness -0.97±0.02 N*s inferior spore added and crushed Ling-zhi added sausages, respectively. Springiness (mm) was an ability of the sample to recover its original form after deforming force was removed. Control and smoked fish sausages with spore have the highest springiness while smoked fish sausages with crushed Ling-zhi were found to have lowest springiness.

Measurement of Peroxide Value

Peroxide value has been used as an indicator of the extent of oxidative rancidity. It is affected by the age of raw material as well as oxidation of fats during processing and storage (AOCS, 1993). Changes in PV value of added Ling-zhi formulated in smoked fish sausage are shown in Figure 4. The initial PV values at 1-3 months of all samples were constant, ranging from 0.90 in Ling zhi added sausages to 1.37 in the control sausage. However, after 3 months of storage, the PV values of Ling zhi added sausages were gradually increased to 2.30-2.51 whereas that of the control was sharply increased (3.4-3.8) almost two fold higher than other samples. In the present study, PV values in all samples were below 25 meq of active O₂/kg, which is considered as limit of

Table 1 Proximate compositions of smoked fish sausages with/without Ling-zhi

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Moisture (ns)</th>
<th>Ash (ns)</th>
<th>Protein (ns)</th>
<th>Fat</th>
<th>Fiber</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>83.17±1.76</td>
<td>1.33±0.21</td>
<td>12.26±0.25</td>
<td>5.08±0.06b</td>
<td>0.71±0.01b</td>
</tr>
<tr>
<td>FSS</td>
<td>80.53±0.51</td>
<td>1.51±0.15</td>
<td>12.89±0.46</td>
<td>7.10±0.38a</td>
<td>0.69±0.01b</td>
</tr>
<tr>
<td>FSW</td>
<td>81.48±0.45</td>
<td>1.62±0.13</td>
<td>11.10±0.90</td>
<td>5.07±0.59b</td>
<td>0.74±0.03b</td>
</tr>
<tr>
<td>FSL</td>
<td>80.74±1.19</td>
<td>1.44±0.21</td>
<td>12.62±0.49</td>
<td>3.37±0.16c</td>
<td>0.87±0.10ab</td>
</tr>
</tbody>
</table>

Mean values and standard deviations with different letters (a, b, c, ... ) in the same column indicate significant differences (P < 0.05)

A The same as Figure 1

Table 2 Texture attributes of smoked fish sausages with/without Ling-zhi

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Hardness (N)</th>
<th>Cohesiveness</th>
<th>Adhesiveness (N*s)</th>
<th>Springiness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.30±0.01b</td>
<td>0.21±0.06b</td>
<td>-0.97±0.02a</td>
<td>14.40±0.52a</td>
</tr>
<tr>
<td>FSS</td>
<td>4.59±0.02c</td>
<td>0.26±0.07ab</td>
<td>-0.53±0.03b</td>
<td>14.38±0.01a</td>
</tr>
<tr>
<td>FSW</td>
<td>5.53±0.02a</td>
<td>0.32±0.01a</td>
<td>-0.96±0.01a</td>
<td>13.66±0.11b</td>
</tr>
<tr>
<td>FSL</td>
<td>1.45±0.04d</td>
<td>0.19±0.04b</td>
<td>-0.04±0.03c</td>
<td>10.98±0.02c</td>
</tr>
</tbody>
</table>

Mean values and standard deviations with different letters (a, b, c, ... ) in the same column indicate significant differences (P < 0.05)
acceptability in fatty foods (Evranuz, 1993; Narasimhan et al., 1986).

Measurement of TBA Value

The oxidative process was evaluated by the peroxide index and TBA values in sausages in order to determine how it was affected by the different storage time. Figure 5 showed the effect of different forms of Ling-zhi on TBA values in the sausage during storage at -18°C. The TBA values of all samples were constant at the first 3 months, ranging from 0.80 in Ling-zhi added sausages to 0.98 in the control sausage. However, after 3 months of storage, the TBA values of the control and smoked fish sausage with crushed Ling-zhi increased rapidly. These values were significantly lower than that of the control and smoked fish sausage with crushed Ling-zhi ($P>0.05$). TBA value is routinely used as an index of lipid oxidation in meat products in storage (Raharjo and Sofos, 1993), and rancid flavor is initially detected in meat products between TBA values of 0.5 and 2.0 (Gray and Pearson, 1987).

Conclusions

This study concluded that added crushed Ling-zhi, Ling-zhi water extract and spore in smoked fish sausage provided antioxidant benefits to smoked fish sausage during cold storage (-18°C) and the effects were concentration dependent. All the Ling-zhi forms studied, crushed Ling-zhi demonstrated the highest scavenging activities and total phenolic content, but such a high concentration may not be acceptable by the consumers because of its bitter flavor and soft, mushy texture. In our present study, addition of 1% Ling-zhi water extract gained the highest score of overall acceptability. Moreover, it could also extend the shelf-life of the product up to 6 month. Therefore, it is suggested that Ling-zhi, as a natural herb, could be used to extend the shelf-life of meat products, providing the consumer with food containing natural additives, which might be seen more healthful than those of synthetic origin.

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Study on Preparation and Quality of Tomato Crispy Crackers

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Abstract
The first purpose of this research was to examine the preparation tomato crispy crackers (Kaw-kreb) from different methods as follows: (1) added with minced fresh tomato; (2) added with boiled tomato; (3) added with tomato ketchup; and (4) added with combined boiled tomato and tomato ketchup. The second purpose was to study the product quality in terms of physical and chemical analysis, microbial count, and sensory evaluation. From the experiment, the accepted preparation used the following ingredients: tapioca flour (57.47%), tomato ketchup (10.06%), water (28.74%), salt (0.57%), pepper powder (0.57%), minced garlic (1.15%), and white sugar (1.44%). The moisture content and water activity of the final product were 1.75% (w.b.) and 0.45, respectively. From the expansion measurement of the crispy crackers, the value was 46.25%. The finding of colour analysis was as follows: 70.36 (L\*), 4.32 (a\*), and 0.28 (b\*) (p<0.05). The hardness of the product was 22.46 Newton force. According to the chemical analysis, the peroxide value of tomato crispy crackers was 12.78 Milliequivalent of peroxide oxygen kg\(^{-1}\) with 58.02% the vegetable oil uptake. Concerning the sensory test, the results showed that the tomato crispy crackers were satisfied in the colour, tomato flavor, taste, crispness, and over all acceptability. The microbial analysis, TPC, \textit{Escherichia coli}, \textit{Staphylococcus aureus}, and mould were confronted the Thailand Community Standard (TCS 107-2003).

Keywords: crispy crackers, Kaw-kreb, tomato ketchup

Introduction
Tomatoes have been traditionally credited as rich sources of carotenoids and vitamins, particularly beta-carotene, provitamin A and vitamin C (Hanson et al. 2004).

The tree produces fruit throughout the year. Tomato is cheap in Thailand. It can be harvested and produced many food products such as tomato ketchup, tomato juice, dried tomato, and tomato grace.

Crispy crackers are popular snack foods in many Southeast Asian countries. They are known as Kaw-Krab in Thailand. Basically, fish crispy crackers are produced by mixing flesh fish with starch and water. The dough is then shaped into round, oblique, stick or longitudinal forms and gelatinized by boiling or steaming. The gelatinized dough is then cooled, sliced, and dried until the moisture content reaches around 10 percent. The sliced dried products is fried in cooking oil before eaten as snack food or together with rice and other daily dishes (Mohamed et al., 1989; Nurul et al., 2009)

Starch flour is one of the essential ingredients for making the special crispy crackers various starches such as tapioca, wheat, corn, rice, and mung bean is available for crisp cracker making (Tangkanakul et al., 1994)

Although, tomato had featured in the diet of Thailand at the household level for centuries, there is a low level of awareness about the food product development of tomato in crispy cracker making.
The objective of this study was to examine the effect of preparation of tomato crispy crackers (Kaw-kreb) from different methods. The study also assessed the product quality in terms of physical and chemical analysis, microbial count, and sensory evaluation.

Materials and Methods

Raw Material

Mature, red ripe and wholesome fruits of tomato was purchased from local market at Mae Rim, Chiang Mai and transported in plastic bags to the processing factory, Agro-industry division, faculty of agricultural technology, Chiang Mai Rajabhat University for intermediate processing. The fruits were kept in a refrigerator at 4 °C before use within 3 days. Tapioca flour (5 suns, Big Tree Inter-trade, Ltd., Bangkok, Thailand), tomato ketchup (Rosa, Hi-Q Food Product company Ltd., Samutprakarn, Thailand), white sugar (Mitephon, Bankok, Thailand), salt (Prung Thip, Nakornratchasima, Thailand), pepper powder (Rai Tip, Bagkok, Thailand), garlic (Mae Rim, Chiang Mai, Thailand), cooking palm olein oil (Tip, Bangkok, Thailand) were obtained from a local market.

Preparation of Tomato

Minced tomato was prepared by mincing the cleaned tomato and squeezing tomato juice, using blender at high speed for 5 mins (Phillip, Thailand).

Boiled tomato was prepared by cleaning tomato and boiling for 30 mins. Then the boiled tomato was blended to be tomato puree.

Preparation of Crackers

The crackers were formulated using different preparation methods (1) added with minced fresh tomato (FT); (2) added with boiled tomato (BT); (3) added with tomato ketchup (KT) and; (4) added with combined boiled tomato and tomato ketchup (BKT). Other ingredients used in this tomato crispy cracker formulation were tapioca flour (57.47%), water (28.74%), salt (0.57%), pepper powder (0.57%), minced garlic (1.15%), and white sugar (1.44%). The added tomato (10.06%) was mixed with half proportion of the flour, sugar, brine and some boiled water, and blended manually for 10 mins. The remaining tapioca flour, pepper powder, minced garlic, and boiled water were added in the first part and blended for 10 mins. The dough was stuffed into banana leaves with a roll of a diameter of 5 cm, a length of 40 cm and both ends were tied. The dough was cooked in steamed at 120°C for 30 minutes, using steamer (Figure 1). Then it was cooled in cold water and chilled storage at 5°C overnight. The chilled dough was sliced manually to a thickness of 1.5 mm using a sharpen knife and dried overnight in an oven with a temperature at 65°C. (Likit Cheewan, Thailand). The 20 gram of dried slices were deep fried in 1 liter of cooking palm oil at 180-200°C for a min using a pan to be the tomato crispy cracker (Figure 2).

Moisture Content and Water Activity Determination

Moisture content was determined by drying the samples overnight at 105°C, cooled, and weighted. Weight loss on drying to a final constant weight was
recorded as moisture content. Water activity was measured by using water activity analyzer (Novasia, Italy). All determinations were carried out in triplicates.

**Hardness**

Hardness was measured by a penetration test using the Texture Analyzer (TA-XT2 Stable Micro system, England). The condition of the texture analyzer were as follow: pre-test and post-test speed, 2.0 mm s⁻¹ distance, 5.0 mm; time, 5.0s; trigger type, auto and trigger force, 10 g. The dried slices were fried in cooking palm oil at 180-200 °C for 1 min. The crackers were put above a support rig and penetrated using cylindrical probes (p-2 stainless steel cylindrical probe). This rig was used to measure the fractubility by means of a penetration test. One parameter was measured: hardness (N).

**Linear expansion measurement**

The percentage linear expansion (LE) was obtained on deep frying the dried crackers in cooking oil at 180-200°C. The un-puffed cracker was ruled with three across using a fine oil pen. Each line was measured before and after puffing. The percentage linear expansion was calculated according to the method used by Yu (1991) as follows:

\[
\text{LE} (%) = \frac{\text{Length after puffing} - \text{length before puffing} \times 100}{\text{length before puffing}}
\]

**Colour**

The colour of the fried cracker after was measured using a colorimeter (Minalta CM 300m, Japan). The colour reading includes lightness (L*), redness (a*), and yellowness (b*). The equipment was standardized with a white colour standard.

**Peroxide Value Determination**

The peroxide value was analyzed by applying the colorimetric method. The result was presented in Milliequivalent of peroxide oxygen kg⁻¹. All determinations were carried out in triplicates.

**Oil Absorption**

Oil absorption (OA) was measured according to the method proposed by Nurul et al. (2009). The crackers were weighed before and after frying in palm oil (at 180-200°C) They were ground and dried in the oven overnight and their moisture content was determined by the equation. More over, the crude lipid content was determined by the soxhlet method too.

\[
\text{OA} (\%) = \frac{\text{W of fried sample} - \text{W of unfired sample}}{\text{W of unfired sample}} \times 100
\]

**Sensory Evaluation**

The tomato crispy crackers were subjected to sensory evaluation by panels of 20 people, which comprise of an equal number of people of both sexes within the age bracket of 18-22 years. The panelists had no prior information about the code test products. For the four crackers, the panelists were asked to evaluate for colour, tomato flavor, taste, crispness (texture).

Furthermore, the panelists were asked to integrate their preference for the specific attributes of colour, tomato flavor, taste, and crispness (texture) with the level of gratification elicited by the coded samples in order of overall acceptability. All the five sensory quality attributes were measured using the 5-point hedonic scale. The scale of values ranged from ‘like extremely’ to ‘dislike extremely’ to the highest and lowest scores of ‘5’ and ‘1’, respectively.

The order of presentation was balanced and randomized to eliminate contrast effect and positional bias (Omobuwajo, 2003).

**Microbial Analysis**

The total plate count, *Staphylococcus aureus*, *Escherichia coli*, and mold were analyzed by FDA's Bacteriological Analytical Manual (2001).

**Statistical Analysis**

The experimental design was completely randomized design (CRD). The data collected was analyzed by analysis of variance (ANOVA). The statistical calculation used the statistical programmes. The means of treatment showing significant
Results and Discussion

The moisture content and water activity of the crackers are shown in Table 1.

Table 1: Moisture content and water activity of the fried crackers with different tomato preparation methods

<table>
<thead>
<tr>
<th>Crackers</th>
<th>Moisture content (%)</th>
<th>Water activity (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>1.33±0.36a</td>
<td>0.42±0.08</td>
</tr>
<tr>
<td>BT</td>
<td>0.97±0.18b</td>
<td>0.40±0.10</td>
</tr>
<tr>
<td>KT</td>
<td>1.75±0.26a</td>
<td>0.45±0.13</td>
</tr>
<tr>
<td>BKT</td>
<td>1.25±0.15a</td>
<td>0.41±0.09</td>
</tr>
</tbody>
</table>

1/ Value are means of 3 determinations.
2/ a-b Mean with the same letter within the same column are not significantly different (p>0.05)
3/ ns not significantly difference (p>0.05)

There were significant differences in the moisture content (p<0.05). As a fried product, the low moisture content of the tomato crackers was expected. The Thailand Community Standard (TCS 107/2003) of crispy crackers indicated that the moisture content of edible crispy crackers should be lower than 4%, which is limited for microbial deterioration. Furthermore, the water activity of the crackers was below 0.60, which inhibited the growth of most pathogenic microorganism such as Escherichia coli and Staphylococcus aureus.

For the addition of boiled tomato in the formula, it effected moisture content of crackers, which was lower than other formulas. This might be due to the loss of cell structure of heated tomato (Brook et al., 2008). When mixed the heated tomato with other ingredients it could not entrap the water in its cell, leading water to evaporate easily.

There were significant differences in linear expansion, oil absorption, and hardness of each sample (p<0.05). In the crackers, the starch component gelatinized and expanded on frying. The degree of gelatinization of the starch is one of the factors which influence the degree of expansion of half-products when immersed in hot oil.

Table 2: Linear expansion, oil absorption, hardness of the fried crackers with different tomato preparation methods

<table>
<thead>
<tr>
<th>Crackers</th>
<th>Linear expansion (%)</th>
<th>Oil absorption (%)</th>
<th>Hardness (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>30.26±0.26d</td>
<td>32.64±0.23d</td>
<td>40.65±0.29d</td>
</tr>
<tr>
<td>BT</td>
<td>34.80±0.34c</td>
<td>40.16±0.46c</td>
<td>30.21±0.87c</td>
</tr>
<tr>
<td>KT</td>
<td>46.25±0.75a</td>
<td>58.02±0.26a</td>
<td>22.46±0.21a</td>
</tr>
<tr>
<td>BKT</td>
<td>39.53±0.19b</td>
<td>47.65±0.21b</td>
<td>25.32±0.32b</td>
</tr>
</tbody>
</table>

1/ Value are means of 3 determinations.
2/ a-d Mean with the same letter within the same column are not significantly different (p>0.05)

The oil adsorption of fried crackers was higher in the sample with higher linear expansion and lower in the sample with lower linear expansion. As shown in Table 2, the more the degree of the expansion of crackers, the more air cells will form and the more oil was trapped; consequently, the higher the degree of oil absorption. (Nurul et al., 2009) Moreover, the homogeneous of all ingredient might be effected the linear expansion. As seen in the cracker added with tomato ketchup, most mixture was blended wholsomely. During frying, the final product could not be interrupted by the non-homogeneous mixture.

The hardness of fried crackers increased with the decrease in linear expansion added cracker thickness (Tangkanakul et al., 1994). The hardness of the product might be resulted from the tomato characteristic. The paste and homogeneous body of tomato ketchup may blend with the tapioca flour throughout which did not interrupt the linear expansion and oil absorption. This had led to the lower hardness than the other preparation methods.

Hardness can also be determined by sensory evaluation and usually by using the terms for crispness with an opposite term for hardness. Low hardness will be
shown as a high crispness score and consumer prefers crackers with a high crispness score.

Peranginangin et al. (1997) found that increasing linear expansion will increase the crispness score of fish crackers which means that the product becomes lower hardness value.

**Table 3** Hunter colour L*, a*,b* values of the fried crackers with different tomato preparation methods.

<table>
<thead>
<tr>
<th>Crackers</th>
<th>L*</th>
<th>a*</th>
<th>b*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>55.68±0.86c</td>
<td>4.98±0.74a</td>
<td>0.16±0.03d</td>
</tr>
<tr>
<td>BT</td>
<td>40.36±0.75d</td>
<td>5.12±0.56a</td>
<td>0.18±0.04c</td>
</tr>
<tr>
<td>KT</td>
<td>70.36±0.96a</td>
<td>4.32±0.37b</td>
<td>0.28±0.02a</td>
</tr>
<tr>
<td>BKT</td>
<td>65.88±0.23b</td>
<td>4.73±0.18a</td>
<td>0.24±0.01b</td>
</tr>
</tbody>
</table>

1/ Value are means of 3 determinations.
2/ a-d Mean with the same letter within the same column are not significantly different (p>0.05)

From Table 3, the lightness of tomato crispy cracker which made form tomato ketchup was highest (p<0.05). That was the consequences of the linear expansion, which contributed to some pigments of tomato paste to the colour of the product. Moreover, the linear expansion of the tomato crackers effected the redness of the supplemented tomato ketchup crackers was lower than the rest formula. While, the effect increased the yellowness of the product together, resulting from the high amount of oil uptake into the porous structure (Ngadi, et al., 2007).

The peroxide values of the product in Table 4 present the highest value in the crackers which added with tomato ketchup. The peroxide values are used as the indicator of the extended oxidative rancidity. It is affected by light, temperature, and moisture content of the products. the Thailand Community Standard (TCS 107/ 2003) of crispy crackers indicated that the peroxide value of edible crackers should be less than 30 Milli equivalent of peroxide oxygen kg-1. Thus, all samples were confronted to the standard.

According the oil absorption of all treatments, it was found that the addition of tomato ketchup in crackers had the highest oil uptake value. This effected the maximum crude fat content, compared to other treatments at 3.52 % (p<0.05), and was possible to increased the peroxide value after deep fat frying.

The mean sensory scores are shown in Table 5. In terms of colour, taste, and crispness, the highest sensory scores were in the crackers which added with tomato ketchup. As discuss previously, the linear and oil absorption effected the hardness and colour of the product.

**Table 5** Mean sensory scores for the fried crackers with different tomato preparation methods

<table>
<thead>
<tr>
<th>Crackers</th>
<th>colour</th>
<th>tomato flavour</th>
<th>taste</th>
<th>crispness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT</td>
<td>2.27±0.36b</td>
<td>1.73±0.14a</td>
<td>2.70±0.28b</td>
</tr>
<tr>
<td>BT</td>
<td>2.47±0.28b</td>
<td>1.82±0.24a</td>
<td>1.93±0.47b</td>
<td>3.13±0.45b</td>
</tr>
<tr>
<td>KT</td>
<td>3.80±0.14a</td>
<td>2.07±0.47a</td>
<td>2.93±0.36a</td>
<td>4.00±0.18a</td>
</tr>
<tr>
<td>BKT</td>
<td>1.73±0.18c</td>
<td>1.40±0.19b</td>
<td>2.13±0.24b</td>
<td>3.26±0.37b</td>
</tr>
</tbody>
</table>

1/ Value are means of 3 determinations.
2/ a-b Mean with the same letter within the same column are not significantly different (p>0.05)

In terms of colour and crispness, the panelists prefer the supplemented tomato ketchup which was score around 4.00 or 'closed to like slightly'. In contract, the score of tomato flavor and taste of all produce was below 3.00 or neither like nor dislike, so it should be further developed the formulae of all treatments especially the addition with tomato ketchup.
Table 6 Mean overall acceptability scores for the fried crackers with different tomato preparation methods

<table>
<thead>
<tr>
<th>Crackers</th>
<th>overall acceptability</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT</td>
<td>3.07±0.38b</td>
</tr>
<tr>
<td>BT</td>
<td>3.03±0.12c</td>
</tr>
<tr>
<td>KT</td>
<td>3.58±0.21a</td>
</tr>
<tr>
<td>BKT</td>
<td>3.13±0.30b</td>
</tr>
</tbody>
</table>

1 Value are means of 20 panelists.
2a-b Mean with the same letter within the same column are not significantly different (p>0.05)

From table 6. In term of mean overall preference, the addition of tomato ketchup was 3.58 or ‘closed to Like slightly’ and the other preparation methods were scored around 3.00 or closed to neither like nor dislike. For the microbial analysis, the total plate count of all samples were in the range 5.6 x 10³-7.96 x 10³ cfu g-1 sample, E coli was less than 3 MPN g-1 sample, Staphylococcus aureus was not detected in all samples. Mold was less than 100 cfu g-1 sample. Thus all tomato crispy crackers were confronted The Thailand Community Standard (TCS 107/2003) of fried crispy crackers, and safe for consumer.

Conclusions

The results of this study showed that the effect of tomato preparation on formulation showed different qualities of crispy crackers. The adding tomato ketchup in crispy crackers formulation had resulted in the highest acceptability in all sensory characteristics. Moreover, the moisture content and peroxide values had confronted the Thailand Community Standard. The data presented in this study have demonstrated the potential for the production of tomato crispy crackers. Processing of tomato into these fast-moving snack food is suitable for community production distribution in local food product.

Acknowledgments

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References


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Effect of the Physical Properties on Consumer Preference of Nuggets

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Abstract

Nowadays, health food consumption is more popular. It helps to increase the new types of health foods. One of them is meat analogue product made from plant protein. One problem of this product is low in consumer acceptance. Therefore, it is less preferred than other types of health foods. It must be developed to satisfy consumer by improving some important characteristics. The objective of this study was to identify the physical properties that impact to preliminary consumer preference between chicken nuggets and chicken nugget analogues in Taiwan using preference mapping. From this technique, the important properties which effect to consumer preference will be known. Measured properties consisted of texture profile analysis, shear force, color value (inside and outside of samples) and white index. The results showed that preference mapping presented two dimensions explaining 79.86% of the variation found. Lightness and white index showed positive relationship with the preference of chicken nuggets and chicken nugget analogues, but not for redness (inside), yellowness (inside) and texture properties. It revealed that chicken nuggets were more preferred than chicken nugget analogues. In addition, it indicated that color values, white index and texture properties of chicken nuggets differed from chicken nugget analogues. A source of color difference came from the native color of raw material. Moreover, texture properties of chicken nugget analogues were needed to improve closely to major brands of chicken nuggets. In conclusion, this study has shown that to develop chicken nugget analogue product, it needs to improve product’s color and texture which will affect to consumer preference. This knowledge provides the information for developing the new formula of chicken nugget analogue which can increase the opportunity of product success.

Keywords: chicken nugget, meat analogue, physical properties, preference mapping

Introduction

Since the global environment is changing, therefore many things have altered such as lifestyle, society and popularity. The change creates innovations including new food products. Yaisomanang (2010) reported that consumers have growing concerns about their health and are very careful in choosing food products. Foods are subjected to consume as medicine for both direct and indirect purpose. In addition, snacks will become more popular because of single living. People who still desire interaction will find snacking as an easier and more economic choice for dining. Moreover, people have no time for full meals because of busy life, so snacks are necessary in this situation.

Kanyingi and Ward (2006) reported that soy is the only plant protein that is equivalent to animal protein and high in fiber content. Moreover, soy contains all nine essential amino acids and omega-3 fatty acid required by the human body. Texturized soy protein is made from
extruded soy flour. It can be used as a meat analogue and sold dried in various forms and then re-hydrated. Nowadays, chicken nugget analogues that are produced from soy protein, vegetable oil and vegetarian seasoning are sold in Taiwan and USA. It is snack from meat analogue which contains higher protein and nutrition than real chicken nugget. However, it is less preferred than chicken nuggets from some reasons such as taste, flavor, color or texture.

Repères - Market Research & Opinion (n.d.) reported that marketing executives cannot be content with knowing if their product is liked or not by consumers. They must also explain this overall judgment; identify the strengths and weaknesses of the product so as to guide its improvement. However, consumers are not capable to explain their judgments precisely and reliably. They have neither the sensorial capacities nor the vocabulary required. Preference mapping enables this problem to be resolved. Therefore, the purpose of this study was to identify the physical properties of chicken nuggets and chicken nugget analogues in Taiwan that impact to consumer preference by applying preference mapping technique.

Materials and Methods

Chicken Nuggets and Chicken Nugget Analogues

There were 3 groups of samples which were 3 major brands of chicken nugget, 3 minor brands of chicken nugget and 2 brands of chicken nugget analogues. All samples (2 kg for each sample) were purchased from local department store (Carrefour, Neipu, Taiwan) and transported to the laboratory which were then stored frozen at –18°C until used for experimental purpose.

Preparation of Samples

The container for deep frying was prepared. Five hundred milliliters of palm oil was used for deep frying. The oil temperature was increased with low level of heating and it was measured with thermocouple during heating. Sixteen pieces of samples were fried when oil temperature reached to 180°C for 4 minutes. Oil on the surface of sample was absorbed using tissue paper. Samples were kept in container that could maintain the sample temperature while waiting for doing the next experiment (sensory evaluation, texture profile analysis and shear force measurement, or color value measurement).

Sensory Evaluation

Sample labeling was replaced with 3 digit code number. The sequence of sample serving was randomized by using the table of random sample serving. Panelists were 32 students in Department of Food Science in National Pingtung University of Science and Technology, Taiwan. The first order of 4 samples was served to panelists. Shape, taste, juiciness, texture and overall liking of samples were evaluated by panelists (Das et al., 2008; Thomas et al., 2007). The evaluation was conducted by using hedonic 9-point scale (1 = dislike extremely and 9 = like extremely). For prevention of tiring during sample evaluating, 8 samples were separated to 2 groups and served to panelists for 2 times (4 samples/time). The results of sensory evaluation were analyzed along with other properties.

Texture Profile Analysis and Shear Force Measurements

The texture profile analysis (TPA) was performed by the twofold compression method using Universal Testing Machine 5564 Instron Canton Mass (USA) connected to a PC equipped with Merlin® software. A cylindrical probe 3.19 mm. was used for analyzing. Cylindrical samples (29 mm. of diameter and 15 mm. of height) were subjected for double compression (Das et al., 2008; Somboonpanyakul et al., 2007; Thomas et al., 2007). Deformation level and velocity were 50% of their height and 120 mm/min, respectively. The following texture parameters were determined
Springiness – ability of the sample to recover its original form after the deforming force was removed; Cohesiveness – the ratio of positive force during the second to that of the first compression cycle (downward strokes only); Gumminess – the force necessary to disintegrate a semisolid sample for swallowing; Adhesiveness – work necessary to pull the compressing plunger away from the sample; Chewiness – work to masticate the sample for swallowing; Hardness – which is the maximum force required to compress the sample for the first time, it represents the hardness of the sample at first bite.

Shear force measurement was performed by the same machine. However, probe for analyzing was changed to a Warner-Bratzler probe. Cylindrical samples (29 mm. of diameter and 15 mm. of height) were subjected for analysis. Samples were analyzed with velocity at 30 mm./min (Das et al., 2008).

Color Values Measurement
Color values were determined in CIE-Lab coordinates by using a HunterLab ColorQuest XE (Hunter associates laboratory, USA). Inside and outside color values of samples were expressed in terms of lightness ($L^*$), redness ($a^*$) and yellowness ($b^*$). Moreover, white index was calculated (both inside and outside of samples). White index (WI)'s equation was (Adiletta, 2009):

$$WI = 100 - \{(100 - L^*)^2 + a^2 + b^2\}^{1/2} \quad (1)$$

Statistical Analysis
Means and standard deviations were calculated. SPSS for Windows version 12.0 (SPSS (Thailand) Co., Ltd., Bangkok, Thailand) was used to analyze significant differences between treatments by MANOVA. Moreover, mean comparisons were tested based on Duncan's Multiple Comparison test ($p \leq 0.05$). For cluster analysis (Agglomerative hierarchical clustering) and preference mapping were conducted by using XLSTAT 2007 (Addinsoft, New York, USA).

Results and Discussion

Instrument and Sensory Evaluations
All samples were prepared with deep frying in hot oil (180 °C) for 4 minutes. Objective of this experiment was to collect the datum for making preference mapping. Collecting data consisted of sensory evaluation and physical properties of samples that could be called “instrumental data”.

Means of sensory evaluation of 8 samples were shown in Table 1. These results of preliminary consumer testing from 32 panelists revealed that panelists preferred chicken nuggets more than chicken nugget analogues. Therefore, this preliminary consumer testing could be analyzed with instrumental data for making preference mapping because there was difference of preference level in each sample.

Means of TPA and shear force of 8 samples were shown in Table 2. The results revealed that cohesiveness and adhesiveness of all samples were not significantly different ($P > 0.05$). In contrast, Springiness, gumminess, chewiness, hardness and shear force of chicken nugget analogues were higher than that of chicken nuggets. However, texture properties could not be used for explaining the difference of samples, because chicken nugget’s group (both major brands and minor brands) and chicken nugget analogue’s group had a wide range in TPA and shear force. For example, chewiness was 2.23 – 7.71 N×mm (Major 1 to Minor 3) and 4.25 – 8.30 N×mm (Analogue 1 and 2), hardness was 1.29 – 2.55 N (Major 1 to Minor 3) and 1.58 – 3.30 N (Analogue 1 and 2).

Means of color values & white index of 8 samples were shown in Table 3. Lightness and White index of chicken nugget analogues were lower than chicken nuggets. However, inside redness and inside yellowness of chicken nugget analogues were higher than chicken nuggets. Since
chicken nugget analogues were made from soy protein which its native color is brown, therefore it could effect to the increase of redness and yellowness, but decrease lightness and white index of chicken nugget analogues.

A common question that normally arises from consumer sensory data is the use of mean calculations to represent the acceptance level of the sample evaluated, from which comparisons are made to infer about preference. This important issue is substantiated by a number of works on sensory consumer science calling attention to segmentation. In this line, multivariate statistical technique should be used as a means of revealing underlying consumer preferences (MIQUELIM et al., 2008). Therefore, cluster analysis and preference mapping were performed with the data for this purpose.

Table 1 Mean liking ratings of chicken nugget and chicken nugget analogue samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Shape</th>
<th>Taste</th>
<th>Juiciness</th>
<th>Texture</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major 1</td>
<td>5.66bc±0.29</td>
<td>5.56abc±0.79</td>
<td>5.56ab±0.52</td>
<td>6.19±0.49</td>
<td>6.03ab±0.69</td>
</tr>
<tr>
<td>Major 2</td>
<td>6.66±0.47</td>
<td>6.31±0.62</td>
<td>6.28±0.14</td>
<td>6.16±0.39</td>
<td>6.53±0.22</td>
</tr>
<tr>
<td>Major 3</td>
<td>6.34ab±0.62</td>
<td>6.00±0.59</td>
<td>6.00±0.27</td>
<td>5.88±0.60</td>
<td>6.28ab±0.44</td>
</tr>
<tr>
<td>Minor 1</td>
<td>5.50±0.46</td>
<td>4.78±0.72</td>
<td>4.63±0.43</td>
<td>4.81±0.64</td>
<td>4.66±0.66</td>
</tr>
<tr>
<td>Minor 2</td>
<td>5.31±0.38</td>
<td>5.75±0.59</td>
<td>6.19±0.47</td>
<td>5.81ab±0.71</td>
<td>6.16abc±0.67</td>
</tr>
<tr>
<td>Minor 3</td>
<td>6.03abc±0.26</td>
<td>4.84bc±0.55</td>
<td>5.38b±0.50</td>
<td>5.19bc±0.47</td>
<td>5.47bcd±0.48</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>3.81d±0.55</td>
<td>3.69d±0.67</td>
<td>3.56d±0.74</td>
<td>3.53d±0.64</td>
<td>3.44e±0.52</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>5.81bc±0.93</td>
<td>5.44abc±0.05</td>
<td>5.94abc±0.50</td>
<td>5.38abc±0.93</td>
<td>5.38cd±0.01</td>
</tr>
</tbody>
</table>

Means with identical letter in the same column are not significantly different (p > 0.05).

Table 2 Texture profile analysis and shear force of chicken nugget and chicken nugget analogue samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Springiness (mm)</th>
<th>Cohesiveness</th>
<th>Gumminess (N)</th>
<th>Adhesiveness (J)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major 1</td>
<td>5.49±0.51</td>
<td>0.55±0.15</td>
<td>1.39ab±0.36</td>
<td>-0.0007±0.00</td>
</tr>
<tr>
<td>Major 2</td>
<td>4.89±0.25</td>
<td>0.52±0.16</td>
<td>0.78±0.27</td>
<td>-0.0005±0.00</td>
</tr>
<tr>
<td>Major 3</td>
<td>3.95d±0.79</td>
<td>0.39±0.15</td>
<td>0.61±0.34</td>
<td>-0.0004±0.00</td>
</tr>
<tr>
<td>Minor 1</td>
<td>5.18±0.51</td>
<td>0.48±0.17</td>
<td>1.13bc±0.43</td>
<td>-0.0009±0.00</td>
</tr>
<tr>
<td>Minor 2</td>
<td>3.96b±0.75</td>
<td>0.43±0.15</td>
<td>0.54±0.23</td>
<td>-0.0004±0.00</td>
</tr>
<tr>
<td>Minor 3</td>
<td>3.84b±0.33</td>
<td>0.62±0.20</td>
<td>0.76±0.21</td>
<td>-0.0002±0.00</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>5.19±0.37</td>
<td>0.53±0.11</td>
<td>1.61a±0.52</td>
<td>-0.0005±0.00</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>5.03±0.37</td>
<td>0.54±0.10</td>
<td>0.84±0.22</td>
<td>-0.0002±0.00</td>
</tr>
</tbody>
</table>

ns = Non significant (P > 0.05).

Means with identical letter in the same column are not significantly different (p > 0.05).

Table 2 (Continue)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chewiness (N x mm)</th>
<th>Hardness (N)</th>
<th>Shear force (kgf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Major 1</td>
<td>7.71a±0.40</td>
<td>2.55±0.57</td>
<td>1.10bc±0.31</td>
</tr>
<tr>
<td>Major 2</td>
<td>3.85bc±0.40</td>
<td>1.52bc±0.42</td>
<td>1.09bc±0.36</td>
</tr>
<tr>
<td>Major 3</td>
<td>2.59bc±0.80</td>
<td>1.50bc±0.37</td>
<td>1.47bc±0.11</td>
</tr>
<tr>
<td>Minor 1</td>
<td>6.02ab±0.59</td>
<td>2.34ab±0.56</td>
<td>0.71c±0.20</td>
</tr>
<tr>
<td>Minor 2</td>
<td>2.23c±0.41</td>
<td>1.29c±0.35</td>
<td>0.97bc±0.30</td>
</tr>
<tr>
<td>Minor 3</td>
<td>2.95c±0.89</td>
<td>1.30c±0.42</td>
<td>1.23bc±0.25</td>
</tr>
<tr>
<td>Analogue 1</td>
<td>8.30±0.66</td>
<td>3.30±0.62</td>
<td>2.21a±0.16</td>
</tr>
<tr>
<td>Analogue 2</td>
<td>4.25bc±0.13</td>
<td>1.58bc±0.34</td>
<td>1.35bc±0.20</td>
</tr>
</tbody>
</table>

Means with identical letter in the same column are not significantly different (p > 0.05).
Table 3 Color values and white index of chicken nugget and chicken nugget analogue samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Outside</th>
<th></th>
<th>Inside</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L*</td>
<td>a*</td>
<td>b*</td>
<td>White index</td>
</tr>
<tr>
<td>Major 1</td>
<td>62.62ab±0.48</td>
<td>4.63c±0.08</td>
<td>22.50c±0.32</td>
<td>56.13ab±0.78</td>
</tr>
<tr>
<td>Major 2</td>
<td>59.51c±0.69</td>
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<td>22.24c±0.50</td>
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<tr>
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<td>29.15a±0.45</td>
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</tr>
<tr>
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<tr>
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Means with identical letter in the same column are not significantly different (p > 0.05).

Table 3 (Continue)

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<th>White index</th>
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<td></td>
<td>L*</td>
<td>a*</td>
<td>b*</td>
</tr>
<tr>
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Means with identical letter in the same column are not significantly different (p > 0.05).

Cluster analysis and preference mapping

Overall liking’s scores, texture profile analysis and shear force, and color values and white index of 8 samples were analyzed with agglomerative hierarchical clustering technique for grouping samples together based on product’s preference and physical properties. Results clearly revealed that samples could be clustered into 2 groups (Figure 1). First group was chicken nugget’s group. Second group was chicken nugget analogue’s group. From those results, it revealed that color values (especially, lightness, inside redness and yellowness) and white index were physical properties that made chicken nugget analogues clearly differed from chicken nuggets, because native color of raw material of chicken nugget differed from chicken nugget analogues.
explained 60.68%, t2 explained 19.18%) of the original data, which can be considered a fairly good solution. The other dimensions derived in the model did not contribute for significant explanations of the preference and therefore were not used. From preference mapping, both inside and outside of lightness and white index were physical properties which showed the positive relationship with preference, but not for redness (inside), yellowness (inside) and texture properties (TPA and shear force). Although health is one of the frequently mentioned motivations when consumers make their food choices (Chen, 2011), these preference mapping revealed that chicken nuggets were more preferred than that of chicken nugget analogues. Since chicken nuggets were produced from chicken meat which its native color is white. Whereas, soy protein which its native color is brown was the main ingredient for manufacturing chicken nugget analogues therefore the color of chicken nugget analogues was darker. Moreover, texture properties that included TPA and shear force of chicken nugget analogues differed from chicken nuggets. It showed that texture properties of chicken nugget analogues could not satisfy to consumer and were required to improve by decreasing TPA (springiness, gumminess, chewiness and hardness) and shear force closely to major brands of chicken nuggets. The result of preference mapping was consistent to cluster analysis, but preference mapping could explain texture properties better than cluster analysis, MANOVA and Duncan's Multiple Comparison test. Therefore, color and texture improvements of chicken nugget analogues would help to satisfy consumer.

![Preference mapping of chicken nuggets and chicken nugget analogues.](image)

**Conclusions**

Samples could be clearly clustered into 2 groups that were chicken nugget’s group and chicken nugget analogue’s group. Preference mapping showed that lightness and white index were physical properties which showed the positive relationship with preference. In contrast, physical properties that showed negative relationship with preference consisted of redness (inside), yellowness (inside) and texture properties. Those reasons made real chicken nugget were more preferred than chicken nugget
analogues. Therefore, chicken nugget analogues need to be improved in lightness, white index and texture properties closely to major brands of chicken nuggets for more accepting by consumer.

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Simple Fed–Batch Technique for the Production of Recombinant Enterokinase Light Chain By *Pichia pastoris*

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Abstract

Enterokinase is a serine proteinase with a sequence-specific cleavage at the (Aps)_4 – Lys. Its high specificity for the recognition site makes enterokinase becoming a useful tool for an in vitro digestion of fusion protein. In this work, a secreted recombinant bovine enterokinase light chain (rEKL) was produced by *Pichia pastoris* Y11430 using a simple fed–batch technique. Different constant specific growth rates (µ_set) were investigated for the production performances of the enzyme during methanol production phase. The best experimental result was obtained at µ_set 0.0075 hr⁻¹ with cell density, total protein concentration, and rEKL specific activity of 128 g.L⁻¹, 341 mg.L⁻¹ and 22,654 U.mg⁻¹ proteins, respectively. Even though µ_set of 0.006 hr⁻¹ did not result in the highest amount of total protein concentration, the highest specific activity was obtained at 36,192 U. mg⁻¹ proteins. In addition an increased of the induction time to 117 hrs for both µ_set of 0.006 and 0.0075 hr⁻¹ resulted in the higher total protein concentration, specially specific activity of rEKL, which was much higher level than that previously reported. With this high level production of rEKL, the purification steps would be easier to obtain pure rEKL with high specific activity.

Keywords: enterokinase, fed-batch, high cell density, *Pichia pastoris*

Introduction

Recently, the enterokinase which also called enteropeptidase is widely utilized as a tool protease in research and production of protein engineering for in vitro cleavage of fusion proteins (Fang et al., 2004) because of its high degree of specificity, its tolerance to a wide range of reaction conditions, and its ability to recognize sequence which lies entirely on the amino terminal side of the scissile bond. It also allows the release of carboxyl-terminal fusion partners from fusion proteins without leaving unwanted amino acid residues on their amino termini (Peng et al., 2004). The light chain is a catalytic subunit which recognize and cleavage of both synthetic substrate and fusion protein carrying the (Asp)_4 – Lys target sequence (Light and Fonseca, 1984).

Many heterologous hosts have been used to produce rEKL. Mammalian COS was first used to produce rEKL but very low levels of secreted protein, and activity were obtained (LaVallie et al., 1993). In prokaryotic expression systems, low activity of bovine rEKL have been shown (Yuan and Hua, 2002; Tan et al., 2007). In addition, production of rEKL in *Aspergillus niger* and *Saccharomyces cerevisiae* using complicated medium gave very low concentration of only 1.9 and 3.8 mg.L⁻¹, respectively (Svetina et al., 2000; Kim et al., 2005). Nowadays, *Pichia* expression system is one of the most successful foreign protein expressions systems. Compared with *E. coli* system, it has additional advantages which can help recombinant protein to achieve correct folding and post-translational modification such as glycosylation (Peng et al., 2004; Zhang et
al., 2008; Fang and Huang, 2004). The yield of recombinant proteins was increased in comparison with A. niger and S. cerevisiae expression systems where the highest enzymatic activity was obtained (Zhang et al., 2008).

The recombinant protein production by P. pastoris was affected by many parameters but the methanol feed control during production phase can lead to either success or failure of the production. The methanol flow rate must be controlled to provide a methanol concentration that is just enough for protein synthesis but not in excess. The high methanol concentrations in fermentation broth might lead to the generation of toxic products such as formaldehyde and hydrogen peroxide which may compromise cell viability and productivity (Rosenfeld, 1999). The methanol feeding strategy, which also dictates the specific growth-rate, is a key parameter for maximizing recombinant protein production (Cos et al., 2005). Though various bioprocess strategies have been studied for methanol feeding, which could be metabolism related, based on kinetics parameters such as oxygen consumption and methanol consumption (Kobayashi et al., 2000; Hellwig et al., 2001), low expression efficiency of target protein would occur because P. pastoris cells were competing for methanol, the only carbon source, for cell growth and protein expression. In contrast to these multi-phase protocols, a simple fed–batch technique is proposed here. The production of recombinant enterokinase based on the methanol fed according to the ratio of methanol to cell concentration was performed.

Material and Methods

Microorganism

P. pastoris Y11430, a wild–type (Mut+ and His+) strain containing the pPICZαB NH8 EKL (Kupradit et al., 2008) was used for production. This construct also contains AOX1 promoter which can be induced by methanol and uses the zeocin resistance gene as a selectable marker.

Inoculum Preparation

Primary inoculum was prepared by picking a colony of P. pastoris from YPD agar into 20 ml YPD broth. The secondary inoculum was prepared by transferring the primary inoculum into a 250 ml shake flask that contained 80 ml glycerol BMGY medium pH 5.0. The culture was incubated at 30 °C, with 200 rpm rotary shaking for 24 h. Zeocin 100 µg. mL⁻¹ was used in all medium.

Fed–Batch Technique

All fed–batch cultures was carried out in a 2 L fermentor (Sartorius, Germany) containing 950 ml GBS medium (containing H₃PO₄ 85% 26.7 ml, CaSO₄ 0.93 g, K₂SO₄ 18.2 g, MgSO₄.7H₂O 14.9 g, KOH 4.13 g, glycerol 40.0 g, PTM1 trace salts 4.35 ml in 1 L of deionized water). The PTM1 trace salts contained: CuSO₄.5H₂O 6.0 g, KI 0.08 g, MnSO₄.7H₂O 3.0 g, Na₂MoO₄.2H₂O 0.2g, H₂BO₃ 0.02 g, ZnCl₂ 20.0 g, FeCl₃ 13.7 g, CoCl₂.6H₂O 0.9 g, H₂SO₄ 5.0 ml, biotin 0.2 g in 1 L of deionized water. Ammonia solution 25% was used as an alkaline to control and adjust the pH. Antifoam was added manually when high level of foam occurred. The fermentation process was divided into 4 stages as followed;

Glycerol batch phase

The fermentation condition was set up and controlled by an automatic temperature controller (Julabo, Germany) at 30 °C, 1 vvm aeration rate, 1,000 rpm agitation and pH 5.5. 5% inoculum (50 ml and OD about 3) was transferred into a 2 L fermentor. The glycerol was completely consumed as indicated by a sharp increase in the DOT signal.

Glycerol fed-batch phase

After glycerol batch phase, glycerol feed (GF) medium (glycerol 500 g.L⁻¹ and PTM1 trace salts 12 ml.L⁻¹) was added to the fermentor with the feeding of 16.46
g.hr⁻¹ during 3.85 h which was calculated and based on the data previously reported to obtain around 40 g/L cell concentration at the end of this phase (Jahic et al., 2002; Charoenrat et al., 2005; Kupradit et al., 2008).

**Methanol induction phase**

The methanol induction phase was started by replacing the GF medium with the methanol feed medium (MF) (12 ml PTM1 trace salts per liter of methanol). For 1 L initial fermentation volume, 0.633 ml of MF medium was injected into fermentor 5 times during 3 hours until the *P. pastoris* can be fully adapted to use methanol as substrate as recognized by the change of DOT.

**Methanol production phase**

The feeding of MF medium was calculated to increase the cell mass concentration exponentially in the bioreactor at growth rates which prevent the accumulation of toxic levels of methanol. The methanol feed was automatically fed to the fermentor by using a syringe pump (Cole Parmer, USA). The temperature was decreased to 20°C after first the 2-3 h and then kept until the end of the process.

**Cell Concentration**

Cell concentration was determined by measuring the optical density at the absorbance of 600 nm (OD₆₀₀). The dry cell weight was determined by centrifugation 5 ml culture samples at 4000 rpm for 10 min. The supernatant was collected for further analyses, and the pellet was washed with distilled water once and dried at 105°C, until constant weight.

**Protein Concentration**

The total protein concentration in the supernatant was analyzed by using Bradford technique. Bovine serum albumin was used as a standard.

**Enterokinase Activity Assay**

The assay of enterokinase activity was modified from the methods of Hermon – Taylor (1970) which determined using the fluorogenic substrate Gly–(Asp)₄–lys–β–naphthylamide (GD₄K–β–naphthylamide). Thirty micro liter of the crude culture supernatant was added to two hundreds micro liter of substrate solution (50 μM GD₄K–β–naphthylamide in 70 mM Tris–Cl pH 8 and 10% Dimethyl Sulfoxide). The enzymatic activity was measured using Spectra Max Gemini EM machine by an increasing of fluorescence at excitation 337, emission 420 nm that caused by the release of β–naphthylamide over one minute interval. The commercial rEK₄₅ from Invitrogen was used as a positive control.

**Residual Methanol Concentration**

Residual methanol concentration was analyzed using a gas chromatograph (GC) equipped with a flame ionization detector (SRD Instrument, USA). Helium, 99.999% pure, was used as carrier gas. The GC column (Carbowax®, Restek, USA) was a 30 m × 0.32 mm bonded phase fused silica capillary column. The injector and detector temperatures were set at 200, and 300 °C, respectively. The oven was operated at programmed temperature, from 41 to 200 °C at the rate of 15 °C. min⁻¹.

**Calculation of the Methanol Feed**

Since the accumulation in the medium and the evaporation of methanol were insignificant in comparison to the feed, the integrated value of this feed rate can be assumed as the total methanol consumption. The feeding rate was calculated to allow the volumetric cell mass concentration to increase exponentially as suggested by a previous work (Korz et al., 1994).

With a desired specific growth rate μ and a given biomass concentration the actual feeding rate of methanol is given by:

$$m_s(t) = F(t)S_F(t) = \left(\frac{\mu(t)}{Y_{X/S}}\right)\nu(t)X(t)$$

Where; $m_s$ is the mass flow of substrate (g.hr⁻¹), $F$ is the volumetric feeding rate (L.hr⁻¹), $S_F$ is the methanol concentration in solution (g.L⁻¹), $\mu$ is the specific growth rate.
\( Y_{XS} \) is the biomass/substrate yield coefficient \((\text{g.g}^{-1})\), \( m \) is the specific maintenance coefficient \((\text{hr}^{-1})\), \( X \) is the biomass concentration \((\text{g.L}^{-1})\) and \( V \) the cultivation volume \((\text{L})\), respectively.

In a fed-batch system:

\[
\frac{d(XV)}{dt} = \mu XV
\]

(2)

If we assume \( \mu \) as time invariant one obtains integration when starting the feeding at time \( t_S \)

\[
X(t) = X(t)\int X(t)\text{e}^{\mu(t-t_S)}
\]

(3)

By introducing Eq. 3 into Eq. 1, the substrate mass feeding rate for a constant specific growth rate \((\mu_{\text{set}})\) can be calculated as follows:

\[
m_f(t) = \frac{\mu_{\text{set}}}{Y_{XS} + m}V_f X_0 e^{\mu_{\text{set}}(t-t_S)}
\]

(4)

In this work, the yield coefficient \( Y_{XS} \) was set at 0.27, \( m \) at 0.035 h\(^{-1}\) was obtained from previous works (Charoenrat et al., 2005; Kupradit et al., 2008), and different constant specific growth rates \((\mu_{\text{set}})\) 0.006, 0.0075 and 0.0105 hr\(^{-1}\) was used to calculate the MF medium feeding rate.

**Results and Discussion**

The results of the cell density cultivation during the first three stages of all fermentations are shown in Fig 1. At the end of the glycerol batch phase, the cell dry weight was approximately 26-28 g.L\(^{-1}\). The glycerol fed-batch phase was started by feeding glycerol feed (GF) medium into the fermentor in order to not only derepress the aox1 promoter, but also to increase cell density to approximately 40 g.L\(^{-1}\) before starting methanol induction phase. In methanol production phase, the result for total methanol consumption at different specific growth rates \((\mu_{\text{set}})\) 0.006, 0.0075 and 0.0105 hr\(^{-1}\) in the three processes are shown in Figure 2. At the beginning, 2.29, 2.51 and 2.95 g.hr\(^{-1}\) of MF medium were fed to the fermentor for the three consecutive values of \( \mu_{\text{set}} \) and then increased every 3 hours until the end of this phase. The optimal feeding of MF medium and induction time to get high cell density, total protein concentration, residual methanol concentration, and enzymatic activity were investigated, respectively.

Accumulation of methanol inside the fermentor at different feeding rates was also illustrated in Figure 2. The residual methanol concentration was detected since the beginning of the process at \( \mu_{\text{set}} \) 0.0105 hr\(^{-1}\) before increased to 1.74 g.L\(^{-1}\) at only 69 h of induction. At \( \mu_{\text{set}} \) 0.006 hr\(^{-1}\), the residual methanol concentration was found after 69 h of induction time and it was only determined after 93 h induction for fermentation at \( \mu_{\text{set}} \) 0.0075 hr\(^{-1}\)

**Cell Density**

The cell densities at three different feeding strategies were significantly different during methanol production phase. The highest cell density of 128 g.L\(^{-1}\) was obtained at the end of the fermentation for
Increased but the total protein concentration decreased rapidly. This may be due to the accumulation of residual methanol which exceeded inhibitory level, thus inhibit the cell growth and reduced total protein concentration. In the other two fermentation processes, total protein concentration was increased overtime. The highest total protein concentration was obtained at approximately 341.13 mg.L\(^{-1}\) for \(\mu_{\text{set}} 0.0075\) hr\(^{-1}\), compared to 187.25 mg.L\(^{-1}\) in the fermentation of \(\mu_{\text{set}} 0.006\) hr\(^{-1}\).

The Enterokinase Accumulation and Apecific Activity
The enterokinase accumulation during methanol production was significantly different between three fermentation processes. The maximum enterokinase activity was found at \(\mu_{\text{set}} 0.0075\) hr\(^{-1}\) \((7.7 \times 10^6 \ \text{U.L}^{-1})\) which was higher than at \(\mu_{\text{set}} 0.006\) hr\(^{-1}\) \((6.0 \times 10^6 \ \text{U.L}^{-1})\) at 117 hours of induction time and much higher than in
fermentation at $\mu_{set}$ 0.0105 h$^{-1}$ (1.0×10$^6$ U. L$^{-1}$) as shown in Figure 5.

**Figure 5** Process profile for rEK$_L$ activity.

The induction time affected the production and enzymatic activity of rEK$_L$. The activity of rEK$_L$ reduced after only 21 hr of induction time for the fermentation at $\mu_{set}$ 0.0105 hr$^{-1}$. In addition, for both $\mu_{set}$ 0.0075 hr$^{-1}$ and 0.006 hr$^{-1}$, after 117 hours of induction time, the enzymatic activity decreased even when the protein concentration still increased (Figure 5). It could be summarized that the effect of methanol concentration on *P. pastoris* culture is that excessive methanol inhibits growth (Zhang *et al*., 2000; Mayson *et al*., 2003) and also cause cell death (Jahic *et al*., 2003) which leads to degradation of proteases. It has been observed that the optimum methanol concentration for cell growth differed significantly from the optimum methanol concentration for production (Mayson *et al*., 2003), and the specific product formation rate ($q_p$) does not correlate to the specific growth rate ($\mu$) (Kupcsulik and Sevella, 2004). However in this work, the optimum methanol concentration for cell growth correlated with the optimum concentration for protein production but differed from the enzymatic activity. The reasons can be explain by the different kind of proteins and strains of *P. pastoris* used for fermentation. From our results, we concluded that the optimum methanol concentration for cell growth correlated to the optimum methanol concentration for production. At $\mu_{set}$ of 0.0075 hr$^{-1}$, the highest cell density, total protein concentration, and enterokinase activity were obtained at 117 hours of induction time. However, the highest specific rEK$_L$ was presented in fermentation at $\mu_{set}$ 0.006 hr$^{-1}$ (3.6×10$^4$ U. mg$^{-1}$ protein), the specific activity of rEK$_L$ at $\mu_{set}$ 0.0075 hr$^{-1}$ was still very high (2.5×10$^4$ U. mg$^{-1}$ protein).

**Figure 6** Process profile for specific enterokinase activity.

The results from SDS–PAGE of the three fermentation processes also indicated that only small amount of the *P. pastoris* own protein was secreted to the culture medium. Thus it is an advantage for further purification, and facilitate separation of the product from most other cellular components. However, the expected protein band in three fermentation process was different. Some foreign protein secreted in *P. pastoris* appeared to be hyperglycosylated (Cregg *et al*., 2000). Little different in size of oligosaccharides could be correlated to composition of the growth medium or cultivating in shake-flask or fermentor (Brethayer and Castellino, 1999). In this research, the different protein size possible may be due to the different glycosylation process at different concentration of methanol feeding rate. In order to have exact answer for this problem, deglycosylation with EndoF and purification steps will be carried out.
Conclusions

Recombinant enterokinase light chain was successfully produced in *P. pastoris* by using a simple fed-batch technique. The results for cell density, protein concentration, enterokinase activity, and specific enterokinase activity were significantly different between the three fermentation processes. Using the feeding strategy at $\mu_{set} = 0.0075$ h$^{-1}$, not only high cell density and overall protein yield but also the highest enterokinase activity was obtained at 117 h. Therefore, this study presented a simple and cost-effective procedure that might be applicable for large-scale production.

Acknowledgments

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Structure Characterization and Molecular Docking Studies of α-Amylase Family-13 Glycrosyl Hydrolases from Lactobacillus plantarum Complexed with Maltoheptaose: a Novel Feature of α-Amylase Catalytic Mechanism

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Abstract

The aim of the work was to contribute to the understanding of the roles played by specific binding interactions and electrostatic effects of the catalytic sites of α-amylase from Lactobacillus plantarum, which reacts with maltoheptaose. The α-amylases (E.C.3.2.1.1) from glycoside hydrolase family 13 (GH13) are one of the most important and oldest industrial enzymes. Amylases are enzymes which hydrolyze the starch molecules into polymers composed of glucose units. Although amylases be derived from several sources, including plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Amylolytic lactic acid bacteria (ALAB) utilize starchy biomass and convert into lactic acid in single step fermentation. Only minority of lactic acid bacterial species have the capacity to produce hydrolysis enzymes. A strain L. plantarum was found to produce amylase enzyme. The results indicated that homology based approach for predicting the three dimensional (3D) structures of α-amylase from L. plantarum using α-amylase Amy 2 as start structure as templates showed a very similar structure as expected from the high sequence identity. Moreover alignment studies raised amino acid substitutions in α-amylase from L. plantarum compare to α-amylase from Bacillus subtilis which may affect the α-amylase putative active site leading to the formation of an extra hydrogen bond between Asp171, Glu200, Asp277 and Asp176, Gln 208, Asp269, respectively. The interactions between α-amylase from L. plantarum and maltoheptaose were predicted by flexible docking including minimization. Further investigations are underway to explore the positions binding site of α-amylase complexed with maltoheptaose.

Keywords: α-amylases; Lactobacillus plantarum; family 13 of glycoside hydrolase; homology modeling; molecular docking.

Introduction

Amylases are enzymes which hydrolyse starch molecules to give diverse products including dextrin and progressively smaller polymers composed of glucose units (Windish et al., 1965). These enzymes are of great significance in present day biotechnology with applications ranging from food, fermentation, textile to paper industries (Pandey et al., 2000). Among starch converting enzymes, the α-amylases (E.C. 3.2.1.1) are of special importance (Nielsen and Borchert, 2000) and extensively studied (MacGregor et al., 2001). α-1,4-Glucan-4-glucanohydrolase
(α-amylase) are widely distributed in plants, mammalian tissues and microorganisms. Various Lactobacillus strains exhibiting amylolytic activity have been isolated and in a few cases the corresponding α-amylase. The α-amylase genes of L. plantarum, L. amylovorus (Giraud and Cuny, 1997) and L. manihotivorans (Morlon-Guyot et al., 2001) have been sequenced. Multiple sequence alignments are now one of the most widely used bioinformatics analyses. They are needed routinely as parts of more complicated analyses or analysis pipelines and there are several very widely used packages, e.g. Clustal W & Clustal X (Thompson et al., 1994 and 1997), T-Coffee (Notredame et al., 2000), MAFFT (Katoh et al., 2002) and MUSCLE (Edgar, 2004). The computed alignment was manually checked and corrected. Pair-wise evolutionary distances were computed using the passion correction algorithm employed and phylogenetic tree was constructed by neighbor-joining method.

The development of powerful search methods that permit the detection of sequence similarity in related proteins has provided a formidable tool to identify proteins that are related in structure and, possibly, in function (FASTA, BLAST; (Altschul et al., 1990 and 1997). A three-dimensional structural model of a protein is a powerful asset in the investigation of its biological function, but producing such a model quickly and inexpensively by crystallography or NMR is not always possible. Many of the rapidly accumulating genomic sequences have detectable homologues in the database of experimentally solved three-dimensional structures from which structural models may be built by theoretical prediction. For individual proteins, the most successful technique is homology modeling. The quality of homology modeling depends on whether these exists one or more protein structures in the protein structure databases that show significant sequence similarity to the target sequence. The description and analysis of protein surfaces has been essential in the study of protein-protein interactions, and in the development of tools for automated docking methods (Strynadka et al., 1996; Ausiello et al., 1997). Docking methods are considered to be the best of the computational approaches to predict the 3D structure of a complex given the 3D structures of the monomers. Since it is outside of the scope of this paper to review the existing docking algorithms, we will simply mention the most popular methods such as GRAMM (Tovchigrechko et al., 2002), ZDOCK (Chen et al., 2002), PPD (Norel et al., 2001), AutoDock (Moriss et al., 1998), ClusPro (Comeau et al., 2005), ICM-DISCO (Carter et al., 2005) and many others (Schneiderman-Duhovny et al., 2005). While docking simulations focus on determining the 3D structure of the complex without modeling the binding pathway, the alternative binding simulations approach (McCammon, 1998) is intended to reveal all of the association details, including the binding constant (Gohlke et al., 2003).

In this paper, we report a homology model of α-amylases using the crystal structures of α amylases from L. plantarum and Bacillus subtilis [PDB code: 3dhu and 1bag, respectively]. Using the three-dimensional (3D) model, we have identified the residues important for α-amylases activity, i.e. the substrate and inhibitor-binding site, which can be exploited in the design of more potent inhibitors. Phylogenetic tree of α-amylases Amy2 from L. plantarum which isolated from starchy waste was constructed using the other homologous α-amylases sequences.

Materials and Methods

Sequence Similarities and Phylogenetic Analysis.

The protein sequence of α-amylases from Lactobacillus plantarum was aligned with 4 sequences of α-amylases which were retrieved from NCBI GenBank. GenBank® is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences provided by the National
Center for Biotechnology Information (NCBI). All sequences were used to align in Clustal W program (Larkin et al., 2007; Thompson et al. (1994) and observed the percent identity of α-amylases sequences. The computed alignment was manually checked and corrected. Pairwise evolutionary distances were computed using the passion correction algorithm employed in Muscle program and phylogenetic tree was constructed by neighbor-joining method. Total of 100 bootstrapped values were sampled to determine a measure of the support for each node on the consensus tree.

Predictions of Three-Dimensional Structure α-Amylases from Lactobacillus plantarum by Homology Modeling Method.

The 3D structures of α-amylases from L. plantarum were constructed. The 3D structures of α-amylases from L. plantarum was generated using the homology module (Needleman and Wunsch, 1970; Fiser and Sali, 2003) of Insight-II based on X-ray structure of template which showed the highest identity. The sequences of α-amylases from L. plantarum and template were aligned by homology module to identify the blocks that are likely to contain structurally conserved region. All atomic coordinates of the residues in those blocks were transferred from template to build the modeled structure of α-amylases from L. plantarum. The homology-modeled structure was then energy-minimized by discover module of Insight-II Accelrys and favored amino acid residue in Ramachandran plot (Ramachandran et al., 1963) its accuracy was considered.

Molecular docking by Autodock

• Binding site definition

The Autodock (Comeau et al., 2005) version 4.0 was used rectangular boxes for the definition of the binding site. In the plug-in, the box center can be defined either by providing explicit coordinates or, more user-friendly. The box center is then calculated from the mean coordinates of the atoms from program. The size and the exact position of the box can also be adjusted to the user’s demands. For visualization purposes the plug-in furthermore allows to chose between two display options and the color of the box frame. Binding site definitions defined here can also be exported to input files for either Autodock.

Setup and Execution of Docking Runs

Autodock need receptor and ligand representations in a file format called pdbqt which is a modified protein data bank (Moriss et al., 1998) format containing atomic charges, atom type definitions and, for ligands, topological information (rotatable bonds). These file preparations are carried out by the plugin using scripts from the Autodock Tools package. Alternatively, run input files can be written to start the docking runs from the command line. Both Autodock allow for flexibility of predefined side chains during docking. Here the plugin facilitates the selection of flexible side chains. Side chains within the docking box can be visualized straightforwardly and can be translated into a flexible receptor definition.

Binding Site Analysis with Interaction Maps

Autodock uses interaction maps for docking. Prior to the actual docking run these maps are calculated by the program autogrid. For each ligand atom type, the interaction energy between the ligand atom and the receptor is calculated for the entire binding site which is discretized through a grid. This has the advantage that interaction energies do not have to be calculated at each step of the docking process but only looked up in the respective grid map. In addition to speeding up a docking runs the grid maps on their own can also provide value hints for ligand optimization. Since a grid map represents the interaction energy as a function of the coordinates their visual inspection may reveal potential unsaturated hydrogen acceptors or donors or unfavourable overlaps between the ligand
and the receptor. Since several maps can be loaded and controlled simultaneously, a rapid inspection of several interaction types can be made very easily (Trott and Olson, 2010).

2TAA_B | PDBID | CHAIN | SEQUENCE
---|---|---|---
ATPDWRSSQSIYFLR1TDARGSTTATCDNTAQDYYCGGWTQWQIDKLDL 50

2GUY_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
ATPDWRSSQSIYFLR1TDARGSTTATCDNTAQDYYCGGWTQWQIDKLDL 50

3DHU_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
MLGLYQGNNMTHYFVYNS---EAGNFGAVTDLO 36

1BAG_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
TLAEGITSGTLNHK--------WSTNLKHHNK 27

1BLI_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
ANLNGTMYQFEWMN--------DSQWHRQLQDSOA 30

2TAA_B | PDBID | CHAIN | SEQUENCE
---|---|---|---
YIQGGMGTAIWFITPVTQPQDCA-------YVDATVGYWTQD1SLYNEN96 50

2GUY_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
YIQGGMGTAIWFITPVTQPQDCA-------YVDATVGYWTQD1SLYNEN96 50

3DHU_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
RKLQGDIYLDLIPFIVEVNR--------KGTLLG1AYKDYRGINFPEY 81

1BAG_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
DIVDAGYTAIQSPV-7QVENQGKDSKMNWQNLTY6TQ15NGRYLT 76

1BLI_A | PDBID | CHAIN | SEQUENCE
---|---|---|---
YLAEGITSGTLNHK--------WSTNLKHHNK 27

Results and Discussion

Finding ways to more explain these features, homology modeling and alignment studies were carried out. We present the sequence alignment of α-amylases from Lactobacillus plantarum with that of α-amylases. A correspondence of a α-amylase from L. plantarum, corresponding to positions 1-449 showed 16% of similarity with 1BAG which was α-amylase from B. subtilis. When the sequence identity is below 30%, the error in the aligned main chain atoms can be estimated from the sequence difference.

Figure 1 Multiple sequence alignments of α-amylases from Aspergillus oryzae (PDB2TAA), Aspergillus niger (PDB2GUY), Lactobacillus plantarum (PDB3DHU), Bacillus subtilis (PDB1BAG) and Bacillus licheniformis (PDB1BLI). Numbering of the sequences starts with N-terminus of the mature protein.
Simple linear relation has been found between the structural difference and sequence difference if the sequence difference is taken to be the average of that between the sets of sequences compatible to the structures (Koehl and Levitt, 2002.

The structure models are constructed from the residuals of the structure template that are aligned to the target sequence in the sequence comparison. The quality of this alignment thus is critical for the accuracy achievable. The aligned residues from sequence comparison are generally different from that from structure-structure comparison though, especially when the sequence identity is low. For comparative modeling, protein prediction methods based on homology have been used. The amino acid differences between α-amylases from *L. plantarum* (PDB3DHU) and α-amylases from *L. plantarum* from homology modeling method were spread out through the whole structure (Figure 2). As to be expected, the model is very similar to the structures of the templates. Moreover, there are no long loop insertions in α-amylases as 0.91%. The score expresses how well the backbone conformations of all residues are corresponding to the known allowed areas in the Ramachandran plot.

![Figure 2A](image1.png)  
**Figure 2A** Superimposed computer-generated backbone, α-amylase (green) were taken from the Protein Data Bank (3DHU) and α-amylase (magenta) from homology modeling method. The three-dimensional structure of α-amylase from Protein Data Bank and from homology modeling method are similar in that they each consist of (α/β)8 barrel in domain A.

![Figure 2B](image2.png)  
**Figure 2B** The protein backbone superimposed structure of α-amylase (green) from the Protein Data Bank and α-amylase (magenta) from homology modeling method showing the side chains of the catalytic site Asp171, Asp277 and Glu200 which is located on the C-terminal side of the (α/β)8 barrel.

The conserved active site residues in α-amylase from *Lactobacillus plantarum* was compared with *Bacillus subtilis* (PDB 1BAG) structure, which also revealed the similar position of active site. According to MacGregor (1988), the conservation of these four amino acids (His122, Asp206, Glu230, Asp297 of *Aspergillus oryzae*) and a few semi-conserved amino acids indicated strong similarities throughout the α-amylase family at the catalytic site. This is quite reasonable since all catalyze the same hydrolytic reaction.

![Figure 3A](image3.png)  
**Figure 3A** Electrostatic surface representation of α-amylase from *L. plantarum*. The surface potentials of color property from blue, positively charged to red, negatively charged. The binding modes were usually bound in the part of subsite of the active site with yellow dashed circle.
The protein backbone structure of \( \alpha \)-amylase from \( L. \) plantarum showing the side chains of the catalytic site Asp171, Asp277 and Glu200. The data for this plot were taken from the Protein Data Bank (PDB3DHU). The structure consists of three domains, the central \((\alpha/\beta)_8\) barrel (domain A), a five stranded antiparallel \( \beta \)-sheet domain C at the bottom, and a protruding loop at the top (domain B).

The protein-ligand complexes give greater insights in structure based drug design, so a protein-ligand complex was developed. It gives a more detailed and accurate picture of interact favorably to a particular receptor based on the predicted free-energy of binding.

The active site pocket of template revealed that the ligand is highly embedded in hydrogen bond donor region of the protein. Docking simulation generated by the docking programs can be directly loaded into. For each docking pose, information containing the docking score is displayed in a small text viewer, allowing direct analysis of configuration/score relationships. Moreover, results from multiple docking runs are shown in Figure 5. The docking poses are ranked according to their docking scores and both the ranked list of docked ligands and their corresponding binding poses may be exported. Comparative active site analysis of \( \alpha \)-amylase from \( Lactobacillus \) plantarum (3DHU) and maltoheptaose shows highly conserved residues Trp(137) : O(42), Trp(137) : O(43), Gly(203) : O(36), Gly(205) : O(26), Phe(206) : O(25), Arg(280) : O(72), His(317) : O(71), Gln(381) : O(55), Gln(318) : O(71) and Gln(318) : O(75), respectively (Figure 5b). The active site residues in homology model Asp171, Asp277 and Glu200 are different from those residues involved in the active site of \( \alpha \)-amylase from \( L. \) plantarum (3DHU). The electrostatic surface of \( \alpha \)-amylase complexed with maltoheptaose from \( L. \) plantarum was shown in Figure 5a. The surface potentials of color property from blue, positively charged to red, negatively charged. The binding modes were usually bound in the
part of subsite of a deep binding groove in yellow area.

Finally, docking study of the model was performed in order to elucidate its structural and functional relevance in terms of binding site. The active subsite and binding site were clearly classified based on molecular docking. The active site of α-amylase from *L. plantarum* on the c-terminal site of the (α/β)8 barrel, with the catalytic residues Asp171, Asp277 and Glu200. Several invariant residues, including Ty137, His317, Arg280, Phe206 and His317 form hydrogen bonds with the maltoheptaose.

**Conclusions**

In the present work the three-dimensional model of α-amylase from *Lactobacillus plantarum* was constructed in order to accomplish its molecular modeling and docking studies. The resulting molecular docking simulations were analyzed for binding interactions of α-amylase which the active site of α-amylase from *L. plantarum* on the c-terminal site of the structure, with the catalytic residues Asp171, Asp277 and Glu 200. Several invariant residues, including Ty137, Glu318, Gly203, Gly205, Arg280, Phe206 and His317 form hydrogen bonds with the maltoheptaose. Protein structure is conserved during evolution much better than protein sequence. There are numerous examples of proteins that show little sequence similarity but still adopt similar structures, contain identical or related amino acid residues in their active sites, and have similar catalytic mechanisms. These shared features support the notion that, despite low sequence similarity, such proteins are homologous. Overall, our results suggest that the simple modeling procedure applied here could help identify and characterize, at least in a preliminary way, protein-protein complexes. The next step is to apply it on a laboratory scale.

**Acknowledgements**

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**References**


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Origin of Proteolytic Enzymes Involved in Production of Malaysian Fish Sauce, Budu

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Abstract

This study focuses on the investigation of the origin of proteolytic enzymes involved during fermentation in the production of Budu from an under-utilised fish, Ilisha melastoma. Budu samples was produced from 3 types of fish parts- the head, consisting of the head and the muscular upper half of the body; the body, comprising of the lower parts of the fish, including the visceral, and also the whole fish. Six analyses which determined the liquid yield, degree of hydrolysis, salt content, pH, protease activity and crude protein content of fish sauce were carried out on these samples. Results indicated that the protease activity, crude protein and degree of hydrolysis of Budu samples from fish body, where the protease enzymes were mainly found in the pyloric caeca and intestines of fish, were significantly higher (P<0.05) than that of fish head at the initial 0 to 14 days of fermentation. Endogenous protease enzymes from the fish were more active in the initial stage of fermentation. There was no significant difference in pH between fish head and fish body samples during fermentation, except at days 60 and 90. Fish head had significantly higher (P<0.05) salt content due to its lower muscle content enable the salt to penetrate inside the head at a faster rate. The results implied that the endogenous fish enzymes, especially from fish viscera were the main contributor of protease action during the initial days of fermentation. Bacterial enzymes may be involved in the later stage of fermentation.

Keywords: Fish sauce; proteases; proteolysis; anchovy

Introduction

Fish is regarded as an excellent source of protein in terms of quality, supply and cost. However, fishes are easily perishable and became unpalatable due to improper handling and storage after capture. Therefore, fish sauce processing appears to be an efficient technique to utilize small, less utilized pelagic fish. The raw materials of fish sauce fermentation can be shellfishes, such as oyster and shrimps or small fishes such as anchovy (Dissaraphong et al., 2006). The fermentation of fish sauce is widely carried out in Southeast Asia and is becoming more popular in Europe, North America (Klomklao et al., 2006).

Fermentation is a very old technique used in food preservation as it extends the shelf life of the product, and also enhances the flavour and nutritional quality of the food. Traditionally, fish sauce was produced by mixing fish with salt at a ratio of 3:1 and then fermented for 6-12 months (Tsai et al., 2006). The fish sauce produced is a clear brown liquid hydrolysate and is used for flavor enhancement and a source of nutrients (Lopetcharat et al., 2001). Fermented anchovy sauce is also used in Korea to improve the taste and flavour of traditional foods (Heu et al., 1997).

Budu is a Malaysian fish sauce which is a thick brownish liquid, pH 5.6, saturated with salt and containing 1.77% organic nitrogen and 0.12% being volatile nitrogen (Orejano and Liston, 1982). The characteristic odor of fish sauce is attributed to the various amino acids,
nucleotides, peptides, ammonia and urea present in the liquid (Klomklao et al., 2006).

Various studies had been conducted to determine the origin of the fish sauce (Orejano and Liston, 1982; Kirshbaum et al., 2000; Siringan et al., 2006). However, the origin of proteinases involved in fish sauce fermentation is poorly understood. It has been widely thought that the origin of proteinases involved in fish sauce fermentation is from the endogeneous proteinases. The objectives of this study were to investigate the origin proteinases involved in the protein hydrolysis of fish.

Materials and Methods

Sample preparation

Fresh anchovies (*Ilisha melastoma*) were purchased from local market in Penang, Malaysia. The anchovies were cut into different parts, body, head with the whole fish as a control, and mixed with salt in ceramic containers with a fish/salt ratio 3:2 (w/w). Body of fish included the gut while head of fish contained only the head and upper muscle part of the fish. The containers were ferment in incubator at 40 °C. Samples were withdrawn at the interval sampling times of 0 day, 14 day, 28 day, 2 months, 3 months, 4 months and 5 months.

Liquid Percentages

After incubating for the designated time, the samples were centrifuged at 710 g for 5 minutes to obtain the liquid portion. The liquid obtained was used for further analysis.

Protease Activity

Protease activity of the fish sauce was determined according to the method of Wang et al., (1974) method. Approximately 1 ml sample was diluted with 9 ml distilled water. Then 0.1ml diluted samples was transferred to a test tube and added with 1 ml Tris buffer (pH 7.0) with 1 % bovine serum albumin and heated at 38 °C for 20 minutes. Then 3 ml trichloroacetic acid solution, 5 % (w/v) was added to stop the reaction. The mixture was centrifuged at 7000 rpm for 10 minutes. Supernatant was used to determine the L-tyrosine with 280 nm wavelength. One unit of protease activity was defined as the amount of enzyme needed to produce 1 µg of tyrosine per minute per mg soluble protein of enzyme extract (U mg protein\(^{-1}\)) under the above conditions, using tyrosine as standards.

Degree of Hydrolysis

Degree of hydrolysis (DH) is defined as the percentage of free amino groups cleaved from protein, which was calculated from ratio α-amino nitrogen divided by total nitrogen. The α-amino content was determined by a modified formol titration method (Nilsang et al., 2005). Approximately 10 ml of sample was mixed with 10 ml distilled water and titrated to pH 7.0 with 0.1 M NaOH. Then 10 ml of formaldehyde solution (38 %) was added. The titration was continued to pH 9.5 with 0.2 N NaOH. Total nitrogen for fish was determined by using Kjeldahl method (AOAC, 2000).

Crude Protein Content

Total nitrogen content in each sample was determined using Kjehdahl method (AOAC, 2000), and expressed as mg nitrogen/mL. Crude protein was calculated by times total nitrogen content with 6.25.

pH

The pH of the fish sauce was determined using pH Meter (Delta 320, Mettler Toledo; Switzerland).

Salt Content

Salt content of the fish sauce was measured by the Volhard method (AOAC, 2000).

Statistical Analysis

Data were analyzed by using One-way analysis of variance (ANOVA) with the software SPSS version 12 for windows. The means were compared using Tukey test with 95 % confidence interval.
Results and Discussion

Liquid Yield

The liquid yields of all samples were not different (P>0.05) (Table 1). In all cases, more liquid was apparently extracted at month 5. Thus, it can be implied that the liquid yield depends on the initial osmotic pressure exerted by the salt. Enzymatic breakdown of fish tissue by proteolysis indicated that the solid component in fish sauce became lower, thus contributing to an increase percentage of liquid yield. At 150 days, the liquid yield of head was significantly lower (P<0.05) than liquid yield of whole and body. This is because the liquid yield of fish sauce depended on the digestible portion of the fish, such as the flesh and viscera (Hariono et al., 2006). Head had lower amount of flesh and no viscera, contributing to a lower liquid yield at days 150 of fermentation.

Table 1: Liquid yield (%) of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12.45 ± 0.79</td>
<td>11.38 ± 1.54</td>
<td>10.30 ± 0.46</td>
</tr>
<tr>
<td>14</td>
<td>13.87 ± 0.22</td>
<td>13.26 ± 2.8</td>
<td>14.05 ± 0.60</td>
</tr>
<tr>
<td>28</td>
<td>13.94 ± 0.83</td>
<td>13.48 ± 1.05</td>
<td>13.88 ± 0.34</td>
</tr>
<tr>
<td>60</td>
<td>15.48 ± 0.69</td>
<td>6.43 ± 2.03</td>
<td>9.76 ± 1.04</td>
</tr>
<tr>
<td>90</td>
<td>14.11 ± 1.06</td>
<td>13.17 ± 0.23</td>
<td>10.63 ± 2.36</td>
</tr>
<tr>
<td>120</td>
<td>17.00 ± 8.95</td>
<td>10.98 ± 2.14</td>
<td>13.31 ± 6.25</td>
</tr>
<tr>
<td>150</td>
<td>22.03 ± 5.70</td>
<td>15.76 ± 0.60</td>
<td>19.65 ± 0.16</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

Protease Activity

Table 2 showed the comparison of protease activity between different parts of fish throughout the fermentation period. Fish head showed the lowest protease activity in the initial 14 days of fermentation (P<0.05). This is due to the autolysis of fish protein by the proteinases and peptidases contained in the stomach and intestines of fish. Most of the fish protease synthesis organs located in the body of fish and viscera are an important source of various proteinases responsible for autolytic activity of small pelagic fish (Dissaraphong et al., 2006; Klomklao et al., 2006). However, the protease activity of all samples showed no significant different after 28 days of fermentation (P>0.05). The protease activity in all samples also became relatively constant after 28 days of fermentation (P>0.05). The head consisted of the muscular upper half of the body. These results imply that the protease enzymes from the viscera such as stomach, pancreas and intestine are more active than that from the muscle. Endopeptidases of trypsin like protease are more active in the initial stage of fermentation (Steinkraus, 1996). The protease activity in all samples were not different at the later stage of fermentation due to the slow release of some exopeptidases from the tissues (Steinkraus, 1996).

Results suggested that the proteolysis during fish sauce fermentation was initiated by endogenous enzymes from the fish viscera. The protein hydrolysis process by the endogenous fish enzymes then provide the necessary nutrient for bacterial fermentation to begin (Nawong, 2006). Since the fish sauces were not produced acceptepctly, it is possible that the protease in the later stage of fermentation was secreted by bacteria. Bacteria have been found to be responsible in protein degradation of fish and meat muscle (Ashie et al., 1996). Molly et al. (1997) stated that the initial hydrolysis of muscle protein is attributed mainly to the endogenous enzymes and is followed by the action of microbial peptides which degrade the protein fragment to small peptides and free amino acid. Further investigations are needed to carry out to determine the involvement and role of microorganisms in fish sauce fermentation.

Degree of Hydrolysis

The degree of hydrolysis of all samples showed a drastic increase during the initial 0 to 14 days of fermentation (P<0.05).
**Table 2**: Protease Activity (IU ml⁻¹) of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.59 ± 0.10ᵇᵃ</td>
<td>0.36 ± 0.03ᵇᵇ</td>
<td>0.38 ± 0.04ᵇᵇ</td>
</tr>
<tr>
<td>14</td>
<td>1.38 ± 0.28ᵇᵇ</td>
<td>0.59 ± 0.26ᵃᵃ</td>
<td>1.59 ± 0.10ᵇᵇ</td>
</tr>
<tr>
<td>28</td>
<td>1.42 ± 0.11ᵇᵇ</td>
<td>1.69 ± 0.12ᵃᵃ</td>
<td>1.65 ± 0.35ᵇᵇ</td>
</tr>
<tr>
<td>60</td>
<td>1.24 ± 0.21ᵇᵇ</td>
<td>1.59 ± 0.15ᵃᵃ</td>
<td>1.61 ± 0.19ᵇᵇ</td>
</tr>
<tr>
<td>90</td>
<td>1.50 ± 0.04ᵇᵇ</td>
<td>1.38 ± 0.16ᵇᵇ</td>
<td>1.75 ± 0.11ᵇᵇ</td>
</tr>
<tr>
<td>120</td>
<td>1.36 ± 0.05ᵇᵇ</td>
<td>0.87 ± 0.10ᵇᵇ</td>
<td>1.18 ± 0.21ᵇᵇ</td>
</tr>
<tr>
<td>150</td>
<td>1.36 ± 0.05ᵇᵇ</td>
<td>1.31 ± 0.15ᵃᵃ</td>
<td>1.39 ± 0.08ᵇᵇ</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

The degree of hydrolysis of fish sauces produced from fish body was significantly higher than head throughout the fermentation period (P<0.05) (Table 3). The higher degrees of hydrolysis in fish sauces produced from body and whole fish were owing to the higher content of digestive enzymes from fish’s viscera that acted on muscle proteins. Enzymes in the fish tissue, particularly the gut, are responsible for hydrolysis of the proteins.

**Table 3**: Degree of hydrolysis (%) of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.71 ± 1.29ᵃᵃ</td>
<td>12.11 ± 0.32ᵇᵇ</td>
<td>12.80 ± 0.65ᵇᵇ</td>
</tr>
<tr>
<td>14</td>
<td>60.34 ± 0.97ᵇᵇ</td>
<td>19.43 ± 0.23ᵇᵇ</td>
<td>53.03 ± 0.65ᵇᵇ</td>
</tr>
<tr>
<td>28</td>
<td>72.57 ± 0.97ᵇᵇ</td>
<td>25.26 ± 1.62ᵃᵃ</td>
<td>71.31 ± 0.32ᵇᵇ</td>
</tr>
<tr>
<td>60</td>
<td>78.40 ± 1.94ᶜᶜ</td>
<td>27.43 ± 0.32ᵃᵃ</td>
<td>73.83 ± 0.97ᵇᵇ</td>
</tr>
<tr>
<td>90</td>
<td>91.20 ± 1.94ᶜᶜ</td>
<td>28.50 ± 1.15ᵃᵃ</td>
<td>68.80 ± 1.62ᵇᵇ</td>
</tr>
<tr>
<td>120</td>
<td>80.52 ± 0.97ᶜᶜ</td>
<td>22.81 ± 0.65ᵃᵃ</td>
<td>75.73 ± 0.65ᵃᵃ</td>
</tr>
<tr>
<td>150</td>
<td>66.97 ± 0.09ᶜᶜ</td>
<td>26.64 ± 1.81ᵃᵃ</td>
<td>48.53 ± 0.61ᵃᵃ</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

**Crude Protein**

Crude protein produced from body and whole fish was higher than crude protein from head (P<0.05) (Table 4). This is because body and whole contained higher amount of flesh and viscera contributing to a higher amount of crude protein content. In addition, the crude protein content of fish sauce from head was lower owing to a lower protease activity in the fish head.

Besides that, more salt was accumulated inside the head of fish which inactivated the protein degradation. Rodger et al. (2006) stated that the salt penetrated into fish tissue depended on various factor like species of fish, temperature, muscle structure, fat content and size of fish. The rate of salt penetrated into the head was faster than body of fish due to the smaller size of the head, thus having a bigger surface area to volume ratio enabling salt to penetrate inside head of fish samples faster than body of fish.

The crude protein of all samples increased significantly during the initial stage of fermentation, then started to decrease after 3 month of fermentation, corresponding with the result of protease activity (P<0.05). This may be due to the combined effect of autolysis and microbial degradation of the fish muscle (Soyiri et al., 2003).

**Table 4**: Crude protein content (%) of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.72 ± 0.39ᵃᵃ</td>
<td>2.29 ± 0.14ᵇᵇ</td>
<td>2.89 ± 0.22ᵇᵇ</td>
</tr>
<tr>
<td>14</td>
<td>11.36 ± 1.55ᵃᵃ</td>
<td>5.56 ± 0.71ᵇᵇ</td>
<td>13.89 ± 2.98ᵇᵇ</td>
</tr>
<tr>
<td>28</td>
<td>16.71 ± 1.70ᵇᵇ</td>
<td>7.61 ± 1.42ᵃᵃ</td>
<td>19.29 ± 2.25ᵃᵃ</td>
</tr>
<tr>
<td>60</td>
<td>12.29 ± 0.17ᵇᵇ</td>
<td>8.06 ± 1.50ᵃᵃ</td>
<td>13.29 ± 2.10ᵃᵃ</td>
</tr>
<tr>
<td>90</td>
<td>18.14 ± 0.54ᶜᶜ</td>
<td>8.32 ± 1.86ᵃᵃ</td>
<td>15.43 ± 0.15ᵇᵇ</td>
</tr>
<tr>
<td>120</td>
<td>16.27 ± 0.21ᵃᵃ</td>
<td>7.91 ± 1.97ᵃᵃ</td>
<td>13.31 ± 2.92ᵃᵃ</td>
</tr>
<tr>
<td>150</td>
<td>12.70 ± 0.80ᶜᶜ</td>
<td>7.29 ± 0.30ᵃᵃ</td>
<td>9.44 ± 0.97ᵃᵃ</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S.D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

**pH**

The pH of the fish sauces produced from this study was between 4.84 and 5.30 during the month’s fermentation (Table 5). Values of pH showed no statistically significant difference between the fish sauce made from the head, whole and body of fish throughout the fermentation period, except at days 60 and 90 (P<0.05), corresponding to the result of salt content discussed later. At days 60 and 90, the salt content fish sauce made from the head parts was higher than that produced from the body parts. Salt
had a highly significant linear decreasing effect on pH (Leroi and Joffraud, 2000). Leroi and Joffraud (2000) in their study also explained that pH decrease in flesh of fish by addition of salt due to the increase of ionic strength of the solution inside the cells.

Table 5: pH of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.81 ± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.85 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.83 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>5.82 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.84 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.82 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>28</td>
<td>5.67 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.62 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.67 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>5.70 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.51 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.49 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90</td>
<td>5.47 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.94 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.40 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>5.32 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.94 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.21 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>5.30 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.84 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.95 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S>D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

The pH of the fish sauces dropped significantly (P<0.05) during the fermentation period. Lopetcharat and Park (2002) stated that the drop in pH might be due to the dissociation of amino acids and small peptides in the presence of salt.

Table 6: Salt content (%) of fish sauce made from different parts of fish

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Tail</th>
<th>Head</th>
<th>Whole</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>26.95 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.00 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.37 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>25.47 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>29.18 ± 1.46&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.34 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>28</td>
<td>28.54 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.87 ± 0.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.03 ± 0.23&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>21.15 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.64 ± 0.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.22 ± 0.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90</td>
<td>22.26 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24.97 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.86 ± 0.71&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>19.71 ± 0.39&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.55 ± 1.87&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.17 ± 1.14&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>150</td>
<td>24.52 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.56 ± 1.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.24 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as means ± S>D; values are means of duplicate from three independent samples. Means in the same column followed by different lowercase and uppercase letters are significantly different (P<0.05).

Salt

There were no statistically significant differences (P>0.05) in the salt concentration of budu from the head, whole and body parts of the fish except for the days 60 and 90 where budu made from the head had significantly (P<0.05) higher salt concentration than that from whole and body of fish (Table 5). Generally, the salt concentration of fish sauce made from head is higher than whole and body fish. This is probably due to the head had lower muscle content and therefore the salt molecules can penetrate the fish tissue easily. Stomach consists of a high amount of connective tissue and smooth muscle. Therefore, the amount of salt penetrated into the tissue will be lower (Dissaraphong, 2005).

Conclusions

Protease activity in fish body was significantly higher than fish head during the initial of fermentation days. Endogenous enzymes, especially from the viscera initiated the protein hydrolysis of fish protein, leading to formation of higher amount of crude protein. High salt content resulted in a lower pH value in the samples.

Acknowledgments

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Simple Determination of Ochratoxin A in Rice by Ultra Performance Liquid Chromatography Coupled with Mass-Spectrometry

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Abstract

Ochratoxin A is secreted from mold, and is a contaminant generally found in several grains. In this study, a non-immunological approach was employed for the extraction of ochratoxin A in rice. The association of ultra performance liquid chromatography–tandem mass spectrometry was used to determine its content via electrospray ionization in positive mode; multiple reactions monitoring was used for the operating mode of analysis. The analyte was isolated using an Acquity™ UPLC bridged ethylene hybrid (BEH) C18 column (100 mm x 2.1 mm, 1.7 µm i.d.) with 0.5% formic acid in 5 mM ammonium formate/acetonitrile by gradient elution within 11 min. Modified QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) was employed using 5% (v/v) formic acid in acetonitrile for solvent extraction. Cleanup was done by dispersive solid phase extraction combination of primary secondary amine (PSA) and C18. Method performance was assessed in a concentration range of 0.01–0.50 mg L⁻¹; acceptable linearity with R² values better than 0.99 were observed. The percent recovery ranged from 93 to 100%, with within-day and between-day measurements at three concentration levels (0.01, 0.05 and 0.1 mg kg⁻¹) showing the percent relative standard deviation (%RSD) values less than 4.8 (n = 10). The limit of detection of the method was 0.5 µg kg⁻¹. Oats were provided as a proficiency testing material for the evaluation of inter-laboratory precision, which attained a z-score of -1.8. This method is not only more effective, but also suitable for the purpose of replacing expensive imported immunoaffinity columns. It could also be employed for routine analysis of ochratoxin A in several grains.

Keywords: electrospray, immunoaffinity column, ochratoxin A, QuEChERS

Introduction

Ochratoxin A (OTA) is a small toxic chemical molecule (MW 403.82) which is produced by the fungus species, including Aspergillus ochraceus and Penicillium verrucosum. (The chemical structure of OTA is shown in Figure 1.) Indeed, the presence of OTA – especially in cereals (e.g. wheat, barley and rice), as well as coffee, dried fruits, etc. – is found worldwide, because OTA can adjust to survive under different conditions of moisture, pH, temperature and time.

OTA can cause serious toxicity by inducing kidney and liver damage, and also can lead to immunosuppressive and carcinogenic problems. In 1993, OTA was classified by the International Agency for Research on Cancer (IARC) as a possible human carcinogen under group 2B. In 2006,
the European Food Safety Authority (EFSA) established a tolerable weekly intake (TWI) of OTA of 120 µg kg⁻¹. Furthermore, the European Community has set a maximum limit (ML) for OTA in all products derived from unprocessed cereal, including processed cereal products and cereals intended for direct human consumption, with the exception of foodstuffs at 3.0 µg kg⁻¹ (EC regulation No.1881/2006).

Nowadays the immunological approach is by far the most popular technique used in routine analysis of OTA by instrumental detection. Immunoaffinity column (IAC) technique (Karbancioglu-Güler and Heperkan, 2008) is a choice of immunoassay which provides reproducibility, is simple and portable, and has low interferences and good recovery. OTA has been extensively investigated in various matrices by the employment of IAC coupled with HPLC-FLD, such as red wine (Olivares-Marín et al., 2009), coffee (Batista et al., 2009), Tunisian cereal (Zaied et al., 2009) and rice (Vega et al., 2009). Nevertheless, IAC (Hamide Z. S. et al., 2010) has limitations in terms of specificity and short lifetimes of antigens; it is also expensive, and requires a specified storage temperature of 2–8 ºC. To overcome these problems in sample preparation technique, the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged and Safe) has been introduced (Anastassiades et al., 2003). The technique uses simple glassware, a minimal amount of organic solvent, and various salt/buffer additives to partition analytes into an organic phase for cleanup by dispersive solid-phase extraction (d-SPE). For the determination of OTA, ultra performance liquid chromatography–tandem mass spectrometry, UPLC-MS/MS (Yiping et al., 2003) has become a valuable technique. It has provided the most efficient confirmatory tool that can discriminate residues at trace levels for reliable and sensitive detection to harmonize with food safety regulations. Consequently, the non-immunoassay method was studied by using modified QuEChERS for OTA extraction, and associated with ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) for the determination of OTA in rice which was used as a representative of grains. The method was developed to replace the classical method of IAC extraction.

Materials and Methods

Standard and Chemical Reagents

Ochratoxin A (99.0% purity) was purchased from Supelco (Bellefonte, PA, USA); acetonitrile (MeCN) LC-MS grade was supplied by Mallinckrodt Baker (Deventer, Holland); MeCN and acetone (pesticide grade) was obtained from Kanto Chemical (Tokyo, Japan); formic acid (FA) and glacial acetic acid (99–100% purity) were from BDH (Briare, France); and magnesium sulfate was supplied by Panreac (Barcelona, Spain). Sodium citrate dibasic sesquihydrate and ammonium formate were purchased from Fluka (Steinem, Germany); sodium citrate tribasic dehydrate was obtained from Riedel-de Haën (Buchs, Switzerland); sodium chloride was supplied by RFCL (New Delhi, India); sodium acetate was from Merck (Darmstadt, Germany); PSA powder (40 µm) was purchased from Varian (Oxfordshire, UK); and C18 powder (40 µm) was purchased from Merck (Darmstadt, Germany).

Standard Preparation

Stock standard solution of OTA was prepared at a concentration level of 200 mg L⁻¹ in MeCN and stored in an amber bottle at below -10 ºC. A working standard solution was diluted to 5 mg L⁻¹ with MeCN. For the matrix-matched calibration curve, the working standard (5 mg L⁻¹) was diluted in blank matrix solution to the lower concentration at 0.01–0.5 mg L⁻¹.

Sample Preparation

The QuEChERS-based method of the European Committee for Standardization (CEN Standard Method EN 15662) was
modified to isolate OTA from milled rice. Ten g samples of milled rice were weighed into 50 mL polypropylene centrifuge tubes (Corning, Lowell, MA, USA); then 10 mL of water and 20 mL of 5% formic acid in MeCN were added to each sample. The mixtures were mechanically shaken for 25–30 min. Then a mixture of 4 g of MgSO₄, 1 g NaCl, 1 g sodium citrate tribasic dehydrate, and 0.5 g sodium citrate dibasic sesquihydrate was added into each centrifuge tube, shaken vigorously by hand for a few minutes, and then centrifuged at 3400 rpm for 5 min. After centrifuging, the entire supernatant was transferred into another tube containing 0.3 g of PSA, 0.1 g of C18 and 1.2 g of MgSO₄. The tube was capped and shaken by hand for 1–2 min. The mixture was again centrifuged at 3400 rpm for 5 min. A 5 mL of extract solution was transferred into a glass tube and evaporated to dryness by using N₂ evaporator at 40–50 ºC. Finally, 1 mL of mobile phase (A/B = 1/1) was reconstituted and filtered through 0.2 µm nylon filter into an amber vial prior to UPLC-MS/MS.

**UPLC condition**

The UPLC consisted of a binary pump system, degasser, autosampler, and column chamber (Waters Corp., Milford, MA, USA). OTA analysis was performed by an Acquity UPLC™ BEH C18 column (2.1 mm x 100 mm, 1.7 µm i.d.) with guard column. The optimized mobile phase composition was 5 mM ammonium formate with 0.5% formic acid (mobile phase A) and MeCN (mobile phase B). The time gradient program was started at 5%A (held for 2 min). This was followed by 70%A at 3.5 min (held for 2 min), 95%A at 6 min (held for 2 min), and 5%A at 8.2 min which was carried on for 11 min with a flow rate of 0.25 mL min⁻¹. The injection volume was 5 µL, with needle-overfill partial loop mode of injection. The column and sample were controlled under temperatures of 40 ºC and 20 ºC, respectively.

**MS/MS Condition**

The Micromass Quattro Premier™ XE (Waters Corp.) is a triple quadrupole mass spectrometer using electrospray ionization interface in the positive mode (ESI+). All MS/MS parameters were optimized as shown in Table 1. For operation in ESI-MS/MS mode, nitrogen gas was used for the cone voltages, and desolvation gas flow for the formation of precursor ions 404.08. Argon gas was also supplied into the collision cell, with collision energies of 25 and 14 V, for breaking up the pseudomolecular ions at 404.08 m/z in order to create other product ions: 239.07 and 358.08, respectively. MassLynx software (version 4.1) was used for data acquisition/processing and instrument control. Multiple reactions monitoring (MRM) was applied for the operating mode of OTA analysis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>ESI+</th>
</tr>
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<tbody>
<tr>
<td>Capillary voltage (kv)</td>
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</tr>
<tr>
<td>Extractor (v)</td>
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<tr>
<td>Cone voltage</td>
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<td>Source temperature (ºC)</td>
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<td>Desolvation temperature (ºC)</td>
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<td>Cone gas flow (L/h)</td>
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<td>Low MS 1 resolution</td>
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<tr>
<td>Ion energy 1</td>
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<td>Low MS 2 resolution</td>
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<td>High MS 2 resolution</td>
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<tr>
<td>Ion energy 2</td>
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<td>Collision gas flow (ml/min)</td>
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<td>Multiplier</td>
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</table>

**Method Validation**

The developed method was validated by following NATA technical note 17 (Guidelines for the validation and verification of chemical test method). The parameters were assessed, such as matrix effect, linearity, accuracy, precision, limit of detection (LOD) and limit of quantitation (LOQ).
Results and Discussion

In order to optimize the performance of the extraction method, Thai rice sample was fortified at concentration level 0.02 mg kg\(^{-1}\) with 3 measurements (n=3) for each parameter investigations.

Acidity of the Extraction Solvent

In the original QuEChERS extraction, MeCN was introduced as the extraction solvent because it was perceived to have more advantages than other solvents. Since OTA is relatively acid (pKa 4.4), it is more stable in an acidified solvent; therefore the acidity of the extraction, which is important, should be suitably controlled. In this study, two acid types (formic acid and acetic acid) were compared for their acidity at 1%, 5% and 10%, respectively. The results are shown in the diagram in Figure 2.

![Figure 2](image)

Figure 2 Assessments of acidified MeCN, extraction solvent (20 mL) for rice sample (10 g) at fortified level 0.02 mg kg\(^{-1}\) (error bar are ± SD., n=3).

The results demonstrate that the recovery percentages were dramatically increased by 5% acid employment. Generally, the choice of acid type that should be selected is volatile acid, which is unlikely to damage mass spectrometry technique instruments. Thus, 5% formic acid in MeCN is recommended for use, because its volatility is superior to that of acetic acid.

Buffer Type

At this stage, the purposes of buffer addition are to induce phase separation and to maintain the pH of the solution. The comparison of two buffer types, citrate buffer and sodium acetate (NaOAc) buffer, were one aspect of this study. The results are shown in Figure 3.

![Figure 3](image)

Figure 3 Comparison of buffer type for studied OTA in rice sample extraction at level 0.02 mg kg\(^{-1}\)(error bar are ± SD., n=3).

The results in Figure 3 indicate that the best percentage mean recovery was obtained when the addition of citrate buffer was employed. Consequently, the use of sodium citrate buffer should be optimal for the maintenance of the acid solution and induced phase separation.

Choice and Effect of Sorbent in Purification

After the phase separation stage, five types of d-SPE (i.e. PSA, NH\(_2\), C18, Al-N and Florisil) were investigated for the best cleanup effect on the extract solution. According to the Figure 4, it was found that there was no significant difference in performance in recovery of OTA by using any of the five sorbents. However, for the evaluation of effective d-SPE – if 100% was determined to be the maximum acceptable recovery – the use of Alumina (neutral, Al-N) and Florisil\(^\circledast\) (Fl) yielded recovery exceeding the criteria value. Notably, PSA, NH\(_2\) and C18 achieved the criteria standard. However, the use of NH\(_2\) might obtain a slightly lower %recovery than PSA or C18. As seen in the results, PSA and C18 were selected for purity of extract solution in this study.
Indeed, the investigation of various d-SPEs was presented so that the different types of d-SPE would have no significant influence on recovery of OTA. Additionally, the main component of rice is carbohydrate (sugar), which is a polar compound (hydroxyl group). For this reason, an anion-exchanger, PSA, was used to remove sugar which existed in the hydroxyl group; it was also used in the trapping of polypeptide chains for amino acid profiles, as well as for several vitamins, etc., in rice samples. Along with C18, it is another reversed phase d-SPE for trapping and removing of non-polar matrices such as starch and saturated fatty acid composition in rice. At this stage, two mixed d-SPEs, such as PSA and C18, are selected for minimized interference.

As PSA is an anion exchanger, it can also trap OTA and causes loss in percent recovery. The optimum amount of PSA should be investigated.

As seen in the results in Figure 5, the trend line declined with increasing PSA amount; also, 0.3 g was the most effective PSA amount and recommended for further analysis. Practically, the amounts of d-SPE C18 were not investigated because there is no effect from used on the OTA. However, 0.1 g of C18 was employed for more cleanups by following the reference AOAC official method of analysis (2007).

For all reasons of the advantage of d-SPE, the mixed d-SPE of 0.3 g of PSA and 0.1 g of C18 were served for this approach.

**Method Validation**

**Matrix Effect**

To assess the matrix effect, a comparison between the standard calibration curve and the matrix-matched calibration curve was evaluated for significant differences in terms of $t$-values ($t$-test: two paired samples for means) at a 95% confidence level. The results indicated that $t$-calculation (9.81) is more than $t$-statistical (2.04) which means there is a significant difference between the dilution of standard in mobile phase and in matrix extract solution. Thus, the matrix-matched standard calibrations have been used for the whole study.

**Linearity**

The linearity of the matrix-matched standard calibration curve was constructed by plotting the ion areas of the primary product (quantitative ion) standard against the corresponding 11 standard concentration levels (0.01, 0.05, 0.1, 0.15, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45 and 0.5 mg kg$^{-1}$). The linear regression was not forced through the origin. The coefficient of determination ($R^2$) was 0.9966, which demonstrates that the standard curves are linear over the investigated concentration range.

**Accuracy**

The accuracy was described in terms of mean recovery at three concentration levels.
(0.01, 0.05 and 0.1 mg kg\(^{-1}\)) of the fortified sample. The recovery results of the fortification sample were over 93%, which were within the acceptable range of the AOAC reference (70–120%).

**Precision**

The precision of the method was evaluated by the percentage of relative standard deviation (%RSD) of 10 replicates (n=10) of fortified blank sample (rice) at three concentration levels (0.01, 0.05 and 0.1 mg kg\(^{-1}\)). The experiments were done within-day, for investigation of repeatability, and between days (3 days) for investigation of reproducibility under the same conditions (the same analyst and the same instrument). The evaluation is dependent on analyte concentration; therefore the %RSD values should be compared to the approximate values from the Horwitz equation. The results are summarized in Table 2. All %RSD results were given satisfactory values of ≤4.8, which did not exceed the acceptable values from the Horwitz equation.

**Table 2** Performance characteristic obtained from fortified rice samples at 3 levels (0.01, 0.05, 0.1 mg kg\(^{-1}\)) with 10 measurements (n=10).

<table>
<thead>
<tr>
<th>Time</th>
<th>Conc. (mg kg(^{-1}))</th>
<th>%Recovery ± SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Within day</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>100 ± 2.4</td>
<td>2.4</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>93 ± 2.3</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>96 ± 1.4</td>
<td>1.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Between days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.01</td>
<td>98 ± 4.6</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>93 ± 3.8</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>0.10</td>
<td>98 ± 4.3</td>
<td>4.4</td>
<td></td>
</tr>
</tbody>
</table>

Furthermore, proficiency testing (PT) was also included via the “Food Analysis Performance Assessment Scheme (FAPAS)”. The analysis of OTA in oats served as the method performance assessment, with a satisfactory factor (z-score) of -1.8 (satisfactory range -2 < z-score < 2).

**Limit of Detection and Limit of Quantitation**

LOD and LOQ of the method were 0.5 µg kg\(^{-1}\) and 1.7 µg kg\(^{-1}\) by calculation of signal-to-noise ratios of 3 times (3 S/N) and 10 times (10 S/N) of the lowest concentration level (0.1 mg kg\(^{-1}\)) of the fortified sample.

**Conclusions**

A simple and effective QuEChERS-based UPLC-MS/MS has been developed for the determination of OTA in rice. The method has achieved a high quality of results (good recovery, within-lab repeatability and reproducibility), with recoveries from 93 to 100% and %RSD ≤4.8. Moreover, the requirements of a confirmatory method have been fulfilled and the applicability of the method verified by successful participation in proficiency testing. This can be regarded as a strong alternative method to current extraction technique, offering simple, inexpensive and real-time extraction within the maximum limit of OTA, while also providing high sample throughput with 40 to 50 analyzed samples per day.

**Acknowledgments**

This research was financially supported by the Thailand Research Fund and the Commission on Higher Education, Research Grant for Mid-Career University Faculty (TRF-CHE-RES-MR) (RMU5180009) (TRF-MAG-WI525S005), the Center for Petroleum Petrochemicals, and Advanced Materials at Chulalongkorn University and the Overseas Merchandise Inspection Co., Ltd. (OMIC-BKK branch).

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Agribusiness and Management
A Comparative Study of Rice Production and Trade Dynamics between Thailand and Vietnam

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Abstract

Concerns over rice export competition between Thailand and Vietnam have been increasingly raised, especially from Thailand, over the last few years, based on recently quantitative data of export volumes, values and markets for rice of various quality grades from Vietnam. Furthermore, many rice export analysts now believe that Thailand cannot compete with Vietnam any more. These studies do focus mainly on macro policies to promote production and export, and economic analysis from input and output data such as production costs and market shares and values, but often overlook various soft factors behind the scene. This study attempts to identify these missing soft factors that contribute to past performance, potentials and constraints of rice production and export dynamics between the two countries from a systematic perspective to consider various food resources, production forces, domestic consumptions, export markets, as well as potential adverse impacts from climate change. Spatio-temporal analyses are conducted qualitatively, based on data availability, for these factors and their dynamics, followed by discussions of future and broader regional perspectives. Preliminary findings from our analysis indicate that Vietnam is unlikely to pose any threats to Thailand, especially if viewed from a long-term basis with all possible factors taken into account. Planned research areas for further in-depth studies are also provided for possible collaboration. Challenges and opportunities for Thailand to sustain its competitive advantages of rice production, export and trade are also discussed based on gaps identified and on a broader regional perspective.

Keywords: rice production and trade, Thailand and Vietnam, climate change, competitive advantages, food resources and consumption

Introduction

Concerns over rice export competition between Thailand and Vietnam have been increasingly raised, especially from Thailand, over the last few years, based on recently quantitative data of export volumes, values and markets for rice of various quality grades from Vietnam. In particular, by the mid-year of 2010 when related data are available, many rice export analysts, especially those from Thailand, believed that Thailand cannot compete with Vietnam any more. Such competitiveness is becoming even more critical according to a very recent study in early October of 2010 by the Center for International Trade Studies of the University of the Thai Chamber of Commerce. A press release of the conclusion of that study (e.g., The Nation on October 6, 2010) highlights that Thailand will lag behind Vietnam in rice exports due to lack of policy focus, and that Thailand will continuously lose competitiveness and export market shares to Vietnam in ASEAN and other markets over the next 10 years due to that country's clear-
cut policy of developing its rice production and marketing.

The mentioned study provides some important comparative points related to estimation of future rice export and policy issues. Namely, Thailand’s rice export from 2010 to 2020 is estimated to have decreased by 14% -- from 10 to 8.6 Mil. tons -- versus a 25% increase -- from 6 to 7.5 Mil. tons -- for Vietnam. Thailand’s performance is due to both much lower rice yield and higher production costs (as compared to Vietnam).

In order to support such findings, a special emphasis on a comparison of related policy issues is placed by identifying that Thailand lacks a long-term plan for rice production and marketing development while Vietnam has ten key strategies to develop its rice industry seriously. These include higher yield but lower production cost; “three-reduction” of use of inputs (seeds, fertilizers, pesticides) and “three-increase” of output (production, quality, profit); export mainly to major rice-consuming countries (such as the Philippines and Malaysia); lower rice prices (at some USD 123 per ton of 5% rice equivalent); setup of a single marketing team (from both the government and private sectors) for export promotion; cooperation with neighboring countries (Cambodia and Burma) on rice production development and plantation; governmental measures to reduce farmer’s production costs (through soft loan, tax exemption, setup of fund for stable long-term rice price); clear policies set to assure rice farmers to make a profit of at least 30% relative to production costs; and setup of a central market for trading rice overseas and warehouses in target markets.

Based on such a comparative analysis and lessons learnt from Vietnam, important recommendations (to ensure export competitiveness) for Thailand are made from that study, including close cooperation (between government and private sectors) needed to develop rice farming and marketing; restructuring of the industry’s organization (and having a single overseas marketing team); the need to identify whether to promote white rice or jasmine rice in each market; reduction of production costs (and increase of rice harvest volume per hectare, improvement of marketing strategies and rice qualities in terms of taste); and finally formulation of governmental policy to help farmers to reach a profit of at least 30%.

It is important to note here that the reason why the updates above need to be reviewed in such considerable depth is that our study is also conducted concurrently on the same hot topic. Besides this study with a focus on macro policy levels, there are several other related studies conducted, but these look mainly at quantitative data comparisons such as on export competition (Shihara, 2000) and production costs (Bunyasiri, 2010). Therefore, our attempt is made in this paper (that was written in mid-November of 2010) to selectively focus on additional findings related to spatio-temporal analyses and identify associated explanatory soft factors.

**Materials and Methods**

Time-series data related to rice statistics is available from several sources such as FAOSTAT and AFSSIS (ASEAN Food Security Information System), although there may be some minor data deviation between sources. However, we used the data from AFSSIS for tendency analysis as the source provides data types that are available for both Thailand and Vietnam although it only covers the time period from 1983 to 2009/2010 (the latest time period of almost three decades). As data types to be used can be of different units, an index (with the starting year set to be 100) is calculated for convenient comparative
purposes. In case of time-series data unavailability, data for certain years that is available from literature review is used for supplementary illustration. Regarding geographical variation analysis, secondary data from different sources is used to identify similarity and differentiation features between the two countries as well as between different regions within each country. In addition, various consultations with several related experts have been conducted along with personal experiences in the both countries in order to make findings more realistic and convincing.

Results and Discussion

Temporal Tendency Analysis
The data analysis for the time period from 1983 to 2009/2010 shows different growth rates on key rice indicators between Thailand and Vietnam, as seen in Figures 1 and 2. The most striking feature is rice production: the growth in Vietnam almost tripled (from 100 to about 265) while the growth in Thailand just slightly doubled (from 100 to about 180). Growth for both nations is based on rice yield per hectare per year (the growth in Vietnam is rather high from 100 to 250 while growth is much lower in Thailand from 100 to 180); and planted area (the growth in Vietnam is moderate from 100 toward a saturate level of about 125 while overall growth remains almost unchanged in Thailand with some deviation from one year to another). The trend that the yield effect in Vietnam contributes more to the total rice production than the area effect was confirmed during the time period from 1965 to 2004, as analyzed earlier by Thanh and Singh (2006). Another distinguished feature is population: the growth in Vietnam is even higher from 100 to 140 than that in Thailand from 100 to only 120.

In terms of the size, it is noted here that the population size is also much higher in Vietnam than in Thailand but the rice growing area is lower in Vietnam. In addition, rice consumption per capita per year is much higher in Vietnam.

Furthermore, it is very interesting to look again at the fluctuation tendency of rice planted areas in Thailand as it is policy-
A comparative study of rice production related. One important reason is with the farmer’s planting decision that is based on and more sensitive to rice price levels sold in the latest harvest season that can be variable over time too. Such planting is consistent with earlier findings by Sachchamarga and William (2004) that “the rice area planted is also found to be marginally more sensitive to current market price than to the price of paddy rice in the period just prior to planting. This result may be a consequence of the guaranteed rice price policy operated by the Thai government”.

Related information to such behavior was derived from AFSIS, and indicates that Thailand has time-series data reports on harvested areas but Vietnam does not. The harvested areas in Thailand are generally lower than the planted areas due to harvest failures from various causes (floods, droughts, diseases, etc.). Thai farmers can also claim for compensation for crop failures from the government.

**Spatial Analysis**

The geographical variation analysis from secondary data from different sources that have been justified with our personal experiences also shows interesting comparative features between the two countries as well as between different regions within each country. There are two major rice growing areas in each country (the North-East Region and the Central Plain in Thailand; the Red River Delta and the Mekong River Delta in Vietnam), as shown in Figures 3 and 4.

First, at the overall level, there are several different striking factors related to production inputs, namely irrigation coverage, planted rice varieties, labor and mechanization, and human skills in these major rice growing areas. Irrigation coverage is very high in Vietnam in both of its rice growing areas while in Thailand it is also high but only in the Central Plain (please note here that some ambitious attempts to divert water from the Mekong River to the North-East region were proposed by several former Thai politicians (including prime ministers) over the past ten year but unrealized so far).

**Figure 3** Major rice growing areas in Thailand. Darkness level indicates rice growing intensification. Source: Adapted from Clayton (2010).

New high-yielding rice varieties adopted (through R&D) in terms of high yield, short-growing duration, tolerance to environmental changing conditions (such as drought, flood, and poor soil conditions) are more diversified in Vietnam than in Thailand. It is widely recognized that Thai farmers themselves generally do not want to change rice types planted, but Vietnamese farmers do seem both willing and able to change crop varieties (Nielsen, 2003).

In terms of labor and mechanization, rice production is still more labor-intensive in Vietnam than in Thailand, and it seems that farming integration level is higher in Vietnam through various integration models (to link crops, gardens, animal raising, and incomes for farmers to stay with their
farming aquaculture) for better waste recycling and hence better jobs.

![Map of Vietnam showing major rice growing areas](image)

**Figure 4** Major rice growing areas in Vietnam. Darkness level indicates rice growing intensification. Source: Adapted from Baroña (2010).

Another important soft factor is human skills and crop care by farmers in rice production in various stages: these seem to be better human skills in Vietnam (perhaps due to the need to adapt to more frequent natural disasters and less opportunities for farmers to move to non-farming jobs). Better crop care can be illustrated through the data on mean pesticide use: 2.08 kg active ingredients a.i.ha$^{-1}$ in the Central Plain of Thailand, which is 0.5-1.5 kg a.i.ha$^{-1}$ higher than in the Mekong and Red River Deltas in Vietnam (IRRI, 2010).

It is interesting to recall here that the Vietnamese culture for rice cultivation has been long recognized as based on the top four factors: water, fertilizer, diligence, and seed. In addition, over the recent decades, Vietnam placed more emphasis on how to maximize (economic) value per unit of land use. This serves as a good basis for better understanding of various factors as mentioned above and also for comparison with other countries. All the key discerning characteristics identified above, and the apparent numerical data mentioned are additional factors to justify why the rice yield in Vietnam is much higher at the national level in terms of both yield per crop and number of crops per year (number of crops per year in Vietnam is about double compared to that in Thailand).

Now if one looks at geographical variations within each country, several important gaps can be identified. In Thailand for example, the key differences between its two major rice growing areas include irrigation, mechanization, labor availability, crop intensity, and rice types grown. In the Central Plain, irrigation and mechanization are both much more satisfactory which allows 2 to 2.5 crops per year (as compared to only more or less one crop per year in the North-East region), but labor shortage is a problem especially in peak times simply because of better non-farming opportunities nearby. In Vietnam, only minor differences can be identified in its two major rice growing areas: number of crops per year in the Mekong River Delta is very higher (3 to 3.5 compared to 2 to 3 in the Red River Delta).

**Future Perspectives**

Both quantitative and qualitative analyses made so far combined with other studies do reveal that Vietnam in general is more advantageous than Thailand in the past several decades due to several key dimensions in relation to clear-cut policies to support the rice farmers, rice culture philosophies and rice cultivation intensity. However, there are always several inherent disadvantages as compared to Thailand, especially viewed from a long-term basis. These include less area for rice cultivation, continuation of land lost to urbanization and industrialization, higher population size and population growth rate. Rice consumption
per person per year is also much higher in Vietnam: 170.3 kg milled rice as compared to only 100.8 kg milled rice in 1999 (IRRI, 2002). Vietnam, due to its specific geographical location and shape, has more natural disasters (floods, droughts, typhoons, etc.), and has already reached a high level of cultivation intensity that might exceed the maximum carrying capacity level and might exert adverse impacts on natural resources and the environment if further intensified. More importantly, under the context of climate change, Vietnam is much more vulnerable to sea level rise because of lower land locations of its major rice growing areas that are along and much closer to the sea. In particular, the Mekong delta, which accounts for more than 50 percent of the total rice production in Vietnam, is highly sensitive to the adverse impacts from both the Mekong River’s upstream (through five other countries) and sea-water intrusion (from the South China sea).

In addition to difficulties for Vietnam in the future to maintain the same continued growth in rice production as in the last three decades, there are new rice growing countries in the region (such as Burma and Cambodia) that have emerged as new competitors for both Vietnam and Thailand, especially in terms of similar types of rice grown for export.

Conclusions

Although there is another concurrent study on a similar and hot topic, our study has provided additional important temporal and spatial factors related to rice production and export dynamics between Thailand and Vietnam, behind macro policy issues and quantitative analysis of data related to export and production as usually used for comparison in various past studies. Striking different factors that have been identified between the two countries are (1) irrigation coverage for crops through public investments; (2) R&D for high-yield rice varieties; (3) farmers’ decision on variety change and planting areas and sizes; (4) farmers’ diligence and crop cares; (5) impacts of climate change and other related issues; and (6) emerging rice producing countries.

Taking into account possible factors, it can be concluded that major rice threats to Thailand from Vietnam are unlikely if gaps identified in the previous sections related to policy, production, and export can be bridged where possible and at feasible levels as compared with Vietnam. These new research areas also call for possible collaborative exchange and research between Thailand and Vietnam.

Due to time and budget limitations, this paper, however, should be considered as a preliminary and complementary study and we have already planned for further in-depth research to (1) re-confirm gaps identified (through ground surveys in the two countries); (2) identify improvement measures and alternatives (through system modeling with best practices elsewhere), especially for Thailand; (3) assess impacts from Climate Change (then identify adaptation measures) on rice production and also from rice production on the resource base and the environment; and (4) identify better possible win-win strategies (to avoid competition and go beyond competition) between Vietnam and Thailand based on broader regional perspectives.

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Thai Consumer Willingness to Pay for Genetically Modified Rice

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Abstract

Even though rice is the most important staple food in Thailand, to date research on genetically modified (GM) rice has not been allowed in field trials. Nevertheless, certain countries currently allow the production of GM rice, in order to prepare themselves for increasing domestic consumption. In the future, imported GM rice could potentially compete with Thai rice in both the international and domestic markets. This paper attempts to quantify Thai consumers’ willingness to pay (WTP) for GM rice. Sixty representative consumers were selected by convenient sampling to participate in the experiment. The demand-revealing mechanism chosen was the \( n \)th price auction. Subjects were asked to bid for GM rice and GM rice with additional advantages, namely additional nutritional value, herbicide-tolerance, longer shelf life, and no environmental hazards. The results show that Thai consumers are generally averse to GM rice, as the WTP for GM rice was discounted by 18.73%. However, Thai consumers do perceive GM rice with additional advantages indifferently from non-GM Jasmine rice; whilst the no environmental hazards GM rice received the highest premium.

Keywords: genetically modified organism, jasmine rice, \( n \)th price auction, willingness to pay

Introduction

Thailand is not a major GM food producer in the world, mainly because the Thai government opposes the use of biogenetical engineering techniques in order to maintain the reputation of its well-known Jasmine rice, which is GMO-free. However, some countries have conducted research on GM rice, seeing it as a solution to ever-increasing demands in domestic consumption. Others believe that GM food could be beneficial to children suffering from malnutrition in poorer countries.

“Golden rice,” for example, was created to prevent vitamin A deficiencies, and by 2011 it is expected that Golden rice will be available to some small-scale farmers in developing countries, free of charge (GMO Compass, 2010). The Philippines, who are currently developing an allergen-free rice, especially designed for those with allergies (GMO Compass, 2010). Focusing on Thai Jasmine rice, scientists have recently patented a new transgenic method to create fragrant rice which is similar to Jasmine rice (Wipatayotin, 2009).

The benefits of GM rice are not limited to a consumers’ perspective, research has been directed towards the supply side. Bayer CropScience has genetically developed rice with a herbicide-tolerant quality; traded under the name of “Liberty Link” (Blue, 2007). Additionally, the International Rice Research Institute in the Philippines has reported that other countries, such as India and Iran, are also developing GM rice to combat herbicides (Japan Times, 2009).

In the near future, GM rice could be commercialized in certain markets. China's Ministry of Agriculture recently declared two kinds of genetically modified rice, named “Hua Hui No.1” and “Bt Shan You
63”, as safe to produce and consume; and issued safety certificates for the period of 5 years (Post Today, 2010). Both types of GM rice have a resistance towards pests and diseases, consequently reducing the use of herbicides by up to 80%, and increasing the yields of paddy by 8%. However, both types can only be cultivated and used in Hubei Province, and are still waiting to be approved for commercial trade.

At present, GM rice is still being limited in field experiments, yet there have been reports of global food supplies being contaminated (Bankokbiznews, 2010). Despite ambiguities over its long term health effects, GM rice will certainly influence the world’s food markets in the coming future; due to an increasingly affluent population in both China and India, the destruction of crop areas due to climate change, and starvation in poorer countries.

Although previous surveys show that Thai consumers are generally not against GMO (Environics International, 2000), research on Thai consumers’ knowledge and acceptance of GMO is still limited. The current issue is not merely about the acceptance of GM food versus GMO-free food, but also on whether the benefits associated with GMO can reduce consumers opposition to GMO food. Moon and Balasubramanian (2001) classify GMO attributes into negative (such as health risks and environmental hazards) and positive (such as higher yields, reduced chemical use, and improved nutrition). The survey conducted in the US and the UK shows that risk perceptions have a stronger impact on consumers’ willingness to pay (WTP) than the benefit perceptions.

Since rice is the most important staple food of Thai consumers, this paper aims to study Thai consumers’ WTP for GM rice, particularly if GM rice is associated with additional positive or negative attributes. Some nongovernmental organizations (NGOs) such as Greenpeace are publicly against the production and consumption of GM rice, as evidenced by their protests at “BioAsia 2007 Thailand” (Manager Online, 2007). They support traditional breeding or the marker-assisted selection (MAS) instead of a genetically engineered process (Greenpeace International, 2009).

**Materials and Methods**

Compared to the traditional survey method, the demand-revealing mechanism of the experimental auction is widely accepted due to its several advantages (Noussair et al., 2004). Firstly, the auction method uses a homogenous unit, namely money, to measure preferences which makes comparison across subjects much easier. Secondly, all subjects are committed to purchasing the item, and will reveal their true value in order to exchange their money for the item. Thirdly, the subject’s willingness to pay can be directly measured. Lastly, subjects must take all of the item’s characteristics into consideration before coming to a decision.

This paper relies on the nth price auction method, similar to VanWechel et al. (2003), Huffman et al. (2003), Rousu et al. (2004), and Chiaravutthi (2010), since it is an efficient method that avoids the problem of insincere bids from off-margin bidders; consequently this ensures that participants reveal their actual demand (Shogren, 2001). The nature of the nth price auction is random, so that all the participants have a homogenous positive probability of purchasing the item. In practice, the bids are ranked from highest to lowest. After that, the experimenter will assign a random number (n), and the (n-1) highest bidders have to purchase the item at the n bidder’s price.

The recruiting process was based on convenient sampling with invitation posters posted at many public areas around the campus. The important requirements were that participants had to be the household’s main shopper, and no information regarding GMO was conveyed to participants prior to the experiment. The experiment was arranged in a closed room at Mahidol University during June and July, 2010.
Sixty representative consumers participated in the experiment which was divided into six sessions, each session consisting of ten participants.

On the day of the experiment, after the subjects had finished reading the information sheet and signed the informed consent form, they were endowed with 500 Baht. In the room, all the subjects were seated in their own cubicle to prevent them from observing the others’ behavior. No actual names were used during the whole process. The experimenter explained the process to them including the \( n \)-th-price auction, and allowed sufficient time for questions to be asked.

The auctions consisted of eight rounds: two training rounds and six actual bidding rounds, as shown in Table 2.1. Both training rounds were set up so that subjects would be familiar with the auction process. In the first training round, a bag of sugar was used, whilst a bag of salt was used in the second training round. Each of the six actual rounds were differentiated by their associated labels.

**Table 2.1** Sequences of the experimental session

<table>
<thead>
<tr>
<th>Round</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training 1</td>
<td>Auction for a bag of sugar</td>
</tr>
<tr>
<td>Training 2</td>
<td>Auction for a bag of salt</td>
</tr>
<tr>
<td>Actual 1</td>
<td>Auction for normal Jasmine rice</td>
</tr>
<tr>
<td>Actual 2</td>
<td>Auction for GM Jasmine rice</td>
</tr>
<tr>
<td>Actual 3</td>
<td>Auction for GM Jasmine rice with additional nutritional value</td>
</tr>
<tr>
<td>Actual 4</td>
<td>Auction for GM Jasmine rice with herbicide tolerance</td>
</tr>
<tr>
<td>Actual 5</td>
<td>Auction for GM Jasmine rice with longer shelf life</td>
</tr>
<tr>
<td>Actual 6</td>
<td>Auction for GM Jasmine rice with no environmental hazards (enclosed farm)</td>
</tr>
</tbody>
</table>

In the actual rounds, all bags of rice were re-packaged in clear plastic bags with newly printed labels, in order to avoid packaging and branding effects on the decision making process. These labels consisted of plain white paper, containing details of the type of rice, the cooking method, net weight, and the expiry date. The rice used throughout this experiment was Jasmine rice with no GMO component, but subjects were told that it was GM rice developed under government sponsored projects. The truth was conveyed to the subjects after the experiment was completed. From the third to the sixth round, additional attributes were introduced. The third round was an auction for GM rice with vitamin A enrichment, similar to Golden rice. The fourth round was an auction for GM rice, certified as having a tolerance toward pests, similar to the ongoing research in certain countries. The fifth round involved GM rice with an extended shelf life of up to 3 years. Although this benefit has never actually been researched in rice, genetically modified tomato known as “Flavr Savr” is an example of GM food with the attribute of a longer shelf life (Schwartz, 2010). The last round was labeled as “no environmental hazards” because the rice is being cultivated on an enclosed experimental farm. Some consumers are afraid that transgenic crops in open fields could contaminate the environment, and lead to long-term unpredictable outcomes to our ecological systems.

At the end of the sixth round, one out of six rounds was randomly selected as binding to ensure that the winner paid less than the endowment. When the winners were announced, they had to use the endowed money to purchase the rice items. Moreover, all six actual rounds were randomly arranged to prevent the sequential effects of a fixed arrangement. At the end of the experiment, all the subjects had to fill in questionnaires that asked about their demographics. Out of sixty participants, ten are males and fifty are females. The average age is 33.65 with the SD of 5.42. Ninety seven percent of them hold the bachelor degree or higher. Forty three participants report the monthly income between Baht 10,000 and 24,999, and fifteen participants report the income of Baht 25,000 and 49,999; while there is one participant in the Baht 5,000 and 9,999 income range and one participant in the Baht 50,000 and 99,999 income range. The number of members in the household is
ranging from one to eight, which twenty one participants have four members and thirteen participants have five members in the household. Thirty five participants have no child, fifteen participants have one child, nine participants have two children, and one participant has three children in the household.

Results and Discussion

Comparisons between actual label and GMO label results show that Thai consumers generally have negative perceptions towards GM rice, as evidenced in Table 3.1. The average bid for GM Jasmine rice in the second round was 19.88 Baht, a decrease of 18.73% from the 24.47 Baht bid for Jasmine rice in the first round. This is statistically supported as shown in Table 3.3.

The Noussair et al. (2004) classification of consumers into four types: unwilling, reluctant, indifferent, and favorable was followed. The majority of Thai consumers can be classified as “reluctant”, as they have negative attitudes towards GMO food, and do not want to risk consuming it. Reluctant consumers are defined as those who bid lower for GM rice than normal Jasmine rice. The percentage of reluctant consumers was 61.67%. The percentage of subjects who bid zero was 6.67%, and these are classified as “unwilling” consumers as they completely rejected GMO rice. Bidding zero reflects the bidder’s such intention since there is (almost) no chance of winning the auction. Only 25% of the participants are “favorable” consumers who had positive perceptions of GMO rice as their bids were higher for GMO; whilst the remaining 13.33% are “indifferent” since their bids between GMO and GMO-free were the same. In addition, the difference between consumers’ acceptance of GM rice can be more clearly understood when only the decreasing bids (of unwilling and reluctant consumers) are considered. The average bid for GM rice then drops to 16.84 Baht, representing a 31.18% decrease on the figure for actual Jasmine rice.

<table>
<thead>
<tr>
<th>Table 3.1 Comparisons between Jasmine rice and GM Jasmine rice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. of Bidders</strong></td>
</tr>
<tr>
<td>Number of all bids</td>
</tr>
<tr>
<td>Decreasing bids for GM Jasmine rice</td>
</tr>
<tr>
<td>Increasing bids for GM Jasmine rice</td>
</tr>
<tr>
<td>Equal bids for GM Jasmine rice</td>
</tr>
<tr>
<td>Zero bids for GM Jasmine rice</td>
</tr>
<tr>
<td>Average bid for actual Jasmine rice</td>
</tr>
<tr>
<td>Average bid for GM Jasmine rice</td>
</tr>
<tr>
<td>Average bid for GM Jasmine rice in Baht (on decreasing bids only)</td>
</tr>
<tr>
<td>Percentage discount for GM Jasmine rice</td>
</tr>
<tr>
<td>Percentage discount for GM Jasmine rice (on decreasing bids only)</td>
</tr>
</tbody>
</table>

The results from rounds three to six are more interesting when additional attributes are attached to the GM rice. As shown in Figure 3.1, the average bids for additional nutrition (pro-vitamin A), herbicide tolerance (reductions in the use of herbicides), longer shelf life (3 years storage) and no environmental hazards (due to its cultivation in an enclosed experimental farm) were 23.27, 22.23, 22.93 and 23.38 Baht, respectively.

![Figure 3.1 Average bids for actual rounds](image-url)
Despite additional advantages, the average bids in the third to sixth rounds were lower than Jasmine rice, but above the GM rice without additional attributes bids. Although the GM rice was stated as containing additional benefits, certain consumers were still “unwilling” to buy, and submitted zero bids. However, this only represented 6.67% of all the participants. Based on Table 3.2, the most favorable attribute is no environmental hazards, which was higher than the average bid of a typical GM rice by 37.49%. The next favorable advantages were longer shelf life, pro-vitamin A, and herbicide tolerance with figures of 27.66%, 12.11%, and 7.60% premiums, respectively.

Table 3.3 confirms that although Thai consumers perceive GM rice and GM-free rice differently, GM rice with additional attributes is viewed indifferently from GM-free rice. Also, GM rice with additional attributes is viewed differently from GM rice. Although the “no environmental hazards” attribute scores the highest premium in absolute terms, all of the attributes do not statistically differ from each other.

**Table 3.2** Comparisons between WTP for GM rice and GM rice with additional attributes

<table>
<thead>
<tr>
<th>Types</th>
<th>Average bid in Baht [SD]</th>
<th>% Discount</th>
<th>% Premium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jasmine rice</td>
<td>24.47 [11.70]</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GM Jasmine rice</td>
<td>19.88 [11.46]</td>
<td>18.73</td>
<td>-</td>
</tr>
<tr>
<td>GM rice with additional nutritional value</td>
<td>23.27 [12.84]</td>
<td>4.90</td>
<td>12.11</td>
</tr>
<tr>
<td>GM rice with herbicide tolerance</td>
<td>22.23 [12.33]</td>
<td>9.13</td>
<td>7.60</td>
</tr>
<tr>
<td>GM rice with a longer shelf life</td>
<td>22.93 [14.63]</td>
<td>6.27</td>
<td>27.66</td>
</tr>
<tr>
<td>GM rice with no environmental hazards</td>
<td>23.38 [15.75]</td>
<td>4.43</td>
<td>37.49</td>
</tr>
</tbody>
</table>

Note: % Discount is from Jasmine rice. % Premium is from GM Jasmine rice.

**Table 3.3** t-Test statistics

<table>
<thead>
<tr>
<th>Difference between an average bid for Jasmine rice and …</th>
<th>t-Test statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM Jasmine rice</td>
<td>3.664*</td>
</tr>
<tr>
<td>GM Jasmine rice with additional nutritional value</td>
<td>0.943</td>
</tr>
<tr>
<td>GM Jasmine rice with herbicide tolerance</td>
<td>1.648</td>
</tr>
<tr>
<td>GM Jasmine rice with a longer shelf life</td>
<td>1.028</td>
</tr>
<tr>
<td>GM Jasmine rice with no environmental hazards</td>
<td>0.770</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Difference between an average bid for GM Jasmine rice and …</th>
<th>t-Test statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM Jasmine rice with additional nutritional value</td>
<td>5.042*</td>
</tr>
<tr>
<td>GM Jasmine rice with herbicide tolerance</td>
<td>2.927*</td>
</tr>
<tr>
<td>GM Jasmine rice with a longer shelf life</td>
<td>2.953*</td>
</tr>
<tr>
<td>GM Jasmine rice with no environmental hazards</td>
<td>2.887*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Difference between an average bid for GM Jasmine rice with no environmental hazards and …</th>
<th>t-Test statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM Jasmine rice with additional nutritional value</td>
<td>0.099</td>
</tr>
<tr>
<td>GM Jasmine rice with herbicide tolerance</td>
<td>1.187</td>
</tr>
<tr>
<td>GM Jasmine rice with a longer shelf life</td>
<td>0.477</td>
</tr>
</tbody>
</table>

Note: *P-value < 0.05.

**Conclusions**

Using the nth price auction, the results show that Thai consumers do not generally accept GM rice, as WTP for GM rice was discounted by 18.73% from GMO-free rice. The average bid for Jasmine rice was Baht 24.47, while the average bid for GM Jasmine rice was Baht 19.88. However, certain Thai consumers could decide to purchase GM rice if it has two characteristics, either the price of GM rice has to be sufficiently lower or the GM rice carries additional benefits.
Premiums for GM rice with the attributes of no environmental hazards (such as cultivation in an enclosed farm), longer shelf life, additional nutritional value, and herbicide tolerance were 37.49%, 27.66%, 12.11%, and 7.60%, respectively. Statistical tests also support the view that the average bid for GM Jasmine rice with additional benefits is not significantly different from that of normal Jasmine rice.

The results show that 25% of the participants bid higher for GM rice; whilst 13.33% were different between GMO rice and GMO-free rice. Since only 6.67% of the participants in this study completely rejected the GM rice, it would seem that an opportunity exists for GM rice producers. However, 61.67% of consumers would demand much lower prices, at a steep discount of 31.18%. The implication is that unless GMO producers can reduce the cost of production, and the selling price considerably, their options are going to be limited. However, if GMO technology is associated with the “right” benefits, and consumers’ concerns can be lessened, new marketing opportunities exist. For example, GM rice with resistance to pesticides and herbicides could lead to higher yields and therefore lower costs.

The consensus from both the private and public sectors regarding the pursuit of GM rice has to be clear. Meanwhile, to protect consumers’ rights prior to the possible allowance of commercialization of imported GM rice, the Thai government must set a clear policy for GM rice sales and promotion. On the other hand, while other countries focus on the research and development of GM rice, there is a large market opportunity for organic or GMO-free rice in the Thai market since it still carries the highest premium.

This study serves as only a preliminary warning regarding the complexity of Thai consumers’ perceptions towards GMO. More studies are needed to ascertain Thai consumers’ attitudes toward GM rice, and indeed other GM food.

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