

Antibacterial Activity of Formulated Fish Snack from Bacterial Cellulose

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Abstract—The antimicrobial activities of *Eugenia caryophyllus* (Clove) and *Zingiber officinale* Roscoe H (Ginger) extracts were studied for the antimicrobial activities in bacterial cellulose fish snacks in order to extend the shelf-life without adding the synthetic preservative. The extracts of clove and ginger were concentrated and tested for the minimum inhibition concentrations (0-100%) against pathogenic bacteria: *Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*. Both species exhibited remarkable bacteriostatic and bactericidal activities. The bacterial cellulose fish snacks were then formulated from the mainly mixtures of fish meat and bacterial cellulose derived from *Acetobacter xylinum* TISTR 998 (75%: 25%) supplemented with the extracts of clove and ginger in the ratio of 0, 5 and 10%. Results showed that both extracts addition significantly reduced the microbial growth with significantly difference ($p < 0.05$) in aerobic plate count over 8 days when compared with control. Nutritional values of formulated fish snack as protein, fat, carbohydrate were analyzed. Physical characteristic in terms of color of formulated fish snacks was also reported in the CIELAB system. Water activities and moisture content changes over the storage period were recorded. Sensory analysis indicated that 10% added ginger extract had a significant stronger flavor than the other bacterial cellulose fish snack formulations. Results suggest that herbal extract are the alternative method to preserve the fish meat derived product.

Keywords—Clove; Ginger; Food pathogen; *Acetobacter xylinum*; Fish snack

I. INTRODUCTION

Thailand is well known for various medicinal plants that have long been used for the treatment of diseases as the investigation of Wiyakrutta *et al.*, 2004[1]. Medicinal plants have long been consumed in traditional foods and beverages and also prescribed for treating various diseases especially for the infections. Among medicinal plants, the studies of the antimicrobial activity of *Zingiber officinale* Roscoe (ginger) [2,3] and *Eugenia caryophyllus* (clove) [4,5] were reported. For food products, microbial spoilage from food pathogenic bacteria is the primary factor in quality deterioration of fresh meats. For examples, *Escherichia coli* is a gram negative bacillus which causes serious infectious diseases such as urinary tract infection and bloodstream infection [6,7]. *Salmonella typhimurium*, a gram negative bacterium, is recognized as one of the leading cause of food borne

bacterial diseases which most people infected with *Salmonella* develop diarrhea, fever, vomiting, and abdominal cramps during 8 to 72 hours after infection [8]. Many problems of human salmonellosis following consumption of contaminated foods have increased dramatically worldwide. Moreover, *Staphylococcus aureus* is one of the most common gram positive bacterium that causes severe nausea, abdominal cramps, vomiting, and diarrhea. Although sanitary processing practices and refrigeration of ready to eat products is often the principle means to control spoilage and food borne pathogens in this type of [9], an attempt to improve their quality of life, some food processors have used medicinal plants as source of alternative food preservation. Though a lot of plants have been used in traditional medicine, little is known about the correlation in the supplementation of the medicinal plants to prolong the shelf of specific food. Therefore, the focus on the alternative preservation using the medicinal Thai plant as the supplement from the processed natural food was investigated. Three different bacterial species as the food borne pathogens were used to investigate the possible antibacterial activities of the process fish snack from bacterial cellulose. Nutritional values of the processed fish snack were analyzed and the prolong shelf life of processed medicinal supplementation of fish snack shelf-life from the chosen two Thai medicinal plants were also reported.

II. MATERIAL AND METHODS

A. Preparation of plant extracts

Clove and ginger were purchased commercially from local market in Thailand. Clove was oven dried under 60 °C for 1 h. Sample was then ground with blender for 10 min and was applied to the oven dryer under 60 °C for 30 min. For the ginger, sample was cut into small pieces and ground into powder. Sample was then oven dried under 60 °C for 6 h. Samples were weighted and prepared at 1% w/v for the hot water extraction for 1 h and were concentrated under vacuum rotary evaporator for 30 min. Concentration process was applied to samples by using vacuum evaporator. All extracts were prepared and tested in triplicate.

B. Cultivation of bacterial cellulose

A filtrated coconut juice substrate from local market in Thailand was aseptically sterilized. *Acetobacter xylinum* TISTR 998 obtained from culture collection of Institute of

Scientific and Technology Research, Bangkok, Thailand was initially grown on nutrient broth under 30 °C for 2 days. A 10% of inoculant volume was transferred into the 100 ml mature sterilized coconut juice under the static batch cultivation. This ready inoculum was further prepared into 1000 ml as those described earlier [10]. The bacterial cellulose layer as depicted in figure 1 was then cleaned thoroughly before cut into dices. The sample was ready to use after blended and squeezed the left over water out.

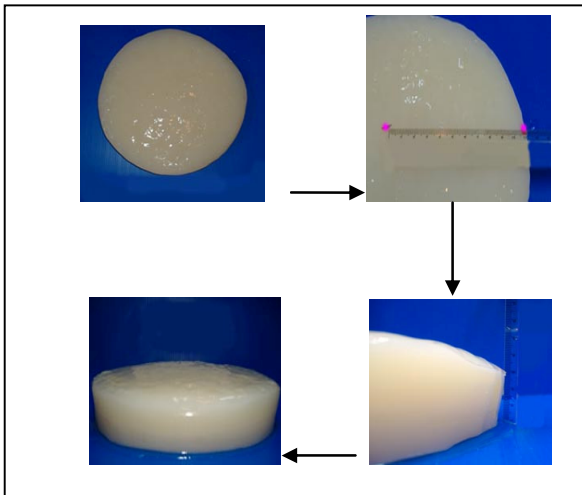


Figure 1. Bacterial cellulose layer derived from *Acetobacter xylinum* TISTR 998 after 3 weeks cultivation.

C. Preparation of pathogenic microorganism

The antibacterial activities of two plant extracts were determined against *Escherichia coli* TISTR 887, *Salmonella typhimurium* TISTR 292 and *Staphylococcus aureus* TISTR 517 which were obtained from the culture collection of Thailand Institute of Scientific and Technology Research, Bangkok, Thailand. All cultures were prepared in Nutrient broth and stored at 4 °C. All cultures were cultured until reached 1.0 unit of $A_{600\text{ nm}}$ before use. Turbidity was adjusted to match that of a 5 McFarland standard (10^8 CFU/ml). Then, this dilution and a 1:10 dilution of the cell suspension were performed to give an inoculum concentration of 10^7 CFU/ml. For the determination of antibacterial activities, all strains were grown in Mueller Hinton broth for 24 hours at 37 °C.

D. Antimicrobial inhibition

Disk diffusion test was performed using the standard procedure as described by Jorgensen *et al*, 1999[10]. The inoculum suspension of each bacterial strain was spread on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 0.1, Biomark™, India). Sterile 6-mm filter paper disc were aseptically placed on MHA surfaces, and two crude extracts were immediately added to disc in volumes of 20 of four concentrations of the crude extract, 0%, 33%, 50% and 100%, respectively. A 20- aliquot of distilled was also added to a sterile paper disc as a control. The plates were incubated at

37 °C for 24 h and 48 h to validate for the inhibition zone. Diameters of inhibition zones were measured. Each experiment was done in duplicate.

E. Fish snack formulation

A 54.35% of fish meat was mixed thoroughly with 18.11% of bacterial cellulose derived from the static batch cultivation of *A. xylinum* TISTR 998 on coconut juice for 2 weeks under room temperature (30 °C). Other ingredients composed of 0.72% salt, 0.72% black pepper powder, 1.45% soy source, 2.17% seasoned flavor, 1.45% monosodium glutamate powder, 1.45% pepper powder, 3.62% Bar-B-Q flavor powder, 7.25% tapioca flour, 4.35% wheat flour and 4.35 % sugar were then added and mixed. Five supplemented medicinal plant fish snack formulas were formulated by adding the crude extract into the mixture as formula 1, 0% extract; formula 2, 5% clove extract; formula 3, 10% clove extract; formula 4, 5% ginger and formula 5, 10% ginger. The blends of all mixtures were made into thin layers and then were boiled for 10 min. After 5 min of immersion thin layers in the cool water, the samples were incubated under 4 °C in the refrigerator for 6 h. The layers were then fried for 10 min. Ready fish snacks were then cut into flakes and packed in the plastic bag. The flow chart of fish snack processing is shown in figure 2.

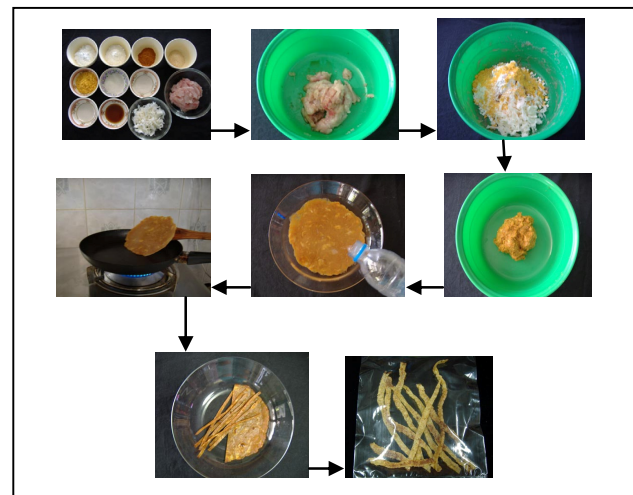


Figure 2. Flow chart of fish snack processing:

- (1) sample preparation, (2) soy sauce and salt addition, (3) seasoning, (4) kneading, (5) spreading before 5 minutes steaming and immersing in cool water, (6) immersing in cool water for 6 hours and frying, (7) cut into flakes and (8) packing.

F. Physical analysis and nutritional evaluation

All medicinal supplemented fish snacks were measured the color characteristic using a colorimeter. The values of L*, a* and b* were estimated using a CIELAB color system. A_w and moisture content changes over the storage time were also detected. The nutritional value in terms of carbohydrate, fat, protein and dietary fiber were analyzed according to AOAC, 1995.

G. Storage time

All formulas of supplemented fish snacks were kept under room temperature (28° C) for evaluation the shelf-life. The flake samples of each formula were collected at interval in 8 days to check the microbial growth. Appropriate dilutions were prepared in peptone water and the plate count method was used for counting the microbial growth on Mueller-Hinton agar under the incubation for 24 hours at 37° C.

H. Sensory analysis

Sensory evaluation for the appearance, color, flavor, taste, texture and preference were performed by 2 panels, male and female, aged 20-25 years old, consisting of 10 panelists for each group using scoring test with nine point hedonic scale. Analyses of variance and the significance of the mean difference were performed by the SPSS version 10. The statement of significance was based on $p < 0.05$ unless otherwise indicated.

III. RESULT AND DISCUSSION

The results of disk diffusion test indicated that crude extracts of clove and ginger showed different degrees of growth inhibition, depending on the bacterial strains (Table 1). The higher concentration of both extract revealed the higher inhibitory effect toward all food borne pathogen. Comparison of the diameter inhibition of three pathogenic bacteria showed that the diameters of ginger extract in day 1 and day 2 inhibited *E. coli* and *S. aureus* with relatively the same value with the clove extract. However, the clove extract at the highest concentration with the higher bacterial growth in the plate cultures showed the broadest antibacterial activity by inhibiting growth of *S. typhimurium* tested (the diameter of inhibition zone, 10-16.25 nm), while the extracts of ginger extract results in lower inhibitory effect. Our result is in agreement with the investigation of Devi *et al.*, 2010 [11]. They found that treatment with eugenol at their MIC (0.0125%) and MBC (0.025%) reduced the viability and resulted in complete inhibition of the *S. typhi* within 60 min exposure by SDS-PAGE, SEM and AFM analysis in order to conclude that eugenol increased the permeability of the membrane. They also confirmed the deformation of macromolecules in the membrane from the eugenol activity by FT-IR spectroscopy. Moreover, Walsh *et al.*, 2003 [12] reported that eugenol exhibits an excellent bactericidal activity against a wide range of organisms like *E. coli*, *S. aureus*, *P. aeruginosa* and *L. monocytogenes* [13]. The microscopic mechanisms of the antibacterial inhibition of this clove extract have been explained by many investigators [14,15]. From these studies, they suggest that the mode of antibacterial action of eugenol is through disruption of cytoplasmic membrane, which increases its non-specific permeability. In addition, the hydrophobic nature of eugenol enables it to penetrate the lipopolysaccharide of the Gram-negative bacterial cell membrane and alters the cell structure, which subsequently results in the leakage of intracellular constituents. Recent evidences reveal that the eugenol had a strong effect on *Enterobacter aerogenes* by using the

hydroxyl group to bind to proteins resulting in the prevention of bacterial enzyme activity.

TABLE I. ANTIBACTERIAL ACTIVITY OF EXTRACTS OF MEDICINAL PLANTS AGAINST FOOD BORNE PATHOGEN USING DISK DIFFUSION TEST

Microorganism and Condition			Diameter of inhibition zone (nm)					
			Clove extract concentration			Ginger extract concentration		
Pathogenic bacteria	Dil.*	Incubation (day)	33%	50%	100%	33%	50%	100%
<i>E. coli</i>	10 ⁻¹	1	7.0	9.0	9.5	7.50	8.75	10.5
		2	7.25	9.0	10.0	8.0	8.75	10.5
	10 ⁻²	1	8.5	9.25	10.0	8.0	8.75	10.5
		2	8.5	9.5	11.25	8.75	9.75	11.5
<i>S. aureus</i>	10 ⁻¹	1	8.5	10.75	13.5	8.0	10.0	12.0
		2	9.0	11.0	13.5	8.5	10.75	12.5
	10 ⁻²	1	11.5	11.5	16.25	11.25	11.75	16.25
		2	11.5	12.0	16.5	11.5	11.75	16.25
<i>S. typhimurium</i>	10 ⁻¹	1	8.75	10.5	12.5	8.0	11.0	13.0
		2	9.0	10.5	12.5	8.0	11.25	13.0
	10 ⁻²	1	11.5	11.5	14.25	9.0	11.0	12.75
		2	11.5	12.0	14.5	9.0	11.5	13.0

Dil.* = Dilution

For ginger, the antibacterial activity and inhibition activity of ginger extracts could be attributed to its chemical properties. The main constituents of ginger are sesquiterpenoids with zingiberene as the main component. Other components include β -sesquiphellandrene, bisabolene and farnesene, which are sesquiterpenoids, and trace monoterpenoid fraction, (β -sesquiphellandrene, cineol and citral) [16]. Chen *et al.*, 2008 [17] reported the antimicrobial activity of Zingiberaceae plants in Taiwan that the extracts from *Z. kawagooi* Hayata and *Z. oligophyllum* K. Schumann species had microbial inhibition activities toward *E. coli*, *S. enteric*, *S. aureus* and *V. parahaemolyticus*.

Normally the medicinal plants such as garlic, cinnamon, black pepper, clove, ginger, red/green chilies and cumin are used extensively in the Thai cuisine. The most active compound in clove is recognized as Eugenol, which is used as an antiseptic and possesses local anaesthetic activity; it is therefore used for toothache [18]. Further application to use as a preservative is preferable approach to use in food industry. The ready to eat fish snack from this experiment is displayed in figure 3. Sensory evaluations on texture and appearance were tested with 30 panelists. We found that our crispy flake fish snack was tasty as same as the popular commercially available fish snack in Thai market. Under the supplement condition with ginger and clove extracts, The essential substances in these two extracts were active in the

reduction of bacterial growth during the storage time. Changing in the bacterial growth during the 8 days storage time is presented in Figure 4. The microbial growth showed profound inhibitory effect toward the supplemented formulas when compared with the control as only 31%, 27%, 21% and 15% microbial growth at the eight day storage time under room temperature of the 5% ginger, 10% ginger, 5% clove and 10% clove, respectively.

Nutritional analysis showed relatively the same values of carbohydrate, lipid, protein and dietary fiber in all formulas approximately, 79.31%, 4.47%, 2.42% and 6.52%, respectively. As shown in Figure 5, all fish snack formulas showed brownish yellow color; however, all formulas color comparison revealed a significantly difference at the 95% confidence interval in color appearance ($p < 0.0001$). Moisture content and the water activity at the 8th day storage time in Figure 6 exhibited relatively same values of moisture content. Sensory analysis between the overall acceptance of male and female panelists showed no significant difference. However, color, flavor, test, texture, physical appearance and overall acceptance of five formulas exhibited a significant difference of these parameters ($p < 0.05$).



Figure 3. Fish snack from bacterial cellulose.

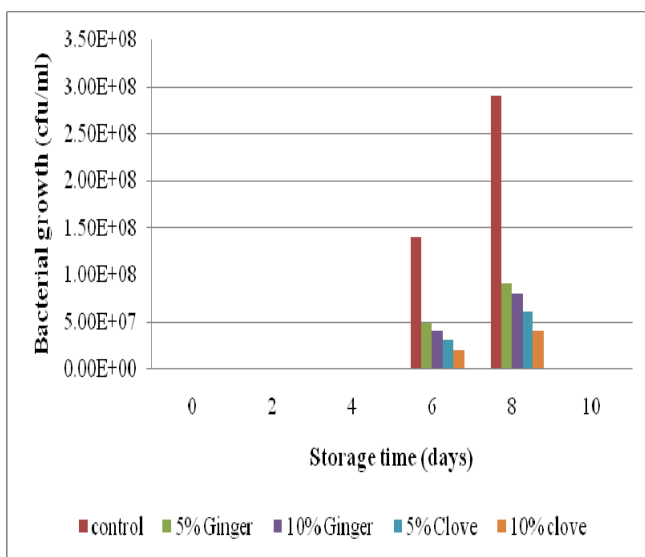


Figure 4. Bacterial growth during the storage time on the formulated fish snacks.

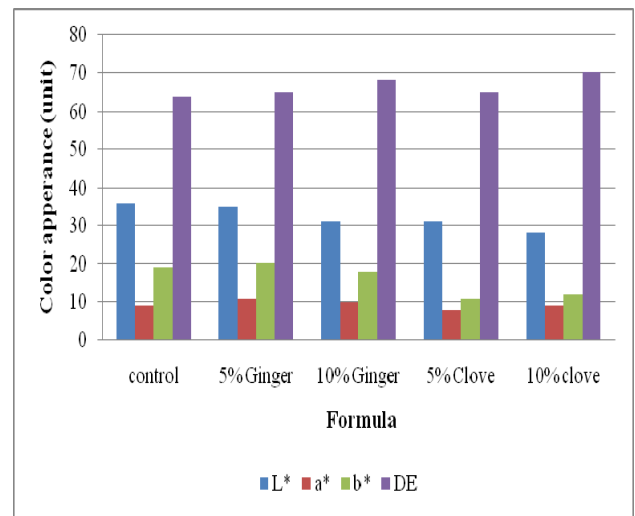


Figure 5. Color appearances of the formulated fish snacks

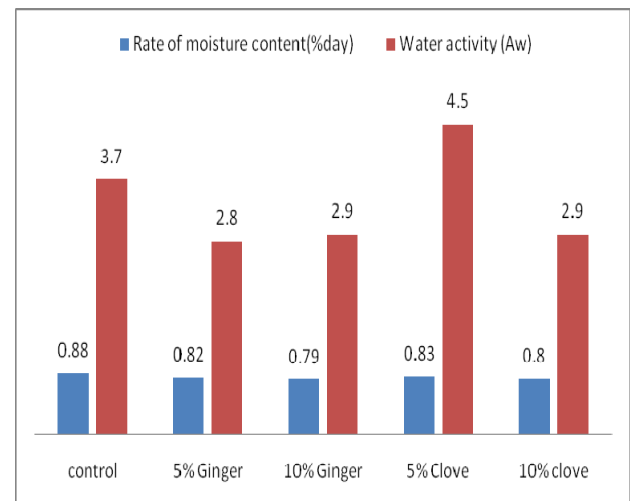


Figure 6. Rate of moisture content of storage time and A_w values at the 8th day of the formulated fish snacks.

Most research previously have focused on the antimicrobial activity of various plant water and oil extracts as well as their components in the field of medicine and therapeutics; however, the focus on the practical application for snack food is very limited. Further investigation on antimicrobial effects of medicinal plant extract against bacteria, yeasts, filamentous fungi, and viruses in food application is challenging and still need further discovery.

ACKNOWLEDGMENT

The author is grateful to Kanokwan Sroykesorn and Arpa Wanleeluk for their assistance in the manuscript preparation.

This work was partly supported by the Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand.

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