



Screening of Halophilic Bacteria and Product Characteristic from Salty-Fermented Fish (Pla-ra)

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Abstract

Salty-fermented fish, Pla-ra or Pa-daek is known in Thailand and Lao, respectively, and recognized as seasoning for traditional food. However, production of Pla-ra is often produced in small and medium scale manufacturing for local consumption and also distribution. The aims of this study was to isolate halophilic bacteria and study product characteristic from Pla-ra obtained from different areas (Mukdahan, Nakhon Phanom, Sakon Nakhon of Thailand, and Savannakhet of Laos) and identify character of products. Pla-ra contained pH values at 4.93 - 6.91, 1.32 - 1.98 % acidity, 16.3 - 30.8 Brix of total soluble solid, and 24.86 - 33.58% sodium chloride. There were 40 isolates of halophilic bacteria, and most of them could grow in culture medium containing 20-25% sodium chloride. However, most of strains that showed high proteolytic activity did not show acid production. The sample contained low sodium chloride showed high acidity, low pH, and short fermentation period. Although the result showed that proteolytic and halophilic bacteria play an important role on Pla-ra production, flavor and consumer preference of product are vary from place to place, ingredients, and raw materials. To preserve the diversity of regional food culture, study of bacterial community affecting on flavor and product preference is also need to be conducted to identify quality of Pla-ra production in different areas.

Keywords: Salty-Fermented Fish, Halophilic Bacteria, Proteolytic Bacteria, Pla-ra, Pa-daek

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1. Introduction

A traditional preservation of fish in Southeast Asia, especially, in Maekong basin is salty-fermented fish. It is an important preservation method for high protein food. Fish rapidly spoils, or goes rotten; however, fermentation is a method which attacks the ability of spoilage microorganism (Dagoo, 2000).

Pla-ra is a traditional northeastern Thai fermented fish. It is called Pa-daek in Lao. Pla-ra or Pa-daek are seasoning produced by fermentation of fish with rice bran or roasted rice powder and salt in a closed container for at least six months (Suriyanont & Chancharoonpong, 2018). Pla-ra is mostly used as ingredient of local food in northeastern Thailand and Lao such as spicy salad, soup, and curry. It has a very strong smell, which is considered unpleasant by some people. Its flavors are salty and sour, depending on the amount of salt put in and lactic acid resulting from fermentation process. Originally, production of Pla-ra aims to household consumption. Because of food culture exchange and migration of people, high demand of traditional food increases. Therefore, high demand of traditional food as Pla-ra in food business, import-export market is required.

Trade value of Pla-ra in 2004 was over 800 million Bath, and 20 million Bath was the value for export product. In present day, production of Pla-ra is produced in small and medium scale manufacturing for local consumption and also export to other countries. Production capacity of

Pla-ra is over 40,000 Tons each year (Saengkaew et al., 2017).

In fermentation product, proteolytic and halophilic bacteria play an important role on protein fermentation and high salinity fermentation product, respectively. Moreover, lactic acid bacteria such *Tetragenococcus* species or *Lactobacillus* species are represented in fermented food as well. Amount of salt in fermentation also play an important role on microbial selection for preferred product character (Hongthongdaeng, 1979; Hui et al., 2004; Marui et al., 2015; Nada et al., 1980; Vichasilp, 2005).

To preserve the diversity of regional food culture, scientific interest to bacterial community and properties of Pla-ra in various regions is suggested to study. The aims of this study was to isolate halophilic bacteria from Pla-ra obtained from 4 different areas (Mukdahan, Nakhon Phanom, Sakon Nakhon of Thailand, and Savannakhet of Laos) and also identify character of products from different area.

2. Materials and Experiment

2.1 Sample Collection

Pla-ra products made in Mukdahan, Nakhon Phanom, Sakon Nakhon of Thailand, and Savannakhet of Laos were obtained from local markets in original area. The information about production as fish materials, and fermentation period for each product was provided by the producer.

The samples were collected from November 2016 to January 2017. After purchase at the markets, the samples were cold-stored in a refrigerated container to minimize microbial activity then all samples were immediately analyzed within 24 h.

2.2 Determination of Sodium Chloride, pH and Titratable Acidity of Pla-ra

Sodium chloride content was determined by using salt analyzer (SAT-500, DKK- TOA Corporation, Japan). One ml of sample was diluted in 9 ml of deionized water then 20 µl of diluted sample was used to analyze. The pH of fermented samples was determined directly in the container using a digital pH meter with aseptic technique. Titratable acidity of samples were determined by titration with 0.1 N sodium hydroxide and phenolphthalein was used as indicator (Jiang et al., 2007).

2.3 Screening of Halophilic Bacteria in Pla-ra

Twenty-five gram of sample was mixed with 0.85% NaCl solution in stomacher for 2 min. Sample was diluted to appropriated dilution then enumerate by spread plate technique with nutrient agar supplemented with 5% sodium chloride. The culture was incubated at 37°C for 24-48 h.

In order to determine the prevalent microorganism and to select representative colonies from each plate in a random manner, the Harrison disc method was used in this study adopted from Harrigan (1998) to be able to calculate the

distribution of various microorganisms present in a sample.

The collected colonies were kept as stock culture in nutrient agar slant supplemented with 5% sodium chloride. The morphology, gram staining and catalase reaction were observed according to methods described in (Sharma, 2007).

2.4 Effects of Sodium Chloride in Culture Media on Growth of Halophilic Bacteria

Growth of isolated halophilic bacteria in different sodium chloride concentration was observed in nutrient broth. Sodium chloride was added into the culture medium to obtain the final concentrations, which were 10, 15, 20, and 25% (W/V). The cultures were incubated at 37°C for 24 h to observe their survival by streak plate technique on nutrient agar with 5% sodium chloride.

2.5. Ability on Proteolytic Enzyme and Acid Production of Isolated Halophilic Bacteria

Isolated halophilic bacteria was cultured in nutrient broth with 5 % sodium chloride at 37°C for 24 h. Optical density of culture was adjusted to 0.5 at OD600 before used. Twenty µl of culture was spotted on nutrient agar supplemented with 5% sodium chloride and 1% skim milk. After incubation at 37°C for 24 h, clear zone was determined for the ability to produce of proteolytic enzyme. To study ability on acid production, 20 µl of culture was spot on Glucose yeast extract (GYE) agar supplemented with 0.5% calcium carbonate and 0.004 % bromocresol purple. The strain presented a yellowish colony and a clear zone due to

acid production and dissolving calcium carbonate were continued to measure acid production in GYP broth.

3. Results and Discussion

3.1 Products Characteristics of Pla-ra

Pla-ra products made in Sakon Nakhon, Nakhon Phanom, and Mukdahan provinces in northeastern Thailand, were obtained from fresh markets in local area. Pa-daek products made in Savannakhet province of Laos were obtained from fresh market in local area. The production area, fish type, and fermentation period of Pla-ra were provided by the sellers. Pla-ra products from different area showed different chemical properties. Pla-ra from Thailand made from different type of fish including mixed fish, mushy fish, scaly fish, and whole fish, while samples from Laos made from mixed fish. Pla-ra from Thailand showed long period of fermentation as 6 - 12 month, while fermentation period of Pla-ra from Laos was 4 - 6 months (Table 1).

All samples showed pH values at 4.93 - 6.91, 1.32 - 1.98 % acidity, 16.3 - 30.8 Brix of total soluble solid, and 24.86 - 33.58% sodium chloride. Pla-ra from Nakhon Phanom showed the highest sodium chloride content (32.97 - 33.58%) with high pH value (5.63 - 6.91), and low acidity (1.32 - 1.56%). In contrast, Pla-ra from Laos showed the lowest sodium chloride (24.86 - 26.67%) with low pH value (4.93 - 5.41), and high acidity (1.42 - 1.98%). The results also showed that Pla-ra with low sodium chloride (01-LAO, and 02-LAO)

showed short fermentation period (4 - 6 months). There was report that Pla-ra in northeastern area of Thailand which contain high sodium chloride need long fermentation period because microorganism in raw material is able to grow in low sodium chloride condition better than in high sodium chloride condition (Suriyanont & Chancharoonpong, 2018). High sodium chloride content product always found in salty-fermented fish products. Traditional salty and fermented seafood in Korea also showed high sodium chloride as 26 - 18% with 4-6 months of fermentation period (Kim & Park, 2014; Ok et al., 2016; Lee & Choe, 1974). Consequently, the fermentation period could be related to the sodium chloride content in Pla-ra production that high sodium chloride content will slow down fermentation of Pla-ra, while the fast fermentation will be encouraged by reducing sodium chloride.

3.2 Microbial Characteristic of Pla-ra

Halophilic bacteria from 8 samples were selected representative colonies via the Harrison disc method. Halophilic activity of each colony was studied in different sodium chloride concentration. Sodium chloride was added into nutrient broth to obtain the final concentrations 10, 15, 20, and 25% (W/V). In total of 40 isolates were identified as halophilic bacteria. Most of the strain was able to grow at high sodium chloride concentration more than 20%. However, some strains as 02-MH-1, 02-SK-5, 01-LAO-1, and 01-LAO-2 were able to grow in sodium chloride concentration not over than 15%. There are 4 strains showed gram negative, with 2 strains of rod shape (01-NP-1 and 02-SK-2)

and 2 strains of cocci shape (01-MH-1 and 02-MH-2) (Table 2&3).

To study proteolytic activity of halophilic bacteria, the culture was inoculated in nutrient agar with 1% skim milk. The results showed that most of strains showed high proteolytic activity did not show acid production (02-NP-2, 01-MH-4, 01-SK-3, 02-LAO-5). The results also showed that all of Pla-ra samples contained both of proteolytic and acid production bacteria (Table 2&3).

The halophilic lactic acid bacteria frequently discovered in fermented food. For example, it was found that lactic acid bacteria, including *Tetragenococcus*, *Pediococcus*, *Weissella*, and *Lactobacillus*, were detected in Pla-ra products. Moreover, halophilic bacteria as *Halanaerobium fermentans* and *Sphingomonas*

roseiflava were found [6]. *Tetragenococcus halophilus* and *T. muriaticus* were found as dominant culture in traditional salted and fermented seafood in Korea (Kim & Park, 2014). Comparing to other fermented fish products, major microorganisms in jeotgal (Korean traditional fermented fish products) were *Bacillus*, *Brevibacterium*, *Micrococcus*, *Pediococcus*, *Pseudomonas*, *Lactobacillus*, *Leuconostoc*, and *Halobacterium* that those microorganisms also showed proteolytic activity and halophilic property (Ok et al., 2016). These also suggest that both proteolytic and lactic acid bacteria are predominant microorganism in salty-fermented fish product and show an important role in salty-fermented fish production in order to control palatability and quality of the finish product.

Table 1 Sample Code, Production Area, Fish Type, and Chemical Properties of Pla-ra

Sample code	Sample origin	Country	Type of Pla-ra	pH	Acidity (%)	Total soluble solid (°Brix)	NaCl (%)	Fermentation period
01-SK	Sakon Nakhon	Thailand	Mushy fish	5.63	1.35	20.7	29.43	12 months
02-SK	Sakon Nakhon	Thailand	Mixed fish	4.96	1.67	16.3	28.84	12 months
01-NP	Nakhon Phanom	Thailand	Mixed fish	6.91	1.32	20.3	32.97	12 months
02-NP	Nakhon Phanom	Thailand	scaly fish	5.63	1.56	23.7	33.58	12 months
01-MH	Mukdahan	Thailand	Whole fish	5.17	1.89	22.4	27.59	6-8 months
02-MH	Mukdahan	Thailand	mixed fish	5.29	1.41	27.8	29.40	6-8 months
01-LAO	Savannakhet	Laos	mixed fish	4.93	1.98	30.8	24.86	4 months
02-LAO	Savannakhet	Laos	mixed fish	5.41	1.46	24.2	26.67	6 months

Table 2 Characteristic of Halophilic Bacteria Isolated from Salty-Fermented Fish in Thailand

Sample code	Bacteria code	Growth in Na Cl (%)*				Catalast test*	Gram stain*	Shape	Proteolytic activity**	Acid production*	Acidity (%)
		10	15	20	25						
01-NP	01-NP-1	+	+	+	+	+	-	Rod	0.81±0.01	-	ND***
	01-NP-2	+	+	+	+	+	+	Rod	1.11±0.01	-	ND
	01-NP-3	+	+	+	+	-	+	Rod	0.22±0.01	+	0.35
	01-NP-4	+	+	+	+	+	+	Rod	0.90±0.05	-	ND
	01-NP-5	+	+	+	+	-	+	Rod	0.30±0.07	+	0.64
02-NP	02-NP-1	+	+	+	+	+	+	Rod	0.79±0.27	-	ND
	02-NP-2	+	+	+	+	+	+	Rod	0.85±0.17	-	ND
	02-NP-3	+	+	+	+	+	+	Rod	0.72±0.01	-	ND
	02-NP-4	+	+	+	+	-	+	Cocci	0.00	+	0.85
	02-NP-5	+	+	+	+	-	+	Cocci	0.00	+	0.72
01-MH	01-MH-1	+	+	+	+	+	-	Cocci	0.95±0.15	-	ND
	01-MH-2	+	+	+	+	+	-	Cocci	0.54±0.23	-	ND
	01-MH-3	+	+	+	+	+	+	Rod	0.46±0.19	-	ND
	01-MH-4	+	+	+	+	+	+	Rod	1.21±0.13	-	ND
	01-MH-5	+	+	+	+	-	+	Rod	0.00	+	0.56
02-MH	02-MH-1	+	-	-	-	+	+	Rod	0.22±0.04	+	0.54
	02-MH-2	+	+	+	+	+	+	Rod	0.73±0.08	-	ND
	02-MH-3	+	+	+	+	+	+	Rod	0.78±0.04	-	ND
	02-MH-4	+	+	+	+	+	+	Rod	0.83±0.08	-	ND
	02-MH-5	+	+	+	+	+	+	Rod	0.89±0.04	-	ND
01-SK	01-SK-1	+	+	+	+	+	+	Rod	0.88±0.44	-	ND
	01-SK-2	+	+	+	+	-	+	Rod	0.15±0.02	+	0.69
	01-SK-3	+	+	+	+	+	+	Rod	0.96±0.44	-	ND
	01-SK-4	+	+	+	+	+	+	Rod	1.01±0.50	-	ND
	01-SK-5	+	+	+	+	+	+	Rod	0.89±0.43	-	ND
02-SK	02-SK-1	+	+	+	+	-	-	Rod	0.77±0.03	+	0.61
	02-SK-2	+	+	+	+	+	+	Cocci	0.53±0.01	-	ND
	02-SK-3	+	+	+	+	-	+	Cocci	0.00	+	0.40
	02-SK-4	+	+	+	+	+	+	Rod	0.75±0.01	-	ND
	02-SK-5	+	+	-	-	-	+	Rod	0.00	+	0.24

* +/- Growth, gram stain or catalase test positive/ negative ; ** Clear zone (cm.) ; ***ND = not detected

Table 3 Characteristic of Halophilic Bacteria Isolated from Salty-Fermented Fish in Laos

Sample code	Bacteria code	Growth in Na Cl (%)*				Catalast test*	Gram stain*	Shape	Proteolytic activity**	Acid production*	Acidity (%)
01-LAO	01-LAO-1	+	+	-	-	-	+	Rod	0.00	+	0.66
	01-LAO-2	+	+	-	-	-	+	Rod	0.00	+	0.38
	01-LAO-3	+	+	+	-	+	+	Rod	0.00	+	0.46
	01-LAO-4	+	+	+	-	+	+	Rod	0.86±0.03	-	ND
	01-LAO-5	+	+	+	-	+	+	Rod	0.59±0.06	-	ND
02-LAO	02-LAO-1	+	+	+	+	+	+	Rod	0.92±0.05	-	ND
	02-LAO-2	+	+	+	-	+	+	Rod	0.22±0.03	+	0.69
	02-LAO-3	+	+	+	-	+	+	Rod	0.91±0.02	-	ND
	02-LAO-4	+	+	+	-	+	+	Rod	0.91±0.01	-	ND
	02-LAO-5	+	+	+	-	+	+	Rod	1.00±0.06	-	ND

* +/- Growth, gram stain or catalase test positive/ negative ; ** Clear zone (cm.) ; ***ND = not detected

4. Conclusion

The results reviewed that salty-fermented fish product in different area showed different character such as type of fish, fermentation period that was related to sodium chloride concentration. Moreover, isolated halophilic bacteria from Pla-ra obtained from 4 different areas might be used as starter culture for fermentation in order to develop production of traditional salty-fermented fish product which should remain their unique character of each area production.

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