

The Use of Selected Lactic Acid Bacteria Isolates for Acceleration of Fermented Fish (Pla-ra) Process

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ABSTRACT

Lactic acid bacteria (LAB) have important influences on fermented fish (Pla-ra) and other fermented foods. By using the selected LAB as a starter culture, Pla-ra fermentation could be accelerated and could yield a good quality product. Thirty seven samples of marine fish Pla-ra bought from four markets in Bangkok, Pathumthani, Samutprakan and Sumutsakorn were used in this experiment. The proteolytic *Lactobacillus plantarum* code number 5.25 and *Lactobacillus brevis* code number 10.5 were isolated from the samples. They exhibited good growth and lactic acid production in a culture medium which contained roasted rice as a carbohydrate source in a 15 % NaCl environment. The code numbers 5.25 and 10.5 were selected as starter cultures in the simulated marine fish (*Rastrelliger neglectus*) fermentation. The products of Pla-ra which were cooked were rated satisfaction scores by taste panelists and pathogenic bacteria could not be detected from the products. It can be concluded that *Lactobacillus plantarum* code number 5.25 and *Lactobacillus brevis* code number 10.5 are potential isolates to be used as starter cultures in commercial marine fermented fish.

Key words : fermented fish (Pla-ra), lactic acid bacteria, process acceleration

INTRODUCTION

Fermented fish or Pla-ra is a well known traditional food especially among people who live in the northeastern and northern regions of Thailand. Pla-ra is also popular among countries in the southern parts of Asia such as Myanmar, Laos, Vietnam and Cambodia.

Most Pla-ra are processed from freshwater fish such as *Trichogaster trichopterus*, *T. microlepis*, *Cyclocheilichthys repasson*, *C. reba*, *Labiobarbus burmanicus*, *L. siamensis*, *Ophicephalus striatus*, *Puntius gonionotus*, and *Anodontostoma chacunda* (National Research Council of Thailand, 1981-1982.). Some marine fish such as *Rastrelliger neglectus*, *Caranx* (*Selaroides*)

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leptolepis, *Johnius argentatus*, and *Rachycentron canadus* are also processed into Pla-ra (Sangjindavong, 2005). Now Pla-ra from marine fish species are as well known as that produced from fresh water fish. The value of Pla-ra produced in Thailand averages 800 million Baht per year, and those exported to other countries have a value of more than 20 million Baht per year (Anonymous, 2000). Pla-ra has the following physical properties and nutritive value: protein: 10-16 %, fat: 2.3-6.10 %, NaCl: 7.77-17.89 %, lactic acid: 0.37-3.15 %, vitamin B₁₂: 2.17±1.18 mg/100 gm, calcium: 1,505.16 mg/100 gm, phosphorous: 661.75 mg/100 gm and pH 4.7-6.2 (National Research Council of Thailand, 1981-1982). Total bacterial count was 2.20×10^6 – 8.85×10^7 CFU/gm and the species of bacteria found were *Tetragenococcus halophilus*, *Pediococcus* sp., *Staphylococcus epidermidis*, *Staphylococcus* sp., *Micrococcus* sp., *Bacillus subtilis* and *B. licheniformis* (National Research Council of Thailand, 1981-1982). Some bacteria such as *Staphylococcus* sp., *Micrococcus* sp. and *Bacillus* sp. were found in Pla-ra from marine fish (Yorsang, 2001). Proteolytic bacteria such as *Bacillus subtilis* and *B. licheniformis* which were isolated from fish paste had strong activity in producing proteolytic enzyme for the fermentation process (Sangjindavong, 2005). The fermentation period of Pla-ra takes, 12-18 months, thus, this research was to conducted develop some biotechnological methods to accelerate the process of Pla-ra production.

MATERIALS AND METHODS

Thirty seven samples of marine fermented fish (Pla-ra) were bought from four markets in Bangkok, Pathumthani, Samutprakan and Samutsakorn and were used in this experiment.

Lactic acid bacteria were isolated from marine fermented fish (Pla-ra) by the pour plate technique. MRS agar with bromocresol purple (0.004 %) and calcium carbonate (0.5 %) was the selected medium for the pour plate technique. The samples were then incubated at 30°C for 24-48 hrs in microaerophilic condition. Lactic acid bacteria were isolated by the ability of acid production, gram negative and producing enzyme catalase. Pure cultures of lactic acid bacteria were kept at -20°C.

Fifteen percent of NaCl was added to MRS broth and the classification of the genera of lactic acid bacteria was analyzed according to Axelsson (1998) and Sneath *et al.*, (1986). API 50 CHL bioMerieux, France was also used for classification of the species of lactic acid bacteria. Starter cultures with code numbers 5.25 and 10.5 were collected for further use.

Pla-ra was prepared from 100 kg of headless *Rastrelliger neglectus* cut into two then 25 % granular salt and 7 % roasted rice were added then mixed together. Samples of 2500 gram were put in plastic jars and then covered. The Pla-ra products were then sterilized by radiation at the Office of Atoms for Peace with a dose of gamma-ray 25 KGy for 18 hours. After radiating, starter cultures code numbers 5.25 and 10.5 with a concentration of 5 log CFU/g dissolved in 0.85 % NaCl were inoculated into the Pla-ra samples at different combinations

as follow :

code number 5.25

code number 10.5

code number 5.25 and 10.5

control

Sensory evaluation and assessment of quality of Pla-ra products were conducted by analysing both their chemical and microbiological properties. Chemical properties of Pla-ra such as pH, Aw, lactic acid content, salt, protein, fat, moisture, ash and histamine were analyzed by AOAC (1995) while microbiological characteristics such as total bacterial count yeast and molds, pathogenic bacteria (*Salmonella* sp, *Vibrio parahaemolyticus*, *Clostridium prefringens*, *Bacillus cereus* and *E. coli*) were studied according to AOAC (1995), A.P.H.A. (1992). Sensory evaluation of uncooked and cooked Pla-ra were conducted by untrained 15 panelist from the Department of Fishery Products, Faculty of Fisheries, Kasetsart University. A five point hedonic scale (5=extremely like, 4=like, 3=neither like nor dislike, 2=dislike, 1-extremely dislike) was used for the appearance, color, flavor and overall liking for each Pla-ra sample. Data were analyzed by analysis of variance (ANOVA) using SAS program. Duncan's multiple range test was used to compare the means at a significant level of $p < 0.05$.

RESULTS AND DISCUSSIONS

Two hundred isolates of lactic acid bacteria were taken from 37 samples of marine Pla-ra based on properties of gram positive and non-production of enzyme catalase. The isolates of lactic acid bacteria had 79 rod shaped isolates with 121 isolates were cocci.

Selection of suitable lactic acid bacteria for a starter was based on the properties of halotolerance, protein digestion and acid tolerant. The growth of 200 isolates of lactic acid bacteria in MRS broth medium with added 15 % sodium chloride were 13 rod shaped isolates and 3 cocci isolates. The genera of the isolates were classified according to Axelsson (1998) and found that these were 2 *Enterococcus* isolates, 13 *Lactobacillus* isolates, and 1 *Pediococcus* isolate. The results of the classification are shown in Table 1.

Table 1 Classification of lactic acid bacteria

Code Number	Shape	Gas from glucose	Growth at 10°C	Growth at 45°C	Growth in 6.5% NaCl	Growth in 18% NaCl	Growth at pH 4.4	Growth at pH 9.6	Genera
1.7	Cocci	-ve	-ve	+ve	+ve	-ve	+ve	-ve	<i>Pediococcus</i>
1.9	Rod	+ve	+ve	-ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
1.10	Cocci	-ve	+ve	+ve	+ve	-ve	+ve	+ve	<i>Enterococcus</i>
5.21	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
5.25	Short Rod	-ve	+ve	-ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
9.9	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
9.11	Rod	-ve	+ve	+ve	-ve	+ve	-ve	-ve	<i>Lactobacillus</i>
9.13	Short Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
10.5	Short Rod	+ve	+ve	-ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
10.7	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
11.6	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
11.10	Rod	-ve	+ve	-ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
11.15	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
12.7	Rod	-ve	+ve	+ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>
12.12	Cocci	-ve	+ve	+ve	+ve	-ve	+ve	+ve	<i>Enterococcus</i>
12.35	Short Rod	+ve	+ve	-ve	+ve	-ve	+ve	-ve	<i>Lactobacillus</i>

Note : -ve no growth at fixed conditions

+ve growth at fixed conditions

The selection of lactic acid bacteria which had the ability to digest protein revealed that 12 isolates could digest gelatin but only 4 could produce enzymes to digest protein in skim milk. The results of this study are shown in Table 2.

Table 2 The ability of lactic acid bacteria to digest gelatin and skim milk

Code Number	Digestion of gelatin	Diameter's ratio of clear zone/colony by digestion of skim milk
1.7	+ve	1.66
1.9	-ve	-ve
1.10	-ve	-ve
5.21	-ve	-ve
5.25	+ve	2.71
9.9	+ve	1.66
9.11	-ve	-ve
9.13	+ve	-ve
10.5	+ve	1.66
10.7	+ve	-ve
11.6	+ve	-ve
11.10	+ve	-ve
11.15	+ve	-ve
12.7	+ve	-ve
12.12	+ve	-ve
12.35	+ve	-ve

Note : +ve positive
-ve negative

Selection of lactic acid bacteria which had the ability to grow in acid and salt with the medium under fixed conditions revealed that code number 5.25 could grow at the maximum fixed condition. The results are shown in Table 3.

Table 3 The ability of lactic acid grown under fixed conditions and production of lactic acid

Code Number	MRS broth + 15% NaCl pH 5.0, OD 600	Lactic acid content in moderate MRS medium (%)
1.7	0.653	0.334
5.25	1.266	0.523
9.9	0.637	0.479
10.5	0.711	0.725

The code numbers 5.25 and 10.5 were classified by biochemical tests, morphological, and API 50 CHL bioMerieux. It was concluded that the code number 5.25 was *Lactobacillus plantarum* and the code number 10.5 was *Lactobacillus brevis* (Vichasilp, 2005).

Two isolates of lactic acid bacteria, *Lactobacillus plantarum* and *L. brevis*, were used as a starter culture in the fermentation of Pla-ra for 6 months. The results of this test are shown in Table 4.

Table 4 Sensory acceptance scores of uncooked pla-ra fermented for 6 months.

Products	Appearance	Color	Odor	Average
Pla-ra + Code No.5.25 (radiated)	4.80 ^a ±0.42	4.40 ^a ±0.70	4.80 ^a ±0.42	4.70 ^a ±0.48
Pla-ra + Code No. 10.5 (radiated)	4.50 ^{ab} ±0.71	4.30 ^a ±0.67	4.70 ^a ±0.48	4.60 ^{ab} ±0.52
Pla-ra + Code No. 5.25 and 10.5 (radiated)	4.30 ^{abc} ±0.82	4.30 ^a ±0.82	4.50 ^a ±0.71	4.50 ^{ab} ±0.53
Traditional Pla-ra (radiated)	3.70 ^c ±0.82	4.30 ^a ±0.67	3.50 ^b ±0.53	3.80 ^c ±0.42
Traditional Pla-ra(Not radiated)	4.00 ^{bc} ±0.82	4.30 ^a ±0.67	3.90 ^b ±0.68	4.20 ^{bc} ±0.42

Value in the same column with different superscripts are significantly different ($P \leq 0.05$)

During the sensory evaluation, Pla-ra with code number 5.25 was mostly accepted followed by Pla-ra with code number 10.5 and Pla-ra with mixed two code numbers 5.25 and 10.5 isolates.

Sensory evaluation of cooked Pla-ra is shown in Table 5 while the histamine content during fermentation process is shown in Table 6

The panelists accepted cooked Pla-ra with starter culture of lactic acid code number 5.25 and 10.5 as well as uncooked Pla-ra.

Table 5 Sensory acceptance scores of cooked Pla-ra fermented for 6 months.

Products	Appearance	Color	Odor	Average
Pla-ra + Code No.5.25 (radiated)	4.80 ^a ±0.34	4.60 ^{ab} ±0.50	4.80 ^a ±0.41	4.80 ^a ±0.41
Pla-ra + Code No. 10.5 (radiated)	4.65 ^a ±0.46	4.7 ^a ±0.47	4.40 ^b ±0.50	4.70 ^a ±0.47
Pla-ra + Code No. 5.25 and 10.5 (radiated)	4.60 ^a ±0.50	4.50 ^{ab} ±0.51	4.35 ^b ±0.67	4.30 ^b ±0.66
Traditional Pla-ra (radiated)	3.95 ^b ±0.76	4.15 ^c ±0.37	3.85 ^b ±0.67	4.00 ^b ±0.46
Traditional Pla-ra (Not radiated)	4.25 ^b ±0.44	4.30 ^{bc} ±0.47	4.15 ^b ±0.49	4.20 ^b ±0.52

Values in the same column with different superscripts are significantly different ($P \leq 0.05$)

Table 6 Histamine content during fermentation process of Pla-ra

Products	Histamine content during fermentation process at different month						
	0	1	2	3	4	5	6
Pla-ra + Code No.5.25 (radiated)	37.257 ^{Aa} ±2.44	39.662 ^{Aa} ±1.87	39.601 ^{Aa} ±1.96	39.167 ^{Aa} ±0.87	38.871 ^{Aa} ±0.49	39.108 ^{Aa} ±0.64	39.094 ^{Ab} ±0.15
Pla-ra + Code No. 10.5 (radiated)	39.558 ^{Aa} ±0.97	39.116 ^{Aa} ±1.88	37.549 ^{Aa} ±0.80	37.625 ^{Aa} ±0.70	38.067 ^{Aa} ±0.08	38.675 ^{Aa} ±0.20	39.071 ^{Ab} ±0.69
Pla-ra + Code No.5.25 and 10.5 (radiated)	35.792 ^{Aa} ±1.88	37.203 ^{Aa} ±0.04	37.743 ^{Aa} ±0.54	37.869 ^{Aa} ±1.24	37.437 ^{Aa} ±1.57	37.979 ^{Aa} ±0.40	38.276 ^{Ac} ±0.38
Traditional Pla-ra (radiated)	38.997 ^{Aa} ±4.42	39.850 ^{Aa} ±1.27	39.340 ^{Aa} ±2.04	39.934 ^{Aa} ±2.74	38.288 ^{Aa} ±0.11	39.233 ^{Aa} ±1.11	40.226 ^{Ab} b±0.90
Traditional Pla-ra (Not radiated)	36.620 ^{Ba} ±3.05	38.575 ^{AB} a±2.19	39.286 ^{AB} a±0.47	39.032 ^{AB} a±0.75	39.061 ^{Ab} a±1.33	39.310 ^{AB} a±0.92	41.428 ^{Aa} ±0.63

A, B Means in the same vertical row followed by different superscripts are significantly different ($P \leq 0.05$)

a, b, c Means in the same horizontal row followed by different superscripts significantly different ($P \leq 0.05$)

The results showed that traditional Pla-ra had the highest histamine content at 41.428 mg/100 gm of sample, (Table 6). The maximum histamine content in scombroid allowed by the United States Food and Drug Organization is 50 mg/100 gm of samples, so every sample of Pla-ra from this study was safe for consumers

Microbiological studies for pathogenic and hygienic bacteria showed that *Clostridium perfringens* and *Bacillus cereus* were less than 10 CFU/gm, *Staphylococcus aureus* was less than 3 CFU/gm, and *Salmonella* sp. and *Vibrio parahaemolyticus* were not found in 25 gram samples. *Escherichia coli* (MPN/g) in the samples was less than 10 CFU/gm and fungi (yeasts and molds) were between $3.5 \times 10^3 - 7.2 \times 10^4$ CFU/gm. It was concluded that the products were safe for consumption. This study showed the possibility to decrease the fermentation process of marine fish Pla-ra, especially *Rastrelliger neglectus*, by using two strains of lactic acid bacteria code numbers 5.25 and 10.5 as starter cultures. This study showed that using code numbers 5.25 and 10.5 within 3 and 4 months of fermentations, respectively, had the highest acceptance scores. The traditional products of Pla-ra and control had to ferment for 6 months. The hedonic score was 4.5 out of 5. Thus code numbers 5.25 and 10.5 are suitable for the fermentation process of Pla-ra.

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