International Research Journal of Biological Sciences _____ ISSN 2278-3202
Vol. **13(4),** 17-24, November **(2024)** *Int. Res. J. Biological Sci.*

Improving Production and Quality of Fermented Fishery Products through Starter Culture Technology

Su Su Mar Lin[1,2,3*], Myo Myint[2] and Ekachai Chukeatirote[1]
[1]Department of Food Science, Mae Fah Luang University, Thailand
[2]Department of Biotechnology, Mandalay Technological University, Mandalay, Myanmar
[3]Department of Advanced Science and Technology, Nay Pyi Taw, Myanmar
susumarlin@gmail.com

Available online at : www.isca.in, www.isca.me
Received 8th July 2024, revised 20th October 2024, accepted 4th November 2024

Abstract

This study was aimed to improve production and quality of fermented fishes through starter culture technology. Total acidity, the pH and salt content in one sample of Plaa-som and two samples of Pla-ra were determined. Total acidity of three samples were 0.18%, 0.207% and 0.234%. pH were 8.34, 6.19 and 5.619 (w/w) respectively. Salt contents of these samples were 5.55%, 4.38% and 4.09% (w/w). Randomly picked 82 colonies on $CaCO_3$ MRS agar plates from the samples were all confirmed to be lactic acid bacteria (LAB) as 44 colonies exhibited Gram-positive, catalase negative and sugar-fermentative characteristics. LAB counts from Pla-ra from Chiang Rai (Sample II) were 10 log CFU/g. According to microscopic morphology, 9.16% are cocci and 90.24% are rod in isolation of lactic acid bacteria from fermented fishes. Only 12 isolates were found to be clear zoned on Skim Milk Agar in screening of LAB for protease activity. These isolates showed antifungal activity against 6 strains of Colletotrichum species. Isolated bacteria 15 III, 133 and 24 III were the best strains for the antifungal activity. The enzyme activity of 24 III was 1.2 U ml^{-1} and the highest in all isolates. This strain was selected for preparation of fermented fishery products. During fermentation, pH dropped slightly to 4.1 and total acidity increased to 1.35% in sample 5. Salt content in the Plaa-som was found to be 1.753% (w/w). Maximum counts of LAB and TVC were 11.5 log CFU/g and 22.81 log CFU/g in sample 5, respectively.

Keywords: Fermented Fishes, Starter Culture Technology, Salt Contents, Skim Milk Culture, Enzyme Activity.

Introduction

Thailand, like the other countries in Asian, has many kinds of indigenous fermented foods which play important role in Thai cooking and consuming. Many fermented products are made from fish, meat, cereals, legumes, vegetable and fruits. Thai people usually keep many fermented foods products in their household[1]. They consume these products everyday in one form or another, either as food or seasoning throughout the country. The main ones are knomjeen, fish sauce, kapi (paste, plaa-ra, plaa-som etc). Pla-som is described as a group of traditional Thai fermented fish products obtained from the fermentation of either whole fish or fish fillets with salt, steamed rice or sticky rice, and garlic until its taste becomes sour[2]. However, plaa-som recipes can be varied depending on local organoleptic preferences and ingredient availability in each region of Thailand. The fermentation relies on a septic fermentation in which the process is performed in an open system without sterilization or selected starter cultures[3]. The fermentation spontaneously occurs due to the presence of natural adventitious microorganisms, mainly lactic acid bacteria (LAB) that are isolated from fermented foods as natural starters to initiate the fermentation process. Food spoilage organisms are also isolated from fermented fish products[4]. Lactic acid productivity and antagonistic activity are screened and these are used to make fermented fish. Appropriate conditions are vital for accomplishment of fermentation by LAB such as the presence of particular carbohydrate and antimicrobial substances containing ingredients; salt and garlic and microaerophilic conditions created by wrap-up package design[5]. These conditions are aimed to promote the growth of LAB instead of other indigenous microorganisms including spoilage and pathogenic groups which also co-contaminate the raw materials and ingredients. Freshwater fishes such as carp are used in fermented fish production[6].

Plaa-som production is usually conducted in small scale or cottage industries thus it entirely depends on spontaneous fermentation by LAB and is without implementation of either Good Manufacturing Practices (GMPs) or Harzard Analysis and Critical Control Point (HACCP) systems. This is considerably very risky[7]. This experiment aimed to determine chemical and microbiological changes during the spontaneous fermentation of plaa-som obtained from GMPs implemented by a local producer. The results can be used to serve as safety control indices to determine additional practices for GMPs and to evaluate possible critical control points (CCPs) for small scale or cottage industries which will lead to better production process practice imparting higher quality and safety standards for pla-som production.

Materials and Methods

Samples: Three samples of fermented fishes (Plaa-som from Chiang Rai, Pla-ra from Chiang Rai and Pla-ra from Roi-et Provice) were collected.

Chemical Analyses: Total acidity and pH were analyzed according to the methods of AOAC[8]. For pH determination, 10 g of homogenized sample were mixed with 90ml of carbon dioxide free distilled water. Direct pH measurement was measured using a standard pH meter while total acidity was determined in the form of titratable acidity. For total acidity, 10 g of homogenized sample were mixed with 40ml of carbon dioxide free distilled water. The homogenate was centrifuged at 3000 rpm for 15 min in an appropriate centrifuge (Avanti® J-25, Beckman coulter, Inc., Connecticut, USA). The supernatant was filtered through a Whatman filter paper No.1, (Whatman plc., Kent, UK). The filtrate was titrated against standard 0.1 M sodium hydroxide (BDH Laboratory Supplies, Poole, UK) with an addition of a few drops of 1% phenolphthalein (Fluka Chemie AG, Buchs, Switzerland) as an indicator. The total acidity was calculated as an equivalent to lactic acid and reported as % (w/w). Salt content was determined as the amount of sodium chloride content using silver nitrate titration according to Mohr method, AOAC[8]. Five grams of homogenized sample were mixed with 25ml distilled water and filtered through a filter cloth and a Whatman filter paper No. 1. The filtrate was titrated against 0.1M silver nitrate (Sigma Chemical Co., Missouri, USA) with an addition of 1ml of 0.25 M potassium chromate (E. Merck Co., Darmstadt, Germany) as an indicator. Salt content was calculated from the volume of 0.1 M silver nitrate used to reach the end point of titration and reported as % sodium chloride (w/w).

Microbiological Analysis: Isolations: 25g of sample were homogenized with 225 ml of 0.85% (w/v) sterile peptone water in a Stomacher lab-blender at high speed for 3 min and serially diluted (10^{-1} to 10^{-8}). Suitable 10 times serial dilutions were prepared with saline peptone water (0.1% (w/v) peptone (Criterion, California, USA) in 0.85% (w/v) sodium chloride and used for colony counting via a standard total viable count technique. LAB counts were determined using microaerobic incubation at 30°C for 3 - 5 days on de Man, Rogosa and Sharpe agar, MRS agar (Pronadisa®, Madrid, Brazil) modified by the addition of 1.0% (w/v) calcium carbonate ($CaCO_3$-MRS), in order to facilitate the observation of clear zones exhibited by acid producing colonies. After an optimal incubation period, PCA and $CaCO_3$-MRS plates with 30 - 300 discrete colonies were counted and the results were reported in log CFU/g of sample. After counting all acid producing colonies surrounded by clear zones on $CaCO_3$-MRS agar plates, colonies were randomly picked for LAB confirmation using Gram staining, catalase test, glucose fermentation test according to Sneath et al.[9].

Technological Properties: Proteolytic activity: Surface-dried plates of milk agar[10] were streaked with 24-h-old cultures, after incubation at 30°C for 4 days, and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

Protease activity assay: Protease activity was measured by a modification of the method of Maeda et al.[11]. Cultures were grown in phytone broth[12] on a rotary shaking incubator at 30°C at 180 rpm for 72h. Cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and the substrate solution containing 1% Azocasein (Sigma Chemical Co., USA) was dissolved in 100 mM phosphate buffer, (pH 6.8) were pre-incubated separately at 37°C for 5 min in a water-bath incubator (RSB-12, Remi, India). The enzyme reaction was started by adding 2 ml of 1% Azocasein to 1ml of enzyme solution and incubated at 37°C for 20 min. The reaction was quenched by the addition of 2.5ml of 10% (w / v) trichloroacetic acid. After centrifugation at 15,000 rpm for 10 min, 2ml of supernatant was neutralized with equal amount of 1N NaOH and the absorbance was measured at 450 nm in UV– VIS Spectrophotometer (Specord 200, Analytik Jena, Germany). One unit of protease activity was defined as the quantity required increasing the absorbance by 0.1 under the above conditions.

Antifungal Activity Assay: 6 strains of *Collectotrichum* species (*Colletotrichum gloeosporiodes, Colletotrichum fructicola, Colletotrichum thailandica, Colletotrichum falcatum, Colletotrichum jasmine-sambee* and *Colletotrichum siamense)* were taken from the Fungus Laboratory in School of Science in Mae Fah Laung University, Thailand. These strains were sub-cultured and incubated in the centre of Petri-dishes for 3-4 days until the length of their mycelia was around 1cm wide. And then antifungal activity was tested by using three isolated bacterial species, which was carried out by inoculating those bacteria apart 2cm from the fungal colonies. As a control of this test, no bacteria were incubated on the other side of the fungal colonies.

Fermented Fish Sample Preparation: A common Thai fresh water SILVER BARB (*Barbodes gonionotus*) fish, locally called *plaa-ta-pien*, weighing approximately 300 g each, was used as the main ingredient. Fish were prepared by scaling, gutting, slitting, thoroughly washing, and soaking in 25% (w/v) brine for approximately 2-3h. They were finally rinsed and drained of all excessive brine before being mixed with the other ingredients. Ten kilograms of fish were then subsequently well mixed with steamed jasmine rice, minced garlic at a ratio of 1:0.5 by weight and inoculated by 10^6CFU/g of selected isolated strain. After mixing of all ingredients, the rest of the steamed jasmine rice and minced garlic were stuffed into the fish bellies. The mixed fish were placed in bowls which were covered with plastic sheets and left at room temperature for 6h in order to initiate the fermentation process. The 6h fermented fish were then kept in a -18°C freezing container for an overnight period.

Immediately after the overnight period, they were packed at approximately 3 fish per plastic bag. Most of the air inside each bag was expelled by pressing before closing the bag tightly with a rubber band. Thereafter, all the packed fish from the same production batch were subjected to further fermentation room temperature. And then chemical and microbiological analyses were determined as Table-1 for 24 hour –interval.

Table-1: Sample Designation and Their Descriptions for Each Microbial Application**.**

Sample Designation	Description of Sample
1	24hr Fermentation
2	48hr Fermentation
3	96hr Fermentation
4	120hr Fermentation
5	144hr Fermentation

Results and Discussion

Chemical Analyses: Fermented Fish samples were analyzed for pH, total acidity, and salt content. Total Acidity, the pH and salt content in samples in one sample of Plaa-som and two samples of Pla-ra are expressed in Table-2. It was found that MJ3 showed the highest acidity with lowest pH and salt content. On the other hand, MJ1 showed the lowest acidity with highest pH and salt content. Chemical compositions of Pla-ra depended on freshness of raw materials. In the study of Mathana Sangjindavong et al.[13], Pla-ra had pH between 4.55-7.48, NaCl between 8.24-21.09%, and lactic acid between 1.25 - 3.53%.

Microbial Analyses: Randomly picked 82 colonies on $CaCO_3$-MRS agar plates from three samples were all confirmed to be LAB as 44 colonies exhibited Gram-positive, catalase negative, and sugar-fermentative characteristics as shown in Table-3. LAB counts in sample 1 were 4 log CFU/ml, those counts in sample 2 were 10 log CFU/ml and those counts in sample 3 were 6 log CFU/ml. According to microscopic morphology, 9.76% are cocci and 90.24% are rod.

Table-2: Chemical Analyses of Total Acidity, pH and Salt Content in Samples.

No.	Samples	Total acidity	pH	Salt Content
1	MJ I	0.18%	8.34	5.55%
2	MJ II	0.207%	6.19	4.38%
3	MJ III	0.234%	5.619	4.09%

MJ I = Plaa-som from Chiang Rai, MJII = Pla-ra from Chiang Rai, MJIII= Pla-ra from Roi-et Provice.

Table-3: Morphological and Biochemical Characteristics of Isolated Bacteria.

Bacterial Strains	Cell Shape	Gram Reaction	Motility	Catalase	Glucose Fermentation
1	Rod	+	+	-	+
2	Rod	+	+	-	+
4	Rod	+	+	-	+
6	Rod	+	+	-	+
9	Rod	+	+	+	+
10	Rod	+	+	-	+
13 PCA	Rod	+	+	-	+
C 15	Rod	+	-	-	+
15 III	Rod	+	-	-	+
16	Rod	+	+	-	+
17 III	Rod	+	-	+	+
18	Rod	+	+	+	+
19	Rod	+	+	-	+
20	Rod	+	-	-	+
21	Rod	+	-	-	+

22	Rod	+	-	-	+
23	Rod	+	+	-	+
24 III	Rod	+	-	-	+
25	Rod	+	-	-	+
26	Rod	+	-	-	+
30 III	Rod	+	-	-	+
32 II	Rod	+	-	-	+
35 III	Rod	+	-	-	+
36	Rod	+	-	+	+
37	Rod	+	-	+	+
38	Rod	+	+	+	+
39	Rod	+	-	+	+
41	Rod	+	-	-	+
42	Rod	+	-	-	+
43	Rod	+	-	-	+
45	Rod	+	-	-	+
46	Rod	+	-	+	+
47	Rod	+	-	+	+
50	Rod	+	-	-	+
51	Rod	+	-	-	+
53	Rod	+	-	-	+
54	Rod	+	-	-	+
55	Rod	+	-	+	+
56	Rod	+	-	-	+
60	Rod	+	-	-	+
24 II	Rod	+	-	+	+
117	Rod	+	-	-	+
118	Rod	+	-	-	+
119	Rod	+	-	-	+
120	Rod	+	-	-	+
121	Rod	+	-	-	+
122	Rod	+	-	-	+
123	Rod	+	-	-	+
124	Rod	+	-	-	+
132	Rod	+	-	-	+
133	Rod	+	-	-	+
134	Rod	+	-	-	+
136	Rod	+	-	-	+
137	Rod	+	+	-	+
138	Rod	+	+	-	+
139	Rod	+	+	-	+
C 13 II	Cocci	+	-	-	+
15 III	Cocci	+	-	-	+

25 III	Cocci	+	-	-	+
26 III	Cocci	+	-	-	+
34 III	Cocci	+	+	+	+
33 III	Cocci	+	+	+	+
35 III	Cocci	+	-	-	+
40 III	Cocci	+	-	-	+
K 1	Rod	+	+	+	+
K2	Rod	+	-	+	+
K 3	Rod	+	+	+	+
K 4	Rod	+	+	+	+
K 5	Rod	+	+	-	+
K 6	Rod	+	+	+	+
K 7	Rod	+	-	-	+
K 8	Rod	+	+	+	+
K 10	Rod	+	+	+	+
K 11	Rod	+	+	-	+
K 13	Rod	+	-	-	+
K 14	Rod	+	-	-	+
K 15	Rod	+	-	-	+
K 16	Rod	+	-	-	+
K 17	Rod	+	-	-	+
K 20	Rod	+	+	-	+
K 21	Rod	+	+	-	+
K 22	Rod	+	+	-	+

Screening of Protease-producing Bacteria: Isolates of LAB were tested for protease activity. Twelve strains of LAB showed protease activities (showing hydrolysis zone in milk agar plate) and the estimated protease activity of these strains were 0.6-1.2 U ml^{-1} according to spectrophotometric method.

Similar to our result, Khusniati et al.[14] found that protease activity of Lacobacillus planturum B110 was 0.44 to 1.03 U/ml at different temperatures and pH.

Antifungal Activity of Isolated Bacteria: The antifungal activity of 44 isolated bacteria confirmed as LAB according to morphological and biochemical tests were tested against 6 strains of *Colletotrichum* species which are phytopathogens. Among the isolated bacteria, 12 isolated strains showed antifungal activity. 24III was the best strain for antifungal activity.

This strain was selected for fermented fish preparation. In agreement with our findings, Barrios-Roblero et al.[15] also found that the LAB strains isolated from fermented beverages showed strong antifungal capacity against *Colletotrichum gloeosporioides.*

Table-4: Enzyme Activity of isolated strains from fermented fish products.

Bacterial Strains	Protease (U ml^{-1})
15 III	0.6
25 II	0.7
26 III	0.8
132	0.5
133	0.7
K 17	0.5
32 III	1.0
117	0.9
118	0.8
30 III	0.6
25 III	0.5
24 III	1.2

Data represent the means of these sets.

Preparation of Fermented Fishes: Chemical Changes during fermentation: Salt content in the plaa-som was found to be 1.753% (w/w) which corresponds to its categorization as being a low salt fermented fish containing less than 8% (w/w) salt[16]. It was also found that the salt content of Plaa-som from Sisaket and Khon Kaen regions varied over narrow ranges of 2–4% and 4–6%, respectively, while those from Ubon Ratchathani and Surin presented higher variations[17].

The pH and total acidity changes during the fermentation are illustrated in Figure-1. The pH was initially found at 6.3 and was found to decrease rapidly from it. Total acidity, calculated as titratable acid equivalent to lactic acid, generally was revealed to have an increasing trend throughout the fermentation, as from 0.333 % to 1.3 %.

Table-5: Inhibition % of Antifungal Activity of Lactic Acid Bacteria.

Bacterial Strains	No. of Fungi/ Inhibition %					
	C 1	C2	C3	C4	C 5	C 6
15 III	39.23	39.585	51.43	43.055	40.065	46.43
25 II	41.65	37.605	41.43	43.055	36.22	43.65
26 III	43.56	46.43	37.855	35.415	32.055	44.155
132	36.035	39.23	45	23.61	29.015	34.52
133	42.725	49.65	50.605	45.835	39.74	38.095
K 17	29.135	41.955	41.665	32.015	32.865	27.76
32 III	37.41	35.415	32.29	48.105	47.725	25.385
117	30.39	40.585	38.75	45.83	47.725	32.855
118	37.41	27.62	29.165	48.105	40.905	34.23
30 III	38.885	31.945	35	37.98	48.105	49.995
25 III	31.25	36.43	35	32.045	48.105	45.45
24 III	45.835	39.61	44.845	43.665	58.33	56.435

Data represent the means of these sets: C1 = *Colletotrichum gloeosporiodes*, C2 = *Colletotrichum fructicola*, C3 = *Colletotrichum thailandica*, C4 = *Colletotrichum falcatum*, C5 = *Colletotrichum jasmine-sambee*, C6 = *Colletotrichum siamense*.

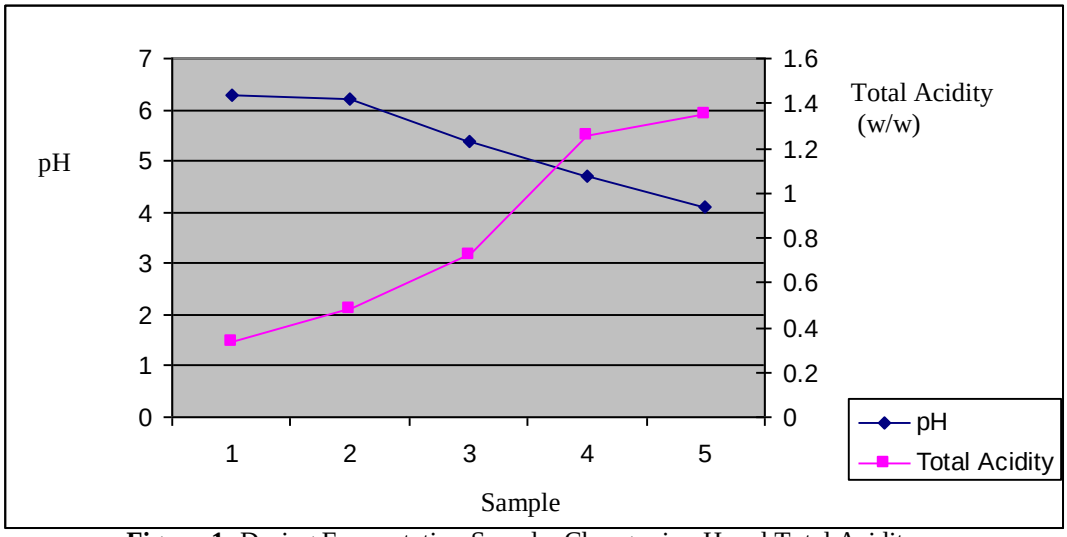

Figure-1: During Fermentation Sample, Changes in pH and Total Acidity.

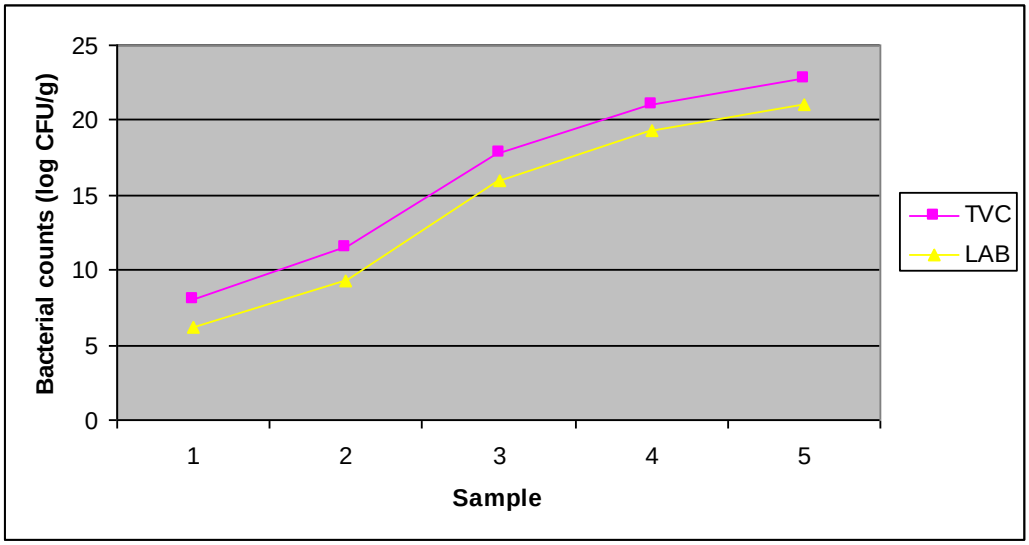

Figure-2: Total Viable Counts and Lactic Acid Bacteria Counts during the Spontaneous Fermentation Process of Plaa-som.

Conclusion

In this experiment, three samples of thai fermented fishes (Plaa-som from Chiang Rai, pla-ra from Chiang Rai and pla-ra from Roi-et Provice) were collected. LAB counts in sample II were the highest which proved that it was the best one to isolate lactic acid bacteria. 44 isolated bacteria were found to be as lactic acid bacteria in microscopic morphology and biochemical tests. In screening of isolated LAB for protease activity, twelve strains of LAB showed protease activities and the estimated protease activity of these strains were 0.6-1.2Uml^{-1}. That of 24 III was 1.2Uml^{-1}. This strain was the best one for antifungal activity against 6 strains of *Colletotrichum* species. So, that isolates was selected for fermented fish preparation.

In the preparation of fermented fish plaa-som, the salt content in plaa-som was found to be 1.753% (w/w) which corresponds to its categorization as being a low salt fermented fish containing less than 8% (w/w) salt. In sample 5, pH slowly dropped to 4 and total acidity increased to nearly 1.4% (w/w). Total viable counts and LAB counts in sample 5 were 22.82log CFU/g and 21.04log CFU/g. It can be concluded that sample 5 (144h fermentation) was the best time for fermentation. Plaa-som production can become biological fermented fish qualified products which provide the human health and nutrition because of inoculating with beneficial bacteria (Lactic acid bacteria).

References

1. Saisithi P (1987). Traditional fermented fish products with special reference to Thai products. *Asean Food J*., 3, 3-10.

2. TISI (2005). Thai Community Products Standard 26 / 2546. In Thai Community Product Standard. Thai Industrial Standards Institute, Kopermsub and Yunchalard 25 Ministry of Industry, Bangkok, Thailand.

3. Motarjemi, Y. (2002). Impact of small scale fermentation technology on food safety in developing countries. *International Journal of Food Microbiology*, 75(3), 213-229.

4. Barile, L. E., Milla, A. D., Reilley, A., & Villadsen, A. (1985). A spoilage patterns of mackerel Rastrelliger faughni Matsui. 1. Delays in icing. *Spoilage of Tropical Fish and Product Development*, 29-40.

5. Gram, L., & Huss, H. H. (1996). Microbiological spoilage of fish and fish products. *International journal of food microbiology*, 33(1), 121-137.

6. Beddows, C. G. (1998). Fermented fish and fish products. *Microbiology of fermented foods*, 416-440.

7. Nout, M. J. R., & Motarjemi, Y. (1997). Assessment of fermentation as a household technology for improving food safety: a joint FAO/WHO workshop. *Food Control*, *8*(5-6), 221-226.

8. Horwitz, W., & Latimer, G. W. (2000). Association of official analytical chemists. *Gaithersburg, MD, USA*.

9. Sneath, P. H., Mair, N. S., Sharpe, M. E., & Holt, J. G. (1986). Bergey's manual of systematic bacteriology. Volume *2* (pp. xxiii+-965).

10. Gordon, R. E., Haynes, W. C., & Pang, C. H. N. (1973). The genus Bacillus. Agricultural handbook no. 427. *Agricultural Research Service, US Department of Agriculture, Washington, DC*.

11. Maeda Y., Takenaga H., Aso S. and Yamanaka Y. (1993). Utilization of heat-dried stipe of mushroom (*Agaricus bisporus* Sing.) for animal feed. *J. Japan. Soc. Grassl. Sci.*, 39(1), 22-27

12. Nagai T., Nishimura K., Suzuki H., Banba Y., Sasaki H., Kiuchi K. (1994). Isolation and characterization of Bacillus

subtilis strain producing natto with strong umami-taste and high viscosity. *Nippon Shokuhin Kogyo Gakkaishi*, 41, 123–128. 10.3136/nskkk1962.41.123

13. Sangjindavong, M., Chuapoehuk, P., Runglerdkriangkrai, J., Klaypradit, W., & Vareevanich, D. (2008). Fermented fish product (pla-ra) from marine fish and preservation. *Kasetsart J.(Nat. Sci.)*, 42(1), 129-136.

14. Syafriana, V. (2019). Characterization of protease crude extract from indigenous lactic acid bacteria and the protein degradation capacity in local tuber and cereal paste flour. *J. Kim. Terap. Indones.*, 21(1), 38-44.

15. Barrios-Roblero, C., Rosas-Quijano, R., Salvador-Figueroa, M., Gálvez-López, D., & Vázquez-Ovando, A. (2019). Antifungal lactic acid bacteria isolated from fermented beverages with activity against Colletotrichum gloeosporioides. *Food bioscience*, 29, 47-54.

16. Tanasupawat, S., & Komagata, K. (1995). Lactic acid bacteria in fermented foods in Thailand. *World Journal of Microbiology and Biotechnology*, 11, 253-256.

17. Punyauppa-Path, S., Kiatprasert, P., Punyauppa-Path, P., Rattanachaikunsopon, P., Khunnamwong, P., Limtong, S., & Srisuk, N. (2022). Distribution of Kazachstania yeast in Thai traditional fermented fish (Plaa-Som) in northeastern Thailand. *Journal of Fungi*, 8(10), 10-29.