Kasetsart J. (Nat. Sci.) 39 : 88 - 97 (2005)

Isolation and Characterization of Salt-Loving Protease Producing Bacteria form Fish Sauce Samples

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ABSTRACT

Fermented fish samples from fish sauce factories in the eastern part of Thailand were used as sources for the isolation of salt-loving protease-producing bacteria. The conditions for optimal growth and salt-loving protease production were established for the extremely halophilic bacteria. Two hundred and eighty five strains of halophilic bacteria were isolated. Among these isolated strains, a totally ten strains of extremely halophilic bacteria exhibited the highest salt-loving proteases producing ability. Maximal growth and salt-loving proteases of these strain occurred in mM73 agar medium at 4M (24 %, w/v) NaCl. On the other hand, bacterial growth and salt-loving protease activity did not occur when NaCl concentration in mM73 agar medium was lowered to 2M (12 %, w/v). All of these strains were further secondary screened in liquid medium. These selected strains were cultured on a rotary shaker at 200 rpm at 37 °C in M73 liquid medium with 4M (24 %, w/v) NaCl at pH 7. The bacteria strain PB407 exhibited the highest salt-loving protease activity. In addition, the strain PB407 was identified and characterized. It was a Gram-negative, rod. The colonies were circle with red pigmentation. Growth occurred in a medium containing 17.5-25 % NaCl. Based on the data obtained in this study, it was concluded that the extremely halophilic bacteria strain PB407 belonged to the genus *Halobacterium*.

Key words: salt-loving protease, extremely halophilic bacteria, isolation, screening, fish sauce

INTRODUCTION

Fish sauce is a brown liquid with unique aroma and flavor and is rich in amino acids (Saisithi, 1994). It is widely used as a condiment and seasoning in most countries of Southeast Asia and is gradually gaining acceptance worldwide. Fish sauce is prepared by adding one part of solar-dried marine salt to three parts of small fish such as anchovies and sardines, and allowing them to solubilise in closed tanks at tropical temperatures. When most of the fish tissue has solubilised, the liquid fish sauce is drained off and filtered to yield a clear amber solution. The residue is extracted with brine to give a second-quality fish sauce. This procedure can be repeated until almost every tissue fraction has solubilised.

The solubilisation, which occurs mainly as a result of autolytic action by the digestive proteases in fish and enzymes from bacteria present on/in the fish and salt, usually takes 6 to 12 months (Beddow, 1998). The activities of the enzyme reduces due to the high salt concentration up to 25% (w/v) NaCl which occurs during fermentation and delayed the fermentation period. This means that a large capacity of storage tanks is required. To reduce this need and hence, capital investment, it is desirable to speed up the solubilisation. This

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Received date : 16/08/04 Accepted date : 30/09/04

situation has led investigator to look for alternatives to the traditional fermentation method. Recently, commercially available enzymes, extracted from various plants, animals and bacteria were used with the aim of decreasing the production time. The results were inconclusive as enzymatic activity decreased significantly at high salt concentrations (Beddow and Ardeshir, 1979; Raksakulthai and Haard, 1992; Thongthai *et al*., 1992).

Extremely halophilic bacteria or halobacteria have been defined as microorganisms which grow best in media containing 2.5–5.2 M (saturated) NaCl (Kushner and Kamekura, 1988). They are found in solar salt, on the surface of dried-salted fish and in fish sauce fermentation tanks (Larsen,1984). Using enzymes from these bacteria in industrial processes has advantage of optimum activities at high salt concentrations (Ventosa *et al*., 1998).

In order to speed up fish sauce production, the addition of proteolytic enzyme was favored since proteolysis is the fundamental reaction that take place during fermentation. On the other hand, enzyme from extremely halophilic bacteria required high salt concentration for activity, stability or both. Consequently, isolation and characterization of the strongest salt-loving protease from extremely halophilic bacteria was investigated.

MATERIALS AND METHODS

1. Isolation of halophilic bacteria

Fish sauce samples were obtained from fish sauce factory in the eastern part of Thailand. Approximately 1 ml of each fish sauce sample was added into 7 ml of Seghal and Gibbons Complex medium (SGC) containing 2, 3 or 4M NaCl (Sehgal and Gibbons, 1960). The inoculated media were incubated on rotary shaker at 200 rpm at 37 °C until red turbidity appeared. The turbid cultures were streaked on SGC broth containing 1.8 % (w/v) agar and incubated at 37 °C for 7 to 10 days. Colonies were selected based on colony appearance and on microscopic inspection of the cells. The most diverse isolates, between five and ten different ones per enrichment were chosen for further purification and characterization.

2. Screening of halophilic protease producing strain on solid medium

All bacterial isolates were subcultured in SGC medium with the appropriate (2, 3 or 4M) NaCl concentrations. They were tested for proteolytic activity on modified M73 (mM73) agar medium containing 0.8 % of sterile skim milk and suitable NaCl concentrations (2, 3 or 4M) for each isolate (Norberg and Hofsten, 1969). The cultures were incubated at 37 °C for10 days. A positive reaction for the proteolytic test was indicated by the clear zone of skim milk around the colony. The width of clear zone was considered to be directly related to the amount of extracellular protease produced. The strain having the highest ratio of clear zone diameter (mm) to colony diameter (mm) was selected.

3. Determination of NaCl concentration on growth and enzyme production

The selected strain was spotted onto mM73 agar medium supplemented with 0, 1, 2, 3, 4 or 5M NaCl and incubated at 37 °C for 10 days. The colonies having both fast growth and high enzyme production were selected based on a ratio of clear zone diameter (mm) to colony diameter (mm). The strain having the highest ratio of clear zone diameter (mm) to colony diameter (mm) at high salt concentration was selected.

4. Screening of highest halophilic protease producing strain in liquid medium

Each of the ten strains obtained as extremely halophilic bacteria was cultured in 250ml Erlenmeyer flasks containing 80 ml M73 (Norberg and Hofsten, 1969) broth medium with 4M NaCl, pH 7.0. Inoculation was performed with 5 % (v/v)

seed culture that had been grown for 3 days in the aforementioned growth medium. The cultures were incubated on a rotary shaker at 200 rpm at 37 °C for 7-10 days. A 5 ml of culture broth was taken out every day and centrifuged at room temperature. Cell free supernatants were used for measuring protease activity. The harvested cells were centrifuged at 6,000xg two times with 4M NaCl solution, and were used for measuring growth. Growth were measured turbidically at 600 nm using a spectrophotometer (U V-1201 Shimadzu, Japan).

5. Assay of the protease activity

The protease activity was assayed using a modified method of Sarath *et al.* (1990). The reaction mixture for assay of protease activity contained 75 µl of a suitably diluted enzyme solution and 125 µl of 2% azocasein in 50mM Tris-HCl buffer, pH 8.0. After incubated for 30 min at 40 °C, the reaction was terminated by adding 600 µl of 10 % (w/v) trichloroacetic acid and then the mixture was centrifuged at 2,500xg in a personal centrifuge minispin (Eppendorf, German) for 10 min to remove the precipitate. After adding 600 µl of the supernatant to 700 µl of 1M NaOH, the absorbance at 440 nm was recorded with a spectrophotometer (UV-1201 Shimadzu, Japan) against a reference tube of control which prepared by mixing enzyme solution, trichloroacetic acid and substrate in that order. One arbitrary unit (AU) of protease activity was defined as the amount of enzyme required to produce an increase in absorbance at 440 nm of 0.1 in a 1 cm cuvette, under the above mentioned assay.

6. Taxonomic study of the selected strain

Identification of the selected strain was determined according to morphological, biochemical, physiological and nutritional characteristics following the standard procedure (Larsen, 1984). Gram stain was performed by using acetic acid-fixed samples as described by Dussault (1955). The utilization of sugar and other compounds as carbon sources and acid production from these compounds were determined in SGC medium modified as follows: the yeast extract and Casamino acid were reduced to 0.25 g/l. Each potential carbon source was added to a final concentration of 5 g/l from a concentrated sterile solution (Oren *et al*., 1995).

RESULTS AND DISCUSSION

1. Isolation and screening of salt-loving protease producing halophilic bacteria

In order to isolate extremely halophilic bacteria which could produce halophilic protease, enrichment cultured of samples from different fish sauce factories in the eastern part of Thailand were plated on a solid medium containing 2, 3 or 4M NaCl. Table 1 shows the source and distribution of all isolates that have ability to grow at different NaCl concentrations added in SGC medium. From a total of 285 isolates, 107, 98 and 80 isolates were grown on SGC medium containing 2, 3 and 4M NaCl concentration, respectively. Significantly in ability to grow at different NaCl concentrations with respect to the sources of the sample were not observed. These results showed that most isolate strains could grow on SGC medium with 2M NaCl more than 3M and 4M NaCl, respectively.

It should be noted that extremely halophilic bacteria required high NaCl concentration for growth. The nutritional requirements of the known species are similar possibly as a consequence of the use of enrichment and isolation conditions: they commonly grow in rich media containing amino acid and yeast extract (Oren, 2002).

All of 285 isolates were examined for their ability to produce protease as described in materials and methods. Estimation of the intensities of enzyme activity was based on a ratio of clear zone diameter to colony diameter. Interestingly, almost 12 % of the isolate were found to secrete protease

Table 1 Sampling places and distribution of halophilic bacteria isolated from fish sauce samples in the eastern part of Thailand.

Sampling [a] Places	Numbers of halophilic bacteria isolated on SGC medium at various NaCl concentrations			Total (isolates)
	2M NaCl	3M NaCl	4M NaCl	
1.	19	27	34	80
2.	16	9	14	39
3.	32	27	21	80
4.	30	23	9	62
5.	10	12	2	24
Total (isolates)	107	98	80	285

a: 1. Pichai Fish Sauce Co. Ltd, Chon Buri
2. Dumrongsin Fish Sauce Factory, Chantaburi
3. Sang-authai Fish Sauce Factory,Trat
4. Pornpimol Fish Sauce Factory,Trat
5. Homemade Fish Sauce, Trat

in the medium with 2-4M (12–24 %, w/v) NaCl added (Table 2). Among these 34 isolates, 16, 8 and 10 isolates could grow on SGC with 2, 3 and 4M NaCl, respectively as shown in Table 2. Therefore, the ones possessing high salt-loving protease activity were selected.

2. Effect of NaCl concentration on growth and enzyme production

The effects of NaCl concentration on growth and enzyme production were determined by selecting 24 high protease producing isolates from Table2. On the basis of growth and protease production, the isolates were divided into three groups as shown in Table 3. The first group (PB201, PB202, PB211, PB213, PB214 and PB215) could grow on mM73 medium with 1 to 4M NaCl whereas group II (PB301, PB302, PB303, PB304, PB305, PB306, PB307, PB308) required 1, 2 and 3M NaCl. On the other hand, the group III (PB401, PB402, PB403, PB404, PB405, PB406, PB407, PB408, PB409, and PB410) could grow only in 3-5M NaCl.

In addition, protease production was further investigated. The results indicated that group I produced protease in the media containing only 2-3 M NaCl, group II 1-3 M NaCl and group III only 4-5 M NaCl. All of these isolates grew on media containing between 1-4 M total salts, Thus, they could be classified as halophilic bacteria according to the classification proposed by Kushner (1985).

It was interesting that all isolates in group III had ability to grow at 3-5M NaCl but a salt-loving protease activity occurred only at 4-5M NaCl on solid medium (Table 3). Among these isolates, the strain PB407 had the highest value of the ratio of colony size to clear zone size, indicating that it exhibited the highest protease ability and excreted extracellular protease that displayed a remarkable salt-loving protease with highest activity at 4M NaCl on mM73 agar medium as shown in Figure1.

The data presented here clearly demonstrated that all of ten isolates in group III having colonies on solid media that were pink to redish pink (Figure 1). Since all isolates had salt requirements for growth above 3M (18 %, w/v) NaCl, they exhibited similar characteristics to

Table 2 Screening of protease producing strains on mM73 agar medium supplemented with 2, 3 and 4M NaCl.

NaCl (M)	Isolated No.	Clear zone (A) (mm)	Colony size (B) (mm)	A/B ratio(mm/mm)
2	PB201	23.80	8.60	2.77
	PB202	30.00	8.40	3.57
	PB203	21.20	7.91	2.68
	PB204	23.20	11.40	2.04
	PB205	12.10	9.90	1.22
	PB206	22.10	12.40	1.78
	PB207	25.05	19.30	1.29
	PB208	11.80	10.50	1.12
	PB209	22.80	15.10	1.51
	PB210	13.70	8.25	1.66
	PB211	17.30	4.40	3.93
	PB212	22.90	11.10	2.08
	PB213	14.70	10.00	2.52
	PB214	16.40	4.30	3.00
	PB215	10.55	4.30	3.41
	PB216	16.40	10.50	1.56
3	PB301	10.55	4.60	2.28
	PB302	13.60	4.30	3.16
	PB303	14.10	5.10	2.76
	PB304	22.10	12.40	1.78
	PB305	23.20	10.94	2.12
	PB306	10.50	4.71	2.23
	PB307	13.10	6.04	2.17
	PB308	14.10	4.91	2.87
4	PB401	15.00	6.25	2.40
	PB402	13.60	4.34	3.13
	PB403	13.10	5.70	2.35
	PB404	10.50	4.75	2.21
	PB405	23.4	6.05	3.86
	PB406	26.9	7.9	3.41
	PB407	28.10	7.11	3.95
	PB408	15.87	6.18	2.57
	PB409	16.30	4.55	3.58
	PB410	14.90	8.18	1.82

Table 3 Effects of sodium chloride concentration on growth and protease production of selected strains cultured on mM73 at 37°c for 10 days.

Isolate no.	Ratio of clear zone diameter (mm) to colony diameter (mm)					
	0M NaCl	1M NaCl	2M NaCl	3M NaCl	4M NaCl	5M NaCl
PB201	-	1.54	2.85	2.99	-	-
PB202	-	5.83	4.59	1.64	-	-
PB211	-	-	2.98	1.95	-	-
PB213	-	-	4.35	2.65	-	-
PB214	-	-	3.75	2.39	-	-
PB215	-	-	2.61	2.04	-	-
PB301	-	4.58	4.04	2.28	-	-
PB302	-	2.02	3.35	2.49	-	-
PB303	-	2.34	2.59	3.24	-	-
PB304	-	2.86	4.51	1.41	-	-
PB305	-	3.79	3.53	1.38	-	-
PB306	-	3.96	4.88	1.77	-	-
PB307	-	2.97	6.58	1.77	-	-
PB308	-	2.17	6.98	-	-	-
PB401	-	-	-	-	2.62	2.09
PB402	-	-	-	-	3.40	2.44
PB403	-	-	-	-	2.81	1.63
PB404	-	-	-	-	2.83	-
PB405	-	-	-	-	4.10	1.53
PB406	-	-	-	-	3.58	1.92
PB407	-	-	-	-	3.84	1.88
PB408	-	-	-	-	2.84	2.36
PB409	-	-	-	-	3.77	2.35
PB410	-	-	-	-	2.63	1.68

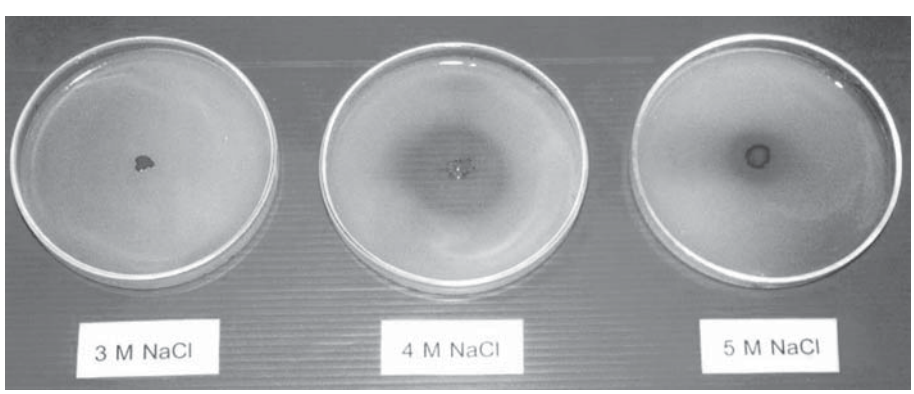

Figure 1 Growth and salt-loving protease production of the strain PB407 on mM73 solid medium with 3,4 and 5 M NaCl incubated at 37 °C for 7 days.

extreme halophiles as reported by Grant and Larsen (1990). Moreover, the characteristics of proteolytic enzymes from this group were similar to proteolytic enzymes from the extremely halophilic bacteria (*Halobacterium* sp.) (Norberg and Hofsten, 1969; Kim and Dordick, 1997). Therefore, the bacteria of this group were selected for characterization in the next experiment.

3. Screening of salt-loving protease producing strain in liquid medium

Ten strains (PB401, PB402, PB403, PB404, PB405, PB406, PB407, PB408, PB409 and PB410) of extremely halophilic bacteria which had an interesting salt-loving protease activity on solid medium were further screened for salt-loving protease activity in liquid medium. All of them were grown at 37 °C in M73 liquid medium on rotary shaker at 200 rpm. The extremely halophilic bacterium strain PB407 also showed the highest salt-loving protease activity as on mM73 solid medium (Figure 2).

Time course production of salt-loving protease and growth of this strain is shown in Figure 3. Salt-loving protease production by this strain was observed after 2 days of incubation, as the culture became slightly red in color. Growth and protease production increased to maximum after 5 days of incubation, followed by a decrease in both growth and enzyme production similar to the report of proteolytic enzyme from *Halobacteium salinarium* (Norberg and Hofsten, 1969; Kamekura and Seno, 1990).

4. Taxonomic studies of a selected strain

The cultural characteristics of the strain PB407 are listed in Table 4. When this strain was grown in the SGC medium, it was rod shaped and Gram negative. Colonies on agar plate were circular with red pigmentation as shown in Figure1. The strain grew aerobically in liquid SGC medium and required high salt concentration for growth. The range of salt concentrations, pH and temperature for growth were 17.5–25 % NaCl, 5-8 and 30-45 ° C, respectively.

The biochemical characteristics of this

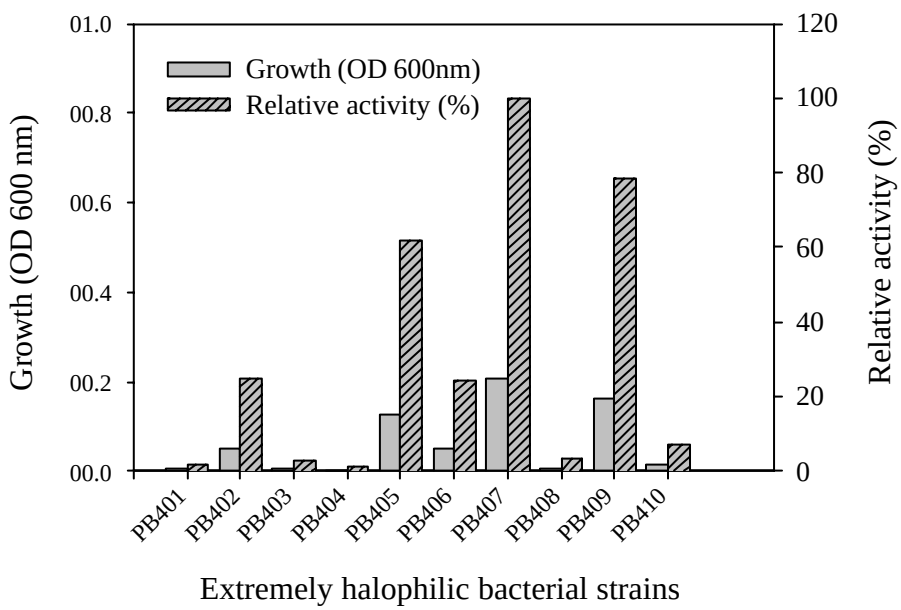

Figure 2 Screening of extremely halophilic bacterial strain producing salt-loving protease in M73 liquid medium pH 7.0 incubated on rotary shaker at 200 rpm at 37 °C for days.

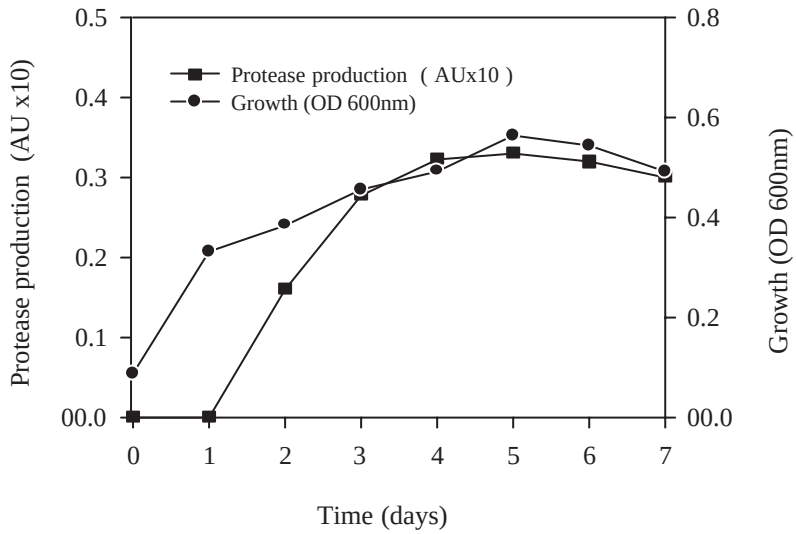

Figure 3 Time course of growth and salt-loving protease production by the strain PB407 in M73 liquid medium pH 7.0.

Table 4 Cultural characteristics of the strain PB407.

Characteristics	Strain PB407
Gram stain	negative
Pigmentation	red
Shape	rod
Cultural characteristics:	
Growth at :	
30, 37 ,45 °C	+
50 °C	-
Growth at pH :	
3, 4	-
5, 6, 7, 8	+
9, 10	-
Growth at NaCl concentrations (%)	
0, 2.5, 5.0, 7.5, 10.0,12.5,15.0	-
17.5, 20.0, 22.5, 25.0	+

Symbols: +, positive; -, negative

strain (PB407) were also investigated, as shown in Table5. It was aerobic and exhibited positive oxidase and catalase reactions, and gelatin liquefaction. It did not grow in any carbon sources listed in Table 5.

Therefore, the bacterial strain PB407 was selected for further studies since it appeared to be the best extracellular protease producer. It had an optimum growth at 17.5-25 %, w/v NaCl and did not grow without NaCl. Based on the definition of Kushner (1985), strain PB407 could be classified as extremely halophilic bacteria. According to

Table 5 Biochemical characteristics of the strain PB407.

Biochemical tests	Strain PB407
Catalase test	+
Oxidase test	+
Gelatin hydrolysis	+
Starch hydrolysis	-
H_2S production	+
Indole production	+
Growth factors requirement	+
Carbohydrate fermentation	
Glucose	-
Galactose	-
Mannose	-
Fructose	-
Ribose	-
Maltose	-
Lactose	-
Sucrose	-
Glycerol	-

Symbols: +, positive; -, negative

morphological, physiological and biochemical characteristics, the strain PB407 was closely related to the species of the genus *Halobacterium* (Grant and Larsen, 1990).

CONCLUSIONS

Totally, 10 of 285 isolates from samples of various fish sauce factories in the eastern part of Thailand exhibited high salt-loving protease activity. All of these strains were secondary screened for salt-loving protease production in M73 liquid medium. The strain PB407 produced the strongest salt-loving protease in liquid medium as well as in the solid medium. Interestingly, it had ability to grow and produce salt-loving protease at 4-5 M NaCl. These facts led to the consideration of using this bacterial strain in production of fish sauce to accelerate the proteolysis and shorten fermentation period. From the investigation of morphological, physiological and biochemical characteristics, it was concluded that, the strain PB407 belonged to the genus *Halobacterium.*

These studies demonstrated that halophilic protease from *Halobacterium* sp. PB407 was a good candidate for application on the biotechnology of fish sauce fermentations. However, further research is needed, including investigation of the in-dept characterizations of this halophilic protease, identification of the strain by using the modern techniques and evaluation of possibility to use this protease in fish sauce fermentation.

ACKNOWLEDGMENTS

We would like to express our thanks to the fish sauce factories: Pichai Fish Sauce Co. Ltd, Chon Buri; Dumrongsin Fish Sauce Factory, Chantaburi; Sang-authai Fish Sauce Factory, Trat ; Pornpimol Fish Sauce Factory, Trat and the Homemade Fish Sauce in Trat for their provided fish sauce samples. This research was partially supported by the Graduate School of Kasetsart University.

LITERATURE CITED

Beddow, C.G. 1998. Fermented fish and fish products, pp. 416-440. *In* B.J.B. Wood (ed.). **Microbiology of Fermented Food, vol.2**. Elsevier Applied Science Publishers. London.

Beddow, C.G. and A.G. Ardeshir. 1979. The production of soluble fish protein solution for use in fish sauce manufacture. I. The use of added enzymes. **J. Food. Technol.** 14 : 603-612.

Dussault, H.P. 1955. An improved technique for staining red halophilic bacteria. **J. Bacteriol.** 70: 484-485.

Grant, W.D. and H. Larsen. 1990. Extremely halophilic archaebacteria order Halobacteriales odr.nov., pp.2216-2233. *In*

J.T. Staley, M.P. Bryant, N. Pfenning and J.G. Holt, (eds.). **Bergey' s Manual of Systematic Bacteria, vol. 3**. Williams and Wilkins, Baltimore, Md.

Izotova, L.S., A.V. Strongig, L.N. Chekulaeva, V.E. Sterkin, V.I. Ostoslavskaya, L.A. Lyubeinskaya, E.A. Timokhina and V.M. Stepanov. 1983. Purification and properties of serine protease from *Halobacterium halobium*. **J. Bacteriol.** 155: 826-830.

Kamekura, M. and Y Seno. 1990. A halophilic extracellular potease from a halophilic archaebacterium strain 172 P1**. Biochem. Cell Biol.** 68: 352-359.

Kim, J.and J.S Dordick. 1997. Unusual salt and solvent dependence of a protease from an extreme halophile. **Biotechnol. Bioeng.** 23: 1219-1236.

Kushner, D.J. 1985. The Halobacteriacea, pp.171-214. *In* C.R. Woese and R.S. Wolfe (eds.). **The Bacteria, vol. 8**. Academic Press, London.

Kushner, D.J. and M. Kamekura. 1988. Physiology of halophilic eubacteria, pp.109-138. *In* F.R. Valeva (ed.). **Halophilic Bacteria, vol.1**. CRC Press, Inc., Florida.

Lanyi,J.K. 1974. Salt dependent properties of proteins from extremely halophilic bacteria. **Bacteriol. Rev.** 38: 272-290.

Larsen, H. 1984. Family Halobactericea, pp261-267. *In* N.R. Krieg and J.G. Holt (eds.). **Bergey's Manual of Systematic Bacteriology, vol.1**. Williams and Wilkins, Baltimore

Norberg, P. and B.V. Hofsten. 1969. Proteolytic enzymes from extremely halophilic bacteria. **J. Gen. Microbiol**. 55: 251-256.

Oren, A., P. Gurevich, R. T.Gemmell and A. Teske. 1995. *Halobaculum gomorrese* gen. Nov., a Novel Extremely Halophilic Archaeon from the Dead Sea. **International Journal of Systematic Bacteriology** 45(4): 747-754.

Oren, A. 2002. Diversity of halophilic microorganisms: Environments, phylogeny, physiology, and applications. **J. Indust. Microbiol. and Biotech**. 28: 56-63.

Raksakulthai, N. and N.E. Haard. 1992. Correlation between the concentration of peptides and the flavor of fish sauce. **ASEN J. Food Sci. Technol.** 7: 86-90.

Saisithi, P. 1994. Traditional fermented fish: fish sauce production, pp. 111-131. *In* A.M. Martin (ed.). **Fisheries Processing: Biotechnological Application**. Chapman and Hall, London.

Sarath, G., R.S.de la Motte and F.W. Wagner. 1990. Protease assay methods, pp 25-54. *In* R.J. Beynon (ed.). Proteolytic Enzymes: A Practical Approach**. IRL Press, Oxford.**

Sehgal, S.N. and N.E. Gibbons. 1960. Effect of metal ion on the growth of *Holobacterium cutrilubrum*. **Can. J. Microbiol**. 6:165-169.

Thongthai, C., T. J. McGenity, P. Suntinanalert and W.D. Grant. 1992. Isolation and characterization of an extremely halophilic archaeobacterium from traditionally fermented Thai fish sauce (Nampla). **Lett. Appl. Microbiol**. 14: 111-114.

Ventosa, A., J.J. Nieto and A. Oren. 1998. Biology of moderately halophilic aerobic bacteria. **Microbiol. Mol. Biol. Rev**. 62: 504-544.