

CHARACTERIZATION OF PROTEASE-PRODUCING BACTERIA ISOLATED FROM TERASI

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ABSTRACT

Total of 117 bacterial strains were isolated from terasi samples and 69% of isolates (71) could perform distinctive proteolytic activity that related to the ability to produce protease enzymes. Their proteolytic activity was further tested using spot incubation technique. Strain S4-5 has shown the highest activity then was selected for further tests in this study. Gram staining test showed that S4-5 is gram positive bacteria and able to grow under anaerobic condition. Based on API biochemical profiles, S4-5 strain bacteria was *Bacillus licheniformis*. Similarity test of genome sequence among *Bacillus* species from gene bank (EMBL Sequence Version) with *Bacillus* spp., strain S4-5 had similarity with *Bacillus licheniformis* genome. The optimal pH of this strain was 6 whereas the optimum temperature for *Bacillus licheniformis* strain S4-5 was 37°C.

Keywords: *Bacillus licheniformis* strain S4-5, protease, terasi, fermented fish

INTRODUCTION

Protease is a group of enzymes that has catalytic function to breakdown proteins. It is unique and naturally occurs in cellular, organ, and organism levels. Protease enzyme is one of the major industrial enzymes that widely used in food, detergent, leather, biochemical, and pharmaceutical industries (Rao *et al.*, 1998). Protease can be derived from various sources such as plants, animals, and microorganisms. Eight thousand tons of protease have been produced by bacteria and fungi each year (Bains, 1998). Microbial proteases sale is approximately 40% from the whole protease sales. Microbial source is preferred than plant and animal sources because it possess almost all desired characteristics for biotechnological applications. Microorganisms represent excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation (Godfrey and West, 1996).

Terasi is fermented solid paste condiment made from shrimp and or fish that usually fermented naturally in high salt condition. In Indonesia, *terasi* is manufactured by local people in small-scale industrial level along the seashore. It is used as an important ingredient in many Indonesian cuisines. However, the information about microorganisms contained in *terasi* especially the protease-producing bacteria is limited. Previously, microflora and their enzyme profile contained in *terasi* starter collected from Bagan Siapi-api in North Sumatra has been reported by Suro and Hosono (1994).

Extracellular protease of *Bacillus subtilis* strain FP-133 has been purified and characterized from *terasi* by Setyorini *et al.* (2006). Halophilic lactic acid bacteria from *terasi* were also has been isolated and characterized by Kobayashi *et al.* (2003). Microbial diversity and enzyme profile from *terasi* resemble products such as *hentak* and *tungtap* from India, *ka-pi* and *pla-ra* from Thailand; and another form of fermented shrimp or fish such as *momoni* from Ghana, *jeot-gal* from Korea had been observed. Lactic acid bacteria found in fermented fish and shrimp in Thailand (Tanasupawat *et al.*, 1998; Sanni *et al.*, 2002; Thapa *et al.*, 2004; Deejing *et al.*, 2005; Ju *et al.*, 2007).

This study was aim to screen and isolate bacterial strains that capable to produce protease in *terasi*. Bacterial isolates that produce the widest clear zone were selected for further test. Further tests were cell morphology and colony characterization, biochemical properties, and molecular taxonomy. Additionally, optimum temperature and pH for cell growth were also investigated.

METHODS

Terasi Samples

Terasi samples were collected from traditional market in three areas in Java Island: Cirebon (northern of West Jawa) for Sample 1 and Sample 2, Malang (middle of East Jawa) for Sample 3, and Tuban (northern of East Jawa) for Sample 4.

Screening and Isolation of Protease-Producing Bacteria from *Terasi*

Terasi sample (10.0 g) was suspended into plastic bag contains 90 ml of sterile distilled water then supplemented with 0.1% (w/v) peptone then homogenized. Diluted sample was put on stomacher (MAYO, Italy) for 2-3 minutes. Series dilution was performed in

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0.1 ml of aliquots with concentration between 10^{-3} - 10^{-6} . Each solution was spread triplicate on casein agar using spread technique. The plates were incubated at 37°C overnight. Representative colonies which produced clear zone then retrieved from plate and picked up randomly to be stroke on nutrient agar plate until the pure cultures were obtained.

All bacterial isolates were tested for their proteolytic activity using spot technique by transferring a bacterial single colony onto casein agar plate which then incubated at 37°C for 24h. Two strains which have the highest ratio of clear zone were observed for further tests in this study.

Morphological Characterization of Bacteria

Morphological characterizations of colony and cell were observed such as configuration, margin, elevations, surface, and pigments for colony; microscopic shape, size, arrangement, and presence of spore or cell (Baghel *et al.*, 2005).

Biochemical Properties of Bacteria

Physiological and biochemical properties were included on Gram-staining, malachite green staining, oxygen requirement (aerobic or anaerobic), catalase test, growth ability in 5 and 7% NaCl, MR-VP test, citrate utilization, starch hydrolysis Nitrate reduction, acid and gas from glucose, acid from mannitol, formation of indole, egg-yolk lechitinase, also growth in NB 50 and 65°C (Norris *et al.*, 1981; Sneath, 1986).

Microbial Identification

Identification using API kit 50 CH (bioMerieux, Inc.) was initialized by culturing full plate of single colony on nutrient agar which then incubated at 37°C for 24h. The culture was picked up using loop and resuspended in 3 ml of 0.85% NaCl, mixed using vortex until become more dense. This suspension was added into ampule of API medium and prepared a suspension with turbidity that equivalent to 2 McFarland (Appendix), then mixed well and dropped into API strips. After sterile mineral oil addition, the API strips was then incubated at 30°C for 3 days, and the results were recorded at 0h, 24h, and 48 h after incubation.

Bacterial strain was identified using molecular taxonomy. We amplified bacterium 16S rRNA gene and determined their sequence. The sequences were then compared to known bacteria sequences in GenBank. Initially, DNA isolate was extracted and its concentration was measured through PCR amplification of 16Sr RNA gene. PCR was done by mixing the chromosomal DNA with master mix (containing all dNTP's, Mg^{++} , BSA, primer) then initiated by the addition of taq polymerase. After amplification, PCR products were examined with agarose gel electrophoresis then purified for sequence reaction. Purified PCR products were submitted to DNA Core Facility for sequencing. Sequencing results then uploaded into the GenBank.

pH Effect on Bacterial Growth

Single colony of pure culture was inoculated in test tube contained nutrient broth supplemented with 0.05% (w/v) casein then incubated at 37°C overnight in shaker with 150 rpm of rotation. 200 µl of fresh culture was then transferred into 250 ml Erlenmeyer flasks contained 100 ml of nutrient broth supplemented with 0.1% (w/v) casein in various pH (pH 5, pH 6, pH 7, pH 8, and pH 9) respectively. pH 5 was prepared using 0.2 M sodium acetate-acetic acid diluted in 100 ml dd water, while pH 6, 7, and 8 were prepared using 0.2 M Na_2HPO_4 - NaH_2PO_4 , and pH 9 was prepared using 0.2 M glycine - NaOH pH medium was adjusted using different buffer before sterilization. Batch fermentation cultures were incubated at 37°C for 3 days in incubator shaker with 150 rpm of rotation.

Samples were taken every 12h. Cell density was observed immediately after culture suspensions were taken from batch fermentation in duplicate and shaken well. Sample then diluted and its absorbance was read using spectrophotometer at 600 nm. 1.5 ml of cell suspensions were transferred into micro-tube for crude enzyme test preparation.

Effect of Temperature on Bacterial Growth

The effect of temperature on microbial growth and enzyme production were measured by incubated bacterial cultures at different temperatures: room temperature (22 - 28°C), 37°C, 45°C, 52°C, and 60°C. Broth medium was adjusted in the optimum pH according to the previous tests (Effect of pH on Bacterial Growth) before bacterial cell inoculation.

Samples were taken every 12h. Cell density was observed immediately after culture suspensions were taken from batch fermentation in duplicate and shaken well. Sample then diluted and its absorbance was read using spectrophotometer at 600 nm. 1.5 ml of culture suspensions were transferred into micro-tube for crude enzyme test preparation.

RESULTS

Preliminary Screening of Protease-Producing Bacteria from Terasi

Four *terasi* samples which collected from three areas in Java Island were screened for their protease-producing bacteria content. Proteolytic activity was determined by the presence of clear zone surround bacteria colonies. Total of 117 bacterial strains were isolated from *terasi* samples and 71 isolates (69%) could produce distinct clear zone as relative index (Table 1).

The relative index of enzyme activity was calculated from the ratio clear zone diameter. However, it was also represent the different proteolytic activity with broad range of relative index (1.04 - 3.13). The distribution and proportion of protease producing bacteria isolated from *terasi* based on their relative index of enzyme activity are shown in Table 2.

Table 1. The distribution of bacterial isolates retrieved from four *terasi* samples

Sample	Location	Total Strain	Protease-Producing Bacteria (%)
1	Branded (Mamasuka)	26	15 (58)
2	Cirebon – West Jawa	32	19 (59)
3	Malang - Cooking <i>terasi</i> -	24	12 (50)
4	Tuban – East Jawa	35	25 (71)

Table 2. Distribution and proportion of protease producing bacteria isolated from *terasi* based on relative index of enzyme activity.

Relative index	Number of isolates	Proportion (%)
0.0 - 1.00	0	0
1.01 - 1.50	42	59
1.51 - 2.00	12	17
2.01 - 2.50	13	18
> 2.50	4	4

Protease-producing bacteria which performed the widest clear zone was selected as indicator. Each strain which purified was further tested using spot incubation technique. The majority of isolated bacteria (42 isolates or ~59%) have enzyme activity relative index between 1.01 and 1.50. S4-5 isolate exhibited highest proteolytic activity which represented by the highest enzyme activity relative index (2.44). This isolate was selected based on these reasons: (1) it has the highest proteolytic activity and (2) the samples were isolated from *terasi* that produced traditionally (fermented naturally).

Table 3. Characterization of morphology colony and cell of S4-5 strains, isolated from *terasi*

Morphological Observation of S4-5	
Morphology of colony	
Configuration	Round
Margin	Undulate
Elevations	Raised
Surface	Rough
Pigments	Cream
Morphology of cell	
Shape	Rod
Size	Long
Arrangement	Single
Presence of Spore	
Position	Central
Shape	Oval

Table 5. Comparison of similarity of 16S rRNA sequence among *Bacillus* species from GenBank (EMBL Sequence Version) with *Bacillus* sp. strain S4-5.

Species	% Similarity (16S rRNA)
<i>Bacillus licheniformis</i>	99.48
<i>Bacillus amyloliquefaciens</i>	99.19
<i>Bacillus subtilis</i> strain M-111	98.23
<i>Bacillus subtilis</i> strain CICC10149	98.23
<i>Bacillus subtilis</i> strain QD9	98.43
<i>Bacillus subtilis</i> strain CICC9011	98.16

Molecular Taxonomy

DNA extraction, PCR, and 16S rRNA gene sequencing of *Bacillus* sp strain S4-5 were conducted at Laboratory of Microbiology of Maejo University-Chiang Mai, Thailand. The comparison of genome sequence (16S rRNA) similarity was performed among *Bacillus* species from GenBank EMBL Sequence Version

Morphological Characterization of Bacteria

S4-5 strain characters were observed based on their colony and cell morphology (Table 3.). S4-5 strain colony morphological characteristics were round configuration, undulate margin, raised elevations, rough surface, and cream pigments. While, Gram-staining of S4-5 strain cells were rod shape, long, and soliter. Malachite green staining showed the presence of spore (Figure 1).

Table 4. Biochemical characteristics of bacteria S4-5 strain.

Characteristic	S4-5	<i>B. licheniformis</i> *
Gram	+	+
Presence of spore	+	+
Catalase	+	+
Voges Proskauer	+	+
Methyl red	-	-
Starch hydrolysis	+	+
Citrate utilization	+	+
Nitrate reduction	+	+
Anaerobic growth	+	+
Growth in 5% NaCl	+	+
Growth in 7% NaCl	+	+
Acid from glucose	+	+
Gas in glucose	-	-
Acid from mannitol	+	+
Formation of indole	-	-
Egg-yolk lechitinase NB	-	-
50°C	+	+
NB 65°C	-	-
API 50 CH kit	<i>B. licheniformis</i>	
Molecular taxonomy	<i>B. licheniformis</i>	

Note: * These characteristics are derived from Sneath (1986) (Bergey's Manual of Systematic Bacteriology).

(<http://www.ebi.ac.uk/Tools/blast2/nucleotide>) with *Bacillus* sp. S4-5 strain. The comparison results show that *Bacillus* sp. S4-5 strain strain was similarity to *Bacillus licheniformis* (Table 5). These results were also supported by biochemical assays that performed before. The sequence data that submitted to NCBI as FJ 317160 for *Bacillus* spp. S4-5 strain.

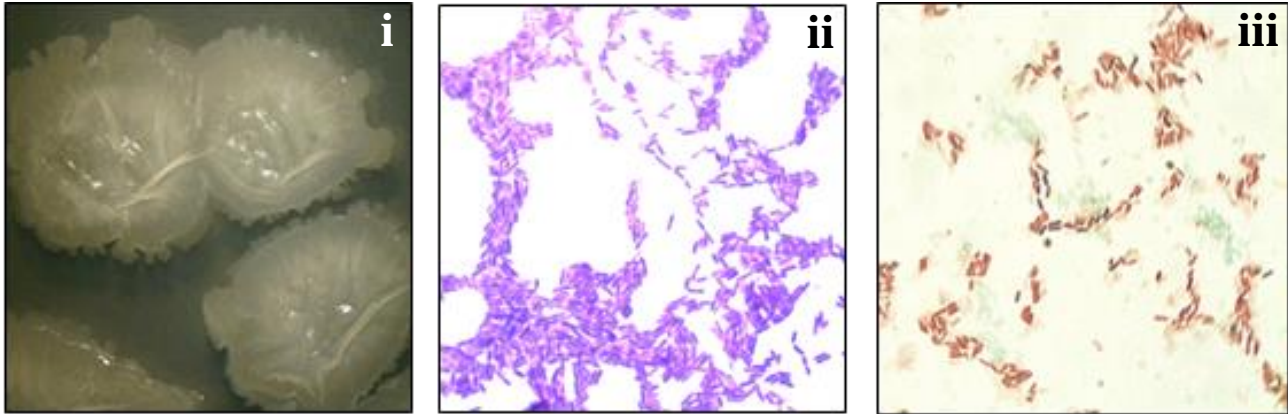


Figure 1. Morphology of S4-5 strain. Colony Morphology(i), Cell morphology by Gram-staining (ii), and Presence of spore (malachite green staining) (iii).

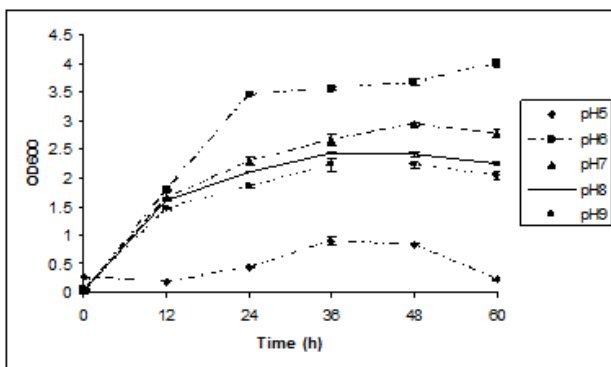


Figure 2. Cell density of *B. licheniformis* S4-5 strain at various pH (incubated at 37°C) for 60h incubation of cultured broth (OD₆₀₀).

Optimum pH for cell growth

Figure 2. shows cell density of *Bacillus licheniformis* S4-5 strain at various pH. The optimum pH was 6. The log phase started in 12 h after incubation and kept continuing until 60 h. The doubling time was about 8.5 h specific growth rate 0.42 h⁻¹ at pH 6. In general, the pattern *Bacillus* sp. S4-5 strain growth on pH change was quite similar with initial pH (7,8 and 9) until 12 h and 60 h of incubation. The cell density was tend to increase until 36 h but did not change much and decreased after 36 h of incubation. The lowest cell growth rate of *Bacillus* sp. S4-5 strain was at pH 5, it grew very slow until 24 h of incubation, then underwent on log phase between 24 h-36 h of incubation then tend to decreased.

DISCUSSION

Four samples that observed in this study had different microbial contents. The microbial contents of *terasi* may differ depend on the geographic condition where *terasi* samples obtained, raw materials quality, and manufacturing methods. Microflora content of *terasi* originated from Bagansiapi-api in North Sumatra, Indonesia has been observed by Surono and Hosono (1994) that composed by *Bacillus brevis*, *Bacillus pumilus*, *Bacillus magetarium*, *Bacillus coagulans*, *Bacillus subtilis*, and *Micrococcus kristinae* with

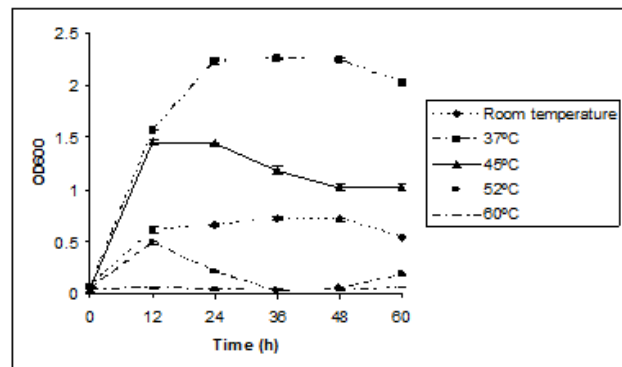


Figure 3. Cell density of *B. licheniformis* S4-5 strain (B) at various temperature (pH 6) for 60h incubation of cultured broth (OD₆₀₀).

proportion 39.1%, 26.1%, 8.7%, 8.7%, 8.7%, and 8.7% respectively. One species that also found was *Sporolactobacillus*. The dominant microflora in that sample was *Bacillus* sp. that classified as halophilic, aerobic, and able to grow in temperature range 10-50°C.

The endospore-forming rods were identified as *Bacillus subtilis* and *Bacillus pumilus*, aerobic cocal bacteria were identified as *Micrococcus*, yeasts were identified as species of *Candida* and *Saccharomycopsis*. Pathogenic contaminants were detected in *hentak* and *tungtap* samples in few amounts (Thapa *et al.*, 2004). Total of 67 microbial strains were isolated from *momoni*. These strains identified as *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Pediococcus*, *Staphylococcus*, *Klebsiella*, *Debaryomyces*, *Hansenula* and *Aspergillus*. *Bacillus* was dominantly found in *momoni* with 37.7% of percentage (Sanni *et al.*, 2002). Four strains of *Bacillus* species that isolated from *ka-pi* and *pla-ra* have high protease activity (Deejing *et al.*, 2005). Ju *et al.* (2007) isolated 188 strains from *jeot-gal*. It has been also screened sixteen strains that showed strong fibrinolytic activities that performed best by *Bacillus licheniformis*.

Preliminary results showed that of 171 strains tested with 169 isolates (99%) could produce protease enzymes. It may be because *terasi* samples were in dry solid paste form and stored for long time before been used as sample for bacterial isolation. This moisture condition was not suitable for microbial growth.

We found that S4-5 strains was Gram-positive and able to grow under anaerobic condition. Based on the API test result and supported by molecular taxonomy of *Bacillus* sp. S4-5 strain was identified as *Bacillus licheniformis*. The discovery of *Bacillus licheniformis* in *terasi* exhibited high proteolytic activity that could be expected to be utilized as pure culture in food industry. Surono and Hosono (1994) found that all of the bacteria in *terasi* released fat hydrolysis enzymes by the presence of esterase (C4) and esterase lipase (C8) activities. Enzyme activity will produce low-molecular-weight fatty acids that are responsible for the cheesy odor.

B. licheniformis is bacteria that can be easily found everywhere because of its importance in environment. This bacteria is the contributor to nature nutrient cycles due to its ability on protease production and amylase enzymes. *B. licheniformis* has been used in the fermentation industries for over decade for production of proteases, amylases, antibiotics, or chemicals. However, recently *B. licheniformis* usage in food industry became dispute. Toxin-producing isolates of *B. licheniformis* were obtained from foods that suspected got involved in food poisoning incidents such as in raw milk and industrially produced baby food. It has been observed that it could inhibit sperm motility, damaged cell membrane integrity, and depleted cellular ATP.

The presence of pathogenic bacteria in *terasi* samples has been expected as contaminants which infected during *terasi* production. However, the contain of pathogenic contaminants of *terasi* in this study it was not observed. It has been reported that pathogenic contaminants were detected in all samples of fermented fish products in North-East India such as *ngari*, *hentak*, and *tungtap*. However, none of those samples contained more than 10^2 CFU/g of *Bacillus cereus*, 10^3 CFU/g of *Staphylococcus aureus*, and enterobacteriaceae population, respectively (Thapa *et al.*, 2004).

Lactic acid bacteria from *terasi* have been isolated and characterized with 104 to 106 cfu/g of counted viable cells on MRS medium. (Kobayashi *et al.*, 2003). All isolates were catalase-negative, gram-positive cocci and able to grow in 15% NaCl. Numerical phenotypic analysis showed that the isolates were clustered into one group. However, they could be classified into two species (*Tetragenococcus halophilus* and *T. Muriaticus*) that revealed by restriction fragment length polymorphism (RFLP) analysis and 16S rRNA gene sequencing. This study is the first to show that both these species of *Tetragenococcus* are distributed in Indonesian fermented foods.

B. licheniformis S4-5 strain could grow in temperature up to 60°C with a broad pH range of 5-9. The optimum pH of *B. licheniformis* S4-5 strain was 6, whereas the optimum temperature was 37°C. The activity of proteases were necessary for bacteria survival because proteases hydrolyze protein to amino acids in the medium and provide energy for bacteria to grow. The growth of microbial population involves cellular macromolecules role that lead to mass increase and asexual cell division.

All nutrients need for microbial growth must available in the environment. The environmental conditions

such as temperature and pH must be in optimum level. The potential inhibitors must be below critical levels. Microbial growth is the result of regulated cell multiplication, enzyme-catalyzed reactions involving biosynthesis of small molecule precursor, the transduction of energy, and the production of DNA, RNA, proteins, and polysaccharides (Hui and Khachatourians, 1995). The effect of environmental conditions on the production of extracellular proteolytic enzymes could play an important role in the induction or repression of enzyme production by specific compounds (Secades and Guijarro, 1999).

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REFERENCES

- Bains, W. (1998) *Biotechnology from A to Z* (2nd edition). Oxford University Press, pp. 146-314.
- Baghel, V.S., Tripathi, R.D., Ramteke, O.W., Gopal, K., Dwivedi, S., Jain, R.K., Rai, U.N. & Singh, S.N. (2005). "Psychotropic proteolytic bacteria from cold environment of Gongotri glacier, Western Himalaya, India" *Enzyme and Microbial Technology* 36, pp. 654-659.
- Chukeatirote, E., Chainun, C., Siengsubchart, A., Moukamnerd, C., Chantawannakul, P., Lumyong, S., Boontim, N. & Thakang, P. (2006). "Microbiology and biochemical changes in thua nao fermentation" *Research Journal of Microbiology* 1, pp. 38-44.
- Deejing, S., Moriguchi, M., Poosaran, N., Chairote, G. and Lumyong, S. (2005). Characterization of various proteases from salt-tolerant bacteria isolated from Thai fermented foods. *Chiang Mai Journal of Science* 32, 149-161.
- Godfrey, T. & West, S. (1996). *Industrial enzymology*. 2nd ed. New York: Macmillan Publishers.
- Hui, Y.H. & Khachatourians, G.G. (1995). *Food biotechnology: microorganisms*. New York: Wiley-IEEE.
- Ju, H.K., Choi, K.H., Kim, M.J., Park, C.S. & Cha, J. (2007). "Purification and characterization of a new fibrinolytic enzyme of *Bacillus licheniformis* KJ-31, isolated from Korean Traditional Jeot-ga". *Journal of Microbiology and Biotechnology* 17, pp. 1469-1476.
- Kobayashi, T., Kajiwaru, M., Wahyuni, M., Kitakado, T., Sato, N.H., Imada, C. & Watanabe, E. (2003). "Isolation and characterization of halophilic lactic acid bacteria isolated from "terasi" shrimp paste: A traditional fermented seafood product in Indonesia" *Journal of General and Applied Microbiology* 49, pp. 279-286.
- Noris, J.R., Berkeley, R.C.W., Logan, N.A. & O'Donnel, A.G. (1981). "The genera *Bacillus* and *Sporolactobacillus*" pp. 1711-1742. In Starr, M.P., Stolp, A., Truper, A.G., Balows, A. & Schlegel, H.G. (ed), *The prokaryotes* Vol 2. Germany: Springer-Verlag Berlin.
- Rao, M.B., Tanksale, A.P., Ghatge, M.S. and Deshpande, V.V. (1998) Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.* 62: 597-635.
- Sanni, A.I., Asiedu, M. & Ayernor, G.S. (2002). "Microflora and chemical composition of Momoni, a Ghanaian fermented fish condiment" *Journal of Food Composition and Analysis* 15, pp. 577-583.
- Secades, P. & Guijarro, J.A. (1999). "Purification and characterization of an extracellular protease from the fish pathogen *Yersinia ruckeri* and effect of culture conditions on production." *Applied and Environmental Microbiology* 65, pp.3969-3975.
- Setyorini, E., Takenaka, S., Murakami, S., & Aoki, K. (2006). "Purification and characterization of two novel halotolerant extracellular protease from *Bacillus subtilis*. strain FP-13"

- Bioscience, Biotechnology and Biochemistry 70, pp. 433-440.
- Sneath, P.H.A. (1986). "Section 13. Endospore-forming Gram-positive rods and cocci". In: Bergey's Manual of Systematic Bacteriology, Vol 2, Holt, J.G. ed. London: Williams & Wilkins.
- Surono, I.S. and Hosono, A. (1994) Microflora and their enzyme profile in "Terasi" starter. Biosci. Biotech. Biochem. 58: 1167-1169.
- Tanasupawat, S., Okada, S. & Komagata, K. (1998). "Lactic acid bacteria found in fermented fish in Thailand." Journal of General and Applied Microbiology 44, pp. 193-200.
- Thapa, N., Pal, J. & Tamang, J.P. (2004). "Microbial diversity in ngari, hentak and tungtap, fermented fish products of North-East India." World Journal of Microbiology and Biotechnology 20, pp. 599-607.