

Production and Partial Characterization of Protease from *Staphylococcus* sp. Isolated from the Mud Crab, *Scylla serrata*

Anandkumar¹, Ramanadevi, V.¹, Ajithkumar, T. T.², Lipton, A. P.³ and Thangaraj, M.^{1*}

¹CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamilnadu- 608 502

²National Bureau of Fish Genetic Resources, Lucknow, U.P-226 002

³VRC of CMFRI, Vizhinjam, Thiruvananthapuram, Kerala-695 521

*Corresponding Author E-mail: coralholder@yahoo.com

ABSTRACT

Proteases are generally used in detergents, food industries, leather, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds. The present study was aimed to isolate the protease producing bacteria from the gut of mud crab (Scylla serrata) and optimize the production and find the molecular weight. The potent protease producing bacteria was identified as Staphylococcus sp. by conventional and molecular methods. The optimum condition for protease production in liquid media was: Temperature, 45°C; Salinity, 3% and pH 7.0. The best nutrient source for high protease production in liquid media was Mannitol as carbon source and Potassium nitrate as nitrogen source. The specific activity of crude enzyme and partially purified was 207.03 and 315.69 U/mg respectively. The molecular weight of the protease enzyme produced by the crab gut associated Staphylococcus sp. was determined as 25 KDa.

Keywords: Mud crab, *Scylla serrata*, Gut flora, *Staphylococcus*, Protease.

INTRODUCTION

Symbiotic bacteria in an animal's digestive tract often produce complement enzymes for digestion of plant foods as well as synthesize compounds that are assimilated by the host^{1,2}. However, the scenario of fermentative nutrition in dominant aquatic animal remains poorly understood, and only little information are available regarding the enzymatic microflora of crab intestine. The worth-mentioning investigations were done by Fong and Mann³ in herbivorous sea urchins, Sochard *et al.*⁴ in copepods, Hood *et al.*⁵ in Penaeid shrimps, Hoshino *et al.*⁶ in Japanese coastal fishes, Bairagi *et al.*⁷ and Ray *et al.*⁸ in some fresh water fishes.

Protease is the most important industrial enzyme of interest accounting for about 60% of the total enzyme market in the world and account for approximately 40% of the total worldwide enzyme sale⁹. These enzymes are found in a wide diversity of sources such as plants, animals and microorganisms but they are mainly produced by bacteria and fungi¹⁰. The molecular weight of proteases ranges from 18 -90 kDa¹¹. Microbial proteases are produced from high yielding strains including species of *Bacillus* sp., *Alcaligenes faecalis*, *Pseudomonas fluorescens* and *Aeromonas hydrophilia* grown under submerged culture conditions. Furthermore studies have showed that nutritional, physical factors can significantly influence protease production^{12,13}.

Fish receive bacteria in the digestive tract from the aquatic environment through water and food that are populated with bacteria. Being rich in nutrient, the environment of the digestive tract of fish confers favorable conditions for the microorganisms. Gut micro flora plays an important role in the digestive process, growth and disease resistant of the host¹⁴. Although few reports concerning microbial enzyme production in the gastrointestinal tract of fish are available^{2,7}, information on the distribution of these enzyme-producing endosymbionts in different regions of the gut are scarce¹⁵. In the present study, an

attempt has been made to isolate the protease producing bacteria from the gut of mud crab, *Scylla serrata* and optimize the production of protease and find the molecular weight.

MATERIALS AND METHODS

Bacterial isolation and identification

Live crab samples of *Scylla serrata* were collected from Mudasalodai, Cuddalore district, Tamilnadu. After 24 hours starvation, the crabs were dissected aseptically and digestive tracts were removed carefully. The digestive tracts were thoroughly rinsed several times in sterile 0.9% saline in order to remove non adherent bacteria.

Serial dilutions of up to 10^{-6} were made from this mixture to get different concentration. From all the diluted samples, 0.1 ml was spread into petriplates containing nutrient agar for isolation of total bacterial population. The plates were incubated at 37°C for 24 hours following which the isolated colonies were picked for pure culture by streak plate method.

The isolates were screening for best strain produced protease by plate assay using protease specific medium containing (g/l) K_2HPO_4 2.0, glucose 1.0, peptone 5.0, gelatin 15.0, and agars 15. The clear zone diameters were measured after 24h of incubation at 28°C by flooded the plates with mercuric chloride solution, this method was referred as gelatin clear zone method¹⁶.

Based on the colony morphology and Gram's staining the protease-producing bacterial strain was identified by standard procedures described in Bergy's manual of determinative bacteriology¹⁷. For molecular identification the genomic DNA was extracted from stored specimens by the standard protocol¹⁸. DNA samples from each individual was diluted to 25 ng/ μ l with deionized distilled water and used for PCR amplification. Polymerase chain reaction (PCR) was carried out using mitochondrial primer 16S rRNA gene (*Eubac 27F* 5'- AGAGTTTGATCCTGGCTCAG-3', *1492RA* 5'- GGTTACCTTGTTACGACTT-3'). The amplification reaction was carried out in a 25- μ l reaction volume containing 10 mM Tris -HCL, pH 8.3, 15 mM $MgCl_2$, 100 μ M each of dATP, dCTP, dGTP, and dTTP, 0.2 μ M of primer, 1U of *Taq* polymerase and 25ng of template DNA. The PCR was performed in a thermalcycler (TechGene, India) for 35 cycles consisting of denaturation at 95°C for 1min., annealing at 56°C for 45 seconds, and extension at 72°C for 1.5 min. The final extension was carried out at the same temperature for 5 minute¹⁹. The cleaned up PCR products were sequenced and the raw 16S rRNA gene sequences of the isolate was aligned and edited using BioEdit²⁰.

Optimization of protease production

The protease activity in the liquid medium was assessed by culturing the bacteria in an enrichment medium containing beef extract (0.3%), peptone (0.5%), NaCl (0.5%), and glucose (0.5%) at pH 7.0 for 24h, and then 10% of enriched culture was inoculated in a 250-ml flask containing 45 ml Basal medium containing (g/l): $(NH_4)_2SO_4$ - 2g, K_2HPO_4 - 1g, KH_2PO_4 - 1g, $MgSO_4 \cdot 7H_2O$ - 0.4g, $MnSO_4 \cdot H_2O$ - 0.01g, $FeSO_4 \cdot 7H_2O$ - 0.01g, yeast extract- 1g and peptone- 10g at pH 7.0. The culture was then incubated for 2 days by reciprocal shaking at 32°C. The cells were then harvested by centrifugation at 10,000g for 15 min. and the supernatant was used for further protease assay.

To 0.25 ml culture supernatant, 1.25 ml Tris buffer (100 mM; pH 7.2) and 0.5 ml 1% aqueous casein solution were added. The mixture was incubated for 30 min at 30°C. Then, 3 ml 5% trichloro acetic acid (TCA) was added to this mixture, whereby it formed a precipitate. The mixture was further incubated at 4°C for 10 min, and then centrifuged at 5,000 g for 15 min. Thereafter, 0.5 ml supernatant was taken, to which 2.5 ml of 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. To this mixture, 0.5 ml folin phenol reagent was added and the absorbance was read at 660 nm using an UV-vis spectrophotometer. The amount of protease produced was measured with the help of a tyrosine standard graph²¹. Based on the tyrosine released, protease activity was expressed in units per gram of substrate (U/g).

Effect of physical factors on protease production

The effect of temperature on protease production was studied by growing the bacteria in fermentation media set at different temperatures (25, 30, 35, 40, 45 and 50°C). The protease activity was assayed by standard protocol²¹.

The effect of pH on protease production was studied by growing the bacteria in fermentation media set at different pH (4, 5, 6, 7, 8, 9 and 10). The protease activity was assayed by standard protocol²¹.

Effect of nutritional factors on protease production

Because the bacteria was isolated from the gut of marine crab, hence the effect of NaCl on protease production was also tested by adding different concentrations of NaCl (1, 2, 3, 4 and 5%) in the 1% carbon and nitrogen source basal medium.

The effect of carbon sources on protease production was tested by using six different carbon sources namely sucrose, mannitol, fructose, lactose, glucose and xylose. They were tested individually at the concentration of 1% in the 1% nitrogen source basal medium.

The effect of nitrogen sources on protease production was tested by using six different nitrogen sources ammonium sulphate, urea, casein, peptone, meat extract and potassium nitrate. They were tested individually at the concentration of 1% in the 1% carbon source basal medium.

Partial purification and characterization of protease

The crude protease extract was partially purified by ammonium sulphate (90%) precipitation followed by dialysis. The precipitate was collected by centrifugation at 12,500 rpm for 20 min at 4 °C, and dissolved in 0.5M Tris HCL buffer (pH 9.0). The solution was dialyzed against the same buffer at 4°C for 8 h with 3 changes of the dialysis buffer, and protein concentration was measured as the method of Lowry *et al.*²² using BSA as standard protein. The molecular weight of partially purified enzyme was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis.

RESULTS

The host crab, *Scylla serrata* is given in Fig.1.

Fig. 1. *Scylla serrata*



Among the three gut associated bacterial strains only one strain showed best result and taken for further analysis. The protease activity of the selected strain in solid medium is given in Fig. 2.

Fig. 2. The plate showing the protease activity by *Staphylococcus* sp.

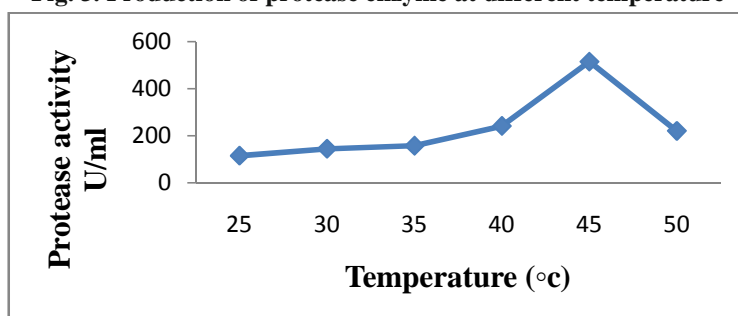


The gram staining result showed the bacterium was gram positive cocci. Based on the morphological and molecular features the bacteria was identified as *Staphylococcus* sp.

Optimization of protease production in liquid medium

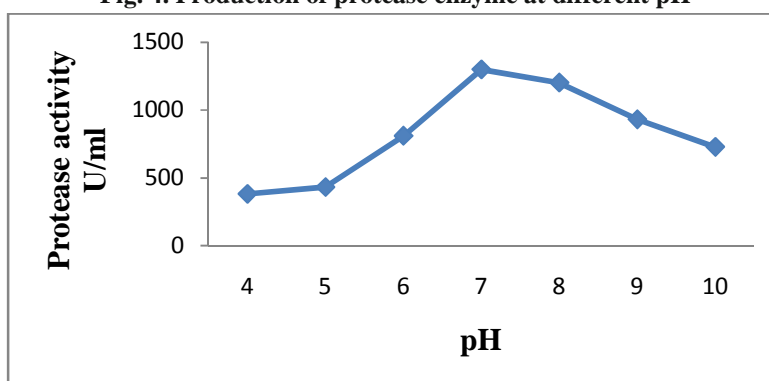
The effect of temperature on protease production is given in Fig. 3. Among the six different temperature conditions 45°C shows higher (514.44 U/ml) enzyme production in the liquid media.

Fig. 3. Production of protease enzyme at different temperature



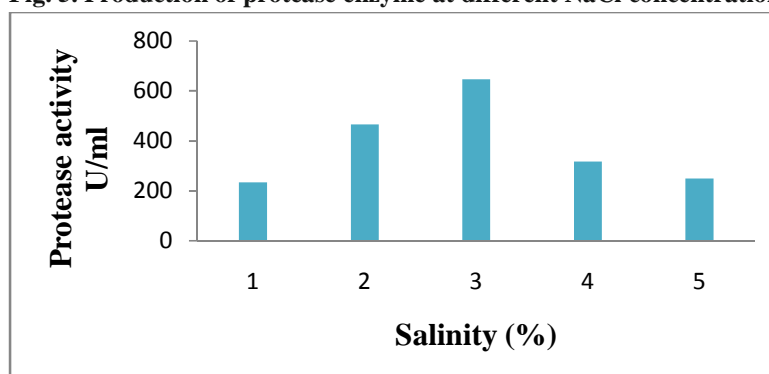
The effect of pH on protease production was studied by growing the bacteria in fermentation media set at different pH (4, 5, 6, 7, 8, 9 and 10). Among the seven pH ranges, pH7 shows high (1299.75 U/ml) protease production (Fig. 4)

Fig. 4. Production of protease enzyme at different pH



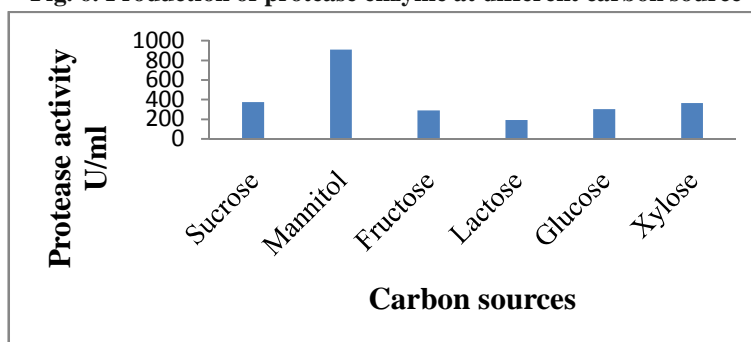
Because the bacteria was isolated from the gut of marine crab, hence the effect of NaCl on protease production was also tested. The effect of salinity on protease production is given in Fig. 5. Among the five different salinity conditions 3% NaCl shows higher (646.72 U/ml) enzyme production in the liquid media.

Fig. 5. Production of protease enzyme at different NaCl concentration



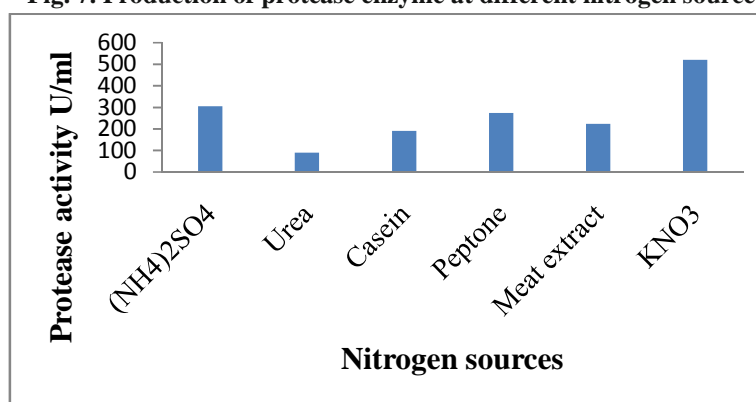
The effect of carbon sources on protease production was tested by using six different carbon sources. Among the six different carbon sources, mannitol shows high (911.29 U/ml) enzyme production (Fig. 6)

Fig. 6. Production of protease enzyme at different carbon source



The effect of nitrogen sources on protease production was tested by using six different nitrogen sources. Among these six different nitrogen sources, potassium nitrate shows high (520.74 U/ml) enzyme production and the result is given in Fig. 7.

Fig. 7. Production of protease enzyme at different nitrogen source



Specific activity of protease enzyme

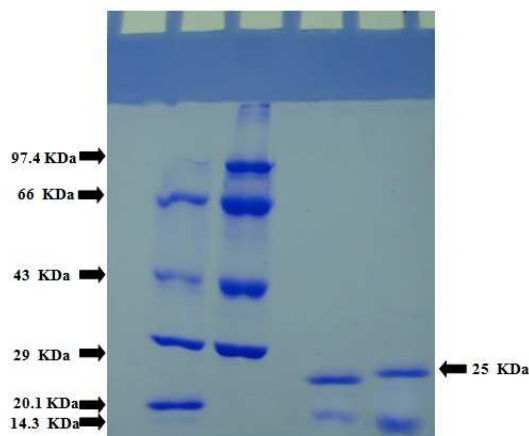
The specific activity of crude and partially purified protease enzyme is given in Table.1.

Table 1. Specific activity of crude and partially purified protease enzyme

Purification steps	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	57968	280	207.03	1	100
(NH ₄) ₂ SO ₄ precipitate	28286	89.6	315.69	1.52	48.8

Partial characterization

This partial purified protease was electrophoresed on SDS-PAGE (12 and 5%) and two bands were observed. Using standard protein markers the size of the partially purified enzyme was found to be about 25kDa (Fig. 8).

Fig. 8. SDS-PAGE zymogram of protease enzyme produced by *Staphylococcus* sp.

DISCUSSION

Here, the attention has been focused on the aerobic gut bacteria of the marine crab species and selected strain was isolated and assayed for protease activities to ascertain their role in exogenous production of digestive enzymes and the activity at various conditions.

To investigate whether the concentration of NaCl affects the production of extracellular protease by *Staphylococcus* sp., it was grown in a medium added with varying concentrations of NaCl (1.0 to 5.0%). As shown in Fig. 11, the extracellular protease production was maximum (646.72 U/ml) in the medium with 3% NaCl. Further, it indicated that *Staphylococcus* sp. is halophilic. It was reported that microorganisms living in the marine environment absolutely require Na⁺ for growth. In general, the salinity of the ocean is close to 3.5%. The maximum protease production registered in the medium added with 3.0% NaCl may be attributed to the isosaline nature of this medium, when compared to seawater. Patel *et al.*²³ reported that extracellular protease production by seawater *Bacillus* sp. isolate was maximum at 10% NaCl₂. Strtyorini *et al.*²⁴ also reported the maximum extracellular protease production at 10% NaCl by halotolerant *B. subtilis* FP133 isolated from salt-fermented fish paste. These results suggest that the extracellular protease production by the halophilic bacteria is much more affected by the Na⁺ electrochemical potential of the immediate environment.

The optimization for growth and protease production increased proteolytic activity and was useful in development of starter culture for acceleration the fermentation process²⁵. Sarkar *et al.*²⁶ reported that maximum protease enzyme production was obtained when pH of the culture media was maintained at pH 6, while in the case of *B. subtilis* the maximum protease production was reported at pH 6.5²⁷. In this present study, it was concluded that the optimum pH was 7.0 for the production of high amount of protease enzyme. At this pH condition, the isolate produced 1299.75 U/ml when compared with other pH ranges. The influence of incubation temperature on protease production by bacteria was studied by some researchers²⁸. The optimum temperature for protease production for *B. licheniformis* was 45°C²⁹. In the present study it was found that optimum temperature of 45°C was effective condition to produce high amount of protease enzyme. The temperature was found to influence extracellular enzyme secretion; possibly by changing the physical properties of cell membrane. Carbon sources greatly influenced the enzyme production and most commonly used substrate was reported to be casein³⁰. It has been noticed that lack of glucose in a media in dramatic decrease in the enzyme production³¹. Qadar *et al.*³² showed maximum protease enzyme production when 0.1% glucose was used as substrate instead of casein in the *Bacillus* culture broth. In the present study, mannitol was found to be the best optimum carbon source for protease production by the bacterial isolate followed by sucrose, lactose, fructose and glucose. But glucose was the best carbon source for protease production by *Aspergillus* sp. as per Gomma *et al.*³³ and by *Bacillus* sp. as per Boominathan *et al.*³⁴. In The effect of nitrogen source on protease production by *Bacillus* sp. was investigated by Qadar *et al.*³². They observed that among the different nitrogen sources, peptone was found to be best followed by meat and yeast extract. Among the different inorganic source, ammonium carbonate was found to be the best for their bacterial isolate in our observation.

The enzyme producing microorganisms isolated from the crab digestive tracts in the present study can be beneficially used as a probiotic while formulating the diet for cultivable crab species, especially in the larval stages. The main strategy to use probiotic is to isolate intestinal bacteria with favorable properties from mature animals and include large numbers of these bacteria in the feed of immature animals of the same species³⁵. The use of probiotic in commercial aquaculture is necessary for formulating diets at larval stages to minimize the cost of feed preparation.

This present study investigates the production of novel halophilic thermostable protease by *Staphylococcus* sp. isolated from the gut of marine crab, *Scylla serrata* using different nutritional sources. Owing its potential to convert waste proteins for protease production, the bacteria can be applied for bioconversion of any organic wastes for aqua feed formulation and subsequent reduction in feed-related waste outputs. Besides this, the thermostability and halotolerency of protease can be used for many industrial purposes including the detergent industry.

Acknowledgements

The authors are grateful to the Authorities of Annamalai University, The Director, CAS in Marine Biology, Annamalai University for encouragement and facilities provided.

REFERENCES

1. Hungate, R. E. The rumen microbial ecosystem. *Annual Rev. Ecol. Syst.*, **6**: 39-66 (1975)
2. Saha, S., Roy, R. N., Sen, S. K. and Ray, A. K. Characterization of cellulase producing bacteria from the digestive tract of tilapia, *Oreochromis mossambica* (Peters) and grass carp, *Ctenopharyngo donidella* (Valenciennes). *Aqua. Res.*, **37**: 380-388 (2006)
3. Fong, G. and Mann, K. H. Role of gut flora in the transfer of amino acids through a marine food chain. *Can. J. Fish. Aqua. Sci.*, **37**: 88-96 (1980)
4. Sochard, M. R., Wilson, D. F., Austin, B. and Colwell, R. R. Bacteria associated with the surface and gut of marine copepods. *App. Env. Microbiol.*, **37**: 570-579 (1979)
5. Hood, M. A., Meyers, S. P. and Colmer, A. R. Bacteria of the digestive tract of the white shrimp, *Penaeus setiferus*. *Bacteriol. Proceed.*, **71**: G-147 (1971)
6. Hoshino, T., Ishizki, K., Sakamoto, T., Kumeta, H., Yumoto, I. and Matsuyama, H. Isolation of a *Pseudomonas* species from fish intestine that produces a proteases active at low temperature. *Lett. App. Micro.*, **25**: 70-72 (1997)
7. Bairagi, A., Ghosh, K. S., Sen, S. K. and Ray, A. K. Enzyme producing bacterial flora isolated from fish digestive tracts. *Aquacul. Int.*, **10**:109-121 (2002)
8. Ray, A. K., Bairagi, A., Ghosh, K. S. and Sen, S. K. Optimization of fermentation conditions for cellulase production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 isolated from fish gut. *Acta Ichthyol. Piscat.*, **37(1)**: 47-53 (2007)
9. Chouyyok, W., Wongmongkol, N., Siwarungson, N. and Prichnont, S. Extraction of alkaline protease using an aqueous two-phase system from cell free *Bacillus subtilis* TISTR 25 fermentation broth. *Process Biochem.*, **40**: 3514-3518 (2005)
10. Raju, K., Jaya, R. and Ayyanna, C. Hydrolysis of casein by Bajara protease importance. *Biotech. Coming Decade.*, **181**: 55-70 (1994)
11. Sidney, F. and Lester, P. Methods in Enzymology. *Academic Press Inc*, New York, (1972)
12. Tobe, S., Nagoh, Y., Watanabe, T. and Mukaiyama, T. 2005. Bacteriolytic activity of detergent protease and its enhancement by detergents materials. *J. Oleo Sci.*, **54**: 389-395 (2005)
13. Yossan, Q., Peng, Y., Li, X., Wang, H. and Zhang, Y. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. *Curr. Microbiol.*, **46**: 169-173 (2006)
14. Olsson, J. C., Westerdahl, A., Conway, P. L. and Kjelleberg, S. Intestinal colonization potential of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*)- associated bacteria with inhibitory effects against *Vibrio anguillarum*. *App.Env.Microbiol.*, **58(2)**: 551-556 (1992)
15. Ringo, E. and Strom, E. Microflora of Arctic charr, *Salvelinus alpinus* the gastrointestinal microflora of free-living fish and the effect of diet and salinity on intestinal microflora. *Aquacul. Fish. Manage.*, **25**: 623-629 (1994)
16. Abdel Galil, O. A. Formation of protease by *Aspergillus fumigates* and *Penicillium* sp. *J. King. Saud. Univ.*, **4(2)**: 127-136 (1992)
17. Holt, J. G. Bergey's Manual of Systematic Bacteriology. 1st edn. Baltimore, Williams and Wilkins (1984)
18. Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular cloning: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989)
19. Espejo, R. T., Feijoo, C. G., Romero, J. and Vasquez, M. PAGE analysis of the heteroduplexes formed between PCR-amplified 16s rRNA genes: estimation of sequence similarity and rDNA complexity. *Microbiology*, **144**: 1611-1617 (1998)

20. Hall, T. A. 1999. Bio Edit: a user friendly biological sequence alignment editor and analysis program for windows 95/98/NT. <http://www.mbio.ncsu.edu/BioEdit/bioedit.html/>.
21. Takami, H., Akiba, T. and Horikishi, K. Production of extremely thermostable alkaline protease from *Bacillus* sp. *App. Microbiol. Biotech.*, **30**:120-124 (1989)
22. Lowry, Q. H., Rosenberough, N. J., Farr, L. A. and Randall, R. J. Protein measurement with the Folin Phenol reagent. *J. Biol. Chem.*, **193**: 256-275 (1951)
23. Patel, R. K., Dodia, M. S., Joshi, R. H. and Singh, S. P. Production of extracellular halo-alkaline protease from a newly isolated Haloalkaliphilic *Bacillus* sp. isolated from seawater in Western India. *World J. Microbiol. Biotech.*, **22(4)**: 375-382 (2006)
24. Strtyorini, E., Takenake, S., Murakami, S. and Aoki, K. Purification and characterization two novel halotolerant extracellular protease from *Bacillus subtilis* strain FP 133. *Biosci. Biotech. Biochem.*, **70(2)**: 433-440 (2006)
25. Rahman, R. N. Z. A., Basri, L. P. G. M. and Salleh, A. B. Physical factors affecting the production of organic solvent tolerant protease by *Pseudomonas aeruginosa* strain K. *Bioresour. Tech.*, **96**: 429-436 (2005)
26. Sarkar, P. K., Cook, P. E. and Owens, J. D. *Bacillus* fermentation of soybeans. *W. J. Microbiol. Biotech.*, **9**: 295-299 (1993).
27. Rahman, R. N. Z. A., Razak, C. N., Ampon, K., Basri, M. and Yunus, W. M. Z. W. Purification and characterization of heat stable alkaline protease from *Bacillus stearothermophilus* F1. *App. Microbiol. Biotech.*, **40**: 822-827 (1994)
28. Hoyoux, C., Zbinden, M., Samadi, S., Gaill, F. and Compere, P. Wood-based diet and gut microflora of a galatheid crab associated with Pacific deep-sea wood falls. *Mar. Biol.*, **156**: 2421-2439 (2009)
29. Sen, S. and Satyanarayana, T. Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. *Indian J. Microbiol.*, **33**: 43-47 (1993)
30. Pastor, M. D., Lorda, G. S. and Balatti, A. Protease abstention using *Bacillus subtilis* 3411 and Amarnath seed meal medium of different aeration rats. *Braz. J. Microbiol.*, **32**: 6-9 (2001)
31. Gajju, H., Bhalla, T. C. and Agarwal, O. H. Thermostable alkaline protease from thermophilic *Bacillus coaguans* PB-77. *Indian J. Microbiol.*, **36**: 153-155 (1996)
32. Qadar, S. A. U., Shireen, E., Iqbal, S. and Anwar, A. Optimization of protease production from newly isolated strain of *Bacillus* sp. PCSIR EA-3. *Indian J. Biotech.*, **8**: 286-290 (2009)
33. Gomma, M. A., Mostafa, M. M., Abouzied, M. M. and El-Habashy, M. Production of semi alkaline proteases by *Aspergillus* sp. *Egyptian J. Food Sci.*, **16**: 9-26 (1989)
34. Boominadhan, U., Rajakumar, R., Sivakumaar, P. K. V. and Joe, M. M. Optimization of protease enzyme production using *Bacillus* sp. isolated from different wastes. *Botany Res. Inter.*, **2 (2)**: 83-87 (2009)
35. Gildberg, A., Mikkelsen, H., Sandaker, E. and Ringo, E. Probiotic effect of lactic acid bacteria in the feed on growth and survival of Atlantic cod (*Gadus morhua*). *Hydrobiologia*, **352**: 279-285 (1997)