Asian Journal of
Biochemistry

ISSN 1815-9923

Academic
Journals Inc.

www.academicjournals.com

Asian Journal of Biochemistry 7 (3): 123-132, 2012
ISSN 1815-9923 / DOI: 10.3923/ajb.2012.123.132

Purification and Characterization of Extracellular Protease from Halotolerant Bacterium *Virgibacillus dokdonensis* VITP14

V. Devi Rajeswari, G. Jayaraman and T.B. Sridharan
Industrial Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore, Tamil Nadu, India

Corresponding Author: T.B. Sridharan, Industrial Biotechnology Division, School of Biosciences and Technology, VIT University, Vellore-14, Tamil Nadu, India

ABSTRACT
In this study extracellular protease was purified with ammonium sulphate precipitation, dialysis, gel filtration and ion exchange chromatography. The purity was checked with MALDI-TOF and SDS-PAGE. Confirmation of protease was done with zymogram. The molecular weight of the protease was found to be 36 kDa. MALDI-TOF analysis of the protein showed the Molecular weight confirmation of the studied protease. Further the enzyme was characterized for its maximal activity with different pH, temperature, salt concentration and inhibitors. The maximal activity was found at pH 7.0, 40°C, 1.5 M salt concentration and found to be serine protease.

Key words: Extracellular protease, *Virgibacillus,* purification, characterization

INTRODUCTION
Haloalkaliphilic bacteria have been mainly focused on its molecular diversity and its phylogeny, in-depth exploration of its enzymatic potential and properties have to be revealed. Till date only few haloalkaliphilic enzymes have been studied and reported about its potential uses (Gupta *et al.*, 2005; Patel *et al.*, 2005; Studdert *et al.*, 1997, 2001; Seitz *et al.*, 1997; Eddy and Jablonski, 2000). Even though there are enzymes used in biotechnological and industrial applications, which will not meet the growing demands. Due to this, researchers have recently focused on isolation and characterization of the enzyme from various microbes. Especially, extremophiles is a valuable source of enzyme for a variety of applications (Herbert *et al.*, 1992; Eichler, 2001; Kathiresan and Manivannan, 2007; Ikram-ul-Haq *et al.*, 2003).

Proteases are important enzymes which dominate two-third of the detergent enzyme market (Niehaus *et al.*, 1999; Gupta and Roy, 2002). I also plays an important role in the hydrolysis of proteins by specific catalytic action (Manivannan and Kathiresan, 2007). *Bacillus* is the major producer of most commercial proteases (Mehrotra *et al.*, 1999; Abdel-Naby *et al.*, 1998; Mabrouk *et al.*, 1999; Beg and Gupta, 2003; Phadatare *et al.*, 1993; Hoq *et al.*, 2005). Despite the presence of proteases, enzymes with specific properties are the major interest for scientists which led them to find new sources of protease with various specific properties and functions (Joo and Chang, 2005; Bezerra *et al.*, 2005; Mei and Jiang, 2005). One such novel source is marine microbes. Due to its extreme physiological characteristics, metabolic patterns and utilization of nutrient these microbes are expected to possess unique properties. Reports with halophilic proteases are very limited in the literature. The present study showed the purification and characterization of halotolerant protease from a marine bacterium *Virgibacillus dokdonensis*.

MATERIALS AND METHODS

Micro organism used: Halotolerant bacterium VITP14 was isolated and previously characterized for its 16S rRNA sequencing was used for purification and characterization of its extracellular protease (GenBank accession number HQ829428).

Preparation of inoculums: Production of enzyme was done in Zobell marine broth. A 5% inoculum was added to Zobell broth from an overnight culture (OD 2.1 at 660 nm) and incubated for 48 h at 37°C (100 rpm); the culture was drawn after incubation and centrifuged at 10,000 rpm for 10 min at 4°C. This collected culture supernatant was used for further analysis.

Ammonium sulphate precipitation: The culture filtrate was precipitated with the addition of ammonium sulphate. The salt was slowly added to the filtrate to obtain 75% saturation with gentle stirring and kept at 4°C for complete precipitation to occur. The precipitate was collected by centrifugation at 10,000 rpm for 15 min at 4°C. The collected precipitate was dissolved in minimum quantity of 1 mM tris-HCl buffer (pH 8.0). This precipitate was lyophilized and further purified in ion exchange and gel filtration chromatography (Padmakar *et al.*, 2005).

Ion exchange chromatography: The precipitated enzyme solution was loaded at a flow rate of 0.2 mL min^{-1} to (2×7 cm), pre-equilibrated with 1 mM Tris-HCl buffer of pH 8.0 and the eluted fractions (1 mL each) were collected. After loading the sample, column was washed with the same buffer until the reading of the eluted buffer at 280 nm was zero. The bound proteins were then eluted with a concentration gradient of sodium chloride (0-0.5 M NaCl) in the same buffer and fractions of 2 mL each were collected at a flow rate of 0.2 mL min^{-1}. Protein concentration of each fraction was determined by measuring the absorbance at nm and each fractions were assayed for protease activity. Fractions with high specific activity were pooled and then concentrated by lyophilization (Singh *et al.*, 2001).

Gel filtration chromatography: One milliliter of partially purified, lyophilized enzyme was loaded to a Sephadex-G-75 (Fluka Chemicals, Buchs, Switzerland) column (1.25×35 cm) pre-equilibrated with 1 mM of Tris-HCl buffer (pH 8.0) and then eluted with the same buffer. Fractions of 2 mL each were collected at a flow rate of 2.5 mL h^{-1}. Protein concentration of each fraction was determined by measuring absorbance at 280 nm and all the collected fractions were assayed for protease activity. The active fractions were pooled, concentrated by lyophilization and purity was checked in SDS-PAGE (Sana *et al.*, 2006).

Enzyme assay: The proteolytic activity was assayed by the modified method of Kunitz using casein as the substrate. The reaction was carried out in a tube containing 500 µL of 2% (w/v) casein in 1 mM Tris-HCl buffer (pH 8.0) with enzyme from the fractions, incubated at 37°C for 10 min. The reaction was terminated by adding 2.5 mL of 10% (w/v) trichloroacetic acid (TCA), kept at room temperature for 30 min and then centrifuged at 10,000 xg for 8 min. The absorbance was measured against a blank at 280 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µmol of tyrosine in 1 min under standard assay conditions. The specific activity is expressed in units of enzyme activity/mg of protein (Ibrahim and Ali-salamah, 2009).

Polyacrylamide gel electrophoresis: The purity and molecular weight of the enzyme was determined in SDS-PAGE using a 5% (w/v) stacking and a 12% (w/v) separating gels.

Samples were prepared by mixing purified enzymes in a ratio of 1:1 (v/v) with sample buffer. Before loading the samples, it was heated at 100°C for 5 min. After the run the gels were stained with 0.25% coomassie brilliant blue R-250 in 45% ethanol-10% acetic acid and destained with 5% ethanol with 7.5% acetic acid. The molecular weight of the enzyme was identified by compared with the commercial standard protein marker (Banik and Prakash, 2006).

Detection of proteolytic activity on polyacrylamide gels-protease zymogram: Zymography was performed in conjunction with PAGE according to the method described by Garcia-Carreno *et al.* (1993) with slight modification. The sample was not heated before electrophoresis. After electrophoresis, the gel was submerged in 100 mM glycine-NaOH buffer (pH 10.0) containing 2.5% Triton X-100 for 30 min, with constant agitation to remove SDS. Triton X-100 was then removed by washing the gel three times with 100 mM glycine-NaOH buffer (pH 10.0). The gel was then incubated with 1% (w/v) casein in 100 mM glycine-NaOH buffer (pH 10.0) for 20 min at 50°C. Finally, the gel was stained with Coomassie Brilliant Blue R-250. The development of clear zones on the blue background of the gel indicated the presence of protease activity.

MALDI-TOF mass spectrometry analysis: Molecular mass was analyzed in MALDI-TOF mass spectrometry using a Voyager-DE PRO MALDI-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). One microliter of sample in TFA 0.1% was mixed with 2 mL of the matrix prepared with α-cyano-4-hidroxy-cinnamic acid (Sigma), 60% acetonitrile and 0.1% v/v TFA. The mass of the protein was analyzed in linear analysis mode using an acceleration voltage of 25 kV, with the laser fixed in 2890 mJ com^2 and a delay of 300 ns.

Effect of pH: To determine the effect of pH, the purified enzyme was incubated at different pH range (6.0 to 9.0). To 500 µL buffer of different pH range, 500 µL of purified enzyme was incubated at 37°C for 30 min. After incubation, casein was added to the reaction and incubated for 10 min and the reaction was stopped by the addition of TCA and measured the absorbance at 280 nm (Anvari and Khayati, 2011).

Effect of temperature: To determine the effect of temperature on the activity of enzyme, the partially purified enzyme (500 µL) was mixed with 500 µL of Tris-HCl buffer (pH 8.0) and incubated at different temperatures (37, 40, 50°C). Casein substrate was added to the above mixture after 30 min and incubated at 37°C for 10 min. The reaction was stopped by the addition of TCA and the supernatant was obtained by centrifugation was measured at 280 nm (Venugopal and Saramma, 2006).

Effect of NaCl: The effect of NaCl on enzyme activity was determined by incubating the partially purified enzyme with different concentrations of NaCl (0 to 2.0 M). The residual activity of the enzyme was checked after incubation. The control without addition of NaCl was maintained (Sana *et al.*, 2006).

Effect of inhibitors: To determine the type of enzyme, the partially purified enzyme was incubated with various enzyme inhibitors like phenylmethylsulfonyl fiuoride (PMSF), ethylenediaminetetraacetic acid (EDTA) and dithiobis-nitrobenzoic acid (DTNB). The enzyme inhibitor mixture was incubated for 30 min at 37°C, the residual activity was measured using casein as a substrate. The activity of the enzyme assayed in the absence of inhibitors was taken as 100% (Sen *et al*., 2011).

RESULTS AND DISCUSSION

Purification of the enzyme: The halotolerant protease was produced and the enzyme was purified through ammonium sulphate fractionation followed by ion exchange and gel permeation chromatography. Further, the purification of the enzyme was confirmed in MALDI-TOF. A summary of the purification was shown in Table 1. The ammonium sulphate fractionation was an effective step in purification which was leading to 2.1-folds purification with specific activity of 40 U mg^{-1}. The ion exchange chromatography with DEAE Sephadex enhanced the specific activity to 63 U mg^{-1} with 3.3 fold purification. During the last step of purification with gel permeation chromatography the specific activity was further enhanced to 102 U mg^{-1} with 5.2 folds purification (Fig. 1, 2).

SDS-PAGE, zymogram and MALDI-TOF analysis: Protein purification was successfully achieved to the homogeneity which was evident by the appearance of single band corresponding to 36 kDa on SDS-PAGE (data not shown). The molecular weight was lower as compared to other halophilic and alkaline proteases where it ranged from 40 to 130 kDa (Studdert *et al*., 2001; Gimenez *et al*., 2000; Kamekura and Seno, 1990; Schmitt *et al*., 1990). The protease activity of the

Table 1: Purification fold and recovery in each purification steps

Purification steps	Activity (U mL^{-1})	Protein (mg mL^{-1})	Specific activity (U mg^{-1})	Purification (Fold)	Recovery(%)
Crude extract	996	52.0	19	1.0	100
Ammonium sulphate precipitation	881	22.0	40	2.1	88
Ion exchange chromatography	443	7.0	63	3.3	44
Gel filtration chromatography	92	0.9	102	5.3	9

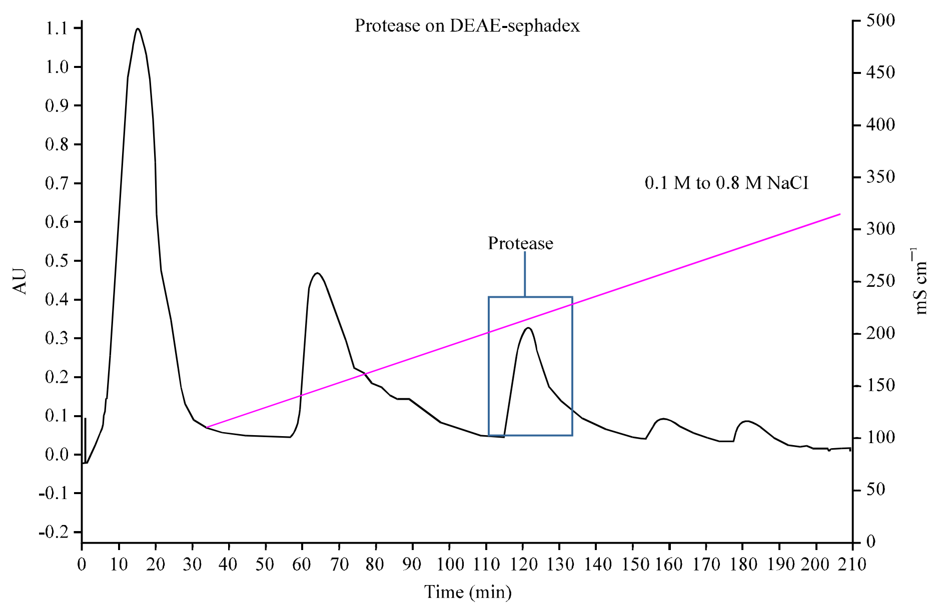

Fig. 1: Elution profile of enzyme from ion exchange chromatography

purified enzyme was confirmed in zymogram which clearly showed the clear zone against the substrate. The degree of purity was further confirmed by MALDI-TOF mass spectrometry, which showed a single peak with 36438.21 kDa (Fig. 3, 4).

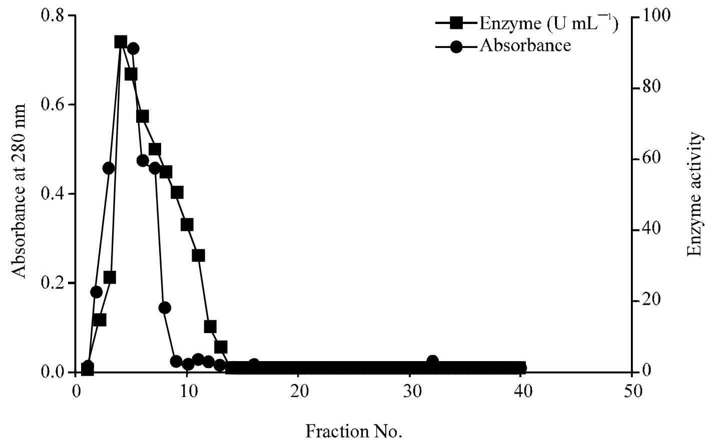

Fig. 2: Elution profile of protease from Sephadex G-75

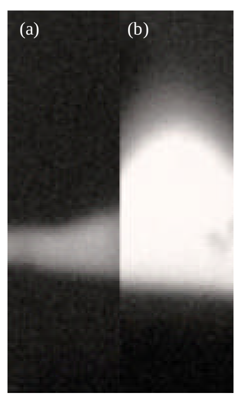

Fig. 3(a-b): Zymogram analysis of protease; (a) Enzyme eluted from Ion Exchange Chromatography and (b) Enzyme eluted from Sephadex G-75

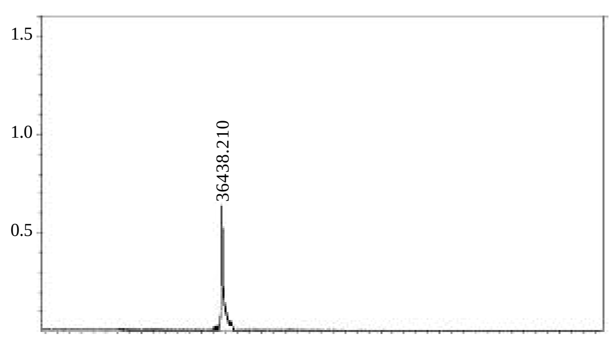

Fig. 4: MALDI-TOF analysis of purified enzyme showing molecular mass of 36438.210

CHARACTERIZATION OF THE ENZYME

Effect of pH: The pH profile of the halotolerant protease was determined at 37°C by incubating the purified enzymes are different pH range (6.0-9.0). The enzyme was active in all the tested pH range and the optimum was found to be at pH 8.0 (Fig. 5). Similarly, a previous study with a protease produced by *Monascus purpureus* grown on marine wastes showed pH 8.0 as optimum pH (Liang *et al.*, 2006). In contrast a study with haloalkaliphilic bacterium *Halogeometricum borinquense* (Vidyasagar *et al.*, 2006), *Bacillus* sp. (Gupta *et al.*, 2005), *B. subtilis* CN215 (Uchida *et al.*, 2004) and *B. subtilis* PE-11 (Adinarayana *et al.*, 2003) showed pH 10.0 as optimum for enzyme activity.

Effect of temperature: The optimum temperature for enzyme activity was 40°C beyond which there was a rapid decline. Similarly, at lower temperature, the enzyme activity sharply decreased (Fig. 6). This result is in accordance with Sana *et al.* (2006) showed 40°C is optimum for a marine bacterium. However, the temperature tolerance of this enzyme is quite lower than many halophilic proteins such as protease from halophilic bacterium *Salinivibrio* sp. showing optimal activity at 65°C and another report (Gupta *et al.*, 2005) showed 60°C as optimum for an solvent tolerant bacterium *Pseudomonas aeruginosa* eventhough it is active from 40 to 65°C.

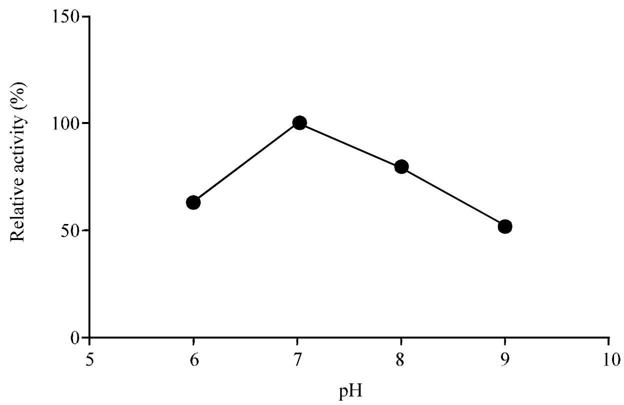

Fig. 5: Enzyme activity at different pH range

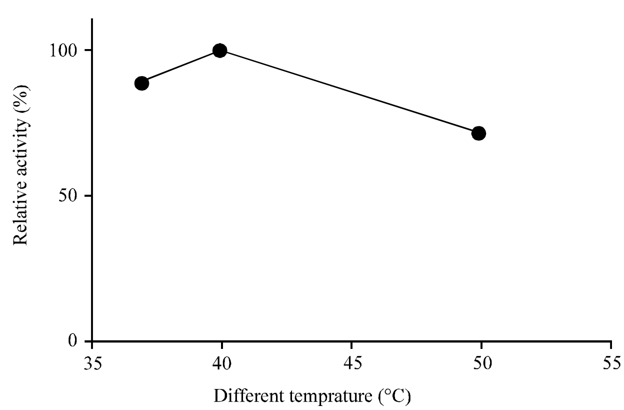

Fig. 6: Enzyme activity at different temperatures

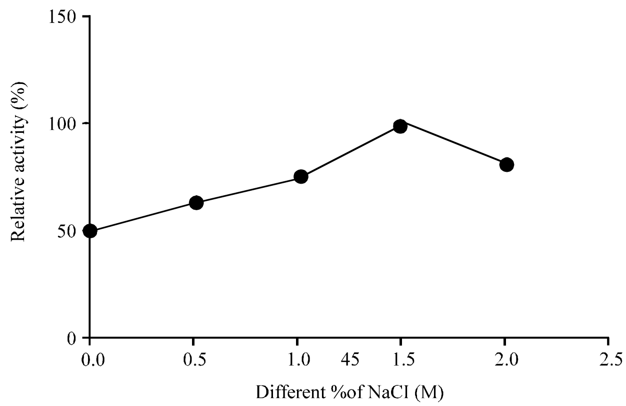

Fig. 7: Enzyme activity at different percentage of sodium chloride

Table 2: Effect of inhibitors on enzyme activity

Inhibitors	Relative activity (%)
PMSF	0
EDTA	100
DTT	89

Effect of NaCl: The effect of NaCl on enzyme activity was tested in different concentrations of NaCl (0 to 2.0 M). Maximum activity was found in 1.5 M (Fig. 7). In contrast, a study with *Salinivibrio* strain showed maximum activity at 0.5 M (Karbalaei-Heidari *et al*., 2007). Further, increase in salt concentration led to reduction in activity this is in accordance with a study showed reduction in activity with increase salt concentration (Patel *et al*., 2006). But a study with a marine bacterium showing optimum activity at even 30% of NaCl and even it is stable at 35% (Sana *et al*., 2006). A study with a marine organism *Halogeometricum borinquense* showed activity with 30% NaCl even at an incubation temperature of 60°C (Vidyasagar *et al*., 2006).

Effect of inhibitors: To determine the type of enzyme, purified enzyme was incubated with different protease inhibitors. Protease showed complete inhibition against PMSF (phenylmethylsulphonyl fluoride) (Table 2). Hence, it is confirmed that this halotolerant enzyme was a serine type. PMSF sulphonated the serine active site which results in the loss of activity (Gold and Fahrney, 1964). There was no inhibition with EDTA (ethylene diamine tetraacetic acid) and DTT (dithiothreitol) which are metallo and thiol protease inhibitors. Similar results showed serine protease *B. licheniformis* (Tang *et al*., 2004), *B. pumilus* (Aoyama *et al*., 2000; Huang *et al*., 2003) and *B. intermedius* (Balaban *et al*., 2004).

CONCLUSION

The extracellular protease from halotolerant bacterium VITP14 was extracted and purified which was further confirmed by zymogram. Purity check and molecular weight identification was done with MALDI-TOF and SDS-PAGE. The enzyme was also characterized for its optimal and maximal activity with different pH, temperature and salt concentration. Thus, this enzyme will be much potential in industries where higher salt concentrations were used during fermentation process.

REFERENCES

Abdel-Naby, M.A., A.S. Ismail, S.H. Ahmed and A.F. Abdel-Fattah, 1998. Optimization of alkaline protease from *Bacillus mycoids*. Bioresour. Technol., 64: 205-210.

Adinarayana, K., P. Ellaiah and D.S. Prasad, 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. AAPS Pharm. Sci. Technol., 4: 1-9.

Anvari, M. and G. Khayati, 2011. Production and characterization of alkaline protease from *Bacillus licheniformis* sp. isolated from Iranian northern soils with ram horn hydrolysate. Trends Applied Sci. Res., 6: 1206-1213.

Aoyama, M., M. Yasuda, K. Nakachi, N. Kobamoto, H. Oku and F. Kato, 2000. Soybean-milk-coagulating activity of *Bacillus pumilus* derives from a serine proteinase. Applied Microbiol. Biotechnol., 53: 390-395.

Balaban, N.P., A.M. Mardanova, M.R. Sharipova, L.A. Gabdrakhmanova, E.A. Sokolova, G.N. Rudenskaya and I.B. Leshchinskaya, 2004. Purification and characterization of serine proteinase 2 from *Bacillus intermedium* 3-19. Biochemistry, 69: 520-526.

Banik, R.M. and M. Prakash, 2006. Purification and characterization of laundry detergent compatible alkaline protease from *Bacillus cereus*. Ind. J. Biotechnol., 5: 380-384.

Beg, Q.K. and R. Gupta, 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. Enzyme Microbial. Technol., 32: 294-304.

Bezerra, R.S., E.J.F. Lins, R.B. Alencar, P.M.G. Paiva, M.E.C. Chaves and L.C.B.B. Coelho, 2005. Alkaline proteinase form intestine of Nile tilapia (*Oreochromis niloticus*). Process Biochem., 40: 1829-1834.

Eddy, M.L. and P.E. Jablonski, 2000. Purification and characterization of a membrane associated ATPase from *Natronococcus occultus*, a haloalkaliphilic archaeon. FEMS Microbiol. Lett., 189: 211-214.

Eichler, J., 2001. Biotechnological uses of archaeal extremozymes. Biotechnol. Adv., 19: 261-278.

Garcia-Carreno, F.L., L.E. Dimes and N.F. Haard, 1993. Substrate gel electrophoresis for composition and molecular weight of proteinases and proteinaceous proteinase inhibitors. Anal. Biochem., 214: 65-69.

Gimenez, M.I., C.A. Studdert, J.J. Sanchez and R.E. De Castro, 2000. Extracellular protease of *Natrialba magadii* purification and biochemical characterization. Extremophiles, 4: 181-188.

Gold, A.M. and D. Fahrney, 1964. Sulfonyl fluorides as inhibitors of esterases. II. Formation and reactions of phenylmethane sulfonyl α-chymotrypsin. Biochemistry, 3: 783-791.

Gupta, A., I. Roy, R.K. Patel, S.P. Singh, S.K. Khare and M.N. Gupta, 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus* sp. J. Chromatogr. A., 1075: 103-108.

Gupta, M.N. and I. Roy, 2002. Applied Biocatalysis: An overview. Indian J. Biochem. Biophys., 39: 220-228.

Herbert, R.A., 1992. A perspective on the biotechnological potential of extremophiles. Trends Biotechnol., 10: 395-402.

Hoq, M.M., K.A.Z. Siddiquee, H. Kawasaki and T. Seki, 2005. Keratinolytic activity of some newly isolated *Bacillus* species. J. Biol. Sci., 5: 193-200.

Huang, Q., Y. Peng, X. Li, H. Wang and Y. Zhang, 2003. Purification and characterization of an extracellular alkaline serine protease with dehairing function from *Bacillus pumilus*. Curr. Microbiol., 46: 169-173.

Ibrahim, A.S.S. and A. Ali-salamah, 2009. Optimization of media and cultivation conditions for alkaline protease production by alkaliphilic *Bacillus halodurans*. Res. J. Microbiol., 4: 251-259.

Ikram-ul-Haq, H. Mukhtar, S. Daudi, S. Ali and M.A. Qadeer, 2003. Production of protease by a locally isolated mould culture under lab conditions. Biotechnology, 2: 30-36.

Joo, H.S. and C.S. Chang, 2005. Production of protease from a new alkalophilic *Bacillus* sp. I-312 grown on soybean meal: Optimization and some properties. Proc. Biochem., 40: 1263-1270.

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

Karbalaei-Heidari, H.R., A.A. Ziaee, J. Schaller and M.A. Amoozegar, 2007. Purification and characterization of an extracellular haloalkaline protease produced by the moderately halophilic bacterium, *Salinivibrio* sp. strain AF-2004. Enzym. Microbial Technol., 40: 266-272.

Kathiresan, K. and S. Manivannan, 2007. Production of alkaline protease by *Streptomyces* sp., isolated from coastal mangrove sediment. Res. J. Environ. Sci., 1: 173-178.

Liang, J., Y. Han, J. Li, X. Xu, H.H. Rees and R. Lai, 2006. A novel bradykinin-like peptide from skin secretions of rufous-spotted torrent frog, *Amolops loloensis*. Peptides, 27: 2683-2687.

Mabrouk, S.S., A.M. Hashem, N.M.A. El-Shayeb, A.M.S. Ismail and A.F. Abdel-Fattah, 1999. Optimization of alkaline protease productivity by *Bacillus lichenformis* ATCC 21415. Bioresour. Technol., 69: 155-159.

Manivannan, S. and K. Kathiresan, 2007. Alkaline protease production by *Penicillium fellutanum* isolated from mangrove sediment. Int. J. Biol. Chem., 1: 98-103.

Mehrotra, S., P.K. Pandey, R. Gaur and N.S. Darmwal, 1999. The production of alkaline protease by a *Bacillus* species isolate. Bioresour. Tech., 67: 201-203.

Mei, C. and X. Jiang, 2005. A novel surfactant and oxidation-stable alkaline protease from *Vibrio metschnikovii* DL 33-51. Proces Biochem., 40: 2167-2172.

Niehaus, F., C. Bertoldo, M. Kahler and G. Antranikiar, 1999. Extremophiles as a source of novel enzymes for industrial application. Applied Microbiol. Biotechnol., 5: 711-729.

Padmakar, B., P. Madhavilatha and V. Ramakrishna, 2005. Purification of alkaline protease from the cotyledons of germinating Indian bean (*Dolichos lablab* L. *var lignosus*) seeds. Biotechnology, 4: 365-369.

Patel, R., D. Mittal and S.P. Singh, 2005. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization. Process Biochem., 40: 3569-3575.

Patel, R.K., M.S. Dodia, R.H. Joshi and S.P. Singh, 2006. Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp. Process Biochem., 41: 2002-2009.

Phadatare, S.U., V.V. Deshpande and M.C. Srinivasan, 1993. High activity alkaline protease from *Conidiobolus caronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. Enzyme Microb. Technol., 15: 72-76.

Sana, B., D. Ghosh, M. Saha and J. Mukherjee, 2006. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma-Proteobacterium isolated from the marine environment of the Sundarbans. Process. Biochem., 41: 208-215.

Schmitt, W., U. Rdest and W. Goebel, 1990. Efficient high performance liquid chromatographic system for the purification of a halobacterial serine protease. J. Chromatogr., 521: 211-220.

Seitz, K.H., C. Studdert, J. Sanchez and R. de Castro, 1997. Intracellular proteolytic activity of the haloalkaliphilic archaeon *Natronococcus occultus*. Effect of starvation. J. Basic Microbiol., 7: 313-322.

Sen, S., V.V. Dasu, K. Dutta and B. Mandal, 2011. Characterization of a novel surfactant and organic solvent stable high-alkaline protease form new *Bacillus pseudofirmus* SVB1. Res. J. Microbiol., 6: 769-783.

Singh, J., N. Batra and C.R. Sobti, 2001. Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. Process. Biochem., 36: 781-785.

Studdert, C.A., M.K.H. Seitz, M.I.P. Gil, J.J. Sanchez and R.E. de Castro, 2001. Purification, biochemical characterization of the haloalkaliphilic Archeon *Natronococcus occultus* extracellular serine protease. J. Genet. Microbiol., 41: 375-383.

Studdert, C.A., R.E. de Castro., K.H. Seitz and J.J. Sanchez, 1997. Detection and preliminary characterization of extracellular proteolytic activities of the haloalkaliphilic archaeon *Natronococcus occultus*. Arch. Microbiol., 168: 532-535.

Tang, X.M., F.M. Lakay, W. Shen, W.L. Shao and H.Y. Fang *et al*., 2004. Purification and characterization of an alkaline protease used in tannery industry from *Bacillus licheniformis*. Biotechnol. Lett., 26: 1421-1424.

Uchida, H., D. Kondo, S. Yamashita, T. Tanaka, H.L. Tran, H. Nagano and T. Uwajima, 2004. Purification and properties of a protease produced by *Bacillus subtilis* CN2 isolated from a Vietnamese fish sauce. World J. Microbiol. Biotechnol., 20: 579-582.

Venugopal, M. and A.V. Saramma, 2006. Characterization of alkaline protease from *Vibrio fluvialis* strain VM10 isolated from a mangrove sediment sample and its application as a laundry detergent additive. Process Biochem., 41: 1239-1243.

Vidyasagar, M., S. Prakash, C. Litchfield and K. Sreeramulu, 2006. Purification and characterization of a thermostable, haloalkaliphilic extracellular serine protease from the extreme halophilic archaeon *Halogeometricum borinquense* strain TSS101. Archaea., 2: 51-57.