Indian Journal of Experimental Biology
Vol. 55, May 2017, pp. 303-310

# Purification and characterization of highly active LasB protease from *Pseudomonas aeruginosa* MCCB 123

Divya Jose[1], Manjusha K[2], Seena Jose[1], A Mohandas[1] & IS Bright Singh[1*]

[1]National Centre for Aquatic Animal Health; [2]Department of Marine Biology, Biochemistry and Microbiology, Cochin University of Science and Technology, Lakeside Campus, Fine Arts Avenue, Cochin-682 016, Kerala, India

*Received 19 May 2014; revised 29 September 2015*

Proteolytic enzymes constitute one of the most important groups of industrial enzymes and have received worldwide attention due to their wide range of biotechnological applications and their sustainability in reducing environmental degradation. In this study, a LasB protease with dual substrate specificities was purified from *Pseudomonas aeruginosa* MCCB 123 with 6.85 fold increase in protease specific activity and 9.05 fold increase in elastase-specific activity. The enzyme revealed the presence of a 33 kDa protein. Optimum temperature and pH of protease activity were 60°C and 9.0, and that of elastase activity were 40°C and 8.0, respectively. Based on the cytotoxicity assay on Hep2 cells, the enzyme has an $IC_{50}$ (inhibitory concentration) value of 47.28 µg $mL^{-1}$. According to the deduced amino acid sequence, the MCCB 123 LasB protease is a zinc dependent metalloendopeptidase belonging to M4 neutral protease GluZincin superfamily. Appreciable protease activity of the enzyme at high temperature and alkaline pH proposes its potential application in detergency. MCCB 123 LasB protease is 3.96 fold more active when compared to commercial protease Savinase suggesting its industrial relevance.

**Keywords**: Detergent, Deproteinization, Microbial protease, Savinase

Enzymes are eco-friendly green chemicals that have wide range of applications in industrial sector[1]. Microbial protease represents largest group of industrial enzymes which account for 65% of total worldwide enzyme sales[2]**.** Among microbial proteases, bacterial alkaline proteases secure a prominent position in the enzyme market[3], and are commonly used due to their relatively high activity and stability at high pH[4]. They are supplemented as ingredients in detergents, used in food processing, leather industry, waste treatment, dairying, food and feed manufacturing, silver recovery[5-11] and peptide synthesis[6,12].

Even though, *Bacillus* spp. has been viewed as a promising group of organisms for protease production towards industrial applications, *Pseudomonas* proteases have also been considered for such uses as they are either similar or more active than those produced by the former. Furthermore, *Pseudomonas aeruginosa* can grow in alkaline conditions and in water-soluble oil and its enzymes are adapted to extreme conditions[13]. But only a few reports exist on the proteolytic enzymes from *Pseudomonas aeruginosa*[14]. Predominant proteases secreted by this bacterium are LasA protease, LasB protease, Protease IV and alkaline proteases[15,16]. This organism has been proposed as antagonistic against aquaculture pathogens[17,18]. The *Pseudomonas* proteases do have applications in deproteinization of shrimp and shell waste[19,20], peptide synthesis[21,22], and also as biocatalysts for enzymatic synthesis in the presence of organic solvents[23,24].

In this study, we purified and characterized LasB protease from *Pseudomonas aeruginosa* MCCB 123; and compared its activity with that of commercial protease Savinase® from *Bacillus* sp.

## Materials and Methods

### Identification of protease producing bacterial isolate

Protease producing isolate coded as MCCB 123, an organism isolated from a coir retting ground (Chellanum North, Kerala, India), was characterized phenotypically for its identity following Baumann and Schubert[25].

### Identification by 16S rRNA gene sequencing

The identity *Pseudomonas* MCCB 123 was confirmed by 16S rRNA gene sequence analysis. DNA extraction was carried out by Phenol-chloroform method[26]. Amplification of 16S rRNA

*Correspondence:
Phone: +91 484 2381120; Fax: +91 484 2381120
E-mail: isbsingh@gmail.com (ISBS); dibhara@gmail.com (DJ)

gene was performed according to Reddy *et al*.[27] using universal primers 16 S1 (GAG TTT GAT CCT GGC TCA) and 16 S2 (ACG GCT ACC TTG TTA CGA CTT). The PCR product was separated on 1 % agarose gel, purified using QIAEX II gel purification kit (Qiagen), cloned into pGEM-T Easy vector (Promega, USA), and sequenced using the primer walking service of Microsynth AG, Switzerland. The sequences obtained were matched with the database in Genbank using the BLAST algorithm and ribosomal database project (RDP) release 9[28].

**Purification of protease**

The protease was purified by ammonium sulphate precipitation followed by DEAE-cellulose chromatography. Ammonium sulphate was added to cell-free culture supernatant up to 30% saturation, precipitates removed by centrifugation at 8260 × *g* for 15 min at 4°C and discarded, and continued up to 80% saturation, collected the precipitate the same way and resuspended in 20 mM Tris-Cl buffer (pH 8.50). The enzyme was dialyzed against the same buffer using Amicon UF stirred cell (Millipore Corporation USA, Model 8010), with a 10 kDa cutoff membrane and used for further purification. The enzyme was then loaded on AKTA Prime protein purification system equipped with a C10 mm × 20 cm DEAE cellulose column equilibrated with 20 mM Tris-Cl buffer (pH 8.5). After washing the column with the same buffer, the enzyme was eluted by applying a linear gradient of NaCL from 0-1000 mM at a flow rate of 0.5 mL $min^{-1}$ and fractions of 2 mL were collected. Active fractions were pooled and concentrated by lyophilization.

**Electrophoresis and zymography**

The lyophilized active fractions of the enzyme were subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) using 5% stacking gel and 15% resolving gel at a constant current of 12 mA[29]. Zymogram was performed with 4% stacking gel and 10% resolving gel co-polymerized with 0.1% gelatin as substrate under native SDS-PAGE conditions.

**Protease assay**

Protease assay was carried out following the method of Khembavi *et al*.[30]. An aliquot of 0.5 mL suitably diluted enzyme solution was mixed with 0.5 mL substrate (1% Hammerstein casein in 50 mM Tris-Cl buffer (pH 9) and incubated at 60°C for 30 min. The reaction was stopped by the addition of 0.5 mL 20% TCA; the mixture was centrifuged and the absorbance was measured at 280 nm. One unit of protease activity is defined as the amount of enzyme required to liberate 1 µg tyrosine per mL per min. Assays were carried out in triplicates and the mean value was expressed as unit protease activity.

**Elastase assay**

Elastase activity was determined using Elastin–Congo red (Sigma – Aldrich Co.) as the substrate according to the method of Kessler *et al*.[31] The reaction mixture consisting of 1 mL enzyme solution, 5 mg elastin-congo red, and 1 mL 50 mM Tris-Cl buffer (pH 8) was incubated at 40ºC for 3 h. The reaction was stopped by the addition of 0.1 mL 100 mM EDTA, centrifuged at 8260 ×*g* for 15 min, and the absorbance measured at $Abs_{495}$ nm. One unit enzyme activity is the amount of enzyme which causes an optical density increase by 1 $unit^{-h}$. Assays were carried out in triplicates and the mean value was expressed as elastase activity.

**Protein assay**

Quantification of protein was carried out according to the method of Hartree[32] using bovine serum albumin (BSA) as the standard. Based on the molecular weight and elastase activity the enzyme was identified as LasB protease and subjected for further studies.

**Effect of pH on LasB protease and elastase activities**

Effect of pH on the protease activity was determined over a pH range of 6-12 using the buffers of 50 mM concentrations: sodium phosphate (pH 6, 7), Tris-Cl (pH 8.0, 9.0), glycine-NaOH (10.0, 11.0, 12.0) for 30 min at 60°C. Effect of pH on elastase activity over the same range was determined using the same buffers for 3 h at 40°C. The substrates (casein and elastin – Congo red) were prepared in the respective buffers and assayed under standard conditions.

**Effect of pH on LasB protease and elastase stabilities**

For the measurement of pH stability, the enzyme was incubated in different buffers for 1 h at room temperature and the residual protease and elastase activities were determined under standard assay conditions. The untreated enzyme was taken as the control (100% activity).

**Effect of temperature on LasB protease and elastase activities**

Effect of temperature on protease activity was tested by carrying out the assay at temperature ranges

of 30, 40, 50, 60, 70 and 80°C for 30 min in 50 mM Tris-Cl buffer (pH 9.0), and on elastase activity at the same range for 3 h in 50 mM Tris-Cl buffer (pH 8.0).

**Effect of temperature on LasB protease and elastase stabilities**

Effect of temperature on protease and elastase stabilities was examined by incubating the enzyme at temperatures ranging from 30, 40, 50, 60, 70 and 80°C for 1 h in 50 mM Tris-Cl buffer at pH 9.0 and 8.0, respectively, and the residual protease and elastase activities were measured under standard assay conditions. The non-heated enzyme was considered as the control (100% activity).

**Comparison of LasB protease activity with commercial proteases Savinase®**

Specific LasB protease activity of the partially purified protease was compared with that of the commercial protease Savinase® from *Bacillus* sp. (Sigma-Aldrich Co., Product no P3111) under the assay conditions at pH 9.0 and temperature 60°C.

**Calculation of specific, relative and residual activities**

Specific activity was calculated by dividing the enzyme units with the protein content,

Specific activity (U $mg^{-1}$) = Total unit activity (U $ml^{-1}$) ÷ Total protein content (mg $mL^{-1}$).

Relative activity is the percentage enzyme activity of the sample with respect to the sample, for which the maximum activity was obtained,

Relative activity = [Total activity (U $ml^{-1}$) × 100] ÷ Maximum activity (U $ml^{-1}$)

Residual activity is the percentage enzyme activity of the sample with respect to the activity of the control (untreated sample),

Residual activity = [Activity of the sample (U) × 100] ÷ Activity of control (U)

**Cytotoxicity analysis of LasB protease**

Hep2 cells were seeded in 96-well plates (Greiner Bio-One) with Eagle's minimal essential medium containing 82 mM glutamine, 1.5 $gL^{-1}$ sodium bicarbonate and 10% fetal bovine serum. Purified enzyme in concentrations of 0 to 500 µg $mL^{-1}$ (v/v) was added to the wells in triplicates. Cells in the growth medium (without the enzyme) were kept as control. After 14 h incubation, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was performed and the percentage of inhibition was determined.

Percentage inhibition of cells at each concentration of the enzyme = [100−(Average absorbance (MTT assay) of cells at a particular concentration of the compound ÷ Average absorbance of control cells without the enzyme) × 100)]. Probit analysis for percentage cell inhibition was done with SPSS 17.0 package for Windows.

**MTT assay**

After replacing the medium, 50 µL of MTT (Sigma Aldrich) solution (5 mg $mL^{-1}$ in PBS) was added to each well and incubated for 5 hours in the dark. The control consists of medium alone with MTT added. After incubation, the medium was removed and MTT-formazan crystals were dissolved in 200 µL dimethylsulfoxide. Absorbance was recorded immediately at 570 nm in a microplate reader (TECAN Infinite Tm).

**PCR amplification, purification and sequencing of LasB protease gene**

Genomic DNA was purified using the phenol-chloroform method as described by Sambrook and Russell[26]. The LasB protease primers were synthesized based on Jellouli *et al*.[33] Amplification of LasB protease gene was performed using the primers AAGCGTCGGCCGAGTACTTCG (forward) and GACCGGCATTCCTTCCTGGAG (reverse). The amplification was performed using DNA Thermal cycler (Eppendorf). Amplified PCR products were analyzed on an agarose gel and purified using gel purification kit of Sigma and sequenced using the primer walking service of Xcelris, Ahmedabad.

## Results and Discussion

**Identification of the bacterial isolate**

The strain MCCB 123 was found to be Gram-negative rods that can denitrify, produce pyocyanin and can grow at 41ºC. Further, the molecular characterization based on 16S rRNA gene sequencing confirmed its identity as *P. aeruginosa* and was deposited in GenBank with the accession no. FJ665510. According to Baumann and Schubert[25] and other researchers[13] morphological and biochemical characteristics of MCCB 123 agrees with that of *P. aeruginosa.* 16S rRNA gene sequencing (accession no. FJ665510) further confirmed this identity.

**Purification and molecular mass determination of the enzyme**

Purified enzyme exhibited dual substrate specificities *viz*. protease and elastase activities. Purification resulted in 6.85 and 9.05 fold increase in protease and elastase-specific activities respectively (Table 1).

Table 1—Purification of LasB protease produced by *Pseudomonas aeruginosa* MCCB 123

| Purification step | PA (U/mL) | EA (U/mL) | Total Protein (mg) | Specific PA (U/mg) | Specific EA (U/mg) | Purification fold (PA) | Purification fold (EA) |
|---|---|---|---|---|---|---|---|
| Culture filtrate | 24754.17 | 654.14 | 8.55 | 2895.22 | 76.50 | 0 | 0 |
| $(NH_4)_2SO_4$ Precipitation | 22737.50 | 574.14 | 4.09 | 5559.29 | 140.37 | 1.92 | 1.83 |
| DEAE-cellulose chromatography | 13695.83 | 478.06 | 0.69 | 19849.02 | 692.84 | 6.85 | 9.05 |

The elution profile of LasB protease on the DEAE-cellulose column is shown in Fig. 1. The enzyme eluted between 0.44 M to 0.68 M NaCl (fractions 44-68). The molecular weight of LasB protease was estimated to be 33 k Da by reducing SDS-PAGE (Fig. 2A). Zymogram of the enzyme showed a clearance zone (Fig. 2B). The purified LasB protease enzyme – with both protease and elastase activities - produced by the strain MCCB 123 showed a 33 kDa band in SDS-PAGE. This molecular mass is in close agreement with the previous reports[21,33]. The purified enzyme also exhibited an increase in protease (6.85 fold) and elastase (9.05 fold) specific activities.

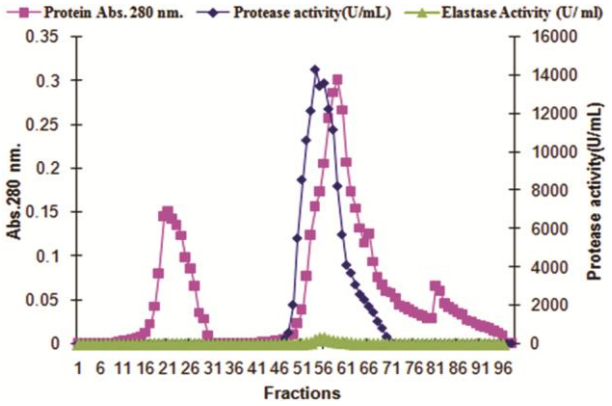


Fig. 1—Elution profile of LasB protease through DEAE – cellulose C 16/40 column

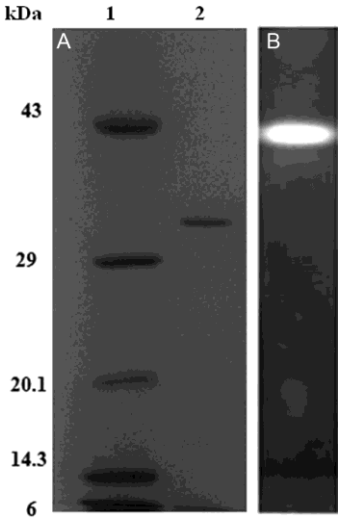


Fig. 2—(A) SDS-PAGE profile of purified LasB protease. Lane 1, molecular weight markers, lane 2, 33 kDa LasB protease; and (B) Zymogram of LasB protease

**Effect of pH on LasB protease and elastase activities and stabilities**

The enzyme showed optimum protease activity at pH 9 and optimum elastase activity at pH 8. The protease activity was stable up to pH 10. The study of the effect of pH on LasB protease stability showed that the enzyme was stable from pH 7.0 to 10 for 1 h and retained more than 90 % of its residual activity from pH 7.0 to 9.0 and 48 % activity at pH 10.0. Elastase activity was stable at pH 7.0, 8.0 and 9.0 with residual activities of 71, 93 and 40%, respectively when compared to the control.

MCCB 123 LasB protease, with its optimum protease activity at pH 9.0, appears to be different from other reported proteases that hydrolyze casein at pH 8[33]. The enzyme is also stable from pH 7.0 to 10.0. The maximum protease activity at pH 9.0 that entails it to be classified under the category of alkaline protease, also makes it suitable in laundry industry, leather processing, and other industrial processes that are carried out in the alkaline pH range. Alkaline proteases are particularly important because they are active and stable under harsh conditions such as high temperatures (50-60°C) and high pH[34-37].

**Effect of temperature on LasB protease and elastase activities and stabilities**

MCCB 123 LasB protease was active over a wide range of temperature from 30 to 80°C with its optimum at 60°C while elastase was active from 30-60°C with its optimum at 40°C. The enzyme retained more than 80 % protease activity and 60% elastase activity from 30 to 50°C.

The protease activity of the enzyme at 30°C makes it useful in detergent industry, which is now looking for alkaline proteases that works well under ambient temperature[38], to maintain fabric quality and also

for reducing the energy demand[39]. The temperature stability profile of the LasB protease activity revealed that the enzyme was highly stable from 30 to 50°C retaining greater than 80% of its residual activity. This high-temperature stability of the enzyme can be utilized in the areas where proteases are exposed to high-temperature conditions such as in the recovery of silver from x-ray films, and in detergent industry.

**Comparison of LasB protease activity with commercial Protease Savinase®**

Activity of MCCB 123 (LasB) protease was compared with commercial proteases Savinase® from *Bacillus* sp. The specific protease activity of purified MCCB 123 LasB protease (14048.91 U $mg^{-1}$) was 3.96 fold higher when compared to Savinase® (3546.40 U $mg^{-1}$). Comparison with the commercial protease Savinase® from *Bacillus* sp. indicates that MCCB 123 LasB protease was 3.96 fold more active suggesting its importance in industrial applications and the possible economic viability.

**Cytotoxicity analysis of purified LasB protease**

The enzyme is nontoxic to Hep2 cells 1-10 µg $mL^{-1}$. $IC_{50}$ value was found to be 47.28 µg $mL^{-1}$ (Fig. 3). Even though 25 µg $mL^{-1}$ concentration of the enzyme inhibited the cells (in terms of MTT assay) morphological changes were absent when compared to the control cells (Fig. 4). Cells exhibited detachment, rounding and shrinkage at 50 µg $mL^{-1}$. Based on MTT assay values the enzyme was nontoxic to Hep2 cells for up to 10 µg $mL^{-1}$ and inhibited 50% ($IC_{50}$) of the cells at 47.28 µg $mL^{-1}$. These results are in consensus with the earlier report of Galloway[40] that elastase (LasB protease) of *P. aeruginosa* is relatively nontoxic with an $LD_{50}$ for mice in the range of 60-400 µg depending upon the root of inoculation.

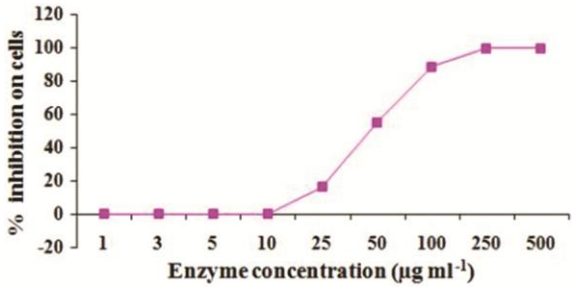


Fig. 3—Toxicity of LasB protease in Hep2 cells in terms of percentage inhibition determined through MTT assay

**Comparison of deduced amino acid sequence of *P. aeruginosa* MCCB 123 elastase with other elastases**

A comparative analysis of deduced amino acid sequence of LasB protease gene of *P. aeruginosa* MCCB 123 with other known proteases is represented in Fig. 5. There are totally 24 amino acids that differ in sequence between MCCB 123 LasB protease and PA7 protease[41], 14 amino acids in propeptide region and 10 amino acids in mature protease region. There is difference in one amino acid in propeptide sequence and 4 amino acids in mature protease region from A2 protease[42] and MN7 protease[33]. MCCB 123 LasB protease gene sequence (accession number JN118955) is 99% similar to that of *P. aeruginosa.* Classification based on the deduced amino acid sequence demonstrated that the MCCB 123 LasB protease is a zinc dependent metalloendopeptidase belonging to M4 neutral protease GluZincin superfamily. The difference in the deduced amino acid sequence of MCCB 123 LasB protease with the other reported proteases[33,41,42] indicates its unlikeness among others.

In conclusion, LasB protease was purified from the culture supernatant of *Pseudomonas aeruginosa* MCCB 123 and characterized. The enzyme exhibited good protease and elastase activities. High protease activity at higher temperatures and alkaline pH, and its higher activity in comparison to commercial protease Savinase suggest its industrial relevance.

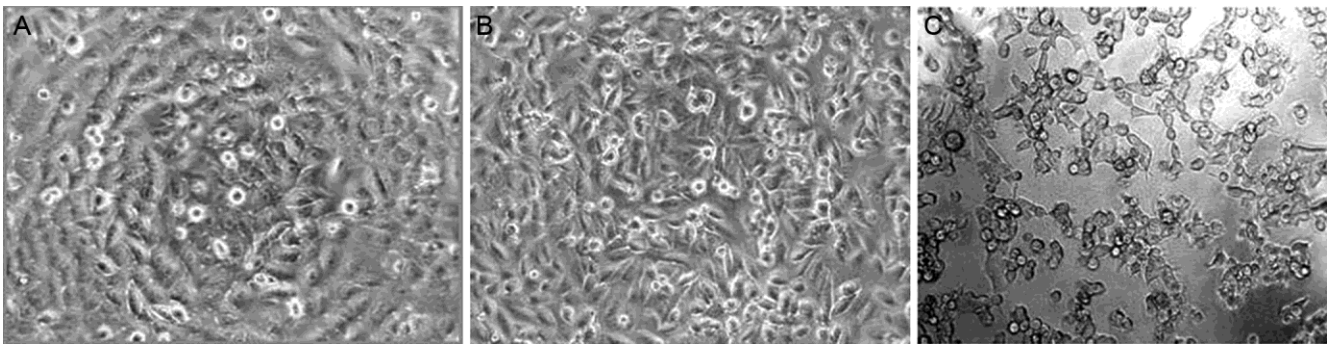


Fig. 4—Effect of LasB protease on Hep2cells (40 x) (A) control without the enzyme; (B) 25 µg $mL^{-1}$ enzyme; and (C) 50 µg $mL^{-1}$ enzyme

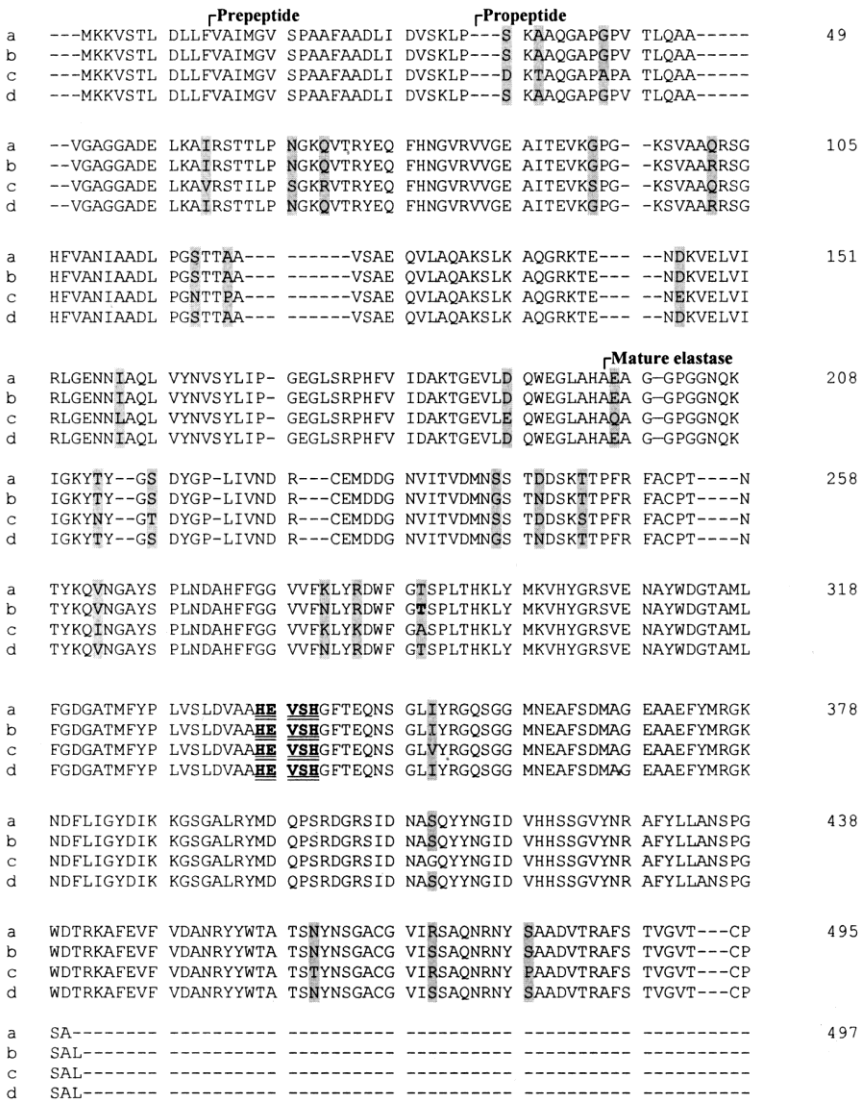


Fig. 5—Aminoacid sequence alignment of *Pseudomonas aeruginosa* MCCB 123 Elastase with other reported elastases. (A) *P. aeruginosa* MCCB 123 elastase; (B) A2 elastase from *P. aeruginosa*[42]; (C) *P. aeruginosa* PA7 elastase[41]; and (D) *P. aeruginosa* MN7 elastase[33]. [The sequences were aligned using Bioedit and multiple alignment was done by CLUSTAL W. Numbers at right hand side correspond to the positions of the individual proteins. Dashes indicate gaps introduced to optimize alignment. The shaded region represents amino acid changes in MCCB 123 LassB protease with other enzymes. The prepeptide, propeptide and mature elastase region are indicated. The double underlined region represents the HEXXH motif (H E V S H) which is the characteristics of M4 metallopeptidase family]

## Acknowledgement

This work was supported by the financial assistance provided by National Centre for Aquatic Animal Health, Cochin University of Science and Technology, and the first author is thankful to Cochin University of Science and Technology for award of Junior and Senior Research Fellowship for the study.

## References

1 Lakshmi BKM & Hemalatha KPJ, Production of alkaline protease from *Bacillus lichemiformis* through statistical optimization of growth media by response surface methodology. *Ferment Technol*, 5 (2016) 1000130.

2 Pathak AP & Rathod MJ, A report on thermostable alkaline protease producing bacteria from terrestrial thermal spring. *Indian J Geo-Mar Sci*, 44 (2015) 1104.

3 Kumar CG & Takagi H, Microbial alkaline protease: from a bio industrial view point. *Biotechnol Adv*, 17 (1999) 561.

4 Baweja M, Tiwari R, Singh PK, Nain L & Shukla P, An alkaline protease from *Bacillus pumilis* MP27: functional analysis of its binding model towards its applications as detergent additive. *Front Microbiol*, 7 (2016) 1195.

5 Sanil JT, Gharibi SOS & Shariati MA, The importance of alkaline protease commercial applications: a short review. *Indian J Res Pharm Biotechnol*, 5 (2017) 5.

6 Sony IS & Potty VP, Screening of protease produced by bacterial isolates of bakery industry. *Int Res J Biol Sci*, 6 (2017) 36.

7 Sharma KM, Kumar R, Panwar S & Kumar A, Microbial alkaline proteases: Optimization of production parameters and their properties. *J Gen Eng Biotechnol*, (2017) http://dx.doi.org/10.1016/j.jgeb.2017.02.001.

8 Anwar A & Saleemuddin M, Alkaline proteases: A review. *Biores Technol*, 64 (1998) 175.

9 Gupta R, Beg QK & Lorenz P, Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl Microbiol Biotechnol*, 59 (2002) 15.

10 Hoikoshi K, Alkaliphiles - from an industrial point of view. *FEMS Microbiol Rev*, 18 (1996) 259.

11 Rao MB, Tanksale AM, Ghatge MS & Deshpandae VV, Molecular and biotechnological aspects of microbiological proteases. *Microbiol Mol Biol R*, 62 (1998) 597.

12 Kumar D & Bhalla TC, Microbial proteases in peptide synthesis: approaches and applications. *Appl Microbiol Biotechnol*, 68 (2005) 726.

13 Karadzic I, Masui A & Fujiwara N, Purification and characterization of protease from *Pseudomonas aeruginosa* grown in cutting oil. *J Biosc Bioeng*, 98 (2004) 145.

14 Bayoudh A, Gharsallah N, Chamkha M, Dhouib A, Ammar S & Nasri M, Purification and characterization of an alkaline protease from *Pseudomonas aeruginosa* MN1. *J Ind Microbiol Biotechnol*, 24 (2000) 291.

15 Caballero AR, Moreau JM, Engel LS, Marquart ME, Hill JM & Callaghan RJO, *Pseudomonas aeruginosa* protease IV assays and comparison to other *Pseudomonas* proteases. *Anal Biochem*, 290 (2001) 330.

16 Tingpej P, Smith L, Rose B, Zhu H, Conibear T, Nassafi KA, Manos J, Elkins M, Bye P, Willcox M, Bell S, Wainwright C & Harbour C, Phenotypical characterization of clonal and non clonal strains *Pseudomonas aeruginosa* strains isolated from the lungs of adults with cystic fibrosis. *J Clin Microbiol*, 45 (2007) 1697.

17 Rattanachuary P, Kanatchote D & Suntinanalert P, Selection of proteolytic bacteria with the ability to inhibit *Vibrio harveyi* during white shrimp (*Litopeaneus vannamei*) cultivation. *Songklanakarin J Sci Technol*, 29 (2007) 235.

18 Chythanya R, Karunasagar I & Karunasagar 1, Inhibition of shrimp pathogenic vibrios by a marine *Pseudomonas* 1-2 strain. *Aquaculture*, 208 (2002) 1.

19 Wang SL & Chio SH, Deproteinization of shrimp and crab shell wastes with the protease of *Pseudomonas aeruginosa* K-187. *Enzyme Microbiol Technol*, 22 (1998) 629.

20 Oh YS, Shih IL, Tzeng YM & Wang SL, Protease produced by *Pseudomoans aeruginosa* K-187 and its application in deproteinization of shrimp and crab shell wastes. *Enzyme Microbiol Technol*, 27 (2000) 3.

21 Ogino H, Gemba Y, Yamada M, Shizuka M, Yasuda M & Ishikawa H, The synthetic rate of dipeptide catalyzed by organic solvent - stable protease from *Pseudomonas aeruginosa* PST-01in the presence of water soluble organic solvents. *Biochem Eng J*, 5 (2000) 219.

22 Ogino H, Yamada M, Watanabe F, Ichinose H, Yasuda M & Ishikawa H, Peptide synthesis catalyzed by organic solvent - stable protease from *Pseudomonas aeruginosa* PST-01 monophasic aqueous - organic solvent systems. *J Biosci Bioeng*, 88 (1999) 513.

23 Geok LP, Razak CNA, Rahman RNZA, Basri M & Salleh AB, Isolation and screening of extracellular organic solvent - tolerant protease producer. *Biochem Eng J*, 13 (2003) 73.

24 Tang XY, Pan Y, Li S & He BF, Screening and isolation of an organic solvent - tolerant bacterium for high yield production of organic solvent - stable protease. *Biores Technol*, 99 (2008) 7388.

25 Baumann P & Schubert RHW, Pseudomonadales, in *Bergy's manual of systematic bacteriology*, edited by NR Krieg & JG Holt (Williams and Wilkins, Baltimore) 1984.

26 Sambrook J & Russell DW, *Molecular cloning: A laboratory manual* (Cold Spring Harbor Laboratory Press, New York) 2001.

27 Reddy GSN, Aggarwal RK, Matsumoto GI & Shivaji S, *Arthrobacter flavus* sp.nov., a psychrophilic bacterium isolated from a pond in McMurdo Dry Valley, Antartica. *Int J Syst Evol Microbiol*, 50 (2000) 1553.

28 Altschul SF, Gish W, Miller W, Myers EW & Lipman DJ, Basic local alignment search tool. *J Mol Biol*, 215 (1990) 403.

29 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227 (1970) 680.

30 Kembhavi AA, Kulharni A & Pant AA, Salt - tolerant and thermostable alkaline protease from *Bacillus subtilis* NCIM No.64. *Appl Biochem Biotechnol*, 38 (1993) 83.

31 Kessler E, Safrin M, Olson JC & Ohman DE, Secreted LasA of *Pseudomonas aeruginosa* is a staphylolytic protease. *J Biol Chem*, 268 (1993) 7503.

32 Hatree E, Determination of protein; a modification of the Lowry method that gives linear photometric response. *Anal Biochem*, 48 (1972) 422.

33 Jellouli K, Bayoudh A, Manni L, Agrebi R & Nasri M, Purification, biochemical and molecular characterization of a metalloprotease from *Pseudomonas aeruginosa* MN7 grown on shrimp wastes. *Appl Microbiol Biotechnol*, 79 (2008) 989.

34 Chen T-L, Chou Y-J, Chen W-M & Young C-C, *Tepidimonas taiwanensis* sp. nov., a novel alkaline-protease-producing bacterium isolated from a hot spring. *Extremophiles*, 10 (2006) 35.

35 Thebti W, Riahi Y & Belhadj O, Purification and characterization of a new thermostable haloalkaline, solvent stable and detergent compatible serine protease from *Geobacillus toebii* strain LBT 77. *BioMed Res Int* (2016) 9178962.

36 Mothe T & Sultanpuram VR, Production, purification and characterization of a thermotolerant alkaline serine protease from a novel species *Bacillus caseinilyticus*. *3 Biotech*, 6 (2016) 53.

37 Sundus H, Mukhtar H & Nawaz A, Industrial applications and production sources of serine alkaline proteases: A review. *J Bacteriol Mycol Open Access*, 3 (2016) 00051.

38 Maurer K-H, Detergent proteases. *Curr Opin Biotech*, 15 (2004) 330.

39 Venugopal M & Saramma AV, 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 (2006) 1239.

40 Galloway DR, *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. *Mol Microbiol*, 5 (1991) 2315.

41 Roy PH, Tetu SG, Larouche A, Elbourne L, Tremblay S, Ren Q, Dodson R, Harkins D, Shay R, Watkins K, Mahamoud Y, Paulsen IT, Complete genome sequence of the multiresistant taxonomic outlier *Pseudomonas aeruginosa* PA7. *PLoS One*, 5(2010): e8842.

42 GhorbelBellaaj O, Hayet BK, Bayoud A, Younes I, Hmidet N, Jellouli K, Nasri M, *Pseudomonas aeruginosa* A2 elastase: Purification, characterization and biotechnological applications. *Int J Biol Macromol*, 50 (2012) 679.