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Biochemical characterization and homology modelling of cold-active alkophilic protease from Northwestern Himalayas and its application in detergent industry

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ABSTRACT

Proteolytic enzymes are one of the largest classes of commercial enzymes used in industrial applications especially alkophilic protease is required by detergent industry. Cold-active alkophilic protease producing psychrotrophic bacterium was isolated from soil sediment of Wular lake Kashmir and identified as *Bacillus subtilis* WLCP1. The molecular weight of purified protease was 38 kDa, determined by SDS-PAGE. The optimal pH of the purified protease was 10.0 and was stable at pH 7.0–11.0. The optimum temperature of the enzyme was 15 °C, lowest to be reported for protease producing *Bacillus* sp. and can work up to 40 °C at pH 10. The protease activity was inhibited by phenylmethylsulfonyl fluoride (PMSF) while improved by Ca^{2+} and Cu^{2+} indicating the enzyme is serine alkaline protease. Purified genomic DNA from *Bacillus subtilis* WLCP1 was cloned and sequenced that encodes a protein of 378 amino acids. Two 3D structure models were built using the structure of Subtilisin E and SUBTILISN BPN' as templates and concluded by Ramachandran Plot. The purified protease from *Bacillus subtilis* WLCP1 with detergent enhanced the removal of blood stains under cold conditions, making it extremely useful as detergent additive for cold washing due to its ability to work in alkophilic pH and low temperature ranges, and first ever to be reported from the North Western Himalayas, exploring the range of habitat for industrial enzymes.

1. Introduction

Protease, a proteolytic enzyme is an important industrial tool capable of degrading proteins that finds its application where ever protein removal is needed (Rao et al., 1998). Alkophilic proteases are type of enzymes that demonstrate maximal activity in alkophilic conditions, mostly between pH 9–11. These proteases have been produced and reported from different sources such as bacteria, plants, fungi, yeast and mammalian tissues but proteases of microbial origin have played crucial role in Biotechnological processes owing to their unrestrained biochemical range (Ellaiah et al., 2002; Prakasham et al., 2005). Globally, *Bacillus* sp. has been one of the most prolific candidate in industrial enzyme market sales, used mainly in detergent formulations, food, pharmaceutical and leather industries (Dias et al., 2008; Saeki et al., 2007). Till now, alkophilic proteases have been largely sourced from *B. subtilis* and *B. licheniformis* strains (Kumar and Takagi, 1999).

With focus growing on microbes present in extreme conditions, temperature is considered as one of the most influential factor that affects the rate of biochemical reactions. Most of the planet earth is covered with vast range of cold-temperature environment, consisting of cold-adapted microorganisms. These microorganisms that adapt and grow at cold temperature regions are known as psychrophiles and psychrotrophs, having the ability to survive within a wide range of temperatures ranging for 0–35 °C (Feller et al., 1996; Margesin, 2009). Psychrotrophs budding at cold regions consist of enzymes that can be vital for broad range of basic and industrial applications mainly due to their metabolic contribution. Enzymes secreted from these psychrotrophs can offer various cost-effective and environmental advantages over the enzymes from microorganisms that work at higher temperatures (Mesophiles) (Brenchley, 1996; Gerday et al., 2000; Gounot, 1991; Huston et al., 2000; Kumar et al., 2002). Enzymes secreted from psychrotrophs have advantage over their *meso*philic counterparts due to their higher specific activity (k_{cat}) and high catalytic efficiency (k_{cat} K_m^{-1}) at temperatures ranging from 0 °C to 30 °C. These characteristics of cold-active enzymes make them an important candidate having large potential for industrial processes and advantageous for

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Biotechnological applications (Acevedo et al., 2013). The high enzymatic activity of cold-active enzymes at low temperatures helps shorten the time process without loss of efficiency, thus reducing the utilization of energy (Kuddus and Ramteke, 2008). Cold-active alkophilic proteases are useful in various industrial applications due to their relative low optimal temperature and stability in alkophilic pH (Kuddus and Ramteke, 2009) and have been reported form numerous microorganisms belonging to different cold habitats. Presently, Biotechnological era demands exploration of various cold habitats in search of novel source for cold-active enzymes with unique properties (Joshi and Satyanarayana, 2013).

In the present study, we are the first to report isolation, purification and characterization along with homology modelling of cold-active alkophilic protease producing psychrotrophic *Bacillus* sp. from one of the largest fresh water lake of Asia (Wular Lake) located in Kashmir, India

2. Materials and methods

Bacteriological media components provided by Hi-Media, India. All other chemicals used were of analytical grade and obtained from leading manufacturers including Sigma and Hi-Media. Reagents for protein estimation, SDS-PAGE and DEAE-Cellulose were purchased from Sigma chemical company, India. *Escherichia coli* strain DH5α and pUC19 were employed for cloning of protease gene and restriction enzymes were provided by Thermo Fisher Scientific, India.

2.1. Isolation and screening of alkophilic protease producing bacterium

Soil samples for isolation of alkophilic protease were collected during the month of December from different parts of Wular Lake (34°20′N 74°36′E) positioned in Kashmir, India. Temperature of the Lake ranges in between –1.5 °C and 8.2 °C in the month of December. The samples were collected aseptically from the upper layer not exceeding 4–5 cm depth. 1 g of each soil sample was serially diluted and plated on nutrient agar medium to attain pure cultures (Hegde et al., 2002).

Bacterial cultures were allowed to grow on alkaline casein agar and skimmed milk agar media, followed by incubation at (4–20 °C). A clear zone around colonies after 48 h incubation indicated protease activity. One isolate showing maximum zone of inhibition was retained for study and additionally purified by repetitive streaking on the same medium for alkophilic protease production. The isolate was designated as WLCP1 and initially identified on the basis morphological, cultural and biochemical characteristics (Bergey et al., 1994).

2.2. Alkophilic protease production and optimization

For production of alkophilic protease by strain WLCP1, a loopful of the bacterial culture was inoculated from an agar plate and transferred into a glass tube containing alkophilic protease production medium with the following composition ($g\,l^{-1}$): Peptone 15.0, Soymeal 5.0, Sodium chloride 5.0, followed by incubation for 48 h at 15 ± 2 °C. The cell-free supernatant was recovered by centrifugation at 8000 *g* for 15 min at 4 °C, filtered through a 0.45 μm pore size membrane filter and used for determining extracellular protease activity.

For evaluating significant factors affecting protease production by strain WLCP1, various sources of nitrogen substrates and carbohydrates (glucose, BSA, casein, skim milk, and malt extract) and incubation time (22, 44, 66, 88, 110, 132 and 154 h) were tested at 15 ± 2 °C using single-factor-at-a time approach and controls were prepared concurrently to compare the data. For optimizing cultural conditions (temperature and pH), broth containing 0.5% tryptone and Soymeal was inoculated with 7 days old broth culture, followed by incubation at different temperatures (4–40 °C) and different pH levels (6–12). The experiments were conceded out in triplicate, and results are the average of three replications.

2.3. Identification of strain WLCP1

Molecular identification of the WLCP1 was achieved by sequence analysis of 16SrRNA gene. The DNA was extracted from the bacterial strain through cell lysis method. The 16S rRNA gene was amplified using the universal primer 27F (5′AGAGTTTGATCMTGGCTCAG3′) and 1492R (5′TACGGYTACCTTGTTACGACTT3′). The PCR reaction was carried out by following thermal cycling conditions: denaturation of DNA template at 94 °C for 3 min followed by primer annealing at 94 °C for 30 s, 50 °C for 60 s and 72 °C for 60 s with final extension at 72 °C for 10 min. Purification of PCR products was carried out by using Montage PCR Clean up kit (Millipore). The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol and sequenced by ABI 3730xl sequencer (Applied Biosystems).

2.3.1. Phylogenetic analysis

16S rDNA sequence of the WLCP1 was made as a query in NCBI blast. Phylogenetic analysis was performed using the software package Molecular Evolutionary Genetics Analysis (MEGA) version 7 (Kumar et al., 2016) after multiple alignment of data by CLUSTAL X (Thompson et al., 1997) and a phylogenetic tree was reconstructed by using the neighbor-joining method of Saitou and Nei (1987).

2.4. Enzyme and protein assay

The protease activity was determined through the modification of the method defined by Takami et al. (1989) by adding 2.5 ml from 0.65% of casein solution prepared within 100 $mmol\,l^{-1}$ Tris-HCl buffer (pH 8.5) onto 0.5 ml enzyme solution, the reaction solution was kept at 15 °C for incubation for 10 min. The control was also performed by using 0.5 ml of Tris-HCl buffer (100 mM, pH 8.5) as a replacement for enzyme solution. The reaction was stopped by adding 2.5 ml of 110 $mmol\,l^{-1}$ trichloroacetic acid (TCA), followed by incubation at 37 °C for 30 min. The reaction mixture was centrifuged at 10,000 *g* for 10 min. By adding 2.5 ml of 0.5 $mol\,l^{-1}$ Na_2CO_3 solution, and 0.5 ml of 0.5 $mol\,l^{-1}$ Folins-Ciocalteus reagent into 1 ml of the supernatant, the mixture was kept at 37 °C for 30 min and absorbance was measured at 660 nm. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg min^{-1} tyrosine. The protein concentration was determined according to the Bradford method, in which bovine serum albumin (BSA) was used as the standard (Bradford, 1976).

2.5. Purification of alkophilic protease

2.5.1. Ammonium sulfate precipitation and dialysis

The cells of WLCP1 were centrifuged at 10,000 *g* for 10 min, and the culture supernatant containing extracellular protease was subjected to ammonium sulfate precipitation. The mixture precipitated through the ammonium sulfate at the range of 0–90% was centrifuged at 5000 *g* for 15 min. The precipitate obtained was dissolved in Tris–HCl buffer (0.05 $mol\,l^{-1}$, pH 8.0) at minimum volume and was dialyzed against the same overnight at 4 °C.

2.5.2. DEAE cellulose column chromatography

Dialysed enzyme (100 ml) was taken and applied to DEAE-cellulose column (2.4 × 45 cm) pre-equilibrated with Tris–HCl buffer (0.05 $mol\,l^{-1}$, pH 8.0). The extraction process was carried with Tris–HCl buffer (0.05 $mol\,l^{-1}$) by maintaining the flow rate of 20 $ml\,h^{-1}$. Enzyme and protein assays were performed on the collected fractions.

2.5.3. SDS - polyacrylamide gel electrophoresis

The molecular weight of the purified alkophilic protease was examined through SDS-PAGE by running 10% polyacrylamide gel for resolving the protein. The Molecular weight was determined with reference to standard molecular weight protein marker, See blue Plus2 Pre-stained Protein Standard (Life Technologies Mol. Wt. 198–3). The staining was done by Coomassie Brilliant Blue R-250 in methanol–acetic acid–water (5:1:5, v/v), and decolorized with 5% methanol and 7% acetic acid in water.

2.6. Characterization of purified alkophilic protease

2.6.1. Effect of pH and temperature on enzyme activity and stability

The optimum pH for enzyme activity was determined by dissolving purified protease with 1% (w/v) solution of casein as a substrate in different buffers: 50 mmol l^{-1} sodium citrate (pH 6.0), 50 mmol l^{-1} sodium phosphate (pH 7.0—10.0) and 50 mmol l^{-1} KCl/NaOH buffer (pH 11.0–12.0) and incubated at 20 ± 1 °C for 1 h. The optimum temperature was measured by studying the hydrolytic activity of purified enzyme at different temperatures (5–50 °C) using 1% casein as a substrate and pH 10. Protease activity was determined by the standard protease assay.

Alkalistability for protease was determined by pre-incubating the purified enzyme without substrate at different pH values (6.0–12.0) followed by 1 h incubation. Temperature stability of enzyme was determined by pre-incubating the enzyme at different temperatures (5–50 °C) and pH 10.0, followed by 3 h incubation. Residual activity was determined by considering maximum activity as the standard reference.

2.6.2. Effect of inhibitors and metal ions on protease activity

Appropriate concentrations of different metal ions and inhibitors were added to the purified enzyme and pre-incubated for 30 min, followed by addition of substrate. Residual activity was determined by the standard protease assay with control considered as maximum (100% of relative activity).

2.7. Statistical analysis

The experiments were conducted in triplicate and were subjected to One-way ANOVA ranked with Duncan's multiple range tests using SPSS 16 Version software. The values are represented as mean with standard errors.

2.8. Construction of DNA library and isolation of alkophilic protease ORF

2.8.1. DNA manipulation techniques

Standard procedures for DNA isolation, restriction enzyme digestion, ligation, competent cell preparation and transformation were used (Sambrook et al., 1989). Genomic DNA was partially digested with EcoRI and Not1 and the DNA fragments were separated with QIA quick gel extraction kit (Qiagen) and cloned into pUC19, resulting in plasmid pWLCP1 which was transformed into *E. coli* DH5α by electroporation using Gene Pulser (Bio-Rad). Transformants were selected on LB agar plates supplemented with 100 μg of ampicillin ml^{-1}, X-gal (20 μg ml^{-1}) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) (40 μg ml^{-1}) and incubated at 37 °C for 48 h. The white recombinant clones were scored and maintained.

2.8.2. Screening the genomic library for proteolytic activity and isolation of ORF of the alkophilic protease

Recombinant clones were screened for functional protease activity on skim milk agar plates. Upon screening 1100 clones, a clone carrying recombinant plasmid was sequenced and the complete ORF of the alkophilic protease was amplified with the primers ALKF (5′-ATGTGCG TCAAGAAAAAAAAATG-3′) and ALKR (5′-TTAGTTAGAAGCTGCTTGA ACG-3′) using the BigDye Terminator sequencing method and an ABI PRISM 3700 sequencer (Applied Biosystems).

2.9. Homology modelling

2.9.1. Target sequence

The primary amino acid sequence of alkophilic protease gene obtained from *Bacillus subtilis* WLCP1 was used as the target sequence for developing the 3D protein models.

2.9.2. Template searching

Template search with BLAST and HHBlits was performed against the SWISS-MODEL (https://swissmodel.expasy.org/) template library (SMTL). The primary amino acid sequence of alkophilic protease gene was searched with BLAST (Altschul et al., 1997) in opposition to the primary amino acid sequence incorporated in the SMTL. HHblits profile was initially built using the procedure outlined in Remmert et al. (2012), followed by 1 iteration of HHblits against NR20. The obtained profile was then searched against all profiles of the SMTL. The highest quality templates were selected for model building.

2.9.3. Model building

On the basis of target-template alignment, models were built using ProMod3. The geometry of the resulting model was regularized by using a force field (Guex and Peitsch, 1997). QMEAN scoring function was used for evaluating the model quality (Benkert et al., 2010).

2.9.3.1. Ligand modelling and oligomeric state conservation. The ligands meeting the certain criteria were included in the model and on the basis of analysis of pair-wise interfaces of the identified template structures, the homo-oligomeric structure of the target protein was predicted (Mariani et al., 2011).

2.9.4. Evaluation of model

In the last step of homology modelling, the model was evaluated for structure validation by the inspection of Ramachandran plot obtained from RAMPAGE (http://www.cryst-bioc.cam.ac.uk/rampage/) (Lovell et al., 2003).

2.10. Application of purified protease on blood stains removal

Application of *Bacillus subtilis* WLCP1 alkophilic protease as a detergent additive was studied as per modified method described by Mothe and Sultanpuram (2016). White cotton cloth pieces were stained with blood and following sets were prepared and then studied:

(a) Blood stained cloth dipped in flask with distilled water (100 ml) + 2 ml of commercial detergent (Surf excel-5 mg ml^{-1}).
(b) Blood stained cloth dipped in flask with distilled water (100 ml) + 2 ml of purified enzyme.
(c) Blood stained cloth dipped in flask with distilled water (100 ml) + 1 ml of commercial detergent (Surf excel-5 mg ml^{-1}) + 2 ml of purified enzyme.
(d) Blood stained cloth dipped in flask with distilled water (100 ml) + 1 ml of commercial detergent (Surf excel-5 mg ml^{-1}) + 2 ml of purified enzyme.

Three sets (a, b, c) were incubated at 15 °C and set (d) was incubated at room temperature for 20 min. After incubation, cloth piece was taken out from each set, washed and dried, followed by examination via digital reflectance meter (Aimil, India). Untreated white cloth piece stained with blood was taken as control.

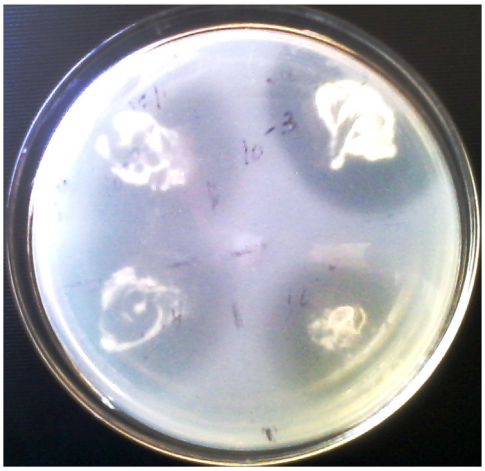

Fig. 1. Protease activity on skim milk.

3. Results

3.1. Isolation and identification of strain

In present study, a total number of 72 pure and morphologically different bacterial isolates were collected and screened on Nutrient agar medium at alkaline pH of 6–12 and temperature 4–10 °C. Out of all the isolates, WLCP1 was selected for further study as it showed maximum clear zone on skimmed milk agar plate (Fig. 1). Strain WLCP1 was found to be Gram positive, rod shaped, non-spore forming and motile bacteria. Biochemical tests indicated that WLCP1 belonged to *Bacillus* species (Data not shown).

3.2. 16S rRNA analysis and phylogenetic studies

The partial 16S rRNA sequence of cold-active alkophilic strain WLCP1 was compared with the existing database by using blastn, showing 99% homology to *Bacillus subtilis* strain MB408 and MB409 ribosomal RNA gene. Phylogenetic tree was constructed using 16S rRNA sequences from closely related species in DNA database of NCBI (Fig. 2). The microorganism was identified as *Bacillus Subtilis* and designated as *Bacillus subtilis* WLCP1.

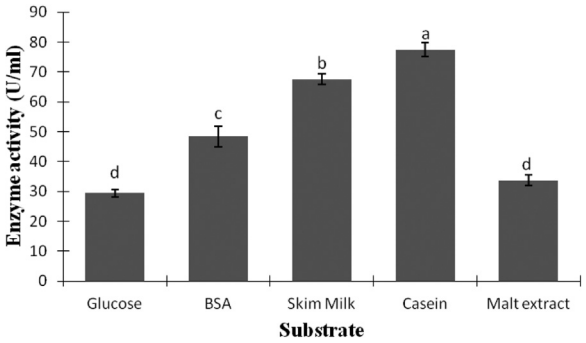

Fig. 3. Alkophilic protease productions in different substrates. Data based on three replications and expressed as mean ± SD.

3.3. Optimization of protease production

A mixture of 6 different carbohydrate and organic nitrogen sources were tested for optimum enzyme yield at 15 °C. The results depicted in Fig. 3 suggest that casein was found to be best substrate for alkophilic protease production with specific activity of 77.5 ± 2.2 U ml^{-1}.

The cells were incubated at different time intervals and maximum enzyme production was recorded at 110 h of incubation (Fig. 4). Isolate WLCP1 was able to grow substantially in a broad temperature range in between 5 and 30 °C, with optimum protease production at 15 ± 2 °C after 110 h incubation (Fig. 5). Similarly, the isolate yielded maximum protease at pH 10 and (15 ± 2) °C (Fig. 6).

3.4. Purification of alkophilic protease

After enzyme was produced, it was precipitated by ammonium sulfate at 55% saturation level under optimum conditions. There was an increase of 2.07 fold in protein purification and yield of 76.53% due to ammonium sulfate saturation. After that, enzyme was subjected to dialysis followed by DEAE cellulose column chromatography where the enzyme was purified to 49.33 fold with a specific activity of 406.12 U mg^{-1} and yield of 29.28% (Table 1).

SDS-PAGE with the help of standard protein marker (Life Technologies Mol. Wt. 198–3) was run against the test sample to confirm the enzyme purity. The molecular weight of the protein was found to be 38 kDa (Fig. 7).

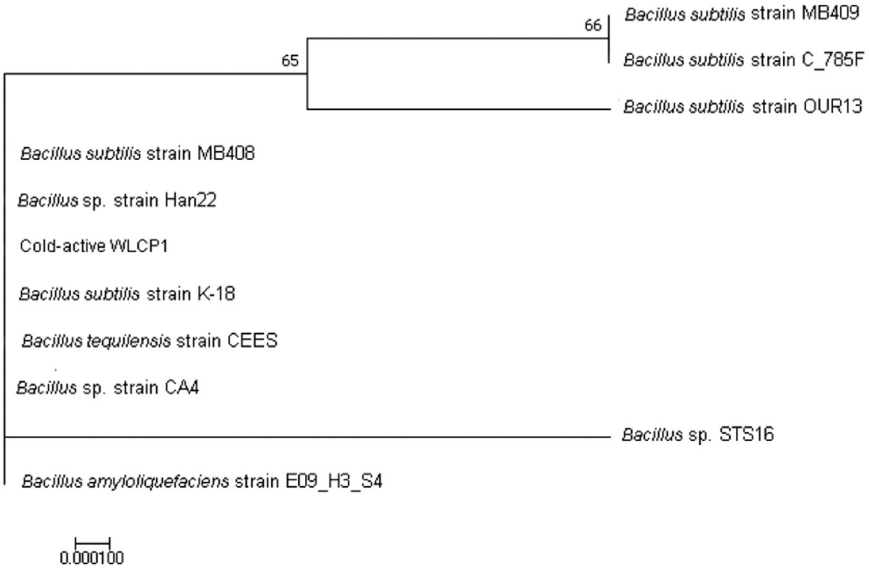

Fig. 2. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA7.

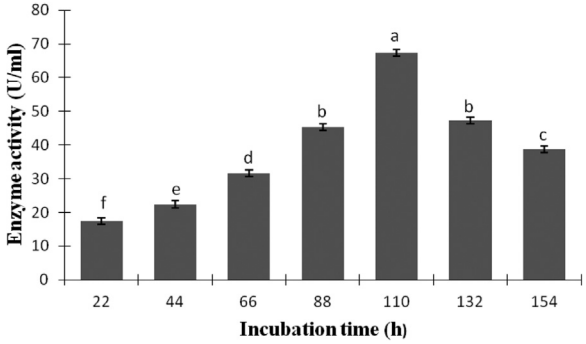

Fig. 4. Protease productions at different time intervals. Data based on three replications and expressed as mean ± SD.

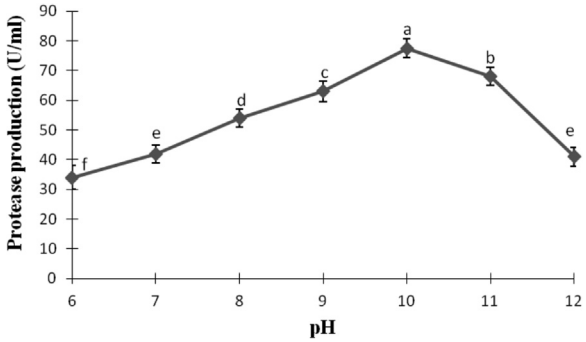

Fig. 5. Effect of temperature on protease productions (110 h incubation). Data based on three replications and expressed as mean ± SD.

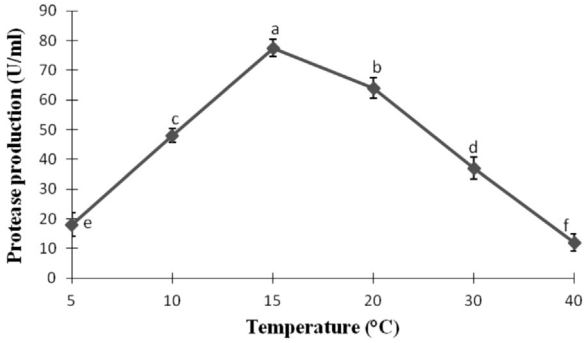

Fig. 6. Effect of pH on protease productions (15 ± 2 °C, 110 h incubation). Data based on three replications and expressed as mean ± SD.

3.5. Effect of pH and temperature on activity and stability of purified alkophilic protease

Purified enzyme showed activity in the wide range of pH (6.0–12.0), with maximum activity at pH 10.0, suggestive of enzyme being alkophilic. The enzyme was stable within the pH range in between 7.0 and 11.0 after 1 h incubation (Fig. 8).

Optimum temperature for purified enzyme was measured by examining the hydrolytic activity at different temperatures and the maximum activity was recorded at 15 ± 2 °C, suggestive of enzyme being cold-active. The enzyme was highly stable within the temperature range in between 5 °C and 30 °C at pH 10.0 after 3 h of incubation (Fig. 9).

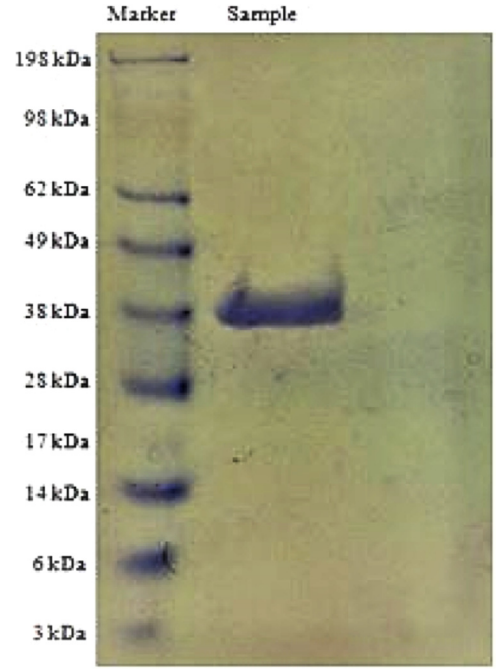

Fig. 7. SDS PAGE of the purified cold-active alkophilic protease from *Bacillus Subtilis* WLCP1.

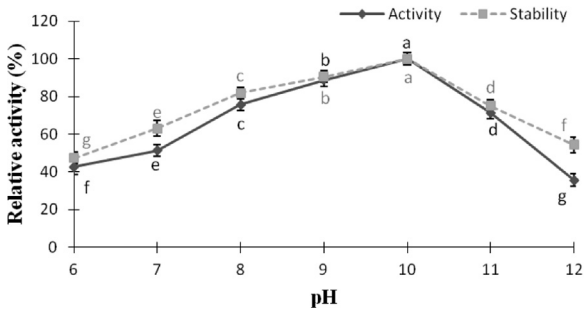

Fig. 8. Effect of pH on purified protease. Maximal protease activity observed was set as 100% relative activity. Data based on three replications and expressed as mean ± SD.

3.6. Effect of inhibitors and metal ions

Purified alkophilic protease was faintly inhibited by EGTA and EDTA but was completely inhibited by PMSF which is a well known serine protease inhibitor. Treatment with cysteine protease inhibitors such as Ni^{2+} and Zn^{2+} had no significant effect. It was partially inhibited by Mg^{2}, Pb^{2+}, Mn^{2+} and Al^{3+}, and completely inhibited by Fe^{2+}. However metals such as Ca^{2+}, Cu^{2+}, enhanced the residual

Table 1
Summary of purification of cold-active alkophilic protease produced by *Bacillus Subtilis* WLCP1.

Purification method	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification Fold	Yield (%)
Crude enzyme	4300	521	8.25	1.00	100
Ammonium sulfate precipitation	3291	192	17.14	2.07	76.53
Dialysis	2658	74.8	35.53	4.30	61.81
DEAE cellulose column chromatography	1259	3.1	406.12	49.22	29.28

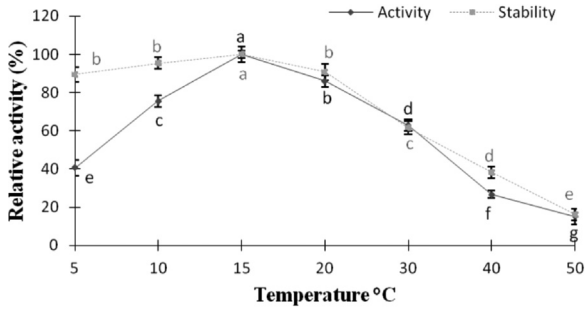

Fig. 9. Effect of temperature on purified protease. Maximal protease activity observed was set as 100% relative activity. Data based on three replications and expressed as mean ± SD.

Table 2
Effect of various metal ions and inhibitors.

Sl. No.	Reagents	Relative activity (%)
1.	Control	100
2.	Mg^{2+}	44 ± 2.1
3.	Pb^{2+}	46 ± 3.5
4.	Mn^{2+}	64 ± 2.3
5.	Al^{3+}	35 ± 3.1
6.	Fe^{2+}	8 ± 2.4
7.	Ca^{2+}	102 ± 1.9
8.	Cu^{2+}	105 ± 3.9
9.	Ni^{2+}	89 ± 3.2
10.	Zn^{2+}	79 ± 2.1
11.	PMSF	0
12.	EDTA	97 ± 2.4
13.	EGTA	94 ± 3.9

Data based on three replications and expressed as mean ± SD.

activity. Results indicate that enzyme is a serine alkaline protease (Table 2).

3.7. Cloning and sequencing of protease encoding gene

Genomic DNA (4 μg ml^{-1}) was isolated using cell lysis method. A cloning vector pUC19 was used to construct a small-insert metagenomic library. Recombinant clones were randomly selected and analyzed, revealing that the average size of ~1.14 kb insert DNA was present within the clones. A total number of 1100 recombinant clones were screened for proteolytic activity. One of the clone carrying recombinant plasmid showed activity against skimmed milk agar by making a clear zone around the colony after 48 h of incubation at 37 °C and was designated as pWLCP1.

The ORF encoding the protease was amplified and sequencing of the insert DNA in this clone revealed an ORF of 1137 bp encoding an alkaline protein with 378 amino acids which showed significant sequence similarity (92%) with serine alkaline proteinase of *Bacillus pumilus* (BAA93474.1) gene as shown in the sequence alignment (Fig. 10).

These results suggested that the cloned protease was closely related to alkaline serin proteinase family. The protein sequence was further used for homology modelling.

3.8. Homology modelling

In order to gain insights about the 3D structure of protein sequence of alkophilic protease from *Bacillus subtilis* WLCP1, homology modelling was carried out using SWISS-MODEL (Fig. 11a). The PDB ID of two similar alkophilic proteinases from Subtilisin E and SUBTILISIN BPN′, 3whi.1.A and 1s01.1.A were taken as templates with 68.69% and 72.96% sequence identity and resolution of 2.4 Å and 1.70 Å respectively. The oligo-state of the target protein was found to be monomer after analyzing both the template structures. The stereo chemical analysis of the modeled proteins was carried out using Pro Mod 3 Version 1.0.2 software. The refined models were analyzed for evaluation of the Ramachandran plot quality by RAMPAGE Software. The Ramachandran plot using RAMPAGE software illustrated that 298 residues (91.1%) were in the most favored region, 21 (6.4%) were in allowed region and 8 residues (2.4%) were in disallowed region, thus making it a stable model (Fig. 11b). Thus these results provided that the predicted 3D structures are satisfactory.

3.9. Wash analysis

Blood stains were partially removed in the presence of detergent only (set A) and enzyme only (set B) whereas blood stains were utterly removed in the combination of detergent and purified enzyme within 20 min of incubation in low temperature at 15 °C (set C). Comparatively the activity was decreased under normal temperature at 40 °C (set D) (Fig. 12a), as suggested by reflectance meter (Fig. 12b).

4. Discussion

Ecosystems with cold temperature are always enthralling for exploration of microorganisms for their cold-adapted proteins and enzymes for the industrial applications (Gounot, 1991). Recently, there has been an increased emphasis on microorganisms existing permanently in cold environment for production of cold active proteases. Different geographical regions have been explored for cold-active protease producing microorganisms regions such as *Bacillus licheniformis* from glacier soil (Baghel et al., 2005); *Stenotrophomonas sp*. from the soil of Gangotri glacier (Western Himalaya, India) and Thajiwas glacier of Kashmir (India) (Kuddus and Ramteke, 2011; Saba et al., 2012). Previously, microbial strains with ability of producing protease have been reported by the extensive application of direct screening by clear zone formation on skimmed milk agar or casein agar medium (Lee et al., 2005; Sookkheo et al., 2000; Zeng et al., 2003; Zhou et al., 2009). Same approach was used in this study to obtain cold-active alkophilic protease-producing microorganism from Wular Lake, Kashmir, identified as *Bacillus subtilis* WLCP1. Psychrotrophic *Bacillus* sp. holds edge over their mesophilic counterparts due to their ability to thrive under extreme conditions. Workers have successfully reported production of cold-active alkophilic proteases from *Bacillus amyloliquefaciens* S94, *Bacillus cereus* and *Bacillus* sp. (Kuddus and Ramteke, 2012). The strain WLCP1 demonstrated characteristics of a cold-adapted microorganism and was classified as psychrotrophic, producing maximum protease at 15 ± 2 °C.

Bacillus subtilis WLCP1 protease had broad pH range with optimal activity at pH 10.0, coinciding with the cold-active alkophilic protease derived from psychrotrophic *Bacillus subtilis* ITRCGG-3 (Baghel et al., 2005) and also with cold-active protease of other microbial species, *Serratia rubidaea* (Doddapaneni et al., 2007). However, the protease was significantly active and stable in a broad pH range (7–11) after 1 h of exposure. This broad range of activity and alkalistability made this protease vital for having wide range of industrial applications.

The *Bacillus subtilis* WLCP1 alkophilic protease showed characteristic features of a cold-active enzyme. The enzyme showed maximal activity at 15 °C, which is lowest to be reported for cold-active alkophilic protease producing *Bacillus* sp; however, only 16% of maximum activity was lost at 20 °C. The enzyme was significantly active at 30 °C; retaining 63% of maximal activity but the protease activity was drastically reduced at 40 °C. The enzyme was sensitive to the temperature at 50 °C. Saba et al. (2012) reported cold-active alkophilic protease from *Stenotrophomonas* sp with maximal activity at 15 °C, but none of the cold-active alkophilic protease has been reported with such low maximal activity from *Bacillus* sp. Enzyme was highly stable within the temperature range of 5–20 °C at pH 10, retaining 94% of activity. The enzyme was relative stable at 30 °C, retaining 63% of maximum activity. However, 73% of activity was lost at 40 °C and nearly completely

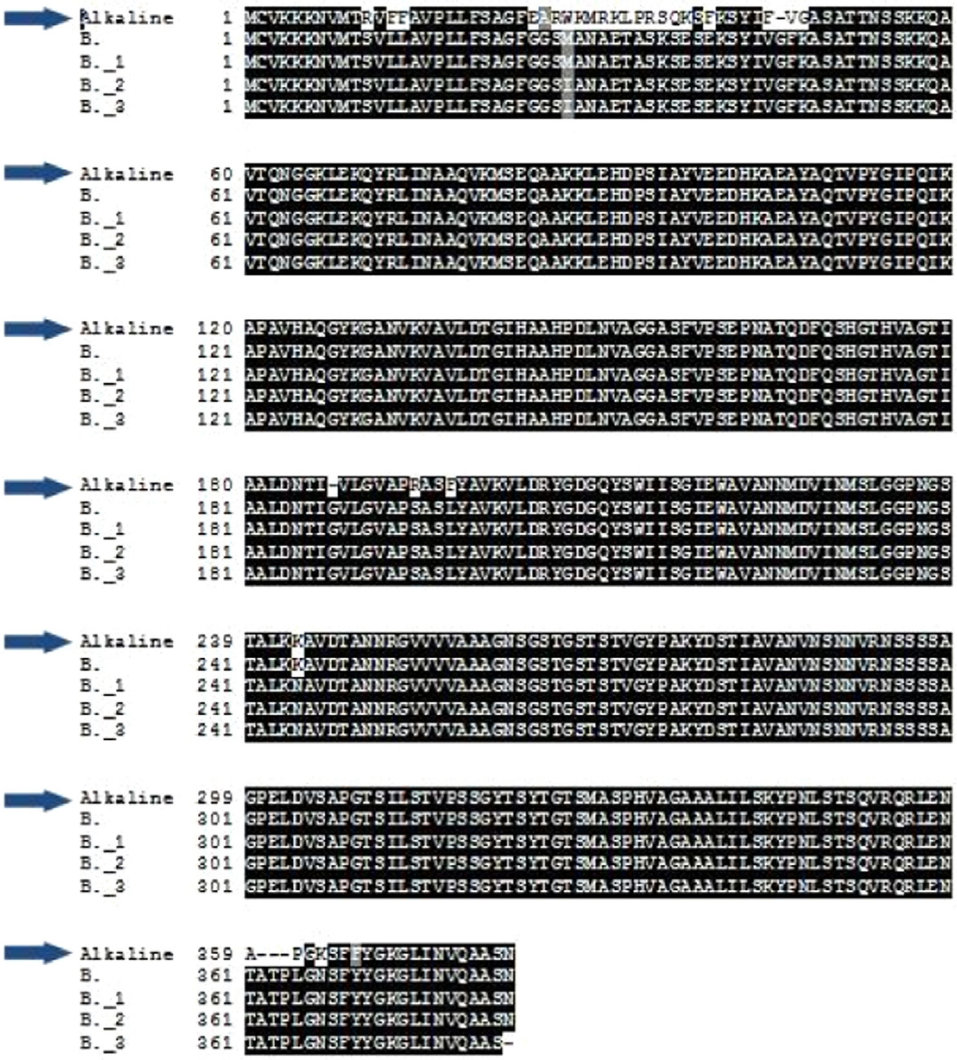

Fig. 10. Multiple sequence alignment of alkophilic protease gene sequence. Proteases used for alignment are alkophilic serine proteinase of *Bacillus pumilus* (BAA93474.1), alkophilic serine protease of *Bacillus pumilus* (BAE79641.1), keratinase Ker1 of *Bacillus pumilus* (ANQ68333.1) and alkophilic serine proteinase of *Bacillus pumilus* (ACO94164.1). The conserved residues are in dark boxes, while partial identity is shown by partially shaded boxes. The alkophilic protease gene sequence is indicated by arrows in the left.

lost at 50 °C after 3 h of exposure. The lowest maximal activity reported from *Bacillus* sp. previously was 20 °C (Joshi et al., 2007). This disparity may likely be related to the relevant genetic and physiological adaptation.

The molecular weight of the protein was found to be 38 kDa. The results coincide with the work reported by Reddy et al. who has reported a 38 kDa organic solvent and detergent stable protease from *Bacillus* sp. RKY3 (Reddy et al., 2008). Moreover, the molecular weight of the isolated enzyme falls in the range of some previously reported work, such as a 34 kDa serine protease has been reported from *B. pumilus* CBS (Jaouadi et al., 2008) and a 35 kDa manganese dependent alkaline serine protease has been reported from *B. pumilus* TMS55 (Ibrahim et al., 2011).

The protease of WLCP1 was tested against various numbers of inhibitors and metal ions. Some of them were well known inhibitors of metallo, cysteine and serine proteases. The results indicate that our protease is a serine alkaline protease because it was totally inhibited by a well known inhibitor of serine protease, PMSF (Kumar and Takagi, 1999).

The cold-active alkophilic protease gene revealed an ORF of 1137 bp encoding an alkophilic protein with 378 amino acids. Similarly, Sadeghi et al. (2010) cloned the serine alkaline protease-encoding gene from *Bacillus subtilis* 168 using pTZ57R revealing an ORF of 1329 bp. The alkaline strain *Bacillus alcalophilus* PB92 gene encoding this high-alkaline serine protease was cloned using pUB110 as cloning vector and sequence analysis revealed an ORF of 380 amino acids (Van der Laan et al., 1991). Cloning of the gene encoding an alkaline protease marks an essential step in the engineering of most efficient producer microorganisms with *E.coli* and *B.subtitlis* being the two microorganism of choice (Gupta et al., 2002). More than half of the enzymes used in various industries are now produced from genetically engineered microorganisms (Hodgson, 1994).

The protein sequence of *Bacillus subtilis* WLCP1 was compared with two template structures of Subtilisin E and SUBTILISIN BPN' for homology modelling using SWISS-MODEL, the model was concluded by Ramachandran plot quality analysis that predicted the stability of the model, being the first to be reported for cold-active alkophilic protease. Similarly a protease model was designed of protein from *Bacillus pumilus* MP 27 compared with the mesophilic bacterium *Bacillus mesentericus* with SWISS-MODEL (Baweja et al., 2016). Homology modelling provides in-depth knowledge of the three-dimensional structure of a protein that in turn helps in understanding the details of a particular protein. Cold-adapted proteins are gaining attention by initially cloning and sequencing the gene and then later building homology

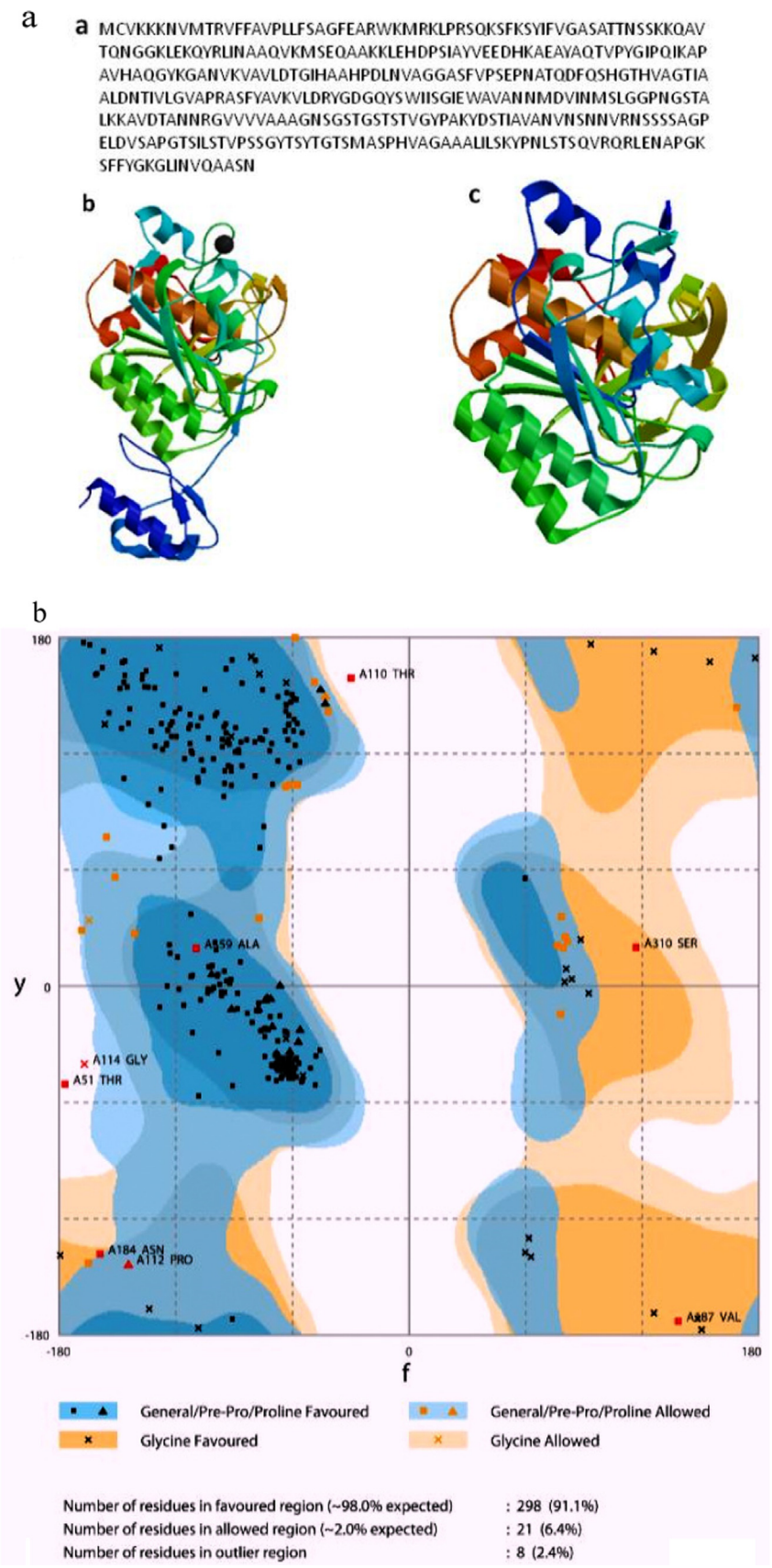

Fig. 11. a (a) Primary amino acid sequence of *Bacillus subtilis* WLCP1 for which templates were searched and models were built. **(b, c)** Secondary structure elements indicated were deduced from template and homology model structures using SWISS-MODEL workspace with crystal structures of template 3whi.1.A and 1s01.1.A from Subtilisin E and SUBTILISIN BPN´. **b** Ramachandran plot analysis of model using RAMPAGE software. It shows the various residues falling in favored, allowed and disallowed region.

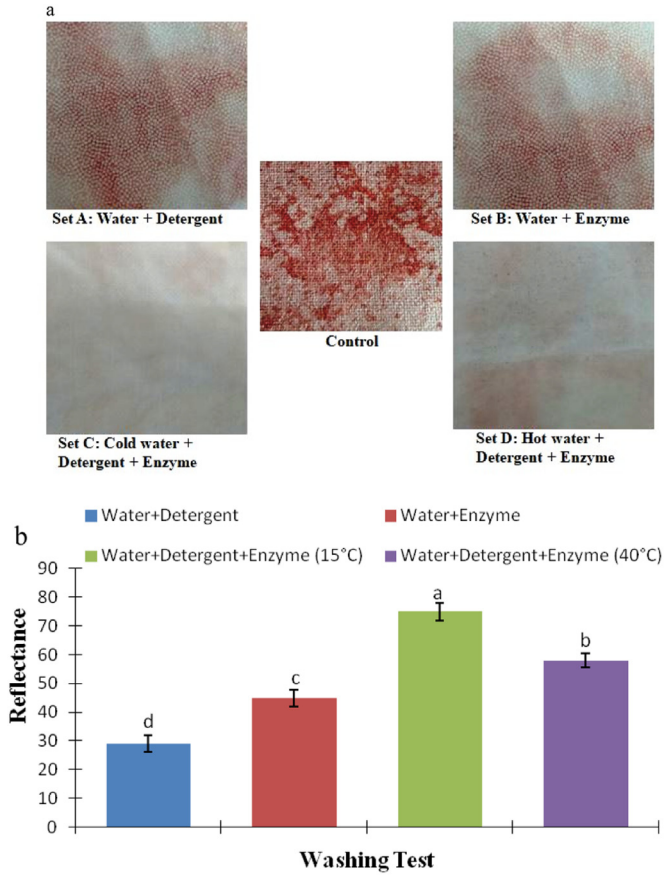

Fig. 12. a Application of purified protease of *Bacillus subtilis* WLCP1 on blood stains removal. Set-A Cloth washed with 2 ml detergent alone, Set-B cloth washed with purified enzyme alone, Set-C cloth washed in cold water with protease (2 ml) + detergent at 15 °C, and Set-D cloth washed in normal water with protease (2 ml) + detergent at 40 °C. Untreated cloth was used as control. **b** Measurement of reflectance of *Bacillus subtilis* WLCP1 alkophilic protease by removing blood stains from the cloth.

models by comparing with other gene sequences of homologous proteins.

The purified enzyme from *B. subtilis* WLCP1 along with detergent completely removed the blood stains from the cloth after 20 min of treatment at 15 °C but the reflectance was decreased by 16% ± 2.4 at 40 °C. The results indicate that the enzyme is more suitable as detergent additive for cold washing, with potential application in low-temperature that may be exploited commercially. Other workers have also reported cold-active proteases derived from various microbial species as potential candidates for detergent industries, requiring low temperatures (Kuddus and Ramteke, 2009; Saba et al., 2012). High protease activity at cold temperature is an imperative characteristic for energy saving in processes performed at low temperature. Moreover, proteases with such ability have become attention grabbing for industrial and biotechnological applications.

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Conflict of interest

The authors declare no conflict of interest.

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