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Thermostable alkaline serine protease from *Bacillus haloduranes* C-125: gene expression and evaluation for its industrial usefulness

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Abstract

Proteases are the group of hydrolytic enzymes which hydrolyzes peptide bonds in proteins showing potential applications in industrial and pharmaceutical sector. Therefore proteases production from novel strain with efficient characteristics of biotechnological interest is significant. A putative protease encoding gene (Apr) from alkaliphilic bacterium *Bacillus haloduranes* C-125 was successfully cloned and expressed in *Bacillus subtilis* expression system. Crude recombinant enzyme with specific activity of 41.5 U mg^{-1} was partially purified using ammonium sulfate precipitation, dialysis and ultrafiltration. The partially purified fraction was 2.8 folds having the specific activity of 116 U mg^{-1} of protein. The molecular weight of mature peptide was estimated as 28 kDa on SDS-PAGE. Zymographic analysis also showed the clear band on the same position. The partially purified enzyme was investigated for activity inhibition in the presence of various protease inhibitors. Serine protease nature of this enzyme was confirmed based on inhibition by PMSF. Biochemical characterization of partially purified enzyme demonstrated that it was highly active in alkaline conditions (pH10-13) and at high optimum temperature (60°C). The protease activity was studied in the presence of various oxidants, surfactants (sodium dodecyl sulphate (SDS), Triton X-100, Tween 20, H_2O_2, sodium perborate) and various metal ions. Enzyme showed high stability in presence of all additives which suggested the potential application of *Bacillus haloduranes* protease in industries specially the detergent market.

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Introduction

Proteases are an important group of enzymes which are ubiquity found in all life forms i.e bacteria, fungi, yeasts, plants and animals. Proteases constitute almost 66% of total enzymes being used in various industrial processes (Gupta *et al.*, 2002). These are commonly classified on the basis of pH optima (acidic, neutral, alkaline) and their functional groups (aspartic, cysteine, metallo, serine).

Alkaline and thermostable proteases are being most widely used in various industries like detergent, food, pharmaceutical and leather (Rao *et al.*, 1998). Among alkaline protease producing bacteria, bacilli are well known potential protease producers of thermostable alkaline protease (Gupta *et al.*, 2002).

Alkaline serine proteases of bacterial sources show high activity at alkaline pH (9-13) and temperature ranging from 35-80°C (Rao *et al.,* 1998; Mokashe *at al.,* 2015).

These characteristics of bacterial alkaline proteases make them potential candidates for various industrial processes where enzymes need to withstand high pH and temperatures. Moreover, alkaline proteases are halotolerant too that has additional advantage of these kinds of enzymes being capable to work efficiently in processes and formulations which involve high concentration of salts (Joo and Chang, 2005; Mokashe *at al.,* 2015) which suggest use of these proteases in application under saline conditions.

For successful application of alkaline proteases in industrial processes, the high stability and activity of enzyme towards anionic surfactants and oxidants is desirable.

Many surfactants and oxidants are common ingredients of detergent formulations now a days. Many alkaline proteases do not fulfill these requirements. So, there is a need for continued exploration of such alkaline protease producers. Thermoalkaline proteases have been reported to show better washing properties which suggest the use of these proteases in detergents for removal of proteinaceous dirt from fabric. If proteases used in detergent formulations are both alkaline and thermostable, the washing performance is enhanced (Banik and Prakash, 2004).

Bacillus halodurans C-125 is reported as an alkaliphilic bacterium with growth pH ranging from 7–10.5. The genome of this *bacillus* strain has been successfully sequenced which revealed that around 4000 protein coding sequences (CDSs) comprise the whole genome. Among these CDSs, several protease genes have been reported, and one of the gene is designated as thermostable alkaline protease. We selected this gene for study because of importance of thermostable alkaline proteases for industrial applications. Interestingly, biochemical characterization of this gene has not been studied yet. In view of this, the present study was designed to biochemically characterize the thermostable alkaline protease from *B. halodurans*-c125 for the specific properties which are desirable in an enzyme for its successful application in detergent industry.

Materials and methods

Reagents, plasmid and bacterial strains

All chemicals used in this study were purchased from Sigma-Aldrich (MO, USA). The strains used as cloning host was *Escherichia coli* JM109 (Promega; WI, USA) and expression host was *Bacillus subtilis* 1A751, a protease deficient strain which was obtained from the BGSC *(Bacillus* Genome Stock Center; OH, USA).

The integration vector used for expression in *B. subtilis* was pSG1154. Bacteria were cultured either in LB (Luria-Bertani) broth (for both *E. coli* and *B. subtilis*) or TBAB (tryptose blood agar base) for propagation of *B. subtilis* media at 37° C.

Vector construction and gene expression

The thermostable alkaline protease was resynthesized by *E. coli* (DNA 2.0; CA, USA) for optimal codon usage. The synthesized gene was then amplified by

PCR using primer pair (Bhal-5; cctaagATGAGACAAAGTCTAAAAGTTATGGTTTTGT CAACA and Bhal-3; cgaagcttggccgcTTATTGTGTTGCACGTCCAGCATG) and further subcloning was done into the pSG1154 vector at *Avr*II and *Hind*III restriction sites which were designed at 5´ and 3´ ends of the gene, respectively, to obtain the expression plasmid pSG1154-B.hal-apr in *E. coli* expression system.

The DNA products were subjected to agarose gel electrophoresis and DNA fragments were purified from the gel using Wizard®SV Gel and PCR Clean-Up System by following the manufacturer's protocol and all constructs were verified through DNA sequencing. The plasmids pSG1154 (vector control) and pSG1154-B.hal-apr (construct) were both transformed into protease deficient *B. subtilis strain* 1A751 by integration into the bacterial chromosome using standard protocols found on the BGSC website (http://www.bgsc.org/ catalogs/Catpart4.pdf).

Induction of protein expression was done with xylose (0.8%) when OD of fresh culture of *Bacillus subtilis* 1A751-B.hal-Apr in LB broth reached at 0.5 at 600 nm. Culture was allowed to grow overnight after induction at 225 rpm at 37°C.

Protease activity assay

Proteolytic activity was calculated by using casein as described by Majeed *et al.,* (2013) (Majeed *et al.*). In general, 1mL of casein (1%) and I mL of purified enzyme were mixed after preincubation for 30 min at selected temperature and pH separately.

The mixture was then incubated for 20 min at 60°C. After incubation the reaction was stopped with the addition of 3mL of 5% trichloroacetic acid (TCA) and allowed to settle for 10 min at room temperature.

The reaction mixture was centrifuged at 11K×g for 5 min and the amount of tyrosine released in the supernatant was measured at 275nm. One unit of proteolytic activity is the amount of protease required to liberate1 μg of tyrosine in one minute.

Fractionation with ammonium sulfate, dialysis and ultrafiltration

One L growth medium was inoculated with *Bacillus subtilis* 1A751-B.hal-Apr and expression of protease was induced overnight by adding xylose (0.8%) at OD_{600} of 0.5.

Culture supernatant was obtained by centrifugation of the culture broth at 5K × g for 20 min and precipitated by slowly adding the ammonium sulfate up to 70 % saturation. The resulting precipitates were separated by centrifugation at 15K × g for 20 min.

The obtained precipitates were resuspended in a minimal amount of buffer (0.1 M Tris/HCl, pH 7.5) and dialysed against the same buffer.

The dialyzed fraction was concentrated by ultrafiltration (Millipore) and assayed for alkaline protease activity. All purification steps were conducted at 4°C. The protein content was determined by measuring the absorbance at 275 nm.

Determination of molecular weight by SDS-PAGE and zymography

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed using NuPAGE® Bis-Tris Precast Gels according to the manufacturer's guidelines. The molecular weight of the protease was determined using BenchMark™ Protein Ladder (Invitrogen Cat. No. 10747-012) as markers.

Zymography was carried out using the method as described by Laemmeli (Laemmli., 1970) using 0.1% gelatin in 10% polyacrylamide running gel and 4% stacking gel without gelatin. After electrophoresis, the gel was incubated in Triton X-100 (2.5%) at 25 °C for two hours to remove SDS and then incubation was done in neutral buffer (Tris-HCl (0.1 M) containing $CaCl_2$ (1 mM), pH 7.8) at 37 °C for 24 h.

The gel was stained using Coomassie brilliant blue-250, and clear bands of protease were observed in the gel after destaining with 40% methanol and 10% acetic acid in H_2O.

Biochemical characterization of the partially purified protease

Determination of optimum pH

To study the effect of pH on protease activity, the pH of the enzyme sample and casein solution (1%) was adjusted at various pH range 6-13 separately. Reaction was initiated by mixing protease with the substrate and incubation was done for 20 min at 60°C. After incubation the reaction was stopped by adding 5% TCA (3 mL) and enzyme activity was calculated by performing assay at 275 nm as described above. Buffers used were 50 mM sodium phosphate buffer (pH 6.0–8.0) 50mM glycine–NaOH buffere (9-10) and 50mM KCl-NaOH buffer (11-13) for the experiments.

Determination of optimum temperature

In order to determine the optimum temperature for maximum enzyme activity, the protease and substrate solution (1% casein) were heat treated pre-incubated at various temperatures (40–90°C) separately at pH 12 for 30 min. After preincubation both were mixed and the assay was performed at the same temperature as described above.

Thermal stability profile

To check the thermal stability of the partially purified protease, the heat treatment of the enzyme was conducted at different temperatures (50–70°C) at pH 12 for 0, 15, 30, 45 and 60 min. After the heat treatment, the residual activity was calculated by performing the enzyme assay as described above. The non-heat treated enzyme sample was taken as control.

Effects of protease inhibitors and metallic ions on protease activity

Effect of protease inhibitors such as dithio-bis-nitrobenzoic acid (DTT), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), β-mercaptoethanol and DMSO was determined at 5mM by measuring relative activity after preincubating the enzyme for 30 min at 60°C. The activity of enzyme without adding any inhibitor was considered as 100%. Effect of various metallic ions i.e Ca^{2+},Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+} and Zn^{2+} on enzyme activity was studied at 5 mM by adding divalent ions to the reaction mixture. Relative activities were estimated after 30 min of incubation at 60°C. The activity of enzyme sample without any metallic ions was considered 100%.

Effect of Calcium Ions on thermal stability

This study was performed by measuring the enzyme activity at different temperature between 50-70 °C with and without incubating the enzyme with calcium ions (5mM) for 30 min. Results were compared by taking the activity of enzyme incubated at 60°C without addition of calcium ion as 100 %

Effect of surfactants and oxidants on protease activity

Proteolytic activity in presence of selected surfactants and oxidants (sodium dodecyl sulphate (SDS), Triton X-100, Tween 20, H_2O_2, sodium perborate) was investigated to check enzyme stability with these additives. The final concentration of all surfactants and oxidants studied were kept 5% (w/v) and all incubations were done at 60°C for 30 min before measuring the residual activity using assay as described above. All incubations occurred at pH 12.

All experiments mentioned above were repeated at least three times, and each value represents the average of three repetitions. Average values and standard deviations for graph bars were calculated using MS Excel program.

Results

A thermostable alkaline protease encoding gene (Apr) from the thermophilic *Bacillus halodurans* C-125 was selected for this study. The gene sequence (NCBI NC_002570) was resynthesized to conduct codon optimization for expression in *E. coli*.

The amino acid sequence of the protein showed the predicted conserved catalytic triad (Asp-124, His-154 and Ser-307) of known serine proteases. The deduced sequence comprised of 361 amino acids which include the signal peptide (1- 24), propeptide (25-93) and mature peptide (94-361) residues (Fig. 1).

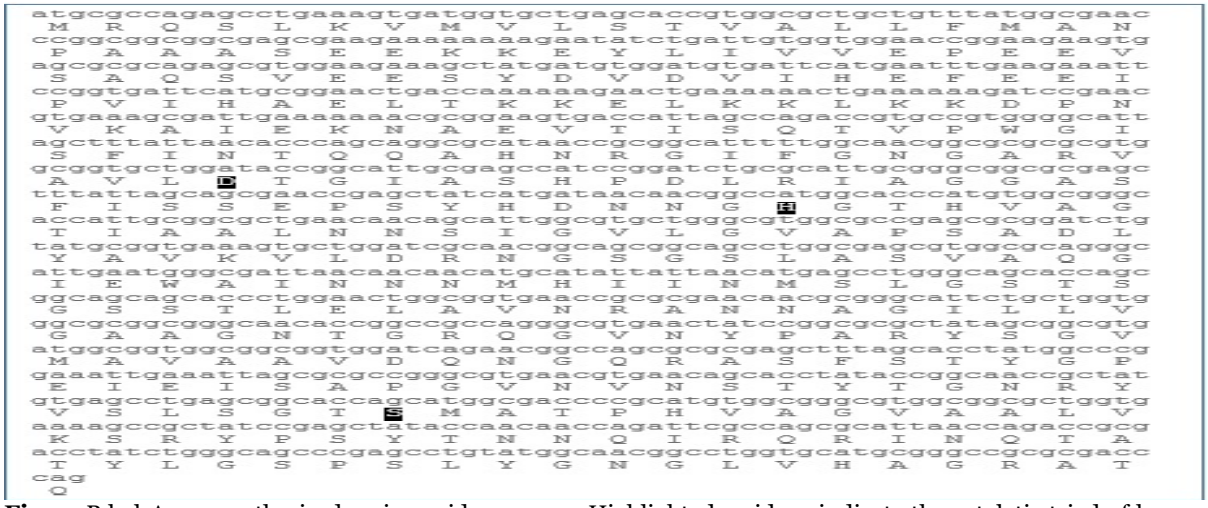

Fig. 1. B.hal-Apr resynthesized amino acid sequence. Highlighted residues indicate the catalytic triad of known serine proteases.

The molecular weight of mature protease deduced from the amino acid sequence was estimated as 27,670 Da. The thermostable alkaline protease gene was cloned in pET29b thus obtaining the recombinant plasmids pET-Apr-hal and the pET-Apr-hal was then expressed under T7 promoter in *E. coli* BL21 (DE3). Cell lysis was done by sonication and after centrifugation, protease activity (data not shown) was detected in the intracellular cell lysate of *E. coli* (pET-Apr-hal). The gene was subcloned into a pSG1154 integration vector under a xylose inducible promoter. Cloning strategy is shown in Fig. 2.

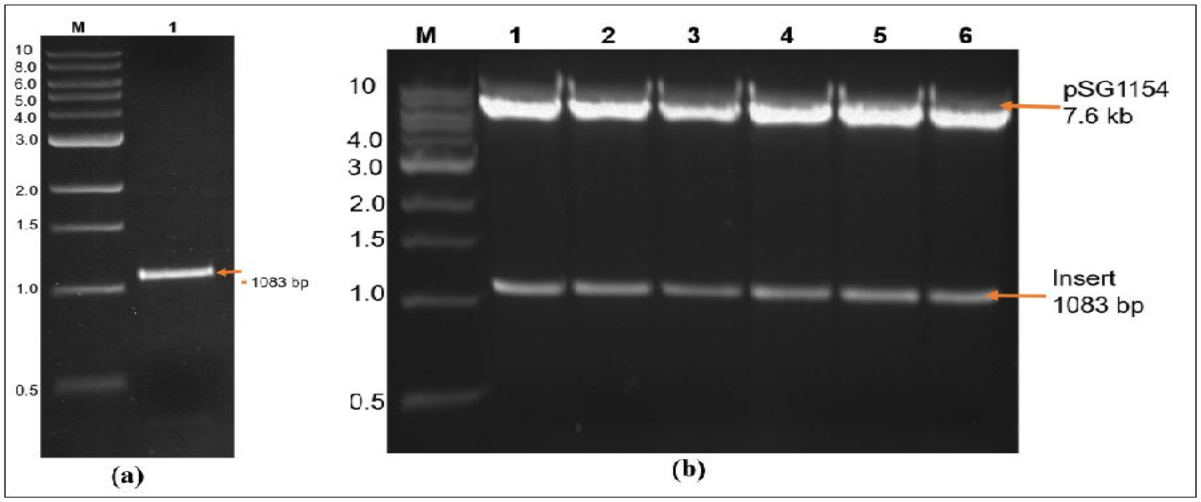

Fig. 2. Agarose gel illustrating the cloning strategy of B.hal-AprT in pSG1154. (a) Amplification of B.hal-Apr gene. Lane 1 represent PCR product for B.hal-Apr. (b) Restriction digestion result of 6 selected colonies. Lane 1-6 represent the corresponding colonies. M: 1kb DNA ladder (NEB, USA).

The expression plasmid (pSG1154-B.hal-Ar) was transformed into *B. subtilis* strain 1A751 and resultant recombinant strain 1A751-B.hal-Apr secrets an active recombinant protease. The screening of transformants for protease production was on LB/agar plates containing spectinomycin (pSG1154-B.hal-Ar harboring the spectinomycin resistance). Further confirmation of recombinants appeared in presence of spectinomycin was done by screening on LB+starch+agar and LB+casein+agar plates. Transformants which formed halo zones on LB+casein+agar plates (Fig. 3) but not on LB+starch+agar plates confirmed the insertion of protease gene.

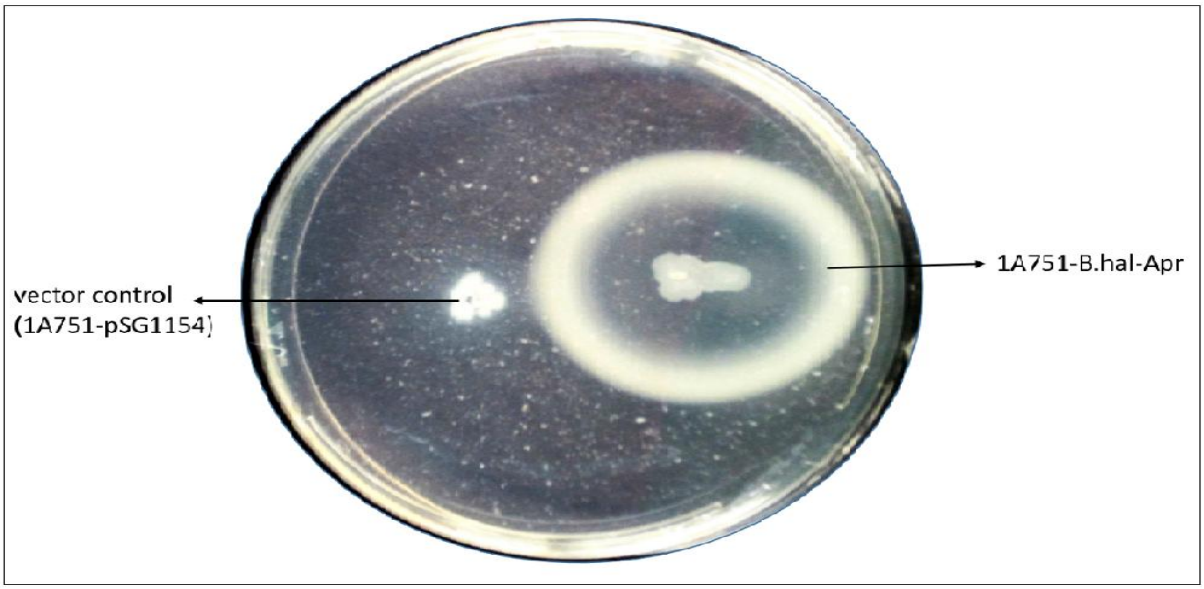

Fig. 3. *B. subtilis* protease expression. Transformed colonies with vector control (left) and pSG1154-B.hal-Apr expression construct (right) were checked on LB agar plates having 1% casein and 0.8% xylose.

Purification of recombinant protease

The overnight bacterial culture (induced with xylose) was harvested by centrifugation and the specific activity of supernatant containing the active recombinant protease enzyme (1A751-B.hal-Apr) was calculated to be 41.5 U mg^{-1}.

This active enzyme was partially purified using ammonium sulfate precipitation method followed by dialysis and ultrafiltration. The partially purified fraction was 2.8 folds having the specific activity of 116 U mg^{-1} of protein. The partially purified enzyme was checked on SDS-PAGE which showed a band of 28 kDa (Fig. 4).

Zymographic analysis

The recombinant thermostable alkaline protease was subjected to SDS/polyacrylamide gel electrophoresis with and without 0.1% gelatin in resolving and stacking gel respectively. A clear zone was observed on gel showing the proteolytic activity on gelatin. The molecular mass of this protein was compared with protease band on SDS-PAGE and was estimated as 28kDa (Fig.4).

Determination of pH and temperature optima

Proteolytic activity of the recombinant enzyme (1A751-B.hal-Apr) was studied over a broad pH range (6-13). The enzyme was found to have the highest activity at pH 12 (Fig. 5(a)). The activity decreased to 77% and 84% when the reaction was carried out at pH 11 and pH 13 respectively.

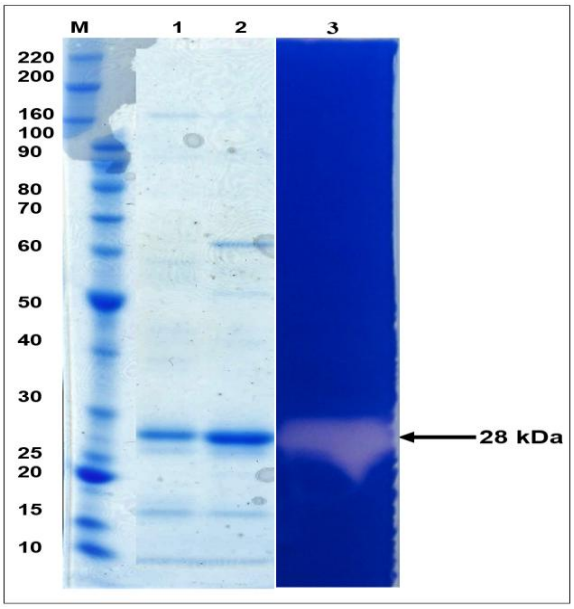

Fig. 4. SDS-PAGE and zymograpy of B.hal-Apr expressed in *B.subtilis*. Lane M: shows BenchMark™ Protein Ladder. Lane 1: represent ammonium sulfate precipitated supernatant, Lane 2: enzyme sample after ultreflitration. Lane 3: Zymography of partially purified Protease.

The optimum temperature optimum was found to be 60°C (Fig. 5b). The protease retained 75% of the

activity at 70°C. Under optimal conditions (pH 12 and 60°C), the enzyme exhibited 274U/mL of activity. Thermo stability of the protease was determined by heat treated the enzyme in a temperature range (50-70°C) for different time intervals and then calculating the residual enzyme activity (Fig. 5c) The enzyme showed high stability at 50 and 55°C retaining 91% and 84% of its activity respectively even after 60 min of incubation.

At 60°C there was a drop in enzyme activity to 67% after one hour. At 65°C, half of the enzyme activity is lost after one hour of incubation while only 30% of the activity remained at 70°C after 60 min.

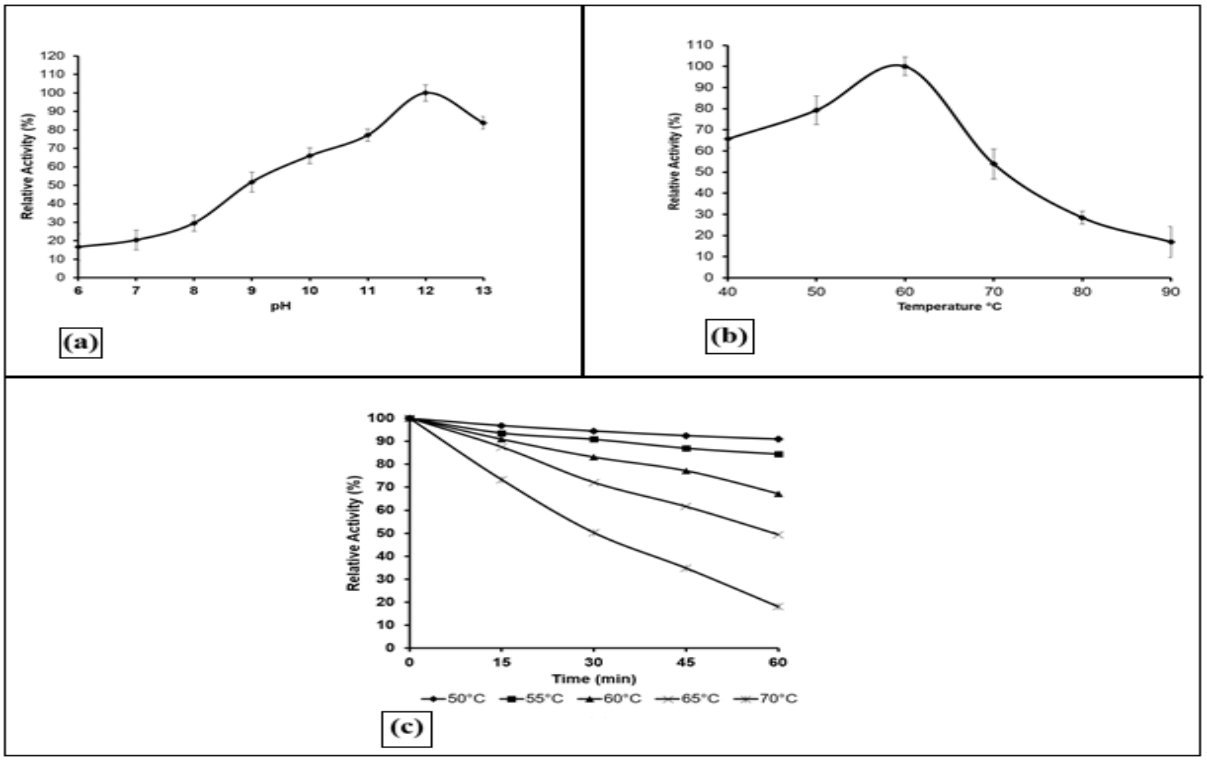

Fig. 5. Effect of pH and Temperature on protease activity. (a) Protease activity at various pH range at 60°C. (b) Protease activity at different temperatures and pH 12. (c) Thermal stability of protease at various temperatures for different time intervals.

Effect of protease inhibitors and metallic ions on protease activity

The nature of the partially purified enzyme was determined by investigating the effect of various inhibitors (β-mercaptoethanol (β-ME), dithiothreitol (DTT), Dimethyl sulfoxide (DMSO), ethylenediaminetetraacetate (EDTA) and phenylmethylsulfonyl fluoride (PMSF) on enzyme activity.

In the presence of the serine protease inhibitor i.e PMSF, the enzyme activity was completely inhibited which indicated that the enzyme under study was serine-type protease (Fig. 6(a)). The proteolytic activity of the enzyme was slightly inhibited in the presence of the DMSO and β-mercaptoethanol while the DTT and EDTA had no effect on enzyme activity.

The effect of various metallaic ions (5 mM) on the activity of partially purified enzyme was investigated (Fig. 6(b)). Enzyme retained almost 100% of its activity in the presence of Co^{2+} and Fe^{2+} and 80% of its activity in presence of Ni^{2+}. However, more inhibition in enzyme activity was recorded with Cu^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} while Ca^{2+} greatly enhanced the enzyme activity to 158% of the control enzyme (without metal ions addition).

As Ca^{2+} dramatically enhanced the enzyme activity, an experiment was designed to find the effect of Ca^{2+} on

thermal stability of enzyme (Fig. 6(c)). Recombinant protease was found more stable in presence of Ca^{2+} even at higher temperature (70°C).

Effect of surfactant and oxidizing agent

The protease exhibited high stability in the presence of all surfactants studied (Fig. 6(d)). All these agents resulted in either small or no effect on enzyme activity. On the other hand the enzyme retained 96% and 90% of its activity in the presence of H_2O_2 and sodium perborate respectively.

Discussion

The importance of alkaliphilic microorganisms in industrial applications is no more a mystery for a long time, and many alkaline enzymes are being commercialized now a days like alkaline proteases, alkaline cellulases and alkaline amylases (Preiss *et al.*, 2015). The major contribution of alkali and heat stable enzymes is in the detergent industry and contributes for almost 30% of the total enzyme production worldwide (Li *et al.*, 2012). An important property of alkaline proteases which are considered goldmine for their industrial application is the stability of protease in presence of various surfactants, oxidants and detergents. Several studies have been reported so far on such kind of proteases (Joo and Chang., 2006; Jaouadi *et al.*, 2008; Haddar *et al.*, 2009; Subba Rao *et al.*, 2009; Anbu., 2013; Majeed *et al.*, 2013).

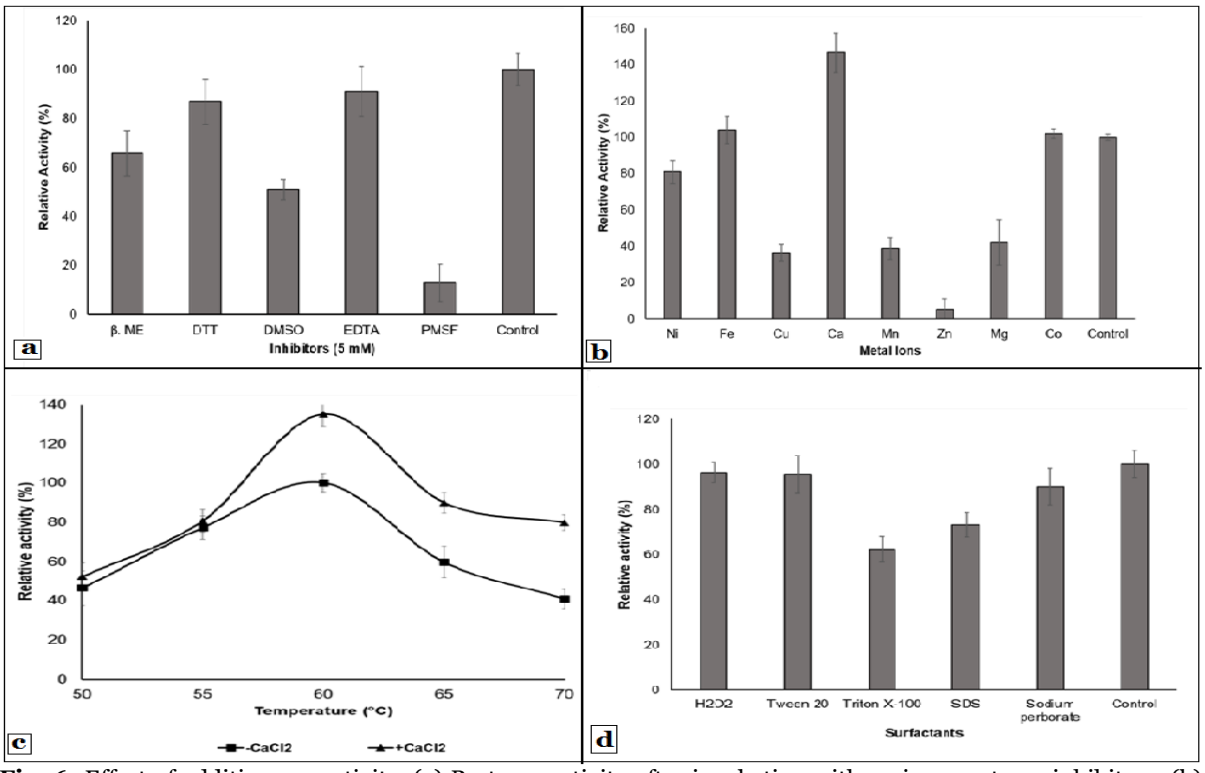

Fig. 6. Effect of additives on activity. (a) Protease activity after incubation with various protease inhibitors. (b) Protease activity after incubation with various metallic ions.(c) Protease Activity after incubation with $CaCl_2$ at various temperatures. (d) Protease activity after incubation with various oxidants and surfactants. Reaction without any additive is taken as control.

Interestingly, very less reports are available on isolation of alkaliphilic microorganisms that can grow at high temperatures as well. The reason behind this is the difficulty of isolating such microorganisms which grow at extreme growth conditions regarding pH and temperature. Moreover, the mechanism to stabilize membranes, cell walls, and proteins at high pH and temperature are not fully understood.

This report described the expression, partial purification, and biochemical characterization of an extracellular thermostable alkaline protease from a *B.*

haloduranes C-125. To the best of our knowledge, this is the first protease which was cloned and expressed in *B.subtilis* expression system from *Bacillus haloduranes* C-125. Expression of the complete protease coding sequence in *B.subtilis* secreted the mature truncated protease 1A751-B.hal-Apr, with a molecular weight of 28 kDa.

The recombinant enzyme was partially purified using ammonium sulfate precipitation method followed by ultrafiltration to concentrate enzyme. The purified protease was subjected to SDS-PAGE and a band of 28kDa confirm the presence of enzyme in extracellular media. Many *Bacillus* species have been reported with variety of molecular masses for proteases: 39.5 kDa alkaline thermostable protease from *Bacillus circulans* (Rao *et al.*, 2009) ; 28 kDa thermostable alkaline protease BPP-A from *Bacillus pumilus* strain MS-1 (Miyaji *et al.,* 2006); 28 kDa *Bacillus megaterium* (Asker *et al.*, 2013) *and* 24 kDa thermostable alkaline protease from *Bacillus sp. MLA64* (Lagzian and Asoodeh., 2012).

Partially purified protease 1A751-B.hal-Apr was studied to determine its pH and temperature optima. The recombinant protease from *B.haloduranes* are among the very few enzymes that are active at high pH (pH 10.0 to 12.0) and high temperature values (60 to 70°C) (Fujiwara *et al.*, 1993; Rao *et al.*, 2009; Olajuyigbe., 2013). Furthermore, *Bacillus haloduranes* protease was confirmed as stable thermally as it retained 50% of its activity at 70 °C for 30 min.

The effect of various additives on enzyme activity were investigated to find its stability and suitability in detergent industry where enzymes need to withstand various surfactants, oxidants and detergent formulations. The recombinant protease exhibited high stability in the presence of all additives tested which suggests that this enzyme may be used as an additive reagent in formulations of laundry detergents. Our results were similar as described by Joo *et al.,* (2003) (Joo *et al.*) and Majeed *et al.,* (2013) (Majeed *et al.*).

1A751-B.hal-Apr was found very stable in the presence of EDTA. The resistance of recombinant enzyme to chelating agents is promising because generally these reagents are indispensable ingredients in detergent formulations (Hagihara *et al.*,2001).

Conclusion

In this study the thermostable alkaline protease from *B. halodurans*-c125 was biochemically characterized for the specific properties which are desirable in an enzyme for its successful application in detergent industry. In conclusion the partially purified recombinant protease 1A751-B.hal-Apr reveals attractive features exhibited by this protease, like the convenient production in *Bacillus subtilis*, the high thermostability, the broad pH range, and its high tolerance to surfactants and organic solvents make this enzyme an attractive candidate for proteolysis under demanding reaction conditions.

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Conflict of interest

The authors of this work declare that they have no conflict of interest.

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