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Partial purification and characterization of metalloprotease of halotolerant alkaliphilic bacterium *Bacillus cereus* from coastal sediment of Goa, India

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We partially purified extracellular metalloprotease of a halotolerant alkaliphilic bacterium isolated from coastal sediment of Goa, India, which was identified as *Bacillus cereus* strain CS1 based on biochemical characteristics and 16S rDNA sequence. Zymogram of partially purified enzyme clearly revealed two prominent protease isozymes of 210 and 215 kDa. Although, protease showed activity over a wide range of pH (pH 8 to 12) and temperature (30, 37, 45 and 50°C), optimum pH and temperature were 9 and 37°C, respectively. Protease exhibited extreme halotolerance upto 8% NaCl making it one of the industrially valuable enzyme. Among different carbon sources, glucose (1%) enhanced maximum enzyme activity (71U/ml), whereas, among the nitrogen sources, tryptone (0.2%) proved best to enhance enzyme activity (158U/ml). Ca^{++} ions enhanced protease activity by 7%, whereas, Cu^{++}, Mg^{++}, Pb^{++} and Hg^{++} ions inhibited activity by 91, 79, 87 and 77%, respectively. Among inhibitors tested, protease activity was significantly inhibited by Na_2–EDTA as residual enzyme activity was only 21%, which clearly confirmed it to be a metalloprotease. The partially purified protease mixed separately with two detergents Rin and Wheel clearly demonstrated significant stain removing capability. Thus, protease produced by *Bacillus* is a potential candidate as an additive in detergent industry.

Key words. Halo-tolerant, isozymes, zymogram, metalloprotease.

INTRODUCTION

Microbes contribute more than 90% of ocean biomass and exhibit great diversity including bacteria, archaea and lower eukaryotes. Marine microbes are reported to play very significant role from being primary producers in ocean and also acting as sources of variety of novel enzymes and antibiotics. A saline environment produces stress to non-halophilic microorganisms by decreasing their water activity and increasing Na^+ concentration inside the cells. However some microorganisms can tolerate and grow from 0 to 10% of NaCl and are referred as halotolerant organisms. Halotolerant organisms constitute a very interesting group of organisms with great potential for use in biotechnology industries because of their optimal activities at high salt concentrations (Setyorini et al., 2006). Recently, considerable interest has been drawn on enzymes of moderately

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Abbreviations: Na_2 –EDTA, Disodium ethylene diamine tetra acetic acid; **PMSF,** phenyl methyl sulphonyl fluoride; **DTT,** dithiothreitol; **SDS,** sodium dodecyl sulphate; **kDa,** kilodalton.

halophilic microorganisms and their biotech-nological potentials (Gupta et al., 2005). This class of halophilic enzymes, while performing similar enzymatic functions as their non-halophilic or extreme halophilic counterparts, exhibits the capability to be active in either presence or absence of salt. Therefore, they have a new potential to be used in some industrial processes where the concentration of salt solutions used would vary with time (Mohapatra et al., 1998).

Among three major groups of industrial enzymes (lipases, amylases, and proteases), proteases alone account for 60% of the total worldwide sale of enzymes, which are derived solely from microbial sources. *Bacillus* is the major producer of most commercial proteases (Beg and Gupta, 2003; Mabrouk et al., 1999). Proteases are among the most commercially significant enzymes used in food, dairy, leather, detergent, pharmaceutical, diagnostics, waste management, silver recovery and protein hydrolysate preparation. Exploration of microbial sources of these enzymes for applications in various industries especially as detergent additives has attracted worldwide attention (Denizci et al., 2004). Microbial alkaline proteases dominate the global market, with a two-third share of the detergent industry (Gupta et al., 2002).

In the present work, we reported partial purification and biological characterization of metalloprotease of halotolerant alkaliphilic bacterium, *Bacillus cereus* strain CS1 isolated from coastal sediment of Goa, India.

MATERIALS AND METHODS

Sample collection and Screening of microorganism

For isolation of alkaline protease producing microorganism, coastal sediment samples were collected from Miramar beach, Goa. 0.1 ml of serially diluted sediment sample was spread plated on 5% skim milk agar (pH 9) and plates were incubated for 24 h at room temperature. Discrete colonies showing clear zones of proteolytic activity around them were picked up and purified through repeated streaking on fresh agar plates. Based on diameter of zone of clearance, the high protease yielding strain was selected for further characterization.

Identification of the bacterial strain

Morphological, cultural and biochemical characteristics of the selected bacterial isolate were studied and recorded as per Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). The bacterial isolate was further confirmed by 16S rDNA sequencing and BLAST search (Sambrook et al., 1989; Altschul et al., 1990). A phylogenetic tree was constructed with MEGA version 4.0 using the neighbor-joining method. The sequence has been deposited in the GenBank database.

Growth V/S Protease activity of the bacterial strain

Growth and protease activity were measured by spectrophotometric method at different time intervals. The bacterial strain was inoculated in nutrient broth (pH 9) and incubated at room temperature (37°C) at shaker speed of 150 rev/min. The culture suspension was withdrawn aseptically every 3 h and growth as well as enzyme activity was monitored spectrophotometrically.

Protease assay

Protease assay of the enzyme, with casein as the substrate, was determined by the modified method of Kunitz (Kunitz, 1947). 200 µl of the enzyme solution was added to 800 µl of the substrate solution (0.5 % casein in 0.1 M Tris-HCl buffer, pH 9) and the mixture was incubated at 37°C. The reaction was stopped by addition of 1 ml of 10% trichloroacetic acid (TCA) and the sample was incubated at room temperature for 15 min. Followed by centrifugation at 12, 000 x *g* for 10 min, the absorbance of the supernatant was measured at 280 nm. A control was run simultaneously, in which TCA was added prior to the addition of enzyme solution. A standard curve was generated with pure tyrosine. All assays were done in triplicates and the mean value was presented along with standard deviation (that is, mean ± S.Date .). Statistical significance (*p*) of the experiments were also determined and expressed in the result. One unit of proteolytic activity was defined as that amount of enzyme, which liberated 1µg of tyrosine/ml/minute under the specific conditions of assay. Protein content of the sample was measured by the method of Lowry (Lowry et al., 1951) using BSA as standard.

Partial purification of the enzyme

Enzyme in the cell free supernatant was precipitated with ammonium sulfate at 70 % (w/v) saturation precipitation and the resulting pellet was dissolved in 0.5 ml of 0.05 M sodium carbonate buffer (pH 10) and dialysed against three changes of the same buffer. The dialysed fraction was tested for protease production and was used for further characterization.

Characterization of extracellular protease

Effect of pH, temperature and salinity on growth and protease activity

Effect of pH on growth and protease activity (Units/ml) of the bacterial strain was determined by growing cells in nutrient broth with different pH in the range of 7 to 12, respectively. Effect of temperature was also determined by growing the isolate at different temperatures (that is, 30, 37, 45 and 50°C) under standard assay conditions. Similarly, effect of salinity (% NaCl) was also monitored using different concentrations of NaCl in the range of 0 to 10%. All experiments were done in triplicates and the mean value was presented along with standard deviation (that is, mean ± S.D.).

Effect of culture components on protease activity

The effect of carbon sources 1% (w/v) and nitrogen sources 0.2% (w/v) on growth and enzyme activity was determined by growing the isolate in minimal salt media with different carbon and nitrogen sources. In this study, various carbon sources such as sucrose, lactose, glucose, fructose and maltose were used. Sources of nitrogen included yeast extract, tryptone, peptone, casein, NH_4NO_3 and KNO_3. All experiments were done in triplicates and the mean value was presented along with standard deviation (that is, mean ± S.D.).

Effect of metal ions and inhibitors on protease activity

The dialysed enzyme was pre incubated for 1 h in various

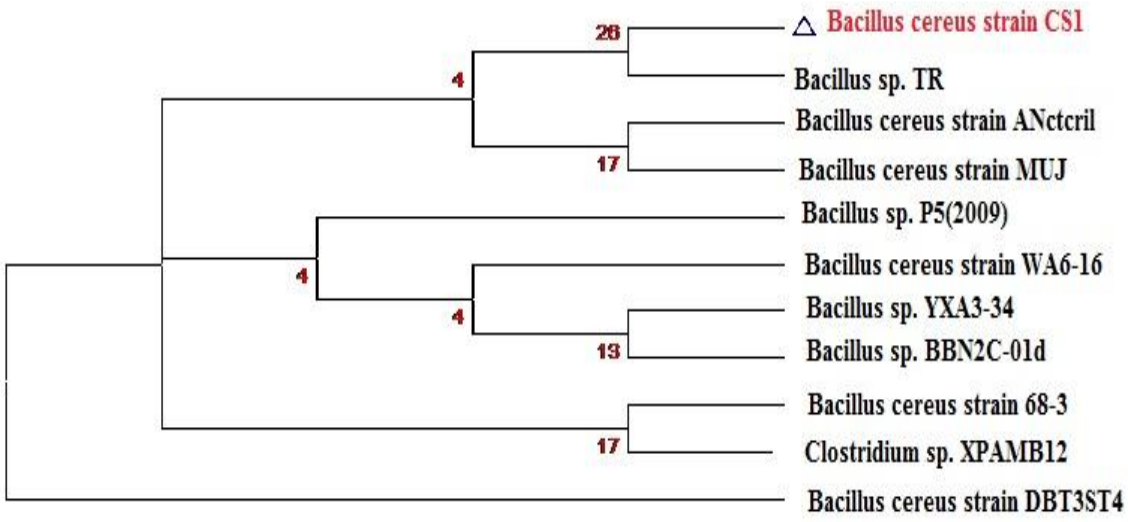

Figure 1. Dendrogram showing phylogenetic relationship between *Bacillus cereus* strain with other *Bacillus* spp. Based on 16S rRNA gene sequences.

concentrations (1, 2.5, 5mM) of different metallic salts ($CuSO_4$, $CaCl_2$, $HgCl_2$, $MnSO_4$, $MgSO_4$, Pb $(NO_3)_2$, $ZnCl_2$) and the enzyme activity (Units/ml) was measured under optimum conditions. For the inhibition studies, the enzyme was pre-incubated for 1 h with 1 mM concentration of different protease inhibitors like phenylmethylsulphonylfluoride (PMSF), β- mercaptoethanol, dithiothreitol (DTT), Na_2-EDTA, and SDS (sodium dodecyl sulphate) and assayed the residual activity (%) at optimum conditions of assay. All experiments were done in triplicates and the mean value was presented along with standard deviation (that is, mean ± S.D.).

SDS- PAGE and zymography

SDS–PAGE was carried out using a 12% (w/v) running gel by the method of Laemmli (1970). For zymography, 0.2% casein (w/v) was co-polymerized with the running gel and samples were then loaded onto the gel without heating followed by electrophoresis with 80 V at 4°C. Following electrophoresis, the gel was incubated in 2.5% (v/v) Triton X-100 (renaturing solution) for 30 min at room temperature with gentle agitation, then decanted and replaced with developing buffer (50 mM Tris, 0.2 mM NaCl, and 5 mM $CaCl_2$, pH 8.0). The gel was equilibrated for 30 min at room temperature with gentle agitation, then was decanted and replaced with developing buffer and incubated at 37°C for at least 4 h and was stained by silver staining.

Activity of partially purified enzyme as detergent additive

Application of protease as a detergent additive was studied on white cotton pieces (5×5 cm) stained with blood and fish curry. The stained cloth pieces were taken in separate flasks (Kanmani et al., 2011) and the following sets were prepared and studied: 1) flask with distilled water (100 ml) + blood stained cloth; 2) flask with distilled water (100 ml) + blood stained cloth + 1 ml (7.0 mg/ml) detergent; 3) flask with distilled water (100 ml) + blood stained cloth + 1 ml (7.0 mg/ml) detergent + 1 ml enzyme preparation.

The above flasks were incubated at 45°C for 15 min. After incubation, cloth pieces were taken out, rinsed with water and dried. Visual examination of cloth pieces exhibited the effect of enzyme in removal of blood stains. Untreated cloth pieces stained with blood and fish curry were taken as control.

RESULTS AND DISCUSSION

Identification of protease producing bacterial strain

The alkaliphilic, facultative aerobic isolate was rod shaped, motile, spore forming and tentatively identified as *Bacillus sp.* based on morphological and biochemical characteristics. This strain interestingly showed significant proteolytic activity at 37°C, as well as, 45°C. Subsequently, BLAST search analysis of 16S rDNA sequence interestingly revealed that this strain showed 100% DNA sequence homology with *Bacillus cereus.* The data of 16S rDNA sequence has already been submitted to GenBank (GenBank accession no. JQ 657725) and the strain has been designated as CS1. Phylogenetic analysis of its16S rRNA gene sequence showed that it is affiliated with the genus *Bacillus* and closely related to *Bacillus* sp. TR (100% sequence identity) (Figure 1).

Growth V/S protease activity

Growth pattern V/S protease activity of *B. cereus* strain CS1 clearly revealed that enzyme activity was highest (that is, 149 Units/ml) during late log phase of growth (12 h) indicating that high level of extracellular alkaline protease enzyme is produced when cells are metabolically more active (Supplementary Figure 1).

Partial purification of protease

It is interesting to note that protease recovery by

Table 1. Purification level (fold) and % Recovery of Alkaline protease activity.

Purification step	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Purification Level (folds)	% recovery of activity
CFS (cell free supernatant) (500ml)	145±3.28	72500	35.63±1.57	17815	4.069	-	-
Ammonium sulphate precipitation 70%, dialysis (5ml)	212 ± 3.05	1060	18.8±1.62	94	11.2	2.8	1.462

*Values are shown as mean ± standard deviation.

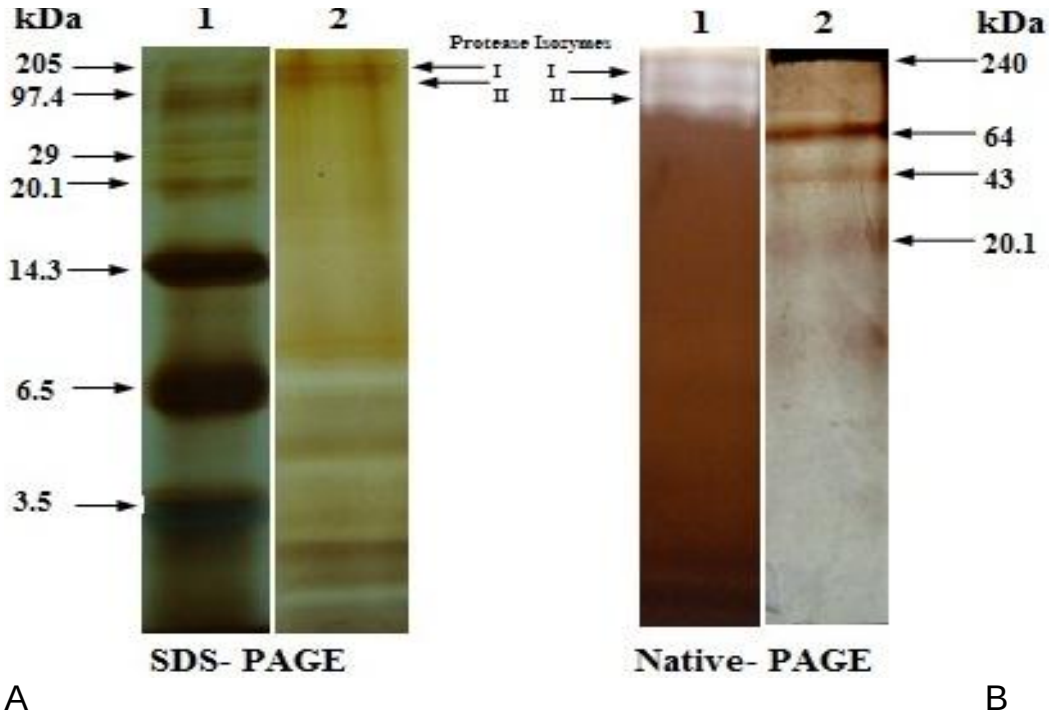

Figure 2. A. SDS- PAGE analysis of partially purified Protease. Lane 1, Broad range protein marker; lane 2, partially purified enzyme sample. **B.** Zymogram of partially purified rotease. Lane 1- Partially purified enzyme; Lane 2- Native PAGE marker.

ammonium sulfate precipitation (70%) was 1.46% with 2.8 fold purification as clearly revealed by the protease experiment (Table 1). Zymogram analysis of the dialysed enzyme sample clearly demonstrated two distinct bands confirming presence of two protease isoenzymes with approximate molecular mass of 210 and 215 kDa respectively (Figure 2). Multiple protease enzymes and isozymes have been reported in a number of microorganisms including bacteria. *Bacillus* sp. produced two protease isozymes of 18 and 66 kDa (Mala and Srividya, 2010), whereas *Bacillus* sp. strain GUS1 has been reported to secrete three alkaline protease isozymes with molecular mass between 30 to 47 kDa (Seifzadeh et al., 2008) and *Pseudoalteromonas* sp. strain P 96 to 47 from King George island, Antarctica produced five metalloprotease isozymes with molecular mass 30.5, 40, 41, 42.5 and 52 kDa respectively (Vazquez et al., 2008).

Effect of pH, temperature and salinity on growth and protease activity

Although growth and enzyme activity was observed in the pH range 7 to 12, significant increase in enzyme activity was observed within pH range 8 to 11 with pH 9.0 being the optimum pH for growth and enzyme activity (140 Units/ml). This clearly indicates that bacterial strain CS1

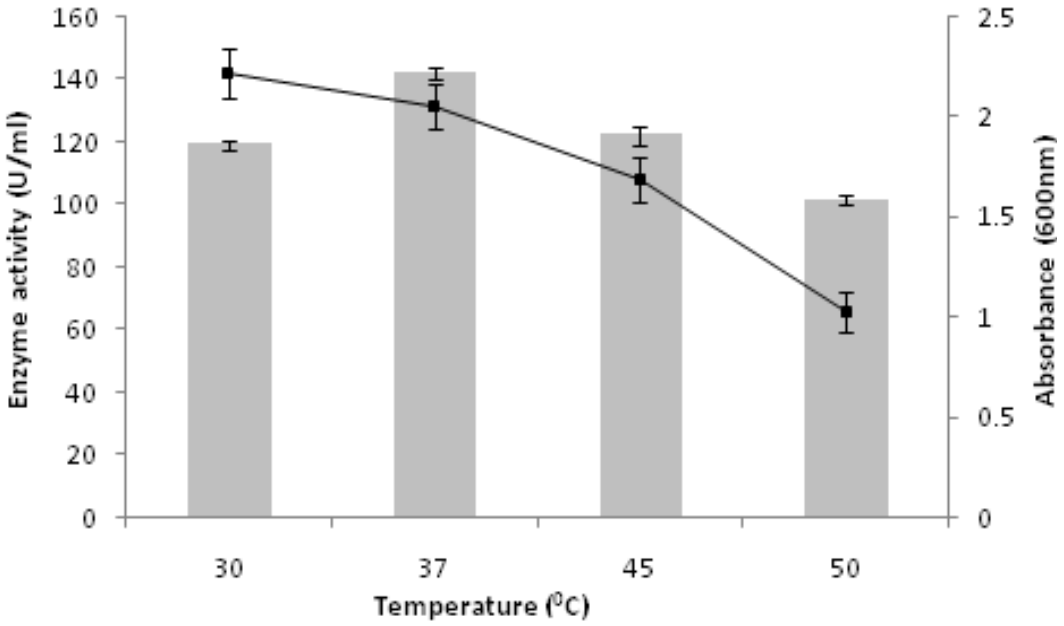

Figure 3. Effect of pH on growth and enzyme activity of *Bacillus cereus* strain CS1 at 37°C.

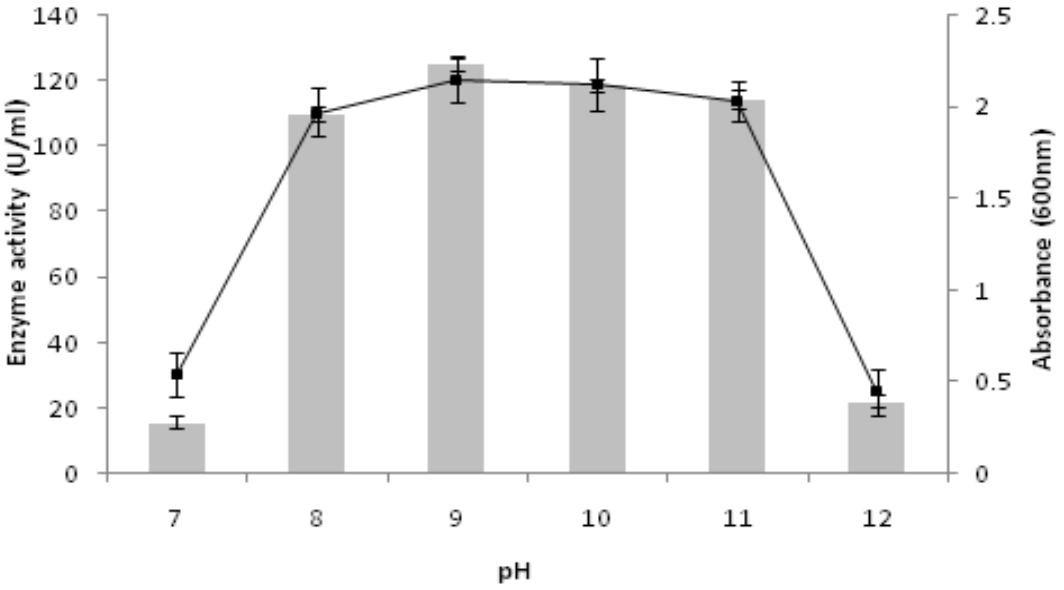

Figure 4. Effect of temperature on growth and enzyme activity of *Bacillus cereus* strain CS1 at pH 9.

is an alkaliphile producing alkaline protease (Figure 3). Similar reports were shown for protease isolated from *Bacillus* sp. APR-4 (Kumar and Bhalla, 2004). Protease undergoes thermal activation above 28°C with maximum growth and enzyme activity (that is, 142 Units/ml) at 37°C followed by thermal inactivation above 45°C (Figure 4).

Similarly, Sharmin et al. (2005) reported highest growth and protease activity at 37°C for *B. amovivorus* isolated from degraded pulse sample and Shumi et al. (2004) showed maximum protease production at 37°C for B. *fastidious* respectively. Alkaline protease enzyme production by *Bacillus* sp. has been previously reported by Feng et al. (2001) and Aoyama et al. (2000). However, here we have reported metalloprotease from alkaliphilic bacterium isolated from coastal sediment sample showing maximum growth and enzyme activity at 37°C.

Although, the isolate *B. cereus* could grow in the range of 0 to 8% NaCl (w/v), protease activity, as well as,

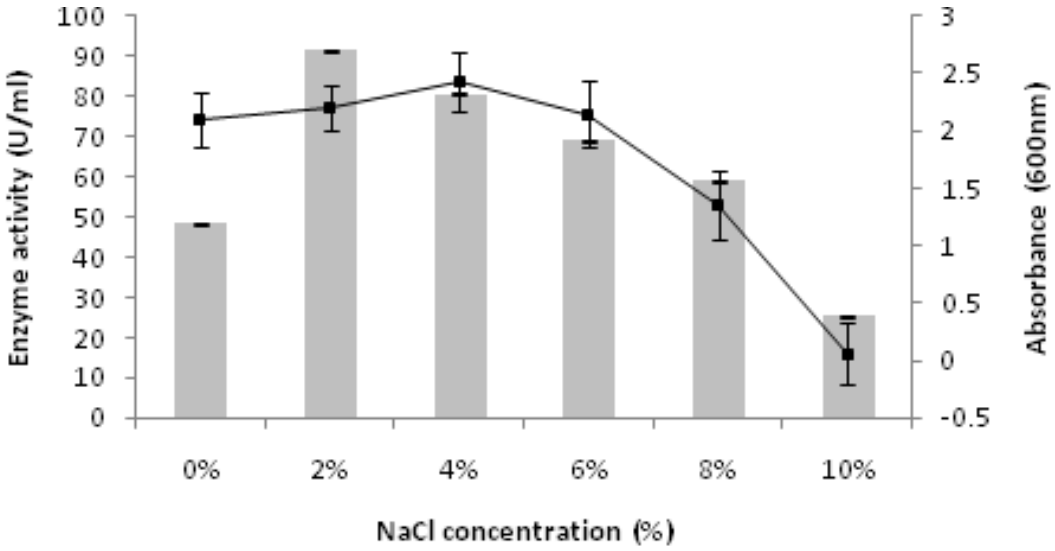

Figure 5. Effect of NaCl concentration on growth and enzyme activity of *Bacillus cereus* strain CS1 at pH 9 and 37°C.

Table 2. Effect of metal ions on alkaline protease residual activity (%) of *Bacillus cereus* strain CS1.

Metal	1 mM	2.5 mM	5 mM
Control	100	100	100
$CuSO_4$	27±1.24	19±0.82	9±0.08
$CaCl_2$	59±2.22	79±2.51	107±3.02
$MgSO_4$	29±1.2	26±0.9	21±1.06
$MnSO_4$	51±1.49	53±1.57	56±1.79
KCl	43±1.63	47±1.71	50±1.82
$Pb(NO_3)_2$	31±1.73	34±1.75	13±0.23
Metal	**0.01 mM**	**0.1 mM**	**1mM**
$HgCl_2$	28±1.25	27±1.27	23±1.12

*The activity in absence of metal ions refers to 100 % residual relative activity of protease; *Values are shown as mean ± standard deviation and statistical significance, $p<0.05$.

growth was maximum in the presence of 2% NaCl as the enzyme activity was 92 Units/ml. It is interesting to mention that enzyme production was significantly reduced above 2% NaCl (Figure 5). These findings clearly indicate the halotolerant nature of *B. cereus* strain CS1. Halotolerance is an added advantage to the alkaline protease enzymes which enhances its potential in industrial processes where the concentration of salt varies (Mohapatra et al., 1998).

Effect of culture components on protease activity

Among the various carbon sources used, glucose (1%) induced maximum enzyme activity (71U/ml) although growth was comparatively less (Supplementary Figure 2). However, sucrose and lactose suppressed both growth and enzyme activity. Nitrogen source had a great influence on protease activity. It was observed that in the presence of inorganic nitrogen sources, that is, KNO_3 and NH_4 $(NO_3)_2$ both growth and enzyme activity ceased. Tryptone showed the maximum enzyme activity (161U/ml), however, there was no significant variation in growth with different nitrogen sources (Supplementary Figure 3). Overall, a change in nitrogen source improved the growth and enzyme activity than a change in carbon source the reason being that production of enzyme required amino acids which were made available by the nitrogen sources.

Effect of metal ions and inhibitors on protease activity

Among the various metal ions tested, Ca^{++} ions enhanced the residual protease activity by 7% (Table 2). These studies suggest that cations activated the protease from *B. cereus*. This result correlates with the findings of Ghorbel et al. (2003) that Ca^{++}, Mn^{++} and Mg^{++} increased the activity of protease from *B. cereus* BG1 at 60°C. While Adinarayana et al. (2003) and Nascimento and Martins (2004) reported that Ca^{++} and Mn^{++} increased the activity of protease from *B. subtilis* PE-11 and *Bacillus* sp. SMIA-2 respectively. whereas Hg^{++}, Cu^{++}, Mg^{++} and Pb^{++} ions strongly inhibited enzyme activity with residual activity of 77, 91, 79 and 87%, respectively. A similar type of result was obtained by other investigators, where alkaline protease from *Bacillus brevis* lost its activity completely in the presence of Hg^{++} (Banerjee et al., 1999)

Table 3. Effect of inhibitors on alkaline protease residual activity (%) of *Bacillus cereus* strain CS1.

Inhibitors (1 mM)	Residual protease activity (%)
PMSF	50.888±1.57
Na_2- EDTA	21.545± 0.99
DTT	24.942±1.05
SDS	52.816±1.59
β- mercaptoethanol	51.559±1.47
Control	100

*Values are shown as mean ± standard deviation and statistical significance, $p<0.05$.

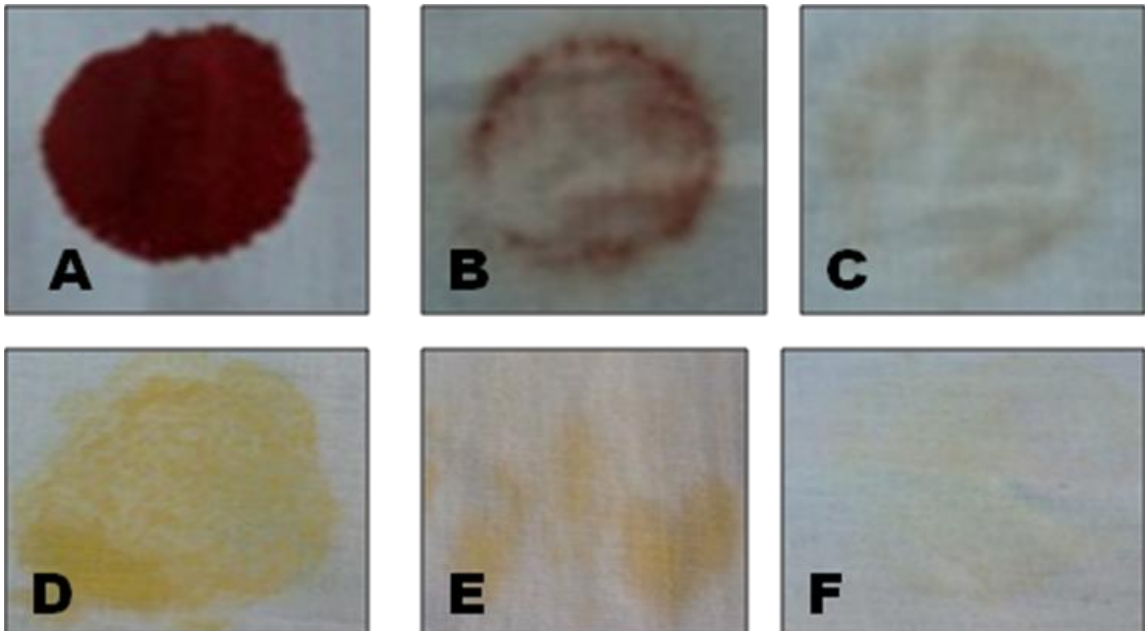

Figure 6. Washing test to assess activity of partially purified alkaline protease preparation from *Bacillus cereus* strain CS1 as a detergent additive. **(A)** Cloth stained with blood. **(B)** Blood-stained cloth washed with detergent (RIN) only. **(C)** Blood-stained cloth washed with detergent(RIN) supplemented with enzyme preparation. **(D)** Cloth stained with fish curry. **(E)** Fish curry stained cloth washed only with detergent (Wheel). **(F)** Fish curry stained cloth washed with detergent (Wheel) supplemented with enzyme preparation.

Among the various inhibitors used, Na_2-EDTA significantly inhibited protease activity of *B. cereus* as % residual activity was only 21%, clearly confirming it to be a metalloproteases (Kuddus and Ramteke, 2008). Therefore it could be a metal-dependent metalloprotease. Calcium cations are known to be stabilizers of many enzymes, protecting them from conformational changes. The Ca^{2+} binding sites examined for some bacterial proteases contain a number of co-coordinating aspartate and glutamate residues (Vordouw et al., 1976). Residual enzyme activity was 52 and 51%, respectively in presence of SDS and β-mercaptoethanol. Similarly, 50 and 45% residual enzyme activity was observed in the presence of PMSF and DTT, respectively (Table 3). Similarly, *Bacillus* sp. protease showed a no inhibition by PMSF, but was strongly inhibited by EDTA (Xin et al., 2011; Lee et al., 2002; Takami et al., 1990; Manachini and Fortina, 1998).

Assessment of efficacy of crude enzyme preparation as detergent additive

The supplementation of partially purified enzyme preparation in detergents such as Rin and Wheel significantly improved their cleansing efficiency as blood and fish curry stains on the cloth disappeared within 15 min (Figure 6). Our finding go hand in hand with earlier findings on *Bacillus licheniformis* strain N-2 which indicated blood stain removing capability of protease

(Nadeem et al., 2008). Similarly, protease from alkaliphilic bacteria and *Pseudomonas aeruginosa* strain PD100 also possessed capability to remove blood stain from the cotton cloth in the absence of detergents (Najafi et al., 2005; Kanekar et al., 2002; and Banerjee et al., 1999). Thus, protease produced by *B. cereus* is a valuable and potential candidate in detergent industry as detergent additive where alkaline conditions, higher processing temperatures and certain additives containing metal ions are necessary to boost the effectiveness of detergents. The supplementation of the enzyme preparation in two detergents that is, Rin and Wheel could significantly improve the cleansing performance towards the blood and fish curry stains.

Conclusion

B. cereus strain CS1 produced two prominent isozymes (210 and 215 kDa) of metalloprotease. This enzyme was active and stable at 37°C and pH 9. Furthermore, the isolate could grew in the range of 0 to 8% NaCl indicating its halotolerant nature; Ca^{++} ions increased the residual activity by 7% indicating it is a metal- dependent enzyme. The enzyme showed promising results in the removal of blood and fish curry stains. Therefore, these characteristics of the protease enzyme suggest that it can be used as detergent additive in detergent industry to improve the performance of the detergent.

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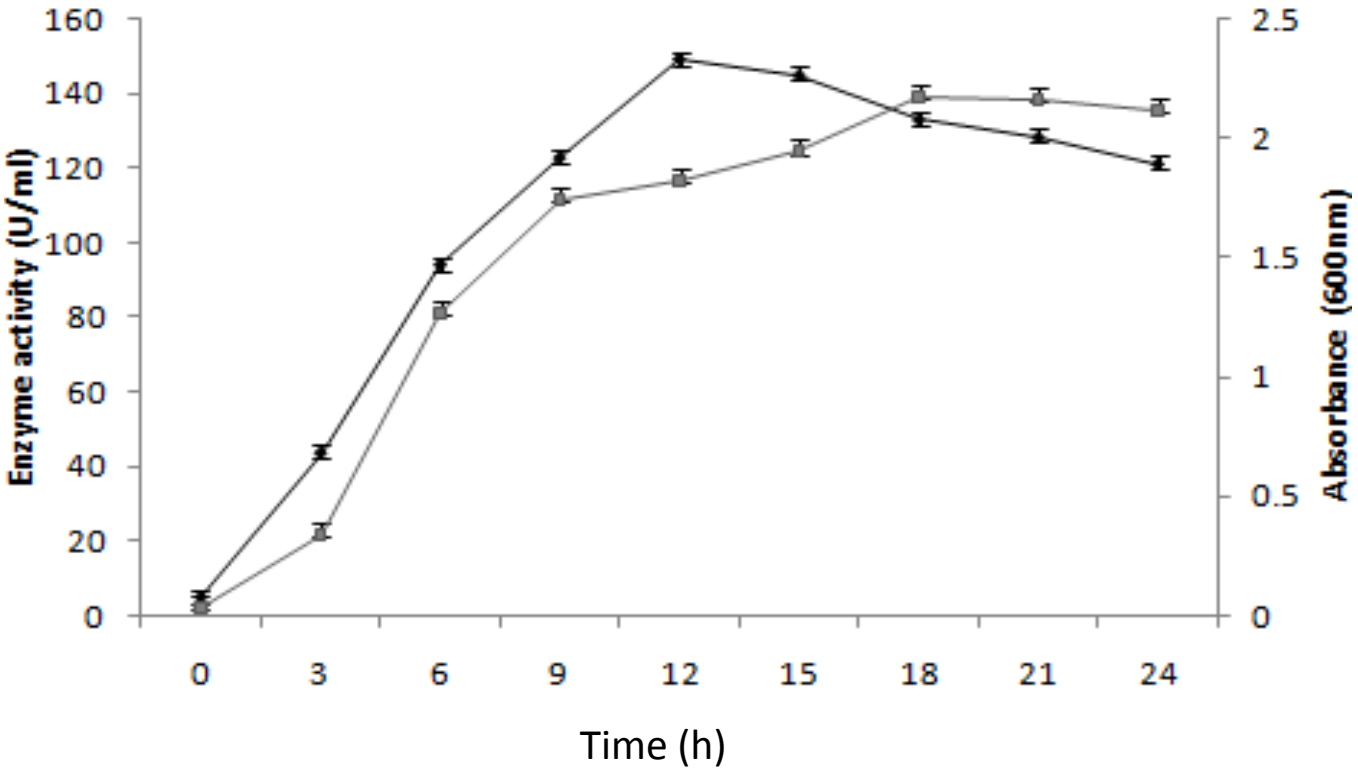
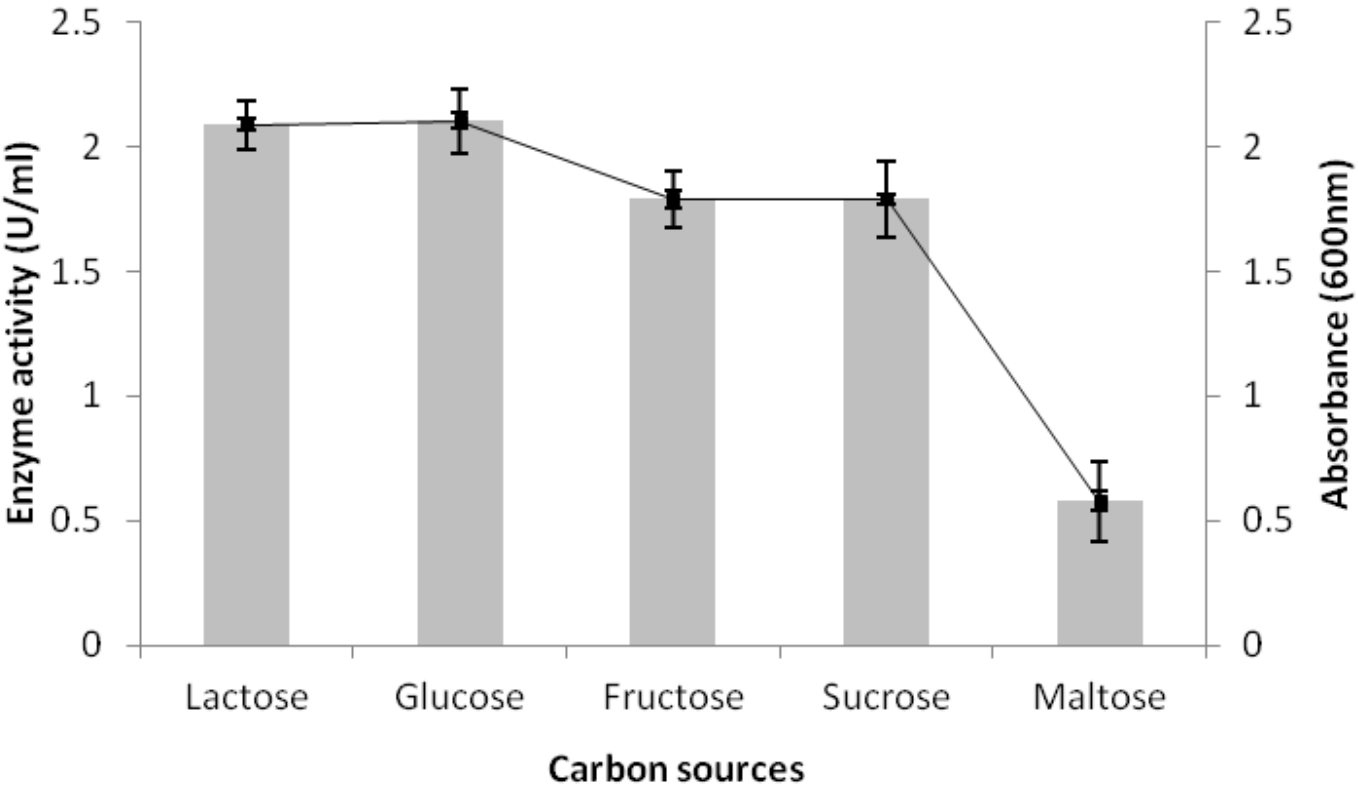
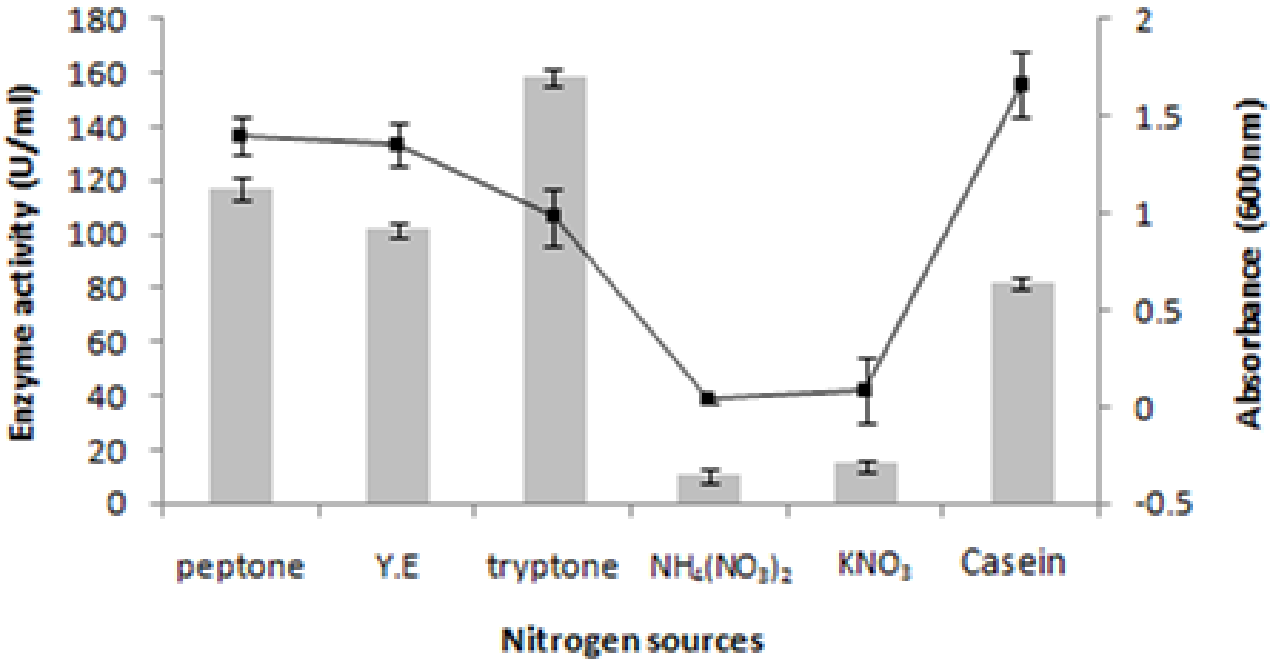


Supplementary Figure 1. Growth and enzyme activity of *Bacillus cereus* strain CS1 at pH 9 at 37°C.



Supplementary Figure 2. Effect of carbon sources on growth and enzyme activity of *Bacillus cereus* strain CS1 at pH 9 at 37°C.



Supplementary Figure 3. Effect of nitrogen sources on growth and enzyme activity of *Bacillus cereus* strain CS1 at pH 9 at 37°C.