

Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated *Bacillus subtilis* PE-11

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ABSTRACT

The purpose of the research was to study the purification and partial characterization of thermostable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. The enzyme was purified in a 2-step procedure involving ammonium sulfate precipitation and Sephadex G-200 gel permeation chromatography. The enzyme was shown to have a relative low molecular weight of 15 kd by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and was purified 21-fold with a yield of 7.5%. It was most active at 60°C, pH 10, with casein as substrate. It was stable between pH 8 and 10. This enzyme was almost 100% stable at 60°C even after 350 minutes of incubation. It was strongly activated by metal ions such as Ca⁺², Mg⁺², and Mn⁺². Enzyme activity was inhibited strongly by phenylmethyl sulphonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP) but was not inhibited by ethylene diamine tetra acetic acid (EDTA), while a slight inhibition was observed with iodoacetate, *p*-chloromercuric benzoate (*p*CMB), and β-mercaptoethanol (β-ME). The compatibility of the enzyme was studied with commercial and local detergents in the presence of 10mM CaCl₂ and 1M glycine. The addition of 10mM CaCl₂ and 1M glycine, individually and in combination, was found to be very effective in improving the enzyme stability where it retained 52% activity even after 3 hours. This enzyme improved the cleansing power of various detergents. It removed blood stains completely when used with detergents in the presence of 10mM CaCl₂ and 1M glycine.

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INTRODUCTION

Proteases are one of the most important industrial enzymes accounting for nearly 60% of the total worldwide enzyme sales.¹⁻⁶ Of these, alkaline proteases are employed primarily as cleansing additives. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases.¹ Ideally, proteases used in a detergent formulation should have a high level of activity over a broad range of pH and temperatures. Alkaline proteases from high yielding strains have been studied extensively. One of the major drawbacks affecting the stability at alkaline pH of enzymes recovered from thermophiles is that enzymes from alkalophiles confer stability in a wide pH range but are usually thermolabile.⁷ Thus it is desirable to search for new proteases with novel properties from as many different sources as possible. In the present study we examined the efficiency of an enzyme, recovered from a newly isolated strain of *Bacillus subtilis* PE-11, in the presence of standard commercial detergents. Further, characterization of this enzyme and the effect of various cofactors or additives on the stability at higher temperatures and in alkaline pH were carried out. The enzyme has been used as an effective additive for the laundry industry and can be exploited commercially.

MATERIALS AND METHODS

Isolation and Cultivation of Microorganisms

The soil samples were collected from sandy soil, milk processing plant, the drainage of a slaughterhouse, the beds of an effluent treatment plant, etc, of Visakhapatnam, India, and were diluted in sterile saline solution.

The diluted samples were plated onto skim milk agar plates containing peptone (0.1% wt/vol), NaCl (0.5% wt/vol), agar (2.0% wt/vol), and skim milk (10% vol/vol). Plates were incubated at 37°C for 24 hours. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the zone of clearance, strain PE-11 was selected for further experimental studies. The isolated proteolytic strain was a spore-forming gram-positive rod, identified as *B subtilis*, and it⁸⁻⁹ was designated as *B subtilis* PE-11.

Production of protease from *B subtilis* PE-11 was carried out in a medium containing the following: glucose, 0.5% (wt/vol); peptone, 0.75% (wt/vol); and salt solution, 5% (vol/vol) (MgSO₄·7H₂O, 0.5% [wt/vol]; KH₂PO₄, 0.5% [wt/vol]; and FeSO₄·7H₂O, 0.01% [wt/vol]) and maintained at 37°C for 48 hours in a shaker incubator (140 rpm). The pH of the medium was adjusted with 1N NaOH or 1N HCl. After the completion of fermentation, the whole fermentation broth was centrifuged using Sorvall RC5C centrifuge (Sorvall, New Delhi, India) at 10 000 rpm at 4°C, and the clear supernatant was recovered. The crude enzyme supernatant was subjected to further studies.

Enzyme Purification

Ammonium Sulphate Precipitation

The organism was grown for 48 hours as described previously. The cells were separated by centrifugation (10 000 rpm, 15 minutes), and the supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1M Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer.

Sephadex G-200 Gel Filtration Chromatography

The protein pellet obtained after saturation with ammonium sulphate between 50% and 70% was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at a NaCl concentration of 0.6M. Fractions (19-23) with high protease activities were pooled, dialyzed, and concentrated by lyophilization (Lyophilizer, Vertis, South Africa) and used for further studies.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

After Sephadex G-200 column chromatography, the fractions (19-23) showing the highest specific activity were dialyzed, lyophilized, and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli¹⁰ using 10% acrylamide.

Analytical Methods

Determination of Alkaline Protease Activity

Alkaline protease activity was determined by a modified procedure based on the method of Tsuchida et al.¹¹ One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine per mL per minute under the above assay conditions.

Protein Assay

Protein was measured by the method of Lowry et al¹² with bovine serum albumin (BSA) as the standard. The concentration of protein during purification studies was calculated from the absorbance at 280 nm.

Characterization of Purified Enzyme

Effect of pH on Purified Enzyme Activity and Stability

The activity of the crude and purified protease was measured at different pH values in the absence and presence of 10mM CaCl₂. The pH was adjusted using the following buffers (0.05M): phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0). Reaction mixtures were incubated at 40°C for 30 minutes, and the activity of the enzyme was measured.

The purified enzyme was diluted in different relevant buffers (pH 5.0-12.0) and incubated at 40°C for 2 and 20 hours for enzyme stability. The relative activity at each exposure was measured as per assay procedure.

Effect of Temperature on Enzyme Activity and Stability

The activity of the crude and purified enzyme was determined by incubating the reaction mixture at different temperatures ranging from 30°C to 90°C in the absence and presence of 10mM CaCl₂ for 30 minutes.

To determine the enzyme stability with changes in temperature, purified enzyme was incubated at differ-

ent temperatures (60°C, 70°C, and 80°C) in the presence of 10mM CaCl₂, and relative protease activities were assayed at standard assay conditions.

Effect of Protease Inhibitors and Chelators on Enzyme Activity

The effect of various protease inhibitors (5mM) such as serine inhibitors (phenylmethylsulphonyl fluoride [PMSF] and diisopropyl fluorophosphate [DFP]), cysteine-inhibitors (*p*-chloromercuric benzoate [*p*CMB] and β-mercaptoethanol [β-ME], iodoacetate), and a chelator of divalent cations (ethylene diamine tetra acetic acid [EDTA]) were determined by preincubation with the enzyme solution for 30 minutes at 60°C before the addition of substrate. The relative protease activity was measured.

Effect of Various Metal Ions on Protease Activity

The effects of metal ions (eg, Ca²⁺, Mg²⁺, Al³⁺, Co⁺², Cd⁺², Fe⁺³, Na⁺, Zn²⁺, Hg²⁺, and Cu²⁺ [5mM]) were investigated by adding them to the reaction mixture. Relative protease activities were measured.

Hydrolysis of Protein Substrates

Protease activity with various protein substrates including BSA, casein, egg albumin, and gelatin was assayed by mixing 100 ng of the enzyme and 200 μL of assay buffer containing the protein substrates (2 mg/mL). After incubation at 60°C for 30 minutes, the reaction was stopped by adding 200 μL of 10% trichloro acetic acid (TCA) (wt/vol) and allowed to stand at room temperature for 10 minutes. The undigested protein was removed by centrifugation and peptides released were assayed. The specific protease activity towards casein was taken as a control.

Detergent Stability

The compatibility of PE-11 protease with local laundry detergents was studied in the presence of 10mM CaCl₂ and 1M glycine. Detergents used were Nirma (Nirma Chemical, India); Henko (Henkel Spic, India); Surf, Surf Excel, Super Wheel, Rin (Hindustan Lever Ltd, India); and Ariel (Procter and Gamble, India). The detergents were diluted in distilled water (0.7% wt/vol) and incubated with protease for 3 hours at 60°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.

Washing Test with Protease Preparation

Application of protease (5000 U/mL) as a detergent additive was studied on white cotton cloth pieces (4 × 4 cm) stained with blood. The stained cloth pieces were taken in separate flasks. The following sets were prepared and studied:

1. flask with distilled water (100 mL) + stained cloth (cloth stained with blood)
2. flask with distilled water (100 mL) + stained cloth (cloth stained with blood) + 1 mL wheel detergent (7 mg/mL)
3. flask with distilled water (100 mL) + stained cloth (cloth stained with blood) + 1 mL wheel detergent (7 mg/mL) + 2 mL enzyme solution

The above flasks were incubated at 60°C for 15 minutes. After incubation, cloth pieces were taken out, rinsed with water, and dried. Visual examination of various pieces exhibited the effect of enzyme in removal of stains. Untreated cloth pieces stained with blood were taken as control.

RESULTS AND DISCUSSION

B subtilis PE-11, maximum growth and maximum enzyme production was observed at 48 hours (**Figure 1**). The zone of hydrolysis of *B subtilis* PE-11 on milk agar is shown in **Figure 2**.

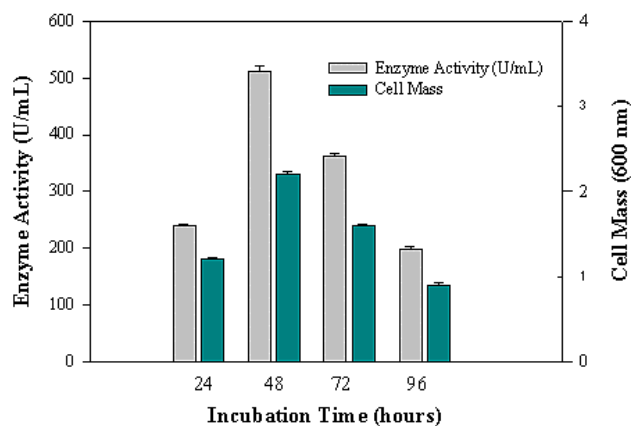


Figure 1. Growth and protease production by *B subtilis* PE-11.



Figure 2. The zone of hydrolysis of *B subtilis* PE-11 on milk agar.

Purification of Extracellular Protease of *B subtilis* PE-11

Sephadex G-200 Gel Filtration Chromatography

The protein pellet obtained after 60% saturation with ammonium sulphate was dissolved in 0.1M Tris-HCl buffer and loaded onto a column of Sephadex G-200 (1.5 × 24 cm) equilibrated with Tris-HCl buffer, pH 7.8. The elution profile of gel filtration chromatography is shown in **Figure 3**.

From the elution profile, it was observed that the protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at NaCl concentration of 0.6M. Fractions (19-23) with protease activities were pooled, dialyzed, and concentrated by lyophilization and used for further studies. The summary of purification steps involved for alkaline protease is presented in **Table 1**.

SDS-PAGE of Culture Filtrate and Purified Protease from *B subtilis* PE-11

When the ammonium sulphate precipitation and purified protease were analyzed by SDS-PAGE, 7 bands were observed in the case of the ammonium sulphate precipitation, while purified protease showed a single band, indicating a homogeneous preparation. The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins. The molecular mass standards were BSA (67 kd), ovalbumin (45 kd), carbonic anhydrase (30 kd),

trypsinogen (24 kd), and α -lactalbumin (14 kd) on SDS-PAGE. The molecular weight was determined by interpolation from a linear semilogarithmic plot of relative molecular mass versus the R_f value (relative mobility). Depending on the relative mobility, the molecular weight of the protein band was calculated to be 15 kd, which coincided with the band of α -lactalbumin marker protein.

Characterization of Purified Enzyme

pH Optimum and pH Stability

For the determination of the pH optimum, phosphate (pH 5.0-7.0), Tris-HCl (pH 8.0), and glycine-NaOH (pH 9.0-12.0) buffers were used in the absence and presence of 10mM CaCl_2 . The highest protease activity was found to be at pH 10.0 using glycine-NaOH buffer (**Figure 4**). These findings are in accordance with several earlier reports showing pH optima of 10.0-10.5 for protease from *Bacillus* sp, *Thermus aquaticus*, *Xanthomonas maltophila*, and *Vibrio metschnikovii*.¹³⁻¹⁶ The important detergent enzymes, subtilisin Carlsberg and subtilisin Novo or BPN¹⁷ also showed maximum activity at pH 10.5.

The stability of the purified protease was also determined by the preincubation of the enzyme in various buffers of different pH values. In the case of 2 hours preincubation group, the enzyme was stable over a broad range of pH 8 to pH 11 (**Figure 5**). On the other hand, in the case of the 20 hours preincubation group, the enzyme was stable between pH 8 and pH 10. However, in an earlier study, the protease from a *Thermus* sp strain Rt41A¹⁸ exhibited stability for at least 4 hours over a pH range of 5 to 10.

Temperature Optimum and Thermal Stability

The activity of the crude and purified enzyme was determined at different temperatures ranging from 30°C to 90°C in the absence and presence of 10mM CaCl_2 . The optimum temperature recorded was at 60°C for protease activity. The enzyme activity gradually declined at temperatures beyond 60°C (**Figure 6**). A similar type of result was observed by other investigators where a maximum temperature of 55°C was recorded for an alkaline protease from *B stearothermophilus* AP-4¹⁹ and 60°C for a protease²⁰ derived from *Bacillus* sp B21-2.

The thermal stability of the purified protease was tested at different temperatures of 60°C, 70°C, and 80°C for different periods (50 to 350 minutes) in the presence of

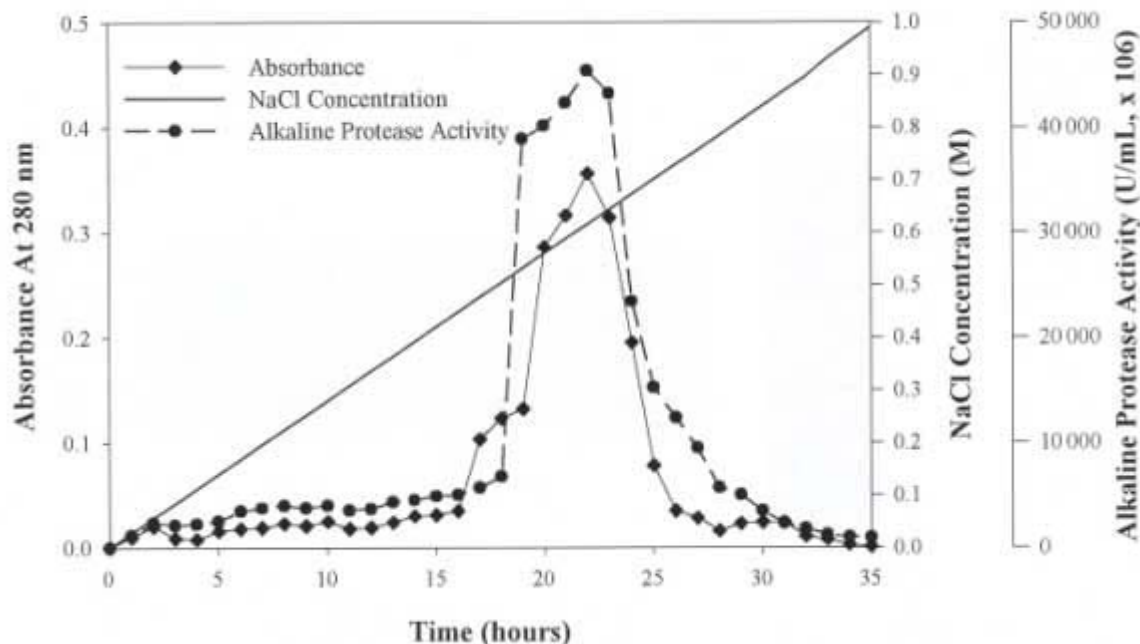


Figure 3. Elution profile of *B subtilis* PE-11 protease by Sephadex G-200 column.

Table 1. Summary of Purification Steps of Alkaline Protease From *Bacillus subtilis* PE-11

Purification Step	Total Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	% Recovery
Crude enzyme	50400	4920	10.2	1.0	100
(NH ₄) ₂ SO ₄ precipitation, dialyzed	36250	3000	12.1	1.2	72.0
Sephadex G-200	3760	17.6	213.6	21.0	7.5

10mM CaCl₂. The PE-11 protease had a half life of 250 and less than 50 minutes at 70°C and 80°C, respectively. The enzyme was almost 100% stable at 60°C even after 350 minutes of incubation (**Figure 7**). Previous reports on thermostability have shown half-lives of >200 minutes at 50°C and 2 to 22 minutes at 60°C for heat stable serine proteases, and half-lives of 3.4 minutes and 2.4 minutes at 50°C have been recorded for subtilisin Carlsberg and subtilisin BPN, respectively.¹³

Effect of Inhibitors and Chelators

Inhibition studies primarily give an insight into the nature of an enzyme, its cofactor requirements, and the nature of the active center.²¹ The effect of different inhibitors on the enzyme activity of the purified protease was studied (**Table 2**). Of the inhibitors tested (at 5mM

concentration), PMSF was able to inhibit the protease completely, while DFP exhibited 94% inhibition. In this regard, PMSF sulphonates the essential serine residue in the active site of the protease and has been reported to result in the complete loss of enzyme activity.²² Our findings were similar to those of Tsuchida et al¹¹ and Yamagata et al,²³ where the protease was completely inhibited by PMSF. This indicated that it is a serine alkaline protease. In the case of other inhibitors, the protease was not inhibited by EDTA, while a slight inhibition was observed with iodoacetate, pCMB, and β-ME.

Effect of Metal Ions

Most of the metal ions tested had a stimulatory effect (Ca²⁺, Mg²⁺, and Mn²⁺) or a slight inhibitory effect

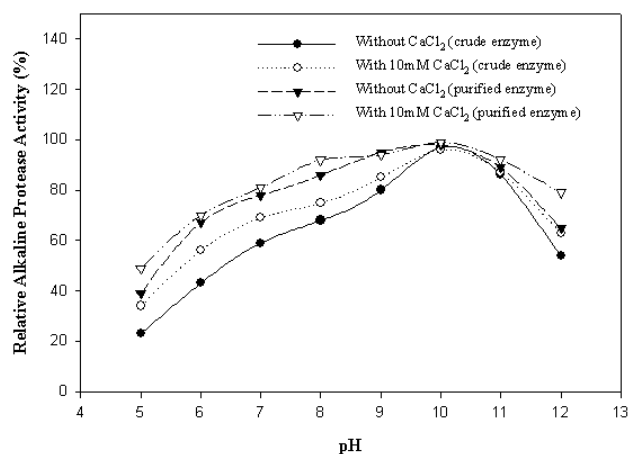


Figure 4. Effect of pH on the activity of alkaline protease in the presence and absence of 10mM CaCl₂.

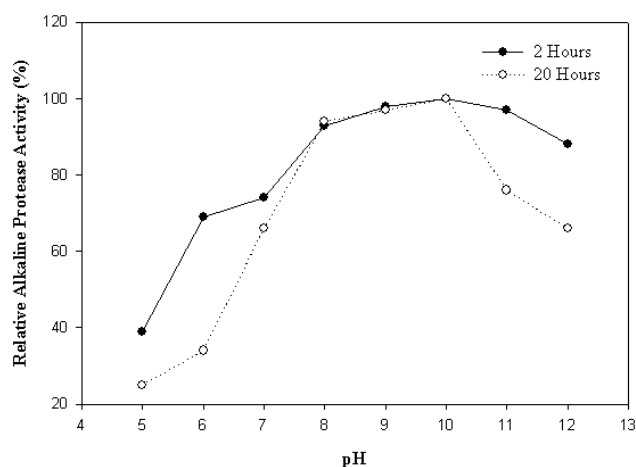


Figure 5. Effect of pH on the stability of alkaline protease in the presence of 10mM CaCl₂.

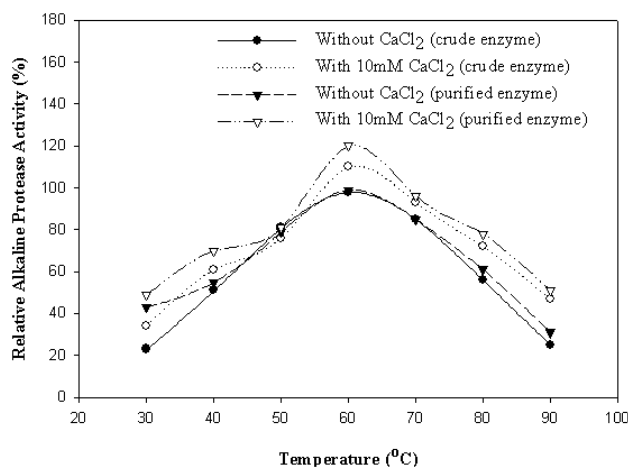


Figure 6. Effect of temperature profiles on the activity of alkaline protease in the presence and absence of 10mM CaCl₂.

(other ions) on enzyme activity (**Table 3**). Some of the metal ions such as Ca²⁺, Mg²⁺, and Mn²⁺ increased and stabilized the protease activity of the enzyme; this is possible because of the activation by the metal ions. These cations also have been reported to increase the thermal stability of other *Bacillus* alkaline proteases.²⁴⁻²⁵ These results suggest that concerned metal ions apparently protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperatures.²⁶⁻²⁸ Other metal ions such as Zn²⁺, Cu²⁺, Hg²⁺, Co²⁺, Na⁺, Cd²⁺, Al³⁺, and EDTA did not shown any appreciable effect on enzyme activity.

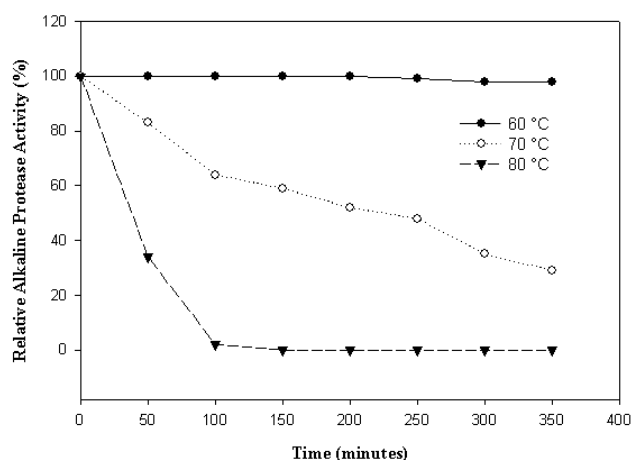


Figure 7. Effect of temperature on the stability of the enzyme in the presence of 10mM CaCl₂.

Table 2. Effect of Protease Inhibitors/Chelators on the Enzyme Activity*

Inhibitor/Chelator (5mM)	% Relative Enzyme Activity
Control	100
β-ME	81
Iodoacetate	90
pCMB	85
PMSF	00
DFP	06
EDTA	106

*β-ME indicates β-mercaptoethanol; DFP, diisopropyl fluorophosphate; EDTA, ethylene diamine tetra acetic acid; pCMB, p-chloromercuric benzoate; and PMSF, phenylmethyl sulphonyl fluoride.

Table 3. Effect of Various Metal Ions on Alkaline Protease Activity*

Metal Ions (5mM)	Residual Alkaline Protease Activity (%)
Control	100
Zn ²⁺ (ZnCl ₂)	95
Cu ²⁺ (CuCl ₂)	96
Mg ²⁺ (MgCl ₂)	116
Hg ²⁺ (HgCl ₂)	93
Co ²⁺ (CoCl ₂)	92
Ca ²⁺ (CaCl ₂)	135
Na ⁺ (NaCl)	98
Cd ²⁺ (CdCl ₂)	92
Al ³⁺ (AlCl ₃)	97
EDTA	99
Mn ²⁺ (MnCl ₂)	108

*EDTA indicates ethylene diamine tetra acetic acid.

Table 4. Alkaline Protease Activity Against Different Natural Substrates*

Substrate (2 mg/mL)	Relative Enzyme Activity (%)
Casein	100
BSA	41
Egg albumin	23
Gelatin	9

*BSA indicates bovine serum albumin.

Hydrolysis of Protein Substrates

When assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein and poor to moderate hydrolysis of BSA and egg albumin, although the hydrolysis was hardly observed with gelatin (**Table 4**).

Compatibility with Detergents

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. The PE-11 protease showed excellent stability and compatibility in the presence of locally available detergents (Nirma, Wheel, Henko, Surf, Surf Excel, Ariel, and Rin).

Protease from *B subtilis* PE-11 showed stability and compatibility with a wide range of commercial detergents at 60°C in the presence of CaCl₂ and glycine as stabilizers. Our protease showed good stability and compatibility in the presence of Wheel followed by Surf excel (**Table 5**). The enzyme retained more than 50% activity with most of the detergents tested even after 3 hours incubation at 60°C after the supplementation of CaCl₂ and glycine. Bhosale et al²⁹ reported high activity alkaline protease from *C coronatus* showing compatibility at 50°C, in the presence of 25mM CaCl₂, with a variety of commercial detergents. Bhosale et al reported 16% activity in Revel, 11.4% activity in Aerial, and 6.6% activity in Wheel. Comparing these results, the *B subtilis* PE-11 enzyme was significantly more stable in commercial detergents.

The compatibility of alkaline protease was studied with Wheel in the presence of 10mM CaCl₂ and 1M glycine for different periods (0.5 to 3 hours) at 60°C. The enzyme retained about 67% activity after 1.5 hours in the presence of Wheel at 60°C and was almost inactivated after 3 hours in the absence of any stabilizer (**Figure 8**). However, the addition of CaCl₂ (10mM) and glycine (1M), individually and in combination, was very effective in improving the stability, where it retained 52% activity even after 3 hours.

As the protease produced by our isolate *B subtilis* PE-11 was stable over a wide range of pH values and temperatures and also showed compatibility with various commercial detergents tested in the presence of CaCl₂ and glycine, it was used as an additive in detergent, to check the contribution of the enzyme in improving the washing performance of the detergent. The supplementation of the enzyme preparation in detergent (ie, Wheel) could significantly improve the cleansing of the blood stains (**Figure 9**).

CONCLUSION

The alkaline protease isolated from *B subtilis* PE-11 is a thermostable serine protease. It is stable at alkaline pH, at high temperatures, and in the presence of commercial detergents and is compatible with commercial and local detergents. These properties indicate the possibilities for use of the protease in the detergent industry. This enzyme can be exploited commercially.

Table 5. Compatibility of Alkaline Protease Activity from *Bacillus subtilis* PE-11 With Commercial Detergents in the Presence of 10mM CaCl₂ and Glycine at 60°C

Time (hour)	Relative Residual Alkaline Protease Activity (%)							
	Control	Nirma	Wheel	Henko	Surf	Surf Excel	Ariel	Rin
0.0	100	100	100	100	100	100	100	100
0.5	96	90	95	92	91	93	88	87
1.0	94	87	92	89	87	90	85	83
1.5	91	85	89	86	83	87	82	81
2.0	87	79	82	79	75	80	74	72
2.5	80	69	73	68	63	70	61	59
3.0	76	58	65	58	51	56	52	53

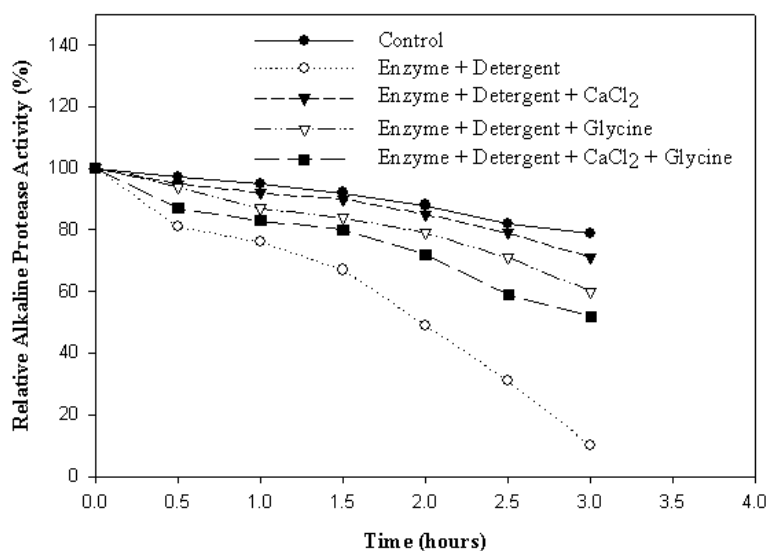


Figure 8. Compatibility of alkaline protease with Wheel in the presence of 10mM CaCl₂ and 1M glycine at 60°C.

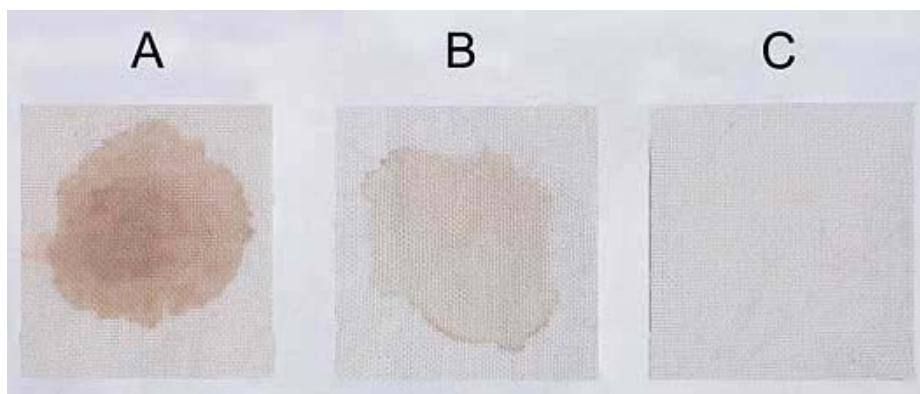


Figure 9. Washing performance of alkaline protease from *B subtilis* PE-11 in the presence of detergent (Wheel). (A) cloth stained with blood; (B) blood-stained cloth washed with detergent only; and (C) blood-stained cloth washed with detergent and enzyme.

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