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RESEARCH ARTICLE

PURIFICATION AND CHARACTERIZATION OF PROTEASE ENZYME FROM NATIVE ISOLATE BACILLUS SUBTILIS AND ITS COMPATABILITY WITH COMMERCIAL DETERGENTS

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Abstract

The present study describes the purification characterization of crude protease from *Bacillus subtilis* and its evaluation in detergent. An alkaline protease producing *B.subtilis* was isolated from local soil samples were collected from Local Garden. The bacterial culture *B.subtilis* showed luxurious growth in Nutrient agar at pH (7.2) and at a temperature 37°C. The bacterium is mesophilic in nature. The enzyme was purified in a 2-step procedure involving ammonium sulfate precipitation and Sephadex G-200 gel permeation chromatography. The molecular weight of the enzyme determined by SDS-PAGE was found to be(30KDa) and was purified (112)fold with a yield of (6%). The highest protease activity was found to be atpH (10.0) using glycine-NaOH buffer. The enzyme was almost 100% stable at60°C even after (5) hours of incubation.Most of the metal ions tested had a stimulatory effect (Ca^{2+}, Mg^{2+}, and Mn^{2+}) or a slight inhibitory effect (other ions) on enzyme activity. The protease showed excellent stability and compatibility in the presence of locally available detergents. The enzyme retained more than 50% activity with most of the detergents tested even after 2 hours incubation at 60°C after the supplementation of(5mM$CaCl_2$)and(1M glycine).The purpose of the research was to study designed to isolate purificationand characterize the protease enzyme from *Bacillus subtilis* and its compatibility with commercial detergents.

INTRODUCTION

Proteases are the most important industrial enzymes accounting for approximately 40% of the total industrial enzyme market [1]. Compared to animal and fungal proteases, bacterial alkaline proteases have more commercial significance in laundry, food, leather and silk [3] due to their high production capacity and catalytic activity [4–7]. However, proteases with high activity at different pH values and at high temperatures have novel application potential in pharma, diagnostic, detergent, tannery, amino acid production, contact-lens cleaning agents, effluent treatment, enzymic debridement, supporting the natural healing process in the skin ulcerations [5-6,8-9]. They also hydrolyze peptide bonds in aqueous solutions and synthesize them in non-aqueous conditions [10]. In addition, their functional and thermal stability of protein chemistry and protein engineering are the most important parameters to be investigated to understand their utility in different sectors. Although proteases producing microorganisms, plants and animals are wide spread in nature, microbial community is preferred due to their growth and simplicity for generation of new recombinant enzymes with desired properties. Physical, biochemical, molecular and catalytic properties of proteases varies with the nature of the organism [6,11-12]. In general, most of the industrial proteases have some limitations [13] and their use highly depends on their stability during isolation, purification and storage in addition to their robustness against solvents, surfactants and oxidants [10,14–16]. Hence, in depth knowledge of

kinetics andcatalytic behavior during protease production from any new strainis a prerequisite for evaluation of its biotechnological potential[17-18]. In this context, a potential alkaline protease producingbacterial strain was isolated in our laboratory [17,19] andevaluated in detail for fermentation parameters and the kinetics of enzyme production with respect to development of low cost andeasy available medium ingredients to fit for industrial use [2,17–19]. 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.

2. Material and Methode:

In all the experiments acid washed (0.4N HCl) Pyrex glassware rinsed with double distilled water was used. For the preparation of culture media and other chemical reagents analytical grade chemicals (E Merk GR/BDH, Analar R) were used. Nutrient agar and nutrient broth mediumwere used in the work.

2.1. Collection of soil sample& isolation of bacteria:

Soil samples were collected from Local Garden, Hyderabad, T.S., India. The samples were collected in sterilized bottles for isolation of *B.subtilis*by serial dilution plate method [20].The identified colonies were sub cultured on nutrient agar slants and preserved at 4ºC. Sub-culturing was performed at one month interval.Plate hydrolysis assaywas performedfor production of protease. A clear zone of skim milk hydrolysis gave an indication of protease producing organisms. Depending upon the zone of clearance, strain *Bacillus subtilis*was selected for further experimental studies. The isolated proteolytic strain was a spore-forming gram-positive rod, identified as *B. subtilis*, and it was designated as *B. subtilis*[21].

2.2. Extracellular protease production:

Production of protease from *B. subtilis* was carried out in a medium containing the following: casein 2g; peptone (5)gm; NaCl (5)gm and pH was adjusted to 8 and maintained at 37°C for 48 hours in a shaker incubator (140 rpm). After the completion of fermentation, the whole fermentation broth was centrifuged at 10000 rpm at 4°C, and the clear supernatant was recovered. Protein was measured by the method of [*22*]with bovine serum albumin (BSA) as the standard.

2.3. Enzyme Purification:

2.3.1. 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.The protein sample was loaded into the activated dialysis membrane tube, whichwas sealed later from both the sides. The dialysis membrane tube was then hung into the 500 mL of buffer (Tris-HCl, pH 8.0) with 10 times lesser strength (i.e., 5mM). The experiment was carried for 8 hours with 1-2 changes in buffer at 4°C[23].

2.3.2 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)[24]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,and used for further studies[25].

2.5. Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis(SDS-PAGE):

The protein sample was mixed with sample buffer (1.5M Tris-HCl (pH 8) 0.625 ml, 20% SDS 1.0 mL, Glycerol 1.0 mL, 2-mercaptoethanol 0.5 mL, 0.2 % Bromophenol blue) in 1:1 ratio and boiled for 2-10 min. the sample was loaded into gel and the gel was run at constant current of 50-150V for 3-4 hr.The gel was removed carefully and stained in a staining solution (methanol 4mL, distilled water 5ml, glacial acetic acid 1mL, 0.2 % Coomassie Brilliant Blue) with constant shaking for 2 hr. the excess stain was removed by destaining solution (Methanol 4ml, distilled water 5mL, Glacial acetic acid 1mL)[26,27].

2.6. Characterization of Purified Enzyme:

2.6.1. Effect of pH on Purified Enzyme Activity and Stability:

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 presence and absence of 5mM $CaCl_2$. 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[28].

2.6.2. 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 5mM $CaCl_2$ for 30 minutes. To determine the enzyme stability with changes in temperature, purified enzyme was incubated at different temperatures (60°C, 70°C, and 80°C) in the presence of 5mM $CaCl_2$, and relative protease activities were assayed at standard assay conditions[29].

2.6.3. Effect of Various Metal Ions on Protease Activity:

The effects of metal ions (eg, Ca^{2+}, Mg^{2+}, Al^{3+}, Co^{2+}, Cd^{2+}, Fe^{3+}, Na^{+}, Zn^{2+}, Hg^{2+}, and Cu^{2+} [5mM]) were investigated by adding them to the reaction mixture. Relative protease activities were measured.

2.6.4. Compatibility with commercial detergents:

The compatibility of protease with local laundry detergents was studied in the presence of 5mM $CaCl_2$ 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 2 hours at 60°C, and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100%.The activity was then assayed by [30].

3. RESULTS AND DISCUSSION

3.1. Bacterial culture:

The bacterial culture *Bacillus subtilis* showed luxurious growth in Nutrient agar at pH 7.2 and at a temperature 37°C (Fig. 1). The bacterium is mesophilic in nature.

Fig 1: *Bacillus subtilis* on Nutrient Agar Medium.

3.2. Casein hydrolysis assay:

The bacterial culture *Bacillus subtilis* showed a distinguishable pattern of hydrolysis of polymers like Casein and Milk powder. On observation after 24hr of incubation, the plate showed the hydrolysis of casein as a clear zone (Fig.2). Thus the culture isolate *B.subtilis* i.e., strain has a proteolytic and spore formi**n**g ability.

Fig. 2: The zone of casein hydrolysis of *B. subtilis*.

3.3. Purification of Extracellular Protease of *B. subtilis*:

3.3.1. Sephadex G-200 Gel Filtration Chromatography:

The protein pellet obtained after 60% saturation with ammonium sulphatewas 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 (Fig 3).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 protease is presented in (Table1).

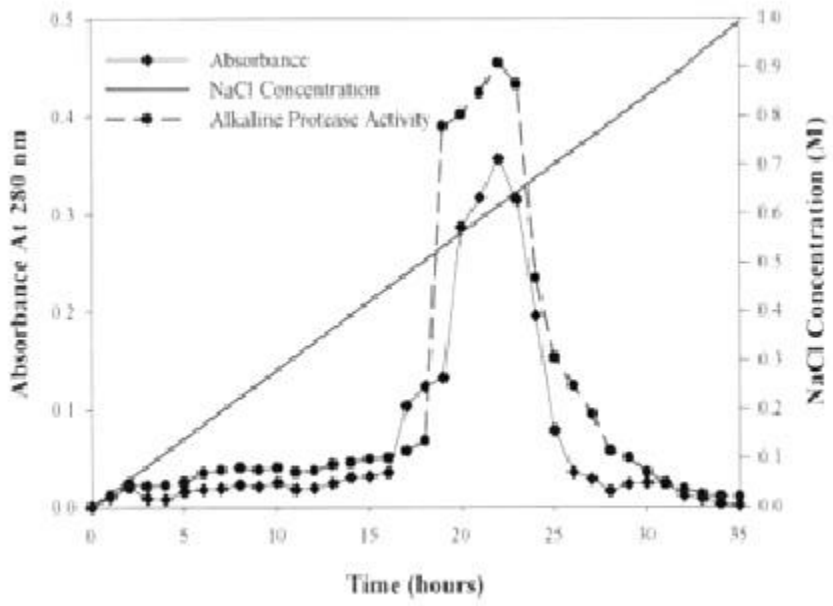


Fig 3: Elution profile of *B. subtilis* protease by Sephadex G-200 column.

Table 1. Summary of Purification Steps of Alkaline Protease From Bacillus subtilis.

Purification Step	Total Enzyme Activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification Fold	Recovery %
Crude enzyme	279000	6100	46	1.0	100

$(NH_4)_2SO_4$ precipitation, dialyzed	90867	59	1545	34	33
Sephadex G-200	15425	3	5142	112	6

3.4. SDS-PAGE of Culture Filtrate and Purified Protease from *B. subtilis*:

When the ammonium sulphate precipitation and purified protease were analyzed by SDS-PAGE, 6 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 werebovine serum albumin(BSA) (66kd), ovalbumin (43kd), carbonic anhydrase (29kd), and α- lactalbumin (14 kd) on SDS-PAGE. The molecular weight was determined by interpolation from a linear semi logarithmic plot of relative molecular mass versus the $R_f$value (relative mobility). The molecular weight of the protein band was calculated to be 30kd, which coincided with the band of carbonic anhydrase marker protein (Fig 4).

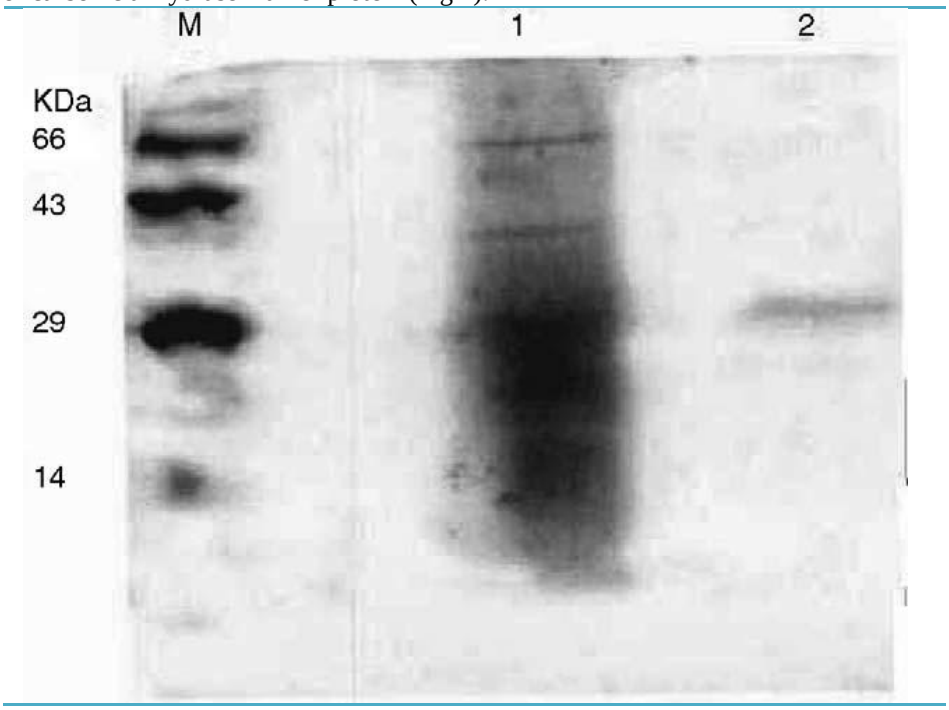


Fig. 4: SDS - PAGE showing the molecularweight of alkaline protease enzyme produced by *Bacillus subtilis* (30kDa) M, molecular weight marker;Line 1crude preparation, line 2, purified.

3.5. Characterization of Purified Enzyme:

3.5.1. pH Optimum and pH Stability:

The effect of pH on protease activity was determined over a pHrange of 7.0–13.0. The enzyme preparation was highly active in thepH range of 7.0–12.0 with an optimum between pH 8.0 and 11.0and the relative activity at pH 12.0 was about 70% (Fig. 5a).The pH stability profile, reported in (Fig. 5b), showed that the A21enzyme preparation is highly stable at a pH range between 6.0 and11.0, maintaining 100% of its original activity after 1 h incubationat 40 ◦C. At pH 5.0, the enzyme preparation retained about 70% ofits initial activity.

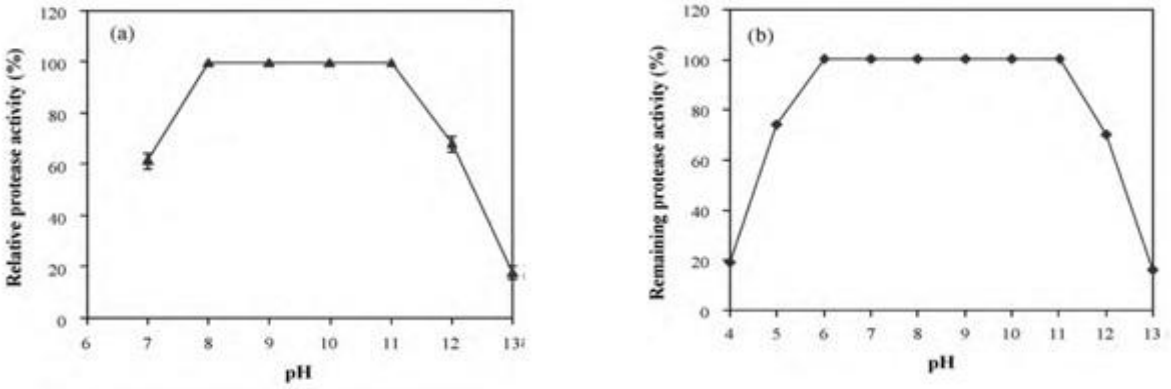


Fig 5: Effect of pH on activity (a) and stability (b) of the crude protease from B subtilis.

3.5.2. Temperature Optimum and Thermal Stability:

The relative activities at various temperatures, using casein as a substrate, in the absence or presence of 5mMCaCl2 are reported in (Fig. 6a). proteolytic preparation retained 89% and 52% of its initial activity after 1 h incubation at 50 and 55 ◦C, respectively, and 24% activity after 1 h incubation at 60 ◦C. The thermostability of the crude enzyme was also investigated at 55 and 60 ◦C in the presence of 5mMCaCl2. As reported in (Fig. 6b), the stability of the crude enzyme was considerably enhanced in the presence of 5mMCaCl2.

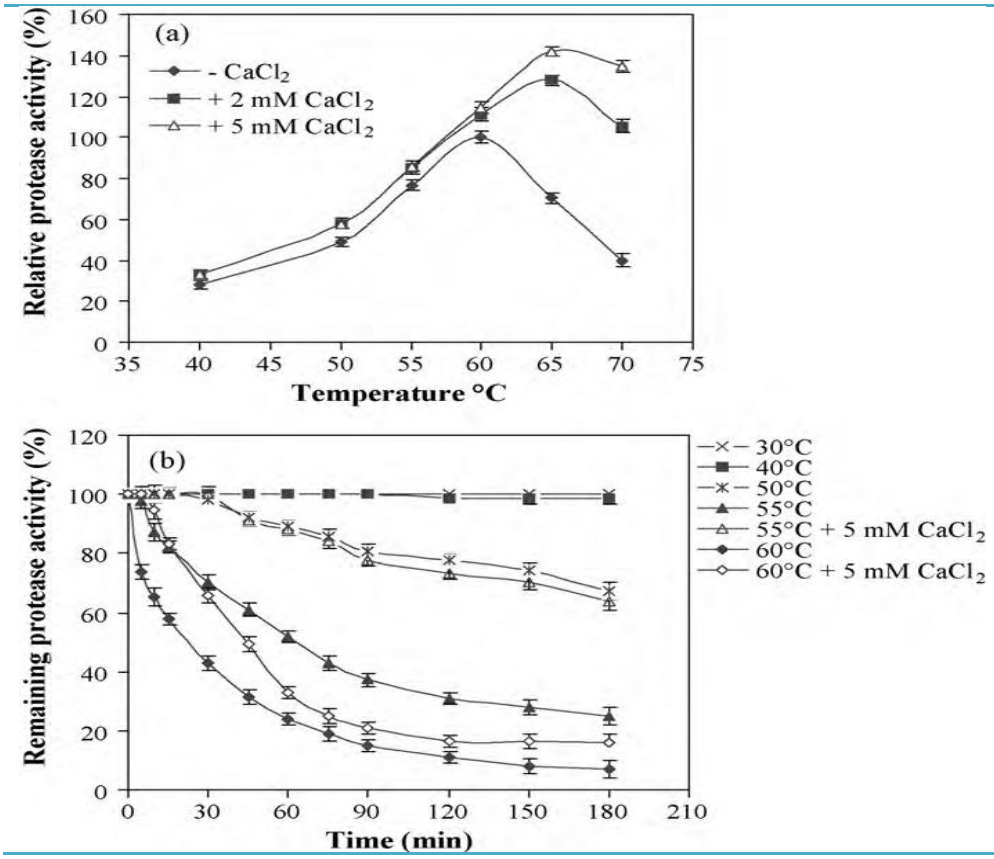


Fig 6: Effect of temperature on activity (a) and stability (b) of crude protease in the presence of 5mMCaCl$_2$.

3.6. Effect of Metal Ions:

Most of the metal ions tested had a stimulatory effect (Ca^{2+}, Mg^{2+}, and Mn^{2+}) or a slight inhibitory effect (other ions) on enzyme activity (Table 2). Some of the metal ions such as Ca^{2+}, Mg^{2+} and Mn^{2+} 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.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.Other metal ions such as Zn^{2+}, Cu^{2+}, Hg^{2+}, Co^{2+}, Na^{+}, Cd^{2+}, Al^{3+} and EDTA did not shown any appreciable effect on enzyme activity.

Table 2. Effect of Various Metal Ions on Alkaline Pro-tease Activity.

Metal Ions (5mM)	Residual Alkaline Protease Activity (%)	Metal Ions (5mM)	Residual Alkaline Protease Activity (%)
Control	100	Ca^{2+} ($CaCl_2$)	135
Zn^{2+} ($ZnCl_2$)	95	Na^{+} (NaCl)	98
Cu^{2+} ($CuCl_2$)	96	Cd^{2+} ($CdCl_2$)	92
Mg^{2+} ($MgCl_2$)	116	Al^{3+} ($AlCl_3$)	97
Hg^{2+} ($HgCl_2$)	93	EDTA	99
Co^{2+} ($CoCl_2$)	92	Mn^{2+} ($MnCl_2$)	108

3.7. Compatibility with Detergents:

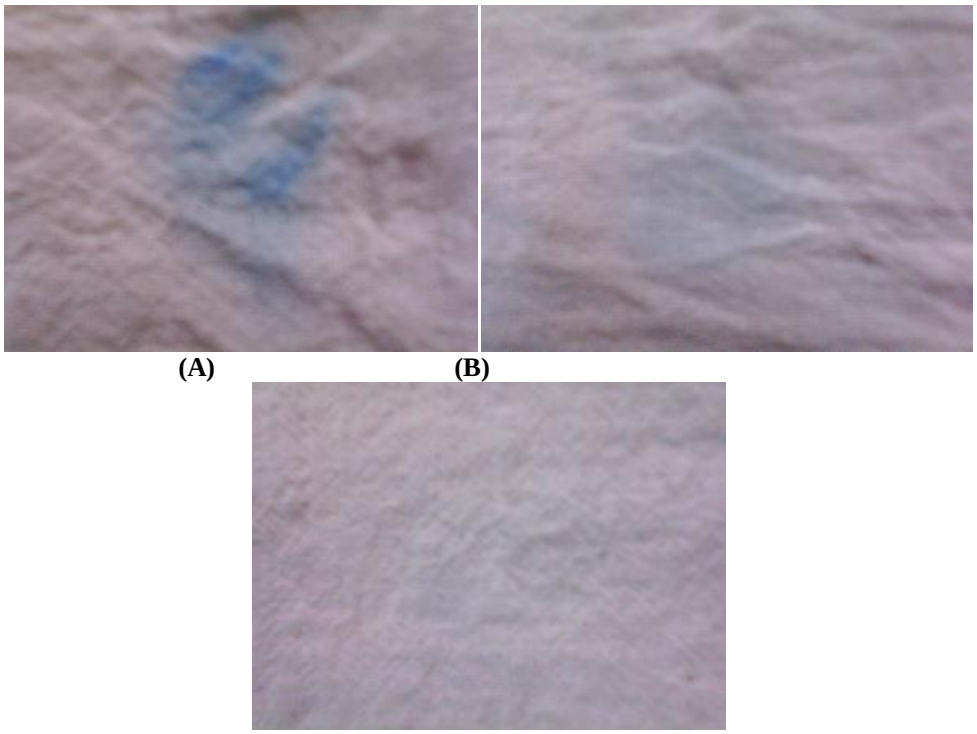
Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. The protease showed excellent stability and compatibility in the presence of locally available detergents (Nirma, Super Wheel, Henko, Surf, Surf Excel, Ariel, and Rin).

Protease from *B subtilis* showed stability and compatibility with a wide range of commercial detergents at 60°C in the presence of $CaCl_2$ and glycine as stabilizers. Our protease showed good stability and compatibility in the presence of Wheel followed by Surf excel (Table 3). The enzyme retained more than 50% activity with most of the detergents tested even after 2 hours incubation at 60°C after the supplementation of $CaCl_2$ and glycine.

The compatibility of alkaline protease was studied with Super Wheel in the presence of 5mM $CaCl_2$ and 1M glycine for different periods (0.5-1-2) hours at 60°C. The enzyme retained about 67% activity after 1.5 hours in the presence of Super Wheel at 60°C and was almost inactivated after 2 hours in the absence of any stabilizer (Figure 7). However, the addition of $CaCl_2$ (5mM) and glycine (1M), individually and in combination, was very effective in improving the stability, where it retained 50% activity even after 2 hours. As the protease produced by our isolate *B subtilis* was stable over a wide range of pH values and temperatures and also showed compatibility with various commercial detergents tested in the presence of $CaCl_2$ 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.

Table 3. Compatibility of Alkaline Protease Activity from Bacillus subtilisWith Commercial Detergents in the Presence of $CaCl_2$ and Glycine at 60°C.

Relative Residual Alkaline Protease Activity (%)								
Time (hour)	Control	Nirma	Super Wheel	Henko	Surf	Surf Excel	Ariel	Rin
0.0	100	100	100	100	100	100	100	100
0.5	98	95	97	98	91	96	88	97
1.0	96	93	94	95	85	94	93	95
1.5	92	90	89	92	83	89	87	92
2.0	87	85	86	84	81	85	85	83



(A) **(B)**

(C)

Fig. 7: Washing performance of protease from *B. subtilis*inthe presence of detergent (Wheel).
(A). Cloth stained with ink.
(B). Ink-stained cloth washed with detergent only.
(C). Ink-stained cloth washed with detergent and enzyme.

4. Conclusion:

It can be concluded from the present results:

1. Characterization and environmental friendly potential application of alkaline protease produced by isolated *B.subtilis*wasstudied.
2. The alkaline protease isolated from *B subtilis* is a thermostable serine. Itwas highly stable and active at high pH and showed optimum activity at pH 8.0–11.0 and at 60 ◦C. The *B.subtilis*crude protease showed excellent stability and compatibility with various commercial
3. Considering its promising properties, *B.* enzymatic preparation may be considered a potential candidate for future use in biotechnological processes, particularly in detergent
4. is a very promising strain for biotechnological applications.
5. The addition of (5mM $CaCl_2$)and (1M glycine), individually and in combination, was found to be very effective in improving the enzyme stability where it retained 50% activity even after 2 hours.
6. The molecular weight of the enzyme determined by SDS-PAGE was found to be (30KDa) and was purified (112)fold with a yield of (6%).

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