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Research article

PURIFICATION AND CHARACTERIZATION OF AN EXTRACELLULAR ALKALINE PROTEASE PRODUCED FROM AN ISOLATED *BACILLUS SUBTILIS*

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ABSTRACT: This paper describes the studies on the purification and partial characterization of serine alkaline protease produced through submerged fermentation process from a locally isolated *Bacillus subtilis*. This strain, grown in a highly alkaline medium (pH 10), produces an extracellular proteolytic enzyme. The alkaline protease was purified in a simple two-step procedure involving ammonium sulphate precipitation and gel filtration. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified alkaline protease indicated an estimated molecular mass of 30KDa. It was more active in the range of 20-60ºC and had an optimum activity at 55ºC with optimum pH of 10.5. Characterization of the protease showed that it required certain cations such as Mg^{++}, Mn++ and Ca^{++} for maximal activity. The serine nature of the alkaline protease was confirmed by PMSF inhibition. The temperature and pH stability of this Alkaline Protease from *Bacillus Subtilis*makes it potentially useful forindustrial applications.

Keywords: *Bacillus subtilis*, Alkaline protease, SmF, Purification, Characterization

INTRODUCTION

Driven by increasing industrial demands for biocatalysts able to cope with industrial process conditions, continuous efforts are being focused on the search for such enzymes (Thumar and singh, 2007). Despite the fact that large numbers of different enzymes have been identified and many are being used in various biotechnological applications, the available enzymatic array is still not sufficient to meet the ever increasing demand (Han and Damodaran, 1998; Burg and Eijsink, 2002). Alkaline protease are one of the most important group of microbial enzymes that find varied uses in various industrial sectors such as leather, detergents, textile, food and feed etc. Industrially important alkaline proteases from bacterial sources have been studied extensively, of which *Bacillus* sp. is most reported as it serves as an ideal source of these enzymes because of rapid growth and limited space required for cultivation.B. subtilis is a highly favourable bacterium for protease production because it is non pathogenic, well explored as a model of gram positive bacteria and have remarkable potential to synthesize various types of protease.

This paper reports about an alkalophilic*Bacillus subtilis* isolated from soil sample collected from proteinaceous waste disposal areas. To elaborate production of large quantities of extracellular alkaline proteases by this locally isolated *Bacillus subtilis*strain identified for its capacity to grow rapidly on submerged condition; it was considered of significance to purify and characterize this enzyme by studying the effect of varying pH, temperature, substrate concentration and activators/inhibitors to explore the factors affecting the activity and determine the possible application for industrial purposes.

MATERIALS AND METHODS

Isolation and cultivation of microorganism

An alkalophilic*Bacillus subtilis* was isolated from soil sample collected from proteinaceous waste disposal areas. About 1g/100 ml of soil suspension was spread on 1% casein agar plates and incubated at 37°C for 7days. The grown up colonies from above plates were transferred to 0.4% gelatin agar plates and incubated for 24 h at 37°C, colonies from clear zone were picked and pure strain was isolated by repeated plating. Isolated microbial strain was grown on agar slants of Reese's medium containing (%) casein-0.5, glucose-0.25, yeast extract- 0.05, pH 8.5 at temperature 37°C and maintained at 4°C in refrigerator and subculture was done at interval of one month.

Alkaline protease production in liquid culture

The inoculum was prepared by transferring a loop full of culture from 24h old slant into 10 ml sterile Reese's medium and incubated at 37°C for 24h then transferred to 100 ml of production medium containing (%) $CaCO_3$-0.3, KH_2PO_4-0.2, $(NH_4)_2SO_4$-0.14, $MgSO_4.7H_2O$-0.03, $CaCl_2$-0.03 and urea- 0.03; with 3% ground nut husk powder (GNHP) at pH 10. GNHP was used as a substrate for production of alkaline protease. The medium was incubated at 37°C under shaking condition on 200 rpm. After the completion of fermentation, the whole fermentation broth was centrifuged at 10000 rpm and the clear supernatant was recovered which was used as crude enzyme.

Enzyme purification

The purification of alkaline protease was carried out by precipitation of the crude enzyme with ammonium sulphate between 30-70% saturation. The precipitation which collected through centrifugation (10,000×g at 4°C) was suspended in to a minimum volume of 0.1M Tris-HCl buffer, pH (8). This preparation was used as partially purified alkaline protease.

The partially purified enzyme was desalted by conventional dialysis against 0.1 M Tris-HCl buffer at 4°C overnight. The enzyme was loaded on to Sephadex G-100 column (55×2.5cm) pre equilibrated with 0.1 M Tris-HCl buffer (pH 8). The column was washed with the same buffer to remove unbound proteins. The bound alkaline protease was eluted with same buffer containing 0.1 M Nacl gradient and 2 ml fractions were collected. The absorbance taken at 280 nm was recorded in spectrophotometer for enzyme activity of alkaline protease. After each step in the purification procedure, specific activity (U/mg protein) were determined.

Analytical methods

Determination of alkaline protease activity

Alkaline protease activity was determined by hydrolyzing casein using standard method (Wongmongkol and Prichanont, 2006). 0.1 ml of fermented broth was added to 1 ml of 0.5% (w/v) casein in 0.1M carbonate-bicarbonate buffer solution (pH 10.5), then 0.9 ml of the same buffer solution was added to it and the content was maintained at 45°C in a water bath for 20 min followed by addition of 2 ml of 10% (w/v) trichloroacetic acid (TCA) to stop the reaction. After well mixing the above content was centrifuged at 3,000 rpm for 10 minutes. The tyrosine content of this clear solution was estimated by spectrophotometer at 280 nm.

For this study, one unit of alkaline protease activity is defined as the amount of enzyme that liberates one microgram of tyrosine per minute using 0.5% (w/v) casein as a substrate in a buffer solution of pH 10.5 at 45°C.

Protein assay

Protein was measured by the method of Lowery (Loweryet. al., 1951) 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

Gel electrophoresis

After Sephadex G-100 column chromatography, the fractions (46-50) showing the highest protease activity were dialyzed and then subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed according to Laemmli's (Laemmli, 1970) using 10% acylamide.

Temperature profile and thermal stability

The optimum temperature for alkaline protease activity was determined by incubating the reaction mixture at different temperature in the range of 28-80°C. The thermal stability of the purified alkaline protease was investigated by incubating it at different temperature (20-80°C) for 30 min than using the enzyme preparation for estimation at 55°C pH 10.5 for 20 min.

pH optima and pH stability

The effect of pH on protease activity was determined at 55°C by using carbonate-bicarbonate buffer pH (8-12). Reaction mixture was incubated at 55°C for 20 min. Similarly, the enzyme stability at different pH was determined by incubating the enzyme in the pH range of (8-12) respective buffers. After incubating the enzyme for 24h at 37°C, activities were determined at 55°C for 20 min.

Inhibition

The enzyme was incubated with various inhibitors [Phenyl methyl sulphonyl fluoride (PMSF), Diisopropyl fluorophosphates (DFP), *p*-chloromercuric benzoate (*p*-CMB), β-mercaptoethanol (β-ME), Iodoacetate, Ethylene diamene tetra acetic acid (EDTA)] At 10 mM concentration for 15 min at 37ºC and the residual activities were determined. Activity of the control not containing any inhibitors was also determined.

Metal ions effect assessment

The effect of metal ions (10 mM) (Mg^{++}, Zn^{++}, Mn^{++}, Co^{++}, Cu^{++}, Ag^{+}, Ca^{++}, Fe^{++}, Hg^{++},Sr^{++}, Na^{+}, K^{+}) on enzyme was assessed by carrying out enzyme assay. The relative activities were estimated with reference to control.

Hydrolysis of protein substrates

Alkaline protease activity with various protein substrates BSA, casein, egg albumin, haemoglobin and gelatin was assayed by mixing 100 ng of the enzyme and 200 µl of assay buffer containing the protein substrates (2mg/ml). After incubation at 55 ºC for 20 minutes, the reaction was stopped by adding 200µl of 10% trichloro acetic acid (TCA) (w/v) and allowed to stand at room temperature for 10 minutes. The undigested protein was removed by centrifugation and peptides released were assayed. The specific alkaline protease activity towards casein was taken as a control.

RESULTS AND DISCUSSION

The bacterial strain isolated from soil of proteinaceous waste disposal areas was identified as *Bacillus subtilis* based on the morphological, physiological and biochemical characteristics by a premier research institute lab of India, IARI (Indian agriculture research institute, New Delhi, India) as under :

Table 1.Taxonomical properties of *B. subtilis*

Cell shape	Long rod in short chain
Endospore	+
Gram reaction	+
Colony colour	Creamy white
Gelatin hydrolysis	-
Blue green diffusible pigment on King's A	-
Yellowish green diffusible pigment on King's B	-
Oxidase	+
H2S	+
Levan	+
Citrate utilization	-
Arginine dyhydrolase_	-
Catalaze	+
KOH test	-

Purification of alkaline protease of Bacillus subtilis

Bacillus subtilis alkaline protease was purified through combination of ammonium sulphate precipitation and gel filtration chromatography. The steps involved in the purification process along with the results are illustrated in Table 1.

Ammonium sulphate precipitation at 60% saturation yielded 1.18 fold purification enhancing the specific activity to 196 U/mg protein with a yield of 91.42%. After dialysis, the 60% ammonium sulphate fraction was subjected to gel filtration chromatography on sephadex G-100 column leading to 12.69 fold purification with the specific activity of 2106 U/mg of protein. The result was quite similar when compared to an alkaline protease from *Bacillus cereus* in which 16.8 fold purification was achieved by mono Q and superdex-200 column chromatography (Banik and Prakash, 2006).

Table 2. Summary of purification steps of alkaline protease from *Bacillus subtilis*

Purification step	Volume (ml)	Unit/ml	Total activity (U)	Total Protein (mg)	Specific Activity (U/mg)	Purification fold	% recovery
Fermented Broth	100	56	5600	33.7	166	1	100
$NH_4(SO_4)_2$ precipitation	10	512	5120	26.0	196	1.18	91.42
Sephadex G-100	10	63.2	632	0.3	2106	12.69	11.28

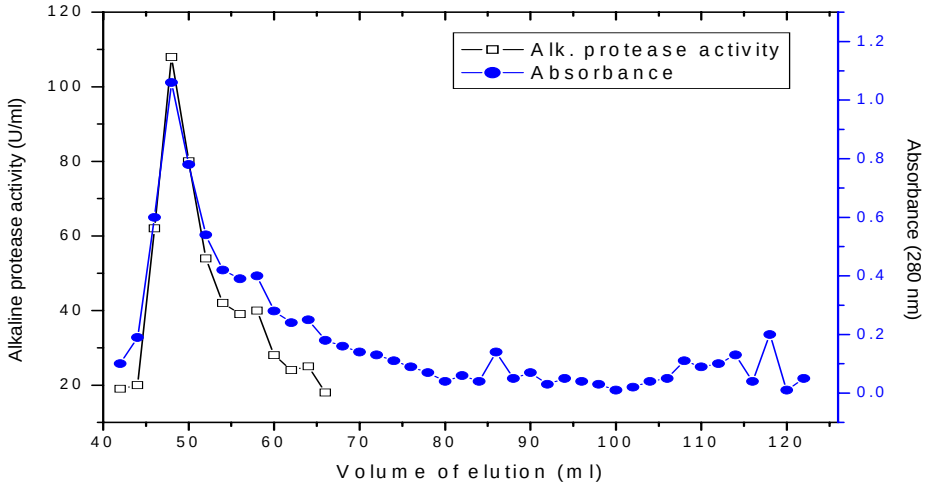

Figure 1.Elution profile of purification of alkaline protease from *Bacillus subtilis*

SDS-PAGE of purified alkaline protease from Bacillus subtilis

The enzyme was successfully purified to the homogeneity as evident by a single band on SDS-PAGE. The apparent molecular mass of purified alkaline protease was estimated as 30 KDa by using Rf values of the reference proteins. The molecular mass standards were BSA (67KDa), oval albumin (45 KDa), carbonic anhydrase (30KDa), Trypsinogen (24KDa) and α-lactalbumin (14KDa) on SDS-PAGE. The molecular weight of the alkaline protease obtained from other *Bacillus* species were reported as 28 KDa from *Bacillus cereus* (Banik and prakash, 2006); 15KDa from *Bacillus subtilis* PE-11 (Adinarayana et al. 2003); 30 KDa from *Bacillus* sp. PN-13 (Ogino et al. 2007).

Characterization of purified alkaline protease

Temperature optima and thermostability

The temperature optima of purified *Bacillus subtilis*alkaline protease was 55ºC. The alkaline protease activity gradually declined at temperatures beyond 55ºC (Fig. 2). This was comparable to alkaline protease from *Bacillus stearothermophilus* AP-14 and 60ºC for alkaline protease derived from *Bacillus subtilis* PE-11 and *Pseudomonas aeruginosa* (Adinarayanaet al. 2003, Karadzic et al. 2004).

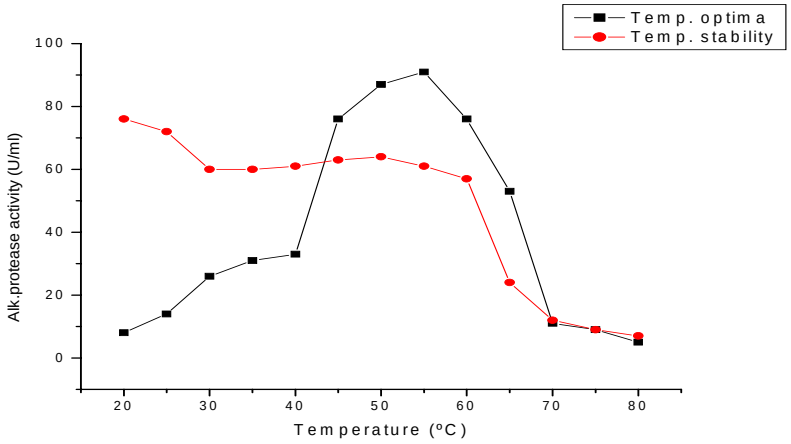

Figure 2. Temperature optima and thermo stability for purified alkaline protease

The thermal stability of purified alkaline protease was tested at different temperatures ranging 20-80ºC for 30 minutes. The enzyme was quite stable up to 60ºC, beyond that it lost (17 to 9%) at 70 to 80ºC respectively. Whereas Alkaline protease from alkaliphilic*Bacillus pumilus* was stable only for 20 min at 55 ºC (Han and Damodaran, 1998).

pH optima and pH stability

The purified alkaline protease was active in the pH range of 9-11 with optimum at 10.5. These findings are in accordance with several earlier reports showing pH optima of 10 to 10.5 for protease from *Bacillus* sp., *Thermusaquaticus*, *Xanthomanasmaltophila* and *Vibrio metscnikovii* (Durham et al. 1987; Matsuzawa et al. 1988; Debette 1991; Kwon 1994).

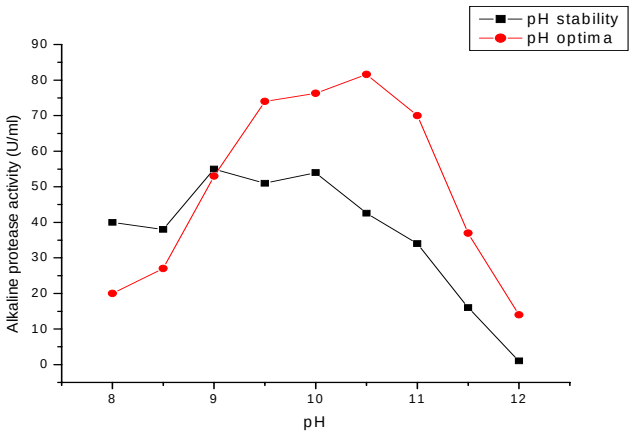

Figure 3.pH optima and pH stability for purified alkaline protease

The greater stability in the alkaline range reflects the alkaliphilic nature of the enzyme. The above results are significant because of the fact that the important detergent enzymes, SubtilisinCarlberg and Subtilisin Novo or BPN also show maximum activity at pH 10.5 (Adinarayanaet al. 2003).

Effect of inhibitors

The effect of different inhibitors (10mM) on the purified alkaline protease was studied. Protease was completely inhibited by Phenyl methyl sulphonyl fluoride (PMSF), a specific inhibitor of serine protease (Fig. 4). Other specific protease inhibitor such asDiisopropyl fluorophosphates (DFP) also exhibited 97% inhibition. In contrast, there was no inhibition by cysteine-type inhibitors viz. *p*-chloromercuric benzoate (*p*-CMB), β-mercaptoethanol (β-ME), Iodoacetate, Ethylene diamene tetra acetic acid (EDTA). No inhibition was detected with metalloprotease inhibitor like EDTA. These results are similar to the results obtained with an alkaline protease from alkaliphilic*Thermoactinomycetes*sp HS682 and *Bacillus cereus*, in which the enzyme was completely inhibited by DFP and PMSF but not by EDTA (Tsuchiya et al. 1997;Banik and Prakash 2006).

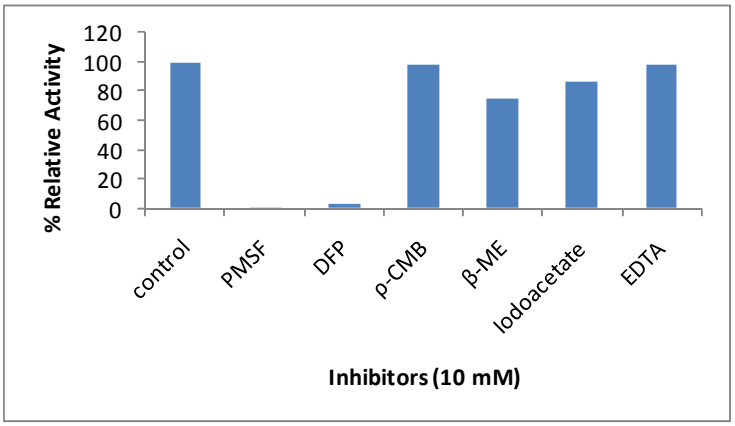

Figure 4. Effect of inhibitors on purified alkaline protease activity

Effect of metal ions

Metal ions had varied effect on purified alkaline protease activity. There was significant increase in protease activity with Ca^{++}, Mg^{++}, Mn^{++} and slightly inhibitory effect with Co^{++}, Hg^{++}, Al^{++}. The maximal activity of the enzyme reached to 130% with these metals. Some of the metal ions such as Ca^{++}, Mg^{++}, Mn^{++}increased and stabilized the protease activity of the enzyme, possibly because of the activation by the metal ions.

These metal ions also have been reported to increase the thermal stability of other *Bacillus* alkaline protease (Rahmanet al. 1990;Paliwalet al. 1994). The findings were significant in the wake of a recent report on two novel halotolerant extracellular protease from *Bacillussubtilis*strain FP-133 which were activated in the presence of Ca^{++} and inhibited by Hg^{++} (Joo and Chang 2005).

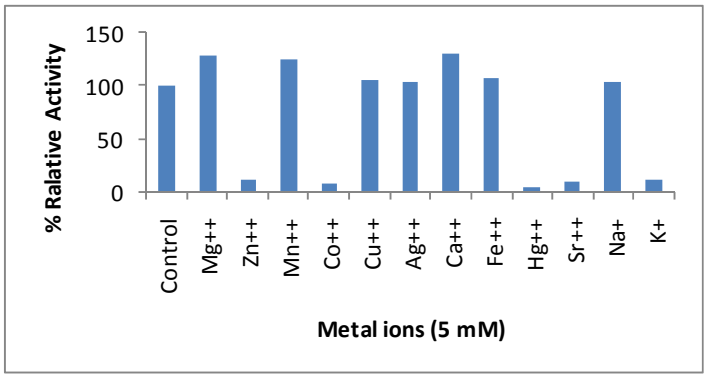

Fig.5. Effect of metal ions on purified alkaline protease activity

Hydrolytic activity

When alkaline protease assayed with native proteins as substrates, the protease showed a high level of hydrolytic activity against casein, moderate hydrolysis against haemoglobin and poor to moderate hydrolysis of BSA (Bovine serum albumin) and egg albumin, but the hydrolysis was hardly observed with gelatin.

Table 3.Alkaline protease activity against different natural substrates

Substrates (2mg/ml)	Relative enzyme activity (%)
Casein	98
Haemoglobin	82
BSA	51
Egg albumin	20
Gelatin	7

CONCLUSION

The studies presented in this paper focussed on two-step purification and characterization of a novel alkaline protease from a bacterial strain of *Bacillus subtilis.*The purified alkaline protease has molecular weight 30KDa with an optimum activity at 55ºC and pH 10.5. Due to these characteristics it can withstand high pH and temperature levels and therefore appears to be a potentially useful enzyme in detergent industries.

Since for a variety of industrial applications relatively high thermostability is an attractive and desirable characteristic of an enzyme, this enzyme can be exploited commercially.

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