Partial purification and properties of a laundry detergent compatible alkaline protease from a newly isolated *Bacillus* species Y

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Abstract Alkaline protease production by a newly isolated *Bacillus* species from laundry soil was studied for detergent biocompatibility. From its morphological and nucleotide sequence (about 1.5 kb) of its 16S rDNA it was identified as *Bacillus* species with similarity to *Bacillus* species Y (Gen Bank entry: ABO 55095), and close homology with *Bacillus cohnii* YN-2000 (Gen Bank entry: ABO23412). Partial purification of the enzyme by ammonium sulfate (50–70% saturation) yielded 8-fold purity. Casein zymography and Sodium dodecylsulphate- Polyacrylamide gel electrophoresis (SDS-PAGE) of the partially purified enzyme revealed two isozymes of molecular sizes approximately 66 kDa and 18 kDa, respectively. The enzyme was most active at pH 12 and 50°C. At pH 12 the enzyme was stable for 5 h and retained 60% activity. The enzyme retained 44% activity at 50°C up to 2 h. The protease showed good hydrolysis specificity with different substrates tested. The presence of Mn^{2+} , Co^{2+} and ethylenediaminetetracetic acid (EDTA) showed profound increase in protease activity. The protease of *Bacillus* species Y showed excellent stability and compatibility with three locally available detergents (Kite, Tide and Aerial) up to 3 h retaining almost 70–80% activity and 10–20% activity at room temperature (30°C) and 50°C, respectively, indicating the potential role of this enzyme for detergent application.

Keywords Alkaline protease · *Bacillus* species Y

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Introduction

It is well known that proteases constitute one of the most important groups of industrial enzymes, as it accounts for at least a quarter of the total global enzyme production [1]. Proteases have a long history of application in food and detergent industries with the detergent alkaline proteases holding the largest share of the enzyme market [2]. Although a wide range of microorganisms are known to date to produce proteases, a large proportion of the commercially available alkaline proteases are derived from *Bacillus* strains because of its ability to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures [3]. Many of the available alkaline proteases exhibited low activity and stability towards detergent formulations. Considering these facts, we attempted to isolate and screen new alkalophilic microorganisms from some local washer men place soil which produced a highly alkaline proteolytic enzyme having potential in detergent industries. The present study describes a detergent compatible alkaline protease from a new *Bacillus* species Y with high alkaline stability at pH 12.

Materials and methods

Isolation and screening of microorganism

Protease-producing isolates were isolated from the laundry wash area using a skimmed milk agar plate NaCl (0.5% w/v), agar (2% w/v) and skim milk (10% v/v) with Na_2CO_3 (0.4% w/v). Plates were incubated at 37°C for 24 h [4].

Among the isolates, *Bacillus* species which exhibited a prominent clear zone was selected for the study of protease production [14]. The isolate was maintained on skim milk agar, stored at 4°C. The above medium without agar constituted the basal culture medium for the protease production. Sodium carbonate solution was sterilized separately, and then added to the medium. The medium (100 ml) was inoculated with 1 ml (containing approximately 10^9 cells/ml) of a 24-hour-old seed culture in 250 ml baffled flasks, and incubated at 37°C with shaking at 120 rev/min for 72 h. The cell-free supernatant was recovered by centrifugation (8,000 rpm, 4°C, 20 min), and used for determining the protease activity.

Characterization of the isolated bacteria

The morphological and physiological properties of the isolate were investigated according to Bergey's manual of determinative bacteriology [5]. The sequencing of 16S rDNA and taxonomic studies of the strain were performed at The Chromous Biotech Pvt. Ltd., Bangalore India. A partial 16S rDNA fragment (~1.5 kb) was amplified using high-fidelity DNA polymerase. The polymerase chain reaction (PCR) product was sequenced bi-directionally using the forward reverse and an internal primer. The sequence data was aligned and analyzed to identify the bacterium and for finding nearest related strains.

Ammonium sulfate precipitation

The organism was grown in basal medium for 72 h at 37°C on shaker incubator, the cells were separated by centrifugation (7,155.2 g, 15 min), and the protease in the cell-free supernatant was fractionated by precipitation with $(\text{NH}_4)_2\text{SO}_4$ between 50 and 70% saturation. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1 M sodium carbonate buffer, pH 10, and dialyzed against the same buffer (0.05 M) for 24 h with two changes of buffer, and then the enzyme was collected and used for further assay [6].

Protease assay

Enzyme was assayed by the using casein as a substrate. Alkaline protease activity was determined in 0.5 M sodium carbonate buffer (pH 10.0) using casein at 1% concentration. The reaction mixture (2 ml) containing 0.5 ml of 1% casein in 0.5 ml of sodium carbonate buffer (0.5 M, pH 10.0) and 1 ml of appropriately diluted enzyme solution were incubated at 30°C for 10 min, and the reaction was stopped with 1 ml of 10% trichloroacetic acid [7]. The tubes were centrifuged

at 11,180 g for 10 min and the degraded products were measured by modified Lowry method. One unit of alkaline protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine/min/ml under the standard assay conditions. The estimations were based on a tyrosine calibration curve.

Protein estimation

Protein estimation was determined by the method of Bradford method using the Bio-Rad protein dye reagent concentrate and Bovine serum albumin (BSA) as standard [8].

Chemicals

Casein for protease assay was purchased from Sigma. All other media components and chemicals used are of purity grade available from Hi-Media.

Gel electrophoresis

Purity of the sample and estimation of molecular mass were determined by 12% SDS-PAGE based on method of Laemmli [9]. Proteins were visualized by silver staining. The molecular mass of the enzyme was estimated by calibrating using standard protein molecular weight markers (medium range) [phosphorylase b (97 kDa), bovine serum albumin (BSA) (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), trypsinogen (20 kDa) and lysozyme (14 kDa)] on SDS-PAGE.

Detection of protease activity by zymography

The alkaline protease produced from the strain was analyzed on 12% SDS-polyacrylamide gel containing 0.1% casein as copolymerized substrate as described by Oliver et al. [10]. The staining and destaining of the gel was performed by Coomassie brilliant blue in a single step based on method given by Leber and Balkwill [11]. The protease activity band was visualized as a clear colorless band against blue background.

Characterization of alkaline protease

The activity of the partially purified protease was measured at different pH values using the following buffers (0.5 M): Sodium phosphate (pH 7–7.5), Tris-HCl (8–9), sodium carbonate (pH 10–12). Stability of the partially purified enzyme at (pH 10 and 12) was studied by preincubating the enzyme in the respective buffers for

2, 4, 5 and 24 h and assaying the residual enzyme activity at optimum assay conditions. Effect of temperature on the enzyme activity was determined by incubating the reaction mixture at different temperature between 4°C and 80°C. The effect of metal ions e.g. (MgCl₂, NaCl, MnCl₂, FeCl₃, MgCl₂, HgCl₂, CaCl₂, CoCl₂, EDTA) (10 mM) were investigated by adding them to the reaction mixture. Relative protease activities were measured under optimum assay conditions. Protease activity with various protein substrates including BSA, casein, hemoglobin, egg albumin and gelatin (2 mg/ml) were assayed by mixing 10 µl of partially purified enzyme (100 µg/ml), 0.5 ml of assay buffer containing the protein substrate (0.5 ml) in the reaction mixture. The protease activity with BSA as the substrate was taken as control and relative activities were measured as described in earlier section. The above experiments were carried out in triplicates and average mean of the values are reported.

Detergent stability

The compatibility of *Bacillus* species Y protease with local laundry detergents was studied under different incubation time periods between 0.5 and 3 h. Detergents used were Kite, Tide and Aerial. The detergents were diluted in sodium carbonate buffer (0.5 M, pH 10) to get a concentration of 0.7% (to simulate washing conditions) and incubated with the enzyme both at room temperature (RT – 30°C) and 50°C, respectively and the residual activity was determined. The enzyme activity of a control sample (without any detergent) was taken as 100% [4]. The experiment was carried out in triplicates and average mean of the values are reported.

Results and discussion

Bacillus-derived alkaline proteases are the major industrial workhorses and the recent trend towards the use of alkaline proteases from these sources in different process applications like detergents, tanning, food, waste treatment and peptide synthesis has increased remarkably because of their increased production capacities, high catalytic activity and high degree of substrate specificity [12, 13].

Isolation and cultivation of microorganisms

Of the total 81 isolates screened, growth and enzyme production was observed at 48 h by five *Bacillus* isolates with maximum zone of hydrolysis by one isolate. The zone of hydrolysis of this strain on milk agar is shown in Fig. 1. The strain which showed appreciable amounts of

Fig. 1 The zone of hydrolysis of *Bacillus* species Y on milk agar.

protease production in terms of higher specific activity (35 U/ml, 101.3 U/mg specific activity) as compared to protease production from other isolates (23 U/ml, 50 U/mg; 10 U/ml, 35 U/mg; 55 U/ml, 98 U/mg; 22 U/ml, 35 U/mg) was further studied. Analysis of the partial nucleotide sequence of the 16S rDNA (~1.5 kb) identified the isolate to be most similar to *Bacillus* species Y (Gen Bank entry: ABO 55095), with a close homology to *B. cohnii* YN-2000 (Gen Bank entry: ABO23412) (Fig. 2).

Partial purification of protease

Ammonium sulfate method of partial purification yielded 8-fold purity and 18.5% recovery as compared to alcohol precipitation method which yielded 1.3-fold purity and 37% recovery (Table 1). The percentage recovery in terms of activity as well as protein is less with ammonium sulfate method as this involved fractional precipitation between 50 and 70% saturation while alcohol precipitation method involved total protein precipitation. However, ammonium sulfate method proved to be better for purification of the enzyme.

This partially purified enzyme was detected and characterized by zymography using casein as substrate (Fig. 3a). Casein zymography showed the presence of two isozymes. The molecular weight of the two isozymes was determined to be approximately 66 kDa and 18 kDa, respectively (Fig. 3b). The molecular masses of microbial alkaline proteases ranged between 15 and 36 kDa, with few exceptions of high molecular mass, such as 42 kDa from *Bacillus* species PS719 [13] and very high (90 kDa) from *Bacillus subtilis* [15]. Similarly, Gouda (2006). reported three major clear zones of proteolytic activities by a marine *Bacillus* species MIG (strain designation) that were active

Fig. 2 Phylogenetic tree representing close homologs of *Bacillus* species Y (unknown). The tree was constructed by Neighbor-Joining on a p distance matrix.

Table 1 Summary of purification steps of alkaline protease from *Bacillus* species Y

Purification step	Enzyme activity (U/ml)	Total enzyme activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% Recovery of activity
Crude enzyme (200 ml)	27	5400	0.26	53.3	101.3	1.0	100
a. Alcohol precipitate (5 ml)	400	2000	2.96	14.8	135	1.33	37
b. Ammonium sulfate precipitate (50–70%), dialyzed (5 ml)	200	1000	0.24	1.2	847	8.36	18.5

over broad range of pH and fourth one that was only active in the alkaline range [16].

Effect of different pH and temperature on alkaline protease activity and stability

The enzyme showed reasonable activity between pH 10 and 13 with maximum activity at pH 12 (Fig. 4). The enzyme was stable at both pH 10 and 12. At pH 10 the enzyme was stable up to 5 h and retained almost 62% activity while further incubation up to 24 h showed activity up to 15%. At pH 12, the enzyme was stable up to 5 h and retained 60% activity while further incubation up to 24 h showed 32% activity (Fig. 5). These findings are in accordance with earlier studies on proteases from other bacteria that the pH range of most alkaline proteases was generally between 9 and 11 [12]. The enzyme showed reasonable good activity between temperatures 30°C and 60°C with maximum activity at 50°C (Fig. 6). As the graph shows (Fig. 7), the

enzyme was stable at 37°C for up to 150 min of testing. At 50°C the enzyme retained 44% activity up to 120 min and was completely inactivated by 150 min. These findings suggest that protease secreted from *Bacillus* species Y is highly pH stable alkaline protease. In general all currently used detergent-compatible proteases are alkalophilic in nature with varying thermostabilities at laundry temperature (50–70°C).

Effect of various metal ions on alkaline protease activity

The presence of Mn^{2+} , EDTA and Co^{2+} were stimulatory (Table 2), confirming that these cations take part in the stabilization of the protease structure and are required for protection against thermal denaturation [17], while the other metal ions did not show any appreciable effect on the protease activity as in previous reports [19]. The stability of the enzyme in the presence of EDTA suggests

Fig. 3a Casein zymogram showing bands of protein cleavage in 12% SDS-PAGE containing 0.1% casein. After electrophoresis the gels were washed in Triton X-100 to remove the SDS, and then incubated at optimum pH. Lane contains ammonium sulfate ppt, dialyzed sample. Arrows indicate the two isozymes of alkaline protease.

Fig. 3b Fractions from ammonium sulfate cut, dialyzed sample on 12% SDS-PAGE: Lane 1: Standard protein molecular weight mass marker, Lane 2: ammonium sulfate ppt, dialyzed sample.

Fig. 4 Optimum pH of the alkaline protease activity. Activity was assayed at 30°C. Buffers used were (0.5 M): sodium phosphate (pH 7–7.5), Tris–HCl (8–9), sodium carbonate (pH 10–13).

that metal cofactors are not required for enzyme activity. This property of the enzyme is very useful for application as detergent additive since chelating agents, which function as water softeners and are involved in the removal of stains are components of the detergent and then specifically bind divalent cations [18, 19].

Hydrolysis of protein substrates

The protease showed a good hydrolytic activity with all the substrates tested. The activity of casein was taken as control (Table 3). The enzyme showed highest specificity with egg albumin, moderate hydrolysis with gelatin > hemoglobin >

Fig. 5 Effect of pH on the stability of the alkaline protease. The enzyme was preincubated in pH 10 and pH 12 for different time intervals at 30°C and the residual activity was assayed at optimum assay conditions.

Fig. 6 Effect of temperature on the alkaline protease activity. The effect of temperature was determined at the range between 4 and 80°C.

BSA, suggesting broad substrate specificity of this enzyme. Similar studies done by Adinarayana et al. [4] showed that *B. subtilis* PE-11 had high specificity for casein, poor to moderate hydrolysis of BSA and egg albumin and no preference for gelatin. In view of other investigators, 0.1% (w/v) gelatin was the best substrate concentration for thermostable protease activity produced by *B. brevis*

geltinoamylolyticus [20]. Many of the previous studies revealed that alkaline proteases showed highest activity towards casein relative to other proteins including BSA, hemoglobin, keratin and azocasein [21, 22]. The ability of the protease produced by the test organism to hydrolyze a wide range of proteins substrate may be advantageous for its use in detergents against a wide variety of stains.

Fig. 7 Temperature stability of the alkaline protease. The stability was determined by preincubating the enzyme at temperatures 30°C and 50°C for different time intervals. The residual activity was estimated under optimum assay conditions.

Table 2 Effect of various metal ions on alkaline protease activity

Metal ions (5 mM)	Residual alkaline protease activity (%)
Control	100
Mg ²⁺ (MgCl ₂)	95
Mn ²⁺ (MnCl ₂)	600
Fe ³⁺ (FeCl ₃)	95
Hg ²⁺ (HgCl ₂)	100
Ca ²⁺ (CaCl ₂)	100
Co ²⁺ (CoCl ₂)	225
EDTA	550

Table 3 Alkaline protease activity against different natural substrates

2 mg/ml (substrate)	% specific activity (U/mg)
Casein (control)	100
Gelatin	175
Hemoglobin	150
Egg albumin	375
BSA	125

Detergent stability

Besides pH, a good detergent protease is expected to be stable in the presence of commercial detergents. Alkaline protease is a promising detergent additive at alkaline conditions and activity over a broad temperature range would facilitate washing even at room temperatures which is a present day requirement to maintain fabric quality at low energy demands. Hence, the stability of the enzyme in presence of three locally available detergents was performed at both room temperature (30°C) and 50°C (temperature optima of the enzyme). The protease of *Bacillus* species Y showed excellent stability and compatibility with three locally available detergents (Kite, Tide and Aerial) at RT up to 3 h retaining almost 70–80% activity. When tested at 50°C, the enzyme retained almost 10–20% residual activity indicating the potential role of

this enzyme for detergent application (Table 4). Protease from *B. subtilis* PE-11 retained more than 50% activity with most of the detergents tested even after 3 h incubation at 60°C after the supplementation of CaCl₂ and glycine [4]. Alkaline protease of *B. circulans* BM15 showed 67–78% activity with various commercial detergents tested [23]. Singh et al. [24] reported that *Bacillus* sp. SSR1 protease retained 40.90% of its activity in the presence of local detergents. Bhosale et al. [25] reported only 16% activity in Revel, 11.4% activity in Aerial, and 6.6% activity in Wheel. Ingale et al. [26] reported a *Basidiobolus* protease which had retained activity only below 40% after 40 min with Surf (3 mg/ml). Comparing these results, the *Bacillus* species Y enzyme was significantly more stable with commercial detergents.

In a report by Banik and Prakash [27] the endogenous protease activity in various commercially available laundry detergents of international companies was studied and the maximum protease activity was found at 50°C in pH range 10.5–11.0 in all the tested laundry detergents.

Table 4 Compatibility of alkaline protease from *Bacillus* species Y with local commercial detergents

Time (h)	Relative residual alkaline protease activity (%)					
	(30°C)			(50°C)		
	Tide	Ariel	Kite	Tide	Ariel	Kite
0.5	84	80.4	87	75	72	70
1.0	80	80	73.3	60	58	54
1.5	77.7	77.7	71.1	50	55	50
2.0	76	73.3	71.1	45	40	46
3.0	75.5	70.5	71.1	20	12	10

Conclusion

Therefore the present study indicates that the alkaline protease from this newly isolated *Bacillus* species Y is valuable for application in detergent formulations owing to its alkaline pH optima (pH 12), temperature optima (50°C), broad substrate specificity and detergent compatibility.

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