Solid-state fermentation for the production of alkaline protease by *Bacillus cereus* 1173900 using proteinaceous tannery solid waste

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Animal fleshing, the major proteinaceous solid waste generated from leather industry, was used as substrate for the production of alkaline protease by *Bacillus cereus* 1173900 in solid-state fermentation (SSF). Maximum protease activity of 12,310 U/g was observed at 60 h in SSF crude extract. The extracted protease enzyme was purified by 53.64-fold through ammonium sulphate precipitation and chromatography separation in Sephadex G-100. The purified protease had a specific activity of 201.6 (U/mg). The molecular weight of the purified enzyme was 66 kDa, determined by SDS–PAGE. The zymogram also revealed a clear hydrolytic zone due to proteolytic activity, which coincided with the band obtained with SDS–PAGE. Enzyme activity was inhibited by EDTA, suggesting that the enzyme belongs to metalloprotease(s).

Keywords: Alkaline protease, animal fleshing, *Bacillus cereus*, solid state fermentation.

THERE are about 3000 leather manufacturing industries in India with processing capacity of 600,000 t/yr. Tamil Nadu, Uttar Pradesh, West Bengal and Punjab have major clusters of tanneries[1]. The solid and liquid wastes discharged from the leather industry contain variable amounts of proteins, which are either utilized or underutilized. Animal fleshing (ANFL), the major solid waste generated during pre-tanning operations of leather processing, is proteinaceous in nature, comprising cutaneous muscle layers and sub-cutaneous adhering tissues that are not required in the subsequent operations of leather manufacture. The generation of ANFL is 35% on the wet weight of raw hides with 80% moisture[2]. The leather industries are confronted with two options for the disposal of solid wastes; either to minimize the quantity of waste generated or maximize the return on by-products[3]. However, recovery of the value-added products from solid wastes has been largely a neglected field. Protein and enzyme production using solid waste as the substrate has been demonstrated as a viable technique for by-product recovery[4]. The microbial proteases are commercially important enzymes having a wide range of applications in various industrial, biotechnological, medicinal and basic research fields, apart from waste treatment[5,6]. Alkaline proteases, an important group of industrial enzymes, are produced by a wide range of microorganisms, including fungi and bacteria. *Bacillus* spp. have been reported to produce alkaline protease from various substrates, viz. green-gram husk[7], mustered-oil cake, wheat bran, rice bran, *Imperata cylindrical* grass, banana leaves, potato peels and used tea leaves[8], wheat bran and lentil husk[9]. However, few reports are available on alkaline proteolytic enzymes from *Bacillus cereus*[10]. Solid-state (substrate) fermentation (SSF) has been defined as the fermentation process occurring in the absence or near-absence of free water. In the past few years, new biotechnological innovations have identified SSF as a promising low-cost technology. SSF has numerous advantages, including productivity and may be preferred to the submerged fermentation due to simple operational procedures, lower levels of catabolite repression and better product recovery[11]. Reports on SSF of ANFL for the production of alkaline protease using *B. cereus* are limited or perhaps not available. Hence, the focal theme of the present study was on the production of alkaline protease by *B. cereus* 1173900 in SSF from ANFL as the substrate, and determination of the optimum conditions for enzyme production.

The bacterial strain was isolated from the wastewater discharged from a tanning industry in Chennai, India. The isolated strain was maintained in nutrient agar plates and the purity of the strain was checked by microscopic examination. The pure isolate was then examined for its proteolytic activity. The 16S rDNA gene was sequenced after genomic DNA extraction and PCR amplification with specific universal primers, and then blasted using the public domain. The base pair sequence obtained was submitted to GenBank (NCBI).

The limed ANFL of goat skins was collected from a commercial tannery, Chennai, India. It was treated with ammonium chloride (1.25% w/v) for 3–4 h to remove the adsorbed calcium salts on the collagen fibres. The delimed ANFL fleshing was suspended in water for 1 h at neutral pH (ref. 12). The square shape ANFL pieces of dimension 0.25 cm were obtained by manual scissoring. The pieces meant for further experiments were packed in polyethylene containers and stored at 4°C until the commencement of the experiments. ANFL was chemically characterized following procedures reported in our previous findings[13].

Ten grams of square shaped ANFL (size 0.25 cm) was transferred into 250 ml Erlenmeyer flask for SSF studies. The minimal salt medium composition (g/l) of KH_2PO_4, 2.0; K_2HPO_4, 7.0; $(NH_4)_2SO_4$, 1.0; glucose, 1.0; sodium citrate, 0.5 and $MgSO_4 \cdot 7H_2O$, 0.5 was autoclaved at 121°C and 15 psi for 15 min, and used for the SSF process. The 24-h grown culture of *B. cereus* was suspended in sterilized minimal medium and poured into the flask containing ANFL and initial moisture content was main-

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tained at 60%. The fermentation flasks were incubated at 37°C. All experiments were carried out in duplicate and repeated thrice.

The effect of pH (6–11) and harvest time (84 h) on protease production by *B. cereus* was monitored in the minimal salt medium containing ANFL at room temperature. Protease activity was determined for 84 h with an interval of 12 h in the SSF process.

The crude proteolytic enzymes were extracted from SSF with 0.1% NaCl solution under agitation. Subsequently, the solids were removed and filtered (0.45 mm, Millipore nitrocellulose membrane) to obtain the culture filtrate. All experiments were carried out in duplicate and repeated thrice.

The protease activity assay was carried out by treating 1 ml of proteolytic enzyme with casein as substrate (1% (w/v)) for 30 min at pH 8 using sodium phosphate buffer at 37°C. The reaction was terminated by the addition of 3 ml of 5% trichloroacetic acid and the mixture was kept at room temperature for 10 min followed by filtration through Whatman No. 1 filter paper. The absorbance of the filtrate was measured at 280 nm. One unit of alkaline protease activity is defined as 1 μg of tyrosine liberated per ml under the assay conditions[14].

The cell-free supernatant was precipitated with 70% (w/v) saturated ammonium sulphate and centrifuged at 12000 *g* for 20 min at 4°C. The collected precipitate was resuspended in 20 mM Tris-HCl buffer (pH 8.0) and dialysed against the same buffer for 24 h at 4°C using nitrocellulose membrane with repeated changes. The dialysed sample was further purified using a Sephadex G-100 column equilibrated and eluted with 20 mM Tris-HCl buffer (pH 8.0). These purified fractions were collected, assayed for enzymatic activity and lyophilized for further studies.

The optimum pH for the purified enzyme was determined by preparing 1% casein solution using 0.2 M buffers of different pH values such as sodium phosphate (pH 6.0–8.0), Tris-HCl (pH 7.0–9.0), and glycine NaOH (pH 9.0–11.0), and 1 ml of suitably diluted proteolytic enzyme was added to the mixture which was then kept at 37°C for 30 min. Similarly, the enzyme stability at different pH values was determined by incubating the enzyme in the pH range 6–11 in the respective buffers as listed above and incubated for 1 h at 37°C. After the incubation period, aliquots were taken and the residual activities were measured according to standard assay procedure.

The optimum temperature for protease activity was determined by measuring the activity of protease at various temperatures (20°–70°C) using 1% casein as the substrate at optimized pH 8. The thermal stability was studied by incubating the enzyme at different temperatures, and appropriate aliquots were withdrawn after 1 h and the residual activities determined at assay temperature.

The purified enzyme was incubated with different salt solutions: NaCl, $FeSO_4$, $CaCl_2$, $MnSO_4$, $CuSO_4$, $ZnSO_4$ at 1, 5 and 10 mM concentrations for 1 h at 37°C. The effect of various inhibitors, viz. phenyl methyl sulphonyl fluoride (PMSF), ethylene diamine tetra-acetic acid (EDTA), ethyleneglicol-*bis*(*β*-aminoethyleter *N*′,*N*′-tetra-acetic acid (EGTA), 1,10-phenathroline and dithiotheitol (DTT) at a concentration of 5 mM for 1 h on purified protease enzyme was studied. The effect of surfactants (1% w/v) like sodium dodecyl sulphate, sodium tripolyphosphate, sodium tetraborate and Tween 80 on protease enzyme for 1 h was also determined. The residual activity was determined by protease activity method.

SDS–PAGE was carried out for the determination of purity and molecular mass of the enzyme[15], using a 5% (w/v) stacking gel and a 12.5% (w/v) separating gel. Zymographic analysis was performed by the enzyme pattern of proteins, obtained by zymogram with 1% casein substrate and detected using coomassie brilliant blue R250 (ref. 16).

All the experiments were carried out in triplicate. Results obtained were analysed using Microsoft Excel program to determine the standard deviations.

Chemical characteristics of ANFL were determined on a dry weight basis and the standard deviations were based on triplicate analysis. The characteristics of ANFL are total Kjeldhal nitrogen (TKN), 102 ± 5.1 (mg/g); total organic carbon (TOC), 380 ± 20.1 (mg/g); fat, 5.0 ± 0.5 (mg/g); moisture content, 75.5 ± 3.5 (%); ash content, 15.2 ± 0.3 (%); total solids, 25.8 ± 2.1 (%) and total protein, 50 ± 1.5 (%).

The BLAST result showed high similarity to a group consisting of several *Bacillus* strains (similarity 98%). The nucleotide sequence reported here has been submitted to the GenBank database and assigned accession number *Bacillus cereus* 1173900. The phylogenetic analysis revealed that the closest relatives of the isolated strain were *B. cereus* 03BB102, *B. cereus* ATCC 10987 and *B. cereus* MCM B-326 (Figure 1).

The alkaline protease was produced by *B. cereus* in SSF using ANFL as the solid substrate and their parameters, viz. time, pH and temperature were optimized. This experiment was carried out in the pH range from 6 to 11

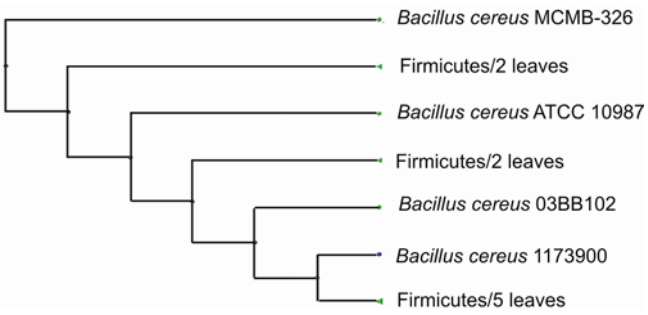

Figure 1. Phylogram obtained based on phylogenetic analysis of 16S rDNA sequence of *Bacillus cereus* 1173900.

Table 1. Purification procedure of alkaline protease from *Bacillus cereus* 1173900

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (*n*-fold)
Culture supernatant	13015	5500	2.36	100	1.0
Ammonium sulphate and dialysed	8500	720	11.80	65.3	5.0
Sephadex G-100	3024	15	201.6	23.2	85.4

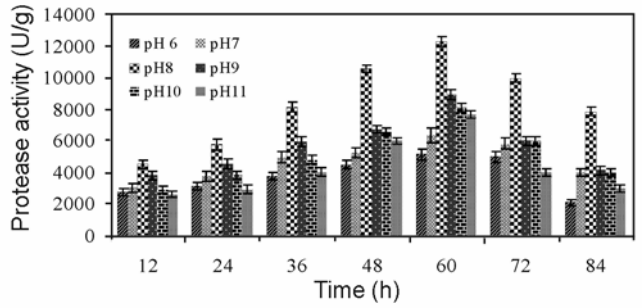

Figure 2. Optimization of pH and incubation period on protease activity of crude enzyme (U/g).

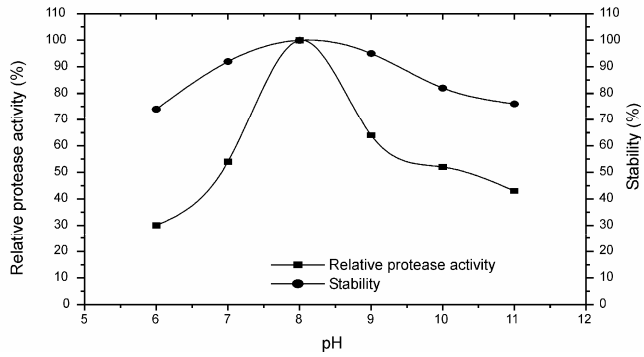

Figure 3. Effect of pH on protease residual activity (%).

and incubation time was up to 84 h (Figure 2). The alkaline protease production by bacterial strains strongly depends on the extracellular pH, as the culture pH steadily influences many enzymatic processes and the transport of various components across the cell membranes, which in turn sustain cell growth and product development[12]. The ability of the bacteria to synthesize maximum crude enzyme (12,310 U/g) was recorded at fermentation period of 60 h and at pH 8, while minimum protease production (2100 U/g) was observed at pH 6. The temperature for SSF was optimized by performing the protease assay at different temperatures, i.e. 20°C, 30°C, 40°C, 50°C, 60°C and 70°C, at the optimum pH 8.

Maximum protease activity of 13,015 U/g was observed at optimum temperature of 40°C. However, the activity decreased at temperatures below 20°C and above 50°C. The optimum temperature of 40°C reported by us corroborates with that of Shumi *et al.*[17] on protease production by *Bacillus* sp.

Protein pellets obtained after precipitation with saturated ammonium sulphate (70% w/v) were dissolved in 20 mM Tris-HCl buffer and loaded onto the Sephadex G-100 column. This was equilibrated with Tris-HCl buffer to elute the enzyme. Purified lyophilized samples were used for molecular-weight determination. The alkaline protease was purified by 53.64-fold with ammonium sulphate precipitation and further chromatographic separation in Sephadex G-100 column to obtain 23.2% yield. The specific activity of the purified enzyme was 201.6 U/mg protein. Table 1 shows the purification steps involved for alkaline protease of *B. cereus*.

Although the enzyme was active over a broad range of pH values, the optimum pH for enzyme activity was found 8.0, and thus the enzyme was considered under alkaline protease. The appropriate pH for protease stability was determined by incubating with buffers of varying pH from 6 to 11 at 37°C for l h (Figure 3). In this study, the relative enzyme activity was 85% in the pH range 7–11. The maximum protease relative activity was 100% at pH 8, whereas the minimum activity of 29.8% was observed at pH 6. These values are in good agreement with those obtained from *B. cereus* BG1 (ref. 18) and *B. cereus* KCTC 3674 (ref. 19).

Results showed that the shape of the pH stability curve is similar to that of the optimal pH values for the highest activity. The *Bacillus* sp. producing alkaline protease showed activity over a broad range of alkaline pH values, whereby more than 85% of enzyme activity was present[20]. Protease stability was recorded the highest as 100% at pH 8 and the lowest at pH 6 with 74% residual activity. These results conclude that the *B. cereus* enzyme can be classified under alkalophilic protease.

The optimum temperature for alkaline protease activity was determined by assay at different temperatures from 20°C to 70°C, using casein as the substrate at optimum pH 8.0. The protease was active in the temperature range from 30°C to 50°C, but gradually decreased at temperature 60°C and 70°C. The optimum temperature for protease activity was 40°C (Figure 4). The stability of the purified enzyme was determined at different temperatures for 1 h at pH 8.0 (Figure 4). The highest protease thermal stability was recorded at 50°C and the lowest at 70°C. Our finding was similar to that Kumar *et al.*[21], who reported that *Bacillus clausii* produced relative protease enzyme activity of 100% after incubation in the temperature range from 30°C to 65°C for 60 min.

The purified enzyme was incubated with different metal ionic solutions of 1, 5 and 10 mM for 1 h at 37°C. The results (Table 2) revealed that the prominent inhibitory effects were by Zn^{2+}, Cu^{2+} and Fe^{2+}, whereas Ca^{2+}, Mg^{2+} and Mn^{2+} exhibited stimulatory effect on protease activity. The presence of metal ions like Ca^{2+} not only protected the enzyme from undergoing denaturation, but also enhanced the activity marginally[22]. The effect of different protease inhibitors (5 mM) on the purified enzyme is summarized in Table 2. Protease inhibitors like EDTA and EGTA (metal chelators) had the highest inhibitory action over the protease enzyme with a relative activity below 20%. The other inhibitors, viz. 1,10-phenanthroline, DDT (cysteine protease inhibitor) and PMSF (serine protease inhibitor) had no significant inhibitory effect on the protease activity, revealing that the alkaline protease enzyme produced from *B. cereus* can be considered as metalloprotease (Table 3). The results corroborate with the observations of Ghorbel *et al.*[10] on the inhibitive effect of EDTA onto *Bacillus* sp. producing enzyme activity.

The stability of *B. cereus* alkaline protease against different detergents (1% w/v) was studied (Table 4). The surfactant-stable properties of alkaline protease make it ideal to be used in detergent-based products. Among the detergents, Tween 80 showed the highest residual activity of 94%, and the least residual enzyme activity of 22% was observed in sodium tripolyphosphate. The results are in agreement with the values reported by other studies which concluded that alkaline protease enzyme was unaffected[5] by Tween 80 and the mechanism of Tween 80 to enhance the extracellular enzyme production[23].

The purified alkaline protease subjected to SDS–PAGE yielded a single band, testifying its homogeneity (Figure 5). The molecular weight of the protease was determined by comparing with the migration distances of standard markers. The molecular mass of protease estimated by comparing the electrophoretic mobility of marker protein showed that the *B. cereus* protease had a molecular mass of 66 kDa. The zymogram activity staining also revealed

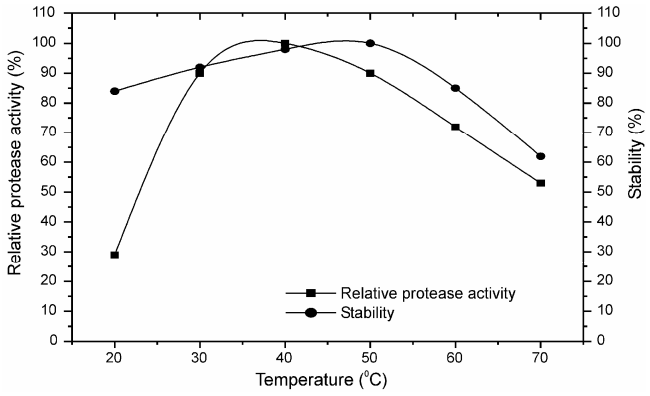

Figure 4. Effect of temperature on protease activity and residual activity (%).

Table 2. Effect of metal ions on protease activity

Metal ion salt	Residual activity (%)		
	1 mM	5 mM	10 mM
Control	100 ± 5.2	100 ± 5.2	100 ± 5.2
Mg^{2+}	82 ± 4.1	118 ± 7.9	107 ± 7.9
Fe^{2+}	62 ± 2.1	51 ± 3.1	33 ± 3.1
Ca^{2+}	99 ± 5.2	120 ± 8.6	125 ± 8.6
Mn^{2+}	95 ± 5.1	113 ± 6.4	101 ± 6.4
Cu^{2+}	21 ± 1.8	19 ± 1.9	12 ± 1.9
Zn^{2+}	33 ± 3.1	25 ± 2.8	14 ± 2.8

Table 3. Effect of inhibitors (5 mM) on protease activity

Inhibitor	Residual activity (%)
Control	100.0 ± 6.2
PMSF	78.65 ± 4.6
EDTA	11.8 ± 1.2
EGTA	13.6 ± 1.4
1,10-Phenanthroline	71 ± 5.2
DTT	30.1 ± 1.5

Table 4. Effect of surfactants (1%) on protease activity

Detergents (1%)	Residual activity (%)
Control	100.0 ± 5.9
Sodium dodecyl sulphate	40 ± 1.5
Sodium tripolyphosphate	22 ± 1.8
Sodium tetraborate	50 ± 2.9
Tween 80	94 ± 4.3
Tween 60	90 ± 3.7

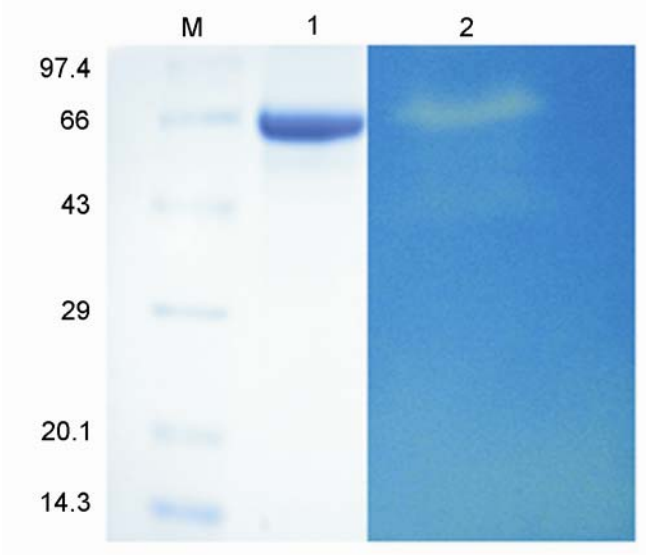

Figure 5. SDS–PAGE (12.5% polyacrylamide) and zymogram (M, marker; lane 1, Purified protease and lane 2, Zymogram) analysis of the purified extracellular proteases from *B. cereus* 117390.

a clear hydrolytic zone of proteolytic activity against the blue background. The molecular mass of calcium-dependent protease from *B. cereus* BG1 was 34 kDa (ref. 5). Another study has reported the molecular weight of purified dehairing protease from *B. cereus* MCM B-326 as 45 kDa (ref. 24).

In the present study, the alkaline protease producing *B. cereus* 1173900 was isolated, which is an extracellular enzyme producer under SSF conditions using ANFL as the substrate. The optimum and maximum alkaline protease production for the enzyme was observed at pH 8 in a 0.2 M buffer for a fermentation period of 60 h. The optimum temperature was 40°C, and the enzyme was thermally stable up to 50°C. The protease activity was increased in the presence of Ca^{2+}, Mg^{2+} and Mn^{2+}. The highest activity inhibition by 11.8% was detected after the incubation of purified enzyme with EDTA, which concluded that *B. cereus* alkaline protease could be classified under metalloprotease. In the presence of Tween 80, maximum residual activity of 94% was observed in the purified enzyme. The molecular weight of *B. cereus* alkaline protease was 66 kDa. Therefore, the SSF process can be considered as a potential method to produce high-yield alkaline metalloprotease using ANFL as the substrate.

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