Purification and characterization of laundry detergent compatible alkaline protease from *Bacillus cereus*

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An alkalophilic bacterium, *Bacillus cereus* produced an extracellular alkaline protease, which was found to be active at high temperature and pH range, suitable for commercial laundry detergents. *B. cereus* protease was partitioned in different aqueous two-phase systems such as PEG/dextran, PEG/potassium phosphate, PEG/sodium citrate, PEG/magnesium sulphate and PEG/sodium dihydrogen phosphate and best separation was found in PEG/potassium phosphate system. Therefore, production of protease was performed by the method of extractive fermentation in aqueous two-phase system composed of PEG 4000/potassium phosphate. Enhanced production was obtained in aqueous two-phase system as it overcomes the limitation of catabolic repression and product inhibition. The enzyme was purified to homogeneity by procedures including ammonium sulphate precipitation, concentration by ultrafiltration, anion exchange chromatography and gel filtration. The purified enzyme had specific activity 3256.05 U/mg and found to be a monomeric protein with a molecular weight of 28 kDa on sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). Its maximum protease activity against casein was found to be at pH 10.5 and at 50°C. Proteolytic activity of the enzyme was detected by casein zymography, which gave a very clear protease activity zone on gel that correspond to the band obtained on SDS-PAGE with a molecular weight nearly 31 kDa. Protease was inhibited by specific serine protease inhibitors, suggesting the presence of serine residues at the active site of the enzyme.

**Keywords**: alkaline protease, *Bacillus cereus*, detergent compatible, ATPS, purification, characterization

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**Introduction**

Proteases are very important industrial enzymes, which contribute about 60% of the total world enzyme market. Alkaline proteases have a large variety of industrial applications, mainly in the detergent, food, pharmaceutical, silk and leather industries. An alkaline protease from an alkalophilic bacterium, *B. cereus* showed better compatibility and better stability in commercial laundry detergents in a wide temperature range in comparison to endogenous proteases present in commercial laundry detergents. Production of protease by fermentation in homogeneous system is inhibited by product inhibition and catabolic repression as cells and product formation takes place in a single phase. Extractive fermentation using aqueous two-phase system (ATPS), provides an alternative approach for enhanced production of protease as cells and product are partitioned into two different phases and so it overcomes the limitation of product inhibition. A prominent increase in alkaline protease production was obtained when the fermentation was carried out in ATPS containing PEG and dextran T500 but the very high viscosity and cost of dextran limits its use. These problems may be overcome by using PEG/salt aqueous two-phase systems. ATPS made by PEG and salts have been reported for the in-situ production of some biological molecules due to economic reasons and wide range of hydrophobic differences between the two-phase systems.

This paper reports the production of *B. cereus* protease by extractive fermentation in optimized cultural condition, its purification and characterization. The protease was partitioned in different ATPS made by PEG and different salts and partition coefficient were determined for each system. In-situ fermentation was carried out in PEG 4000/potassium phosphate system with highest partition coefficient. The enzyme was purified to homogeneity and characterized by casein and gelatin zymography. Further characterization of the enzyme was carried out by testing the effect of various protease class inhibitors on the activity of enzyme at its optimum pH and temperature.

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Materials and Methods

Bacterial Strain and Culture Conditions

*Bacillus cereus* MTCC 3105 was maintained at 4°C on nutrient agar medium. The medium (pH 8.5) used for alkaline protease production consists of (g L\(^{-1}\)): corn starch, 50; soybean meal, 20; CaCl\(_2\), 0.4; and MgCl\(_2\), 0.2. The flasks were incubated for 72 h in a temperature-controlled (30°C) shaking incubator (200 rpm). The contents were centrifuged (10,000 \(\times\) g, 30°C, 20 min) and the cell free supernatant was used for determining extracellular protease activity.

Assay of Protease Activity

The alkaline protease activity was estimated by the procedure of modified Hagihara method using casein as substrate\(^9\). Enzyme activity was expressed as protease unit, where, one unit of protease activity was defined as the quantity of the enzyme that liberated the digestion product not precipitated by protein precipitating reagent and gave absorbance at 275 nm equivalent to 1 \(\mu\)g/mL of tyrosine per min, under assay conditions.

Protein Assay

Protein content in culture filtrates was determined by the method of Bradford using Bio-Rad protein dye reagent concentrate and bovine serum albumin as standard\(^10\).

Partitioning of Alkaline Protease in Different ATPSs

Binodial curves were plotted between polyethylene glycol (PEG) 4000 and different polymer/salts viz. potassium phosphate (KH\(_2\)PO\(_4\)+K\(_2\)HPO\(_4\)), sodium citrate, sodium dihydrogen phosphate, dextran, magnesium sulphate, sodium sulphate, sodium potassium tartarate according to the method reported by Albertsson\(^11\). ATPSs were prepared by taking plait point of the binodial curves and known amount of alkaline protease added in each system. Samples from each phase were collected and analyzed for alkaline protease activity.

Extractive Fermentation Using ATPS

Production of alkaline protease by *B. cereus* was carried out by using ATPS in the production media. Phase system was constructed by adding requisite amounts of PEG-4000 and potassium phosphate (KH\(_2\)PO\(_4\)+K\(_2\)HPO\(_4\)) to the production media. Aliquots were removed from the fermentation broth at regular time intervals for alkaline protease estimation. The microbial cells formed a pellet at the bottom of the centrifuge tube and phase separation occurred. Samples of top and bottom phases were analyzed for alkaline protease activity.

Purification of Alkaline Protease

The fermented broth (200 mL) of extractive fermentation was centrifuged at 10,000 rpm for 30 min at 4°C. Ammonium sulphate fractionation was carried out at 80% saturation and centrifuged at 4°C for 20 min. The precipitated sample was desalted and concentrated on Amicon Ultra-15 filter unit (10 kDa cut-off). The desalted sample was loaded on a Mono-Q spin column (Vivapure) for anion exchange chromatography, equilibrated with the loading buffer of 25 mM Tris/HCl (pH 8.0). The enzyme was eluted with a linear gradient of 0-0.5 M NaCl. Fractions containing protease activity were pooled.

The concentrated enzyme sample was then subjected to a size exclusion chromatography on a Superdex-200 (2\(\times\)60 cm) gel filtration column (Pharmacia) equilibrated with 25 mM Tris/HCl buffer (pH 8.0) and eluted with the same buffer containing 150 mM NaCl. Fractions of 1 mL each were collected at the flow rate of 30 mL/h. The active fractions were pooled out and analyzed for protease activity.

Gel Electrophoresis

Purity of the sample and estimation of molecular mass were determined by 12.5% SDS-PAGE (Bio-Rad) based on Laemmli method\(^12\). Proteins were visualized by Coomassie brilliant blue staining.

Detection of Protease Activity by Zymography

The alkaline protease produced from *B. cereus* was analyzed on 12% SDS-polyacrylamide gel containing 0.1% casein as copolymerised substrate as described by Oliver *et al*\(^13\). Staining and destaining of the gel was performed by Coomassie brilliant blue (Novagen stain kit) in a single step based on the method given by Leber and Balkwill\(^14\). The protease activity band was visualized as a clear colourless band against blue background.

Effect of Protease Inhibitors

Inhibitors known to be specific to different classes of proteases were used to determine the nature of protease produced from *B. cereus*. The enzyme was incubated with each inhibitor at different concentration for 2 h at 37°C followed by the measurement of residual activity under the standard protease assay condition.
Results and Discussion

The results of the partitioning experiments of alkaline protease in different ATPSs are presented in Table 1. It was observed that most of the protease was partitioned in upper PEG phase in PEG/potassium phosphate, PEG/citrate, PEG/sodium sulphate and PEG/sodium dihydrogen phosphate systems, while it was partitioned in bottom phase in other systems. Microbial cells were partitioned in lower salt phase in each system. ATPS composed of PEG 4000 (13.71%) and potassium phosphate (12.12%) with a highest partition coefficient 4.35 and volume ratio 1:28 was chosen for conducting extractive fermentation. An aliquot of 1 mL sample from the homogeneous and each phase of ATP fermentations were withdrawn aseptically at 12 h intervals from 36 to 84 h, centrifuged and assayed for protease activity. The result of extractive fermentation is presented in Fig. 1. It showed that total production of enzyme in PEG-4000/phosphate system was 2.41 times higher than the homogeneous fermentation. This finding is corroborated with results reported by Lee and Chang\(^6\) who showed 1.33 times enhanced protease production using *Bacillus licheniformis* in an ATPS containing PEG 6000 and dextran T 500\(^5\). However, dextran is a costly polymer and hence uneconomical at industrial scale. In the present study, KH\(_2\)PO\(_4\)+K\(_2\)HPO\(_4\) (7:3) was found to be superior to dextran to make a successful and cheaper two-phase system.

The alkaline protease produced from *B. cereus* by extractive fermentation was purified to its homogeneity and the results of purification study are summarized in Table 2. Analysis of the active peak fractions on SDS-PAGE revealed a homogeneous protease with molecular weight 28 kDa (Fig. 2). A similar molecular weight of proteases from other *Bacillus* strain had already reported\(^15,16\). Approximately 16-fold purification of alkaline protease from the initial culture broth was achieved, with a recovery of 13%. The specific activity of the purified enzyme was 3256.05 U/mg (Table 2).

The enzyme was found active over a broad pH range and maximum activity was found at pH 10.5 (Fig. 3). The influence of temperature optimization studies at pH 10.5 showed optimum alkaline protease activity at 50°C, which rapidly decreased at higher temperature (Fig. 4). These characteristics of the

<table>
<thead>
<tr>
<th>Systems</th>
<th>PEG %</th>
<th>Salt %</th>
<th>Phase volume ratio (V_t/V_b)</th>
<th>Enzyme activity (u/mL) Top</th>
<th>Bottom</th>
<th>Partitioning coefficient (k)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG 4000/Potassium phosphate</td>
<td>13.71</td>
<td>12.12</td>
<td>1.28</td>
<td>708.56</td>
<td>162.80</td>
<td>4.35</td>
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<td>PEG 4000/Sodium sulphate</td>
<td>14.80</td>
<td>10.60</td>
<td>1.09</td>
<td>680.00</td>
<td>224.00</td>
<td>3.03</td>
</tr>
<tr>
<td>PEG 4000/tri-sodium citrate</td>
<td>14.0</td>
<td>11.98</td>
<td>1.02</td>
<td>645.80</td>
<td>251.00</td>
<td>2.57</td>
</tr>
<tr>
<td>PEG 4000/Sodium dihydrogen phosphate</td>
<td>14.33</td>
<td>15.40</td>
<td>1.14</td>
<td>510.60</td>
<td>368.80</td>
<td>1.38</td>
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<tr>
<td>PEG 4000/dextran</td>
<td>11.44</td>
<td>9.36</td>
<td>1.78</td>
<td>424.90</td>
<td>543.40</td>
<td>0.78</td>
</tr>
<tr>
<td>PEG 4000/Magnesium sulphate</td>
<td>12.60</td>
<td>24.51</td>
<td>0.42</td>
<td>357.00</td>
<td>742.00</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Fig. 1 — Comparison of protease production by extractive fermentation in PEG 4000/potassium phosphate aqueous two-phase system with homogeneous fermentation (♦, homogeneous fermentation; ▲, ATPS fermentation).
enzyme are essential for its use in detergent industry$^{3,16}$.

The purified protease was detected and characterized by zymography using casein as substrate (Fig. 5). Molecular weight of $B.\ cereus$ protease on zymography gel was found to be 31 kDa. Casein zymography showed very clear increase in band thickness with increase in concentration of enzyme loaded on gel. In order to determine nature of the $B.\ cereus$ protease, activity was measured in the presence of different protease inhibitors (Table 3). Protease was strongly inhibited by the serine protease inhibitors PMSF and pefabloc at 5 mM. In contrast, there was no inhibition by cysteine-type inhibitors viz. $p$-chloromercuribenzoic acid (pCMB), $\beta$-mercapto-
ethanol (β-ME) and iodoacetic acid. No inhibition was detected when metalloprotease inhibitor like EDTA and aspartate protease inhibitor, i.e. aprotinin was added to B. cereus protease.

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