Research Article

Optimization of Bacterial Protease from Yercaud Hills

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Abstract

Protease is an industrially important enzyme having wide applications in pharmaceutical, leather, laundry, food and waste processing industries. Industrial enzyme production is effective only if the organism and the target enzyme are capable of tolerating different variables of the production processes. In this present study, totally 20 strains were isolated from Yercaud hills and they were screened for protease production using casein agar medium based on the zone formation. Based on their zone formation, Alcaligenes faecalis, A. eutrophus, Bacillus cereus, B. subtilis, B. megaterium, B. coagulans, Micrococcus roseus and Pseudomonas aeruginosa were selected and their protease was precipitated with ammonium sulphate and among the eight strains Bacillus megaterium was more potential and the protease produced by the B. megaterium have the maximum stability at 60°C temperature, pH 8, 3% of salt concentration, and in the metal ions, Ca²⁺ showed the maximum stability. These results show the thermal stability of the protease enzyme could be used for various biotechnological processes.

Keywords: Protease; Bacillus; Optimization; Yercaud hills

INTRODUCTION

Proteases are one of the important enzymes for industry. The sale of proteases constitutes around 60% of the total sales of industrial enzymes (Adinarayana et al., 2003) and around 500 tons of protease enzymes are produced every year to fulfill demand coming from industries (Crueger and Crueger 1984). They are economically essential for the detergent, protein, brewing, meat, photographic, leather, and dairy industries. At present, the overall cost of enzyme production is very high (due to high cost of substrate and medium used) and therefore, development of novel processes to increase the yield of proteases with increasing the production cost is highly appreciable from the commercial point of view. To achieve these goals, during the recent years, effects have been directed to expose the means to reduce the protease production costs through improving the yield, and the use of either cost free of low cost feed stocks or agricultural by product substrates for protease production. Proteases are common enzymes in plant and animal tissues, fungi, and bacteria. Microorganisms are the preferred proteases producers, as they grow rapidly, require small cultivation space, and can easily be subjected to genetic manipulation. Bacterial proteases are industrially the most significant compared to animal and fungal proteases (Ward, 1985). Alkaline proteases produced by different kinds of microorganisms; from bacteria (Najafi et al., 2005; Nadeem et al., 2009; Pawar et al., 2009), fungi (Khan et al., 1979; Charles et al., 2008; Sindhu et al., 2009), yeast (Chen et al., 1996; Chi et al., 2007) and actinomycetes (Thumar and Singh, 2007; Vinothini et al., 2008; Vishalakshi et al., 2009), in addition to its production from plants (Zhu et al., 1999;
Quiroga et al., 2005) and animals (Boyer, 1971; Hoffman, 1974). The present study was aimed at the isolation, purification and optimization of protease enzyme from bacterial strains isolated from Yercaud hills, Salem, Tamil Nadu, India.

MATERIALS AND METHODS

Sample collection and Processing

Soil samples were collected from Yercaud hills, Salem, Tamil Nadu, India. Serial dilution has been done for each sample and each dilution was used to inoculate the isolation medium described by Aftab et al. (2006). Agar plate medium (15 cm diameter) containing 50 mL solidified medium was inoculated with 20 μL from each dilution. The plates that showed considerable single colonies were selected for this purpose.

Screening for proteolytic activity

Individual bacterial colonies isolated above were further screened for proteolytic activity on Skim milk agar medium containing: Casein enzymatic hydrolysate, 5.00 g/L; Yeast extract, 2.50 g/L; Dextrose,1.00 g/L; Skim milk powder, 28.00 g/L; Agar,15.00 g/L. The pH of the medium was adjusted at 8 using 1N NaOH. Individual colonies were spot inoculated and were incubated at 30°C for 24 hours. After incubation period, based on the clear zone, potential strains were selected for purification study.

Determination of protease activity

The reaction mixture containing 1 mL of 1% casein solution in 0.05 M glycine - NaOH buffer having pH 10 and 1 mL of a given enzyme solution were incubated at 40°C for 20 min and the reaction was then stopped with 3 mL of 10% tri-chloroacetic acid. The mixture was allowed to stand for some time and was then centrifuged at 10,000 rpm for 10 min at 4°C. 0.5 mL of the filtrate was then diluted with 4.5 mL of glycine - NaOH buffer having pH 10. The absorbance of the liberated tyrosine in the filtrate was measured against a blank (non-incubated sample) at 280 nm. One proteolytic unit was defined as the amount of the enzyme that released 1μg of tyrosine under the assay conditions.

Purification of protease enzyme

About 100 mL of culture filtrate was treated with ammonium sulphate of 20%, 40%, 60% and 80% saturation and most of the proteins were precipitated out. The precipitate was collected by centrifugation in a super speed refrigerated centrifuge. The precipitate was redissolved in Tris-HCl (0.05 M, pH 8.0) buffer. The enzyme was dialyzed 3 times against the buffer. The enzyme activity was determined in both the precipitated and lyophilized enzyme and enzyme activity was assayed under standard conditions.

Optimization of culture conditions

Effect of the pH on protease activity and stability

The pH optimum was determined with azocasein 1% (w/v) as substrate, dissolved in different buffers (citrate phosphate, pH 4–6, sodium phosphate, pH 7.0, Tris-HCl, pH 8.0 and glycine NaOH, pH 9–10). The effect of pH on enzyme stability was determined by pre-incubating the crude enzyme preparation without substrate at different pH values (6.0–10.0) for 2 h at room temperature and measuring the residual activity at 70°C.

Effect of temperature on the proteolytic activity and stability

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure at pH 7.5 within a temperature range of 30–70°C at 5°C interval. Thermostability was determined by incubation of crude enzyme preparation at temperature ranging from 30–70°C for 2 h in a constant temperature water bath. After treatment the residual proteolytic activity was assayed.

Stability of protease in sodium chloride

Crude enzyme preparation was pre-incubated in phosphate buffer (0.05 M, pH 8.5) containing various NaCl concentrations (1 to 5%), at 45°C for 1 and 2 h. In each case the activity of the enzyme was measured in the same way as mentioned earlier.

Metal ions

The crude extract was incubated with different ionic solutions CaCl₂, MnSO₄, ZnSO₄, FeSO₄ and NaCl at 0.1 mM. Residual proteolytic activity was determined according to the standard conditions cited above.
RESULTS AND DISCUSSION

In the present study to isolate protease producing bacterial populations, the water and sediment were plated on casein containing nutrient agar medium and they were incubated at room temperature at 30°C. Bacterial density was found to be 2.9×10^8 CFU/g in sediment sample and 1.8×10^7 CFU/mL in water sample. Compared to water samples, sediment samples harbored bacteria which may be due to more substrate availability along with the abundance of other nutrients. Like that of our study, Marshall (1985), Marty et al., (1980) and Jayalakshmi (1992) reported high level of nutrients availability in sediment. A sum of 20 strains was selected for the proteolytic activity based on their morphology. All the 20 bacterial strains isolates were qualitatively assayed by well diffusion assay method in water agar medium supplemented with 1% casein. Seven isolates exhibited a large zone of hydrolysis (Figure 1) and were identified based on the morphological and biochemical characteristics as Alcaligenes faecalis, A. eutrophus, Bacillus cereus, B. subtilis, B. megaterium, B. coagulans, Micrococcus roseus and Pseudomonas aeruginosa. All the seven potential strains were precipitated with ammonium sulphate with various concentration 20%, 40%, 60% and 80%. Except A. eutrophus all the strains gave more enzyme activity in 80% (Figure 2). Based on the zone formation and enzyme activity Bacillus megaterium was selected, optimized for production characterization of protease.

![Figure 1: Screening of protease producing bacteria in casein agar plate](image1.png)

![Figure 2: Selection of Potential strains by ammonium sulphate precipitation in different concentration](image2.png)

Figure 1: Screening of protease producing bacteria in casein agar plate

Figure 2: Selection of Potential strains by ammonium sulphate precipitation in different concentration

The initial medium pH ranging from 4–10 was studied for the stability of protease, pH 8 supported the maximum protease activity (230 U/mL) and the minimum enzyme activity (121 U/mL) was observed at pH 10 (Figure 3). This observation is comparable with the findings of (Boominadhan and Rajkumar, 2009) using Bacillus sp. where the optimum pH of the medium was 8.0. Most commercial proteases mainly neutral and alkaline have been reportedly produced from the genus of Bacillus (Rao et al., 1998). Different temperatures varying from 30–70°C were observed in the present study for the detection of optimum temperature required for the stability of the protease enzyme. 60°C seemed to be the most favorable as the maximum production of about 236 U/mL was obtained. Minimum protease activity was observed at 30°C (145 U/mL) (Figure 4). The optimum temperature of this protease was 60°C which was similar to that described for other Bacillus proteases (Manachini et al., 1988, Janssen et al., 1994, Horikoshi, 1990 and Banerjee et al., 1999).

![Figure 3: Protease activity at different pH](image3.png)
The effect of varying percentage of NaCl concentration on protease stability was studied. At 3% of salt concentration, maximum protease activity (259 U/mL) was observed. Likewise minimum enzyme activity was obtained at 5% of salt concentration (171 U/mL) (Figure 5). The crude extract was incubated with different solution of salts (final concentration of 0.1 mM) at 28°C for 1 h. The enzyme could be released by elution with sodium chloride, or by growing the organism at high salts concentration, suggesting that the enzyme was probably bound to the cell by weak ionic bonds. In another study with a few marine strains of Pseudomonas, Corpe and Winters (1972) opined that, found most of the protease activity to be cell-bound, largely being associated with the cell envelope. Results as recorded in (Figure 6) showed that the activity was reduced in the presence of Hg²⁺ and Fe²⁺ ions, while the presence of Ca²⁺ resulted in increase of proteolytic activity (264 U/mL). This observation strongly suggested the requirement of some metal ions for protease production by this organism. These results are in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions (Adinarayana et al., 2003; Thangam and Rajkumar, 2002).

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