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OPTIMIZATION OF EXTRACELLULAR ALKALINE PROTEASE ENZYME FROM *BACILLUS* SP

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ABSTRACT
An alkalophilic bacterium, *Bacillus sp* produced an extracellular alkaline protease, which was found to be active at high temperature and pH range, suitable for commercial laundry detergents. The four substrates viz., soyabean meal, egg albumin, groundnut cake and bengal gram powder were used for increasing the production of enzyme. Among the four substrate used bengal gram, the best substrate for optimization, showed the highest enzyme activity. The physical and chemical parameters were also optimized. The maximum enzyme activity under optimum conditions was obtained with incubation period 24 - 48h incubation temperature 50ºC, initial pH 10.0, inoculum level 2%. The enzyme was purified to homogeneity by procedures including ammonium sulphate precipitation and acetone precipitation. The purified enzyme had specific activity 7355μg of amino acid released per mg of protein.

INTRODUCTION

Proteases are very important industrial enzymes, which contribute about 60% of the total world enzyme market. Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (Rahman *et al*., 1994) and among bacteria, *Bacillus sp* are specific producers of extra-cellular proteases (Coolbear *et al*., 1992; Banerjee *et al*., 1999). These enzymes have wide industrial application, including pharmaceutical industry, leather industry, manufacture of protein hydrolizates, food industry and waste process in industry (Pastor *et al*., 2001). Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in the solubility of non gaseous reactants and products, and reduced incidence of microbial contamination by mesophilic organisms. Enzymes from extreme thermophiles are not only stable at high temperature, but also to organic solvents, detergents and charotropic agents. This opens up the possibility of using enzymes in the presence of denaturing substrates or products, and in stringent conditions generally (Coolbear *et al*., 1992; Beg and Gupta 2003). Proteases secreted from thermophilic bacteria are thus of particular interest and have become increasingly useful in a range of commercial applications (Singh *et al*., 2001; Adams and Kelly 1998). The present investigation describes the isolation and selection of thermophilic bacteria capable of producing extracellular alkaline protease and the effect of different sources of protein on the production of protease.

MATERIALS AND METHODS

Isolation and maintenance of cultures: Thermophilic bacterial cultures were isolated from field soil samples collected around coimbatore. A quantity of 5g of the soil sample was suspended in 10mL sterile distilled water and serially diluted using sterile water blanks up to 10^{-5} dilution. One mL of the 5th diluted sample was spread on yeast extract glucose agar (YEGA) plates containing glucose 0.5 per cent, yeast extract 0.5 per cent, peptone 0.3 per cent, sodium chloride 0.3 per cent and 1.5 per cent agar, pH 10.0. The plates were incubated in a thermostatically controlled incubator at 50ºC. Colonies that appeared on the isolation plates were selected by using the bacterial isolation kit and only the isolated colonies of *Bacillus sp*. were streaking on fresh YEGA plates. Single colonies were picked up and maintained on YEGA slants.

Cultivation: Erlenmeyer flasks (250 mL) containing 50mL basal medium (Glucose 1 per cent, Casein 0.2 per cent, $MgSO_4$ 0.01 per cent, K_2HPO_4 0.02 per cent, KCL 0.03 per cent, pH 10.0) were inoculated with 2 loops of inoculum. After 7 days of cultivation at 200 rpm, the broth was centrifuged at 5000 x g and the cell free culture filtrate was used as crude enzyme extracellular protease.

Assay of alkaline protease: To a quantity of 1.0mL of the culture filtrate taken in a test tube, 2.0 mL of potassium phosphate buffer (0.1M, pH7.0) was added. Then 1.0 mL of substrate (casein 0.5 per cent) was added and mixed thoroughly the contents were incubated at 28ºC over a water bath with occasional shaking for 3 hrs. The enzyme activity was terminated by the addition of one drop of 10 per cent trichloro acetic acid (TCA); blanks were mainitained with 1.0ml of buffer solution instead of culture filtrate. Then, the reaction mixtures were taken for the amino nitrogen releasing assay

and protein estimation by Lowry's *et al.,* (1951) method. Enzyme (specific) activity was calculated as μg of amino nitrogen released per mg of enzyme protein.

Effect of different incubation period: Different incubation periods (24, 48, 72 and 96 h) were employed to study their effect on protease production. The fermentation was carried out at 50ºC. The optimum incubation period achieved by this step was fixed in subsequent experiments.

Enzyme production in various growth media: Commercial grade of soybean meal, Bengal gram powder, egg albumin, groundnut cake powder, were procured from the local market and used as the substrate to study their effect on the production of protease at basal medium conditions including initial moisture content of 60 per cent and 2 per cent inoculum level. Protease production was carried out for 48 hrs at 50ºC. The best substrate achieved by this step was fixed in subsequent experiments.

Enzyme purification: The crude enzyme extract was treated with acetone and ammonium sulphate precipitation. The precipitate was recovered by centrifugation (9000x G, 20 minutes) dissolved in 0.1 M potassium phosphate buffer (pH 7.0) and used as enzyme solution. The purified enzyme solution collected from acetone and ammonium precipitate was taken for the amino nitrogen releasing assay and the protein estimation were done by lowry's method. Enzyme (specific) activity was calculated by μg of amino nitrogen released per mg of protein.

RESULTS AND DISCUSSION

The growth pattern of *Bacillus sp*. and protease production was observed for 12 hrs in liquid medium with 0.2% casein as a carbon source in a 250 mL Erlenmeyer flask (Fig. 1). *Bacillus sp* grew very fast and the formation of protease started from 24h of the growth and reached a maximum in 48 hours and then began to fall. This suggests that protease production was directly linked to the culture being metabolically active. Ward (1985) reported that *Bacillus* sp usually produce more protease during the late exponential phase. The function of this enzyme is obscure, but its production is correlated with the onset of a high rate of protein turnover during sporulation in certain bacilli. Dhandapani and Vijayaragavan (1994) obtained similar results and reported that the maximum activity of protease from thermostable *Bacillus sterothermophillus* strain AP-4 was after 36hrs of growth in broth at 55ºC.

Of the four media used, Dye's medium with 0.2 per cent Bengal gram powder supported maximum protease production. The enzyme production was slightly lower in 0.2 per cent soybean meal and groundnut cake powder compared to that in Dye's medium with 0.2 per cent Bengal gram powder (Fig. 2). Protease production by Bacillus sp was reduced to half its maximum level when 0.2 per cent Egg albumin was present as the nitrogen source (Ferrero *et al.,* 1996). Kobayashi *et al.,* (1995) reported that egg albumin as a poor source of nitrogen for protease activity in alkalophilic *Bacillus* sp. KSM-K16.

Different concentrations of various organic nitrogen sources tested, 0.2 per cent egg albumin, 0.2 percent soybean meal, 0.2 per cent groundnut cake powder and 0.2 per cent Bengal gram powder were added to Dye's medium (Dye, 1962). The synthesis of alkaline protease is not constitutive, different levels of production were found with respect to growth and enzyme production. Of the four substrates, Bengal gram powder supported maximum protease production. Protease from alkalophilic and thermophilic isolates of *Bacillus* sp was

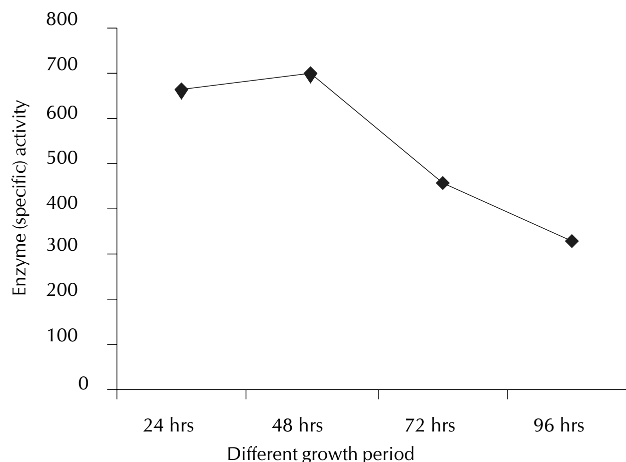

Figure 1: Protease activity at different growth period, Enzyme activity expressed as microgram of amino nitrogen released per mL of enzyme protein per hr at room temperature

Table 1: Enzyme purification

Purification method	Quantity of aminoacid(μg mL^{-1}) released	Protein content (mg mL^{-1})	Enzyme (specific) activity*	Purifi-cation
Culture filtrate	238	0.444	537	-
Acetone precipitation	132	0.477	3599	6 folds
Ammonium (50%) sulphate precipitation	127	0.173	7355	13 folds
Ammonium sulphate precip-itation (100%)	179	0.285	6255	11 folds

*Data expressed as μg of amino acid released per mg of enzyme protein.

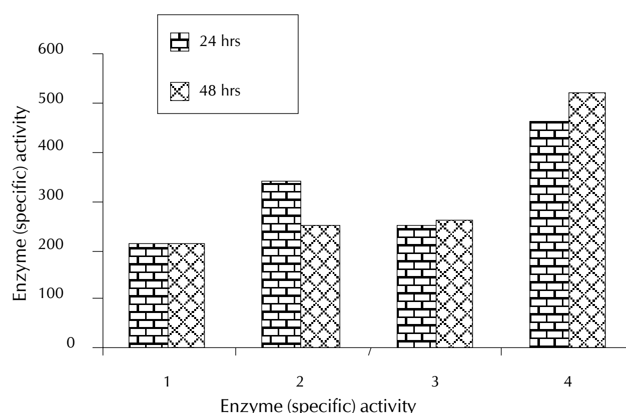

Figure 2: Effect of different source of protein substrates on the production of protease, 1. Egg albumin, 2.Soybean meal, 3.Groundnut cake, 4.Bengal gram powder, *Enzyme activity expressed as microgram of amino nitrogen released per mL of enzyme protein per hour at room temperature

purified by acetone precipitation and ammonium sulphate precipitation (50 per cent and 100 per cent saturation). The specific activity of the purified protease was estimated to be 7355 μg of amino nitrogen released per mg of protein in 50 per cent and 6255 μg mL^{-1} in 100 per cent saturation, which is about 13 folds and 11 folds higher than that of the crude enzyme preparation (Fig. 3).

Acetone precipitation recorded 6 folds with the specific activity of 3599 μg of amino nitrogen per mg of protein. Sampath *et al*., (1997) stated that the proteases from *Streptomyces sp* G157 was purified 4.2 folds with a specific activity of 92 units per mg of protein. The specific activity of the purified protease was estimated to be 7355 as μg of amino nitrogen released per mg of protein which is about 13 fold higher than that of the crude enzyme precipitation. The alkalophilic *Bacillus sp* had high enzyme activity and pH range of 6 -10 with a maximum value at pH 10.0 and the optimum temperature for protease activity was found to be 50ºC. The pH and thermal stability as well as high specific activity of this enzyme can be exploited for industrial applications.

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