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As the most important industrial enzymes, proteases account for nearly 60% of the total worldwide enzyme sales, with two third of them being of microbial origin (Kalisz, 1988). The most important microbial proteases are the alkaline proteases (mainly of bacterial origin). Although they are being produced at commercial scale since many years, emerging market requirements have forced the industrial enzymologists to look for the new microorganisms which are able to produce proteases with improved performance or novel characteristics.

In the present study, a new bacterium was isolated from alkaline soil samples of Leh and Ladakh (India). This isolate was identified as *Bacillus sphaericus* and deposited in MTCC, Institute of Microbial Technology, Chandigarh where it was given an accession number MTCC-B-0014. This isolate was found to be a strict alkalophile as it could not grow in agar plates of medium at neutral pH.

This isolate could utilize a wide range of carbon and nitrogen sources for growth and enzyme production. But the maximum enzyme activity of 550-600 U/ml could be obtained when a combination of biopeptone and yeast extract were used as nitrogen sources alongwith glucose as carbon source.

Alkaline proteases are a high volume low value product. Hence a cheap and preferably agro-based nutrient medium is required for its production so as to make the process economically viable. Hence an optimized medium, using starch and PH-Soyatose, was developed which could result in enhanced protease activity of 1000-1200 U/ml at the end of fermentation. This represented an increase of almost 100% over that obtained in Horikoshi medium.

Various physical, chemical and physiological parameters of the alkaline production process were optimized. It was observed that this isolate could optimally

produce alkaline protease at 30°C and at a pH of 10.0. Innoculum age of 12-18 hours, depending upon the medium used, was found to be optimal for enzyme production. Similarly the use of innoculum at 10% (v/v) level gave the best results. Carbonate ions were observed to play a significant role in enzyme production. A strong repression of both enzyme production and microbial growth in the presence of NH_4Cl was observed. Higher concentrations of glucose were also inhibitory, but in a non-specific manner which was quite different from catabolite repression because catabolite repression is observed at rather low concentrations.

During the batch production of this enzyme an agitation rate of 350 rpm and aeration at 1 vvm level gave the best results, although good enzyme production could also be observed when 0.5 vvm aeration level was used. In a typical batch run the protease production appeared to occur in both growth associated and non-growth associated manner. Maximum dP/dt and q_p were observed during exponential phase of growth, but the major share of protease activity was synthesized in post-exponential and stationary phases of growth.

Although alkaline protease are being produced since many decades, still there are only a few studies regarding the kinetics of the process. Hence the kinetic analysis of the protease production by this strain using various models was done. Using Kono-Asai method, the enzyme production process was found to be of mixed type with both growth associated and non-growth associated enzyme production. Values of various kinetic constants were calculated and both k_{P1} and k_{P2} had a positive value. Theoretically calculated values of cell dry weight and enzyme activity were found to be quite in agreement with the observed values, indicating the suitability of the Kono-Asai model for explaining the protease production process.

Higher substrate concentrations were observed to inhibit the enzyme production. But dP/dt was observed to fall to zero as soon as glucose was exhausted. In light of both these observations it was decided to go for fed batch mode of enzyme production so as to get higher product concentration in the broth, which will make subsequent downstream processing more cost-effective.

During fed batch studies, feed medium composition was varied to find out an optimum composition of the feed medium. Over feeding of glucose was found to be deleterious for enzyme production. Both the productivity and the substrate economy suffered in such cases. Only a low concentration of glucose (5 g/500 ml) in feed medium was observed to give the best productivity and product yield coefficient over substrate utilization. Feeding of concentrated medium at the same feed rate also gave poor results. During typical fed batch run enzyme activity of 968 U/ml could be achieved compared to 670 U/ml in a batch mode. Fed batch run could increase the productivity from 27916 $Ul^{-1}h^{-1}$ in batch mode to 40333 $Ul^{-1}h^{-1}$. Yield coefficient of product formation over glucose also registered an improvement from 67000 U/g to 96800 U/g.

Two proteases could be purified from the culture broth which differ by their degree of hydrophobicity and in their molecular weights. Major fraction of protease activity belonged to protease B which had a M_r of 29.3 kD compared to 28.7 kD for protease A under denaturing conditions. Protease B was apparently a multimeric protein. Both proteases were poorly stained by both silver and coomassie blue staining methods. Major protease i.e. protease B had a pI value of 8.6 which is quite suitable for detergent applications as it is quite near the pH of detergents.

This enzyme was observed to be optimally active at 50-55°C and a pH of 10.5. The optimum temperature was found to increase to 60°C in presence of Ca^{++}. Ca^{++}

was also observed to result in an increase in thermal stability at various temperatures. The enzyme was found to be quite stable at a wide range of pH values (7.5-11.0). Salt precipitated powdered enzyme preparation was found to be stable for 18 months at 37°C.

Protease activity in the culture broth was characterized to be a serine protease of chymotrypsin type with metal ion requirements. Best p-nitroanilide substrate for both these proteases was also a chymotrypsin specific substrate i.e. N-suc-ala-ala-pro-phe-pNA. None of the metal ions tested could significantly enhance the protease activity, however Fe^{++}, Cu^{++} and Zn^{++} were inhibitory.

This enzyme was observed to be quite stable towards various detergent components. It showed excellent results compared to commercially available enzyme preparations in the presence of locally available commercial detergent powder preparations. This enzyme could also be successfully used for another important industrial application i.e. recovery of silver from used X-ray films. It was found that 25 U/ml of enzyme could be used at a temperature of 50°C and pH 11.0 for best results.

In conclusion it can be said that this newly isolated alkalophilic *Bacillus sphaericus* produces an alkaline protease, using a relatively less expensive production medium, which seems to have a very good industrial potential for a variety of applications.