



**A** study was carried out with the objective of isolation, purification and characterization of potentially useful alkaline proteases from bacterial sources.

For the selection of production strains with stable high yielding properties extensive screening of proteolytic bacteria was carried out. The proteolytic bacteria for this purpose were isolated from alkaline soils. These strains were studied for the yield of alkaline protease by both submerged and solid state fermentation. The two strains yielding highest by submerged fermentation, K 147 and K 25 were identified as *Bacillus licheniformis* and *Bacillus* sp. respectively. On monitoring the ability to maintain the high yielding nature, *B. licheniformis* K 147 was found to be unstable. The other bacterium, *Bacillus* sp. K 25 was maintaining its high yielding nature and was used for further submerged fermentation studies.

The proteolytic bacteria isolated were tested for the yield of alkaline protease by SSF in two steps. In the first step, for the preliminary selection of some high yielding strains, the proteolytic bacteria were subjected to SSF studies under the arbitrarily selected conditions supposed to be suitable for the majority of the strains. In the second step these high yielding strains were subjected to detailed SSF studies varying the moisture levels and incubation periods; and the highest yielding one was selected. The strain K 242, found to be the highest yielding, was identified as *Bacillus pumilus*. Since this strain was stable also, it was used for further SSF studies.

Various factors influencing the alkaline protease production by *Bacillus* sp. K 25 by submerged fermentation were studied. The production was maximum when the culture was in the early stationary phase. The optimum temperature of incubation and the optimum initial pH requirement

were 45°C and pH 9.0 respectively. The best carbon source for the production was starch. Soya bean meal was the best nitrogen source. In general the organic nitrogen sources were better than the inorganic ones for the production. The optimum level of both starch and soya bean meal for the maximum production was found to be 2% (w/v). The use of sodium chloride at a level of 1% (w/v) and calcium chloride at a level of 0.02% (w/v) was found to be favouring the production. The age of inoculum was having only very little effect on the yield of the enzyme. The optimum level of inoculum for the production was in the range 1-8% (v/v). Agitation (200-300 r.p.m.) was found to be increasing the production significantly. Providing these optimum conditions for the production, the incubation period required for the maximum accumulation of alkaline protease was 96 h. Under optimized conditions the yield obtained by *Bacillus* sp. K 25 was 6.6 u ml<sup>-1</sup>.

Factors influencing the production of alkaline protease by *Bacillus pumilus* K 242, by solid state fermentation were studied. Of the different solid substrates tried wheat bran was found to be the best one. The maximum production was obtained with the use of wheat bran of particle size 500-710  $\mu$ . A combination of conditions such as 64% moisture level, incubation temperature 37°C and incubation time 72 h was found to be suitable for getting the maximum yield. The optimum pH of the moistening solution for the maximum production was 9.0. Supplementation of the medium with some carbon sources was found to be enhancing the production. The best carbon source for supplementation was glucose at 2% (w/w of the moistened substrate) level. The different nitrogen sources tested were found to be having either no significant effect or an inhibitory effect on the production. Incorporation of sodium chloride at a concentration of 0.5% (w/v), into the moistening solution was enhancing the production slightly. The age of

inoculum was having no significant effect on the production. An inoculum level in the range 2-8% (v/w of moistened substrate) was required to get the maximum yield. The ratio of medium volume to flask volume was having an influence on the yield. An increase in yield could be obtained by reducing this ratio from 1:14.7 to 1:1.47. Under optimized conditions the yield obtained by *Bacillus pumilus* K 242 was 120  $\mu$ /g DBB.

The alkaline protease of *Bacillus* sp. K 25 was purified from the culture supernatant by employing methods such as ammonium sulphate precipitation, negative adsorption by DEAE cellulose, CM cellulose chromatography and gel filtration using Sephadex G-100. More than ten-fold purification could be achieved with the recovery of more than 40%.

For the purification of alkaline protease from the bacterial bran extract of *Bacillus pumilus* K 242, methods such as ammonium sulphate precipitation, DEAE Sephadex A-50 chromatography and gel filtration using Sephadex G-100 were employed. There was five-fold increase in the purity with 40% recovery.

Both the proteases were found to be homogenous when examined by native PAGE.

Some properties of the purified proteases of *Bacillus* sp. K 25 (Protease K 25) and *Bacillus pumilus* K 242 (Protease K 242) were studied. While the optimum pH and temperature for the activity of protease K 25 were found to be 11 and 60°C respectively those for protease K 242 were 10.5 and 55°C respectively. Both the enzymes were inhibited strongly by PMSF, which indicated the presence of active serine residue in the enzyme molecules. A slight inhibition of protease K 242 at a high concentration (10 mM) of EDTA could also be observed. The effects of different metal ions on the activity of the

enzymes were studied. None of them was found to be having considerable enhancing effect on the activity of these enzymes.  $Mn^{2+}$  was increasing the activity of protease K 25 slightly. Of the different metal ions inhibiting the activity of these proteases  $Hg^{2+}$  was the most inhibitory one. Protease K 25 and protease K 242 were found to be stable in the pH ranges 6-11 and 6-10 respectively. At high pH (pH 12.0) protease K 25 was exhibiting a better stability than the protease K 242. The thermostability exhibited by protease K 25 was also better than that exhibited by protease K 242. Both the enzymes were not losing any activity after incubating them at 45°C for 30 min. They showed reduction in activity when incubated at 50°C or above for 30 min and complete loss of activity when incubated at 70°C for 30 min. The presence of calcium chloride was found to be improving the thermostability of both the enzymes.

In order to test the suitability of the proteases of *Bacillus* sp. K 25 and *Bacillus pumilus* K 242 for use in commercial detergents, their stability in presence of detergents and the activity on insoluble substrate in presence of detergents were studied. Results were evaluated in comparison with the protease of *Bacillus licheniformis* which is the predominantly used detergent protease at present. The proteases were tested in five commercial detergents. There were no considerable differences in the stability shown by these three proteases in four out of the five detergents tested. The activities of these proteases on insoluble substrates in presence of detergents were studied using blue casein-PAG, a substrate obtained by the immobilization of dyed casein in the structure of polyacrylamide gel. From the results, the protease of *Bacillus pumilus* K 242 could be found to be better than the other two proteases in cleaving the insoluble substrate. Due to this reason the protease of *B. pumilus* K 242 could be suggested as the most suitable one for the use in

detergents. The protease of *Bacillus* sp. K 25 also could be suggested to be utilizable as detergent enzyme because, its ability to act on the insoluble substrate was generally better than that of the protease of *B. licheniformis* NCIM 2042.

The results of the present studies on bacterial alkaline proteases are of great relevance from an industrial microbiological viewpoint. The high yielding strains, *Bacillus* sp. K 25 and *Bacillus pumilus* K 242 are having stable high yielding nature and can be used for the large scale production of alkaline proteases.

Soya bean meal and starch which are cheap and easily available can be used in submerged fermentation for alkaline protease production by *Bacillus* sp. K 25. This indicates the feasibility of economical production of alkaline protease using this strain. For alkaline protease production by *Bacillus pumilus* K 242, by solid state fermentation, wheat bran a very cheap agricultural waste can be used. When the economy and the other advantages of the SSF process are taken into account, the SSF using *Bacillus pumilus* K 242 can be suggested as suitable for obtaining high yield of alkaline protease at low cost. The techno-economic importances of such SSF processes have to be given due attention in a developing country like India.

The yield of alkaline protease by both the strains were very high even without any genetic modification. The possibility for obtaining better yields by genetic modifications of these strains cannot be ruled out. Such attempts will be fruitful only if the improved strains are capable of maintaining their high yielding nature. Detailed studies are required to make any progress in this line.

The alkaline proteases of both the bacteria were exhibiting desirable properties such as high pH and temperature optima, high pH and thermostability and insensitivity to chelating agents. So these enzymes can be suggested as possibly suitable for many commercial applications. Of the two strains that can be used as potential sources of alkaline proteases, *Bacillus pumilus* K 242 deserves special mention because the protease of this bacterium can be produced very economically by SSF process. This will be of great significance, especially when the alkaline protease is required in high yield at low cost, as required in the case of detergent proteases.

The results of studies on the enzyme properties such as stability in detergents and activity in presence of detergents are suggestive of possible usefulness of these proteases as detergent additives. It may be possible to recommend these enzymes for application in detergent industry, after proper evaluation of their performance in field trials. The suitability of these proteases for other commercial applications were not investigated. However, considering the desirable properties exhibited by these enzymes, it is reasonable to assume that further studies on the utility of these enzymes for other commercial applications also can be promising.