SUMMARY

This study was undertaken (i) to isolate a thermostable α-amylase which can be used in the starch processing industry and (ii) to develop a strain for industrial production of the enzyme. Following are the main conclusions of this study.

1. Thermophilic microorganisms were isolated from various geothermal areas in the northern India and were screened by using a multi-step screening regimen to select an isolate which produces an extracellular thermostable α-amylase. The selected isolate, named MK 716, produced an α-amylase which showed optimal activity at 70°C and was active upto 100°C. The enzyme retained about 80% activity after one hour exposure at 80°C, a temperature which is frequently used for liquefaction of starch in industrial processes. The performance of the enzyme was evaluated by a Corporate R&D establishment and was found to be impressive.

2. Morphological, physiological and biochemical characteristics of the isolate MK 716 revealed that it belonged to the genus *Bacillus* and was, therefore, regarded as *Bacillus* sp. MK 716. Although *Bacillus* sp. MK 716 showed 90% similarity with *Bacillus stearothermophilus* and *Bacillus acidocaldarious*, it could not be placed in any of these species because of some significant differences. *Bacillus* sp. MK 716 also differed from other thermophilic species of the genus *Bacillus*. Thus, *Bacillus* sp. MK 716 appeared to be a novel thermophilic strain of the genus *Bacillus*.

3. The success of the strain improvement programme was very impressive. Classical as well as modern genetic engineering tools were used to increase the productivity of various strains. Mutagenesis of *Bacillus* sp. MK 716 with ethylmethane sulphonate yielded a mutant, named E1. The enzyme yield of the mutant E1 was about 5,300 units/ml as compared to 130 units/ml of the parent strain *Bacillus* sp. MK 716. Thus, about 40-fold increase in the α-amylase yield was obtained through mutagenesis. Cloning of the E1 α-amylase gene, which has been manipulated as described later, into

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Bacillus subtilis by using a multicopy vector resulted in 2.5 fold increase in the enzyme yield. The α-amylase yield of the recombinant strain, *B. subtilis* BGAT 9, was 13,900 units/ml. The recombinant plasmid, pAmy B9, was found to possess excellent structural and segregational stability.

4. In order to clone and characterize the α-amylase gene of the mutant E1, representative genomic DNA library of the mutant was constructed in *E. coli* TB 1 by using pBR 322 as vector. The library was screened on starch-agar plates and 51 clones, which formed starch hydrolysis zones, were obtained. The size of the inserts in the clones varied from 2.3 kb to 5.5 kb and the inserts were present in both the orientations. α-Amylase was produced in both the orientations, thereby indicating that the inserts carried the α-amylase gene along with its regulatory elements which were recognized by *E. coli*.

5. The α-amylase yields of all the clones were determined and the clone, GAT 11, which produced maximum amount of the enzyme, was selected for further studies. pGAT 11, the recombinant plasmid in GAT 11, contained a 4.8 kb insert. Restriction endonuclease map of the 4.8 kb insert was developed. The minimum size of the fragment required for α-amylase expression was found to be 2.0 kb. The 2.0 kb fragment was found to destabilize the plasmid whereas a 2.4 kb fragment, which contained the extra 0.4 kb on one end, did not destabilize the plasmid. The α-amylase yield of the *E. coli* TB 1 clones carrying the 2.0 kb fragment was more than the yield with 2.4 kb fragment. Thus, hyperexpression appeared to be the cause of plasmid instability.

6. The nucleotide sequence of the E1 α-amylase gene (*amyE1*) differed from the sequences of other *Bacillus* α-amylases. The E1 α-amylase (AmyE1) showed 91.1-93.4% homology with *B. stearothermophilus* and 60-65% homology with *B. licheniformis* and *B. amyloliquefaciens* α-amylases. The size of AmyE1 (487 amino acid residues) was similar to the size of *B. licheniformis* (483 residues) and *B. amyloliquefaciens* (483 residues) α-amylases. Multiple alignment with *B. stearothermophilus* α-amylase sequences showed that most of the amino acids were missing from the C-terminal end of the AmyE1. Two promoter like elements, P1 and P2, were located upstream of the structural gene. The promoter P2 was highly similar to the *Escherichia coli* σ^{70} and *B. subtilis* σ^{43} consensus promoters and thus appeared stronger as compared to the promoter P1. The highly homologous *B. stearothermophilus* sequences

were found to contain only one complete promoter (equivalent to P2).

7. The 2.0 kb fragment, discussed above, contained the promoter P2 whereas the 2.4 kb fragment contained both, P1 and P2, the promoters. Both the fragments have been cloned in *Bacillus subtilis* 1A 289 using pRB 373 as vector. The clone carrying the 2.0 kb fragment (*B. subtilis* BGAT 9) produced more enzyme than the clone carrying the 2.4 kb insert (*B. subtilis* BGAT 1). However, unlike in *E. coli* TB 1, the plasmid carrying the 2.0 kb insert (pAmy B9) was stable in *B. subtilis*.

8. Optimization of the process conditions resulted in further increase in α-amylase yield. The strain BGAT 9 produced 13,900 units/ml in M9 minimal medium containing soluble starch as carbon source and amino acids to satisfy the auxotrophic requirements of the host strain. Replacement of the amino acids with soyabean meal (0.5% w/v) resulted in about four-fold increase in enzyme production. Replacement of the soluble starch with crude corn starch did not affect the enzyme yield of BGAT 9. Optimization of inoculum age and volume increased the yield to 1,80,000 units/ml. The net outcome of the strain and process improvement was about 1380-fold increase in α-amylase yield. The performance of the strain BGAT 9 in a laboratory scale fermenter was satisfactory.

9. The improvement in the enzyme yield was accompanied by the improvement in the process economics. The recombinant *B. subtilis* produced α-amylase at 37°C. Production of the enzyme at mesophilic temperature will require less energy as compared to production at thermophilic temperature. The production medium consisted of crude corn starch, soyabean meal and inorganic salts. These materials are relatively cheap and abundantly available in India.