1.0 ABSTRACT

Thermostable α-amylases are commercially important in various starch processing industries. The composition of the medium was optimized to improve α-amylase production by *Bacillus licheniformis* ATCC 6346. Enzyme production was improved to 44.1 UmL^{-1} from 20.08 UmL^{-1} in the medium containing (gL^{-1}) starch 4.0, $(NH_4)_2SO_4$ 5.0, K_2HPO_4 7.5, KH_2PO_4 4.0, peptone 6.0, $CaCl_2.2H_2O$ 0.01, $MgCl_2.6H_2O$ 0.01 and $FeCl_3$ 0.01. Addition of NaCl (0.2-4.0 gL^{-1}) to the growth medium inhibited the production of α-amylase. Peptone ($6gL^{-1}$) was replaced with the same amount of commercial (Tryptone, yeast extract and casamino acids) and local nitrogen sources (mustard, ground nut & soyameal powder and sesamum & coconut seed cake powder). Replacing peptone ($6gL^{-1}$) with same amount of soymeal powder has increased the enzyme production from 44.43 to 52.27 UmL^{-1}. A negligible amount of (0.691 UmL^{-1}) was produced in the medium containing coconut seed cake powder ($6gL^{-1}$) while the fat free seed cake increased the production of α-amylase to 14.74 UmL^{-1}. Maximum enzyme activity was obtained (29.61 UmL^{-1}) in the media having $24gL^{-1}$ defatted coconut seed cake powder. Similarly the effect of mustard, ground nut & soymeal powders and sesamum seed cake powder were studied. Replacing peptone ($6gL^{-1}$) with same amount of mustard powder showed 32.95 UmL^{-1} of enzyme activity while the defatted mustard powder increased enzyme production to 46.01 UmL^{-1}. Maximum enzyme production (58.14 UmL^{-1}) was obtained in the medium having $18gL^{-1}$ defatted mustard powder. When the peptone ($6gL^{-1}$) was replaced with $6gL^{-1}$ sesamum seed cake powder, 21.11 UmL^{-1} of enzyme activity was obtained while defatted sesamum seed cake powder increased enzyme production to 46.97 UmL^{-1}. Maximum enzyme production (56.64 UmL^{-1}) was obtained in the media having $18gL^{-1}$ defatted sesamum seed cake powder. Replacing peptone ($6gL^{-1}$) with same amount of ground nut powder showed 21.97 UmL^{-1} of enzyme activity while the defatted ground nut powder increased the enzyme production to 34.42 UmL^{-1}. Maximum enzyme production (39.36 UmL^{-1}) was obtained in the media having $14gL^{-1}$ defatted ground nut powder. Replacing peptone ($6gL^{-1}$) with same amount of soymeal powder showed 52.27 UmL^{-1} of enzyme activity while the defatted soymeal powder decreased enzyme production to 41.75 UmL^{-1}. Maximum enzyme production (44.62 UmL^{-1}) was obtained in the media having $18gL^{-1}$ defatted soymeal powder at 48h and 42°C. The decreased enzyme production could be due to the variation in carbohydrate content of the media. However supplementation of soluble starch to the media did not improve the production of α-amylase. Another alternative cause for the difference in the enzyme

production could be the variation in the amino acid compositions of the media. As enzyme production was high in the defatted mustard powder containing medium, the amino acids equivalent to that present in mustard ($18gL^{-1}$) powder were supplemented to the sesamum seed cake powder ($18gL^{-1}$) or coconut seed cake powder ($24gL^{-1}$) containing media. Among the different amino acids supplemented, tryptophan supplemented to sesamum and coconut seed cake powder increased the production of α-amylase (57.42 and 58.26 UmL^{-1} respectively).

Production of α-amylase was scaled up from 25mL to 2L. When the media volume to flask volume ratio was maintained at 1:10, α-amylase production was decreased from 46.30 to 30.24 UmL^{-1} with the increase in the volume of fermentation medium from 25 to 200mL. When the media volume to flask (2L flask) volume ratios were 1:10, 1.5:10, 2:10 and 2.5:10, highest α-amylase activity (30.24 UmL^{-1}) was obtained at the ratio of 1:10. When the speed of agitation in the fermenter (3L) was increased from 100 to 200rpm, α-amylase production was increased from 13.59 to 36.76 UmL^{-1} at 42°C and 0.6vvm aeration. At 0.6vvm aeration and 200rpm, the dissolved oxygen level has immediately reduced to zero and increased after 57 hours. When the agitation speed was increased from 200 to 400rpm, highest α-amylase (51.17 UmL^{-1}) production was obtained at 300rpm, 42°C and 1.2vvm aeration. Under controlled conditions (300rpm and 1.2vvm aeration), 51.17 UmL^{-1} enzyme activity was obtained at 28 hours of fermentation at 42°C while at 37°C, highest (52.45 UmL^{-1}) enzyme activity was obtained at 32 hours.

Effects of metal ions on the activity and stability of α-amylase were studied. α-Amylase activity was increased by Ca^{2+} and Na^{+} while inhibited by Cu^{2+}, Hg^{2+} and Mn^{2+} and less affected by Mg^{2+} and Ba^{2+}. In the presence of either 0.1M Na^{+} or 1mM Ca^{2+}, 33 and 100% of initial activity were retained respectively at 60min and 85°C. The α-amylase, in the presence of 0.1M Na^{+} and 1mM Ca^{2+} retained 94, 64 and 17.3% of the initial activity at 85, 90 and 95°C respectively at 180min.

Purification of α-amylase was carried out. The spent medium contained 37.5 UmL^{-1} α-amylase activity and 1.77 mgL^{-1} protein. Highest specific activity ($65.54Umg^{-1}$) was obtained at 50% $(NH_4)_2SO_4$ saturation and 66.6% recovered. The precipitated and dialyzed enzyme was purified using DEAE-Sephorose at pH 8.0, and eluted with the 0.01M Tris

buffer containing 0-0.8 M NaCl. Pooled fractions (11) contained 38.33UmL^{-1} enzyme activity and 0.2mgmL^{-1} protein. The recovery of α-amylase by ion-exchange chromatography was 7.5%, with 8.2 fold purification, showing the specific activity of 173.8Umg^{-1} protein. The purified α-amylase was tested for purity by SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis). The purified enzyme showed a single band with an apparent molecular weight of 55.54 kDa.

Kinetic properties of crude and purified α-amylases were compared with commercial α-amylase. The optimum time for first order kinetics was optimized as 5min for all type enzyme preparations at 85°C and pH 7.0. The optimum pH for the activity of crude, purified and commercial α-amylases at 85°C was 7.0. Crude and purified enzymes showed the highest activities at 85°C while the commercial enzyme showed at 90°C at pH 7.0. The commercial enzyme was more stable than crude and purified enzymes. Crude enzyme was more stable than purified enzyme. Substrate specificity indicated, that both purified and crude α-amylases were able to hydrolyse starch, amylose and amylopectin at 85°C and pH 7.0.