Chapter-5
Summary and Conclusions
5. SUMMARY AND CONCLUSIONS

Starchy residues appear to be the cheapest future raw material of the Indian alcohol industry in view of the rising cost and deteriorating quality of fermentable molasses due to government’s decontrol policy and technological advancements in the sugar recovery process. Starch has thus become a very important biopolymer for transformation to many useful, value added biochemicals including sweeteners and ethanol at industrial scale. In the course of conventional enzymatic hydrolysis, starch is first gelatinized by cooking at a very high temperature and then is liquefied at a slightly lower temperature which results in the formation of dextrins. This raises the viscosity and poses problems in mixing and pumping of the slurry. This precooking necessary for gelatinization of the raw material requires a large amount of heat energy, which is 30-40% of all energy spent for alcohol production. In view of energy costs, effective utilization of natural starchy resources and viscosity problems, direct hydrolysis of raw starch below the gelatinization temperature is desirable. The use of raw starch digesting amylases does not require precooking of starch for enzymatic hydrolysis. In view of this, the present study entitled “STUDIES ON A RAW STARCH LIQUEFYING α-AMYLASE FROM Bacillus sp. FOR THE DIRECT LIQUEFACTION OF RAW STARCHES WITHOUT GELATINIZATION AND ITS EVALUATION IN SOME BIOTEchnOLOGICAL APPLICATIONS” has been designed after taking into consideration the need of reducing the cost of starch hydrolysis and its major thrust has been aimed at obtaining a bacterial isolate for the production of direct raw starch liquefying α-amylase, its purification and characterization, and its evaluation for the direct liquefaction of natural starchy substrates for ethanol production. The major findings of the study are summarized below:

1) Of a large number of raw starch degrading amylolytic colonies obtained from different locations, one isolate P₁ which showed the ability to grow as solid state and submerged cultures and degraded insoluble starches effectively was selected for further studies. The selected isolate produced 128549U/g and 1405U/ml of α-amylase under solid and submerged fermentation respectively. It degraded insoluble potato, rice, wheat and maize starches to the extent of 99.9, 99.5, 97.3 and 95.6% respectively. On
the basis of various morphological, physiological, biochemical characteristics and 16s rRNA sequence, the bacterium was identified as *Bacillus subtilis* subsp. *spizizenii*.

2) The α-amylase production was substantially increased in solid state fermentation by standardization of various environmental and cultural parameters. Highest yield of 3,12,744 U/g of the substrate was achieved at 37°C, after 48 h of incubation, in the solid media consisting of wheat bran (5g) moistened with 7.5ml of moistening agent (5ml tap water and 2.5 ml inoculum) and supplemented with glycerol (2.0% w/w) as carbon source, soyabean meal (2% w/w) as nitrogen source, sodium chloride (2mM) as the metal ion source, B-complex (0.015% w/w) as vitamin source, threonine (0.0125% w/w) as amino acid source and Tween 40 (0.0125% v/w) as surfactant. The enzyme recovery from the fermented bran was done by using Tween 40 (0.0175% v/w).

3) The α-amylase production also increased in submerged fermentation by standardization of various environmental and cultural parameters. Highest yield of 9711 U/ml was achieved at 50°C, after 48 h of incubation, in the liquid media consisting of glycerol (2% w/v) as carbon source, soyabean meal (2.5% w/v) as nitrogen source, sodium chloride (2.25mM) as the metal ion source, B-complex (0.0175% w/v) as vitamin source and threonine (0.01% w/v) as amino acid source. The inoculation of this medium was done by 10% inoculum.

4) Comparison of enzyme production by solid state fermentation and submerged fermentation revealed that that SSF yielded 42 times more production of α-amylase than SmF from 1 g of cells. Therefore SSF technique is more efficient when compared to SmF technique for the production of α-amylase by *Bacillus subtilis* subsp. *spizizenii*.

5) Response surface methodology was used to study the cumulative effect of the nutritional parameters including carbon source (glycerol), nitrogen source (soyabean meal), metal ion (sodium chloride), amino acid (threonine) and vitamin source (B-complex) to enhance the production of extracellular α-amylase in solid-state fermentation. For obtaining the
mutual interaction between the variables and optimizing these variables, a $2^5$ factorial central composite design using response surface methodology was employed. The optimal calculated values of tested variables for maximal production of $\alpha$-amylase were: glycerol, 2.037%; soyabean meal, 2.346%; sodium chloride, 2.033mM; threonine, 0.017%; and B-complex solution, 0.0146% with a predicted $\alpha$-amylase activity of 384820U/g of wheat bran. These predicted optimal parameters were tested in the laboratory and the final $\alpha$-amylase activity obtained (381111 U/g of wheat bran) was very close to the predicted value. This value is still greater than the yield obtained by synergistic effect of all the nutrients at their optimum concentrations obtained by varying ‘one variable at a time’ (with 312744 U/g) suggesting the important role of response surface methodology in the optimization studies. A comparison of the results obtained both theoretically and experimentally revealed that $\alpha$-amylase production increased considerably with the increase of soyabean meal and glycerol concentration.

6) The enzyme production was scaled-up by employing solid state fermentation in three different vessels: Erlenmeyer flasks of variable capacity (250 ml to 1000 ml), Enamel coated metallic trays (38.5cmx30cmx4.2cm) and sieve trays (30cmx45cmx5 cm). The highest yield of 310237 U/g was achieved in 250 ml Erlenmeyer flask having 5 g wheat bran and the same decreased slightly with an increase in the quantity/size of the flask, yielding 274890 U/g with 40 g of wheat bran in 1000 ml flask.

Different quantities of wheat bran ranging from 50 to 500 g were taken in enamel trays and the yields were maximum upto 200g of the bran, and the same got declined with the further increase in the wheat bran quantity after 48h of incubation. An average yield of 276560 U/g was observed per tray, in a batch of 2 kg involving 10 such stacked trays.

Wheat bran ranging from 400-1000 g was also taken in specially designed wooden sieve trays and the yields were maximum upto 750g of the bran, and the same got declined with the further increase in the wheat bran
quantity after 72h of incubation. An average yield of 199136 U/g was observed per tray, in a batch of 18 kg involving 24 such trays stacked in two groups of 12 each.

7) The enzyme production was scaled-up by employing submerged fermentation in Erlenmeyer flasks of variable capacity (100 ml to 1000 ml). The production volume (containing optimized medium) with respect to flask size was varied in the ratio of 1:5 and 1:4 and 3:10. Highest enzyme yield of 9681 U/ml was obtained when 20ml of the optimized medium was taken in 100ml flask and the same decreased slightly with an increase in the volume of production medium/size of the flask, yielding 8643 U/ml with 300ml of optimized production medium in 1000 ml flask.

α-Amylase study was scaled up from shake flask to fermenter level in 7.5L fermentor. Optimized medium, 4.5L was used as the production medium and seed medium, 0.5L was inoculated aseptically into the production medium. The enzyme production reached a maximum of 9287 U/ml after 40h of incubation at 50°C.

8) The studies on the localization of the α-amylase from Bacillus subtilis subsp. spizizenii from both the fermentation conditions revealed that although the enzyme was localized in the intracellular and cell associated fractions but most of it was excreted outside (~90%) by the organism which was obtained in the extracellular fractions.

9) The expression of α-amylase from Bacillus subtilis subsp. spizizenii from both the fermentation conditions was studied by noting the electrophoretic banding pattern, which revealed that expression of α-amylase protein was higher after every 12h showing that more and more of α-amylase should be present in the extracellular cell extracts till 84h of incubation. But the maximum activity under both the fermentation conditions was observed after 48h of incubation. This could also be related with the probability of expression of other proteins like protease which interfered with the activity.

10) The α-amylase preparation was purified to homogeneity using (i) Ammonium sulphate precipitation (ii) Gel filtration using sephadex G-200 (iii) DEAE sephadex ion exchange chromatography and (iv) preparative gel
The purified α-amylase preparation was characterized by studying the temperature and pH activity and stability profiles, effect of various metal ions, effector molecules and substrate concentration, its nature, affinity of it towards the different raw starches and determining its catalytic domain.

a) The enzyme had a pH optima of 6.5 and temperature optima of 70°C.

b) The enzyme was most stable at 50°C and had a half life of 5 and 2h at 60 and 70°C respectively which improved significantly in presence of NaCl where it retained a residual activity of 96.3 and 72.6% respectively after 6h of incubation. At 90 and 100°C, the residual activity was 32.6 and 28.9 after 0.5h of incubation in the presence of NaCl.

c) The enzyme was most stable at pH 6.5 retaining 54 and 40.1% activity after 4 and 6h of incubation respectively.

d) The α-amylase was found to be glycosylated metallo-protein and its activity got promoted in the presence of sodium chloride, sodium molybdate, calcium chloride, sodium tungstate, potassium chloride and copper sulphate,

e) The enzyme activity got inhibited in presence of certain metal salts including silver nitrate, barium chloride, surfactants including Tween 20, Tween 40, Tween 60, Tween 80, TxitonX-100 and SDS, oxidizing agents including hydrogen peroxide and sodium azide, reducing agents including β-mercaptoethanol and cysteine HCl and chelating agents including EDTA and sodium citrate.

f) The $K_m$ values for the α-amylase were 1.11 and 1.25 mg/ml respectively on soluble starch and amylpectin and $V_{max}$ values were 166.66 and 125U/ml/min respectively.

g) The adsorption rate of the enzyme onto different raw starch granules was almost negligible showing the enzyme to be non adsorbing raw starch degrading α-amylase.

h) The chemical modification of the enzyme suggested the involvement of Asp, Glu, Tyr, Trp, Cys, Lys and His for substrate binding and catalytic activity of the enzyme.
The crude α-amylase preparation was evaluated in the hydrolysis of various concentrations of insoluble rice, potato, wheat and maize starches at 50, 60, 70, 80 and 90°C. An enzyme dose of 2.5U/mg in a total volume of 25ml was found to be sufficient in the complete liquefaction of all the insoluble raw starches. The α-amylase preparation was found to be very active in the rapid hydrolysis of different raw starches with raw potato starch granules being hydrolysed the best, followed by raw wheat, rice and maize starch granules. It worked well on all the temperatures revealing more than 90% liquefaction and up to 45% saccharification of different insoluble starches in a short incubation time.

a) The pattern of degradation of various insoluble starches by α-amylase preparation was studied by scanning electron microscopy. A different pattern of degradation was observed for each type of starch.

b) The end product of hydrolysis of all the insoluble starches under study was mainly maltose with little amount of glucose when acted upon by raw starch liquefying α-amylase from *Bacillus subtilis* subsp. *spizizenii*. Whereas it was mainly glucose when acted upon by the combination of α-amylase preparation and glucoamylase from *Aspergillus* sp.

The potential application of the direct raw starch liquefying α-amylase preparation was evaluated by using it in the direct liquefaction of raw starchy substrates at moderate temperatures without involving the process of gelatinisation. The process of direct liquefaction of various raw starchy biomass including maize, rice, sorghum and wheat was standardized by altering various parameters including incubation temperature, enzyme dose, incubation time, enzyme dose, metal ions and its concentration and total solid concentration.

a) The optimum temperature of the hydrolysis of insoluble rice, wheat, sorghum and maize flour was 80, 70, 80 and 60°C respectively above which the CE and FE remained almost constant.

b) Maximum liquefaction by α-amylase preparation at the respective optimised temperatures occurred when a dose of 1.50, 1.50, 2.50 and 2.50
lac units/100g of flour were used for rice, wheat, sorghum and maize flour respectively.

c) An incubation period of 2, 1½, 2 and 3h was found to be the best for the direct liquefaction of rice, wheat, sorghum and maize flour respectively at respective optimized temperature and α-amylase dose.

d) Sodium chloride at 5, 5, 15 and 10mM concentration was found to be the best for the direct liquefaction of rice, wheat, sorghum and maize flour at all other optimized conditions.

e) The optimum solid concentration of 25, 30, 25 and 25% was found to be the best for the direct liquefaction of rice, wheat, sorghum and maize flour respectively by α-amylase from *Bacillus subtilis* subsp. *spizizenii*.

f) A comparison of the conventional method of ethanol production from different flours (by using standard commercial enzymes including Termamyl™ and AMG™ involving gelatinization of raw flours) and by using raw starch liquefying α-amylase preparation from *Bacillus subtilis* subsp. *spizizenii* under optimized conditions of hydrolysis, followed by simultaneous saccharification by standard commercial glucoamylase AMG™ was done. Ethanol yields of 343.6, 462.42, 412.62 and 383.96ml/kg of flour and an overall efficiency of 74.26, 85.70, 74.36 and 83.39 were obtained by conventional method for the hydrolysis of maize, rice, sorghum and wheat flour respectively. The yields and overall efficiency were quite comparable in the process involving raw starch liquefying α-amylase from *Bacillus subtilis* subsp. *spizizenii* for ethanol production from various flours including maize, rice, sorghum and wheat. The yields were 334.24, 456.52, 395.71 and 381.90 ml/kg of raw flour and the O.E. were 72.01, 84.12, 71.96 and 80.96 respectively.

14) Ethanol production from different starchy grains including maize, rice, sorghum and wheat was scaled up from 100g of grains as substrate to ½, 1, 1½, 2, 2½ and 3kg of grains.

15) The α-amylase preparation was evaluated for its ability to remove the size from the fabrics and the process was standardized by altering various parameters including temperature, metal ions, surfactants, and enzyme
dose. The enzyme preparation could effectively remove the size after 10 min of incubation at 60°C when an enzyme dose of 1500U was used in 25 ml of distilled water containing 0.01%(v/w) of commercially used surfactant and 10mM sodium chloride. The results obtained were comparable to the process of desizing involving commercial α-amylase Palkozyme which desized the fiber after 10 min of incubation at 70°C when an enzyme dose of 1500U was used in 25 ml of distilled water containing 0.01%(v/w) of commercially used surfactant and 10mM calcium chloride.

The present study was initiated to develop a simple and indigenous solid state fermentation or liquid fermentation based system for in-house production of raw starch liquefying α-amylase for the efficient hydrolysis of raw/insoluble starchy biomass for Indian alcohol industry which is spending a huge amount in importing these enzymes from Novo Nordisk, Denmark and other foreign sources. The study has yielded a bacterial strain of Bacillus subtilis subsp. spizizenii capable of producing very high levels of raw starch hydrolysing α-amylase in both solid state and liquid fermentation system. However the enzyme productivity was more in solid state fermentation as compared to submerged fermentation. The enzyme has been found to be optimally active at 70°C, pH 6.5 and was quite thermostable at high temperatures. The enzyme preparation could effectively desize the textile fibres. It could also completely liquefy the insoluble wheat, maize, potato and rice starches at moderate temperatures in a short duration of time of 1-2h. Moreover, the enzyme preparation has also been found to effectively hydrolyse the various raw flours including wheat, rice, maize and sorghum at moderate temperatures within 1-2 h. Comparable ethanol yields were obtained when our enzyme preparation was used in combination with glucoamylase from Apergillus sp. as obtained by commercial enzymes (which require prior gelatinization of starch before its action). On the basis of the application studies of the in-house enzyme preparation(s), a process has been designed for the effective and direct liquefaction of raw starch biomass followed by simultaneous saccharification and fermentation for alcohol production. In conclusion the laboratory strain of Bacillus subtilis subsp. spizizenii can be the potential candidates for developing a simple solid state fermentation based process for in-house production raw starch liquefying α-amylase which can be

292
effectively employed for direct hydrolysis of raw starchy biomass for ethanol production. Although the process of ethanol production was scaled up till 5 kg of the raw substrate and as this study was purely on a laboratory scale, there is a need to further scale up the production and application trials of the enzyme at pilot plant level before any conclusion can be drawn for the commercial utilization of this enzyme.