SUMMARY

In the present work, filamentous fungi *Aspergillus oryzae* was for the production of carbohydrase enzymes viz. amylases, cellulases and xylanase by solid state fermentation (SSF). Abundantly available agro residues generally considered challenge in solid waste management were chosen as main media component to explore their utilization in enzyme production. The process parameters were optimized for maximum yield of carbohydrase cocktails and subsequently their physico-chemical properties were determined. Along with the natural solid substrate poly urethane foam (PUF) was also attempted as inert support for solid culture production of cellulase and xylanase to compare the SSF process to submerged fermentation process (SmF). During downstream processing (DSP) the crude enzyme extract was concentrated at vacuum and was taken for stability assessment after formulating with stabilizers to check industrial usability.

Among the eight agro residues viz. wheat bran (WB), rice bran (RB), rice flake waste (RFW), rice hull (RH), lentil bran (LB), green gram bran (GGB), black gram bran (BGB), corn residue (CR) used as substrate independently for SSF, wheat bran was found to be the superior to show maximum enzyme titers of carbohydrase enzymes of interest viz. α-amylase (4680±200 IU gds⁻¹), amylglucosidase (1257±40 IU gds⁻¹), endo-glucanase (27±2 IU gds⁻¹), β-glucosidase (112±15 IU gds⁻¹), exo-glucanase (2.6±0.5 IU gds⁻¹) and xylanase (103±15 IU gds⁻¹). The optimized process parameters for carbohydrase enzymes production during SSF were found as temperature: 30°C, pH: 5.0, initial moisture content: 50%, inoculum level: 20% with the solid to flask volume ratio of 0.05. From the results it was evident that the enzymes production was stimulated more in presence of organic nitrogen sources than inorganic nitrogen sources. In addition non-ionic surfactant, Brij-96, showed a stimulatory effect on production of all kinds of carbohydrases. Catabolite repression studies revealed the presence of 150 g gds⁻¹ glucose was detrimental to the fermentative production of enzymes by SSF. It was found during this study that the SSF was significantly more resistant to the catabolite repression than SmF process for the production of carbohydrase enzymes by *A. oryzae*.

The physico-chemical characterization of the crude carbohydrase enzymes revealed that acidic pH (pH:5.0) was favourable for the activities of amylases, cellulases and xylanase. All the carbohydrase enzymes produced by *A. oryzae* during SSF were found to be stable in the pH range of 4.5-5.5 indicating their usability for various industrial applications. The temperature optima for the carbohydrase enzymes were found in the range of 50°C to 60°C. Amylases showed their maximum activity at 50°C and the cellulases and xylanase activities were found to be highest at 60°C. So in next stage of experiments the amylases were assayed at 50°C, cellulases and xylanase were assayed at 60°C and pH-5.0. The higher
value found for the activation energy for enzyme inactivation; $E_{ad}$ in comparison with activation energy for substrate hydrolysis; $E_a$, which was an indication of higher energy requirement for enzyme denaturation than substrate catalysis. The melting temperature ($T_m$) of all the enzyme components was also in the higher side 68-72°C which was in agreement with increased spectrum for their usability. The induction of amylase activities in presence of divalent cation as, Co$^{2+}$, and inhibition in presence of chelating agent as EDTA indicated metal ion requirement during their reaction. The cellulosytic components were induced maximally in presence of L-Cys indicating the presence of thiol group in their active sites.

The maximum total yield of both kind of fibrolytic enzymes viz. cellulase (402 IU) and xylanase (1459 IU) were noticed when 2g of PUF was saturated with 100ml of TKP based medium. Activities of both the enzymes viz. cellulase (4.87 IU ml$^{-1}$) and xylanase (16.84 IU ml$^{-1}$) were found to be highest on 96h of fermentation using optimized amount of PUF during SSF. More or less 4.6-5.0 fold increased yield was observed for both the fibrolytic enzymes during scale up study. However comparative study between SSF and SmF revealed that SSF system was more resistant to catabolite repression in presence of added glucose than SmF system. During SSF the enzyme yield was found to be 1132.1 IU g$^{-1}$ of biomass in presence of 150g l$^{-1}$ added glucose whereas lower enzyme yield (211.6 IU g$^{-1}$) was found during SmF at the same conditions. Initially the maximum growth rate was found to be higher in SmF compared to SSF but with the increase in glucose concentration the growth rate decreased but in SSF the fungal growth rate was found to be quite stable. Consequently other important kinetic parameters, maximum biomass production was also found to be better in SSF system. Present data may be useful for industrial production of enzymes, because of the advantage of using concentrated fermentation broth in PUF based SSF system with a lower risk of contamination and a lower recovery cost related to higher enzyme titres as compared to SmF system.

Enzyme extraction from the fermented mold bran was carried out using different solvents viz. distilled water, tap water, acetate buffer (pH:5.0), normal saline, 10% glycerol, 10% ethanol and 10% acetone to select best extractant. Distilled water was found to be superior for maximum enzyme recovery of crude enzyme in two hours. Higher dielectric constant of water than other solvents might help in reducing the interactive forces between the enzymes and substrate result better enzyme recovery. The extraction conditions using distilled water were optimized and the conditions found for maximum enzyme recovery were: time: 120min, temperature: 30°C, mold bran to water ratio: 1:4, shaker speed: 120 rpm. It was found that two repetitive cycles of extraction at optimized conditions was sufficient to recover most of the extracellular enzymes. To increase the enzyme protein in the extract crude cell free extract was fivefold concentrated under vacuum at 40-50°C temperature. The specific activity of α-amylase, amylglucosidase, endo-glucanase, β-glucosidase, exo-glucanase and xylanase were increased 1.5, 1.2,
1.2, 1.5, 2.0 and 1.3 times respectively in the concentrate of carbohydrases. During storage stability studies of the carbohydrase concentrate no activities were detected in enzyme concentrate when it was stored at room temperature for 30 days but storage at 4°C for the same time period helped to restore activity of α-amylase, amyloglucosidase, endo-glucanase, β-glucosidase, exo-glucanase and xylanase of 55% (initial 162 IU mg⁻¹ after 30 days 89.1 IU mg⁻¹), 43% (initial 39.2 IU mg⁻¹ after 30 days 16.856 IU mg⁻¹), 42% (initial 0.4 IU mg⁻¹ after 30 days .164 IU mg⁻¹), 42% (initial 3.9 IU mg⁻¹ after 30 days 1.638 IU mg⁻¹), 42% (initial 0.054 IU mg⁻¹ after 30 days 0.02268 IU mg⁻¹) and 40%(initial 3.1 IU mg⁻¹after 30 days 1.24 IU mg⁻¹) respectively of the original. The enzyme concentrate with 5% glycerol when stored at 4°C for 30 days, the residual activity found for α-amylase, amyloglucosidase, endo-glucanase, β-glucosidase, exo-glucanase and xylanase were 75% (initial 162 IU mg⁻¹ after 30 days 121.5 IU mg⁻¹), 72% (initial 39.2 IU mg⁻¹ after 30 days 28.224 IU mg⁻¹), 54% (initial 0.4 IU mg⁻¹ after 30 days 0.216 IU mg⁻¹), 61% (initial 3.9 IU mg⁻¹ after 30 days 2.379 IU mg⁻¹), 53% (initial 0.054 IU mg⁻¹ after 30 days 0.02862 IU mg⁻¹) and 58% (initial 3.1 IU mg⁻¹ after 30 days 1.798 IU mg⁻¹) respectively of the original activities. Glycerol (1-5%) may be used as a protectant during storage of enzyme concentrate (5 fold) at 4°C.

Various starchy and cellulosic substrates viz. crude rice starch (RS), crude potato starch (PS), crude cassava starch (CS), sugarcane bagasse (SCB), corn cobs (CC) and rice straw (RSW) were taken to conduct the hydrolysis study using the concentrated carbohydrase enzymes. The cellulosic substrates were treated with alkali prior to enzymatic hydrolysis whereas the starchy substrates were gelatinized before enzymatic treatment. The hydrolysis reaction was carried out on 15% substrate at 50°C at shaking condition (120 rpm) for 72h. The enzyme volume was varied from 10 to 25 ml. For all the three cellulosic substrates as SCB, CC abd RSW the rate of carbohydrate conversion in terms of sugar released was found to be the highest during first 24h followed by a plateau up to 72h. Maximum conversion noted for SCB, CC and RSW were 32%, 40% and 30% respectively. With increase in enzyme volume (10-20 ml) in the reaction mixture containing pretreated substrates viz. SCB, CC and RSW the conversion efficiencies were increased significantly but beyond that there was no significant increase in conversion. However, in case of SCB and RSW using higher dose of enzyme (20ml) conversion efficiency increased 3-5% compared to 15ml dose where as ~6-7% increase observed on CC which conferred better accessibility of the enzyme towards CC than SCB and RSW. Maximum substrate conversion for RS, PS and CS were 71.5%, 80% and 62% respectively. During the studies on the hydrolysis of starchy substrates it was evident that the PS was most susceptible to enzyme attack followed by RS and CS respectively. With the increase in substrate concentration the yield in reducing sugar as well as the conversion was found to be reduced.

When SCB, SC and RSW used as substrate (Concentration:20%) the maximum conversion was found to be 18, 23 and 17% respectively after 72h. For the same substrates the conversion efficiency with
the 20ml enzyme was found to be 36, 42 and 35% respectively at substrate concentration of 10%. Intermediate conversion efficiency was observed at 15% initial substrate concentration. However for all cases first 24h conversion rate was found to be maximum followed by a stationary trend.

The same experiment was carried out with starchy substrate viz. RS, PS and CS. It was noticed that during enzymatic hydrolysis of RS the conversion efficiencies were 74, 65 and 50% when the initial substrate concentration was 10, 15 and 20% respectively after 72h of hydrolysis. For the PS and CS the data obtained were 82 and 75% at 10% substrate concentration respectively.