Summary and conclusions
Chapter 5

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The fungi as enzyme sources have many advantages that, the enzymes produced are normally extracellular, making easier for downstream process. The development of economically feasible technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials will enable to utilize the large quantities of biomass such as the residues of both food industries and agriculture. Fungi are the main cellulase producing microorganisms, even though a few bacteria and actinomycets have also been reported to produce cellulases (Varma et al., 1994; Miyamoto, 1997).

Cellulases from several fungi and certain bacteria have been studied extensively, but little attention has been given to cellulases from *Aspergillus flavus*. Thus the present investigation was selected to conduct an extensive study on cellulases from *A. flavus*. Present study was aimed at isolation of promising cellulase producing *Aspergillus* sp. and its identification, optimization of cultural conditions for production of cellulolytic enzymes in submerged and solid state fermentation, and partial purification and characterization of cellulolytic enzymes. An attempt was also made to find the behavior of immobilizaed cellulolytic enzymes for the hydrolysis of agricultural by-products.

**Isolation and identification of cellulase (endoglucanase) producing fungi**

Fungal cultures were isolated from solid waste effluents from wheat flour mill and saw mill industries. Microbial populations were enumerated in terms of colony forming units. Low fungal population was observed from saw mill solid waste samples than from flour mill solid waste effluents. Fungal cultures were initially identified as species of the genera of *Aspergillus* and *Penicillium* based on cultural, morphological, microscopic characteristics.
The cellulolytic activity of these cultures was studied by standard CM-cellulose and congo red plate assay method. The promising primary fungal isolates showing significant clearing hydrolytic zone on CM-cellulose agar and congo red plate assay were further studied by flask scale culturing to determine the production of cellulolytic enzymes. Highest numbers of cellulolytic fungi were isolated from wheat flour mill solid waste as demonstrated by plate assay procedure and shake-flask-culture studies. Such studies have led to recognition of one promising fungal isolate that produced maximum endoglucanase (6.37 U/ml), exoglucanase (0.72 U/ml) and β-D-glucosidase (7.3 U/ml). The cultural and microscopic characteristics of this promising fungal isolate proved Aspergillus sp., and it was further confirmed by Microbial Culture Identification Service IMTECH, Chandigarah as Aspergillus flavus. This culture was used for further studies.

**Optimization of cultural conditions for endoglucanase (CMCase),

exogluconase (FPase) and β-D-glucosidase (Cellbiohydrolase)

production by submerged fermentation**

The process development is the key step in fermentation processes. The studies related to process development involves optimization of different fermentation conditions (physical and nutritional) towards enhancement of cellulolytic enzymes production. Shake-flask cultural conditions (physical and nutritional factors) for cellulolytic enzymes production by the isolated promising Aspergillus flavus isolate were optimized. Maximum endoglucanase and exoglucanase production was found in the culture filtrate of 2nd-day incubation, where as maximum β-D-glucosidase and fungal dry weight was observed at 6th-day of incubation. The optimum incubation temperature and initial medium pH were found to be at 30°C and 6, respectively. The carbon sources such as CMC at 1% and lactose at 2% were found to be optimum for maximum production of enzymes. The organic nitrogen
compounds stimulated higher cellulase yield than inorganic nitrogen source. Among tested nitrogen sources like yeast extract, tryptone and ammonium sulphate at different concentrations, yeast extract at 0.4 % was found to be optimum for cellulolytic enzymes production.

**Partial purification and characterization of cellulolytic enzymes**

In the present study the partial purification of cellobololytic enzymes from the culture filtrate was performed by ammonium sulphate salt precipitation followed by desalting through dialysis. For purification of endoglucanase and exoglucanase culture filtrates were harvested at 2nd-day of incubation, where as for β-D-glucosidase purification at 6th-day of incubation. Purification of cellulolytic enzymes produced by *A. flavus* was achieved to purification factor (fold) of 1.44, 1.435 and 1.16 for endoglucanase, exoglucanase and β-D-glucosidase, respectively. The enzyme recovery was 81.0, 80.2 and 89.8 for endoglucanase, exoglucanase and β-D-glucosidase, respectively. The calculated specific activity of partially purified endoglucanase is 3.743 U/mg protein as compared to 1.90 U/mg protein of crude culture filtrate. The specific activity of partially purified endoglucanase was 0.514 U/mg protein as compared to 0.358 U/mg protein of crude culture filtrate. The specific activity of partially purified β-D-glucosidase was high (3.47 U/mg) when compared to the crude culture filtrate (2.99 U/mg). The SDS-PAGE and zymogram analysis revealed three isoforms of endoglucanase with Mr of 24, 33 and 40.5 kDa. In similar analyses, β-D-glucosidase resolved as a monomer with Mr of 52.5 kDa. The lack of standard protocol for exoglucanase (zymogram) analyses limited its molecular weight determination. The endoglucanase activity of the present study was found to be stable up to temperature of 40°C and pH of 5-7. The optimum temperature and pH for its activity
were 50°C and 5, respectively. The exoglucanase was found to be less stable to temperature (30°C) and pH (4). The optimum temperature and pH for its activity were 50°C and 5, respectively. The β-D-glucosidase was found to be stable up to temperature of 50°C and pH of 5-7. The optimum temperature and pH for its activity were 50°C and 5, respectively. The endoglucanase activity was not affected by the surfactants such as Tween-20, Tween-80 and Triton X-100, whereas SDS inhibited its activity. Among the tested metal ions, Cu²⁺, Hg²⁺ and Pb²⁺ at 5 and 10 mM concentrations were found to be inhibitory. The exoglucanase activity was not affected by the surfactants such as Tween-20, Tween-80 and Triton X, whereas SDS inhibited its activity. Among the tested metal ions, Cu²⁺, Hg²⁺ and Pb²⁺ at 5 and 10 mM concentrations were found to be inhibitory. The β-D-glucosidase activity was not affected by the surfactants such as Tween-20, Tween-80, Triton X and SDS at 0.5 and 1.0% concentration. Among the tested metal ions, Cu²⁺, Hg²⁺ and Pb²⁺ at 5 and 10 mM concentrations were found to be inhibitory. The β-D-glucosidase activity was resistant to product inhibition up to 50 mM glucose and further increase in its concentration decreased the enzyme activity.

**Optimized cultural conditions for endoglucanase and β-D-glucosidase production by solid state fermentation**

The solid substrates such as wheat bran, oat bran, rice bran, sugarcane bagasse and sawdust were screened for endoglucanase and β-D-glucosidase by *A. flavus* in solid state fermentation (SSF). Among the solid substrates used in the present study, maximum endoglucanase and β-D-glucosidase production and secretion of extracellular protein was found with wheat bran. In view of maximum endoglucanase and β-D-glucosidase production with wheat bran, further optimization was carried with wheat bran only. The traditional ‘one variable at a time’ approach was used to select the significant factors affecting the
endoglucanase and β-D-glucosidase production. The optimized cultural conditions for wheat bran SSF are as: a) time course – 2\textsuperscript{nd}-day for endoglucanase production and 6\textsuperscript{th}-day for β-D-glucosidase production. b) substrate moisture content - 60% moisture content in wheat bran and c) inoculum level – 0.8 ml. Once critical factors were identified via ‘one variable at a time’, the response surface methodology was adopted to obtain a quadratic model consisting of 20 runs to estimate quadratic effects and central points for endoglucanase and β-D-glucosidase production as response. The R\textsuperscript{2} value for endoglucanase was 0.88 (88%) and for β-D-glucosidase 0.94 (94%). The maximum production of endoglucanase (93.24 U/g FMB) was attained with optimized conditions of wheat bran at 7.5 g, incubation time at 3\textsuperscript{rd}-day and inoculum level at 0.75 ml. The maximum production of β-D-glucosidase (116 U/g FMB) was attained with optimized conditions of wheat bran at 7.5 g, incubation time at 6\textsuperscript{th}-day and inoculum level at 0.75 ml.

Hydrolytic activity of immobilized cellulolytic enzymes

Cellulolytic enzymes produced by \textit{A. flavus} were immobilized by physical entrapment method using sodium alginate mix and hydrolysis was carried out in shake flasks. The hydrolysis of cellulosic (CM-cellulose, Cellobiose) and lignocellulosic substrates (wheat bran, rice bran oat bran) was carried in the presence of immobilized cellulase enzymes. The results demonstrated that immobilized cellulolytic enzymes are effective for cellulose hydrolysis. The desirable characteristics such as prolonged enzyme retention and better stability up to 5-6 batches were observed. The relative activity declined upto 35%, for the first three batches and remained relatively stable for the next batches (4 and 6\textsuperscript{th}), and declined sharply after 6\textsuperscript{th} batch of hydrolysis.