ABSTRACT

Rationale of the study

β-Glucosidases and β-xylosidases are the critical components of total cellulolytic complex that catalyze the final step in hydrolysis of cellulose and hemicellulose in lignocellulosic biomass. The endoglucanases and exoglucanases hydrolyze cellulose to cellobiose and cellooligosaccharides, which are finally converted to glucose by β-glucosidases. Both endo and exoglucanases are inhibited by cellobiose, and hence it is necessary to degrade cellobiose to achieve complete cellulose degradation. Complete degradation of cellulose requires the synergistic action of all the enzymes in the cellulase complex. β-Glucosidase produces glucose from cellobiose, reducing the cellobiose inhibition, which allows the endoglucanase and exoglucanase enzymes to function efficiently. In addition, β-glucosidases are useful in the flavor industry since they release aromatic compounds from glycoside precursors present in fruits and fermenting products.

β-xylosidases are necessary for the complete hydrolysis of xylan. Endoxylanases hydrolyze β-1,4-linkages in insoluble xylans to produce soluble xylooligosaccharides. β-xylosidases cleave alkyl- and aryl- glycosides, xylobiose, and xylooligosaccharides to xylose. These enzymes are employed in wine making because they hydrolyze bitter compounds present in grape juice during extraction and liberate aroma from grapes during wine making. Filamentous fungi such as Aspergillus niger, Aspergillus awamori, Trichoderma reesei, Talaromyces emersonii are known to be efficient producers of β-xylosidases.

In order to investigate the biotechnological application, it would be desirable to purify and characterize the enzymes. Most of the purification techniques are reported which constitute two or more purification steps. The present work describes a development of a simple method for purification of three enzymes. The characterization of enzymes may help in understanding the molecular details.
Aspergillus niger NCIM 1207 was isolated which produces very high levels of β-glucosidase and β-xylosidase activities. The further work on production and purification was continued with the following objectives.

1) Production of β-glucosidase and β-xylosidase of Aspergillus niger NCIM 1207 in the media supplemented with suitable carbon source and their optimization studies.
2) Characterization of crude β-glucosidases and β-xylosidase.
3) Purification of β-glucosidases and β-xylosidase to their homogeneity.
4) Physical characterization of purified enzymes such as molecular weight, pI determination.
5) Characterization of purified enzymes. (Optimum pH, optimum temperature, pH and temperature stability, enzyme kinetics, effect of heavy metals, organic solvents, substrate specificity)
6) Chemical modification studies of purified enzymes to determine active sites and substrate binding sites)

Chapter 1: Introduction

This chapter deals with the literature survey on microbial β-glucosidases and β-xylosidases with reference to cellulases, their mechanism of action and applications. More emphasis was given on two terminal key enzymes β-glucosidase and β-xylosidase. The literature survey includes the information on their occurrence, localization, isoenzyme forms, multienzyme functions, induction, regulation and repression, catalytic mechanism, classification, methods for assay, production, and purification of enzymes, characterization and applications with appropriate references.

Chapter 2: Production of β-glucosidases and β-xylosidase by Aspergillus niger NCIM 1207.

Production of β-glucosidase in submerged fermentation using Aspergillus niger 1207 was studied. The different substrates such as Cellulose-123, Solka floc, Avicel, Sigma cellulose, oat spelt xylan, Birch wood xylan, in presence and absence of urea & glycerol or glucose were studied for enzyme production. Xylan (oat spelt) was found to be most suitable for production of
high amounts of both \(\beta\)-glucosidase and \(\beta\)-xylosidase activities. Hence, further optimization studies were carried out using xylan as substrate.

It was reported earlier that \(\beta\)-glucosidase production was enhanced in presence of glucose and urea supplemented media using cellulose as carbon source. Hence optimization studies were carried out using xylan as carbon source in the medium supplemented with glucose / glycerol and urea. Higher activities of both the enzymes (13 IU/ml each) were obtained when organism was grown at 30\(^{\circ}\)C in media containing 3% xylan, 0.5%urea and 2.5% glycerol. Highest activities of both the enzymes (18-20 IU/ml) were obtained when the organism was grown at two different temperatures (30\(^{\circ}\)C for first 5 days followed by incubation at 36\(^{\circ}\)C for 9 days).

Aspergillus niger NCIM 1207 produced significantly high levels of \(\beta\)-glucosidase and \(\beta\)-xylosidase activities in submerged fermentation. Cellulose induced predominantly \(\beta\)-glucosidase, while xylan induced both \(\beta\)-glucosidase and \(\beta\)-xylosidase activities. Both the enzymes of this strain were found to undergo catabolite repression in the presence of high concentrations of glucose and glycerol. The sudden drop in pH of the fermentation medium below 3.5 caused the inactivation of enzymes when the fungus was grown in glycerol containing media at lower temperatures. The growth of the organism at 36\(^{\circ}\)C led to an increase in pH of the fermentation medium above 6.0 that affected \(\beta\)-xylosidase activity significantly. Highest levels of \(\beta\)-glucosidase ((19 IU/ml or 633 IU/g of substrate) and \(\beta\)-xylosidase (18.7 IU/ml or 620 IU/g of substrate) activities were detected when \(A. \) niger was grown at 30\(^{\circ}\)C for first five days followed by further incubation at 36\(^{\circ}\)C. Such a process of growing the organism at lower temperatures (growth phase) followed by growth at higher temperatures (production phase) in case of fungal systems has not been reported so far. \(A. \) niger NCIM 1207 is a potential candidate to produce both \(\beta\)-glucosidase and \(\beta\)-xylosidase activities in high amounts that can be used for supplementation of commercial cellulase preparations that are deficient in \(\beta\)-glucosidase and \(\beta\)-xylosidase.

The \(\beta\)-glucosidases and \(\beta\)-xylosidase were active at pH 4.5 and were found to be stable over a pH range between 3 – 7.5 and 3 – 6.5 respectively. The \(\beta\)-glucosidases and \(\beta\)-xylosidase exhibited maximum activity at 65\(^{\circ}\)C. Both, cellulose induced and xylan induced \(\beta\)-glucosidases showed stability at 60\(^{\circ}\)C for 5 h and lost total activity at 70\(^{\circ}\)C within 1 h. \(\beta\)-Xylosidase was found to be comparatively more stable that retained 100% of its original activity even after 5
hours of exposure at 70°C. Solka floc, xylose as well as xylan, induced only one isoform of \( \beta \)-glucosidase which is evident from zymogram staining. All the three enzymes were stable in most of the organic solvents except 1,4 Dioxane with enhancement in enzyme activities (20-80%) in some of the solvents such as methanol, iso-amyl alcohol, iso-octane, propanol and hexanol.

Chapter 3: Purification and characterization of \( \beta \) glucosidase and \( \beta \)-xylosidase by *Aspergillus niger* NCIM 1207.

The extracellular \( \beta \)-glucosidases (cellulose and xylan induced) and xylan induced \( \beta \)-xylosidase from *Aspergillus niger* NCIM 1207 were purified to homogeneity. The protocols were based on fractional ethanol precipitation, pH and thermal stability, separation of impurities by thermal denaturation and solubility differences in solvents etc. Purified enzymes showed a prominent single band on SDS-PAGE as well as on native gel. The molecular weights of all three enzymes were estimated by SDS-PAGE and also confirmed by HPLC and gel permeation chromatography and found to be 122 and 336 kDa respectively suggesting a trimeric structure of native molecule. These molecules were glycoprotein in nature and constitute approximately 35% carbohydrate moiety in \( \beta \)-glucosidases and 38% carbohydrate moiety in \( \beta \)-xylosidase. The isoelectric point (pI) of all three enzymes was around 4.6 which are evident from isoelectric focusing. The pH and temperature optima for all three enzymes were 4.5 and 65°C respectively. They were stable over pH range from 3.5 to 6.0. For \( \beta \)-glucosidases \( t_{1/2} \) at 70°C was 10 minutes while for \( \beta \)-xylosidase it was 45 minutes. The purified \( \beta \)-glucosidases and \( \beta \)-xylosidase could be stored for at least three-four months at 4°C and pH 4.5 without any loss of catalytic activity.

Cellulose and xylan induced \( \beta \)-glucosidases showed high stability in presence of various organic solvents except 1, 4 dioxane. \( \beta \)-xylosidase was also inhibited by chloroform whereas there was 1.2 to 1.5 fold increase in its activity when the enzymes were preincubated in methanol, ethanol, propanol, hexanol and iso-octane for 24 h at room temperature. Especially \( \beta \)-xylosidase was strongly inhibited by Hg\(^{2+} \) as compared to \( \beta \)-glucosidases. Cellulose and xylan induced \( \beta \)-glucosidases obeyed Michalis Menten kinetics and the Km and Vmax for \( p \)NPG were 1.42mM, 1250\( \mu \)moles/min/mg and 1.08mM, 714\( \mu \)moles/min/mg for cellulose and xylan induced \( \beta \)-glucosidases respectively. The Km and Vmax for \( p \)NPX was 1.3mM and 645\( \mu \)moles/min/mg for xylan induced \( \beta \)-xylosidase. The \( \beta \)-glucosidases and \( \beta \)-xylosidases showed more affinity to cellobiose and xylobiose respectively as compared to \( p \)NPG and \( p \)NPX. Both \( \beta \)-glucosidases
showed no cross reactivity with other p-nitrophenyl derivatives except with pNPX (2-5%). There was no reactivity with other diasaccharides such as sucrose, lactose, maltose or polysaccharides such as cellulose 123, Avicell, Solka floc and xylan. β-Xylosidase showed 2 to 5%, 24%, 115% reactivity towards pNPG, p-nitrophynl arabinofuranoside and oNPX. The two β-glucosidases and β-xylosidase were inhibited by glucose or xylose respectively.

Chemical modification studies revealed that tryptophan and carboxylate may be involved in catalysis in case of β-glucosidases. Substrate protection studies in β-glucosidases suggested that tryptophan and arginine may have a role in substrate binding. In case of β-xylosidase, cysteine and carboxylate may be involved in catalysis and tryptophan in substrate binding. Mass spectrometric analysis revealed that cellulose induced β-glucosidase showed 24% homology with β-glucosidase A of Aspergillus niger CBS 513.88/FGSC A1513 and 5% homology with glucoamylase of Aspergillus shirousami. Xylan induced β-glucosidase showed 12% homology with β-glucosidase A of Aspergillus niger CBS 513.88/FGSC A1513. Xylan induced β-xylosidase exhibited 35% homology with probable exo-1,4-β-xylosidase of Aspergillus niger CBS 513.88/FGSC A1513 and 11% homology with β-glucosidase A of Aspergillus niger CBS 513.88/FGSC A1513.