MICROBIAL ENZYMES RELATED TO AGRO-WASTE MATERIAL DEGRADATION

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In

MICROBIOLOGY

BY

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Affectionately dedicated

to

my caring Parents

and

Husband.

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DECLARATION

This is to certify that the work incorporated in the thesis entitled **"Microbial enzymes related to degradation of agro-waste material,"** submitted by **Smt. Ujwala Vinayak Khisti** was carried out under my supervision at NCIM resource centre, National Chemical Laboratory, Pune, 411008, Maharashtra, INDIA. Materials obtained from other sources have been duly acknowledged in the thesis.

Dr. D. V. Gokhale
(Research Guide)

DECLARATION BY RESEARCH SCHOLAR

I hereby declare that the work of the thesis entitled **"Microbial enzymes related to degradation of agro-waste material"** submitted for the degree of ***Doctor of Philosophy*** to the University of Pune, has been carried out by me at NCIM resource centre, National Chemical Laboratory, Pune, 411 008, Maharashtra, INDIA, under the supervision of Dr. D. V. Gokhale (Research Supervisor). The work is original and has not been submitted in part or full by me for any degree or diploma to any other university.

Ujwala Vinayak Khisti
(Research Scholar)

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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 ß-glucosidase and ß-xylosidase activities. The further work on production and purification was continued with the following objectives**.

1) Production of ß-glucosidase and ß-xylosidase of *Aspergillus niger* NCIM 1207 in the media supplemented with suitable carbon source and their optimization studies.
2) Characterization of crude ß-glucosidases and ß-xylosidase.
3) Purification of ß-glucosidases and ß-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 ß-glucosidases and ß-xylosidases with reference to cellulases, their mechanism of action and applications. More emphasis was given on two terminal key enzymes ß-glucosidase and ß-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 β-glucosidase and β-xylosidase activities. Hence, further optimization studies were carried out using xylan as substrate.

It was reported earlier that β-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^{o}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^{0}C for first 5 days followed by incubation at 36^{0}C for 9 days).

Aspergillus niger NCIM 1207 produced significantly high levels of β-glucosidase and β-xylosidase activities in submerged fermentation. Cellulose induced predominantly β-glucosidase, while xylan induced both β-glucosidase and β-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^{0}C led to an increase in pH of the fermentation medium above 6.0 that affected β-xylosidase activity significantly. Highest levels of β-glucosidase ((19 IU/ml or 633 IU/g of substrate) and β-xylosidase (18.7 IU/ml or 620 IU/g of substrate) activities were detected when *A. niger* was grown at 30^{0}C for first five days followed by further incubation at 36^{0}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 β-glucosidase and β-xylosidase activities in high amounts that can be used for supplementation of commercial cellulase preparations that are deficient in β-glucosidase and β-xylosidase.

The β-glucosidases and β-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 β-glucosidases and β-xylosidase exhibited maximum activity at 65^{0}C. Both, cellulose induced and xylan induced β-glucosidases showed stability at 60^{0}C for 5 h and lost total activity at 70^{0}C within 1 h. β-Xylosidase was found to be comparatively more stable that retained 100% of its original activity even after 5

hours of exposure at 70^0C. Solka floc, xylose as well as xylan, induced only one isoform of β-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 β glucosidase and β-xylosidase by *Aspergillus niger* NCIM 1207.

The extracellular β-glucosidases (cellulose and xylan induced) and xylan induced β-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 β-glucosidases and 38% carbohydrate moiety in β-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 65^0C respectively. They were stable over pH range from 3.5 to 6.0. For β-glucosidases t ½ at 70^0C was 10 minutes while for β-xylosidase it was 45 minutes. The purified β-glucosidases and β-xylosidase could be stored for at least three-four months at 4^0C and pH 4.5 without any loss of catalytic activity.

Cellulose and xylan induced β-glucosidases showed high stability in presence of various organic solvents except 1, 4 dioxane. β-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 isooctane for 24 h at room temperature. Especially β-xylosidase was strongly inhibited by Hg^{2+} as compared to β-glucosidases. Cellulose and xylan induced β-glucosidases obeyed Michalis Menten kinetics and the Km and Vmax for *p*NPG were 1.42mM, 1250μmoles/min/mg and 1.08mM, 714μmoles/min/mg for cellulose and xylan induced β-glucosidases respectively. The Km and Vmax for *p*NPX was 1.3mM and 645μmoles/min/mg for xylan induced β-xylosidase. The β-glucosidases and β-xylosidases showed more affinity to cellobiose and xylobiose respectively as compared to *p*NPG and *p*NPX. Both β-glucosidases

showed no cross reactivity with other *p*-nitrophenyl derivatives except with *p*NPX (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 *p*NPG, p-nitrophnyl arabinofuranoside and *o*NPX. 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.

LIST OF ABBREVIATION

AMM	*Aspergillus* minimal medium
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl suphoxide
DTNB	2,2-Dithiobisnitrobenzoic acid
EDAC	1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide
EDTA	Ethylene diamine tetra acetic acid
GRAS	Generally Regarded As Safe
HEPES	2-(2-Hydroxyethyl) Piperazine-N-(4-Butanesulfonic acid)
IEF	Isoelectric Focusing
IEF-PAGE	Isoelectric Focusing Polyacrylamide Gel Electrophoresis
MES	2-(N-Morpholinolethanesulfonic acid)
NAI	N-acetylimidazole
NBS	N-Bromosuccinimide
NCIM	National Collection of Industrial Microorganisms
NEM	N-ethylmaleimide
PAGE	Polyacrylamide Gel Electrophoresis
PCMB	*p-Chloromercuribenzoate*
PDA	Potato Dextrose Agar
PEG	Polyethylene glycol
pI	Isoelectric pH
PMSF	Phenyl methyl sulfonyl fluoride
*p*NPG	*p*-Nitrophenyl-β–D-glucopyranoside
*p*NPX	*p*-Nitrophenyl-β–D -xylopyranoside
SDS	Sodium dodecyl sulphate
SmF	Submerged fermentation
SsF	Solid state fermentation
TFA	Trifluroacetic acid
TNBS	2,4,6-trinitrobenzenesulfonic acid

CHAPTER 1

INTRODUCTION

Presently, petroleum represents a basic feedstock for production of commodity chemicals and fuels. Rapid depletion of this finite resource and increase in emission of CO_2 levels encouraged a replacement of petroleum with renewable resources such as lignocellulosic biomass as feedstock (Dukes, 2003). With the advent of conversion technologies, biomass resources have regained the potential as feedstock for fuels and chemicals. Many countries are engaged in research and development programs that can provide biofuels from lignocellulosic biomass. US department of Energy Office developed a program which may provide 60 billion gallons per year of biofuels by 2030. Corn starch and sugars from sugarcane and beets are currently being used directly for biofuels such as ethanol. Brazil has been using sugarcane as raw material for large scale bio-ethanol production for more than 30 years (Goldemberg, 2007). Chemicals such as 5-hydroxymethylfurfural (HMF) obtained by dehydration of glucose is a platform chemical for synthesis of variety of useful acids, aldehydes, alcohols and amines, as well as 2, 5-dimethylfuran which is similar to gasoline. In fact, all biofuels and commodity chemicals are based on such food resources and oils because it is easy to convert them into valuable products. Utilization of structural sugars in biomass is comparatively difficult due to its recalcitrance nature. Biomass processing can commence only when we improve the slow kinetics of breaking down biomass to release sugars with high yields. Recent advances in synthetic biology (Pleiss, 2006), metabolic engineering (Lee et al., 2006; Keasling and Chou, 2008) and system biology approach (Mukhopadhyay et al., 2008) have generated microbial cell factories for synthesis of commodity chemicals. These microbial cell factories are constructed by modulating gene expression to fine tune microbial metabolism and also by engineering the proteins to impart new catalytic activities or to improve native properties.

Lignocellulosic biomass structure and enzymatic degradation

Biomass consists of three types of polymers: cellulose, hemicellulose and lignin. Cellulose has strong physico-chemical interaction with hemicelluloses and lignin. Cellulose, a linear glucose polymer, is highly ordered polymer of cellobiose (D-glucopyranosyl-β-1,4-D-glucopyranose) representing about 50% of the wood

mass. Native cellulose has about 10,000 glycosyl units in the cellulose chain that form fibrils which are stabilized by strong intermolecular hydrogen bonds between hydroxyl groups of the adjacent molecules. Cellulosic materials have crystalline domains separated by less ordered, amorphous, regions. These amorphous regions are the potential points for chemical and enzymatic attacks. The crystalline cellulose is highly resistant to chemical and enzymatic hydrolysis due to its structure in which chains of cellodextrins are precisely arranged. Several different crystalline structures of cellulose are known, corresponding to the location of hydrogen bonds between and within strands. Natural cellulose is cellulose I, with structures Iα and Iβ. Cellulose produced by bacteria and algae is enriched in Iα while cellulose of higher plants consists mainly of Iβ. Cellulose in regenerated cellulose fibers is cellulose II. The conversion of cellulose I to cellulose II is irreversible, suggesting that cellulose I is metastable and cellulose II is stable. With various chemical treatments it is possible to produce the structures of cellulose III and cellulose IV (Nishiyama et al., 2002)

Hemicellulose is the second most abundant polysaccharide fraction available in nature. Xylan is the one of the major structural polysaccharide in plant cell. Xylans are heteropolysaccharides with a homopolymeric backbone chain of β-1, 4-linked β-D-xylopyranose units. The backbone consists of O-acetyl, α -L-arabinofuranosyl, α-1,2-linked glucuronic or 4-O-methylglucuronic acid substituents. Wood xylans exist as O-acetyl-4-O-methylglucuronoxylans in hardwoods or as arabino-4-O methyl glucuronoxylans in softwoods. The cereal xylans are made up of D-glucuronic acid and/or its 4-Omethyl ether and arabinose. Based on side chain the xylans are classified as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. The β-1,4-linked β -D-xylopyranose units is substituted at positions C-2, C-3 and C-5 to varying degrees depending upon the plant and the stage of development of the plant when the polymer is obtained. In monocots, at the C-2 positions 1,3-linked α -D-glucronic acid or 4-Omethyl- α -D-glucuronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1,3 linked a-L arabinofuranose. In some xylans, particularly in hardwoods, xylopyranose residues may be O-acetylated at the C-2 or (more commonly) at the C-3 positions. Again, small amounts of phenolic components,

such as ferulic and p-coumaric acids are esterified to xylan *via* their carboxyl groups to C-5 of xylose ring. (Bastawde, 1992, Kulkarni et al., 1999)

Lignin is made up of phenylpropanoid units derived from the corresponding p-hydroxycinnapyl alcohols. These phenylpropanoid units are made up of dimethoxylated (syringyl), monomethoxylated (guaiacyl) and nonmethoxylated (p-hydroxyphenil) alcohols. Lignin is hydrophobic and highly resistant to chemical and biological degradation. It is present in the middle lamella and acts as cement between the plant cells. It is also located in the layers of the cell walls, forming, together with hemicelluloses, an amorphous matrix in which cellulose fibrils are embedded and protected against biodegradation. This association between cellulose and hemicelluloses and lignin makes the plant cell wall resistant to mechanical and biological degradations. The processing of lignocellulosic biomass will make the lignin enormously available for conversion into value added products, rather than its fuel value. The partially hydrolyzed lignin has excellent properties for use as substitutes for phenol-formaldehyde resins, polyurethane foams, adhesives, insulation materials, rubber processing, antioxidants, etc. It also provides a cheap source for vanillin and syringol for the flavor and fragrance industry.

Cellulases

Cellulose can be hydrolyzed by many different enzymes that are known as cellulases which hydrolyze the β-1,4 linkage in the cellulose. Cellulases hydrolyze cellulose by two different catalytic mechanisms, the retaining and the inverting mechanisms. Inverting type mechanism produces the α-sugar and a retaining type mechanism releases a product in the β-configuration (Davies and Henrissat, 1995; Zechel et al., 2000). Both the mechanisms involve two carboxylate residues (aspartic acid and/or glutamic acid) and catalyze the reaction by acid-base catalysis. Depending upon the type of reaction catalyzed and substrate specificity, the cellulases are classified in to endo-cleaving (endoglucanases) and exo-cleaving (exo-glucanases). Endo-glucanases cleave glycosidic bonds internally in cellulose chain releasing oligosaccharides of various lengths. Exo-glucanases act in a processive manner on reducing or non-reducing ends of the cellulose chain liberating either glucose

(glucanohydrolases) or cellobiose (cellobiohydrolases) as major end products. The cellobiose or cellooligosaccharides are further hydrolyzed by third group of enzymes called β-glucosidases. Cellulases have a folded carbohydrate binding modules (CBM) that are connected to the catalytic domain by a flexible linker. These CBMs bind the enzyme to the crystalline cellulose that results in enhancement in enzyme activity.

Endoglucanases (Endo-1,4-β-glucanase, EC 3.2.1.4) are also called as carboxymethylcellulases (CMCase) because they cleave artificial substrate, carboxymethylcellulose, that is used for determining enzyme activity. These enzymes attack cellulose at amorphous regions making cellulose more accessible to cellobiohydrolases by providing new free chain ends. Fungal endoglucanases are generally monomers with no or low glycosylation. They have pH optima between 4.0 and 5.0 and are active at temperatures between 50 to 70 ^{o}C. Multiple endoglucanases have been reported in many fungi such as *T. reesei* (Baldrian and Valaskova, 2008) and *Penicillium chrysogenum* (Abbas et al., 2005). Some endoglucanases possess cellulose binding domains (CBM) and some are without CBM (Sandgren et al., 2005). Exo-glucanases (CBHs) are monomers with no or low glycosylation with pH optima between 4.0 and 5.0. However, they possess wider temperature optima from 37 to 60 ^{o}C (Cantarel et al., 2009). CBHs act on β-1,4-glycosidic bonds from chain ends, releasing cellobiose as the major end product. Some CBMs hydrolyze cellulose from non-reducing ends and other act from reducing ends of the cellulosic chains. This helps in increasing the synergy between opposite acting enzymes which results in degradation of cellulose more efficiently (Sandgren et al., 2005). The cellobiose released by action of CBHs acts as a competitive inhibitor which limits the ability of the enzymes to hydrolyze cellulose molecules (Baldrian and Valaskova, 2008).

Mechanism of cellulose Hydrolysis

First Reese and his coworkers suggested the mode of cellulose hydrolysis involving a C_1 and Cx components (Reese et al., 1950). They reported that the conversion of native cellulose into soluble sugars is a two step process. The C1 component was believed to disaggregate or activate the cellulose chains so that the enzymes classified as Cx could carry out the depolymerization. They proposed that

microorganisms capable of growing only on soluble forms of cellulose, such as carboxymethyl cellulose (CMC), synthesized only the Cx component, whereas microorganisms growing on highly ordered forms of cellulose produced both C_1 and Cx. Due to inability to produce culture filtrates active against crystalline cellulose, the early studies were focused on the Cx components. However, the discovery in 1964-1965 that, culture filtrates prepared from *T. viride* and *T. koningii* were capable of extensive hydrolysis of native cellulose, was a turning point in the study of cellulases. This discovery led to the beginning of search for a C_1 component. In 1972, three independent research groups made the important discovery that the C1 component was, in fact, a hydrolytically active enzyme, cellobiosyl hydrolase (cellobiohydrolase) (Pettersson et al., 1972; Nisizawa et al., 1972, Wood and McCrae, 1972). Cellobiohydrolase was found to act synergistically with the Cx components to degrade crystalline cellulose. It was therefore proposed that Cx (CMCase) acts as an endoglucanase to produce available chain ends on cellulose which are substrates for cellobiohydrolase. It turned out to be the Cx component that initiates the cellulose breakdown rather than the C1 proposed by Reese and coworkers (Pettersson et al., 1972; Nisizawa et al., 1972; Wood and McCrae, 1972). Further, the widely accepted mode of enzymatic hydrolysis of cellulose involves synergistic actions of three enzymes i.e. endoglucanases, exoglucanases and β-glucosidase. Endoglucanases hydrolyses the intermolecular β-1,4-glucoside bond of cellulose chain randomly to produce new chain ends, exoglucanases processively cleave cellulose chain ends to release soluble glucose and/or cellobiose and β-glucosidases hydrolyzes cellobiose to glucose. The feedback inhibition by cellobiose and glucose is also observed during cellulose hydrolysis. As compared to glucose, cellobiose causes severe feedback inhibition, i.e. it strongly inhibits the cellobiohydrolases.

There are two major mechanisms of enzymatic glycosidic bond hydrolysis first proposed by Koshland. The retaining mechanism involves initial protonation of the glycosidic oxygen via the acid/base catalyst with concomitant formation of a glycosyl-enzyme intermediate through the nucleophile. The resulting glycosyl enzyme is hydrolyzed by a water molecule and this second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate.

The nucleophile and acid/base are always found 5-6 Å apart on all systems studied so far. The inverting mechanism involves the single step protonation of the glycosidic oxygen via the acid/base catalyst and concomitant attack of a water molecule activated by the nucleophile. The consequent product shows a stereochemistry opposite to that of the substrate. The type of mechanism is conserved within a given glycosyl hydrolase family and dictated by the active site architecture and atomic distance between the acid/base and nucleophilic residues (aspartic acid and/or glutamic acid). The acid and base are typically located about 7-13 Å apart in order to accommodate the nucleophilic water `below' the pyranoside ring. During the hydrolysis of native cellulose, inverting type enzyme produces the α-sugar and a retaining enzyme produces a product in the β-configuration (Davies and Henrissat, 1995; Zechel et al., 2000). The recent concept of mode of cellulose hydrolysis by cellobiohydrolase is the concept of "processivity." Processive enzyme action can be defined as the sequential cleavage of a cellulose chain by an enzyme. In effect, exoglucanases /cellobiohydrolases are by nature and structure, processive enzymes. This topology allows these enzymes to release the product while remaining firmly bound to the cellulose chain, thereby creating the conditions for processivity. Their tunnel-like active site thus allows processive action on the cellulose chain (Fig 1.1b). Another cleft or groove like 'open' structure (Fig. 1.1a) allows a random binding of several sugar units in polymeric substrates and is commonly found in endo-acting cellulases.

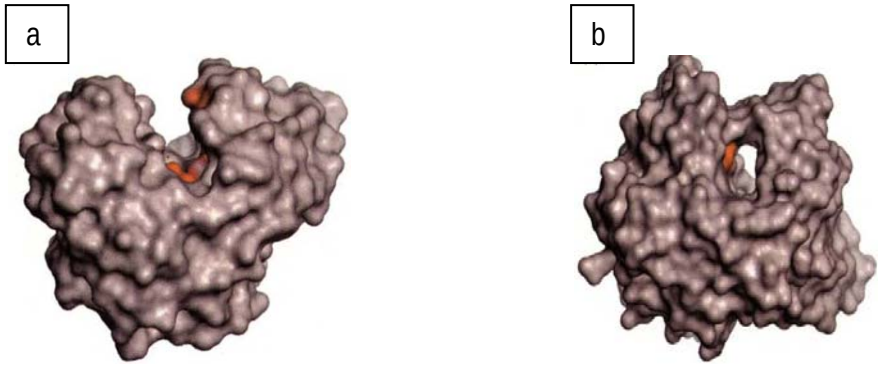


Fig. 1.1 a) The cleft type active site of endoglucanase E2 from *T. fusca,* b) The tunnel type active site cellobiohydrolase II from *T. reesei* creating the conditions for processivity.

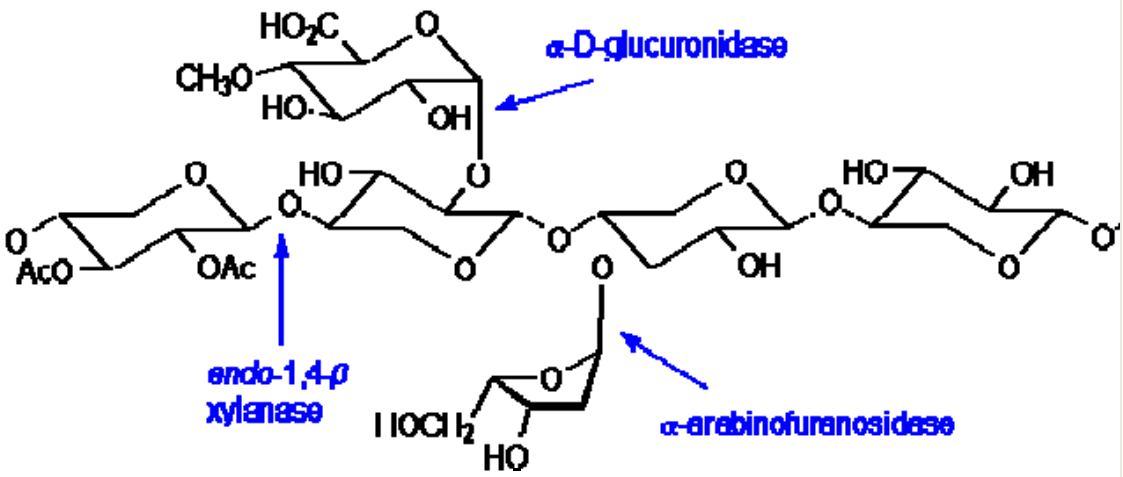
Xylanases

Xylanases are glycosidases (O-glycoside hydrolases, EC 3.2.1.x) which catalyze the endohydrolysis of 1,4-β-D-xylosidic linkages in xylan. They are a widespread group of enzymes, and are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and anthropods (Prade, 1995]. First reported in 1955 (Whistler and Masek, 1955), they were originally termed pentosanases, and were recognized by the International Union of Biochemistry and Molecular Biology (IUBMB) in 1961 when they were assigned the enzyme code EC 3.2.1.8. Their official name is endo-1,4-β-xylanase, but commonly used synonymous terms include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4- β -D-xylanase, β-1,4-xylanase and β-xylanase. The main enzymes involved in the fractionation of xylan polysaccharides are:

1. Endo-β-(1, 4)-D-Xylanase (EC 3.2.1.8): These enzymes act randomly on xylan to produce large amounts of xylo-oligosaccharides of various chain lengths.
2. Exo-β-(1, 4)-D-Xylanase: These enzymes remove the single xylose or xylobiose units from the non-reducing end of the xylan chain.
3. β-Xylosidase or Xylobiase. (EC 3.2.1.37*)*: These enzymes hydrolyse disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.
4. Other enzymes such as α-arabinofuranosidase (EC 3.2.1.55), acetylxylan esterase (EC 3.2.1.72), α-glucuronidase (EC3.2.1.139) and feruloyl esterase (EC 3.2.1.73) remove side groups in heteroxylans.

Most of the fungi and bacteria are known to express functionally diverse multiple forms of xylanases. This multiplicity could be a result of post-translational modifications (glycosylation, auto-aggregation or/and proteolytic digestion), genetic redundancy or differential mRNA processing. The structure of xylan with action of different xylanolytic enzymes is given in Fig 1.2.

Fig.1.2 A Representation of Xylan with side chains



Strain improvement

Cellulases are complex enzymes consisting of several hydrolytic enzymes and no single enzyme source is capable of hydrolysis of cellulose. These enzymes offer a good starting point for the improvement of cellulase which helps in economics of their production. The economic viability of biomass conversion depends on the enzyme cost, which triggers the search for high cellulase producing organisms. Hypercellulolytic strains can be developed using classical mutagenesis, genetic engineering, enzyme engineering using advanced biotechnological techniques like directed evolution and rational design studies. The improved enzyme preparations are expected to have desirable properties such as higher catalytic efficiencies, increased stabilities at higher temperatures and higher tolerance to end product inhibitions (Zhang et al., 2006).

Strain improvement by mutation using physical and chemical mutagens is a traditional method used with great success for isolating mutants producing enhanced levels of cellulases. Initial work focused on random mutagenesis of the wild strains to develop hyper-producing mutants of *T. reesei* namely QM 9414 and Rut C-30. Many studies have employed 2-deoxyglucose and obtained several cellulase hyper-producers which were found to be glucose derepressed mutants (Dillon et al., 2008). *A. terreus* was subjected to UV and NTG treatments which resulted in isolation of mutants

having 3.5, 4.6 and 3.3 fold increase in filter paper, β-glucosidase and CMCase activity compared to wild type parental strain (Araujo et al., 2006). The strain of *P. janthinellum* NCIM 1171 was subjected to mutation involving treatment of EMS followed by UV-irradiation (Adsul et al., 2007). The mutants showed enhanced cellulase production, clearance zone on Avicel containing media and rapid growth on Walseth cellulose agar plates containing 0.2% 2-deoxy glucose. All mutants showed approximately two-fold increase in activity of both filter paper and CMCase. *T. reesei* C-30 was subjected to NTG treatment followed by UV-irradiation which resulted in isolation of mutants capable of producing two fold increase in FPase as well as CMCase activities (Jun et al., 2009). A mutant of *Acremonium cellulolyticum* designated as CF-2612 was isolated by UV and NTG treatments which produced very high titres of FPase (17.8 IU/ml) with improved β-glucosidase activity (Fang et al., 2009). EI-Bondkly et al., (2010) have constructed β-glucosidase hyper producers of *Trichoderma harzianum* by using mutagenesis. The UV irradiation and ethylmethane sulphonate treatment followed by exposure to colchicines treatment has resulted in strain improvement with 186% increase in the production of β-glucosidase. Mutations leading to insensitization of repression by easily metabolizable carbon or glucose resulted in higher production of β-glucosidase and other cellulases even in presence of glucose (Kotchoni and Shonukan, 2002). Bokhari and coworkers (2008) isolated 2 deoxyglucose-resistant-mutants (M7) of *Humicola lanuginosa* by exposure of conidia to γ-rays and obtained high yield (10 fold) of β-glucosidases. Protoplast fusion has also been used to improve the strains for enzyme production. *T. reesei* produces more amount of endoglucanase and exoglucanases while *A. niger* produces more amount β-glucosidase. The protoplast fusion of these two strains was shown to be helpful in the production of high yield of complete set of cellulolytic enzymes (Ahmed and Berkly, 2006). Protoplast fusion between *P. echinulatum* and *T. harzianum* resulted in the strain with high β-glucosidase and FPA activities (Dillon et al., 2008).

Cloning the genes encoding the enzymes and heterologously expressing them in commonly used industrial strains has become a common practice. Such heterologous expression has become a powerful tool to improve yields and titers of enzymes. Novel genes encoding enzymes active in termite larvae gut and in the cow rumen can be

inserted in filamentous fungi after insertion in to suitable vectors (Nevalainen et al., 2003). Very recently, endo-β-glucanase II gene (egl2) from *T. ressei* was cloned and inserted into a silkworm (*Bombyx mori*) nucleopolyhedrovirus (BmNPV) genome using BmNPV/Bac-toBac expression vector (Zhou et al., 2010). Many fungal cellulases were cloned and expressed to develop robust fungal strains producing enhanced levels of cellulases. The β-glucosidase from *Talaromyces emersonii* was expressed in *T. reesei* RUT-C30 using strong cbh1 promoter that resulted in expression of highly thermostable β-glucosidase with high specific activity (Murray et al., 2004). Protein engineering of biomass degrading enzymes, including mutagenesis of potential active site center residues, has been employed as a tool for elucidating the catalytic mechanisms and also improving the enzyme properties (Schulein, 2000). Zhang et al., (2010) have improved *T. reesei* strain by over-expression of β-glucosidase gene under the control of cellobiohydrolase 1 promoter. The resultant recombinants produced high levels of β-glucosidase and filter paper activities. Similarly, cellobiohydrolase I & II were over-expressed using additional copies of the genes cloned under cbh1 promoter. This resulted in 1.5 fold increase in cellobiohydrolase I activity and 4 fold increase in cellobiohydrolase II expression (Miettinen-Oinonen et al., 2005). In addition, chimeric proteins have also been developed, for example, the endoglucanase from *Acidothermus cellulolyticus* was fused with *T. reesei* cellobiohydrolase and expressed in *T. reesei*. This bifunctional cellulase (endo-and exo-acting) has been demonstrated to improve the saccharification yields (Bower et al., 2005). *Penicillium echinulatum* is effective for bioconversion processes. However, nothing is known about the molecular biology of its cellulolytic system. Rubini et al., (2010) described, for the first time, the isolation, cloning and expression of a *P. echinulatum* cellulase cDNA (Pe-egl1) encoding a putative endoglucanase. Development of effective technologies based on biomass feedstock is challenging. Degradation of cellulosic substrates requires enzymes which hydrolyze completely these substrates to their respective monomers. This is possible only when new strains with high cellulase activity profiles will be developed using system biology, recombinant DNA technology, synthetic biology and metabolic engineering approaches. These strains/enzymes must be robust enough to tolerate extreme

conditions employed during cellulose hydrolysis which may reduce the further down-streaming cost. There are 99% of the microbes that are uncultivable and hence remain untapped for their potential applications (Singh, 2010). These untapped sources can be exploited for isolating efficient cellulase producers with desirable properties. This can be possible using metagenomic approach which is considered to be the most viable method to search for desirable enzymes such as cellulases.

Protoplast fusion is an important approach and has been widely used in the fungal genetic modification since 1976, and it could induce DNA recombination between the two strains. Genome shuffling is established on the basis of protoplast fusion, but it is actually the recursive fusion of multiple parents with the combination of suitable screening method. Genome shuffling offers great potential for the improvement of industrially important micro-organisms through protoplast fusion. Genome shuffling is a process that could efficiently combine the advantage of multiparental crossing with the recombination of entire genomes normally with conventional breeding or through recursive protoplast fusion that greatly increases recombination, compared to the general protoplast fusion. Additionally, genome shuffling can accelerate directed evolution by facilitating recombination between members of a diversely selected population. Since this technique was first successfully used in bacterial system (Zhang et al., 2002) especially to improve acid tolerance in *Lactobacillus* sp. (Patnaik et al., 2002). It has been widely applied in improving some important phenotypes of micro-organisms, such as lipase production in *Penicillium expansum,* improvement of tylosin production in *Streptomyces fradiae*, acceleration of screening and breeding of high taxol-producing *Nodulisporium sylviform,* pentachlorophenol degradation in *Sphingobium chlorophenolicum* etc. Genome shuffling accelerated combination of the advantages distributed in multiparents. Thus, it is more efficient and saves lots of energy and time consumption. Genome shuffling provides a new tool for cell and metabolic engineering and requires no sequence information or sophisticated genetic tools.

The conversion of cellulosic materials to ethanol by intergeneric fusants between *T. reesei* and *S. cerevisiae* would be the appropriate approach. Such intergeneric protoplast fusion was performed between *T. reesei* and *A. niger* resulting

in fusants producing high levels of endoglucanases, exoglucanases and β-glucosidases (Ahmed and Burkley, 2006). Protoplast fusion between *P. echinulatum* and *T harzianum* led to isolation of fusants producing higher FPase and β-glucosidase activities compared to parental strains. These fusants were morphologically similar to *P. echinulatum* (Dillon et al., 2008). Genome shuffling improves production of cellulase by *Penicillium decumbens* JU-A10. After two rounds of genome shuffling, three fusants, GS2-15, GS2-21 and GS2-22, were obtained, showing 100%, 109% and 94% increase in FPase activity than JU-A10 respectively. The cellulase production of the fusants on various substrates, such as corn stover, wheat straw, bagasse and the corncob residue, was studied. The maximum productivities of GS2-15, GS2-21 and GS2-22 were 92·15, 102·63 and 92·35 FPU l^{-1} h^{-1} on the corncob residue at 44 h respectively, which were 117%, 142% and 118% higher than that of JU-A10 (42·44 FPU l^{-1} h^{-1}, at 90 h). The improvements of cellulase production in the fusants could be due to their enhanced growth rates, earlier cellulase synthesis and higher secretion of extracellular proteins (Cheng et al., 2009).

Strain improvement by random mutagenesis has been used so as to increase the production of β-xylosidase (Rodriguez and Alea, 1992; Bajpai, 1997). Basaran and coworkers (2001) have isolated a high growth rate mutant obtained after NTG treatment. This mutant assimilates xylose faster than the wild type and is able to use a larger portion of xylan sources than the wild strain (25 to 29 mU/ml/h) in three days old culture supernatant. Bokhari and coworkers (2010) used random mutagenesis in *Humicola lanuginosa* with two exposures to γ-rays. They isolated a mutant M7D which produced high β-xylosidase and was able to produce enzymes in presence of 50 mM glucose.

Ungchaitham and coworkers (2001) isolated a *Strptomyces spp.* which was capable of producing β-xylosidase (0.9 U/mg of protein) in 1% xylan at pH 7.0 at 40^0C for 24h. Its gene has been cloned in *E. coli* with pUC18 as a cloning vector. A recombinant plasmid containing 3.6 kb insert was found to express β-xylosidase activity. Sub cloning of the insert into pUC19 indicated the cloned gene with its own promoter which was able to function in *E. coli*. In another study, a recombinant plasmid carrying β-xylosidase gene from the extremely thermophilic and aerobic

bacterium *Caldocellum saccharolyticum* was transformed into *E. coli* strain and expressed. Xue and coworkers (2003) cloned and expressed an arabinofuranosidase / xylosidase gene of *Thermoanaerobacter ethanolicus* in *E. coli*. To develop new enzymes to decompose agricultural by-products, multifunctional fusion proteins can be constructed. Xue and coworkers (2009) have fussed *T. ethanolicus* XAR and *T. lanuginosus* XYN to obtain trifunctional XAR – XYN protein that exhibited β-xylosidase, α-arabinosidase and xylanase activity when XYN was fussed down stream of XAR (Stop codon removed).

β-Glucosidase

Occurrence

β-Glucosidase is widely distributed in nature and can be found in animals (Mc Mahon et al., 1997), plants (Heyworth et al., 1962), bacteria (Hans and Srinivasan, 1969), fungi (Deshpande et al., 1978) and yeast (Fleming et al., 1967) that indicate its general importance to life. β-Glucosidases have a variety of functions in different organisms such as biomass conversions in microorganisms (Fowler, 1993), activation of defense compounds (Poulton, 1990;), production of phytohormones (Brzobohaty et al., 1993), aromatic volatiles (Mizutani et al., 2002) & metabolites (Barleben et al., 2005). β-Glucosidases are predominantly produced by microorganisms such as molds, fungi and bacteria (Bayer et al., 1998). β-glucosidases from fungi and bacteria have been studied extensively (Saha et al., 2002). Due to higher extracellular yields, the most important sources for industrial production of β-glucosidases are *Pyromyces, Thermoascus, Termitomyces, Talaromyces, Thermomyces* (Eyzaguirre et al., 2005). The hypersecretory fungi are *Trichoderma reesei* (Kubicek, 1992), *Penicillium funiculosum* (Lachke et al., 1983), *Penicillium purpurogenum* (Steiner et al., 1994), *Fusarium oxysporum* (Kumar et al., 1991; Christakopoulos et al., 1995), *A. niger* (Hurst et al., 1977), *Sclerotium rolfsii* (Lachke and Deshpande, 1988), *Humicola spp* (Hayashida et al., 1988).

Cellulases from thermophillic fungi are important because of their thermostability and wide applications. These genera include *Chaetomium thermophilum, H. insolens, H. grisea, Myceliopthora thermophila, Taleromyces*

emersonni and *Thermoascus aurontiacus* (Maheshwari et al., 2000). Only two aerobic thermophilic bacteria have been described that produce cellulases; *Rhodothermus* and *Acidothermus cellulolyticus* (Halldorsdottir et al., 1998). Extremophilic cellulases could be obtained either by isolating extremophilic microorganisms or by protein engineering (Antony et al., 2003). The β-glucosidase purified from hyperthermophilic archea of *Pyrococcus furiosus* was active optimally at 105^0C with half life of 85h at 100^0C and 13h at 110^0C (Kengen at al., 1993).

Localization

The endocellulases and exocellulases produced by fungi constitute the cellulase complex that is produced extracellularly. In addition to extracellular β-glucosidases, intracellular (Eberhart and Beck, 1973; Smith and Gold, 1979) and cell or mycelium bound (Deshpande at el., 1978; Smith and Gold, 1979) β-Glucosidases have been found in microorganisms. Intracellular β-glucosidases have been implicated in the germination of basidospores of *Schizophyllum commuse* (Wilson and Niederpruem, 1967) and together with the extracellular enzymes and possibly the mycelium bound enzyme, appear to be involved in cellulose degradation.

The cell wall bound β-glucosidases are also extracellular since they exist external to the plasma membrane, in the periplasmic space. These enzymes are induced by cellobiose and cellulose (Berg and Petterson, 1977) and repressed by glucose. A species of *Monilia* produced extracellular, intracellular and mycelium bound β-glucosidases when grown on cellulose (Dekker, 1981). Several forms of β-glucosidases have been found in *T. reesei*, either in culture supernatants or bound to the cell wall or membrane. *T. reesei* releases low amounts of β-glucosidase to the culture medium (Sternberg et al., 1977). This has been explained by the observation that a large part of the enzymes remains bound to the cell wall during fungal growth (Rogalski et al., 1991). Kubicek (1992) has postulated that membrane bound β-glucosidases participate in the formation of sophorose. It has already been mentioned that β-glucosidases from *T. reesei* may be involved in cell wall metabolism during conidiogenesis and therefore they are a true component of cellulolytic enzyme system. Inglin & coworkers (1980) have isolated an intracellular β-glucosidases which could

have two possible functions: 1) in metabolic control of cellulase induction and 2) as a proenzyme before transport through the cell membrane to the external environment.

Isoenzyme forms

Many fungal strains have been found to produce multiple forms of β-glucosidases. The origin of multiplicity is yet not clear even though some explanations have been postulated. Multiplicity in cellulases including β-glucosidases can be attributed to the events transcriptional / translational level or may be due to posttranslational events such as glycosylation or proteolysis (Badhan et al., 2007). β-Glucosidase heterogeneity *in S. pulverulentum* has been attributed to proteolysis (Deshpande et al., 1978). Mutiple β-glucosidases of *Talaromyces emersonii* were found to be the result of posttranslational modification such as glycosylation (McHale et al., 1982). In *Schizophyllum commune*, two β-glucosidases with molecular mass of 95,700 and 93,800 Da were originated from single gene but the multiplicity observed could be due to heterogeneity in transcription and glycosylation (Willick and Seligy 1985). However, the different isoenzyme forms of β-glucosidases could be the products of separate genes. The β-glucosidases from *A. fumigatus* were identified based on zymogram staining using methylumbelliferyl β-D-glucoside for developing a proteome strategy to discover novel β-glucosidases from this fungus (Kim et al., 2007). The multiplicity of β-glucosidases increased with complex substrates used for growth which indicated that the heterogeneity is substrate dependent. Three β-glucosidases were purified from *A. terreus* with different molecular weights. The expression of different β-glucosidases was found to be substrate dependent (Nazir et al., 2009). *P. janthinellum* mutant EU2D-21 produced two β-glucosidases under submerged fermentation conditions. One of the β-glucosidase (BGL1) is more thermostable than other one (BGL2). Cellulase preparation derived from solid state fermentation contained predominantly less thermostable species of β-glucosidase (Singhvi et al., 2011).

The isoenzymes may have different locations in the cell and the locations change depending on the age and nutritional conditions (Deshpande et al., 1978). Thus the localization of β-glucosidase activity depends on the carbon source used for

growth of *S. pulverulentum* (Deshpande et al., 1978). The cellobiose produced only cell wall bound β-glucosidase while cellulose could be necessary for extracellular enzyme production. In *T. reesei* QM 9414, isoenzymes appeared very early in the cultivation suggesting that they are inherent properties of the fungus (Labudova and Farkas, 1983). The fungus *Botryodiplodia theobromae* produced high molecular weight β-glucosidase (350 to 380 kDa) at early stage of cultivation while smaller forms (45 to 47 kDa) were found to be predominant in older culture filtrates (Umezurike, 1979).

Induction

A synergistic action of cellulolytic enzymes is required for cellulose degradation and expression of these enzymes is coordinately regulated. The same compound may provoke expression of both cellulases and hemicellulases albeit to different extents (Kubicek et al., 1993). Mandels et al., (1962) suggested that *T. reesei* produces constitutive level of cellulases that are bound onto the surface of conidia (Kubicek et al., 1988). The low level of cellulases is formed constitutively to yield soluble hydrolyzed products of cellulose which can be transported into the cell and functions as inducers (Badhan et al., 2007). Inducible cellulolytic activity has been identified in many organisms and their synthesis is repressed by the presence of glucose and other readily metabolizable sugars in the medium (Ilmen et al., 1997). The role of glucose, cellobiose, sophorose and other soluble sugars as inducers or repressors vary with microorganisms (Setala and Penttiala, 1995). Production of cellulases by *T. reesei* is regulated at the transcriptional level and the expression of cellulase genes in *T. reesei* QM 9414 is coordinated through transcriptional factors (Ilmen et al., 1997). In *T. fusca*, expression of celluase genes is regulated at two levels, induction by cellobiose and catabolite repression in the presence of glucose (Wilson, 2004). Kaur et al., (2006) have reported fructose and ethanol as best inducers for the production of celluloytic enzymes in *Melanocarpus spp*. and *Scytalidium thermophilum* respectively. A high level of β-glucosidase was induced by cellobiose in *Humicola grisea* (Polizeli et al., 1996) and *Kluyveromyces marxianus* (Rajoka et al., 2004).

β-Glucosidase can catalyze transglycosylation to produce positional isomers which can act as an inducer for cellulases in *Hypocrea jecorina* and *Penicillium purpurogerum* (Suto and Tomita, 2001). Cellulose was found to act as an inducer in *Cytophaga hutchinosonii* while glucose and cellobiase repressed its formation (Louime et al., 2006). In case of *Hypocrea jecorina,* β-glucosidase was found to get induced by sophorose (Fowler and Brown, 1992, IImen et al., 1997). Recently Nazir et al., (2009) have reported fructose and cellobiose as good inducer of β-glucosidase activity in *A. terreus* as compared to glucose and polysaccharide like Avicel, solka floc, CMC, and lignocellulosic substrates except corn cobs (8.85 U/ml). Ahmed and coworkers (2009) have reported production of β-glucosidase from *T. harzianum* grown on different carbon sources such as glucose, CMC, corn cobs, birch wood xylan and wheat bran under submerged fermentation. Maximum β-glucosidase was produced on CMC (0.92 IU/ml) while there was no induction of β-glucosidase on glucose (0.05IU/ml). In case of fungus *Daldinia eschscholzii*, CMC, Avicel PH-101 and filter paper were found to be the most effective inducers of β-glucosidase activity while microcrystalline cellulose (Avicel PH-101) and filter paper were also fairly good inducers (Karnchanatat, 2007). The study of induction of β-glucosidase and other cellulases by *A. niger*, in Czapek-Dox medium supplemented with different carbon sources, such as glucose, cellulose, cellobiose and CMC was reported by Narasimha and coworkers. Maximum production was obtained with CMC (1.12 IU/ml). Glucose in the medium has supported maximum growth but resulted in minimal production of β-glucosidase (0.46 IU/ml) and less secretion of extracellular proteins. Similar results have been reported by Gautam and coworkers (2010). They found that CMC produced maximum β-glucosidase (1.03IU/ml) as compared to that on cellulose while glucose resulted in less production of β-glucosidase. In the study of biosynthesis of cellulolytic enzymes by *Tricothecium roseum*, Shanmugam and coworkers (2008) have demonstrated that maximum β-glucosidase production (1.82 IU/ml) was achieved when citric acid was added as an inducer to the potato dextrose yeast extract medium containing CMC.

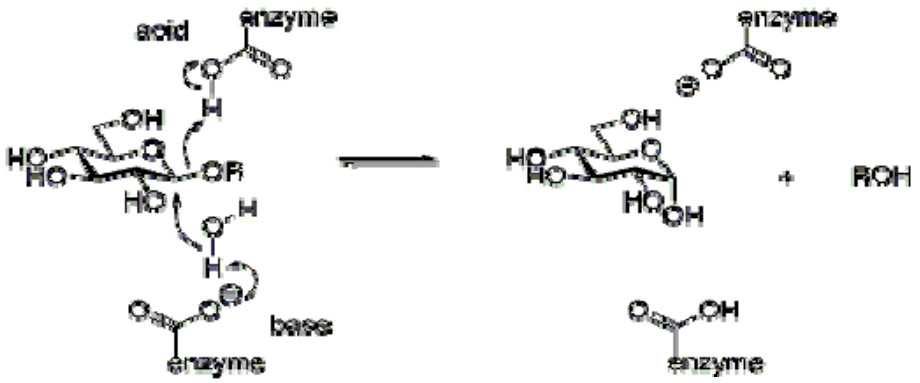
Repression

Inducible cellulolytic activity has been observed in many microorganisms and the expression of these enzymes is regulated by repression in presence of glucose or other readily assimilable sugars in the medium (IImen et al., 1997). It was regulated by Cre A protein that functions as a transcriptional repressor of glucose repressible genes. It has been found that the mRNAs corresponding to cellulase genes were found to be regulated at the level of transcription by a mechanism analogous to catabolite repression (Mishra et al., 1991). These numbers of mRNA transcripts per cell were found to be decreased with increase in growth rate. The number of mRNA transcripts was found to be higher for growth under cellobiose limitation than for growth under nitrogen limitation and this control involved housekeeping Sigma A factor (Dror et al., 2003 a and b). Based on the inverse correlation observed between the growth rate and the synthesis of key cellulosome gene transcripts, as well as the apparent absence of key components of catabolite repression system (catabolite responsive elements (CRE) sequences), Dror and coworkers (2003 b) have inferred that the growth rate plays a role in regulation of the cellulosome related genes that have been studied but, known mechanisms of catabolite repression do not play a role. Stricker and coworkers (2008) have reviewed the regulatory mechanism of cellulase and hemicellulase expression in *A. niger* and *H. jecorina* and highlighted major similarities and differences in regulation mechanisms. The transcriptional factor xylanase regulator Xln R in *A. niger* have been identified as responsible for transcriptional activation for cellulolytic and xylanolytic enzymes. In recent review, Kubicek et al., (2009) has also discussed the role of GPCR in the induction and regulation of cellulases.

Catalytic mechanism

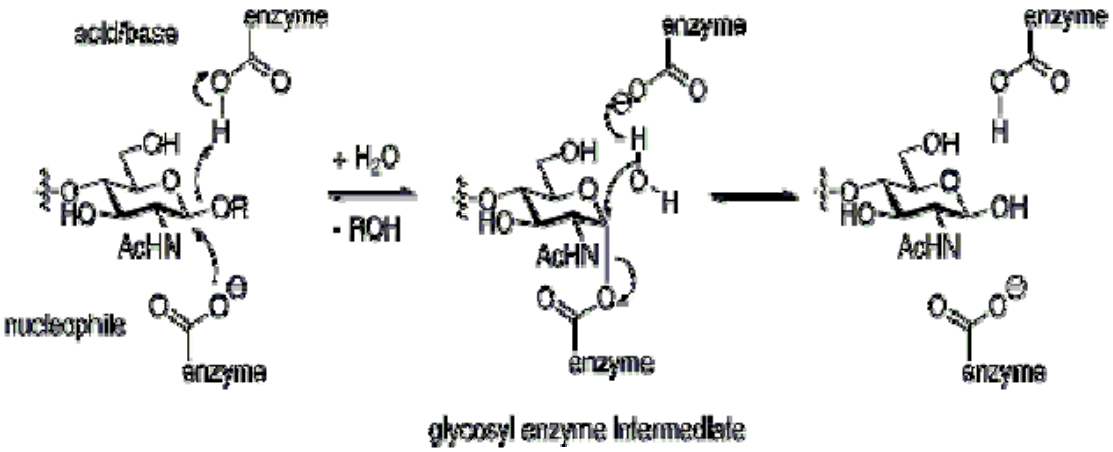
β-glucosidase (all glycosidases) hydrolyze the glycosidic bonds by one of the two mechanisms giving rise to either an overall retention or an overall inversion of the configuration of the anomeric substrate carbon (Sinnot et al., 1990). Thus glycosidases can be divided into two classes viz. inverting and retaining glycosidases based on the anomeric configuration of the released monosaccharide or oligosaccharide in

respective glycoside as compared to substrate (McCartar and Withers, 1994). In both the mechanisms, two carboxylic acids that are conserved within each glycoside hydrolase family are required for hydrolysis which proceeds through the formation of oxocarbonium ion-like transition state. Inverting glycosidases use a single displacement mechanism in which a water molecule directly displaces the aglycon through the involvement of carboxylate residues. These enzymes utilize two carboxylate residues that can act as acid and base respectively as shown below for β-glucosidase.



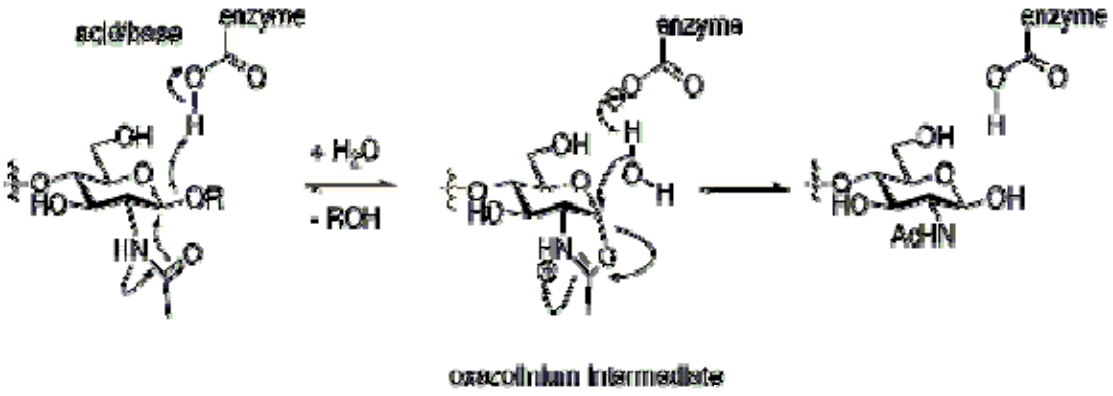
Mechanism used by inverting glucosidases

Retaining glucosidases operate through a two step double displacement mechanism, with each step resulting in inversion leading to net retention of stereochemistry. Again two carboxylate residues are required which are usually enzyme born. One acts as a nucleophile and the other as an acid / base. In the first step of the reaction (glycosylation), the nucleophile attacks the anomeric center resulting in the formation of glycosyl-enzyme intermediate. In the second step (deglycosylation), the deprotonated acidic carboxylate acts as a base and assists a nucleophilic water to hydrolyze the glucosyl-enzyme intermediate releasing the hydrolyzed product. The mechanism is illustrated below.



Retention mechanism with formation of glycosyl-enzyme intermediate

An alternative retention mechanism can occur which proceeds through a nucleophilic residue that is bound to the substrate, rather than being attached to the enzyme. Such mechanisms are common for certain N-acetylhexosaminidases possessing acetamido group allowing the participation of neighboring group to oxazoline or oxazolinium ion. This mechanism also involves two steps with individual inversions leading to net retention of configuration. This alternative mechanism id illustrated below.



Retention mechanism with formation of oxazolinium intermediate

Classification

Glycoside hydrolases have been classified into EC 3.2.1 as enzymes hydrolyzing O- or S-glycosides. These glycosides hydrolases are classified based on the stereochemical outcome of the hydrolysis reaction. They are classified as inverting or retaining enzymes. They are also classified as exo or endo acting enzymes depending upon the action on the end (usually non-reducing) or in the middle of the polysaccharide chain.

Sequence based classification is the most powerful predictive method for suggesting the function for the newly sequenced enzymes. Glycoside hydrolase enzymes can be classified by sequence or structure based methods. Classification system based on sequence similarity led to the definition of more than 100 families (Henrissat et al., 1995; Henrissat and Davies 2000). This classification is available at http://afmb.cnrs-mrs.fr/CAZY. List of the families and the enzymes included in these families can be accessed using this web site. This database provides a series of regularly updated sequence based classification which allows reliable predictions of the mechanism. β-Glucosidases are found in families 1, 3 and 9 and the enzymes from filamentous fungi are present in families 1 and 3.

The β-glucosidases from sp., *A. niger, H. grisea, Orpinomyces spp., Pyromyces T. emersonii, T. reesei* and *T. viridae* are included in family 1. The conserved motif NEP (residue 430 to 433) includes the glutamate residue functioning as acid–base catalyst (Keresztessy et al., 1994), while I (Y, X) (V, I) TENG motif (Residue 893 to 899) contains the glutamate which acts as nucleophile (Withers et al., 1990). The structures of several bacterial and plant enzymes have been published (Chi et al., 1999, Hakulincn et al., 2000) which is a $(\alpha/\beta)_8$ barrel found in all cases. However, no three dimensional structures of family 1 fungal β-glucosidase have been described so far.

The family 3 fungal β-glucosidases include sequences from *A. aculeatus, A. kawachii, Agaricus bisporus, Botryotinia fuckelania*, *Coccidioides posadasii* (Bgl 1 and Bgl 2), *Phaeosphaeria avenaria*, *Phanerochaete chrysosporium* K3 and OGC 101*, Pyromyces spp*., *Septoria lycopersici, T. emersonii*, *T. reesei* (Cel 3 b and Cel 3 A/ Bgl1) and *Volvariella volvacea*. The alignment of enzymes belonging to Family 3

has been divided into two groups which show high internal sequence similarity. The first group includes β-glucosidases from basidiomycetes such as *A. bisporus* and *V. volvacea,* which show sequence similarity to that of the enzyme from slime mold *Dictyostelium discoidenum.*

Methods for assay of β-glucosidase

There are several methods that are sensitive and easy to use, for determination of β-glucosidase activity. The most common methods are those which use alkyl or aryl glucosides as substrates. These synthetic substrates, upon hydrolysis, release colored or fluorescent products that can be measured spectrophotometrically. The most commonly used substrate is *p*-nitrophenyl- β-D-glucoside (*p*NPG), which releases *p*-nitrophenol. *p*NPG may be replaced by the ortho isomer, but several fungal enzymes such as β-Glucosidases from *Trichoderma koningii* and *T. reesei* hydrolyse this isomer slowly (Chen et al, 1992). Other substrates used are methyl-β-D-glucopyranoside, the natural glycosidases such as salicin, esculin, amygdalin and the cellobiose (Wood and Bhat, 1988). Activity towards cellobiose is measured by determining free glucose by glucose oxidase – peroxidase method (Day and Workman, 1982) or by coupled hexokinase / glucose 6 –phosphate dehydrogenase assay (Bergmeyer et al., 1974). For cellulose degradation, cellobiose should be the substrate of choice for determining cellobiose degrading ability of the enzyme.

β-Glucosidase products can be analyzed by high performance liquid chromatography (HPLC). The techniques such as pulse amperometric detection, thin layer chromatography (TLC), high performance thin layer chromatography (HPTLC) have been used. These techniques also allow detection of transglycosylation products. The β-glucosidase can also be detected by zymogram techniques using substrates 4-methyl umbeliferyl -β-D- glucoside or 6-bromo-2-naphthyl- β-D-glucoside and 5-bromo-4-chloro-3-indolyl- β-D-glucopyranoside (Eyzaguirre et al., 2005). These zymogram techniques are useful in detecting multiple β-glucosidases which may have different electrophoretic mobility or isoelectric points.

Production of β-glucosidase

Carbon source used in cultivation is one of the important factor affecting the cost and yield of cellulase production. Agro industrial residues are generally considered for solid state fermentation (SSF). Juhasz and coworkers (2005) studied use of steam pretreated spruce, willow, corn stover and delignified lignocelluloses (Solka floc) for production of enzymes by *T. reesei*. They demonstrated that steam pretreated corn stover is good substrate for enzyme production as well as hydrolysis. Lignocellulose from agriculture, food processing (Bisaria and Ghosh, 1981), sugarcane bagasse, wheat bran, rice straw, wheat straw, rice husk, saw dust, corn cobs, sweet sorghum pulp etc. have been used for production of different enzymes (Chandel et al., 2007). Agroindustrial waste produce such as orange bagasse, sugarcane, corn cob, and oat husk, wheat straw are effective substrates for enzymes production by SSF.

The effect of carbon sources on production of β-glucosidase by *Aureobasidium pullulans* under submerged fermentation was reported by Saha et al., (1994). The highest level (230mU/ml) of total β-glucosidase activity was produced in corn bran medium rather than cellobiose. Lactose was also a good carbon source for production of β-glucosidase activity. Ahmed and coworkers (2003) studied the induction of xylanase and cellulase in *T. harzianum* in presence of 1% glucose, 1% CMC and 1% Xyaln and they have reported the maximum β-glucosidase production in 1% xylan (0.629 IU/ml) as compared to that in 1% CMC (0.062 IU/ml). Bokhari and coworkers (2008) used *Humicola lanuginosa* in submerged fermentation for production of β-glucosidase and found that enzyme production was apparently growth associated and lignocellulosic substrates, xylan and xylose induced the cells to produce β-glucosidase activity and the best performance was found with corn cobs (20 g/L) at 45^0C in 64 h (17.93 U/ml). Production of high activities of thermostable β-glucosidase and endoglucanase by *Thermoascus aurantiacus* was reported using solka floc as carbon source (Gomes et al., 2000). The combination of two different carbon sources can also give better production of enzymes. Dried kinnow pulp and wheat bran in the ratio of 4:1 resulted in high yield of FPase activity in *T. reesei* C-30 whereas, those

substrate in 3:2 proportion yield maximum CMCase and β-glucosidase activity (Oberoi et al., 2008)

Organic or inorganic nitrogen sources exert significant effect on the production of cellulolytic enzymes. Bhatia and coworkers (2002) reviewed that inorganic nitrogen sources resulted in equal or higher β-glucosidase production than organic nitrogen sources. Bokhari and coworkers (2008) have studied the effect of various nitrogen sources such as ammonium nitrate, ammonium sulphate, diammonium phosphate, urea and corn steep liquor to medium containing corn cobs (2% w/v) on production of β-glucosidase by *Humicola lanuginosa*. Corn steep liquor favored maximum β-glucosidase production followed by urea. Use of corn steep liquor alone as a carbon and nitrogen sources resulted in less production of β-glucosidase. Ammonium sulphate has been shown to facilitate β-glucosidase and other cellulases production in *A. niger, A. terreus* and *S. thermophilum* (Chahal et al., 1996, Fadal, 2000, Kaur et al., 2006c). Activities of β-glucosidase and CMCase in culture filtrate of *A. niger* were increased in presence of urea (Narasimha et al., 2006). Ammonium sulphate was reported to give maximum yield of all the three cellulolytic enzymes. Among the organic nitrogen sources addition of yeast extract resulted in increased production of cellulolytic enzymes (Ganguly and Mukharjee 1995). Ammonium sulphate and wheat bran were reported for optimum enzyme production by *Trichoderma koningii* (PeiJun et al., 2004). Shanmugam and coworkers (2008) have reported effectiveness of nitrogen sources in production of β-glucosidase and other cellulases by *Tricothecium roseum*. They found peptone to be more effective as compared to yeast extract and $NaNO_3$. Ellouz et al., (1995) demonstrated that crude complex substrates used in combination with cellulose give high cellulase yields. In contrast, the growth of *T. reesei* in production medium without nitrogen source increased cellulase production (Turker and Mavituna, 1987).

The environmental factors such as temperature, pH, aeration, growth period and additives are the most important parameters in production of cellulolytic enzymes. Fadal (2000) have shown that biosynthesis of β-glucosidase and endoglucanases by *A. niger* F119 was inhibited at low or high temperature (24^0C and 40^0C). The optimum temperature for production of all the three cellulolytic enzymes was reported to be

32^0C. Asquicri and Park (1992) have reported 37^0C as an optimum temperature for production of β-glucosidase and CMCase from thermostable *Aspergillus spp*. Maximum cellulases production were observed when SSF was performed at 35^0C and 45^0C using *Penicillium chrysogenum* (Sharma et al., 1996). Bokhari and coworkers (2008) have reported 45^0C as the optimum temperature for maximum production of β-glucosidase using *Humicola lanuginosa* under submerged conditions. The temperature requirement varies according to organisms. Maximum production of cellulolytic enzymes by *T. aureoviridae* was 28^0C (Zaldivar et al., 2001), whereas PeiJun et al., (2004) found that the ramping of temperature from 32^0C to 27^0C after 30 h of incubation enhanced production of cellulases by *T. koningii*. In case of thermophilic fungi, the optimal enzyme production temperatures are slightly lower than optimal growth temperature (Maheshwari et al., 2000; Soni et al., 2008).

The hydrogen ion concentration has a marked effect on enzyme production. It may be due to the stability of extracellular enzyme at particular pH and the rapid denaturation at lower or higher pH values (Kalra and Sandhu, 1986). The β-glucosidase and cellulase synthesis was inhibited at low pH below 4.0 and at high pH above 5.5 in case of *A. niger* under SSF (Fadal, 2000). There are many reports on the requirements of pH of culture medium for extracellular enzyme production by fungi and bacteria and in most cases the maximum production was observed between pH 4.5 and 5.5 (Coughlan, 1985).

Purification of β-glucosidases

Fungal β-glucosidases are found in culture supernatants, bound to the cell wall and cell membrane and in the cytoplasm. Most commonly, these enzymes are extracellular and secreted to the medium. General purification steps involve removal of cells or mycelium from the fermented broth, concentration, ammonium sulphate or organic solvent precipitation followed by a combination of several chromatographic methods. In case of intracellular enzymes, an additional step of cell lysis is required to extract the enzyme. The chromatographic methods include ion exchange, affinity and size exclusion chromatography. However, the specific procedure and its efficiency differ from case to case. Ammonium sulphate or organic solvent precipitated enzyme

preparations give high average yields albeit with limited purification (Aires-Barros et al., 1994). Such precipitated enzyme preparations are suitable for use in commercial formulations. Purification procedures are under continuous development and several steps are usually necessary based on different separation principles.

To obtain enzymes in their native form without modification, the number of purification steps should be minimum to avoid proteolytic cleavage. To facilitate the purification, it is advantageous to use culture broths obtained under specific culture conditions where the amount of impurities is minimized. Some investigators have used adsorption and desorption from hydroxylapetite for purification of β-glucosidase from *Streptomyces* sp. (Kusama et al., 1986). *T. reesei* β-glucosidase was purified using controlled porosity glass activated by aminopropyltriethoxysilane and oxiranes and linked to salicin and cellobiose (Rogalski et al., 1991). Isoelectrofocusing (Hidalgo et al., 1992) and chromatofocusing are the two methods that allowed the isolation of β-glucosidase isozymes with different pI values, which cannot be easily separated by conventional techniques. The methods employed for purification of β-glucosidase are given in Table 1.1.

Characterization of β-glucosidases

Native fungal β-glucosidases show molecular weight in the broad range from 40 to 640 kDa. SDS gel electrophoresis gives single polypeptide chains from 35 kDa to 250 kDa. Quaternary structures from monomers to tetramers were also reported. The molecular weights, isoelectric points, carbohydrate content and Km for various purified β-glucosidases are given in Table 1.2.

Table 1.1. Purification strategies employed for filamentous fungal β-glucosidase.

Organism	Purification step	Total protein (mg)	Total activity (IU)	Specific activity (U/mg)	Yield (%)	Purification (fold)	Ref
A. niger	Ammonium sulphate precipitation, 1st CM sepharose 2nd CM Sepharose Q- Sepharose Sephacryl S-300	0.41	150	366	22	2.6	Yan et al., 1998
A. terreus ß-GlucosidaseI	Ultra filtration, DEAE-sepharose, hydrophobic interaction	1.5	275.9	183.9	9.19	76.6	Nazir et al., 2009
A. terreus ß-GlucosidaseII	Ultra filtration, DEAE-sepharose, hydrophobic interaction, gel filtration	4	256	64	8.5	26.6	Nazir et al., 2009
A.terreus ß-Glucosidase III	Ultra filtration, DEAE-sepharose, hydrophobic interaction, gel filtration	0.05	15.35	3.07	0.51	127.9	Nazir et al., 2009
*Aspergillus sp.*MT0204	Ammonium sulphate precipitation Ion exchange chromatography, hydrophobic interaction	0.56	5.12	9.14	24.5	23.44	Qi et al., 2009
Trichoderma harzianum	Ammonium sulphate precipitation, gel filtration, Sephadex G-200, Sephadex G-50,	-	-	0.35	73.7	1.74	Ahmed et al., 2009
Paecilomyces thermophila	DEAE 52 Sephacryl S-200	0.7	56.4	80.6	21.7	105	Yang et al., 2008
Melanocarpus sp. MTCC 3922	Ultra- filtration DEAE-Sepharose, PBE-94	77.47	778.0	10.04	4.08	15.9	Kaur et al., 2007
Daldinia eschscholzii	Ammonium sulphate precipitation, ion exchange, hydrophobic interaction, gel filtration	0.87	67.74	77.86	6.28	50.2	Karnchanatat et al., 2007
Aureobasidium pullulans	Ammonium sulphate, CM Bio-Gel A-0.5m, sephacryl S-200	0.37	46	124	3	129	Saha et al., 1994

Table 1.2. Physical properties, carbohydrate content and K_M values for fungal ß-glucosidase

Organism	M_w ($X10^3$)		pl	Carbohydrate Content (%)	K_M (mM)		Ref.
	Native	SDS-PAGE			PNPG (ONPG)	Cellobiose	
Aspergillus sp. MT-0204	-	42	-	-	22.47	-	Qi et al., 2009
A. terreus	-	29 43 98	2.8 3.7 3.0	-	-	-	Nazir et al., 2009
A. fumigatus	-	120 95 70	8.5 5	-	1.52	-	Kim et al., 2007
A. tubingensis I II III IV	-	131 126 54 54	4.2 3.9 3.7 3.6	-	0.76 0.35 3.2 6.2	-	Decker, et al., 2001
A.niger Cellobiase A Cellobiase B Cellobiase C	-	88 80 71		8.8 9.4 7.2	-	0.90 1.63 1.0	Abdel-Naby et al., 1999
Aspergillus sojae	250	118	3.8	23.8	0.14	-	Kimura et al., 1999
A.kawashii EX1 EX2 CB-1	-	145 130 120	-	Glycoprotein Glycoprotein Glycoprotein	-	-	Iwashita, et al., 1999
A. niger ß Glucosidase A ß Glucosidase B	-	118 109	-	-	0.43 0.11	0.50 0.27	Le TragonMasson et al., 1998
Aspergillus niger	-	-	-	-	0.63		Spagna et al., 1998
A.niger ß Glucosidase II	360	120	4.0	-	2.2	15.4	Yan et al., 1998
A.oryzae	40	43	4.2	-	0.55	7	Riou et al., 1998
Acremonium persicinum	140	128	4.3	1.5	0.3	0.91	Pitson et al., 1997
A.niger	137	-	3.8	12.5	-	0.85	Yazaki et al., 1997
A.niger	330	110		-	1.11	-	Rashid and Siddiqui, 1997
A.niger ß Glucosidase I	105	49	3.2	-	21.7	-	Yan and Lin, 1997

Applications of β-glucosidases

The most important application of β-glucosidase is in the saccharification of cellulose for the production of glucose and eventual fermentation to ethanol (Lee et al., 1997). This technology is not yet economically feasible because of the high cost of the enzymes involved in cellulose degradation. Endoglucanases and exoglucanases attack cellulose and generate cellobiose, which is an inhibitor of cellulases and cannot be fermented by yeasts. β-Glucosidase converts cellobiose to glucose facilitating the action of endo- and exoglucanases. Glucose, in turn, inhibits β-glucosidase and hence it is necessary to develop glucose tolerant β-glucosidases with high k_i values for glucose. Many β-glucosidases with high K_i values have been isolated from different strains of *Aspergillus* (Decker et al., 2001) and *Paecilomyces varioti* MG3 (Joseph et al., 2010). The most commonly used cellulase system of *T. reesei* contains low levels of β-glucosidase and the accumulation of cellobiose will lead to product inhibition (I-Son et al., 2010). Therefore addition of external β-glucosidase for effective saccharification is necessary (Sternberg et al., 1977). More specifically, addition of thermostable β-glucosidases to commercial cellulase enzyme preparations resulted in synergistic effect and increased hydrolysis of cellulose Using β-glucosidases as additives in cellulose based feeds is beneficial for single stomach animals, such as pigs and chickens which results in enhancement in the digestibility of the feed (Coenen et al., 1995; Zhang et al., 1996). The efforts are being made to reduce the enzyme cost by US department of energy in collaboration with biotechnology companies such as Genecore and Novozyme.

β-Glucosidases are produced at industrial scale using *A. niger* which is a GRAS (Generally Regarded As Safe) microorganism and can be used for processing of food and beverages (Spagna et al., 1998). These enzymes play a key role in the enzymatic release of aromatic compounds from glycosidic precursors present in fruit juices, musts and wines. These enzymes can be used in flavor enhancement of fruit juices and wines by liberating flavor compounds from glucosidic precursors. In addition to the free volatile odorous terpenols, some fruit juices contain some aroma precursors (terpenylglycosides) that are non-odorous and nonvolatile. These precursors are bound to glucose residues by β-linkages. The natural process by

endogenous plant β-glucosidases is very time consuming and hence supplementation of exogenous β-glucosidases is necessary to release the terpenic residues during wine making (Gueguen et al., 1999). In tea beverages treated with immobilized β-glucosidases, the essential oil content is reported to be increased by 6-20% (Sub et al., 2010). The β-glucosidases which are active and stable at acidic pH values (3-4) in presence of ethanol are suitable for flavor enhancement in wine making. Red fruit juices and wines contain antocyanins (β-glucosides of antocyanidins) which impart color to the juice preparations. Isoflavons in soy-based foods have phytoestrogenic properties which can relieve menopausal symptoms and help prevent several chronic diseases and certain cancers. However, isoflavons are mainly in the inactive form of glycosides and hydrolysis of these glycosides to their aglycon forms by β-glucosidases is highly desirable (Hu et al., 2009). In soy-milk, treatment with β-glucosidases (Pham and Shah, 2009) or fermentation with β-glucosidases producing *Lactobacillus* strains (Marazza et al., 2009) increased the aglycon content significantly. A novel thermostable β-glucosidase (Te BglA) from *Thermoanaerobacter ethanolicus* JW200 and two β-glucosidases from *Thermotoga maritime* (Tm BglA and Tm BglB) are reported to have potential applications in converting isoflavon glycosides into their aglycans (Song et al., 2011).

Some of the β-glucosidases with low activity on cellobiose and terpenyl glucosides are used for decolorization of products from red fruits (Le Tragon-Masson and Pellerin 1998). Citrus fruits contain glucosidic compounds such as prumine and naringin which are responsible for bitter taste to their juices. The enzymes such as α-rhamnosidases, β-glucosidases (Roitner et al., 1984) are employed to hydrolyze these glucosides to reduce bitterness.

The synthetic activity of β-glucosidases is exploited for the biosynthesis of oligosaccharides and alkyl-glycosides. Oligosaccharides are growth promoting agents for probiotic bacteria and also used as therapeutic agents and diagnostic tools. They also have very important functions in biological systems such as fertilization, embryogenesis and cell proliferation. Alkyl-glycosides are nonionic surfactants with high biodegradability. Their antimicrobial properties allowed them to be used in pharmaceutical, chemical, cosmetic, food and detergent industries (Bankova et al.,

2006). Some β-glucosidases catalyze transglycosylation reactions (Christakopoulous et al., 1995) which can lead to the production of oligosaccharides and glycoconjugates. Several β-glucosidases have been used for this purpose to synthesize alkyl-β-glucosides and cellooligosaccharides (Jun et al., 2008; Tako et al., 2010). Applications of this property of β-glucosidases have been discussed in details by Bhatia and coworkers (2002).

β -Xylosidase

Occurrence

Reese and coworkers found a large number of producers in the five genera of fungi – *Aspergillus, Penicillium, Botryodiploidia, Pestalotoca and Trichodermea* (Reese et al., 1973). It has also been reported in species of *Absidia, Mucor, Rhozyms, Rhermoascus*, *Thermomyces* (Flannign and Sellaos, 1977), yeast *Cryptococcus* (Notario et al., 1976), *Bacillus spp* (Lajudie and De B arjac 1976) and some plant pathogenic bacteria such as *Agrobacterium, Corynebacterium, Xanthomonas* (Hayward, 1977) Rumen bacteria , *A. niger , Coniphora cerebella* (King and Fuller, 1968). Bacteria such as *P. citrea and P. issachenkonii* were isolated from degraded thallus of the brown alga *Fucus evanescens* (Ivanova et al., 2002) and were found to produce several enzymes catalyzing hydrolysis of complex polysaccharides found in the brown alga thallus including β–xylosidase. An apparently analogous enzyme has been purified to homogeneity from rat kidney that hydrolyzed hemicelluloses to yield β-D-glucoside, β-D-galactoside, α-L-arabinoside, β-D-fucoside and β-D-xyloside (Glew et al., 1976).

β-D-xylosidases are also associated with disease diagnosis. In Creutzfeldt-Jakob Disease (CJD), there are prominent ultra structural alterations in plasma membrane and found to show increase in β-xylosidase, β-glucoronidase, and N-acetyl-β- D- galactominidase activities in CJD patients (Kim et al., 1988). Chiao et al., (1978) described a patient with sub acute neuropathic (Type 3) Gauchers disease, where tissues displayed a profound lack of β-xylosidase activity as well as the expected deficiency of glucocerebrosidase activity.

Filamentous fungi are widely used as enzyme producers and are generally considered more potent xylanolytic producers than bacteria and yeast (Haltrich et al., 1996, Polizeli et al., 2005).

Localization

β-Xylosidases may be extracellular or cell bound depending on the microorganism and the culture conditions (Lenartovicz et al., 2003). β-Xylosidases in almost all bacteria and yeasts are cell associated and are considered to occur in the cytosol in a soluble form (Bajpai, 1997). In contrast to this, fungal β-xylosidases remain associated with the mycelia during early stages of growth and then released into the medium. These mycelia associated enzymes are released either by true secretion or after cell lysis (Wong and Saddler, 1992) and as a result, several fungal β-xylosidases are made available extracellularly (Bhattacharyya et al., 1997; Kiss and Kiss, 2000; Rizzatti et al., 2001; Lenartovicz et al., 2003; Yan et al., 2008). Some fungal β-xylosidases remain cell associated during all growth period (Katapodis et al., 2006; Kumar and Ramon, 1996; Ito et al., 2003; Lembo et al., 2006).

Induction and regulation

Though xylose is an inhibitor of β-xylosidases (Deleyn and Claeyssens, 1977), it still can act as an inducer of xylanolytic enzymes. Significant amount of β-xylosidase was observed when *T. reesei* (Kristufek et al., 1995), *A. nidulans* (Perez-Gonzalez et al., 1998) and *A. versicolor* (Andrade et al., 2004) were grown on xylan. It has also been shown that xylose is required in less concentration for induction of β-xylosidases. Li et al., (2000) have shown that the presence of 0.1% xylose and 1% xylan oat spelt enhanced the induction of β-xylosidase in *Trichoderma koningii.* This observation was also supported in case of *T. reesei* (Kristufek et al., 1995). In *A. niger*, along with expression of xln R, presence of xylose is absolutely required for induction of xylanolytic gene complex (Tamayo et al., 2008). In some cases, disaccharides or higher molecular weight substrates can act as the best inducer of β-xylosidases (Rajoka et al., 1997). Xylan was found to be the best inducer for β- xylosidase in filamentous fungi (Ito et al., 2003; Krogh et al., 2004). Substrates derived from xylan were found to play an important role in induction of β-xylosidases (Kulkarni et al.,

1999). The disaccharides or high molecular weight substrates proved to be the best inducers of this enzyme (Rajoka et al., 1997). The substances derived from xylan also play an important role in the induction of β-xylosidases. Xylan was found to be the best inducer of β-xylosidase in fungi (Ito et al., 2003; Krogh et al., 2004). Lignocellulose substrates were reported to be better inducers than xylan and xylose for production of β-xylosidase (Haltrich et al., 1996). B-D-xylopyranosyl residues (Reese et al., 1973, Rizzatti et al., 2001), synthetic structural analogues such as β-methyl xyloside (Saraswat and Bisaria, 1997) have been used as β-xylosidases inducers. Alcohols in presence of glycerol (Ito et al., 2003), xylitol were also found to act as inducer for β-xylosidase production.

Though the regulation of xylanolytic gene expression is still poorly understood, the enzyme production studies using different carbon sources and growth conditions have elucidated it to some extent. It is reported that β-xylosidase gene expression is regulated at the transcriptional level as revealed from studies at cellular and molecular levels (Strauss et al., 1995; van Peij et al., 1997). The expression of xylanolytic enzymes in most of the fungi is subject to specific induction in presence of xylan or xylose and to carbon catabolite repression involving CreA repressor (de Groot et al., 2003; Prathumpai et al., 2004). The transcriptional activator XlnR also play an important role in regulation which is known to regulate the expression of number of genes such as those encoding for β-xylosidase, xylanase, α-glucuronidase, arabinoxylan arabinofuranohydrolase and D-xylose reductase that are involved in xylan degradation (Hasper et al., 2002; Stricker et al., 2008). Carbon sources modulate the *xlnR* gene expression and its repression is mediated by CreA (Tamayo et al., 2008). Thus it is the balance between the transcription of the factor and the CreA repressor which regulates the xylanolytic genes transcription (Tamayo et al., 2008).

Repression

β-Xylosidase genes are subject to catabolite repression (Kulmburg et al., 1993; Van Peij et al., 1997), a mechanism which plays an important role in regulation and secretion of inducible enzymes. The presence of easily metabolizable carbon sources such as glucose or xylose, represses the synthesis xylan degrading enzymes by carbon catabolite repression mechanism (Ronne, 1995; Tonukari et al., 2002). Carbon

catabolite repression alters the transcription and it is regulated by CreA protein that functions as a transcriptional repressor of glucose repressible genes (de Vries et al., 1999). The molecular studies demonstrated that the catabolite repression of β-xylosidases is associated with the binding sites for CreA in their promoters. Such CreA binding sites were found upstream, in non-coding region of *A. niger* β-xylosidase gene (*xlnD*) indicating that upstream repressing sequences directly control the *xlnD* transcription (van Peij et al., 1997). Six CreA binding sites located in the upstream regulatory sequence (URS) of the *T. emersonni* β-xylosidase gene caused its repression by glucose (Reen et al., 2003). Very recently, CreA mediated indirect repression of xlnR gene was observed in *A. niger* (Tamayo et al., 2008). Such CreA mediated catabolite repression was also observed in other fungal genes encoding for xylanolytic enzymes (Mach et al., 1996; Perez-Gonzalez et al., 1998).

Though xylose is an inducer of xylanolytic enzymes, at higher concentrations, it also triggers CreA mediated repression. The β-xylosidases of *T. emersonni* and *T. reesei*, are inhibited by D- xylose and the inhibition was found to be competitive and K_i was 1.3 and 2.4mM respectively using *p*NPx as substrate (Poutanen and Pulls, 1988; Rasmussem et al., 2006). Repression effect was also observed in *H. grisea* (de Almeida et al., 1995) and *A. phoenicis* (Rizzatti et al., 2001). On the other hand, some β-xylosidases have been found to be xylose tolerant such as β-xylosidases from *S. thermophilus* (Zanoelo et al., 2004) and *P. thermophila* (Yan et al., 2008) which were not affected by higher xylose concentrations. Such xylose tolerant β-xylosidases are essential for the efficient hydrolysis of hemicelluloses in a developed process. Glycerol and other alcohols are also known to repress β-xylosidases production in some cases through catabolite repression mechanism (Ito et al., 2003; Katapodis et al., 2006). The studies on inductive or repressive effect of different nitrogen sources suggested the existence of another regulatory mechanism (Rajoka et al., 2007).

Multifunctional enzymes and iso-enzyme forms

β-D-Xylosidases (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) are exoglycosidases that hydrolyze xylooligosaccharides and remove successive D-xylose residues from non-reducing terminal ends and useful for the complete saccharification of xylan (Belfaquih and Penninckx, 2002). Generally, purified β-xylosidases are unable to hydrolyze xylan. Still there are some reports suggesting that β-xylosidases are able to attack xylan slowly to produce xylose (Dekker and Richards, 1976). This enzyme is active on small xylooligosaccharides, aryl-β-D-xylopyranosides, aryl-α-L-arabinopyranoside, aryl-β-D-glucoopyranoside, aryl-β-D-quinovopyranosides (Claeyssans et al., 1971); xylibiitol (Takenishi et al., 1973), xylotriitol and L-serene xylopyranoside (Reese et al., 1973). Many β-xylosidases have transxylosidation (Transferase) activity especially at high substrate concentration resulting in the formation of products of higher molecular weight than that of the substrate (Conrad and Noethen, 1984). β-Xylosidases possessing α-arabinosidase activity have been reported, i.e. enzyme from *T. reesei* (Poutanen and Pulls, 1988), *T. ethanolicus* (Shao and Wiegel, 1992). Hemicellulases from different anaerobes primarily ruminant bacteria have been reported to have both β-xylosidase and α-L-arabinosidase activities encoded by single gene product. Some of the examples of β-xylosidases with α-L-arabinosidase activity are: *Bacteriodes avatus* xsA (Hespell and Whitehead, 1990), *Butyrivibrio fibrosolvens* xylB (Utt et al., 1991), *Clostridium stercorarium* syl A (Sakka et al., 1993) and *P. ruminicola* xynB genes, all of which contained numerous regions of sequence identity (Gasparic et al., 1995). β-Glucosidase from fungus *Sclerotinia sclerotiorum* was found to be strongly associated with β-xylosidase activity, suggesting that both activities could be represented in a single protein complex (Waksman, 1988). Similarly β-glucosidase from *A. sojae* has β-xylosidase activity and a single protein in pig kidney has both β-glucosidase and β-xylosidase activity (Robinson and Abraham, 1967).

β-Xylosidases hydrolyze glycosidic bonds by one of the two mechanisms which involve either retention or overall inversion of the configuration of the anomeric substrate carbon (Sinnot, 1990). These two mechanisms have already been described in earlier section. Majority of the xylosidases as verified in *A. niger* and *A.*

awamori hydrolyze glycosidic bonds using double displacement mechanism with retention of the anomeric center configuration. The β-xylosidases from *P. herquei* and *Cochliobolus carbonum* show uniqueness in that they operate with the inversion of anomeric center.

Filamentous fungi produce multiple β-xylosidases. Typical examples include β-xylosidases from *A. pulverulentus*, *A. niger*, *Neocallimastrix patriciarum* and *P. herquei* that existed in two forms (Ito et al., 2003). However, *Penicillium wortmanni* produced four β-xylosidases. These enzymes show differentiation in number of physicochemical properties, structures, specific activities, yields and particularly specificity leading to enhanced xylan degradation. Isoenzymes may present different effectiveness in hydrolyzing xylobiose, substituted xylooligosaccharides, xylosyl substituents or oligosaccharides containing xylosyl and other residues.

Substrate specificity

Most of the β-xylosidases are specific for the synthetic substrates, xylopyranosides such as *p*-nitrophenyl β-D-xylopyranoside (Bhattacharyya et al., 1997; Saha, 2003a; Ito et al., 2003; Lembo et al., 2006). Some of the β-xylosidases cleave *p*-nitrophenyl-α-L-arabinofuranoside, *p*-nitrophenyl-β-L-arabinopyranoside or *p*-nitrophenyl β-D-galactopyranoside (Kiss and Kiss, 2000; Ito et al., 2003; Zanoelo et al., 2004). Most of the purified β-xylosidases were not active on oat spelt xylan (Polizeli et al., 2005; Katapodis et al., 2006.), except *T. reesei* β-xylosidase which hydrolyzed xylan to form xylose (Herrmann et al., 1997). This enzyme is a multifunctional enzyme which is also known as β-D-xylan hydrolase. The true β-xylosidases are those which hydrolyze xylobiose and xylooligosaccharides to xylose in an exoenzyme fashion. The product analysis studies on the xylooligosaccharides hydrolysis by various β-xylosidases revealed that most of the enzymes hydrolyze xylobiose, xylotriose and xylotetraose. The β-xylosidase of *A. phoenicis* is reported to hydrolyze only up to xylotriose (Rizzatti et al., 2001). Some of the β-xylosidases hydrolyzed up to xylotetraose (Zanoelo et al., 2004), xylopentaose, xylohexaose (Saha, 2003b). According to Yan et al., (2008), xylosidases hydrolyzing up to xylopentaose seem to be more applicable for xylan hydrolysis.

The rate of xylose released from xylooligosaccharides by purified enzyme was increased with chain length (Saha et al., 2001; Yan et al., 2008). The opposite was verified for *A. nidulans, Trichoderma viride* β-xylosidases (Kumar and Ramon, 1996). There was no effect of increase in chain length and rate of hydrolysis by *Sporotrichum thermophilus* β-xylosidase (Katapodis et al., 2006).

Classification

β-Xylosidases are grouped into families based on their amino acid sequence similarities. According to Carbohydrate Active Enzyme data base (CAZy), β-xylosidases are divided into families 3, 30, 39, 43, 52, 54 of glycoside hydrolases (GHs) (Cantarel et al., 2009). Filamentous fungal β-xylosidases have been described only for families 3, 43, and 54 (Ito et al., 2003; Wakiyama et al., 2008). Members of glycosidase families 3 and 54 operate with retention of the anomeric configuration while GH43 family contains "inverting" glycoside hydrolases. Considering that protein fold is more conserved than their sequences, families with related 3D structure are grouped into higher hierarchial levels, denominated clans (Davies and Henrissat, 1995).

The catalytic residues of GH 3 are Glu and Asp. Study of cloned and sequenced genes have indicated involvement of these gene products in macromolecular degradation (Faure, 2002). Many family 3 β-xyolosidases exhibit a combination of different activities, especially association of β-xylosidases and β-glucosidases activities. They also show transglycosylation activity (Wakiyama et al., 2008).

The β-xylosidases from family 43 are analytically most efficient and do not exhibit transglycosylation at high substrate concentration (Jordan et al., 2007). Similarities in three dimensional structures are found in families in 43 and 62, thus both are grouped into clan GH-A. The family 43 β-xylosidases show a 5 fold β-propeller and operate with inversion of the annomeric centre (Cantarel et al., 2009). Site directed mutagenesis revealed Asp and Glu residues involved in catalysis (Yanase et al., 2002).

Glycosyl hydrolase family 54 includes only two different glycosyl hydolases i.e. α-L-arabinofuranosidase (EC 3.2.1.55) and β-xylosidase (EC 3.2.1.37). GH 54 is unique from all existing clans. Based on protein structure and mutagenic studies, Glu and Asp are the candidates for the nuclephile and are the general acid or base catalytic residues respectively (Wan et al., 2007).

Production of β-xylosidase

Filamentous fungi are widely used for β-xylosidase production and generally considered as more potent producers than bacteria and yeast (Haltrich et al., 1996; Polizeli et al., 2005). Genus *Aspergillus* is more important because of its thermo-tolerance and production of thermostable enzymes (Castro et al., 1997). Members of *Aspergillus* section Nigri are efficient producers of several types of extracellular enzymes (Serra et al., 2006).

Lemos and coworkers (2000) have reported considerable level of β-xylosidase (1.3 U/ml) produced by *Aspergillus awamori*, when grown in milled sugarcane bagasse under submergerd fermentation (SMF). The organisms such as *Aspergillus ochraces, Aspergillus sydowii, Aspergillus tamarii* have also been reported to produce β-xylosidase induced by sugar cane bagasse (Biswas et al., 1988; Gosh et al., 1993). Rajoka and Khan (2005) have studied production of β-xylosidase by a cycloheximide and 2-deoxy-D-glucose resistant mutant of *Kluyveromyces marxianus* PPY 125 in growth media containing galactose, glucose, xylose, cellobiose, sucrose and lactose as carbon sources. They have reported maximum product yield in 2% xylose containing media and a basal level was observed in non induced culture grown on glucose. Similar observation was reported by Perez-Gonzalez et al., (1998) for β-xylosidase production from *Aspergillus nidulans*. In other enzyme systems, disaccharide or high molecular weight substrates have been found to be the best inducer of β-xylosidase (Rajoka et al., 1997). *K. marxianus* produces β-xylosidase without any accompanying cellulases (Belem and Lee, 1998).

Basaran and Ozcan (2008) obtained *P. stipitis* NP 54376 a high growth rate mutant by NTG treatment. Bokhari et al., (2010) used a mutant derivative of *Humicola lanuginosa* M7D to achieve maximum production of β-xylosidase (728 IU/g substrate)

using Vogel's medium containing xylan. Thermophilic strains of *Thermomyces lanuginosus* produced phytase, β-xylosidase, β-galactosidase and α-L-arabinofuranosidase (Singh et al., 2000a,b; Sonia et al., 2005) but their production levels of β-xylosidase were quite low (< 3 IU/g substrate). Ungchaitham and coworkers (2001) have reported β-xylosidase activity of about 0.9 U/mg of protein produced from *Streptomyces spp*. CH7 when grown in a medium containing 1% xylan as a carbon source at pH 7.0 and 40^0C for 24 h. Its gene has been cloned in *E. coli* with pUC 18 as a cloning vector. A recombinant plasmid containing 3.6 kb insert was found to express β-xylosidase activity. Clarke et al., (1996) have cloned genes encoding β-xylosidase and α-L-arabinofuranosidase from *T. reesei* and expressed it in *Saccharromyces cerevisiae*.

β-xylosidase production study using *Humicola lanuginosa,* Bokhari and coworkers (2010) have studied the effect of carbon sources such as bagasse, corn cobs, wheat straw, xylan and nitrogen sources such as sodium nitrate, ammonium sulphate, corn steep liquor, ammonium sulphate, ammonium nitrate and urea. Xylan and corn steep liquor at pH below 6.0 were found to support maximum production of enzymes at 55^0C with initial pH 6.5 under SSF. It was comparable to a thermotolerant *Aspergillus spp*. reported by Rizzatti and coworkers (2001).

Corn steep liquor and soybean were the best nitrogen sources followed by sodium nitrate, urea and peptone (Rajoka et al., 2005). It was also observed that $NaNO_3$ concentration greatly increased cellulase synthesis in *Cellulomonas biazotea* (Rajoka et al., 1998). Production of β-xylosidase in xylose yeast medium using corn steep liquor as N_2 source in presence of glucose has resulted in enhancement of enzymes synthesis and there was no inhibition of enzyme synthesis by catabolite repression (Rajoka et al., 2005). Similar results of mixed inductive and or repressive effect have been observed in other organisms (Li and Ljungdahl, 1994). It has been suggested that corn steep liquor may not have supported the formation of Cre A protein as observed in its absence (Lockington et al., 2002). Bokhari et al., (2010) have reported corn steep liquor followed by urea and ammonium nitrate to be suitable for production of β-xylosidase produced by a mutant if *Humicola lanuginosa* in SSF.

Temperature is known to affect microbial growth, cell biomass and enzyme production significantly. Rajoka and coworkers (2005) have observed maximum specific productivity of β-xylosidase at fermentation temperature of 35^0C. At higher temperature, enzyme production by the cells was decreased. At lower temperature, the transport of substrate is affected resulting, in lower enzyme production (Aiba et al., 1973). At higher temperature, the maintenance energy requirement for cellular growth is high due to thermal inactivation of enzymes involved in metabolic pathways resulting in lower amount of enzyme production (Aiba et al., 1973). This low production at high temperature may also be due to reversible denaturation of enzymes formed on optimized medium (Converti and Dominguez, 2001). Abdeshahian et al., (2010) have reported cultivation of *A. niger* FTCC 5003 on palm kernel cake as a substrate to produce high activity of β-xylosidase in SSF (6.13 u/g substrate) at 32.5^0C, 60% moisture and 1.5 L/min aeration rate.

Panagiotou et al., (2003) showed that the peak level of β-xylosidase was produced by *Fusarium oxysporum* on corn stover at 30 to 33^0C rather than at 27^0C. Kalogeris and coworkers (2003) obtained high level of β-xylosidase by *Thermoascus aurantiacus* on wheat straw at 49^0C. The elevated temperature may affect the membrane of vesicles in the filamentous fungi and may lead to initiation of metabolic change and product formation (Tao et al., 1997). Still higher temperature causes the reduction of microbial growth and metabolic activity (Pandey et al., 2001). Bokhari et al., (2010) have reported maximum production of β-xylosidase by *Humicola lanuginosa* at 45^0C under SSF. A thermotolerant *Aspergillus phoenicis* was also reported to produce high β-xylosidase activity at 45^0C (Rizzatti et al., 2001). Several studies have reported initial pH below 6 to be suitable for the production of β-xylosidase (Singh et al., 2000a; Sonia et al., 2005). Bokhari and coworkers (2010) have shown that initial pH 6.5 to be optimal for maximum production of β-xylosidase using *Humicola lanuginosa.* Rizzatti and coworkers (2001) have reported pH 5.5 to be suitable for production of β-xylosidase by *A. phoenicis*.

Purification

Most of the purification schemes for xylanolytic enzymes adopt a three step strategy (Sa-Pereira et al., 2003). β-Xylosidase from *B. thermantarcticus* was purified to homogeneity by Sephacryl-S-200, Q-Sepharose FF and Phenyl-Sepharose column (Lama et al., 2004). Various purification procedures also use ammonium sulphate precipitation and /or ultrafiltration (Ximenes et al., 1996; Sa-Pereira et al., 2003). The mycelial β-xylosidase from *Scytalidium thermophilum* was purified by ammonium sulphate fractionation and chromatography on Sephadex G-100 and DEAE Sephadex A-50 (Zanoelo et al., 2004). Rizzatti and coworkers (2001) dialyzed the filtrate overnight and applied to DEAE cellulose column equilibrated with buffer. β-D-xylosidase was eluted with a linear gradient of NaCl (0 to 0.4 M) in buffer. Active fractions were pooled, dialyzed against water and lyophilized. The protein sample was redissolved in 100 mM sodium acetate buffer, pH 5.5 and applied to Sephadex G 100 column (55.5 X 1.3 cm). Fractions with β-xylosidase activity were pooled and dialyzed against distilled water. Several methods for purification of β-xylosidase from different organisms are summarized in Table 1.3.

The properties such as molecular weight, isoelectric point and glycosylation are given in Table 1.4.

Table 1.3. Purification strategies employed for filamentous fungal β-xylosidase

Organism	Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)	Ref
Humicola lanuginosa (Parent)	Ammonium sulphate precipitation, gel filtration-sephadex G75, ion exchange Q –sepharose	7.7	1053	136	27	13	Bokhari et al., 2010
Humicola lanuginosa (mutant)	Ammonium sulphate precipitation, gel filtration-sephadex G75, ion exchange Q –sepharose	5.8	2421	417	35.1	22.9	
Aspergillus japonicus	Ammonium sulphate precipitation, DEAE-Toyoperal 650S, Superdex200pg	1.03	115	112	12.3	59.4	Wakiyama et al., 2008
Humicola grisea	Sephacryl S-300, DEAE-Sepahrose	0.0034	0.067	19.6	9.2	27	Iembo et al., 2005
Streptomyces CH-7	Ammonium sulphate precipitation, DEAE -biogelA, DEAE-biogel A Sephadex G200	15	185	12.3	30	9.3	Pinphanic hakarn et al., 2004
Fusarium proliferatum	DEAE-Sepharose CL-6B,Cmbio-elA,Biogel A0.5m Gel filtration, Biogel HTP hydroxyl apatite,column chromatography			53			Saha et al., 2003b
Thermoanaero-bacter ethanolicus	DEAE-cellulose, phenyl-Sepharose, DEAE-Sepharose	2.3	152	66	14	72	Shao and Wiegel, 1992
Neurospora crassa	Isoelectric focusing, polyarylamide gel electrophoresis	1.5	0.4	0.26			Deshpande et al., 1986
Aspergillus niger	Ethanol, fractionation, Chromatography-1Sephadex G50, 2Cellulose DE50, 3 Sephadex CPC-50 4 Sephadex G200	1	35.2	35.2	42.5	199	Tavobilov et al., 1983

Table 1.4. Extracellular β-xylosidase properties from filamentous fungi

Species	**Native form**	**MW (kDa)**	**Glycosylation (%)**	**pI**	**References**
Talaromyces thermophilus	Monomeric	97	-	-	Guerfali et al., 2008
Aspergillus japonicus	Monomeric	113.2	27.6		Wakiyama et al., 2008
Paecilomyces thermophila	Monomeric	53.5	61.5	4.8	Yan et al., 2008
Talaromyces emersonii	-	-	86.9-100	High amount	Rasmussem et al., 2006
Trichoderma reesei		87.6-102	High amount	3.0-3.5	Rasmussem et al., 2006
Aspergillus fumigatus	Monomeric	72.5			Lenartovicz et al., 2003
Fusarium proliferatum	Monomeric	91.2	-	7.8	Saha et al., 2003b
Aspergillus phoenicis	Monomeric	132	43.5	3.7	Rizzatti et al., 2001
Fusarium verticillioides	Monomeric	94.5	-	7.8	Saha et al., 2001
Trichoderma koningii G-39	Monomeric	104	Glycosylated	4.6	Li et al., 2000
Aspergillus oryzae	Monomeric	110			Kitamoto et al., 1999
Aspergillus pulverulentus Xyl I Xyl II	Trimeric Dimeric	180 190	4.2 4.6	4.7 3.5	Sulistyo et al., 1995
Neocallimastix frontalis	Dimeric	180		4.35	Hebraud and Fevre, 1990
Penicillium wortmanni IFO 7237 I II III IV	Monomeric Probably dimeric	110 195 210 180	-	3.7 4.3 4.6 4.8	Matsuo et al., 1987
Aspergillus niger	Monomeric	78			John et al., 1979
Penicillium wortmanni QM 7322	Monomeric	96-102	23	5.0	Deleyn and Claeyssens, 1977
Termitomyces clypeatus	Monomeric	94	-	-	Bhattacharyya et al., 1997
Cochliobolus carbonum	Monomeric	42	Glycosylated		Ransom and Walton, 1997

Application

Abundant xylan rich agricultural or agro-industrial waste (about 40 million ton per year) represents an important biomass source in world which is not properly used due to the lack of proper technologies. Large amount of this biomass is dumped in rivers streamlet that causes damage to economic activities in the agro-industrial sector and environment as well (Cano and Palet, 2007). This xylan rich biomass offers possibilities to be utilized for its bioconversion to xylooligosaccharides (XOS) and xylose which can be diverted to many commodity chemicals such as ethanol, lactic acid, succinic acid. The conversion of xylan to their monosaccharides is mediated by two processes: the acid and enzymatic hydrolysis. Though acid hydrolysis of raw lignocellulosic materials offers great advantage, it releases toxic substances such as furfural, hydroxymethylfurfural, lignin subproducts and other fermentation inhibitors. In addition, acid hydrolysis process is corrosive with environmental and economic problems (Ladisch, 1979; Tsao, 1986). Thus the enzymatic process offers great advantages, because it is more specific, that can be carried out in mild conditions and the final products obtained are always without the presence of undesirable products. The enzymatic process involves the use of xylanolytic systems with β-xylosidase as the key enzyme. Over the years, β-xylosidases have been used in several conventional industrial processes since these enzymes catalyze hydrolyzing and transglycosylating reactions. Enzyme preparations with β-xylosidases are commercially produced worldwide and the most important producers are *T. reesei* and *Humicola insolens*.

Much interest has been shown to develop technologies to produce economically important products from lignocellulosic biomass. Hemicellulose is a heterogeneous polymer which liberates pentoses (C_5 sugars) such as xylose and arabinose. Most of the naturally occurring microbe show less efficiency in fermentation of these pentoses (Girio et al., 2010). The ethanol production from pentoses, such as xylose has been very well studied (Katahira et al., 2004). Xylose fermenting yeasts, such as *Candida shehatae, Pichia stipitis*, *Pachisolen tannophilus* could utilize xylose in hemicellulosic hydrolysates but their tolerance to inhibitory compounds in undetoxified lignocellulosic hydrolysates is low (Roberto et al., 1991). Feasibility of cellulosic ethanol plants at industrial scale is hindered by the inability of

yeasts or bacterial strains to convert all sugars efficiently to ethanol. This could be possible by constructing the strains with both hexose and pentose fermentation capabilities under robust operating conditions. Among the sugars used for ethanol production, xylose represents 10-20% which can also be used for xylitol production. Xylitol finds wide applications as a natural food sweetener, dental caries reducer and also sugar substitute for diabetics (Saha, 2003a). Additionally, the lignocellulosic biomass can be converted to other commodity chemicals, food additives etc using suitable biocatalysts (Sorensen et al., 2005; Adsul et al., 2011).

Enzymes can be used to synthesize oligosaccharides, glycoconjugates since enzymatic synthesis has advantages over organic synthesis (Wong et al., 1994). Alkyl-β-xylosides, ascorbid glucosides can be synthesized by β-xylosidase mediated transfer reactions (Pan et al., 2001; Gargouri et al., 2004). Transxylosylation activity exhibited by β-xylosidases has been exploited for synthesis of xylanase substrates (Eneyskaya et al., 2007) and oligosaccharides with chromophoric and fluorogenic groups (Zeng et al., 2000). The fungal β-xylosidases are more suitable for this purpose since they are more stable and less costly than those from other microorganisms and plants. Enzyme complexes with xylanases and β-xylosidases are used for synthesis of xylooligosaccharides (XOS) of different chain lengths with usually 2-5 residues. These XOS are the most desirable for application in food industry, pharmaceutical industry. These XOS are considered prebiotics since they selectively support the growth of probiotic organisms such as *Lactobacillus* sp. and *Bifidobacterium bifidum*.

The use of exogenous enzymes into feed is known to improve the nutritive quality of feed and also to reduce the feed cost. The addition of xylanase preparations with β-xylosidases feed helps in hydrolyzing the hemicellulose present in wheat, corn and other cereals. These, in turn, promote the nutrient digestibility and reduce the manure, nitrogen and phosphorous excretion. These enzymes increase the metabolizable energy and reduce the food viscosity, leading to animal weight gain (Polizeli et al., 2005). Additionally, the use of enzymes in feed improves the meat and milk production efficiency (Ahuja et al., 2004; Graminha et al., 2008).

The other applications of xylanases and β-xylosidases include the use in pulp and paper industries, brewing industry, wine making, coffee processing, vegetable

maceration, etc. The use of xylanolytic systems in pulp pretreatment reduces the requirement of chemical products, especially chlorine and chlorine oxide (Viikari et al., 1994). β-Xylosidases along with other enzymes such as cellulases, pectinases and xylanases are used in extraction and clarification of juices (Polizeli et al., 2005). In brewing industry, xylanases and β-xylosidases are used to cleave long chains of arabinoxylan present in wheat meal which reduces the viscosity and thereby removing the beer turbidity (Dervilly et al., 2002). In wine making, β-xylosidases can be applied along with xylanases and cellulases to reduce the concentration of β-glucans concentration which poses a problem in filtration step due to high must viscosity. Additionally, the use of β-xylosidases liberates compounds that develop specific desirable odor to wine preparations (Bhat, 2000).

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CHAPTER 2

PRODUCTION OF β-GLUCOSIDASE AND β-XYLOSIDASE BY *A. NIGER* NCIM 1207

ABSTRACT

Aspergillus niger NCIM 1207 produced significantly high levels of β-glucosidase and β-xylosidase activities in submerged fermentation. Cellulose induced only β-glucosidase, while xylan induced both β-glucosidase and β-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 36^0C led to an increase in pH of the fermentation above 6.0 that affected β-xylosidase activity significantly. Highest levels of β-glucosidase ((19 IU/ ml or 633 IU/ g of substrate) and β-xylosidase (18.7 IU/ml or 620 IU/ g of substrate) activities were detected when *A. niger* was grown at 30^0C for first five days followed by further incubation at 36^0C. 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 β-glucosidase and β-xylosidase in high amounts that can be used for supplementation of commercial cellulase preparations.

The β-glucosidases and β-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 β-glucosidases and β-xylosidase exhibited maximum activity at 65^0C. Both, cellulose induced and xylan induced β-glucosidases showed stability at 60^0C for 5 h and lost total activity at 70^0C within 1 h. β-Xylosidase was found to be comparatively more stable that retained 100% of its original activity even after 5 hours of exposure at 70^0C. Solka floc, xylose as well as xylan, induced only one isoform of β-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.

INTRODUCTION

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 (Gueguen et al., 1996). Fungal strains are known to be efficient β-glucosidase producers; for instance *Trichoderma* and *Aspergillus* sp. thermophilic fungi (*Chaetomium thermophilum, Humicola insolens, Sporotrichum thermophile* (Sonia et al., 2005; Badhan et al., 2007; Kaur et al., 2007) are good sources of novel β-glucosidases.

β-xylosidases are necessary for the complete hydrolysis of xylans. 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 (Manzanares et al., 1999). Filamentous fungi such as *Aspergillus niger, Aspergillus awamori, Trichoderma reesei, Talaromyces emersonii* are known to be efficient producers of β-xylosidases.

Earlier, hyperproduction of β-glucosidase (Gokhale et al., 1984) and β-xylosidase (Gokhale et al., 1986) by *Aspergillus niger* NCIM 1207 have been reported. Cellulases of *A. niger* NCIM 1207 were found to undergo catabolite repression in presence of glucose and glycerol accompanied by sudden drop in pH of the fermentation medium below 2.0. This sudden drop in pH caused inactivation of the cellulase enzymes (Gokhale et al., 1991). The pH inactivation was reversed by addition of urea in the growth medium, which helps to maintain the pH of the fermentation medium between 4.0-5.0 (Gokhale et

al., 1992). Here the production of β-glucosidase and β-xylosidase enzymes by *A. niger* NCIM 1207 in xylan containing media supplemented with glycerol and urea has been described. In addition, it is also shown that growth of the strain first at 30^0C followed by incubation at 36^0C resulted in maximum production of both β-glucosidase and β-xylosidase enzymes. The characterization of crude enzymes in relation to their optimum pH and temperature, pH and temperature stability, solvent stability, and multiplicity using zymogram staining is also described.

MATERIALS AND METHODS

Materials

Yeast extract, bacto-peptone were obtained from Difco Chemical Co. Detroit, USA. Cellulose -123 was obtained from Carl Scheicher and Schull Co. Dassel, FRG. Solka Floc SW 40 was from Brown Co, Berlin. *p*-Nitrophenyl-β–D-glucopyranoside (*p*NPG), *p*-Nitrophenyl-β–D xylopyranoside (*p*NPX) and Oat spelt xylan were obtained from Sigma Chemical Company, USA. All the other chemicals were of analytical grade. Xylan birchwood was from Fluca AG, Switzerland.

Microbial Strains and Enzyme Production by Submerged Fermentation

Aspergillus niger NCIM 1207 was obtained from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The culture was maintained on Potato Dextrose Agar (PDA) slopes and sub-cultured once in every two months. The organism was preserved at 4 ^{0}C during the research work.

The Aspergillus minimal medium (AMM) for enzyme production contained (g/L) $NaNO_3$ 0.5; $MgSO_4.7H_2O$ 0.5; KCl 0.5; KH_2PO_4 2.0; yeast extract 1.0 and bacto-peptone 5.0. Enzyme production was performed in 100 ml Erlenmeyer flasks with 75 ml of the Aspergillus minimal medium (AMM) with cellulose or xylan as carbon source. The medium was inoculated with the spore suspension (1 ml) containing 10^7 spores from 7 day old culture grown on PDA slope and incubated at 30^0C on a rotary shaker (150 rpm) for 14 days. The mycelium was separated by centrifugation at 8000 rpm for 15 min. and the supernatant was used as a source of extracellular enzyme.

Enzyme Assays

β-Glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activity was estimated as reported earlier (Gokhale et al., 1984) using *p*NPG as substrate. The total assay mixture of 1 ml consisted of 0.9 ml of *p*NPG (1 mg/ml) in 50 mM citrate buffer, pH 4.5 and 0.1 ml of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 65^0C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm, after developing the color with 2% sodium carbonate.

β-Xylosidase (β-D-xylan xylohydrolase, EC 3.2.1.37) activity was estimated as reported earlier (Gokhale et al., 1986) using *p*NPX as a substrate. The total assay mixture of 1ml consisted of 0.9 ml of pNPX (1mg/ml) in 50 mM citrate buffer, pH 4.5 and 0.1 ml of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 65^0C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm, after developing the color with 2% sodium carbonate.

One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *p*-nitrophenol from the substrate.

Protein Estimation

Protein was estimated according to Bradford's Coomassie Brilliant Blue method (1976) with bovine serum albumin as standard (20-150 µg protein). Aliquote of 0.1 ml of the test sample was mixed with 5 ml of Coomassie Blue reagent and absobance was measured after 10 min incubation at room temperature spectrometriclly at 595 nm.

Characterization of Crude Enzymes

Native Polyacrylamide Gel Electrophoresis (Native PAGE) and zymogram

Native PAGE was performed as described by Laemmli (1970) at pH 8.3 using 10% acrylamide as resolving gel with 5% stacking gel. Aliquotes of 15 µl were loaded into sample wells and electrophoresed at a constant voltage of 150 volt for 2 h. The gels were stained with 0.1% w/v Coomassei Brillient Blue R 250 for one hour followed by destaining solution (30% v/v methanol and 10% v/v acetic acid) for 2-4 hours. Unstained gel was cut into 0.5 cm pieces and the pieces were immersed in 50 mM citrate buffer, pH 4.5, incubated for one hour and the enzyme activity was determined under standard assay conditions.

For developing zymogram, crude enzyme preparations (50-70 µg) were fractioned by Native Polyacrylamide Gel Electrophoresis using 10% acrylamide as resolving gel and 5% stacking gel as described above. The β-glucosidase activity in gels was detected by developing zymogram against 10 mM 4-methyl umbelliferyl - β-D-Glucoside (Sigma) as a substrate prepared in 50 mM sodium citrate buffer, pH 4.5 (Van Tilbeurgh, H 1988). After electrophoresis, the gel was immersed in substrate solution for 45 min at 50^0C in the dark. The β-glucosidase bands in the gel were detected under UV light using gel documentation system (Syngene).

Effect of pH on enzyme activity and stability

The optimum pH of the enzymes (β-glucosidases and β-xylosidase) was determined by estimating the activities at 65^0C in 50 mM citrate buffer at various pH values (3.0 – 6.5). The pH stability was studied by incubating the enzymes in 50 mM buffer systems with pH ranging from 2.0 to 10.0. Buffer systems used were citrate phosphate buffer (pH 2.5 to 6.0), phosphate buffer (pH 7.0), borate buffer (pH 8.0 to 9.0) and bicarbonate buffer (pH 9.0 to 11.0). The residual activity was then assayed under standard assay conditions.

Effect of temperature on enzyme activity and stability

The optimum temperature of enzymes was determined by measuring the enzyme activity at various temperatures (50 to 80^0C) in 50 mM citrate buffer, pH 4.5. The temperature stability was determined by incubating the enzymes in 50 mM citrate buffer, pH 4.5 at 60^0C and 70^0C followed by measuring the residual activity under standard assay conditions.

Effect of organic solvents on crude enzyme stability

The effect of different organic solvents on enzyme stability was determined by preincubating 3 ml of crude enzyme in 1 ml of organic solvent for 24 h at 30 ^{0}C with shaking at 150 rpm. Residual activity of suitably diluted enzyme was measured by standard assay method.

RESULTS

Production of β-glucosidase and β-xylosidase on cellulose and xylan containing media supplemented with different additives

A. niger NCIM 1207 was grown in AMM containing cellulose and xylan and it was found that cellulose and xylose induced only β-glucosidase and little amount of β-xylosidase while xylan induced both the activities in equal amounts (Table 2.1). Maximum enzyme activities were produced at 3% xylan concentrations. Hence further studies were performed using xylan as substrate at 3% concentration. To study the effect of glucose and glycerol on β-glucosidase and β-xylosidase production, the organism was grown in a medium containing different concentrations of glucose and glycerol along with oat spelt xylan. The results showed that no repression of enzyme production was observed at 1% glucose. Glucose at higher concentration and glycerol at all concentrations suppressed both the enzyme activities (data not shown). The growth of *A. niger* in a medium containing higher concentrations of glucose or glycerol caused the pH to suddenly drop below 3.0. To investigate whether the addition of urea to glycerol containing medium helps to maintain the pH of the medium, the culture was grown in a medium containing xylan (3%) in combination with glycerol (2.5%) and urea (0.5%). It is clear from the results that activities of both β-glucosidase and β-xylosidase were increased.

Table 2.1: Production of β-glucosidase and β-xylosidase activities on cellulose and xylan as carbon sources

Aspergillus minimal medium (AMM) supplemented with	pH	Enzyme activity	
		β-glucosidase IU/mL	β-xylosidase IU/mL
1%Glucose	3.0	1.2	0.2
2% Cellulose 123	3.0	3.2	0.3
2%Cellulose +1% Glucose+1% Urea	7.0	4.8	0.6
1% Xylose	3.0	2.0	0.1
1% Xylan	3.0	2.2	2.3
2% Xylan	3.1	3.0	3.2
3% Xylan	3.2	5.0	3.4
3% Xylan + 1% glucose	3.2	6.1	4.2
3% Xylan + 1% glucose +1% urea	3.4	8.4	7.8
3% Xylan + 0.5% urea +2% glycerol	3.4	12.2	9.8
3% Xylan + 0.5% urea +2.5% glycerol	3.4	13.6	13.3
3% Xylan + 0.5% urea +3.0% glycerol	3.5	12.0	5.7

The enzyme activities were calculated after 14 days of incubation. The values are the average of three independent experiments with 4-6% standard deviation.

Effect of temperature on enzyme production

To study the effect of temperature on enzyme production, the organism was grown at three different temperatures viz, 25, 30 and 36^0C and the results are given in Fig. 2.1 & 2.2). In addition, the organism was also grown at 30^0C for first five days followed by incubation at 36^0C for further 8 days. The production of both the enzymes was monitored up to 16 days and the values of enzyme activities are given in Table 2.2. The optimum temperature was found to be 30^0C since maximum production of both the enzymes was observed at this temperature. It was observed that the pH of the fermentation medium was maintained between 3.5 and 4.5 throughout the period of fermentation (Fig. 2.1). This supports our earlier observation that the sudden drop in pH value below 3.0 of the fermentation medium was responsible for inactivation of the

enzymes. It was also observed that growth of *A. niger* at 36^0C caused the pH of the medium to rise above 6.0 which specifically affected β-xylosidase activity more than β-glucosidase (Fig. 2.2). Highest levels of β-glucosidase ((19.0 IU/mL or 633 IU/g of substrate) and β-xylosidase (18.7 IU/mL or 620 IU/g of substrate) activities were detected when *A. niger* was grown at 30 ^{o}C for first five days followed by further incubation at 36^0C (Table 2.2). The profile of the pH changes demonstrated that no inactivation of enzymes occurred since the pH of the medium fluctuated between 3.5 and 4.5 (Fig 2.3).

Table 2.2 Effect of temperature on production of β-glucosidase and β-xylosidase by *Aspergillus niger* NCIM 1207.

Temperature	Enzyme activity (IU/mL)		pH	Protein mg/mL
	β-glucosidase	β-xylosidase		
25^0C ± 1.0	7.6	7.6	3.8	0.124
30^0C ± 1.0	13.1	13.3	3.8	0.168
36^0C ± 1.0	8.8	0.01	7.7	0.259
30^0C ± 1.0 (0-5 days) 36^0C ± 1.0 (6-14 days)	19.0	18.7	4.5	0.205

The fungus was grown in AMM medium containing 3% xylan, 0.5% Urea and 2.5% glycerol with shaking at 150 rpm. The enzyme activities were calculated after 14 days of incubation at various temperatures. The values are the average of three independent experiments with 5% variation.

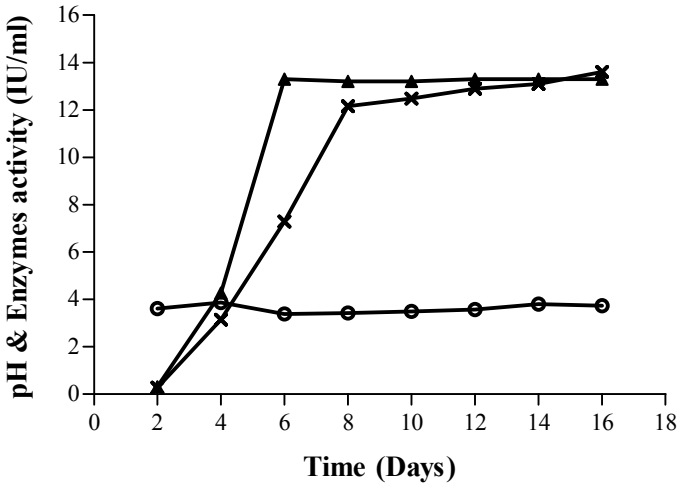


Fig. 2.1. Production of β-glucosidase (×), β-xylosidase (▲) and pH profile (o) in *Aspergillus* minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 30^0C

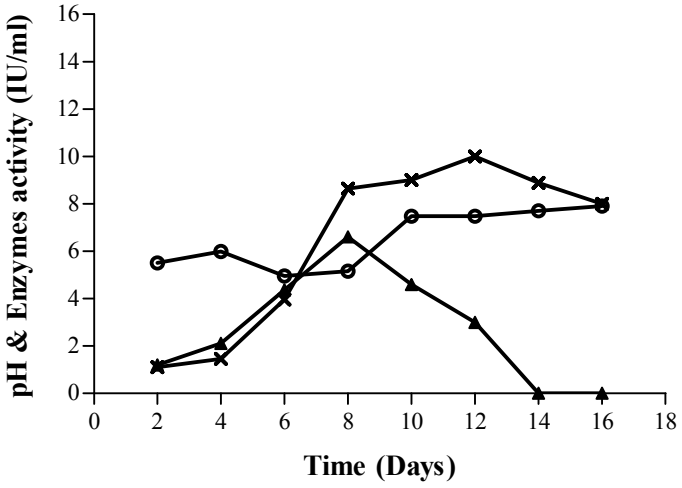


Fig. 2.2. Production of β-glucosidase (×), β-xylosidase (▲) and pH profile (o) in *Aspergillus* minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 36^0C

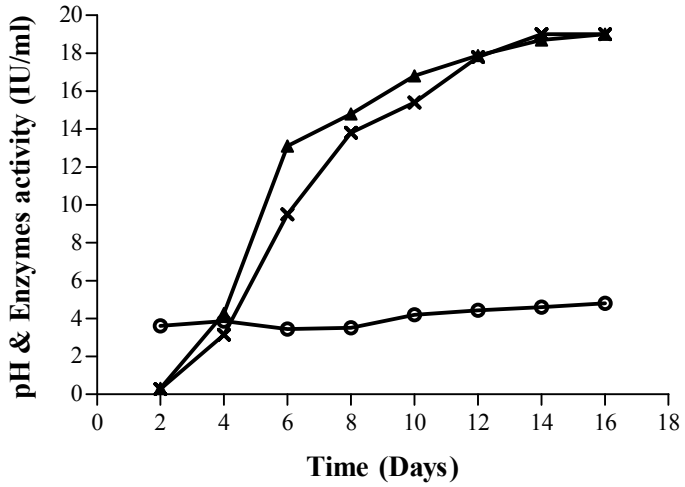
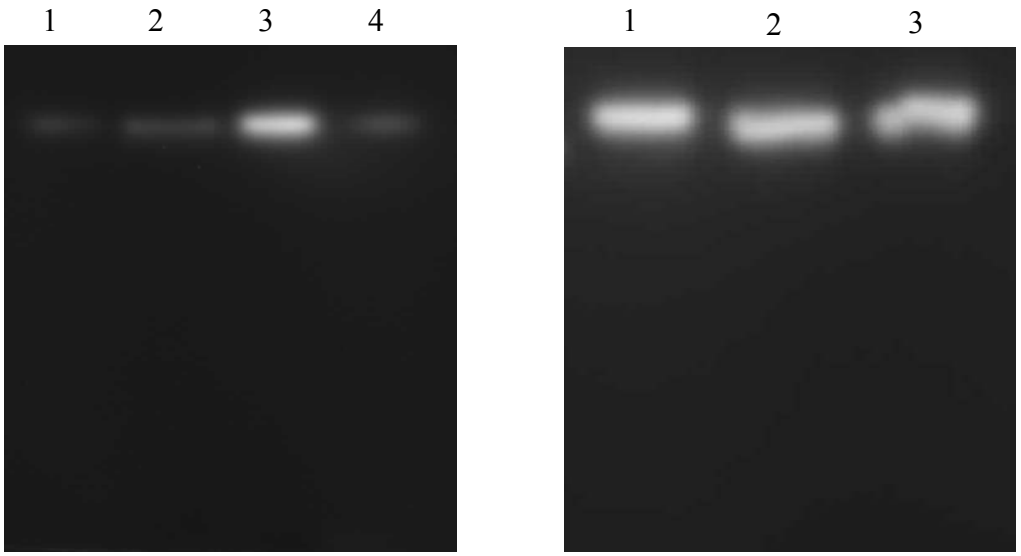


Fig. 2.3. Production of β-glucosidase (×), β-xylosidase (▲) and pH profile (o) in Aspergillus minimal medium supplemented with 3% xylan, 0.5% urea and 2.5% glycerol at 30^0C (days 0 to 5) and 36^0C (days 6 to 16).

Characterization of crude enzymes

Native gel electrophoresis and zymogram for β-Glucosidase:

After native gel electrophoresis, the enzyme bands were identified by cutting unstained corresponding gel pieces and found that in Xylan induced broth, both β-glucosidase and β-xylosidase resulted in a single band. This indicated that both the enzymes have approximately similar charge. Zymogram for β-glucosidase was developed by using methyl umbelliferyl glucoside as substrate which revealed the presence of only one isoform expressed in presence of both cellulose and xylan indicating that *A. niger* NCIM 1207 produced only one form of β-glucosidase (Fig. 2.4).



Crude broth with 25 µg of protein per well Crude broth with 5 IU of enzyme per well

Fig. 2.4. Zymogram showing the expression of single species of β-glucosidase in *A. niger* NCIM 1207 grown on medium supplemented with cellulose, xylan and xylose

Lane 1: Cellulose induced β-Glucosidase
Lane 2: Xylan induced β-Glucosidase
1.0 % urea, 1% glucose
Lane 3: Xylan induced β-Glucosidase
2% Glycerol, 0.5% urea
Lane 4: Xylose induced β-Glucosidase

Lane 1: Cellulose induced β-Glucosidase
Lane 2: Xylan induced β-Glucosidase
0.5% urea, 2% glycerol
Lane 3: Xylan induced β-Glucosidase
1% glucose, 1% urea

Effect of pH on enzyme activity and stability

Cellulose and xylan induced β-glucosidases and β-xylosidase assays were performed from pH 2.5 to 8.0 using 50 mM buffer systems at 65^0C for 30 min. The maximum activity of β-glucosidases (Figure 2.5 a, b) and β-xylosidase (Fig. 2.5c) was observed at pH 4.5 with a sharp decline in activity above pH 5.5. Negligible enzyme activity was observed at pH 3.0. The β-glucosidases (cellulose and xylan induced) were stable at a broad pH range from 3.0 to 7.5 even after 120 h incubation at room temperature (Fig. 2.6 a, b). The β-xylosidase was stable in a pH range from 3.0 to 6.5 when incubated for 120 h at room temperature and retained 85% activity even after 120 h at pH 6.5 at room temperature (Fig. 2.6c).

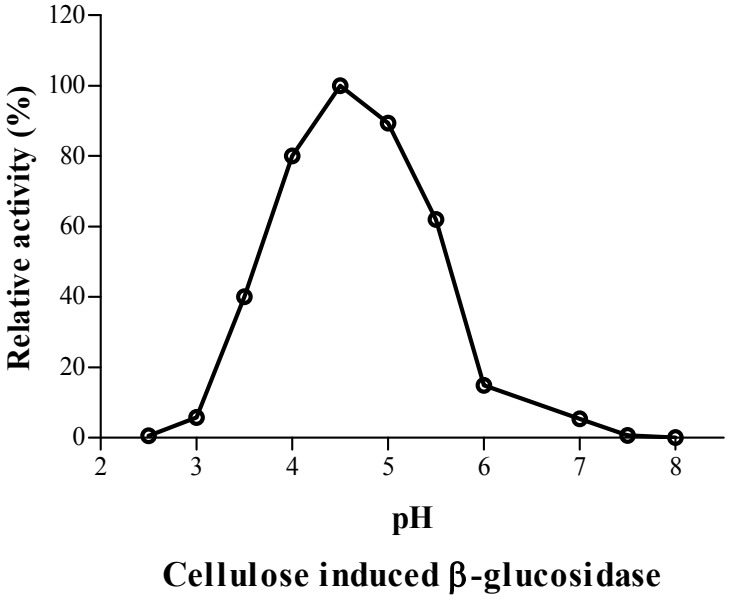


Fig. 2.5 a. Effect of pH on activity of cellulose induced β-glucosidase.

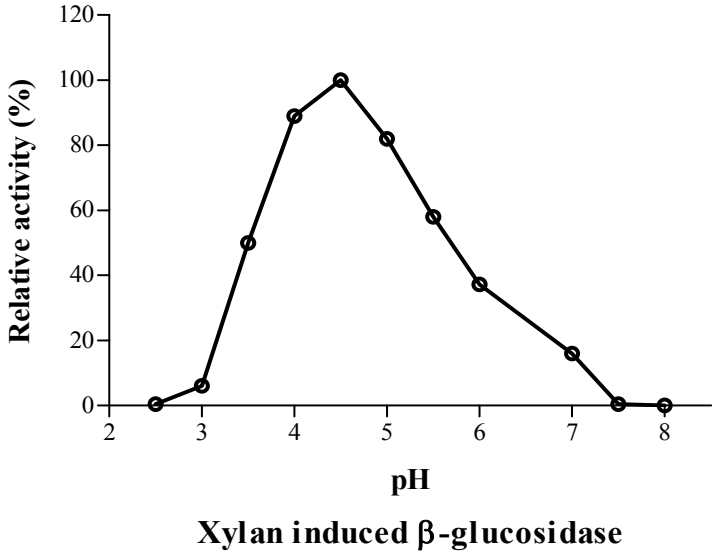


Fig. 2.5 b. Effect of pH on activity of xylan induced β-glucosidase.

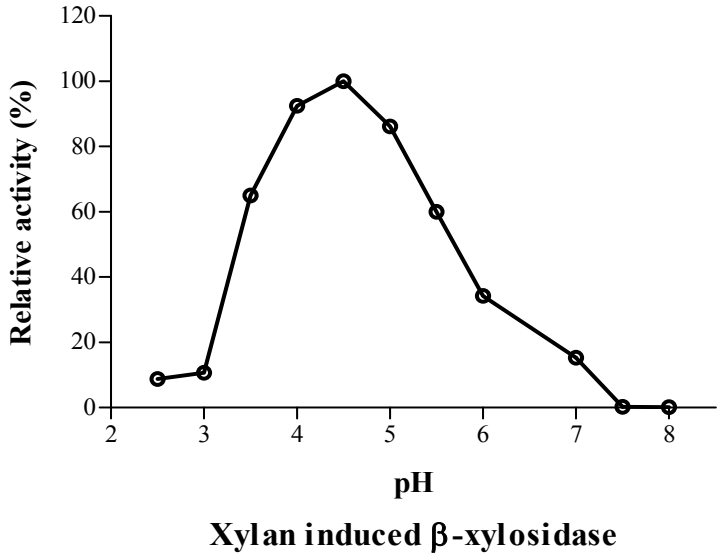


Fig. 2.5 c. Effect of pH on activity of xylan induced β-xylosidase.

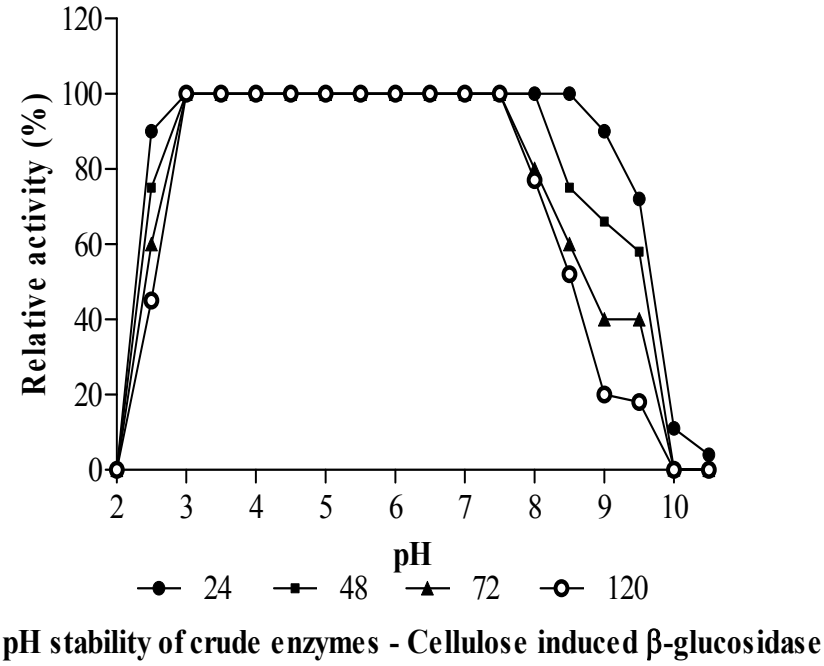


Fig. 2.6 a. Effect of pH on stability of cellulose induced β-glucosidase.

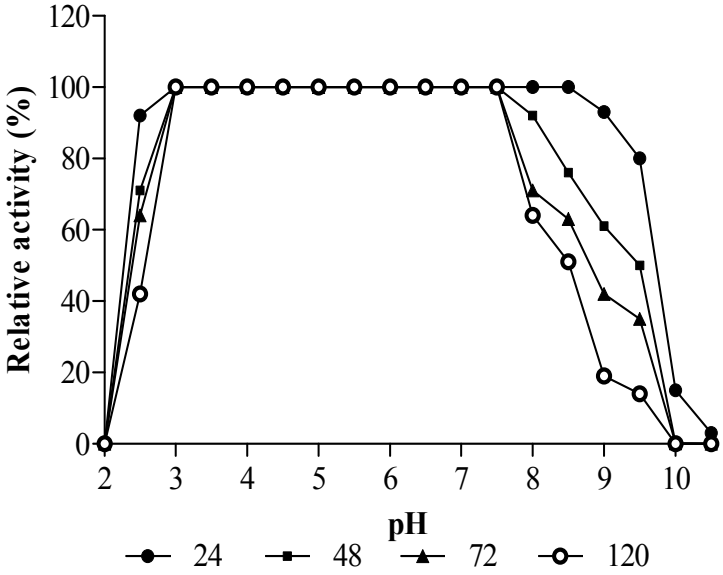


Fig. 2.6 b. Effect of pH on stability of xylan induced β-glucosidase.

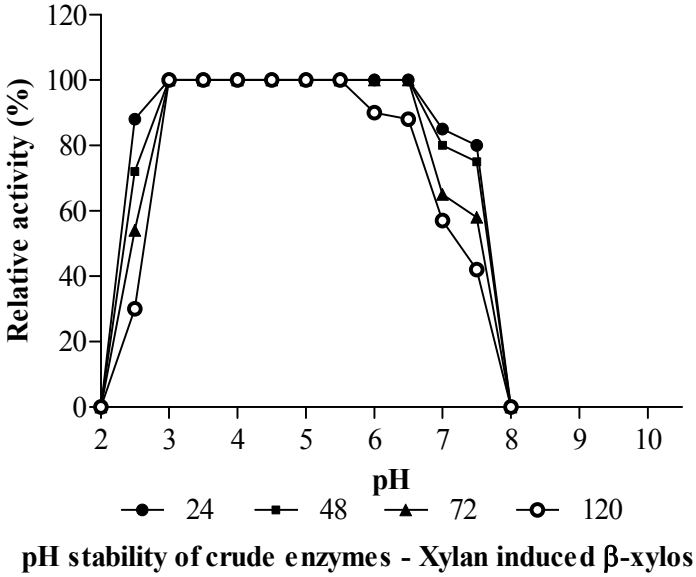


Fig. 2.6 c. Effect of pH on stability of xylan induced β-xylosidase

Effect of temperature on enzyme activity and stability

Enzyme activities were determined at different temperatures 50^0C to 80^0C under standard conditions. Cellulose induced β-glucosidase exhibited broad temperature optima (55^0C -65^0C) (Fig. 2.7a) while xylan induced β-glucosidase was active at 65^0C (Fig. 2.7b). Xylan induced β-xylosidase exhibited maximum activity at 65^0C with significant activity (90%) even at 70^0C (Fig. 2.7c). Studies on stability of enzymes at different temperatures (60^0C and 70^0C) revealed that both β-glucosidases and β-xylosidase were stable at 60^0C even after 5 h. Both β-glucosidases were totally inactivated after 1 h of incubation at 70^0C (Fig. 2.8a,b). Xylan induced β-xylosidase retained 100% of its original activity even after 5 h exposure at 70^0C (Fig. 2.8c).

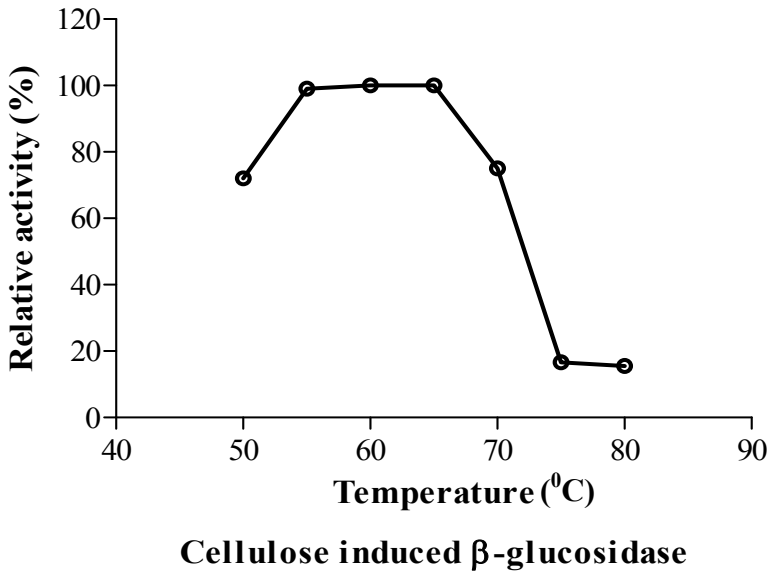


Fig. 2.7 a. Effect of temperature on activity of cellulose induced β-glucosidase.

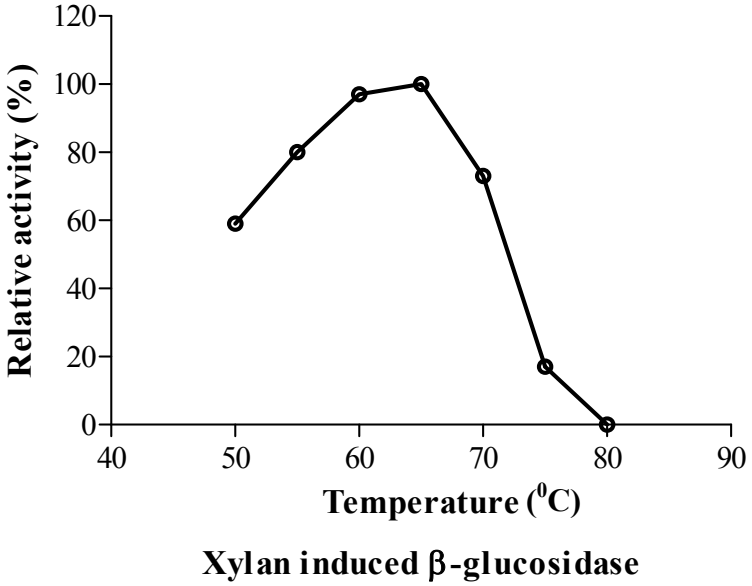


Fig. 2.7 b. Effect of temperature on activity of xylan induced β-glucosidase

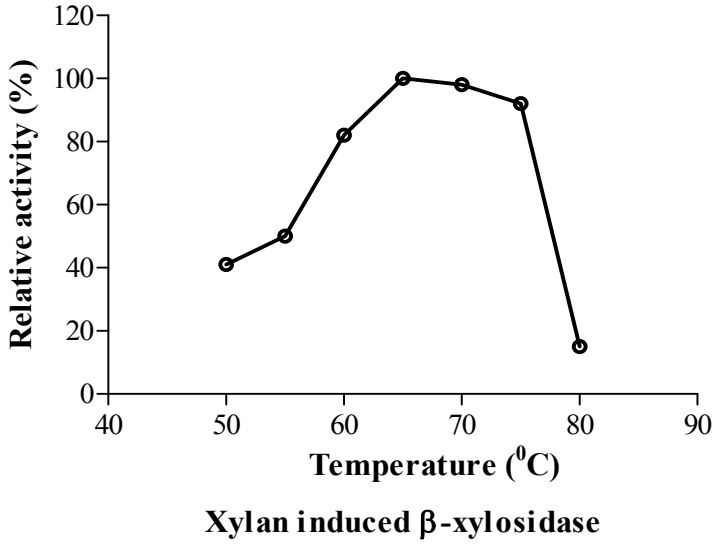


Fig. 2.7 c. Effect of temperature on activity of xylan induced β-xylosidase

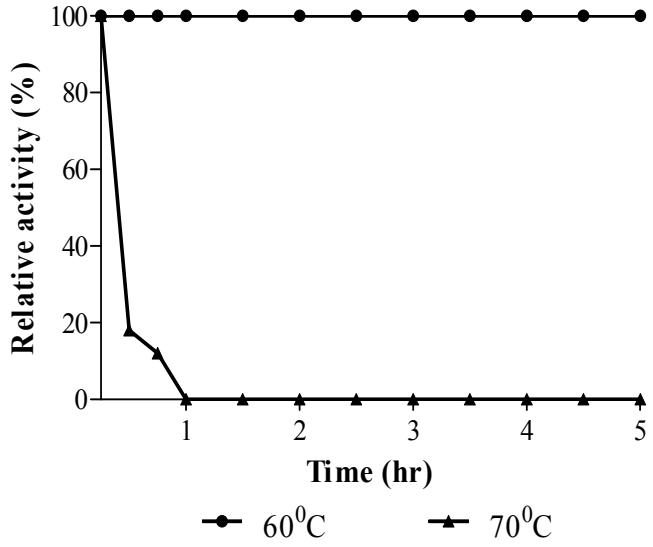


Fig. 2.8 a. Effect of temperature on stability of cellulose induced β-glucosidase.

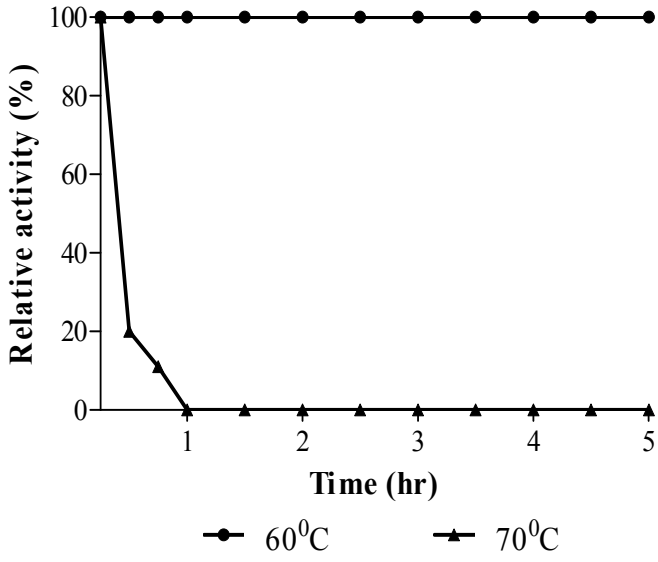


Fig. 2.8 b. Effect of temperature on stability of xylan induced β-glucosidase.

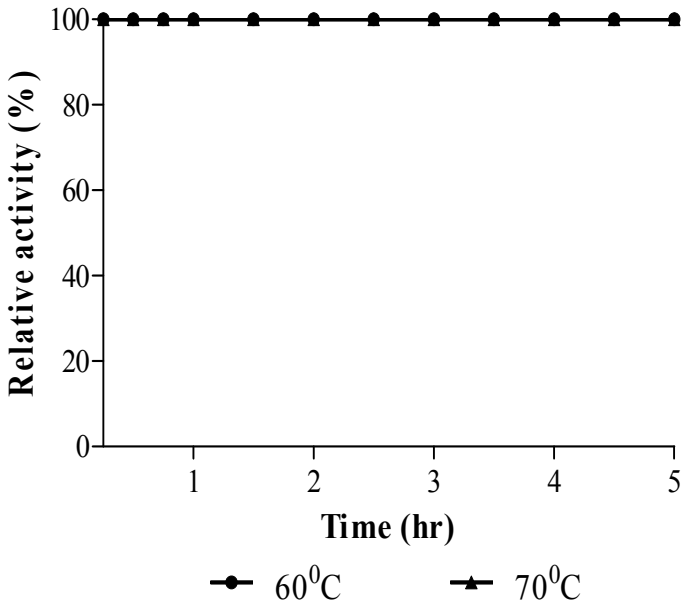


Fig. 2.8 c. Effect of temperature on stability of xylan induced β-xylosidase

Effect of different organic solvents on stability of crude enzymes

The effect of water miscible and water immiscible solvents at 25% concentration on stability of enzymes was studied and the results are shown in Table 2.3. Crude enzymes showed more than 90% of its original activity even after 24 h of incubation in hexane, chloroform, DMSO, acetonitrile, ethyl acetate, isooctane, toluene and hexadecane. Both cellulose and xylan induced β-glucosidases and β-xylosidase were not stable in 25% 1-4 dioxane with loss of total activity. In general, some of the solvents such as methanol, isoamyl alcohol, isooctane, propane diol, penta-1-ol showed 20-60% enhancement in enzyme activity. Especially, hexanol showed 80% enhancement in β-xylosidase activity.

Table 2.3. Effect of different organic solvents on stability of crude enzymes.

Organic solvents (25%)	Relative activity after 24 hrs incubation at 30^0C %		
	β-glucosidase (Cellulose induced)	β-glucosidase (Xylan induced)	B-xylosidase (xylan induced)
Control	100	100	100
Hexane	100	102	103
Chloroform	96	104	81
2-propanol	98	99	114
Methanol	130	122	146
Isoamyl alcohol	130	128	131
Xylene	98	94	101
Acetonitrile	100	102	98
DMSO	92	100	98
1-4 dioxane	00	00	00
Isooctane	158	149	141
Propan-1:2- Diol	130	131	138
Butane-1- ol	100	102	100
Tertiary Butyl alcohol	90	92	90
Cyclohexanol	90	92	116
Hexanol	127	134	180
Pentan-1-ol	130	131	142

DISCUSSION

A. niger NCIM 1207 is a potential fungal strain which produces high amounts of both β-glucosidase and little amount of β-xylosidase when grown on cellulose and xylan respectively (Gokhale et al., 1984; 1986). Glucose and fructose are known to support appreciable levels of β-glucosidase production in *A. terreus* (Kaur et al., 2007; Nazir et al., 2009). The present study demonstrates that this fungal strain produced only β-glucosidase and little amount of β-xylosidase when grown on cellulose or xylose containing media. However, it is reported that the best yield of β-xylosidase was obtained on xylose in case of *T. reesei* (Kristufek et al., 1995), *A. nidulans* (Perez-Gonzalez et al., 1998) and *A. versicolor* (Andrade et al., 2004). The growth of *A. niger* NCIM 1207 on glucose resulted in low levels of β-glucosidase production. It was also shown that both β-glucosidase and β-xylosidase were produced when xylan was used as substrate. Xylan is degraded by xylanolytic enzymes which produce disaccharide or high molecular weight substrates which have been found to be the best inducers of β-xylosidase. The xylose liberated by the action of β-xylosidase could act as inducer of β-glucosidase in *A. niger* NCIM 1207. This could be the reason for production of high amounts of both the enzymes when *A. niger* NCIM 1207 was grown in xylan. Xylan was also proved to be best β-xylosidase inducer for various filamentous fungi (Ito et al., 2003; Krogh et al., 2004).

The effect of addition of glucose and glycerol in xylan containing media on the production of both the enzymes was studied. The results showed no repression of enzyme production at 1% glucose but the higher concentration of glucose and glycerol suppressed both the enzyme activities. The growth of *A. niger* in a medium containing higher concentrations of glucose or glycerol caused the pH to suddenly drop below 3.0. This sudden drop could be responsible for the inactivation of the enzymes. Similar observations were made earlier in the case of β-glucosidase production by *A. niger* NCIM 1207 in cellulose-containing media (Gokhale et al., 1991). The supplementation of urea was shown to maintain the pH of the fermentation medium between 3.0 and 4.0 and to protect β-glucosidase from pH inactivation (Gokhale et al., 1992). To investigate whether the addition of urea to glycerol containing medium helps to maintain the pH of the medium, the culture was grown in a medium containing xylan (3%) in combination with

glycerol (2.5%) and urea (0.5%). It is clear from the results that activities of both β-glucosidase and β-xylosidase were increased.

The growth of fungus at 30^0C helped to maintain the pH between 3.5 and 4.5 throughout the period of fermentation. This supports our earlier observation that the pH must be maintained between 3.5 and 4.5 to obtain maximum enzyme production. The growth of *A. niger* at 36^0C caused the pH of the medium to rise above 6.0, which specifically affected β-xylosidase activity more than β-glucosidase. Highest levels of β-glucosidase and β-xylosidase activities were obtained when *A. niger* was grown at 30^0C for the first five days followed by further incubation at 36^0C. These results suggest that *A. niger* NCIM 1207 could be grown at lower temperature for the first five days (growth phase) followed by incubation at higher temperature (production phase) to achieve the highest enzyme activities. Such a procedure for growing the organism at lower temperatures followed by producing the enzymes at higher temperatures in case of fungal systems has not been reported so far.

It has been reported that *A. niger* KK2 produces β-glucosidase (100 IU/ g) and β-xylosidase (193 IU/g) activities in 6 days when grown on rice straw in solid state fermentation (Kang et al., 2004). *Trichoderma atroviride* TUB F-1663 produced only β-glucosidase (7.6 IU/g) when grown on steam pretreated spruce under submerged conditions (Kovacs et al., 2009). Vu et al., (2009) subjected *Aspergillus* sp., to, two rounds of repeated γ-irradiation of Co^{60} treatment and four rounds of treatment with N-methyl-N'-nitro-N-nitrosoguanidine. The best mutant designated as *Aspergillus* sp. XTG-4 was selected, and it produced 27.12 IU/mL of β-glucosidase activity on wheat bran. Thermophilic strains such as *Thermomyces lanuginosus* produce both β-glucosidase and β-xylosidase (Sonia et al., 2005), but the levels of β-xylosidase are low. Bokhari et al., (2010) reported the production of β-xylosidase by a newly isolated mutant of *Humicola lanuginosa*, M7D. It produced maximum β-xylosidase (728 IU/g substrate) when grown on Vogel's medium with xylan as carbon source. The same mutant produces remarkably high β-glucosidase activity (17.93 IU/ml) during growth on corncobs containing medium at 45^0C (Bokhari et al., 2008). Our strain *A. niger* NCIM 1207 produced significant levels of both β-glucosidase (18.6 IU/ml) and β-xylosidase (19.0 IU/ml) when grown on xylan containing media. This is the first report on production of both of these enzymes in high

amounts using xylan as substrate. This enzyme preparation could be efficiently used to supplement commercial cellulase preparations from *Trichoderma reesei* that are deficient in β-glucosidase and β-xylosidase. Very recently, Qing et al., (2010) showed for the first time that xylooligomers were far more inhibitory to cellulase than glucose, cellobiose, and xylose, thereby reducing the cellulose hydrolysis. These results suggest that hemicellulose removal from lignocellulosic materials prior to enzymatic hydrolysis is necessary to achieve higher saccharification. The results also reinforce the importance of β-xylosidase activities in cellulase and β-glucosidase enzyme preparations to hydrolyze hemicellulose to xylose, which is less inhibitory. The supplementation of commercial cellulases with such enzyme complex may also help to reduce enzyme doses needed to achieve complete hydrolysis of cellulose. We feel that *A. niger* NCIM 1207 is a potential candidate to produce both β-glucosidase and β-xylosidase in high amounts that can be used to supplement commercial cellulase preparations.

Most of the the β-glucosidases and β-xylosidases show optimum pH in the range of 4.5 to 5.5 (Eyzaguirre et al., 2005, Knob et al., 2010). However, some of the β-glucosidases such as from *Talaromyces emersonii* (McHale et al., 1981), *Botrytis cinerea* (Sakaki et al., 1994, 1995), and *A. aculeatus* (Sakamoto et al., 1985) showed optimum activity at acidic pH. Most of the fungal β-xylosidases have acidic pH optima ranging from 4.0 to 6.0. However, β-xylosidase from *S. thermophile* and *T. thermophilus* presented optimum activity at neutral pH (Katapodis et al., 2006; Guerfali et al., 2008). All the three enzymes were stable in majority of organic solvents such as hexane, propanol, methanol, isoamyl alcohol, xylene, acetonitrile, DMSO, iso-octane, propane-1: 2-diol, butane-1-ol, cyclohexanol, pentanol, acetone alcohol, etc. and activated by methanol, isoamyl alcohol, iso-octane, pentanol and hexanol.

A.niger NCIM 1207 was found to produce only one β-glucosidase when grown on either cellulose, xylan or xylose which is evident from zymogram developed using methylumbelliferyl β-D-glucoside. Nazir et al., (2009) reported that *A. terreus* produced multiple β-glucosidases. The study also showed that higher numbers of β-glucosidases were expressed on complex substrates as compared to fructose, glucose and cellobiose where only one β-glucosidase was expressed. This differential expression could be due to structural heterogeneity of the cellulosic substrates in addition to culture conditions.

CONCLUSION

Aspergillus niger NCIM 1207 produced high levels of both β-glucosidase and β-xylosidase when grown on xylan containing medium supplemented with glycerol and urea. Though the optimum temperature for enzymes production was 30^0C, the growth of the organism at 30^0C for first five day followed by further incubation at 36^0C enhanced the production of both the enzymes. The pH of the fermentation medium appears to play an important role in the production of enzymes. This enzyme preparation can be used for supplementation of commercial cellulases that are deficient in β-glucosidase and β-xylosidase production to achieve complete degradation of cellulosic materials.

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CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF β-GLUCOSIDASE AND β-XYLOSIDASE

ABSTRACT

The extracellular β-glucosidases (cellulose and xylan induced) and xylan induced β-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 the 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 β-glucosidases and 38% carbohydrate moiety in β-xylosidase. The isoelectric point (pI) of all three enzymes was around 4.6 which is evident from isoelectric focusing. The pH and temperature optima for all three enzymes were 4.5 and 65^0C respectively. They were stable over pH range between 3.5 to 6.0. For β-glucosidases t ½ at 70^0C was 10 minutes while for β-xylosidase it was 45 minutes. The purified β-glucosidases and β-xylosidase could be stored for 3-4 months at 4^0C and pH 4.5 without any loss of catalytic activity.

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

INTRODUCTION

β-Glucosidase (1,4- β-D glucohydrolase, EC 3.2.1.21) and β-xylosidase (1, 4- β-D xylohydrolase, EC 3.2.1.37) are glycoside hydrolases, a wide spread group of enzymes that hydrolyze glycosidic bonds between two or more carbohydrates or between a carbohydrate and a non carbohydrate moiety (Davies and Henrissat, 1995). They hydrolyze glycosidic bonds by one of two major mechanisms giving rise to either overall retention or overall inversion of the configuration of the anomeric substrate carbon (Sinnott, 1990). The glycosidic bond is the most stable and the enzymes accelerate the hydrolysis of glycosidic bonds by more than 10^{17} folds and thus are most efficient catalysts (Davies et al., 1998). β-glucosidase is a key enzyme regulating the whole cellulolytic process by hydrolyzing cellobiose which is a strong inhibitor of endoglucanase and exoglucanase (Harhangi et al., 2002). The bioethanol production from lignocellulosic biomass such as sugarcane bagasse has been studied for more than two decades, but it is still not economically feasible (Clomburg and Gonzalez, 2010, Rodriguez and Gonzalez, 2010).

The enzymatic hydrolysis of xylan involves the action of endoxylanases that hydrolyze internal β-(1, 4) xylosidic linkages in the insoluble xylan back bone to yield soluble oligosaccharides and xylobiose which are further hydrolyzed to produce xylose. β-xylosidase is rate limiting enzyme because it acts on xylobiose which is an inhibitor of endoxylanase. It has also shown that xylooligosaccharides are strong inhibitors of cellulose hydrolysis than xylose (Qing et al., 2010). Majority of these enzymes also showed transglycosylation activity (Xie et al., 2004). In addition to saccharification, β-glucosidases also have biotechnological applications in wine industry (Riou et al., 1998), for removal of terpenic residues of glucose (Yan and Lin, 1997), processing of food and beverages (Martino et al., 1994, Spagna et al., 1998), flavor improvement of beverages, decolorization of red fruit juices (Francis, 1989), debittering of citrus fruit juices (Roitner et al., 1984), synthesis of oligosaccharides and glycoconjugates by transglycosylation reaction (Christakopoulos et al., 1994, Harhangi et al., 2002). β-xylosidases are also used in ethanol production from pentoses, xylitol, polyalcohols which find wide applications as natural food sweetener, dental caries reducer and sugar substitute in diabetes (Parajo et al., 1998, Saha, 2003), in conversion of lignocellulose to food additives, chemical products (Skoog and Hahn-Hagerdal, 1988).

The role of enzymes in saccharification of lignocellulosic mass, production of transglycosylated molecules and potential biotechnological applications of the two enzymes have increased the interest of the researchers in hyper production, purification and characterization of these enzymes. Efforts are being made to reduce the cost of enzymes by understanding the production during fermentation and by development of simple method of purification. Many fungal strains are known to be efficient producers of β-glucosidase and β-xylosidase (Lynd et al., 2002, Knob et al., 2010). There are number of reports on production of β-glucosidase producing yeasts such as *S. cervisiae*, *Pichia etchellshii* and mesophilic fungi (*Trichoderma harzianum* and *Aspergillus* sp.) (Iwashita et al., 1998, Van Rensburg et al., 1998, Oh et al., 1999, Kaur et al., 2007).

The enzymes have been purified from fungal and bacterial sources using variety of methods involving ammonium sulphate precipitation. Some investigators have employed various methods of purification of β-glucosidases such as adsorption and desorption from hydroxylapetite (Kusama et al., 1986), controlled porosity glass activated by aminopropyltriethoxysilane and oxiranes and linked to salicin or cellobiose (Rogalski et al., 1991), Some reports demonstrated the use of concanavalin A coupled to Sepharose to purify a cellobiase from *T. viride*, taking advantage of its glycoprotein nature (Kminkova and Kucera, 1982), cellobiamine linked to epoxy – activated Eupergit C-30 N (Watanabe et al., 1992), isoelectric focusing (Deshpande et al., 1978; Schmid, and Wandrey, 1987) and chromatofocusing (Watanabe et al., 1992).

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.

MATERIALS AND METHODS

Materials

Cellulose -123 was obtained from Carl Scheicher and Schull Co. Dassel, FRG. Solka Floc SW 40 was from Brown Co, Berin. *p*-Nitrophenyl-β–D-glucopyranoside (*p*NPG), *p*-Nitrophenyl-β–D xylopyranoside (*p*NPX) and Oat spelt xylan were obtained from Sigma Chemical Company, USA. 3, 5-dinitrosalysilic acid, SDS PAGE markers, N-ethylmaleimide (NEM), Iodoacetate, phenyl methylsulfonyl fluoride (PMSF), *p*-chloromercuribenzoate (PCMB), diethyl-pyrocarbonate (DEPC), 1 ethyl-3-(3 dimethyl aminopropyl) carbidiimide (EDAC), 2-4-6trinitrobenzenesulfonic acid (TNBS), 5- bromosuccinimide (NBS), N-acetylimidazole (NAI), 2-3 butanedione, citraconic anhydride, acetic anhydride, phenyl glyoxal, HEPES and MES (Sigma – Aldrich, St. Louis, USA). Ampholytes (pH range 3-10) (Bio-rad). Aryl glycosides (*p*NP-β-D cellobioside, *p*NP-α-D-glucoside, *o*NP-β-D-galactopyranoside, *p*NP-β-D-glucoside, *p*NPα-galactopyranoside, phenyl Sepharose CL4B, Coomassie Brillient Blue G-250, Bromophenol Blue, Ampholytes – broad pH range (Sigma). All other chemicals were of analytical grade.

Enzyme assays

β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) activity was estimated as reported earlier (Gokhale et al., 1984) using *p*NPG as substrate. The total 1 ml of reaction mixture consisted of 0.9 ml of *p*NPG (1mg/ml) and 0.1 ml of suitably diluted enzyme. The reaction was initiated by the addition of enzyme followed by incubation at 65^0C for 30 min. The *p*-nitrophenol liberated was measured at 410 nm, after developing the color with 2% sodium carbonate. β-xylosidase (β-D-xylan xylohydrolase, EC 3.2.1.37) activity was estimated by the same method as above using *p*NPX (1mg/ml) as a substrate. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmole of *p*-nitrophenol from the substrate.

Protein determination

Protein was estimated according to Bradford's method (1976) with bovine serum albumin as standard. However, during gel permeation chromatography technique, protein concentrations were determined by using the formula $1.7A_{280}$ = 1mg/ml protein.

Production of enzymes

For cellulose induced β-glucosidase, submerged fermentation was carried out in AMM supplemented with 1% glucose, 1% urea and 2% solka flok at 30^0C for 13 days on a rotary shaker (150 rpm). The mycelium was separated by centrifugation at 8000 rpm for 15min.at 4^0C and the supernatant was used as a source of extracellular enzyme. For xylan induced β-glucosidase, submerged fermentation was carried out in AMM supplemented with 0.5% urea, 2% glycerol and 3% Xylan (Oat spelt) at 30^0C for 13 days on a rotary shaker (150 rpm). The mycelium was separated by centrifugation at 8000 rpm for 15min. at 4^0C and the supernatant was used as a source of extracellular enzyme. For xylan induced β-xylosidase, submerged fermentation was carried out in AMM supplemented with 1% glucose, 1% urea, and 3% Xylan (birch wood) at 30 ^{0}C for 6 days on a rotary shaker (150 rpm). The mycelium was separated by centrifugation at 8000 rpm for 15min.at 4^0C and the supernatant was used as a source of extracellular enzyme.

Purification of enzymes

Cellulose induced β-Glucosidase

First the precipitation was carried out by mixing fermented broth and ethanol at a proportion of 1:1.1 at 4^0C for 15min. It was centrifuged at 8000 rpm for 10 min at 4^0C. The supernatant was further precipitated by making the proportion of broth to ethanol as 1: 1.3 by addition of ethanol at 28^0C for 10 min and centrifuged at 5000 rpm for 5 min. The precipitate was suspended in citrate buffer (50mM) pH 4.5, centrifuged at 12,000 rpm for 8 min and supernatant was analyzed for enzyme activity, protein content and purity. The protocol for purification of cellulose induced β-glucosidase is given in Fig. 3.1.

Method : Fractional Ethanol Precipitation

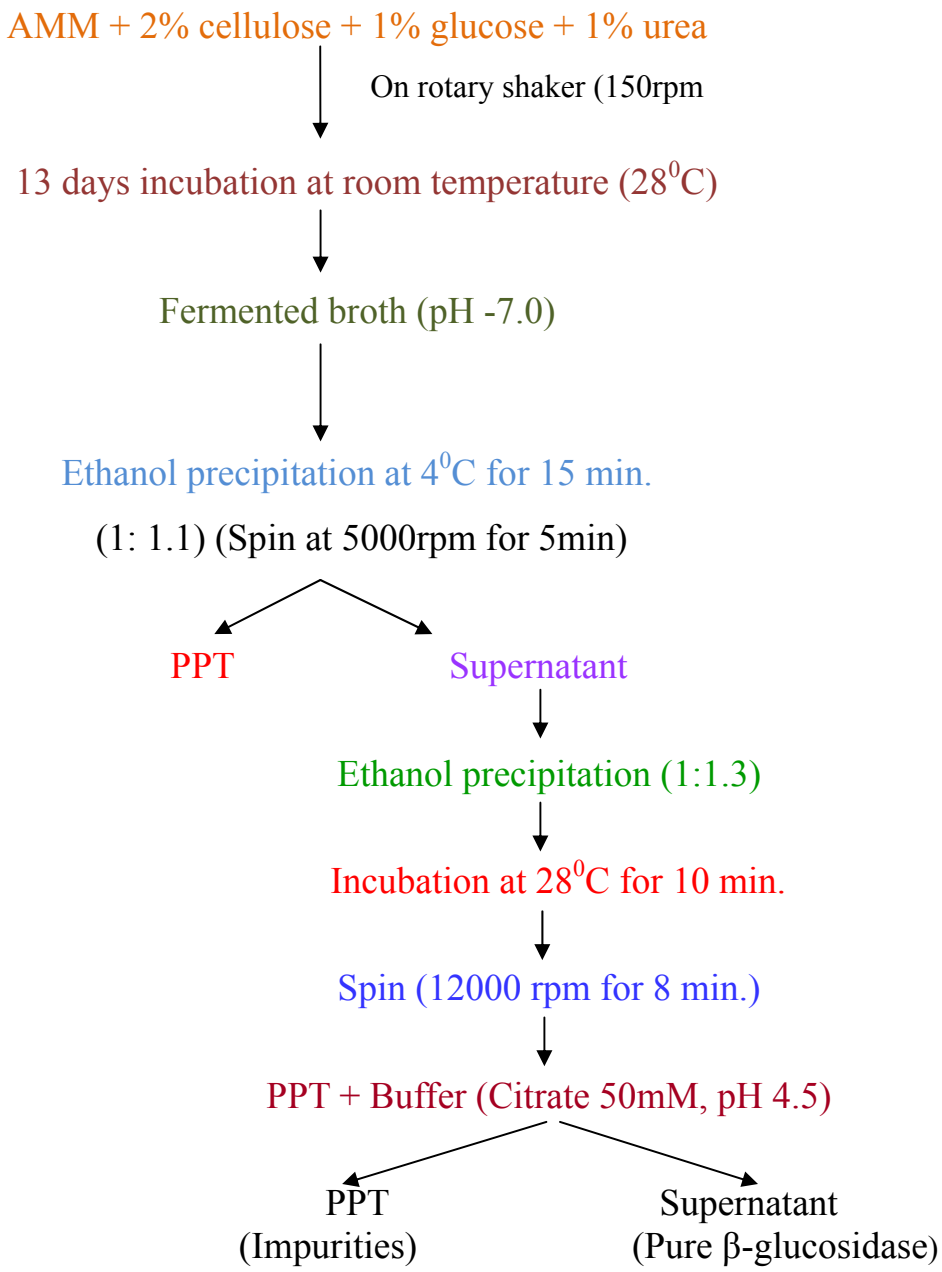


Fig. 3.1. Purification method for cellulose induced β-glucosidase

Xylan induced β-Glucosidase

The harvested broth (pH 4.5) was precipitated with ethanol at a proportion of 1:1.3 in water bath at 40^0C for 15min and centrifuged at 5000 rpm for 10 min. The precipitate obtained was suspended in water ethanol in a proportion of 1:1.4 (freshly prepared), mixed well and kept for 30 min at room temperature followed by centrifugation at 12,000 rpm for 15 min. The supernatant containing β-glucosidase was concentrated by rotavapour initially at 25^0C and then at 30^0C so as to remove ethanol and to concentrate the enzyme. This was further analyzed for enzyme activity, protein content and purity. The protocol for purification of xylan induced β-glucosidase is given in Fig. 3.2.

Method : Fractional Ethanol Precipitation

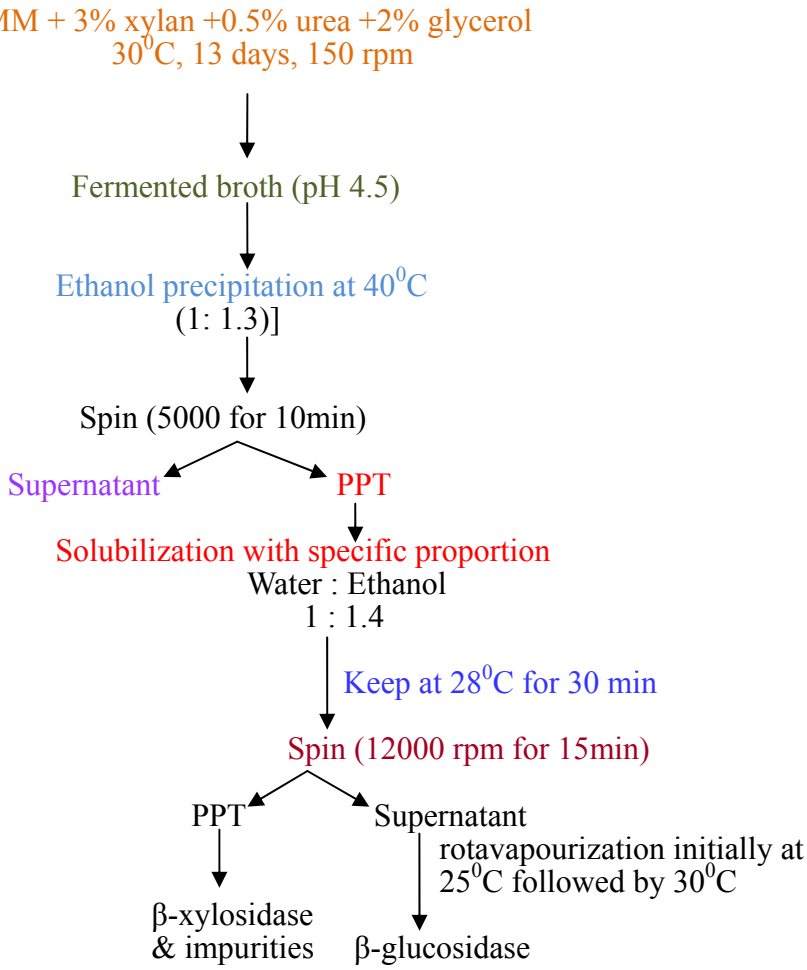


Fig. 3.2. Purification method for xylan induced β-glucosidase

Xylan induced β-Xylosidase:

The harvested broth (pH 3.0) was precipitated with ethanol at a proportion of 1:1.3 in water bath at 35^0C for 15min. and centrifuged at 5000rpm for 5min. The precipitate obtained was suspended in citrate buffer (250mM, pH 4.5), centrifuged at 12000rpm for 5min. The supernatant was kept at 72^0C for 45 min. and then centrifuged at 12,000 rpm for 5 min. This supernatant was analyzed for β-xylosidase activity, protein content and purity. The protocol for purification of xylan induced β-xylosidase is given in Fig. 3.3.

Methods : Fractional Ethanol Precipitation

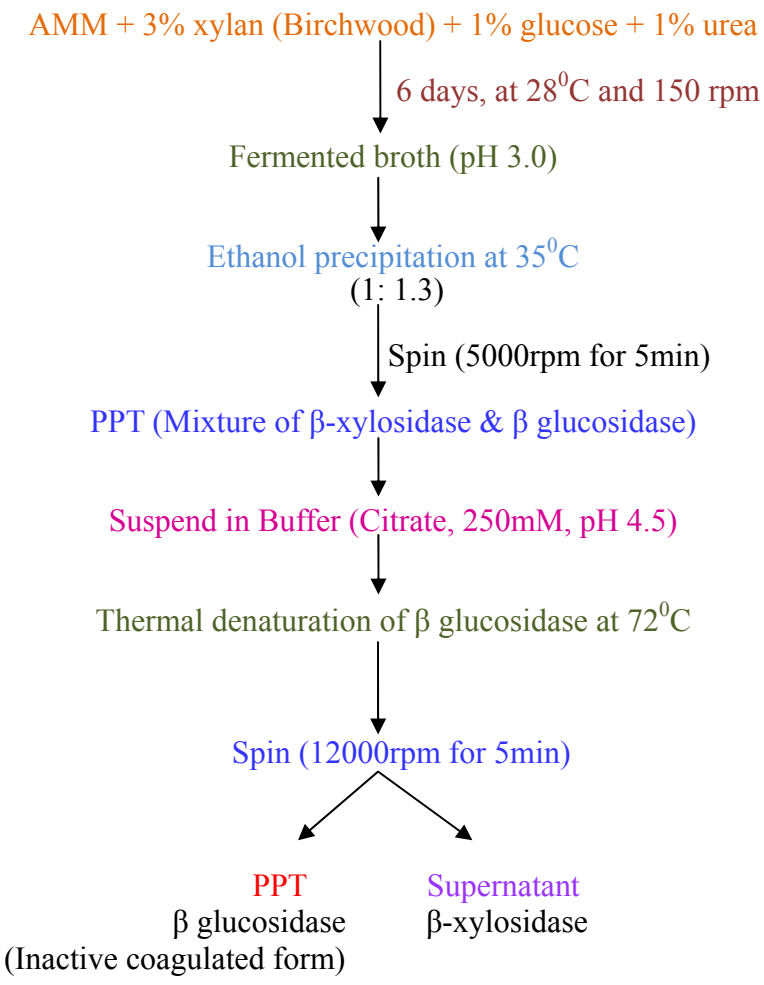


Fig. 3.3. Purification method for xylan induced β-xylosidase

Characterization of β-glucosidase and β-Xylosidase

SDS-PAGE

The homogeneity and molecular mass of β-glucosidase and β-xylosidase were determined by SDS-PAGE. This was performed using 8% acrylamide gel (Laemmli, 1970). High and low molecular weight standard markers (Sigma) were used to determine the molecular weight of the enzymes. After electrophoresis protein bands were visualized by silver staining. The Mr (relative molecular mass) of β-glucosidase and β-xylosidase were determined using the plot of log Mr of standard protein markers (Sigma) versus their relative mobilities (Hames, 1990).

Isoelectric Focusing (IEF)

IEF was performed using 8% acrylamide gel containing 2.4 % Ampholyte carrier with broad pH range (3-10). Ampholyte (Sigma) in a mini protein system (Biorad), Sodium hydroxide (20mM) and Trichloroacetic acid were used as catholyte and anolyte solutions respectively (O'Farrell, 1975). Isoelectric focusing was carried out for 1 ½ hour at constant voltage at 200V followed by 400 V for 1 ½ hour (Robertson et al., 1987). The gel was fixed in 10% trichloroacetic acid for 10 min. and soaked in 1% trichloroacetic acid overnight and then stained with Coomassie Blue staining.

Determination of isoelectric pH (pI)

The Isoelectric Focusing Polyacrylamide Gel Electrophoresis (IEF-PAGE) was performed (8% concentration) in mini gel apparatus (Biorad) using wide range (pH 3-10) ampholyte. Approximately 5µg of purified protein was applied to the gel and focused at 200V for 1 ½ hour and 400V for 2 hours. After running the gel it was cut into 0.5cm pieces and immersed in 1ml KCl (10mM), socked for 30 min. and these fractions were checked for pH.

The purified enzymes (500 µg) were loaded on to a phenyl sepharose CL4B column (1.6 X 90 cm) pre-equilibriated with 10mM citrate buffer pH 4.5, and fractionated at a flow rate of 12ml /h. Fractions of 2 ml were collected and those exhibiting protein and enzyme activity were identified. The molecular weight standard markers were also fractionated in a similar way. The molecular weight standard markers used were thyroglobulin (663 kDa), apoferrritin- horse spleen (443 kDa), β amylase from sweet potato (200kDa), bovine serum albumin (66kDa), carbonic anhydrase (29kDa) were used. K_{av} values for molecular weight

standards were plotted against log of molecular weight (Dalton) and standard graph was obtained and used to calculate the molecular weights of the native β-glucosidase and β-xylosidase (Whitaker, 1963)

Mass spectrometric analysis of the purified proteins

Proteins in gel bands were reduced, carboxyamidomethylated and digested with trypsin Gold (Promega) on a robotic platform for protein digestion (MassPREP station, Waters Ltd) as follows. The samples were incubated in 100 µl of de-stain solution (50 mM ammonium bicarbonate, 50% acetonitrile) for 10 minutes three times at 40^0C and, following removal of the final aliquot, the sample was dehydrated by incubation at 40^0C in 50 µl of acetonitrile for 5 minutes, the acetonitrile was removed and incubation was continued for 10 minutes to allow evaporation to occur. The sample was further processed by incubation at 40^0C in 50 µl of reducing solution (10 mM dithiothreitol, 100 mM ammonium bicarbonate) for 30 minutes and, following removal of the reducing solution, incubation at 40^0C in 50 µl of alkylation solution (55 mM iodoacetamide, 100 mM ammonium bicarbonate) for 20 minutes. The gel pieces were then washed at 40^0C in 50 µl of 100 mM ammonium bicarbonate for 10 minutes, 50 µl of acetonitrile for 5 minutes and dehydrated by double wash at 40^0C in 50 µl of acetonitrile for 5 minutes and evaporation for 5 minutes. The microtitre plate containing the gel slices was cooled to 6^0C for 10 minutes before addition of 30 µl per well of trypsin gold (Promega), diluted to 10 ng µl-1 in 50 mM ammonium bicarbonate. The plate was incubated at 6^0C for further 20 minutes to permit trypsin entry into the gel plugs with minimal autocatalysis before incubation at 37^0C for 4 hours. Samples were stored at 4^0C until MS analysis.

Resulting peptides were analysed by ESI-MS/MS after on-line separation on a PepMap C18 reverse phase, 75 µm i.d., 15 cm column (LC Packings) on a CapLC system attached to a Q-TOF2 mass spectrometer equipped with a nanolockspray source (Waters Ltd) and operated with MassLynx Version 4.0 acquisition software. At least two blank runs were run between each sample run to ensure carry-over contamination did not occur.

Tandem MS data were acquired using an automated data-dependent switching between the MS and MS/MS scanning based upon ion intensity, mass and charge state. In this automated acquisition type of experiment, a method was created in the MassLynx 4.0

software in which charge state recognition was used to select doubly, triply and quadruply charged precursor peptide ions for fragmentation. Up to four precursor masses at a time were chosen for tandem MS acquisition. A collision energy was automatically selected based on charge and mass of each precursor and varied from 15 to 55 eV.

ProteinLynxGlobalServer version 2.0 (Waters, Ltd) was used to process the uninterpreted MS data into peak list (pkl) files which were searched against all entries in the Swissprot and/or NCBInr databases (version 2010_12) using the web version of the MASCOT MS/MS ions search tool (http://www.matrixscience.com) (Perkins et al., 1999 or http://www.matrixscience.com). Carbamidomethylation of cysteine and oxidation of methionine were set as variable modifications. One missed cleavage by trypsin, and semitryptic fragements were accepted. Other than file type (Micromass pkl) and instrument type (ESI-QUAD-TOF), all remaining search values were the preset defaults. Only protein identifications with probability-based MOWSE scores above a threshold of $P<0.05$ were accepted. In addition, some fragmentation data was analyzed manually for selected peptides and de novo sequencing performed using the PepSeq function of the MassLynx 4.0 software.

Effect of pH on enzyme activity and stability

The effect of pH on enzyme activity was determined by assaying enzyme activities at 65^0C in a pH range of 2.0 -11.0 using following 50mM buffer systems; KCl-HCl buffer (pH 2.0), citrate phosphate buffer (pH 2.5 - 6.0), phosphate buffer (pH 7.0) and glycine NaOH buffer (pH 8.0 - 11.0). Stability assays were performed by incubating the purified enzymes at 30^0C for 24 hours in 50mM buffers at different pH values such as KCl-HCl buffer (pH 2.0), citrate phosphate buffer (pH 2.5 - 6.0), phosphate buffer (pH 7.0) and glycine NaOH buffer (pH 8.0 - 11.0). The residual enzyme activity was then assayed under standard assay conditions considering the enzyme activity at 0 time as 100%.

Effect of temperature on enzyme activity and stability

Measurement of enzyme activities against *p*NPG were carried out at pH 4.5 under standard assay conditions. The reaction mixture was incubated at different temperatures in a range of 30-80^0C. Temperature stability studies were performed by incubating the enzyme in 50mM citrate buffer, pH 4.5, for 3 h at different temperatures (40-80^0C). The residual enzyme activity was determined under standard assay conditions.

Effect of organic solvents on stability

Enzyme stability in water miscible organic solvents was determined by incubating the enzyme in 50mM citrate buffer (pH 4.5) with 25% (v/v) organic solvent for 24 hour at 30^0C. Enzyme stability in water immiscible organic solvents was determined by incubating the enzyme solutions prepared in 50mM citrate buffer (pH 4.5) with 25% (v/v) organic solvents for 24 hour at 30^0C shaking at 100 rpm on rotary shaker. The residual activity was estimated under standard assay conditions.

Effect of metal ions and EDTA on enzyme activity

The effect of metal ions and EDTA on enzyme activity was determined by enzyme assays in presence of various metal ions and EDTA at final concentration of 0.1mM and 1mM using *p*NPG and *p*NPX for β-glucosidase and β-xylosidase respectively.

Substrate specificity study

Substrate specificities of β-glucosidase and β-xylosidase were determined against *p*NP-β-glucopyranoside (3mM), *p*NP-α-D-glucopyranoside (3mM), *p*NP-β-D galactopyranoside (3mM), *p*NP-α-D galactopyranoside (3mM), *p*NP-α-D mannoside (3mM), *p*NP-α-L-arabinopyranoside (3mM), *p*NP-β-xylopyranoside (3mM), *p*NP-β-D-cellobioside (3mM), *o*NP-α-D galactopyranoside (3mM) as substrates (Sigma) using the standard assay described for β-glucosidase and β-Xylosidase. The activities against polysaccharides such as CMC (1%), Avicel (1%), solka floc (1%), xylan birch wood (1%), xylan oat spelt (1%) were determined by incubating the reaction mixture (1ml) containing 100 µl enzyme, 400 µl citrate buffer (50mM, pH 4.5) and 500 µl of respective substrate (1% w/v) at 65^0C for 1h. The reducing sugars released were quantified by 3:5 dinitrosalicylicacid (DNAS) method (Fischer and Stein 1961). The activities on sucrose (1%), lactose (1%), maltose (1%) and D-cellobiose (1%) were estimated by assaying the amount of glucose released using GOD-POD method (Lin et al., 1999).

Effect of mono/disaccharides and alcohols on enzyme activities

The effect of mono/disaccharides (1mg/ml) and alcohol (methanol, ethanol and propan-2-ol) with 20 % (v/v) on β-glucosidase and β-xylosidase activities was studied using *p*NPG and *p*NPX substrate.

Determination of Michalis – Menten constant

The values for the K_m and V_{max} for all the three purified enzymes were determined from double reciprocal plots of substrate concentration [S] versus initial reaction rates [V] (Lineweaver and Burk, 1934). Enzyme assays with appropriately diluted β-glucosidase and β-xylosidase were performed using *p*NPG and *p*NPX as a substrate respectively under standard assay conditions. The concentrations of substrates were 0.33 -13.20 mM for β-glucosidase and 0.369 – 14.76 mM for β-xylosidase (Workman and Day 1982; Wase et al., 1985).

Chemical modification studies using group specific reagents

Purified β-glucosidases and β-xylosidase (5µg each) were incubated with specific chemical reagents to different amino acid functional groups with specific concentration. Reaction conditions were given in Table 3.11 after 30 min incubation at 30^0C, residual activity of enzyme samples was determined by standard assay method.

Carboxylate residues

Modification of carboxylate residue was performed by incubating β-glucosidase and β-xylosidase (10µg) with varying concentrations of EDAC (10µg to 500µg) in 1ml of 50mM MES/HEPES buffer (75: 25) pH 6.0 at 30 ^{0}C. The control was kept without addition of EDAC. Samples were withdrawn after suitable time intervals and the reaction was terminated by addition of 1 mL of 50 mM citrate buffer pH 4.5. The residual activity of modified enzyme was determined under standard assay conditions.

Chemical modification of tryptophan

Tryptophan residues were modified by incubating purified enzyme with increasing concentrations of NBS (0.1 to 1.0 mM) in 50 mM of sodium citrate buffer pH 4.5 at room temperature. After 10 min, the aliquots were removed for analysis of residual enzyme activity. The NBS mediated inactivation was also monitored spectroscopically by monitoring the decrease in absorbance at 280nm.

Substrate protection study

The protective effect of the substrate on chemical modification reactions was studied by incubating enzymes (10µg) with excess amount of substrate i.e. *p*NPG or *p*NPX (1mg/ml)

followed by treatment with corresponding modified reagents. The residual enzyme activity was assayed periodically under standard assay conditions.

RESULTS

Production of β-glucosidase and β-xylosidase

The submerged fermentation in AMM supplemented with 1% glucose, 1% urea and 2% solka floc was carried out for 13 days at 30^0C. The broth with pH 7.0 and 13 IU/ml enzyme activity was further used for purification of cellulose induced β-glucosidase.

For purification of xylan induced β-glucosidase, the submerged fermentation was performed in AMM supplemented with 0.5% urea, 2.5% glycerol and 3% oat spelt xylan. The flasks were incubated initially at 30^0C for 5 days followed by 35^0C for further 8 days. The broth with pH 4.6, containing 13 IU/ml of β-glucosidase and β-xylosidase enzyme activities was used for further purification.

The submerged shake flask fermentation in AMM supplemented with 1% glucose, 1% urea and 3% xylan (birch wood) for 6 days resulted in production of 3.5 IU/ml β-glucosidase and 7.5 IU/ml β-xylosidase enzymes. The fermented broth containing xylan induced β-xylosidase with pH 2.8 -3.0 was used for purification. Such a low pH broth containing comparatively less activity of β-glucosidase was found to be suitable for purification of β-xylosidase.

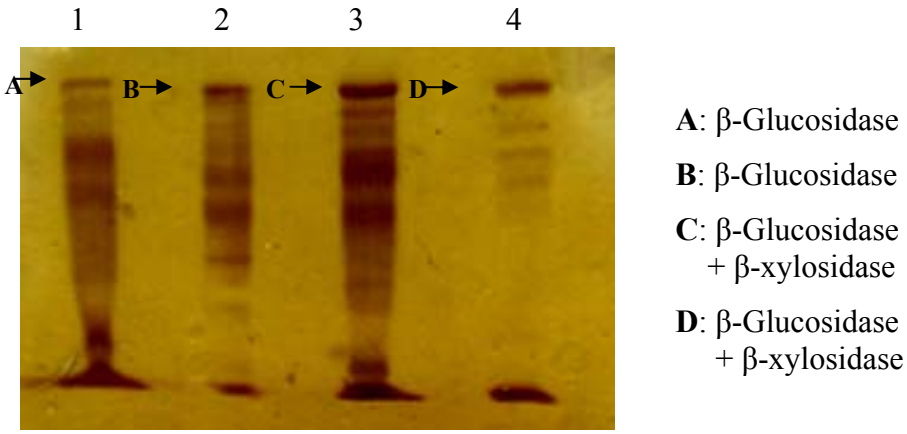


Fig. 3.4. SDS PAGE of crude broth. The samples were electrophoresed on 8 % w/v of SDS PAGE and stained with silver stain. Lane 1 : AMM+cellulose, Lane 2 : AMM+Xylose, Lane 3 : AMM+Xylan, Lane 4 : AMM+Xylan 6 day

Purification of β-glucosidase and β-Xylosidase

Cellulose induced β-glucosidase

The fractional ethanol precipitation of fermented broth as given in methods led to purification of β-glucosidase to homogeneity. The results of purification of cellulose induced β-glucosidase are summarized in Table 3.1. The enzyme was purified approximately 16 fold with overall yield of 23 % and specific activity 751U/mg protein. The purified enzyme was stored in 50 mM citrate buffer, 4.5 at -20^{0}C for 3 to 4 months without any apparent loss in its initial activity. The purified enzyme was analyzed by SDS-PAGE which revealed a single band of a protein as shown in Fig. 3.5.

Table 3.1. Purification of cellulose induced β glucosidase

Purification step	**Total activity (IU)**	**Total protein (mg)**	**Specific Activity (IU/mg)**	**Fold purification**	**Yield (%)**
Culture filtrate	800	18	44.4	1	-
Fractional ethanol precipitaion	184	0.245	751	16	23

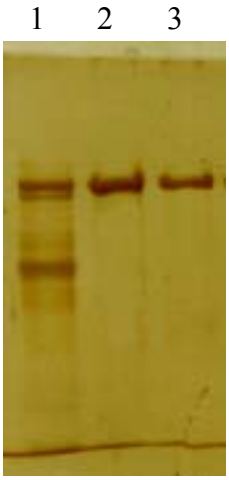


Fig. 3.5. SDS-PAGE of purified cellulose induced β-Glucosidase. The purified protein was electrophoresed on 8% w/v of SDS PAGE and stained with silver stain. Lane 1 : Crude broth, Lane 2 : β-glucosidase 2µg, Lane 3 : β-glucosidase 1µg.

Xylan induced β-glucosidase

The xylan induced β-glucosidase was purified to homogeneity as given in method. The enzyme was purified 13.4 fold with 22 % yield and specific activity of about 645 IU/mg protein. The enzyme was stored at -20^{0}C for 3 to 4 months without any apparent loss in its initial activity. The results of the procedure for the purification of xylan induced β-glucosidase are summarized in Table 3.2. The purified enzyme was analyzed by SDS-PAGE which revealed a single band of a protein as shown in Fig. 3.6.

Table 3.2. Purification of xylan induced β glucosidase

Purification step	**Total activity (IU)**	**Total protein (mg)**	**Sp. Activity IU/mg**	**Fold purification**	**Yield (%)**
Culture filtrate	1300	27	48	1	-
Ethanol ppt. & Solubilization with specific proportion of water & ethanol	286	0.443	645	13.4	22

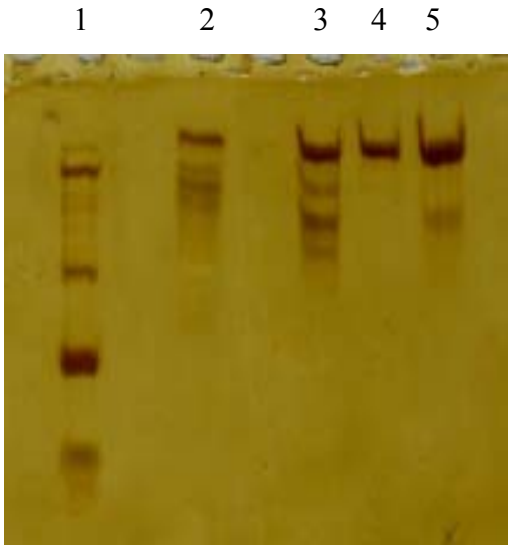


Fig. 3.6. SDS-PAGE of purified xylan induced β-glucosidase (Lane 4). The samples were electrophoresed on 10 % w/v of SDS PAGE and stained with silver stain. Lane 1 : molecular weight standard, Lane 2 : crude broth, Lane 3 : 1:1.3 precipitate at 4^{0}C, Lane 4 : purified β-Glucosidase, Lane 5 : 1:1.3 precipitate at 40^{0}C.

Xylan induced β-xylosidase

The xylan induced β-xylosidase was purified to homogeneity as given in the method. The purification of β-xylosidase was 6.4 fold with 20% yield. The specific activity of the enzyme was 545 IU/mg protein. The results of the procedure for purification of xylan induced β-xylosidase are summarized in Table 3.3. The purified enzyme was stored in 50 mM citrate buffer, pH 4.5 for 3 to 4 months at -20^{0}C without any loss of activity. The purity of enzyme was checked by SDS- PAGE which showed a single band (Fig.3.7).

Table 3.3. Purification of Xylan induced β-xylosidase

Purification step	Total activity (IU)	Total protein (mg)	Sp. Activity IU/mg	Fold purification	Yield (%)
Culture filtrate	780	9.2	84	1	-
Ethanol ppt.	372	0.350	1065	12.6	47
Thermal treatment for 45 min	109	0.200	545	6.4	14

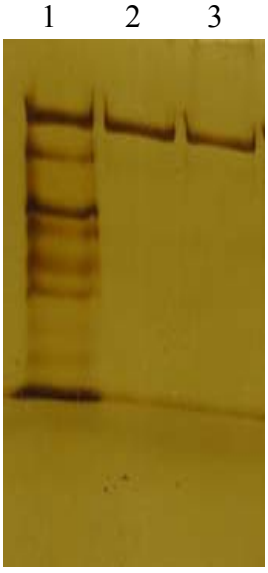
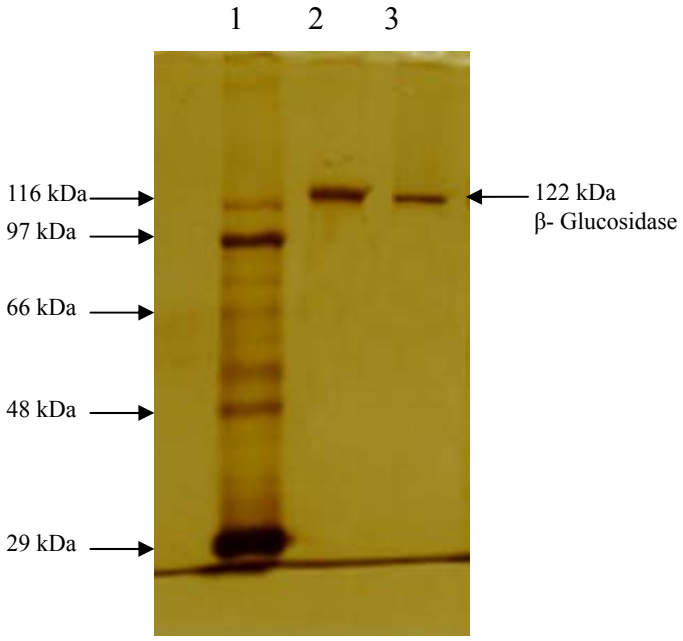


Fig. 3.7. SDS-PAGE of purified xylan induced β-xylosidase. The samples were electrophoresed on 10 % w/v of SDS PAGE and stained with silver stain. Lane 1 : crude broth, Lane 2 : 1:1.3 precipitate at 35^{0}C, Lane 3 : purified β-xylosidase.

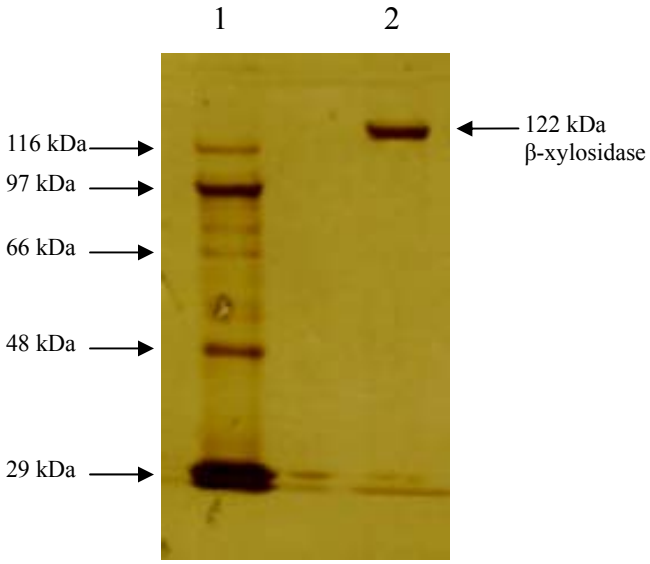
Molecular properties of enzymes

The apparent molecular weights of the purified β-glucosidases induced by cellulose and by xylan were determined by SDS-PAGE and it was estimated to be around 122 kDa (Fig. 3.8). The molecular weight of xylan induced β-xylosidase was observed to be 122kDa (Fig. 3.9). The purity of both β-glucosidases as well as β-xylosidase was further confirmed by 8% w/v native gel electrophoresis with Coomassie Brilliant blue stain as a single protein band as shown in Fig.- 3.10



Lane 1- Molecular weight markers
Lane 2- Cellulose induced β- glucosidase 1.5 µg
Lane 3- Xylan induced β- glucosidase

Fig. 3.8. SDS-PAGE of cellulose and xylan induced β- glucosidase with molecular weight standards. The purified protein was electrophoresed on 8% (w/v) of SDS PAGE and was stained with silver stain. The markers used were β- galactosidase (116.0 kDa), phosphorylase b (97.4 kDa),bovine serum albumin (66kDa) fumerase (48.0 kDa) and carbonic anhydrase (29.0 kDa).



Lane 1- Molecular weight markers
Lane 2- Xylan induced β-xylosidase 2.0 µg

Fig. 3.9. SDS PAGE of xylan induced β- xylosidase with molecular weight standards

The purified protein was electrophoresed on 8% (w/v) of SDS PAGE and stain with silver stain. The markers used were β-galactosidase (116.0 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66kDa), fumerase (48.0 kDa) and carbonic anhydrase (29.0 kDa).

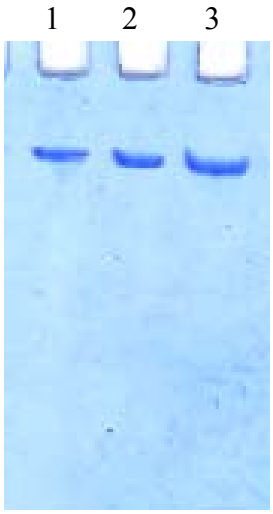


Fig. 3.10. Native PAGE of purified enzymes. The protein samples (1–2 µg) were electrophoresed on 8 % w/v of native PAGE and stained with Coomassie Brilliant Blue stain. Lane 1 : cellulose induced β-glucosidase, Lane 2 : xylan induced β-glucosidase, Lane 3 : xylan induced β-xylosidase.

Molecular weight of native enzymes

Native molecular weights of purified β-glucosidases and β-xylosidase from *A. niger* 1207 were determined by gel permeation chromatography using Sepharose CL4B column equilibriated with 10mM citrate buffer (pH 4.5). The partition coefficients (k_{av}) for purified enzymes and standard molecular weight markers such as thyroglobulin (663kDa), apoferritin from horse spleen (443 kDa), β-amylase from sweet potato (200kDa), bovine serum albumin (66kDa) and carbonic anhydrase (29kDa) were determined. A graph of K_{av} verses log of molecular weight (Da) was plotted. The molecular weights of native β-glucosidases (cellulose and xylan induced), β-xylosidase (xylan induced) were approximately 336 kDa.

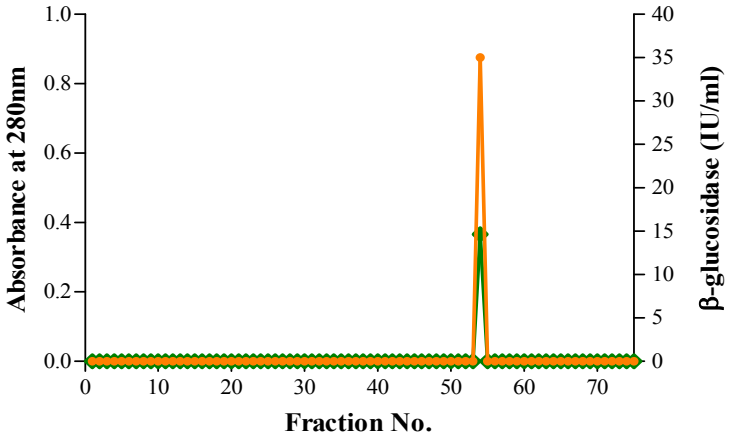


Fig. 3.11. Elution profile of the purified β-glucosidase through Sepharose CL4B column

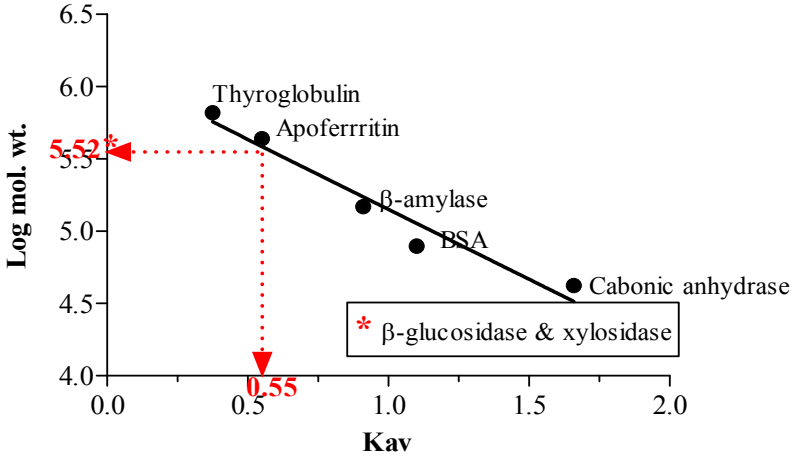


Fig. 3.12. Determination of molecular weight of native protein by gel permeation chromatography

Isoelectric focusing

Analytical IEF data demonstrated that β-glucosidases and β-xylosidase are the acidic proteins. The cellulose as well as xylan induced β-glucosidases and β-xylosidase show acidic pI of 4.6. (Fig. 3.13).

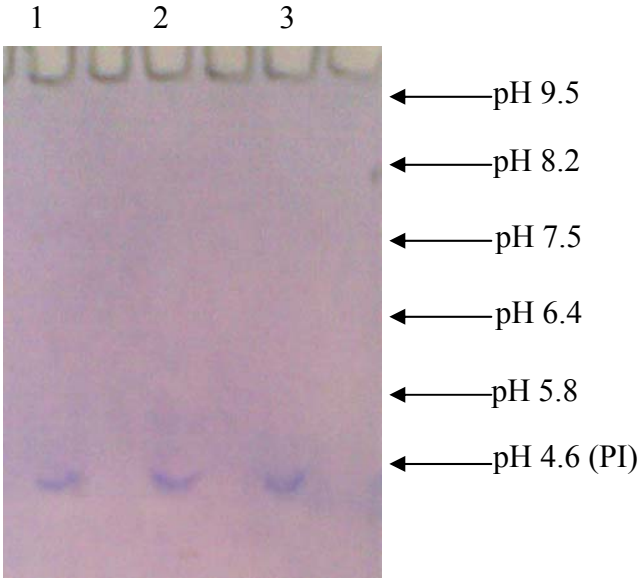


Fig. 3.13. IEF - PAGE of purified enzymes from *A. niger* NCIM 1207. All three purified enzyme samples, 5 μg each were electrophoresed using wide range ampholyte pH (3-10) as described in method. After electrophoresis, gel was stained with Coomassie Brilliant Blue R – 250 stain (Deutscher, 1990). Lane 1: Cellulose induced β-glucosidase, Lane 2: xylan induced β-glucosidase, Lane 3: xylan induced β-xylosidase

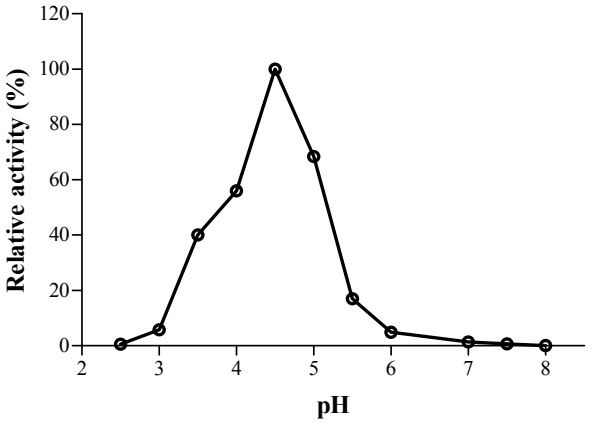
Mass spectrometric analysis

Mass spectrometric analysis (Please see Annexure I) 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

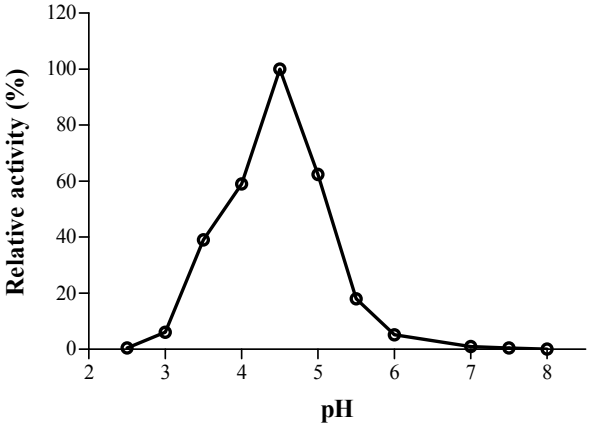
Biochemical properties of β-glucosidases and β-xylosidase

pH optimum and stability

Maximum activity of purified β-glucosidases (cellulose and xylan induced) was obtained at pH 4.5. The activity was decreased significantly at pH 3.0 and 6.0. (Fig. 3.14). β-xylosidase was also found to be active at pH 4.5. The activity was mush affected at pH 7.0.



Cellulose induced β-glucosidase



Xylan induced β-glucosidase

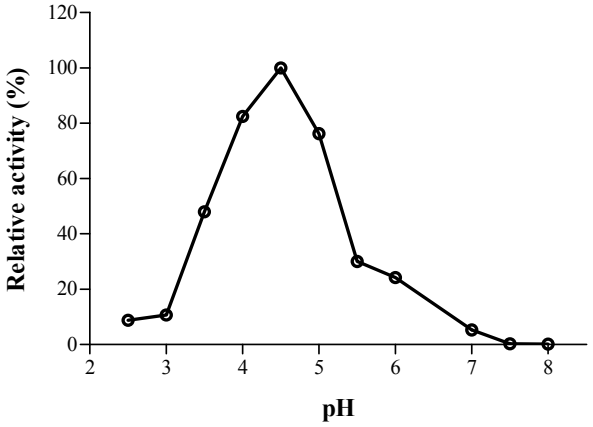
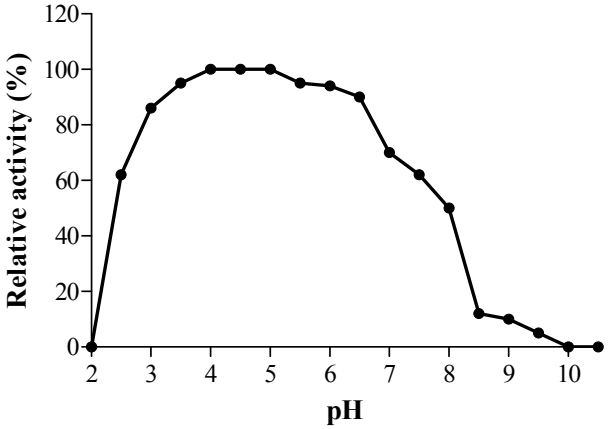
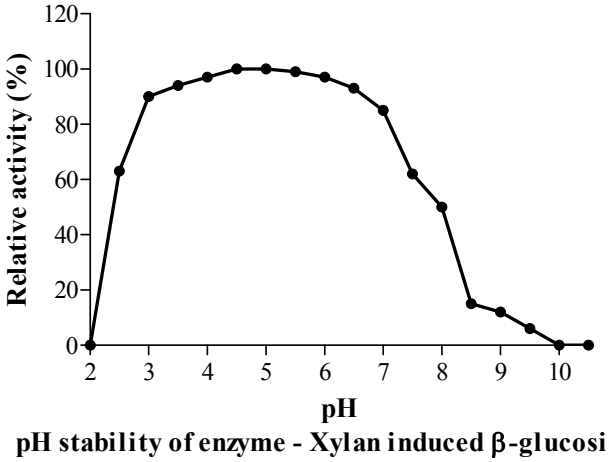


Fig. 3.14. Effect of pH on activity of purified β-glucosidases and β-xylosidase from *A. niger* NCIM 1207. A suitably diluted purified enzyme was incubated in a series of pH 2.0 – 8.0 at 30^0C for a period of 24 h and the residual activity was determined under standard assay conditions as described in methods. Maximum activity obtained was taken as 100%.

The β-glucosidases (cellulose and xylan induced) were stable at broad pH range (3.0 to 7.5) even after 120 h of incubation at room temperature. The β-xylosidase was stable in a pH range 3 to 6.5 when incubated for 24h at room temperature and retained 85% activity even after 120h at pH 6.5 at room temperature. However, β-xylosidase lost 60% of its initial activity at pH 7.0.





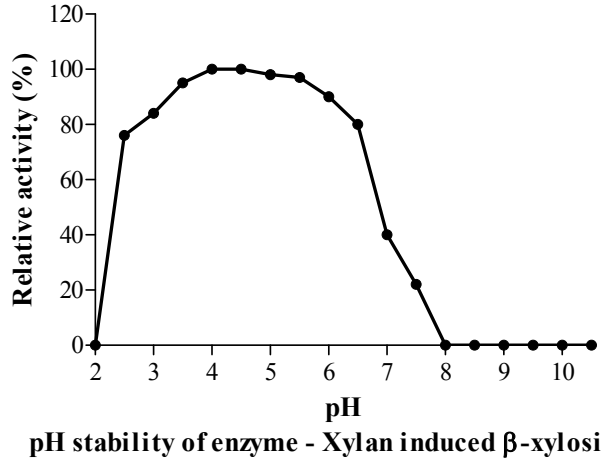
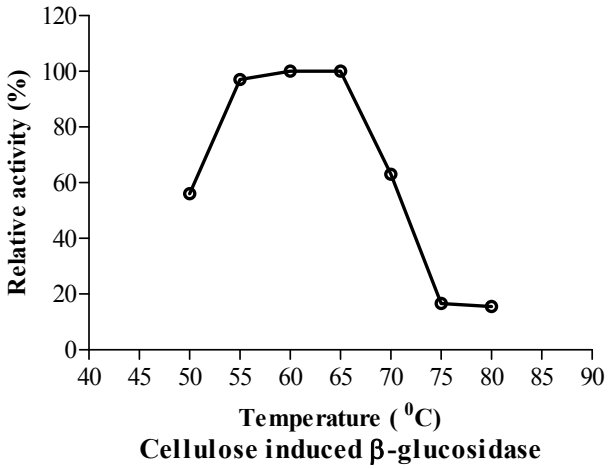
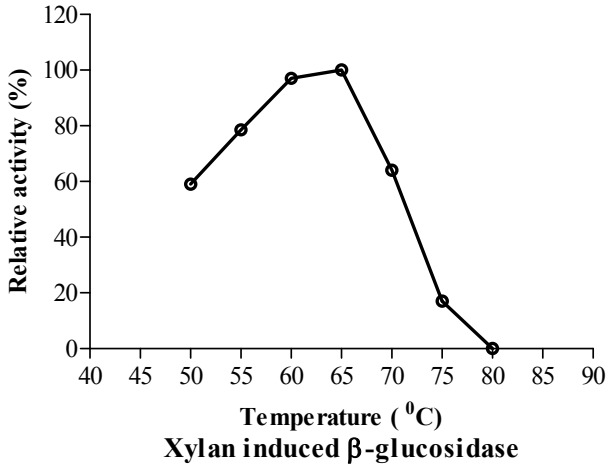


Fig. 3.15 Effect of pH on stability of purified β-glucosidases and β-xylosidase from *A. niger* NCIM 1207.

Effect of temperature on enzyme activity and stability

Cellulose and xylan induced β-glucosidases exhibited maximum activity at 65^0C with substantial activity between 55^0C to 75^0C (Fig. 3.16) with almost 90% loss in activity at 75^0C. Xylan induced β-xylosidase exhibited maximum activity at 65^0C with retention of more than 90% activity between at 75^0C. Studies on stability of enzymes at different temperatures (50^0C, 60^0C and 70^0C) revealed that both β-glucosidases (cellulose and xylan induced) and β-xylosidase were stable at 50^0C even after 5 h. Both β-glucosidases were stable at 60^0C for 2 ½ h while β-xylosidase was stable for 3h. Both β-glucosidases have lost 85% of the initial activity after 15 min exposure at 70^0C while β-xylosidase lost about 50% of its activity at 70^0C after 45 min of exposure.





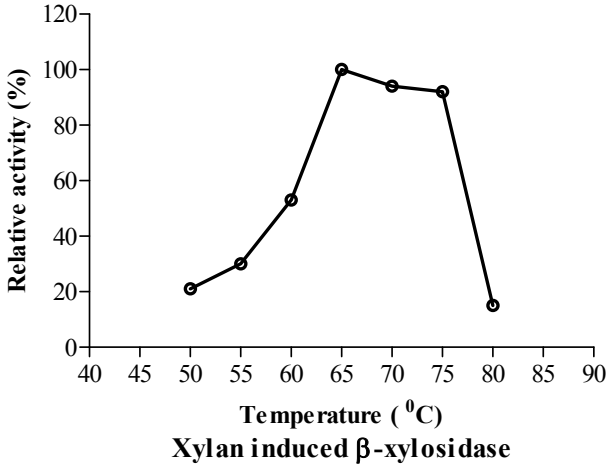
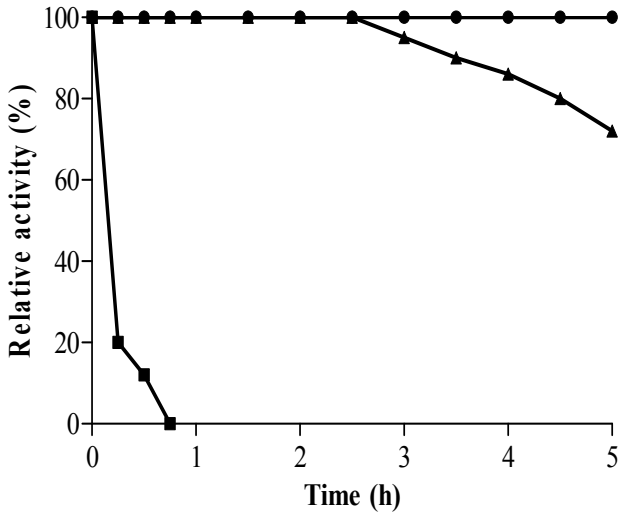
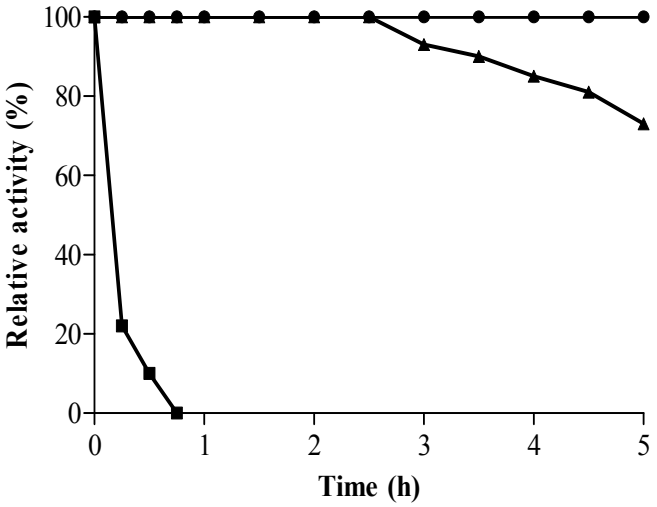


Fig. 3.16. Effect of temperature on activity of purified enzymes from *A. niger* NCIM 1207.

Enzyme activities were assayed at different temperature 45^0C – 80^0C as described in methods. Maximum enzyme activities obtained were taken as 100%.



Temperature stability of Enzyme - Cellulose induced β-glucosidase



Temperature stability of Enzyme - Xylan induced β-glucosidase

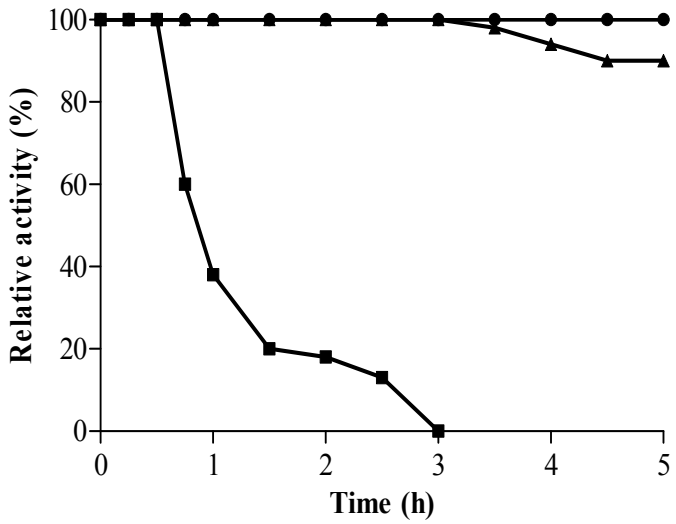


Fig. 3.17. Effect of temperature on stability of activity of purified enzymes from *A. niger* NCIM 1207. Purified enzymes were incubated at 50^0C (●), 60^0C (▲) and 70^0C (■). Aliquots were removed at suitable time interval and assayed for residual activities.

Effect of different organic solvents on stability of enzymes

The effect of organic solvents on enzymatic activities are summarized in Table 3.4. Both cellulose and xylan induced β-glucosidases were stable in all water miscible and water immiscible solvents except 1,4 dioxane. Incubation of β-xylosidase with chloroform led to loss of 52% of its initial activity. Ethylene glycol, sorbitol, manitol had no effect on β-glucosidase and β-xylosidase activity. Methanol, isoamyl alcohol, isooctane, propane-1-2 diol, pentan-1-ol and hexanol at 25% concentration have shown enhancement in the activities of β-glucosidases as well as β-xylosidase.

Table 3.4. Effect of different organic solvents on stability of crude enzymes

Organic solvents (25%)	Relative activity after 24 h incubation at 30^0C (%)		
	β-glucosidase (Cellulose induced)	β-glucosidase (Xylan induced)	B-xylosidase (xylan induced)
Control	100.0	100.0	100.0
Hexane	105.8	107.2	**127.2**
Chloroform	**110.0**	**116.1**	**47.8**
2-propanol	95.0	98.7	**134.0**
Methanol	**140.0**	**135.5**	**166.0**
Isoamyl alcohol	**137.2**	**135.5**	**135.3**
Xylene	115.0	120.4	102.7
Aceto nitrile	100.0	97.4	115.1
DMSO	94.0	94.4	93.7
1-4 dioxane	**0.0**	**3.0**	**0.0**
Isooctane	**160.0**	**163.0**	**150.2**
Propan-1:2- Diol	**134.2**	**132.0**	**142.0**
Butane-1- ol	96.0	95.5	100.2
Tertiary Butyl alcohol	85.5	84.6	89.9
Cyclohexanol	92.2	93.8	135.2
Hexanol	**137.2**	**140.0**	**191.0**
Pentan-1-ol	**140.5**	**138.5**	**154.5**
Acetone	105.1	109.2	99.1
Ethanol	108.5	110.0	**156.0**
Glycerol	104.0	108.2	102.2
Ethylene glycol	102.5	102.6	76.2
Sorbitol	96.5	90.9	78.5
Manitol	90.6	98.8	78.5

Effect of metal ions

The effects of various cations, EDTA and SDS on β-glucosidases and β-xylosidase activities were studied. The β-glucosidases and β-xylosidase did not show an obligate requirement of metal ions for their activity and EDTA did not affect activity of enzymes suggesting that they are neither metal requiring nor metaloenzymes. $HgCl_2$ at 1 mM concentration inhibited cellulose induced β-glucosidase (47%) and xylose induced β-glucosidase (41%). However, 90.8% inhibition of β-xylosidase activity was observed in presence of 1mM $HgCl_2$. Some of the metal ions such as Fe^{2+} and Mn^{2+} enhanced the β-glucosidase and β-xylosidase activities.

Table 3.5. Effect of metal ions on *Aspergillus niger* NCIM 1207 β-glucosidase and β-xylosidase

Metal Ion & chemicals (1 mM/ml)	**Relative activity (%) in presence of metal ions**					
	Cellulose induced β-glucosidase		**Xylan induced**			
			β-glucosidase		**β-xylosidase**	
	0.1mM	**1.0mM**	**0.1mM**	**1.0mM**	**0.1mM**	**1.0mM**
Control	100.0	100.0	100.0	100.0	100.0	100.0
$AgNo_3$	98.2	92.2	96.0	94.0	100.0	97.8
$BaCl_2$	102.0	103.0	108.1	105.0	100.0	93.4
$CaCl_2$	94.2	91.8	96.4	90.6	99.0	96.0
$CuSO_4$	90.2	85.3	91.3	86.5	94.6	86.1
$CoCl_2$	98.8	95.3	98.0	95.0	100.0	100.0
$FeCl_3$	110.2	110.1	100.0	112.0	109.2	115.0
$FeSO_4$	120.5	123.5	118.2	122.3	118.3	130.0
$HgCl_2$	**69.0**	**53.1**	**71.0**	**58.8**	**20.5**	**9.7.4**
$MgSO_4$	98.0	90.2	97.2	92.8	90.8	84.6
$MnCl_2$	115.0	126.0	112.2	128.2	96.9	89.8
$NiCl_2$	103.2	104.4	102.6	106.2	98.5	92.8
$ZnCl_2$	97.2	77.8	91.8	82.9	100.0	99.6
NaCl	98.3	95.5	100.0	96.6	100.4	84.5
EDTA	100.5	101.1	100.1	103.1	90.3	78.6

Effect of monosaccharides and disaccharides

The effect of different mono and disaccharides on all three enzymes is summarized in Table 3.6. No significant stimulatory or inhibitory effect was observed in presence of monosaccharides and disaccharides except glucose and cellobiose on β-glucosidase and xylose and xylobiose on β-xylosidase activity.

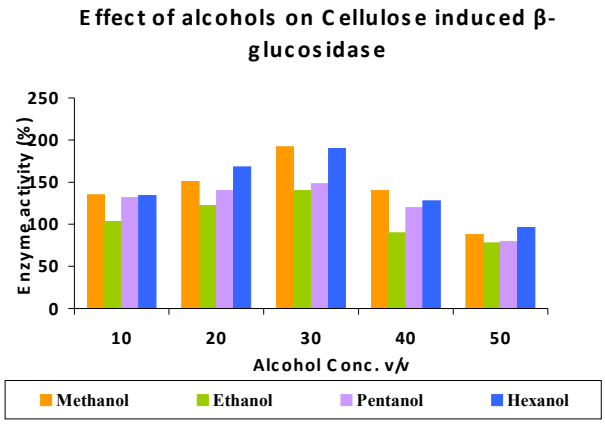
Table 3.6. Effect of monosaccharides and disaccharides on activity of enzymes.

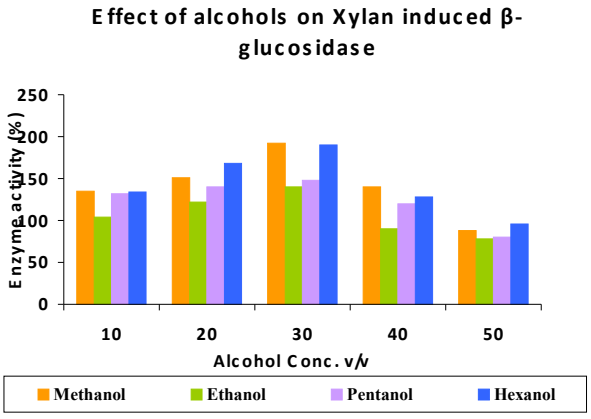
Substrate (1 mg/ml)	Relative activity (%)		
	Cellulose induced	Xylan induced	
	β-Glucosidase	β-Glucosidase	β-Xylosidase
Control	100	100	100
Monosaccharides			
Glucose	60.2	62.2	95.4
Arabinose	102.0	99.0	104.2
Xylose	98.3	101.0	67.4
Galactose	99.2	100.0	101.2
Fructose	94.2	98.2	96.0
Disaccharides			
Sucrose	101.2	102.0	98.0
Cellobiose	62.0	70.2	102.0
Xylobiose	99.6	102.2	57.0

Enzyme assay was performed in presence of various monosaccharides and disaccharides.

Effect of methanol, ethanol, pentanol and hexanol on activity of enzymes

The enzyme activities in presence of different concentrations (10, 20, 30, 40 and 50% v/v) of methanol, ethanol, pentanol and hexanol were studied under standard conditions. The result in Fig. 3.18 revealed that presence of methanol, pentanol and hexanol positively influenced the activities of both β-glucosidases (cellulose and xylan induced) and β-xylosidase. Appreciable increase in β-glucosidase was observed at 30% concentration of methanol, ethanol, pentanol and hexanol by 2.1, 2.0, 2.1, 2.12 fold respectively.





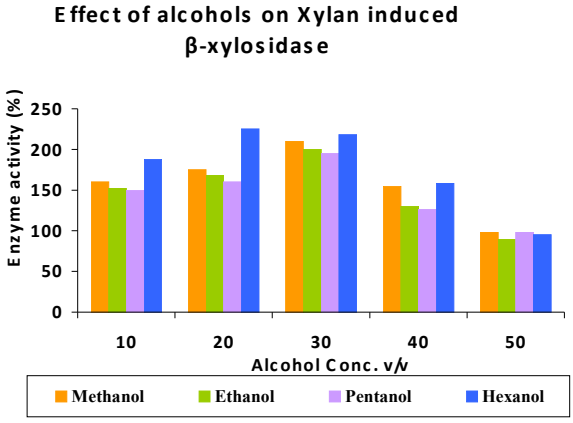


Fig. 3.18. Effect of different conc. - % v/v of methanol, ethanol, pentanol & hexanol on β-glucosidases (Cellulose & Xylan induced) and β-xylosidase (xylan induced)

Substrate specificities of enzymes

Substrate specificity studies were performed using p-nitrophenyl derivatives of different sugars. The action of purified β-glucosidases (cellulose and xylan induced) and β-xylosidase was tested against different substrates with α and β configuration as well as mono, disaccharides and polysaccharides. The results are summarized in the Table 3.7. Both β-glucosidases showed highest activity with cellobiose followed by pNP-β-D-glucopyranoside. The highest activity of β-xylosidase was obtained with xylobiose followed by oNP-β-D-xylopyranoside and *p*NP-β-D-xylopyranoside. The β-xylosidase also showed preference (24%) for *p*NP- α-L arabinipyranoside.

Table 3.7 : Substrate specificities of purified enzymes of *A. niger* NCIM 1207.

Substrate	**Relative activity (%)**		
	Cellulose induced β-glucosidase	**Xylan induced**	
		β-glucosidase	**β-xylosidase**
pNP **β-D glucopyranoside** (3mM)	100	100	05
pNP β-D xylopyranoside (3mM)	02	02	100
pNP β-D galactopyranoside (3mM)	00	00	00
oNP β-D xylopyranoside (3mM)	00	00	**115**
pNP β-D cellobiside (3mM)	00	00	00
pNP α-L arabinipyranoside (3mM)	00	00	**24**
pNP α- D galactopyranoside (3mM)	00	00	00
pNP α- D mannoside (3mM)	00	00	00
oNP α- D galactopyranoside (3mM)	00	00	00
Sucrose (1mg/ml)	00	00	00
Lactose (1mg/ml)	00	00	00
Maltose (1mg/ml)	00	00	00
D-Cellobiose (1mg/ml)	140	134	00
β-D xylobiose (1mg/ml)	00	00	146

The substrate specificity were determined by estimating the enzyme activities using p-nitrophenyl derivatives as well as disaccharides.

Kinetic studies - Michalis Menten constants

The reaction kinetics of purified β-glucosidases and β-xylosidase were determined from Lineweaver-Burk plot under optimal conditions (30 min, pH 4.5 at 65^0C). The effect of different concentrations of *p*NPG (0.25mM -14.0mM) on the reaction rate was studied and K_m and V_{max} were calculated which are summarized in Table 3.8 & Fig.3.19 and 3.20. Results indicated that and K_m and V_{max} of cellulose induced β-glucosidase were higher as compared to xylan induced β-glucosidase.

Table 3.8. Kinetic parameters of β-glucosidases and β-xylosidase.

Enzyme	Substrate	K_m (mM)	V_{max} (µmoles/ min/mg)	K_{cat}/min	K_{cat}/K_m (mM/min)
Cellulose induced β-glucosidase	pNP β-D-glucopyranoside	1.42	1150	1.4×10^5	1.18×10^5
Xylan induced β-glucosidase	pNP β-D-glucopyranoside	1.08	714	8.8×10^4	8.1×10^4
Xylan induced β-xylosidase	pNP β-D-xylopyranoside	1.30	645	7.9×10^4	6.1×10^4

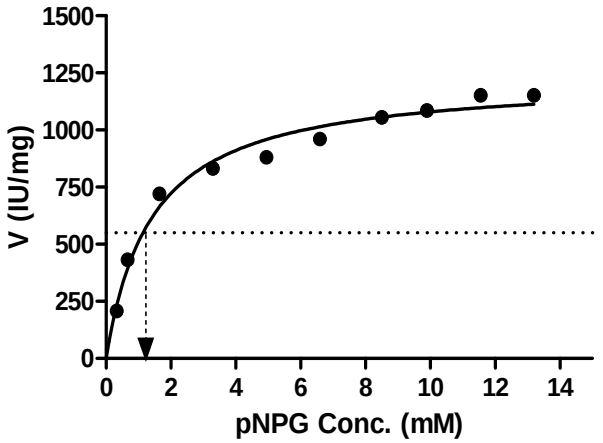


Fig. 3.19. Michaelis Menten Graph for cellulose induced β-glucosidase with pNPG

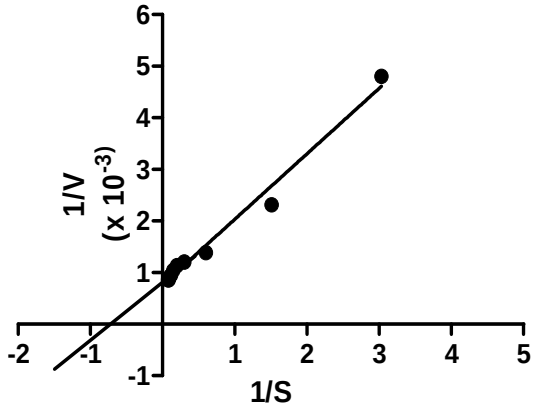


Fig. 3.20. Lineweaver - Burk plot for cellulose induced β-glucosidase with pNPG

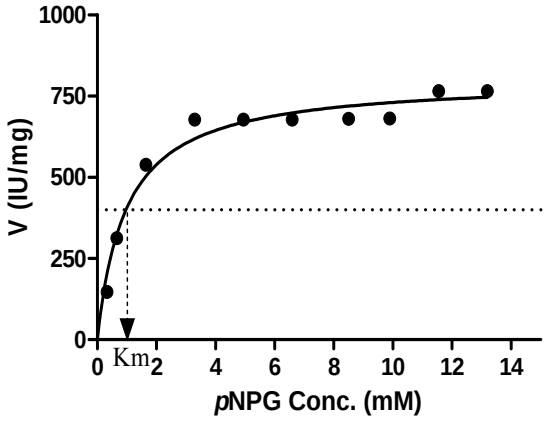


Fig. 3.21. Michaelis Menten Graph for Xylan induced β-glucosidase and pNPG

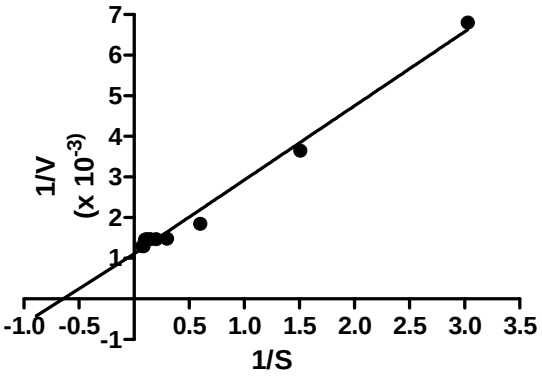


Fig. 3.22. Lineweaver - Burk plot for Xylan induced β-glucosidase and pNPG

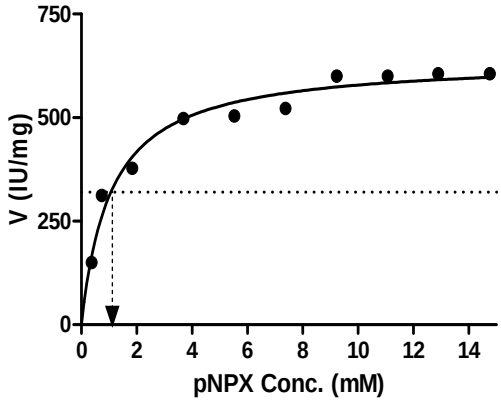


Fig. 3.23. Michaelis Menten Graph for Xylan induced β-xylosidase and pNPX

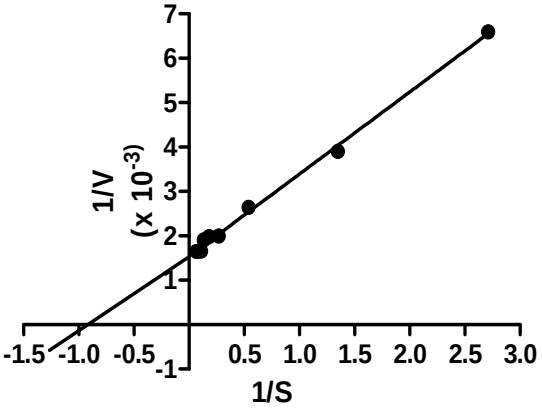


Fig. 3.24. Lineweaver - Burk plot for Xylan induced β-xylosidase and pNPX

Chemical modification studies

The importance of amino acid functional groups for the activity of both β-glucosidases and β-xylosidase was determined by chemical modification studies using chemical agents with restricted amino acid specificity. The results of the inactivation studies are given in Table 3.11. The enzymes were not inhibited by DEPC, PMSF, NAI suggesting the non-involvement of histidine, lysine, serine, tyrosine residues in catalytic site. Strong inhibition of β-glucosidases by EDAC, NBS and phenylglyoxal indicated the involvement of carboxylate, tryptophan and arginine for their catalytic activity. The β-xylosidase was strongly inhibited by EDAC, NBS and N-ethyl malemide indicating involvement of carboxylate, tryptophan and cysteine for its the catalytic activity. In view of these observations, the role of above-mentioned amino acid residues for catalysis was further investigated.

Table 3.11. Effect of group specific chemical modifiers on enzymes

Chemical Reagent	Conc. (mM)	Possible reaction site	Buffer	Residual activity (%)		
				Cellulose induced β-glucosidase	Xylan induced	
					β-glucosidase	β-xylosidase
EDAC	100	Asx/Glx	MES/HEPES, 75 : 25, 50 mM pH 6	40	40	42
DEPC	5.0	His	Sodium phosphate 50 mM, pH 6	100	100	100
NBS	0.5	Try	Sodium acetate 50 mM, pH 4.5	00	00	00
NEM	10 .0	Cys	Sodium phosphate 50 mM, pH 7.5	100	100	00
Iodoacetate	10 .0	Cys	Sodium phosphate 50 mM, pH 8	100	100	00
NAI	10 .0	Tyr	Sodium borate 50 mM, pH 7.6	100	100	100
PMSF	10 .0	Ser	Sodium phosphate 50 mM, pH 7.5	100	100	100
Phenylglyoxal	10 .0	Arg	Sodium bicarbonate 50 mM, pH 8.5	33	35	100

Substrate protection study

Carboxylate residue

The EDAC mediated inactivation was not prevented by incubating the β-glucosidase and β-xylosidase with excess of substrate prior to modification which suggests that carboxylate residues may be involved in catalytic activity and not in substrate binding.

Table 3.12. Effect of carboxylic modification on enzyme activity and substrate protection study

Reaction mixture	Residual activity (%)	Inhibition (%)	Protection (%)
Cellulose induced β-glucosidase activity			
Enzyme (Control)	100	0	100
Enzyme + EDAC 100 mM	44.2	55.8	0
Enzyme + *p*NPG 0.2 mM + EDAC 100 mM	44.2	55.8	0
Enzyme + *p*NPG 0.4 mM + EDAC 100 mM	44.0	56.0	0
Enzyme + *p*NPG 0.6 mM + EDAC 100 mM	44.0	56.0	0
Xylan induced β-glucosidase			
Enzyme (Control)	100	0	100
Enzyme + EDAC 100 mM	36	64	0
Enzyme + *p*NPG 0.2 mM + EDAC 100 mM	35.8	64.2	0
Enzyme + *p*NPG 0.4 mM + EDAC 100 mM	35.8	64.0	0
Enzyme + *p*NPG 0.6 mM + EDAC 100 mM	34.8	64.1	0
Xylan induced β-xylosidase			
Enzyme (Control)	100	0	100
Enzyme + EDAC 100 mM	31.5	68.5	0
Enzyme + *p*NPX 0.2 mM + EDAC 100 mM	31.6	68.4	0
Enzyme + *p*NPX 0.4 mM + EDAC 100 mM	31.6	68.4	0
Enzyme + *p*NPX 0.6 mM + EDAC 100 mM	31.6	68.4	0

Tryptophan modification

NBS mediated inactivation of both β-glucosidases and β-xylosidase was partially prevented by pre- incubating the enzyme with excess amount of substrate (*p*NPG and *p*NPX) prior to modification reaction (Table 3.13). It indicates that tryptophan residues may have a role in substrate binding in case of cellulose and xylan induced β-glucosidase and xylan induced β-xylosidase.

Table 3.13. Effect of tryptophan modification on enzyme activity and substrate protection study

Reaction mixture	Residual activity (%)	Inhibition (%)	Protection (%)
Cellulose induced β-glucosidase activity			
Enzyme (Control)	100	0	100
Enzyme + NBS 0.5 mM	0	100	0
Enzyme + *p*NPG 0.2 mM + NBS o.5 mM	28	72	28
Enzyme + *p*NPG 0.4 mM + NBS 0.5 mM	32	68	32
Enzyme + *p*NPG 0.6 mM + NBS 0.5 mM	32	68	32
Xylan induced β-glucosidase			
Enzyme (Control)	100	0	100
Enzyme + NBS 0.1 mM	0	100	0
Enzyme + *p*NPG 0.2 mM + NBS 0.5 mM	28	73	28
Enzyme + *p*NPG 0.4 mM + NBS 0.5 mM	46	66	46
Enzyme + *p*NPG 0.6 mM + NBS 0.5mM	46	66	46
Xylan induced β-xylosidase			
Enzyme (Control)	100	0	100
Enzyme + NBS 0.1 mM	0	100	0
Enzyme + *p*NPX 0.2 mM + NBS 0.5 mM	61	39	61
Enzyme + *p*NPX 0.4 mM + NBS 0.5 mM	69	31	69
Enzyme + *p*NPX 0.6 mM + NBS 0.5 mM	69	31	69

Modification of arginine in β-glucosidases

Phenylglyoxal 10mM concentration inactivated both β-glucosidases and the inactivation is reversed by prior incubation of enzymes with excess of substrates. These results indicated the involvement of arginine residue for substrate binding.

Table 3.14. Effect of arginine modification on enzyme activity and substrate protection study

Reaction mixture	Residual activity (%)	Inhibition (%)	Protection (%)
Cellulose induced β-glucosidase activity			
Enzyme (Control)	100	0	100
Enzyme + phenylglyoxal 10 mM	33	77	0
Enzyme + *p*NPG 0.2 mM + phenylglyoxal 10 mM	78.5	21.5	45.5
Enzyme + *p*NPG 0.4 mM + phenylglyoxal 10 mM	80.0	20.0	47.0
Enzyme + *p*NPG 0.6 mM + phenylglyoxal 10 mM	80.0	20.0	47.0
Xylan induced β-glucosidase			
Enzyme (Control)	100	0	100
Enzyme + phenylglyoxal 10 mM	35	65	0
Enzyme + *p*NPG 0.2 mM + phenylglyoxal 10 mM	65.5	34.5	30.5
Enzyme + *p*NPG 0.4 mM + phenylglyoxal 10 mM	67.2	32.8	32.2
Enzyme + *p*NPG 0.6 mM + phenylglyoxal 10 mM	70.0	30.0	35.0

Modification of cysteine in β-xylosidase

N-ethyl malemide at 10 mM concentration inactivated β-xylosidase. The preincubation of enzyme with substrate before modification did not protect the enzyme significantly suggesting the role of cystein residue only for catalytic activity.

Table 3.15. Effect of cystiene modification on β-xylosidase activity and substrate protection study

Reaction mixture	Residual activity (%)	Inhibition (%)	Protection (%)
Xylan induced β-xylosidase			
Enzyme (Control)	100	0	100
Enzyme + NEM 10 mM	0	100	0
Enzyme + *p*NPX 0.2 mM + NEM 10 mM	10.2	89.8	10.2
Enzyme + *p*NPX 0.4 mM + NEM 10 mM	12.4	87.6	12.4
Enzyme + *p*NPX 0.6 mM + NEM 10 mM	12.4	87.6	12.4

DISCUSSION

Most of the fungal cellulases are extracellular and produced under submerged conditions. Purification protocols are under constant development and several steps are usually necessary based on different separation principles. The general protocols used for cellulase purification involve the separation of mycelium from the fermented broth, precipitation of proteins by ammonium sulphate or organic solvents, followed by ion exchange, affinity or hydrophobic chromatography and gel filtration. In addition, the other purification methods like adsorption and desorption from hydroxyapetite, affinity chromatography have been employed that yielded good results.

We developed the protocols for purification of β-glucosidases (cellulose and xylan induced) and β-xylosidase (xylan induced) using fractional ethanol precipitation method. All the three enzymes have been purified to homogeneity with good average yields and specific activity. The recovery of cellulose induced β-glucosidase was 23% with 16 fold purification. Xylan induced β-glucosidase was purified with 22% yield, 13.4 fold purification and specific activity of 645 IU/mg protein. Xylan induced β-xylosidase was purified to homogeneity with 14% yield, 6.4 fold purification and specific activity of 545 IU/mg of protein. From xylan induced broth, β-glucosidases and β-xylosidase were recovered by ethanol precipitation. Based on the solubility differences, β-glucosidase was solubilized using a mixture of water:ethanol (1:1.4) and recovered in pure form without any impurity. This method of separation of β-glucosidase is very simple and such purification protocol is not yet reported. Xylan induced β-xylosidase was purified and separated from other proteins and β-glucosidase on the basis of temperature stability. In all the cases, the fermented broth with high specific activity of individual enzyme and obtained under specific conditions was processed that resulted in purification of individual enzymes with higher yields and specific activity.

There are very few reports of one or two steps methods for purification of β-glucosidases and β-xylosidases (Gonzalez et al., 2008, Sanghi et al., 2010). Gonzalez and coworkers have purified intracellular β-glucosidase from *Metschnikowia pulcherrima* by amino agarose gel (monoamino ethyl-n-aminoethyl agarose) column chromatography with 38% yield. Johansson and Reczey (1998) used aqueous two phase (dextran and polyethylene glycol) partitioning method for concentration and obtained 3.3 fold purification and 54%

recovery of β-glucosidase from *A. niger*. Yan et al., (1998) have used 70% ethanol precipitation followed by CM sepharose column chromatography and sepharose and sephacryl S-300 column chromatography for purification of β-glucosidases from *Aspergillus niger* with specific activity of 360 IU/mg of protein. Parry et al., (2001) have purified β-glucosidase from *Thermoascus aurantiacus* by DEAE sepharose, Ultrogel AcA 44 column chromatography and Mono-P-chromatofocusing. Karnchanatat et al., (2007) have used ammonium sulphate precipitation, SP sepharose (ion exchange), phenyl sepharose (hydrophobic interaction) and sephadex-200 (gel filtration) for purification of β-glucosidase from *Daldinia eschscholzii* with 6.28% recovery, 50.23 fold purification and 77.86U/mg specific activity. Wie et al., (1996) purified β-glucosidase from *Monoascus purpureaus* by ammonium sulphate precipitation, ion exchange and gel filtration chromatography. Dariot et al., (2008) have purified β-glucosidase from *Monoascus purpureaus* by gel filtration chromatography with 13 fold purification. The β-glucosidase from novel strain of *Aspergillus* sp. MT0204 was purified to homogeneity in four steps (Qi et al., 2009). The steps involved ammonium sulphate precipitation, anion exchange chromatography (High QIEX column), followed by hydrophobic interaction chromatography using methyl HIC which resulted in high recovery (24.5%) of purified enzyme.

Exo-1, 4-β-xylosidase from *A. niger* 15 was purified by ethanol fractionation and chromatography on column of Sephadex G-50, cellulose DE-52, Sephadex C-50 and Sephadex G-200. The enzyme was isolated with 42.5% yield and specific activity of 35.2 IU/mg (Tavobilov et al., 1982). Purified β-xylosidase from *A. japonicus* strain MU-2 was obtained by ammonium sulphate precipitation followed by anion exchange chromatography (DEAE – Toyopearl 650s) and gel permeation chromatography with 0.5 IU/mg specific gravity and 12.3% yield (Wakiyama et al., 2008). The β-D-xylosidase from thermo-tolerant strain of *A. phoenicis* was purified by DEAE– cellulose column chromatography followed by Sephadex G-100 gel permeation chromatography which resulted in 17.7 fold purification and 9.8 % yield (Rizzatti et al., 2001). Purification of β-xylosidase from *Strptomyces species* CH7 was performed with ammonium sulphate precipitation, anion exchange chromatography with DEAE – Biogel (twice) followed by gel permeation chromatography using Sephadex G-200. The enzyme was obtained with specific activity 12.3 IU/g protein and 30.3% yield. The use of liquid–liquid extraction by reversed micelles to recover and to increase the purity of β-

xylosidase from *Penicillium janthinellum* with 43.5 recovery and 5.34 fold purification was reported (Hasmann et al., 2003).

The cellulose and xylan induced β-glucosidases and β-xylosidase of *A. niger* NCIM 1207 were found to have molecular weight of 122 kDa as determined by SDS-PAGE. The native molecular weight of all the three enzymes measured by gel filtration chromatography was found to be approximately 336 kDa suggesting that the enzymes are likely to function as trimers. *A. niger* β-glucosidases exist as trimers with a molecular weight of 360 kDa (Yan et al., 1998) and 325 kDa (Hoh et al., 1992) with a monomeric molecular weight of 120 kDa. The β-glucosidase from *A. oryzae* is a monomeric protein with molecular weight 40 kDa (Riou et al., 1998). *A. pullulanse* produces dimeric β-glucosidase possessing a native molecular mass of 340kDa with a monomeric unit of 165kDa (Saha et al., 1994). Watanabe et al., (1992) have reported β-glucosidase II from *A. niger* with a subunit of 120kDa and dimeric molecular weight with 240 kDa. Rashid and Siddhiqui (1997) purified β-glucosidase from *A. niger* which is a trimer possessing a native molecular weight of 330kDa. The each monomeric unit has the molecular weight of 110kDa. The β-glucosidase purified from *Thermoascus aurantiacus* was found to be a homo trimer of 350kDa with subunit of 120kDa (Parry et al., 2001). The high molecular weight of the β-glucosidases (Cellulose and xylan induced) is similar to those of many extracellular β-glucosidases reported from other fungal sources.

Molecular weight of fungal β-xylosidases ranges from 37.5 to 250kDa (Knob et al., 2010). The lowest molecular weight (37.5kDa) of β-xylosidase was reported from *Penicillium herquue* (Ito et al., 2003). Monomeric (Lembo et al., 2006, Yan et al., 2008) and dimeric forms Eneyskaya et al., 2007) are widespread among β-xylosidases reported so far from microorganisms. There are reports of various fungal cultures such as *A. phoenics* (Rizzatti et al., 2001) and *A. japonicas* (Wakiyama et al., 2008) producing extracellular β-D-xylosidases with molecular weights of 132 kDa and 113.2 kDa respectively. There are several monomeric β-xylosidases reported from *Fusarium proliferatum, (*91 kDa) (Saha, 2003), *Penicillium wortmanmi* IFO (210 kDa) (Matsuo et al., 1987). Exo-1, 4-β-xylosidase from *A. niger* 15 with a molecular mass of 253 kDa is a dimeric protein with a monomeric unit of 122 kDa (Rodionova et al., 1983). *Aspergillus awamori* X-100 produces β-xylosidase with molecular weight 250 kDa by gel permeation chromatography with a monomer of 125 kDa by

(Eneyskaya et al., 2007). The xylan induced β-xylosidase appears to be a trimer with native molecular weight of 340 kDa with 122 kDa monomer. Such high molecular weight trimeric β-xylosidase is not reported so far in the literature. There is only one report of trimeric β-xylosidase from *A. pulverulentus* possessing molecular weight of 180 kDa with monomer of 60 kDa (Sulistyo et al., 1995).

All the three enzymes from present study showed acidic pI of 4.6. Majority of β-glucosidases and β-xylosidases show acidic isoelectric pH values in the range of 4-6 (Eyzaguirre et al., 2005, Knob et al., 2010). Some glucosidases are very acidic (pI 3.1) or basic (pI 8.0) suggesting significant differences in amino acid composition. β-glucosidase from aerobic fungi generally have acidic pI (Coughlan, 1985). The pI for β-xylosidases have been reported from 3.4 in *A. nidulans* (Kumar and Ramon, 1996) and as high as 7.8 in *Fusarium proliferatum* (Saha, 2003) but many β-xylosidases have pI in a range 4-5 (Knob et al., 2010). β-xylosidase from *T. emersonni* had pI 8.9 (Tuohy et al., 1993).

Fungal β-glucosidases are mostly glycoproteins and the carbohydrate content varies from 1.5 % in *Acremonium persicinum* (Pitson et al., 1997) to as high as 75 % in *Trichoderma spp*. (Fadda et al., 1994). Many β-xylosidases were found to have high level of carbohydrate content. It ranges from 4.2 % (Sulistyo et al., 1995) to 61.5% in *Pecilomyces thermophilia* (Yan et al., 2008). These carbohydrates did not affect catalytic activity but promote further enzyme stability under denaturing conditions (de Almeida et al., 1995). The β-glucosidases and β-xylosidase from *A. niger* NCIM 1207 were found to be glycoproteins with approximately 35% and 38% carbohydrate content respectively.

In present study, cellulose and xylan induced β-glucosidases and xylan induced and β-xylosidase were found to be active in a pH range of 4 - 5.5 with optimal activity at pH 4.5. The majority of the fungal β-glucosidases show optimum pH over a range of pH 4 to 5.5. However, some β-glucosidases were found to be active at pH as low as 2.5 (McHale and Coughlan, 1981) up to 8.0 (Yoshioka and Hayashida, 1981). The majority of fungal β-xylosidases are active in a pH range 4.0 to 5.5 with exceptions of β-xylosidase from *Penicillium sclerotonium* which is active at acidic pH 2.5 (Knob and Carmona, 2009). Both the β-glucosidases of *A. niger* NCIM 1207 were stable in a pH range from 3.5 – 7 up to 48 hours at room temperature while β-xylosidase was stable in a range from 3.5 – 6.0 for 48 hours.

The optimum temperature of β-glucosidases ranges from 35 to 80^0C but most of the enzymes have an optimum temperature of 55^0C and above. The intracellular β-glucosidase of T. emersonii showed optimal activity at 35^0C (McHale and Coughlan, 1981). The β-glucosidases of *A. niger* NCIM 1207 were found to be active at 65^0C and were stable at 60^0C for 3 h. The extracellular fungal β-glucosidases from mesophilic fungi are thermostable up to 60^0C, while those of thermophilic fungi are stable even at higher temperatures (Stutzenberger, 1990). β-xylosidases presented maximal activity in temperature from 30-70^0C. β-xylosidases from thermophilic fungi such as *A. niger, Aspergillus brasiliensis* (Pedersen et al., 2007) and from thermotolerant fungus *A. phoenicis* (Rizzatti et al., 2001) presented optimal activity at 75^0C. The β-xylosidase of *A. niger* 1207 was active at 65^0C and stable at 70^0C for 3 h suggesting that this enzyme is more thermostable than β-glucosidases. Other thermophilic strains which produce β-xylosidase are *Humicola grisea* (Lembo et al., 2006), *S. thermophilum* (Zanoelo et al., 2004, *S. thermophile* (Katapodis et al., 2006) and *P. thermophila* (Yan et al., 2008).

Several ions are known to activate β-glucosidase activity suggesting its possible role as a cofactor in enzyme substrate reaction. It also has a stabilizing effect on the β-glucosidases. Some metal ions activated β-glucosidases of *A. niger* NCIM 1207. Mn^{2+} activated both β-glucosidases but there was no effect on β-xylosidase. $HgCl_2$ at 1 mM concentration strongly inhibited β-xylosidase. The chealating agent EDTA did not affect the enzyme activities indicating that they are not metaloproteins. Activation by Mn^{2+} may be explained by stabilization of enzyme structure. Similar results were reported for β-glucosidase from *Daldinia eschscholzii* (Karnchanatat et al., 2007) and β-glucosidase II and III from *A. terreus* (Nazir et al., 2009). The β-xylosidase from *Streptomyces spp.* CH7 was found to be totally inhibited by Hg^{2+} as well as D-Sulfhydryl affinity agent N-ethylmaleimide suggesting the involvement of sulfhydryl group in catalytic activity.

β-glucosidases as well as β-xylosidase were stable in majority of polar as well as non polar organic solvents even after 24 h exposure except 1-4, Dioxane. There was no effect of chloroform on β-glucosidases while β-xylosidase was inhibited by 52%. There were stimulatory effects of methanol, isoamyl alcohol, isooctane, propane 1-2 diol, hexanol and pentanol on β-glucosidase as well as β-xylosidase. Similar effects have been reported by Saha et al., (1994) and Nazir and coworkers (2009). The activity of β-glucosidase from *A. oryzae*

was stimulated by 30% in presence of 15% (v/v) ethanol (Riou et al., 1998). In general β-glucosidase can preferentially utilize alcohols rather than water as acceptors for glucosyl moiety during catalysis of pNPG, resulting in elevated reaction rate (Pemberton et al., 1980; Saha et al., 1994). The β-glucosidases were stable and activated by up to 30% (v/v) concentration of alcohol. The alcohols not only provided better hydrolysis conditions but also provided themselves as better acceptors for the β-glucosyl residues. At higher concentration, the enzyme was inhibited by these alcohols, probably because of protein precipitation and further due to denaturation. The β-xylosidase S1, purified from *Penicillium herquuel* showed transxylosylation activity using xylobiose as donor and alcohols (methanol, ethanol, isoprpanol, butanol and glycerol) as acceptors (Ito et al., 2003).

Fungal β-glucosidases hydrolyze cellobiose during the saccharification of cellulose. These enzymes show diversity in the substrate specificity and are classified into two groups based on their relative activities towards cellobiose (cellobiase) and *p*NPG or *o*NPG (aryl- β-glucosidases). In the present study, the substrate specificity studies demonstrated that the purified β-glucosidases of *A. niger* NCIM 1207 were active on cellobiose and *p*NPG with more preference to cellobiose. Few enzymes show significantly higher affinities for cellobiose such as enzymes from *N. frontalis* (Li et al., 1991), *Piromyces* sp. (Teunissen et al., 1992). Some of the β-glucosidases show absolute specificity for *p*NPG that are classified as aryl- β-glucosidases (Nazir et al., 2009). β-xylosidases are active against p-nitrophenyl glucosides artificial substrates. However, most of these enzymes prefer xylopyranosides such as pNPX (Saha, 2003, Lembo et al., 2006). Some of these enzymes are also able to cleave p-nitrophenyl-α-L-arabinofuranoside, p-nitrophenyl-β-L-arabinopyranoside, p-nitrophenyl-β-D-galactopyranoside or p-nitrophenyl-α-D-glucopyranoside (Kiss and Kiss, 2000; Ito et al., 2003; Zanoelo et al., 2004). Purified β-xylosidase of *A. niger* NCIM 1207 is active against xylobiose, *o*NPX, *p*NPX and *p*NP-α-L arabinopyranoside in decreasing preference. To our knowledge, this is the first report on β-xylosidase showing preference to *o*NP-β-D-xylopyranoside (*o*NPX). β-xylosidases show no action against xylans with an exception of multifunctional β-xylosidase from *T. reesei* which was able to release xylose from xylan (Herrmann et al., 1997). Broad substrate specificity is common among enzymes which are involved in the hydrolysis of hemicellulases. The similarity between D-xylopyranose and L-arabinopyranose leads to bifunctional xylosidase-arabinosidase enzymes (Li et al., 2000; Mai

et al., 2000). β-xylosidase with bifunctional activity of (aryl) xylobiose and (Aryl) arabinoside is of interest for the utilization of substituted xylans. Many fungal β-xylosidase with additional α-L-arabinofuranosidase activity have been reported (Deleyn and Claeyssens, 1977; Herrmann et al., 1977; Ransom and Walton, 1997; Andrade et al., 2004; Katapodis et al., 2006; Yan et al., 2008).

The cellulose and xylan induced β-glucosidases as well as β-xylosidase obeyed Michaelis Menten equation. The cellulose induced and xylan induced β-glucosidases have K_m values of 1.4 mM and 1.5mM of pNPG respectively. These were similar to those of other fungal β-glucosidases such as *A. fumigates* (Kitpree et al., 1986) *A. wentii* (Srivastava, 1984), *Sclerotium rolfsii* (Sadana et al., 1983) with K_m values 1.4, 1.6, 1.38 mM of pNPG respectively. The K_m values of many purified fungal β-glucosidases are in a range of as low as 0.055mM pNPG from *Stachibotrys atrta* (De Gussem et al., 1978) to as high as 34 mM pNPG from *Phytopthora infectans* (Bodenmann et al., 1985). In case of *A. niger,* the lowest K_m reported for β-glucosidase was 0.11 mM of *p*NPG (Le Traon-Masson and Pellerin, 1988) and highest K_m was 21.7mM of *p*NPG (Yan and Lin, 1997). In present study, the V_{max} values for cellulose and xylan induced β-glucosidases were high 1150 mM/min/mg and 714mM/min/mg protein respectively. Thus the enzymes show good affinity towards *p*NPG and catalytically efficient and exhibited high K_{cat} as well as K_{cat}/K_m values. Aryl β-xylosidase from cellulolytic thermophile expressed in *E. coli* showed K_m of 10mM for p-NPX (Hudson et al., 1991), Exo-1, 4- β-xylosidase of *A. niger* 15 showed Km 0.23 mM for pNPX and xylose was a competitive inhibitor with K_i=2.9mM (Tavobilov et al., 1983). β-xylosidase from *Fusarium proliferatum* had K_m value of 0.77mM of pNPX at 4.5 pH and 50^0C and completely inhibited by xylose with K_i value 5mM (Saha et al., 2003). β-xylosidase from *Humocola lanuginose* exhibited K_m value of 1.8 mM (Bokhari et al., 2010). β-xylosidase from *Humicola grisea var thermoidea* showed K_m value of 1.37 mM of *p*NPX (Iembo et al., 2005).

We employed chemical modification method to determine amino acids responsible for catalysis. EDAC mediated modification led to inhibition of β-glucosidases and β-xylosidase suggesting the involvement of carboxylate residue, aspartic acid or glutamic acid in catalytic function. In addition, we also found that Trp plays an important role in catalytic activity. In addition to carboxylate residues, cysteine also appears to be involved in catalysis in case of β-xylosidase. The studies on phenylglyoxal mediated inactivation reveals that arginine is also

involved in catalysis in case of β-glucosidases. The substrate protection studies revealed that Trp and Arg are involved in substrate binding. Tryptophan may also be involved in stabilization of tertiary configuration of the molecules. Asp and Glu from glycosyl hydrolases act either as proton donors in their protonated form or as nuleophile or oxocarbonium stabilizing agents in their charged forms (Sinnott, 1990; Clarke et al., 1993). β-xylosidase of *Humicola grisea* was strongly inactivated by NBS and slightly activated by DTT and mercaptoethanol. It was 25% inactivated by EDAC indicating role of tryptophan and glutamic acid in active site (Iembo et al., 2005). Evidence for involvement of L-tryptophan residue at the catalytic site is given by high inhibition of β-xylosidase activity by NBS, which is strong inhibitor for cellulolytic and xylanolytic enzymes (Medeiros et al., 2003).

Identification of purified molecules was performed by peptide analysis. The cellulose induced β-glucosidases was shown to match with BGLA ASPNC (β-glucosidases A of *A. niger* strain CBS 513.88) with peptide coverage of 24 % while xylan induced β-glucosidase was found to match with BGLA ASPNC with sequence coverage of 12%. Xylan induced β-xylosidase was found to match with exo-1, 4- β-xylosidase with 35% sequence coverage.

CONCLUSION

The extracellular β-glucosidases (cellulose and xylan induced) and β-xylosidase (xylan induced) were purified to homogeneity by ethanol fractionation method. These enzymes are glycoprotein in nature, with molecular weight of approximately 336 kDa. These enzymes were trimeric molecules with subunit of 122 kDa. The xylan induced β-glucosidase and β-xylosidase were of, more or less equal molecular weights and isoelectric points but differed in temperature and pH stability. The xylan induced β-glucosidase and β-xylosidase were found to be different molecules as identified by mass spectrometric analysis.

These enzymes were found to be stable in presence of metal ions and solvents except Hg^{2+} and 1-4-dioxane and therefore may be suitable in flavour and food industries. The thermostability and stability in different solvents may be useful in saccharification of cellulose and hemicellulose.

The β-glucosidase and β-xylosidase activity were enhanced in presence of alcohols. This property may be useful in application of these enzymes in production of transglycosylated products.

The β-glucosidases (cellulose and xylan induced) and β-xylosidase (xylan induced) were found to contain carboxylic acid residues, aspartic acid or glutamic acid in catalytic function. In addition, tryptophan plays an important role in catalytic activity. In case of and β-glucosidase, tryptophan and arginine may be involved in substrate binding. In β-xylosidase cysteine also appears to be involved in catalytic activity.

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CONCLUSIONS

- *Aspergillus niger* NCIM 1207 produces high levels of both β-glucosidase and β-xylosidase activities when grown on xylan containing media with glycerol and urea at 30^{o}C.
- It produced highest amounts of both β-glucosidase (19.0 IU/ml) and β-xylosidase (18.7 IU/ml) activities when grown at 30^{o}C for first five days followed by further incubation at 36^{o}C for 9 days.
- Cellulose and xylan induced β-glucosidase and xylan induced β-xylosidase were purified to homogeneity by ethanol fractionation method. Molecular mass of all these purified enzymes was 336 kDa. These are trimeric proteins with individual subunit of 122 kDa.
- The β-glucosidases (cellulose and xylan induced) and β-xylosidase (xylan induced) were found to contain carboxylic acid residues, aspartic acid or glutamic acid in catalytic function. In addition, tryptophan plays an important role in catalytic activity. In case of and β-glucosidase, tryptophan and arginine may be involved in substrate binding. In β-xylosidase cysteine also appears to be involved in catalytic activity.
- Identification of purified molecules was performed by peptide analysis. The cellulose induced β-glucosidases was shown to match with BGLA ASPNC (β-glucosidases A of *A. niger* strain CBS 513.88) with peptide coverage of 24 % while xylan induced β-glucosidase was found to match with BGLA ASPNC with sequence coverage of 12%. Xylan induced β-xylosidase was found to match with exo-1, 4- β-xylosidase with 35% sequence coverage.

Publications

Khisti, U., Bastawde, K.B. and Gokhale, D.V. (2011). Hyper-production of β-glucosidase and β-xylosidase by Aspergillus niger NCIM 1207 in xylan containing media. BioResources. (Accepted).

ANNEXURE-I

Dr. Gokhale National Chemical Laboratory, India.

Gel Bands MP2310 Lab book LIDDELL AS170 p 164

Peptide analysis of purified proteins :

Excised 1D gel bands diced into cubes (~1 mm^3) placed into individual wells of a microtitre plate, processed (destained, reduced, alkylated) and trypsin digested using standard procedures on the MassPREP station.

Delivered resulting peptides via nanoLC- to the Q-ToF2 for ESI-MSMS analysis. An automated experiment (DDA = data dependent acquisition) was run where selected peptides automatically enter MSMS for fragmentation.

The data was searched against the public databases using MS/MSIONS search on the MASCOT web site (http://www.matrixscience.com/search_form_select.html) using standard default settings (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=MIS) against the Swissprot database (SwissProt 2010_12) with standard variable modifications of carbamidomethylation of Cysteine and oxidation of Methionine; semi-tryptic digestion and additional modifications are also occasionally incorporated into searches. I attach the pkl files to the e-mail – these can be used for further searches. The web links below will take you to the results. There are hot links on the pages which will take you further into the data (e.g. coverage map etc – note that the Mascot site help pages are very good for aiding in interpretion of the results).

I strongly recommend that you read up about the MASCOT software and how to properly interpret the mascot output.

Note that autolysis fragments of the digestion enzyme trypsin will be seen in the MASCOT hit list, fibrinogen (peptide ion 785.8) is sometimes seen too, just ignore it, this it is an internal standard for the MS.

SUMMARY OF IDENTIFICATIONS

Sample ID	MP sample ID	proteins identified
I	**1F_MP2310**	**B-glucosidase and glucoamylase**

II	**1G_MP2310**	**B-glucosidase**
III	**1H_MP2310**	**exo-1,4-beta-xylosidase and beta-glucosidase**

Details for each sample follow on separate pages

1G_MP2310 (sample II) **B-glucosidase**

Mascot MSMSionssearch x Swiss-Prot database

semi-trypsin, 1 missed tryptic cleavage,
Carbamidomehtylation on C, oxidation on M, deamidation [NQ]
http://www.matrixscience.com/cgi/master_results.pl?file=../data/20101222/FttAieewh.dat
Please use the link above to view the entire data output of the search, the top hit is summarised here:-

1. BGLA_ASPNC **Mass:** 93171 **Score:** 304 **Matches:** 9(3) **Sequences:** 8(3)

Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
6	461.2541	920.4936	920.4967	-0.0031	0	41	7.8	1		R.LYDELIR.V
7	480.2581	958.5016	958.5084	-0.0067	0	63	0.048	1		I.QDAGVVATAK.H
10	493.2595	984.5044	984.5240	-0.0196	0	17	1.6e+03	7		K.NDGALPLTGK.E
13	508.8020	1015.5894	1015.5914	-0.0019	0	63	0.048	1		R.IGADSTVLLK.N
17	696.8750	1391.7354	1391.7521	-0.0167	0	72	0.0054	1		K.GADIQLGPAAGPLGR.S
21	835.9193	1669.8240	1669.8280	-0.0040	0	(10)	9.9e+03	1	U	R.LGIPGMCAQDSPLGVR.D + Carbamidomethyl (C)
22	843.9205	1685.8264	1685.8229	0.0035	0	31	73	1	U	R.LGIPGMCAQDSPLGVR.D + Carbamidomethyl (C); Oxidation (M)
23	996.0054	1989.9962	1990.0371	-0.0409	1	56	0.23	2	U	R.ITLQPSKETQWSTTLTR.R + Deamidated (NQ)
24	1035.0486	2068.0826	2068.0477	0.0350	0	13	4.3e+03	1		K.VAGDEVPQLYVSLGGPNEPK.I

Protein View

Match to: **BGLA_ASPNC** Score: **304**
Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1
Found in search of 1G_MP2310_22DEC10.pkl
Nominal mass (M_r): **93171**; Calculated pI value: **4.64**
NCBI BLAST search of BGLA_ASPNC against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88

Variable modifications: Carbamidomethyl (C),Oxidation (M),Deamidated (NQ)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **12%**
Matched peptides shown in **Bold Red**

1 MRFTSIEAVA LTAVSLASAD ELAYSPPYYP SPWANGQGDW AEAYQRAVDI
51 VSQMTLAEKV NLTTGTGWEL ELCVGQTGGV PR**LGIPGMCA QDSPLGVR**DS
101 DYNSAFPAGV NVAATWDKNL AYLRGQAMGQ EFSDK**GADIQ LGPAAGPLGR**
151 SPDGGRNWEG FSPDPALSGV LFAETIKGI***Q DAGVVATAK***H YIAYEQEHFR
201 QAPEAQGYGF NITESGSANL DDKTMHELYL WPFADAIRAG AGAVMCSYNQ
251 INNSYGCQNS YTLNKLLKAE LGFQGFVMSD WAAHHAGVSG ALAGLDMSMP
301 GDVDYDSGTS YWGTNLTISV LNGTVPQWRV DDMAVRIMAA YYKVGRDRLW
351 TPPNFSSWTR DEYGFKYYYV SEGPYEKVNQ FVNVQRNHSE LIRR**IGADST**
401 **VLLKNDGALP LTGK**ERLVAL IGEDAGSNPY GANGCSDRGC DNGTLAMGWG
451 SGTANFPYLV TPEQAISNEV LKNKNGVFTA TDNWAIDQIE ALAKTASVSL
501 VFVNADSGEG YINVDGNLGD RRNLTLWRNG DNVIKAAASN CNNTIVIIHS
551 VGPVLVNEWY DNPNVTAILW GGLPGQESGN SLADVLYGRV NPGAKSPFTW
601 GKTREAYQDY LYTEPNNGNG APQEDFVEGV FIDYRGFDKR NETPIYEFGY
651 GLSYTTFNYS NLQVEVLSAP AYEPASGETE AAPTFGEVGN ASDYLYPDGL
701 QRITKFIYPW LNSTDLEASS GDASYGQDAS DYLPEGATDG SAQPILPAGG
751 GAGGNPR**LYD ELIR**VSVTIK NTGK**VAGDEV PQLYVSLGGP NEPK**IVLRQF
801 ER**ITLQPSKE TQWSTTLTR**R DLANWNVETQ DWEITSYPKM VFAGSSSRKL
851 PLRASLPTVH

de novo sequenced peptides

493 +2 **M^{SO}VFVGSSSR** corresponds to residues 840-848 note that the 2nd valine in the de novo sequence is a V in this database entry (BGLA_ASPNC) but it is an V in BGLA_ASPKA Beta-glucosidase A OS=Aspergillus kawachi and BGL1_ASPAC Beta-glucosidase 1 OS=Aspergillus aculeatus (the other 2 mascot hits)

996 +2 **LTLQPSEETKWSTTLTR** confirms mascot hit peptide. Note that L can be I or L, and the 10th residue can be Q or K (see attached de novo sequence interpretation notes at the end of this document), the different Aspergillus species entries have K and Q, so we cannot be sure which residue (K OR Q) is present in this peptide in this sample.

480 +2 ***QDAGVVATAK*** confirms mascot hit peptide.
Note that this is not a true tryptic peptide, it is listed in the database search output because the parameter setting “semitrypsin” was used.

1F_MP2310 (sample I) **B-glucosidase and glucoamylase**

Mascot MSMSionssearch x Swiss-Prot database
semi-trypsin, 1 missed tryptic cleavage,
Carbamidomehtylation on C, oxidation on M, deamidation [NQ]
http://www.matrixscience.com/cgi/master_results.pl?file=../data/20101222/FttAieHEL.dat

Please use the link above to view the entire data output of the search, the top hit is summarised here:-

1. BGLA_ASPNC **Mass:** 93171 **Score:** 887 **Matches:** 23(11) **Sequences:** 16(10)

Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
1	403.1980	804.3814	804.3800	0.0015	0	25	2.1e+02	1		R.VDDMAVR.I
3	430.2226	858.4306	858.4309	-0.0003	0	10	6.1e+03	7		R.IMAAYYK.V
4	461.2544	920.4942	920.4967	-0.0025	0	44	2	1		R.LYDELIR.V
5	461.2555	920.4964	920.4967	-0.0003	0	(21)	4.5e+02	1		R.LYDELIR.V
6	480.2590	958.5034	958.5084	-0.0049	0	93	3.1e-05	1		I.QDAGVVATAK.H
10	493.2607	984.5068	984.5240	-0.0172	0	24	2.1e+02	3		K.NDGALPLTGK.E
11	493.2638	984.5130	984.5240	-0.0110	0	(15)	1.7e+03	3		K.NDGALPLTGK.E
12	508.7997	1015.5848	1015.5914	-0.0065	0	66	0.014	1		R.IGADSTVLLK.N
14	508.8050	1015.5954	1015.5914	0.0041	0	(12)	3.9e+03	5		R.IGADSTVLLK.N
18	552.2990	1102.5834	1102.5883	-0.0049	0	68	0.0094	1	U	K.VNQFVNVQR.N
22	565.3145	1128.6144	1128.6139	0.0006	0	58	0.076	1		K.GIQDAGVVATAK.H
25	607.2585	1212.5024	1212.5081	-0.0057	0	64	0.017	1		R.GQAMGQEFSDK.G + Oxidation (M)
27	696.8794	1391.7442	1391.7521	-0.0079	0	76	0.0011	1		K.GADIQLGPAAGPLGR.S
28	696.8807	1391.7468	1391.7521	-0.005	0	(27)	1.1e+02	1		K.GADIQLGPAAGPLGR.S

	29	696.8829	1391.7512	1391.7521	3 - 0.0009	0	(28)	83	2		K.GADIQLGPAAGPLGR.S
	30	696.8860	1391.7574	1391.7521	0.0053	0	(17)	1e+03	2		K.GADIQLGPAAGPLGR.S
☑	35	702.8723	1403.7300	1403.7330	- 0.0030	0	86	0.00013	1		R.AVDIVSQMTLAEK.V
☑	41	835.9191	1669.8236	1669.8280	- 0.0044	0	88	6.8e-05	1	U	R.LGIPGMCAQDSPLGVR.D + Carbamidomethyl (C)
☑	45	1035.0317	2068.0488	2068.0477	0.0012	0	68	0.0057	1		K.VAGDEVPQLYVSLGGPNEPK.I
☑	46	1089.0507	2176.0868	2176.0800	0.0068	0	75	0.001	1		K.NGVFTATDNWAIDQIEALAK.T
☑	47	1089.5342	2177.0538	2176.0800	0.9738	0	(62)	0.024	1		K.NGVFTATDNWAIDQIEALAK.T
☑	56	1139.5844	2277.1542	2277.1317	0.0225	0	74	0.0012	1		R.NWEGFSPDPALSGVLFAETIK.G
☑	66	1205.5397	3613.5973	3612.5957	1.0016	0	25	56	1	U	R.EAYQDYLYTEPNNGNGAPQEDFVEGVFIDYR.G

Protein View

Match to: **BGLA_ASPNC** Score: **887**
Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1
Found in search of 1F_MP2310_22DEC10.pkl
Nominal mass (M_r): **93171**; Calculated pI value: **4.64**
NCBI BLAST search of BGLA_ASPNC against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **24%**
Matched peptides shown in **Bold Red**

1 MRFTSIEAVA LTAVSLASAD ELAYSPPYYP SPWANGQGDW AEAYQR**AVDI**
51 **VSQMTLAEK**V NLTTGTGWEL ELCVGQTGGV PR***LGIPGMCA QDSPLGVR***DS
101 DYNSAFPAGV NVAATWDKNL AYLR**GQAMGQ EFSDKGADIQ LGPAAGPLGR**
151 SPDGGR**NWEG FSPDPALSGV LFAETIKGIQ DAGVVATAK**H YIAYEQEHFR
201 QAPEAQGYGF NITESGSANL DDKTMHELYL WPFADAIRAG AGAVMCSYNQ
251 INNSYGCQNS YTLNKLLKAE LGFQGFVMSD WAAHHAGVSG ALAGLDMSMP
301 GDVDYDSGTS YWGTNLTISV LNGTVPQWR**V DDMAVRIMAA YYK**VGRDRLW
351 TPPNFSSWTR DEYGFKYYYV SEGPYEK**VNQ FVNVQR**NHSE LIRR**IGADST**
401 **VLLKNDGALP LTGK**ERLVAL IGEDAGSNPY GANGCSDRGC DNGTLAMGWG
451 SGTANFPYLV TPEQAISNEV LKNK**NGVFTA TDNWAIDQIE ALAK**TASVSL
501 VFVNADSGEG YINVDGNLGD RRNLTLWRNG DNVIKAAASN CNNTIVIIHS
551 VGPVLVNEWY DNPNVTAILW GGLPGQESGN SLADVLYGRV NPGAKSPFTW
601 GKTR**EAYQDY LYTEPNNGNG APQEDFVEGV FIDYR**GFDKR NETPIYEFGY
651 GLSYTTFNYS NLQVEVLSAP AYEPASGETE AAPTFGEVGN ASDYLYPDGL
701 QRITKFIYPW LNSTDLEASS GDASYGQDAS DYLPEGATDG SAQPILPAGG
751 GAGGNPR**LYD ELIR**VSVTIK NTGK**VAGDEV PQLYVSLGGP NEPK**IVLRQF
801 ERITLQPSKE TQWSTTLTRR DLANWNVETQ DWEITSYPKM VFAGSSSRKL
851 PLRASLPTVH

de novo sequenced peptides

835 +2 hit ***GLLPGMCAQDSPLGVR*** confirms mascot peptide sequence

note that the first two residues could be in either order (GL or LG),
and the usual caveats regarding I/L etc apply

552 +2 hit **VNQFVNVQR** confirms mascot peptide sequence

the amylase hit is summarised here:-

6. AMYG_ASPSH **Mass: 68089 Score: 103 Matches: 2(1) Sequences: 2(1)**
Glucoamylase OS=Aspergillus shirousami GN=glaA PE=3 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
40	817.4142	1632.8138	1632.8107	0.0031	0	12	2.6e+03	4	U	R.ATLDSWLSNEATVAR.T
43	983.9914	1965.9682	1965.9643	0.0039	0	92	2.2e-05	1	U	R.SIYTLNDGLSDSEAVAVGR.Y

Proteins matching the same set of peptides:

AMYG_ASPKA **Mass: 68230 Score: 101 Matches: 2(1) Sequences: 2(1)**
Glucoamylase I OS=Aspergillus kawachi GN=gaI PE=1 SV=1
AMYG_ASPAW **Mass: 68267 Score: 100 Matches: 2(1) Sequences: 2(1)**
Glucoamylase OS=Aspergillus awamori GN=GLAA PE=1 SV=1
AMYG_ASPNG **Mass: 68267 Score: 100 Matches: 2(1) Sequences: 2(1)**
Glucoamylase OS=Aspergillus niger GN=GLAA PE=1 SV=1

Protein View

Match to: **AMYG_ASPSH** Score: **103**
Glucoamylase OS=Aspergillus shirousami GN=glaA PE=3 SV=1
Found in search of 1F_MP2310_22DEC10.pkl
Nominal mass (M_r): **68089**; Calculated pI value: **4.30**
NCBI BLAST search of AMYG_ASPSH against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus shirousami
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **5%**
Matched peptides shown in **Bold Red**

```
   1 MSFRSLLALS GLVCSGLASV ISKRATLDSW LSNEATVART AILNNIGADG
  51 AWVSGADSGI VVASPSTDNP DYFYTWTRDS GIVLKTLVDL FRNGDTDLLS
 101 TIEHYISSQA IIQGVSNPSG DLSSGGLGEP KFNVDETAYA GSWGRPQRDG
 151 PALRATAMIG FGQWLLDNGY TSAATEIVWP LVRNDLSYVA QYWNQTGYDL
 201 WEEVNGSSFF TIAVQHRALV EGSAFATAVG SSCSWCDSQA PQILCYLQSF
 251 WTGSYILANF DSSRSGKDTN TLLGSIHTFD PEAGCDDSTF QPCSPRALAN
 301 HKEVVDSFRS IYTLNDGLSD SEAVAVGRYP EDSYYNGNPW FLCTLAAAEQ
```

```
351 LYDALYQWDK QGSLEITDVS LDFFKALYSG AATGTYSSSS STYSSIVSAV
401 KTFADGFVSI VETHAASNGS LSEQFDKSDG DELSARDLTW SYAALLTANN
451 RRNSVVPPSW GETSASSVPG TCAATSASGT YSSVTVTSWP SIVATGGTTT
501 TATTTGSGGV TSTSKTTTTA SKTSTTTSST SCTTPTAVAV TFDLTATTTY
551 GENIYLVGSI SQLGDWETSD GIALSADKYT SSNPPWYVTV TLPAGESFEY
601 KFIRVESDDS VEWESDPNRE YTVPQACGES TATVTDTWR
```

de novo sequenced peptides

817 +2 --LDSWLSNEATVAR partial sequence which confirms the low scoring peptide sequence mascot hit

Note BLAST search top 2 hits:

```
gi|308390265|gb|ADO32576.1|  ginsenoside-beta-D-glucosidase precursor [Aspergillus
niger]
Length=640
 Score = 44.3 bits (97),  Expect = 0.002
 Identities = 13/13 (100%), Positives = 13/13 (100%), Gaps = 0/13 (0%)

Query  1   LDSWLSNEATVAR  13
           LDSWLSNEATVAR
Sbjct  27  LDSWLSNEATVAR  39


gi|261278645|pdb|3EQA|A  Chain A, Catalytic Domain Of Glucoamylase From
Aspergillus Niger
Complexed With Tris And Glycerol
Length=470
 Score = 44.3 bits (97),  Expect = 0.002
 Identities = 13/13 (100%), Positives = 13/13 (100%), Gaps = 0/13 (0%)

Query  1   LDSWLSNEATVAR  13
           LDSWLSNEATVAR
Sbjct  3   LDSWLSNEATVAR  15
```

984 +2 --*TLNDGLSDSEAVAVGR* partial sequence which confirms the high scoring peptide sequence mascot hit

Note BLAST search top 2 hits:-

```
gi|308390265|gb|ADO32576.1|  ginsenoside-beta-D-glucosidase precursor [Aspergillus
niger]
Length=640
 Score = 54.5 bits (121),  Expect = 2e-06
 Identities = 17/17 (100%), Positives = 17/17 (100%), Gaps = 0/17 (0%)

Query  1    YTLNDGLSDSEAVAVGR  17
            YTLNDGLSDSEAVAVGR
Sbjct  313  YTLNDGLSDSEAVAVGR  329


gi|261278645|pdb|3EQA|A  Chain A, Catalytic Domain Of Glucoamylase From
Aspergillus Niger
Complexed With Tris And Glycerol
Length=470

 Score = 54.5 bits (121),  Expect = 2e-06
 Identities = 17/17 (100%), Positives = 17/17 (100%), Gaps = 0/17 (0%)

Query  1    YTLNDGLSDSEAVAVGR  17
            YTLNDGLSDSEAVAVGR
Sbjct  289  YTLNDGLSDSEAVAVGR  305
```

1H_MP2310 (sample III) exo-1,4-beta-xylosidase and beta-glucosidase

Mascot MSMSionssearch x Swiss-Prot database
semi-trypsin, 1 missed tryptic cleavage,
Carbamidomehtylation on C, oxidation on M, deamidation [NQ]
http://www.matrixscience.com/cgi/master_results.pl?file=../data/20101223/FttAlrsnh.dat
Please use the link above to view the entire data output of the search, the top two hits are summarised here:-

1. XYND_ASPNC **Mass:** 87157 **Score:** 1345 **Matches:** 46(20) **Sequences:** 19(10)
Probable exo-1,4-beta-xylosidase xlnD OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=xlnD PE=3 SV=1

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
8	467.7446	933.4746	933.4742	0.0005	0	10	6.5e+03	1	U	N.APYMISPR.A
12	481.2575	960.5004	960.5029	-0.00024		48	0.82	1	U	V.PVEVGSFAR.V
14	493.2346	984.4546	984.4552	-0.00006		59	0.061	1	U	R.AAFEEAGYK.V
15	493.2378	984.4610	984.4552	0.0058	0	(7)	1e+04	3	U	R.AAFEEAGYK.V
16	493.2409	984.4672	984.4552	0.0120	0	(44)	2.1	1	U	R.AAFEEAGYK.V
17	493.2419	984.4692	984.4552	0.0140	0	(44)	2	1	U	R.AAFEEAGYK.V
20	522.7674	1043.5202	1043.5247	-0.00045		57	0.11	1	U	R.DDIEQGVIR.L
21	522.7684	1043.5222	1043.5247	-0.00025		(35)	17	1	U	R.DDIEQGVIR.L
22	522.7704	1043.5262	1043.5247	0.0015	0	(48)	0.8	1	U	R.DDIEQGVIR.L
24	530.7672	1059.5198	1059.5713	-0.05015		70	0.0061	1	U	R.VPVEVGSFAR.V
25	530.7909	1059.5672	1059.5713	-0.00041		(60)	0.05	1	U	R.VPVEVGSFAR.V
26	530.7922	1059.5698	1059.5713	-0.00015		(45)	1.6	1	U	R.VPVEVGSFAR.V
27	530.7924	1059.5702	1059.5713	-0.00011		(53)	0.3	1	U	R.VPVEVGSFAR.V
28	530.7925	1059.5704	1059.5713	-0.00009		(68)	0.0083	1	U	R.VPVEVGSFAR.V
29	530.7938	1059.5730	1059.5713	0.0017	0	(49)	0.63	1	U	R.VPVEVGSFAR.V
31	552.2698	1102.5250	1102.5295	-0.00044		43	2.7	1	U	N.TSDAGPAPYPK.K

☑	36	614.8214	1227.6282	1227.6459	-0.01076	0	58	0.085	1	U	K.NSNNVLPLTEK.A
☑	38	615.3213	1228.6280	1227.6459	0.9822	0	(50)	0.47	1	U	K.NSNNVLPLTEK.A
☑	39	671.8748	1341.7350	1341.7391	-0.00041	0	(78)	0.00072	1	U	K.VVLEGEEEVVLK.W
☑	40	671.8750	1341.7354	1341.7391	-0.00037	0	(75)	0.0017	1	U	K.VVLEGEEEVVLK.W
☑	41	671.8750	1341.7354	1341.7391	-0.00037	0	81	0.00034	1	U	K.VVLEGEEEVVLK.W
☑	42	671.8757	1341.7368	1341.7391	-0.00023	0	(65)	0.016	1	U	K.VVLEGEEEVVLK.W
☑	43	671.8769	1341.7392	1341.7391	0.0001	0	(79)	0.00059	1	U	K.VVLEGEEEVVLK.W
☑	44	671.8772	1341.7398	1341.7391	0.0007	0	(65)	0.016	1	U	K.VVLEGEEEVVLK.W
☑	48	546.6464	1636.9174	1636.9260	-0.00087	0	(77)	0.00075	1	U	R.TLIHQIASIISTQGR.A
☑	49	819.4687	1636.9228	1636.9260	-0.00032	0	91	3.7e-05	1	U	R.TLIHQIASIISTQGR.A
☑	50	821.9160	1641.8174	1641.8151	0.0024	0	(16)	1e+03	1	U	R.YGLDVYAPNINTFR.H
☑	51	821.9176	1641.8206	1641.8151	0.0056	0	39	5.1	1	U	R.YGLDVYAPNINTFR.H
☑	53	821.9222	1641.8298	1641.8151	0.0148	0	(22)	2.9e+02	1	U	R.YGLDVYAPNINTFR.H
☑	54	839.3819	1676.7492	1676.7464	0.0028	0	90	4e-05	1	U	R.SHLICDETATPYDR.A + Carbamidomethyl (C)
☑	55	856.4177	1710.8208	1710.8941	-0.07032	0	45	1.3	1	U	R.ESIAWPGNQLDLIQK.L
☑	57	856.4556	1710.8966	1710.8941	0.0026	0	(13)	2.2e+03	1	U	R.ESIAWPGNQLDLIQK.L
☑	60	675.6843	2024.0311	2024.0479	-0.01069	0	49	0.45	1	U	R.LGLPAYQVWSEALHGLDR.A
☑	61	675.6863	2024.0371	2024.0479	-0.01009	0	(9)	4.4e+03	1	U	R.LGLPAYQVWSEALHGLDR.A
	65	737.3665	2209.0777	2208.0997	0.9779	0	(13)	1.7e+03	2	U	Y.NAVNGVPACADSYFLQTLLR.D + Carbamidomethyl (C)
☑	66	1105.5538	2209.0930	2208.0997	0.9933	0	73	0.0018	1	U	Y.NAVNGVPACADSYFLQTLLR.D + Carbamidomethyl (C)
☑	70	840.7529	2519.2369	2519.2333	0.0036	0	(73)	0.0015	1	U	R.VNEDGDWVVFPGTFELALNLER.K
☑	71	1260.6282	2519.2418	2519.2333	0.0086	0	89	3.8e-05	1	U	R.VNEDGDWVVFPGTFELALNLER.K
☑	74	1302.1315	2602.2484	2602.2308	0.0176	0	61	0.026	1	U	M.CAYNAVNGVPACADSYFLQTLLR.D + 2 Carbamidomethyl (C)

☑	75	878.4757	2632.4053	2632.4072	-0.00019	(86)	7.3e-05	1	U	R.AASLISLFTLDELIANTGNTGLGVSR.L
☑	76	878.4780	2632.4122	2632.4072	0.00050	136	6.9e-10	1	U	R.AASLISLFTLDELIANTGNTGLGVSR.L
☑	80	1171.2318	3510.6736	3510.6678	0.00058	(46)	0.43	1	U	R.GQETPGEDVSLAAVYAYEYITGIQGPDPESNLK.L
☑	81	1171.2349	3510.6829	3510.6678	0.01510	(24)	81	1	U	R.GQETPGEDVSLAAVYAYEYITGIQGPDPESNLK.L
☑	82	1171.2366	3510.6880	3510.6678	0.02020	133	1e-09	1	U	R.GQETPGEDVSLAAVYAYEYITGIQGPDPESNLK.L
☑	84	1234.9026	3701.6860	3702.7148	-1.02088	177	3.5e-14	1	U	R.LVTTQYPASYAEEFPATDMNLRPEGDNPGQTYK.W
☑	85	1235.2533	3702.7381	3702.7148	0.02333	(13)	8.8e+02	1	U	R.LVTTQYPASYAEEFPATDMNLRPEGDNPGQTYK.W

Proteins matching the same set of peptides:

XYND_ASPNG **Mass:** 87157 **Score:** 1345 **Matches:** 46(20) **Sequences:** 19(10)

Exo-1,4-beta-xylosidase xlnD OS=Aspergillus niger GN=xlnD PE=1 SV=2

Protein View

Match to: **XYND_ASPNC** Score: **1345**
Probable exo-1,4-beta-xylosidase xlnD OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=xlnD PE=3 SV=1
Found in search of 1H_MP2310_22DEC10.pkl
Nominal mass (M_r): **87157**; Calculated pI value: **4.76**
NCBI BLAST search of XYND_ASPNC against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **35%**
Matched peptides shown in **Bold Red**

1 MAHSMSRPVA ATAAALLALA LPQALAQANT SYVDYNIEAN PDLYPLCIET
51 IPLSFPDCQN GPLR**SHLICD ETATPYDRAA SLISLFTLDE LIANTGNTGL**
101 **GVSRLGLPAY QVWSEALHGL DR**ANFSDSGA YNWATSFPQP ILTTAALNR**T**
151 **LIHQIASIIS TQGR**AFNNAG R**YGLDVYAPN INTFR**HPVWG R**GQETPGEDV**
201 **SLAAVYAYEY ITGIQGPDPE SNLK**LAATAK HYAGYDIENW HNHSRLGNDM
251 NITQQDLSEY YTPQFHVAAR DAKVQSVM**CA YNAVNGVPAC ADSYFLQTLL**
301 **R**DTFGFVDHG YVSSDCDAAY NIYNPHGYAS SQAAAAAEAI LAGTDIDCGT
351 TYQWHLNESI AAGDLSR**DDI EQGVIR**LYTT LVQAGYFDSN TTKANNPYRD
401 LSWSDVLETD AWNISYQAAT QGIVLLK**NSN NVLPLTEK**AY PPSNTTVALI
451 GPWANATTQL LGNYYGN**APY MISPRAAFEE AGYK**VNFAEG TGISSTSTSG
501 FAAALSAAQS ADVIIYAGGI DNTLEAEALD R**ESIAWPGNQ LDLIQK**LASA
551 AGKKPLIVLQ MGGGQVDSSS LKNNTNVSAL LWGGYPGQSG GFALRDIITG
601 KKNPAGR**LVT TQYPASYAEE FPATDMNLRP EGDNPGQTYK** WYTGEAVYEF

651 GHGLFYTTFA ESSSNTTTKE VKLNIQDILS QTHEDLASIT QLPVLNFTAN
701 IRNTGKLESD YTAMVFAN**TS DAGPAPYPK**K WLVGWDRLGE VKVGETRELR
751 **VPVEVGSFAR VNEDGDWVVF PGTFELALNL ER**KVRVK**VVL EGEEEVVLK**W
801 PGKE

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2. BGLA_ASPNC **Mass:** 93171 **Score:** 456 **Matches:** 10(5) **Sequences:** 9(4)

Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1

	Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
	3	403.1950	804.3754	804.3800	-0.00045		30	65	2		R.VDDMAVR.I
	6	461.2585	920.5024	920.4967	0.0057	0	20	5.6e+02	4		R.LYDELIR.V
☑	10	480.2602	958.5058	958.5084	-0.00025		55	0.18	1		I.QDAGVVATAK.H
☑	19	508.8030	1015.5914	1015.5914	0.0001	0	64	0.022	1		R.IGADSTVLLK.N
☑	32	565.3123	1128.6100	1128.6139	-0.00038		64	0.023	1		K.GIQDAGVVATAK.H
☑	35	607.2592	1212.5038	1212.5081	-0.00043		34	19	1		R.GQAMGQEFSDK.G + Oxidation (M)
☑	46	696.8783	1391.7420	1391.7521	-0.01001		93	2.2e-05	1		K.GADIQLGPAAGPLGR.S
☑	63	1089.0494	2176.0842	2176.0800	0.0042	0	94	1.3e-05	1		K.NGVFTATDNWAIDQIEALAK.T
☑	64	1089.5438	2177.0730	2176.0800	0.9930	0	(67)	0.0066	1		K.NGVFTATDNWAIDQIEALAK.T
☑	68	1155.0443	2308.0740	2308.0648	0.0093	0	37	6.1	1	U	R.DLANWNVETQDWEITSYPK.M

Protein View

Match to: **BGLA_ASPNC** Score: **456**
Probable beta-glucosidase A OS=Aspergillus niger (strain CBS 513.88 / FGSC A1513) GN=bglA PE=3 SV=1
Found in search of 1H_MP2310_22DEC10.pkl
Nominal mass (M_r): **93171**; Calculated pI value: **4.64**
NCBI BLAST search of BGLA_ASPNC against nr
Unformatted sequence string for pasting into other applications
Taxonomy: Aspergillus niger CBS 513.88
Variable modifications: Carbamidomethyl (C),Oxidation (M)
Semi-specific cleavage, (peptide can be non-specific at one terminus only)
Cleavage by semiTrypsin: cuts C-term side of KR unless next residue is P

Sequence Coverage: **11%**
Matched peptides shown in **Bold Red**

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  1 MRFTSIEAVA LTAVSLASAD ELAYSPPYYP SPWANGQGDW AEAYQRAVDI
 51 VSQMTLAEKV NLTTGTGWEL ELCVGQTGGV PRLGIPGMCA QDSPLGVRDS
101 DYNSAFPAGV NVAATWDKNL AYLRGQAMGQ EFSDKGADIQ LGPAAGPLGR
151 SPDGGRNWEG FSPDPALSGV LFAETIKGIQ DAGVVATAKH YIAYEQEHFR
201 QAPEAQGYGF NITESGSANL DDKTMHELYL WPFADAIRAG AGAVMCSYNQ
251 INNSYGCQNS YTLNKLLKAE LGFQGFVMSD WAAHHAGVSG ALAGLDMSMP
301 GDVDYDSGTS YWGTNLTISV LNGTVPQWRV DDMAVRIMAA YYKVGRDRLW
351 TPPNFSSWTR DEYGFKYYYV SEGPYEKVNQ FVNVQRNHSE LIRRIGADST
401 VLLKNDGALP LTGKERLVAL IGEDAGSNPY GANGCSDRGC DNGTLAMGWG
451 SGTANFPYLV TPEQAISNEV LKNKNGVFTA TDNWAIDQIE ALAKTASVSL
501 VFVNADSGEG YINVDGNLGD RRNLTLWRNG DNVIKAAASN CNNTIVIIHS
551 VGPVLVNEWY DNPNVTAILW GGLPGQESGN SLADVLYGRV NPGAKSPFTW
601 GKTREAYQDY LYTEPNNGNG APQEDFVEGV FIDYRGFDKR NETPIYEFGY
651 GLSYTTFNYS NLQVEVLSAP AYEPASGETE AAPTFGEVGN ASDYLYPDGL
701 QRITKFIYPW LNSTDLEASS GDASYGQDAS DYLPEGATDG SAQPILPAGG
751 GAGGNPRLYD ELIRVSVTIK NTGKVAGDEV PQLYVSLGGP NEPKIVLRQF
801 ERITLQPSKE TQWSTTLTRR DLANWNVETQ DWEITSYPKM VFAGSSSRKL
851 PLRASLPTVH
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Manual sequencing by ESI tandem MS with low energy collision induced dissociation (CID), even with the assistance of specialized software (such as Waters/Micromass PepSeq and MasSeq which is what we use), is often open to interpretation of the mass spectra produced. Sometimes the spectrum is clean and the results clear, but in other cases some of the residues are not easy to call unambiguously. Extra information can assist in the interpretation of the resultant spectra and amino acid sequences. However, it is also important to back up the information by examination of any similar protein sequences and re-check the assignments made. Here are some notes to assist in your understanding and interpretation of the de novo sequence information generated using the Q-ToF2.

On the spectrum section of the BioLynx Peptide Sequencing report, where 2 residues appear "joined together" rather than spaced out separately with a line down to the x-axis denoting the position of the allocated ion, the residues can be in either order.

Leucine (L) and Isoleucine (I) are isobaric (both have a mono-isotopic mass value of 113.08406) and they cannot be differentiated in this type of analysis.
Therefore, when you see I or L called in a *de novo* sequence, the residue can be <u>either</u> I or L. Often an alignment with related protein sequences helps to call the residue more definitively, or examination of nucleic acid sequence, where available.

Similarly, glutamine (Q) and lysine (K) differ by only 0.036 u.
Also, phenylalanine (F) and oxidized methionine (M^{SO}) differ by only 0.033 u.
It can be difficult to differentiate between these pairs of residues, so for example, if you see an F called, it could be an oxidised methionine residue, and vice versa.

Common derivatives used for Cys include iodoacetamide, iodoacetic acid and vinyl pyridine - all result in unique amino acid residue masses (159, 160, and 208, respectively). We routinely use iodoacetamide during processing, resulting in the modification of cysteine with a mass of 160 (denoted C^{AM} or CAM)

If there are two or more consecutive glycine residues in a sequence, there is often no abundant ion from cleavages between them.
Similarly, sometimes where Gly-Ala appear consecutively there is no abundant ion from cleavages between them.
Gly-Gly is isomeric to asparagine (Asn) (N).
Gly-Ala is isomeric to glutamine (Gln) (Q) and isobaric to lysine (Lys) (K).
It's therefore possible to assign the mass between two sequence ions as Asn or Gln/Lys, instead of Gly-Gly or Gly-Ala, respectively.

Losses of water (18) ammonia (17) and carbon monoxide (28) from the sequence fragment ions are often present.

The amino acid Pro is associated with very abundant y-type fragment ions. Therefore in C-terminal fragment ions, the y ion that results from cleavage adjacent to Pro is often easily identifiable because of its intensity.

To obtain the entire sequence of any peptide from tandem MS data it is necessary to obtain fragmentation at every peptide bond, a situation that often does not occur. It is common to <u>not</u> find any fragments resulting from cleavage between the first and second amino acids (in which case only the combined mass of the 2 terminal amino acids can be determined). Also, cleavages

on the C-terminal side of Proline are often absent or of low intensity, which can preclude determination of the entire sequence.

For a more detailed understanding of de novo peptide *de novo* sequencing by MS, look at the excellent tutorial on the

IonSource.Com web site

http://www.ionsource.com/tutorial/DeNovo/DeNovoTOC.htm

and see

The ABC's (and XYZ's) of peptide sequencing.
Steen H, Mann M.
Nat Rev Mol Cell Biol. 2004 Sep;5(9):699-711. Review.PMID: 15340378
[PubMed - indexed for MEDLINE]