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 CO₂ 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 cellohexaoses 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 I\(\alpha\) and I\(\beta\). Cellulose produced by bacteria and algae is enriched in I\(\alpha\) while cellulose of higher plants consists mainly of I\(\beta\). 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 one of the major structural polysaccharide in plant cell. Xylans are heteropolysaccharides with a homopolymeric backbone chain of \(\beta-1, 4\)-linked \(\beta\)-D-xylopyranose units. The backbone consists of O-acetyl, \(\alpha\)-L-arabinofuranosyl, \(\alpha\)-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-O-methyl ether and arabinose. Based on side chain the xylans are classified as linear homoxylan, arabinoxylan, glucuronoxylan and glucuronorabinoxylan. The \(\beta-1,4\)-linked \(\beta\)-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 \(\alpha\)-D-glucronic acid or 4-Omethyl- \(\alpha\)-D-glucuronic acid might occur, while at C-3 of xylopyranose, one frequently finds 1,3 linked \(\alpha\)-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-hydroxycinnamyl alcohols. These phenylpropanoid units are made up of dimethoxylated (syringyl), monomethoxylated (guaiacyl) and nonmethoxylated (p-hydroxyphenyl) 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 °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 °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 C₁ 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 C₁ 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 C₁ 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 anomic 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 xyloooligosaccharides 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.
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 *Humincola 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 into suitable vectors (Nevalainen et al., 2003). Very recently, endo-β-glucanase II gene (egl2) from *T. reesei* 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 I 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-egl11) 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.
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°C 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 fused *T. ethanolicus* XAR and *T. lanuginosus* XYN to obtain trifunctional XAR – XYN protein that exhibited β-xylanolase, α-arabinosidase and xylanase activity when XYN was fused downstream of XAR (Stop codon removed).

**β-Glucosidase**

**Occurrence**

β-Glucosidase is widely distributed in nature and can be found in animals (McMahon 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*, *Talaromyces*
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°C with half life of 85h at 100°C and 13h at 110°C (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 basidiospores of *Schizophyllum commune* (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). Multiple 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 Penttila, 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 cellulolytic 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, Ilmen 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 (Ilmen 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.

![Image of 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 is 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](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, Hakulinen et al., 2000) which is a (α/β)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°C 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 \( \beta \)-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 \( \beta \)-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 \( \beta \)-glucosidase by *Humicola lanuginosa*. Corn steep liquor favored maximum \( \beta \)-glucosidase production followed by urea. Use of corn steep liquor alone as a carbon and nitrogen sources resulted in less production of \( \beta \)-glucosidase. Ammonium sulphate has been shown to facilitate \( \beta \)-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 \( \beta \)-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 \( \beta \)-glucosidase and other cellulases by *Trichothecium 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 \( \beta \)-glucosidase and endoglucanases by *A. niger* F119 was inhibited at low or high temperature (24\(^0\)C and 40\(^0\)C). The optimum temperature for production of all the three cellulolytic enzymes was reported to be
32°C. Asquicri and Park (1992) have reported 37°C 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°C and 45°C using *Penicillium chrysogenum* (Sharma et al., 1996). Bokhari and coworkers (2008) have reported 45°C 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°C (Zaldivar et al., 2001), whereas PeiJun et al., (2004) found that the ramping of temperature from 32°C to 27°C 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 hydroxylapatite 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.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (IU)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. niger</em></td>
<td>Ammonium sulphate precipitation, 1st CM sepharose</td>
<td>0.41</td>
<td>150</td>
<td>366</td>
<td>22</td>
<td>2.6</td>
<td>Yan et al., 1998</td>
</tr>
<tr>
<td></td>
<td>2nd CM Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q- Sepharose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sephacryl S-300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A. terreus</em> ß-Glucosidase</td>
<td>Ultra filtration, DEAE-sepharose, hydrophobic interaction</td>
<td>1.5</td>
<td>275.9</td>
<td>183.9</td>
<td>9.19</td>
<td>76.6</td>
<td>Nazir et al., 2009</td>
</tr>
<tr>
<td><em>A. terreus</em> ß-Glucosidase II</td>
<td>Ultra filtration, DEAE-sepharose, hydrophobic interaction, gel filtration</td>
<td>4</td>
<td>256</td>
<td>64</td>
<td>8.5</td>
<td>26.6</td>
<td>Nazir et al., 2009</td>
</tr>
<tr>
<td><em>A. terreus</em> ß-Glucosidase III</td>
<td>Ultra filtration, DEAE-sepharose, hydrophobic interaction, gel filtration</td>
<td>0.05</td>
<td>15.35</td>
<td>3.07</td>
<td>0.51</td>
<td>127.9</td>
<td>Nazir et al., 2009</td>
</tr>
<tr>
<td><em>Aspergillus sp. MT0204</em></td>
<td>Ammonium sulphate precipitation, Ion exchange chromatography, hydrophobic interaction</td>
<td>0.56</td>
<td>5.12</td>
<td>9.14</td>
<td>24.5</td>
<td>23.44</td>
<td>Qi et al., 2009</td>
</tr>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>Ammonium sulphate precipitation, gel filtration, Sephadex G-200, Sephadex G-50,</td>
<td>-</td>
<td>-</td>
<td>0.35</td>
<td>73.7</td>
<td>1.74</td>
<td>Ahmed et al., 2009</td>
</tr>
<tr>
<td><em>Paecilomyces thermophila</em></td>
<td>DEAE 52, Sephacryl S-200</td>
<td>0.7</td>
<td>56.4</td>
<td>80.6</td>
<td>21.7</td>
<td>105</td>
<td>Yang et al., 2008</td>
</tr>
<tr>
<td><em>Melanocarpus sp. MTCC 3922</em></td>
<td>Ultra- filtration DEAE-Sepharose, PBE-94</td>
<td>77.47</td>
<td>778.0</td>
<td>10.04</td>
<td>4.08</td>
<td>15.9</td>
<td>Kaur et al., 2007</td>
</tr>
<tr>
<td><em>Daldinia eschscholzii</em></td>
<td>Ammonium sulphate precipitation, ion exchange, hydrophobic interaction, gel filtration</td>
<td>0.87</td>
<td>67.74</td>
<td>77.86</td>
<td>6.28</td>
<td>50.2</td>
<td>Karnchanat et al., 2007</td>
</tr>
<tr>
<td><em>Aureobasidium pullulans</em></td>
<td>Ammonium sulphate, CM Bio-Gel A-0.5m, sephacryl S-200</td>
<td>0.37</td>
<td>46</td>
<td>124</td>
<td>3</td>
<td>129</td>
<td>Saha et al., 1994</td>
</tr>
</tbody>
</table>
Table 1.2. Physical properties, carbohydrate content and $K_M$ values for fungal $\beta$-glucosidase

<table>
<thead>
<tr>
<th>Organism</th>
<th>$M_w$ ($10^3$)</th>
<th>pl</th>
<th>Carbohydrate Content (%)</th>
<th>$K_M$ (mM)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Native</td>
<td>SDS-PAGE</td>
<td>PNPG (ONPG)</td>
<td>Cellobiose</td>
<td></td>
</tr>
<tr>
<td><strong>Aspergillus sp. MT-0204</strong></td>
<td>-</td>
<td>42</td>
<td>-</td>
<td>22.47</td>
<td>Qi et al., 2009</td>
</tr>
<tr>
<td><strong>A. terreus</strong></td>
<td>-</td>
<td>29</td>
<td>2.8</td>
<td>-</td>
<td>Nazir et al., 2009</td>
</tr>
<tr>
<td>A. terreus</td>
<td></td>
<td>43</td>
<td>3.7</td>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>A. terreus</td>
<td></td>
<td>98</td>
<td>3.0</td>
<td>3.7</td>
<td></td>
</tr>
<tr>
<td><strong>A. fumigatus</strong></td>
<td>-</td>
<td>120</td>
<td>8.5</td>
<td>-</td>
<td>Kim et al., 2007</td>
</tr>
<tr>
<td><strong>A. tubingensis</strong></td>
<td>II</td>
<td>131</td>
<td>4.2</td>
<td>0.76</td>
<td>Decker, et al., 2001</td>
</tr>
<tr>
<td><strong>A. tubingensis</strong></td>
<td>III</td>
<td>126</td>
<td>3.9</td>
<td>0.35</td>
<td></td>
</tr>
<tr>
<td><strong>A. tubingensis</strong></td>
<td>IV</td>
<td>54</td>
<td>3.7</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td><strong>A. tubingensis</strong></td>
<td></td>
<td>54</td>
<td>3.6</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td>-</td>
<td>88</td>
<td>8.8</td>
<td>-</td>
<td>Abdel-Naby et al., 1999</td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td></td>
<td>80</td>
<td>9.4</td>
<td>0.90</td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td></td>
<td>71</td>
<td>7.2</td>
<td>1.63</td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td><strong>Aspergillus sojae</strong></td>
<td>250</td>
<td>118</td>
<td>3.8</td>
<td>23.8</td>
<td>Kimura et al., 1999</td>
</tr>
<tr>
<td><strong>A. kawashii EX1</strong></td>
<td>-</td>
<td>145</td>
<td>Glycoprotein</td>
<td>-</td>
<td>Iwashita, et al., 1999</td>
</tr>
<tr>
<td><strong>A. kawashii EX2</strong></td>
<td></td>
<td>130</td>
<td>Glycoprotein</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>A. kawashii CB-1</strong></td>
<td></td>
<td>120</td>
<td>Glycoprotein</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong> $\beta$ Glucosidase A</td>
<td>-</td>
<td>118</td>
<td>-</td>
<td>0.43</td>
<td>Le TragonMasson et al., 1998</td>
</tr>
<tr>
<td><strong>A. niger</strong> $\beta$ Glucosidase B</td>
<td>-</td>
<td>109</td>
<td>-</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td><strong>Aspergillus niger</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.63</td>
<td>Spagna et al., 1998</td>
</tr>
<tr>
<td><strong>A. niger</strong> $\beta$ Glucosidase II</td>
<td>360</td>
<td>120</td>
<td>4.0</td>
<td>2.2</td>
<td>Yan et al., 1998</td>
</tr>
<tr>
<td><strong>A. oryzae</strong></td>
<td>40</td>
<td>43</td>
<td>4.2</td>
<td>-</td>
<td>Riou et al., 1998</td>
</tr>
<tr>
<td><strong>Acremonium persicinum</strong></td>
<td>140</td>
<td>128</td>
<td>4.3</td>
<td>1.5</td>
<td>Pitson et al., 1997</td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td>137</td>
<td>-</td>
<td>3.8</td>
<td>12.5</td>
<td>Yazaki et al., 1997</td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td>330</td>
<td>110</td>
<td>-</td>
<td>1.11</td>
<td>Rashid and Siddiqui, 1997</td>
</tr>
<tr>
<td><strong>A. niger</strong> $\beta$ Glucosidase I</td>
<td>105</td>
<td>49</td>
<td>3.2</td>
<td>21.7</td>
<td>Yan and Lin, 1997</td>
</tr>
</tbody>
</table>
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 variotii* 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 Thermoaerobacter 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.,
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 celooligosaccharides (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, Botryodiplodia, Pestalotocca and Trichodermea* (Reese et al., 1973). It has also been reported in species of *Absidia, Mucor, Rhizopus, Rhermoascus, Thermomyces* (Flannign and Sellaos, 1977), yeast *Cryptococcus* (Notario et al., 1976), *Bacillus spp* (Lajudie and De Barjac 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. emersonii* 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. emersonii* 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 pNPx 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 (Belfaqzih 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), Butyribibrio fibrosolvens xylB (Utt et al., 1991), Clostridium stercorarium sylA (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 (Sinnit, 1990). These two mechanisms have already been described in earlier section. Majority of the xylidosidases 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-xylosidases 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 anomeric 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 hydrolases 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 nucleophile 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 thermostolerance 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 submerged fermentation (SMF). The organisms such as *Aspergillus ochraceus*, *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).

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°C 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 *Saccharomyces 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°C 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 NaNO₃ concentration greatly increased cellulase synthesis in *Cellulomonas biazoatea* (Rajoka et al., 1998). Production of β-xylosidase in xylose yeast medium using corn steep liquor as N₂ 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°C. 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°C, 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°C rather than at 27°C. Kalogeris and coworkers (2003) obtained high level of β-xylosidase by Thermoascus aurantiacus on wheat straw at 49°C. 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°C under SSF. A thermotolerant Aspergillus phoenicis was also reported to produce high β-xylosidase activity at 45°C (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. thermaantarcticus* 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 *Scytaeidium 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

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

43
<table>
<thead>
<tr>
<th>Species</th>
<th>Native form</th>
<th>MW (kDa)</th>
<th>Glycosylation (%)</th>
<th>pI</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Talaromyces thermophilus</em></td>
<td>Monomeric</td>
<td>97</td>
<td>-</td>
<td>-</td>
<td>Guerfali et al., 2008</td>
</tr>
<tr>
<td><em>Aspergillus japonicus</em></td>
<td>Monomeric</td>
<td>113.2</td>
<td>27.6</td>
<td></td>
<td>Wakiyama et al., 2008</td>
</tr>
<tr>
<td><em>Paecilomyces thermophila</em></td>
<td>Monomeric</td>
<td>53.5</td>
<td>61.5</td>
<td>4.8</td>
<td>Yan et al., 2008</td>
</tr>
<tr>
<td><em>Talaromyces emersonii</em></td>
<td>-</td>
<td>-</td>
<td>86.9-100</td>
<td>High amount</td>
<td>Rasmussem et al., 2006</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td></td>
<td>87.6-102</td>
<td>High amount</td>
<td>3.0-3.5</td>
<td>Rasmussem et al., 2006</td>
</tr>
<tr>
<td><em>Aspergillus fumigatus</em></td>
<td>Monomeric</td>
<td>72.5</td>
<td></td>
<td></td>
<td>Lenartovicz et al., 2003</td>
</tr>
<tr>
<td><em>Fusarium proliferatum</em></td>
<td>Monomeric</td>
<td>91.2</td>
<td>-</td>
<td>7.8</td>
<td>Saha et al., 2003b</td>
</tr>
<tr>
<td><em>Aspergillus phoenicis</em></td>
<td>Monomeric</td>
<td>132</td>
<td>43.5</td>
<td>3.7</td>
<td>Rizzatti et al., 2001</td>
</tr>
<tr>
<td><em>Fusarium verticillioides</em></td>
<td>Monomeric</td>
<td>94.5</td>
<td>-</td>
<td>7.8</td>
<td>Saha et al., 2001</td>
</tr>
<tr>
<td><em>Trichoderma koningii G-39</em></td>
<td>Monomeric</td>
<td>104</td>
<td>Glycosylated</td>
<td>4.6</td>
<td>Li et al., 2000</td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>Monomeric</td>
<td>110</td>
<td></td>
<td></td>
<td>Kitamoto et al., 1999</td>
</tr>
<tr>
<td><em>Aspergillus pulverulentus</em></td>
<td>Trimeric</td>
<td>180</td>
<td>4.2</td>
<td>4.7</td>
<td>Sulistyo et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Dimeric</td>
<td>190</td>
<td>4.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Neocallimastix frontalis</strong></td>
<td>Dimeric</td>
<td>180</td>
<td></td>
<td>4.35</td>
<td>Hebraud and Fevre, 1990</td>
</tr>
<tr>
<td><em>Penicillium wortmanni</em></td>
<td>Monomeric</td>
<td>110</td>
<td>-</td>
<td>3.7</td>
<td>Matsuo et al., 1987</td>
</tr>
<tr>
<td>IFO 7237</td>
<td>Probably dimeric</td>
<td></td>
<td></td>
<td>4.3</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td></td>
<td>195</td>
<td></td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td></td>
<td>210</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td></td>
<td>180</td>
<td></td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus niger</em></td>
<td>Monomeric</td>
<td>78</td>
<td></td>
<td></td>
<td>John et al., 1979</td>
</tr>
<tr>
<td><em>Penicillium wortmanni</em></td>
<td>Monomeric</td>
<td>96-102</td>
<td>23</td>
<td>5.0</td>
<td>Deleyn and Claeyssens, 1977</td>
</tr>
<tr>
<td>QM 7322</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Termitomyces clypeatus</em></td>
<td>Monomeric</td>
<td>94</td>
<td>-</td>
<td>-</td>
<td>Bhattacharyya et al., 1997</td>
</tr>
<tr>
<td><em>Cochliobolus carbonum</em></td>
<td>Monomeric</td>
<td>42</td>
<td>Glycosylated</td>
<td></td>
<td>Ransom and Walton, 1997</td>
</tr>
</tbody>
</table>
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 (C₅ 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-\(\beta\)-xylosides, ascorbid glucosides can be synthesized by \(\beta\)-xylosidase mediated transfer reactions (Pan et al., 2001; Gargouri et al., 2004). Transxylosylation activity exhibited by \(\beta\)-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 \(\beta\)-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 \(\beta\)-xylosidases are used for synthesis of xyloooligosaccharides (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 \(\beta\)-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 \(\beta\)-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).
References


1,4-β-xylosidase of *Aspergillus niger* 15. Prikl. Biochim. Mikrobiol. 19, 232-239.

on the expression of Cochliobolus carbonum xylan degrading enzyme genes. Afr.

Tsao, G., T. (1986). Conversion of cellulosics: structures of cellulosic material and
their hydrolysis by enzymes. In: Alani DI, Moo- Young M (eds) Perspectives in

Turker, M., Mavituma, F. (1987). Production of cellulase by freely suspended and

Umezurike, G., M. (1979). The cellulolytic enzymes of *Botryodiplodia theobromae
Pat*: separation and characterization of cellulases and β-glucosidases, Biochem. J.
177, 9–19.

Ungchaitham Sumalee, Chirawan Thana, Yuwadee Talawanich and Pairoh
Pinphanichakarn. (2001). β-Xylosidase from *Streptomyces spp.* CH7 and Its Gene

*Butyvibrio fibrisolvens* xylB gene encoding a novel bifunctional protein with β-D-
57:1227-34.

Xylosidase activity, encoded by xlnD, is essential for complete hydrolysis of xylan
by *Aspergillus niger* but not for induction of the xylanolytic enzyme spectrum.


properties of an extracellular β-xylosidase from *Aspergillus japonicus* and


