INVESTIGATIONS OF THE BIOPROCESS PARAMETERS FOR THE PRODUCTION OF HEMICELLULASES BY *Thermomyces lanuginosus strains*

SANTHOSH KUMAR KUTTAN PILLAI

Submitted in fulfilment for the requirement of a Degree of Doctor of Technology: Biotechnology, in the Department of Biotechnology and Food Technology, Faculty of Applied Sciences, Durban University of Technology, Durban, South Africa

FINAL APPROVED FOR SUBMISSION

16/3/2010

Supervisor **Date**

Professor S. Singh

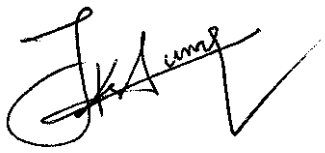
16/03/2010

Supervisor **Date**

Professor K. Permaul

DECLARATION

I hereby declare that this dissertation is my own, unaided work. It is being submitted for the degree of Doctor of Technology, to the Durban University of Technology, Department of Biotechnology and Food Technology, Durban, South Africa. It has not been submitted before for any degree or dissertation to any other institution.



_____

Santhosh Kumar Kuttan Pillai

March 2010

ACKNOWLEDGEMENTS

The author would like pay tribute to the following people who have been instrumental in providing help, direction, encouragement and support throughout the Doctoral research.

Prof. Suren Singh and Prof Kugen Permaul (Durban University of Technology) for their support and guidance throughout the study.

Dr. A. Manimaran for great company, inputs and constant encouragement for the study.

Dr. Adinarayana Kunamneni for guidance and support during the initial phase of the study.

Dr. Umasankar Akula for friendship and assistance.

Dr. Peter Biely (Slovak Academy of Sciences) and Prof. B. A. Prior (Stellenbosch University) for their technical advice and review of manuscripts.

Mr. Ray Burger, Mr. Teddy Sadayan and Ms Sarika Bihari (SAPPI, Stanger) for assisting in pulp analysis.

Mr. Elliot Ncayiana (Durban University of Technology) for assistance in the lab.

My friends, Pren, Viresh, Munnir, John, Elaine, Leroo, Sam, Pre, Kammy and Dawn for their company and support.

All staff members of the Department of Biotechnology and Food Technology who directly or indirectly helped me to complete this work successfully.

My uncle Dr. Pillai and family and my cousin Dr. Kumar for their encouragement and support.

My parents, wife and children for their patience, support; love and prayers that have been my strength throughout this long journey.

And most of all I humbly bow my head before God Almighty, who blessed me with the power to complete this endeavor successful.

CONTENTS

ABBREVIATIONS

DNS	3,5 Dinitrosalicylic acid
gds	Gram dry substrate
GH	Glycosyl hydrolases
IU	International unit
KDa	Kilo dalton
nkat	Nano katal
nm	Nano meter
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
PDA	Potato dextrose agar
pnp	*p* -nitrophenol
SD	Standard deviation
SEM	Scanning electron microscopy
SmC	Submerged cultivation
SSC	Solid state cultivation
UV	Ultraviolet
vvm	Volumes of air per minute per volume

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Due to several environmental and economic issues applicable to conventional chemical processes in many industries, including the pulp and paper industry which is subjected to considerable scrutiny, biotechnology is gaining rapid ground as it offers several advantages over conventional technologies. Industrial enzymes, which represent the heart of biotechnology is now experiencing major research initiatives, aiming at the development of a number of new products and improvement in the process and performance of several existing products.

Biocatalysis that exploits the catalytic activities of enzymes has emerged as a promising approach to chemical synthesis of novel and industrially significant compounds (Schmid *et al*., 2001). Enzymes can catalyze reactions exhibiting enantioselectivity and regioselectivity under appropriate conditions (Shoemaker *et al*., 2003). Enzymes have been used in several manufacturing processes since ancient times, in the production of food products, such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo and linen. The development of fermentation processes during the later part of the last century, made it possible to produce enzymes on a large scale. The continuously expanding applications of enzymes for the chemical, pharmaceutical and food industries is creating a growing demand for biocatalysts that exhibit improved or new properties. Advancements in biotechnology, especially in the area of genetics and protein engineering have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions,

enabling a further expansion of their industrial use. Based on such favorable properties, enzymes are widely used as catalysts and processing aids in many industrial processes.

In 2005, there were 4200 biotechnology companies worldwide and total revenue amounted to $63 billion (Demain and Adrio, 2008). The global market for industrial enzymes was estimated to $2 billion in 2004, and its annual growth rate predicted to be between 4% and 5% (Turner *et al*., 2007). Despite the fact that more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the present enzyme toolbox is still not sufficient to meet all demands. A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. As a result, the characterization of thermophilic microorganisms that are able to thrive in extreme environments has received a great deal of attention. Many advantages such as reduced contamination risk and faster reaction rates have been confirmed for the use of thermophiles as potential enzyme source. In general, parameters such as temperature, pH and enzymatic stability are important for the industrial application of any enzyme.

Among the various hydrolases, hemicellulases are a diverse group of enzymes that are widely used in industry. Hemicellulases, xylanases in particular, have been applied in industrial processes such as pulp bleaching, baking, clarification of juices, extraction of coffee, plant oils and starch and as a feed supplement to improve digestion in animals (Singh *et al*., 2003). Other potential applications include the conversion of xylan in wastes from food and agricultural industries into xylose and xylooligosaccharides (Akpinar *et al*., 2009), and in the bioconversion of lignocellulosic materials to fuels and chemical feedstocks (Maalej *et al*., 2009). The commonly used industrial enzymes and their application are highlighted in Table 1.1.

1.2 STRUCTURE OF HEMICELLULOSE

An annual yield of plant biomass on earth is estimated to be more than 120 billion tons comprising of approximately 50% of various forms of lignocelluloses (Smith, 1996), which represents the most abundant renewable organic resource (Sánchez, 2009). It is a composite material in which cellulose (35 – 50%), hemicellulose (20 – 30%) and lignin (20 – 30%) are found in close association (Puls, 1997; Bissoon *et al.,* 2002). Hemicelluloses, the second most abundant polysaccharide, are a heterogeneous group of branched and linear polysaccharides that are bound via hydrogen bonds to the cellulose microfibrils in the plant cell wall, crosslinking them into a robust network (Subramaniyan and Prema, 2002).

Hemicelluloses are usually classified according to the main sugar residues in the backbone of the polymer, e.g., xylans (β-1,4-linked D-xylose units) and mannans (β-1,4-linked D-mannose units), which are the main group of hemicelluloses, and arabinans (α-1,5-L-linked L-arabinose unit) and galactans (β-1,3-linked D-galactose units) which are less abundant (Sjöström, 1981; Fengel and Wegener, 1984).

1.2.1 Xylan

Xylan represents a significant resource of renewable biomass and comprises up to 20 – 35% dry weight of wood and agricultural wastes (Cui *et al*., 2009). Xylans, present in all terrestrial plants, are found in large quantities in hardwoods from angiosperms (15 – 30% of the cell wall content) and softwoods from gymnosperms (7 – 10%), as well as in annual plants (<30%) (Viikari *et al*., 1993).

Table1.1 Enzymes used in various industrial sectors and their applications (Kirk *et al*., 2002)

Industry	Enzyme	Application
Detergent (laundry and dish wash)	Protease	Protein stain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannan stain removal (reappearing stains)
Starch and fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin methyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
	Transglutaminase	Laminated dough strengths
Animal feed	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	β-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	β-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
	Amylase	Juice treatment, low calorie beer
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semi synthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, bating
	Lipase	De-pickling
Personal care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

Xylan is typically located in the secondary cell wall of plants, but is also found in the primary cell wall, in particular in monocots (Wong *et al*., 1988). Consistent with their structural chemistry and side-group substitutions, the xylans seem to be interspersed, intertwined, and covalently linked at various points with the overlying 'sheath' of lignin, while producing a coat around underlying strands of cellulose (Biely, 1985) via hydrogen bonding (Joseleau *et al*., 1992). The xylan layer with its covalent linkage to lignin and its non-covalent interaction with cellulose may be important in maintaining the integrity of the cellulose *in situ* and in helping protect the fibers against degradation by cellulases (Uffen, 1997).

Most xylans occur as heteropolysaccharides, containing different substituent groups in the backbone chain and in the side chain (Biely, 1985). Xylan varies in structure between different plant species, and the homopolymeric backbone chain of 1,4-linked β -D-xylopyranosyl units can be substituted to varying degrees with glucuronopyranosyl, 4-*O*-methyl-D-glucuronopyranosyl,α-L-arabinofuranosyl, acetyl, feruloyl and/or *p*-coumaroyl side-chain groups (Kulkarni *et al*., 1999; Li *et al*., 2000). A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases are depicted in Fig. 1.1.

Wood xylan exists as *O*-acetyl-4-*O*-methylglucuronoxylan in hardwoods and as arabino-4-*O*-methylglucuronoxylan in softwoods, while xylans in grasses and annual plants are typically arabinoxylans (Kulkarni *et al*., 1999). Linear unsubstituted xylan has also been reported, e.g., in esparto grass (Chanda *et al.,* 1950), tobacco (Eda *et al*., 1976) and certain marine algae (Nunn *et al*., 1973), with the latter containing xylopyranosyl residues linked by both 1,3- β and 1,4- β linkages (Nunn *et al*., 1973). The degree of polymerization in xylans is also variable, with, for example, hardwood and softwood xylans generally

consisting of 150 – 200 and 70 – 130 β -xylopyranose residues, respectively (Kulkarni *et al*., 1999).

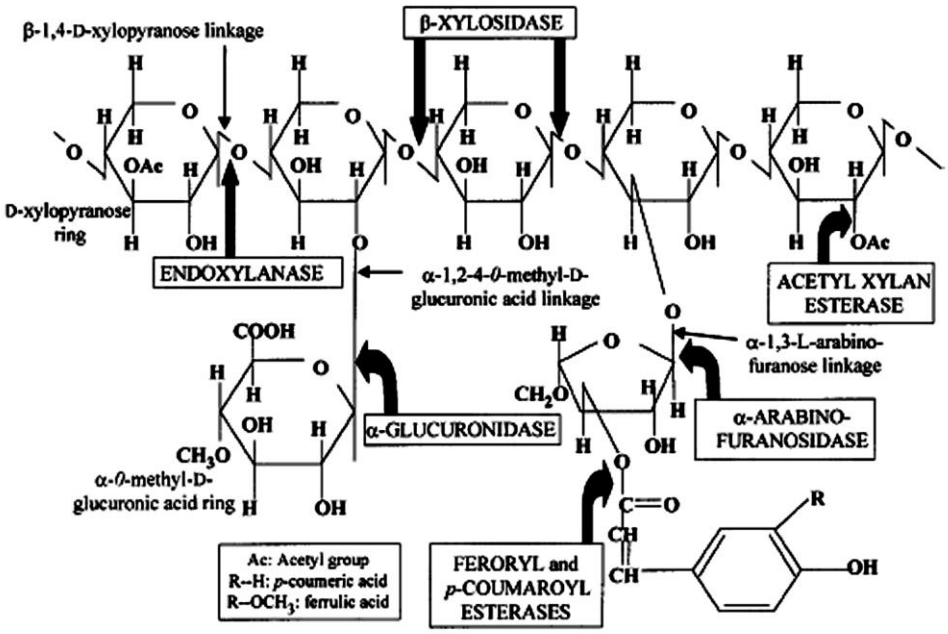


Fig.1.1 A hypothetical plant xylan structure showing different substituent groups with sites of attack by microbial xylanases (Beg *et al*., 2001)

Hardwood xylans are highly acetylated and acetylation is more frequent at the C – 3 than at the C – 2 position (e.g., birchwood xylan contains more than 1 mol of acetic acid per 2 mol of xylose). The presence of these acetyl groups is responsible for the partial solubility of xylan in water. These acetyl groups are readily removed when xylan is subjected to alkali extraction (Sunna and Antranikian, 1997).

Softwood xylans are not acetylated, and instead of an acetyl group they have α-L-arabinofuranose units linked by α-1,3-glycosidic bonds at the C – 3 position of the xylose (Puls and Schuseil, 1993). The arabinosyl substituents occur on almost 12% of the

xylosyl residues (Wong *et al*., 1988). The ratio of β-D-xylopyranose, 4-*O*- methyl-α-D-glucuronic acid and L-arabinofuranose is 100:20:13 (Puls and Schuseil, 1993). Softwood xylans are shorter than hardwood xylans, with a DP between 70 – 130 and are also less branched (Sunna and Antranikian, 1997).

1.3 ENZYMATIC HYDROLYSIS OF XYLAN

1.3.1 Hemicellulases

The variable structure and organization of hemicellulose require the concerted action of many enzymes for its complete degradation. Hemicellulases are key enzymes in the degradation of plant hemicellulose and carbon flow in nature (Shallom and Shoham, 2003). Hemicellulases are either glycoside hydrolases or carbohydrate esterases which are outlined below.

1.3.1.1 Xylanases

Xylanases are glycosidases (*O*-glycoside hydrolases), which catalyze the endohydrolysis of 1, 4-β-D-xylosidic linkages in xylan backbone resulting in the conversion of the polymeric substance into xylooligosaccharides and xylose (Subramaniyan and Prema, 2002; Shallom and Shoham, 2003). However, the extend of xylan hydrolysis depends upon its type, solubility, degree of polymerism and substitution. Degree of substitution on xylan side chains has been found to create steric hindrance for endoxylanases. Endoxylanases have been also reported to catalyze intermolecular transglycosylation in presence of high concentrations of xylooligomers (Kormelink *et al.*, 1993a). First xylanase purified from *Aspergillus foetidus* 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. The common synonyms of endo-1, 4-β-xylanase include xylanase, endoxylanase, 1,4-β-D-xylan-xylanohydrolase, endo-1,4-β-D-xylanase, β-1,4-xylanase and β-xylanase (Collins *et al*., 2005).

1.3.1.2 Classification of xylanases

The heterogeneity and complexity of xylan has resulted in an abundance of diverse xylanases with varying specificities, primary sequences and folds, and hence has lead to limitations with the classification of these enzymes by substrate specificity alone (Collins *et al*., 2005). Dekker and Richards (1976) divided endo-1,4-β-xylanases into two groups *viz*., i) those that did not liberate arabinose from arabinoxylans and arabinoglucuronoxylans, and ii) those that liberated arabinose from these substrates. Only in a few cases, however the absence of an α-L-arabinofuranosidase activity as a contaminant was established. Wong *et al*. (1988) also classified xylanases on the basis of their physicochemical properties and proposed two groups: those with a low molecular weight (<30 kDa) and basic p*I* and those with a high molecular weight (>30 kDa) and acidic p*I*. However, several exceptions to this pattern have been found (Matte and Forsberg, 1992; Sunna and Antranikian, 1997) and approximately 30% of presently identified xylanases, in particular fungal xylanases, cannot be classified by this system. Various strains of *T. lanuginosus* have been reported to produce xylanase with molecular masses varying between 23 and 29 kDa and p*I* values between 3.7 and 4.1. *Thermomyces lanuginosus* DSM 5826 produced a xylanase of 25.5 kDa and a p*I* of 4.1 (Cesar and Mrša, 1996). *T. lanuginosus* SSBP and *T. lanuginosus* 46882 were reported to produce xylanases of 23.6 and 25.7 kDa and pI values of 3.8 and 3.7, respectively (Singh *et al*., 2003).

Henrissat *et al*. (1989) introduced a classification system which allowed the classification of not only xylanases, but glycosidases in general and which has now become the standard means for the categorization of these enzymes. This system is based on primary structure comparisons of the catalytic domains only and group enzymes in families of related sequences (Henrissat and Coutinho, 2001). The initial classification grouped cellulases and xylanases into 6 families (A – F) (Henrissat *et al*., 1989), which was updated to 77 families in 1999 (1 – 77) (Henrissat and Coutinho, 2001) and which continues to grow as new glycosidase sequences are identified.

Coutinho and Henrissat (1999) classified endo-1,4-β-xylanase into glycosyl hydrolase families 5, 8, 10, 11, 16, 43, and 62. The majority of the endo-1,4-β-xylanase sequences included in families 5, 8, 10, 11, and 43 were found to be monospecific (containing only one EC number) with only one distinct catalytic domain. However, some endo-1,4-β-xylanase sequences classified in families 10, 11, and 43 were also found to be polyspecific (containing at least two EC numbers) with two catalytic domains. The endo-1,4-β-xylanase sequences reported in families 16 and 62 were polyspecific with two catalytic domains (Coutinho and Henrissat, 1999; Collins *et al*., 2005).

Enzymes within a particular family have a similar molecular mechanism (Gebler *et al*., 1992) and similar three-dimensional structure (Henrissat and Coutinho, 2001) and it has also been suggested that they may have a similar specificity of action on small, soluble, synthetic substrates (Claeyssens and Henrissat, 1992). Family 10 xylanases have a cellulose binding domain and a catalytic domain connected by flexible linker region (Henrissat and Bairoch, 1993) and exhibit lower substrate specificity (Fig. 1. 2). They are capable of cleaving glycosidic linkages in xylan main chains closer to side-chain substituents resulting in the release of short oligosaccharides (Kolenová *et al*., 2006).

Crystallization of xylanases of family 10 showed a tertiary fold with a typical 8-fold α/β barrel $(\alpha/\beta)_8$ resulting in a 'salad bowl' shape of the molecule (Derewenda *et al*., 1994).

Family 11 xylanases are highly specific and preferentially cleave in unsubstituted regions of the xylan backbone and have deep clefts in the substrate binding sites (Fig.1. 2) and form mainly β-sheets (Törrönen *et al*., 1994).

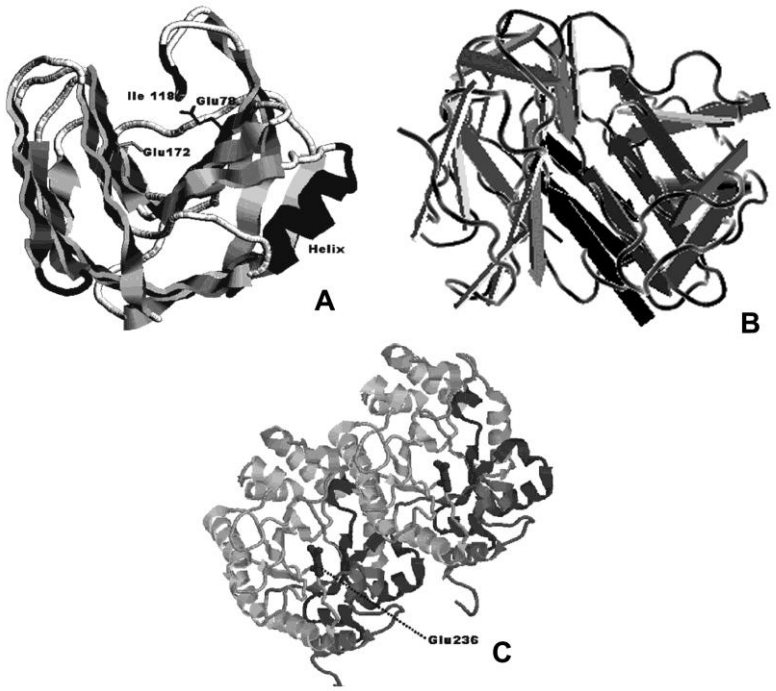


Fig. 1. 2 The three-dimensional structures of Family 11 xylanase from A) *Bacillus circulans* (Subramaniyan and Prema, 2002); B) *T. lanuginosus* (Gruber *et al*., 1998) and C) Family 10 Xylanase from *Streptomyces lividans* (Subramaniyan and Prema, 2002).

1.3.1.3 Multiple forms of xylanases

There are several reports regarding fungi and bacteria producing multiple forms of xylanases (Wong *et al*., 1988; Tsujibo *et al.,* 1997). *Streptomyces* sp. B-12-2 produced five endoxylanases when grown on oatspelts xylan (Elegir *et al*., 1994). Around 15 xylanases have been reported from the culture filtrates of *A. niger* and 13 xylanases from *T. viride* (Biely *et al*., 1985). The most outstanding case regarding multiple forms of xylanases was production of more than 30 different protein bands separated by analytical electrofocusing from *Phanerochaete chrysosporium* grown on Avicel (Dobozi *et al*., 1992). Due to the complex structure of heteroxylans, all of the xylosidic linkages in the substrates are not equally accessible to xylan-degrading enzymes. Because of the above, hydrolysis of xylan requires the action of multiple xylanases with overlapping but different specificities (Wong *et al*., 1988).

1.3.1.4 Mode of action of xylanases

Several models have been proposed to explain the mechanism of xylanase action leading to the hydrolysis of xylan. Generally, hydrolysis may result either in the retention or inversion of the anomeric center of the reducing sugar monomer of the carbohydrate. This suggests the involvement of one or two chemical transition states. Glycosyl transfer usually results in nucleophilic substitution at the saturated carbon of the anomeric center and takes place with either retention or inversion of the anomeric configuration. Most of the polysaccharide hydrolyzing enzymes such as cellulases and xylanases are known to hydrolyze their substrates with the retention of the C1 anomeric configuration. There is involvement of a double displacement mechanism for the anomeric retention of product (Clarke *et al*., 1993). The double displacement mechanism involves the following features, (i) an acid catalyst that protonates the substrate, (ii) a carboxyl group of the enzyme

positioned on a covalent glycosyl enzyme intermediate with this carboxylate in which the anomeric configuration of the sugar is opposite to that of the substrate, (iii) this covalent intermediate is reached from both directions through transition states involving oxo carbonium ions and (iv) various non-covalent interactions providing most of the rate enhancement (Clarke *et al*., 1993).

Based on the crystallographic study of xylopentaose binding to *Pseudomonas fluorescens* xylanase A, Leggio *et al*. (2000) proposed a suitable enzyme mechanism that combines the classic concepts listed above and facts derived from their study such as (i) xylan is recognized and bound by xylanase as a left-handed threefold helix, (ii) the xylosyl residue at subsite –1 is distorted and pulled down toward the catalytic residues, and the glycosidic bond is strained and broken to form the enzyme-substrate covalent intermediate, (iii) the intermediate is attacked by an activated water molecule, following the classic retaining glycosyl hydrolase mechanism and the product is released (Leggio *et al*., 2000).

There are several reports regarding the hydrolytic pattern of xylanases from *Bacillus* sp., and the most of them are mainly releasing xylobiose, xylotriose, and xylotetraose, while formation of xylose occurred only during prolonged incubation. Xylanases A and B, from *T*. *reesei* and C and D from *T*. *harzianum* under different combinations, showed synergistic interactions on different xylan substrates. Xylanase combinations were more effective than single xylanase for hydrolyzing pine holocellulose (Wong and Maringer, 1999). Xylanase II of *B. circulans* WL-12 (p*I* 9.1) (Esteban *et al.,* 1982) hydrolyzed xylan principally to xylobiose, xylotriose, and xylotriose. This enzyme was shown to require a minimum of four xylopyranoside residues to form the productive complex, thus xylotetraose out of other substrates tried was the most preferred substrate to saturate all binding sites of the enzyme. However, the xylanase I from the same source degraded xylan rapidly to

xylotetraose and prolonged incubation resulted in xylose, xylobiose, and xylotriose as the main end products.

1.3.2 ACCESSORY ENZYMES

1.3.2.1 β- mannanases

β-1,4-Mannanases (EC 3.2.1.78) hydrolyze mannan-based hemicelluloses and liberate short β-1,4-manno-oligomers, which can be further hydrolyzed to mannose by β-mannosidases (EC 3.2.1.25). β-Mannanases cleave randomly within the 1,4-β-D mannan main chain of galactomannan, glucomannan, galactoglucomannan and mannan (McCleary and Matheson, 1986). Mannanases are generally larger proteins (30 – 90 kDa) and have acidic isoelectric points. Apart from their ability to hydrolyze different mannans, some β-D-mannanases have also been reported to transglycosylate manno-oligosaccharide substrates (Schroder *et al*., 2004). The main hydrolysis products from galactomannans and glucomannans are mannobiose, mannotriose and various mixed oligosaccharides (Dhawan and Kaur, 2007). The hydrolysis yield is dependent on the degree of substitution as well as on the distribution of the substituents (Suurnäkki *et al*., 1997). There are currently about 50 β-mannanase gene sequences in GH families 5 and 26, and about 15 β-mannosidase gene sequences in families 1, 2 and 5 (Shallom and Shoham, 2003). The production of β-mannanases is usually induced by mannan-rich substrates such as locust bean gum, or by cellulose, which has proven to be an effective inducer of β-mannanases in several fungi (Johnson, 1990). Mannanases of microbial origin have been reported to be both induced as well as constitutive enzymes and are usually being secreted extracellularly into the medium (Dhawan and Kaur, 2007).

A vast variety of microorganisms including bacteria, actinomycetes, yeasts and fungi are known to be mannan degraders (Puchart *et al*., 2004). Although a number of mannanase-

producing bacterial sources are available, only a few are commercially exploited as wild or recombinant strains, of these, the important ones includes *Bacillus* sp., *Caldibacillus cellulovorans* and *Caldicellulosiruptor* sp. Rt8B (Hatada *et al*., 2005; Zhang *et al*., 2006). Among fungi, the most mannanolytic group belongs to the genera *Aspergillus, Agaricus, Trichoderma* and *Sclerotium* (Chen *et al*., 2007; Huang *et al*., 2007). Mannanases have also been reported in higher plants (Marraccini *et al*., 2001) and animals (Yamaura *et al*., 1996).

1.3.2.2 α-L-Arabinofuranosidases

Enzymes hydrolysing L-arabinose linkages have been classified into two major groups: endo-1,5-α-L-arabinases (EC 3.2.1.99) that hydrolyze the α-1,5-L-arabinofuranosidic linkages of arabinans (Kaji, 1984) and α-L-arabinofuranosidases (EC 3.2.1.55) catalyze the hydrolysis of terminal non-reducing α-L-arabinofuranosyl residues from arabinoxylan, L-arabinan, and other L-arabinose containing polysaccharides, releasing arabinose as the only hydrolysis product (Subramaniyan and Prema, 2002). The systematic name is α-L-arabinofuranoside arabinofuranohydrolase, but commonly used synonyms include α-L-arabinofuranosidase. α-L-arabinofuranosidases are found in GH families 3, 43, 51, 54 and 62 (Saha, 2000). A number of microorganisms, including fungi, actinomycetes, and bacteria, have been reported to produce α-arabinosidases.

The first α -L-arabinofuranosidase was purified by Kaji *et al*. (1967) from the culture filtrate of *Aspergillus niger*. Multiple forms of arabinosidases have been reported from some microorganisms. *A. niger* produces three enzymes with different specific activities such as α-L-arabinofuranosidase A, which is active only on PNPA and oligomeric substrates, α-L-arabinofuranosidase B which is active on PNPA and both oligo and polymeric substrates and an endo-1,5- α -L-arabinase (Kaji, 1984; Rombouts *et al*., 1988; van der

Veen *et al*., 1991). On the other hand, *Penicillium capsulatum* (Filho *et al*., 1996) and *A. terreus* (Luonteri *et al*., 1998) produce 2 and 3 enzymes, respectively, with similar substrate specificities.

Most fungal α-L-arabinofuranosidases are able to release arabinose from polymeric substrates acting alone. However, the enzymes acting alone on polymeric substrates seem to prefer arabinose-substituted oligosaccharides as substrates, since in most of the cases the amounts of arabinose released by the enzymes are enhanced when xylanases are present (Sinha and Sengupta, 1995).

1.3.2.3 α-D-Glucuronidases

α-D-Glucuronidases (EC 3.2.1.1) cleave the α-1,2-glycosidic bond of the 4-*O*-methyl-D-glucuronic acid side chain of xylans (Puls *et al*., 1987). α-D-glucuronidases hydrolyze the α-1, 2-glycosidic linkages between xylose and D-glucuronic acid and are found exclusively in family 67. The first crystal structure for a GH67 glycosidase was solved for α-glucuronidase from *Cellvibrio japonicus* (Nurizzo *et al*., 2002). The hydrolysis of the far stable α-(1, 2)-linkage is the bottleneck in the enzymatic hydrolysis of xylan and the reported α-glucuronidases have different substrate requirements. Similar to the lignin carbohydrate linkage, 4-*O*-methyl-glucuronic acid linkage forms a barrier in wood degradation (Subramaniyan and Prema, 2002).

1.3.2.4 β-Xylosidases

β-Xylosidases (EC 3.2.1.37) are exo-type glycosidases that catalyze the hydrolysis of 1,4-β-D-xylooligosaccharides by removing successive xylose residues from the non-reducing termini. It also releases xylose from branched or substituted xylooligosaccharides produced by the action of endo-1, 4-β-xylanases (Subramaniyan and Prema, 2002). The

systematic name is 1,4-β-D-xylan xylohydrolase, but a commonly used name include β-xylosidase and are found in families 3, 39, 43, 52 and 54 (Shallom and Shoham, 2003).

Several β-xylosidases have however been reported to attack polymeric xylan in an exo fashion (Biely, 2003). Kormelink *et al*. (1993b) reported that fungal β-xylosidases from *A. awamori* had a specific requirement for structural arrangement of branched oligosaccharides when tested on different arabinose-substituted xylooligosaccharides. The β-xylosidases from *A. awamori* was able to remove terminal xylopyranosyl residues from the non-reducing end of branched oligosaccharides only when two contiguous unsubstituted xylopyranosyl residues were linked to singly or doubly substituted xylopyranosyl residues. Similar observations on the behaviour of β-xylosidase from *T. reesei* have been reported by Hermann *et al*. (1997). In addition to the formation of hydrolysis products, many β-xylosidases have substantial transferase activity, especially at high substrate concentrations, producing oligosaccharides of higher molecular weight than the original substrate (Matsuo and Yasui, 1984).

1.3.2.5 β-Mannosidase

β-Mannosidase (β-1,4-D-mannoside mannohydrolase, EC 3.2.1.25) catalyses the hydrolysis of mannose units from the non-reducing end of mannosides. However, some β-mannosidases are active both on glucosides and mannosides (Bauer *et al*., 1996). The most commonly employed substrate for analysis of β-mannosidase activity is a chromogenic monosaccharide. In addition, several β-mannosidases are also capable of degrading longer manno-oligosaccharides, with DP over 4 (Akino *et al*., 1988; Arai *et al*., 1995). However, only a few β-mannosidases have been shown to release mannose from the non-reducing end of mannan-based polymers (Araujo and Ward, 1990; Kulminskaya *et al*., 1999).

Although there are functional differences, many β-mannosidases are related to each other and are classified in family 2 of glycoside hydrolases, which is included in the GH-A clan (Henrissat, 1991; Henrissat and Davies, 1997). The molecular weights of most β-mannosidases, are in the range 50 – 130 kDa. However, some β-mannosidases appear to consist of several subunits (Parker *et al*., 2001). The isoelectric points of most β-mannosidases are in the acidic range, except for some bacterial mannosidases with isoelectric points near neutrality.

1.3.2.6 α-Galactosidase

α-Galactosidase (EC 3.2.1.22) catalyses the cleavage of terminal α-galactose residues from α-*O*-galactosides including galactose-containing oligosaccharides and branched polysaccharides, such as galactomannans and galactoglucomannans. These enzymes have been classified into families 4, 27, 36 and 57 of glycoside hydrolases (Henrissat, 1991). In hemicellulose degradation, α-galactosidases release galactosyl side-groups from oligomeric and polymeric mannan substrates. It may have an application in digestion of guar gum, which contains about 40% galactoses with α-1,6-linkages on a β-mannosyl backbone. α-galactosidase can be used in modification of wood-derived materials because galactomannans and galactoglucomannans are the main groups of hemicelluloses in softwoods (Shabalin *et al*., 2002; Golubev *et al*., 2004).

1.3.2.7 β-Glucosidase

β-Glucosidase (EC 3.2.1.21) is a ubiquitous enzyme found in most of the polysaccharide degrading microbes. In hemicellulose degradation, β-Glucosidase catalyses the hydrolysis of non-reducing terminal glucose from oligosaccharides released by β-mannanase. In a few cases it has been shown to release glucose residues from polymeric glucomannan. β-Glucosidase is also important in the degradation of cellulose; it degrades cellobiose

released by cellobiohydrolase and endoglucanase. Most β-glucosidases have been classified into families 1 and 3 of glycoside hydrolases (Yamamoto *et al*., 2000).

1.3.2.8 Hemicellulolytic esterases

Hemicellulolytic esterases include acetyl xylan esterases (EC 3.1.1.72) that hydrolyze the acetyl substitutions on xylose moieties, and feruloyl esterases (EC 3.1.1.73), which hydrolyze the ester bond between the arabinose substitutions and ferulic acid. This latter ester bond is involved in crosslinking of xylan with lignin (Shallom and Shoham, 2003). Acetylxylan esterases represent a group of carbohydrate esterases with great potential in biotechnology and carbohydrate chemistry. They deacetylate partially acetylated 4-*O*-methyl-D-glucuronoxylan, the main hardwood hemicellulose, or its fragments generated upon the action of endo-1,4-β-xylanases (Hakulinen *et al*., 2000). The complete hydrolysis of natural glucuronoxylans requires esterases to remove the bound acetic and phenolic acids. Esterases break the bonds of xylose to acetic acid [acetyl xylan esterase], arabinose side chain residues to ferulic acid (feruloyl esterase) and arabinose side chain residue to *p*-coumaric acid (*p*-coumaroyl esterase). Cleavage of acetyl, feruloyl, and *p*-coumaroyl groups from the xylan are important for the complete removal of lignin. They may contribute to lignin solubilization by cleaving the ester linkages between lignin and hemicelluloses (Subramaniyan and Prema, 2002).

The catalytic modules of hemicellulases are either glycoside hydrolases (GHs) that hydrolyze glycosidic bonds, or carbohydrate esterases (CEs), which hydrolyze ester linkages of acetate or ferulic acid side groups. Various hemicellulolytic enzymes, their substrates, classification into GH and CE families are given in Table 1. 2.

Table 1.2 Hemicellulolytic enzymes and their classification (Shallom and Shoham, 2003)

Enzyme*	Substrates	EC	Family
Endo-β-1,4-xylanase	β-1,4-xylan	3.2.1.8	GH 5 8 10 11 43
Exo-β-1,4-xylosidase	β-1,4-xylooligomers xylobiose	3.2.1.37	GH 3 39 43 52 54
α-L-Arabinofuranosidase	α-Arabinofuranosyl (1⟶ 2) or (1⟶ 3) xylooligomers α-1,5-arabinan	3.2.1.55	GH 3 43 51 54 62
Endo-α-1,5-arabinanase	α-1,5-arabinan	3.2.1.99	GH 43
α -Glucuronidase	4-O-methyl-α-glucuronic acid (1⟶ 2) xylooligomers	3.2.1.139	GH 67
Endo-β-1,4-mannanase	β-1,4-mannan	3.2.1.78	GH 5 26
Exo- β-1,4-mannosidase	β -1,4-mannooligomers mannobiose	3.2.1.25	GH 1 2 5
α -Galactosidase	α-galactopyranose (1⟶6) mannooligomers	3.2.1.22	GH 4 27 36 57
β-Glucosidase	β-glucopyranose (1⟶ 4) mannopyranose	3.2.1.21	GH 1 3
Endo-galactanase	β-1,4-galactan	3.2.1.89	GH 53
Acetyl xylan esterase	2- or 3-*O*-acetyl xylan	3.1.1.72	CE 1 2 3 4 5 6 7
Acetyl mannan esterase	2- or 3-*O*-acetyl mannan	3.1.1.6	
Ferulic and *p*-cumaric acid esterases		3.1 .1 .73	CE 1

*****Endo-glycoside hydrolases cleave the glycosidic bond within the polysaccharide backbone, whereas exo-glycoside hydrolases cleave off mono- or di-saccharides either from the reducing or the non-reducing end of the polysaccharide chain

1.4 PRODUCTION OF XYLANASE

The basic factors for efficient production of enzymes are the choice of an appropriate inducing substrate and an optimum medium composition. Xylanases may be industrially produced in submerged liquid culture or on a solid substrate and 80 – 90% of all xylanases are produced in submerged culture (Polizeli *et al*., 2005). Various waste and underutilized lignocellulosic agricultural residues can serve as low-cost substrate for production of industrial enzymes. Hydrolysates of many different bulk materials have been used for xylanase and cellulase production for decades. Use of such inexpensive substrates in xylanase production makes the process commercially viable since the use of pure xylan is very expensive. Moreover, it is a judicious alternative for utilization and disposal of agricultural lignocellulosic residues.

A large number of lignocellulosic materials are in frequent use in the production of xylanases from fungi. These include wheat bran, wheat straw, rice husk, rice straw, barley straw, corn cobs, sugarcane bagasse, beet pulp, beechwood shavings and apple pomace etc., (Archana *et al.*, 1999). Co-produced cellulases tend to be a major limitation for application of fungal xylanase in pulp bleaching which can be overcome by using a strain that does not produce cellulase or by using xylan as substrate so that cellulase production remains suppressed during the growth of fungi (Gomes *et al.*, 1993). Notably, some filamentous fungi are known to produce higher xylanase when cultured on wood pulp than on pure xylan (Royer and Nakas, 1989).

1.5 INDUCTION AND REGULATION OF HEMICELLULASES

Xylanases are usually inducible enzymes secreted in medium containing pure xylan or xylan-rich residues (Balakrishnan *et al*., 1997). However, constitutive production of xylanase has also been reported (Segura *et al*., 1998). Induction is mostly by xylan in

Trametes trogii (Levin and Forschiassin, 1998), *A*. *awamori* (Siedenberg *et al*., 1998), and *Streptomyces* sp. QG-11-3 (Beg *et al*., 2000). However, in *Cellulomonas flavigena*, xylan is a poor inducer (Avalos *et al*., 1996). Induction of xylanase by several other compounds, such as L-sorbose, various xylooligosaccharides, xylose, and lignocellulosic residues, has been reported. L-sorbose in medium induced the xylanase production in *Sclerotium rolfsii* (Sachslehner *et al*., 1998) and *Trichoderma reesei* PC-3-7 (Xu *et al*., 1998). In *Bacillus* sp. BP-7 (Lopez *et al*., 1998) and *Trichosporon cutaneum* SL409 (Liu *et al*., 1999), xylanase was induced by xylose and repressed in the presence of glucose.

Several reports have shown xylanase induction by lignocelluloses such as wheat bran, rice straw, corncobs, and sugarcane bagasse (Keskar, 1992; Kuhad *et al*., 1998; Puchart *et al*., 1999; Beg *et al*., 2000; Gupta *et al*., 2001). Another compound often used as a potent inducer is methyl β-xyloside (BMX), a non-metabolizable structural analogue of xylobiose (Simão *et al.*, 1997). Induction of the xylanolytic system by other synthetic compounds, such as 2-*O*-β-D-xylopyranosyl D-xylose (Xylβ1-2Xyl), 3-*O*-β-D-xylopyranosyl D-xylose (Xylβ1-3Xyl) and 2-*O*-β-D-glucopyranosil D-xylose (Glcβ1-2Xyl), has also been described (Hrmová *et al*., 1991). The xylobioses, which are homodisaccharides (Xylβ1-2Xyl e Xylβ1-3Xyl), are potent inducers of endo-1,4-β-xylanase but fail to induce cellulolytic endo-1,4-β-glucanase complex. The opposite was true for the heterosaccharide Glc β1-2Xyl. Such synthetic substrates serve as hybrid inducers, promoting the synthesis of both the enzyme complexes. The results of these studies suggest that the existence of separate regulatory systems for the synthesis of cellulases and xylanases (Polizeli *et al*., 2005).

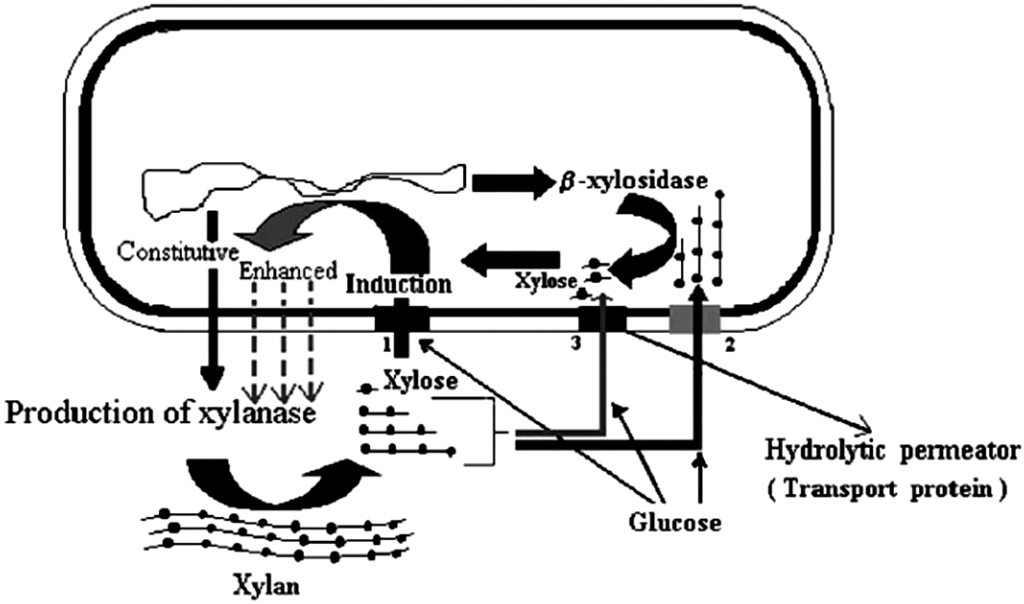


Fig. 1.3 Hypothetical model for xylanase gene regulation in bacteria (Subramaniyan and Prema, 2002).

The regulation of xylanase secretion by microorganisms is still not completely understood. In general, most systems are inducible, and the molecular inducers are the corresponding monosaccharides or disaccharides (e.g., xylose or xylobiose), or larger oligosaccharides such as aldotetraouronica acid. Nearly all systems are also regulated by carbon-source availability (i.e., catabolite regulatory elements). A low basal level of expression of some extracellular enzymes and permeases must exist to allow the uptake of the corresponding inducers. In fact, in many microorganisms some of the hemicellulolytic related genes are expressed constitutively at low levels (Shallom and Shoham, 2003).

In most of the reports regarding xylanases there is the occurrence of constitutive enzyme production (Wang *et al*., 1992; Zhao *et al*., 1997). Xylanase degrades xylan, comparatively a large heteropolysaccharide, which is prevented from entering the cell matrix by the cell

membrane. The products of xylan hydrolysis are small-molecular-weight molecules (xylose, xylobiose, xylotriose, and other oligo-saccharides) and these molecules easily enter the microbial cells and sustain the growth by acting as energy and carbon source (Biely, 1985; Wang *et al*., 1992; Zhao *et al*., 1997, Singh *et al*., 2003). The products of hydrolysis can stimulate xylanase production by different methods (Fig 1.3). Xylose being a small pentose molecule can enter the bacterial and fungal cells easily and induce xylanase production (Biely, 1985; Zhao *et al*., 1997). However, the larger molecules pose a problem in transportation, which questions the direct induction role of these macromolecules on enzyme synthesis (Zhao *et al*., 1997).

There are two plausible explanations for the inductive role of larger molecules based on the reports of Wang *et al*. (1992) and Gomes *et al*. (1994). One of the explanations is that the xylo-oligomers formed by the action of xylanase on xylan are directly transported into the cell matrix where they are degraded by the intracellular β-xylosidase that releases the xylose residues in an exo-fashion from the xylo-oligomers. The above concept is supported by the universal occurrence of intracellular β-xylosidases (Kulkarni *et al.*, 1999; Fontes *et al*., 2000; La Grange *et al*., 2000) in microorganisms. The other possibility is that the oligomers are hydrolyzed to monomers during their transportation through the cell membrane into the cell matrix by the action of hydrolytic transporters having exo β-1,4-bond cleaving proteins like the β-xylosidases. The above idea stemmed from the reports on β-xylosidases with transferase activity (Conrad and Nothen, 1984). In both ways the resulting xylose molecules as mentioned earlier, result in the enhanced production of xylanase. However, there are rare cases where the xylose molecules repress the xylanase production in *Bacillus thermoalkalophilus* (Rajaram and Varma, 1990). If glucose, the most effective carbon source, is present in the growth medium there is repression of synthesis of catabolic enzymes that may be occurring at the transcriptional level or by mere inducer

exclusion of the respective inducers of these enzymes. The first one, that is, the catabolic repression at the transcriptional level has been clearly explained by Saier and Fagan (1992).

1.6 XYLANASE PRODUCING ORGANISMS

Xylanases are produced by a plethora of organisms including bacteria, algae, fungi, protozoa, gastropods and arthropods (Prade, 1996) and some members of higher animals, including fresh water mollusks (Yamura *et al*., 1997). Most of the microbial xylanases have been reported from bacteria and fungi (Wong *et al.,* 1988; Kulkarni *et al*., 1999). However, there are reports on xylanase from plants, for e.g., Japanese pear fruit (during over-ripening period) and European wheat (*Triticum aestivum*) flour (Cleemput *et al*., 1997).

Several microorganisms including bacteria, yeasts and fungi have been investigated for the production of xylanases for industrial purposes. Whilst a number of non-pathogenic, 'generally recognised as safe (GRAS) microbial organisms are used as producers of enzymes, the filamentous fungi are particularly important due to their relative ease and economy of use and the variety and levels of the enzymatic activities that they are able to produce. This undoubtedly stems from their inherent metabolic diversity which is reflected in their ability to grow on a wide range of substrates and under a wide variety of conditions in the natural environment. Moreover, filamentous fungi are particularly interesting producers of xylanases since they secrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria.

During last three decades, many fungi having varied physiological characteristics have been investigated for xylanase production by different workers. These include mesophiles (Tan *et al*., 1985; Belancic *et al*., 1995; Park *et al*., 2002), thermotolerants (Keskar *et al*., 1989; Mendicuti Castro *et al.*, 1997; Rizzatti *et al*., 2004), thermophiles (Wiegel *et al*.,

1985; Dusterhoft *et al*., 1999; Maalej *et al*., 2008), alkalophiles (Honda *et al*., 1985; Raj and Chandra, 1995; Taneja *et al.*, 2002; Dwivedi *et al*., 2009) and plant pathogens (Poutanen *et al*., 1987; Christakopoulos *et al.*, 1997; Giesbert *et al.*, 1998; Gomez-Gomez *et al.*, 2002).

The mesophilic fungus *Aspergillus* has been extensively studied for hemicellulase production. Among them**,** *A. ochraceous* (Biswas *et al.*, 1990), *A*. *tamarii* (Simão *et al.*, 1997), *A*. *niger* (Frederick *et al.*, 1985; Qy *et al.*, 1996) and *A*. *nidulans* (Mendicuti Castro *et al.*, 1997) have been reported for xylanase production. *A*. *nidulans* produced a xylanolytic complex of at least three endo-1,4-β-D-xylanases of molecular weights 22, 24 and 34 kDa and a β-xylosidase when grown in the presence of xylan or xylooligosaccharides (Fernández-Espinar *et al*., 1992; Piñaga et al., 1994). Park *et al.* (2002) indicated production of 5484 U mg^{-1} of xylanase from *A. niger* using rice straw as substrate under solid state fermentation (SSF). Alkalitolerant *A*. *fischeri* Fxn 1 (Raj and Chandra, 1995) and *A*. *nidulans* KK-99 (Taneja *et al.*, 2002) has been found to produce alkalistable xylanases. Many more species of *Aspergillus such* as *A*. *fumigatus*, *A*. *terreus, A*. *sydowii*, *A*. *kawachi* and *A*. *sojae* have also been found to secrete xylanolytic enzymes *in vitro* (Hrmova *et al.*, 1989; Ghosh and Nanda, 1994; Kimura *et al.*, 1995). Garcia-Kirchner *et al.* (2002) found increments in the yield of cellulase and xylanase during co-culturing (mixed-culture) of *Penicillium* sp. and *A*. *terreus.*

Several strains of *Trichoderma* have been characterized for production of xylanase (Defaye *et al.*, 1985; Durand *et al.*, 1988). Gomes *et al.* (1992) reported 188 IU ml^{-1} xylanase activity from *T*. *viride*. Relatively much higher yield (960 IU ml^{-1}) of xylanase has been noticed from *T*. *reesei* (Bailey *et al.*, 1993). Production of very high titres of xylanase has been noticed in *Schizophyllum commune* cultures by Haltrich *et al*. (1993) while

Steiner *et al*. (1987) reported 1244 IU ml^{-1} of xylanase along with co-secreted cellulase (65 IU ml^{-1}) from *S*. *commune*.

Xylanase production has also been noticed in basidiomycetous fungi by several workers (Elisashvili *et al.*, 1999; Inglis *et al.*, 2000). Milagres *et al.* (2001) have reported xylanase production by two basidiomycetes *Poria-medula panis* and *Wolfporia cocos* on wheat bran. Besides above reports on xylanase production by common fungi, it has also been reported and characterized from certain plant pathogens such as *Fusarium oxysporum* (Gomez-Gomez *et al.*, 2002), *Chochilobolous carbonum* (Apel-Birkhold and Walton, 1996), Wood rot fungi, *Phanerochaete chrysosporium* (Castanares *et al*., 1995; Khalil, 2002) and *Heterobasidion annosum* (Maijala *et al.*, 1995). *Aureobasidium pullulans*, a yeast, has been reported to produce 398 IU ml^{-1} of xylanase on xylan and β-methyl xyloside (BMX) containing medium (Leathers *et al*., 1986). Xylanase production by various microorganisms is presented in Table 1.3. and characters of xylanase produced by some microorganisms are given in Table 1.4.

Table 1.3 Xylanase production by various microorganisms

Microorganism	Xylanase (U ml^{-1})	Reference
Aspergillus niger KKS	138	Kang *et al*. (1995)
A. fumigatus	125.1	Lenartovicz *et al*. (2002)
A. niger B03	996.3	Dobrev *et al*. (2007)
Chaetomium globosum 11-Ch.g./5	65.3	Zychlinska *et al*. (1992)
Pencillium oxalicum SAU_E-3.510	488.5	Dwivedi *et al*. (2009)
Pycnoporus coccineus	135	Elisashvili *et al*. (2008)
Phanerochaete chrysosporium	15-20	Copa-Patiño *et al*. (1993)
Pseudomonas sp. WLUN024	450	Xu *et al*. (2005)
Schizophyllum commune	1244	Steiner *et al*. (1987)
Sclerotium rolfsii	267	Sachslehner *et al.* (1998)
Sporotrichium pulverulentum	20.4	Eriksson and Johnsrud (1983)
Talaromyces emersonii CBS 814.70	56	Tuohy *et al*. (1990)
Thielavia terrestris ATCC 26917	25.5	Merchant *et al.* (1988)
Trichoderma harzianum	450	Saddler *et al.* (1985)
T. reesei	960	Bailey *et al.* 1993)
T. viride	188.1	Gomes *et al*. (1992)
Bacillus circulans	400	Ratto *et al.* (1992)
Bacillus sp.	120	Balakrishnan *et al.* (1992)
Cellulomonas fiavigena NIAB 441	16	Rajoka and Malik (1984)
Cellulomonas sp. (GS2)	9.33	Saxena *et al.* (1991)
Streptomyces roseiscleroticus NRRL-B-11019	16.2	Grabski and Jeffries (1991)
Thermomyces lanuginosus	650-780	Gomes *et al.* (1993)
T. lanuginosus SSBP	3575	Singh *et al*. (2000a)
T. lanuginosus RM-B	2449	Puchart *et al*. (1999)
T. lanuginosus IMI 84400	2460	Puchart *et al*. (1999)
T. lanuginosus ATCC 16455	2600	Puchart *et al*. (1999)
T. lanuginosus ATCC 46882	2840	Bennet *et al*. (1998)
T. lanuginosus IOC-4145	850	Damaso *et al*. (2004)
T. lanuginosus IMI 84400	226	Puchart and Biely (2008)
T. lanuginosus MC134	3299	Kumar *et al*. (2009)

Table 1.4 Characteristics of some xylanases produced by different microorganisms

Microorganisms	Molecular weight (kDa)	Optimal temperature (°C)	Optimal pH	Reference
Aspergillus aculeatus	18, 26, 52	50, 50, 70	4.0, 4.0, 5.0	Fujimoto *et al*. (1995)
A. awamori	39, 23, 26	45 – 55	4.0 – 5.5	Kormelink *et al*. (1993b)
A. fischeri	31	60	6.0	Raj and Chandra (1996)
A. fumigatus	19, 8.5	55	5.5	Silva *et al*. (1999)
A. kawachii	35, 26, 29	60, 55, 50	5.5, 4.5, 2.0	Ito *et al*. (1992)
A. nidulans	22, 34	62, 56	5.5, 6.0	Fernández-Espinar *et al.* (1994)
A. nidulans KK-99	ND	55	8.0	Taneja *et al*. (2002)
A. oryzae	35	60	5.0	Kitamoto *et al*. (1999)
A. sojae	33, 36	60, 50	5.0, 5.5	Kimura *et al*. (1995)
A. sydowii	33	50	4.0	Ghosh and Nanda (1994)
A. versicolor	19	55	6.0	Carmona *et al*. (1998)
Acrophialophora nainiana	22	55	7.0	Salles *et al*. (2000)
Aureobasidium pullulans	25	54	4.4	Li *et al*. (1993)
Bacillus sp.	99	75	6.0	Bataillon *et al*. (2000)
Chaetomium cellulolyticum	25, 47, 57	50	5.0–7.0	Baraznenok *et al*. (1999)
Cryptococcus sp.	22	40	2.0	Iefuji *et al*. (1996)
Fusarium oxysporum F3	20.8, 23.5	60, 55	6.0	Christakopoulos *et al*. (1996)
H. grisea var *thermoidea*	23	70	5.5	Monti *et al*. (1991)
Myceliophthora sp.	53	75	6.0	Chadha *et al*. (2004)
Penicillium brasilianum	31	ND	ND	Jørgensen *et al*. (2003)
P. capsulatum	22	48	3.8	Ryan *et al*. (2003)
Penicillium sp.	25	50	2.0	Kimura *et al*. (2000)
Streptomyces sp.	24.5, 37.5, 38	55 – 60	6.0 – 8.0	Georis *et al*. (2000)
Thermoascus aurantiacus	ND	70 – 75	4.0 – 5.0	Kalogeris *et al*. (1998)
Thermomyces lanuginosus	24.7	70	6.0 – 6.5	Singh *et al*. (2000b)
Trichoderma harzianum	20	50	5.0	Tan *et al*. (1985)

1.6.1 Thermophiles

Microorganisms, during the period of their evolution become adapted to survive in a wide array of environmental niches. Accordingly microorganisms are found in almost all habitats including those exhibiting extremely variable conditions. Extreme thermophily is witnessed in eubacteria or archaea, some of which are able to grow near or above 100°C in thermal springs, solfatara fields, or hydrothermal vents (Brock, 1995; Blochl *et al.*, 1997).

A number of thermophilic (optimal growth at 50 – 80°C) and hyperthermophilic (optimal growth at >80°C) xylanase producing microorganisms have been isolated from a variety of sources, including terrestrial and marine solfataric fields, thermal springs, hot pools and self-heating decaying organic debris (Harris *et al*., 1997; Sunna *et al*., 1997; Vieille and Zeikus, 2001; Singh *et al*., 2003; Sunna and Bergquist, 2003).The majority of the xylanases produced have been found to belong to families 10 and 11, with as yet, no report on thermophilic xylanases belonging to any of the other glycoside hydrolase families.

Family 10 xylanases have been isolated from various thermophilic and hyperthermophilic organisms, including *Thermotoga* sp. (Zverlov *et al*., 1996), *Caldicellulosiruptor* sp. (Luthi *et al*., 1990), *Rhodothermus marinus* (Abou-Hachem *et al*., 2003), *Bacillus stearothermophilus* (Khasin *et al*., 1993), *Thermoascus aurantiacus* and *C. thermocellum* (Leggio *et al*., 1999). Indeed, a family 10 xylanase, XynA from *Thermotoga* sp. strain FjSS3-B.1 is one of the most thermostable xylanases reported to date with an apparent optimum temperature for activity of 105°C and a half-life of 90 min at 95°C (Simpson *et al*., 1991), while less frequent, family 11 thermophilic xylanases have also been isolated, with those from *Paecilomyces varioti* (Kumar *et al*., 2000), *Caldicellulosiruptor* sp Rt69B.1. (Morris *et al.*, 1999), *Dictyoglomus thermophilum* (McCarthy *et al*., 2000), *Chaetomium*

thermophilum, *Nonomuraea flexuosa* (Hakulinen *et al*., 2003), *Bacillus* strain D3 (Connerton *et al*., 1999) and *T. lanuginosus* (Schlacher *et al.,* 1996; Singh *et al*., 2003) being the most thoroughly investigated.

Many endoxylanases from thermophiles have some degree of structural homology with those from mesophiles. A number of authors have tried to explain the thermostability observed in enzymes from thermophiles in terms of extra disulphide bridges, an N-terminal proline residue causing a reduction in conformational freedom, salt bridges and presence of hydrophobic side-chains (Turunen *et al*., 2001). Hakulinen *et al*. (2003) described some minor modifications responsible for the increased thermal stability of xylanases: (i) higher Thr/Ser ratio; (ii) increased number of charged residues, especially Arg, resulting in enhanced polar interactions; and (iii) improved stabilization of secondary structures involving a higher number of residues in the beta-strands, and stabilization of the alpha-helix region. Some xylanases improve their stability by compacting the protein structure with a higher number of ion pairs or aromatic residues on the protein surface, resulting in enhanced interactions. However, no definite conclusion has been reached, since a phenomenon displayed by one microorganism may not occur in another.

Xylanases with high thermostability are better candidates for industrial applications, particularly for enzymatic hydrolysis at elevated temperatures (e.g., biopulping) where mesophilic xylanases fail to meet desired results. Several thermophilic strains have been tested for production of thermostable enzymes and have been found more apt for industrial applications as compared to their mesophilic counterparts (Satyanarayana *et al.,* 1992; Maheshwari *et al.,* 2000). Among the reported xylanase producers, strains of *Thermomyces lanuginosus* have been found to produce high titres of thermostable xylanolytic enzymes (Kitpreechavanich *et al.*, 1984; Anand and Vithayathil, 1990; Gomes *et al.*, 1993; Alam *et al.*, 1994; Hoq *et al.*, 1994; Purkarthofer and Steiner, 1995; Chadha

et al., 1999; Singh *et al*., 2003; Kumar *et al*., 2009). The thermostability of β-xylanases produced by nine thermophilic *T. lanuginosus* strains in coarse corn cob medium was assessed by Singh *et al*. (2000b). The xylanase produced by *T. lanuginosus* SSBP retained 100% of its activity after 6 h at temperatures up to 65°C. In comparison, seven ATCC strains and the DSM 5826 strain of *T. lanuginosus* only retained 100% xylanase activity at temperatures up to 60°C. The T1/2 of all strains at 70°C at pH 6.5 varied greatly from 63 min for strain ATCC 28083 to 340 min for strain SSBP (Table 1.5). T1/2 determinations are more accurate and reliable especially when comparing stability properties of enzymes among strains.

Table 1.5 Estimated half-lifes and inactivation rate constants of β-xylanase from *T. lanuginosus* strains at 70°C and pH 6.5 (Singh *et al*., 2000b)

Strains	**$T_{1/2}$ (min)**	**Inactivation rate (h)**
T. lanuginosus SSBP	232	–0.0015 ± 0.0001
T. lanuginosus DSM 5826	201	–0.0018 ± 0.0001
T. lanuginosus ATCC 16455	54	–0.0056 ± 0.0002
T. lanuginosus ATCC 22083	81	–0.0040 ± 0.0002
T. lanuginosus ATCC 26909	79	–0.0037 ± 0.0001
T. lanuginosus ATCC 28083	79	–0.0052 ± 0.0004
T. lanuginosus ATCC 34626	126	–0.0028 ± 0.002
T. lanuginosus ATCC 36350	121	–0.0030 ± 0.0002
T. lanuginosus ATCC 58160	116	–0.0032 ± 0.0002

1.6.1.1 *Thermomyces lanuginosus*

T. lanuginosus (formerly known as *Humicola lanuginosa*) is a widely distributed thermophilic fungus generally appears in the self-heating masses of organic debris

(Emerson, 1968). It was first isolated in 1899 by Tsiklinskaya from a potato, which had been inoculated with garden soil and grown on white bread kept at 52 – 53°C (Cooney and Emerson, 1964). This thermophilic fungus has been isolated in the Brazil, British Isles, Canada, Denmark, Ghana, India, Indonesia, Italy, Japan, Nigeria, South Africa, and USA. *T. lanuginosus* strains have been reported to occur in dry and waterlogged grassland, loamy garden soil and aquatic sediments but the fungus is more specifically associated with organic substrates such as the culms, roots and leaves of grasses, composts of various plant materials and the dung of various birds and mammals (Singh *et al*., 2003). It has also been trapped from air in Indonesia and the British Isles where it was the second most abundant thermophilic species, and was surpassed only by *A*. *fumigatus* in abundance (Cooney and Emerson, 1964).

1.6.1.2 Morphology and taxonomy

T. lanuginosus belongs to the class Deuteromycetes (imperfect fungi), with unicellular or septate mycelia and reproduces asexually by forming aleurioconidia (Hudson, 1992) borne single at the tips of aleuriophores. In young colonies, spores are colourless and smooth walled but as maturation proceeds, they turn dark brown and the thick exospore becomes characteristically wrinkled. Mature spores are spherical, irregularly sculptured and range from 5.5 – 12 µm in diameter. Dipicolonic acid has been found in aleurioconidia, which plays an important role in heat resistance. Aleuriospores are generally unbranched, but occasionally they branch once or twice near the base and appear to cluster. Septations commonly occur in the aleuriospores, but they are difficult to observe. The mycelium is partly superficial, and partly immersed while there are no stroma, setae and hyphopodia. Aleuriospores are micronematous, straight or flexous, colourless or brown and smooth (Cooney and Emerson, 1964).

T. lanuginosus grow rapidly on various media reaching 2.5 to over 5 cm in diameter at 45 – 50°C within 2 days. Initially the colonies appear white and felt-like and are less than 1 mm high, but soon turn grey or greenish-grey, commencing from the centre of the colony. Subsequently the colony turns purplish brown and the agar substratum stains a deep pink or wine colour, due to diffusible substances secreted by the colony. Mature colonies appear dull dark brown to black (Cooney and Emerson, 1964).

1.7 APPLICATION OF HEMICELLULASES

Global markets for industrial enzymes grew from €1 billion in 1995 (Godfrey and West, 1996) to almost €2 billion in 2001 (Godfrey, 2003) and continue to increase as new enzymes and applications are discovered. Presently the technical industries, dominated by the detergent, starch, textile and biofuel industries, account for the majority of the total enzymes market, with the feed and food enzymes together totaling only about 35% (Collins *et al*., 2005). Hydrolases constitute approximately 75% of the markets for industrial enzymes, with the glycosidases, including cellulases, amylases and hemicellulases, constituting the second largest group after proteases (Bhat, 2000). Xylanases constitute the major commercial proportion of hemicellulases but represent only a small percentage of the total enzyme sales. The sales figures are expected to increase however, as these enzymes have attracted increasing attention due to their potential for use in several applications.

Xylanolytic enzymes from microorganisms have attracted a great deal of attention in the last decade, particularly because of their biotechnological potential in various industrial processes (Kuhad and Singh 1993; Bajpai, 1999; Niehaus *et al*., 1999; Bhat, 2000), such as food, feed, and pulp and paper industries. Other less well documented putative applications include: brewing, to increase wort filterability and reduce haze in the final

product (Tikhomirov *et al*., 2003); in coffee extraction and in the preparation of soluble coffee (Wong *et al*., 1988) in detergents (Kamal Kumar *et al*., 2004); in the protoplastation of plant cells (Kulkarni *et al*.,1999); in the production of pharmacologically active polysaccharides for use as antimicrobial agents (Christakopoulos *et al*., 2003) or antioxidants (Katapodis *et al*., 2003); in the production of alkyl glycosides for use as surfactants (Matsumura *et al*., 1999); and in the washing of precision devices and semiconductors (Imanaka and Sakurai, 1992).

1.7.1 Food industry

Xylanases are used in the food industry in a number of applications including baking, juice preparation, and starch processing (MacCabe *et al*., 2002). Xylanases may be employed in bread-making, together with α-amylase, malting amylase, glucose oxidase and proteases. The xylanases, like the other hemicellulases, break down the hemicellulose in wheat-flour, helping in the redistribution of water and leaving the dough softer and easier to knead (Polizeli *et al*., 2005). Xylanases also have an application in rye baking where the addition of xylanase makes the doughs soft and slack (Dervilly *et al*., 2001; Dervilly *et al*., 2002; Harbak and Thygesen, 2002). Xylanases are used as dough strengtheners because they provide excellent tolerance to the dough toward variations in processing parameters and in flour quality. They also significantly increase the volume of the baked bread (Harbak and Thygesen, 2002). Also, a larger amount of arabinoxylooligosaccharides in bread would be beneficial to health. In biscuit-making, xylanase is recommended for making cream crackers lighter and improving the texture, palatability and uniformity of the wafers (Polizeli *et al*., 2005). Xylanase is also used in curing of coffee (Turner *et al*., 2007). The main desirable properties for xylanases for use in the food industry are high stability and optimum activity at low pH (acidic) conditions.

Hydrolysis of hemicellulosic residues yields a mixture of sugars, primarily D-xylose. Complete hydrolysis of xylan to xylose by xylanolytic saccharification of agricultural residues, woody biomass, hemicellulosic waste from municipal and industrial sources, provides an efficient method for yielding sugar syrups (Biely, 1985; Christov *et al*., 1999; Akpinar *et al*., 2009). These can be further utilized for animal or human consumption or can be used in fermentation industries for microbial growth.

Synergistic action of xylanase and cellulase mixtures could result in complete and efficient method of yielding sugars from lignocelluloses (Wong *et al.*, 1988). Degradation of lignocellulosic biomass by xylanase in synergism with other enzymes can be used for generation of biofuels (ethanol), xylitol (sweetener), and organic acids, via fermentation. (Kuhad and Singh, 1993; Olson and Hann-Hagerdel, 1996; Dominguez, 1998). Moreover, the saccharified liquor can also be used as substrate to produce protein rich biomass or SCP (Ericksson *et al.*, 1990). In juice making processes, xylanases, in conjunction with cellulases, amylases and pectinases, help to improve yield of juice by means of liquefaction of fruit; stabilization of the fruit pulp; increased recovery of aromas, essential oils, vitamins, mineral salts, edible dyes, pigments etc. It also helps in the reduction of viscosity, hydrolysis of substances that hinder the physical or chemical clearing of the juice, or that may cause cloudiness in the concentrate (Polizeli *et al*., 2005). Some commercial xylanases and its applications are given in Table 1.6.

1.7.2 Animal feed industry

The use of enzymes in the production of feed is an important sector of agri-business, with an annual world production exceeding 600 million tons and a turnover of >50 billion dollars (Polizeli *et al*., 2005). Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. These

enzymes break down arabinoxylans in the ingredients of the feed, reducing the viscosity of the raw material (Twomey *et al*., 2003). These enzymes eliminate sticky excreta problems and contribute greatly to decrease waste problems by reducing the water content of the droppings. In addition, the economical use of grain reduces the manure output; which is beneficial to the environment (Bedford, 1995). The nutritional quality can also be enhanced in ruminant animal feed by the addition of xylanase. This is believed to be due to an increase in cellulose accessibility to ruminant digestion by removing the xylan component of feed making digestion complete and improving the feed conversion efficiency (Beauchemin *et al*., 2003).

1.7.3 Pulp and paper industries

Chlorinated phenolic compounds as well as polychlorinated biphenyls, produced during conventional pulp bleaching are toxic and highly resistant to biodegradation, form one of the major sources of environmental pollution (Subramaniyan and Prema, 2002). During the last 30 years, the pulp and paper industry has intensified its efforts to minimize pollution arising from pulping and bleaching processes. Substantial improvements were achieved for the already established delignification processes. Moreover, new pulping methods were developed in order to produce pulps, which are more easily bleachable using only chlorine free bleaching agents (Khristova *et al*., 2006).

Table 1.6 Commercial xylanases produced by microorganisms (Polizeli *et al*., 2005)

Commercial name	Distributors	Microorganism	Fermentation	Optimal pH	Optimal temperature (°C)	Application
Allzym PT	Alltech	*A. niger*	SbmF	5.3	65	Animal feed improvement
Amano 90	Amano Pharmaceuticals	*A. niger*	SSF wheat raw	4.5	50	Pharmaceutical analysis, food industry
Bio-Feed Plus	Novo Nordisk	*Humicola insolens*	SbmF	n.c.	n.c.	Animal feed
Bleachzyme F	Biocon, India	n.c.	n.c	6.5 – 7.0	40 – 50	Pulp bleaching
Cartazyme	Clariant, UK	*Termomonospora fusca*	n.c.	5.0	45 – 55	Pulp bleaching
EcopulpX-200	Primalco	*T. reesei*	SbmF	5.0 –6.0	50 – 55	Cellulose pulp bleaching
Ecosane	Biotec	*T. reesei*	SbmF	n.c.	n.c.	Animal feed
Ecozyme	Thomas Swan, UK	n.c.	n.c.	7.0	50	Cellulose and paper industry
Grindazym GP e GV	Danisco Ingredients	*A. niger*	SbmF	n.c.	n.c.	Bird and pig feed
Irgazyme 40	Nalco-Genencor, Ciba, -Geigy	*T. longibrachiatum*	SbmF	n.c.	n.c.	Paper industry and animal feed
Luminase	Diversa Corp, USA		Diversa, 2004	6-8	50	Pulp and paper Industry
Multifect XL	Genencor	*T. longibrachiatum*	SbmF	5.0 – 5.5	55 – 60	Food industry
Pulpzyme	Novozymes, Denmark	*Bacillus* sp		9.5	50	Cellulose and paper industry
Resinase	A/S	n.c.	n.c.	n.c.	n.c.	Cellulose and paper industry
Solvay pentonase	Solvay Enzymes	*T. reesei*	SbmF	5.3 – 5.5	55	Starch and bread-making industries
Sternzym HC46	Stern-Enzym		SSF	n.c.	n.c.	Bread-making
Sumizyme X	Shin Nihon	*T. koningii*	SSF wheat raw	5.0	55	Manufacture of mushrooms and vegetables extracts, bread-making, enzymatic peeling of cereals, animal feed
Xylanase	Seikagaku	*Trichoderma* sp	SbmF	n.c.	n.c.	Carbohydrate structural studies
Xylanase	Granotec do Brazil	n.c.	n.c.	n.c.	n.c.	Weight decreasing in Cream-Crackers, better texture and taste, Wafer's uniformity improvement
Xylanase GS35	Iogen	*T. reesei*	SbmF	4.5	40	Cellulose pulp bleaching, animal feed

n.c: not cited; SbmF : Submerged fermentation; SSF : Solid substrate fermentation

Xylanases are fast becoming a major group of industrial enzymes, finding significant application in the paper and pulp industry. Xylanases are of great importance to the pulp and paper industries because the hydrolysis of xylan facilitates the release of lignin from paper pulp and reduces the level of usage of chlorine as the bleaching agent (Shoham *et al.,* 1992). Viikari *et al.* (1986) were the first to demonstrate that xylanases are applicable for delignification in the bleaching process. The applicability of xylanases increases daily because Rayon, cellophane, and several chemicals, such as cellulose esters (acetates, nitrates, propionates and butyrates) and cellulose ethers (carboxymethyl cellulose, methyl and ethyl cellulose), are all produced from the dissolving pulp, that is, the pure form of cotton fibre free from all other carbohydrates (Subramaniyan and Prema, 2002).

To obtain pulp of very high brightness and brightness stability, all the lignin must be removed from the pulp. For that chemical pulping is more effective than mechanical pulping. However, there is the formation of residual lignin, which has to be removed by bleaching process. The residual lignin in chemical pulp is dark in color because it has been oxidized extensively and modified in the cooking process. This residual lignin is difficult to remove due to its covalent binding to the hemicellulose and perhaps to cellulose fibers. The bleaching of pulp in paper industries is a purification process involving the destruction, alteration or solubilization of lignin, coloured organic matters and other undesirable residues on the cellulosic fibres (Madlala *et al.*, 2001). Residual xylan and lignin get covalently linked to degradation products generated during pulping, get trapped in the cellulose fibre pulp matrix and impart it brownish colour. (Ziboro, 1990; Kantelinen *et al*., 1991). These colour imparting chromophores are hard to extract and elemental chlorine and chlorine dioxides are used to bleach these for enhancing brightness of pulp (Gierer and Wännström, 1984). The dominant role of chlorine in bleaching is to convert the residual lignin in the pulp to water or alkali-soluble products. Chlorine and chlorine based

chemicals form toxic and recalcitrant organochloro compounds that are released in environment by way of effluent and present dangers of toxicity and bioaccumulation (Subramaniyan and Prema, 2000). Environmental agencies and consumers are increasingly becoming discreet regarding use of chlorine in bleaching. Numerous reports indicate that xylanase pretreatment can reduce chlorine requirements in bleaching of both softwood and hardwood kraft pulps (Tolan and Canovas, 1992; Onysko, 1993; Scott *et al*., 1993). Several weighty benefits of xylanase pretreatment of pulp include higher brightness ceilings, significant reduction in the amounts of chlorine based bleaching chemicals thus avoiding consequential release of organochlorine compounds in bleach plant effluents. Xylanase pretreatment brings about depolymerization of xylans and this helps in release of otherwise occluded chromophoric material. The loosening of pulp structure by xylanase treatment enhances lignin extraction with minimum chemicals (Viikari *et al*., 1990).

1.7.4 Mode of action of xylanase on pulp

During the kraft process part of the xylan is relocated on the fiber surfaces. A considerable amount of xylan is present in the fibers after the pulping process. Two types of phenomena are involved in the enzymatic pretreatment. The major effect is due to hydrolysis of reprecipitated and readsorbed xylan or xylan-lignin complexes that are separated during the cooking process. Enzymatic hydrolysis of the reprecipitated and relocated xylans on the surface of the fibers apparently renders the structure of the fiber more permeable. The increased permeability allows the passage of lignin or lignin-carbohydrate molecules in higher amounts and of high molecular masses in the subsequent chemical reactions (Subramaniyan and Prema, 2002). A minor effect is due to the enzymatic hydrolysis of the residual non-dissolved hemicellulose by endoxylanases. Residual lignin in unbleached pulp (Kraft pulp) is linked to hemicellulose and that cleavage of this linkage will allow the lignin to be released (Lundgren *et al*., 1994). Biobleaching of pulp is reported to be more

effective with xylanases than with lignin-degrading enzymes. This is because the lignin is cross-linked mostly to the hemicellulose, and the hemicellulose is more readily depolymerized than lignin (Subramaniyan and Prema, 2000).

The removal of even a small portion of the hemicellulose can be sufficient to open up the polymer and facilitate removal of the residual lignin by mild oxidants. The principal objective of the application of biotechnological methods is the achievement of selective hemicellulose removal without degrading cellulose. The degradation of cellulose is the major problem associated with the conventional pulping process, which invariably affects the cellulose fiber, and thus the quality of the paper (Shoham *et al*., 1992; Madlala *et al*., 2001). The removal of xylan from the pulp leads to a decrease in energy demand during bleaching (Bajpai and Bajpai, 2001). Therefore; enzymatic treatments of pulp using xylanases have a better prospect in terms of both lower costs and improved fiber qualities.

1.7.5 Desired characteristics of xylanase for biobleaching

For effective biobleaching using xylanases from microbial sources, it is desirable to have cellulase-free xylanase otherwise celluolse hydrolysis may result in lowering pulp viscosity while cellulase-free xylanase pretreatment enhances pulp viscosity since low molecular weight xylans are removed.

The alkaline pH and high temperature regimes are part of pulp cooking and thus the xylanase should be stable at alkaline pH and must have good thermal stability (Viikari *et al*., 1992). Xylanases with varying properties impart different effects and therefore effective molecular weight, net ionic properties and specific action patterns of xylanase suiting to the microenvironment of pulp are essential (Biely *et al*., 1997; Clarke *et al.*, 1997). Buchart *et al.* (1992) have shown that two *T*. *reesei* xylanases having different p*I* of 5.5 and 9.0 behave differently. At pH 7.0 former liberated more of reducing sugars while

the latter hydrolyzed small amounts of xylan but conferred more brightness to the pulp. At the given pH, xylanase with p*I* 9.0 is positively charged and gets well absorbed onto negatively charged pulp fibres while xylanase with p*I* 5.5 may not get absorbed at all. Presence of metal ions in pulp has also been reported to positively alter xylanase action while pulps devoid of metal ions are poorly hydrolyzed (Senior *et al*., 1990).

The use of non-chlorine reagents in conjunction with xylanases offers an eco-friendly alternative to the use of chlorine based bleaching reagents. Several workers have examined the effect of xylanase pretreatment on bleaching efficiency of non-chlorine reagents (Poppius *et al.*, 1989; Yang *et al.*, 1992). Ragauskas *et al*. (1994) obtained optimal bleaching with ozone while Yang *et al.* (1992) found that xylanase pretreatment can activate kraft pulp toward bleaching with another non chlorine reagent, H_2O_2. In general, it has been noticed that xylanase pretreatment enhances bleaching efficiency of non-chlorine reagents such as ozone, peracetic acid, peroxides etc. Use of xylanases has also been indicated in deinking or toner removal of recycled paper waste (Jeffries *et al*., 1998). Jeffries *et al*. (1996) have examined use of commercial enzyme preparations, Novozyme 342TM, Pulpzyme HBTM (Novo Industries), LiftaseTM Exp A40 (Genencor) etc., containing cellulase and xylanase in removal of toner from office waste paper and found much reduced levels of residual ink in enzyme treated test sheets (26 ppm) as compared to controls (258 ppm).

1.8 AIM OF THE STUDY

The aim of this study was to evaluate *T. lanuginosus* for the production of hemicellulases, its yield enhancement using mutagenesis and application of a selected xylanase on bagasse pulp to assess the improvement of pulp properties.

The objectives were

i. To determine the localization of hemicellulases in *T. lanuginosus* strains,

ii. To develop high yielding strains of *T. lanuginosus* through mutagenesis,

iii. To investigate the synthesis of xylanase by *T. lanuginosus* MC134,

iv. To optimize the medium components and cultural conditions of *T. lanuginosus* MC134 strain,

v. To study the influence of agitation and aeration on the production of xylanase by *T. lanuginosus* MC134 in a fermenter,

vi. To evaluate the bleach boosting abilities of *T. lanuginosus* xylanase on bagasse pulp,

vii. To evaluate simultaneous xylanase production and biobleaching potential of *T. lanuginosus.*

CHAPTER 2

INDUCIBLE CHARACTER OF β-XYLANASE IN A HYPERPRODUCING MUTANT OF *Thermomyces lanuginosus*

Published: Engineering in Life Sciences 2009, 9: 298 – 302

ABSTRACT

Ten strains of *Thermomyces lanuginosus* from various culture collections were evaluated for extracellular endo-β-1,4-xylanase production. The best xylanase producer (5771 ± 173 nkat ml^{-1}) *T. lanuginosus* SK, was subjected to UV and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis. A mutant strain *T. lanuginosus* MC134, that showed on oatspelts xylan a 1.5 fold higher xylanase production than the parent strain SK, was subjected to a study of the regulation of xylanase synthesis during growth on various carbohydrates and during induction in glucose-grown cells. In the growth experiments the highest production of xylanase was observed in the presence of xylans, however, an appreciable amount of the enzyme, about 10%, was also produced during growth on xylose. Xylobiose was found to be the most efficient xylanase inducer in the glucose-grown cells. Its induction efficiency was followed by xylose, beechwood and birchwood xylan. Xylanase induction by polysaccharides started several hours later but proceeded for a longer time than that induced by the low molecular mass inducers, indicating that the polysaccharides serve as more sustainable source of inducers and that they have to be first hydrolyzed by the low level of constitutively synthesized xylanase. The repression of the induction of xylanase by glucose confirmed that the xylanase synthesis in the mutant strain is similar to the parent strain and exhibits an induction-repression regulation mechanism.

2.1 INTRODUCTION

Xylan is a major hemicellulosic polysaccharide and abundant renewable resource in nature (Singh *et al*., 2003). It consists of a backbone of β-1,4-linked D-xylopyranosyl residues with branches of other pentoses and hexoses, and uronic, ferulic, cinnamic and acetic acids (Biely, 1985; Gomes *et al*., 1994). Endo-1,4- β-xylanases, (EC 3.2.1.8) which randomly cleave the β - 1,4 backbone of xylan, are important group of glycoside hydrolases that are being applied in the food, feed, pulp and paper industries and biorefineries (Wong *et al*., 1988; Kuhad *et al*., 1997).

In the class Deuteromycetes, a thermophilic fungus *T. lanuginosus* has been reported as one of the best naturally occurring cellulase-free endo- 1, 4-β-xylanase producers (Singh *et al*., 2000). Although most of the *T. lanuginosus* strains produce high levels of xylanase, previous studies have shown variations in the expression of xylanases among strains of different origin (Chadha *et al*., 1999; Singh *et al*., 2003). For enhancement of productivity leading to a reduction in the production cost, it is necessary to improve the strain efficiency and optimize the fermentation conditions. Enzyme production can also be increased by introducing more potent strains and by generating mutants secreting higher levels of the enzyme.

Two classical methods, *viz*., UV light and chemical mutagenesis have been used to improve enzyme production (Kumar *et al*., 2009). Generally, in microorganisms xylanase is an inducible extracellular enzyme produced during growth on xylan or xylan derived fragments, and repressed in the presence of glucose. In this work, the effect of a number of soluble sugars on xylanase production in a new xylanase hyperproducing mutant of *T. lanuginosus* was investigated. In addition, the induction of xylanase in repressed glucose-grown cells was studied by compounds structurally related to xylan and the effect of glucose on xylanase synthesis was also assessed.

2.2 MATERIALS AND METHODS

2.2.1 Organisms and culture conditions

T. lanuginosus SK was obtained from the culture collection of the Department of Microbiology, University of Kwa-Zulu Natal, Durban, South Africa. *T. lanuginosus* SSBP is one of our own isolates (Singh *et al*., 2003). The remaining *T. lanuginosus* strains were obtained from the American Type Culture Collection, ATCC: 16455, 22083, 34626, 36350, 38905, 58157, 58158 and 58160). All *T. lanuginosus* strains were periodically subcultured on potato dextrose agar (PDA) and incubated at 40 – 50°C for 5 days and thereafter stored at room temperature. For xylanase production, medium (50 ml in 250 ml Erlenmeyer flasks, pH 6.5) containing (g l^{-1}) oatspelts xylan (15.0), yeast extract (15.0) and KH_2PO_4 (5.0) was used. An agar disc (9 mm in diameter) of an actively growing 5-day-old culture was used as inoculum. After 5 days of growth at 50°C and shaking at 150 rpm, the culture was centrifuged (10 000 g, 10 min) and the supernatant was assayed for xylanase activity.

2.2.2 Xylanase and protein assays

Xylanase activity was assayed using birchwood xylan as substrate (Roth 7500, Karlsruhe, Germany) (Bailey *et al*., 1992**)**. A 1.8 ml of the substrate solution (1%, w/v) prepared in 0.05 M sodium citrate buffer, pH 6.5, was mixed with 0.2 ml of appropriately diluted enzyme and incubated for 5 min at 50°C. The reaction was terminated by adding the DNS reagent. The enzyme activity is expressed in nkat. One nkat is defined as the amount of enzyme required to liberate 1 nmol of reducing sugars (xylose equivalents) per second. One unit of enzyme (U) is equivalent to 16.67 nkat.

Protein concentration in the culture supernatant was determined using bovine serum albumin as standard (Lowry *et al*., 1951).

2.2.3 Accessory enzyme assays

Accessory enzymes such as xylosidases, arabinofuranosidases, glucosidases, and mannosidase activities were determined by measuring the release of *p*-nitrophenol from *p*-nitrophenol-β-D-xylopyranoside (3 mg ml^{-1}), *p*-nitrophenol -α-L- arabinofuranoside (3 mg ml^{-1}), *p*-nitrophenol-β-D-glucopyranoside (4 mg ml^{-1}), and *p*-nitrophenol-β-D-mannopyranoside (2 mg ml^{-1}) (Sigma, USA), respectively. Mannanase activity was determined at 50°C (pH 6.5) using locust bean gum (Sigma, USA) as a substrate. Reducing sugars were quantified using the DNS reagent method (Miller, 1959).

2.2.4 Strain improvement through mutagenesis

T. lanuginosus SK strain was suspended in 0.1 M sodium phosphate buffer, pH 6.5, containing Tween 80 (0.025%, v/v). The mycelium was separated from spores by filtration through a sterile muslin cloth. Four ml aliquot of the spore suspension (1 x 10^7 spores ml^{-1}) was transferred aseptically into a sterile Petri dish and exposed to UV light (254 nm) using a Philips germicidal lamp (30 W) at a distance of 26.5 cm for 180 min. Chemical mutagenesis was carried out using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG, 350 µg ml^{-1}) to treat the spore suspension (1 x 10^7 spores ml^{-1}) for 180 minutes (Kumar *et al*., 2009). The mutagenized spores were washed three times with sterile distilled water and then plated on PDA. More than 300 colonies were analyzed for xylanase production in the liquid oatspelts xylan medium as described above.

2.2.5 Effect of carbon sources and search for inducers

Xylanase production was evaluated by cultivating the *T. lanuginosus* mutant MC134 in a medium (Purkarthofer and Steiner, 1995) containing various sugars and xylan as a main carbon source at 50°C (150 rpm) for 5 days. An agar disc (9 mm in diameter) of a 5 - day-old culture was used as inoculum.

For induction and repression studies, mycelium obtained by growing *T. lanuginosus* mutant strain MC134 in glucose medium (Purkarthofer and Steiner, 1995) for 3 days was harvested at the late exponential phase on a nylon cloth under sterile conditions, washed in phosphate buffer (0.1 M, pH 6.5) and then suspended in the same buffer containing inducers at concentrations of 10, 50, 100, 250 and 500 µg ml^{-1}. The final biomass concentration was 4.0 mg ml^{-1} (dry weight). D-xylose, xylobiose, methyl β-D-xylopyranoside, beechwood and birchwood xylan (Sigma) were tested as inducers. All induction experiments were carried out in 250 ml flasks containing 50 ml of the induction solution. The flasks were incubated at 50°C for 10 h at 150 rpm. The samples were taken at various time intervals, cooled on ice and centrifuged (10 000 g, 10 min). Xylanase activity was determined in the supernatant and the collected mycelial pellet was washed thrice with distilled water prior to drying at 105°C to constant weight.

For glucose repression studies, mycelium suspended in the phosphate buffer containing xylose as inducer (100 µg ml^{-1}) was tested for xylanase production at various concentrations of glucose. Sampling and analysis were done as described in section 2. 2. 2.

2.3 RESULTS AND DISCUSSION

2.3.1 Screening of *T. lanuginosus* strains for hemicellulases production

T. lanuginosus strains are known as excellent producers of xylanase (Hoq *et al*., 1994; Puchart *et al*., 1999; Singh *et al*., 2003). Ten *T. lanuginosus* strains were analysed for xylanase and other hemicellulases production on oatspelts xylan medium and the results are presented in Tables 2.1, 2.2 and 2.3. All strains utilized oatspelts xylan to secrete good levels of β-xylanase (Table 2.1). The results of the present study indicated the common occurrence of xylanolytic system in all the tested *T. lanuginosus* strains, while the amount of xylanase production among these varied significantly. The xylanase that is produced by these strains are similar to most xylanases of fungal

origin; these enzymes are the major extracellular product of these organisms. The strains showed varying levels of extracellular β-xylanase in oatspelts xylan medium under shake flask conditions, even though good mycelial growth was noticed with all the strains. The maximum xylanase activity (5771 ± 173 nkat ml^{-1}) was observed with *T. lanuginosus* SK strain after 5 days of incubation. This was followed by SSBP (4690 ± 188 nkat ml^{-1}). All other strains produced relatively good titres of extracellular xylanase, with strain ATCC 58160 producing the lowest level of 2670 ± 74 nkat ml^{-1}. Good levels of protein were also noticed in culture filtrates (Table 2.1).

Oatspelts xylan medium containing yeast extract was found to support good growth and xylanase production by these *T. lanuginosus* strains. In oatspelts xylan, the most abundant carbohydrate is xylose (52.5%) followed by arabinose (22.3%) (Li *et al*., 2000). The secretion of good levels of xylanase on to the medium by all the tested strains could be attributed to these inducing substances. Purkarthofer and Steiner (1995) also demonstrated the induction of xylanase in *T. lanuginosus* DSM 5826 using oatspelts xylan. The difference in the xylanase producing abilities among groups of similar fungi reinforces the fact that abilities of taxonomically similar fungi of different origin may be found to vary immoderately.

However, all the tested *T. lanuginosus* strains did not produce cellulase upon grown on oatspelts xylan media. Puchart *et al*. (1999) also reported on the non-cellulolytic nature of 17 *T. lanuginosus* strains. The cellulase-free β-xylanase from *T. lanuginosus* strains has great potential in pulp and paper industry for selective removal of xylan from cellulosic materials in biobleaching where cellulosic fibre length should be preserved in papermaking.

Even though high levels of extracellular xylanase activities were noticed for all the strains, intracellular xylanase activities were very low (Table 2. 2). It was noticed that intracellular xylanase activity was found to be five to ten times lower than extracellular

xylanase activity after 5 days of incubation in oat spelts xylan media. ATCC 34626 showed the highest intracellular xylanase activity of 632 ± 16.6 nkat ml^{-1} which was 90% lower than extracellular xylanase produced by strain SK. Among the strains analysed for intracellular xylanase production, *T. lanuginosus* SK was the second best intracellular xylanase producer (572 ± 11.6 nkat ml^{-1}). All other strains displayed similar trend for intracellular xylanase levels, with strain ATCC 16455 producing the lowest level of 80 ± 2.24 nkat ml^{-1}.

Table 2.1 Extracellular β-xylanase production by *T. lanuginosus* strains

***T. lanuginosus* strains**	**Xylanase activity (nkat ml^{-1})***	**Soluble protein (mg ml^{-1})***
SK	5771 ± 173	0.83 ± 0.02
SSBP	4690 ± 188	0.76 ± 0.01
ATCC 16455	3862 ± 135	0.88 ± 0.02
ATCC 34626	3446 ± 127	0.74 ± 0.02
ATCC 36350	3422 ± 95	0.79 ± 0.01
ATCC 22083	3178 ± 82	0.78 ± 0.01
ATCC 38905	3160 ± 107	0.74 ± 0.02
ATCC 58158	3096 ± 100	0.72 ± 0.01
ATCC 58157	2980 ± 68	0.77 ± 0.03
ATCC 58160	2670 ± 74	0.73 ± 0.02

*Each value represents a mean of three replicate determinations with standard deviation (± SD)

In comparison with the high levels of extracellular xylanase, other hemicellulases were exceptionally low in the culture filtrates of all the strains. ATCC 58160 was the best β-xylosidase producer (5.20 ± 0.13 nkat ml^{-1}) followed by ATCC 34626 and ATCC 58157, which also displayed the same levels of activity on oatspelts xylan media. ATCC 58158 and SSBP showed xylosidase activity levels of 3.96 ± 0.09 nkat ml^{-1} and 3.10 ± 0.04 nkat ml^{-1}, respectively. All other strains tested showed activity levels less than 2.5 nkat ml^{-1}. However, the intracellular xylosidase levels were extremely low for all the strains tested, the highest being strain SSBP showing 0.145 ± 0.003 nkat ml^{-1}.

α-L- arabinosidase activity analysis of both extracellular and intracellular fractions for all the 10 strains showed very low enzyme activity for all the strains tested on oatspelts xylan media. ATCC 34626, ATCC 58160 and ATCC 58157 showed almost the same levels of α-L-arabinosidase extracellularly (Table 2.2 and 2.3). The highest intracellular arabinosidase producer was strain SK (0.109 ± 0.002 nkat ml^{-1}). ATCC 38905 was the lowest arabinosidase producer both extracellularly and intracellularly.

For β-galactosidase, all strains showed almost same levels of enzyme activity ranging between 1.62 and 1.34 nkat ml^{-1} in the culture filtrates. However, there was no similar trend for intracellular β-galactosidase activity. The activity levels were extremely low and strains ATCC 38905 and ATCC 16455 did not show any intracellular β-galactosidase activity (Table 2.3).

In comparison to β-galactosidase production levels, α-galactosidase activity was slightly superior for all the strains tested. Among the strains analysed for α-galactosidase production, strain SK was the leading producer, showing an enzyme activity of 3.84 ± 0.09 nkat ml^{-1}. Four other strains also showed enzyme activity levels above 2.5 nkat ml^{-1} (Table 2.2). However, the highest intracellular activity level was 0.473 ± 0.01 nkat ml^{-1} displayed by ATCC 58158. ATCC 16455 did not show any intracellular α-galactosidase activity.

All strains exhibited low levels of β-glucosidase activity in the culture filtrates when grown on oatspelts xylan media. The maximum extracellular β-glucosidase activity was displayed by strain SK (1.80 ± 0.03 nkat ml^{-1}). β-glucosidase activity for all the strains were between 1.8 and 0.76 nkat ml^{-1}. Intracellular β-glucosidase activities also showed a similar trend (Table 2.3). The maximum intracellular β-glucosidase activity (1.89 ± 0.03 nkat ml^{-1}) was displayed by strain ATCC 58158 and ATCC 16455 (0.28 ± 0.001 nkat ml^{-1}) showed the lowest.

Mannan degrading enzymes such as β-mannanase and β-mannosidase were analysed in both extracellular and intracellular fractions. Among the accessory enzymes produced by various *T. lanuginosus* strains in oatspelts xylan media, β-mannanase was the most promising (Table 2.2). An enzyme activity of 15.10 ± 0.37 nkat ml^{-1} was recorded by strain SK. Most of the strains exhibited enzyme activities above 10 nkat ml^{-1}. However, ATCC 58157 showed the lowest β-mannanase activity of 5.84 ± 0.029 nkat ml^{-1}. Intracellular β-mannanase activity levels were also comparatively higher than other accessory enzymes; the highest being 7.85 ± 0.19 nkat ml^{-1} shown by ATCC 34626 followed by ATCC 22083 (5.75 ± 0.18 nkat ml^{-1}).

In comparison to β-mannanase activity levels, β-mannosidase activities were significantly lower in extracellular fractions. All the strains displayed activities within the range 2.36 and 1.81 nkat ml^{-1} (Table 2.2). The highest β-mannosidase activity (2.36 ± 0.07 nkat ml^{-1}) was recorded by ATCC 58160. However most of the strains analysed, did not show any intracellular β-mannosidase activity. The enzyme activities were either extremely low or beyond detectable limits (Table 2.3).

Table 2.2 Production of extracellular hemicellulases by *T. lanuginosus* strains

T. lanuginosus **strains**	**Extracellular hemicellulases activity (nkat ml^{-1})**						
	Xylosidase	**Arabinosidase**	**Galactosidase**	**Galactosidase**	**Glucosidase**	**Mannanase**	**Mannosidase**
ATCC 58160	5.20 ± 0.13	1.91 ± 0.03	1.46 ± 0.01	2.76 ± 0.05	0.88 ± 0.002	14.65 ± 0.43	2.36 ± 0.07
ATCC 34626	5.18 ± 0.12	2.05 ± 0.03	1.46 ± 0.02	1.76 ± 0.03	1.03 ± 0.01	13.36 ± 0.32	1.91 ± 0.04
ATCC 58157	5.07 ± 0.14	1.90 ± 0.03	1.38 ± 0.00	2.84 ± 0.07	1.75 ± 0.02	5.84 ± 0.029	1.98 ± 0.03
ATCC 58158	3.96 ± 0.09	1.73 ± 0.03	1.36 ± 0.00	2.98 ± 0.06	0.93 ± 0.003	8.19 ± 0.040	1.94 ± 0.04
SSBP	3.10 ± 0.04	1.20 ± 0.01	1.58 ± 0.01	2.12 ± 0.06	1.40 ± 0.02	10.56 ± 0.25	1.94 ± 0.04
ATCC 36350	2.43 ± 0.06	1.46 ± 0.01	1.51 ± 0.03	2.52 ± 0.08	1.45 ± 0.02	12.63 ± 0.23	2.16 ± 0.06
SK	2.38 ± 0.04	0.80 ± 0.01	1.60 ± 0.02	3.84 ± 0.09	1.80 ± 0.03	15.10 ± 0.37	1.97 ± 0.03
ATCC 38905	1.26 ± 0.02	0.23 ± 0.00	1.62 ± 0.04	1.98 ± 0.04	0.85 ± 0.004	12.29 ± 0.21	1.88 ± 0.02
ATCC 22083	1.24 ± 0.03	1.57 ± 0.02	1.59 ± 0.04	2.18 ± 0.06	1.04 ± 0.02	12.39 ± 0.22	1.92 ± 0.03
ATCC 16455	1.20 ± 0.02	0.55 ± 0.01	1.34 ± 0.03	1.68 ± 0.02	0.76 ± 0.01	13.40 ± 0.34	1.81 ± 0.02

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Table 2.3 Production of intracellular hemicellulases by *T. lanuginosus* strains

***T. lanuginosus* strains**	**Intracellular hemicellulases activity (nkat ml^{-1})**							
	Xylanase	**Xylosidase**	**Arabinosidase**	**Galactosidase**	**Galactosidase**	**Glucosidase**	**Mannanase**	**Mannosidase**
ATCC 34626	632 ± 16.6	0.074 ± 0.002	0.041 ± 0.00	0.016 ± 0.00	0.037 ± 0.00	1.49 ± 0.02	7.85 ± 0.19	0.01 ± 0.00
SK	572 ± 11.6	0.096 ± 0.001	0.109 ± 0.002	0.054 ± 0.001	0.149 ± 0.002	1.04 ± 0.01	3.40 ± 0.07	0.00 ± 0.00
ATCC 58158	532 ± 10.6	0.082 ± 0.002	0.057 ± 0.00	0.078 ± 0.001	0.473 ± 0.01	1.89 ± 0.03	1.01 ± 0.002	0.03 ± 0.00
SSBP	506 ± 10.3	0.145 ± 0.003	0.092 ± 0.002	0.046 ± 0.00	0.113 ± 0.001	1.46 ± 0.03	1.04 ± 0.02	0.03 ± 0.00
ATCC 38905	326 ± 9.4	0.025 ± 0.00	0.026 ± 0.00	0.00 ± 0.00	0.035 ± 0.00	0.42 ± 0.004	3.72 ± 0.04	0.00 ± 0.00
ATCC 36350	306 ± 8.3	0.064 ± 0.001	0.065 ± 0.001	0.041 ± 0.001	0.114 ± 0.001	1.11 ± 0.01	1.08 ± 0.02	0.06 ± 0.00
ATCC 58157	249 ± 5.45	0.06 ± 0.001	0.044 ± 0.00	0.036 ± 0.00	0.072 ± 0.00	1.45 ± 0.02	4.41 ± 0.12	0.65 ± 0.003
ATCC 58160	213 ± 6.65	0.121 ± 0.003	0.051 ± 0.00	0.057 ± 0.001	0.141 ± 0.002	1.47 ± 0.02	2.71 ± 0.03	0.02 ± 0.00
ATCC 22083	119 ± 2.95	0.106 ± 0.001	0.072 ± 0.001	0.030 ± 0.00	0.188 ± 0.002	1.48 ± 0.02	5.75 ± 0.18	0.00 ± 0.00
ATCC 16455	80 ± 2.24	0.028 ± 0.00	0.018 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.28 ± 0.001	1.02 ± 0.02	0.00 ± 0.00

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Singh *et al.* (2000) reported on the production of low levels of accessory enzymes by T*. lanuginosus* SSBP. Moreover, enzyme production is apparently strain dependent. *T. lanuginosus* strain DSM 5826 did not produce accessory enzymes such as mannanase and mannosidase after growth on corn cobs (Purkarthofer *et al*., 1993a), while strain SSBP, on the other hand, produced detectable levels of both enzymes (Singh *et al*., 2000). Puchart *et al*. (1999) reported that only three of the 17 *T. lanuginosus* strains tested were able to produce mannanase on locust bean gum. This clearly indicates that enzyme production is dependent on the carbon source and the strain used.

Since accessory enzymes produced by all *T. lanuginosus* strains were very low and *T. lanuginosus* SK strain produced maximum extracellular xylanase, it was chosen for strain improvement studies. UV mutagenesis of *T. lanuginosus* SK aleurospores and subsequent screening yielded 118 colonies. A mutant, designated M13, obtained by UV mutagenesis, showed a 17% higher xylanase production than the parent strain. A second generation mutant obtained by NTG mutagenesis of the strain M13, designated MC134, produced xylanase (8598 ± 132 nkat ml^{-1}) under the same conditions, which was 1.5-fold higher than the enzyme level produced by the parent strain *T. lanuginosus* SK.

2.3.2 Growth and xylanase production by *T. lanuginosus* MC134

To evaluate the inducing and repressing effects of various carbohydrates, growth experiments were performed with 15 different carbohydrate compounds and the results are presented in Table 2.4. Low constitutive level of endo-xylanase was detected even in the absence of a carbon source. Most of the mono- and disaccharides tested as carbon sources favoured good mycelial growth of the mutant MC134, but did not induce xylanase production, suggesting that abundant growth does not necessarily translate to a high enzyme production. Although D-Glucose was the best carbon

source for growth of the organism, it was a poor substrate for the enzyme production. The specific activity of xylanase referred to mycelium dry weight was the lowest with easily metabolized hexoses, such as glucose, mannose and fructose (Table 2.4). An approximate 20-fold increase in specific activity was recorded on sugars which are structurally unrelated to plant xylans, D-arabinose and D-ribose. A further 200-fold higher specific activity was observed on xylose in comparison to glucose, which suggested that xylose should be considered for a xylan fragment interacting positively with the regulatory system of xylanase production. Xylanase production was the highest during growth on birchwood xylan (11 751 ± 232 nkat ml^{-1}) and beechwood xylan (10 200 ± 186 nkat ml^{-1}), with the specific activities of the enzyme attaining a 10-fold increase in comparison to that obtained on xylose and a 2468-fold higher specific activity than on glucose (Table 2.4). These results indicated that xylanase production in *T. lanuginosus* mutant MC134 is most efficiently triggered by the corresponding polysaccharides serving as a source of xylan fragments functioning as the enzyme inducers. Similar observations were noticed with a naturally occurring strain of *T. lanuginosus* when cultivated on various carbon sources (Purkarthofer and Steiner, 1995).

Table 2.4 Growth and xylanase production by *T. lanuginosus* MC134 on various carbon sources

Carbon sources	Xylanase activity (nkat ml^{-1})*	Dry weight (mg ml^{-1})*	Xylanase/dry weight (nkat mg^{-1})*
Birchwood xylan	11751 ± 232	5.6 ± 0.14	2098.39 ± 66
Beechwood xylan	10200 ± 186	ND	ND
Oatspelts xylan	8569 ± 124	ND	ND
D-Xylose	960 ± 26	4.6 ± 0.12	208.7 ± 4.3
D-Arabinose	130 ± 5.2	4.8 ± 0.16	27.08 ± 0.88
D-Ribose	116 ± 4.6	4.2 ± 0.09	27.62 ± 0.78
Sucrose	15 ± 0.12	5.2 ± 0.13	2.88 ± 0.06
L-Arabinose	14 ± 0.15	4.2 ± 0.08	3.33 ± 0.07
D-Galactose	11 ± 0.22	4.9 ± 0.15	2.24 ± 0.05
Maltose	9 ± 0.16	4.8 ± 0.11	1.88 ± 0.02
D-Mannose	7 ± 0.14	5.0 ± 0.16	1.40 ± 0.02
D-Glucose	5 ± 0.08	5.9 ± 0.12	0.85 ± 0.01
D-Fructose	4 ± 0.06	5.1 ± 0.09	0.78 ± 0.01
Cellobiose	3 ± 0.02	4.4 ± 0.08	0.68 ± 0.02
Lactose	3 ± 0.01	4.3 ± 0.11	0.70 ± 0.01
None	3 ± 0.02	1.4 ± 0.04	2.14 ± 0.06

*Each value represents a mean of three replicate determinations with standard deviation (± SD)

ND: Not determined

2.3.3 Inducers on xylanase synthesis

To study the synthesis of xylanase in the presence of a certain inducer, glucose-grown mycelium was incubated for 0 to 10 h with various soluble sugars and xylan. D-Arabinose, D-ribose, D-xylose, xylobiose, methy-β-D-xylopyranoside, beechwood and birchwood xylan were examined for induction of xylanase in *T. lanuginosus* MC134 in glucose-grown mycelium. Xylobiose was found to be the best inducer at all concentrations tested. At a concentration of 50 µg ml^{-1} or 500 µg ml^{-1} or at equi-molar concentration (1.6 mM) the dimer induced 3 to 5 fold higher levels of xylanase than the monomer and about 1.5 to 2 fold higher levels than the polymers (Figs. 2.1 and 2.2). Moreover, the onset of xylanase synthesis by xylobiose commenced earlier compared to the other inducers tested. Xylobiose also supported the early synthesis of xylanase in *T. lanuginosus* MC134 compared to the xylans used.

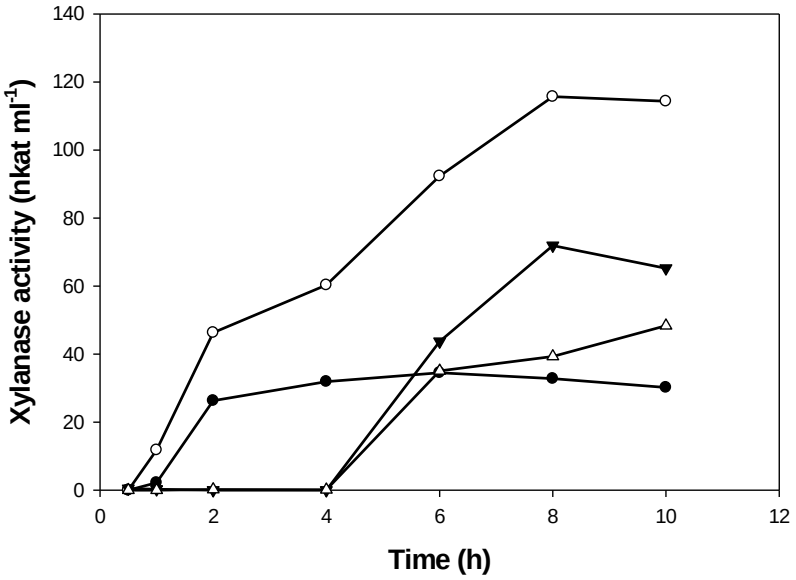


Fig. 2.1 Xylanase synthesis by *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on D-Xylose (●), xylobiose (○), beechwood xylan (▼) and birchwood xylan (Δ) at 50 µg ml^{-1} concentration.

There was a four hour delay in the onset of xylanase synthesis for beechwood and birchwood xylans at 50 µg ml^{-1} concentration and for birchwood xylan, only a 2 h delay was observed at 500 µg ml^{-1} concentration. The longer induction times with polysaccharides, clearly indicate that xylans do not act as direct inducers. Since xylans are too large polymers to penetrate the plasma membrane, their induction effect is due to the formation of low molecular mass fragments such as xylose and xylooligosaccharides upon the action of constitutively produced low level of xylanase (Liu *et al*., 1999). Considering the inducer specificity for synthesis of xylanase, xylose acts most probably as a direct inducer and not after its transformation in the cells to D-xylulose or xylitol (Ghosh and Nanda, 1994).

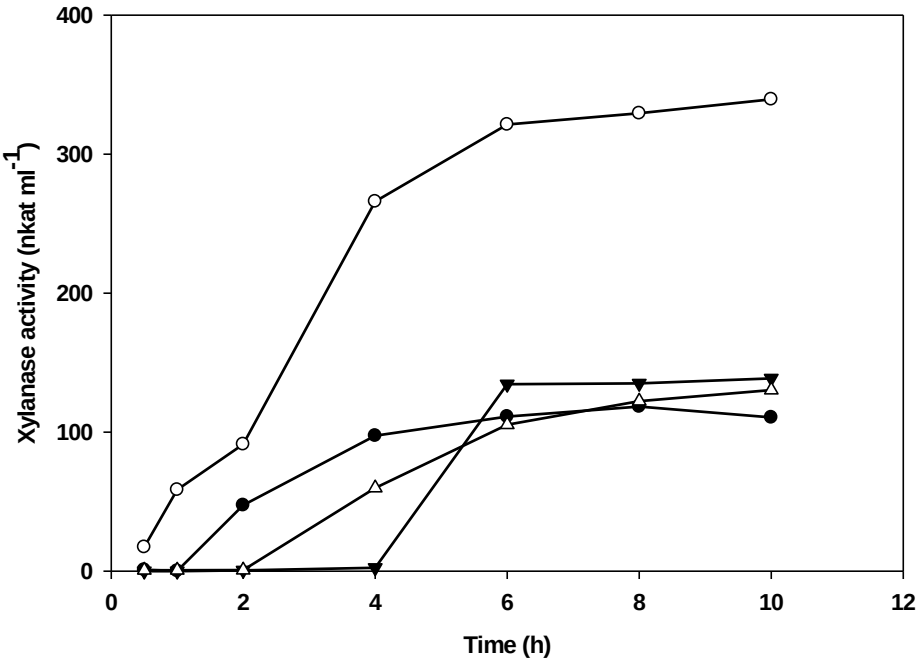


Fig. 2.2 Xylanase synthesis by *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on D-Xylose (●), xylobiose (○), beechwood xylan (▼) and birchwood xylan (∆) at 500 µg ml^{-1} concentration.

D-Arabinose and D-ribose also supported xylanase induction in the mutant strain MC134 to a lesser degree. A higher concentration of D- Arabinose (500 µg ml^{-1}) gave the highest xylanase activity of 17 nkat ml^{-1} after 4 h and declined thereafter (Fig. 2.3). There was a delay of almost 6 h for xylanase formation when lower concentrations of D-arabinose (10 and 50 µg ml^{-1}) were used. D-Ribose gave a better induction of xylanase (33 nkat ml^{-1}) than D-arabinose, after 10 h of incubation at a concentration of 500 µg ml^{-1} (Fig. 2.4). The induction rate decreased with a decrease in inducer concentration.

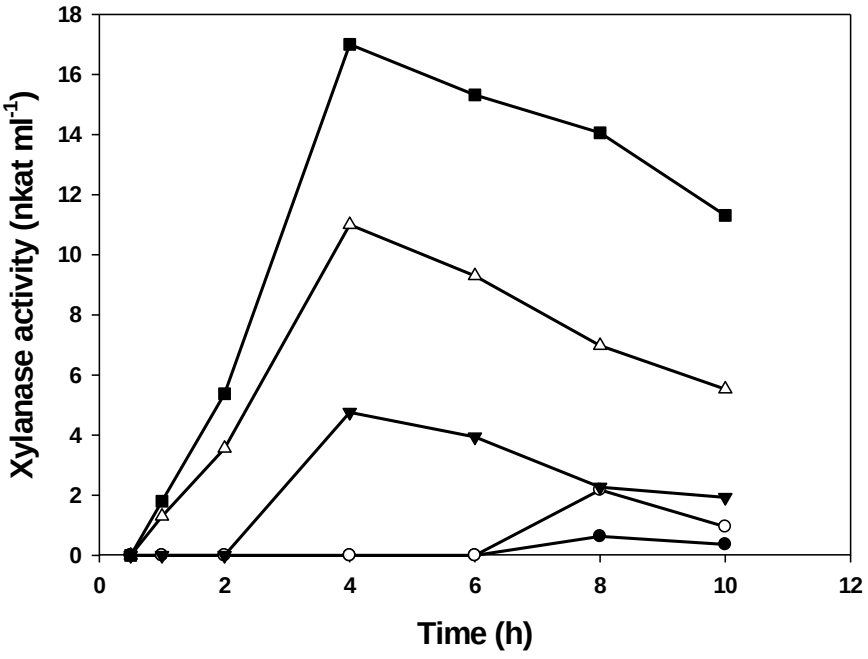


Fig. 2.3 Xylanase synthesis by *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on D-arabinose at 10 µg ml^{-1} (●), 50 µg ml^{-1} (○),100 µg ml^{-1} (▼), 250 µg ml^{-1} (Δ) and 500 µg ml^{-1} (■) concentrations.

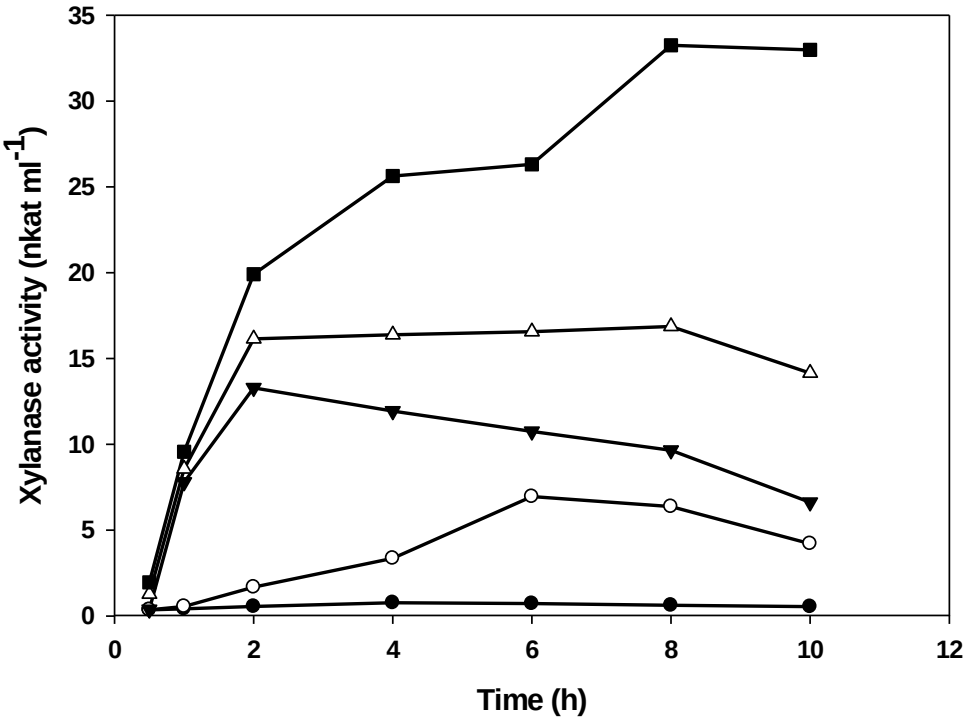


Fig.2.4 Xylanase synthesis by *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on D-ribose at 10 µg ml^{-1} (●), 50 µg ml^{-1} (○),100 µg ml^{-1} (▼), 250 µg ml^{-1} (Δ) and 500 µg ml^{-1} (■) concentrations.

In contrast to the inducible effect of methyl β-D-xylopyranoside, a synthetic xyloside, on xylanase synthesis in numerous microorganisms (Leathers *et al*., 1986; Gomes *et al*., 1994; Simão *et al*., 1997), it did not serve as a xylanase inducer in *T. lanuginosus* MC134 (Fig. 2.5). This result is difficult to understand mainly in connection with the fact that both xylose and xylobiose serve as a xylanase inducers and methyl β-D-xylopyranoside can be considered to be their structural intermediate. However, this result is not unique in literature (Liu *et al*., 1999; Hidalgo-Lara *et al*., 2005).

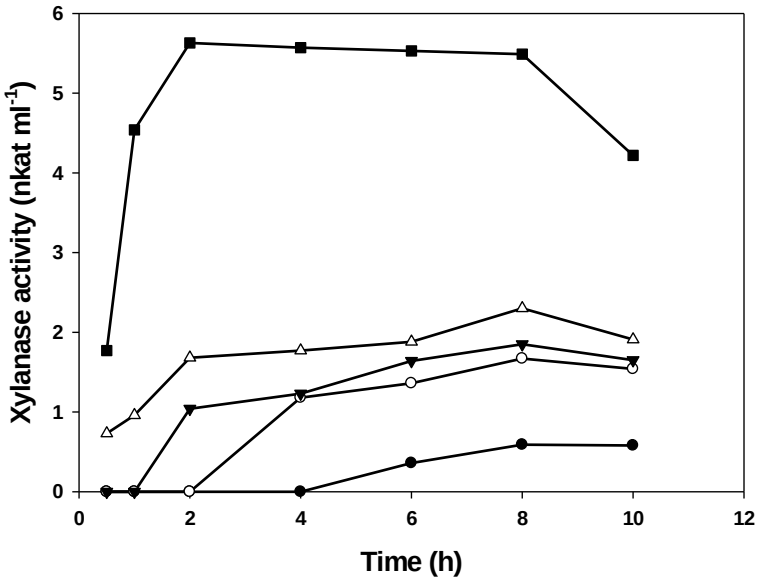


Fig. 2.5 Xylanase synthesis by *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on methyl *β*-xylopyranoside at 10 µg ml^{-1} (●), 50 µg ml^{-1} (○),100 µg ml^{-1} (▼), 250 µg ml^{-1} (Δ) and 500 µg ml^{-1} (■) concentrations.

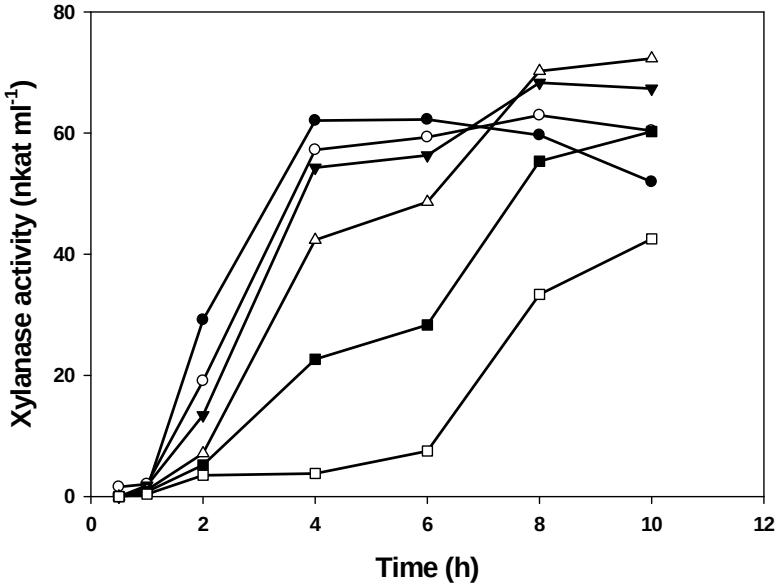


Fig. 2.6 Xylanase synthesis in *T. lanuginosus* MC134 mycelium grown at 50°C and 150 rpm for 10 h on 100 µg ml^{-1} xylose in the absence of glucose (●); presence of glucose at concentrations of 10 µg ml^{-1} (○); 50 µg ml^{-1} (▼); 100 µg ml^{-1} (Δ); 250 µg ml^{-1} (■) and 500 µg ml^{-1} (□).

2.3.4 Xylanase repression

To reveal the glucose repression of xylanase production, the mycelium pregrown on glucose was incubated with a fixed concentration of xylose as an inducer (100 µg ml^{-1}) in the absence and presence of glucose as a repressor. The concentration range of glucose varied from 10 to 500 µg ml^{-1}. The results presented in Fig. 2.6 shows that glucose does not affect the enzyme yields as strongly as the time of its appearance in the medium. The higher the glucose concentration, the longer the xylanase induction time. The delay in xylanase induction in the presence of glucose suggests that xylose induction does not take place in cells before the disappearance of glucose. In summary, we can conclude that xylanase production by the hyperproducing *T. lanuginosus* mutant MC134 is controlled by the induction-repression mechanism as reported for naturally occurring strains of this thermophiic fungus (Purkarthofer and Steiner, 1995; Singh *et al*., 2003). At the same time we can suggest that mutation of the strain SK leading to the mutant MC134, producing 50% more xylanase activity does not seem to have altered the regulatory mechanisms of xylanase gene expression. A question remains to be answered, whether the higher xylanase level is due to increase of its specific activity or increase in the amount of the produced enzyme or to its faster secretion. Further studies will be therefore directed to purification of the mutant xylanase and comparison of its specific activities with that of the wild-type enzyme. Comparison of the amino acid sequences or protein foldings could also reveal further information on the reasons for the increased xylanase activity of the mutant strain. The three-dimensional structure of the *T. lanuginosus* xylanase has already been solved (Gruber *et al*., 1998).

.

2.4 CONCLUSIONS

T. lanuginosus SK was the best xylanase producer among the ten strains used in this study. No cellulase activity was noticed and accessory enzymes very low for all the strains. Intracellular hemicellulase activity was extremely low in all the strains. *T. lanuginosus* mutant MC134 evolved through mutagenesis showed a 1.5 fold increase in xylanase production than the wild type, *T. lanuginosus* SK. The xylanase from *T. lanuginosus* MC134 was inducible during growth on substrates containing xylan. Xylobiose was the best inducer followed by xylose. The xylanase induction was repressed in the presence of higher concentrations of glucose.

CHAPTER 3

PRODUCTION OF β-XYLANASE BY A *Thermomyces lanuginosus* MC134 MUTANT ON CORN COBS AND ITS APPLICATION IN BIOBLEACHING OF BAGASSE PULP

Published: Journal of Bioscience and Bioengineering 2009, 107: 494 – 498

ABSTRACT

The production of hemicellulases by *T. lanuginosus* SK using oatspelts xylan was examined during submerged cultivation. A high level of extracellular xylanase (5771 ± 173 nkat ml^{-1}) production was observed after five days, however, accessory enzyme levels were low. *T. lanuginosus* SK was further subjected to UV and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine mutagenesis. The *T. lanuginosus* MC134 mutant showed a 1.5 fold increase in xylanase production on oatspelts xylan, compared to the wild type strain. Xylanase production was further enhanced to 54994 ± 1584 nkat ml^{-1} using corn cobs under optimized growth conditions. Lower levels of xylanase production was observed in a 5 L fermenter. The biobleaching efficiency of crude xylanase was evaluated on bagasse pulp, and a brightness of 46.07 ± 0.05% was observed with the use of 50 U of crude xylanase per gram of pulp. This brightness was 3.6 points higher than that of the untreated samples. Reducing sugars (25.78 ± 0.14 mg g^{-1}) and UV-absorbing lignin-derived compound values were considerably higher in xylanase-treated samples. *T. lanuginosus* MC134 demonstrated good potential for application in the pulp and paper industries.

3.1 INTRODUCTION

Xylan, the most important hemicellulosic heteropolymer, is composed of a backbone of 1,4-linked β-D-xylopyranose residues and branches of L-arabinofuranose, D-glucuronic acid, or 4-*O*-methyl-D-glucuronic acid (Viikari *et al*., 1994; Beg *et al*., 2001; Collins *et al*., 2005). Hydrolysis of xylan by microbial enzymes is highly specific and an environmentally friendly option when compared to chemical hydrolysis. The unpredictable structure and organization of hemicellulose requires the concerted actions of many enzymes for its complete hydrolysis, including the key enzymes, endo-xylanase (EC 3.2.1.8), and β-xylosidase (EC 3.2.1.37) (Wong *et al*., 1988). Xylanases and the associated debranching enzymes are produced by a variety of microbes, including bacteria, yeast, and filamentous fungi (Maheshwari *et al*., 2000; Collins *et al*., 2005). Among them, the thermophilic fungus *T. lanuginosus* has been reported as one of the superior cellulase-free xylanase producers (Singh *et al*., 2003).

To reduce the bioprocessing cost and enhance xylanase production level in microbes, different strategies have been developed. Quantitative enhancement is the foremost approach, and involves strain improvement and optimization of growth parameters to achieve maximum productivity. Examples of spectacular success in strain improvement in industry are mostly attributed to the extensive application of mutation and selection. UV light (Ellaiah *et al*., 2002) and chemical mutagenesis (Rubinder *et al*., 2002) using *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (NTG) have been reported as successful methods for mutation.

Large quantities of agro-industrial by-products accumulate in the environment, and can be processed for food, fuel, and a variety of chemicals. These negative value agro-wastes can be utilized for cost effective enzyme production at the industrial level. Several inexpensive substrates have been used as a carbon source for microbial xylanase production (Asha Poona and Prema, 2006; Manimaran and Vatsala, 2007).

In the paper production process, residual lignin is removed by multistep chemical bleaching processes, resulting in the release of hazardous adsorbable organic halogens into the environment (Bajpai, 1999). Microbial enzymes are an alternative option that involves the use of hemicellulolytic enzymes to increase the brightness of pulp samples. The main goals of enzyme-aided bleaching are to increase the brightness of the final pulp and to reduce or replace the harmful chlorine compounds used in the bleaching processes (Suurnakki *et al*., 1997). Most studies on biobleaching have been confined to kraft and sulphite pulps. Reports on the biobleaching of non-woody plants are limited. Bagasse, one of the most important non-woody materials, is used for paper production in many developing countries. However, reports on the enzymatic prebleaching of bagasse pulp are scant (Kulkarni and Rao, 1996; Christopher *et al*., 2005). In the present study, xylanase production in *T. lanuginosus* was enhanced via UV/NTG mutagenesis. Fungal growth parameters were optimized for maximum xylanase production using corn cobs as a cheap carbon source in shake flasks and in a lab-scale fermenter. The biobleaching efficiency of the crude xylanase was also evaluated on bagasse pulp.

3.2 MATERIALS AND METHODS

3.2.1 Microorganism and growth conditions

T. lanuginosus SK was obtained from the culture collection of the Department of Microbiology, University of KwaZulu-Natal, Durban, South Africa. The strain was periodically subcultured on potato dextrose agar (Oxoid, UK) and stored at room temperature. Fifty milliliters of growth medium (pH 6.5) containing (g l^{-1}) oatspelts xylan (15.0), yeast extract (15.0), and KH_2PO_4 (5.0) were prepared in each 250 ml Erlenmeyer flask. An agar disc (9 mm diameter) of an actively growing 5 day old culture was used as an inoculum. The flasks were kept at 50°C in a shaking incubator (150 rpm). After 5 days, the culture broth was centrifuged (10 000 g for 10 min), and

the supernatant was used to determine the extracellular hemicellulase activities. For intracellular enzyme extraction, 1 g of washed mycelium (wet weight) suspended in 50 ml of sodium citrate buffer (0.1 M, pH 6.5) was kept in an ice bath and sonicated using a Virsonic 100 (Virtis, USA) ultrasonic processor at 5 kilocycles s^{-1} for 5 min. After sonication, the biomass was removed by filtration followed by centrifugation (10 000 g for 10 min), and the clear supernatant was used as a crude intracellular enzyme source.

3.2.2 Strain improvement through mutagenesis

T. lanuginosus SK culture grown on a PDA plate was scraped into sodium phosphate buffer (0.1 M, pH 6.5). The spores were uniformly suspended using Tween 80 (0.025%, v/v) and separated from the mycelium by filtering through a sterile muslin cloth. Four ml of spore suspension (10^7 spores ml^{-1}) was transferred aseptically onto a Petri dish and exposed to UV light (254 nm) using a Philips germicidal lamp (30 W) at a distance of 26.5 cm for 180 min (Ellaiah *et al*., 2002). The UV exposed spores were stored in dark conditions overnight to avoid photo-reactivation and plated on PDA. The colonies were checked for xylanase production in submerged culture using oatspelts xylan. The best xylanase-producing mutant obtained through UV mutation was further subjected to NTG (350 µg ml^{-1}) mutagenesis (Rubinder *et al*., 2002). The treated spores were washed three times with sterile distilled water and plated on PDA. Colonies were again tested for xylanase production using oatspelts xylan as a carbon source.

3.2.3 Optimization of growth parameters for xylanase production by *T. lanuginosus* MC134 during shake flask cultivation

3.2.3.1 Effect of various carbon and nitrogen sources on xylanase production

The carbon sources (1% w/v) used were glucose, fructose, lactose, maltose, sucrose, xylose, trehalose, mannitol, sorbitol, soluble starch, cane sugar, beechwood xylan, birchwood xylan, oatspelts xylan, coarse corn cob (2 – 3 mm), fine corn cob (<1 mm), or wheat bran (0.5 mm). All commercial xylans were purchased from Sigma, USA. Corn cobs (coarse and fine) were prepared by stripping the kernels from maize; they were then dried, ground using a mill grinder, and sieved. Corn cobs were autoclaved at 121°C for 15 min and stored at 4°C.

The nitrogen sources (1% w/v) used were beef extract, malt extract, meat extract, yeast extract, casein, bacteriological peptone, fish peptone, proteose peptone, vegetable peptone, tryptone, ammonium phosphate, ammonium sulphate, potassium nitrate, sodium nitrate, or urea. For the time course study, 50 ml of the optimized medium containing corn cob and yeast extract was dispensed into 250 ml Erlenmeyer flasks, inoculated with 5 day old fungal cultures, and kept at 150 rpm (50°C) for 9 days. Samples were withdrawn every 24 h to determine the xylanase activity.

3.2.3.2 Effect of inoculum size, initial pH, temperature, and agitation rates on xylanase production

T. lanuginosus MC134 mycelial discs (9 mm diameter; 5 day old culture) were transferred into 250 ml flasks containing 50 ml of media and grown for 6 days at 50°C (150 rpm). The optimal initial pH for xylanase production was determined by adjusting the pH of the culture medium from 3 to 8 using 1 N HCl or 1 N NaOH. For temperature studies, cultures were grown between 30 and 70°C (at 5°C intervals) in a shaking

incubator at 150 rpm for 6 days at pH 6.5. The effect of agitation on xylanase production was examined by growing cultures at different agitation rates (0, 50, 100, 150, 200 and 250 rpm) in separate experiments. Xylanase activity and soluble protein were determined as described in section 2.2.2.

3.2.4 Xylanase production in a laboratory-scale fermenter

Xylanase production was carried out in a 5 L vertical glass fermenter (Minifors, Infors HT, Switzerland) with 3 L (working volume) of the optimized xylanase production medium (pH 6.5) at 50°C. A 10% (v/v) culture of the *T. lanuginosus* mutant MC134 grown in potato dextrose broth was used as an inoculum. The effect of agitation on xylanase production was examined by growing cultures at different agitation rates (200 to 500 rpm) over a period of 7 days. Aeration was set at 1 vvm (maintained using the cascade mode). Foaming was controlled by the addition of silicon antifoam (Fluka) at a concentration of 0.5 ml l^{-1}. Samples (20 ml) were withdrawn through the sampling port every 24 h and centrifuged (10 000 g for 10 min), and the clear supernatant was used to determine the xylanase activity and amount of soluble protein as described in section 2.2.2.

3.2.5 Thin layer chromatography (TLC)

For enzymatic hydrolysis of xylan, 50 ml (1%) of birchwood xylan (0.5 M citrate buffer, pH 6.5) was incubated with 5 U of crude xylanase obtained from the fermenter after 24 h. Samples were withdrawn at different time intervals (2, 6, 12, and 24 h) and 2 µl aliquots were spotted on silica gel F 60 (Merck, Germany) plates. The chromatogram was developed in ethyl acetate:acetic acid:2-propanol:formic acid:water (25:10:5:1:15, v/v). Sugars were detected by pouring the detecting reagent (1% orcinol in 10% H_2SO_4 in ethanol) on the plates, which were then heated at 100°C for 5 min. Xylose (Sigma, USA), xylobiose (Sigma, USA), and oligosaccharides (Megazyme, Ireland) were used as standards.

3.2.6 Materials used for biobleaching

Unbleached sugar cane bagasse pulp (SAPPI Fine Paper, Stanger, South Africa) was washed with tap water until a neutral pH was observed in the wash water. The pulp was dried in an oven (40°C) and stored at 4°C until further use. For biobleaching, pulp (10 g) was treated with 500 U of crude xylanase at 50°C (pH 6.5) for 3 h at 10% pulp consistency. The reaction was terminated by heating to 100°C for 20 min. To determine the presence of reducing sugars, 10 ml of test and control samples were centrifuged at 10 000 g for 15 min and the supernatant was used (Miller *et al*., 1959). Lignin-derived compounds in the supernatant were also monitored by measuring the absorption spectrum from 200 to 465 nm using UV–Vis Spectrophotometer (Varian, Cary 100, USA); a control supernatant from sample treated with denatured enzyme was also monitored. The pulp was subjected to standard handsheet-making processes and the final paper product was analyzed (TAPPI, 1996).

3.2.7 Analytical methods

3.2.7.1 Enzyme and protein assays

β-Xylanase, cellulase and protein

β-Xylanase, and protein assays were carried out as described in section 2.2.2. Cellulase assay was done in the same way as xylanase, except that carboxymethylcellulose was used as substrate.

Accessory enzymes

Accessory enzyme assays were carried out as described in section 2.2.3.

3.2.7.2 Analysis of pulp samples

Standard paper sheets were made from enzymatically pretreated and untreated pulps using the handsheet former (PTI Lab equipment, Austria), and air dried according to TAPPI methods (TAPPI, 1996). The brightness and opacity of the final paper product was measured using a reflectance meter (Technidyne, USA). The ceramic reference TAPPI corresponding to a brightness of 47.5% was used as a standard. The tensile index, burst factor, and tear index of the paper samples were also tested (TAPPI, 1996). Microscopic evaluations of xylanase-pretreated and control pulp samples were assessed by mounting these samples on stubs, sputter coated with gold palladium, and examined under a scanning electron microscope (Philips, USA) at 10 kV.

3.2.7.3 Optimization of biobleaching process

Optimization of enzyme doses (5, 10, 50, or 100 U g^{-1} pulp) and reaction times (1, 2, 3, or 5 h) were carried out by treating bagasse pulp with crude xylanase of *T. lanuginosus* MC134 at 50°C (pH 6.5). The biobleaching efficiency of crude xylanase (50 U g^{-1} pulp) on bagasse pulp was also examined at various temperatures (30, 40, 50, 60 and 70°C) and pH (5, 6, 7, 8 and 9) conditions for 3 h.

3.3 RESULTS AND DISCUSSION

3.3.1 Hemicellulases production by the *T. lanuginosus* SK wild type strain

T. lanuginosus SK produced high levels of cellulase-free xylanase (5771 $\pm$ 173 nkat ml^{-1}) on oatspelts xylan medium after 5 days of cultivation. Accessory enzyme levels were found to be minimal, with the highest being an extracellular β-mannanase (15.10 $\pm$ 0.37 nkat ml^{-1}). The activity of accessory enzymes such as xylosidases, arabinofuranosidases, glucosidases, and mannosidases varied between 0.80 $\pm$ 0.01 and 3.84 $\pm$ 0.09 nkat ml^{-1}. A high level of extracellular xylanase production was observed in the culture filtrate, whereas intracellular xylanase levels were very low

(572 ± 11.6 nkat ml^{-1}). Accessory enzyme levels in the intracellular fraction were negligible in *T. lanuginosus* SK and similar observations were made in *T. lanuginosus* SSBP (Singh *et al*., 2000).

3.3.2 Strain improvement

Mutagenesis, a well-recognized strain improvement approach, was employed to enhance xylanase production in *T. lanuginosus* (Chadha *et al*., 1999). One hundred and eighteen colonies obtained through a UV mutagenesis study were tested for xylanase production using a medium containing oatspelts xylan. Maximum xylanase production was observed in mutant M13, which showed 17% increase in xylanase (7040 ± 183 nkat ml^{-1}) production after 5 days, compared to the wild-type fungus. The level of xylanase production in the mutant M13 was also stable even after being subjected to repeated transfers and further NTG mutagenesis. The resulting mutant, MC134, showed a 1.5 fold increase in xylanase (8598 ± 132 nkat ml^{-1}) production when compared to the wild type *T. lanuginosus* SK strain.

3.3.3 Effect of carbon and nitrogen sources on xylanase production

Maximum xylanase production was observed when *T. lanuginosus* MC134 cultures were grown on coarse corn cob (20038 ± 700 nkat ml^{-1}) at 1% (w/v) concentration, whereas, fine corn cob showed a production of 10436 ± 352 nkat ml^{-1} (Table 3.1). Polymeric pure substrates such as birchwood (8647 ± 267 nkat ml^{-1}), beechwood (8282 ± 217 nkat ml^{-1}), and oatspelts xylan (7879 ± 235 nkat ml^{-1}) demonstrated appreciable xylanase production. Wheat bran also supported moderate xylanase production (3479 ± 100 nkat ml^{-1}). Although, mono- and di-saccharides favored good fungal growth, the xylanase production level was minimum (Table 3.1). These results indicate that β-xylanase was induced in the presence of the substrate containing xylan polymer. Highest soluble protein concentrations in culture filtrates were found in cultures with the highest level of β-xylanase production.

Coarse corn cob was further optimized for maximum xylanase production under shake flask conditions (Table 3.2). There was an increase in xylanase production when coarse corn cob concentration was increased in the medium. Xylanase activity peaked at a coarse corn cob concentration of 3.25%. Further increase in corn cob concentration did not influence in higher enzyme production. There was a decline in xylanase secretion when corn cob concentration was increased above optimum level. Final pH values were in alkaline range (8.23 to 8.63) for all the corn cob concentrations tested. Good levels of extracellular protein were also noticed in all the corn cob concentrations analysed.

Table 3.1 Xylanase production and final medium pH by *T. lanuginosus* MC134 during growth on various carbon sources

Carbon source (1%, w/v)	**Xylanase activity (nkat ml^{-1})**	**Final medium pH**
Corn cobs (coarse)	20038 ± 700	8.21 ± 0.18
Corn cobs (fine)	10436 ± 352	8.12 ± 0.26
Birch wood xylan	8647 ± 267	7.35 ± 0.21
Beech wood xylan	8282 ± 217	7.56 ± 0.25
Oat spelts xylan	7879 ± 235	7.69 ± 0.21
Wheat bran	3479 ± 100	8.06 ± 0.19
Xylose	1376 ± 0.51	6.06 ± 0.14
Sucrose	19.2 ± 0.63	6.64 ± 0.16
Maltose	12.8 ± 0.42	7.34 ± 0.24
Mannitol	11.1 ± 0.38	7.46 ± 0.16
Cane sugar	10.6 ± 0.36	6.38 ± 0.11
Trehalose	10.3 ± 0.34	7.01 ± 0.17
Glucose	10.1 ± 0.27	5.34 ± 0.19
Sorbitol	8.8 ± 0.31	7.80 ± 0.22
Fructose	8.3 ± 0.28	7.45 ± 0.18
Lactose	6.8 ± 0.19	6.14 ± 0.21
Soluble starch	6.6 ± 0.16	6.47 ± 0.21
None	6.6 ± 0.21	6.01 ± 0.22

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Most of the nitrogen sources favored xylanase production when at a concentration of 1% (w/v) and yeast extract (24988 ± 883 nkat ml^{-1}) was found to be the best nitrogen source. Comparatively xylanase production was lower when inorganic salts were used as nitrogen sources (Table 3.2).

Table 3.2 Xylanase production and final medium pH by *T. lanuginosus* MC134 during growth on various nitrogen sources

Nitrogen source (1%, w/v)	**Xylanase activity (nkat ml^{-1})**	**Final medium pH**
Yeast extract	24988 ± 883	8.36 ± 0.20
Casein	22915 ± 558	8.58 ± 0.29
Beef extract	22736 ± 696	8.32 ± 0.20
Vegetable peptone	22053 ± 688	8.38 ± 0.29
Fish peptone	22030 ± 621	8.28 ± 0.26
Bacteriological peptone	21941 ± 587	8.38 ± 0.19
Proteose peptone	21253 ± 601	8.25 ± 0.28
Tryptone	20816 ± 588	8.45 ± 0.30
Meat extract	19372 ± 611	8.54 ± 0.22
Potassium nitrate	8436 ± 277	6.63 ± 0.26
Ammonium phosphate	8349 ± 226	7.08 ± 0.24
Malt extract	8254 ± 217	6.75 ± 0.18
Urea	7464 ± 198	7.52 ± 0.19
Sodium nitrate	4764 ± 166	6.24 ± 0.23
Ammonium sulphate	3427 ± 88	5.54 ± 0.19
Corn steep liquor	3140 ± 76	6.71 ± 0.22

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Further studies on the optimization showed an increase in xylanase production to 50312 ± 1612 and 52460 ± 1467 nkat ml^{-1} when coarse corn cob (3.25%) and yeast extract (3.25%) were used as carbon and nitrogen sources, respectively. These enzyme levels were much higher than the xylanase activity levels of 47343 and 36207 nkat ml^{-1} produced by *T. lanuginosus* strains ATCC 46882 (Bennett *et al*., 1998) and DSM 5826 (Purkarthofer *et al*., 1993b), respectively, when grown on the same substrate as shake flask cultures. Good xylanase production has also been reported in other microorganisms when corn cob was used as the carbon source. *Streptomyces olivaceoviridis* E-86 yielded 23088 nkat ml^{-1} xylanase using corn cob media (Ding *et al*., 2004). Due to the fact that the use of pure xylan is uneconomical for commercial xylanase production, the current impetus is the utilization of xylan-rich, low-cost agricultural by-products. Corn cob xylan consists mainly of xylose (84%), arabinose (10%), glucose (3.7%), galactose (2.0%), and uronic acid (3.0%) (Garcia *et al*., 2001). The reason for high level of xylanase production in coarse corn cob xylan could be due to the presence of inducers, such as xylose and arabinose, together with the xylan polymer. The larger particle size of corn cob might have reduced the rate of sugars solubilization and also created a good support system for fungal growth (Gomes *et al*., 1993). The advantages of using corn cob as a substrate include higher enzyme titers and no need for additional physical or chemical pretreatments.

3.3.4 Effect of time course, inoculum size, pH, temperature, and agitation on xylanase production

The extracellular xylanase production by *T. lanuginosus* MC134 was monitored for 9 days. The xylanase production peaked after 6 days (54895 ± 1384 nkat ml^{-1}) and declined thereafter (Table 3.3). The xylanase production level increased when inoculum size was raised from 1 to 2 discs. Further increase in the inoculum load did not enhance xylanase production. Optimum growth temperature for xylanase production by *T. lanuginosus* MC134 was found to be 50°C (54560 ± 1600 nkat ml^{-1}). A slight decrease in xylanase production (50360 ± 1367 nkat ml^{-1}) was observed when growth temperature was raised to 55°C. Higher growth temperatures resulted in the decline of enzyme production.

The mutant produced a high level of xylanase (54228 ± 1637 nkat ml^{-1}) at pH 6.5 when coarse corn cob was used as the carbon source. A significant level of xylanase production was observed at pH 8.0 (35574 ± 1217 nkat ml^{-1}), indicating that the fungus has the ability to produce xylanase over a wide range of pHs. The fungus produced a low level of xylanase under static culture conditions (Table 3.3). There was a remarkable increase in xylanase production when the agitation rate was increased up to 150 rpm, but it declined thereafter. The enzyme yield was low at higher agitation rates, which could be attributed to shearing of fungal mycelium that interrupted the fungal growth and xylanase production. The occurrence of increased protein amounts in the culture filtrate was evident at higher agitation rates (data not shown).

Table 3.3 Effect of different growth parameters on xylanase production by *T. lanuginosus* MC134

Time course		Inoculum size		Initial medium pH		Growth temperature		Agitation rate	
Days	**Xylanase activity (nkat ml^{-1})***	**Disc no.**	**Xylanase activity (nkat ml^{-1})***	**pH**	**Xylanase activity (nkat ml^{-1})***	**°C**	**Xylanase activity (nkat ml^{-1})***	**Rpm**	**Xylanase activity (nkat ml^{-1})***
1	600 ± 25	1	52110 ± 1650	3	1034 ± 33	30	6585 ± 267	0	850 ± 31
2	1867 ± 67	2	54927 ± 1434	4	6635 ± 233	35	11669 ± 467	50	7586 ± 317
3	21788 ± 767	3	41692 ± 1384	5	31322 ± 1117	40	30006 ± 1117	100	36442 ± 783
4	40141 ± 984	4	37074 ± 1150	5.5	42308 ± 1067	45	44742 ± 1533	150	54994 ± 1584
5	52494 ± 1650	5	32939 ± 850	6	48960 ± 1600	50	54560 ± 1600	200	23471 ± 900
6	54895 ± 1384			6.5	54228 ± 1637	55	50360 ± 1367	250	9686 ± 233
7	49009 ± 1600			7	44258 ± 1434	60	21054 ± 700		
8	47577 ± 1617			8	35574 ± 1217	70	6951 ± 283		
9	45209 ± 487								

*Each value represents a mean of three replicate determinations with standard deviation (± SD)

3.3.5 Xylanase production in a lab-scale fermenter

Xylanase production by *T. lanuginosus* MC134 in a fermenter (Fig. 3.1) was studied using the optimized production medium containing coarse corn cob over a period of 7 days (Tables 3.4 to 3.7). The xylanase production increased linearly for up to 6 days and declined thereafter (Fig. 3.2). The highest xylanase production of 33706 ± 1245 nkat ml^{-1} was observed at 400 rpm after 6 days. However, the xylanase production in the fermenter was 40% lower than in shake flask culture. Profuse growth of the fungus (formation of a thick mycelial mat) was observed after 3 days. However, the biomass could not be measured as the coarse corn cob was bound to mycelium and caused a blockage in the sampling port. At lower (200, 300 rpm) agitation rates, the enzyme production level was comparatively less. Mycelial growth also was found to decrease when the stirrer speed was reduced to 200 rpm. There was 34% reduction in xylanase production at 300 rpm after 6 days of fermentation. At higher agitation rates of 500 rpm, an increase in extracellular protein with a concomitant reduction in xylanase activity and cellular growth was noticed. This could be due to the rupturing of fungal mycelial cells noted when the shearing force increases (Fig. 3.3). The pH of the culture medium reached the alkaline range at the end of cultivation. A similar phenomenon was also observed in the shake flask experiments. Xylanase production by several microbial strains using corn cob has been reported. Xylanase induction by corn cob xylan largely depends on rheological factors and the types of extraction methods (Hoq and Deckwer, 1995).

Fig. 3.1 Time-course of a batch cultivation of *T. lanuginosus* MC134 in a 5 litre fermenter using 32.5 g l^{-1} coarse corn cob, at 50°C and pH 6.5 with aeration at 1 vvm and stirrer speed of 400 rpm (cascade mode).

Table 3.4 Effect of agitation rate (200 rpm) on xylanase production by *T. lanuginosus* MC134 in a lab scale fermenter

Cultivation (days)	Xylanase activity (nkat ml^{-1})	Extracellular protein (mg ml^{-1})	Final medium pH
0	2082 ± 68	0.11 ± 0.002	6.38 ± 0.14
1	6220 ± 125	0.24 ± 0.008	6.45 ± 0.24
2	10781 ± 507	0.37 ± 0.014	7.10 ± 0.21
3	15227 ± 326	0.52 ± 0.022	7.18 ± 0.26
4	17644 ± 571	0.56 ± 0.024	7.49 ± 0.18
5	18871 ± 491	0.64 ± 0.025	7.89 ± 0.19
6	17391 ± 421	0.58 ± 0.023	8.08 ± 0.28
7	15007 ± 193	0.53 ± 0.019	8.33 ± 0.30

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Table 3.5 Effect of agitation rate (300 rpm) on xylanase production by *T. lanuginosus* MC134 in a lab scale fermenter

Cultivation (days)	Xylanase activity (nkat ml^{-1})	Extracellular protein (mg ml^{-1})	Final medium pH
0	2489 ± 72	0.12 ± 0.002	6.10 ± 0.19
1	6513 ± 139	0.24 ± 0.004	6.15 ± 0.23
2	11524 ± 124	0.41 ± 0.008	6.31 ± 0.22
3	17608 ± 707	0.60 ± 0.02	6.62 ± 0.18
4	19279 ± 638	0.66 ± 0.024	7.15 ± 0.22
5	22430 ± 749	0.76 ± 0.021	7.57 ± 0.21
6	22236 ± 653	0.74 ± 0.026	8.03 ± 0.28
7	18265 ± 458	0.62 ± 0.024	8.31 ± 0.29

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Table 3.6 Effect of agitation rate (400 rpm) on xylanase production by *T. lanuginosus* MC134 in a lab scale fermenter

Cultivation (days)	Xylanase activity (nkat ml^{-1})	Extracellular protein (mg ml^{-1})	Final medium pH
0	2628 ± 98	0.14 ± 0.005	6.51 ± 0.18
1	9440 ± 324	0.38 ± 0.014	6.62 ± 0.20
2	15571 ± 437	0.52 ± 0.012	7.15 ± 0.24
3	19477 ± 683	0.65 ± 0.026	7.57 ± 0.19
4	22604 ± 539	0.79 ± 0.022	8.03 ± 0.28
5	29599 ± 900	0.94 ± 0.032	8.26 ± 0.25
6	33706 ± 1245	1.09 ± 0.031	8.28 ± 0.29
7	30562 ± 1027	0.99 ± 0.035	8.32 ± 0.24

Each value represents a mean of three replicate determinations with standard deviation (± SD)

Table 3.7 Effect of agitation rate (500 rpm) on xylanase production by *T. lanuginosus* MC134 in a lab scale fermenter

Cultivation (days)	Xylanase activity (nkat ml^{-1})	Extracellular protein (mg ml^{-1})	Final medium pH
0	2229 ± 78	0.15 ± 0.004	6.46 ± 0.16
1	6262 ± 258	0.38 ± 0.015	6.55 ± 0.20
2	12518 ± 476	0.69 ± 0.028	6.78 ± 0.19
3	15727 ± 553	0.86 ± 0.016	6.85 ± 0.22
4	18557 ± 443	0.99 ± 0.032	6.97 ± 0.19
5	19889 ± 380	1.07 ± 0.036	7.10 ± 0.24
6	20398 ± 664	1.11 ± 0.029	7.18 ± 0.25
7	20063 ± 475	1.11 ± 0.038	7.14 ± 0.20

Each value represents a mean of three replicate determinations with standard deviation (± SD)

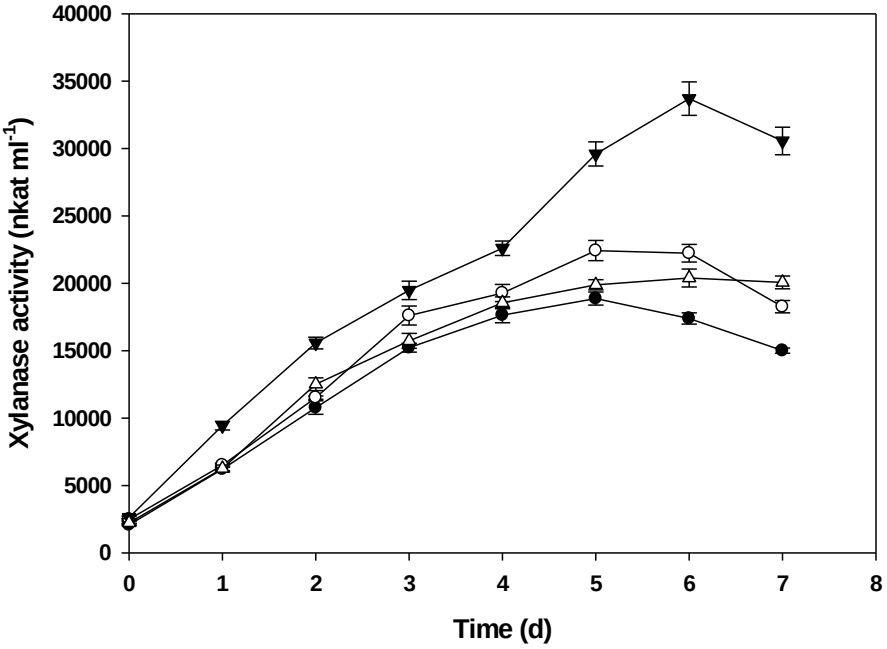


Fig. 3.2 Effect of different agitation rates on xylanase production by *T. lanuginosus* MC134 in a fermenter at 50°C: 200 rpm (●); 300 rpm (○); 400 rpm (▼); and 500 rpm (∆). Each value represents the mean of triplicate determinations with standard deviation (± SD).

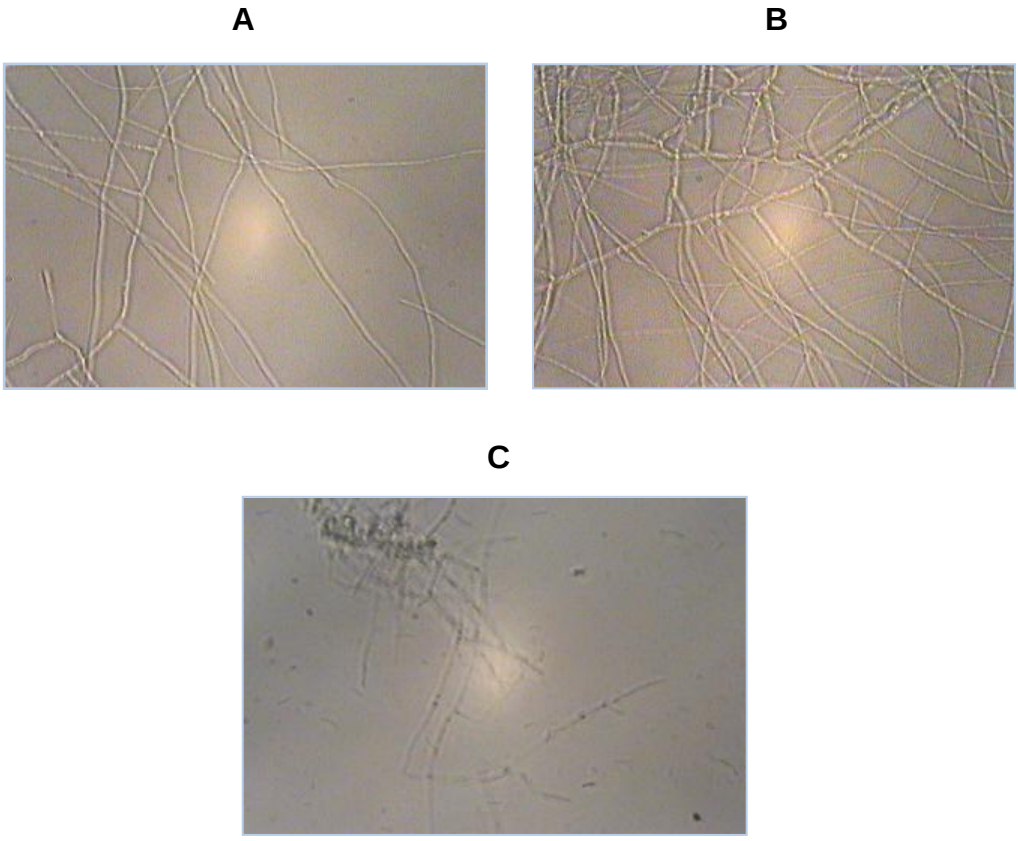


Fig. 3.3 Effect of different stirrer speed on the growth and shearing of *T. lanuginosus* MC134 mycelium in a fermenter. (A) at 200 rpm, (B) 400 rpm and (C) 800 rpm.

3.3.6 Hydrolysis properties of xylanase

The hydrolysis products analyzed by TLC showed that xylose and xylobiose were the predominant end products (Fig. 3.4). Acidic oligosaccharides and xylotriose were observed after 2 h. The hydrolysis pattern clearly indicated that the xylanase is endo-β-1,4-xylanase. After the prolonged enzyme hydrolysis (more than 6 h), the xylotriose spot disappeared and the xylobiose spot intensity increased. The conversion of xylotriose to xylobiose might have occurred through a glycosyl transfer reaction (Bennett *et al*., 1998). The endoxylanases of the glycosyl hydrolase family 11 (GH 11) release short chain acidic oligosaccharides, such as aldotetraouronic acid, aldopentaouronic acid, and aldohexaouronic acid from glucurono xylan. Family 11

endoxylanase cannot act on aldotetraouronic acid and aldopentaouronic acid; whereas it converts aldohexaouronic acid into aldopentaouronic acid and xylobiose through a xylosyl transfer reaction (Kolenova *et al*., 2006). The two acidic oligosaccharide spots (Fig. 3.4) observed after 2 h could be aldopentaouronic acid and aldohexaouronic acid. After 24 h, the intensity of aldopentaouronic acid increased due to slow conversion of aldohexaouronic acid by endoxylanase. The formation of xylose (trace amount) after 6 h could be due to the conversion of xylobiose by the xylosidase present in the crude enzyme extract.

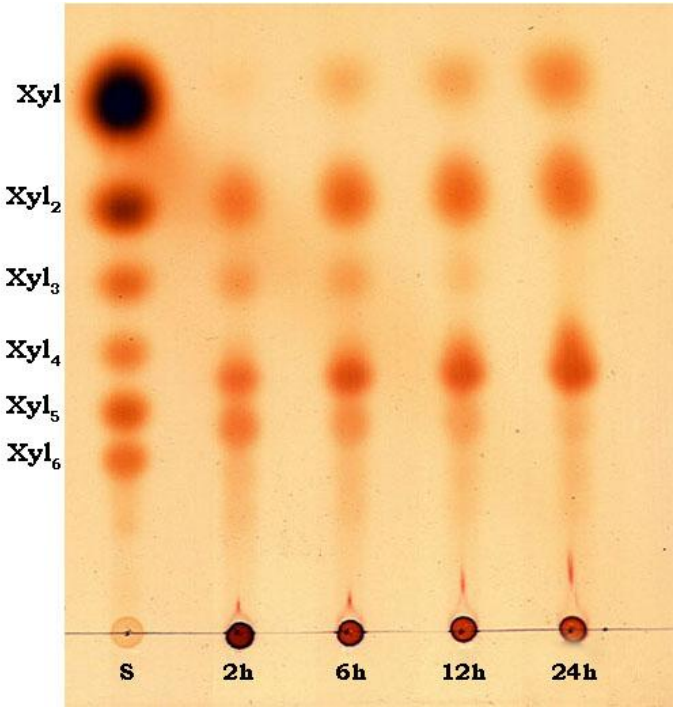


Fig. 3.4 TLC analysis of hydrolysis of birchwood xylan by crude xylanase from *T. lanuginosus* MC134 at 50°C for 24 h. The standards (S) used are: Xyl (xylose): Xyl_2 (xylobiose): Xyl_3 (xylotriose): Xyl_4 (xylotetraose): Xyl_5 (xylopentaose); and Xyl_6 (xylohexaose). The incubation times (h) are indicated below.

3.3.7 Biobleaching of bagasse pulp

Several studies have been carried out on the biobleaching of kraft pulp with different microbial xylanases, mostly to reduce the amount of chlorine needed for bleaching, to reduce the kappa number, and to enhance the brightness (Tremblay and Archibald, 1993; Damiano *et al*., 2003). However, reports on biobleaching of non-woody plants are scarce (Li *et al*., 2005; Gonçalves *et al*., 2008). Of the various dosages of xylanase tested, a maximum brightness of 46.07 ± 0.05%, 3.6 points higher than untreated samples, was obtained when pulp samples were treated with 50 U of crude xylanase. Reducing sugars (25.79 ± 0.14 mg g^{-1}) and lignin-derived compounds released from the pulp further substantiated the brightness increase in the pulp samples pretreated with crude xylanase. On the other hand, an increased dosage of the enzyme (100 U g^{-1} of pulp) did not yield any increase in the brightness (Table 3.8). The results of the biobleaching of bagasse pulp showed that the xylanase might have played a role either in releasing pentosans from bagasse pulp, or by hydrolyzing the xylans precipitated onto the surface of cellulosic fibers. The biobleaching efficiency of *T. lanuginosus* MC134 xylanase was better than alkalophilic *Bacillus* sp. Xylanase, which showed a 2.5% increase in the brightness of bagasse pulp (Kulkarni and Rao, 1996).

The results of the biobleaching of bagasse pulp for varying reaction times showed that the maximum brightness and release of lignin-derived compounds and reducing sugars occurred after 3 h (Table 3.9). However, the extended reaction time did not contribute to further enhancement of the brightness. The treatment of bagasse pulp with crude xylanase from *T. lanuginosus* MC134 lead to a 3.6 point increase in the brightness and a 3.2 point decrease in kappa numbers as compared to untreated samples. The minor differences in tensile strength, breaking length, and burst factor of the paper product

are encouraging (Table 3.10). SEM studies showed that the application of crude xylanase caused morphological changes in the bagasse pulp fiber (Fig. 3.5). The xylanase pre-treated bagasse pulp became unequal and heterogeneous with the formation of protruding peeled fibers on the surface, whereas the control bagasse pulp fibers were smooth with a uniform surface. This result indicated that the enzyme was not only involved in xylan removal, but also assisted in fiber modification. Similar observations on bagasse pulp after xylanase pretreatment have been reported (Christopher *et al*., 2005; Manimaran *et al*., 2009).

Table 3.8 Treatment of bagasse pulp with different dosages of *T. lanuginosus* MC134 xylanase at 50°C for 3 h

Xylanase dose (U g^{-1} pulp)	**Lignin derived compounds**			**Reducing sugars released (mg g^{-1} pulp)**	**Brightness (%)**
	Ab_{237}	**Ab_{280}**	**Ab_{465}**		
0	0.098 ± 0.01	0.071 ± 0.01	0.002	0.00	42.51 ± 0.08
5	0.926 ± 0.02	0.553 ± 0.03	0.018	9.06 ± 0.10	43.22 ± 0.05
10	1.016 ± 0.03	0.696 ± 0.02	0.022	11.8 ± 0.02	43.82 ± 0.03
50	1.67 ± 0.03	0.922 ± 0.02	0.028	25.79 ± 0.14	46.07 ± 0.05
100	1.75 ± 0.03	1.083 ± 0.03	0.026	26.12 ± 0.12	46.09 ± 0.04

Each value represents the mean of triplicate determinations with standard deviation (± SD).

Table 3.9 Optimization of reaction time for biobleaching of bagasse pulp with 50 U g^{-1} xylanase at 50°C (pH 6.5) for 5 h

Reaction time (h)	Lignin derived compounds			Reducing sugars released (mg g^{-1} pulp)	Brightness (%)
	Ab_{237}	Ab_{280}	Ab_{465}		
0	0.15 ± 0.01	0.098 ± 0.01	0.001	0.00	42.5 ± 0.05
1	1.08 ± 0.02	0.553 ± 0.02	0.022	15.28 ± 0.10	43.58 ± 0.04
2	1.45 ± 0.02	0.699 ± 0.01	0.028	21.32 ± 0.15	44.94 ± 0.06
3	1.67 ± 0.03	0.920 ± 0.02	0.027	25.78 ± 0.14	46.07 ± 0.05
5	1.69 ± 0.03	0.948 ± 0.01	0.029	25.82 ± 0.16	46.21 ± 0.03

Each value represents the mean of triplicate determinations with standard deviation (± SD).

Table 3.10 Properties of paper made from bagasse pulp pretreated with xylanase (50 U g^{-1}) pulp in 0.5 M sodium citrate buffer (pH 5 – 6), sodium phosphate buffer (pH 7 – 8) and glycine-NaOH buffer (pH 9) at 50°C for 3 h

Paper properties	**pH**					
	Control	**5**	**6**	**7**	**8**	**9**
Brightness (%)	42.5 ± 0.06	46.1 ± 0.05	46.27 ± 0.05	46.1 ± 0.07	45.95 ± 0.06	45.21 ± 0.05
Kappa number	12.48 ± 0.02	9.4 ± 0.1	9.3 ± 0.1	9.28 ± 0.09	9.54 ± 0.06	9.8 ± 0.08
Opacity	98.31 ± 0.01	98.30 ± 0.06	98.44 ± 0.1	97.74 ± 0.06	98.54 ± 0.08	98.88 ± 0.1
Tensile strength	47.32 ± 0.06	43.8 ± 0.08	44.92 ± 0.03	44.88 ± 0.1	43.50 ± 0.08	43.41 ± 0.12
TEA	49.30 ± 0.08	48.2 ± 0.05	47.5 ± 0.1	47.6 ± 0.14	47.56 ± 0.08	47.24 ± 0.09
Break	2.47 ± 0.06	2.26 ± 0.02	2.13 ± 0.08	2.3 ± 0.05	2.45 ± 0.07	2.35 ± 0.06
Stretch	2.4 ± 0.05	2.3 ± 0.06	2.4 ± 0.08	2.3 ± 0.07	2.3 ± 0.08	2.28 ± 0.04
CD-Residual	3.15 ± 0.06	2.87 ± 0.07	2.79 ± 0.05	2.81 ± 0.07	2.76 ± 0.06	2.81 ± 0.06
Burst Factor	221.4 ± 5	201.3 ± 6	193.5 ± 4	191.2 ± 6	193.1 ± 8	192.6 ± 4
Reducing sugar released (g^{-1} pulp)	0.00	24.8 ± 0.06	25.82 ± 0.05	24.7 ± 0.08	24.16 ± 0.04	23.8 ± 0.06

Each value represents the mean of triplicate determinations with standard deviation (± SD).

3.3.8 Properties of paper made from bagasse pulp pretreated with xylanase from various *T. lanuginosus* strains

Several studies have been carried out on the biobleaching of kraft pulp with different microbial xylanases, mostly to reduce the amount of chlorine needed for bleaching, to reduce the kappa number, and to enhance the brightness (Damiano *et al*., 2003). The treatment of bagasse pulp with crude xylanase from various *T. lanuginosus* strains showed an improvement in brightness by 3 points for all the strains tested. Mutant MC134 showed a brightness of 46.27% followed by SSBP and ATCC 38905 (Table 3.11). However, there were not many variations in improving the brightness of the pulp irrespective of xylanase from various *T. lanuginosus* strains. The kappa number also showed the same trend and was between 9.0 and 9.96 points for all the enzymes tested. There were only minor differences in the release of reducing sugars released. SEM studies showed that the application of crude xylanase caused morphological changes in the bagasse pulp fiber (Fig. 3.5).

In order to adopt the biobleaching process in the existing pulp and paper industries, the proposed technology must not compromise with the final paper quality. Although, the results obtained through xylanase pretreatment did not help in improving the physical properties of paper, it did not affect pulp properties.

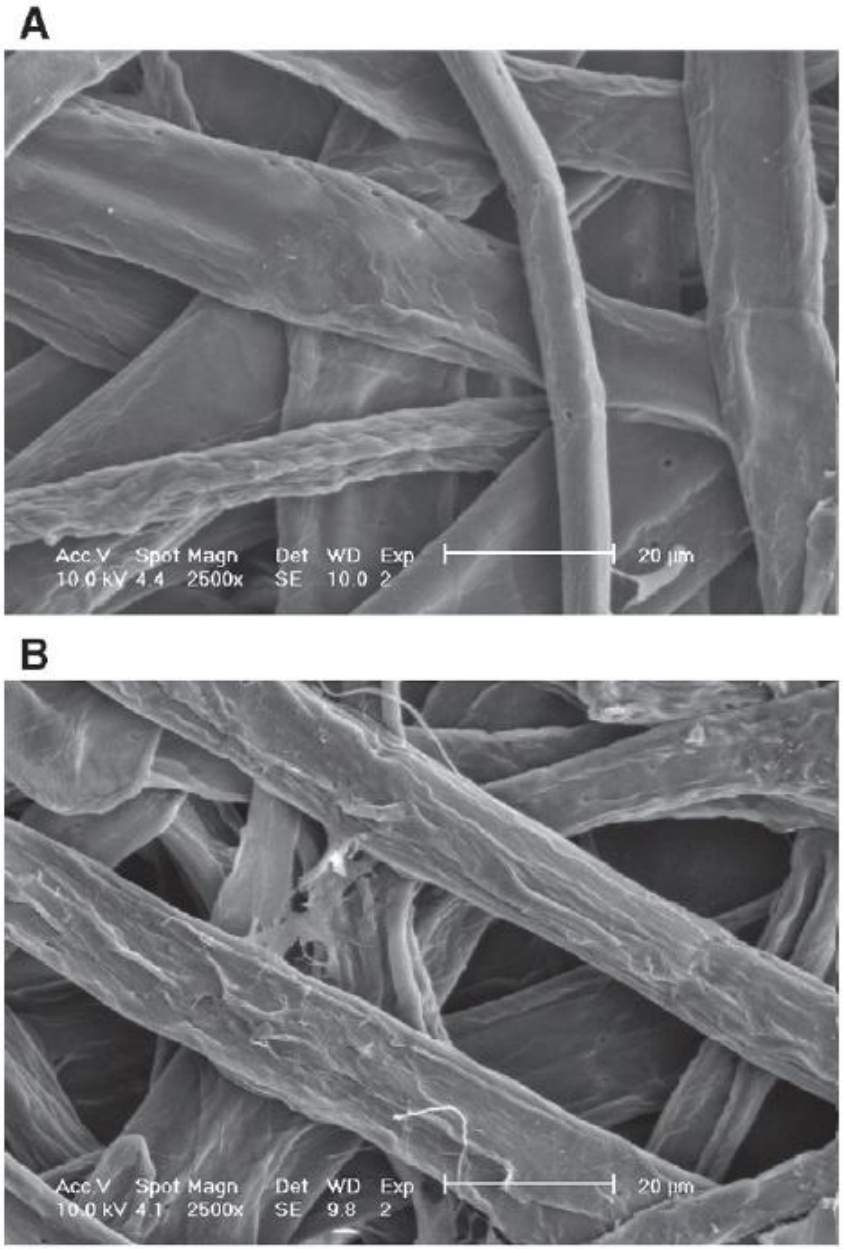


Fig. 3.5 SEM of bagasse pulp samples. (A) Control pulp; (B) Pulp treated with crude xylanase (50 U g^{-1} pulp) at 50°C for 3 h.

Table 3.11 Properties of paper made from bagasse pulp pretreated with xylanase (50 U g^{-1} pulp) in 0.5 M sodium citrate buffer (pH 6) at 50°C for 3 h

T. lanuginosus strain	Brightness (%)	Kappa number	Opacity	Tensile strength	TEA	Break	Stretch	CD-Residual	Burst Factor	Reducing sugars released (g^{-1} pulp)	Lignin derived compounds		
											Ab 237	Ab 280	Ab 465
MC134	46.27	9.3	98.44	44.92	47.5	2.13	2.4	2.79	193.5	25.82	1.74	0.78	0.025
SSBP	46.20	9.00	98.3	44.3	47.7	2.2	2.3	2.66	201	26.0	1.80	0.84	0.020
38905	46.09	9.31	98.33	43.42	47.61	2.32	2.3	2.85	197	25.63	1.75	0.80	0.020
58160	45.81	9.36	98.52	43.65	47.52	2.22	2.4	3.16	197	23.88	1.75	0.81	0.027
36350	45.73	9.36	98.39	43.46	47.85	2.2	2.3	2.81	198	25.95	1.91	0.86	0.030
58158	45.42	9.45	98.39	43.34	48.02	2.32	2.2	2.88	199	26.99	1.80	0.83	0.019
22083	45.37	9.44	98.48	43.59	47.98	2.32	2.2	3.06	201	23.76	1.75	0.76	0.021
34626	45.10	9.96	98.56	43.81	47.75	2.25	2.1	2.85	199	26.99	1.74	0.82	0.023
16455	44.88	9.81	98.42	44.56	47.14	2.12	2.2	3.05	194	26.71	1.87	0.77	0.020
58157	44.83	9.83	98.48	43.48	47.52	2.35	2.3	3.02	197	26.11	0.15	0.098	0.001

Christopher *et al*. (2005) reported that a marginal improvement in the brightness of the bagasse pulp was observed after treatment with 50 U of crude xylanase as compared to previous studies. The brightness of wheat straw pulp pretreated with xylanase from *T. lanuginosus* CBS 288.54 showed a 7.8 point increase in brightness over untreated samples (Li *et al*., 2005). The brightness improvement of *T. lanuginosus* MC134 xylanase was lower when compared to the reported biobleaching results on non-woody plants. A possible reason may be the complex nature of the hemicellulose found in bagasse, which requires xylanases with different characteristics for the total hydrolysis of xylan.

The *T. lanuginosus* MC134 mutant has the potential to produce a high level of xylanase from corn cob at a low cost. Biobleaching results of the bagasse pulp showed that crude xylanase has a possible application in the enzymatic bleaching of non-woody plant fibers. Further studies on the biobleaching by xylanase, combined with other microbial accessory enzymes such as α-arabinofuranosidases and non-chlorinated processes, will help to develop a cost effective alternative bleaching process.

3.4 CONCLUSIONS

T. lanuginosus SK produced good levels of extracellular xylanase on oatspelts xylan media; however, intracellular xylanase was very low. Accessory enzyme levels secreted by the fungus were negligible. The strain was subjected to UV-NTG mutagenesis and xylanase production was further improved by 1.5 fold. The enzyme production was further enhanced by growing the fungus on corn cob medium under optimized growth parameters. However, a 40% reduction in xylanase production was noticed in a 5 L fermenter. The biobleaching potential of crude xylanase was evaluated on bagasse pulp and the brightness was improved by 3.6 points.

Chapter 4

HYPER PRODUCTION OF CELLULASE-FREE XYLANASE BY *Thermomyces lanuginosus* SSBP ON BAGASSE PULP AND ITS APPLICATION IN BIOBLEACHING

Published: Applied Microbiology and Biotechnology 2009, 81: 887 – 893

ABSTRACT

A cellulase-free xylanase production by *T. lanuginosus* SSBP using bagasse pulp was examined under submerged (SmC) and solid-state cultivation (SSC). Higher level of xylanase activity (19320 ± 37 U g^{-1} dried carbon source) was obtained in SSC cultures than in SmC (1772 ± 15 U g^{-1} dried carbon source) after 120 h with a 10% inoculum. The biobleaching efficacy of crude xylanase was tested on bagasse pulp, and a maximum brightness of 46.1 ± 0.06% was observed with 50 U of crude xylanase per gram of pulp, which was 3.8 points higher than the brightness of untreated samples. Reducing sugars (26 ± 0.1 mg g^{-1}) and UV-absorbing lignin-derived compounds in the pulp filtrates were observed as maximum in 50 U of crude xylanase treated samples. *T. lanuginosus* SSBP has potential applications due to its high productivity of xylanase and its efficiency in pulp bleaching.

4.1 INTRODUCTION

Microbial xylanases have attracted much attention owing to their potential applications in food, feed, pulp and paper, and biofuel industrial sectors. In general, paper-making processes utilize large quantities of chemicals resulting in problems of disposal of hazardous effluents. The need for safer and environmentally sound technologies has become more imperative. Owing to the heterogeneity and complex chemical nature of plant hemicellulose, its complete breakdown requires the action of several hydrolytic enzymes with diverse substrate specificity, namely, β-1,4- endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid (ferulic and *p*-coumaric acid) esterase (Singh *et al*., 2003; Collins *et al*., 2005). Among hemicellulolytic enzymes, the use of cellulase-free xylanases has a wide range of biotechnological applications, especially in the pulp and paper industries. They are used primarily as bleaching agents to reduce the amount of chlorine required to achieve desirable levels of paper brightness (Viikari *et al*., 1994; Kenealy and Jeffries, 2003; Singh *et al*., 2003; Bajpai, 2004; Collins *et al*., 2005). The properties of many commercial xylanases are unknown and are difficult to apply into the existing pulp bleaching process. Therefore, industries need enzymes which should function efficiently on pulp materials in order to compete with the bleaching chemicals.

T. lanuginosus, a thermophilic fungus, has been reported as one of the best cellulase-free xylanase producers (Singh *et al*., 2003). Several studies have been reported on xylanase production by submerged cultivation of this strain and application of its enzymes in biobleaching of softwood and hardwood pulps (Tremblay and Archibald, 1993; Bim and Franco, 2000; Duarte *et al*., 2003; Oakley *et al*., 2003). However, studies on the application of xylanases in the biobleaching of non-woody plant pulps such as wheat straw and banana fibre are scant (Roncero *et al*., 2003; Li *et al*., 2005; Jiang *et al*., 2006; Ayyachamy and Vatsala, 2007).

The potential value of the enzyme for diverse applications is feasible only through the development of cost-effective enzyme production methods. Use of inexpensive agricultural by-products for enzyme production through solid-state cultivation (SSC) is an efficient method being used at larger scale and has many advantages over submerged cultivation (Gessesse and Mamo, 1999). In the present study, we report on unusually high production of xylanase by *T. lanuginosus* SSBP during growth on bagasse pulp under optimized solid-state and submerged cultivation. The biobleaching efficiency of crude xylanase was also tested on bagasse pulp. An attempt was also made to attain simultaneous xylanase production and biobleaching of bagasse pulp.

4.2 MATERIALS AND METHODS

4.2.1 Organism and media used

T. lanuginosus SSBP, one of our own isolates, was used in this study (Singh *et al*., 2003). This strain was periodically subcultured on potato dextrose agar medium (Sigma) and maintained at –70°C. Bagasse pulp (unbleached), after subjecting to soda pulping process, was obtained from Sappi Fine Paper, Stanger, South Africa, and was used as a carbon source for enzyme production. The pulp was thoroughly washed with tap water, until a neutral pH of wash was attained, and dried at 45°C overnight prior to use.

4.2.2 Xylanase production by *T. lanuginosus* SSBP using bagasse pulp

Submerged cultivation (SmC) was carried out in 500 ml Erlenmeyer flasks containing 10 g of oven-dried (45°C) pulp in 300 ml of medium containing yeast extract (2 g) and KH_2PO_4 (0.5 g). Flasks were inoculated with 0.5 ml of a 36 h *T. lanuginosus* culture (grown on potato dextrose broth) per gram of bagasse and incubated at 50°C with shaking (150 rpm). After 5 days, the SmC cultures were centrifuged (10 000 g at 4°C for 10 min), and the supernatant was used to determine the xylanase activity.

For SSC, 10 g of bagasse pulp was placed in 500 ml Erlenmeyer flasks, and stock nutrient solution (4 ml) containing 2 g yeast extract and 0.5 g KH_2PO_4 was added. Flasks were inoculated with 0.5 ml of a 36 h *T. lanuginosus* culture (grown on potato dextrose broth) per gram of bagasse and incubated at 50°C under static condition with periodical mixing. After 120 h, enzyme was extracted twice with 100 ml sodium citrate buffer (50 mM, pH 6.5) by squeezing the entire content through a muslin cloth. The extract was then centrifuged at 10000 g for 10 min, and the clear supernatant was used as a source of crude xylanase.

For the SmC and SSC time course study, the produced enzyme was extracted every 24 h over a period of 120 h and checked for xylanase activity. The effect of the inoculum level on xylanase production in SmC and SSC was studied by varying the load of 36 h *T. lanuginosus* SSBP cultures (0.5, 1.0, 1.5 or 2.0 ml g^{-1} bagasse).

4.2.3 Simultaneous enzyme production and biobleaching

T. lanuginosus SSBP was grown in SmC and SSC under optimized growth conditions. After 5 days, the enzyme was extracted as described in section 4.2.2., and used as a source of crude xylanase for biobleaching of bagasse pulp. The solid portion (pulp plus fungal cultures) was washed with hot tap water (80°C) and dried at 45°C. The dried pulp was weighed and then disintegrated using a beater (Lorrentzen and Wettre, Sweden) for 10 min. A standard handsheet was made, and the final paper product was analyzed by Technical Association of the Pulp and Paper Industry (TAPPI) methods (1996).

4.2.4 Enzyme assays

β-Xylanase and cellulase assays were carried out as described in section 2.2.2. Accessory enzyme assays were carried out as described in section 2.2.3.

4.2.5 Materials used for biobleaching

Unbleached sugar cane bagasse pulp was washed with tap water to remove the fines and water-soluble compounds. For biobleaching, pulp sample (10 g) was pretreated with 500 U of crude xylanase obtained from SmC or SSC at 50°C (pH 6.5) for 3 h at 10% pulp consistency. The reaction was terminated by heating the enzyme treated pulp samples at 100°C for 20 min. Ten milliliters of biobleaching test and control samples were centrifuged at 10 000 g for 15 min and the supernatant analyzed for reducing sugars and lignin-derived compounds as described in section 3.2.6. The pulp was subjected to standard handsheet-making processes, and the final paper product was analyzed as described in section 3.2.6.

4.2.6 Analytical methods

Standard paper sheets were analyzed as described in section 3.2.7.2.

4.2.7 Optimization of biobleaching process

Optimization of the enzyme dose (5, 10, 50, or 100 U g^{-1} pulp) and reaction time (1, 2, 3, or 5 h) was carried out by treating the bagasse pulp with the crude xylanase of *T. lanuginosus* SSBP obtained from SmC and SSC at 50°C (pH 6.5). Biobleaching efficiency of crude xylanase (50 U g^{-1} pulp) on bagasse pulp was also examined at various temperatures (30 – 70°C) and pH (5 – 9) conditions for 3 h.

4.3 RESULTS

4.3.1 Xylanase production by *T. lanuginosus* SSBP under SmC and SSC

The inoculum optimization results indicated that the xylanase production by *T. lanuginosus* SSBP was maximized with an inoculum concentration 1 ml per gram dried carbon source (Fig. 4.1) after 120 h in SSC (19320 ± 37 U g^{-1} dried carbon source) and SmC (1772 ± 15 U g^{-1} dried carbon source). The fungus produced

significant levels of cellulase-free xylanase when bagasse pulp was used as carbon source. Among the two culture growth conditions, a high level of xylanase production (17800 ± 23 U g^{-1} dried carbon source) by *T. lanuginosus* SSBP was obtained when bagasse pulp was used as a solid growth support (Fig. 4.2), whereas SmC yielded 1767 ± 26 of xylanase U g^{-1} dried carbon source after 120 h with 5% inoculum. Maximum enzyme production was observed after 120 h in both cases. Cellulase activity was absent, and accessory enzymes *viz*., β-xylosidase, β-gluosidase, α-L-arabinofuranosidase, mannanases and β-mannosidase level were considerably low in either culture flasks. Hence, bagasse pulp did not help in inducing other accessory xylanolytic systems in *T. lanuginosus* SSBP.

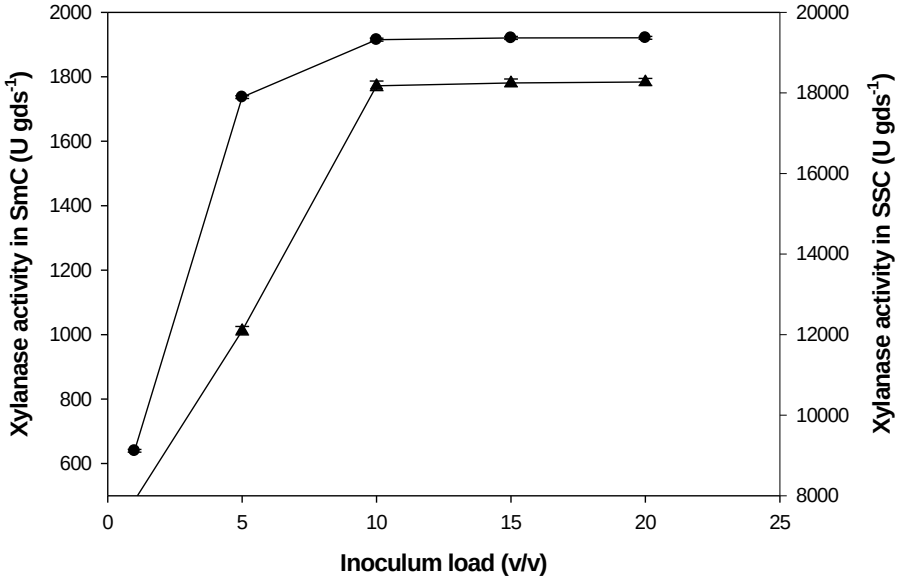


Fig. 4.1 Effect of inoculum load on xylanase production by *T. lanuginosus* SSBP in SSC (●) and SmC (▲) for 120 h at 50°C. Each value represents a mean of three replicate determinations with standard deviation (± SD).

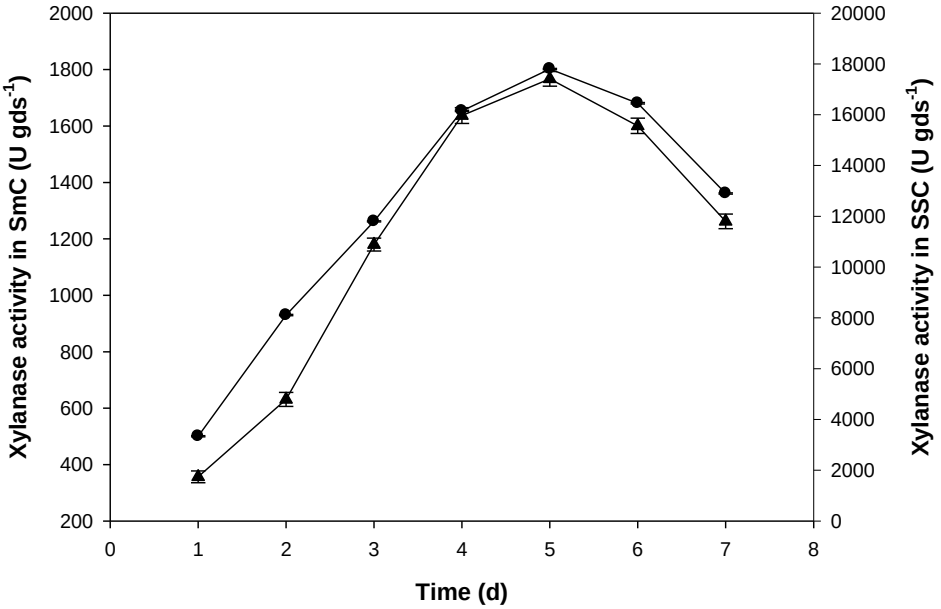


Fig. 4.2 Time course of xylanase production by *T. lanuginosus* SSBP in SSC (●) and SmC (▲) for 8 days at 50°C. Each value represents a mean of three replicate determinations with standard deviation (± SD).

4.3.2 Simultaneous enzyme production and biobleaching

T. lanuginosus SSBP produced a high level of cellulase-free xylanase during growth on a cheap bagasse pulp carbon source after 120 h. To the best of our knowledge, this is the highest value of xylanase (19320 ± 37 U g^{-1} dried carbon source) produced from bagasse pulp by a fungus to date. A handsheet made from residual pulp containing the grown fungus showed a reduction in kappa number (9.5 ± 0.2), but the brightness (37.4 ± 0.04%) was lower than the crude xylanase pretreated pulp (46.2 ± 0.07%) and control samples (42.4 ± 0.05%).

4.3.3 Biobleaching of bagasse pulp

4.3.3.1 Optimization of xylanase dose and reaction time for biobleaching of bagasse pulp

Crude xylanase produced from both SmC and SSC yielded identical levels for reducing sugars and lignin-derived compounds following hydrolysis of bagasse pulp during bleach trials. Of the various dosages of enzyme tested, the maximum brightness of 46.1 ± 0.06% was obtained with 50 U of crude xylanase used pulp samples, which was 3.8 points higher than untreated samples (Table 4.1). Reducing sugars (26 ± 0.1 mg g^{-1}) and lignin-derived compounds released from the pulp further substantiated the brightness increase in the crude xylanase pretreated pulp samples (Table 4.1). Nevertheless, an increased dosage of crude xylanase (100 U g^{-1} of pulp) showed only a 0.1% increase in the brightness when compared with 50 U of enzyme tested. The bleaching of bagasse pulp with crude xylanase at various time intervals showed a maximum brightness and release of lignin-derived compounds and reducing sugars after 3 h.

Table 4.1 Lignin derived compounds, reducing sugar released and brightness of bagasse pulp treated with different doses of crude xylanase in 50 mM sodium citrate buffer (pH 6.5) at 50°C for 3 h

Xylanase dose (IU g^{-1} pulp)	Lignin derived compounds*			Reducing sugar released* (mg g^{-1} pulp)	Brightness* (%)
	Ab_{237}	Ab_{280}	Ab_{465}		
0	0.16 ± 0.01	0.090 ± 0.01	0.001 ± 0	0	42.4 ± 0.06
5	1.05 ± 0.02	0.40 ± 0.04	0.017 ± 0	10.2 ± 0.10	43.3 ± 0.04
25	1.37 ± 0.05	0.60 ± 0.03	0.022 ± 0	16.8 ± 0.05	44.4 ± 0.03
50	1.75 ± 0.07	0.76 ± 0.03	0.021 ± 0	26.0 ± 0.10	46.1 ± 0.06
100	1.82 ± 0.02	0.82 ± 0.01	0.022 ± 0	26.0 ± 0.12	46.2 ± 0.03

* Each value represents a mean of three replicate determinations with standard deviation (± SD)

4.3.3.2 Biobleaching efficiency of crude xylanase at various temperatures and pH values

Kappa number and pulp brightness were not improved significantly following biobleaching trials at 50°C, 60°C, and 70°C, respectively (data not shown). Nevertheless, a slight increase in the removal of lignin-derived compounds from the pulp was observed at 60°C (Fig. 4.3). There was a reduction in the efficiency of enzymatic bleaching at temperatures lower than 50°C (data not shown). The biobleaching efficiency of crude xylanase at various pH values showed that the maximum brightness, kappa number reduction, and reducing sugar release in the pulp supernatant occurred at pH 6.0 (Table 4.2). The bagasse pulp treated with crude xylanase showed a 3.8-point increase in the brightness and a 3.5-point decrease in kappa number at pH 6. Although there was not much difference in terms of brightness increase between pH 5 and 9, the spectrum of pulp supernatant showed a minor difference in lignin-derived compounds released from the bagasse pulp (Fig. 4.3). SEM investigations clearly showed that the application of crude xylanase caused morphological changes in the bagasse pulp fiber (Fig. 4.4). This clearly confirmed that enzyme was not only involved in xylan hydrolysis but also helped in fiber modification. Slightly lower values in tensile strength, breaking length, and burst factor were found post enzyme treatment at all pH values (5 – 9); however, the paper was adjudged to be of good quality based on the data obtained from the evaluation of paper properties (Table 4.2).

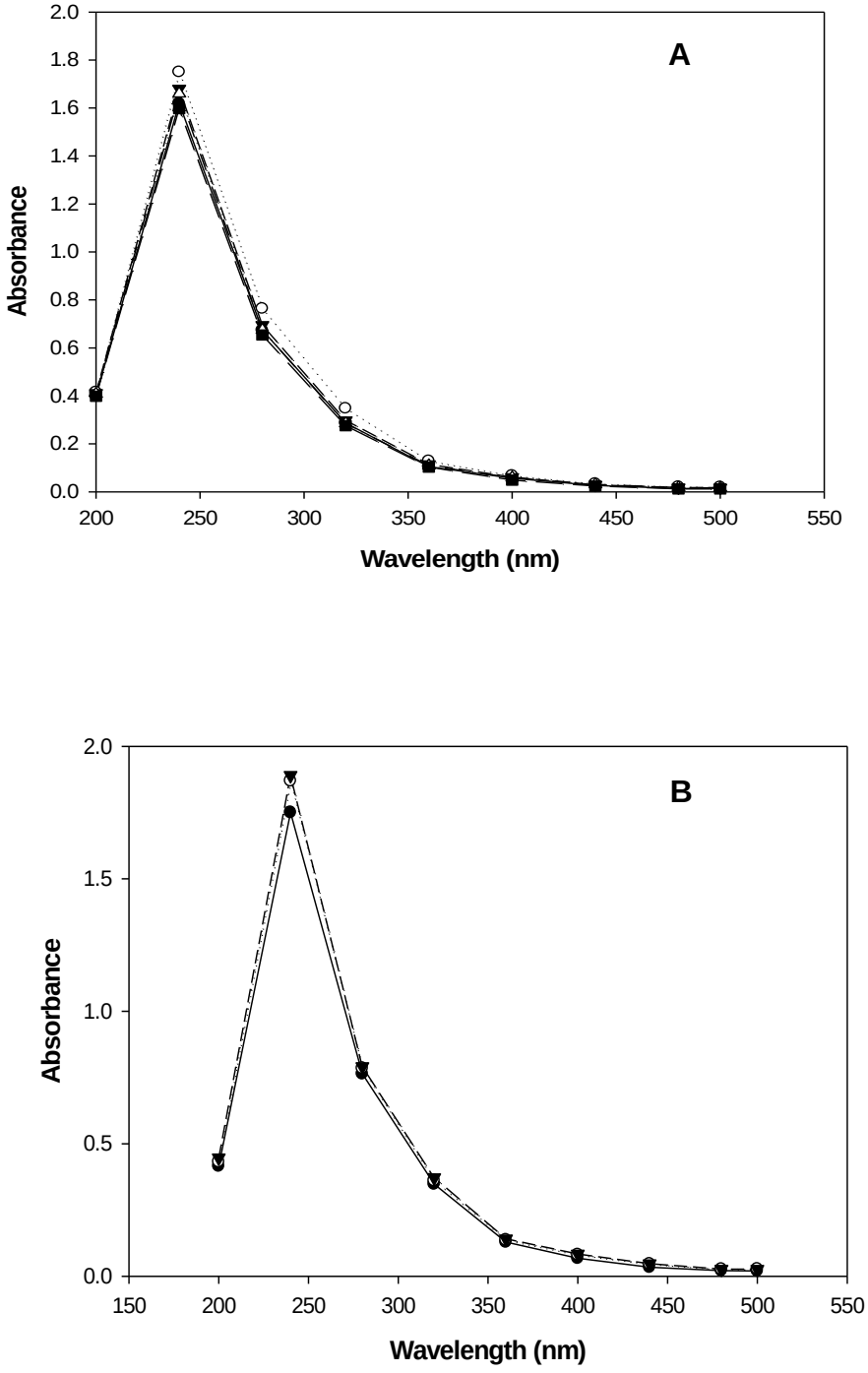


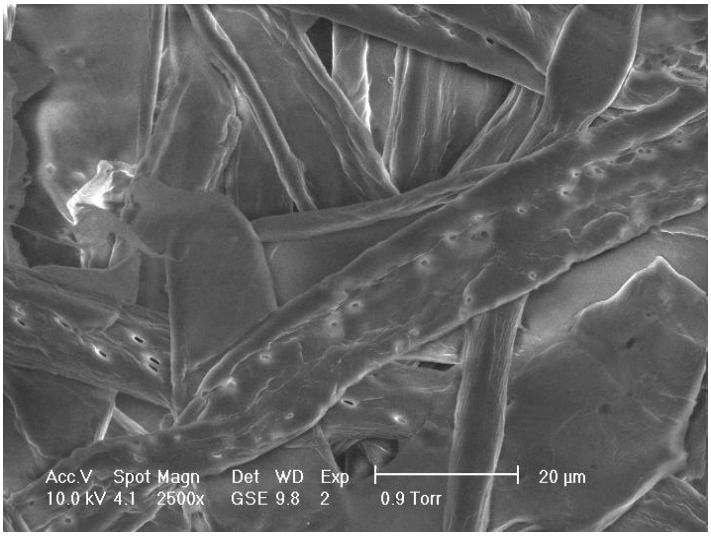
Fig. 4.3 UV–Vis spectrum of lignin-derived compounds released during biobleaching of bagasse pulp (50 U g^{-1} pulp) at various pH values and temperatures. A) pH, 5.0 (●), 6.0 (□), 7.0 (▲), 8.0 (Δ), 9.0 (■) and B) Temperature, 50°C (●), 60°C (□), 70°C (▲). Each value represents a mean of three replicate determinations with standard deviation (± SD).

Table 4.2 Properties of paper made from bagasse pulp pretreated with xylanase (50 U g^{-1} pulp) in 50 mM sodium citrate buffer (pH 5 – 6), sodium phosphate buffer (pH 7 – 8) and glycine-NaOH buffer (pH 9) at 50°C for 3 h

	Control	pH 5	pH 6	pH 7	pH 8	pH 9
Brightness (% ISO)	42.4 ± 0.05	45.9 ± 0.06	46.2 ± 0.07	45.9 ± 0.04	46.0 ± 0.07	45.5 ± 0.06
Kappa number	12.5 ± 0.1	9.3 ± 0.1	9.0 ± 0.2	9.3 ± 0.01	9.2 ± 0.07	9.9 ± 0.06
Opacity	98.5 ± 0.1	98.3 ± 0.2	98.3 ± 0.2	97.8 ± 0.2	98.1 ± 0.1	98.4 ± 0.2
Tensile strength (N/15 mm)	47.1± 0.09	43.2 ± 0.08	44.3 ± 0.05	43.8 ± 0.06	43.5 ± 0.08	43.3 ± 0.07
TEA (J/M^2)	49.1 ± 0.07	47.3 ± 0.07	47.7 ± 0.14	47.5 ± 0.15	47.6 ± 0.09	47.7 ± 0.15
Break	2.4 ± 0.04	2.0 ± 0.03	2.2 ± 0.04	2.3 ± 0.03	2.2 ± 0.01	2.2 ± 0.02
Stretch (%)	2.4 ± 0.07	2.2 ± 0.02	2.3 ± 0.07	2.3 ± 0.05	2.3 ± 0.01	2.3 ± 0.05
CD- Residual	3.07 ± 0.02	2.56 ± 0.03	2.66 ± 0.05	2.49 ± 0.03	2.55 ± 0.06	2.75 ± 0.03
Burst factor ($kPa/m^2/g$)	211 ± 4	195 ± 3	201 ± 4	198 ± 5	194 ± 4	194 ± 4.00
Reducing sugars released g^{-1} pulp	0	24.6 ± 0.2	26.0 ± 0.17	24.5 ± 0.15	25.8 ± 0.2	23.9 ± 0.15

Each value represents a mean of three replicate determinations with standard deviation (± SD)

A



B

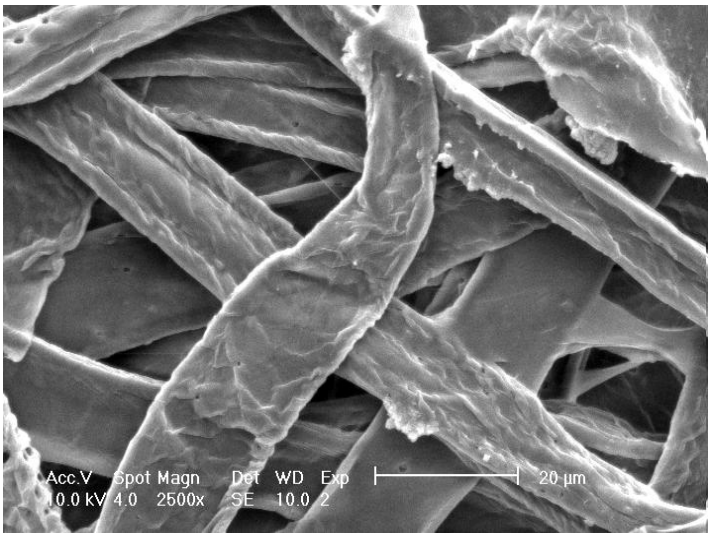


Fig. 4.4 SEM of bagasse pulp samples. A) Control pulp; B) Pulp treated with crude *T. lanuginosus* SSBP xylanase (50 g^{-1}pulp) at 50°C for 3 h.

4.4 DISCUSSION

Using bagasse as a carbon source, xylanase production of 500 U g^{-1} dried carbon source by *Thermoascus aurantiacus* (Santos *et al*., 2003) and 2675 U g^{-1} dried carbon source by *Aspergillus oryzae* (Szendefy *et al*., 2006) have been reported. A xylanase production level of 5098 U g^{-1} dried carbon source by *T. lanuginosus* ATCC 46882 has been previously reported from our group (Christopher *et al*., 2005). In the present study, the xylanase production level of *T. lanuginosus* SSBP was significantly enhanced under optimized SSC conditions and was 3.8 fold higher than the level described for *T. lanuginosus* ATCC 46882. The possible reason for increased enzyme yield could be due to a supplementation of nutrients with bagasse pulp and also a high initial inoculum load.

From the simultaneous enzyme production and biobleaching study, it was expected that there will be an indirect removal of lignin-derived compounds from the bagasse pulp if the fungal cells utilize the hemicellulosic constituents for their growth and xylanase production. Although a handsheet made from residual pulp showed a reduction in kappa number, the brightness was lower than the xylanase pretreated pulp and control samples. *T. lanuginosus* produced pigments during the late log phase of its growth, which was adsorbed by the pulp and changed the color of the pulp, which in turn decreased the original brightness. These problems can be resolved by creating albino strains (which do not synthesize pigments) through efficient strain-improvement methods; thereby, xylanase production and bleaching can be achieved at the same time. Held *et al*. (2003) have developed albino strains of *Ophiostoma* sp. for biological control of sapstaining fungi on pine through ascospore matings.

Results on crude xylanase-aided bleaching of bagasse pulp showed that the enzyme might have played a role either in releasing the reducing sugars from bagasse or by hydrolyzing the xylans precipitated onto the surface of cellulosic fibers. The

biobleaching efficiency of *T. lanuginosus* SSBP xylanase was better than bagasse pulp bleaching by *Bacillus* sp. xylanase which showed only a 2.5% increase in the brightness (Kulkarni and Rao, 1996). The enzyme-aided bleaching of soft and hard wood pulps with family 10 and 11 xylanases and family 26 mannanases showed that xylanases belonging to family 11 were most effective in improving the final brightness of pulp (Clarke *et al*., 1997). The xylanase of *T. lanuginosus* SSBP also belongs to family 11 and is found to be very efficient in biobleaching. The low molecular weight (24.7 kDa) of *T. lanuginosus* SSBP xylanase (Singh *et al*., 2003) might have assisted in penetrating the enzyme into the interior part of the fiber, which in turn could result in the removal of lignin-derived compounds from the bagasse pulp (Beg *et al*., 2001). Since the *T. lanuginosus* SSBP xylanase is cellulase-free (Singh *et al*., 2003), there was a 2.5% weight loss (on account of hydrolyzed arabino-xylan) in the bagasse pulp samples observed after enzymatic treatment. This would be an added advantage of *T. lanuginosus* SSBP compared to enzymatic bleaching with other fungal xylanases (associated with cellulases), which can result in a 16% weight loss (Gübitz *et al*., 1997).

The brightness of bagasse pulp was improved by two units with 50 U g^{-1} pulp of crude xylanase as compared to our previous studies (Christopher *et al*., 2005). The brightness of the wheat straw pulp pretreated with xylanase of *T. lanuginosus* CBS 288.54 showed 7.8 points increase in brightness over untreated samples (Li *et al*., 2005), whereas *Thermotoga maritima* xylanase showed 5.5 points increase in the brightness (Jiang *et al*., 2006). The brightness improvement of *T. lanuginosus* SSBP xylanase on bagasse pulp was lower as compared to biobleaching results of other non-woody plant fibers. The possible reason could be due to the heterogeneity of bagasse xylans, which is composed of 60.9% xylose, 6.1% glucose, 3.5% of arabinose, and trace amounts of galactose and mannose (Rials and Wolcott, 1997; Gonçalves *et al*., 2008). Therefore, xylanases (different characteristics) and β-xylosidases (different substrate specificity) are required for the entire hydrolysis of

hemicellulosic components. In addition, α-L-arabinofuranosidases are also needed to hydrolyze the xylan backbone, which is either mono- or di-substituted with single α-L-arabinofuranose residues. So α-L-arabinofuranosidases is required to hydrolyze L-arabinose containing xylooligosaccharides. Since, *T. lanuginosus* SSBP does not have the capability of producing the required level of accessory xylanolytic enzymes, the combination of aforementioned enzymes with crude xylanase can result in the hydrolysis of entire xylan, and thereby, the brightness of bagasse pulp can be improved substantially.

SEM results clearly indicated that the control bagasse pulp fibers were smooth and showed a uniform surface devoid of protruding fiber formation, whereas xylanase-pre-treated bagasse pulp became irregular and heterogeneous with the occurrence of cracked and peeled fibers on the surface. Similar observations on pulp fiber after xylanase treatment have been reported (Christopher *et al*., 2005; Jiang *et al*., 2006). The capability of pulp bleaching with crude xylanase preparation is an attractive option to complement chemical bleaching because the enzyme can be easily produced on inexpensive carbon sources at low cost.

4.5 CONCLUSIONS

T. lanuginosus SSBP has the potential to produce high level of xylanase from bagasse pulp under optimized SSC conditions. Biobleaching of bagasse pulp results showed that crude xylanase of *T. lanuginosus* SSBP could be applied in enzymatic bleaching of non-woody plant fibers. Further studies on combination of this xylanase with other microbial accessory enzymes such as α-arabinofuranosidases and non-chlorinated processes could help to develop a cost-effective environmentally friendly bleaching process.

CHAPTER 5

GENERAL DISCUSSION

Microbial biotechnology is directed towards the improvement of resource utilization, developing new processes for meeting industrial demands as well as the reduction of environmental impact of large-scale, well-established industries. A rising demand for ecofriendly processes and products urge many industries to opt for environment friendly processes and technologies. These technologies include treatment of raw materials using microbes or their enzymes. Enzymes mediate metabolic reactions with great specificity and rate enhancements. Such catalytic property of enzymes together with their environmentally compatible and easy disposal nature provides tremendous opportunities for industrial applications.

Microbial enzymes have been evaluated for their potential applications in several manufacturing processes since ancient times, *viz*., in the production of wine, cheese, bread, modification of starch etc. The second half of the twentieth century witnessed an unprecedented increase in the use of microorganisms, their metabolic products, and enzymes in a wider area of basic research and their potential industrial applications. During the past two decades, microbial enzymes have been successfully used commercially in many industrial applications including the pulp and paper industry. They have also been widely used in the recycling of lignocellulosic wastes owing to their high specificity, mild reaction conditions and minimum substrate loss (Dashtban *et al*., 2009).

The interest in hemicellulolytic enzymes has increased remarkably during recent years. For complete decomposition of lignocelluloses, three major groups of enzymes are required which include lignolytic enzymes, cellulases and hemicellulases (Lopez *et al*., 2007). Microorganisms produce an array of enzymes for decomposition of plant xylan and the key enzyme for xylan depolymerization is endo-β-1,4- xylanase, which

acts in the regions of low substitution. Owing to the potential applications in industry, microbial xylanases have received a great deal of attention over the years. The basic approach behind use of xylanases involves removal of undesired xylan, as in the case of pulp bleaching and clarification of fruit-juices or wines while applications such as bio-fuels (ethanol production) or SCP production imply utilization of xylan hydrolysates (containing xylose and xylooligosaccharides) for generation of value-added products (Beg *et al.*, 2001; Polizeli *et al*., 2005).

The use of xylanases in the pulp and paper industry has increased significantly with the discovery of enzymatic bleaching by Viikari *et al*. (1986). Since then researchers worldwide have focused their attention toward identifying newer microbial isolates, the xylanases from which can be applied in the pulp and paper industries. Over the years the number of possible applications of xylanases in the pulp and paper industry has increased steadily, and several have become, or are approaching, commercial use. While many applications of enzymes in paper industries are still in the research and developmental stage, several applications have found their way into the mills in an unprecedented short period of time in the last decade (Bajpai, 2004). To satisfy the specific conditions of the bleaching process in the pulp and paper mills, xylanases that are active and stable at elevated temperatures and alkaline pH are critical.

β-xylanases are produced by numerous microorganisms, among which the fungi are the most potent producers. While enzymatic prebleaching requires a thermostable and alkalitolerant enzyme, only a few xylanases are reportedly active and stable at both alkaline pH and elevated temperatures. For the commercial realization and economic viability of xylanase production, it is necessary to identify organisms that produce high levels of this enzyme. A thermophilic fungus, *T. lanuginosus* has been found to be a good cellulase-free xylanase producer with xylanases of good thermostability and pH stability (Puchart *et al*., 1999). Although most of the *T. lanuginosus* strains produce good levels of xylanase, variations in the expression of xylanases among strains of

different origin have been reported (Singh *et al*., 2003). Moreover, the levels of enzymes located inside the cell wall of this fungus are not widely investigated

Strain improvement is an essential part of process development for fermentation products. Genetically modified strains can be cost effective with increased productivity and can possess some specialized desirable characteristics. Strain improvement can be achieved by inducing genetic variation in the natural strain and subsequent screening. Mutation is the primary source of all genetic variation and has been used extensively in industrial improvement of enzyme production (Ghisalba *et al*., 1984). The use of mutation and selection to improve the productivity of cultures has been strongly established for over fifty years and is still recognized as a valuable tool for strain improvement of many enzyme producing organisms. The two classical methods have been used to produce mutants, which include the use of UV light (Ellaiah *et al*., 2002) and NTG (Rubinder *et al*., 2002) for higher enzyme production.

In many microorganisms, xylanase synthesis is regulated by induction repression mechanisms (Purkarthofer and Steiner, 1995; Liu *et al*., 1999). Even though, several thermophilic organisms have been reported to produce thermostable xylanase, the information on the biosynthetic regulation of xylanases in thermophilic organisms is limited (Maheshwari *et al*., 2000; Collins *et al*., 2005).

Most of the xylanases from fungi are inducible extracellular enzyme and this property has great significance in the nutrient medium selection. The xylanase biosynthesis is induced by its substrate, xylan (Kulkarni *et al*., 1999; Subramaniyan and Prema, 2002). The high xylan content in some of the agricultural refuse like corn cobs and wheat bran makes them an accessible and cheap source of inducers. Developing a low-cost method for xylanase production is valuable since it has wide range of applications and holds considerable promise for converting discarded and underutilized agricultural residues to useable products. The inclusion of these negative value substrates in the

nutrient medium is the main strategy in microbial xylanase biosynthesis (Dobrev *et al*., 2007). Apart from nutrient constituents, several factors including pH, temperature, dissolved oxygen concentration and shear stress can influence the production of fungal xylanases.

The main reason for investigating xylanase production by microorganisms is its wide variety of biotechnological applications such as in food processing, poultry feeds, degumming of plant fibers, biobleaching of pulp and paper etc. Xylanases have been widely reported to improve the effectiveness of conventional bleaching chemicals in removing lignin from hardwood and softwood kraft pulps (Viikari *et al*., 1990; Bajpai 1999; Beg *et al*., 2001). Since the last two decades, the chemical bleaching of pulp has become an issue of great concern primarily because of its environmental impact. The chemicals used for bleaching process have resulted in the release of large amounts of chlorinated organic compounds that are known to have toxic, mutagenic and carcinogenic effects (Battan *et al*., 2007). Thus, the use of enzymes for biobleaching has provided an alternative and cost-effective method for process change.

The use of microbial enzymes for bleaching of paper and pulp is now gaining momentum. The microbial enzymes have high specificity for their substrates, the reaction conditions are mild and there is no substrate loss due to chemical modifications. Several criteria are essential for choosing the suitable xylanase producing microorganism for biobleaching of pulp. It is essential that the enzyme should be active at neutral and alkaline pH and should be thermostable. Cellulase-free xylanase is preferred for biobleaching owing to the serious economic implications in terms of cellulose loss, degraded pulp quality, and increased effluent treatment cost.

Not much literature has been reported on the localization of xylanolytic enzymes and high level production and process economy of utilizing such enzymes. Wide scale industrial applications of xylanase require their cost effective production to make the

process economically viable. Thus, the aim of the present study was to reduce the cost of xylanase production by improving the enzyme production of *T. lanuginosus* through mutagenesis, analyzing the xylanase inducing factors, optimizing the cultivation medium for improved production and also to study the biobleachability of xylanase.

The screening of *T. lanuginosus* strains for the production of hemicellulases showed that all 10 tested strains were xylanolytic. All grew well on oatspelts xylan and produced good titres of extracellular xylanase. Among the tested strains, *T. lanuginosus* SK yielded the highest β-xylanase activity after growth on oatspelts xylan. The results of the present study indicated the common occurrence of xylanolytic system in all the tested *T. lanuginosus* strains while the amount of xylanase production among these varied significantly. The xylanase produced by these strains are similar to most xylanases of fungal origin; these enzymes are the major extracellular product of these organisms. Extracellular xylanase activity was found to be five to ten times higher than intracellular xylanase activity after 5 days of incubation. Oatspelts xylan medium containing yeast extract was found to support good growth and xylanase production by these *T. lanuginosus* strains. In oatspelts xylan, the most abundant carbohydrate is xylose (52.5%) with arabinose (22.3%) next (Li *et al*., 2000). The secretion of good levels of xylanase on to the medium by all the tested strains could be attributed to these inducing substances. Purkarthofer and Steiner (1995) also demonstrated the induction of xylanase in *T. lanuginosus* DSM 5826 using oatspelts xylan. The difference in the xylanase producing abilities among groups of similar fungi reinforces the fact that abilities of taxonomically similar fungi of different origin may be found to vary immoderately.

However, all the tested *T. lanuginosus* strains did not produce cellulase upon grown on oatspelts xylan media. Puchart *et al*. (1999) also reported on the non-cellulolytic nature of 17 *T. lanuginosus* strains. The fungus *T. lanuginosus* has received considerable attention as an industrially attractive producer of cellulase-free xylanase (Haki and

Rakshit, 2003). This cellulase-free β-xylanase has great potential in pulp and paper industry for selective removal of xylan from cellulosic materials in biobleaching where cellulosic fibre length should be preserved in papermaking.

Upon analysis for the production of a complete range of hemicellulases, it was found that all the selected strains produced low levels of extracellular hemicellulases apart from xylanase when grown on oatspelts xylan. Singh *et al.* (2000) reported that the production of low levels of accessory enzymes using different carbon sources. It was also noticed that induction level of accessory enzymes varied with different carbon sources used. *T. lanuginosus* DSM 5826 produced different levels of accessory enzymes with various carbon sources (Purkarthofer *et al*., 1993). Moreover, enzyme production is apparently strain dependent. *T. lanuginosus* strain DSM 5826 did not produce accessory enzymes such as mannanase and mannosidase after growth on corn cobs (Purkarthofer *et al*., 1993), while strain SSBP, on the other hand, produced detectable levels of both enzymes (Singh *et al*., 2000). Puchart *et al*. (1999) reported that only three of the 17 *T. lanuginosus* strains tested were able to produce mannanase on locust bean gum. This clearly indicated that enzyme production is dependent on the carbon source and the strain used.

Based on the induction study using various carbohydrates, it can be concluded that the synthesis of xylanase is inducible in *T. lanuginosus* mutant. This is in agreement with several other fungi that have been closely investigated with respect to the regulation of xylanase biosynthesis (Gomes *et al*., 1994; Purkarthofer and Steiner, 1995). The readily metabolizable substrates like arabinose, glucose, lactose, and xylose and its epimers are known to induce the enzyme in some microorganisms (Royer and Nakas, 1989; Liu *et al*., 1999). However these compounds have been proved to be ineffective as inducers of xylanase with *T. aurantiacus* (Gomes *et al*., 1994). Growth of *T. lanuginosus* MC134 on various carbon sources suggests that a good growth does not indicate a good enzyme production. These results showed that xylanase production

in *T. lanuginosus* mutant MC134 was most efficiently trigged by the corresponding polysaccharides serving as a source of xylan fragments functioning as the enzyme inducers. Purkarthofer and Steiner (1995) made similar observations with a naturally occurring strain of *T. lanuginosus* when cultivated on various carbon sources. Gomes *et al*. (2000) reported that among the polysaccharides tested for production of xylanase, birchwood xylan was most effective, followed by beechwood and oat spelt xylans in *Rhodothermus marinus*. The difference among the xylans in their enzyme inducing effect may be due to the presence of different side groups (e.g., 4-*O*-methylglucuronic acid, glucose, etc.) on the xylan backbones (Gomes *et al*., 2000). Polizeli *et al*. (2005) reported that for induction of the xylanolytic enzymes to occur, there has to be physical contact between part of the regulatory machinery of the cell and the inducer; which suggests the existence of some recognition site on the cell surface.

The longer induction period with polysaccharides, clearly indicated that xylans do not act as direct inducers. Since xylans are too large polymers to pass through the cell membrane and trigger the response in the microbial cell leading to the enhanced synthesis of xylanase, it is generally accepted that low-molecular-mass soluble catabolites, such as xylose and xylooligosaccharides, which are released from the polymeric compound by the action of low, constitutive amount of xylanase and which can easily enter the cell, signal the presence of an extracellular substrate and provide the stimulus for the accelerated synthesis of the enzyme (Sachslehner *et al*., 1998; Liu *et al*., 1999). Some authors considered that the xylobiose formed to be the true inducer of endoxylanase synthesis (Haltrich *et al*., 1996; Sunna and Antranikian, 1997).

Considering the inducer specificity for synthesis of xylanase, xylose acts most probably as a direct inducer and not after its transformation in the cells to D-xylulose or xylitol (Ghosh and Nanda 1994). Methyl β-D-xylopyranoside, a synthetic xyloside has been reported as one of the best inducers of xylanase in numerous microorganisms.

(Leathers *et al*., 1986; Simão *et al.*, 1997; Reen *et al*., 2003). However, it did not serve as xylanase inducer in *T. lanuginosus* MC134. This result is difficult to understand mainly in connection with the fact that both xylose and xylobiose serve as xylanase inducers and methyl β-D-xylopyranoside can be considered to be their structural intermediate. However, this result is not unique in the literature (Liu *et al*., 1999; Hidalgo-Lara *et al*., 2005). D-Arabinose and D-ribose also failed to serve as potent inducers, although they supported a slight production of xylanase during the growth experiments.

The delay in xylanase induction in the presence of glucose indicated that xylose induction does not take place in cells before the complete assimilation of glucose. In summary, we can say that the xylanase production by the hyperproducing *T. lanuginosus* mutant MC134 is controlled by the induction-repression mechanism as reported for other naturally occurring *T. lanuginosus* strains (Purkarthofer and Steiner, 1995; Singh et al. 2003). Moreover, we presume that the mutation of the strain SK leading to the mutant MC134 producing 50% more xylanase production does not seem to have altered the regulatory mechanisms of xylanase gene expression.

Production of hydrolytic enzymes is dependent upon several parameters such as pH, temperature, inoculum, agitation, carbon and nitrogen sources etc. Following the one factor at a time approach, optimization of medium and process conditions has been tried by many workers so as to achieve the maximum xylanase production (Haltrich *et al*., 1993). *T. lanuginosus* MC134 produced good levels of β-xylanase activity after growth on coarse corn cobs (20038 ± 700 nkat ml^{-1}) and 10436 ± 352 nkat ml^{-1} on medium containing fine corncobs. There were significant differences in β-xylanase production when *T. lanuginosus* MC134 was cultivated on various carbon sources. Good cellular growth was observed on almost all carbon sources tested. It was noticed that the easily metabolizable sugars such as glucose, sucrose, fructose, lactose maltose etc., repressed the formation of β-xylanase. Several studies on xylanase

production by *T. lanuginosus* also reveal that this species has inherent ability to produce high titres of xylanase (Anand and Vithayathil, 1990; Gomes *et al.*, 1993; Purkarthofer and Steiner, 1995; Lin *et al.*, 1999; Singh *et al*., 2000). Hoq and Deckwer (1995) have investigated xylanase production by *T. lanuginosus* RT9 and *T. lanuginosus* MH4 on wheat bran and corncobs supplemented with basal medium in shake cultures (120 rpm) at 55°C and found xylanase productivity of 773 $Ul^{-1}h^{-1}$ and 54.2 $Ul^{-1}h^{-1}$, respectively, on wheat bran containing medium while on corncob containing medium they noted three fold enhanced production of xylanase by *T. lanuginosus* RT9. In the present study coarse corncobs emerged as a better carbon source in supporting xylanase production by *T. lanuginosus* MC134. A better production of xylanase on corn cob than pure xylan served as evidence that the strain preferred natural heterogeneous substrates as carbon sources rather than isolated individual polysaccharides. The good xylanase yields showed the inherent ability of *T. lanuginosus* MC134 to utilize lignocelluloses from corn cob and wheat bran for xylanase production. This view may be associated to the ecology of *T. lanuginosus* since strains of this organism are often isolated from hay and compost (Emerson, 1968). The results are very important in terms of utilization of locally available lignocellulosic waste as substrates (corn cob and wheat bran) to obtain products of commercial value. However, increase in the concentration of coarse corn cob in the medium above 3.25% resulted in reduction in xylanase yield. Xylanase synthesis suppression at high concentration of xylan containing lignocellulosic material could be due to accumulation of more end products (Gomes *et al*., 1994). The β-xylanase production level on polymeric substrates such as birchwood xylan, beechwood xylan, oatspelts xylan or xylan-containing carbon sources, e.g., corn cobs (coarse and fine) and wheat bran, suggests that xylan is necessary for the effective induction of β-xylanase by strain MC134. However, xylan may not be the direct inducer since it cannot enter the cells and its initial product hydrolysed by a constitutive xylanase level may act as an inducer. Owing to the fact that the use of pure xylan is uneconomical for

commercial enzyme production, therefore, the current impetus is the utilization of xylan-rich low-cost agricultural by-products. Corn cob xylan consists mainly of xylose (84%), arabinose (10%), glucose (3.7%), galactose (2%) and uronic acid (3%) (Garcia *et al*., 2001). The reason for high level of xylanase production in coarse corn cob xylan could be due to the occurrence of inducers such as xylose and arabinose together with xylan polymer. The larger particle size of corn cob reduced the rate of sugars solubilization and also created a support for fungal growth and enzyme release (Gomes *et al*., 1993). The advantage of using corn cob as substrate includes higher enzyme titres and no need for an additional physical or chemical pre-treatment.

Changes in pH of the culture medium after cultivation using various carbon sources were observed. Coarse and fine corn cobs, birchwood xylan, beechwood xylan and oatspelts xylan showed an increase in pH after 5 d growth. The increase in pH during growth on xylan containing carbon sources might be an indication of the presence of high β-xylanase activity. Culture filtrates of *T. lanuginosus* grown on xylan containing substrates were found to be free of cellulase activity as reported previously (Gomes *et al*., 1993; Purkarthofer *et al*., 1993; Alam *et al*., 1994; Hoq and Deckwer, 1995).

Coarse corn cob as a substrate displayed the potential of *T. lanuginosus* MC134 for producing at least 2-3 fold more β-xylanase than with other carbon sources. This result was in line with that of Gomes *et al*. (1993); Purkarthofer *et al*. (1993) and Singh *et al*., 2000). Age and particulate size of the corn cobs are proposed to have an influence on β-xylanase production (Gomes *et al*., 1993; Purkarthofer *et al*., 1993). Our results are also comparable with Purkarthofer e*t al*. (1993), where strain MC134 favoured higher particle size (2 – 3 mm). This might be due to the fact that higher particle size results in slower solubilization of reducing sugars and also creates an immobilised support system with the fungus during growth thereby enhancing β-xylanase release. It was also noted that corn cobs of similar origin with identical cultivation conditions being

used showed significant differences in β-xylanase production. This might be attributed to genotypic variations or different phase of maturity associated with the corn cobs.

The effect of various organic and inorganic nitrogen compounds on the production of β-xylanase by *T. lanuginosus* MC134 showed that almost all organic nitrogen sources promoted good growth of the fungus with yeast extract being the most effective, which was the same for strain DSM 5826 (Purkarthofer *et al*.,1993) and *T. lanuginosus* SSBP (Singh *et al*., 2000) . However among the organic nitrogen sources, urea was less effective and corn steep liquor completely failed to induce xylanase formation. This was in line with the previous report (Ninawe and Kuhad, 2005) on the complete inhibition of xylanase production by *S. cyaneus* SN32 in the presence of corn steep liquor. The different peptones tested as nitrogen sources also gave good results, however, since yeast extract assisted the maximum xylanase yield it was chosen for further optimization. The reason why yeast extract support such a good enzyme release remains to be answered. Piñaga *et al*. (1993) reported on the importance of yeast extract as a critical component of the medium for xylanase production by *Bacillus polymyxa*, likely supplying some essential growth factor. It was further confirmed that yeast extract added to the medium at the end of the stationary phase of growth resulted in a sharp increase in the extracellular protein which correlated with an increase in β-xylanase activity (Piñaga *et al*., 1993). It could be possible that organic nitrogen contains all kinds of amino acids and these amino acids can be absorbed directly by the mycelia (Qinnghe *et al*., 2004). Though organic nitrogen sources supported for good xylanase production, the inorganic nitrogen sources tested, failed to induce xylanase formation. Several reports demonstrated the use of organic nitrogen sources leading to higher enzyme production than inorganic nitrogen sources (Kumar and Takagi, 1999).

In the present study, the extracellular xylanase production by *T. lanuginosus* MC134 was monitored for 9 days. The results on optimization of incubation time indicated that

xylanase production by *T. lanuginosus* MC134 was declined after 6 days of incubation, which may be probably due to proteolysis (Flores *et al*., 1997). The cultivation conditions such as initial medium pH, temperature and agitation rates applied obviously favoured xylanase production suggesting that temperature and/or initial pH in the culture have profound influence on xylanase production by this fungus. The initial medium pH has been found to influence many enzyme systems and their transportation across the cell membrane (Moon and Parulekar, 1991). Production of xylanase by *T. lanuginosus* MC134 was found to be dependent on pH. Interestingly, the final pH of the medium was shifted towards alkaline range when there was good enzyme production observed. This finding is not unique and also observed in *Aspergillus fumigatus* (Anthony *et al*., 2003) when the final pH of culture medium approached towards alkaline pH (7.8 – 8.3) grown at a wide range of initial growth pH (5 – 10). The optimum pH for growth (6.5) of strain MC134 was comparable to the β-xylanases of other *T. lanuginosus* strains (Singh *et al*., 2000). Xylanase production by other microorganisms such as *Aspergillus, Fusarium, Penicillium* and *Streptomyces* has also been shown to be markedly dependent on pH (Smith and Wood 1991; Kuhad *et al*., 1998).

In addition higher oxygen transfer rates, accompanying the high agitation rates (150 rpm) compared to lower agitation rates were also found to be influential for better enzyme production. Mechanical agitation is crucial, because of its effectiveness in mixing the contents of the medium, uniform aeration and prevention of cell clumping (Papagianni, 2004). The shaking rate seemed to have a profound effect on enzyme production by *T. lanuginosus* MC134. This may be due to reduction in oxygen levels in the medium that adversely affect enzyme production (Palma *et al*., 1996).

Optimum growth temperature for β-xylanase production by strain MC134 was 50°C. The preference of higher temperature by *T. lanuginosus* MC134 for xylanase production qualified it as a thermophilic xylanase producer. Temperatures above or

lower than the optimum, there was a reduction in xylanase secretion. This might be due to the fact that at lower temperature, the transport of substrate across the cells is suppressed and lower yield of products are attained. At higher temperature, the maintenance of energy requirement for cellular growth is high due to thermal denaturation of enzymes of the metabolic pathway (Rajoka, 2004) resulting in minimum amount of product formation. There are many reports on *T. lanuginosus* strains previously been grown at 50°C (Anand *et al*., 1990; Singh *et al*., 2003), whereas Gomes *et al.* (1994) reported growing the DSM 5826 strain at 55°C.

The xylanase production was observed to be maximum when 2 discs (9 mm diameter) of 5 days old culture of *T. lanuginosus* MC134 was added to the medium. There was a reduction in enzyme production when inoculum level was increased. Higher concentration of inoculum is not preferable in industrial fermentations (Lincoln, 1960).

When bioreactors are used in the cultivation of filamentous fungi for industrial enzyme production, the agitation rate and aeration levels influence the fungal growth and secretion of enzymes. The enzyme production by *T. lanuginosus* MC134 was affected by agitation. In this study, stirrer speeds ranging from 200 – 500 rpm were used to check the effect on xylanase production in a lab scale fermenter. When using coarse corn cob as the substrate in a 5 L fermenter, the optimal agitation rate was found to be 400 rpm. Low xylanase activities were obtained at 200 rpm, most probably due to oxygen or mass transfer limitations, while at 500 rpm also lower levels of xylanase was produced. The latter result could be explained by a low production rate caused by the increased shear stress. An increase in extracellular protein with a concomitant reduction in xylanase activity and cellular growth was noticed when the agitation rate was increased to 500 rpm. From the results, it can be concluded that the shear stress within the medium, which is related to the stirrer speed, has a marked influence on xylanase production by *T. lanuginosus* MC134. The shearing action of the impellers on the morphology and productivity of filamentous fungi has been reported (Ilias and Hoq,

1998; Gibbs, 2000). The lower production at high shear rates could be caused by hyphae disruption, which leads to leakage of intracellular compounds (Purkarthofer *et al*., 1993). Too strong agitation and aeration have been shown to be harmful for the production of xylanase. In large scale cultivations, the stirrer speed had an even more pronounced effect on the xylanase production. Highest xylanase activities were obtained in 20,000 L reactor study with *T. lanuginosus* when the stirrer was turned off after a certain cultivation time and then used only periodically (Haltrich *et al*., 1996).

The results of the biobleaching of bagasse pulp using xylanases from different *T. lanuginosus* strains showed that the xylanase might have played a role either in releasing pentosans from bagasse pulp, or by hydrolyzing the xylans precipitated onto the surface of cellulosic fibers. The treatment of bagasse pulp with crude xylanase from *T. lanuginosus* MC134 lead to a 3.6 point increase in the brightness and a 3.2 point decrease in kappa numbers as compared to untreated pulp samples. However, xylanases from all the strains tested showed almost same pattern of biobleaching. This might be due to the fact that the genus *Thermomyces* produces xylanases of glycoside hydrolase family 11, and all the tested dosages were the same (50 U ml^{-1}). Moreover testing the pulp samples at different pH's did not show any significant variation in biobleaching among the different strains. It might be due to the fact that xylanases from *T. lanuginosus* strains are stable over a wide range of pH and temperatures.

SEM studies revealed that the xylanase pre-treated bagasse pulp became unequal and heterogeneous with the formation of protruding peeled fibers on the surface, whereas the control bagasse pulp fibers were smooth with a uniform surface. This result indicated that the enzyme was not only involved in xylan removal, but also assisted in fiber modification. Similar observations on bagasse pulp after xylanase pretreatment have been reported. In order to adopt the biobleaching process in the existing pulp and paper industries, the proposed technology must not compromise with the final paper quality. Although, the results obtained through xylanase pretreatment did not help in

improving the physical properties of paper, it did not affect pulp properties. A slight improvement in the brightness of the bagasse pulp was observed after treatment with 50 U of crude xylanase as compared to previous studies (Christopher *et al*., 2005). However, the brightness improvement of xylanases from *T. lanuginosus* strains was lower when compared to the reported biobleaching results on non-woody plants. A possible reason may be the complex nature of the hemicelluloses found in bagasse, which requires xylanases with different characteristics for the total hydrolysis of xylan.

5.1 FUTURE PROSPECTS

An increasing demand for ecofriendly processes and products drive many industries to opt for environmentally sound technologies. Currently most studies are focused on the use of microbial xylanases in various industrial sectors. The use of purified xylan is highly expensive for industrial scale production of xylanases; therefore the current impetus is on the utilization of xylan rich and easily available alternate substrates such as corncobs. Further studies on xylanase production using corncobs at industrial scale and testing the biopulping and bleaching efficiency of xylanase on various plant secondary fibres (non-woody plants) would help in exploiting its immense biotechnological potential. Optimization of cost effective enzyme extraction methods would also make this process more economic and commercially viable. Since *T. lanuginosus* does not synthesize the required level of accessory xylanolytic enzymes, the combination of α-L-arabinofuranosidases and β-xylosidases with crude xylanase could result in the complete hydrolysis of xylan thereby the brightness of pulp can be improved substantially. In addition, *T. lanuginosus* also produces an array of branched xylooligosaccharides from arabino and glucurono xylans. Conversely, xylooligosaccharides titre could be further improved by optimizing the physiological conditions of the fungus and thereafter their probable application in food, biofuels and biorefineries should be evaluated.

REFERENCES

Abou-Hachem, M., Olsson, F. and Karlsson, E. N. 2003. Probing the stability of the modular family 10 xylanase from *Rhodothermus marinus*. *Extremophiles* **7:** 483 – 491.

Akino, T., Nakamura, N. and Horikoshi, K. 1988. Characterization of β-mannosidase of an alkalophilic *Bacillus* sp*. Agricultural and Biological Chemistry* **52:** 1459 – 1464.

Akpinar, O., Erdogan, K. and Bostanci, S. 2009. Production of xylooligosaccharides by controlled acid hydrolysis of lignocellulosic materials. *Carbohydrate Research* **344:** 660 – 666.

Alam, M. M., Gomes, I., Mohiuddin, G. and Hoq, M. M. 1994. Production and characterization of thermostable xylanase by *Thermomyces lanuginosus* and *Thermoascus aurantiacus* grown on lignocellulose. *Enzyme and Microbial Technology* **16:** 298 – 302.

Anand, L. and Vithayathil, P. J. 1990. Production of extracellular xylanase by thermophilic fungus *Humicola lanuginosa* Bunce. *Indian Journal of Experimental Biology* **25:** 434 – 437.

Anand, L., Krishnamurthy, S. and Vithayathil, P. J. 1990. Purification and properties of xylanases from thermophilic fungus, *Humicola lanuginosa* (Griffon and Maublanc) Bunce. *Archives of Biochemistry and Biophysics* **276:** 246 – 553.

Anthony, T., Raj, K. C., Rajendran, A. and Gunasekaran, P. 2003. High molecular weight cellulase-free xylanase from alkali-tolerant *Aspergillus fumigatus* AR1. *Enzyme and Microbial Technology* **32:** 647 – 654.

Apel-Birkhold, P. C. and Walton, J. D. 1996. Cloning, disruption, and expression of two endo-beta 1,4-xylanase genes, XYL2 and XYL3, from *Cochliobolus carbonum*. *Applied and Environmental Microbiology* **62:** 4129 – 4135.

Arai, M., Fujimoto, H., Ooi, T., Ogura, S. and Murao, S. 1995. Purification and properties of β-mannosidase from *Aspergillus aculeatus. Oyo Toshitsu Kagaku* **42:** 49 – 51.

Araujo, A. and Ward, O. P. 1990. Purification and some properties of the mannanases from *Thielavia terrestris. Journal of Industrial Microbiology and Biotechnology* **6:** 269 – 274.

Archana, A., Sharma, A. and Satyanarayana, T. 1999. Xylanolytic enzymes. In: *Thermophilic Moulds in Biotechnology* (Eds: Johri, B. N., Satyanarayana, T. and Olsen, J.) Kluwer Academic Publishers, Dordrecht, pp. 169 – 190.

Asha Poorna, C. and Prema, P. 2006. Production and partial characterization of endoxylanase by *Bacillus pumilus* using agro industrial residues. *Biochemical Engineering Journal* **32:** 106 – 112.

Avalos, O. P., Noyola, T. P., Plaza, I. M. and Torre, M. 1996. Induction of xylanase and β-xylosidase in *Cellulomonas flavigena* growing on different carbon sources. *Applied Microbiology and Biotechnology* **46:** 405 – 409.

Ayyachamy, M. and Vatsala, T. M. 2007. Production and partial characterization of cellulase free xylanase by *Bacillus subtilis* C 01 using agriresidues and its application in biobleaching of non-woody plant pulp. *Letters in Applied Microbiology* **45:** 467 – 472.

Bailey, M. J., Biely, P. and Poutanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* **23:** 257 – 270.

Bailey, M. J., Buchert, J. and Viikari, L. 1993. Effect of pH on production of xylanase by *Trichoderma reesei* on xylan and cellulose based media. *Applied Microbiology and Biotechnology* **40:** 224 – 229.

Bajpai, P. 1999. Application of enzymes in the pulp and paper industry. *Biotechnology Progress* **15:** 147 – 157.

Bajpai, P. 2004**.** Biological bleaching of chemical pulps. *Critical Reviews in Biotechnology* **24:** 1 – 58.

Bajpai, P. and Bajpai, P. K. 2001. Development of a process for the production of dissolving pulp using xylanase enzyme. *Appita Journal* **54:** 381 – 384.

Balakrishnan, H., Dutta-Choudhury, M., Srinivasan, M. C. and Rele, M. V. 1992. Cellulasefree xylanase production from an alkalophilic *Bacillus* sp. *World Journal of Microbiology and Biotechnology* **8:** 627 – 631.

Balakrishnan, H., Srinivasan, M. C. and Rele, M. V. 1997. Extracellular protease activities in relation to xylanase secretion in an alkalophilic *Bacillus* sp. *Biotechnology Letters* **19:** 599 – 601.

Baraznenok, V. A., Becker, E. G., Ankudimova, N. V. and Okunev, N. N. 1999. Characterization of neutral xylanases from *Chaetomium cellulolyticum* and their biobleaching effect on eucalyptus pulp. *Enzyme and Microbial Technology* **25:** 651 – 659.

Bataillon, M., Cardinali, A. P. N., Castillon, N. and Duchiron, F. 2000. Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. *Enzyme and Microbial Technology* **26:** 187 – 192.

Battan, B., Sharma, J., Dhimana, S. S. and Kuhad, R. C. 2007. Enhanced production of cellulase-free thermostable xylanase by *Bacillus pumilus* ASH and its potential application in paper industry. *Enzyme and Microbial Technology* **41:** 733 – 739.

Bauer, M. W., Bylina, E. J., Swanson, R. V. and Kelly, R. M. 1996. Comparison of a β-glucosidase and a β-mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Purification, characterization, gene cloning and sequence analysis. *Journal of Biological Chemistry* **271:** 23749 – 23755.

Beauchemin, K. A., Colombatto, D., Morgavi, D. P. and Yang, W. Z. 2003. Use of exogenous fibrolytic enzymes to improve feed utilization by ruminants. *Journal of Animal Science* **81:** 37 – 47.

Bedford, M. R. 1995. Mechanism of action and potential environmental benefits from the use of feed enzymes. *Animal Feed Science and Technology* **53:** 145 – 155.

Beg, Q. K., Bhushan, B., Kapoor, M. and Hoondal, G. S. 2000. Production and characterization of thermostable xylanase and pectinase from a *Streptomyces* sp. QG-11-3. *Journal of Industrial Microbiology and Biotechnology* **24:** 396 – 402.

Beg, Q. K., Kapoor, M., Mahajan, L. and Hoondal, G. S. 2001. Microbial xylanases and their industrial applications: a review. *Applied Microbiology and Biotechnology* **56:** 326 – 338.

Belancic, A., Scarpa, J., Peirano, A., Diaz, R., Steiner, J. and Eyzaguirre, J. 1995. *Penicillium purpurogenum* produces several xylanases: purification and properties of two of the enzymes. *Journal of Biotechnology* **41:** 71 – 79.

Bennett, N. A., Ryan, J., Biely, P., Vrsanska, M., Kremnicky, L., Macris, B. J., Kekos, D., Christakopoulos, P., Katapodis, P., Claeyssens, M., Nerinckx, W., Ntauma, P. and Bhat, M. K. 1998. Biochemical and catalytic properties of an endoxylanase purified from the culture filtrate of *Thermomyces lanuginosus* ATCC 46882. *Carbohydrate Research* **306:** 445 – 455.

Bhat, M. K. 2000. Cellulases and related enzymes in biotechnology. *Biotechnology Advances* **18:** 355 – 383.

Biely, P. 1985. Microbial xylanolytic systems. *Trends in Biotechnology* **3:** 286 – 290.

Biely, P. 2003. Xylanolytic enzymes. In: Handbook of food enzymology (Eds: Whitaker, J. R., Voragen, A. G. J. and Wong, D. W. S.) Marcel Dekker, Inc., New York, NY, USA. pp. 879 – 915.

Biely, P., Cotè, G. L., Kremnicky, L., Greene, R. V. and Tenkanen, M. 1997. Action of acetylxylan esterase from *Trichoderma reesei* on acetylated methyl glycosides. *FEBS Letters* **420:** 121 – 124.

Biely, P., Markovič, O. and Mislovičová, D. 1985. Sensitive detection of endo-1,4,-β-glucanases and endo-1,4-β-xylanases in gels. *Analytical Biochemistry* **144:** 147 – 151.

Bim, M. A. and Franco, T. T. 2000. Extraction in aqueous two-phase systems of alkaline xylanase produced by *Bacillus pumilus* and its application in kraft pulp bleaching. *Journal of Chromatography B* **743:** 349 – 356.

Bissoon, S., Christov, L., and Singh, S. 2002. Bleach boosting effects of purified xylanase from *Thermomyces lanuginosus* SSBP on bagasse pulp. *Process Biochemistry* **37:** 567 – 572.

Biswas, S. R., Jana, S. C., Mishra, A. K. and Nanda, G. 1990. Production, purification and characterization of xylanase from a hyperxylanolytic mutant of *Aspergillus ochraceus*. *Biotechnology and Bioengineering* **35:** 244 – 251.

Blochl, E., Rachel, R., Burggraf, S., Hafenbradl, D., Jannasch, H. W. and Stetter, K. O. 1997. *Pyrolobus fumarii*, gen. and sp. nov., represents a novel group of archaea, extending the upper temperature limit for life to 113°C. *Extremophiles* **1:** 14 – 21.

Brock, T. D. 1995. The road to Yellowstone - and beyond. *Annual Review of Microbiology* **49:** 1 – 28.

Buchart, J., Ranua, M., Kantelinen, A. and Viikari, L. 1992**.** The role of two *Trichoderma reesei* xylanases in bleaching of kraft pulp. *Enzyme and Microbial Technology* **37:** 825 – 829.

Carmona, E. C., Brochetto-Braga, M. R., Pizzirani-Kleiner, A. A. and Jorge, J. A. 1998. Purification and biochemical characterization of an endoxylanase from *Aspergillus versicolor*. *FEMS Microbiology Letters* **166:** 311 – 315.

Castanares, A., Hay, A. J., Gordon, A. H., McCrae, S. L. and Wood, T. M. 1995. D-xylan-degrading enzyme system from *Phanerochaete chrysosporium*: isolation and partial characterization of an α- (4-O-methyl)-D-glucuronidase. *Journal of Biotechnology* **43:** 183 – 194.

Cesar, T. and Mrša, V. 1996. Purification and properties of the xylanase produced by *Thermomyces lanuginosus. Enzyme and Microbial Technology* **19:** 289 – 296.

Chadha, B. S., Ajay, B. K., Mellon, F. and Bhat, M. K. 2004. Two endoxylanases active and stable at alkaline pH from the newly isolated thermophilic fungus, *Myceliophthora* sp. IMI 387099. *Journal of Biotechnology* **109:** 227 – 237.

Chadha, B. S., Jaswinder, K., Rubinder, K., Saini, H. S. and Singh, S. 1999. Xylanase production by *Thermomyces lanuginosus* wild and mutant strains. *World Journal of Microbiology and Biotechnology* **15:** 217 – 221.

Chanda, S. K., Hirst, E. L., Jones, J. K. N. and Percival, E. G. V. 1950. The constitution of xylan from esparto grass (*Stipa tenacissima* L.). *Journal of Chemical Society* **50:** 1287 – 1289.

Chen, X., Cao, Y., Ding, Y., Lu, W. and Li, D. 2007. Cloning, functional expression and characterization of *Aspergillus sulphureus* beta-mannanase in *Pichia pastoris*. *Journal of Biotechnology* **128:** 452 – 461.

Christakopoulos, P., Katapodis, P., Kalogeris, E., Kekos, D., Macris, B. J., Stamatis, H. and Skaltsa, H. 2003. Antimicrobial activity of acidic xylo-oligosaccharides produced by family 10 and 11 endoxylanases, *International Journal of Biological Macromolecules* **31:** 171 – 175.

Christakopoulos, P., Nerinckx, W., Kekos, D., Macris, B. and Claeyssens, M. 1996. Purification and characterization of two low molecular mass alkaline xylanases from *Fusarium oxysporum* F3. *Journal of Biotechnology* **51:** 181 – 189.

Christakopoulos, P., Nerinekx, W., Kekos, D., Marcis, B. and Claeyssens, M. 1997. The alkaline xylanase III of *Fusarium oxysporum* belongs to family F/10. *Carbohydrate Research* **302:** 191 – 195.

Christopher, L., Bissoon, S., Singh, S., Szendefy, J. and Szakacs, G. 2005. Bleach-enhancing abilities of *Thermomyces lanuginosus* xylanases produced by solid state fermentation. *Process Biochemistry* **40:** 3230 – 3235.

Christov, L. P., Myburgh, J., O'Neill, F. H., VanTonder, A. and Prior, B. A. 1999. Modification of the carbohydrate composition of sulfite pulp by purified and characterized beta-xylanase and beta-xylosidase of *Aureobasidium pullulans*. *Biotechnology Progress* **15:** 196 – 200.

Claeyssens, M. and Henrissat, B. 1992. Specificity mapping of cellulolytic enzymes: classification into families of structurally related proteins confirmed by biochemical analysis. *Protein Science* **1:** 1293 – 1297.

Clarke, A. J., Bray, M. R. and Strating, H. 1993. β-Glycanases and xylanases. Their mechanism of action, in *ACS Symposium* Series No: 533, American Chemical Society, USA. 27.

Clarke, J. H., Rixon, J. E., Ciruela, A., Gilbert, H. J. and Hazlewood, G. P. 1997. Family-10 and family-11 xylanases differ in their capacity to enhance the bleachability of hardwood and softwood paper pulps. *Applied Microbiology and Biotechnology* **48:** 177 – 183.

Cleemput, G., Hessing, M., van Oort, M., Deconynck, M. and Delcour, J. A. 1997. Purification and characterization of β-D-xylosidase and an endo-xylanase from wheat flour. *Plant Physiology* **113:** 377 – 386.

Collins, T., Gerday, C. and Feller, G. 2005. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiology Reviews* **29:** 3 – 23.

Connerton, I., Cummings, N., Harris, G. W., Debeire, P. and Breton, C. 1999. A single domain thermophilic xylanase can bind insoluble xylan: evidence for surface aromatic clusters. *Biochimica et Biophysica Acta* **1433:** 110 – 121.

Conrad, D. and Nothen, W. 1984. Hydrolysis of xylodextrins (DP 2-6) by xylanolytic enzymes from *Aspergillus niger*. *Proceedings: 3rd European Congress of Biotechnology* Vol. 2, 169 – 177.

Cooney, D. G. and Emerson, R. 1964. Thermophilic Fungi: An Account of their Biology, Activities and Classification. W. H. Freeman and Company, San Francisco, CA. pp. 80 – 88.

Copa-Patiño, J. L., Zhang, Y., Padmaperuma, B., Marsden, I., Broda, P. and Sinnott, M. L. 1993. Polarimetry and 13C n.m.r. show that the hydrolyses of beta-D-glucopyranosyl fluoride by beta (1→3) -glucanases from *Phanerochaete chrysosporium* and *Sporotrichum dimorphosporum* have opposite stereochemistries. *Biochemical Journal* **293:** 591 – 594.

Coutinho, P. M. and Henrissat, B. 1999. Carbohydrate-active enzyme server (CAZY) at URL: http://afmb.cnrs-mrs.fr/~cazy/ CAZY/.

Cui, F., Li, Y., Liu, Z., Zhao, H., Ping, L., Ping, L., Yang, Y., Xue, Y. and Yan, L. 2009. Optimization of fermentation conditions for production of xylanase by a newly isolated strain, *Penicillium thiersii* ZH-19. *World journal of Microbiology and Biotechnology* **25:** 721 – 725.

Damaso, M. C. T., De Castro, A. M., Castro, R. M., Andrade, C. M. M. C. and Pereira, N. Jr. 2004. Application of Xylanase from *Thermomyces lanuginosus* IOC-4145 for enzymatic hydrolysis of corncob and sugarcane bagasse. *Applied Biochemistry and Biotechnology* **113:** 1003 – 1012.

Damiano, V. B., Bocchini, D. A., Gomes, E. and Silva, R. D. 2003. Application of crude xylanase from *Bacillus licheniformis* 77–2 to the bleaching of Eucalyptus kraft pulp. *World Journal of Microbiology and Biotechnology* **19:** 139 – 144.

Dashtban, M., Schraft, H. and Qin, W. 2009. Fungal bioconversion of lignocellulosic residues; opportunities and perspectives. *International Journal of Biological Sciences* **5:** 578 – 595.

Defaye, J., Driguez, H., John, M., Schmidt, J. and Ohleyer, E. 1985. Induction of D-xylan-degrading enzymes in *Trichoderma lignonum* by non-metabolizable inducers. A synthesis of 4-thioxylobiose. *Carbohydrate Research* **139:** 123 – 132.

Dekker, R. F. H. and Richards, G. N. 1976. Hemicellulases, their occurrence, purification, properties and mode of action. *Advances in Carbohydrate Chemistry and Biochemistry* **32:** 277 – 352.

Demain, A. L. and Adrio J. L. 2008. Contributions of microorganisms to industrial biology. *Molecular Biotechnology* **38:** 41 – 55.

Derewenda, U., Swenson, L., Green, R., Wei, Y., Morosoli, R., Shareck, F., Kluepfel, D. and Derewenda, Z. S. 1994. Crystal structure at 2.6 Å resolution, of the *Streptomyces lividans* xylanase A, a member of the family of β-1,4-D-glycanases. *Journal of Biological Chemistry* **269:** 20811 – 20814.

Dervilly, G., Leclercq, C., Zimmerman, D., Roue, C., Thibault, J. F. and Saulnier, L. 2002. Isolation and characterization of high molecular mass water-soluble arabinoxylans from barley malt. *Carbohydrate Polymers* **47:** 143 – 149.

Dervilly, G., Thibault, J. F. and Saulnier, L. 2001. Experimental evidence for a semi-flexible conformation for arabinoxylans. *Carbohydrate Research* **330:** 365 – 372.

Dhawan, S. and Kaur, J. 2007. Microbial Mannanases: An overview of production and applications. *Critical Reviews in Biotechnology* **27:** 197 – 216.

Ding, C. H., Jiang, Z. Q., Li, X. T., Li, L. T. and Kusakabe, I. 2004. High activity xylanase production by *Streptomyces olivaceoviridis* E-86. *World Journal of Microbiology and Biotechnology* **20:** 7 – 10.

Dobozi, M. S., Szakács, G. and Bruschi, C. V. 1992. Xylanase activity of *Phanerchaete chrysosporium. Applied and Environmental Microbiology* **58:** 3466 – 3471.

Dobrev, G. T., Pishtiyski, I. G., Stanchev, V. S. and Mircheva, R. 2007. Optimization of nutrient medium containing agricultural wastes for xylanase production by *Aspergillus niger* B03 using optimal composite experimental design. *Bioresource Technology* **98:** 2671 – 2678.

Dominguez, J. M. 1998. Xylitol production by free and immobilized *Debaryomyces hansenii*. *Biotechnology Letters* **20:** 53 – 56.

Duarte, M. C., Silva, E. C. D., Gomes, I. M. D. B., Ponezi, A. N., Portugal, E. P., Vincente, J. R. and Davanzo, E. 2003. Xylan-hydrolyzing enzyme system from *Bacillus pumilus* CBMAI 0008 and its effects on *Eucalyptus grandis* pulp for pulp bleaching improvement. *Bioresource Technology* **88:** 9 – 15.

Durand, H., Clanet, M. and Tiraby, G. 1988. Genetic improvement of *Trichoderma reesei* for large-scale cellulase production. *Enzyme and Microbial Technology* **10:** 341 – 346.

Dusterhoft, E. M., Linssen, V. A. J. M., Voragen, A. G. J. and Beldman, G. 1999. Purification, characterization and properties of two xylanases from *Humicola insolens*. *Enzyme and Microbial Technology* **20:** 437 – 445.

Dwivedi, P., Vivekanand, V., Ganguly, R. and Singh, R. P. 2009. *Parthenium* sp. as a plant biomass for the production of alkalitolerant xylanase from mutant *Penicillium oxalicum* SAUE-3.510 in submerged fermentation. *Biomass and Bioenergy* **33:** 581 – 588.

Eda, S., Ohnishi, A. and Kato, K. 1976. Xylan isolated from the stalk of *Nicotiana tabacum*. *Agricultural and Biological Chemistry* **40:** 359 – 364.

Elegir, G., Szakacs, M. and Jeffries, T.W. 1994. Purification, characterization and substrate specificities of multiple xylanases from *Streptomyces* sp. strain B-12-2. *Applied and Environmental Microbiology* **60:** 2609 – 2615.

Elisashvili, V. I., Khardziani, T. S., Tsiklauri, N. D. and Kachlishvili, E. T. 1999. Cellulase and xylanase activities in higher basidiomycetes. *Biochemistry (Moscow)* **64:** 718 – 722.

Elisashvili, V., Kachlishvili, E. and Penninckx, M. 2008. Effect of growth substrate, method of fermentation, and nitrogen source on lignocellulose-degrading enzymes production by white-rot *basidiomycetes. Journal of Industrial Microbiology and Biotechnology* **35:** 1531 – 1538.

Ellaiah, P., Prabhakar, T., Ramakrishna, B., Taleb, A. T. and Adinarayana, K. 2002. Strain improvement of *Aspergillus niger* for the production of lipase. *Indian Journal of Microbiology* **42:** 151 – 153.

Emerson, R. 1968**.** The thermophiles. In: *The Fungi - An Advanced Treatise* (Eds: Answorth, G. C. and Sussman, A. S.) Academic Press. London. pp.105 – 128.

Ericksson, K. E. L., Blanchette, R. A. and Ander, P. 1990. Biodegradation of lignin. In: *Microbial and Enzymatic Degradation of Wood and Wood Components* (Ed: Timmel, T. E.) Springer-Verlag, Berlin. pp. 215 – 232.

Eriksson, K. K. and Johnsrud, S. C. 1983. Mutants of white – rot fungus *Sporotrichum pulverulentum* with increased cellulase and β-glucosidase production. *Enzyme and Microbial Technology* **38:** 425 – 429.

Esteban, R., Villanueva, I. R. and Villa, T. G. 1982. β-D-Xylanases of *Bacillus circulans* WL-12. *Canadian Journal of Microbiology* **28:** 733 – 741.

Fengel, D. and Wegener, G. 1984. Wood: chemistry, ultra structure, reactions. Berlin: Walter de Gruyter 614 p.

Fernández-Epsinar, M. T., Ramon, D., Pinaga, F. and Valles, S. 1992. Xylanase production by *Aspergillus nidulans. FEMS Microbiology Letters* **91:** 91 – 96.

Fernández-Espinar, M., Piñaga, F., de Graaff, L., Visser, J., Ramón, D. and Vallés, S. 1994. Purification, characterization and regulation of the synthesis of an *Aspergillus nidulans* acidic xylanase. *Applied Microbiology and Biotechnology* **42:** 555 – 562.

Filho, E. X. F., Puls, J., Coughlan, M. P. 1996. Purification and characterization of two arabinofuranosidases from solid-state cultures of the fungus *Penicillium capsulatum*. *Applied and Environmental Microbiology* **62:** 168 – 173.

Flores, M. E., Perez, R. and Huitron, C. 1997. β-Xylosidase and xylanase characterization and production by *Streptomyces sp.* CH-M-1035. *Letters in Applied Microbiology* **24:** 410 – 416.

Fontes, C. M., Gilbert, H. J., Hazlewood, G. P., Clarke, J. H., Prates, J. A., McKie, V. A., Nagy, T., Fernandes, T. H. and Ferreira, L. M. 2000. A novel *Cellvibrio mixtus* family 10 xylanase that is both intracellular and expressed under noninducing conditions. *Microbiology* **146:** 1959 – 1967.

Frederick, M. M., Chin-Hen, K., Frederick, J. R. and Reilly, P. 1985. Purification and characterization of endoxylanases from *Aspergillus niger* I: Two isozyme active on xylan backbone near branch points. *Biotechnology and Bioengineering* **27:** 525 – 532.

Fujimoto, H., Ooi, T., Wang, S. L., Takizawa, T., Hidaka, H., Murao, S. and Arai, M. 1995. Purification and properties of three xylanases from *Aspergillus aculeatus*. *Bioscience Biotechnology and Biochemistry* **59:** 538 – 540.

Garcia, R. B., Nagashima Jr. T., Praxedes, A. K. C., Raffin, F. N., Moura, T. F. A. L. and do Egito, E. S. T. 2001. Preparation of micro and nanoparticles from corn cobs xylan. *Polymer Bulletin* **46:** 371 – 379.

Garcia-Kirchner, O., Munoz-Aguilar, M., Perez-Villalva, R. and Huitron-Vargas, C. 2002. Mixed submerged fermentation with two filamentous fungi for cellulolytic and xylanolytic enzyme production. *Applied Biochemistry and Biotechnology* **98:** 1105 – 1114.

Gebler, J., Gilkes, N. R., Claeyssens, M., Wilson, D. B., Beguin, P., Wakarchuk, W. W., Kilburn, D. G., Miller Jr., R. C., Warren, R. A. and Withers, S. G. 1992. Stereoselective hydrolysis catalyzed by related beta-1,4-glucanases and beta-1,4-xylanases. *Journal of Biological Chemistry* **267:** 12559 – 12561.

Georis, J., Giannotta, F., de Buyl, E., Granier, B. and Frère, J. M. 2000. Purification and properties of three endo-β-1,4-xylanases produced by *Streptomyces* sp. strain S38 which differ in their ability to enhance the bleaching of kraft pulp. *Enzyme and Microbial Technology* **26:** 178 – 186.

Gessesse, A. and Mamo, G. 1999. High-level xylanase production by an alkalophilic *Bacillus* sp. by using solid-state fermentation. *Enzyme and Microbial Technology* **25:** 68 – 72.

Ghisalba, O., Auden, J. A. L., Schuup, T. and Nuesch, J. 1984. The rifamycins: properties, biosynthesis and fermentation. In: *Biotechnology of Industrial Antibiotics* (Ed: E. J. Vandamme) Marcel Dekker, New York pp. 281 – 327.

Ghosh, M. and Nanda, G. 1994. Physiological studies on xylose induction and glucose repression of xylanolytic enzymes in *Aspergillus sydowii* MG49. *FEMS Microbiology Letters* **117:** 151 – 156.

Gibbs, P. A. 2000. Growth of filamentous fungi in submerged culture: Problems and possible solutions. *Critical Reviews in Biotechnology* **20:** 17 – 48.

Gierer, J. and Wännström, S. 1984. Formation of alkali stable C-C bonds between lignin and carbohydrate fragments during kraft pulping. *Holzforschung - International Journal of the Biology, Chemistry, Physics and Technology of Wood* **38:** 181 – 184.

Giesbert, S., Lepping, H., Tenberge, K. B. and Tudzynski, P. 1998. The xylanolytic system of *Claviceps purpurea*: Cytological evidence for secretion of xylanases in infected rye tissue and molecular characterization of two xylanase genes. *Phytopathology* **88:** 1020 – 1030.

Godfrey, T. 2003. The enzymes market for grain processing In: *Recent Advances in Enzymes in Grain Processing* (Eds: Courtin, C. M., Veraverbeke, W. S. and Delcour, J. A.) Katholieke University, Leuven. pp. 401 – 406.

Godfrey, T. and West, S. 1996. *Industrial Enzymology*, Second ed. Macmillan Press Ltd., London.

Golubev, A. M., Nagem, R. A., Brandao Neto, J. R., Neustroev, K. N., Eneyskaya, E. V., Kulminskaya, A. A., Shabalin, K. A., Savel'ev, A. N. and Polikarpov, I. 2004. Crystal structure of alpha-galactosidase from *Trichoderma reesei* and its complex with galactose: implications for catalytic mechanism. *Journal of Molecular Biology* **339:** 413 – 422.

Gomes, D. J., Gomes, J. and Steiner, W. 1994. Factors influencing the induction of endo-xylanase by *Thermoascus aurantiacus. Journal of Biotechnology* **33:** 87 – 94.

Gomes, I., Gomes, J., Steiner, W. and Esterbauer, H. 1992. Production of xylanolytic enzymes by a wild strain of *Trichoderma viride*. *Applied Microbiology and Biotechnology* **36:** 701 – 707.

Gomes, J., Gomes, I., Terler, K., Gubala, N., Ditzelmüller, G. and Steiner, W. 2000. Optimisation of culture medium and conditions for α-L-Arabinofuranosidase production by the extreme thermophilic eubacterium *Rhodothermus marinus*. *Enzyme and Microbial Technology* **27:** 414 – 422.

Gomes, J., Purkarthofer, H., Hayn, M., Kapplmiiller, J., Sinner, M. and Steiner, W. 1993. Production of a high level of cellulase-free xylanase by the thermophilic fungus *Thermomyces lanuginosus* in laboratory and pilot scales using lignocellulosic materials. *Applied Microbiology and Biotechnology* **39:** 700 – 707.

Gomez-Gomez, E., Ruiz-Roldan, M. C., Di Pietro, A., Roncero, M. I. and Hera, C. 2002. Role in pathogenesis of two endo-beta-1,4-xylanase genes from the vascular wilt fungus *Fusarium oxysporum*. *Fungal Genetics and Biology* **35:** 213 – 222.

Gonçalves, R. A., Moriya, R. Y., Oliveira, L. R. M. and Saad, M. B. W. 2008. Xylanase recycling for the economical biobleaching of sugarcane bagasse and straw pulps. *Enzyme and Microbial Technology* **43:** 157 – 163.

Grabski, A. C. and Jeffries, T. W. 1991. Production, purification and characterization of L-(1-4)-endoxylanase of *Streptomyces roseiscleroticus*. *Applied and Environmental Microbiology* **57:** 987 – 992.

Gruber, K., Klintschar, G., Hayn, M., Schlacher, A., Steiner, W. and Kratky, C. 1998. Thermophilic xylanase from *Thermomyces lanuginosus*: high resolution X-ray structure and modeling studies. *Biochemistry* **37:** 13475 – 13485.

Gübitz, G. M., Haltrich, D., Latal, B. and Steiner, W. 1997. Mode of depolymerization of hemicellulose by various mannanases and xylanases in relation to their ability to bleach softwood pulp. *Applied Microbiology and Biotechnology* **47:** 319 – 326.

Gupta, S., Bhushan, B., Hoondal, G. S. and Kuhad, R. C. 2001. Improved xylanase production from a haloalkalophilic *Staphylococcus* sp. SG-13 using inexpensive agricultural residues. *World Journal of Microbiology and Biotechnology* **17:** 5 – 8.

Haki, G. D. and Rakshit, S. K. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technology* **89:** 17 – 34.

Hakulinen, N., Tenkanen, M. and Rouvinen, J. 2000. Three-dimensional structure of the catalytic core of acetylxylan esterase from *Trichoderma reesei*: insight into the deacetylation mechanism. *Journal of Structural Biology* **132:** 180 – 190.

Hakulinen, N., Turunen, O., Janis, J., Leisola, M. and Rouvinen, J. 2003. Three-dimensional structures of thermophilic beta-1,4-xylanases from *Chaetomium thermophilum* and *Nonomuraea flexuosa*. Comparison of twelve xylanases in relation to their thermal stability. *European Journal of Biochemistry* **270:** 1399 – 1412.

Haltrich, D., Nidetzky, B., Kulbe, K. D., Steiner, W. and Župančič, S. 1996. Production of fungal xylanases. *Bioresource Technology* **58:** 137 – 161.

Haltrich, D., Preiss, M. and Streiner, W. 1993. Optimisation of a culture medium for increased xylanase production by a wild strain of *Schizophyllum commune*. *Enzyme and Microbial Technology* **15:** 854 – 860.

Harbak, L. and Thygesen, H. V. 2002. Safety evaluation of a xylanase expressed in *Bacillus subtilis. Food and Chemical Toxicology* **40:** 1 – 8.

Harris, G. W., Pickersgill, R. W., Connerton, I., Debeire, P., Touzel, J. P., Breton, C. and Perez, S. 1997. Structural basis of the properties of an industrially relevant thermophilic xylanase. *Proteins* **29:** 77 – 86.

Hatada, Y., Takeda, N., Hirasawa, K., Ohta, Y., Usami, R., Yoshida, Y., Ito, S. and Horikoshi, K. 2005. Sequence of the gene for a high-alkaline mannanase from an alkaliphilic *Bacillus sp.* strain JAMB-750, its expression in *B. subtilis* and characterization of the recombinant enzyme. *Extremophiles* **9:** 497 – 500.

Held, B., Thwaites, J., Farrel, R. and Blanchette, R. 2003. Albino strains of *Ophiostoma* species for biological control of sap-staining fungi. *Holzforschung* **57:** 237 – 242.

Henrissat, B. 1991. A classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **280:** 309 – 316.

Henrissat, B. and Bairoch, A. 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochemical Journal* **293:** 781 – 788.

Henrissat, B. and Coutinho, P. M. 2001. Classification of glycoside hydrolases and glycosyltransferases from hyperthermophiles. *Methods in Enzymology* **330:** 183 – 201.

Henrissat, B. and Davies, G. 1997. Structural and sequence-based classification of glycoside hydrolases. *Current Opinion in Structural Biology* **7:** 637 – 644.

Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. and Mornon, J. P. 1989. Cellulase families revealed by hydrophobic cluster analysis. *Gene* **81:** 83 – 95.

Hermann, M. C., Vrsanka, M., Juricková, M., Hirsch, J., Biely, P. and Kubicek, C. P. 1997. The β-D-xylosidase of *Trichoderma reesei* is a multifunctional β-D-xylan xylohydrolase. *Biochemical Journal* **321:** 375 – 381.

Hidalgo-Lara, M. E., Amelia Farrés, G. S. and Montes-Horcasitas, M. C. 2005. β-Methyl-xyloside: positive effect on xylanase induction in *Cellulomonas flavigena. Journal of Industrial Microbiology and Biotechnology* **32:** 345 – 348.

Honda, H., Kudo, T., Ikura, Y. and Horikoshi, K. 1985. Two types of xylanases of alkalophilic *Bacillus* sp. no. C-125. *Canadian Journal of Microbiology* **31:** 538 – 542.

Hoq, M. M. and Deckwer, W. 1995. Cellulase-free xylanase by thermophilic fungi: a comparison of xylanase production by two *Thermomyces lanuginosus* strains. *Applied Microbiology and Biotechnology* **43:** 604 – 609.

Hoq, M. M., Hempel, C. and Deckwer, W. 1994. Cellulase-free xylanase by *Thermomyces lanuginosus* RT9: Effect of agitation, aeration, and medium components on production. *Journal of Biotechnology* **37:** 49 – 58.

Hrmova, M., Biely, P. and Vrsanska, M. 1989. Cellulose and xylan- degrading enzymes of *Aspergillus terreus* and *Aspergillus niger*. *Enzyme and Microbial Technology* **11:** 610 – 616.

Huang, S. P., Wang, C. L., Zhang, G. M. and Ma, L. X. 2007. Construction of a double functional recombinant strain of *Pichia pastoris* coexpressing phytase and mannanase and the enzymatic analysis. *Wei Sheng Wu Xue Bao* **47:** 280 – 284.

Hudson, H. J. 1992. Fungal Biology, Cambridge University Press, Cambridge. pp. 106 – 170.

Iefuji, H., Chino, M., Kato, M. and Iimura, Y. 1996. Acid xylanase from yeast *Cryptococcus* sp. S-2: purification, characterization, cloning, and sequencing. *Bioscience Biotechnology and Biochemistry* **60:** 1331 – 1338.

Ilias, M. and Hoq, M. M. 1998. Effect of agitation rate on the growth and production of xylanase free of cellulase by *Thermomyces lanuginosus* MH4 in bioreactor. *World Journal of Microbiology and Biotechnology* **14:** 765 – 767.

Imanaka, T. and Sakurai, S. 1992. Method of washing super precision devices, semiconductors, with enzymes. United States Patent 5,078,802.

Inglis, G. D., Popp, A. P., Selinger, L. B., Kawchuk, L. M., Gaudet, D. A. and McAllister, T. A. 2000. Production of cellulases and xylanases by low-temperature basidiomycetes. *Canadian Journal of Microbiology* **46:** 860 – 865.

Ito, K., Iwashita, K. and Iwano, K. 1992. Cloning and sequencing of the xyn C gene encoding acid xylanase of *Aspergillus kawachii*. *Bioscience Biotechnology and Biochemistry* **56:** 1338 – 1340.

Jeffries, T. W., Davis, M., Rosin, B. and Landucci, L. 1998. Mechanisms for kappa reduction and color removal by xylanases. In: 7th international conference on Biotechnology in the Pulp and Paper Industry, Vol. C, Vancouver, Canada pp. 41 – 43.

Jeffries, T. W., Sykes, M. S., Rutledge-Cropsey, K., Klungness, J. H. and Abubakr, S. 1996. Enhanced removal of toners from office waste papers by microbial cellulases. In: *Biotechnology in the Pulp and Paper Industry*: *Recent Advances in Applied and Fundamental Research* (Eds: Rebotnik, E. S. and Messner, K.) Vienna, Austria pp. 141–144.

Jiang, Z. Q., Li, X. T., Yang, S. Q., Li, L. T., Li, Y. and Feng, W. Y. 2006. Biobleach boosting effect of recombinant xylanase B from the hyperthermophilic *Thermotoga maritima* on wheat straw pulp. *Applied Microbiology and Biotechnology* **70:** 65 – 71.

Johnson, K. G. 1990. Exocellular β-mannanases from hemicellulolytic fungi. *World Journal of Microbiology and Biotechnology* **6:** 209 – 217.

Jørgensen, H., Eriksson, T., Börjesson, J., Tjerneld, F. and Olsson, L. 2003. Purification and characterization of five cellulases and one xylanase from *Penicillium brasilianum* IBT 20888. *Enzyme and Microbial Technology* **32:** 851 – 861.

Joseleau, J. P., Comtat, J. and Ruel, K. 1992. Chemical structure of xylans and their interactions in the plant cell walls. In: *Xylans and xylanases* (Eds: Visser, J., Beldman, G., vanSomeren, M. A. K. and Voragen, A. G. J.) Elsevier Science Publishers, Amsterdam. pp. 1 – 15.

Kaji, A. 1984. α-L-arabinofuranosidase. *Advances in Carbohydrate Chemistry and Biochemistry* **42:** 383 – 394.

Kaji, A., Tagawa, K. and Matsubara, K. 1967. Studies on the enzymes action on araban. Part VIII. Purification and properties of arabanase produced by *Aspergillus niger*. *Agriculture Biological Chemistry* **31:** 1023 – 1028.

Kalogeris, E., Christakopoulos, P., Kekos, D. and Macris, B. J. 1998. Studies on solid-state production of thermostable endoxylanases from *Thermoascus aurantiacus*. Characterization of two isozymes. *Journal of Biotechnology* **60:** 155 – 163.

Kamal Kumar, B., Balakrishnan, H. and Rele, M. V. 2004. Compatibility of alkaline xylanases from an alkaliphilic *Bacillus* NCL (87-6-10) with commercial detergents and proteases. *Journal of Industrial Microbiology and Biotechnology* **31:** 83 – 87.

Kang, S. W., Kim, S. W. and Lee, J. S. 1995. Production of cellulase and xylanase in a bubble column using immobilized *Aspergillus niger* KKS. *Applied Biochemistry and Biotechnology* **53:** 101 – 106.

Kantelinen, A., Sundquist, J., Linko, M. and Viikari, L. 1991. The role of reprecipitated xylan in the enzymatic bleaching of kraft pulp. Proceedings: *6th International Symposium on Wood Chemistry and Technology* pp. 493 – 500.

Katapodis, P., Vardakou, M., Kalogeris, E., Kekos, D., Macris, B. J. and Christakopoulos, P. 2003. Enzymic production of a feruloylated oligosaccharide with antioxidant activity from wheat flour arabinoxylan. *European Journal of Nutrition* **42:** 55 – 60.

Kenealy W. R. and Jeffries, T. W. 2003. Enzyme process for pulp and paper: a review of recent developments. In: Wood deterioration and preservation: advances in our challenging world. American Chemical Society, Washington DC pp. 210 – 239.

Keskar, S. S. 1992. High activity xylanase from thermotolerant *Streptomyces* T7, cultural conditions and enzyme properties. *Biotechnology Letters* **14:** 481 – 486.

Keskar, S. S., Srinivasan, M. C. and Deshpande, V. V. 1989. Chemical modification of a xylanase from a thermotolerant *Streptomyces*. Evidence for essential tryptophan and cysteine residues at the active site. *Biochemical Journal* **261:** 49 – 55.

Khalil, A. I. 2002. Production and characterization of cellulolytic and xylanolytic enzymes from the ligninolytic white-rot fungus *Phanerochaete chrysosporium* grown on sugarcane bagasse. *World Journal of Microbiology and Biotechnology* **18:** 753 – 759.

Khasin, A., Alchanati, I. and Shoham, Y. 1993. Purification and characterization of a thermostable xylanase from *Bacillus stearothermophilus* T-6. *Applied and Environmental Microbiology* **59:** 1725 – 1730.

Khristova, P., Kordsachia, O., Pattb, R., Karar, I. and Khider, T. 2006. Environmentally friendly pulping and bleaching of bagasse. *Industrial Crops and Products* **23:** 131 – 139.

Kimura, L., Sasahara, H. and Tajima, S. 1995. Purification and characterization of two xylanases and an arabinofuranosidase from *A. sojae*. *Journal of Fermentation and Bioengineering* **80:** 334 – 339.

Kimura, T., Ito, J., Kawano, A., Makino, T., Kondo, H., Karita, S., Sakka, K. and Ohmiya, K. 2000. Purification, characterization, and molecular cloning of acidophilic xylanase from *Penicillium* sp. 40. *Bioscience Biotechnology and Biochemistry* **64:** 1230 – 1237.

Kirk, O., Borchet, T. V. and Fuglsang, C. C. 2002. Industrial enzyme applications. *Current Opinion in Biotechnology* **13:** 345 – 351.

Kitamoto, N., Yoshino, S., Ohmiya, K. and Tsukagoshi, N. 1999. Purification and characterization of the overexpressed *Aspergillus oryzae* xylanase, XynF1. *Bioscience Biotechnology and Biochemistry* **6310:** 1791 – 1794.

Kitpreechavanich, V., Hayashi, M. and Nagai, S. 1984. Production of xylan degrading enzymes by thermophilic fungi *A. fumigatus* and *Humicola lanuginosa. Journal of Fermentation Technology* **62:** 415 – 420.

Kolenová, K., Vršanská, M. and Biely, P. 2006. Mode of action of endo-β-1,4-xylanases of families 10 and 11 on acidic xylooligosaccharides. *Journal of Biotechnology* **12:** 338 – 345.

Kormelink, F. J. M., Leeuwen, M. G. F. S. L., Wood, T. M. and Voragen, A. G. J. 1993. Purification and characterization of three endo (1,4)-β-D-xylanases and one β-xylosidase from *Aspergillus awamori*. *Journal of Biotechnology* **27:** 249 – 253.

Kormelink, F. J. M., Gruppen, H., Viëtor, R. J. and Voragen, A. G. J. 1993. Mode of action of the xylan-degrading enzymes from *Aspergillus awamori* on alkali-extractable cereal arabinoxylans. *Carbohydrate Research* **249:** 355 – 367.

Kuhad, R. C. and Singh, A. 1993. Lignocellulose Biotechnology: current and future prospects. *Critical Review in Biotechnology* **13:** 151 – 172.

Kuhad, R. C., Manchanda, M. and Singh, A. 1998. Optimization of xylanase production by a hyper-xylanolytic mutant strain of *Fusarium oxysporum*. *Process Biochemistry* **33:** 641 – 647.

Kuhad, R. C., Singh, A. and Eriksson, K. E. L. 1997. Microorganisms and enzymes involved in the degradation of plant fibre cell wall. *Advances in Biochemical Engineering/Biotechnol*ogy **57:** 47 – 125.

Kulkarni, N. and Rao, M. 1996. Application of xylanase from alkaliphilic thermophilic *Bacillus* sp. NCIM 59 in biobleaching of bagasse pulp. *Journal of Biotechnology* **51:** 167 – 173.

Kulkarni, N., Shendye, A. and Rao, M. 1999. Molecular and biotechnological aspects of xylanases. *FEMS Microbiology Reviews* **23:** 411 – 456.

Kulminskaya, A. A., Eneiskaya, E. V., Isaeva-Ivanova, L. S., Savel'ev, A. N., Sidorenko, I. A., Shabalin, K. A., Golubev, A. M. and Neustroev, K. N. 1999. Enzymatic activity and β-galactomannan binding property of β-mannosidase from *Trichoderma reesei*. *Enzyme and Microbial Technology* **25:** 372 – 377.

Kumar, C. G. and Takagi, H. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnology Advances* **17:** 561 – 594.

Kumar, K. S., Manimaran, A., Permaul, K., and Singh, S. 2009. Production of β-xylanase by a *Thermomyces lanuginosus* MC134 mutant on corn cobs and its application in biobleaching of bagasse pulp. *Journal of Bioscience and Bioengineering* **107:** 494 – 498.

Kumar, P. R., Eswaramoorthy, S., Vithayathil, P. J. and Viswamitra, M. A. 2000. The tertiary structure at 1.59 Å resolution and the proposed amino acid sequence of a family-11 xylanase from the thermophilic fungus *Paecilomyces varioti bainier*. *Journal of Molecular Biology* **295:** 581 – 593.

La Grange, D. C., Claeyssens, M., Pretorius, I. S., and van-Zyl, W. H. 2000. Coexpression of the *Bacillus pumilus* beta-xylosidase (xynB) gene with the *Trichoderma reesei* beta-xylanase-2 (xyn2) gene in the yeast *Saccharomyces cerevisiae. Applied Microbiology and Biotechnology* **54:** 195 – 200.

Leathers, T. D., Detroy, R. W. and Bothast, R. J. 1986. Induction and glucose repression of xylanase from a color variant strain of *Aureobasidium pullulans. Biotechnology Letters* **8:** 867 – 872.

Leggio, L. L., Jenkins, J., Harris, G. W. and Pickersgill, R. W. 2000. X-ray crystallographic study of xylopentose binding to *Pseudomonas fluorescens* xylanase A. *Proteins: Structure, Function, and Genetics* **41:** 362 – 373.

Leggio, L. L., Kalogiannis, S., Bhat, M. K. and Pickersgill, R. W. 1999. High resolution structure and sequence of *T. aurantiacus* xylanase I: implications for the evolution of thermostability in family 10 xylanases and enzymes with (beta) alpha-barrel architecture. *Proteins* **36:** 295 – 306.

Lenartovicz, V., Souza, C. G. M., Moreira, F. G. and Peralta, R. M. 2002. Temperature effect in the production of multiple xylanases by *Aspergillus fumigatus*. *Journal of Basic Microbiology* **42:** 388 – 395.

Levin, L. and Forschiassin, F. 1998. Influence of growth conditions on the production of xylanolytic enzymes by *Trametes trogii*. *World Journal of Microbiology and Biotechnology* **14:** 443 – 446.

Li, K., Azadi, P., Collins, R., Tolan, J., Kim, J. S. and Eriksson, K. E. L. 2000. Relationships between activities of xylanases and xylan structures. *Enzyme and Microbial Technology* **27:** 89 – 94.

Li, X. L., Zhang, Z., Dean, I. F. D., Eriksson, K. L. and Ljungdahl, L. G. 1993. Purification and characterization of a new xylanase APX-II from the fungus *Aureobasidium pullulans* Y-2311-1. *Applied and Environmental Microbiology* **59:** 3212 – 3218.

Li, X. T., Jiang, Z. Q., Li, L. T., Yang, S. Q., Feng, W. Y., Fan, J. Y. and Kusakabe, I. 2005. Characterization of a cellulase-free, neutral xylanase from *Thermomyces lanuginosus* CBS 288.54 and its biobleaching effect on wheat straw pulp. *Bioresource Technology* **96:** 1370 – 1379.

Lin, J., Ndlovu, L. M., Singh, S. and Pillay, B. 1999. Purification and biochemical characteristics of β-D-xylanase from a thermophilic fungus, *Thermomyces lanuginosus*-SSBP. *Biotechnology and Applied Biochemistry* **30:** 73 – 79.

Lincoln, R.D. 1960. Control of stock culture preservation and inoculum build up in bacterial fermentation. *Journal of Biochemical and Microbiological Technology and Engineering* **2:** 481 – 500.

Liu, W., Lu, Y. and Ma, G.1999. Induction and glucose repression of endo- β-xylanase in the yeast *Trichosporon cutaneum* SL 409. *Process Biochemistry* **34:** 67 – 72.

Lopez, C., Blanco, A. and Pastor, F. I. J.1998. Xylanase production by a new alkali-tolerant isolate of *Bacillus*. *Biotechnology Letters* **20:** 243 – 246.

Lopez, M. J., Vargas-García, M. C., Suárez-Estrella, F., Nichols, N. N., Dien, B. S. and Moreno, J. 2007. Lignocellulose-degrading enzymes produced by the ascomycete *Coniochaeta ligniaria* and related species: Application for a lignocellulosic substrate treatment. *Enzyme and Microbial Technology* **40:** 794 – 800.

Lowry, O. H., Rosebrough, N. H., Farr, A. L. and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193:** 265 – 275.

Lundgren, K. R., Bergkvist, L., Hogman, S., Joves, H., Eriksson, G., Bartfai, T., Laan, J. V. D., Rosenberg, E. and Shoham, Y. 1994. TCF Mill Trial on softwood pulp with Korsnas thermostable and alkaline stable xylanase T6. *FEMS Microbiology Reviews* **13:** 365 – 368.

Luonteri, E., Beldman, G. and Tenkanen, M. 1998. Substrate specificities of *Aspergillus terreus* α-L-arabinofuranosidases. *Carbohydrate Polymer* **37:** 131 – 141.

Luthi, E., Jasmat, N. B. and Bergquist, P. L. 1990. Xylanase from the extremely thermophilic bacterium *Caldocellum saccharolyticum*: overexpression of the gene in *Escherichia coli* and characterization of the gene product. *Applied and Environmental Microbiology* **56:** 2677 – 2683.

Maalej, I., Belhaj, I., Masmoudi, F. N. and Belghith, H. 2008. Highly thermostable xylanase of the thermophilic fungus *Talaromyces thermophilus*: purification and characterization. *Applied Biochemistry and Biotechnology* **158:** 200 – 212.

Maalej, I., Guerfali, M., Gargouri, A. and Belghith, H. 2009. Production of xylo-oligosaccharides from agro-industrial residues using immobilized *Talaromyces thermophilus* xylanase. *Journal of Molecular Catalysis B-Enzymatic* **59:** 145 – 152.

MacCabe, A. P., Orejas, M., Tamayo, E. N., Villanueva, A. and Ramón, D. 2002. Improving extracellular production of food-use enzymes from *Aspergillus nidulans*. *Journal of Biotechnology* **96:** 43 – 54.

Madlala, A. M., Bissoon, S., Singh, S. and Christov, L. 2001. Xylanase induced reduction of chlorine dioxide consumption during elemental chlorine-free bleaching of different pulp types. *Biotechnology Letters* **23:** 345 – 351.

Maheshwari, R., Bharadwaj, G. and Bhat, M. K. 2000. Thermophilic Fungi: Their Physiology and Enzymes. *Microbiology and Molecular Biology Reviews* **64:** 461 – 488.

Maijala, P., Raudaskaski, M. and Viikari, L. 1995. Hemicellulolytic enzymes in P- and S- strains of *Heterobasidion annosum*. *Microbiology* **141:** 743 – 750.

Manimaran, A. and Vatsala, T. M. 2007. Biobleaching of banana fibre pulp using *Bacillus subtilis* CO1 xylanase produced from wheat bran under solid-state cultivation. *Journal of Industrial Microbiology and Biotechnology* **34:** 745 – 749.

Manimaran, A., Santhosh Kumar, K., Permaul, K. and Singh, S. 2009. Hyper production of cellulase-free xylanase by *Thermomyces lanuginosus* SSBP on bagasse pulp and its application in biobleaching. *Applied Microbiology and Biotechnology* **81:** 887 – 893.

Marraccini, P., Rogers, W. J., Allard, C., Andre, M. L., Caillet, V., Lacoste, N., Laussane, F. and Michaux, S. 2001. Molecular and biochemical characterization of endo-beta-mannanases from germinating coffee (*Coffea arabica*) grains. *Planta* **213:** 296 – 308.

Matsumura, S., Sakiyama, K. and Toshima, K. 1999. Preparation of octyl β-D-xylobioside and xyloside by xylanase catalyzed direct transglycosylation reaction of xylan and octanol. *Biotechnology Letters* **21:** 17 – 22.

Matsuo, M. and Yasui, T. 1984. Purification and some properties of β-xylosidase from *Trichoderma viride*. *Agricultural and Biological Chemistry* **48:** 1845 – 1852.

Matte, A. and Forsberg, C. W. 1992. Purification, characterization, and mode of action of endoxylanases 1 and 2 from *Fibrobacter succinogenes* S85. *Applied and Environmental Microbiology* **58:** 157 – 168.

McCarthy, A. A., Morris, D. D., Bergquist, P. L. and Baker, E. N. 2000. Structure of XynB, a highly thermostable beta-1,4- xylanase from *Dictyoglomus thermophilum* Rt46B.1, at 1.8 Å resolution. *Acta Crystallographica Section D: Biological Crystallography* **56:** 1367 – 1375.

McCleary, B. V. and Matheson, N. K. 1986. Enzymatic analysis of polysaccharide structure. *Advances in Carbohydrate Chemistry and Biochemistry* **44:** 147 – 176.

Mendicuti Castro, L. P., Trejo-Aguilar, B. A. and Aguilar Osorio, G. 1997. Thermostable xylanases produced at 37°C and 45°C by a thermotolerant *Aspergillus* strain. *FEMS Microbiology Letters* **146:** 97 – 102.

Merchant, R., Merchant, F. and Margaritis, A. 1988. Production of xylanase by the thermophilic fungus *Thielavia terrestris*. *Biotechnology Letters* **10:** 513 – 516.

Milagres, A. M. F. and Sales, R. M. 2001. Evaluating the basidiomycetes *Poria medula-panis* and *Wolfporia cocos* for xylanase production. *Enzyme and Microbial Technology* **28:** 522 – 526.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* **31:** 426 – 428.

Monti, R., Terenzi, H. F. and Jorge, J. A. 1991. Purification and properties of an extracellular xylanase from thermophilic fungus *Humicola grisea* var. *thermoidea*. *Canadian Journal of Microbiology* **37:** 675 – 681.

Moon, S. H. and Parulekar, S. J. 1991. A parametric study of protein production in batch and fed-batch culture of *Bacillus firmus*. *Biotechnology and Bioengineering* **37:** 467 – 483.

Morris, D. D., Gibbs, M. D., Ford, M., Thomas, J. and Bergquist, P. L. 1999. Family 10 and 11 xylanase genes from *Caldicellulosiruptor* sp. strain Rt69B.1. *Extremophiles* **3:** 103 – 111.

Niehaus, F., Bertoldo, C., Kähler, M. and Antranikian, G. 1999. Extremophiles as a source of novel enzymes for industrial application. *Applied Microbiology and Biotechnology* **51:** 711 – 729.

Ninawe, S. and Kuhad, R. C. 2005. Use of xylan-rich cost effective agro-residues in the production of xylanase by *Streptomyces cyaneus* SN32. *Journal of Applied Microbiology* **99:** 1141 – 1148.

Nunn, J. R., Parolis, H. and Russel, I. 1973. Polysaccharides of the red algae *Chaetangium erinaceum* Part I: Isolation and characterization of the water- soluble xylan. *Carbohydrate Research* **26:** 169 – 180.

Nurizzo, D., Nagy, T., Gilbert, H. J. and Davies, G. J. 2002.The structural basis for catalysis and specificity of the *Pseudomonas cellulosa* α-glucuronidase, GlcA67A. *Structure* **10:** 547 – 556.

Oakley, A. J., Heinrich, T., Thompson, C. A. and Wilce, M. C. 2003. Characterization of family 11 xylanase from *Bacillus subtilis* B230 used for bleaching. *Acta Crystallographica D Biological Crystallographica* **59:** 627 – 636.

Olson, L. and Hahn-Hagerdal, B. 1996. Fermentation of lignocellulosic hydrolysates for ethanol production. *Enzyme and Microbial Technology* **18:** 312 – 331.

Onysko, K. A. 1993. Biological bleaching of chemical pulps- A review. *Biotechnology Advances* **11:** 179 – 198.

Papagianni, M. 2004. Fungal morphology and metabolite production in submerged mycelia processes. *Biotechnology Advances* **22:** 189 – 259.

Park, Y. S., Kang, S. W., Lee, J. S., Hong, S. I. and Kim, S. W. 2002. Xylanase production in solid state fermentation by *Aspergillus niger* mutant using statistical designs. *Applied Microbiology and Biotechnology* **58:** 761 – 766.

Parker, K. N., Chhabra, S., Lam, D., Snead, M. A., Mathur, E. J. and Kelly, R. M. 2001. β-Mannosidase from *Thermotoga* species. *Methods in Enzymology* **330:** 238 – 246.

Piñaga, F., Fernández-Espinar, M. T., Vallés, S. and Ramón, D. 1994. Xylanase production in *Aspergillus nidulans*: Induction and carbon catabolite repression. *FEMS Microbiology Letters* **115:** 319 – 324.

Piñaga, F., Pena, L. J. and Vallés, S. 1993. Xylanase production by *Bacillus polymyxa*. *Journal of Chemical Technology and Biotechnology* **57:** 327 – 333.

Polizeli, M. L. T. M., Rizzatti, A. C. S., Monti, R., Terenzi, H. F., Jorge, J. A. and Amorim, D. S. 2005. Xylanases from fungi: properties and industrial applications. *Applied Microbiology and Biotechnology* **67:** 577 – 591.

Poppius, K., Hortling, B. and Sundquist, J. 1989. Chlorine-free bleaching of chemical pulps- The potential of organic peroxyacids. In: Proceedings of the International Pulp and Bleaching Chemistry, Raleigh, NC. pp. 145 – 150.

Poutanen, K., Ratto, M., Puls, J., and Viikari, L. 1987. Evaluation of different microbial xylanolytic systems. *Journal of Biotechnology* **6:** 49 – 60.

Prade, R. A. 1996. Xylanases: from biology to biotechnology. *Biotechnology and Genetic Engineering Reviews* **13:** 100 – 131.

Puchart, V. and Biely, P. 2008. Simultaneous production of *endo*-β-1,4-xylanase and branched xylooligosaccharides by *Thermomyces lanuginosus. Journal of Biotechnology* **137:** 34 – 43.

Puchart, V., Katapodis, P., Biely, P., Kremnicky, L., Christakopoulos, P., Vrsanska, M., Kekos, D., Marcis, B. J. and Bhat, M. K. 1999. Production of xylanases, mannanases, and pectinases by the thermophilic fungus *Thermomyces lanuginosus*. *Enzyme and Microbial Technology* **24:** 355 – 361.

Puchart, V., Vrsanska, M., Svoboda, P., Pohl, J. and Biely, P. 2004. Purification and characterization of two forms of endo-β-1, 4-mannanase from the thermotolerant fungus, *Aspergillus fumigatus* IMI 385708 (formerly *T. lanuginosus* IMI 158749) *Biochemica et Biophysica Acta* **1674:** 239 – 250.

Puls, J. 1997. Chemistry and biochemistry of hemicelluloses: Relationship between hemicellulose structure and enzymes required for hydrolysis. *Macromolecular Symposia* **120** (Functional Polysaccharides II), 183 – 196.

Puls, J. and Schuseil, J. 1993. Chemistry of hemicelluloses: relationship between hemicellulose structure and enzyme required for hydrolysis. In: *Hemicellulose and hemicellulases* (Eds: Coughlan, M. P. and Hazlewood, G. P.) Portland Press, London pp.1 – 28.

Puls, J., Schmidt, O. and Granzow, C. 1987. Glucuronidase in two microbial xylanolytic systems. *Enzyme and Microbial Technology* **9:** 83 – 88.

Purkarthofer, H. and Steiner, W. 1995. Induction of endo-β-xylanase in the fungus *Thermomyces lanuginosus*. *Enzyme and Microbial Technology* **17:** 114 – 118.

Purkarthofer, H. and Steiner, W. 1995**.** Induction of endo-β-xylanase in the fungus *Thermomyces lanuginosus. Enzyme and Microbial Technology* **17:** 114 – 118.

Purkarthofer, H., Sinner, M. and Steiner, W. 1993a. Cellulase-free xylanase from *Thermomyces lanuginosus*: optimization of production in submerged and solid state culture. *Enzyme and Microbial Technology* **15:** 677 – 681.

Purkarthofer, H., Sinner, M. and Steiner, W. 1993b. Effect of shear rate and culture pH on the production of xylanase by *Thermomyces lanuginosus*. *Biotechnology Letters* **15:** 405 – 410.

Qinnghe, C., Xiaoyu, Y., Tiangui, N., Cheng, J. and Qiugang, M. 2004. The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*. *Process Biochemistry* **39:** 1561 – 1566.

Qy, Y., Gao, P., Wang, D., Zhao, X. and Zhang, X. 1996. Production, characterization and application of the cellulase-free xylanase from *Aspergillus niger*. *Applied Biochemistry and Biotechnology* **57:** 375 – 381.

Ragauskas, A. J., Poll, K. N. and Cesternino, A. 1994. Effects of xylanases pretreatment procedures on non-chlorine bleaching. *Enzyme and Microbial Technology* **16:** 492 – 495.

Raj, K. C. and Chandra, T. S. 1995. A cellulase-free xylanase from alkali-tolerant *Aspergillus fischeri* Fxn1. *Biotechnology Letters* **17:** 309 – 314.

Raj, K. C. and Chandra, T. S. 1996. Purification and characterization of xylanase from alkali-tolerant *Aspergillus fischeri* Fxn1. *FEMS Microbiology Letters* **1453:** 457 – 461.

Rajaram, S. and Varma, A. 1990. Production and characterisation of xylanase from *Bacillus thermoalkalophilus* grown on agricultural wastes. *Applied Microbiology and Biotechnology* **34:** 141 – 144.

Rajoka, M. I. 2004. Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena*. *Electronic Journal of Biotechnology* **7:** 259 – 266.

Rajoka, M. I. and Malik, K. A. 1984. Cellulase and hemicellulase production by *Cellulomonas flavigena* NIAB 441. *Biotechnology Letters* **6:** 597 – 600.

Ratto, M., Poutanen, K. and Viikari, L. 1992. Production of xylanolytic enzymes by an alkalitolerant *Bacillus circulans* strain. *Applied Microbiology and Biotechnology* **37:** 470 – 473.

Reen, F. J., Murray, P. G. and Tuohy, M. G. 2003. Molecular characterisation and expression analysis of the first hemicellulase gene (bxll) encoding β-xylosidase from the thermophilic fungus *Talaromyces emersonii*. *Biochemical and Biophysical Research Communications* **305:** 579 – 585.

Rials, T. G. and Wolcott, M. C. 1997. *Physical and mechanical properties of agro based fibres*. In: Paper and composites from agro-based resources. (Eds: Rowell, R. M., Young, R. A. and Rowell, J. K.) Lewis, Boca Raton pp. 63 – 82.

Rizzatti, A. C. S., Sandrim, V. C., Jorge, J. A., Terenzi, H. F. and Polizeli, M. L. T. M. 2004. Influence of temperature on the properties of xylanolytic enzymes of the thermotolerant fungus *Aspergillus phoenicis*. *Journal of Industrial Microbiology and Biotechnology* **31:** 88 – 93.

Rombouts, F. M., Voragen, A. G. J., Searl-van Leeuwen, M. F., Geraeds, C. C. J. M., Schols, H. A. and Pilnik, W. 1988. The arabinanases of *Aspergillus niger*– Purification and characterisation of two α-L-arabinofuranosidases and an endo-1,5-α-L-arabinase. *Carbohydrate Polymer* **9:** 25 – 47.

Roncero, M. B., Torres, A. L., Colom, J. F. and Vidal, T. 2003. TCF bleaching of wheat straw pulp using ozone and xylanase. Part A: paper quality assessment. *Bioresource Technology* **87:** 305 – 314.

Royer, J. C. and Nakas, J. P. 1989. Xylanase production by *Trichoderma longibrachiatum. Enzyme and Microbial Technology* **11:** 405 – 410.

Rubinder, K., Chadha, B. S., Singh, N., Saini, H. S. and Singh, S. 2002. Amylase hyperproduction by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus. Journal of Industrial Microbiology and Biotechnology* **29:** 70 – 74.

Ryan, S. E., Nolan, K., Thompson, R., Gubitz, G. M., Savage, A. V. and Tuohy, M. G. 2003. Purification and characterization of a new low molecular weight endoxylanase from *Penicillium capsularium*. *Enzyme and Microbial Technology* **33:** 775 – 785.

Sachslehner, A., Nidetzky, B., Kulbe, K. D. and Haltrich, D. 1998. Induction of mannanase, xylanase and endoglucanase activities in *Sclerotium rolfsii*. *Applied and Environmental Microbiology* **64:** 594 – 600.

Saddler, J. N., Hogan, C. M. and Louis-Seize, G. 1985. A comparison between the cellulase systems of *Trichoderma harzianum* E58 and *Trichoderma reesei* C30. *Applied Microbiology and Biotechnology* **22:** 139 – 145.

Saha, B. C. 2000. α-L-Arabinofuranosidases: biochemistry, molecular biology and application in biotechnology. *Biotechnology Advances* **18:** 403 – 423.

Saier, M. H. Jr. and Fagan, M. J. 1992. Catabolic repression In: *Encyclopaedia of Microbiology*, Vol.1 (Ed: Lederberg, J.) Academic Press, San Diego, CA pp. 431 – 442.

Salles, B. C., Cunha, R. B., Fontes, W., Sousa, M. V. and Filho, E. X. F. 2000. Purification and characterization of a new xylanase from *Acrophialophora nainiana*. *Journal of Biotechnology* **81:** 199 – 204.

Sánchez, C. 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. *Biotechnology Advances* **27:** 185 – 194.

Santos, E. D., Piovan, T., Roberto, I. C. and Milagres, F. A. M. 2003. Kinetics of the solid state fermentation of sugarcane bagasse by *Thermoascus aurantiacus* for the production of xylanase. *Biotechnology Letters* **25:** 13 – 16.

Satyanarayana, T., Johri, B. N. and Klein, J. 1992. Biotechnological potential of thermophilic fungi. In: Handbook of Applied Mycology, Vol. 4, Fungal Biotechnology (Eds: Arora, D. K., Elander, R. P. and Mukerji, K.G.) Marcel Dekker Inc., New York pp. 729 – 761.

Saxena, S., Bahadur, J. and Varma, A. 1991. Production and localisation of carboxymethylcellulase xylanase and L-glucosidase from *Cellulomonas* and *Micrococcus* spp *Applied Microbiology and Biotechnology* **34:** 668 – 670.

Schlacher, A., Holzmann, K., Hayn, M., Steiner, W. and Schwab, H. 1996. Cloning and characterization of the gene for the thermostable xylanase XynA from *Thermomyces lanuginosus*. *Journal of Biotechnology* **49:** 211 – 218.

Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M. and Witholt, B. 2001. Industrial biocatalysis today and tomorrow. *Nature* **409:** 258 – 268.

Schroder, R., Wegrzyn, T. F., Bolitho, K. M. and Redgwell, R. J. 2004. Mannan transglycolase: A novel enzyme activity in cell walls of higher plants. *Planta* **219:** 590 – 600.

Scott, B. P., Young, F. and Paice, M. G. 1993. Mill-scale enzyme treatment of a softwood kraft pulp prior to bleaching. *Pulp and Paper Canada* **94:** 57 – 61.

Segura, B. G. D., Durand, R. and Fevre, M. 1998. Multiplicity and expression of xylanases in the rumen fungus *Neocallimastix frontalis*. *FEMS Microbiology Letters* **164:** 47 – 53.

Senior, D. J., Mayers, P. R., Breuil, C. and Saddler, J. N. 1990. The interaction of xylanase with pulps: Non-selective adsorption and inactivation of xylanase. In: Biotchnology in Pulp and Paper Manufacture (Eds: Kirk, T. K. and Chang, H. M.) Butterworth-Heinemann, Boston pp. 169 – 182.

Shabalin, K. A., Kulminskaya, A. A., Savel, A. N., Shishlyannikov, S. M. and Neustroev, K. N. 2002. Enzymatic properties of α-galactosidase from *Trichoderma reeei* in the hydrolysis of galacto-oligosaccharides. *Enzyme and Microbial Technology* **30:** 231 – 239.

Shallom, D. and Shoham, Y. 2003. Microbial hemicellulases. *Current Opinion in Microbiology* **6:** 219 – 228.

Shoemaker, G. K., Juers, D. H., Coombs, J. M. L., Matthews, B. W. and Craig, D. B. 2003. Crystallization of β- galactosidase does not reduce the range of activity of individual molecules. *Biochemistry* **42:** 1707 – 1710.

Shoham, Y., Schwartz, Z., Khasin, A., Gat, O., Zosim, Z. and Rosenberg, E. 1992. Delignification of wood pulp by a thermostable xylanase from *Bacillus stearothermophilus* strain T-6. *Biodegradation* **3:** 207 – 218.

Siedenberg, D., Gerlach, S. R., Schugerl, K., Giuseppin, M. L. F. and Hunik, J. 1998. Production of xylanase by *Aspergillus awamori* on synthetic medium in shake flask cultures. *Process Biochemistry* **33:** 429 – 433.

Silva, C. H. C., Puls, J., de Sousa, M. V. and Ferreira-Filho, E. X. 1999. Purification and characterization of a low molecular weight xylanase from solid-state cultures of *Aspergillus fumigatus* Fresenius. *Revista de Microbiologia* **30:** 114 – 119.

Simão, R. C. G., Souza, C. G. M. and Peralta, R. M. 1997. Induction of xylanase in *Aspergillus tamarii* by methyl β-D-xyloside. *Applied Microbiology and Biotechnology* **47:** 267 – 271.

Simpson, H. D., Haufler, U. R. and Daniel, R. M. 1991. An extremely thermostable xylanase from the thermophilic eubacterium *Thermotoga*. *Biochemistry Journal* **277:** 413 – 417.

Singh, S., Madlala, A. M. and Prior, B. A. 2003. *Thermomyces lanuginosus*: properties of strains and their hemicellulases. *FEMS Microbiology Reviews* **27:** 3 – 16.

Singh, S., Pillay, B., Dilsook, V. and Prior, B. A. 2000a. Production and properties of hemicellulases by a *Thermomyces lanuginosus* strain. *Journal of Applied Microbiol*ogy **88:** 975 – 982.

Singh, S., Pillay, B. and Prior, B. A. 2000b. Thermal stability of β-xylanases produced by different *Thermomyces lanuginosus* strains. *Enzyme and Microbial Technology* **26:** 502 – 508.

Sinha, N. and Sengupta, S. 1995. Simultaneous production of α-arabinofuranosidase and xylanase by *Termitomyces clypeatus*. *World Journal of Microbiology and Biotechnology* **11:** 359 – 360.

Sjöström, E. 1981. In: Wood chemistry, Fundamentals and Applications. New York Academic Press, Inc. 223 p.

Smith, D. C. and T. M. **Wood.** 1991. Xylanase production by *Aspergillus awamori*. Development of a medium and optimization of the fermentation parameters for the production of extracellular xylanase and β-xylosidase while maintaining low protease production. *Biotechnology and Bioengineering* **38:** 883 – 890.

Smith, J. E. 1996. Substrates for Biotechnology In: *Biotechnology*. 3rd ed. Cambridge University Press, Cambridge pp. 19 – 30.

Steiner, W., Lafferty, R. M., Gomes, I. and Esterbauer, H. 1987. Studies on a wild strain of *Schizophyllum commune*: Cellulase and xylanase production and formation of the extracellular polysaccharide Schizophyllan. *Biotechnology and Bioengineering* **30:** 169 – 178.

Subramaniyan, S. and Prema, P. 2000. Cellulase-free xylanases from *Bacillus* and other microorganisms. *FEMS Microbiology Letters* **183:** 1 – 7.

Subramaniyan, S. and Prema, P. 2002. Biotechnology of microbial xylanases: enzymology, molecular biology, and application. *Critical Reviews in Biotechnology* **22:** 33 – 64.

Sunna, A. and Antranikian, G. 1997. Xylanolytic enzymes from fungi and bacteria. *Critical Reviews in Biotechnology* **17:** 39 – 67.

Sunna, A. and Bergquist, P. L. 2003. A gene encoding a novel extremely thermostable1,4-beta-xylanase isolated directly from an environmental DNA sample. *Extremophiles* **7:** 63 – 70.

Sunna, A., Moracci, M., Rossi, M. and Antranikian, G. 1997. Glycosyl hydrolases from hyperthermophiles. *Extremophiles* **1:** 2 – 13.

Suurnä**kki, A., Tenkanen, M., Buchert, J. and Viikari, L.** 1997. Hemicellulases in the bleaching of chemical pulps. *Advances in Biochemical Engineering/ Biotechnology* **57:** 261 – 287.

Szendefy, J., Szakacs, G. and Christopher, L. 2006. Potential of solid-state fermentation enzymes of *Aspergillus oryzae* in biobleaching of paper pulp. *Enzyme and Microbial Technology* **39:** 1354 – 1360.

Tan, L. U. L., Wong, K. K. Y., Yu, E. K. C. and Saddler, J. N. 1985. Purification and characterization of two D-xylanases from *Trichoderma harzianum*. *Enzyme and Microbial Technology* **7:** 425 – 430.

Taneja, K., Gupta, S. and Kuhad, R. C. 2002. Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspergillus nidulans* KK-99. *Bioresource Technology* **85:** 39 – 42.

TAPPI Test Methods. 1996. Technical association of the pulp and paper industry, TAPPI, Atlanta.

Tikhomirov, D. F., Sinitsyn, A. P., Zorov, I. N. and Williams, C. 2003. Non-starch polysaccharide hydrolysing microbial enzymes in grain processing In: Recent Advances in Enzymes in Grain Processing (Eds: Courtin, C. M., Veraverbeke, W. S. and Delcour, J. A.) Katholieke University, Leuven pp. 413 – 418.

Tolan, J. S. and Canovas, R. V. 1992. The use of enzymes to decrease the Cl_2 requirements in pulp bleaching. *Pulp and Paper Canada* **93:** 39 – 42.

Törrönen, A., Harkki, A. and Rouvinen, J. 1994. Three-dimensional structure of endo-β-1,4-xylanase II from *Trichoderma reesei*: two conformational states in the active site. *EMBO Journal* **13:** 2493 – 2501.

Tremblay, L. and Archibald, F. 1993. Production of a cloned xylanase in *Bacillus cereus* and its performance in kraft pulp prebleaching. *Canadian Journal of Microbiology* **39:** 853 – 860.

Tsujibo, H., Ohtsuki, T., Iio, T., Yamazaki, I., Miyamoto, K., Sugiyama, M. and Inamori, Y. 1997. Cloning and sequence analysis of genes encoding xylanases and acetyl xylan esterases from *Streptomyces* OPC-520. *Applied and Environmental Microbiology* **63:** 661 – 664.

Tuohy, M., Coughlan, T. L. and Coughlan, M. P. 1990. Solid-state versus liquid cultivation of *Talaromycs emersonii* on straw and pulps: enzyme productivity. In: Advances in Biological Treatment of Lignocellulosic Materials (Eds: Coughlan, M. P. and Amaral Collaco, M. T.) Elsevier Applied Science, London pp.153 – 175.

Turner, P., Mamo, G. and Karlsson, E. N. 2007. Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories* **6:** 1 – 23.

Turunen, O., Etuaho, K., Fenel, F., Vehmaanperä, J., Wu, X., Rouvinen, J. and Leisola, M. 2001. A combination of weakly stabilizing mutations with a disulfide bridge in the α-helix region of *Trichoderma reesei* endo-1,4-β-xylanase II increases the thermal stability through synergism. *Journal of Biotechnology* **88:** 37 – 46.

Twomey, L. N., Pluske, J. R., Rowe, J. B., Choct, M., Brown, W., McConnell, M. F. and Pethick, D. W. 2003. The effects of increasing levels of soluble non-starch polysaccharides and inclusion of feed enzymes in dog diets on faecal quality and digestibility. *Animal Feed Science and Technology* **108:** 71 – 82.

Uffen, R. L. 1997. Xylan degradation: a glimpse at microbial diversity. *Journal of Industrial Microbiology and Biotechnology* **19:** 1 – 6.

van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. and Viseer, J. 1991. Induction, purification and characterization of arabinases produced by *Aspergillus niger. Archives of Microbiology* **157:** 23 – 28.

Vieille, C. and Zeikus, G. J. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiology and Molecular Biology Reviews* **65:** 1 – 43.

Viikari, L., Kantelinen, A., Poutanen, K. and Ranua, M. 1990. Characterization of pulps treated with hemicellulolytic enzymes prior to bleaching. In: Biotechnology in Pulp and Paper Manufacture (Eds: Kirk, T. K. and Chang, H. M.) Butterworth Heinemann, Boston, M A. p.145.

Viikari, L., Kantelinen, A., Sundquist, J. and Linko, M. 1994. Xylanases in bleaching: from an idea to the industry. *FEMS Microbiology Reviews* **13:** 335 – 350.

Viikari, L., Ranua, M., Kantelinen, A., Sundiquist, J. and Linko, M. 1986. Bleaching with enzymes. Proceedings: 3rd International Conference on Biotechnology in the Pulp and Paper Industry. Stockholm pp. 67 – 69.

Viikari, L., Tenkanen, M., Buchert, J., Rättö, M., Bailey, M., Saiikaho, M. and Linko, M. 1993. Hemicellulases for Industrial Applications. In: Bioconversion of Forest and Agricultural Plant Residues (Ed: Saddler, J. N.) CAB International, Wallingford, United Kingdom **9:** 131 – 182.

Viikari, L., Tenkanen, M., Rättö, M., Buchert, J. Kantelinen, A., Bailey, M., Sundquist, J. and Linko, M. 1992. Important properties of xylanases for use in the pulp and paper industry. Proceedings: 5th International Conference on Biotechnology in Pulp and Paper Industry (Eds: Kuwahara, M. and Shimada, M.) Uni Publishers Co. Ltd., Tokyo, Japan pp. 101 – 106.

Wang, P., Ali, S., Mason, J. C., Sims, P. F. G. and Broda, P. 1992. Xylanases from *Streptomyces cyaneus*. In: Xylans and Xylanases (Eds: Visser, J., Beldman, G., Kusters-van Someren, M. A. and Voragen, A. G. J.) Elsevier Science Publishers, Amsterdam pp. 225 – 234.

Whistler, R. and Masek, E. 1955. Enzymatic hydrolysis of xylan. *Journal of the American Chemical Society* **77:** 1241 – 1243.

Wiegel, J., Mothershed, C. P. and Puls, J. 1985. Differences in xylan degradation by various noncellulolytic thermophilic anaerobes and *Clostridium thermocellum. Applied and Environmental Microbiology* **49:** 656 – 659.

Wong, K. K. Y. and Maringer, U. 1999. Substrate hydrolysis by combinations *Trichoderma* xylanases. *World Journal of Microbiology and Biotechnology* **15:** 23 – 26.

Wong, K. K. Y., Tan, L. U. L. and Saddler, J. N. 1988. Multiplicity of β-1, 4-xylanase in microorganisms: functions and applications. *Microbiology Reviews* **52:** 305 – 317.

Xu, J., Nogawa, M., Okada, H. and Morikawa, Y. 1998. Xylanase induction by L-sorbose in a fungus *Trichoderma reesei* PC-3–7. *Bioscience Biotechnology and Biochemistry* **62:** 1555 – 1559.

Xu, Z., Bai, Y., Xu, X., Shi, J. and Tao, W. 2005. Production of alkali-tolerant cellulase-free xylanase by *Pseudomonaas* sp. WLUN024 with wheat bran as the main substrate. *World Journal of Microbiology and Biotechnology* **21:** 575 – 581.

Yamamoto, K., Li, S. C. and Li, Y. T. 2000. Microbial glycosidases. In: Carbohydrates in Chemistry and Biology (Eds: Ernst, B., Hart, G.W. and Sinay, P.) Wiley-VCH, Weinheim, Germany pp. 497 – 511.

Yamaura, I., Nozaki, Y., Matsumoto, T. and Kato, T. 1996. Purification and some properties of an *endo*-1,4-β-D-mannanase from a marine mollusc *Littorina brevicula. Bioscience Biotechnology and Biochemistry* **60:** 674 – 676.

Yamura, I., Koga, T. and Matsumoto, T. 1997. Purification and some properties of endo-1,4-β xylanase from a fresh water mollusk *Pomacea insularus* (de Oringny). *Bioscience Biotechnology and Biochemistry* **61:** 615 – 620.

Yang, J. Y., Lou, G. and Ericksson, K. E. 1992. The impact of xylanase on bleaching of kraft pulps. *Tappi Journal* **75:** 95 – 101.

Zhang, Q., Yan, X., Zhang, L. and Tang, W. 2006. Cloning, sequence analysis and heterologus expression of a β-mannanase gene from *Bacillus subtilis* Z-2. *Molecular Biology* **40:** 368 – 374.

Zhao, Y., Chany II, C. J., Sims, P. F. G., and Sinnott, M. L. 1997. Definition of the substrate specificity of the 'sensing' xylanase of *Streptomyces cyaneus* using xylooligosaccharide and cellooligsaccharide glycosides of 3,4-dinitrophenol. *Journal of Biotechnol*ogy **57:** 181 – 190.

Ziboro, G. C. 1990. Origin and nature of Kraft color 1. Role of aromatics. *Journal of Wood Chemistry and Technology* **10:** 133 – 149.

Zverlov, V., Piotukh, K., Dakhova, O., Velikodvorskaya, G. and Borriss, R. 1996. The multidomain xylanase A of the hyperthermophilic bacterium *Thermotoga neapolitana* is extremely thermoresistant. *Applied Microbiology and Biotechnology* **45:** 245 – 247.

Zychlinska, A. W., Czakaj, J., Jedrychowska, B. and Zukowska, R. S. 1992. Production of xylanases by *Cheatomium globosum*. In: Xylans and Xylanases. Progress in Biotechnology, Vol. 7 (Eds: Visser, J., Beldman, G., Kursters-van Someran, M. A. and Voragen, A. G. J.) Elsevier Science Publishers, Amsterdam. pp. 493 – 496.