PREPARATION AND CHARACTERIZATION OF XYLO-OLIGOSACCHARIDES FROM OIL PALM FROND (*ELAEIS GUINEENSIS*)

by

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Thesis submitted in fulfilment of the requirements for the degree of Master of Science

JULY 2011

ACKNOWLEDGEMENT

Throughout this long journey, I have gained a lot by learning to persevere despite hardship. I am grateful for all of the support and contribution along this journey. I would never have successfully completed this thesis without the assistance of numerous people who I am indebted to.

First and foremost, all praises to Allah S.W.T for who bestowed me the knowledge, health and strength to complete this Msc. research. I would like to express my deep and sincere gratitude to my supervisor, Dr. Fazilah Bt. Arifin for her understanding, encouragement, personal guidance and wide knowledge throughout this study. Thank you Dr.

I wish to express my warm and sincere thanks to Prof. Mohd Azemi Mohd Noor as my field supervisor for his constructive ideas and excellent advice during the preparation of this thesis.

This thesis would not have been possible without the help and support of my colleagues and technicians in Malaysian Institute of Chemical and Bioengineering Technology (MICET), UniKL for helping me get through the difficult times, and for the entertainment and caring they provided. Special thanks to my dearest friend, Rinani Shima, for the shared knowledge and friendship which has been invaluable on both an academic and a personal level, for which I am extremely grateful.

I would also like to acknowledge the research grant I received from UniKL that provided the necessary financial support for this research.

My sincerest thanks and appreciation goes to my parents, Hussin Mohd Zain and Fuziyah Hj Hasbi, for their prayers and emotional support to keep me going.

I owe my loving thanks to my husband, Fitri, for his patience and always telling me to be positive in doing my work. I wish to give my heartfelt thanks to my beloved son, Ariff, who put a smile on my face whenever I was feeling down. As time passes by, you have grown up watching me study and juggle with family and work. I hope I have been a good mother and that I have not lost too much during the tenure of my study. You are the best thing that's ever been mine.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.

FARAH SALINA BINTI HUSSIN

JULY, 2011

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LIST OF ABBREVIATION

XOS	Xylo-oligosaccharides
OPF	Oil palm fronds
T.viride	*Trichoderma viride*
T.lanuginosus	*Thermomyces lanuginosus*
XOS_{TV}	Xylo-oligosaccharides produced by *Trichoderma* viride xylanase
XOS_{TL}	Xylo-oligosaccharides produced by *Thermomyces lanuginosus* xylanase
NaOH	Sodium hydroxide
KOH	Potassium hydroxide
$Ca(OH)^2$	Calcium hydroxide
DP	Degree of polymerization
FOS	Fructo-oligosaccharides
SCFA	Short chain fatty acid
HPLC	High performance liquid chromatography
NDO	Non-digestible oligosaccharide
FOSHU	Food for specified health use
WHC	Water holding capacity
OBC	Oil binding capacity
MPOB	Malaysian Palm Oil Board
CH_3COOH	Acetic acid
KH_2PO_4	Potassium dihydrogen phosphate
AOAC	Official methods of analysis
AAS	Atomic Absorption Spectrophotometer

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PENYEDIAAN DAN PENCIRIAN XILO-OLIGOSAKARIDA DARI PELEPAH KELAPA SAWIT *(ELAEIS GUINEENSIS)*

ABSTRAK

Projek ini dijalankan untuk mengkaji faktor-faktor relevan bagi hidrolisis enzim ke atas bahan mentah lignosellulosa dalam pelepah kelapa sawit (PKS). Dua enzim xilanase digunakan ; *Trichoderma viride* (xilanase *T.Viride*) dan *Thermomyces lanuginosus* (xilanase *T.lanuginosus)* pada pH optima masing-masing pH 5.5 dan pH 6.5. Kajian utama penyelidikan ini adalah pemencilan xilo-oligosakarida (XOS) daripada hemiselulosa selepas proses delignifikasi melalui kaedah hidrolisis enzim. Proses delignifikasi PKS dijalankan melalui kaedah pra-rawatan peroksida alkali pada suhu 80 ^{o}C dan diikuti dengan pengekstrakan hemiselulosa menggunakan kaedah pengekstrakan alkali. Keputusan menunjukkan kandungan Klason lignin menurun dari 24.17 % (PKS) ke 15.25 % (hemiselulosa kasar) dan 1.28 % (hemiselulosa selepas delignifikasi). Penghasilan xilo-oligosakarida yang tertinggi ditentukan dengan membandingkan kondisi optima kedua-dua enzim ini dari segi suhu (40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C dan 60 ^{o}C), masa (10 jam tempoh pengeraman), kepekatan enzim (0.05 U/mL, 1 U/mL, 2 U/mL, 3 U/mL, 4 U/mL dan 5 U/mL) dan juga kepekatan substrat (5 %, 10 %, 15 %, 20 % dan 25 % w/v). Xilanase *T.Viride* menghasilkan XOS dengan maksimum sebanyak 57.6 % (2.42 g/kg xylotriose dan 3.34 g/kg xylobiose) pada jam ketujuh, suhu 50 ^{o}C, 4 U/mL enzim dan 10 % b/i substrat. Manakala xilanase *T.lanuginosus* menghasilkan XOS dengan maksimum sebanyak 27.0 % (2.44 g/kg xylotriose dan 2.95 g/kg xylobiose) pada jam ketiga, suhu 55 ^{o}C, 1 U/mL enzim dan 20 % b/i substrat. Data kapasiti pengikatan air oleh XOS_{TL} menunjukkan nilai yang lebih tinggi dan signifikan berbanding XOS_{TV}. Selain itu, XOS_{TL} mempunyai kapasiti pengikatan minyak yang lebih tinggi

berbanding $XOS_{TV.}$ Data menunjukkan kapasiti pengikatan kalsium dan magnesium bagi XOS daripada kedua-dua enzim meningkat apabila pH larutan ditingkatkan. Data analisis keterlarutan menunjukkan XOS_{TV} lebih larut berbanding XOS_{TL} iaitu sebanyak 88.70 %.

PREPARATION AND CHARACTERIZATION OF XYLO-OLIGOSACCHARIDES FROM OIL PALM FROND *(ELAEIS GUINEENSIS)*

ABSTRACT

This study focused on factors relevant for enzymatic hydrolysis of lignocellulosic raw materials derived from oil palm frond (OPF). Two origins of xylanase enzyme were used ; *Trichoderma viride* (*T.viride* xylanase) and *Thermomyces lanuginosus* (*T.lanuginosus* xylanase) at its own optimum pH 5.5 and pH 6.5. The major interest of this work is the isolation of xylo-oligosaccharides (XOS) from delignified hemicellulose by using enzymatic hydrolysis method. Delignification of OPF was done by alkaline peroxide treatment at 80 ^{o}C and the extraction of hemicellulose by using alkaline extraction method. The results of Klason lignin showed a reduction of lignin content from 24.17 % in OPF samples to 15.25 % in crude hemicellulose and 1.28 % in delignified hemicellulose. The maximum yield of XOS was determined by comparing the conditions of both enzyme in terms of temperature (40 ^{o}C, 45 ^{o}C, 50 ^{o}C, 55 ^{o}C and 60 ^{o}C), time (10 hours of reaction), enzyme concentration (0.05 U/ml, 1 U/ml, 2 U/ml, 3 U/ml, 4 U/ml and 5 U/ml) and also substrate concentration (5 %, 10 %, 15 %, 20 % and 25 %). For *T.viride* xylanase, the maximum yield of XOS were obtained at the seventh hour, 50 ^{o}C, 4 U/ml enzyme and 10 % w/v substrate by yielding 57.6 % (2.42 g/kg of xylotriose and 3.34 g/kg of xylobiose). As for *T.lanuginosus* xylanase, the maximum yield of XOS were obtained at the third hour, 55 ^{o}C, 1 U/ml enzyme and 20 % w/v substrate by yielding 27.0 % (2.44 g/kg of xylotriose and 2.95 g/kg of xylobiose). Water binding capacities showed significant differences with XOS_{TL} showed higher water binding capacity than XOS_{TV}. XOS_{TL} has a higher oil binding capacity than XOS_{TV}. Data also showed the calcium and magnesium binding capacity of XOS continued to

increase as the pH was increased. In solubility test, XOS_{TV} was proven to be more soluble than XOS_{TL} with 88.70 % of solubility rate.

CHAPTER ONE

INTRODUCTION

1.1. Research background

The growing demand for functional foods and the potential for product development open promising markets for xylo-oligosaccharides (XOS) in many fields, including pharmaceuticals, agricultural, food and feed applications (Moura *et al*., 2007). XOS are sugar oligomers produced during the hydrolysis of xylan which is the major component of plant hemicellulose, a heteropolysaccharide with homopolymeric backbone of xylose units (Saha, 2003). Okazaki, Fujikawa, and Matsumoto (1990) pointed out superior effect of XOS on promoting the growth of Bifidobacterium to other oligosaccharides and was supported by Chung, Hsu, Ko and Chan (2007) who concluded that XOS have better promoting effect on the growth of Bifidobacterium in the intestine of the elderly than for fructooligosaccharide and inulin.

Hemicellulose extracted from oil palm fronds (OPF) can be further hydrolysed to produce XOS. Fractionation of hemicellulose into the main components could be of interest in obtaining separate streams useable for different product application (Kabel, 2002). In the recent years, there is an increasing interest in the potential application of hemicelluloses both in food and non-food areas. Promising results have been obtained in the field of papermaking, baking and food additives (Ebringerova & Heinze, 2000).

OPF contributes 70 % of the overall oil palm industry waste in Malaysia (Eng *et al*., 2004). It is reported that Malaysia alone produced, during the past years, about 30 million tones annually of oil-palm biomass, including trunks, fronds and empty fruit

bunched (Abdul Khalil & Rozman, 2004). For that reason, OPF is used as raw material for this research work.

Several methods have been proposed to produce XOS from suitable feedstock, including direct enzymatic treatments, chemical fractionation followed by enzymatic hydrolysis of hemicellulose isolates, and hydrolytic degradation of hemicellulose to XOS by dilute solution of mineral acids, steam or water (autohydrolysis) (Nabarlatz *et al*., 2007; Akpinar *et al*., 2009). Endo-1,4-β-xylanases (EC 3.2.1.8), from *Trichoderma viride and Thermomyces lanuginosus* are chosen because these enzymes have been reported to degrade hemicellulose to short-chain XOS of varying lengths.

1.2 Research objectives

The general aim of this study was to produce xylo-oligosaccharides (XOS) by enzymatic hydrolysis using two origins of xylanase enzymes; *Trichoderma viride* (*T.viride* xylanase) and *Thermomyces lanuginosus* (*T.lanuginosus* xylanase). Therefore the specific objectives in this research were:

1. To study the effect of temperature, enzyme concentration and substrate concentration to produce the maximum yield of XOS by both enzymes.

2. To compare the yield of XOS using delignified and crude hemicellulose based from the conditions obtained.

3. To study the physical properties of XOS in terms of solubility, water binding capacity, oil binding capacity and mineral binding.

CHAPTER TWO

LITERATURE REVIEW

2.1 Oil palm

An oil palm (*Elaeis guineensis Jaq*.) (Figure 2.1) is the golden crop that has helped to change the scenario of Malaysia's agriculture and the Malaysian economy (Dahlan, 2000; Kamarulzaman *et al*., 2004).

Figure 2.1: Photograph of oil palm tree (Law *et al*., 2007)

Oil palm can reach 60-80 feet in height in nature, but is rarely more than 20 or 30 feet in cultivation. The trunks are stout, straight and grow about 50-70 cm per year. Leaf color is green and leaf bases are persistent for years. Old or dead leaves snap and remain attached on wild palms, but are pruned off for cultivated palm to facilitate harvest in plantations. Leaves are up to 6-8 m in length, with leaflets numbering 200-300 per leaf. The fruits are oval in shape, and it ripens about 5-6 months after pollination (Anon, 2004).

Wan Zahari *et al*. (2004) reported in 2006, Malaysia is the world's leading palm oil producer and exporter, accounting for 47 % of global production and 89 % of exports (Sumathi *et al*., 2007). Oil palm produces about 55 t/ha/yr of total dry matter (TDM). Currently the main product is the oil; palm oil and palm kernel oil which is averaging about 4.3 t/ha/yr. This constitutes about 8 % of the TDM whereas 92 % has not been converted into high value-added products. In Malaysia, the oil palm industry generates more than 1.3 million tones of oil palm trunks and 2.4 million tones empty fruit bunches (Jaafar & Sukaimi, 2001) and 36 million tones of pruned and felled fronds.

2.1.1 Oil palm frond (OPF)

According to Dahlan (2000), oil palm frond (OPF) is the leaf-like part produced continuously from the oil palm plant (Figure 2.2). OPF grows in tightly clustered bunches or heads. Unlike the trunk, OPF are obtained during felling on replanting and at pruning during harvesting time. Pruning is the process of cutting fronds (usually 2-3 fronds per palm tree), and it was done to facilitate harvesting since fresh fruit bunches (FFB) are compactly packed and hidden in the leaf axils.

According to Dahlan (1996), the total production of dried OPF is about 8.2 millions t/yr, and this amount is increasing every year. The production of felled fronds is only about 20 % of the trunks, but unlike pruned fronds, they are concentrated in a replanted area together with the trunks. Thus, felled fronds intensify the problems created by felled trunks (Mohammad *et al*., 1986).

Figure 2.2: Photograph of oil palm fronds

In order to avoid this problem, OPF needs to be shredded and pulverized mechanically to enhance the decomposition. However, this will only add to the cost, but also requires intensive labour (Dahlan, 2000). Therefore, OPF had been studied to be used as various value added-products such as animal feed (Dahlan, 2000), paper (Wan Rosli *et al*., 2007), and feedstock for production of ethanol and furfural (Basiron & Sameh, 2005).

2.2 Lignocellulosic materials

The major component of lignocellulose materials is cellulose, along with lignin and hemicellulose. Cellulose and hemicellulose are macromolecules from different sugars; whereas lignin is an aromatic polymer synthesized from phenylpropanoid precursors. The composition and percentages of these polymers vary from one plant species to another. Moreover, the composition within a single plant varies with age, stage of growth, and other conditions (Jeffries, 1994). Long cells enveloped by a characteristic cellular wall form wood. This wall is a complex structure that acts at the same time as plant skin and backbone (Figure 2.3).

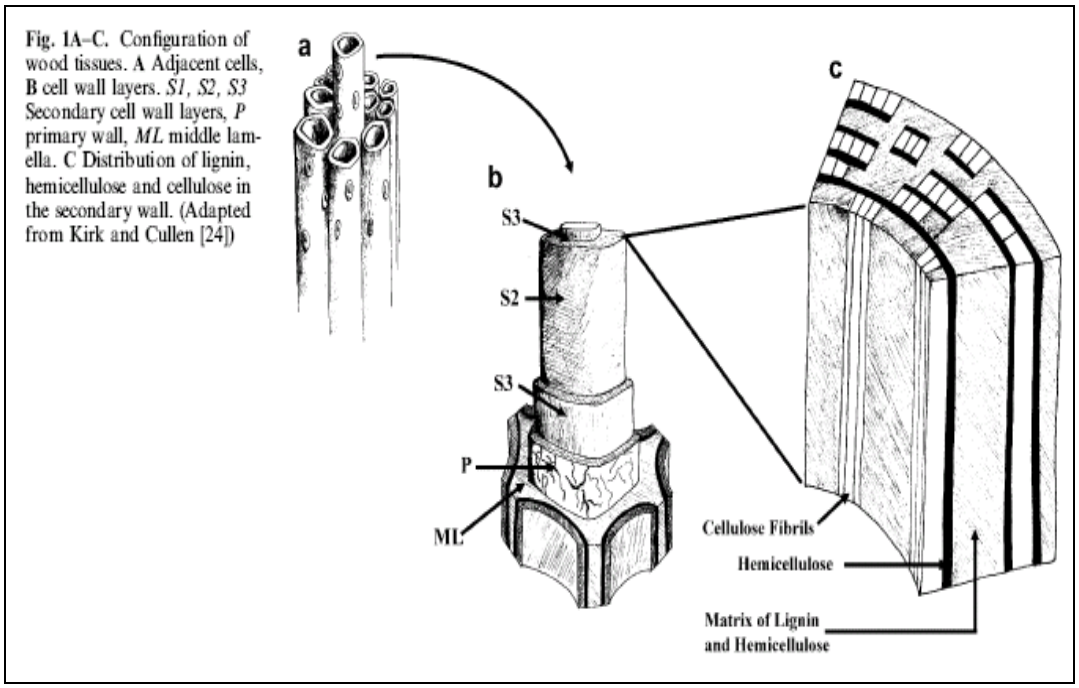

Figure 2.3: Configuration of wood tissues (Perez *et al*., 2002).
A:adjacent cells, B:cell wall layers, S1,S2,S3:secondary cell wall layers, P:primary wall, ML:middle lamella, C:distribution of lignin, hemicellulose and cellulose in the secondary wall

2.2.1 Cellulose

Cellulose makes up about 45 % of the dry weight of wood. This lineal polymer is composed of D-glucose subunits linked by β-1,4 glycosidic bonds forming cellobiose molecules. These form long chains (called elemental fibrils) linked together by hydrogen bonds and van der Waals forces. Hemicellulose and lignin cover microfibrils (which are formed by elemental fibrils). The orientation of microfibrils is different in the different wall levels. Microfibrils were grouped together to constitute the cellulose fiber. Cellulose can appear in crystalline form, called crystalline cellulose. In addition, there is a small percentage of non-organized cellulose chains, which form amorphous cellulose. In this conformation, cellulose is more susceptible to enzymatic degradation (Beguin & Aubert, 1994).

Most wood-derived cellulose is highly crystalline regions. The remaining portion has a lower packing density and is referred to as amorphous cellulose (Roger *et al*., 2005). Cellulose is the major component in plant cell walls and constitutes up to 50 % of the dry weight of wood. Cellulose and hemicellulose can be hydrolysed to sugars that can be further converted, either microbially or chemically, into energy carriers such as ethanol and butanol, or various other products such as organic acids, acetone, or glycerol (Wyman, 2002).

2.2.2 Hemicellulose

Hemicellulose is a complex carbohydrate polymer and makes up 25–30 % of totalwood dry weight. It is a polysaccharide with a lower molecular weight than cellulose and consists of homopolymer or heteropolymers backbone, such as arabinans, galactans, mannans and xylan units (Sun & Sun, 2002). The polymer chains of hemicelluloses have short branches and are amorphous. Because of the amorphous morphology, hemicelluloses are partially soluble or swellable in water. However, most of the hemicelluloses fraction is soluble in water after alkaline extraction (Doner & Hicks, 1997). Hemicelluloses have a much lower degree of polymerization (100-200) compared with that of cellulose (9000-15000) (Xiao *et al*., 2001).

The backbone of the chains of hemicellulose can be a homopolymer (generally consisting of single sugar repeat unit) or a heteropolymer (mixture of different sugars). Formulas of the sugar component of hemicellulose are listed in Figure 2.4. Among the most important sugar of the hemicellulose component is xylose. Hemicellulose includes xylan, mannan, galactan, and arabinan as the main

heteropolymers. The classification of these hemicellulose fractions depends on the types of sugar moieties present. The principal monomer present in most of the hemicellulose is D-xylose, D-mannose, D-galactose, and L-arabinose. The polymer chains of hemicellulose have short branches and are amorphous, consisting of a few hundred sugar residue. Hemicellulose is partially soluble in water because of the amorphous morphology (Coughlan & Hazlewood, 1993).

About 70 % of the dry mass in lignocellulosic biomass consists of cellulose and hemicellulose. If these two carbohydrates were utilized in an efficient hydrolysis process, the hemicellulose would be completly hydrolyzed to D-xylose (50-70 % w/w) and L-arabinose (5-15 % w/w), and the cellulose would be converted to glucose (Ladish 1989; Cao *et al.,* 1995). Hemicellulose can constitute up to 39 % of agricultural residues by dry weight, with the aldopentose D-xylose (usually not less than 95 %) (Winkelhausen & Kuzmanova, 1998) forming the main constituent of this fraction when derived from hardwood or agricultural residues (Singh & Mishra, 1995).

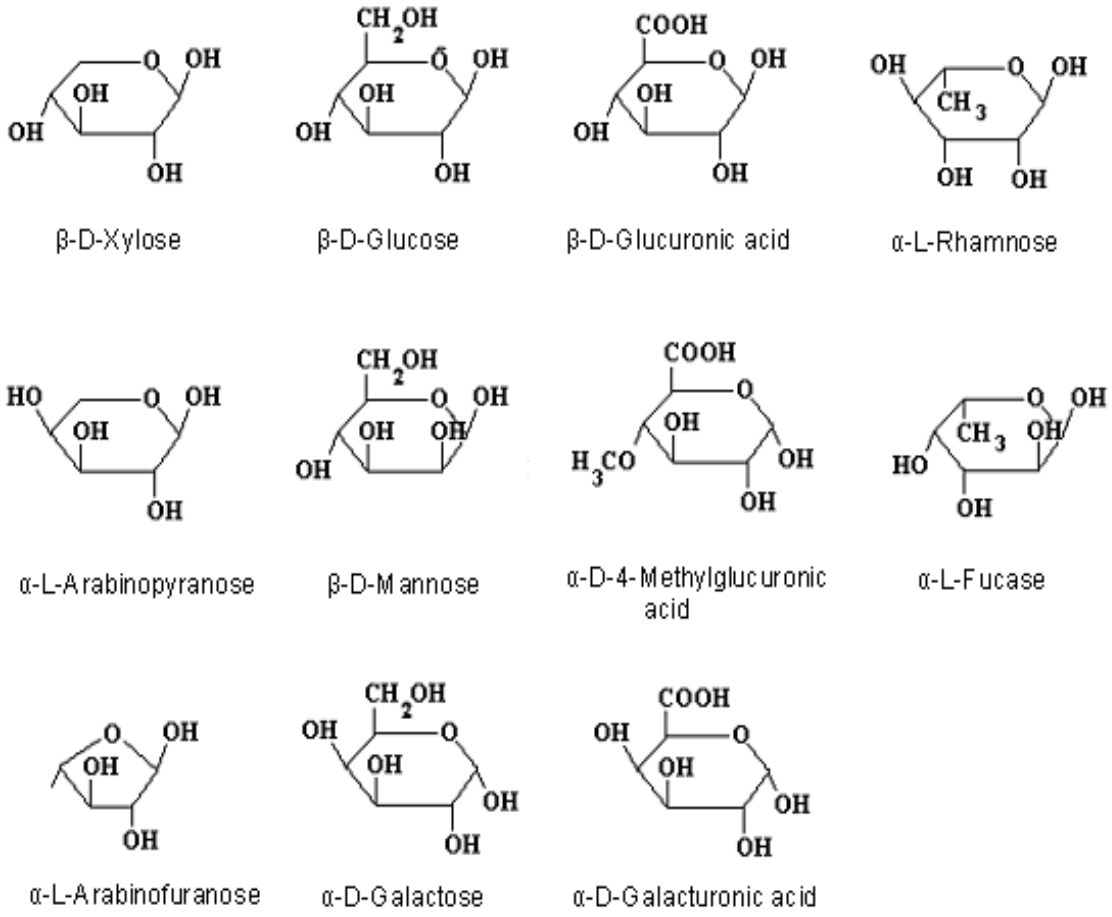

Figure 2.4: Chemical structure of sugar units of hemicelluloses (Fengel & Wegener, 1989)

2.2.3 Xylan

Xylan, which comprises 20-35 % by weight of wood and agricultural wastes, is the major component of the hemicellulose portion. Xylan therefore represents a significant resource of renewable biomass which can be utilized as a substrate for the preparation of many useful products such as fuels, solvents, and chemicals. Xylans in hardwoods and softwoods have different side group patterns. Hardwood xylan is O-acetyl-4-O-methylglucuronoxylan, in which the xylan backbone is substituted at random intervals with acetyl and 4-O-methylglucuronic acid side groups. The ratio of xylose to α-1,2-linked 4-O- methylglucuronic acid is about 10:1, twice as high as in softwoods, and 60-70 % of the xylose units are acetylated at the C2 and or C3 positions (Lindberg *et al*., 1973).

Softwood xylan is mainly arabino-4-O-methylglucuronoxylan, in which L-arabinose units are α-1,3-linked to the xylan backbone. The ratio of xylose to 4-O-methylglucuronic acid is 5:1, and the xylose to arabinose ratio is 8:1 (Timell, 1967). There are no acetyl groups in softwood xylan, but L-rhamnose and galacturonic acid residues have been identified in the main chain linked to the reducing end, producing a more alkali-resistant end group. Mannans are also typical hemicelluloses in both softwoods and hardwoods. There are two main groups, namely galactoglucomannans and glucomannans, of which the former is mainly found in softwoods and the latter in hardwoods. Galactoglucomannans are composed of β-1,4-linked glucose and mannose units in the ratio of 1:1-2 and neither galactose nor acetyl groups are present (Timell, 1967).

2.2.4 Lignin

Lignin is the most abundant polymer in nature. It is present in the cellular cell wall, conferring structural support, impermeability and resistance against microbial attack and oxidative stress (Perez *et al*., 2002). Lignin is the main non-carbohydrate component (phenolic polymer) present in the mature plant cell wall that gives wood its characteristics of brown color, density and mass (Armstrong, 2006). Structurally, lignin is very distinct from the other major structural components of wood material. There are neither typical chains of repeating units nor easily hydrolysable bonds in lignin, as there are in wood carbohydrates. Lignin has a highly irregular three dimensional structure and it is a water insoluble high molecular mass compound (Brunow *et al*., 1998).

The monomeric building units of lignin are shown in Figure 2.5. The guaiacyl unit is dominant in the softwoods. In contrast, syringyl units are dominant in hardwoods (Roger *et al*., 2005). It has been found that softwood is more resistant to lignin removal by alkaline extraction than hardwood. It has been suggested that guaiacyl lignin restricts fibre swelling and thus the enzymatic accessibility more than syringyl lignin (Ramos *et al*., 1992). Lignin seems to consist of amorphous regions and structured forms such as oblong particles and globules (Novikova *et al*., 2002). Besides 20 different types of bonds present within the lignin itself, lignin seems to be particularly associated with the hemicellulosic polysaccharides (Xiao *et al*., 2001). Lignin can be isolated using several methods such as Klason lignin. Klason lignin is obtained after removing the polysaccharides from the extracted wood by hydrolysis with 72 % sulfuric acid (Sjostrom, 1981).

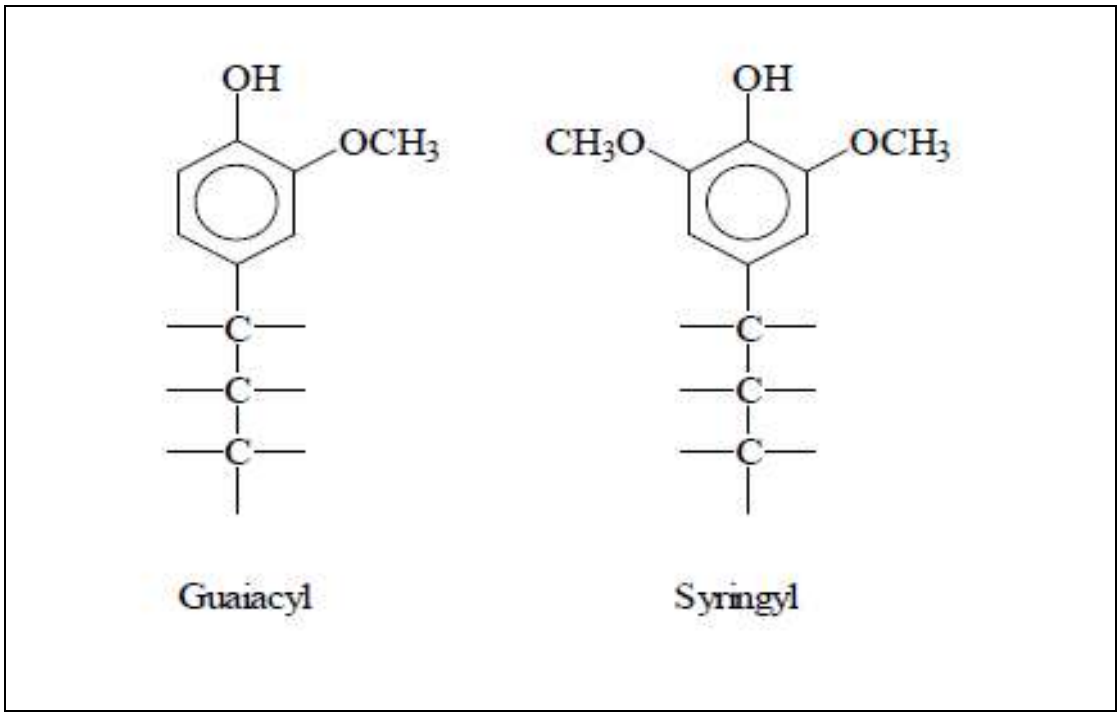

Figure 2.5: Schematic illustration of building units of lignin (Mazlan, 1998)

2.3 Enzyme degradation of hemicelluloses

In wood, the two most common hemicelluloses are xylans and glucomannans. For the total enzymatic hydrolysis of hemicellulosic polysaccharides, several synergistically acting enzymes are needed (Viikari *et al*., 2001). Hemicelluloses are widely distributed heteropolysaccharides. The enzymes that degrade them are ubiquitous and diverse. In nature, xylans have L-arabinose, acetyl, glucuronic, 4-O-methylglucuronic, and *p*-coumaric side chains and ferulic acid cross linkages (Jeffries, 1994). Biological routes are built around using enzymes to break down cellulose (cellulase) and perhaps hemicellulose (hemicellulase) to sugars. These sugars are then fermented to ethanol or other products, which are recovered and purified to meet market requirements. Cellulosic biomass must be pretreated to realize high yields vital to commercial success in biological conversion (Mosier *et al*., 2005).

Enzymatic degradation of lignocellulose involves a set of different enzymes. A wide variety of cellulolytic fungi and bacteria have been reported to date and this number is continually increasing. Presently, enzymatic hydrolysis is considered the most promising technology for converting biomass into sugars and to be used as raw material for the production of various other biotechnical bulk chemical products. Degradation studies have mainly been performed with isolated soluble substrates. Hydrolysis proceeds by the synergistic action of different hemicellulases. The main enzymes needed in the depolymerisation of hemicelluloses are endoenzymes, i.e β-xylanases and β-mannanases. The endoxylanases and endomannanases attack randomly at the internal linkages of xylans and glucomannans, respectively, releasing substituted oligomeric products. In the subsequent steps these intermediary products are further hydrolysed by a set of exo-enzymes (β-xylosidase and β-mannosidase) and side group cleaving enzymes (α-arabinose, α-galactosidase, α-glucuronidase and esterase), resulting in the final monomeric end products (Viikari *et al*., 2001).

Several species of fungi and bacteria produce a wide variety of hemicellulolytic activities needed in the total hydrolysis of hemicellulosic materials (Viikari *et al*., 1993; Sunna & Antranikian, 1997). The best characterized fungal hemicellulases are those produced by *Aspergillus, Penicillium* and *Trichoderma* (Torronen & Rouvinen, 1997; Biely *et al*., 1997) and bacterial hemicellulases produced by *Caldocellum, Bacillus* and *Streptomyces* (Breccia *et al*., 1998; Dupont *et al*., 1998). Furthermore, xylanases are the most thoroughly studied of all hemicellulolytic enzymes.

2.3.1 Pretreatment in bioconversion process

The major steps in biomass to sugars process include raw material preparation (cleaning and size reduction), pretreatment and hydrolysis. There are several desirable goals for pretreatment processes, however, in practice not all of them are achieved with any current treatment. Generally the pretreatment should promote high product yields in a subsequent enzymatic hydrolysis and fermentation operations with minimal costs. The formation of degradation products from lignin and sugars, such as furfural and hydroxymethyl furfural, which are known to inhibit the fermenting organisms, should be minimized. The utilization of lignocellulosic residues in a bioconversion process involving enzymatic hydrolysis requires a pretreatment of raw material. Pretreatment, recognized from an economic point of view as a key step in the bioconversion process, must improve the formation of sugars (both from the hemicellulose and cellulose fractions), avoid the degradation or loss of carbohydrates, avoid the formation of inhibitory products to the subsequent hydrolysis and fermentation processes and, finally, be cost-effective (Sun & Cheng, 2002).

Bioconversions of lignocellulosic materials to useful, higher value products normally require multi-step processes which include: (i) pretreatment (mechanical, chemical or biological) (Grethlein & Converse, 1991; Grethlein, 1984), hydrolysis of the polymers to produce readily metabolizable molecules (e.g. hexose or pentose sugars), (iii) bio-utilization of these molecules to support microbial growth or to produce chemical products and (iv) the separation and purification (Smith *et al*., 1987). In the steam pretreatment of softwood, the recovery of a maximal amount of sugars from both hemicellulose and cellulose has been shown to require a two step pretreatment procedure (Tengborg *et al*., 1998; Soderstrom *et al*., 2002). The highest overall

yields of hemicellulose and cellulose derived sugars in one-step pretreatment have been 66 % and 67 % respectively (Tengborg *et al*., 1998).

Lynd (1996) has summarized the desirable properties for an ideal lignocelluloses material after chemical pretreatment, i.e. it should (a) produce reactive fibers, (b) yield pentoses in non-degraded form, (c) not release the compounds that significantly inhibit fermentation, (d) work in reactors of reasonable size with moderate cost, (f) produce no solid residues, (g) have a high degree of simplicity, and (h) be effective at low moisture contents. A number of pretreatment options are available: acid pretreatment, alkaline treatment, steam explosion, wet oxidation, organic solvent pretreatment, and hot water.

It is well known that alkaline pretreatment provides the effective delignification and chemical swelling of the fibrous cellulose (Zhao *et al*., 2009). These effects enhance the accessibility of enzymes and the digestibility of holocellulose components because of the salvation and the saphonication during alkaline pretreatment. At the same time, the alkaline pretreatment can also cause condensation of lignin and modification of the crystal structure, which can introduce unwanted effects for lignin removal and cellulose degradation (Gregg & Saddler, 1996). Additionally, a lot of researchers have reported on alkaline peroxide treatment for various kind of lignocelluloses biomass claiming that it improved enzymatic saccharification significantly (Chen *et al*., 2008; Sun *et al*., 2000 & Yang *et al*., 2001).

Similarly, Cara *et al*. (2006) have shown that the alkaline peroxide treatment on olive tree wood is an effective delignification strategy to enhance enzymatic hydrolysis. More recently, by using both wheat straw (Saha & Cotta, 2006) and rice hulls (Saha

& Cotta, 2007), alkaline peroxide treatment has been shown to be an effective treatment for the enzymatic saccharification of these lignocellulosic feedstocks.

2.3.2 Effect of lignin removal on xylo-oligosaccharides production

A part of lignin is known to be covalently bound to xylan, and phenolic acids particularly ferulic acid, appears in side chains of heteroxylans from grasses and cereals (Ebringerová & Heinze, 2000). Therefore, some xylo-oligosaccharides might contain such phenolics. A study was done by Nabarlatz *et al*. (2007) on autohydrolysis of agricultural by-products for the production of xylo-oligosaccharides had showed the increase in lignin and organic extractives content of the solid caused the material less accessible to enzyme reaction, yielding less xylo-oligosaccharides in the liquid.

The presence of lignin-derived species in the liquid product calls for the inclusion of additional purification treatments to isolate xylo-oligosaccharides with adequate purity, well-defined, repetitive composition and molar mass. However, the lower the concentration of lignin products relative to xylo-oligosaccharides in the autohydrolysis liquid, the simpler the purification sequence and the higher its effectiveness should be (Nabarlatz, 2007). Another study by Yang *et al*. (2004), also showed the pretreatment of the corncob for xylo-oligosaccharides production helps in breaking down the xylan-lignin complex, liberating xylan available for the action of enzyme.

2.4 Xylanase

Xylanases (E.C.2.8.1.8), a group of hemicellulolytic enzymes, are required for the hydrolysis of β-1, 4-xylans present in lignocellulosic materials (Kheng & Omar, 2005). Xylanases are the microbial enzymes that have aroused great interest recently due to their potential application in many industrial processes such as production of hydrolysates from agro-industrial wastes (Gessesse & Gashe, 1997), nutritional improvement of lignocellulosic feed stuff (Wallace *et al*., 2001), clarification of juices and wines (Gable & Zacchi, 2002) and biobleaching of craft pulp in paper industry (Yinbo *et al*., 1996).

Xylanases catalyze the hydrolysis of xylans. Xylanases are produced from many different fungi and bacteria.and take part in the breakdown of plant cell walls, along with other enzymes that hydrolyze polysaccharides, and also digest xylan during the germination of some seeds (e.g. in the malting of barley grain). Xylanases also can be found in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna & Antranikian, 1997). Xylanase deconstructs plant structural material by breaking down hemicellulose, a major component of the plant cell wall. Most commercial xylanases are produced by *Trichoderma*, *Bacillus*, *Aspergillus*, *Penicillium*, *Aureobasidium*, and *Talaromyces* spp. (Li *et al*., 2000).

According to Wong *et al*. (1988), xylanases can be classified at least in three ways. The first is classified as microbial xylanases categorized into two groups on the basis of their physicochemical properties such as molecular mass and isoelectric point (pI), rather than on their different catalytic properties. One group consists of high molecular mass enzymes with low pI values and the other group of low molecular mass enzymes with high pI values. The second is based on crystal structure. This

can be derived indirectly by a determination of DNA sequence. Xylanases can be structurally classified into family F or (known as glycosidase family 10), and family G (known as family 11). The third classification is based on kinetic properties, substrate specificity, or product profiles. Virtually all xylanases are "endo" acting, as readily determined by chromatography, but the more detailed determination of kinetic properties such as measuring the relative reaction rates on various substrates and determining the kinetics of intermediate product formation is much less common.

The high molecular weight endoxylanases with low pI values belong to glycanase family 10 while the low molecular mass endoxylanases with high pI values are classified as glycanase family. Greater catalytic versatility is found in family 10 than in family 11 xylanases whereas family 10 xylanases hydrolyse naturally occurring polysaccharides to a greater extent (Bennett *et al*., 1998). Recently, xylanases have attracted considerable research interest because of their potential industrial applications. One of the exciting applications of xylanases is the production of xylo-oligosaccharides (Beg *et al*., 2001).

2.4.1 Xylanase mechanism of action

Due to the complex structure of hemicelluloses, several different enzymes are needed for their enzymatic degradation or modification. The two main glycosyl hydrolases depolymerising the hemicellulose backbone are endo-1,4- L-D-xylanase and endo-1,4-L-D-mannanase (Suurnakki *et al*., 1997). Since xylan is a complex component of the hemicelluloses in wood, its complete hydrolysis requires the action of a complete enzyme system, which is usually composed of L-xylanase, L-xylosidase, and enzymes such as K-L-arabinofuranosidase, K-glucuronidase, acetylxylan

esterase, and hydroxycinnamic acid esterases that cleave side chain residues from the xylan backbone. All these enzymes act cooperatively to convert xylan to its constituents (Sunna & Antranikian, 1997).

Xylanases attack randomly the backbone of xylan to produce both substituted and non-substituted shorter chain oligomers, xylobiose and xylose (Eriksson *et al*., 1990). Xylosidases are essential for the complete breakdown of xylan as they hydrolyse xylo-oligosaccharides to xylose (Poutanen & Puls, 1988). The enzymes arabinosidase, K-glucuronidase and acetylxylan esterase act in synergy with the xylanases and xylosidases by releasing the substituents on the xylan backbone to achieve a total hydrolysis of xylan to monosaccharides (Eriksson *et al*., 1990).

Endo-1,4-β-xylanase (EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate. Xylan is not attacked randomly, but the bonds selected for hydrolysis depend on the nature of the substrate molecule, i.e. chain length, degree of branching, and presence of substituents (Poutanen & Puls, 1989; Li *et al*., 2000). Synergistic and cooperative effects among the xylan-degrading enzymes enhance the susceptibility of the heteropolymeric xylan to be attacked by endoxylanases (Van Peij *et al*., 1997; De Vries *et al*., 2000).

Endo-1,4-β-xylanase also randomly cleave xylan main chain and generate variously substituted xylo-oligosaccharides (Biely, 1985). Endo-1,4-β-xylanase have been classified into families 5, 8, 10, 11 and 43 of glycoside hydrolases, based on hydrophobic cluster analysis and similarities in their amino acid sequences (Coutinho & Henrissat, 1999). Members of two best known families, GH-10 and GH-11, differ

in their physicochemical properties such as molecular mass, isoelectric point, as well as in their three-dimensional structure (Wong *et al*., 1988; Campbell *et al*., 1993).

According to Biely *et al*. (1997), after extensive study on the differences in catalytic properties among the xylanase families concluded that endoxylanases of family 10 in contrast to the members of family 11 are capable of attacking the glycosidic linkages next to the branch points and towards the non-reducing end. While endoxylanases of family 10 require two unsubstituted xylopyranosyl residues between the branches, endoxylanases of family 11 require three unsubstituted consecutive xylopyranosyl residues. Endoxylanases of family 10 also possess several catalytic activities, which are compatible with β-xylosidases. The endoxylanases of family 10 liberate terminal xylopyranosyl residues attached to a substituted xylopyranosyl residue, but the enzymes also exhibit aryl-β-D-xylosidase activity.

According to Pulls, (1997), xylan is recognised and bound by xylanase as a left-handed three fold helix (Bissoon *et al*., 2002). 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 (Shoham *et al*., 1992). 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). The optimum pH and temperature for the activity of xylanase have been reported to be in the range of pH 4.5–6.5 and 50–65 ^{o}C, respectively (Bakir *et al*., 2001; Cardoso & Filho, 2003).

2.4.2 Applications of xylanase

The biotechnological use of xylans and xylanases has grown remarkably (Beg *et al*., 2000, 2001; Techapun *et al*., 2003). The end-products of xylan degradation of considerable importance in commercial applications are furfural and xylitol (Parajó *et al*., 1998). Currently, xylanase and cellulase, together with pectinases, account for 20 % of the world enzyme market (Polezeli *et al*., 2005). Xylanolytic enzymes can improve the baking process and modify baked products (Maat *et al*., 1992). In the baking industry xylanases are used for improving desirable texture, loaf volume and shelf-life of bread. A xylanase, Novozyme 867, has shown excellent performance in the wheat separation process (Christopherson *et al*., 1997).

Sá-Pereira *et al*. (2002) had revealed that the optimal pH for bacterial xylanases was in general, slightly higher than the pH optima of fungal xylanases. When the effect of reaction time was investigated, Seyis & Aksoz, (2005) had observed that xylanase activity was maximal at 50 minutes. As the enzyme substrate interaction period increases, the amount of products also increases up to a certain point. There are reports on variation in incubation time for the determination of xylanase activity. This was due to the fact that microorganisms and xylan sources were different (Royer & Nakas, 1991; Gomes *et al*., 1993).

Xylanases may be employed in bread-making, together with á-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. During the bread-baking process, they delay crumb formation, allowing the dough to grow. With the use of xylanases, there has been an increase in bread volumes, greater

absorption of water and improved resistance to fermentation (Harbak & Thygesen, 2002; Camacho & Aguilar, 2003). Also, a larger amount of arabinoxylo-oligosaccharides in bread would be beneficial to health.

Addition of xylanase at the correct dosage to cereal based foods such as bread, pasta and noodles yields more flexible, easy-to-handle dough thereby improving the final baked product. A baking process based on the use of xylanolytic enzymes from *T. lanuginosus* strains has been patented and was reported to improve the baking properties of dough (Jorgensen *et al*., 1997). Xylanases free of cellulases are applied also in pulp and paper industry, where they facilitate lignin extraction and reduce the consumption of toxic chemicals required for pulp bleaching (Madlala *et al*., 2001).

2.4.3 Types of xylanase

a) *Trichoderma Viride*

Trichoderma xylanases have been found to be active on xylans from different sources, usually producing xylo-oligomers, xylobiose and xylose. Xylose is not usually the major product and it is typically produced after an accumulation of xylo-oligomers. Among the xylanases characterized by Wong *et al*. (1988), one isolated from *T. pseudokoningii* (Baker *et al*., 1977) and two isolated from *T. viride* (Dean *et al*., 1991) were reported to be unable to produce xylose. Two non-specific glycanases from *T.viride* were also found to be unable to produce xylose during xylan hydrolysis (Beldman *et al*., 1988). One of these glycanases produces xylobiose as an initial product, indicating that it acts like an exoxylanase (Shikata & Nisizawa, 1975). Hydrolysis pattern of *Trichoderma* xylanases has however suggested that most are endoxylanases.

Xylans are not completely hydrolysed by crude culture filtrates (Poutanen *et al*., 1987) or purified xylanases from *Trichoderma species* (spp.) (Poutanen *et al*., 1989). However, hydrolysis yields from certain xylans could be improved by using mixtures of three different xylanases purified from *T. harzianum* (Wong *et al*., 1986). Xylose yields obtained using a purified xylanase from *T. reesei* were, increased when a purified β-xylosidase was added (Poutanen *et al*., 1989). They were further increased when the relevant debranching enzymes were added to the hydrolysis reaction. When acetylated xylo-oligomers were partially deacetylated by freeze-drying over ammonia, they became more accessible to hydrolysis by xylanase.

Trichoderma xylanases are known to solubilize carbohydrates from cell wall preparations, holocellulose substrates and kraft pulps. Only 20 % of the xylosyl residues from corn shoot cell walls and 10 % of those from bean shoot cell walls were solubilized by a xylanase purified from *T. pseudokoningii* (Baker *et al*., 1977). Much lower levels of other sugar residues were solubilized, suggesting the high degree of selectivity observed in certain *Trichoderma* xylanases (Clark *et al*., 1991). Although xylose was not detected in the cell wall hydrolysates obtained using the *T. pseudokoningii* xylanase, it was found in holocellulose and pulp hydrolysates obtained using xylanases from *T. harzianum, T. reesei* and *T. viride.* These latter enzymes could solubilize 11-71 % of the xylan in hardwood and softwood holocellulose (Sinner *et al*., 1979), 9-25 % of that in kraft pulps and 54 % of that in a bleached hardwood kraft pulp (Senior *et al*., 1988). There was a decrease in the degree of polymerization of the xylan remaining in kraft pulps after xylanase treatment (Miller *et al*., 1991).

Xylanase preparations from *Trichoderma* spp. have been used for studying bleach boosting (Viikari *et al*., 1990). In laboratory experiments, the reduction of the active chlorine loading required during the chlorination stage has been reported to range from 35-41 % for hardwoods and 10-26 % for softwoods. Minor changes in pulp quality after xylanase prebleaching were deemed to be within acceptable limits. These xylanase pretreatments were found to be effective using enzyme loadings as low as 1-5 mg/g dry pulp and incubation times as short as 0.5-3 hours (Cultor, 1991).

The optimal conditions for activity *of Trichoderma* xylanases range from 45-65 °C and from pH 3.5-6.5 (Wong *et al*., 1988). As would be expected, the xylanases with higher optimum temperature are relatively more thermally stable (Irie *et al*., 1990) than those with lower optimum temperature (John & Schmidt, 1988). Two xylanases have been reported to be stable at 50 °C for 1 hour (Irie *et al*., 1990) and one at 60 °C for 20 minutes (Wood & McCrae, 1986). These properties are relatively moderate when compared to xylanases isolated from thermophilic microorganisms. For example, a xylanase isolated from *Thermostoga* sp. strain has optimum temperature of 105 °C at pH 5.5 and a half-life of 90 minutes at 95 °C (Simpson *et al*., 1991). Furthermore, alkaline tolerant xylanases have been isolated from *Bacillus* spp. which had a broad range of pH optima and stabilities, ranging up to pH 10 (Okazaki *et al*., 1985).

b) *Thermomyces Lanuginosus*

Thermomyces lanuginosus (*T. lanuginosus*, formerly known as *Humicola lanuginosa*) is a widely distributed thermophilic fungus commonly isolated from 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 ^{o}C (Cooney, 1964). By definition, a

thermophilic fungus is one that thrives at temperatures up to 60 ^{o}C and fails to grow below 20 ^{o}C (Hudson, 1992). A further characteristic feature of thermophiles is that their enzymes are more heat stable than those of mesophiles when extracted and tested in cell-free systems (Deacon, 1997). *T. lanuginosus* has attracted considerable interest due to its production of thermostable enzymes, especially the xylanase belonging to family 11 of glycosyl hydrolases (Henrissat & Davies, 1997). Furthermore, the xylanase production in *T. lanuginosus* is not accompanied by cellulase production. In addition very low levels of other hemicellulases are found in the culture media of this fungus (Gomes *et al*., 1993; Singh *et al*., 2003).

T. lanuginosus strains are particularly attractive producers of thermostable xylanases from the industrial point of view, due to the fact that they excrete high-level of cellulase-free xylanases into medium (Singh *et al*., 2003). These enzymes are responsible for hydrolysis of hemicellulosic materials, which are heteropolysaccharides found in association with cellulose constituting about 20-30 % of the wood dry weight (Suurnakki *et al*., 1997). Microbial xylanases (1,4-L-D-xylan xylanohydrolase, EC 3.2.1.8) have been the most widely studied of all hemicellulases and are the preferred catalysts for hydrolysis of xylan, the most common hemicellulosic polysaccharide, due to their high specificity, mild reaction conditions, negligible substrate loss and minimal side product formation (Kulkarni *et al*., 1999). Crude and purified xylanases and several other hemicellulases produced by most strains of *T. lanuginosus* are stable at very high temperatures and over a broad pH range (Singh *et al*., 2000).

T. lanuginosus occurs worldwide and has been isolated in many countries from a variety of decaying plant material (Puchart *et al*., 1999). By comparison,