XYLOOLIGOSACCHARIDE PRODUCTION FROM COTTON AND SUNFLOWER STALKS

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ABSTRACT

XYLOOLIGOSACCHARIDE PRODUCTION FROM COTTON AND SUNFLOWER STALKS

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In this study, the aim was enzymatic xylooligosaccharide production from cotton and sunflower stalks, two of main agricultural residues in Turkey. In first two parts of the study, alkali extracted xylan from both of the stalks was hydrolyzed by commercial xylanases Veron and Shearzyme. The effect of temperature, pH, enzyme and substrate concentrations were investigated to determine optimum enzymatic hydrolysis conditions of xylan. Sunflower and cotton stalk xylans were hydrolyzed by Shearzyme more efficiently than Veron under the conditions studied. Shearzyme produced different product profiles containing xylobiose (X2), xylotriose (X3), xylotetrose (X4) and xylopentose (X5) from cotton and sunflower stalk xylan. On the other hand, Veron hydrolyzed both xylan types to

produce X2, X3, X5, X6 and larger xylooligosaccharides without any change in product profiles.

In the third part of the study, home produced xylanase from *Bacillus pumilus* SB-M13, was also investigated for the production of xylooligosaccharides from both cotton and sunflower stalk xylan. The main products obtained by hydrolysis of both substrates by pure *B. pumilus* xylanase were X5 and X6, while crude *B. pumilus* xylanase generated X4 and X5 as the main products.

Xylooligosaccharide production from pretreated cotton stalk without alkali extraction of xylan was the final part of the study. Three different pretreatment methods including biomass pretreatment by *Phanerochaete chrysosporium* fermentation, cellulase pretreatment and hydrothermal pretreatment were investigated to break down complex lignocellulosic structure of cotton stalk to improve the subsequent enzymatic hydrolysis of xylan in pretreated cotton stalk for xylooligosaccharide production. However, xylooligosaccharide was not effectively produced from pretreated cotton stalk. Shearzyme inhibiton was observed after all the pretreatment methods during further hydrolysis of pretreated cotton stalk probably due to production of inhibitory compounds of the enzyme.

Keywords: Cotton stalk, sunflower stalk, xylan, xylooligosaccharide, xylanase, prebiotic, *Phanerochaete chrysosporium*.

ÖZ

PAMUK VE AYÇİÇEĞİ SAPINDAN KSİLOOLİGOSAKKARİT ÜRETİMİ

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Bu çalışmada amaç, Türkiye'nin önemli tarımsal atıklarından olan pamuk ve ayçiçek sapından ksilooligosakkarit üretilmesidir. Çalışmanın ilk iki bölümünde, alkali özütleme yöntemi ile izole edilen ksilan, ticari enzimler olan Veron ve Shearzyme ile hidroliz edilmiştir. Pamuk ve ayçiçek sapı ksilanının, Veron ve Shearzyme ile optimum hidroliz koşullarını belirlemek amacıyla farklı sıcaklık, pH değerleri, enzim ve sübstrat konsantrasyonları çalışılmıştır. Çalışılan tüm koşullarda, ayçiçek ve pamuk sapı ksilanı, Shearyzme ile daha verimli hidroliz edilmiştir. Shearzyme, ayçiçek ve pamuk sapı ksilanından, ksilobioz (K2), ksilotrioz (K3), ksilotetroz (K4) and ksilopentoz (K5) olmak üzere farklı ürün profili oluşturmuştur.

Diğer yandan, Veron, iki tip ksilanı, K2, K3, K5, K6 ve daha büyük ksilooligosakkaritlere hidroliz etmiştir.

Çalışmanın üçüncü bölümünde, laboratuvarımızda *Bacillus pumilus* –SB M13 'den üretilen ksilanızın, ayçiçek sapı ve pamuk sapı ksilanından ksilooligosakkarit üretimi araştırılmıştır. Saflaştırılmış *B. pumilus* ksilanazının pamuk ve ayçiçek sapı ksilanını hidrolizi ile elde edilen ana ürünler K5 ve K6 iken ham *B. pumilus* ksilanazı ana ürün olarak K4 ve K5 oluşturmuştur.

Alkali özütleme yapılmadan, önişlem görmüş pamuk sapından ksilooligosakkarit üretimi çalışmanın son bölümüdür. Önişlem görmüş pamuk sapından ksilooligosakkarit üretmek için, pamuk sapının kompleks lignoselülozik yapısını açmak ve önmuamele sonrası enzim ile hidrolizini geliştirmek amacıyla, *Phanerochaete chrysosporium* ile biyokütle önişlemi, selülaz önişlemi ve ısıl önişlemden oluşan 3 yöntem araştırılmıştır. Ancak, önişlem görmüş pamuk sapından, etkili ksilooligosakkarit üretimi gerçekleştirilemedi. Shearzyme inhibisyonu, bütün önişlem yöntemlerinden sonra, önişlem görmüş pamuk sapının ileri hidrolizasyonu sırasında, muhtemel enzim inhibitörlerinin muhtemel üretimi nedeniyle gözlenmiştir.

Anahtar Kelimeler: Pamuk sapı, ayçiçek sapı, ksilan, ksilooligsakkarit, ksilanaz, prebiotik, *Phanerochaete chrysosporium.*

To My Family

and

In Loving Memory of My Father

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ABBREVIATIONS

AFEX	Ammonia fiber expansion
BSA	Bovine serum albumin
CS	Cotton stalk
CSX	Cotton stalk xylan
DNSA	Dinitrosalicylic acid
DP	Degree of polymerization
HPLC	High performance liquid chromatography
SS	Sunflower stalk
SSX	Sunflower stalk xylan
TLC	Thin Layer Chromotgraphy
X1	Xylose
X2	Xylobiose
X3	Xylotriose
X4	Xylotetrose
X5	Xylopentose
X6	Xylohexose
XOs	Xylooligosaccharides
U	Enzyme Unit

CHAPTER 1

INTRODUCTION

1.1 Agricultural Waste in Turkey

Lignocellulosic agricultural waste material such as rice hull, sugarcane bagasse, cotton stalk (CS), sunflower stalk (SS) and wheat straw are produced in huge amounts and regarded as abundant, inexpensive and readily available natural resources for various industries (Orlando *et al.*, 2002).

Agricultural waste materials accumulate every year in large quantities, which cause a deterioration of the environment and loss of potentially valuable resources. The green house effect is one of the worlds' wide environmental problems, rising from the rapid increase in the atmospheric CO_2 concentration due to men-made causes, one of which is burning of agricultural waste materials that are left in the field after harvesting and burned by the farmers to clear up the field. The use of this waste is an important way to recycle carbon to energy and food. Currently, more and more effort is directed towards the reuse of such wastes, considering economic values and environment. As an alternative, enzymatic degradation of agricultural wastes into soluble sugars by multienzyme complexes is gaining increasing acceptance as the solution of choice for the treatment of agricultural wastes.

The released sugars can be used as a carbon source for fermentations generating chemicals, pharmaceuticals and fuels, particularly alcohol (Chen *et al.*, 2003). The main component of the hemicellulose in the agricultural waste is xylan and thus fractionation of xylan results in a variety of differently substituted xylooligosaccharides (XOS) which can be used in many fields, pharmaceuticals, agricultural applications, and food-related applications (Sjöström 1993).

Crops and livestock represent almost 90% of the agricultural sector in Turkey, with forestry and aquaculture contributing the rest. The type and quantity of crops (wheat, barley, tobacco, cotton, rice, sunflower etc.) that form the basis of the agricultural sector in Turkey give rise to huge amounts of agricultural residues. The agricultural total land of Turkey is about 26.350 million ha, from which: 38.4% sown area, 44.1% forest, 10.4% fallow land, 7.1% cultivated with fruit and vegetable areas as shown in Figure 1.1. Total annual crops production and residues in Turkey is represented in Table 1.1 (Exploitation of Agricultural Residues in Turkey, Ankara, Turkey, 29 June – 1 July 2005, Funded by the European Commission under the LIFE Programme-EC Contract Number LIFE03 TCY/TR/000061) (State Planning Organization, 2007).

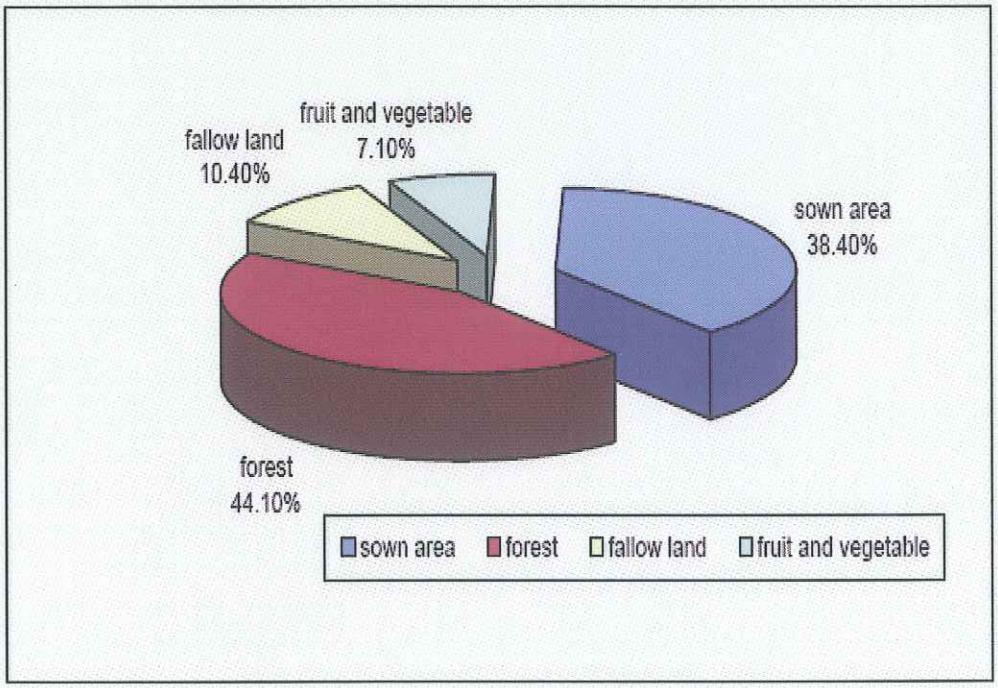


Figure 1.1. Agricultural land distribution in Turkey (Exploitation of Agricultural Residues in Turkey, Ankara, 29 June – 1 July 2005, Funded by the European Commission under the LIFE Programme-EC Contract Number LIFE03 TCY/TR/000061).

Agricultural residues have been considered in three categories:

1. Annual crop residues that remain in the field after the crops are harvested. The main annual crops in Turkey are cereals, maize, cotton, rice, tobacco, sunflower, groundnuts and soybeans.
2. Perennial residues in Turkey that remain in the field after pruning of trees, shells, kernels etc.
3. Agro-industrial residues such as; cotton-ginning, seed oil, olive oil, rice, corn and wine industries.

Table 1.1. Total crops production and residues in Turkey (Exploitation of Agricultural Residues in Turkey, Ankara, 29 June – 1 July 2005, Funded by the European Commission under the LIFE Programme (EC Contract Number LIFE03 TCY/TR/000061).

Crops	**Residues**	**Production (tons)**	**Available Residues (tons)**
Wheat	Straw	22 439 042	3 514 486
Barley	Straw	8 327 457	1 344 452
Rye	Straw	253 243	53 706
Oats	Straw	322 830	48 185
Maize	Straw	2 209 601	2 982 155
	Cop		1 144 384
Rice	Straw	331 563	125 719
	Husk		62 198
Tobacco	Stalks	181 382	246 467
Cotton	Stalks	2 550 000	1 512 169
	Ginning Residues		585 776
Sunflower	Stalks	1.118.000	1 355 472
Groundnuts	Straw	25 167	
	Shell		22 910
Soybeans	Straw	15 064	13 123

The complete utilization of the main fractions of agro-industrial and forest wastes can be accomplished by a variety of strategies involving both chemical and biotechnological processing. Owing to their chemical composition, they show great potential as a renewable raw material for producing a variety of value added chemicals (Yoon *et al.*, 2006).

1.1.1 Cotton Stalk

Cotton is one of the most important agricultural crops of the country and Turkey is also one of the 8 countries producing 85% of the world's cotton. Today, the annual production of cotton stalks (CS) in Turkey is 2.550 million t (State Planning Organization, Prime Ministry, TR, 2007) and gradually will increase to about 4.86 million t by 2020 with the completion of the GAP (South-eastern Anatolian Project) (Pütün *et al.*, 2005).

Cotton is cultivated primarily for textile fibers, and there is not any usage of the cotton plant stalk and its storage is a problem. The CS is plagued with parasites, and stored stalks can serve as a breeding ground\ for the parasites to winter over for next year's crop. Until now, CS has not been effectively utilized in value- added products in spite of the large annual amounts produced; collection and storage of CS limits converting of these residues as raw material into useful products. The chopped CS can be a combined solution to the following problems:

(1) Lack of energy and gradual expansion of energy needs.
(2) Emissions of greenhouse gases like CO_2.

(3) Parasite sources in cotton fields; after the seed cotton collection, the chopped cotton stalks are gathered in bulks, and gradually become parasite sources, so the farmers usually burn them uncontrollably.
(4) Unemployment of people in rural areas after the seed cotton harvesting and collection (Tatsiopoulos and Tolis, 2003).

CS, as wood and agricultural residues, consists of cellulose, lignin, and hemicellulose polymers. The lignocellulosic characteristic of CS makes it a promising resource for converting to value-added products thereby providing a solution to these problems and benefiting both the environment and economy.

The CS has about 25% lignin, 37.9% cellulose, 20.4% hemi-cellulose, 4% extractives, and 4% ash (Fahmy et al., 2000). However, there is no information reported on literature about sugar composition of cotton stalk xylan. The cotton-seed xylan sugar composition has been performed and it has been found that the cotton-seed xylan is a glucuronoxylan with a simple chemical structure containing 64.7% xylose and 9.4% uronic acid. Table 1.2 shows the composition of cotton-seed xylan compared with other xylans. The total sugar content of cotton-seed xylan was 75%, and the value was almost the same as those of birchwood and oat-spelt xylans. By contrast, oat-spelt xylan contained not only xylose and uronic acid but also arabinose and glucose (Sun *et al.,* 2002).

Table 1.2 Sugar composition of three kinds of xylans (Sun *et al.,* 2002).

Source of xylan	Sugar content (%)	Component sugars (%)[a]			
		Xyl	Ara	Glc	Uronic acid
Cotton seed	75.0	64.7	-	-	9.4
Birchwood	72.0	56.0	-	-	10.3
Oat spelt	73.1	52.8	6.7	7.3	1.8

[a] -, not detected.

1.1.2 Sunflower Stalk

The amount of sunflower (*Helianthus* spp.) produced annually in Turkey is estimated to be about 1.118.000 tons (State Planning Organization, Prime Ministry, TR, 2007). It has been reported that sunflower is the fourth most important source of oil-seeds worldwide, with a production of 2.518×10^7 metric tons of seeds in 1995/1996 (Marechal and Rigal, 1999). The main lignocellulosic residues of sunflower are its stalk and head. Sunflower heads consist of three main parts: the neck (part between head and stalk), which is not readily separated at harvest, the bract (lower part) and the pith (core of head). These waste materials are either left in the fields or burnt in both Turkey and in many other countries. Moreover, the burning of heads and stalks results in large amounts of smoke (containing CO_2) and is not environmentally friendly.

For this reason, residues of sunflower are considered as low cost and abundant raw materials for different purposes (Iglesias and Lozano, 2003). Crop residues can inhibit growth of succeeding crops by the release of substances contained in the residues or produced when they decompose. These inhibitory phenomena belong to a widespread group of plant and microbial relationships referred to as allelopathy. Sunflower has been known to be allelopathic, affecting old-field succession (Kaya *et al.*, 2006).

Sunflower stalk (SS) typically consist of cellulose (38%), hemicelluloses (29%) and lignin (11%) (Jimenez and Bonilla, 1993).

1.2 Lignocellulosic Biomass

The structural materials that plants produce to form the cell walls, leaves, stems, stalks, and woody portions of biomass are composed mainly of three biobased chemicals called cellulose, hemicellulose, and lignin. Together, they are called lignocellulose, a composite material of rigid cellulose fibers embedded in a cross-linked matrix of lignin and hemicellulose that bind the fibers. Lignocellulose plant structures also contain a variety of plant-specific chemicals in the matrix, called extractives (resins, phenolics, and other chemicals), and minerals (calcium, magnesium, potassium, and others) that will leave ash when biomass is burned. Some variation in the composition can be seen among different species and within the same species depending on environmental and genetic variability. For example, in softwood and hardwood in general, the contents of the main components vary in the ranges shown in Table 1.3. (Sjöström, 1993).

Table 1.3 Contents of the main components, % of dry wood (Sjöström, 1993).

	Cellulose	Glucomannan	Xylan	Other polysaccharides	Lignin
Softwood	33-42	14-20	5-11	3-9	27-32
Hardwood	38-51	1-4	14-30	2-4	21-31

The chemical properties of the components of lignocellulosics make them a substrate of enormous biotechnological value. Large amounts of lignocellulosic "waste" (Table 1.4) are generated through forestry and agricultural practices, paper-pulp industries, timber industries and many agroindustries and they pose an environmental pollution problem. However, the huge amounts of residual plant biomass considered as "waste" can potentially be converted into various different value added products including biofuels, chemicals, cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard *et al.*, 2003).

Table 1.4. Types of lignocellulosic materials and their current uses (Howard *et al.*, 2003).

Lignocellulosic Material	**Residues**	**Competing Use**
Grain harvesting		
Wheat, rice, oats barley and corn	Straw, cobs, stalk, husks	Animal feed, burnt as fuel, compost
Processed grains		
Corn, wheat,rice, soybean	Waste water, bran	Animal feed
Fruit and vegetable harvesting	Seeds, peels, husks, shells, stones	Animal and fish feed
Fruit and vegetable processing	Seeds, peel, waste water, husks, shells, stones	Animal and fish feed
Sugar cane other sugar products	Baggase	Burnt as fuel
Oils and oilseed plants Nuts, cotton seeds, plives	Shells, husks, lint, fibre, sludge, prescake	Animal feed, fertiliser, burnt fuel
Animal Waste	Manure, other waste	Soil conditioner
Foresty-paper and pulp		
Harvesting of logs	Wood residuals, barks	Soil conditioner burnt
Saw-and plywood waste	Woodchips, wood shavings, saw dust	Pulp and paper industries
Pulp and paper mills	Fiber waste, sulphite liquor	Reused in pulp and board industry as fuel

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. 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. 1.2) (Pe´rez *et al.*, 2002).

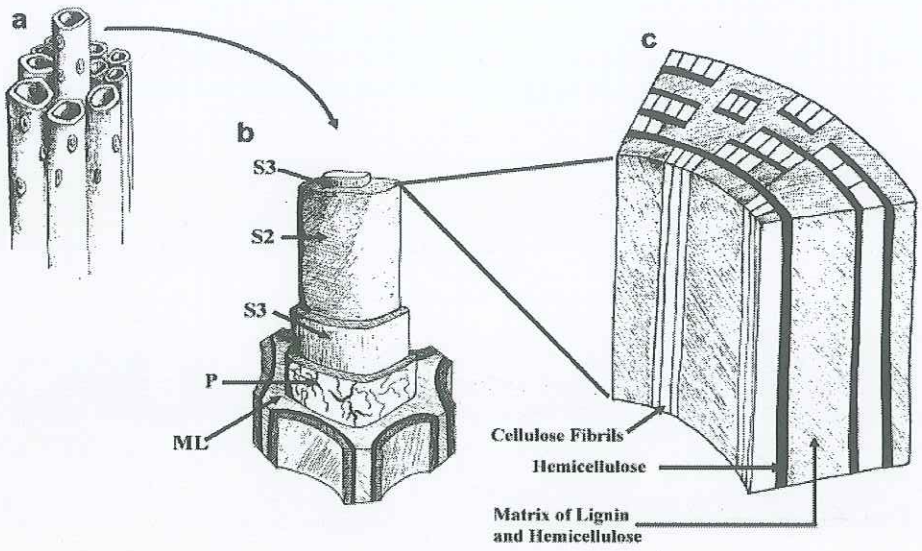


Figure 1.2 a–c. Configuration of wood tissues. a: Adjacent cells, b: cell wall layers. S1, S2, S3 Secondary cell wall layers, P primary wall, ML middle lamella. c: Distribution of lignin, hemicellulose and cellulose in the secondary wall (Pe´rez *et al.*, 2002).

1.2.1 Lignin

Lignin typically ranges from 5 to 30% of plant dry weight (Lynd *et al*, 2002). It performs important functions in the life of plant as a permanent bonding agent imparting rigidity, protecting the hemicelluloses and cellulose from microbial degradation, also play roles as an antioxidant, and a water-proofing agent. There is no single repeating bond between the subunits but a random distribution of at least 10 different types of bond, the most common being the β-aryl ether (β-O-4) bond. This complicated structure of high molecular weight (about 100 kDa) and with no-hydrolysable bonds prevents lignin uptake inside the microbial cells. Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes (Kuhad *et al.*, 1997).

Structurally, lignin is a non-water soluble and optically inactive amorphous heteropolymer. The polymer is synthesized by the generation of free radicals, which are released in the peroxidase-mediated dehydrogenation of three phenyl propionic alcohols: coniferyl alcohol (guaiacyl propanol), coumaryl alcohol, and sinapyl alcohol (syringyl propanol). Coniferyl alcohols are the principal component of softwood lignins, whereas guaiacyl and syringyl alcohols are the main constituents of hardwood lignins. The final result of this polymerization is a heterogeneous structure whose basic units are linked by C-C and aryl-ether linkages, with aryl-glycerol β-arylether being the predominant structure (Figure 1.3) (Pe´rez *et al*, 2002). Grass lignins contain guiacyl-, syringyl-, and *p*-hydroxyphenyl-units. (Jeffries, 1994).

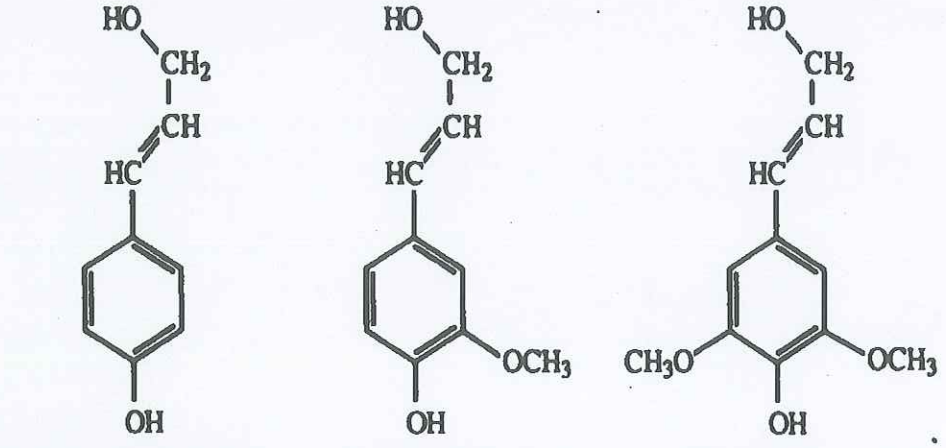


Figure 1.3. Lignin building blocks (Pe´rez *et al.*, 2002).

1.2.2 Cellulose

Cellulose typically ranges from 35 to 50% of plant dry weight (Lynd *et al.*, 2002). Cellulose is a linear homopolymer, with a degree of polymerization (DP) of up to about 15,000 units, made of glucose subunits linked by β-1,4 glucosidic bonds (Kuhad *et al.*, 1997). The additive effect of the bonding energies of the hydrogen bonds increases the rigidity of cellulose and causes it to be highly insoluble as well as highly resistant to most organic solvents (Figure 1.4) (Sjöström 1993).

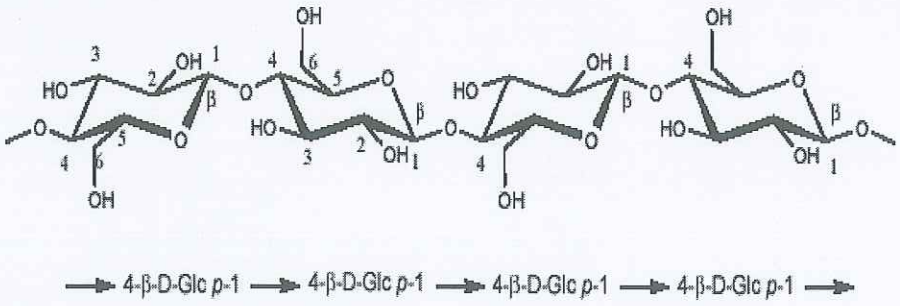


Figure1.4. Structure of cellulose (Sjöström 1993).

Each glucose residue is rotated by 180° relative to its neighbors, so that the basic repeating unit is in fact cellobiose (β - 1, 4-D-glucosyl-D-glucose) (Béguin and Aubert, 1994). Cellulose is classified due to its different intermolecular hydrogen bonding patterns as α (the form of cellulose insoluble in 17.5% NaOH) and β (the form of cellulose soluble in 17.5% NaOH) forms (Kuhad *et al.*, 1997). Cellulose is synthesized in nature as individual molecules. Approximately 30 individual cellulose molecules are assembled into larger units known as protofibrils, which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibers. Hemicellulose and lignin cover microfibrils. An important feature of cellulose is its crystalline structure. Cellulose chains form numerous intra and intermolecular hydrogen bonds, which account for the formation of rigid, insoluble microfibrils. The chains are oriented in parallel and form highly ordered, crystalline domains interspersed by more disordered, amorphous regions (Béguin and Aubert, 1994). Although cellulose forms a distinct crystalline structure, cellulose fibers in nature are not purely crystalline,

and its degree of crystallization varies among species. As crystallinity increases, cellulose becomes increasingly resistant to further hydrolysis (Kuhad *et al.*, 1997).

1.2.3 Hemicellulose

Hemicelluloses are heteropolysaccharides that typically range from 20 to 35% of plant dry weight. Hemicelluloses are composed of complex carbohydrate polymers (DPs of 100 to 200 units) with xylans and mannans as the main components. D-xylose and L-arabinose are the major components of the pentosans (xylans), while D-glucose, D-galactose, and D-mannose are the constituents of the hexosans (mannans). The major class of hemicelluloses is xylans. Xylans are characterized by the presence of L-arabinose (in greases and cereals) or 4-O-methyl-D-glucoronic acid, L-arabinose or acetyl groups (in soft and hardwoods) as a single unit substitute to a D-xylose backbone. There have been observations which suggest that cellulose is protected from enzymatic attack by xylan and mannan. Also, it has been shown that some hemicelluloses (mainly xylan units) are linked by covalent bonds to lignin (Kuhad *et al.*, 1997).

The main difference with cellulose is that hemicellulose has branches with short lateral chains consisting of different sugars. In contrast to cellulose, they are easily hydrolyzable polymers. They do not form aggregates, even when they are co-crystallized with cellulose chains (Pe´rez *et al.*, 2002).

1.2.3.1 Xylan

Xylan polymer consists of a β-D-1,4- linked D-xylose backbone substituted with acetyl, arabinosyl, and glucuronosyl side chains. The composition of xylan shows difference depending on its origin (Table 1.5) (Saha, 2000).

Table 1.5. Composition of xylan from different sources (Saha, 2000).

Xylan Composition	Birchwood Xylan	Rice Bran Xylan	Wheat Xylan
Xylose (%)	89.3	46.0	65.8
Arabinose (%)	1.0	44.9	33.5
Glucose (%)	1.4	1.9	0.3
Galactose (%)	0	6.1	0.1-0.2
Mannose (%)	0	0	0.1-0.2
Anhydrouronic acid (%)	8.3	1.1	0
Glucouronicacid (%)	0	0	0

The principal component of hardwood hemicellulose is glucuronoxylan. The glucuronoxylan of most hardwoods and graminaceous plants consists of a main β-(l-4)-D-xylan backbone with α - (1-2)-4-*O*-methyl-D-glucuronic acid (or α -(1-2)-D-glucuronic acid) substituents on about 10% of the xylose residues (Figure 1.5). Softwood xylans and xylans from most graminaceous plants differ from hardwood xylans in that they have arabinofuranose units linked α -(1-3) to the xylan backbone (Figure 1.6). The glucomannoxylans (usually referred to as glucomannans and galactomannans) are made up of β -(1-4)-D-glucopyranose and β -D-mannopyranose residues in linear chains (Figure 1.7). Hardwood glucomannans consist of β -(l-4)-linked glucose and mannose units forming chains that are slightly branched. The ratio of mannose:glucose is about 1.5:1 or 2:1 in most hardwoods. Softwood glucomannans have occasional galactose side branches linked α -(1-6) to the mannose main chain (Figure 1.8). The α -(1-6) linkage of galactose is very sensitive to acid and alkali and maybe cleaved during alkaline extraction. Softwood xylans and xylans from most graminaceous plants have single L-arabinofuranosyl units attached through α linkages to some *O*-3 positions of the main chain. About 60% to 70% of the xylopyranosyl residues of hardwood xylans are acetylated through ester linkages at position 2 or 3 (Jeffries, 1994).

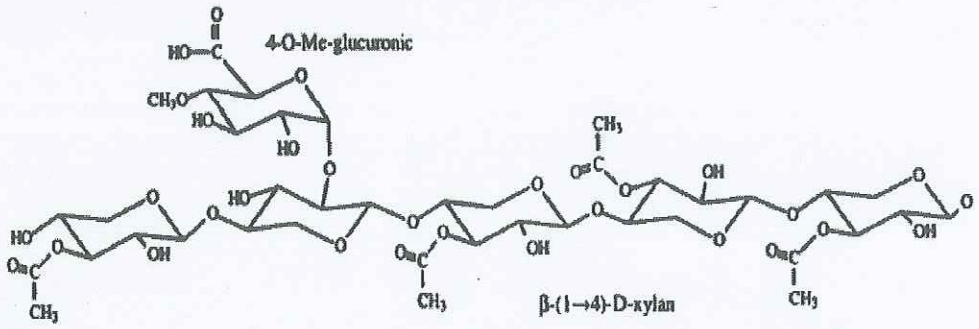


Figure 1.5. O-Acetyl4-O-methyl- D-glucuronoxylan from angiosperms (Jeffries, 1994).

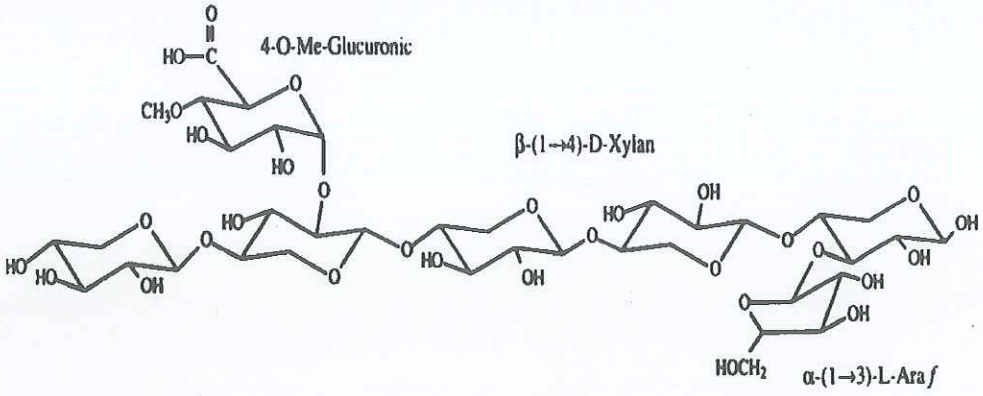


Figure 1.6. Arabino-4-O-methylglucuronoxylan from gymnosperm (Jeffries, 1994).

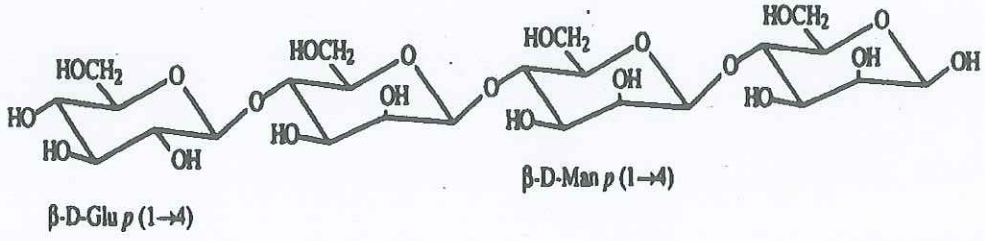


Figure 1.7. Glucomannan from angiosperms (Jeffries, 1994).

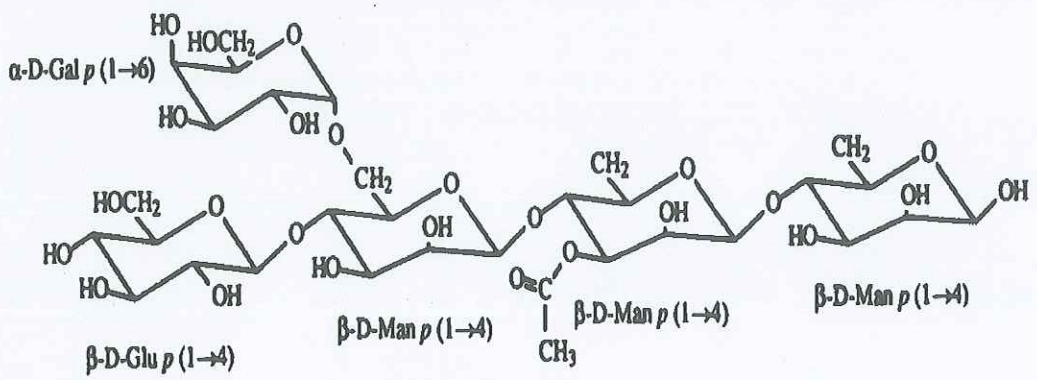


Figure 1.8. O-Acetylgalactoglucomannan from gymnosperm (Jeffries, 1994)

In spite of these general characteristics, the source from which the xylan is extracted strongly determines the specific features with regard to the type, the amount, position and distribution of substituents over the xylan-backbone. Furthermore, within one plant source, different populations of xylans may occur. For example, in arabinoxylan extracted from wheat flour rather high substituted populations as well as less substituted populations have been described (Figure 1.9). In contrast with the xylan from monocotyles, xylan from dicotyles (hard woods, herbs and woody plants) is an *O*-acetylated 4-*O*-methyl- α -D-glucuronoxylan almost without any arabinose substitution. On average every tenth xylosyl residue carries an α -4-*O*methylglucuronic acid residue substituted at the 2-*O*-position. *O*-acetyl substituents can be located at the 2-*O*- and/ or 3-*O*- positions of the xylosyl residues present. In general, the content of *O*-acetyl is 3-5 % (w/w) of the total

wood. As an example, the structural models of xylans extracted from various sources are presented in Figure 1.9 (Gruppen *et al.*,1993) (Verbruggen *et al.*, 1998).

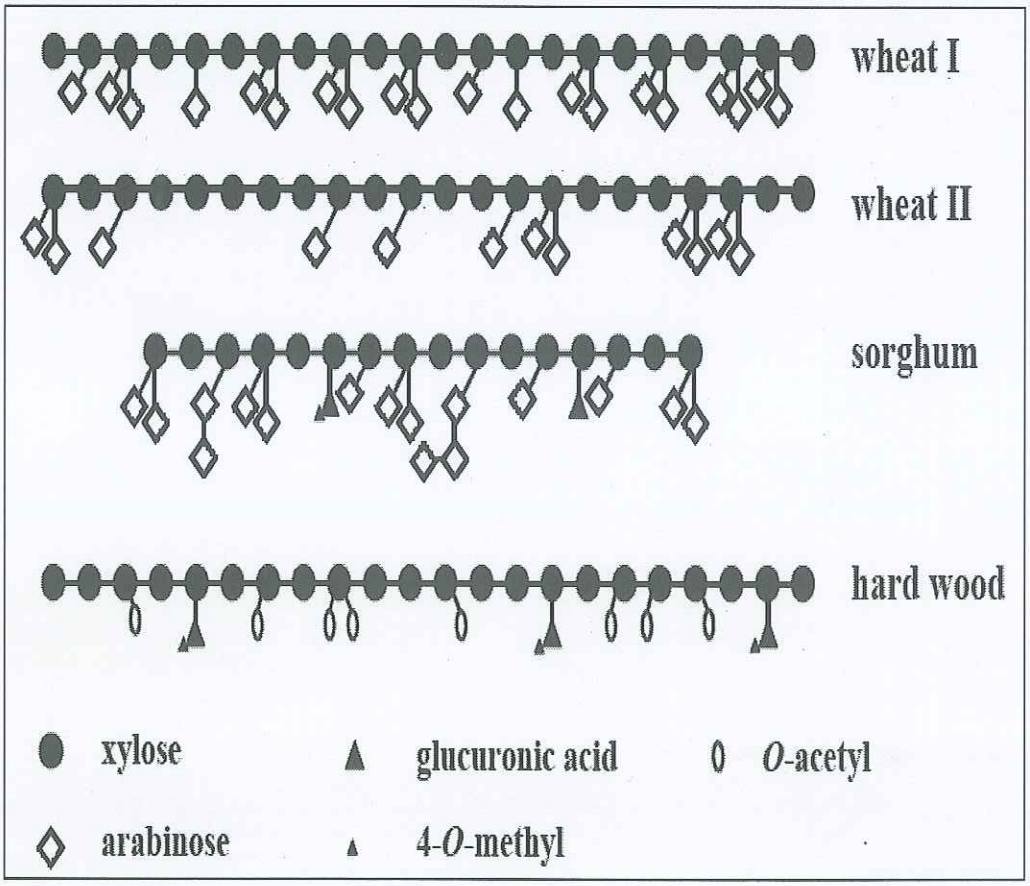


Figure 1.9. Compilation of structural models for xylans extracted from wheat, sorghum and hardwood (Gruppen *et al.*,1993).

1.3. Utilization of Lignocellulosic Biomass

Lignocellulosics biomass is an underutilized renewable resource that is produced in large amounts. Lignocellulosics are poorly biodegradable; therefore some type of pretreatment processing is necessary to disrupt its natural chemico-physical barriers thereby increasing its biodegradability to obtain novel products. After pretreatment, the utilization of lignocellulosic material in agricultural and industrial processes can be enhanced by treatment with enzymes (Brownell and Saddler, 1987).

1.3.1 Pretreatment Methods for Lignocellulosic Biomass

A pretreatment step is essential to effectively prepare lignocellulosic material for enzymatic hydrolysis with high yields. Pretreatment of lignocellulosic biomass is necessary to obtain high sugar yields by enzyme catalysis. An effective pretreatment disrupts cell wall physical barriers as well as cellulose crystallinity and the association with lignin so that hydrolytic enzymes can access the biomass macrostructure. Schematic of goals of pretreatment on lignocellulosic material is given in Figure 1.10 (Mosier *et al.*, 2005). Although many biological, chemical, and physical methods have been tried over the years, pretreatment advances are still needed for overall costs to become competitive with conventional commodity fuels and chemicals (Wyman, 1999).

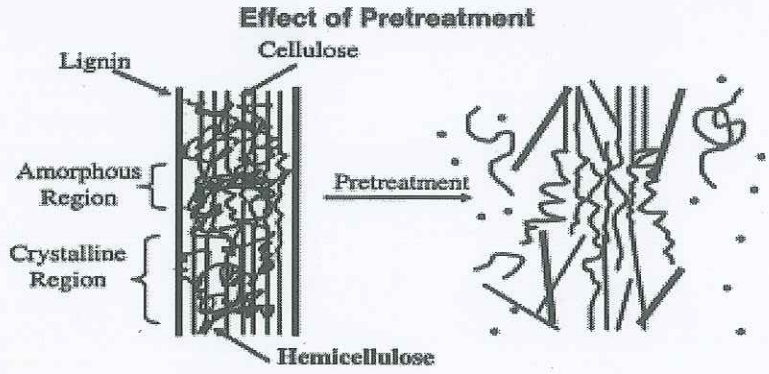


Figure 1.10. Schematic of goals of pretreatment on lignocellulosic material (Mosier *et al.*, 2005).

The pretreatment process itself utilizes pretreatment additives and/or energy to form solids that are more reactive than native material and/or to generate soluble oligo- and monosaccharides (Figure 1.11) (Mosier *et al.*, 2005).

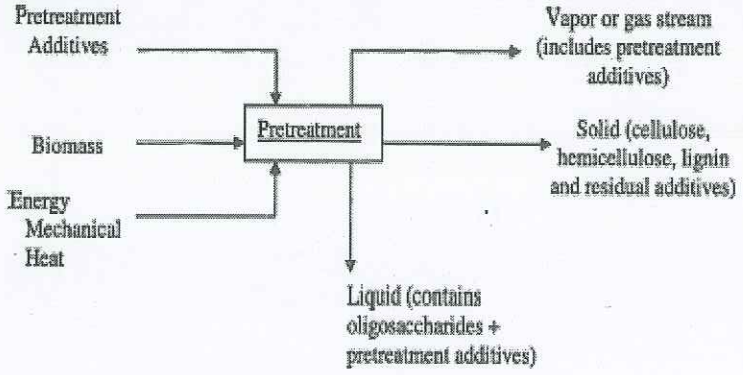


Figure 1.11. Schematic of pretreatment process (Mosier *et al.*, 2005).

There are several pretreatment methods that can expose the polysaccharide components to enzymatic hydrolysis, including high-concentration alkaline extraction, low-concentration alkaline treatment, acidic pretreatment and cooking. In particular, pretreatment by dilute sulfuric acid, with pH control, by ammonia, and with lime appear among the most promising options, with favorable processing conditions summarized for each in Table 1.6 (Wyman *et al.*, 2005).

Table 1.6. Technologies and representative reaction conditions for biomass preparation by pretreatment (Wyman *et al.*, 2005).

Pretreatment Technology	Chemicals used	Temperature °C	Pressure atm absolute	Reaction times min	Concentrations of solids, wt%
Dilute sulfuric acid-cocurrent	0.53% sulfiric acid	130-200	3-15	2-30	10-40
Flowthrough pretreatment	0-0.1% sulfuric acid	190-200	20-24	12-24	2-4
pH controlled water pretreatment	Water or stillage	160-190	6-14	10-30	5-30
AFEX/FIBEX	100% (1:1) anhydrous ammonia	70-90	15-20	<5	60-90
ARP	10-15% ammonia	150-170	9-17	10-20	15-30
Lime	0.05-0.15 g $(CaOH)_2$/g biomass	70-130	1-6	1-6 h	5-20

Dilute-acid (~0.5–1.0% sulfuric) at moderate temperatures (~140–190°C) effectively removes and recovers most of the hemicellulose as dissolved sugars, and glucose yields from cellulose increase with hemicellulose removal to almost 100% for complete hemicellulose hydrolysis (Knappert *et al.*, 1981). Although little lignin is dissolved, data suggest that lignin is disrupted, increasing cellulose susceptibility to enzymes (Yang and Wyman, 2004). Use of SO_2 enhances yields in a way similar to dilute sulfuric acid, but many currently prefer dilute sulfuric acid because it is cheap. Through dilute sulfuric acid, up to 90% hemicellulose yields are achieved, and enzymatic hydrolysis yields of glucose can be over 90%. Co-current/ batch reactors are typical, but percolation reactors reduce times for sugars to degrade. Forcing liquid through a packed biomass bed enhances hemicellulose and lignin removal rates and gives high yields of hemicellulose and cellulose sugars even without acid addition. However, percolation or flowthrough are challenging to implement commercially, and the high amounts of water used result in high energy requirements for pretreatment and product recovery (Liu and Wyman, 2003).

A process known as ammonia recycle percolation passes aqueous ammonia (10–15 wt %) through biomass at elevated temperatures (150–170°C) and then recovers it for recycle. At high temperatures, aqueous ammonia swells biomass, depolymerizes lignin, and breaks lignin–hemicellulose bonds without degrading carbohydrates (Kim *et al.*, 2003).

Pretreatment with lime increases pH and provides a low-cost alternative for lignin removal. Typical lime loadings are 0.1 g $Ca(OH)_2$/g biomass. A minimum of about 5 g H_2O/g biomass is required. Additional water can

be added, but it is neither helpful nor harmful. Lime pretreatment can be performed at a variety of temperatures, ranging from 25 to 130°C, and the corresponding treatment time ranges from weeks (25°C) to hours (130°C) (Chang *et al.*, 1998).

In AFEX pretreatment, biomass is treated with liquid anhydrous ammonia at moderate temperatures (60–100 ° C) and high pressure (250–300 psi) for 5 min. Then, the pressure is rapidly released. In this process the combined chemical and physical effects of lignin solubilization, hemicellulose hydrolysis, cellulose crystallization, and increased surface area, enables near complete enzymatic conversion of cellulose and hemicellulose to fermentable sugars (Teymouri *et al.*, 2005).

Pretreatment under mild operational conditions (145±190°C, liquor to solid ratio 6±10 g /g, reaction times up to 7.5h) has also been performed without adding acid that is often called autohydrolysis, but hemicellulose sugar yields are lower than when acid is added and hemicellulose sugars are primarily in oligomeric form (Garrote *et al.*, 1999).

Fungal pretreatment, another important preatment method, could potentially lower the severity requirements of acid, temperature and time. These reductions in severity are also expected to result in less biomass degradation and consequently lower inhibitor concentrations compared to conventional thermochemical pretreatment. Furthermore, potential advantages of fungal pretreatment of agricultural residues, such as corn stover, are suggested by its effectiveness in improving the cellulose digestibility of many types of forage fiber and agricultural

wastes. Keller *et al.* showed a three- to five-fold improvement in enzymatic cellulose digestibility of corn stover after pretreatment with *Cyathus stercoreus* (Keller *et al.*, 2003) .

1.3.2 Degradation of Lignocellulosic Biomass

Most of the fungi able to produce the necessary enzymes for the degradation of lignocellulosic materials belong to the Ascomycetes, Deuteromycetes, or Basidiomycetes groups. Fungi living on dead wood that preferentially degrade one or more of the wood components by secretion of synergic cellulolytic enzymes to the surrounding medium, cause three types of wood rot (soft rot, brown rot and white rot). Fungi causing soft-rot and brown-rot type of decay, efficiently attacks wood carbohydrates but modify lignin only to a limited extent. White-rot fungi (most Basidiomycetes) are the only wood-rotting fungi which, to any extent, can attack all the components of plant cell walls (Kuhad *et al.*, 1997).

When compared to fungi, bacteria generally degrade wood slowly due to the "lack" of penetrating ability and to the requirement of surfaces with high moisture content. However, some bacteria have been found to degrade lignified plant cells. Rumen bacteria are major degraders of plant fibers (e.g. some bacteria of genus *Ruminococcus, Clostridium, Fibrobacter*, etc.). These bacteria have a complete set of polysaccharide-degrading enzymes and also the ability to adhere to fibers using cell surface protuberance known as cellulosome.

Some actinomycetes, different species of *Streptomyces*, also actively degrade lignocellulosic plant materials (Kuhad *et al.*, 1997).

A large number of microorganisms have the ability to produce cellulose-degrading enzymes. However, relatively few are capable of producing all the necessary enzymes for degradation of crystalline cellulose. The hydrolytic breakdown of cellulose in nature is catalysed by the extracellular enzymes cellobiohydrolases, endoglucanases and β-glucosidases (Lynd, 2002). Glucose and cellodextrins are the products of cellulose hydrolysis. *Trichoderma reesei* is an important fungus that produces at least three endoglucanases, two exoglucanases and two β-glucosidases (enough for degradation of crystalline cellulose) (Kuhad *et al.*, 1997).

According to Kuhad (1997), the cellulolytic enzyme systems of bacteria are not directly comparable to those of fungi. The main reasons are that bacteria often produce cellulases in small amounts and degradation of cellulose takes place by a cluster of multyenzyme complexes that are difficult to disrupt without loss of total activiy. The most studied bacteria with respect to their cellulose system are species of *Clostridium*, *Cellulomonas*, *Bacillus* and *Pseudomonas*.

Because of complex structure of hemicellulose, several different types of enzymes are required for the complete enzymatic degradation of these heteropolymer. Among these, the two major hemicellulose-degrading enzymes are endo-1,4-β-D-xylanase and endo-1,4-β-D-mannanase. Xylobiose, xylotriose and xylose are the major end

products of hemicelluloses hydrolysis. Hemicelluloses are, relative to cellulose, easily hydrolyzed (Kuhad *et al.*, 1997).

In the complex ligninolytic enzyme system, peroxidases, laccases, and H_2O_2-producing oxidases are the most studied. Peroxidases and laccase are defined as phenol oxidase. The lignin-degrading enzymes known so far are extracellular and nonspecific, participating in different oxidative reactions where the aromatic structure of lignin and bonds between the basic units are broken. The reactions catalyzed by these enzymes are very similar. They oxidize phenolic compounds, thereby creating phenoxy radicals, while non-phenolic compounds are oxidized to the corresponding cation radicals. Some of the substrates routinely assayed to determine ligninolytic activity are veratryl alcohol, vanillylacetone, phenol red, ferulic, syringic and vanillic acids and vanillin (Kuhad *et al.*, 1997).

1.4 Xylanases

Due to xylan heterogeneity, the enzymatic hydrolysis of xylan requires different enzymatic activities. Two enzymes, β-1,4-endo-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37), are responsible for hydrolysis of the main chain, the first attacking the internal main-chain xylosidic linkages and the second releasing xylosyl residues by endwise attack of xylooligosaccharides (XOs). These two enzymes are the major components of xylanolytic systems produced by biodegradative microorganisms such as *Trichoderma*, *Aspergillus*, *Schizophyllum*, *Bacillus*, *Clostridium* and *Streptomyces* species. For complete hydrolysis of the molecule, side-chain cleaving enzyme activities are also necessary. The side groups present in xylan are liberated by α-L

arabinofuranosidase, α –D glucuronidase, galactosidase and acetyl xylan esterase (Subramaniyan and Prema, 2002). Ruminal microorganisms are also known to be potent xylanase producers, possibly due to the high dietary hemicellulose content of the feed of ruminant animals (Kulkarni *et al.*, 1999).

Xylanases have several different industrial applications which includes biodegradation of lignocellulose in animal feed, foods, and textiles, as well as biopulping in the paper and pulp industry (Bakir *et al.*,2001). In the pulp and paper industry, xylanases enhance the bleaching of pulp, thereby decreasing the amount of chlorine-containing compounds in the process and the subsequent discharge of organochlorines in the effluent. In the food industry, xylanase enzymes are used to accelerate the baking of cookies, cakes, crackers, and other foods by helping to break down polysaccharides in the dough. In animal feeds, xylanase aids in the digestibility of wheat by poultry and swine, by decreasing the viscosity of the feed (Li *et al.*, 2000).

In most of the industrial applications, especially paper and pulp industries, low pH is required for the optimal growth and activity of xylanase. There has been increased use of xylanase preparations having an optimum pH < 5.5 produced invariably from fungi. The optimum pH for xylan hydrolysis is around 5 for most of the fungal xylanases although they are normally stable at pH 3 – 8 (Subramaniyan, and Prema, 2002). The pH optima of bacterial xylanases are in general slightly higher than the pH optima of fungal xylanases (Khasin, *et.al.*, 1993). The optimum temperature for endoxylanase from bacterial and fungal sources varies between 40 and 60°C. Fungal xylanases are generally less thermostable than bacterial xylanases (Kulkarni *et al.*, 1999).

Endoxylanases and β-xylosidases as well as their encoding genes, have been characterized from many organisms. Various endoxylanases have been identified in *Aspergillus.* Although variation is detected in their molecular mass or pH optimum, the major difference between the enzymes is in their pI, which ranges from 3.5 to 9.0. Endoxylanases also differ in their specificity toward the xylan polymer. Some enzymes cut randomly between unsubstituted xylose residues, whereas the activity of other endoxylanases strongly depends on the substituents on the xylose residues neighboring the attacked residues. Purification and characterization of endo-1,4-β-xylanase from *Aspergillus niger* str. 14. resulted mainly in xylobiose, xylotriose, and xylose, but hydrolysis of an arabinoxylan by the same enzyme resulted mainly in oligosaccharides with a degree of polymerization of more than 3. This suggests that the action of this endoxylanase is reduced by the presence of arabinose residues on the xylan backbone (Vries and Visser, 2001).

1.5 Xylooligosaccharides

Xylooligosaccharides (XOs) are sugar oligomers made up of xylose units and naturally present in fruits, vegetables, bamboo, honey and milk. XOs can be produced from agricultural wastes of lignocellulosic nature such as hardwoods, brewery spent grain, corn cobs, and wheat bran. The structures of obtained XOs depend on the structural features of the xylan originally present in the byproducts used (Alonso *et al.*, 2003) (Kabel *et al.*, 2002). Three different approachs have been used for XO production from these feedstocks:

a. Enzyme treatments of native, xylan-containing lignocellulosic material
b. Chemical fractionation of a suitable lignocellulosic material for isolation (or for solubilization) of xylan, with further enzymatic hydrolysis of this polymer to XOs; and
c. Hydrolytic degradation of xylan to XOs by steam, water or dilute solutions of mineral acids.

Figure 1.12a shows the production of XOs by combined chemical-enzymatic methods in which xylan (or soluble xylan fragments) is obtained from lignocellulosic materials by alkali treatment (with solutions of NaOH, KOH, $Ca(OH)_2$, ammonia or a mixture of these compounds). The processing of xylan-containing lignocellulosic materials in alkaline media is favoured by the pH stability of this polymer, and the solubilized fraction can be recovered from liquors by further processing. In some cases, the raw material has been pretreated with oxidizing agents, salts or alcohols to remove lignin. In case the xylan has been solubilized in caustic liquors, precipitation with organic compounds (including acids, alcohols or ketones) allows the recovery of dissolved hemicelluloses and hemicellulose-degradation products. When the xylan has been isolated or degraded to extracted, further DP reduction can be accomplished by hydrolysis with xylanases (Vazquez *et al.*, 2000).

During enzymatic production of XOs, low exo-xylanase and/or β-xylosidase activity are desired to prevent the production of xylose. The enzymes can be directly added to the reaction media, immobilized or produced in situ by microorganisms (Pellerin *et al.*, 1991). Alternatively, XOs can be produced from lignocellulosic materials in a single step by reaction with steam or water through hydronium-catalysed degradation of xylan, according to the procedure known as autohydrolysis,

hydrothermolysis or water prehydrolysis (Figure 1.12b). Acid prehydrolysis can be used for hydrolytic degradation of hemicelluloses but in this case the oligosaccharides behave as reaction intermediates and the main reaction products are monosaccharides. Aside from the degradation of xylan, several side-processes take place in such kinds of treatment, including extractive removal, solubilization of acid-soluble lignin and neutralization of ash. All of them contribute to the presence of comparatively high concentrations of undesired, non-saccharide compounds in liquors from hydrothermal processing. Because of this, the purification of XOs is of major importance. In order to simplify the purification of XOs, the feedstock can be pretreated before the water treatment (Figure 1.12b). Usually, mild operational conditions are preferred in order to achieve high extent of xylan solubilization (to give XOs with DP typically ≤ 20). If the final distribution of molecular weights is unfavourable for the desired application (for example, for food-related purposes), further DP reduction can be achieved by enzymatic treatments (Vazquez *et al.*, 2000).

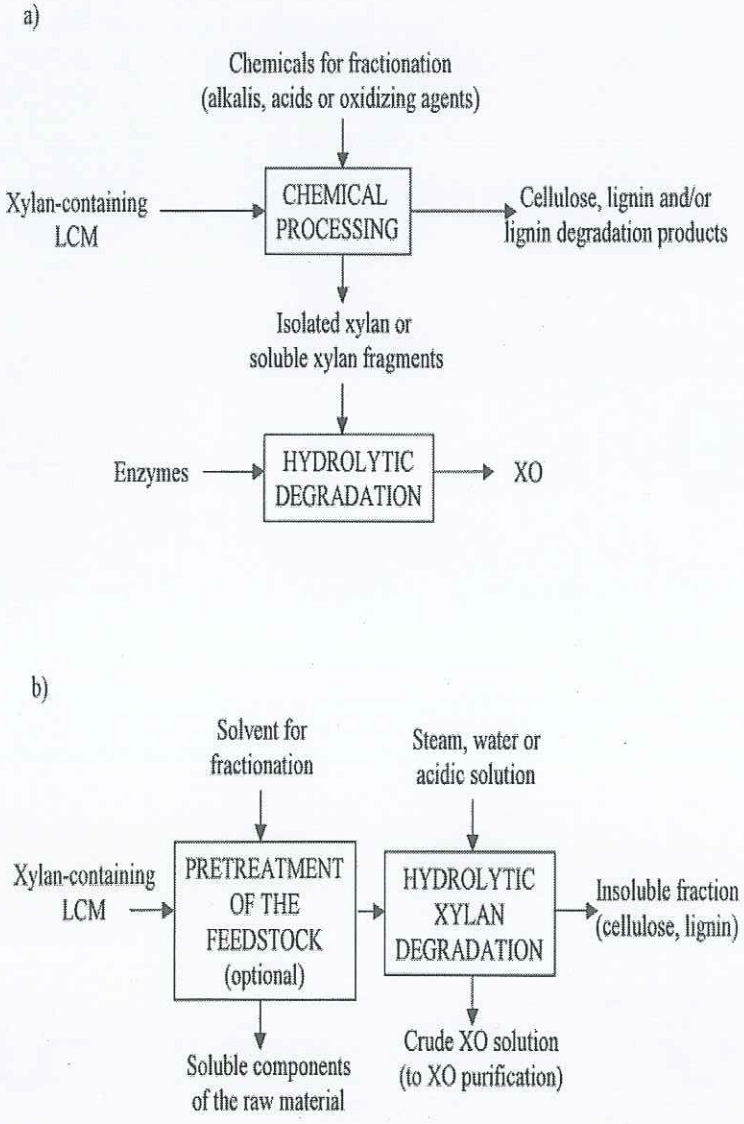


Figure 1.12. XO manufacture methods (a) General idea of XO manufacture by chemical enzymatic methods. (b) Procedure for XO manufacture by hydronium- catalyzed process (Vazquez *et al.*, 2000).

In order to remove undesired compounds and/or to select XOs within a given DP range from crude liquors, many refining strategies have been used. Vacuum evaporation might be the first stage in the processing of crude oligosaccharide solutions. During this stage, expected increase in concentration and the removal of acetic acid and flavours or their precursors occur (Vazquez *et al.*, 2000). Membrane techniques are also used for the separation of XOs within a given degree of depolymerization range from the undesired degree of depolymerization range and non-saccharide compounds (Crittenden and Playne, 1996). Adsorption (using adsorbents such as activated charcoal, acid clay, bentonite, diatomaceous earth, aluminium hydroxide or oxide, titanium, silica and porous synthetic materials) is another method used for purification of XO-containing liquors. In the first stage, XOs are retained by charcoal, and the degree of depolymerization range of the fractions coming from ethanol elution depends on the alcohol concentration, allowing a fractionation of the XOs on the basis of their molecular weight (Vazquez *et al.*, 2000).

XOs show a remarkable potential for practical utilization in many fields, including pharmaceuticals, feed formulations and agricultural applications, but their most important market developments correspond to food-related applications (for example, in combination with soya milk, soft drinks, tea or cocoa drinks, nutritive preparations, dairy products with milk, milk powder and yoghurts, candies, cakes, biscuits, pastries, puddings, jellies, jam and honey products, and special preparations for health food for elder people and children) or as active components of synbiotic preparations (Figure 13) (Vazquez *et al.*, 2000) (Moure *et al.*, 2006).

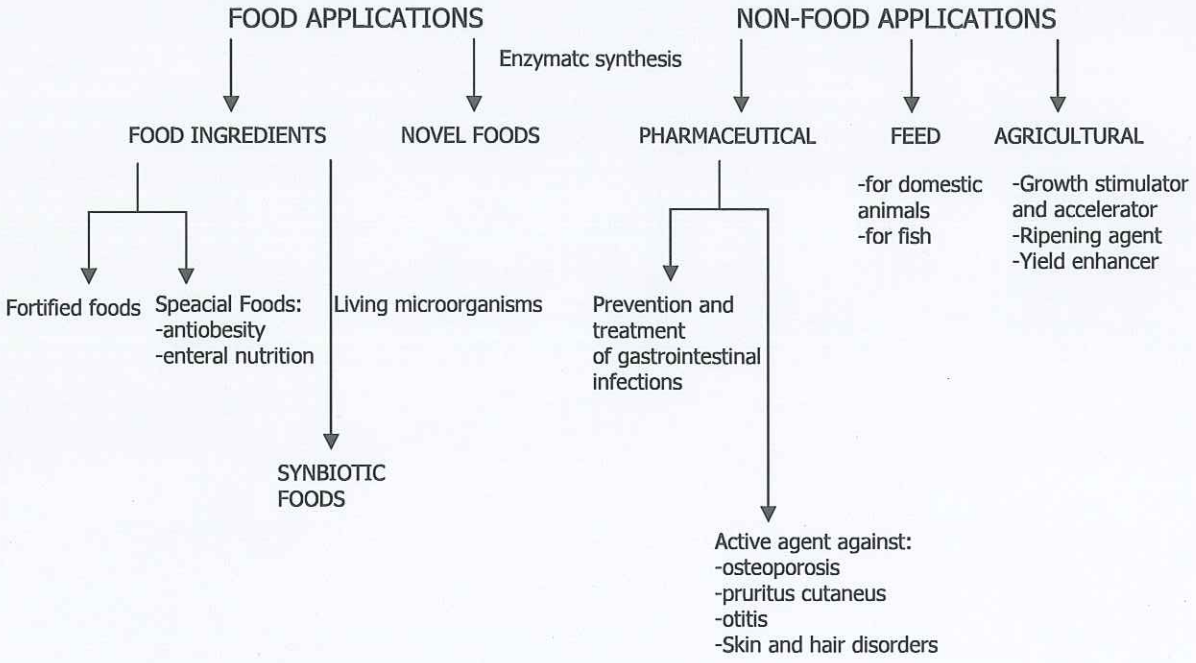


Figure 1.13. Applications of XOs (Vazquez *et al.*, 2000).

The most extensively studied function of XOs is their prebiotic activity. They have other glycosidic linkages than α - 1, 4-bonds and therefore are not hydrolyzed, or hydrolyzed very slowly by our intestinal enzymes (Taniguchi, 2004). From a nutritional point of view XOS usually are considered to be nondigestible oligosaccharides (NDOs), which are not degradable by the low-pH gastric fluid or by human and animal digestive enzymes and will therefore reach the large bowel intact (Zeng *et al.*, 2007).

The human gastrointestinal tract constitutes a complex microbial ecosystem comprising several hundred species of bacteria. The colon in particular is densely populated with more than 10^{11} bacteria per gram

of contents. The majority of bacterial species in the colon belongs to the genera *Bacteroides, Ruminococcus, Bifidobacterium, Eubacterium* and *Clostridium* and are able to ferment sugars. Within the intestinal microflora some of these bacteria are believed to be beneficial to the host while others are potentially pathogenic. One of the strategies to selectively increase the number of health promoting bacteria in the colon, mainly *Bifidobacterium* and *Lactobacillus*, is to supply them with oligosaccharides, which are not degraded in the upper gastrointestinal tract and are less assimilated by undesirable flora present (Gibson and Roberfroid, 1995). XOs are considered to specifically increase the number of (beneficial) bifidobacteria in the colon. In the large bowel the oligosaccharides can be fermented by the intestinal flora into mainly short chain fatty acids (acetate, propionate and butyrate), lactate, CO_2 and H_2 (Campbell, *et al.*, 1997). Both the production of short chain fatty acids and the increase in bifidobacteria are related with a number of health effects, e.g. bowel function, calcium absorption, lipid metabolism and reduction of the risk of colon cancer (Gibson and Wang, 1994). In vitro assays proved that *Bifidobacterium spp.* and *B. adolescentis* are able to utilize both xylobiose (X2) and xylotriose (X3), whereas a mixture containing X2 as the main component was utilized by *B. adolescentis, B. infantis* and *B. longum*. XOs are readily utilized by *B. bifidum*, and the oral ingestion of XO promotes the proliferation of *B. bifidum* in intestines. Contrarily, *Staphylococcus, Escherichia coli* and many *Clostridium* spp. cannot utilize XO (Vazquez *et al.*, 2000).

CHAPTER 2

LITERATURE SURVEY

2.1 Agricultural Waste

Biomass is a major source of energy in developing countries, where it provides 35% of all the energy requirements. Biomass materials with high energy potential include agricultural residues such as straw, bagasse, coffee husks and rice husks as well as residues from forest-related activities such as wood chips, sawdust and bark. Over three billion tonnes of agricultural residues were generated world-wide. The production of straw (from cereals like wheat, barley and oat) was distributed fairly uniformly world-wide, both in developing and developed countries, whereas the production of rice husks, bagasse and coffee husks is predominantly found in the developing countries. (Werthera, *et al.*, 2000).

Total energy consumption in Turkey in 2004 was 87.818 Mton. Of this amount, 67% was provided through imported resources such as petroleum, coal, and natural gas. Domestic and renewable energy resources accounted for 27% of the supplied demand, while wind, solar, hydro, and biomass energy accounted for 16.5%. Agricultural waste, which is included under the biomass energy classification, has

been used on a very small scale. However, Turkey has a great potential for supplying and applying agro waste and other renewable energy resources. By using these resources, Turkey can decrease not only its dependence on imported resources but also environmental emissions (Kaya *et al.*, 2008).

Various agricultural residues such as grain dust cotton stalk, sunflower heads and stalks are available in Turkey as the source of biomass energy and chemicals. The amount of sunflower produced annually in Turkey is estimated to be about 1.118.000 tons (State Planning Organization, 2007). The main lignocellulosic residues of sunflower are its stalk and head. These waste materials are either left in the fields or burnt. Moreover, the burning of heads and stalks results in large amounts of smoke (containing CO_2) and is not environmentally friendly. For this reason, residues of sunflower are considered as low cost and abundant raw materials for different purposes (Erzengin and Küçük, 1998).

Crop residues can inhibit growth of succeeding crops by the release of substances contained in the residues (Kaya, *et al.*, 2006). These inhibitory phenomena belong to a widespread group of plant and microbial relationships referred to as allelopathy. Sunflower has been known to be allelopathic, affecting old-field succession. Residues of cultivated sunflower have been shown to inhibit the growth of subsequent crops in rotation (Morris and Parrish, 1992)

Kaya and co-workers aimed to find the suitability of biodegradation products of sunflower heads as a fertilizer for some agricultural plants. This work was an attempt to decrease the allelochemical content and

increase the nutritive value of sunflower heads by biodegradation and to determine the effect of biodegradation products of sunflower heads on germination and growth of some plants. It was found that the use of biodegradation products of sunflower heads at low concentrations appears suitable for growth of agricultural plants. At least, the pollution effect and allelochemical effect of plant residues will reduce by such applications (Kaya *et al.*, 2006).

Various crop residues rich in lignocellulosics like wheat straw, rice straw, corn stalks and cobs, groundnut shells etc. have been exploited for ethanol production (Olsson and Hahn-Hagerdal, 1996; Bothast and Saha, 1997). In a study, ethanol production from pretreated and enzymatically saccharified SS was optimized in shake flasks and then production was performed in 15 l fermenter. The results showed that SS are an alternative lignocellulosic agricultural waste available in plenty for bioethanol production on a commercial scale (Sanjeev *et al.*, 2002).

SS, which have no specific use, are normally piled up and burnt. The potential energy of these residues can be transformed into other types of more useful and direct energy via physico-chemical or biochemical processes. Therefore, production of alcohols from SS by using hydrochloric and sulphuric acid at various concentrations and temperatures was studied. It was found that maximum yields were obtained in shorter times when using HCl, and these times decreased with increasing acid concentration and temperature (Jimenez and. Bonilla, 1993).

Marechal and Rigal (1999) investigated the developing of a new method for fractionating by-products of sunflower cultivation to find application for the non-oil by-products. They have drawn up a scheme for fractionation of byproducts of sunflower cultivation (Figure 2.1). It combines applications for the sunflower heads, the pith, and fibers from the stalks. Harvesting could be modified to enable recovery of heads along with the seeds in the first pass of the harvester. A second pass with the combine harvester with a lower cut would pick up the stalks. The fibers and pith could be separated with suitable cyclone type equipment. The resulting fractions could be utilized for the manufacture of cardboard, and low density agromaterials (Marechal and Rigal, 1999).

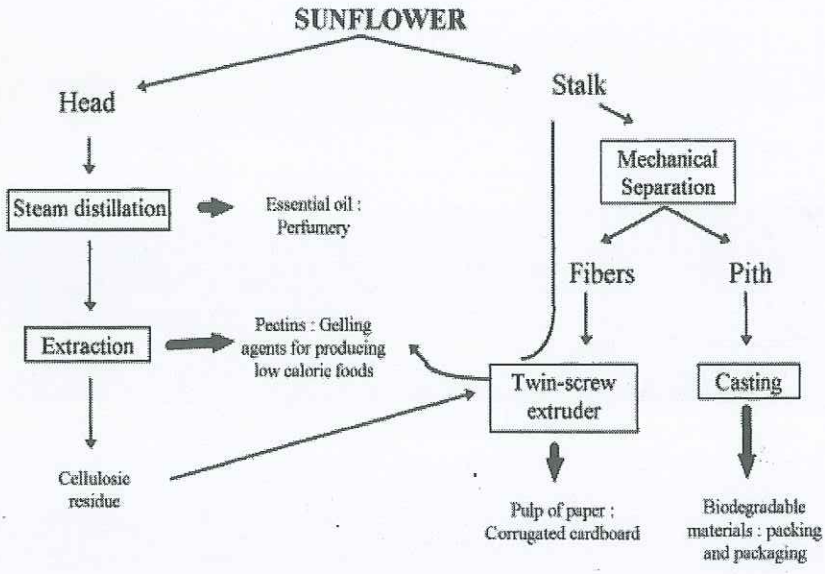


Figure 2.1. Possible commercial uses of various sunflower products (Marechal and Rigal, 1999).

Cotton stalk is another agricultural waste material produced in huge amounts in Turkey ranked seventh in the world with 2,570,000 tonnes of cotton. This production is obtained from Southeastern Anatolian region (50%), Aegean region (30%), Mediterranean region (19%), and Marmara region (1%) of Turkey (Exploitation of Agricultural Residues in Turkey LIFE 03 TCY/TR/000061).

As the potential of the cotton waste started to draw researchers' attention, the usefulness and feasibility of using cotton waste for various applications became the subject of many studies in the last years. Various studies have focused on the use of cotton waste as livestock feed (Poore and Rogel, 1995) composting, paper production (Ververis *et al.*, 2004) and energy production (Holt *et al.*, 2004). In spite of the new methods for its use, today much of the available cotton waste in the world is still disposed of by returning it back to the originating cropland. The field operations, on the other hand, are energy intensive and tend to destroy soil structure and increase the potential for erosion. Therefore, cotton waste must be utilized if sustainable agriculture is targeted. Even though high cotton waste potential for energy production is known, researchers generally focused on the production of ethanol and the production of fuel pellets or briquettes. There were a few studies on the subject of biogas production from cotton wastes. The anaerobic treatability and methane generation potential of three different cotton wastes namely, cotton stalks, cotton seed hull and cotton oil cake were determined in batch reactors. The results revealed that cotton wastes can be treated anaerobically and are a good source of biogas (İşçi and Demirer, 2006).

CS is abundant and renewable, with a high content of cellulose (32–46%) and hemicellulose (20–28%). It is also a potential material for pulping and papermaking. To reduce the pollution of discharged effluent, some new techniques, such as biopulping, have been also developed in the paper-making process (Hadar *et al.* 1993).

Zheng *et al.* (2003) investigated the degradation of the exploded cotton stalk by an alkalophilic *Bacillus* sp. and a white-rot fungus and characterized the properties of degraded CS. They found that steam explosion increased bioavailability of CS. Alkalophilic *Bacillus* NT-19 could preferentially degrade the non-cellulose components of cotton stem but the white rot fungus, *P. chrysosporium*, had a lack of selectivity. The strain of *Penicillium janthinellum* grew well in these hemicellulosic agricultural residues hydrolysates, but higher xylanase activities were found in corn cob and oat husk (Oliveira, *et al.*, 2006).

Esterification of CS with chlorosulfonic acid, phosphorus oxychloride, and their mixture was carried out with the aim to prepare cation exchangers for the removal of heavy metal ions from industrial waste water. The efficiency of the prepared cation exchangers to remove some heavy metal ions (strontium, arsenic, copper, and nickel) at different metal ion concentration, pH, contact time, and temperature was studied. The results showed that CS that contain both phosphate and sulfonate groups (phosphosulfonated cotton stalks) showed higher adsorption of the different heavy metal ions than CS containing sulfonate or phosphate on a separate basis (Nada *et al.*, 2005).

The pyrolysis of CS was studied for determining the main characteristics and quantities of liquid and solid products. According to the

experimental results, the liquid products can be used as liquid fuels, whereas the solid products can be transformed to activated carbon for adsorption processes studied for determining the main characteristics and quantities of liquid and solid products (Pütün *et al.*, 2005).

The agricultural waste is known to be an excellent carbon source for microbial enzyme production. Various agricultural substrates:byproducts and microbial cultures have been used successfully in solid state fermentation for cellulase production. *Aspergillus niger* 38 was cultivated under solid state fermentation for the production of cellulolytic enzyme using as carbon source available agricultural wastes (Jecu, 2000).

Three species of Pleurotus, *P. sajor-caju*, *P. platypus* and *P. citrinopileatus* were cultivated on CS, coir fibre, sorghum stover and mixtures of these wastes in polythene bags. The yield of mushrooms, biological efficiency, nutrient composition of the fruit bodies, energy value of the substrates and energy recovery of the mushrooms were analyzed. It was observed that the *Pleurotus* spp. could be cultivated economically on agro-residues. CS could be used for cultivation of *P. sajor-caju*, corn fibre for *P. platypus* and sorghum stover for *P. citrinopileatus*. The fruit bodies are rich in nutrients and minerals with low fat content. Moreover, the cultivation of *Pleurotus* spp. on agro-residues helps in effective disposal of these wastes (Ragunathan and Swaminathan, 2003).

2.2 Prebiotics

Non-digestible oligosaccharides are oligosaccharides that resist digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. These carbohydrates help to maintain regularity of colonic functions and could possibly contribute to human health by reducing the risk of chronic diseases. A lot of non-digestible oligosaccharides are considered as prebiotics, which are non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of beneficial bacteria in the colon, and thereby improving the host's health (Gibson *et al*, 1995).

Research on the production of oligosaccharides for foods was started around 1970–1975 in Japan, and several oligosaccharides such as glycosylsucrose, fructooligosaccharides, maltooligosaccharides, isomaltooligosaccharides (branched-oligosaccharides), galacto-oligosaccharides, xylooligosaccharides, isomaltulose (palatinose), and lactosucrose have been developed. Several Japanese companies in the food industry, in the pharmaceutical industry, and in others industries produced and sold oligosaccharides from different substrates such as fructose, galactose and xylan. More than 400 prebiotic food products are on the market (mainly in Europe and Asia). The functional foods segment, with U.S. sales estimated at between $10 billion and $20 billion, reflects the trend toward healthier eating and lifestyles (Teruo, 2002).

Fructooligosaccharides are the best studied non-digestible oligosaccharides with prebiotic properties. Fructooligosaccharides are a

diverse family of fructose polymers that vary in length. They can be produced from fructan hydrolysis or from the action of fructosyltransferases on sucrose The enzymatic production of fructooligosaccharides was industrially performed with the aid of fructosyltransferase (EC 2.4.1.9) and ß- fructofuranosidase (EC 3.2.1.26) prepared from *Aspergillus niger* ATCC 20611 (Hidaka *et al.*, 1988) and *Aureobasidium pullulans* KFCC 10524 (Jung *et al.*, 1987, Yun *et al.*, 1994; Yun, 1996). Fructosyltransferase are the enzymes responsible for the microbial production of fructooligosacharides. Fructosyltransferase produces fructooligosaccharides from sucrose in a disproportionate mode, thereby forming 1-kestose initially, then 1-nystose, followed by 1-fructofuranosyl nystose (Yun, 1996). Microbial fructosyltransferase are derived from bacterial and fungal sources (Sangeetha, *et al.*, 2005).

Hirayama (2002) has investigated the novel physiological functions and the practical applications of oligosaccharides. Figure 2.2 shows the difference in the metabolic pathway between digestible and indigestible saccharides. The left column in shows the digestive tract, and the right rectangle shows the inside of the body. Food moves from the mouth to the stomach, then to the small intestine, and then to the large intestine. In the small intestine, digestible saccharides, such as sucrose and starch, undergo the digestion and absorption process. Digestible enzymes convert the saccharides to monosaccharides, which are eventually metabolized, and exhaled as breath carbon dioxide or excreted in urine. On the other hand, indigestible oligosaccharides pass through the small intestine tract. In the large intestine, they enter a fermentation and absorption process. In this process, intestinal microbes transform the oligosaccharides into short-chain fatty acids.

Acetate, propionate, and butyrate are the most common components. Subsequently, the short-chain fatty acids are absorbed and metabolized into carbon dioxide (Hirayama, 2002).

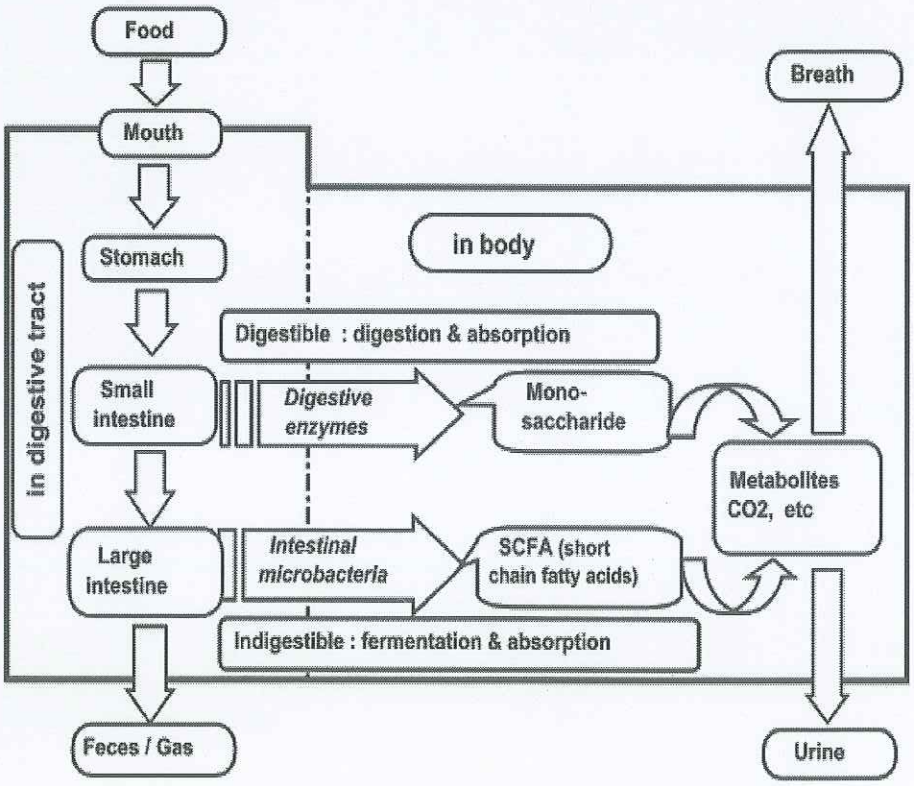


Figure 2.2. Difference in the metabolic pathway between digestible and indigestible saccharides (Hirayama, 2002).

Figure 2.3 summarizes the metabolism of nondigestible sugar substitutes in the large intestine. Sugar substitutes that are not digested in the small intestine reach the large intestine where they are

completely fermented by intestinal bacteria. Short-chain fatty acids such as acetic acid, propionic acid and butyric acid, carbon dioxide, methane, and hydrogen are produced as end-products of the fermentation. A part of the sugar substitute is incorporated into the component of intestinal bacteria and then metabolized (Oku and Nakamura, 2002).

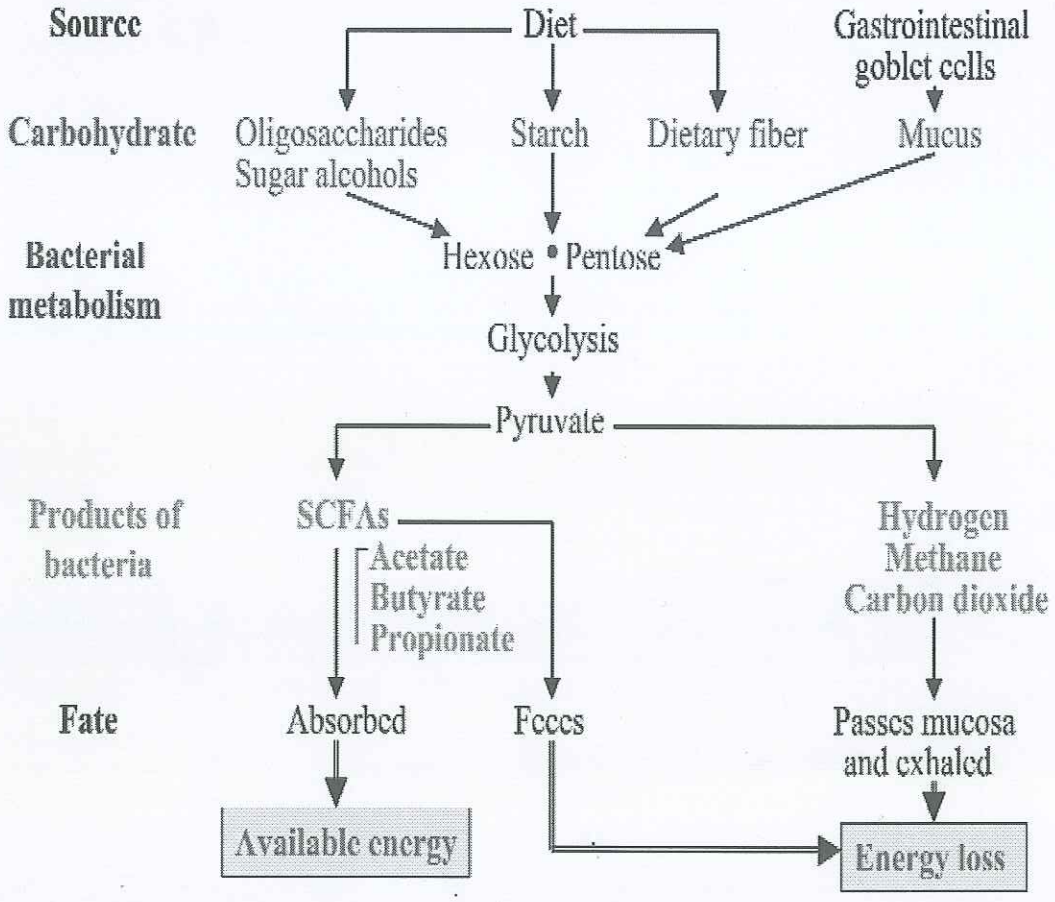
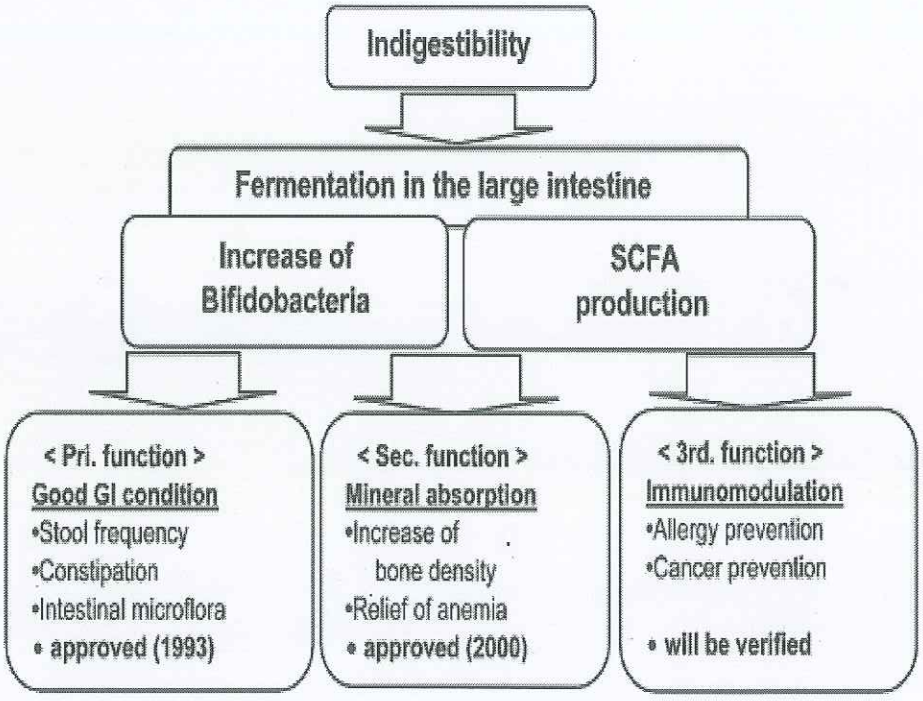


Figure 2.3. Nondigestible sugar metabolism in the large intestine (Oku and Nakamura, 2002).

Figure 2.4 shows an overview of physiological functions and their key properties of oligosaccharides. Indigestibility gives rise to fermentation in the large intestine, followed by an increase of *Bifidobacteria* and short chain fatty acid production. The indigestibility leads to several kinds of physiological functions, and the functions are classified into three types as shown in Figure 2.4. The primary function encourages a good gastrointestinal condition, including a normal stool frequency, less constipation, and healthy intestinal microflora. The second is related to better mineral absorption, including an increase in bone density and relief of anemia (Hirayama, 2002).



Figue 2.4. An overview of physiological functions of oligosaccharides and their key properties (Hirayama, 2002).

Another important group of nondigestible oligosaccharides are the XOs, which can be produced in an industrial scale from xylan rich materials (Vazquez, *et al.*, 2005). Several methods have been proposed for producing XO from suitable feedstocks, including direct enzymatic treatments, chemical fractionation followed by enzymatic hydrolysis of hemicellulose isolates and hydrolytic degradation of xylan to XO by dilute solutions of mineral acids, steam and water (Vazquez, *et al* 2002). Vazquez and co-workers aimed to enhance the potential of xylooligosaccharides from corncob autohydrolysis as prebiotic food ingredients (Vazquez, *et al.*, 2006). Garrote *et al.*, studied the possibility of obtaining xylooligosaccharides in high yield from *Eucalyptus* wood (Garrote *et al.*, 1999).

In a study performed by Mauro *et al.* (2006) assessment and comparison the capability of XOs produced by autohydrolysis of corncob to support the growth of bifidobacteria and lactobacilli were investigated. Four strains, *Bifidobacterium adolescentis DSM 20083*, *B. longum DSM 20097*, *Lactobacillus brevis DSM 20054* and *L. fermentum ATCC 9338*, were used to perform fermentations with culture media containing selected XOs mixtures. Commercial XOs produced by enzymatic hydrolysis and the monomeric constituents of the XOs mixtures were used for comparative purposes. It was shown that autohydrolysis constitutes a promising approach for the production of oligosaccharides from corcob capable of supporting the growth of *B. adolescentis* in comparison to commercial XOs. This arises from *B. adolescentis* capability to grow and utilize XO mixtures from autohydrolysis, mainly constituted by xylotriose (X3) and xylotetrosee (X4), as well as mixtures containing essentially xylobiose (X2). The different XOs utilization patterns observed among the tested strains

might be of relevance for the design of future species-specific prebiotic nondigestible oligosaccharides and symbiotic preparations (Moura *et al.*,2006).

The microflora of the gastrointestinal tract is a key for nutrition and health of the host. Modulation of the microflora can occur through diets that contain prebiotics. The approach of using diet to induce microbial change offers a very straightforward approach towards improved health. In terms of new developments, it is important that the definitive health bonuses associated with prebiotic intake be determined. The health benefits that have been suggested are varied but also very important. In addition to good human volunteer studies, there is a need to enhance the mechanistic understanding of the health effects of prebiotics (Manning, 2004).

The effects of XOs on the cecal microbiota, fecal pH level, cecal weight, and serum lipid levels in rats were investigated. It was concluded that, dietary supplementation with XOs and fructooligosaccharides inhibited the development of precancerous lesions, promoted the growth of bifidobacteria, and lowered the cecal pH in rats. Therefore, diets containing XOs and fructooligosaccharides may be beneficial to gastrointestinal health. Furthermore, XOs supplementation was more effective than fructooligosaccharides supplementation (Hsu *et al.*, 2004).

With increasing consumer health consciousness and also increasing awareness of physiologically functional foods, the future for products containing oligosaccharides seems to be greatly promising. These functional oligosaccharides may play an important role especially for

the reduction of lifestyle related diseases in the near future, as well as the improvement of human health (Nakakuki, 2002).

2.3 Xylooligosaccharide Production

XOs can be produced from a variety of lignocellulosic materials (including hardwoods and agricultural byproducts) having xylan as the main hemicellulose polymer. Recent studies have been reported on the manufacture of XOs from sugarcane bagasse (Jacobsen and Wyman, 2002), hardwoods (Garrote and Parajo 2002), corncobs (Nabarlatz *et al.*, 2004; Parajo *et al.*, 2004), rice hulls (Vegas *et al.*, 2004), flax shive (Jacobs *et al.*, 2003), wheat straw (Sun *et al.*, 2005).

Several methods have been proposed for producing XOs from a variety of xylan-containing raw materials, including direct enzymatic treatment, chemical fractionation followed by enzymatic hydrolysis of hemicellulose isolates and hydrolytic degradation of xylan to XO by dilute solutions of mineral acid, steam or water (Vazquez *et al.* 2000).

Autohydrolysis, carried out under mild operational conditions allow the selective depolymerization of the xylan backbone, leading to XO as major reaction products. In a study performed by Garrote *et al.* (1999), *Eucalyptus globulus* wood samples were subjected to hydrothermal treatments under mild operational conditions and it was found that hydrothermal treatments of *Eucalyptus* wood had little effect on either cellulose or lignin. The main effects of hydrothermolysis were deacetylation and polysaccharide hydrolysis. Xylose and XO were the major reaction products, but sugar degradation compounds (furfural

and hydroxymethylfurfural) also appeared in the reaction media (Garrote *et al.*, 1999).

In another study, Parajo *et al.* (2004) have assayed selected xylan-containing raw materials (*Eucalyptus* globulus wood, corn cobs, rice husks and barley huska) for chemical composition and XO production by autohydrolysis.The highest XO yield was determined with barley husk (27.1%) and corncobs (24.8%), in comparison with 18.0 and 15.4 % for rice husks and Eucalyptus wood (Parajo *et al*, 2004). When xylooligosaccharides for food applications are produced by autohydrolysis, the reaction products have to be refined to decrease the contents of non-carbohydrate compounds. Therefore another study was focused on the refining of corncob autohydrolysis liquors by physicochemical methods and with the characterisation of the final products for application as food ingredients. Main reaction products of hydrothermal treatments of milled corncobs were substituted oligosaccharides but other saccharide and non-saccharide compounds were also present in the reaction media. After that liquors were concentrated and purified, a final isolate containing 88.7 weight percent of saccharides and 11.3 weight percent of nonsaccharide components (mainly made up of phenolic substituents and melanoidins) were obtained (Vazquez *et al.*, 2006)

However, the molecular weight distribution of XOs coming from autohydrolysis treatments shows a substantial fraction of high DP compounds, which should be transferred into low molecular weight compounds by the action of enzymes. Because the preferred DP range of XOs is 2-5 which can be used as active ingredients of functional foods.Therefore the evaluation of the suitability of commercial enzyme

preparations was used to degrade the substituted XO produced by *Eucalyptus* wood autohydrolysis. The results showed that the potential of the commercial enzymes tested for producing XOs with DP values in the range of those acting as prebiotics (Vazquez *et al*, 2002).

Yoon *et al.* (2006) also carried out a study to determine the efficacy of three commercial xylanase preparations as a sole enzyme source for the production of pentoses and XOs from the hemicellulose fraction of corn residues. Products of the enzymatic reaction were identified as arabinose, xylose, X2, and X3. The results of this study indicate that commercial xylanases produced from *Aspergillus niger* and *Trichoderma reesei*, could serve as a sole enzyme source for the production of pentoses and XOs from corn residues (Yoon *et. al*, 2006).

The production of XO from corncob by steaming and enzymatic hydrolysis followed by down-stream processing incorporated with nanofiltration technology was conducted by Yuan *et al.* (2004). They have reported the pilot-plant production of XO from corncob by steaming, and the total yield of XO based on the dry corncob meal was 16.9% and the XO syrup produced contained 74.5% X2 and X3.

A new process consisting of aqueous extraction of xylan using steaming and enzymatic hydrolysis of the extracted xylan for the production of XOs from corncob has been developed. HPLC analysis of the monosaccharides and oligosaccharides produced during the reaction showed that before enzymatic hydrolysis, the content of xylooligosaccharides was very low. After the addition of xylanase, the relative content of some xylose-containing oligosaccharides (especially X2 and X3) increased rapidly. The enzymatic hydrolysis released a small amount of xylose. X3 would be further hydrolysed into xylose and

X2. The relative content of X4 remained at a very low level. This means that if X4 was released, it would be hydrolysed into X2 rapidly (Yang *et al.*, 2005).

Many microorganisms are known to produce different types of xylanases used for production of XO. Among fungi, strains of *Trichoderma* spp have attracted considerable attention as rich sources of xylanolytic enzymes.The xylanase from *Trichoderma longibrachiatum CS-185* hydrolyzed oat spelt xylan to mixtures of XOs with X2, X4 and X5 being major ones (Chen *et al.*, 1997).

The preparation of a series of X*n*s from cotton-seed xylan and the suitability of cotton-seed xylan compared with birchwood and oat-spelt xylans were studied by Sun *et al.* (2002) and they concluded that cotton-seed xylan is suitable for use as a starting material for the production of xylose and X*n*s because xylans with high xylose content and simple chemical structure are limited in nature. By comparing with birchwood xylan, cotton-seed xylan is easy to prepare because the lignin in cotton-seed cake is removed by using sodium hypochlorite (Sun *et al.*, 2002).

Yang *et al.* (2007) have studied XO production from lignocellulolytic agricultural waste (bagasse, corncob, wheat bran, and peanut shell) by using xylanolytic enzymes from *Thermobifida fusca NTU22*. The xylanolytic enzymes including xylanase, β-xylosidase, and acetyl esterase were simultaneously accumulated. β-ylosidase activity was eliminated by treating the crude enzymes at 70 °C for 30 min.

This means that the heat-treated crude xylanase preparation could successfully be used for the production of XOs. Among the

lignocellulosic agricultural wastes, the corncob and bagasse xylans had better XOs yields with 29.5% and 23.7% respectively.

Antimicrobial activity of acidic XOs obtained from birchwood xylan by treatment with XYL I and XYL A was investigated. The antimicrobial activity of the XOs was examined against three Gram (-) and three Gram (+) bacteria, growing aerobically. The compounds were also tested against *H. pylori*, a Gram (-) curved rod bacterium withstanding the stomach's hostile ambience by microaerophilic growth capacity. The results show that isolated pure acidic XOs have antimicrobial activity and aldopentauronic acid was proved more active against the Gram (+) bacteria and against *H. pylori* (Christakopoulos *et al.*, 2003).

XOs cause prebiotic effects when ingested as part of the diet (for example, as active ingredients of functional foods) through the modulation of colonic microflora. XOs (alone or as active components of pharmaceutical preparations) exhibit a range of biological activities different from the prebiotic effects related to gut modulation (Moure *et al.*, 2006).

XOs with various substituents were fermented in vitro by fecal inocula from four human volunteers to study the influence of substitution on the ability and rate of fermentation and on the production of short-chain fatty acids and lactate. By all fecal inocula used nonsubstituted XOs and arabino xylooligosaccharides were fermented more quickly than the more complex structures of acetylated xylooligosaccharides and XOs containing α 4-O-methylglucuronic acid group.

The fermentations of AcXOS and GlcAmeXOs resulted in a lower lactate production, whereas the concentration of propionate and butyrate

increased. These results indicated that the differences in the structural features of XOs affect their behavior in fermentation (Kabel *et al.*, 2002).

XOs show a remarkable potential for practical utilization in many fields, including pharmaceuticals, feed formulations and agricultural applications, but their most important market developments correspond to food-related applications. In this field, XOs present advantages compared with other oligosaccarides in terms of health effects and concentration thresholds, but their comparatively high production costs are hindering a wider and faster market development. For this purpose, further improvements in processing technology would be necessary. The interest in XOs for food applications is demonstrated by the dramatic increase in demand observed, since 1994. For example, production by Suntory increased from 70 t in 1994 to 300 t in 1996. On the other hand, most of the leading companies develop functional foods, confirming the tendency of this market to grow. Other data (such as the wide variety of biological, nutritive and technological properties and applications of XOs, or the variety of the potential sources for their production) also support this idea. Important remarks on this issue are the current trend toward health-care cost reduction and the growing consumer interest in preventive health, which reinforce the expectancies that the dietary supplement industry will continue to exhibit strong growth. Novel foods (through enzymatic synthesis involving reducing oligosaccharides) open new and exciting opportunities in this field (Vazquez *et al.*, 2000).

Table 2.1 summarizes some of representative applications claimed in the last few years, including antioxidant activity (conferred by phenolic substituents), blood- and skin-related effects, antiallergy, antimicrobial, anti-infection and anti-inflammatory properties, selective cytotoxic activity, immunomodulatory action, cosmetic and a variety of other properties oligosaccharides (Moure *et al.*, 2006).

Table 2.1. Biological activities of XO additional to those related to their modulatory gut effects (Moure *et al.*,2006).

Manufacture, Substrate	**Biological effect and application**
Enzymatic hydrolysis of wheat flour arabinoxylan	Antioxidant (DPPH-radical scavenging) Activity
Enzymatic hydrolysis of wheat bran	Antioxidant (erythrocyte hemolysis assay) Activity
Hyrothermal processing of baggase and enzymatic processing	Antioxidant Activity
Feruloyl xylooligosaccharides from enzymatic reactions	Protective effect against lipid (LDL) peroxidation
Active principles of pharmaceutical preparation	Prevention and treatment of oxidative stres
Mixtures of XO and tea catechins	Prevention and control of anemia and arteriosclerosis
Active component of symbiotic preparations with *Lactobacillus* strains	Treatment of vaginal and urogenital infections
Enzymatic hydrolysates of rice bran	Cosmetics
Active component pharmaceutical preparations with flavonoids	Low-glycemic index carbohydrate substitute
Enzymatic hydrolysates of wheat flour arabinoxylan	Prevention of atherosclerosis
Long chain XO from enzymatic processing of lignocellulosic materials	Hypolipemic activity (against cholesterol and phospholipids)

Table 2.1.(Continued). Biological activities of XO additional to those related to their modulatory gut effects (Moure *et al.*,2006).

Acidic XOs	Antihyperlipidemic activity
Acidic XOs containing uronic acid residues	Hair growth stimulation
Acidic XOs from chemical and enzymatic processing of hardwood pulp	Inhibition of melanin and inhibiton of melanoma cell proliferation
Acidic XOs from the enzymatic processing of broadleaf pulp	Collagen production enhancer
Acidic XOs containing uronic acid residues	Anti-inflammatory activity

XOs have advantageous over other nondigestible oligosaccharides in terms of both health and technological related properties (Alonso, *et al.*, 2003). In food processing, XOs show advantages over inulin in terms of resistance to both acids and heat, allowing their utilization in low-pH juices and carbonated drinks. The digestibility of XOs by digestive tract juices and the effect of XOs on the absorption of bile acids were compared with the effects of fructooligosaccharides and isomaltooligosaccharide. Most of the isomaltooligosaccharide and a part of the fructooligosacharides were digested by the small intestinal juice, but XOs was not digested at all by any digestive enzymes. The retardation effect of XOs on bile acid absorption compared with isomaltooligosaccharide and fructooligosaccharide has been proved by in vitro experiments. Owing to the fact that the disaccharidase activity

in the XOs group was lower than in the other dietary oligosaccharide groups, carbohydrate hydrolysis in the digestive tract may be retarded and blood glucose levels may be effectively controlled by dietary XOs (Vazquez *et al.*, 2000). The sweetnees of X2 is equivalent to 30% that of sucrose and the sweetness of other XOs is moderate and possess no off-taste. XOs are stable over a wide range of pH (2.5–8.0, an advantage compared with fructooligosaccharides, particularly in the acidic range, even at the relatively low pH value of the gastric juice) and temperatures (up to 100°C). As food ingredients, XOs have an acceptable odour, and are non-cariogenic and low-calorie, allowing their utilization in anti-obesity diets. The most important world market is located in Japan, where XOs are prized at about 2500 yen/kg (Caparroas *et al.*, 2007) The Japanese market pioneered the incorporation of XOs into foods (Crittenden and Playne, 1996). Today, about 60 companies use XOs in about 100 products. In this field, XOs present advantages compared with other oligosaccarides in terms of health- effects and concentration thresholds, but their comparatively high production costs are hindering a wider and faster market development. For this purpose, further improvements in processing technology would be necessary (Vazquez *et al.*, 2000).

2.4 The Scope of the Study

The aim of the study was the production of XOs from agricultural waste by enzymatic hydrolysis and characterization of the produced XOs. For this purpose, cotton and sunflower stalks, major abundant and renewable resources in Turkey, were used as agricultural wastes. Because, both of these agricultural wastes are major abundant and

renewable resources in Turkey and there is no information about utilization of cotton stalk and sunflower stalk for XO production.
XOs production was firstly performed by using xylan extracted from CS and SS. However, during alkali extraction of xylan, high amount of KOH was used causing serious corrosion and alkali pollution which is not economical and environmental friendly. Therefore, fungal pretreatment by *Phanerochaete chrysosporium*, cellulase pretreatment and high pressure/high temperature pretreatment methods have been conducted and compared to find the suitable method for improving the subsequent enzymatic hydrolysis of xylan in pretreated CS. Finally, XOs were characterized by TLC and HPLC and the results were comparatively evaluated.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Cotton stalk and sunflower stalk were obtained from local producers in Urfa and Kütahya, respectively. Chemicals used in this study were analytical grade, either purchased from Sigma (N.Y., USA) or Merck Chemical Companies (Deisenhofen, Germany). Aluminum-backed silica gel thin layer chromatography plates were from Sigma Chemical Co. (USA).

Xylooligosaccharide standards, xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5), xylohexose (X6) Were purchased from Megazyme (Bray, Ireland).

3.2 Enzymes

Commercial xylanase, VERON 191 having 7500 U/mg specific acitivity from *Aspergillus niger,* was obtained from AB Enzymes-Abitec Group (Finland). Shearzyme (11500 U/mg specific acitivity) from *Aspergillus oryzae* was obtained from Novozymes (Denmark). Cellulase from *Trichoderma reseei* was supplied from Orba Biochemistry Ltd. (Turkey).

3.3 Microorganisms

3.3.1 *Bacillus pumilis*

Bacillus pumilis SB M-13 isolation from soil samples was performed by Suzan Biran (Biran *et al.* 2006). The isolate was maintained at 37°C on agar plate containing 1.0% glucose, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4$ and 2.0% agar to which sterile 0.01%; Na_2CO_3 added separately.

Home-produced xylanase from *Bacillus pumilis* SB M-13 was produced in our laboratory. Xylanolytic enzyme production by *B. pumilus* SB M-13 was performed in 250-ml shake-flask containing 100 ml medium at 35°C, 170 rpm. *Bacillus pumilus* SB M-13 fermentation medium containing 0.5% NaCl, 0.25% yeast extract, 0.1% KH_2PO_4, 0.02% $MgSO_4$, 0.1% Na_2CO_3 and 3% of ground corncobs as sole carbon source. After inoculation of medium with *B. pumilus*, fermentation was performed for 4 days and the cells were harvested by centrifugation at 4^{th} day of cultivation for 40 min at 11,000 x g. The supernatant was used as crude xylanase source. Xylanase purification was performed by Dr. Ayşegül Ersayın Yaşinok (Yaşinok, 2006). Crude and purified xylanase having 0.22 U/mg and 40.9 U/mg specific acitivies respectively, were used for hydrolysis of xylan from cotton stalk and sunflower stalk for XO production.

3.3.2 *Phanerochaete chrysosporium*

Phanerochaete chrysosporium was provided by Prof. Dr. Nazif Kolonkaya from Hacettepe Univesity. *P. chrysosporium* was grown on potato dextrose agar petri dishes and incubated at 35 °C for 4-5 days until sporulation. The microoroganism was stored on agar plates at 4 °C for maximum 2 weeks to use as stock culture.

3.4 Sugar and Phenolic Compunds Analysis

3.4.1 Determination of Reducing Sugar

Dinitrosalicylic acid (DNSA) method was used for the determination of reducing sugar concentration released during the enzymatic reaction (Miller, 1959). DNSA reagent is prepared as described in Appendix A. In DNSA Method, reducing sugars within the samples were determined by using xylose as standard (Appendix B).

3.4.2 Thin Layer Chromotography (TLC)

Samples periodically taken from enzymatic hydrolysis mixtures were applied on silica plates to observe XOs. The solvent system was ethylacetate: acetic acid: water (2:2:1) (Chen *et.al.* 1997). After development of plates in TLC tank, they were dried at room teperature and spried by ethanol: sulfuric acid (9:1) mixture. The silica plates were baked at 110 °C for 10 min.

3.4.3 High Performance Liquid Chromotography (HPLC)

HPLC analysis was performed in in the Central Laboratory of METU. In xylooligosaccharide analysis, HPX-42A (300 x 7.8 mm, BIO-RAD Cat No: 125-0097) column was used with Refractive Index detector. The column temperature was 80°C, and the mobile phase was distilled water with flow rate of 0.6 ml/min. Standarts curves of X6, X5, X4, X3, X2 and xylose used were presented in Appendix C- H.

In analysis of phenolic compunds, Inertsil ODS-3 (250 x 4.6 mm, VARIAN Cat No. A0396250x046) column was used with PDA dedector (280 nm). The column temperature was 30°C, and the mobile phase was methanol and 0.5% CH_3COOH with flow rate of 0.5 ml/min. Standarts curves of gallic acid, catechin and epicatechin used were shown in Appendix I-K.

Glucose and cellobiose were analysed by using MetaCarb 87C (300 x 7.8 mm, VARIAN, Cat No. A5200) column with Refractive Index detector. The column temperature was 80°C, and the mobile phase was distilled water with flow rate of 0.6 ml/min. Standarts curves were presented in Appendix L and M.

3.5 Protein Determination

Protein concentration was determined according to Bradford Method by using bovine Serum Albumin (BSA) as standard (Bradford, 1976), (Appendix N-O).

3.6 Xylanase Assay

The reaction was carried out by mixing one volume of appropriately diluted enzyme solution with ten volumes of 1% birchwood solution at 40 °C. Samples were taken at different time intervals and the reaction was immediately stopped by boiling for 5 min. Dinitrosalicylic acid (DNSA) method was used for the determination of reducing sugar concentration released during the enzymatic reaction (Miller, 1959). One unit of xylanase was defined as the amount of enzyme releasing 1µmole of xylose equivalent per min under assay conditions.

3.7 Cellulase Assay

The reaction was carried out by mixing 250 µl cellulase with 5% substrate in 50 mM citrate buffer, pH 4.8 at 40 °C. Samples were taken at different time intervals and the reaction was immediately stopped by boiling for 5 min. Reducing sugar concentration released during the enzymatic reaction was assayed by DNSA (Miller, 1959). One unit of cellulase is defined as the amount of enzyme that liberates 1 µmole of glucose equivalents per minute under the assay conditions.

3.8 Lignocellulosic Pretreatment

3.8.1 Pretreatment of CS by *Phanerochaete chrysosporium*

Spore supension prepared from *P. chrysosporium* stock culture were inoculated to liquid medium described Appendix P (Tien and Kirk, 1988).

Liquid medium contained 5% CS (autoclaved at 121 °C for 1 h and incubated at 100 °C for overnight) as sole carbon source. Cultures were grown in 2 L and 250 ml erlenmeyer flasks containing 200 ml and 20 ml of medium, respectively at 39 °C with and without shaking. Daily samples were taken. Xylanase activities were assayed by using 1% birchwood xylan and determimation of reducing sugar concentration were performed by DNSA. XO, glucose, cellobiose and phenolic compounds were also analysed by HPLC. Media were filtered to recover CS at 4 th and 7 th days of fermentations. CS taken from each medium was washed with 350 ml of ethylalcohol and 500 ml of distiled water. Finally CS was dried at 60 °C for overnight and used in enzymatic hydrolysis by Shearzyme for XO production.

3.8.2 Pretreatment of CS by Cellulase

During pretreatment of CS by cellulase, 5 % (w/v) of CS (autoclaved at 121 °C for 1 h and incubated at 100 °C for overnight) in 50 ml of 50 Mm citrate buffer at pH 4.8 was stirred for overnight on magnetic stirrer at room temperature and then cellulase was added to CS suspension to initate the reaction. Samples were periodically taken and boiled for 5 min to stop the reaction. Reducing sugar concentration in samples was analysed by DNSA.

3.8.3 Hydrothermal Pretreatment of CS

CS and distilled water were mixed (liquid : solid mass ratio=8) and reacted in the autoclave at 121 °C for 1 h and 135 °C for 30 min. The solid and liquid phases were separated by fitration. The solid residue

was washed by 100 ml of distilled water and dried at 60 °C for overnight. The filtrate, washing water and solid residue was incubated by shearzyme for XO production. Before incubation with shearzyme, reducing sugar concentration in filtrate and washing water were also tested by DNSA.

3.9 Xylan Extraction

The method used by Zilliox and Debeire (1998) was modified for the xylan extraction from cotton stalk and sunflower stalk. Dried cotton stalk and sunflower stalk was milled to 2.5 mm. The milled CS and SS (2 g) was mixed with 100 ml of distilled water sample and the sample was swelled at 60°C for 16 h in an incubator (Nüve, Turkey). The swollen sample was filtered by means of gauze and the pellet was stirred in 17 ml of 24 % w/v KOH+ 1 % $NaBH_4$ solution for 3 h at room temperature. Afterwards, the suspension was filtered by using gauze and the supernatant was centrifuged at 7000g for 10 min. After centrifugation step, the supernatant was filtered by means of a filter paper (Whatman 41) and then xylan was washed with cold ethanol: acetic acid (10:1) solution. The volume of the combination of cold ethanol and acetic acid was 2.5 times higher than supernatant volume. The pellet was dried at 45°C for 24 h and used as xylan source.

3.10 Enzymatic Hydrolysis of Xylan and Pretreated CS

During the enzymatic hydrolysis of xylan, 2% xylan from CS and SS were dissolved in 80 ml in 50mM citrate buffer at pH 5.4 for Veron,

50 mM citrate citrate buffer at pH 4.6 for Shearzyme and 50 mM phosphate buffer for *B. pumilis* xylanase. Xylan solutions were mixed and heated up to boiling point. After boiling 2-3 minutes, these solutions were stirred overnight on a magnetic stirrer at room temperature. The solutions were diluted to 100 ml with the same buffers, mixed well and centrifuged at 2740xg for 20 minutes to clear of the insoluble xylan particles. (Bailey *et al.*, 1992). The hydrolysis reaction was carried out by mixing one volume of appropriately diluted enzyme solution with ten volumes of substrate solution at 40 °C. Samples were taken at different time intervals and the reaction was immediately stopped by boiling for 5 min and reducing sugar concentration in samples were determined by DNSA method. Effects of different temperature, pH, substrate and enzyme concentrations on XO production were investigated using the same method described above.

Pretreated CS (10%) was mixed with 50 mM citrate buffer at pH 4.6 and strirred overnight at room temprerature. The hydrolysis reaction mixture contained 1 ml of properly diluted Shearzyme and 10 ml of 10% pretreated CS. Periodically withdrawn samples were centrifuged 11000 g and supernatant was used for reducing sugar analyss by DNSA method.

3.11 Determination of Sugar Composition of Xylan

Uronic acid and sugar composition in cotton stalk xylan were determined with *m*-hydroxydiphenyl reagents (Melton and Smith, 2002) and Chemical Analysis and Testing Procedure, respectively (Ruiz and Ehrman, 1996).

CHAPTER 4

RESULTS AND DISCUSSIONS

This study focuses on xylooligosaccharides (XOs) production from agricultural waste materials. For this purpose, cotton stalk (CS) and sunflower stalk (SS), readily available and abundant agricultural waste materials in Turkey, have been selected for XO production. Commercial xylanases, Veron and Shearzyme, and home-produced xylanase from *Bacillus pumilis* have been used for enzymatic production of XOs. Optimization of the production conditions of XOs from cotton stalk xylan (CSX) and sunflower stalk xylan (SSX), with commercial xylanase preparations and characterization of XOs were investigated. Therefore, first two parts of the study are composed of XO production from CSX and SSX by using both Veron and Shearzyme. In the third part, home-produced xylanase from *B. pumilus* has been tested for XO production. Finally, investigation of CS pretreatment for opening complex structure and then enzymatic hydrolysis of pretreated CS was conducted for XO production.

4.1 Enzymatic Production XOs from Alkali Extracted Xylan

To fully utilize CS and SS as a raw material for XO production, alkaline extraction is one of the methods used to render the xylan more amenable to the action of xylanase. Alkaline extraction utilizes alkaline

solution to cause lignocellulosic material to swell, leading to increase in its internal surface area, decrease in crystallinity and degree of polymerization of cellulose fraction. It also causes disruption of lignin structure, and separation of structural linkages between lignin and carbohydrates. The basic mechanism of alkali extraction is saponification of intermolecular ester bonds cross-linking xylan hemicellulose and other polymeric materials. Compared to other pretreatment processes, alkaline extraction has its own advantage in breaking down the crystalline structure of cellulose and in decomposing lignin. Besides, inhibitory substances like furfural and hydroxymethylfurfural are not produced unlike in most other pretreatment methods. Therefore, xylan used in the first three part of this study was alkali extracted from CS and SS. Enzymatic production of XOs were performed by using alkali extracted xylan.

4.1.1 Hydrolysis of Cotton Stalk Xylan by Veron

At the beginning of the study, birchwood xylan was firstly used for XO production by Veron. Birchwood xylan (1%, w/v) prepared in 50mM citrate buffer at pH 5.4 was incubated with Veron. The reaction at 30°C was carried out for 48 h. Reducing sugar concentration was 16.5 µmole/ml at the 48th h at which the maximum hydrolysis was observed (Figure 4.1). Then, hydrolysis of 1 % xylan extracted from CS was also performed by Veron. However, negligible hydrolysis was observed (Figure 4.2). Although extracted xylan was mixed with 50mM citrate buffer at pH 5.4, the final pH of xylan suspension was measured as about 11. After adjusting the pH to 5.4 by washing the xylan with more solvent, enzyme activity increased significantly (Figure 4.2). It means

that enzyme was inactivated at high pH. Therefore, the volume of the washing solvent (combination of cold ethanol and acetic acid) was increased from 0.05 fold to 2.5 fold during xylan extraction to adjust pH.

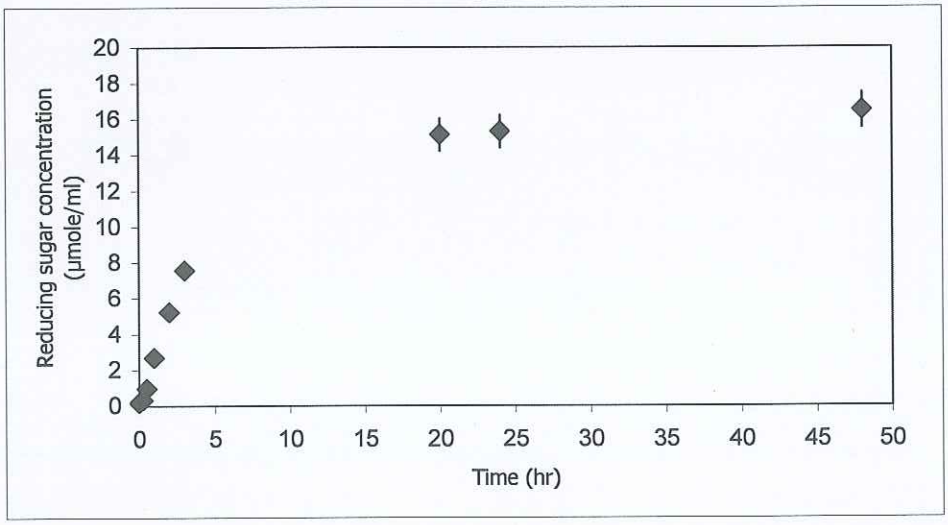


Figure 4.1. Birchwood xylan hydrolysis by Veron. Hydrolysis conditions: 1% birchwood xylan, 30 °C, pH 5.4, 50 Mm citrate buffer.

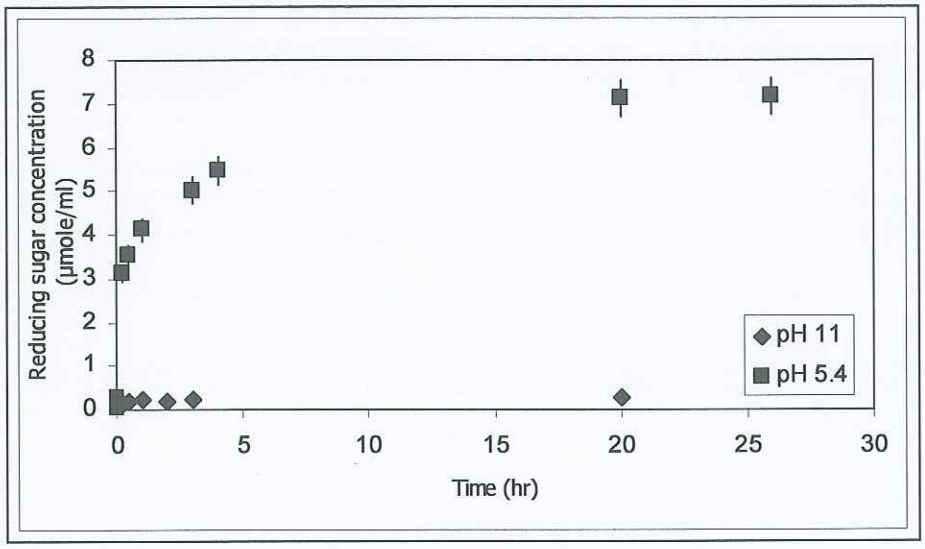


Figure 4.2. Effect of pH on hydrolysis of CSX by Veron. Hydrolysis conditions: 1% CSX, 30 °C, pH 5.4, 50 Mm citrate buffer.

When the results obtained from hydrolysis of birchwood xylan and CSX by Veron were compared, it was observed that reducing sugar concentration was approximately 2 times higher by using birchwood xylan although the initial hydrolysis rate of CSX was higher at the same experimental conditions (Figure 4.3). This was not a very surprising result because birchwood xylan was commercial xylan and it might be purer than xylan extracted from CS, which might contain some impurities that affects the enzyme activity. In addition, since the source of xylans are different and they have some differences in their structure, which could affect the hydrolysis by Veron.

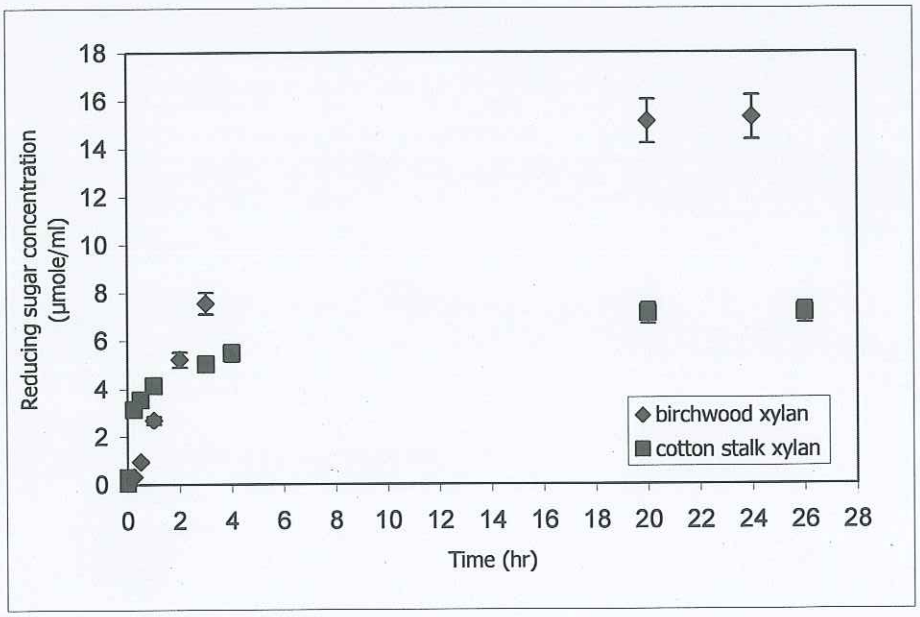


Figure 4.3 Comparison of xylan hydrolysis from birchwood xylan and CSX. Hydrolysis conditions: 1% birchwood and cotton stalk xylan, 40°C, pH 5.4

4.1.1.1 The Effect of Temperature on XO Production from CSX by Veron and Shearzyme

The effect of temperature on XO production from 2% CSX was performed using both Veron and Shearzyme at the same enzyme concentration, 0.21 U/ ml enzyme activity. At the first 8 h, the amount of XO produced by Veron at 50°C was higher than other temperatures studied. However, afterwards, the amount of XO produced at 50 ^{0}C was almost the same as the amount of XO produced at 45 ^{0}C. The concentrration of XO produced at 40 ^{0}C was lower with slight differences (Figure 4.4). The amount of XO produced increased by

increasing temperature, when Shearzyme was used. The higher amount of XO was yielded at all temperatures studied by Shearzyme compared with Veron. However, the difference between the amounts of reducing sugars produced at 45^0C and 50^0C was insignificant (Figure 4.5).

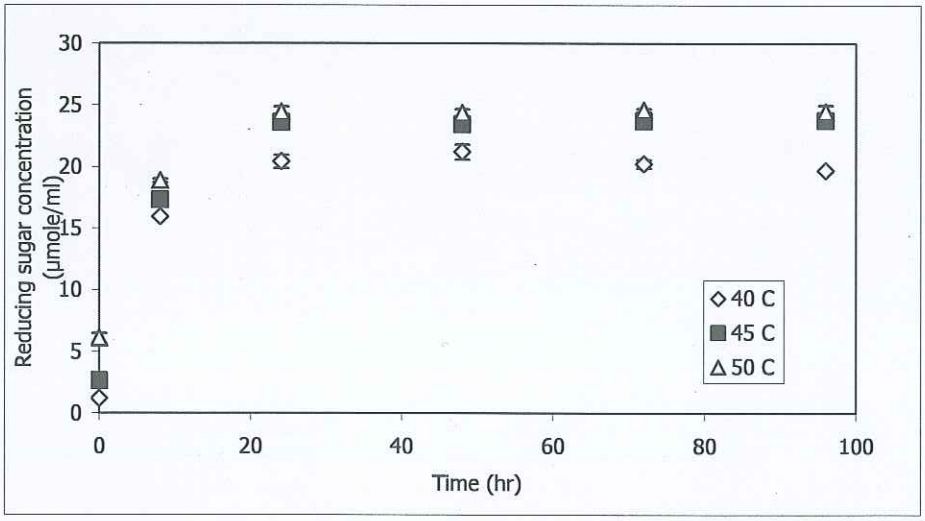


Figure 4.4 Effect of temperature on XO production from CSX by Veron. Hydrolysis conditions: 2 % CSX 0.21 U/ml Veron, pH 5.4, 50 Mm citrate buffer.

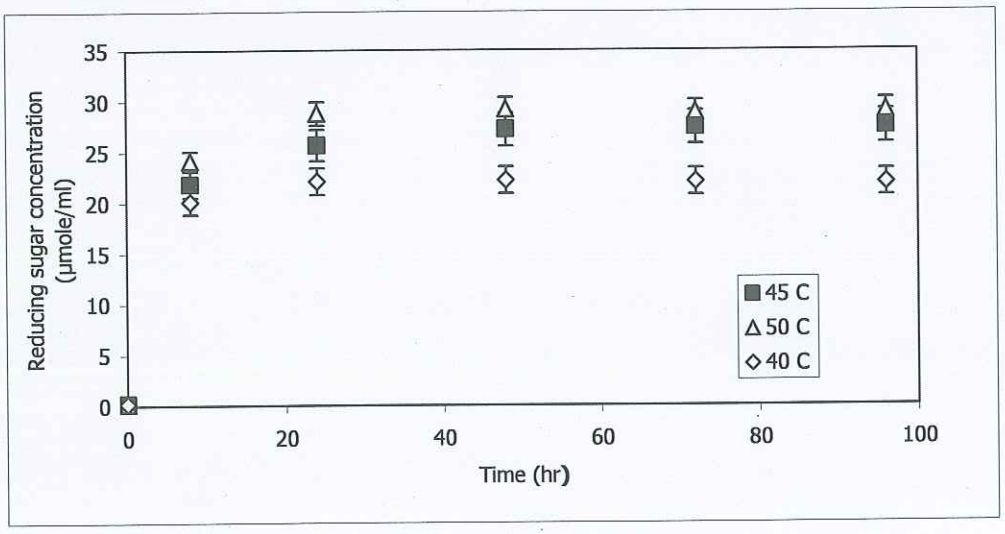


Figure 4.5 Effect of temperature on XO production from CSX by Shearzyme. Hydrolysis conditions: 2% CSX, 0.21 U/ml Shearzyme, pH 4.6, 50 Mm citrate buffer.

TLC profiles of the XOs obtained using Veron and Shearzyme are given in Figure 4.6 and 4.7. Xylan extracted from CS hydrolyzed by Veron mainly to xylobiose (X2), xylotriose (X3), xylopentose (X5), xylohexose (X6). It was observed that at the end of hydrolysis reaction, X2, X5 and X6 were the main products. Among the products, the concentration of X4 was very little. It means that even if X4 was produced; it would hydrolyse to X2 very rapidly. When temperature was increased from 40 ^{0}C to 50 ^{0}C, concentration of smaller XOs increased whereas concentration of larger XOs decreased due to higher action of xylanase activity at higher temperatures (Figure 4.6). As observed from Figuere 4.7, CSX hydrolyzed by Shearzyme to X2, X3, X4 and X5 but the main products were X2 and X3. The concentration of X2 increased after 24th

hour, while the concentration of X3 decreased at all temperatures studied. The product profiles of Veron and Shearzyme were different. X4 formation by Veron was not obsrved on TLC plate. On the other hand, larger XOs than X5 was not observed when CSX hydrolyzed by Shearzyme. However, in none of the experiments, X1 formation was observed using either Veron and Shearzyme on TLC plates.

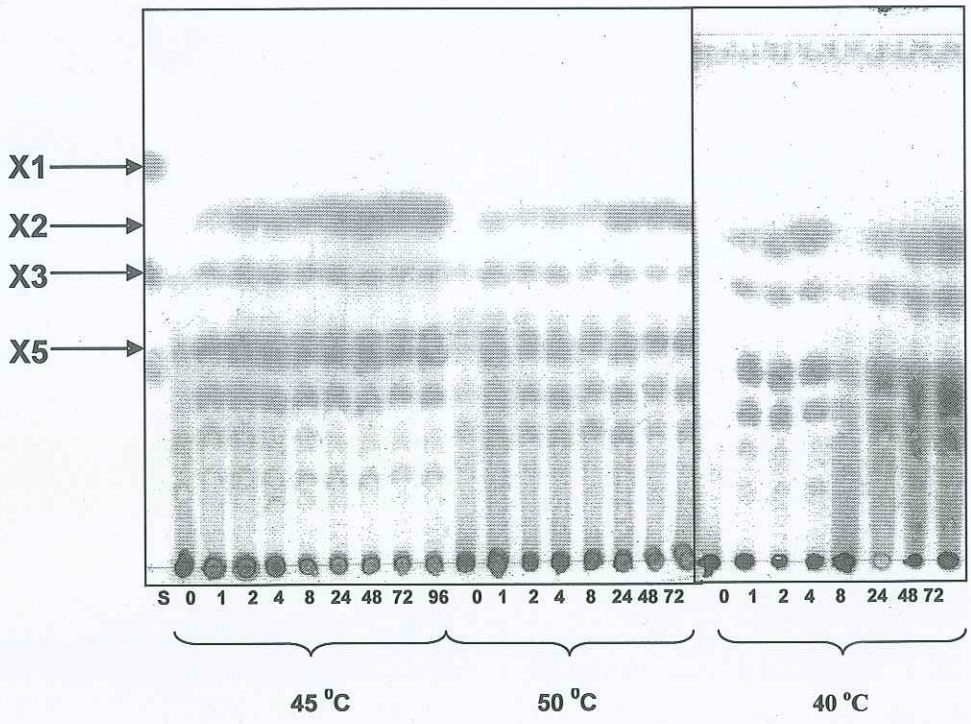


Figure 4.6 TLC of XO produced from CSX by Veron at different temperatures. Hydrolysis conditions: 2 % CSX 0.21 U/ml Veron, pH 5.4.

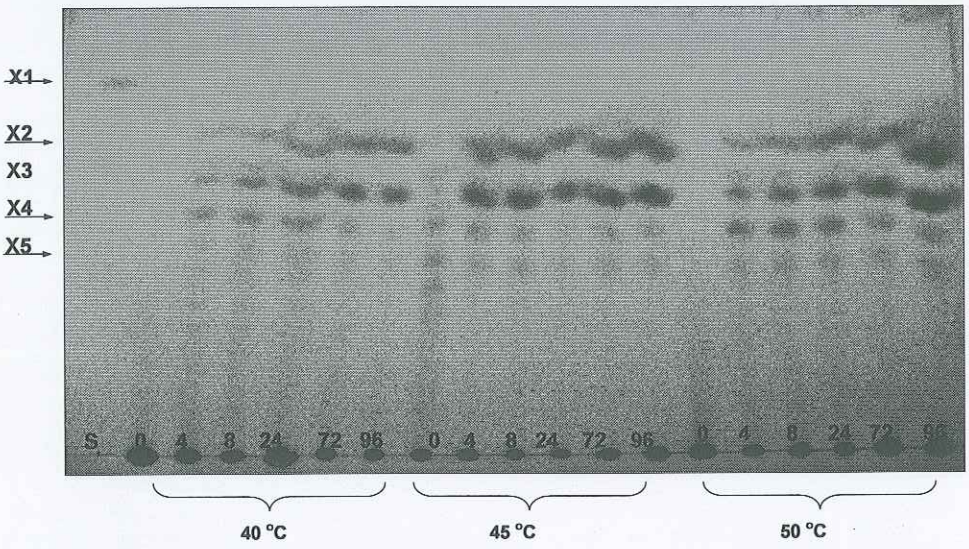


Figure 4.7 TLC of XO produced from CSX by Shearzyme at different temperatures. Hydrolysis conditions: 2% CSX, 0.21 U/ml Shearzyme, pH 4.6.

4.1.1.2 The Effects of Veron and Shearyzme Concentration on XO Production from CSX

In the following stage, the effect of different concentrations of enzymes on hydrolysis of xylan from CS was examined for XO production. 2% xylan from CS was incubated with different concentrations of both Veron and Shearzyme, 0.06, 0.12, 0.21, 0.41, 0.96 U/ml. As observed from Figure 4.8 and Figure 4.9, the amount of XO produced increased by increasing enzyme concentration and incubation time. For all the enzyme concentrations of Veron and Shearzyme investigated, reducing sugar concetration increased rapidly up to 8 h and the increase was not very significant after 24 h. However, Shearzyme yielded the higher

amount of XOs than veron at all enzyme concentrations studided. On the other hand, enzyme efficiencies, µmole product / unit enzyme activity decreased by increasing enzyme concentration significantly for both xylanases (Table 4.1). In the following experiments, 0.21 U/ml enzyme concentration was selected. Because, in higher enzyme concentrations than 0.21 U/ml, hydrolysis rate was slow down and so usage of 0.21 U/ml enzyme concentration was reasonable and more economical.

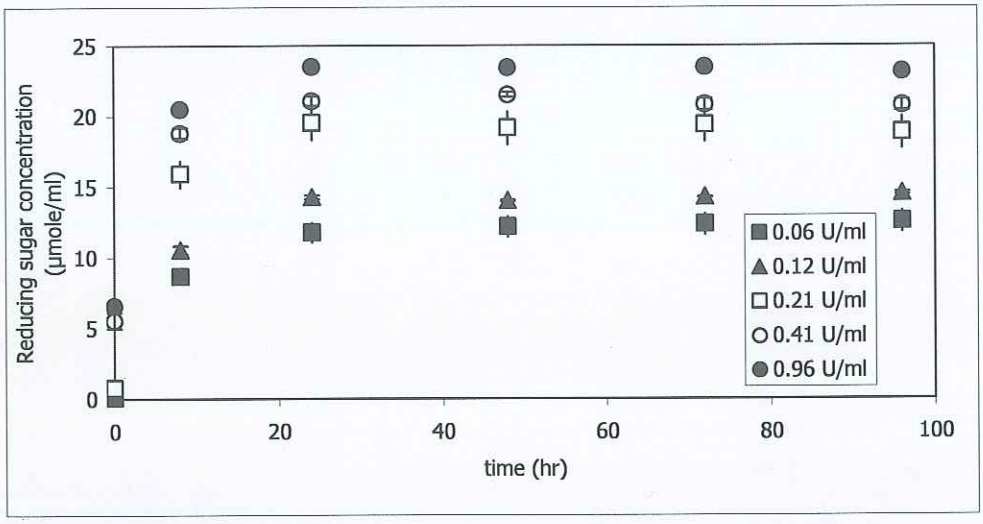


Figure 4.8 Effect of Veron concentration on XO production from CSX. Hydrolysis conditions: 2 % CSX, pH 5.4, 40°C, 50 Mm citrate buffer.

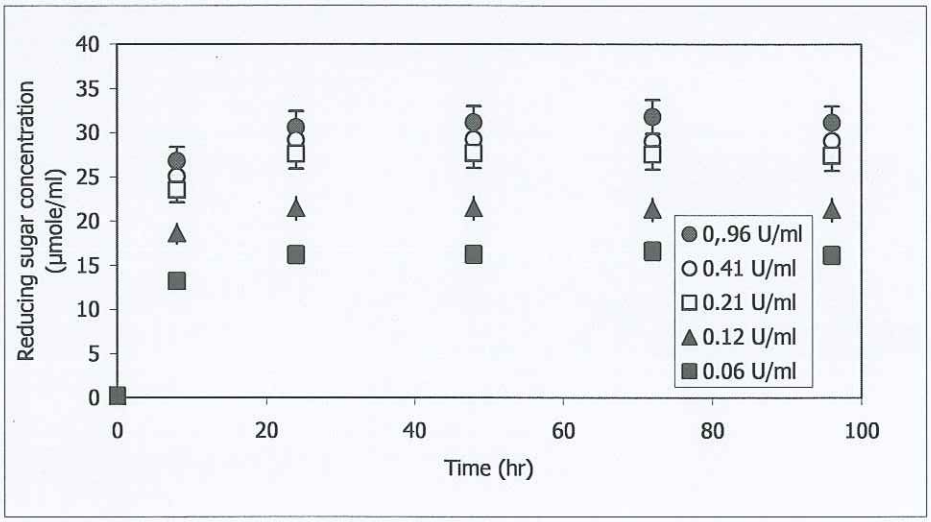


Figure 4.9 Effect of Shearzyme concentration on XO production from CSX. Hydrolysis conditions: 2 % CSX, pH 4.6, 40°C, 50 Mm citrate buffer.

Table 4.1 Enzyme efficiency for XO production from CSX.

	Enzyme Efficiency	
Enzyme Concentration (U/ml)	**Veron (µmole reducing sugar/ U)**	**Shearzyme (µmole reducing sugar/ U)**
0.06	196	269
0.12	119	178
0.21	93	131
0.41	51	71
0.96	25	32

4.1.1.3 The Effect of CSX Concentration of XO Production by Veron and Shearzyme

Hydrolysis of different concentrations (1%-10%) of xylan extracted from CS was performed both by Veron and Shearzyme for XO production. Shearzyme hydrolyzed CSX more efficiently than Veron at all the xylan concentrations that have been used. Up to 9% xylan concentration, no inhibition effect was observed. However, at 10% xylan concentration, an inhibition was observed and enzyme activity decreased significantly (Figure 4.10). On the other hand, XO production increased up to 9% by using Shearzyme but at 9% and 10% xylan concentrations, enzyme activity also decreased significantly. This decrease in enzyme activity for both enzymes might be as a result of different factors such as substrate inhibition, inhibition by impurities, molecules or increase in viscosity (Figure 4.11).

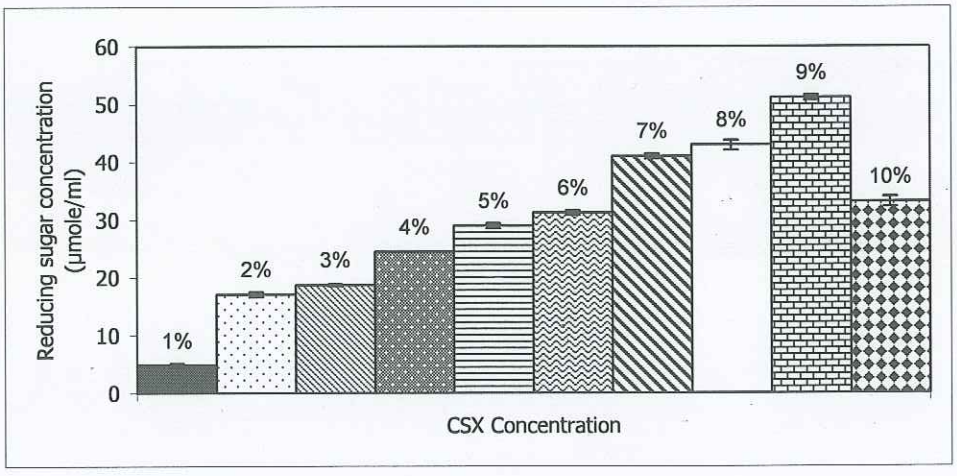


Figure 4.10 Effect of CSX concentration on XO production by Veron. Hydrolysis conditions: 0.21 U/ml Veron, pH 5.4, 40 °C.

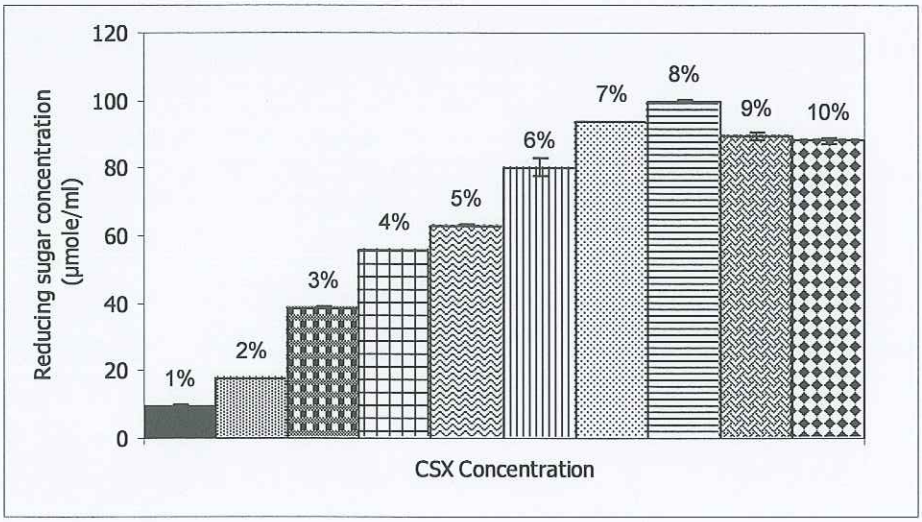


Figure 4.11 Effect of CSX concentration on XO production by Shearzyme. Hydrolysis conditions: 0.21 U/ml Shearzyme, pH 4.6, 40 °C Shearzyme.

4.1.1.4 The Effect of pH of XO Production from CSX by Veron and Shearzyme

The effect of pH on hydrolysis of xylan from CS by Veron and Shearzyme was examined for XO production. The highest XO production by Veron was observed at pH 5.4. Enzyme acitivity and reducing sugar concentration decreased at pH 6.5 significantly (Figure 4.12). Therefore, pH 5.4 is accepted as the optimum pH for hydrolysis of CSX by Veron to produce XOs.

The amount of XO produced by Shearzyme was higher at pH 4.6 than other pH values studied at the first 8 hours of the reaction. However, afterwards, the higher XO production was obtained at pH 5.5 with a

slight difference (Figure 4.13). While Shearzyme was rapid at pH 4.6, it was slow and long lasting at pH 5.5. This result may suggest that shearzyme is more stable at pH 5.5.

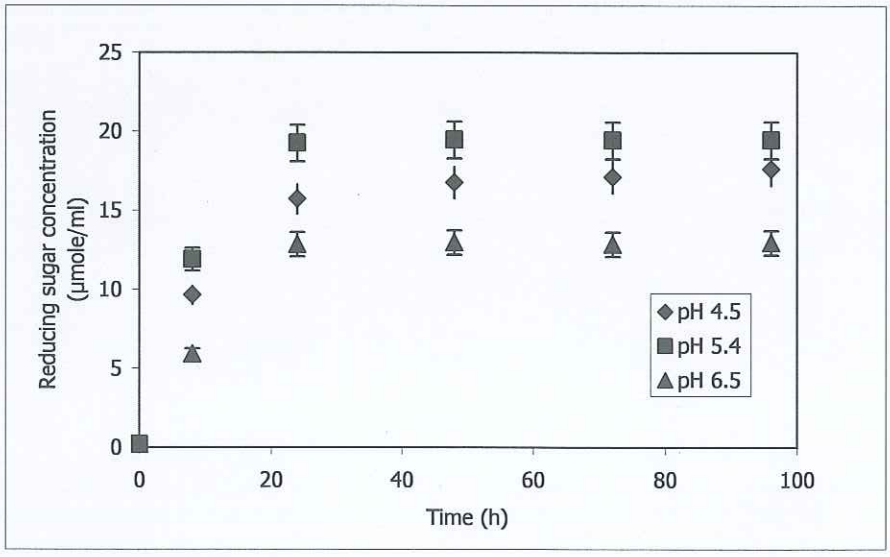


Figure 4.12 Effect of pH on XO production from CSX by Veron. Hydrolysis conditions:0.21 U/ml Veron, 2% CSX, 40 °C

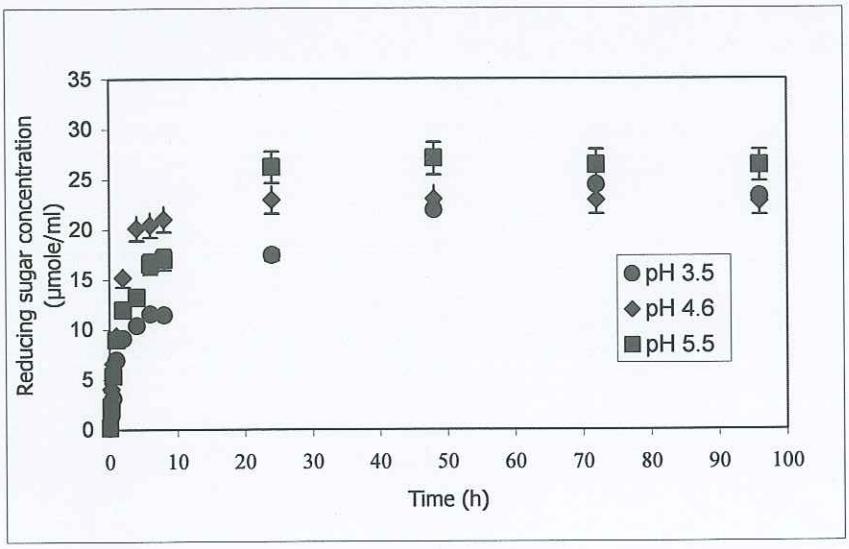


Figure 4.13 Effect of pH on XO production from CSX by Shearzyme. Hydrolysis conditions: 0.21 U/ml shearzyme, 2% CSX, 40 °C

All results showed that XOs could be produced enzymatically from xylan extracted from CS by commercial xylanases, Shearzyme as well as Veron. However Shearzyme yielded higher amount of XO than Veron. Their product profiles showed some differences. While CSX was hydrolyzed to a greater range of XOs by Veron, it was hydrolyzed to smaller XOs by Shearzyme.

4.1.2 Hydrolysis of Sunflower Stalk Xylan by Veron and Shearzyme

In this part of study, xylan extacted from SS was hydrolyzed by Veron and Shearzyme. The optimum SSX hydrolysis conditions and composition of XOs produced were investigated.

SSX, 2%, prepared in 50mM citrate phosphate buffer at pH 4.6 and pH 5.4 was incubated with Shearzyme and Veron having 0.21 U/ml enzyme activity. The reaction at 40°C was carried out for 96 h for both xylanases to observe hydrolysis profile and product composition (Figure 4.14). The maximum reducing sugar concentrations obtained by Shearzyme and Veron were about 25 µmole/ml and 16 µmole/ml, respectively which were obtained at 24 h.

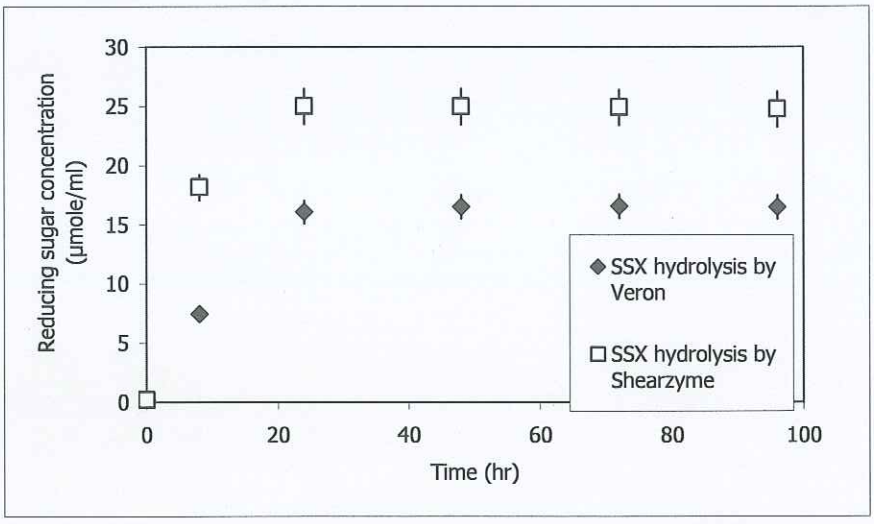


Figure 4.14 Hydrolysis of 2% SSX by Shearzyme and Veron.

The degree of polymerizations of XOs produced by hydrolysis of SSX by Shearzyme were observed in between X2-X5 on TLC plate (Figure 4.15a). The main products were X3 and X4. The larger XOs were not observed on the contrary of XOs produced by Veron.

On the other hand, SSX was hydrolyzed by Veron to X2, X3 X5, X6 and larger XOs than X6. Substrate specificity of xylanases is also a key factor that determine composition of XOs produced. Because as seen from Figure 4.15b, Veron could not hydrolyzed XOs>X5-X6 to smaller ones due to its substrate specificity. In this case, considering industrial applications of XO which may require smaller XOs, larger XOs might be hydroyzed to smaller XOs by other xylanase preparations having substrate specificity for large XOs. Although different product profiles were obtained from hydrolysis of SSX by Shearzyme and Veron, no X1 formation was observed on TLC plates of XOs produced by both enzymes (Figure 4.15a-b).

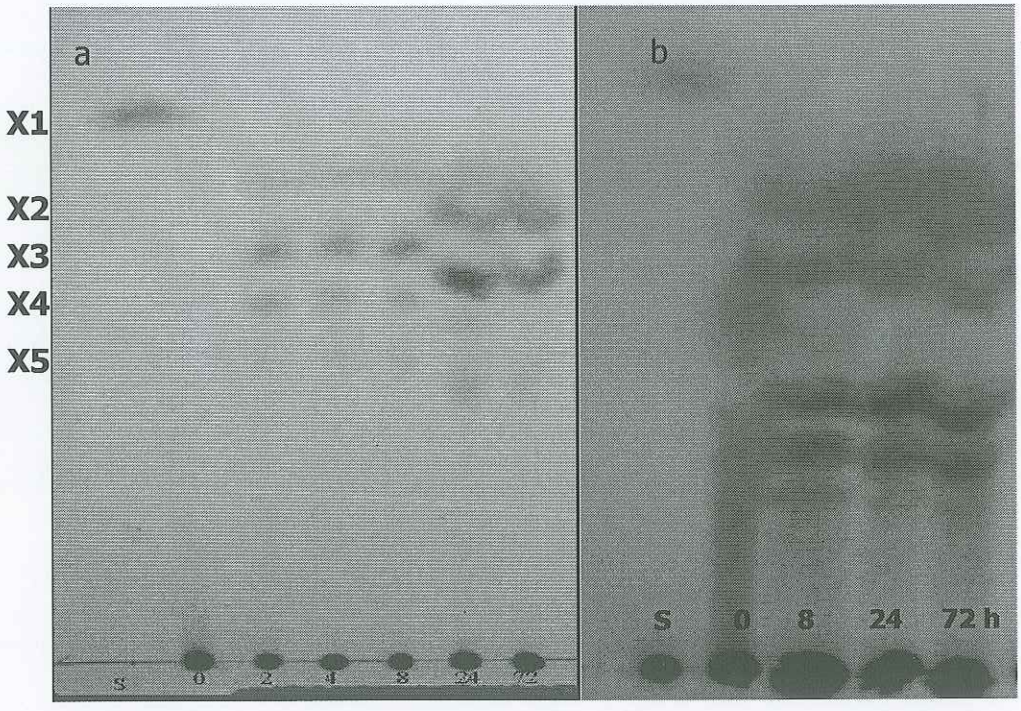


Figure 4.15 TLC plate of XOs produced by hydrolysis of 2% SSX by Shearzyme (a) and Veron (b)

4.1.2.1 The Effect of Temperature on XO Production from SSX by Veron and Shearzyme

The investigation of the effect of temperature on the production of XO from 2% SSX by Veron and Shearzyme was carried out at 40°C, 45°C and 50°C. As seen from Figure 4.16 and Figure 4.17, the hydrolysis profiles of both Veron and Shearzyme were similar at all temperatures studied. The amount of XOs produced at 45°C and 50°C were found almost the same for both xylanases. The concentration of XO formed by Veron and Shearzyme at 40 °C was slightly lower than at 45°C and 50°C. However, higher XO yield was obtained by shearzyme at all temprature. The concentrations of reducing sugar obtained by Shearzyme were 20% and % 32 higher than Veron at 45 °C and 50 °C, respectively.

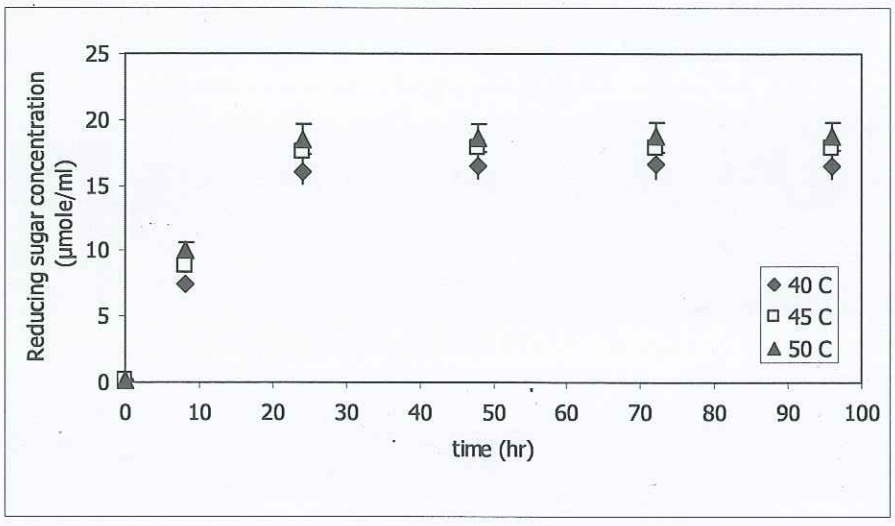


Figure 4.16 Effect of temperature on XO production from SSX by Veron. Hydrolysis conditions: 2 % SSX, 0.21 U/ml Veron, pH 5.4.

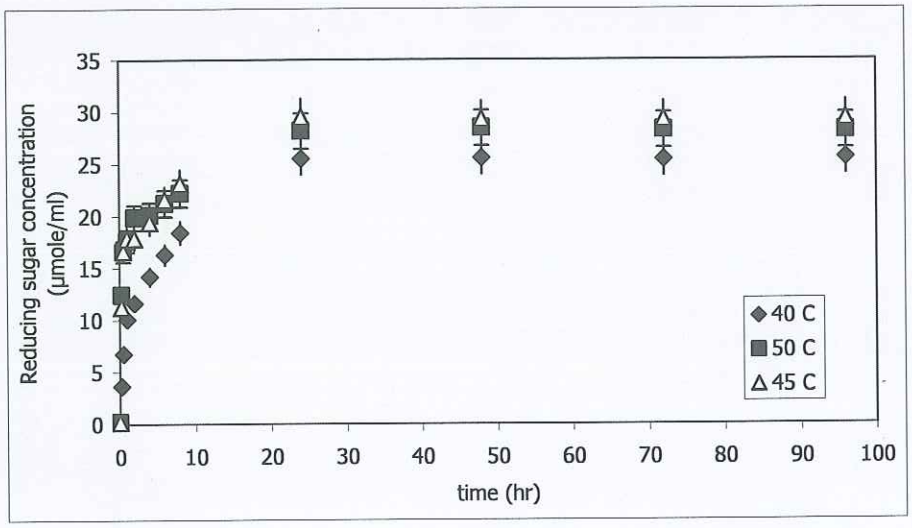


Figure 4.17 Effect of temperature on XO production from SSX by Shearzyme. Hydrolysis conditions: 2 % SSX, 0.21 U/ml Shearzyme, pH 4.6.

4.1.2.2 The Effects of Enzyme Concentration on XO Production from SSXy Veron and Shearzyme

In order to investigate the effect of xylanase concentrations on the production of XO, 2% SSX hydrolysis was conducted with different enzyme concentrations of Veron and Shearzyme, ranging from 0.06 to 0.96 U/ml. It was seen that reducing sugar concentration increased rapidly up to 8 h for all enzyme concentrations of Veron and Shearzyme and the increase was not very significant after 24 h (Figure 4.18, Figure 4.19). As seen from Table 4.2, the reducing sugar yield obtained by Shearzyme was quite higher than the reducing sugar yield obtained by veron. However, increasing enzyme concentrations of Veron and Shearzyme after 0.21 U/ml resulted in a significant decrease, 50 %, in reducing sugar yield.

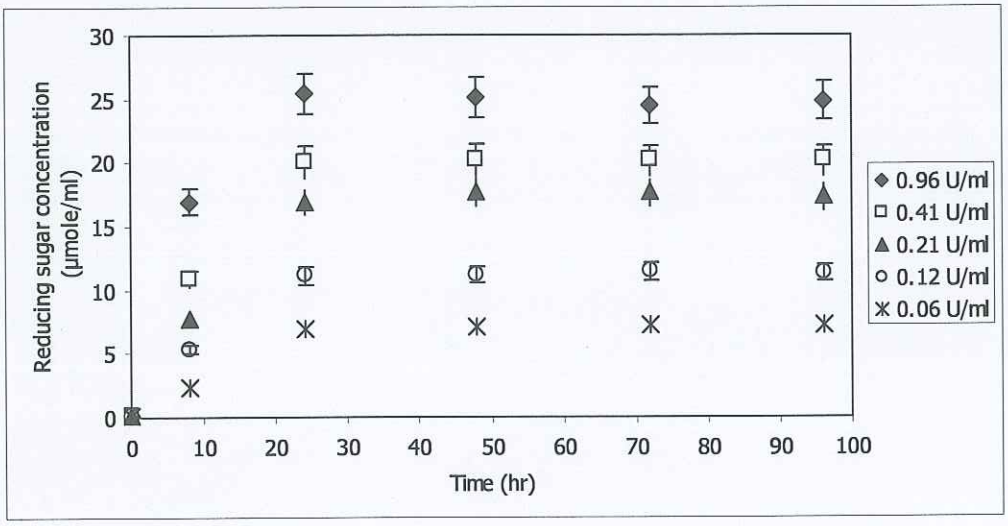


Figure 4.18 Effect of Veron concentration on XO production from SSX . Hydrolysis conditions: 2 % SSX, pH 5.4, 40 °C.

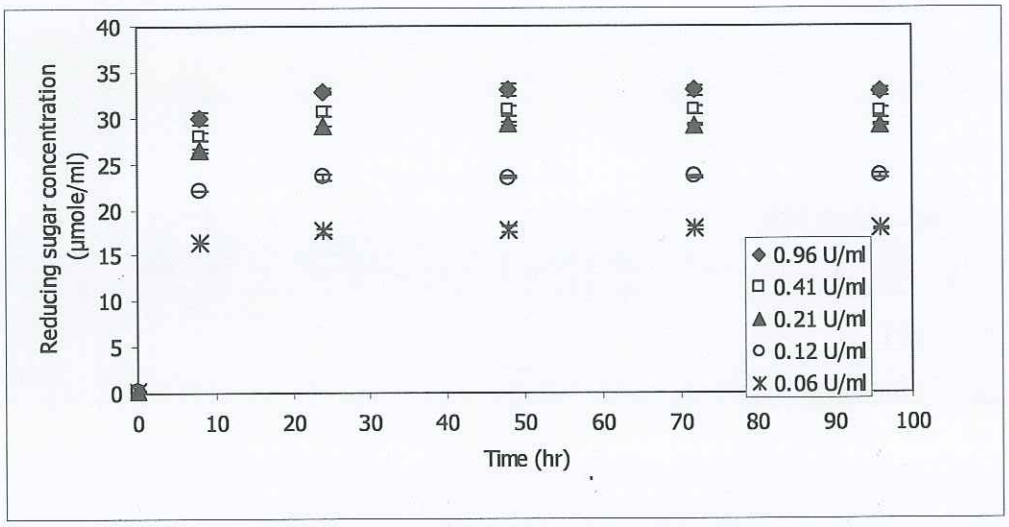


Figure 4.19 Effect of Shearzyme concentration on XO production from SSX. Hydrolysis conditions: 2 % SSX, pH 4.6, 40°C.

Table 4.2 Enzyme efficiency for XO prodcution from SSX.

	Enzyme Efficiency	
Enzyme Concentration (U/ml)	**Veron (µmole reducing sugar/ U)**	**Shearzyme (µmole reducing sugar/ U)**
0.06	116	295
0.12	93	196
0.21	80	138
0.41	50	75
0.96	26	34

4.1.2.3 The Effect of SSX Concentration on XO Production by Veron and Shearzyme

Different concentrations (1%-10%) of SSX were hydrolyzed by Veron and Shearzyme to find the effect of xylan concentration on XO production.

SSX was hydrolyzed by Shearzyme more efficiently than Veron at all the xylan concentrations that have been used. When concentration of SSX increased to 9% inhibition effect was not observed. However, 10 % xylan concentration inhibited the enzyme so the reducing sugar concentreation decreased about 40% (Figure 4.20).

As shown in Figure 4.21, reducing sugar concentration increased depending on the increasing xylan concentration and increasing SSX concentration up 10% did not affect XO production negatively. In other words, neither substrate inhibiton nor inhibiton by any concominant molecules were not observed. However using xylan at higher concentrations than 5% was difficult because of increase in density and viscosity.

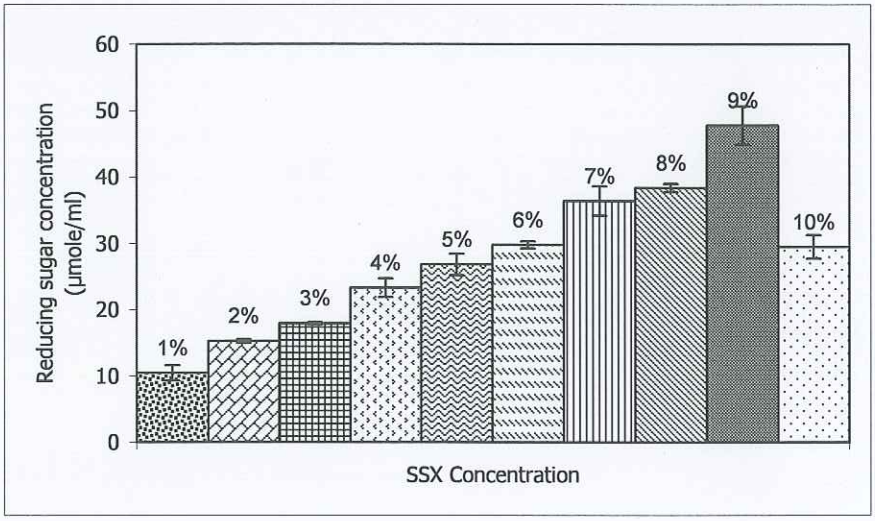


Figure 4.20 Effect of SSX concentration on XO production by Veron. Hydrolysis conditions: 0.21 U/ml Veron, pH 5.4, 40 °C

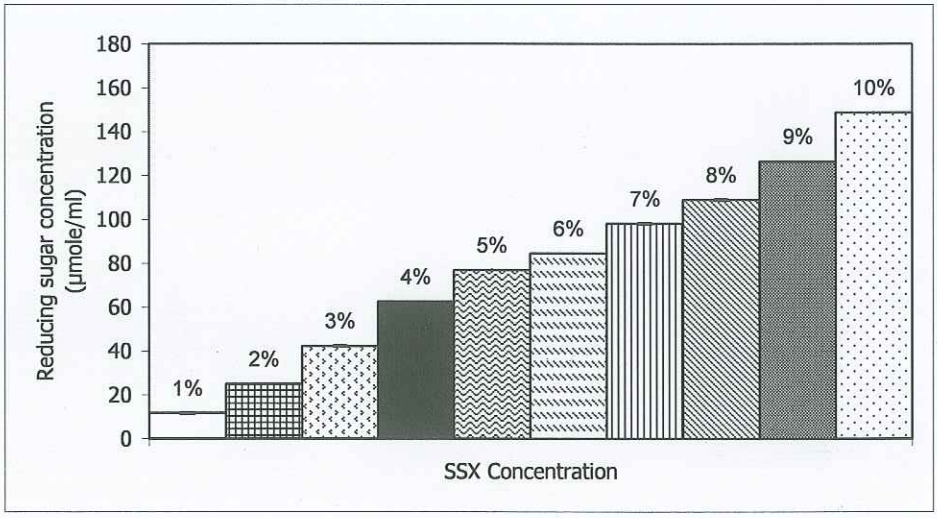


Figure 4.21 Effect of SSX concentration on XO production by Shearzyme. Hydrolysis conditions: 0.21 U/ml Shearzyme, pH 4.6, 40 °C

4.1.2.4 The Effect of pH of XO Production from SSX by Veron and Shearzyme

The effect of pH on hydrolysis of xylan from SS by Veron and Shearzyme was examined for XO production. The highest XO production by veron was observed at pH 5.4 and enzyme acitivity and reducing sugar concentration decreased at pH 6.5 significantly (Figure 4.22). While there was a slight difference between the amount of reducing sugar concentration at pH 4.6 and pH 5.5, Shearzyme yielded larger amount of XO from SSX at pH 5.5. The lowest reducing sugar concentration was obtained at pH 3.5 (Figure 4.23). Therefore, pH 5.4 and pH 5.5 may be accepted as the optimum pH for hydrolysis of SSX by Veron and shearzyme respectively, to produce XOs.

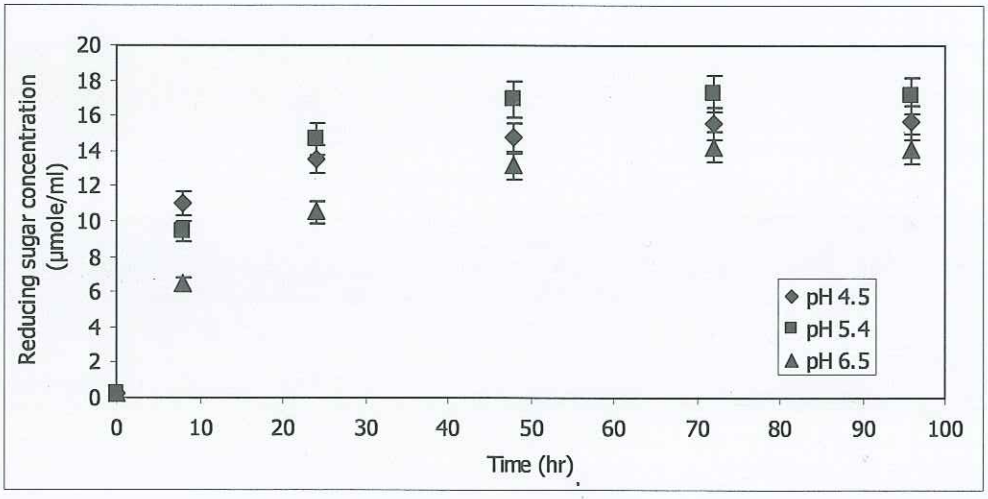


Figure 4.22 Effect of pH on XO production from SSX by Veron. Hydrolysis conditions:0.21 U/ml Veron, 2% SSX, 40 °C

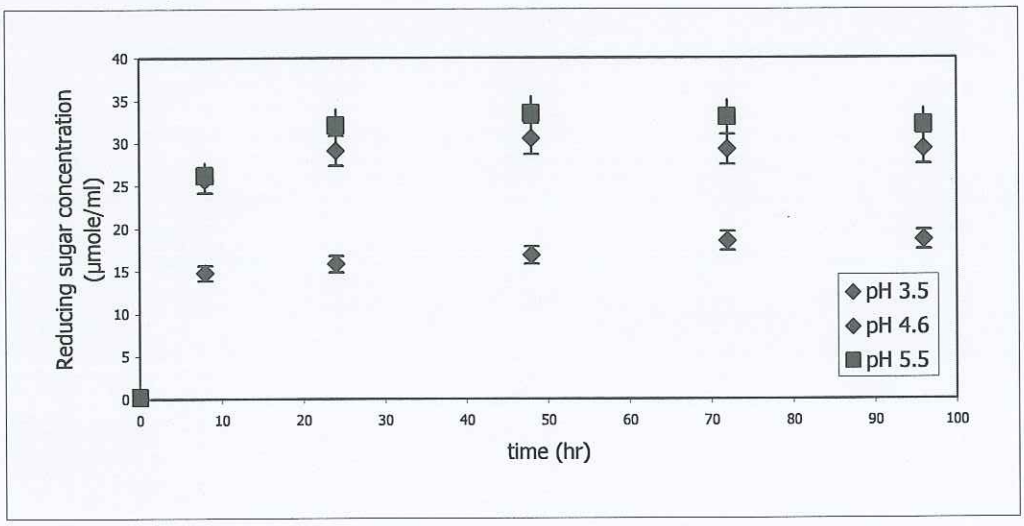


Figure 4.23 Effect of pH on XO production from SSX by Shearzyme. Hydrolysis conditions:0.21 U/ml Shearzyme, 2% SSX, 40 °C

4.1.3 Comparative Efficiency of Veron and Shearzyme on CSX and SSX

Xylan extracted from CSX and SSX were hydrolyzed by two commercial xylanases, Veron and Shearzyme for XO production. Shearzyme was found to yield the larger amount of reducing sugar concentration from CSX and SSX than Veron. In addition, product profiles of Veron and Shearzyme were different. CSX and SSX were hydrolysed by Veron to X2, X3, X5, X6 and XOs > X6. The main products were X5 and X6, which were followed by X2 and X3. On the other hand, Shearzyme yielded a different product profile, X2, X3, X4, X5 from CSX and SSX. The larger XOs were not observed, on TLC plate, when Shearzyme hydrolyzed CSX and SSX compared with Veron which depends on mode of action and substrate specifity of xylanases. However, conversion yieldsy of 2% CSX to XOs at 40 °C and pH 5.4 by both

Veron and Shearzyme were found as about 33%. This is the same with conversion yield of 2% SSX to XOs by Veron. However, hydrolysis of 2% SSX by Sheazyme yielded in the higher conversion yield with 40%.

In some other experiments performed on XO production by xylanases, the effect of addition fresh enzyme or fresh substrate after 24th were investigated (Akpinar *et al.*, 2007). Addition of fresh substrate increased of XO concentration. However, addtion of fresh enzyme to the reaction mixture did not increase XO yield showing formation of xylanase-resistant XOs>X6. Certainly, these amount of efficiences are not sufficient for industrial application. However, it is possible to increase by using different xylanase preparations having different mode of action and substrate specifity. Besides, use of xylan side-chain cleaving enzymes can increase the yield. Because, as seen from TLC result, XOs larger than X6 were produced especially by Veron. According to potential industrial applications of XOs, larger XOs might be hydrolyzed to smaller ones by using enzyme mixtures and conversion efficiency also can be increased.

4.1.4 Hydrolysis of CSX and SSX by Xylanase from *Bacillus pumilis* SB-M13

In addition to commercial xylanases, a home produced xylanase from *Bacillus pumilus* SB-M13, was also tested for XO production. In this part of the experiments, *B. pumilus* was cultivated in a fermentation medium containing 3% of ground corn cobs and then xylanase in culture supernatant at 4th day was assayed for XO production from CSX and SSX using standard assay conditions.

CSX and SSX were hydrolyzed by crude and pure xylanase from *B. pumilus* (BAX). Crude BAX was found to yield the larger amount of XOs from CSX. The amount of XO produced by pure BAX and crude BAX were 10 and 14 µmole/ml, respectively at 24^{th} h, although crude BAX had lower enzyme acitivity (Table 4.3). One of the reasons for this might be instability of pure BAX which was more dilute than crude BAX, accordingly it might possibly be subjected to deactivation. Another reason of higher activity of the crude enzyme preparation could be due to the presence of glucuronosidase and its synergistic action with xylanase on CS xylan which is known as glucuronoxylan.The chemical and sugar composition of the CSX were given in Table 4.4 and Table 4.5 (Akpinar *et al*, 2007a). As seen from the Table 4.5, arabinose, mannose, and galactose were not observed and main constituents were 83.6% xylose, 9.28% uronic acid and 7.12% glucose, which shows glucuronoxylan properties. Consequently, the efficient hydrolysis of CSX may require synergytic act of both xylanase and glucuronosidase. *B. pumilus* might produce extracellular glucouronisidase which may action together with xylanase to enhance xylan hydrolysis efficiency. Therefore, during hydrolysis of CSX, crude BAX produced rather higher level of XO than pure BAX. As expected, in pure BAX preparation could not contain glucuronosidase, so XO yield produced by pure BAX was lower.

Table 4.3. Hydrolysis of CSX and SSX by CBAX and PBAX.

	Reducing sugar concentration (µmole / ml)			
Time (hr)	Hydrolysis of CSX by CBX	Hydrolysis of CSX by PBX	Hydrolysis of SSX by CBX	Hydrolysis of SSX by PBX
0	0.187 ± 0	0,187 ± 0	0.187 ± 0	0.187 ± 0
8	12.84 ± 0.52	9.78 ± 0.06	9.07 ± 0.37	9.68 ± 0.25
24	13.70 ± 0.12	10.31 ± 0.14	9.54 ± 1.25	10.43 ± 0.4

Table 4.4 Chemical composition of CS (Akpinar *et al*, 2007a).

Component	**Content[a] (g/100g of CS)**
Ash	3.81 ± 0.09
Xylan	21.42 ± 1.96
Cellulose	35.68 ± 0.76
Lignin	27.83 ± 1.05
Others (by diff)	11.26

[a]Mean ± standart deviation of at least three replicate determinants

Table 4.5 Sugar composition of CSX (Akpinar *et al*, 2007a)

CSX	Content[a]	CSX	Content[a] (%)
Arabinose	0	Galactose	0
Xylose	83.60 ± 0.66	Glucose	7.12 ± 0.73
Mannose	0	Uronic acid	9.28 ± 1.29

[a]Mean ± standart deviation of at least three replicate determinants

On the other hand, under the same conditions used for hydrolysis of CSX, crude BAX produced same amount of reducing sugar from SSX as pure BAX produced (Table 4.3). In other words, hydrolysis profile of sunflower xylan by both crude and pure BAX was similar to hydrolysis profile of CSX pure BAX. Although, SSX is also glucuronoxylan it is known that there is a great heterogeneity in the structure of xylan from different sources (Tabel 4.6, Table 4.7) (Akpınar *et al*, 2007b) The chain length, degree and type of substitutions may be the key factors that affect the rate of xylan hydrolysis. In the present case, uronic acid substitution, uronic acid to xylose ratio in sunflower stalk xylan might be different from cotton stalk xylan so SSX hydrolysis efficiency and XO production by crude and pure BAX was lower. Difference in hydrolysis efficiency can be explaned by sunflower stalk xylan structure itself. Because possible presence or absence of glucuronisidase activity in enzyme preparations did not affect the SSX hydrolysis profile due to its chemical structure. Consequently, SSX hydrolysis was dependent on xylan structure and BAX substrate specificity.

Table 4.6 Chemical composition of SS (Akpinar *et al*, 2007b)

Component	**Content[a] (g/100g of SS)**
Ash	2.09 ± 0.01
Xylan	16.86 ± 2.73
Cellulose	41.05 ± 3.37
Lignin	34.09 ± 0.49
Others (by diff)	5.92

[a]Mean ± standart deviation of at least three replicate determinants

Table 4.7 Sugar composition of SSX (Akpinar *et al*, 2007b)

SSX	**Content[a]**	**SSX**	**Content[a] (%)**
Arabinose	0	Galactose	0
Xylose	85.73 ± 0.93	Glucose	3.79 ± 3.30
Mannose	0	Uronic acid	11.12 ± 0.46

[a]Mean ± standart deviation of at least three replicate determinants

The composition of XOs produced from CS and SS xylan by both crude and pure BAX is shown in Figure 4.24a-b. The profiles of XOs produced from SSX and CSX by both the crude BAX and pure BAX were slightly different from each other. This result indicated that crude BAX preparations may contain possible debranching enzyme acitivies like glucuronisidase, acetyl xylan esterase, produced extracellularly by *Bacillus pumilus*, which might act synergistically with xylanase and resulted in different product profile formation. X2 formation was only seen on TLC of XOs produced from CSX and SSX by crude BAX (Figure 4.24b). It means that there was ß -1, 4-xylosidase acitivity of crude BAX preparation. The main products obtained by hydrolysis of both SSX and CSX by pure BAX were X5 and X6, while crude BAX produced X4 and X5 (Figure 4.24a). This was also the result of possible presence of debranching enzymes in crude BAX preparations. Substrate specificity of xylanases is also important factor that determine composition of XOs produced. According to the TLC results, xyolooligosaccharides containing 6 or more xylose units were also obtained by both enzymes, crude and pure BAX meaning that they could not hydrolyze them to smaller XOs because of their substrate specificity. In this case, considering industrial applications of XO which may require smaller XOs, larger XOs might be hydroyzed to smaller XOs by other xylanase preparations having substrate specificity for large XOs.

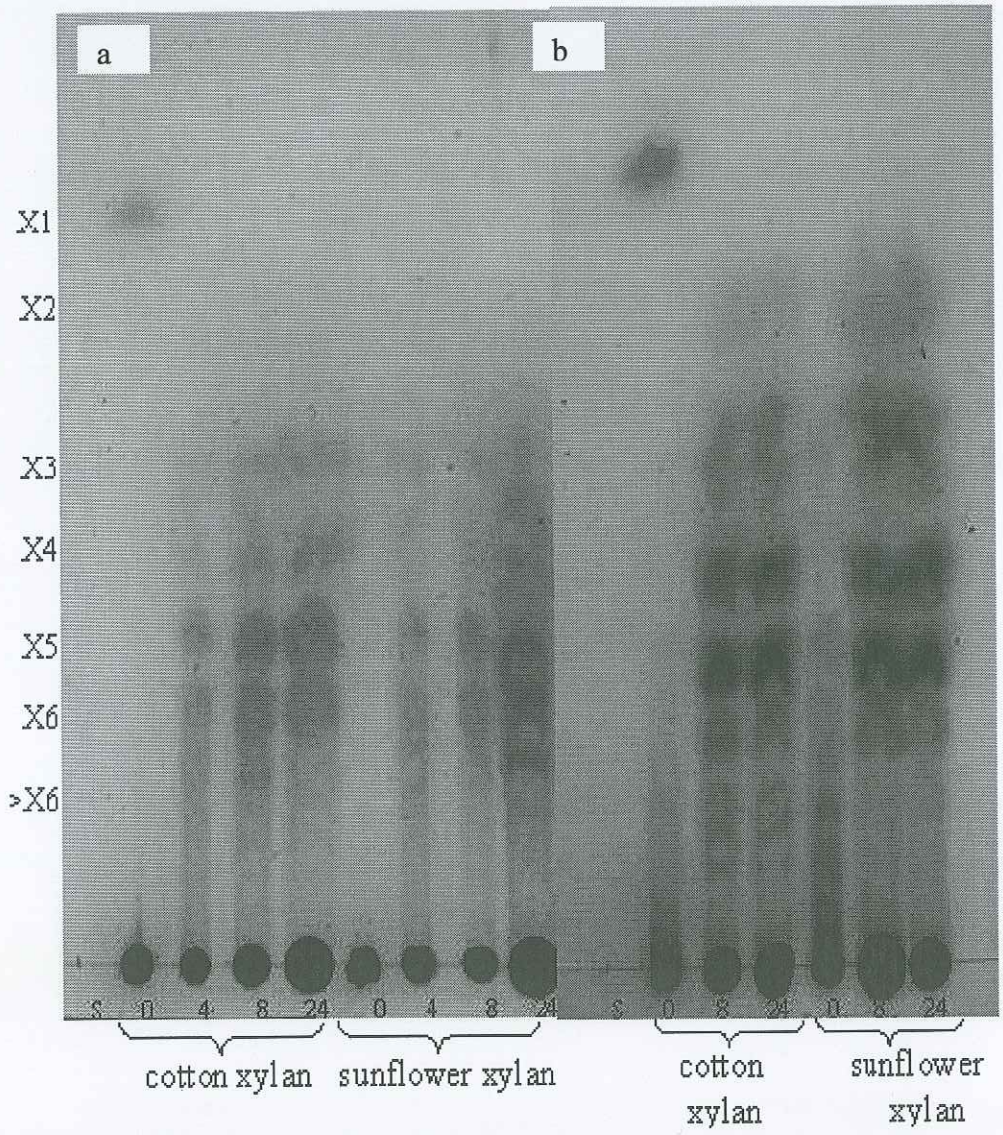


Figure 4.24 TLC of XOs produced from CSX and SSX by *Bacillus pumilis* xylanase, a. TLC of XOs produced by purified BAX, b. TLC of XOs produced by crude BAX.

4.2 XO Production directly from Lignocellulosic Material without Xylan Extraction

In the previous parts of the study, enzymatic XO production was performed by using xylan extracted from CS and SS. Alkali extraction method has some advantages that inhibitory substances like furfural

and hydroxymethylfurfural are not produced unlike in most other pretreatment methods, however, it requires utilization of large amount of alkali solutions (KOH, NaOH, etc.) which mainly make the process hazardous to the environment besides their additional cost. Although there are altervative methods to alkali extraction for separation of xylan, they either require harmful chemicals; produce toxic and/or harmful products or these processes are not energy efficient. So, to find an efficient biological pretreatment method will be highly beneficial considering general characteristics of biological methods such as being mild, green, safe and less energy consuming processes.

To choose a particular pretreatment method, there are many considerations such as:

1) the pretreatment process itself should be economical,
2) the pretreatment process should be a green process,
3) the pretreatment must help to remove lignin efficiently,
4) the pretreatment must help to utilize hemicellulose portion of CS, which should be readily hydrolyzed by enzymatically for XO production.

Therefore, in this part of the study, different biological pretreatment methods were investigated:

1) Microbial pretreatment; A white-rot fungus, *Phanerochaete chrysosporium* is used.

2) Enzymatic pretreatment; Cellulase treatment was performed to break up to open fiber pores and increase accessibility of hemicellulose

3) Hydrothermal pretreatment, to break up complex structure by heat and pressure.

4.2.1 Pretreatment of CS by *Phanerochaete chrysosporium*

White-rot fungi and their enzymes are receiving increasing attention for biotechnological applications. Many efforts have been made to investigate the application of these fungi for the removal of lignin in the pulping and bleaching process. It was first reported by Kirk and Yang (1979) that *Phanerochaete chrysosporium* was able to partially delignify unbleached Kraft pulp.

The aim of this part of the study was to improve utilization of xylan in CS by removing lignin selectively from CS by means of *P. chrysosporium* fermentation for further enzymatic production of XOs from microbially-treated biomass. For this purpose, the fermentation was analyzed in terms of lignin and hemicellulose degradation. In this pretreatment method, utilization of lignin was required as much as possible. However, the utilization of hemicellulose was not desired since it would be later used for XO production. Therefore, *P. chrysosporium* was grown in liquid media contained 5% CS (autoclaved at 121 °C for 1 h and incubated at 100 °C for overnight) as sole carbon source.

Cultures were grown in 2 L and 250 ml erlenmeyer flasks containing 200 ml and 20 ml of medium, respectively at 39 °C with and without shaking. Daily samples were taken and analysed for xylanase activities by using 1% birchwood xylan (w/v) and formation of soluble phenolic compounds from lignin. Then, the media were filtered to recover pretreated CS at 4 th and 7 th days of fermentations. CS taken from each medium was washed with 350 ml of ethylalcohol and 500 ml of distiled water to remove all the solubles especially lignin products. Finally, CS was dried at 60 °C for overnight and used in enzymatic hydrolysis by Shearzyme for XO production.

The fermentations were conducted by static and shaking cultures to observe the effect of shaking on *P. chrysosporium* growth and pretreatment. However, *P. chrysosporium* could not be cultivated in fermentation medium incubated by shaking at 170 rpm. In addition, for the unshaken cultures, the effect of air/liquid volume ratio were also tested by using two different air/liquid volume ratios, 0.08 and 0.1. Xylanase activity in 7th day fermentation medium having air/liquid volume ratio of 0.08 was found as 0.45 U/ml . However, when CS recovered from the 7^{th} day of fermentation medium (air/liquid=0.08) was enzymatically hydrolyzed by incubated by shearzyme, insignificant amount of reducing sugar, about 0.7 µmole/ml, was obtained. This amount was about 35% of reducing sugar concentration obtained by hydrolysis untreated CS (control) (Figure 4.25). So, Shearzyme hydrolyzed xylan in biomass pretreated CS insignificantly and XO production was not observed.

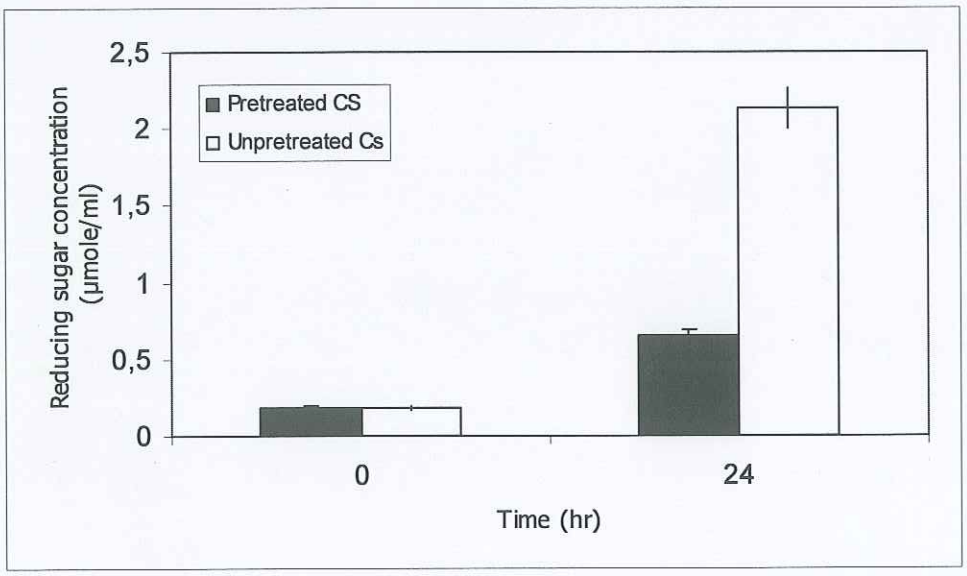


Figure 4.25 Hydrolysis of biomass pretreated and unpretreated CS (10%) by Shearzyme at 40 °C, pH 4.6.

As seen from Figure 4.26, xylanase activiy was also detected in *P. chrysosporium* cultures having air/liquid volume ratio of 0.01 which were performed without shaking. Fermentations were performed in 14 flasks and two fermentation flasks were harvested each day to analyze biodegradation. The solid residue (pretreated CS) were recovered from each flask and used in enzymatic XO production studies.

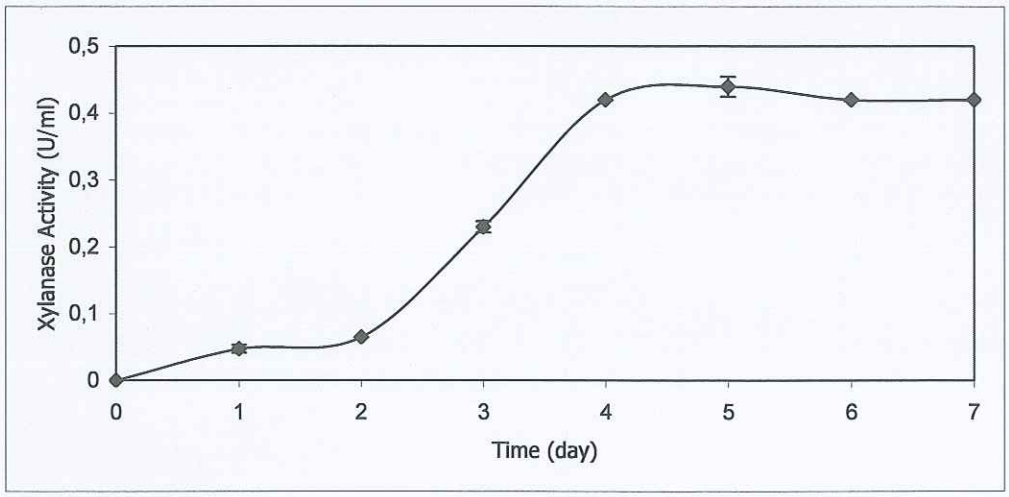


Figure 4.26 Xylanase activity in 200 ml *P. chrysosporium* cultures

CS (10%) recovered from the media for each day up to 4th day and 7th day of the fermentation were incubated with Shearzyme (0.21 U/ml) at 40 ^{o}C for XO production. CS, not pretreated, was also hydrolyzed by Shearzyme as control and as in the previous case, larger amount of reducing sugar was determined. The recovered CS from 7th day fementation medium resulted in the amount of reducing sugar about 40 % of reducing sugar obtained from unpretreated CS (Figure 4.27). Actually, the aim was to selectively remove lignin and produce XO from xylan remained in CS after biomass pretreatment by further Shearzyme hydrolysis. However, the pretreated CS during 7 days were ineffective in enzymatic XO productions. Shearzyme probably might be inhibited by some products and/or contaminants especially lignin based phenolics, during biomass pretreatment. Therefore, CS recovered from media were extra washed to remove contaminants and used for enzymatic hydrolysis. However, the result did not change.

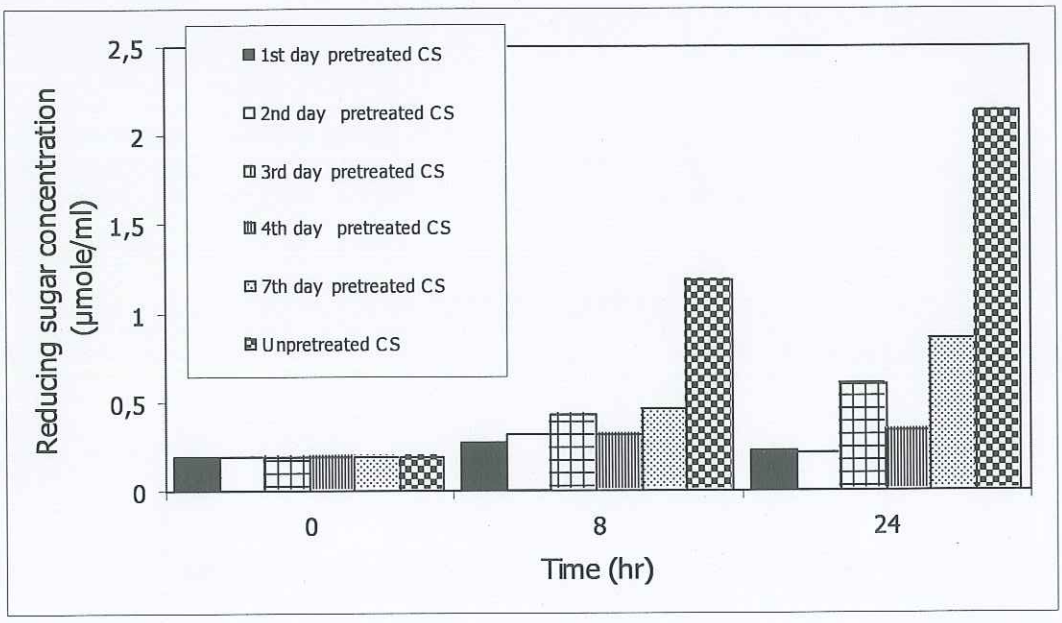


Figure 4.27 Hydrolyis of 10% pretreated and unpretreated CS by Shearzyme (0.21 U/ml) at 40 °C, pH 4.6

The daily samples taken from the fermentation medium were also analysed for reducing sugar content to understand whether xylan is hydrolyzed to XO or not assuming no significant cellulase activity of this microorganism. Reducing sugar concentration was very low during the first 2 days. Afterwards, the reducing sugar concentration increased up to 4th day and then decreased again (Figure 4.28). When characterization of the reducing sugar was performed by TLC, only glucose formation was observed on the 3rd day on TLC plate and its concentration decrased after 5th day which was consistent with the result obtained by DNSA. However, no xylose or XO were observed on the TLC plate. These results suggested glucose production from cellulose by cellulolytic activity of *P. chrysosporium* up to 4th day of

fermentation and then the microorganism started to use glucose in the medium or release of glucose from xylan structure (Figure 4.29).

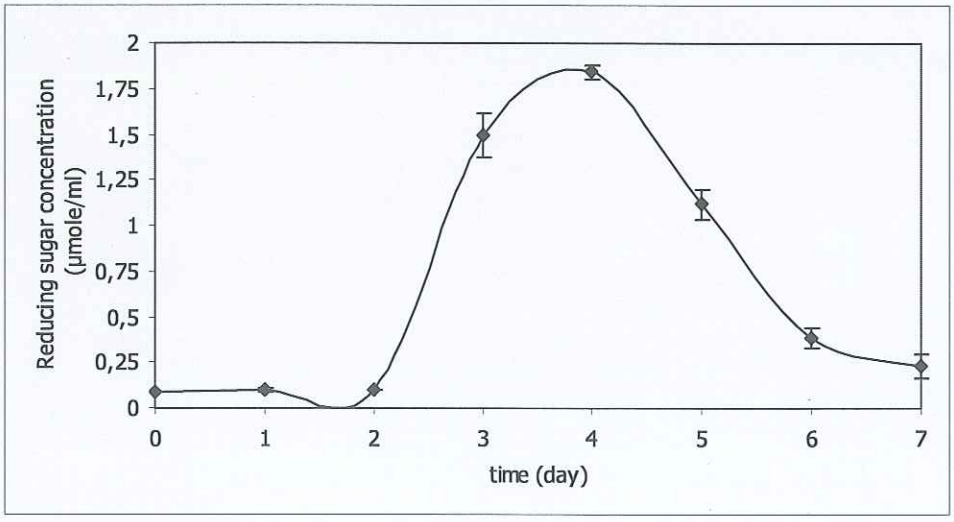


Figure 4.28 Reducing sugar concentrations in *P. chrysosporium* growth media used for biomass pretreatment.

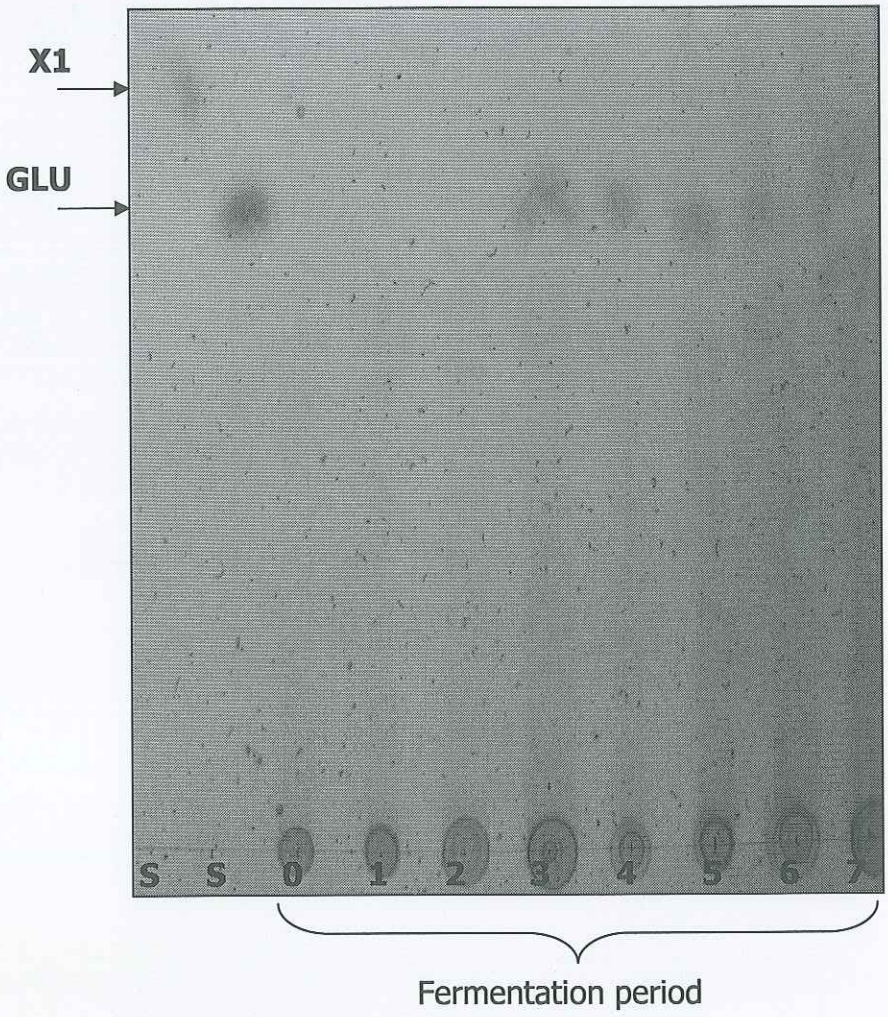


Figure 4.29 TLC of reducing sugar formed in *P. chrysosporium* growth media used for biomass pretreatment.

HPLC analysis of daily samples taken from *P. chrysosporium* fermentation medium used for biomass pretreatment were also conducted to determine of cellobiose, glucose, xylose, XO and phenolic compounds which are formed during biomass pretreatment and then may affect Shearzyme activity during enzymatic hydrolysis of pretreated CS. As observed from Table 4.8, at the beginning of fermentation,

cellobiose, glucose, gallic acid, catechin and epicatechin were determined on *P. chrysosporium* growth medium which means that they formed during sterilization of medium at 121 °C on autoclave. Glucose production was consistent with the result of TLC. At the end of fermentation, all cellobiose produced converted to glucose and X1 also started to appear from the 4th day of fermentation. Phenolic compounds, gallic acid, catechin and epicatechin, found in the medium at the beginning of the biomass pretreatment was used by *P. chrysosporium* up to 4th day of fermentation and then their concentration were again increased because of lignin degradation which is required for efficient further enzymatic hydrolysis of CS for XO production. Production of XO by xylanase activity of *P. chrysosporium* during fermentation might be another method which may be also quite environment friendly and economical. Xylanase activity might be increased by medium optimization to increase amount of XO produced. However, XOs produced should be separated from the medium by using an appropriate membrane system to prevent utilization of XOs by *P. chrysosporium.* Besides xylanases are produced as the second product during the fermentation

Table 4.8 HPLC analysis of sugar and phenolic compounds in *P. chrysosporium* growth media used for biomass pretreatment.

	Concentrations (µg/ml)					
Fermentation day	Cellobiose	Glucose	Xylose	Gallicacid	Catechin	Epicatechin
0	1.1	16.5	0	210.3	5.9	8.1
1	23.4	18.4	0	1.08	5.3	6.17
2	14.8	32.6	0	0.1	4	5.6
4	1.2	244.6	5.7	298	5.1	2
5	5.7	198.6	6.9	158	6.6	2.6
6	0	75	8.8	24.3	6.6	2.7
7	0	44.5	6.1	3.9	6.4	2.9

HPLC analysis of samples taken from enzymatic hydrolysis reaction of CS were presented in Table 4. 9 and 10. It is interesting that at the beginnig of the reaction, glucose, X1 and phenolics were present in the reaction mixture although washing of CS after recovering from the medium. CS in citrate buffer was stirred on magnetic stirrer for overnigt to prepare it as substrate for enzymatic hydrolysis reaction. During overnight mixing, probable degradation might occur. For the period of the reaction, amount of phenolics decreased due to possible binding of these compound on Shearzyme which might cause activity losses. In addition, low amount of XOs production, X5 and X3, were also detected by HPLC. These results suggested that Shearzyme has lost activity but was not completely inhibited. Therefore, formation of phenolics during CS preparation as substrate can be prevented by using another method or formed phenolics can be removed from the substrate solution before initiation of the enzymatic reaction. On the other hand, although some amount of lignin was degraded, CS used in enzymatic hydrolysis still contained lignin and Shearzyme did not sufficiently reach to xylan in CS because of possible presence of lignin in CS structure. Morever, glucose formation by cellulase activity in medium might affect delignification of CS negatively. In a study done by Carvalho *et al* (1998) it was found that an increase in the amount of glucose in the media resulted in a delay of the laccase production which requires for lignin removal in lignocellulosic material. During pretreatment, the glucose released from cellulose might be degraded to 5-hydroxymethylfurfural, levulinic acids and formic acid, whereas the xylose formed from hemicellulose may converted to furfural and formic acid (Taniguchi *et al.*, 2005). These degradation products, together with lignin degradation products might act as inhibitors in the enzymatic hydrolysis stage.

Consequently, biomass pretreatment of CS by *P. chrysosporium* should be improved by further studies. Biomass pretreatment studies in literature has mostly focused on cellulose utilization in lignocellulosic material for ethanol and/or other some chemicals production. Delignification of lignocelulosic material without degradation of xylan by biomass pretreatment has recently become attractive. It was seen from the results in this study that *P. chrysosporium* has cellulolytic and xylanolylitic activity. Therefore, XO production might be carried in fermentation medium by using membrane system to remove XOs from the medium. Another method may be inhibition of xylanase production and increasing lignolytic activity of *P. chrysosporium* by testing different growt medium components and concentrations.

There is a link between lignin degradation and secondary metabolism of *P. chrysosporium*. It means that ligninase activity appears only during secondary metabolism (Fasion *et al.*, 1986). Therefore, fermentation period might be prolonged to increase and observe lignolytic acitivity. Lignolytic acitvity of *P. chrysosporium,* lignin peroxidase, Mn peroxidase and laccase, might be daily assayed during fermentation period together with cellulase and xylanase activities to observe the rate of delignification, hemicellulose and/or cellulose depolymerization in different growth media tested.

In the study conducted by Dobozi *et al.* (1992), *P. chrysosporium* showed minimun xylanase activity in xylose containing medium among the media tested because of end product inhibition. Different concentrations of xylose might be tested for inhibition effect on xylanase production. As a result, biomass pretreatment is a safe and environment friendly method but it should be improved by further studies focusing on hemicellulose utilization to produce XOs.

Table 4.9 HPLC analysis of sugars and phenolic compound formed in enzymatic hydrolysis reaction of biomass pretreated of CS.

	Concentrations (µg /ml)					
Time (hr)	Cellobiose	Glucose	Xylose	Gallicacid	Catechin	Epicatechin
0	0	92	1.6	3.6	1.7	1
24	0	118	2.3	0.55	1.1	0.7

Table 4.10 HPLC analysis of XOs in enzymatic hydrolysis reaction of biomass pretreated of CS.

	µg/ml					
Time (hr)	X6	X5	X4	X3	X2	X1
0	0	0	0	0	0	22
8	0	639	0	0	0	22
24	0	641	0	41	0	30

4.2.2 CS Pretreatment by Cellulase

Lignin, which consists of a three-dimensional polymer of propyl-phenol that is imbedded in and bound to the hemicellulose. Hemicellulose and lignin cover cellulose microfibrils. Treatment of CS with cellulase might help to open fiber pores, increase accessibility of hemicellulose and increase penetration of xylanase. Therefore CS was pretreated by cellulase. During pretreatment of CS by cellulase, 2.5 g of CS (autoclaved at 121 °C for 1 h and incubated at 100 °C for overnight) in 50 ml of 50 Mm citrate buffer at pH 4.8 was incubated with 2.5 ml cellulase at 40 °C. Samples were periodically taken and analysed for reducing sugar concentration.

As seen from Figure 4.30, the amount of reducing sugar increased depending on time and reached to about 17 µmole/ml at 6th day of the reaction. Large amount of reducing sugar was glucose with 96%

followed by xylose with 3.4% and cellobiose with 0.6% which were determined by HPLC (Table 4.11). On the other hand, gallic acid, catechin and epicatechin were also determined in reaction liquor which means that some amount of lignin degradation has also occured.

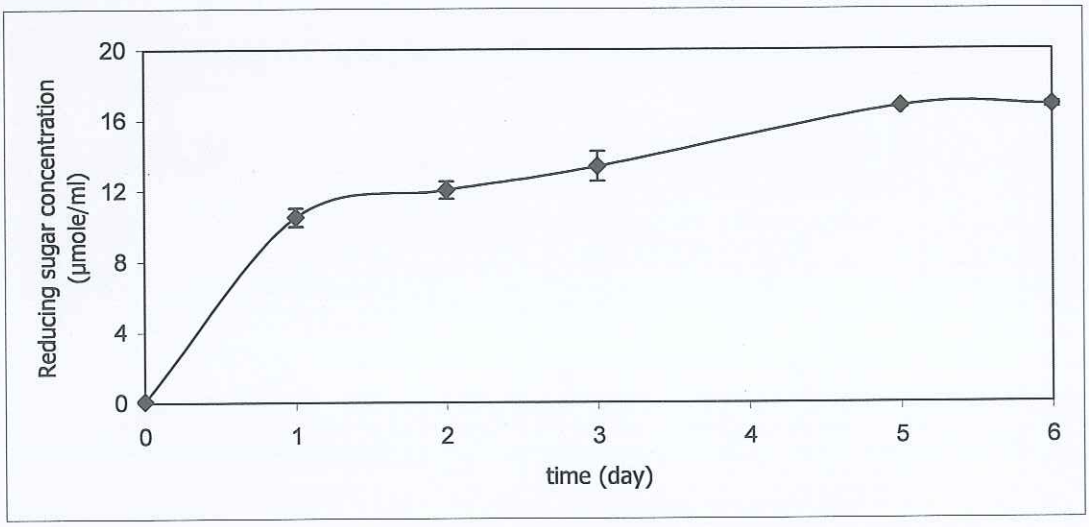


Figure 4.30 Hydrolysis of of 5% CS by cellulase at 40 °C, pH 4.8.

Table 4.11 HPLC analysis of samples from cellulase pretreatmet reaction of CS.

	µg/ml					
Time (day)	Cellobiose	Glucose	Xylose	Gallicacid	Catechin	Epicatechin
0	76.9	137.3	4.3	0.1	3.3	6.5
1	13	643.5	16.7	0.3	2.3	11.9
2	8.9	860	25.7	0.9	2.2	11.9
3	7.5	1187	42.5	1.5	2	4.9

At the end of the pretreatment reaction, CS was recovered from reaction medium, washed and centrifuged. Dried CS (10%) was incubated with Shearzyme at 40 °C for XO production. Unpretreated CS was also incubated with Shearzyme as control. Unfortunately, CS hydrolysis was not observed by xylanase. Cellulase pretreatment did not provide to increase the amount of reducing sugar obtained by hydrolysis of unpretreated CS due to possibly shearzyme inhibition (Figure 4.30). During reaction, cellobiose and glucose concentration decreased and little amount of xylose was determined. The reason of decreasing cellobiose is converting of cellobiose to glucose. However, absorption of glucose to biomass might cause decrease in glucose concentration (Table 4.12).

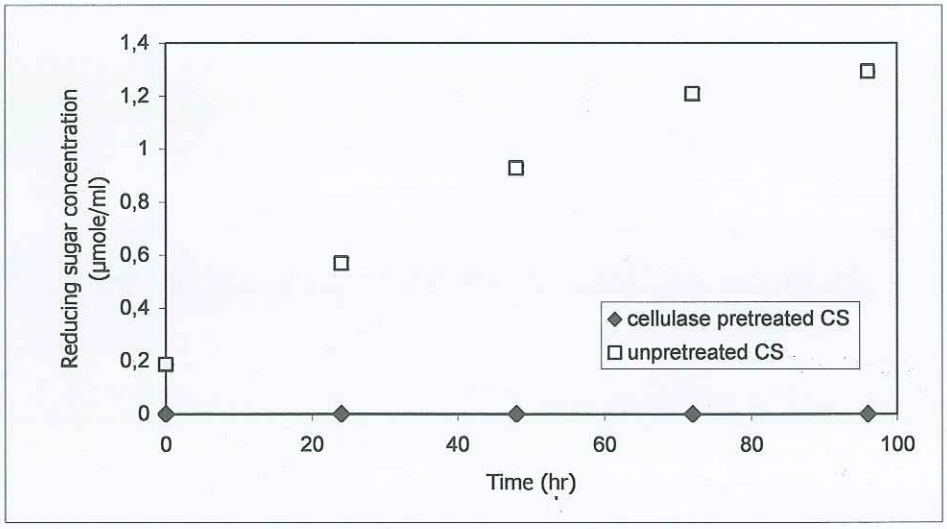


Figure 4.31 Hydrolysis of 10% of unpretreated and cellulase pretreated CS at 40 °C, pH 4.8.

Table 4.12 HPLC analysis of samples from Shearzyme hydrolysis reaction of cellulase pretreated CS.

	Concentrations (µg/ml)					
Time (hr)	Cellobiose	Glucose	Xylose	Gallicacid	Catechin	Epicatechin
0	2	8.2	0	0	0.1	1.6
24	2.1	4.3	2.7	0.1	0	0.3
48	2.6	2.5	7.5	0	0	0
72	1.8	1	8.3	1.5	3.8	0.2

In order to investigate the effect of cellulase treatment on Shearzyme activity, unpretreated CS was incubated both cellulase and xylanase together and the amount of reducing sugars was determined. It was seen that the reducing sugar concentration obtained by cellulase treatment decreased as 50% when both cellulase and Shearyzme were used together (Table 4.13). There might be several reason for intraction of these two enzymes or inhibition of Shearzyme. During pretreatments, a variety of side-reactions which could affect enzyme acitvity might take place, including removal of extractives from solid phase, partial dissolution of lignin and ashes. Lignin and its derivatives might also inhibit Shearzyme activity.

Table 4.13 The effect of cellulase pretreatment on Shearzyme activity.

	Reducing sugar concentration (µmole/ml)	
Time (hr)	Pretreatment of CS by cellulase	Pretreatment of CS by cellulase and Shearzyme
0	0.09 ± 0.05	2.7 ± 1.3
24	10.51 ± 1.4	4.8 ± 2.3

4.2.3 Hydrothermal Processing of CS

CS are treated in aqueous media at 121 °C for 1 h and 135 °C for 30 min at 2 atm and 3 atm, respectively, for efficient enzymatic hydrolysis to produce XOs. The aim was distruption of the compact lignocellulosic structure and increase susceptibility of xylanase for further enzymatic hydrolysis.

After pretreatment of CS, the solid and liquid phases were separated by fitration. The solid residue was washed by 100 ml of distilled water and dried at 60 °C for overnight. Before enzymatic process, both the filtrate and washing solution were tested for their reducing sugar content.

The amount of reducing sugar concentration in filtrate and washing solution were given Table 4.14. Total concentration of reducing sugar in filtrate and washing water after pretreatment at 121 °C for 1 h and 135 °C for 30 min were 6,45 µmole/ml and 7,69 µmole/ml, respectively. Increasing temperature and pressure increased reducing sugar concentration as expected. However, our aim was just open up lignocellulosic structure, not to solubilize and/or depolymerize xylan in CS. Therefore, harsher operational conditions were not used. The results showed that 5% of xylan depolymerized during the treatment and large amount of xylan remained in solid residue. Therefore, dried solid residue, the filtrate and washing solution were incubated by shearzyme in 0.21 U/ml activity at 40 °C for XO production. As seen from Table 4.15, enzyme hydrolysis of hydrothermally pretreated of CS at both conditions, 121°C with 2 atm and 135 °C with 3 atm, resulted negligible increase in the amount of reducing sugar concentration at 24th h of the reactions compared with the amount of reducing sugar, 2 µmole/ml, obtained hydrolysis of unpretreated CS.

On the other hand, pretreatment opened up CS structure to some extent. Because the amount of reducing sugar concentration in filtrate and washing solution after pretreatment was higher than the amount of reducing sugar concentration obtained by hydrolysis of CS by Shearzyme. However, xylan remained in solid residue after pretreatment could not be hydrolysed to XOs efficiently.

Upon pretreatment, CS might undergoes considerable changes in its physical and chemical composition. During these changes, there could be a number of factors that affect and/or inhibit activity of Shearzyme. Most prereatments change biomass composition, particularly the hemicellulose and lignin which might affect enzyme digestbility. Lignin recondensation and/or lignin-carbohydrate binding might be major factors that inhibit enzyme activity (Grohmann et al 1989).

Table 4.14 Reducing sugar concentration in filtrate and washing solution obtained after hydrothermal pretreatment.

	Reducing sugar concentration (µmole/ml)
Filtrate (121 °C for 1h)	5.4 ± 0.24
Washing Solution (121 oC for 1h)	1.05 ± 0.46
Filtrate (135 °C for 30 min)	7.3 ± 0.32
Washing Solution (135 °C for 30 min)	0.39 ± 0.17

Table 4.15 Reducing sugar concentration obtained during Shearzyme incubation of filtrate, washing solution and hydrothermally pretreated CS

	Reducing sugar concentration (µmole/ml)	
	t=0	t= 24 h
Filtrate (121 °C for 1h) + Shearzyme	5.3 ± 0.21	5.8 ± 0.23
Washing Solution (121 °C for 1h) + Shearzyme	1.02 ± 0.44	1.13 ± 0.46
Solid residue (121 °C for 1h) + Shearzyme	0.4 ± 0.19	0.45 ± 0.2
Filtrate (135 °C for 30 min) + Shearzyme	7.3 ± 0.28	7.4 ± 0.3
Washing Solution (135 °C for 30 min) + Shearzyme	0.39 ± 0.16	0.45 ± 0.18
Solid residue (135 °C for 30 min) + Shearzyme	0.27 ± 0.13	0.3 ± 0.14
Cotton stalk + Shearzyme	0.19 ± 0.02	0.57 ± 0.04

CHAPTER 5

CONCLUSIONS

The aim of this study was xylooligosaccharide (XOs) production from cotton (CS) and sunflower stalks (SS). In the first three parts of the study, alkali extracted xylan from CS and SS were hydrolyzed by commercial xylanases, Veron, Shearzyme, and a home produced xylanase from *Bacillus pumilus* (BAX). In the final part of the study, three different pretreatment methods including microbial, enzyme (cellulase) pretreatment and hydrothermal pretreatments were investigated to break down the complex lignocellulosic structure of CS to improve the subsequent enzymatic xylan hydrolysis.

Main conclusions can be summurized as given below:

- Both of the SS and CS xylan was hydrolyzed by commercial and home-produced xylanases.

- SS xylan and CS xylan were hydrolyzed by Shearzyme more efficiently than Veron under the conditions studied.

- In none of the experiments, xylose (X1) formation was observed on TLC plates which was a desirable result in this study.

- The degree of polymerization of XOs produced was started from X2 . The major products changed depending on substrate and enzymes that have been used.

- Conversion yields of CS and SS xylan to XOs by commercial enzymes were found about 40%. This yield is low. However, it is possible to increase the yield by using different xylanase preparations having different mode of action and substrate specifity and debranching enzymes acting on subunits on xylan chain.

- Crude BAX preparation was more efficient in XO production because of the presence of some complementary enzymes.

- XOs were not effectively produced from microbial pretreated CS because of xylanase production during the fermentation and Shearzyme inhibition during xylan hydrolysis. Microbial pretreatment of CS by *Phanerochaete chrysosporium* should be improved by further studies

- The other two pretreatment methods, cellulase pretreatment and hydrothermal pretreatment at 121 °C, 2 atm and 135 °C, 3 atm, were not satisfactory for XOs production. Shearzyme inhibiton was also observed after these pretreatment methods during further hydrolysis of pretreated CS. These approaches should also be investigated in detail.

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APPENDIX A

COMPOSITION OF DNSA REAGENT

- 36% Potassium-sodium tartrate ($C_4H_4KnaO_6.4H_2O$)
- 1% NaOH
- 1% DNSA (dinitrosalicylic acid)
- 0.2% Phenol
- 0.05% Sodium sulfite (Na_2SO_3)

The reagent must be stored in dark bottle at 4 °C in refrigerator.

APPENDIX B

XYLOSE STANDARD CURVE FOR DNSA METHOD

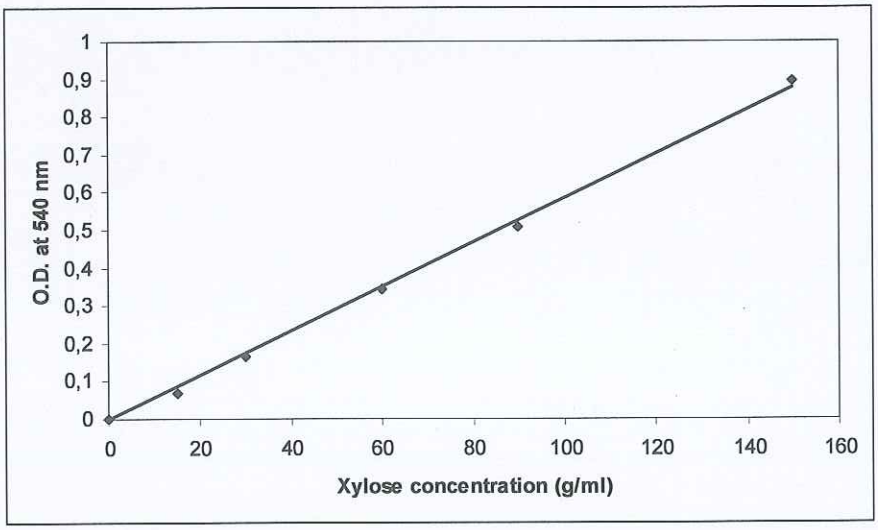

Figure B.1 Xylose standard curve for DNSA method.

APPENDIX C

STANDART CURVE FOR XYLOHEXOSE

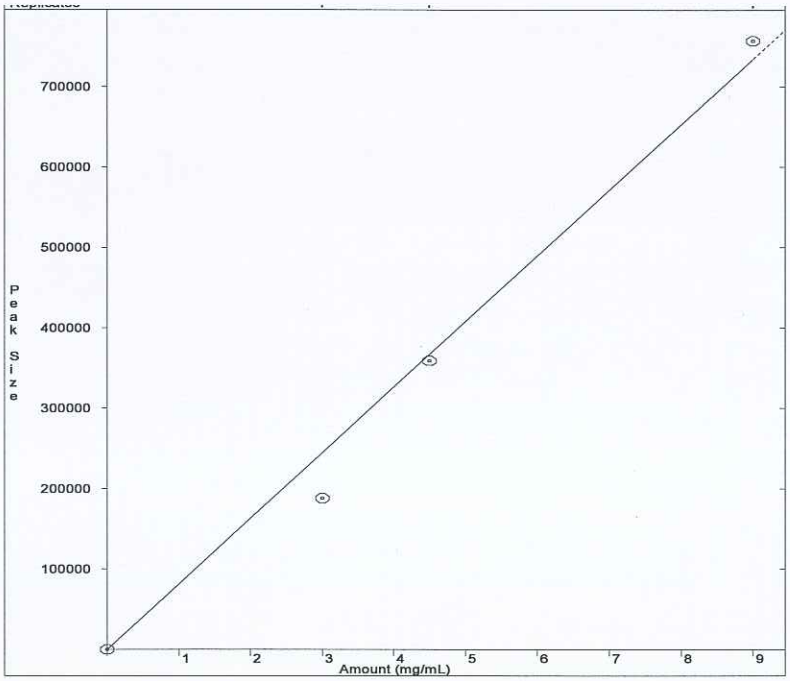

Figure C.1 Standart curve for xylohexose.

APPENDIX D

STANDART CURVE FOR XYLOPENTOSE

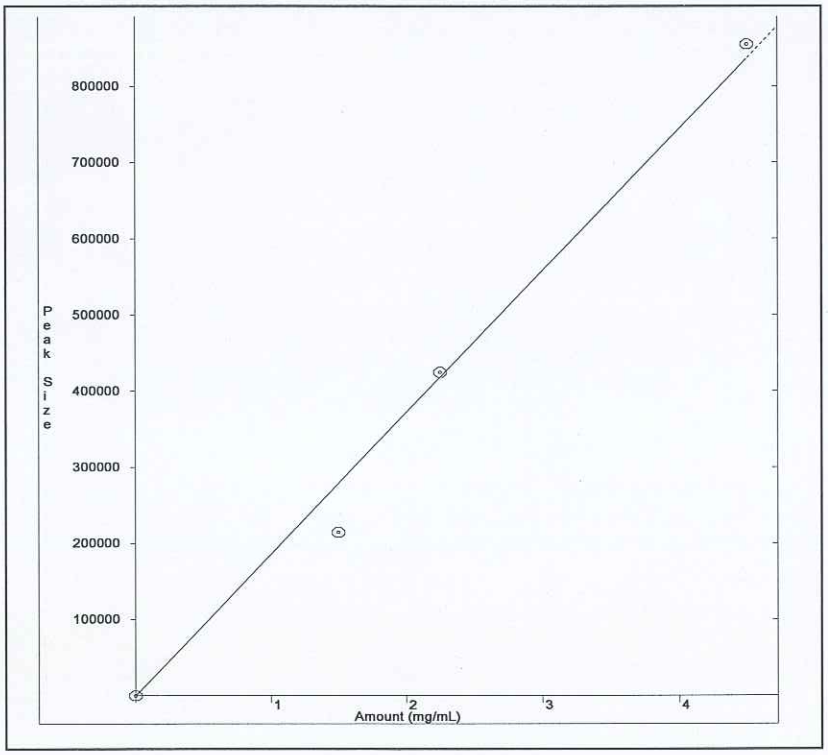

Figure D.1 Standart curve for xylopentose.

APPENDIX E

STANDART CURVE FOR XYLOTETROSE

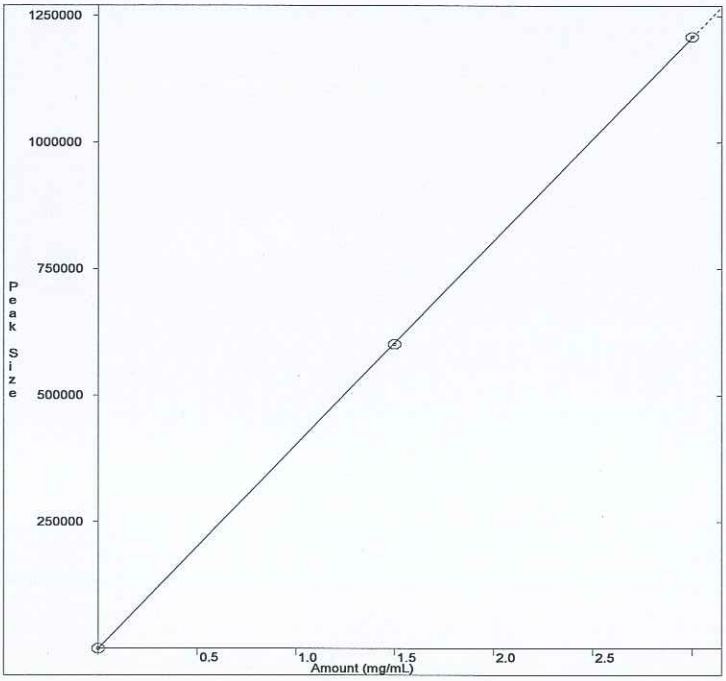

Figure E.1 Standart curve for xylotetrose.

APPENDIX F

STANDART CURVE FOR XYLOTRIOSE

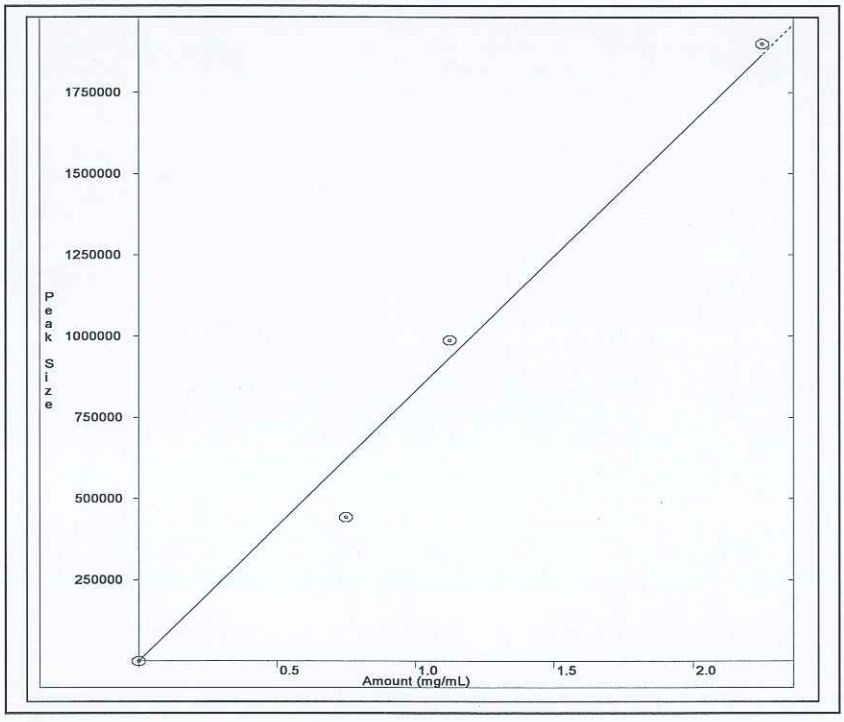

Figure F.1 Standart curve for xylotriose.

APPENDIX G

STANDART CURVE FOR XYLOBIOSE

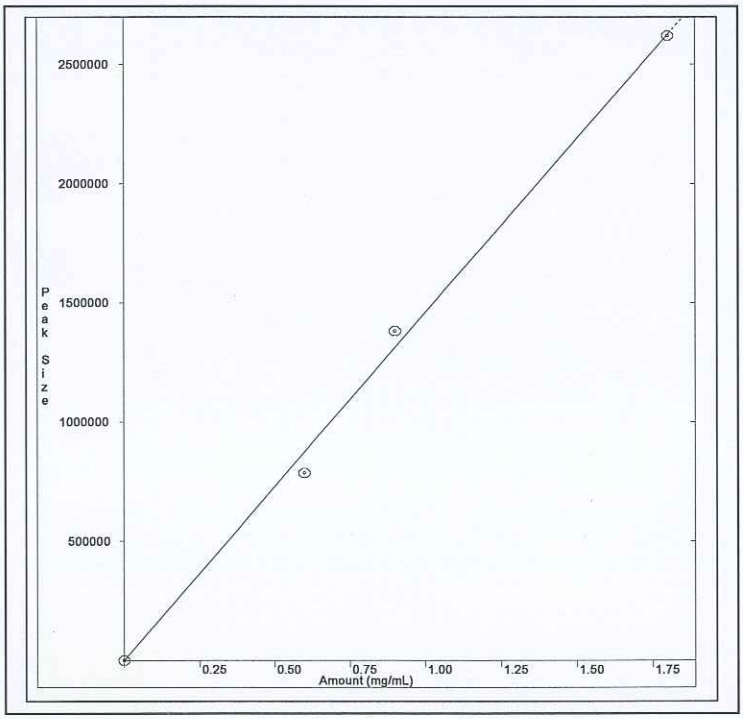

Figure G.1 Standart curve for xylobiose.

APPENDIX H

STANDART CURVE FOR XYLOSE

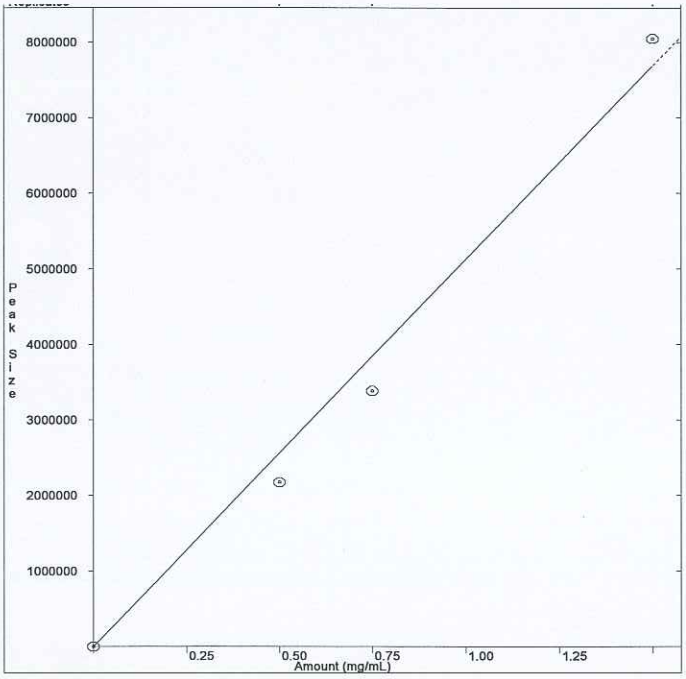

Figure H.1 Standart curve for xylose.

APPENDIX I

STANDART CURVE FOR GALLIC ACID

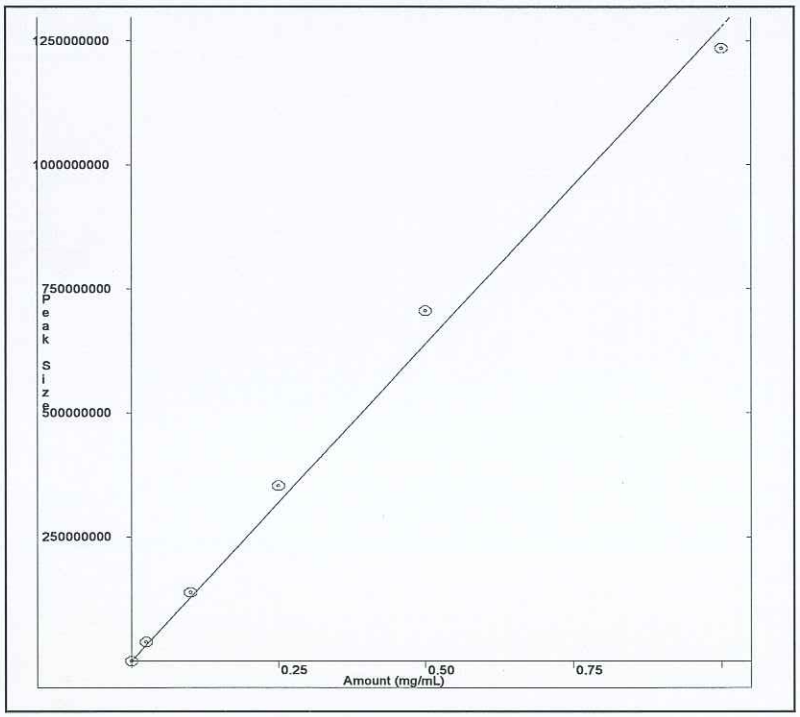

Figure I.1 Standart curve for gallic acid.

APPENDIX J

STANDART CURVE FOR CATECHIN

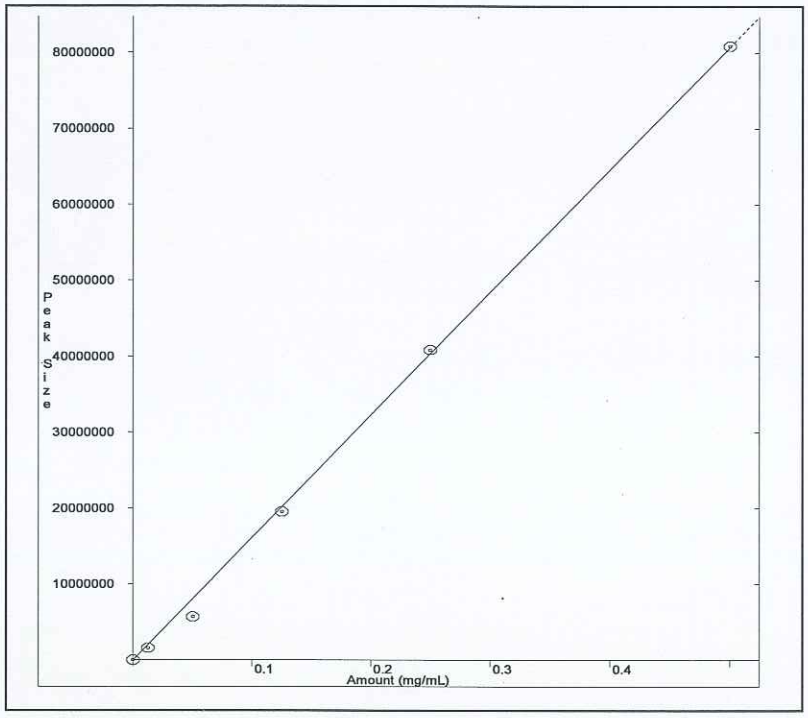

Figure J.1 Standart curve for catechin.

APPENDIX K

STANDART CURVE FOR EPICATECHIN

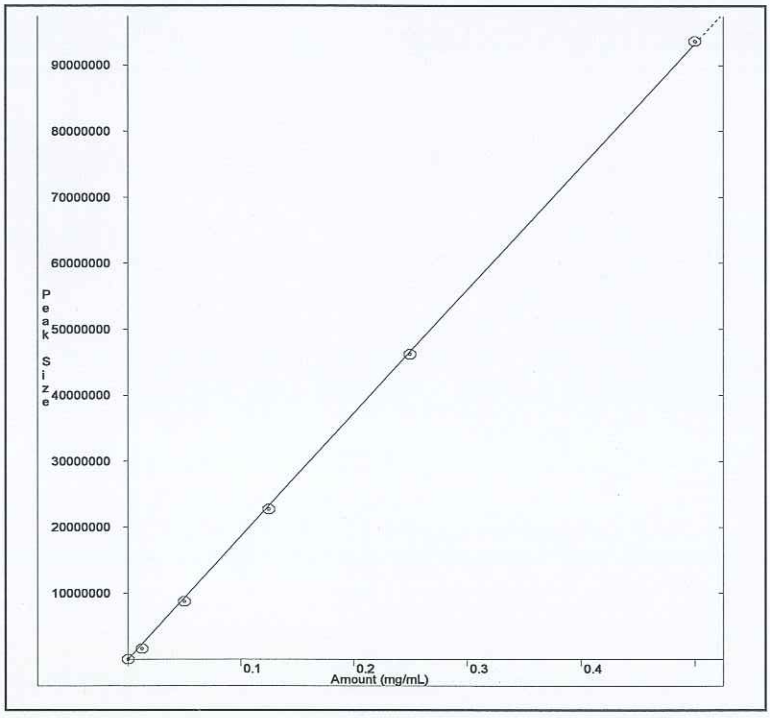

Figure K.1 Standart curve for epicatechin.

APPENDIX L

STANDART CURVE FOR GLUCOSE

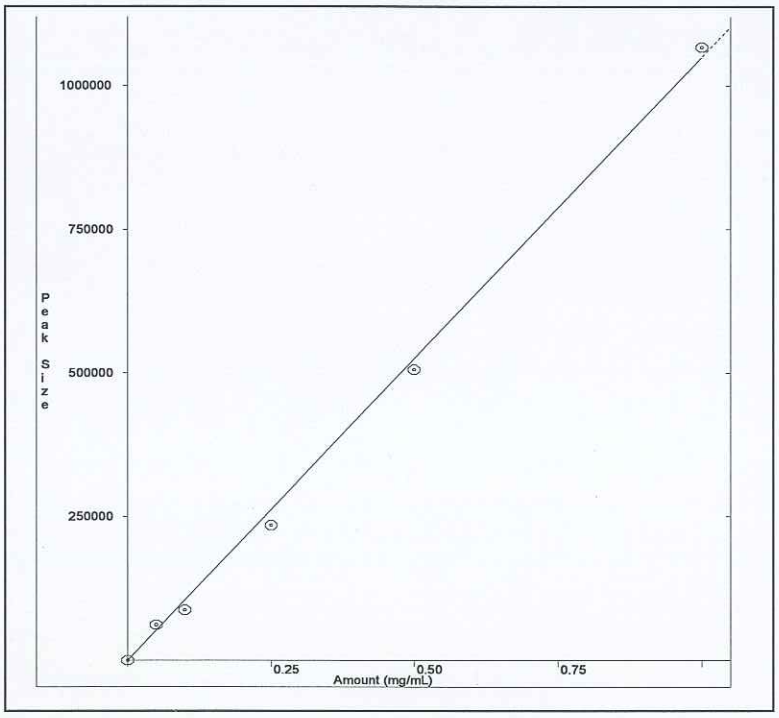

Figure L.1 Standart curve for glucose.

APPENDIX M

STANDART CURVE FOR CELLOBIOSE

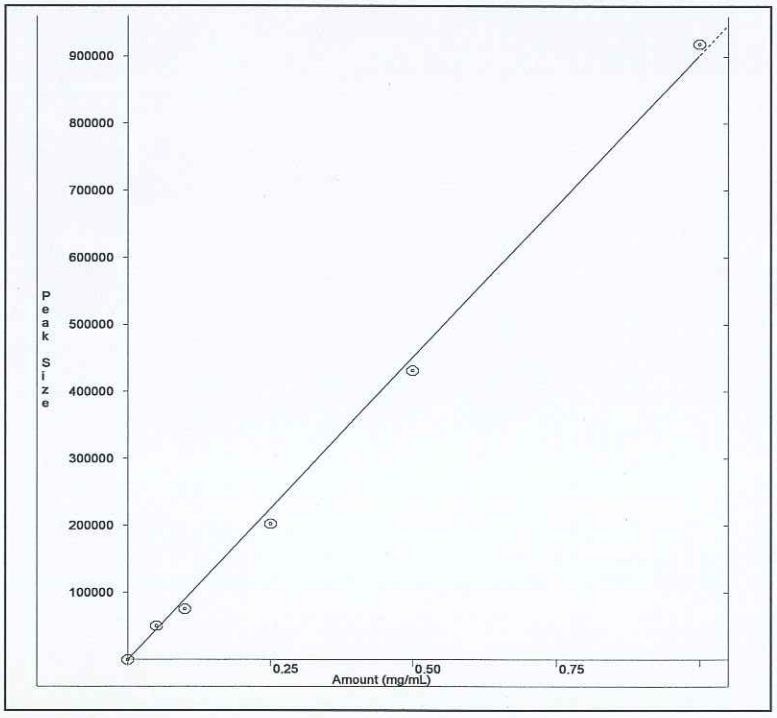

Figure M.1 Standart curve for cellobiose.

APPENDIX N

PREPARATION OF BRADFORD REAGENT

To prepare 5x concentrated stock solution following chemicals are mixed:

- 250 ml 95% ethanol (spectroscopic)
- 500 mg Brilliant Blue G dye (SERVA)
- 500 ml 85% ortho-posphoric acid.

After mixing chemicals, the mixture is diluted to total volume of 1L with distilled water. The stock solution must be stored in a dark bottle at refrigeration temperature.

The diluted sample is prepared by mixing 1 volume of concentrate with 4 volumes of distilled water. After mixing well and filtration of solution, it should wait at least 24 hour at room temperature before usage.

APPENDIX O

STANDARD CURVE FOR BRADFORD METHOD

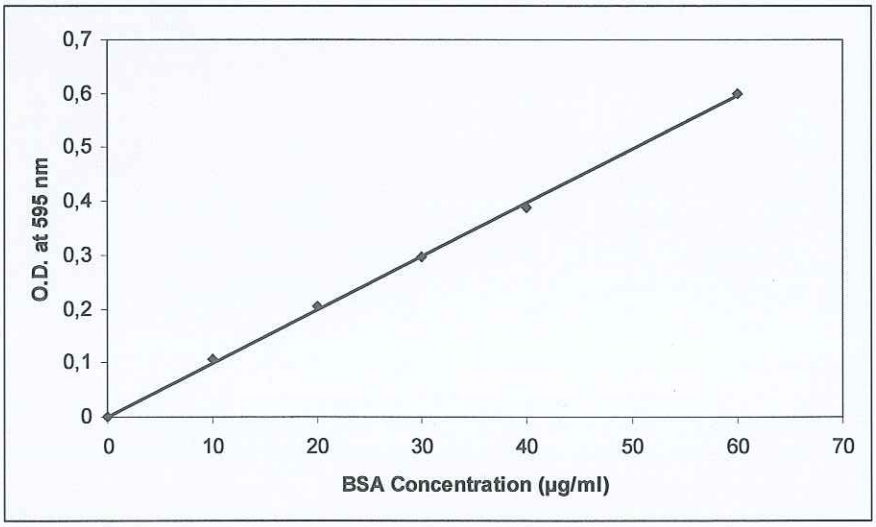

Figure O.1 Standard curve for Bradford method.

APPENDIX P

GROWTH MEDIUM FOR *Phanerochaete chrysosporium*

Stock Reagents

1. Basal III medium (per liter):

KH_2PO_4, 20 g

$MgSO_4$, 5 g

$CaCl_2$, 1 g

Trace elements solution, 100 ml

2. Trace element solution (per liter):

$MgSO_4$, 3 g

$MnSO_4$, 0.5 g

NaCl, 1.0 g

$FeSO_4 \cdot 7H_2O$, 0.1 g

$COCl_2$, 0.1 g

$ZnSO_4 \cdot 7H_2O$, 0.1 g

$CuSO_4$, 0.1 g

$AlK(SO4)_2 \cdot 12H_2O$, 10 mg

H_3BO_3, 10 mg

$Na_2MoO4 \cdot 2H_2O$, 10 mg

Nitrilotriacetate, 13 1.5 g

3. Culture Composition (L)

Basal III medium (filter sterilized), 100 ml

5% cotton stalk

0.1 *M* 2,2-dimethylsuccinate, pH 4.2 (autoclaved), 100 ml

Thiamin (100 mg/liter stock, filter sterilized), 10 ml

Ammonium tartrate (8 g/liter stock, autoclaved), 25 ml

Veratryl alcohol (4 m *M* stock, filter sterilized), 100 ml

Trace elements (filter sterilized), 60 ml

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Molecular Biology Summer Program	Tampere University, Institute of Medical Technology, Finland	1999
MS	METU Biotechnology Department	1997
BS	Hacettepe University, Biology Department	1993
High School	ŞMG High School, Bandırma	1989

WORK EXPERIENCE

Year	Place	Enrollment
2006- Present	Elvin Textile Co., Bursa	Researcher*
2004-2006	Hemosoft IT Co., METU Technolopolis, Ankara	Researcher
2000-2004	Literature Publishing Co., Ankara	Branch Manager
1997-1999	McGraw-Hill Publishing Co., Ankara	Sales Representative

*** European Patent Application for the research performed in Elvin Textile Co. (in progress).**

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Advanced English

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3. Ak O., Bakir U., Guray T., 1998, Production, Purification and Characterization of Chitosanase from *Penicillium spinulosum*, Biochem. Archives, 14:221-225.

POSTER PRESENTATIONS

1. Ak, Ö., Akpınar, Ö., Bakır, U., "Ayçiçek Atıklarından Ksilooligosakkarit Üretimi", XV. Ulusal Biyoteknoloji Kongresi, 2007, Antalya.

2. Akpınar, Ö., Erdoğan, K., **Ak, Ö.**, Kavas, A., Bakir U., Yilmaz, L., "Ligonoselülozik Materyallerden Kontrollü Asit Hidroliz ile Ksilooligosakkarit Üretimi, ", XV. Ulusal Biyoteknoloji Kongresi, 2007, Antalya.

3. Ak, O., Akpinar, O., Bakir, U., "Xylooligosaccharide Production from Agricultural waste", 13th European Congress on Biotechnology, Barcelona, 2007.

4. Akpinar, O., Erdogan, K., **Ak, O.**, Bakir U., Yilmaz, L., "Production of Xylooligosaccharides from Cotton and Tobacco Stalks", 13th European Congress on Biotechnology, Barcelona, 2007.

5. Bakır U., Akpınar **Ö., Ak** Ö, Kocabaş A. "Hasat Sonrası Pamuk Bitkisinin Katma Değeri Yüksek Ürünlere Biyodönüşümü". GAP V. Mühendislik Kongresi, Urfa, 2006.

6. Akpınar Ö., **Ak Ö.**, Bakır U., Yılmaz L. "Pamuk Atıklarından Ksilooligosakkarit Üretimi". 4. Gıda Mühendisliği Kongresi, Ankara, 2005.

7. Ak, Ö., Bakır, U. ve Güray, T. "Penicillium Spinulosum Küfünden Kitosanaz Enziminin Üretilmesi, Saflaştırılması ve Karakterize Edilmesi". 10. KÜKEM Kongresi, Mersin, 1997.