CO-PRODUCTION OF XYLANASE AND ITACONIC ACID BY *Aspergillus terreus* NRRL 1960 ON AGRICULTURAL BIOMASS AND BIOCHEMICAL CHARACTERIZATION OF XYLANASE

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

JUNE 2010

Approval of the thesis:

CO-PRODUCTION OF XYLANASE AND ITACONIC ACID BY *Aspergillus terreus* NRRL 1960 ON AGRICULTURAL BIOMASS AND BIOCHEMICAL CHARACTERIZATION OF XYLANASE

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ABSTRACT

CO-PRODUCTION OF XYLANASE AND ITACONIC ACID BY *Aspergillus terreus* NRRL 1960 ON AGRICULTURAL BIOMASS AND BIOCHEMICAL CHARACTERIZATION OF XYLANASE

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June 2010, 170 pages

Production of xylanase and itaconic acid (IA) from *Aspergillus terreus* NRRL 1960 from agricultural residues was investigated in this study. Two different media were tested and the medium having itaconic acid inducing capacity was chosen for further studies due to its high xylanase and IA production capacity. The best xylan concentration was found as 2% (w/v). Addition of commercial xylanase to production culture resulted in higher initial simple sugar concentration which increased IA production slightly but decreased xylanase production. Among tested agricultural residues; corn cob, cotton stalk and sunflower stalk, the highest xylanase production was obtained on corn cob. Increasing the corn cob concentration and applying wet heat pretreatment increased the xylanase production level. In a two-step fermentation process, 70000 IU/L xylanase

production was achieved in a medium containing 7% wet heat treated corn cob followed by 17 g/L IA production in a medium containing 10% glucose.

Molecular weight and isoelectric point of xylanase were found as 19 kDa and pH 9.0, respectively. The enzyme was optimally active at 50°C and pH 6.5-7.0. Kinetic experiments at 50°C and pH 7.0 resulted in apparent K_m and V_{max} values of 2.5±0.05 mg xylan/mL and 50.2±0.4 IU/µg protein, respectively. The major products of birchwood xylan hydrolysis were determined by thin layer chromatography as xylobiose and xylotriose. These findings indicate that the enzyme could be advantageous for use in different industrial applications due to its low molecular weight and its potential use for xylooligosaccharide production.

Keywords: *Aspergillus terreus* NRRL 1960, xylanase, itaconic acid, corn cob

ÖZ

TARIMSAL ATIKLAR KULLANILARAK *Aspergillus terreus* NRRL 1960 SUŞUNDAN KSİLANAZ VE İTAKONİK ASİT ÜRETİMİ VE KSİLANAZIN BİYOKİMYASAL KARAKTERİZASYONU

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Doktora, Biyoteknoloji Bölümü

Tez Yöneticisi: Prof. Dr. Ufuk Bakır

Ortak Tez Yöneticisi: Prof. Dr. Zümrüt B. Ögel

Haziran 2010, 170 sayfa

Bu çalışmada tarımsal atıklar kullanılarak *Aspergillus terreus* NRRL 1960 suşundan ksilanaz ve itakonik asit (IA) üretimi araştırılmıştır. İki farklı besi yeri test edilmiş ve yüksek ksilanaz ve IA üretim kapasitesi nedeniyle, itakonik asit indükleyici besi yeri ileri çalışmalar için seçilmiştir. En iyi üretimin sağlandığı ksilan değeri %2 (a/h) olarak bulunmuştur. Üretim kültürüne ticari ksilanaz eklenmesi başlangıç basit şeker konsantrasyonunu artırmış; bunun sonucunda, itakonik asit üretimi de artmıştır, fakat ksilanaz üretimi düşmüştür. Denenen tarımsal atıklar; mısır koçanı, pamuk sapı ve ayçiçeği sapı arasında, en yüksek ksilanaz üretimi mısır koçanı kullanımında elde edilmiştir. Mısır koçanı konsantrasyonu artırılarak ve sulu ısı ön işlem uygulanarak ksilanaz üretimi yükseltilmiştir. İki aşamalı fermentasyon yöntemiyle, önce %7 sulu ısı ön işleme tabii tutulmuş mısır koçanı içeren besi yerinde 70000 IU/L ksilanaz üretimi

gerçekleştirilmiştir bunu takiben %10 glikoz içeren besi yerinde 17 g/L IA elde edilmiştir.

Ksilanazın moleküler ağırlığı 19 kDa ve izoelektrik noktası pH 9.0 olarak tespit edilmiş, enzimin glikanlanmadığı bulunmuştur. Enzimin optimum çalışma koşulları 50°C ve pH 6.5-7.0 olarak gözlemlenmiştir. 50°C sıcaklık ve pH 7.0 koşullarında gerçekleştirilen kinetik çalışmalar sonucunda K_m and V_{max} değerleri, sırasıyla, 2.5±0.05 mg ksilan/mL ve 50.2±0.4 IU/µg protein olarak tespit edilmiştir. Huş ağacı ksilanının ksilanaz ile hidrolizinin ana ürünleri, ince film kromatografisi yöntemiyle, ksilobioz ve ksilotirioz olarak gözlemlenmiştir. Bu bulgular, düşük molekül ağırlığa ve ksilooligosakkaritlerin üretiminde kullanılabilme potansiyeline sahip olması bakımından, *A. terreus* NRRL 1960 ksilanazının endüstride farklı uygulamalar için avantajlı bir konuma sahip olduğunu göstermektedir.

Anahtar Kelimeler: *Aspergillus terreus* NRRL 1960, ksilanaz, itakonik asit, mısır koçanı

To my family

ACKNOWLEDGEMENT

I deem it a great privilege and pleasure in expressing my obligations to my most respected, learned and proficient research supervisor Prof. Dr. Ufuk Bakır for her able guidance, skilled advice and constructive criticism which inspired me to carry on my research work.

I extend my deepest gratitude towards Prof. Dr. Zümrüt Begüm Ögel for her guidance and co-supervision during the course of this reasearch project.

I express my deepest and sincerest gratitude to my Ph.D. Committee Members Prof. Dr. Meral Yücel and Prof. Dr. Pınar Çalık for their enthusiastic guidance and constant care with positive suggestions and criticism, where and when I needed during the entire period of my research work.

Special thanks to Dr. Leo H. de Graaff for his enthusiasm and guidance during my research in Wageningen University, the Netherlands.

All my colleagues and lab fellows especially Dr. Serpil Apaydın, Arzu Kanca, Beril Korkmaz Erdural, Betül Söyler, Erinç Bahçegül, Esra Uçkun, Gizem Sober, Gülden Avcı, Işık Haykır, Merve Çınar, and Zeynep Eker are also appreciated for their friendship and encouragement.

I wish to express my sincerest gratitude to my family, Kocabaş and Sutay families for their love, patience and generous helps and to my wife, Dr. Didem Sutay Kocabaş. Her encouragment, inspiration and love throughout the course of my studies has allowed me to reach more than I thought possible.

I express my heartiest gratitude to all my friends especially to A. Tahir Bayraç, Arda Büyüksungur, Gökhan Sadi, Murat Yatağan, Mustafa Sünger, Özlem Darcansoy İşeri and Sefa Denizoğlu for their great friendship.

Finally, the financial assistance of the Middle East Technical University Scientific Research Found Project BAP-08-11-DPT2002K120510 towards this reasearch hereby acknowledged.

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LIST OF ABBREVIATIONS

AE: Acetyl Esterase (AE)

AFase: α-L-Arabinofuranosidase

AFEX: Ammonia Fiber Explosion

BSA: Bovine Serum Albumin

CAD: cis-Aconitate Decarboxylase

CC: Corn Cob

CDW: Cellular Dry Weight

CFE: Cell Free Extract

CS: Cotton Stalk

DNSA: Dinitrosalicylic Acid

DPs: Degrees of Polymerization

ECP: Extracellular Protein

FPU: Filter Paper Unit

GHG: Greenhouse Gas

HMF: 5-hydroxymethylfurfuran

HPLC: High Pressure Liquid Chromatography

IA: Itaconic Acid

IEF: Isoelectric Focusing

IU: International Unit

mRNA: Messenger Ribonucleic Acid

NAD: Nicotinamide Adenine Dinucleotide

NADP: Nicotinamide Adenine Dinucleotide Phosphate

NMR: Nuclear Magnetic Resonance

NRRL: Northern Regional Research Laboratories

SDS-PAGE: Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis

SS: Sunflower Stalk

SSF: Solid-State Fermentation

SubF: Submerged Fermentation

TCA: Tricarboxylic Acid

TLC: Thin Layer Chromatography

XOs: Xylooligosaccharides

CHAPTER 1

INTRODUCTION

Itaconic acid is an unsaturated dicarboxylic acid having broad application in the industrial field of synthetic resins and plastics. Its usage can be increased by reducing the production cost (Willke and Vorlop, 2001). Besides, the biotechnological production of lactic acid, acetic acid and ethanol, utilizing renewable resources via fermentation, are the only processes which can compete with petrochemical routes (Herbert Danner and Rudolf Braun, 1999).

Petroleum, which is today's most frequently used industrial raw material, is neither sustainable, because it is limited, nor environmentally friendly (Kamm and Kamm, 2007). On the other hand, approximate amount of biomass on the earth is 10^{12} tons, and the main sources are plants which are lignocellulosic renewable sources of carbon building blocks. Biomass provides great potential for its industrial conversion into a diverse array of biobased products (Teter *et al*., 2006).

Lignocellulosic raw materials containing cellulose and hemicellulose have come into the focus of research in recent years, and comprehensive studies are concentrated on these raw materials as source for fermentable carbohydrates. Agricultural residues (e.g. straw, stems, bagasse, husks as well as corn stalks, cobs and stover), crops grown specifically for non-food biomass use (e.g. cereals, grasses, short rotation trees), wood and woody biomass, biomass wastes (e.g. green waste, vegetable and fruit wastes, paper mill sludge, sorted municipal solid

waste, paper) are considered as lignocellulosic biomass. Agricultural residues are holding the first place for large-scale industrial fermentations (Peters, 2007).

The main carbon sources in industrial fermentations are sugar or plant derived carbohydrates (Teter *et al.*, 2006). Currently, sucrose (molasses) or glucose from starch hydrolyzates is the most important raw material for the production of chemical bulk products. Even though, the quantity of these materials is not sufficient to replace fossil resources by biomass on the long term, furthermore, because of their usage in food processing, their prices challenge with those of the food industry. Therefore, lignocellulosic biomass is a possible alternative, which is abundant and comparably low-priced. On the other hand, because of difficulties in the conversion of lignocelluloses into fermentable sugars, the cost increases (Neureiter *et al.*, 2004). Expansion of the range and form of the raw materials will reduce the cost of fermented products. To do this, all the available sugars in the lignocellulose sources should be used, since conventionally, the major fermentation feedstocks are hexose sugars (glucose). Hence, reduction of the product cost can be achieved by hydrolyses of hemicellulose and cellulose and the usage of pentoses (xylose) (Danner and Braun, 1999).

Furthermore, although agricultural residues are accepted as fermentation feedstock, they are mostly still under investigation. These investigations are focused on the processing steps: pretreatment, hydrolysis, fermentation and product separation in order to develop competitive and economic processes. Particular effort of investigations is concentrated on the enzymatic treatment of the lignocellulosic feedstocks, the utilization of pentoses for fermentation, and the development of optimized microorganisms (Peters, 2007).

In 2004, US Department of Energy published a list of the most promising building block chemicals with potential use in the production of numerous value-added chemicals, one of which is itaconic acid (Figure 1.1) (Cherubini, 2010). Commercial production of itaconic acid is currently performed by glucose fermentation. Therefore, if the production cost is reduced and resolved in

industrial level, IA can become an economically feasible product. To fulfill this goal, one of the primary economical barriers is conversion of biomass into fermentable sugars.

Xylan in lignocellulosic biomass, source of xylose, is the second abundant polysaccharide found in plant cell wall after cellulose. The key enzyme in the hydrolysis of xylan is endo-β-(1,4)-xylanase (EC 3.2.1.8) (Kulkarni *et al*., 1999). Because of their potential applications in biotechnology such as bioconversion of lignocellulosic biomass into fermentative sugars, interest in xylanolytic enzymes has been increased (Collins *et al*., 2005). Nowadays, it is well known that xylanases are produced by a variety of microorganisms, including filamentous fungi and bacteria (Beg *et al*., 2001).

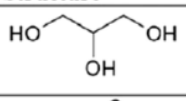
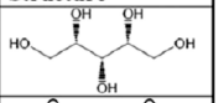
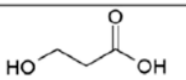
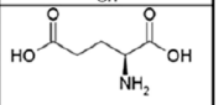
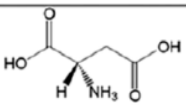
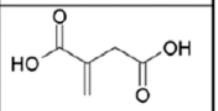
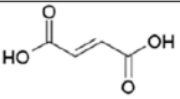
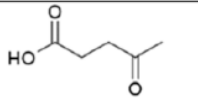
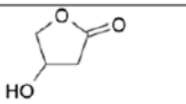
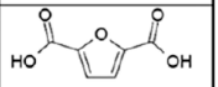
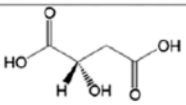
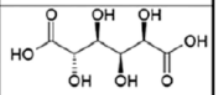
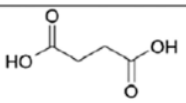
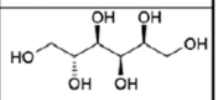
Bio-PM	Structure	Bio-PM	Structure
glycerol		(S, R, R)-xylitol	
3-hydroxy propionic acid		L-glutamic acid	
L-aspartic acid		itaconic acid	
fumaric acid		levulinic acid	
3-hydroxy butyrolactone		2,5-furan-di-carboxylic acid	
L-malic acid		glucaric acid	
succinic acid		sorbitol	

Figure 1.1 Potential chemical building blocks from biomass identified by DOE (Cherubini, 2010)

The major industrial application area of xylanases is the paper and pulp industry in prebleaching of kraft pulp to minimize the use of harsh chemicals (Polizeli *et al*., 2005). In addition, a growing interest has been raised for xylanases due to their potential in xylooligosaccharides (XOs) production. Xylanases are also used as food additives to poultry, in wheat flour, for the extraction of coffee, plant oils and starch, in the improvement of nutritional properties of agricultural silage and grain feed, for clarification of fruit juices and wine, degumming of plant fiber sources, release of aroma and anti-oxidant molecules, production of biopharmaceuticals and for bioconversion of lignocelluloses to sugar, ethanol and other useful substances. Due to the huge industrial usage area, identifying and obtaining new xylanases with different specificities and properties became very important (Beq *et al*., 2001; Polizeli *et al*., 2005; Khokhlova and Ismailova, 1975).

The Kyoto Protocol and the shortage in reserves of fossil resources (not reneweable sources) attracted the attention of researchers to renewable energy sources. Therefore, the aim of this study is to provide some perspectives and point out a few potentially important issues likely to come into focus in a near future of extensive use of biomass as a renewable feedstock in industry. Hence, it has been shown that renewable biomass such as agricultural and forestry by-products can be used as raw materials and converted into value-added products.

Commercially, itaconic acid is produced from *Aspergillus terreus* NRRL 1960 by using glucose or molasses as carbon sources, which causes a high production cost. Thus, it is estimated that decrease in production cost could be maintained by using cheaper carbon sources which would increase the market demand for itaconic acid (Willke and Vorlop, 2001). In addition to that, xylanase production capability of *A. terreus* strains has been reported in literature. Therefore, this study was aimed to produce itaconic acid from *A.terreus* NRRL 1960 using renewable lignocellulosic materials as carbon sources with co-production of xylanase. By this way, the production cost could be reduced, another high

volume product is produced simultaneously and it will provide the usage of renewable and sustainable agricultural residues having no commercial value. For this purpose, xylanase was biochemically characterized in terms of its molecular weight, isoelectric point, kinetic properties, and XOs production capacity.

CHAPTER 2

LITERATURE SURVEY

2.1 Overview of Lignocelluloses

Biomass is an organic material that has stored sunlight in the form of chemical energy. All biomass is produced by green plants converting sunlight into plant material through photosynthesis (Demirbas, 2008). Woody plant materials constitute a large fraction of organic matter on the earth. These materials are the remnants of plant cell walls which provide a strength and rigidity for the plant and help maintain osmolarity of the plant cell. These residues correspond to one of the most energy-rich resources available on the planet. Unless they are correctly discharged or used, added to environmental pollution. From various economic activities, large amounts of agro-industrial residues are generated every year (Francis *et al.*, 2003). It has been estimated that during photosynthesis, plants fix over 100 billion tons of carbon per annum (Kulkarni *et al*., 1999).

Agricultural (e.g., corn stover and sugarcane bagasse) and forestry (e.g., sawdust, thinnings, and mill wastes) residues, portions of municipal solid waste (e.g., waste paper), and herbaceous (e.g., switch-grass) and woody (e.g., poplar trees) crops are considered as lignocellulosic biomass. These materials are abundant, renewable and sustainable resources and competitive in price with petroleum. Production of lignocellulosic materials is widespread all over the world

especially in the regions where there are not much petroleum, thus opening up a new route to manufacturing organic fuels and chemicals (Wyman *et al*., 2005).

2.2 Lignocellulose Chemistry

Plant materials hold captive huge amount of carbon sources. The structural portion of lignocellulosic biomass is composed mainly of cellulose (40–45%), hemicellulose (30–35%) and lignin (20–23%) (Bakir, 2004; Teter *et al*., 2006). Long cellulose chains are joined together by hydrogen bonding and hold together with hemicellulose and lignin, giving the strength and rigidity of the plants and protection against microorganisms and insects (Figure 2.1) (Wyman *et al*., 2005). The composition is dependent on the plant type, age and collected residues (Bakir, 2004; Teter *et al*., 2006). The contents of cellulose, hemicellulose and lignin in common agricultural residues and wastes are given in Table 2.1 (Sun and Cheng, 2002).

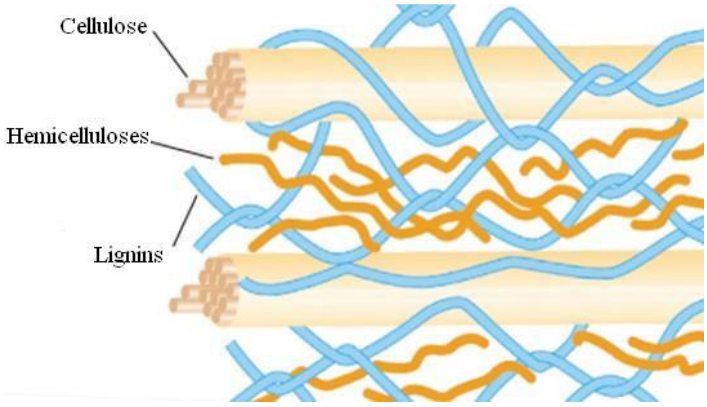


Figure 2.1 Lignocellulose structure of secondary cell wall (Boudet *et al*., 2003)

Table 2.1 The contents of cellulose, hemicellulose and lignin in common agricultural residues and wastes (Sun and Cheng, 2002)

Lignocellulosic materials	Cellulose (%)	Hemicellulose (%)	Lignin (%)
Hardwoods stems	40-55	24-40	18-25
Softwoods stems	45-50	25-35	25-35
Nut shells	25-30	25-30	30-40
Corn cobs	45	35	15
Grasses	25-40	35-50	10-30
Paper	85-99	0	0-15
Wheat straw	30	50	15
Sorted refuse	60	20	20
Leaves	15-20	80-85	0
Cotton seed hairs	80-95	5-20	0
Newspaper	40-55	25-40	18-30
Waste papers from chemical pulps	60-70	10-20	5-10
Primary wastewater solids	8-15	NA	24-29
Swine waste	6.0	28	NA
Solid cattle manure	1.6-4.7	1.4-3.3	2.7-5.7
Coastal Bermuda grass	25	35.7	6.4
Switch grass	45	31.4	12.0

2.2.1 Cellulose

The most abundant polysaccharide in plant cell walls is cellulose, which is a linear homopolymer of glucose linked by β-1,4 glycosidic bonds (Anand, 2004; Ragauskas *et al*., 2006). The cellulose fibers are firmly set in a matrix of other structural biopolymers, primarily hemicellulose (mostly xylan) and lignin (Anand *et al*., 2004). In addition to their significant role in maintaining the structural unity of plants, cellulose and hemicellulose have duty as a major source of nutrients for herbivores and as renewable substrates for the production of food, animal feed, paper and pulp as well as textiles (Kulkarni *et al*., 1999).

Secondary and tertiary structure of cellulose resulted from intra- and intermolecular hydrogen bonding staggered highly recalcitrant and water-insoluble amorphous and crystalline regions (Peters, 2007; Teter *et al*., 2006). Cellobiose is the basic repeating unit of cellulose because within a cellulose chain, each glucosyl residue is rotated by approximately 180 relative to its nearest neighbor residue (Figure 2.2) (Teter *et al*. 2006; Wyman *et al*., 2005). The molecular weights of different celluloses can range from 200-2000kDa where the degrees of polymerization (DPs) of approximately 10000 for cellulose from wood and around 15000 for cellulose from cotton and degree of crystallinity is relatively high averaging 50-70% (Teter *et al*. 2006; Wyman *et al*., 2005; Ragauskas *et al*., 2006).

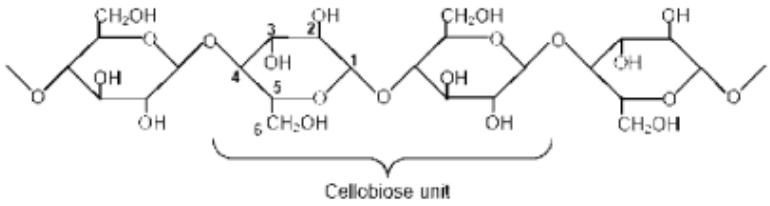
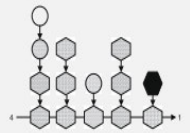
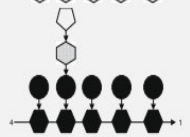
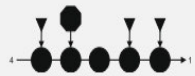
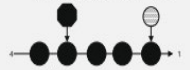
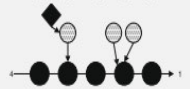
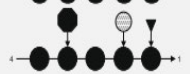


Figure 2.2 Cellulose chain structure (Kumar *et al*., 2009)

2.2.2 Hemicelluloses

Hemicelluloses are low molecular weight polysaccharides and mainly found in close association with cellulose and lignin in the plant cell wall. Hemicelluloses, unlike cellulose, are branched heteropolymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, and galactose) and sugar acids containing fewer than 200 'head to tail' β 1–4 linkages of five-carbon sugar D-xylose (Ryan *et al*., 2003, Teter *et al*., 2006; Evans and Furlong, 2003). The function of hemicelluloses in plants is to serve as a matrix between cellulose and lignin to increase the strength of plant cell walls by forming covalent and non-covalent linkages with other cell-wall constituents, for example pectin, glucans and proteins. Generally, the main sugar residue in the backbone defines the classification of hemicellulose and they are highly branched. Main hemicelluloses found in plants depending on the plant species, developmental stage, and tissue type are xylan, xyloglucan, glucuronoxylan, arabinoxylan, glucomannan, and galactoglucomannans (Figure 2.3) (Teter *et al*., 2006; Evans and Furlong, 2003).

Polysaccharide type	Biological origen	Abbreviation	Amount[a]	Units			DP[b]	Schematic representation
				Backbone	Side chains	Linkage		
Arabinogalactan	Softwoods	AG	1–3;35[*]	β-D-Galp	β-D-Galp α-L-Araf β-L-Arap	β-(1 → 6) α-(1 → 3) β-(1 → 3)	100–600	
Xyloglucan	Hardwoods, grasses	XG	2–25	β-D-Glcp β-D-Xylp	β-D-Xylp β-D-Galp α-L-Araf α-L-Fucp Acetyl	β-(1 → 4) α-(1 → 3) β- (1 → 2) α-(1 → 2) α-(1 → 2)		
Galactoglucomannan	Softwoods	GGM	10–25	β-D-Manp β-D-Glcp	β-D-Galp Acetyl	α-(1 → 6)	40–100	
Glucomannan	Softwoods and hardwoods	GM	2–5	β-D-Manp β-D-Glcp			40–70	
Glucuronoxylan	Hardwoods	GX	15–30	β-D-Xylp	4-O-Me-α-D-GlcpA Acetyl	α-(1 → 2)	100–200	
Arabinoglucuronoxylan	Grasses and cereals, softwoods	AGX	5–10	β-D-Xylp	4-O-Me-α-D-GlcpAβ-L-Araf	α-(1 → 2) α-(1 → 3)	50–185	
Arabinoxylans	Cereals	AX	0.15–30	β-D-Xylp	α-L-Araf/Feruloy	α-(1 → 2) α-(1 → 3)		
Glucuronoarabinoxylans	Grasses and cereals	GAX	15–30	β-D-Xylp	α-L-Araf 4-O-Me-α-D-GlcpA Acetyl	α-(1 → 2) α-(1 → 3)		
Homoxylans	Algae	X		β-D-Xylp[c]				

[a] %, dry biomass.
[b] Degree of polymerization.
[c] May also present β-(1 → 3) linkages on the backbone; [*] (up to) in the heartwood of larches ⬭, β-D-Galp; ●, β-D-Glcp; ⬡, β-D-Manp; ●, β-D-Xylp; ⬭, β-L-Araf; ⬡, β-L-Arap; ⬡, α-L-Fucp; ●, 4-O-Me-α-D-GlcpA; ▼, Acetyl;

Figure 2.3 Types of hemicelluloses (Ragauskas *et al*., 2006)

The major hemicelluloses are galactoglucomannans and arabinoglucuronoxylan in softwoods and glucomannan and glucuronoxylan in hardwoods. Although both the hardwood glucuronoxylan and softwood arabinoglucuronoxylan have a backbone of (14)-linked β-D-xylopyranosyl units, the hardwood glucuronoxylan are partially acetylated (i.e., 3.5–7.0 acetyl groups/10 xylose). Moreover, the amounts of (12)-linked pyranoid 4-O- methyl-α-D-glucuronic acid units in hardwood glucuronoxylan is lesser than softwood arabinoglucuronoxylan and branching in softwood is with (12)-linked pyranoid 4-O-methyl-α-D-glucuronic acid and (13)-linked α-L-arabinofuranosyl units, with an arabinose:uronic acid:xylose ratio of ~ 1:2:8 (Ragauskas *et al*., 2006).

The side groups of xylan have important roles in the bonding of lignin to hemicellulose and also in physicochemical properties and biodegradability of soluble or matrix bound hemicellulose (Figure 2.4).

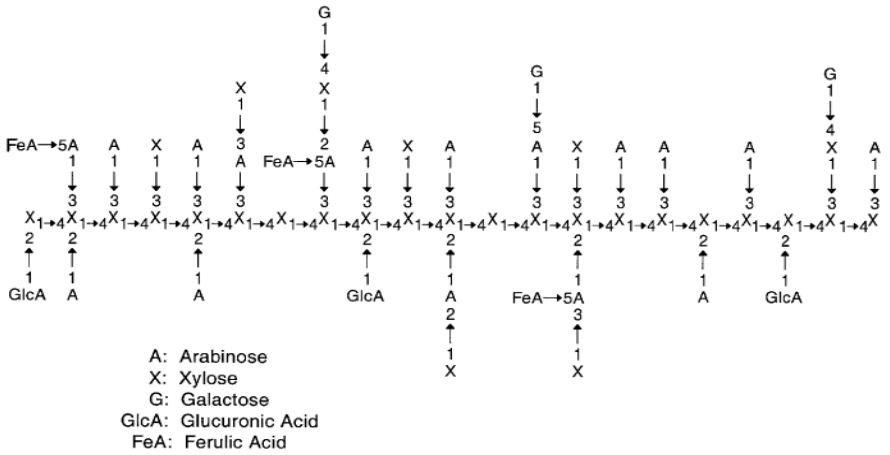


Figure 2.4 Schematic representation of corn fiber heteroxylan (Saha, 2003)

It is already shown that there are ester linkages between lignin and methylglucuronic acid residues and ether bonds from lignin to arabinosyl groups. Furthermore, covalent bonds between ferulic acid and p-coumaric acid

components of lignin and hemicelluloses are reported. It is known that acetylation increase the solubility of polysaccharides in water, whereas, the presence of ester-linkage to noncarbohydrate residues decreases the enzymatic degradability of polysaccharides (Wyman *et al*., 2005).

2.2.2.1 Availability and Uses of Hemicelluloses

Xylan is the main hemicellulolytic polysaccharide exist in plant cell walls and constitute a remarkable renewable biomass comprising up to 20–35 % dry weight of agricultural wastes (Heck *et al.*, 2002). This makes the D-xylose, main constituent sugar of xylan, and the second most plentiful renewable monosaccharide in nature. Xylan especially presents in large quantities in tissues that have undergone secondary thickening. Heteroxylans are characterized by a main chain composed of β-1,4-linked D-xylopyranose residues, found in three-fold left-handed helical conformation, and may be substituted by other sugars, and/or phenolic acid groups (Atkins, 1992). The source and species from which the xylan is isolated are the factors that affect the degree of substitution and the nature of side-chain substituent. The schematic representation of xylan (Fig. 2.4) also lists the different structures which can be attached to the xylan backbone and which cause the large variety of xylan structures found in plants (Joseleau *et al*., 1992). The xylans of hardwoods and cereals are acetylated, while softwood heteroxylans are not. In cereal xylans, arabinofuranosyl substituent esterifications may occur with phenolic groups such as *p*-coumaric and ferulic acids (Haan *et al.*, 2003). Their degree of polymerization varies from 70 to 130 in softwood xylans and 150–200 in hardwood xylans (Salles *et al*., 2005). Some of the xylosyl residues are acetylated at C-2 or C-3; α-1,2-linked to glucuronic or 4-*O*-methylglucuronic acid groups; α-1,3-linked to arabinofuranosyl residues; and may contain ferulic or coumaric acids esterified to C-5 of arabinofuranosyl residues (Salles *et al*., 2000).

Guar seed husk, esparto grass, and tobacco stalks have homopolymer linear xylans (Kulkarni *et al.*, 1999). Xylans can be categorized in four main groups;

linear homoxylan, arabinoxylan, glucuronoxylan and glucuronoarabinoxylan. One of the complex heteroxylan is corn fiber xylan comprise β-(1,4)-linked xylose residues (Saha *et al.*, 1999). The xylan backbone is highly substituted (about 80%) with monomeric side-chains of arabinose or glucuronic acid linked to O-2 and/or O-3 of xylose residues, and also by oligomeric side chains including arabinose, xylose, and sometimes galactose residues. Xylan may play a crucial role in the structural integrity of cell walls by both covalent and non-covalent associations. Ester linkages between glucuronoxylan and lignin via benzyl ester bonds with the carboxylate group of 4-O-methylglucuronic acid are the most reported presentation of this covalent bond. The second most reported one is ether linkage between xylan and lignin via L-arabinose side chains (Joseleau *et al*., 1992).

2.2.3 Lignin

Lignin is completely different from the other major macromolecules constituting the plant. Lignin is a non-carbohydrate "glue" in a matrix which imbeds the polysaccharide fibers (Anand *et al*., 2004). Lignin, complex polyphenolic structure, arises from three precursor alcohols: *p*-hydroxycinnamyl (coumaryl) alcohol, which gives rise to p-hydroxyphenyl units in the polymer; 4-hydroxy-3-methoxycinnamyl (coniferyl) alcohol, the guaiacyl units; and 3,5-dimethoxy-4-hydroxycinnamyl (sinapyl) alcohol, the syringyl units (Lee, 1997) (Figure 2.5).

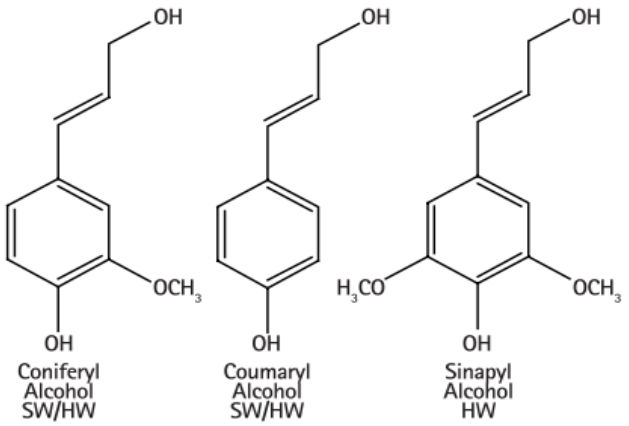


Figure 2.5 Precursors for lignin (Ragauskas *et al*., 2006)

Lignin is accepted as a composite of physically and chemically heterogeneous materials because it lacks the regular and ordered repeating units and its structure may be represented as in Figure 2.6 and 2.7 for spruce and beech lignins, respectively (Lin and Dence, 1992). These illustrations placed to show the complex structure of lignin.

Its extensive cross linking makes it resistant to enzymatic and chemical degradation and its biodegradation is performed mainly and most efficiently by white-rot basidiomycetes, and also by certain actinomycetes as a secondary metabolic process (Lee, 1997; Teter *et al*., 2006).

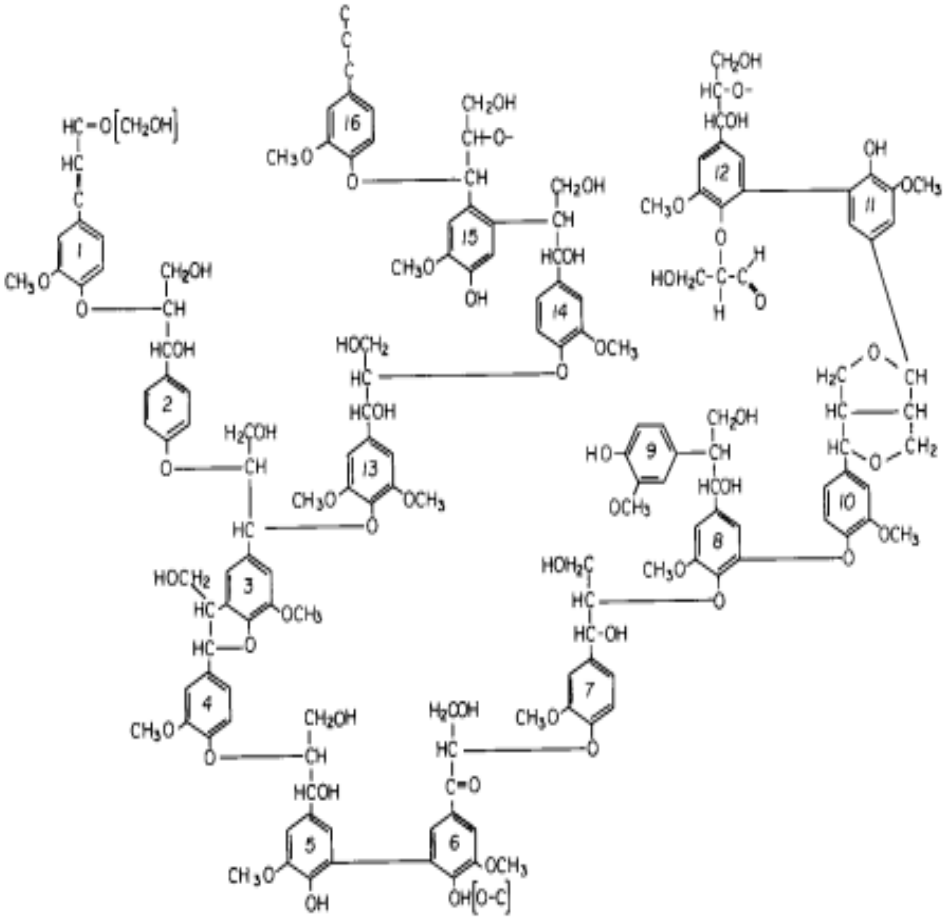


Figure 2.6 Structural model of spruce lignin (Lin and Dence, 1992).

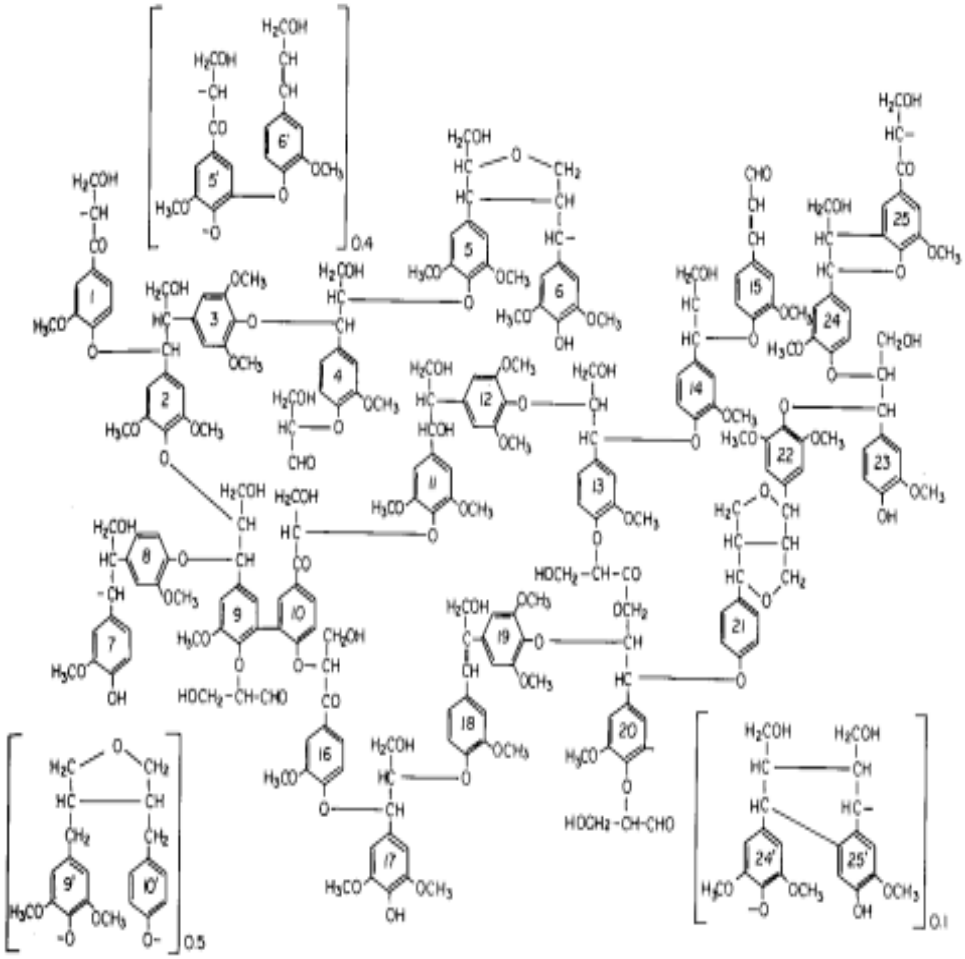


Figure 2.7 Structural model of beech lignin (Lin and Dence, 1992)

2.3 Lignocellulose Hydrolysis

Efficient conversion of lignocellulosic feedstocks such as forest and agricultural residues to ethanol, methane and, in the last years, also to hydrogen became a world priority for producing environmentally friendly renewable energy (Hendricks and Zeeman, 2009). However, enzymatic hydrolysis of lignocelluloses without a pretreatment step is generally not so effective due to high stability of the biomass to microbial attack. To overcome the hydrolysis process bottlenecks such as crystallinity, degree of polymerization, moisture

content, available surface area and lignin content, a pretreatment step is necessary. Since different lignocellulosic biomass have different physicochemical characteristics, it is important to employ suitable pretreatment technology of each raw material (Alvira *et al*., 2010).

Since all pretreatment methods have advantages and disadvantages, it is not possible to point out a method as an ideal one, where selection of a pretreatment method depends on various factors such as the composition of the material and the aim of the process.

2.3.1. Pretreatment Methods for Lignocellulosic Biomass

Prior to enzymatic hydrolysis, an effective and economical pretreatment method should meet the following requirements: production of reactive cellulosic fiber for enzymatic attack, avoiding destruction of hemicelluloses and cellulose, avoiding formation of possible inhibitors for hydrolytic enzymes and fermenting microorganisms, minimizing the energy demand, reducing the cost of size reduction for feedstocks, reducing the cost of material for construction of pretreatment reactors, producing less residues and consumption of little or no and a cheap chemical (Taherzadeh and Karimi, 2008).

Because of the diverse nature of biomass feedstocks, different methods, with their own advantages and disadvantages, have been developed to meet the effective and economical pretreatment requirements. These methods are classified into four groups, namely; physical, chemical, physicochemical and biological pretreatment techniques (Alvira *et al*., 2010).

2.3.1.1 Physical Pretreatment Methods

Physical pretreatment can increase the available surface area and pore size, and decrease the crystallinity and degrees of polymerization of cellulose. Different types of physical pretreatment methods such as milling, chipping, grinding and extrusion are developed to improve the enzymatic hydrolysis of lignocellulosic

materials (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Alvira *et al*., 2010).

Milling

The aim of the milling, chipping and grinding methods is to reduce the particle size and crystallinity by cutting the lignocellulosic biomass into smaller pieces. The reduction in particle size leads to an increase in available surface area and a reduction in degrees of polymerization. Milling also causes shearing of the lignocellulosic materials. All of these effects increase the total hydrolysis yield of the biomass. The energy requirement of milling, chipping and grinding depend on the final particle size and the biomass characteristics. Generally, due to the high energy requirement, these pretreatment methods were found not economically feasible (Sun and Cheng, 2002; Hendricks and Zeeman, 2009).

Extrusion

Extrusion process, which is a novel and promising physical pretreatment method, covers heating, mixing and shearing of the biomass during the passage through the extruder, resulting in physical and chemical modifications. The aim is to increase the accessibility of biomass to enzymatic attack by disrupting the lignocellulose structure by causing defibrillation, fibrillation and shortening of the fibers. In recent studies, application of enzymes during extrusion process is being considered as a novel promising technology (Alvira *et al*., 2010).

2.3.1.2 Chemical Pretreatment Methods

Chemical pretreatment methods cover alkali and acid processes, ozonolysis, organosolv, wet oxidation and ionic liquid pretreatment (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Alvira *et al*., 2010).

Alkali Pretreatment

Alkali methods address the biomass to swallow in the alkali environment such as sodium, potassium and calcium hydroxide, lime and ammonia, by solubilizing

and breaking down of the ester linkages. It makes the biomass more accessible for microorganisms and enzymes. The advantages of alkali pretreatment are making cellulose more dense and stable by changing its structure and can be used to selectively extract hemicellulose. Xylan can be selectively removed with potassium hydroxide pretreatment. Nevertheless, it has a disadvantage of 'peeling' effect of alkali conditions that means loss of polysaccharides because of the degradation and decomposition. Keeping temperature low (room temperature or lower) could help to prevent the peeling effect. Alkali pretreatment can be performed at low temperatures but a relatively long time and high concentration of the base is required (Taherzadeh and Karimi, 2008; Hendriks and Zeeman, 2009; Souse *et al*., 2009).

Sodium hydroxide causes swelling, increasing the internal surface of cellulose and decreasing the degree of polymerization and crystallinity, which provokes lignin structure disruption. Sodium hydroxide has been reported to increase hardwood digestibility from 14% to 55% by reducing lignin content from 24–55% to 20%. Lime pretreatment removes amorphous substances such as lignin, which increases the crystallinity index. Pretreatment with lime has lower cost and less safety requirements compared to sodium or potassium hydroxide pretreatments and can be easily recovered by reaction with carbondioxide. Addition of an oxidant agent such as oxygen or hydrogen peroxide to alkaline pretreatment can improve the performance by favoring the lignin removal (Alvira *et al*., 2010).

Acid Pretreatment

Acid pretreatments can be performed either with dilute or strong acids. Dilute acid method aimed to mainly hydrolysis of hemicellulose and increasing the cellulose availability, whereas, strong acid usage provokes the hydrolysis of both cellulose and hemicellulose. Solubilized hemicelluloses (oligomers) can be subjected to hydrolytic reactions producing monomers, furfural, HMF and other (volatile) products in acidic environments. During acid pretreatment, lignin

precipitates because of the acidic conditions. Strong acid methods have the advantage of low operating temperatures reducing the process cost, however, it has high risk of inhibitory chemical production (Hendriks and Zeeman, 2009; Grio *et al*., 2010).

On the other hand, high acid concentration in the concentrated acid process makes it extremely corrosive and dangerous. Therefore, specialized non-metallic constructions or expensive alloys are required in this process. The acid recovery, which is necessary in the concentrated acid process for economical reasons, is an energy demanding process. Therefore, the high investment, maintenance and energy costs reduce the commercial interest in this process (Taherzadeh and Karimi, 2008). Compared to the concentrated acid hydrolysis, dilute acid hydrolysis has advantages of is the relatively low acid consumption, limited problem associated with equipment corrosion and less energy demanding for acid recovery. Under controlled conditions, the levels of the degradation compounds generated can also be low (Grio *et al*., 2010).

Ozonolysis

Ozonolysis method, covering the pretreatment of lignocellulosic materials with ozone, can effectively degrade lignin and a part of hemicellulose. Ozone serves as a powerful oxidant with high delignification efficiency. The operation conditions of the method is generally room temperature and normal pressure and no formation of inhibitory compounds is observed (Taherzadeh and Karimi, 2008; Alvira *et al*., 2010). However, a large amount of ozone is required which makes the process expensive (Sun and Cheng, 2002).

Organosolv

Organosolv method is a new technique aimed to hydrolyse the hemicellulose and solubilize the lignin by usage of water dissolved organic solvent and inorganic acid catalyst. Hydrochloric acid, sulphuric acid or organic acids such as oxalic, acetylsalicylic and salicylic acid can be used as catalysts in the organosolv

process. The organic solvents used in the process include methanol, ethanol, acetone, ethylene glycol, triethylene glycol and tetrahydrofurfuryl alcohol. For economic reasons, the use of low molecular weight alcohols such as ethanol and methanol has been favored over alcohols with higher boiling points. Removal of solvents is necessary because of the inhibitory effect of solvents for the microbial growth, enzymatic hydrolysis and fermentation. The formation of inhibitory compounds and solvent recycling are the disadvantages of this pretreatment method (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Souse *et al*., 2009; Zhao *et al*., 2009; Grio *et al*., 2010).

Ionic Liquid Pretreatment

Ionic liquids have recently received much attention as solvents for pretreatment of cellulosic biomass. Ionic liquids are salts, typically composed of large organic cations and small inorganic anions. They exist as liquids at relatively low temperatures, often at room temperature. Ionic liquids exhibit interesting properties such as chemical and thermal stability, non-flammability, very low vapour pressures and a tendency to remain liquid in a wide range of temperatures. Their high thermal stability, negligible vapour pressure and no toxic or explosive gas formation allow to classify ionic liquids as ‘‘green solvents” (Alvira *et al*., 2010; Grio *et al*., 2010).

Ionic liquids such as 1-butyl-3-methylimidazolium chloride [bmim][Cl] and 1-allyl-3-methylimidazolium chloride [amim][Cl], are found to be particularly useful to dissolve cellulose. ILs have been shown as very effective in cellulose solubilization, although the solubility study of hemicellulose and lignin in ionic liquids was reported rarely and it is required to be investigated in detail. One of the main drawbacks of using ILs is the limited amount of data about their toxicity and biodegradability (Grio *et al*., 2010). On the other hand, for the large-scale application of ionic liquids, development of energy efficient recycling methods is necessary. Toxicity to enzymes and fermentative microorganisms must be also studied before ionic liquids can be considered an option for

industrial biomass pretreatment. Despite of the current limitations, advanced research such as synthesis of ionic liquids from carbohydrates, may play a role in reducing their cost. Development of pretreatment methods based on ionic liquids offers a great potential for future lignocellulose biorefinering processes (Alvira *et al*., 2010).

2.3.1.3 Physicochemical Pretreatment Methods

Pretreatments that combine both chemical and physical processes are defined as physicochemical processes. Different types of physicochemical methods are developed such as steam explosion, liquid hot water, ammonia fiber explosion (AFEX), wet oxidation, microwave, ultrasound and supercritical fluids pretreatment (Taherzadeh and Karimi, 2008; Alvira *et al*., 2010; Girio *et al*., 2010).

Steam Explosion

Steam explosion is the most widely used physico-chemical pretreatment method for lignocellulosic biomass. In this technique, biomass is subjected to pressurized steam for a period of time ranging from seconds to several minutes, and then suddenly depressurized. Due to the mechanical effects caused by suddenly reduced pressure, fibers are separated. The most important factors affecting the effectiveness of steam explosion are particle size, temperature, residence time and the combined effect of both temperature and time. Steam explosion process has several advantages such as the potential for significantly lower environmental impact, lower capital investment, more potential for energy efficiency, less hazardous process chemicals and conditions and complete sugar recovery (Alvira *et al*., 2010). Steam explosion is recognized as one of the most cost effective pretreatment processes for hardwoods and agricultural residues, but it is less effective for softwoods. Addition of hydrochloric acid, sulphur dioxide or carbon dioxide in steam explosion can effectively improve enzymatic hydrolysis, decrease the production of inhibitory compounds, and lead to more complete removal of hemicellulose (Sun and Cheng, 2002).

Liquid Hot Water

Liquid hot water pretreatment method, covering cooking of lignocellulosic materials, is used since several decades in e.g. pulp industries. In this method, hot water under high pressure penetrates into the biomass, hydrate cellulose, and remove hemicellulose and part of lignin. The major advantages of liquid hot water pretreatment are no addition of chemicals and no requirement of corrosion-resistant materials for hydrolysis reactors. Since the hot water processing removes mainly hemicellulose, a two-stage process combining the hot water for hemicellulose removal and a treatment for delignification (e.g. ammonia treatment) was also suggested for further improvement of enzymatic hydrolysis (Taherzadeh and Karimi, 2008). In comparison to steam explosion, higher pentosan recovery and lower formation of inhibitors are obtained, however, water demanding in the process and energetic requirement are higher and it is not developed at commercial scale (Alvira *et al*., 2010).

Ammonia Fiber Explosion (AFEX)

The AFEX process can significantly improve the enzymatic hydrolysis by either modifying or effectively reducing the lignin fraction of the lignocellulosic materials, while the hemicellulose and cellulose fractions may remain intact. The optimum conditions for AFEX depend on the lignocellulosic materials (Taherzadeh and Karimi, 2008). In the AFEX process, biomass is treated with liquid anhydrous ammonia at temperatures between 60 and 100°C and high pressure for a variable period of time. The pressure is then released, resulting in a rapid expansion of the ammonia gas that causes swelling and physical disruption of biomass fibers and partial decrystallization of cellulose (Alvira *et al*., 2010). One of the major advantages of AFEX pretreatment is no formation of inhibitors, however, some disadvantages exist such as AFEX is more effective on the biomass that contains less lignin, and the AFEX pretreatment does not significantly solubilize hemicellulose. Furthermore, ammonia must be recycled after the pretreatment to reduce the cost and protect the environment (Taherzadeh and Karimi, 2008).

Wet Oxidation

Wet-oxidation is an oxidative pretreatment method, which uses oxygen as oxidator. In this process, the materials are treated with water and air or oxygen at temperatures above 120°C for a period of e.g. 30 min. The temperature, reaction time and oxygen pressure are the most important parameters in wet oxidation. This process is an effective method in separating the cellulosic fraction from lignin and hemicellulose (Taherzadeh and Karimi, 2008). Low formation of inhibitors and efficient removal of lignin are the advantages of the wet oxidation pretreatment. On the other hand, cost of oxygen and catalyst are the main disadvantages for wet oxidation development technologies (Alvira *et al*., 2010).

Microwave

Microwave pretreatment is a physicochemical process involving both thermal and non-thermal effects. Pretreatments were carried out by immersing the biomass in dilute chemical reagents and exposing the slurry to microwave radiation. Alkalis are found to be suitable chemical reagents for microwave pretreatment, where sodium hydroxide was determined as the most effective alkali reagent (Alvira *et al*., 2010).

Ultrasound

Ultrasound pretreatment on lignocellulosic biomass is used for extracting hemicelluloses, cellulose and lignin. In addition to research on ultrasound pretreatment from lignocellulose, some researchers have also shown that saccharification of cellulose is enhanced efficiently by ultrasonic pretreatment. In this method, ultrasound field is introduced into the enzyme processing solution which causes cavitation. This effect enhances the transport of enzyme macromolecules toward the substrate surface and higher enzymatic hydrolysis yields are observed. Furthermore, mechanical impacts produced by the collapse of cavitation bubbles, cause opening up the surface of solid substrates to the action of enzymes (Alvira *et al*., 2010).

Supercritical Fluids

Supercritical fluids, mainly carbon dioxide, have been considered as an extraction solvent for non-extractive purposes, due to several advantages such as availability at relatively low cost, nontoxicity, nonflammability, easy recovery after extraction and environmental acceptability. Supercritical carbon dioxide displays gas-like mass transfer properties, besides a liquid-like solvating power. In the presence of water, supercritical carbon dioxide can efficiently improve the enzymatic digestibility of hardwood and softwood. The delignification with carbon dioxide at high pressures can be improved by co-solvents such as ethanol-water or acetic acid-water and can efficiently increase the lignin removal (Taherzadeh and Karimi, 2008). Operation at low temperatures prevents monosaccharides degradation, but sugar yields are lower in comparison to steam and ammonia explosion methods. Nevertheless a comparison of different pretreatment methods on several substrates showed that carbon dioxide explosion was more cost-effective and formation of inhibitors was lower. Besides the advantages, a very high pressure requirements is specially a concerning issue (Alvira *et al*., 2010).

2.3.1.4 Biological Pretreatment Methods

Biological pretreatments employ microorganisms to treat the lignocelluloses and enhance the enzymatic hydrolysis. Mainly used microorganisms are brown, white and soft-rot fungi, which are responsible for degradation of lignin and hemicellulose. A very little part of cellulose can be degraded by these fungi because of its more resistance nature than the other components. White-rot fungi are among the most effective microorganisms for lignocellulose biological pretreatment. Lignin degradation by white-rot fungi occurs through the action of lignin-degrading enzymes such as peroxidases and laccases (Sun and Cheng, 2002; Taherzadeh and Karimi, 2008; Alvira *et al*., 2010).

Several white-rot fungi such as *Phanerochaete chrysosporium*, *Ceriporia lacerata*, *Cyathus stercolerus*, *Ceriporiopsis subvermispora*, *Pycnoporus*

cinnarbarinus and *Pleurotus ostreaus* have been examined on different lignocellulosic biomass showing high delignification efficiency (Alvira *et al*., 2010). Bacteria can also be employed for biological pretreatment of lignocellulosic biomass. Two bacterial strains; *Sphingomonas paucimobilis* and *Bacillus circulans* are studied by Kurakake *et al*. (2007) for their ability on biological pretreatment of office paper for enzymatic hydrolysis.

Biological pretreatment processes have advantages such as low-capital cost, low energy requirement, no chemicals requirement and mild environmental conditions. However, the main drawback of the biological methods is the low hydrolysis rate. To develop a cost-competitive biological pretreatment and improve the hydrolysis, there is a need to keep on studying and testing more basidiomycetes fungi for their ability to delignify the plant material quickly and efficiently (Taherzadeh and Karimi, 2008; Alvira *et al*., 2010).

2.3.2 Hemicellulose Hydrolysis

Hemicellulose hydrolysis mainly performed by two different methods; either chemical processes (acid hydrolysis) or enzymatic degradation.

2.3.2.1 Acid Hydrolysis of Hemicelluloses

Dilute or strong acid hydrolysis is used for hemicellulose hydrolysis and both processes were explained under the acid pretreatment method.

2.3.2.2 Enzymatic Hydrolysis of Hemicelluloses

The hemicelluloses are classified according to their backbone sugar residues and main types of hemicelluloses are xylan, xyloglucan, glucuronoxylan, arabinoxylan, glucomannan, and galactoglucomannans (Teter *et al*., 2006; Evans and Furlong, 2003). Hence, main hemicellulolytic enzymes are endo-1,4-β-xylanase (EC 3.2.1.8) and β-xylosidase (EC 3.2.1.37) for xylan depolymerization and endo-1,4-β-mannanase (EC.3.2.1.78) and 1,4-β-mannosidase (EC 3.2.1.25) for hydrolysis of the main chain of hetero-mannans. The accessory and

debranching enzymes are α-L-arabinofuranosidase, α-glucuronidase, acetyl (xylan) esterase, and ferulic and p-coumaric acid esterase, and galactosidase (Sunna and Antranikian, 1997; Beq *et al*. 2001; Subramaniyan and Prema, 2002; Cobos *et al.*, 2003; Bakir, 2004; Raweesri *et al*., 2008; Magalhaes and Milagres, 2009).

Being a second most plentiful renewable polymer, hydrolysis of xylan plays an important role in the breakdown process of plant material in nature. Because of the complex chemical nature and heterogeneity of xylan, complete degradation of this complex structure requires the synergistic action of a range of different enzymes (Table 2.2). These enzymes includes the backbone degrading enzymes (β-1,4-endoxylanase, β-xylosidase) and accessory enzymes (a-glucuronidase, acetyl esterase (AE), a-L-arabinofuranosidase (AFase), phenolic acid (ferulic and p-coumaric acid esterase) (Sunna and Antranikian, 1997; Beq *et al*. 2001; Subramaniyan and Prema, 2002; Cobos *et al.*, 2003; Bakir, 2004; Raweesri *et al*., 2008). The actions of the enzymes involved in the breakdown of hemicellulose are shown in the Figure 2.8.

The main xylanolytic activities are catalyzed by endo-β-1,4-xylanases (E.C. 3.2.1.8, 1,4-β-D-xylanxylanohydrolase), which depolymerize xylan by the random hydrolysis of β-1,4-bonds between D-xylose residues in the main chain yielding xylo-oligosaccharides. Exo-1,4-β-D-xylosidases (1,4,β-D-xylan xylohydrolase E.C.3.2.1.37) split off small xylo-oligosaccharides to D-xylose from the nonreducing end (Biely, 1985; Cobos *et al.*, 2003; Subramaniyan and Prema, 2002).

However, β-l-arabinofuranosidase, β-glucuronidase, acetyl (xylan) esterase, ferulic and *p*-coumaric acid esterase all have specific cooperative functions in the complete degradation of xylan (Cobos *et al.*, 2003). α-Arabinofuranosidases (EC 3.2.1.55) function in the hydrolysis of non-reducing α-L-arabinofuranosyl residues. α-D-glucuronidases (EC 3.2.1.1) are required for the cleavage of the α-1, 2-glycosidic linkages between xylose and D-glucuronic acid and esterases take

role in the hydrolyses of the bond between xylan and acetyl,feruloyl, and p-coumaroyl groups (Raweesri et al, 2008; Subramaniyan and Prema, 2002).

Table 2.2 Enzymes in the hydrolysis of complex heteroarabinoxylans (Saha, 2003)

Enzyme	EC Number	Mode of action
Endo-xylanase	3.2.1.8	Hydrolyzes mainly interior β-1,4-xylose linkages of the xylan backbone
β-Xylosidase	3.2.1.37	Releases xylose from xylobiose and short chain xylooligosaccharides
α-Arabinofuranosidase	3.2.1.55	Hydrolyzes terminal nonreducing α-arabinofuranose from arabinoxylans
α-Glucuronidase	3.2.1.139	Releases glucuronic acid from glucuronoxylans
Acetylxylan esterase	3.1.1.72	Hydrolyzes acetylester bonds in acetyl xylans
Ferulic acid esterase	3.2.1.73	Hydrolyzes feruloylester bonds in xylans
ρ-Coumaric acid esterase	3.2.1.73	Hydrolyzes ρ–coumaryl ester bonds in xylans

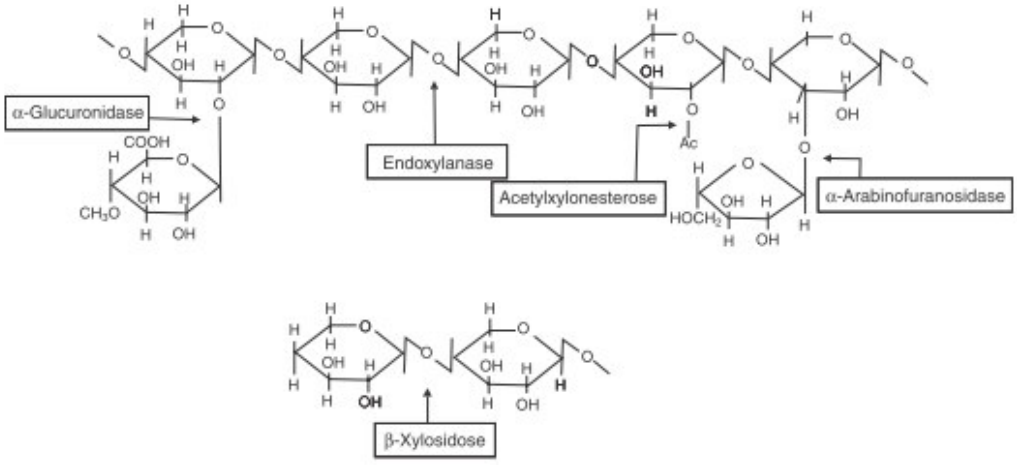


Figure 2.8 The actions of the enzymes involved in the breakdown of xylan (Bakir, 2004)

2.3.2.3 Classification and Structural Description of Xylanase Families

Xylanases are a group of glycosidic enzymes (*o*-glycoside hydrolases, EC 3.2.1.x) which are responsible for the endohydrolysis of 1,4-β-D-xylosidic linkage in xylan (Collins *et al*., 2005). Their official name given in International Union of Biochemistry and Molecular Biology Enzyme Nomenclature Database is endo-1,4-β-xylanase with the classification number of EC 3.2.1.8. Commonly used synonyms are xylanase; endo-(1,4)-β-xylan 4-xylanohydrolase; endo-1,4-xylanase; β-1,4-xylanase; endo-β-1,4-xylanase; endo-1,4-β-D-xylanase; 1,4-β-ylan xylanohydrolase; β-xylanase; β-1,4-xylan xylanohydrolase; endo-1,4-β-xylanase and β-D-xylanase. Although there are differences in optimum conditions for prokaryotic and fungal xylanase activity, there is a strong relationship between their molecular weight and isoelectric point values. Hence, Xylanases (EC 3.2.1.8) have been classified into two groups based on their sequence and structural homology; family-10 (formerly family-F) and family-11 (formerly family-G). Xylanases of family-10 have high molecular weight (>30 kDa) with low pI values, whereas family-11 xylanases have low molecular weight (<30 kDa) with high pI values (Wong *et al*., 1988; Biely *et al*., 1997; Flatman *et al*., 2002). In terms of protein structure, the most conserved region of a family is the catalytic domain and regarding the catalytic properties, family-10 xylanases exhibit greater catalytic versatility or lower substrate specificity than family-11 xylanases (Biely *et al*., 1997). Microorganisms generally produce more than one type of xylanase in multiple forms as a result of differential mRNA processing, partial proteolysis or differences in the degree of amidation and glycosylation, differing in substrate binding site and in the number of unsubstituted xylose units in the polysaccharide backbone (Biely, 1985).

2.3.2.4 Synergism in the Xylanolytic Enzyme System

The complete saccharification of heteropolysaccharide xylan needs diverse action and specifities of enzymes. It has already mentioned that in many bacteria and fungi have the capacity to produce multienzyme system of main chain

cleaving enzymes (β-1,4-endoxylanase and β-xylosidase) and side chain cleaving enzymes (α-L-arabinofuranosidase, acetyl xylan esterase and β-glucuronidase) (Sunna and Antranikian, 1997; Beq *et al*., 2001; Subramaniyan and Prema, 2002; Cobos *et al.*, 2003;Raweesri *et al*., 2008). The effective and complete hydrolysis of xylan not only depends on production of various kinds of enzymes but also their cooperative or synergistic action on xylan. Synergistic action can be observed either between two or more different type main chain cleaving enzymes (as in the case between xylanase and xylosidase) or two or more different type of side chain cleaving enzymes (as in the case between acetyl xylan esterase and arabinofuranosidase) or between main chain and side chain cleaving enzymes (like between xylanase and acetyl xylan esterase) (Bhat and Hazlewood, 2001). In the former case, xylosidases are preventing the end-product inhibition of xylanase during xylan hydrolysis and xylan is degraded to xylose completely by synergistic action of xylanases and xylosidases (Sunna and Antranikian, 1997). In latter case, the synergistic action of main chain and side chain cleaving enzymes resulted in efficient degradation of xylan. The accessibility of xylan backbone for xylanase action is increased by side chain cleaving enzyme (acetyl xylan esterase for acetylated xylan (Biely, 1985) and arabinofuranosidase for arabinoxylans (Raweesri *et al*., 2008)). Cleavage of ester linkages between lignin and hemicelluloses by side chain cleavage enzymes results in solubilization of the lignin and disrupt and loosen the cell wall, which increases the accessibility of xylan for xylanases. This synergistic action is helpful in the biobleaching of pulps to reduce the kappa number and increase the brightness (Sunna and Antranikian, 1997; Subramaniyan and Prema, 2002).

2.3.3 Microbial Production of Xylanolytic Enzymes

Because of being a heteropolysaccharide, xylan breakdown necessitate participation of complex distinct enzymes. The production of these xylanolytic enzyme systems is broad among the fungi, actinomycetes, and bacteria (Table 2.3) (Beq *et al*., 2001, Badhan *et al*., 2007). There are reports on microbial production of endoxylanases beginning from 1960. Early reports are regarding

plant pathology that xylanases play a role during primary invasion of the host tissues (Subramaniyan and Prema, 2002). The investigations showed that plant cell wall degrading enzymes are produced by most of the plant pathogen fungi. The interest on xylanases is increased after finding of their applications in the paper and pulp and food industries (Beq *et al*., 2001; Subramaniyan and Prema, 2002).

Table 2.3 Common xylanase-producing microorganisms (Bakir, 2004)

Fungi	**Bacteria**	**Yeast**
Filamentous fungi *Aeromanas* sp. *Aspergillus* sp. *A. awamori* *A. fumigatus* *A. kawachii* *A. nidulans* *A. niger* *A. oryzae* *Chaetomium globosum* *Humicola insolens* *Neurospora crassa* *Penicillium* sp. *P. chrysogenum* *P. purpurogenum* *Rhizoctonia solani* *Rhizomucor pusillus* *Rhizopus oryzae* *Schizophyllum commune* *Trichoderma* sp. *T. harzianum* *T. koningii* *T. longibrachiatum* *T. reesei* *Thermomyces lanuginosus*	*Aeromonas caviae* *Bacillus* sp. *B. acidocaldarius* *B. circulans* *B. pumilus* *B. stearothermophilus* *B. subtilis* *Cellulomanas fimi* *Clostridium* sp. *C. acetobutylicum* *C. stercorarium* *Micrococcus* sp. *Staphylococcus* sp. *Thermoanaerobacterium* sp. *Thermotoga* sp. *T. maritima* *T. thermarum* Actinomycete *Streptomyces* sp. *S. thermoviolaceus* Archaea *Thermococcus zilligii* *Pyrodictium abyssi*	*Aureobasidium pullulans* *Pichia stipitis* *Trichosporon cutaneum*

Increasing researches on xylanases revealed that they are widespread all over the nature and xylanase production is observed from diverse organism mainly from bacteria, fungi, actinomycetes and yeast (Sunna and Antranikian, 1997; Beq *et al*., 2001; Subramaniyan and Prema, 2002). Marine algae, protozoa, snails, crustaceans, insects, gastropods, arthropods and seeds of terrestrial plants can also produce xylanases (Sunna and Antranikian, 1997; Bakir, 2004).

Chen and co-workers (1986) indicated that six different xylanases with total activity of 0.4 IU/mL were investigated from a single organism *A.terreus* A-07. They confirmed their results by purifying and characterizing those xylanases (Chen *et al*., 1986). Another study was carried out by Hrmova and coworkers at 1989 with two different species of *Aspergillus*, *A.terreus* and *A. niger*. They find out *A.terreus* having higher xylanolytic activity then *A. niger* and they observed 3 IU/mL xylanase, 0.08 IU/mL glucosidase and 0.02 IU/mL xylosidase on 1% carbon source xylan without coproduction of cellulase (Hrmova *et al*., 1989). Furthermore, two different galactosidase were found from a strain of *A. niger* by Manzanares and friends (Manzanares *et al*., 1998). Kiss and Kiss worked on β-xylosidase from four different *Aspergillus* species on wheat bran. *A.carbonarius*, *A. nidulans, A.niger* and *A. oryzae* showed 0.149, 0.033, 0.022and 0.017 IU/mL, respectively. *A.carbonarius* also had arabinofuranosidase activity (Kiss and Kiss, 2000). Similar results were presented by Wakiyama and friends. They investigated about *A. japonicus*` β-xylosidase on oat spelt xylan and indicating that it also showed glucosidase and arabinofuranosidase activity (Wakiyama *et al*., 2008).

One of the optimization studies was performed by Gawande and Kamat (1999) to increase xylanase production by using lignocellulosic wastes. They used *A. terreus* and *A. niger* as an organism in both SSF and SubF and wheat bran, sugarcane bagasse, soybean hull and rice straw as lignocellulosic wastes. They demonstrated that SSF was better than SubF and could result in 2 fold increase in xylanase activity. For both organisms, wheat bran was the best inducing substrate for xylanase production with undetectable amount of cellulase.

Another point they indicated the inoculums size. It should be adequate to colonize all the substrate (Gawande and Kamat, 1999). Jeya and coworkers also stated similar results with *A. versicolor* that highest xylanase production was obtained with wheat bran in SSF with very low cellulase activity (Jeya *et al*., 2005). Another experimental design on lignocellulosic wastes with *A. terreus* was carried out by Ghanem and coworkers (2000). They observe that the organism produced 16.16 IU/mL xylanase on 10 g wheat straw and 10 IU/mL on corn cob with minimal cellulase activity (Ghanem *et al*., 2000). Marques and friends analyzed the *A.terreus* on oat spelt xylan. The results demonstrated 26.9 IU/mL xylanase after 15 days without xylosidase and mannanase activity (Marques *et al*., 2003). Lakshmi and coworkers applied a statistical optimization on *A.terreus* in SSF and claimed that moisture content, inoculum concentration and incubation time major parameters (about 85%) among the incubation temperature medium pH, particle size and xylose (Lakshmi *et al*., 2009).

Aspergillus fumigatus and *A. niveus* were examined on alternative carbon sources in SSF by Nogueira and friends. They pointed out that best xylanase production was observed on powdered corn cob or wheat bran (15 IU/mL) from *A. fumigatus* but on birchwood xylan from (4 IU/mL) *A. niveus*. They also emphasized that best growth was observed with agitation but best xylanase production was obtained at static condition (Peixoto-Nogueira *et al*., 2009). Three different species (*A. niger, A. niveus, A. ochraceus*) were investigated for xylanase production under SSF by Betini and coworkers. According to their results, xylanase production without cellulase activity on wheat bran was higher than on the corn cob. Based on their results, they claimed that higher xylanase production on wheat bran resulted from higher concentration of hemicellulose of wheat bran about 45% than corn cob about 35 % (Betini *et al*., 2009).

2.4 Commercial Applications of Xylanases

Xylan-degrading enzymes (especially cellulose-free xylanases) have a wide range of potential biotechnological applications in various industrial processes,

involving the modification of cereal-based foodstuffs, enhancing the digestibility of animal feedstocks, the delignification of paper pulp. In addition they take role in textile manufacture, in baking, in the release of aroma and anti-oxidant molecules, in the production of biopharmaceuticals and for bioconversion of lignocelluloses to sugar, ethanol, and other useful substances. The huge industrial usage area has provided an increased drift to identify and obtain new xylanases with different specificities and properties (Kulkarni *et al.*, 1999).

Interest in xylanases has increased in recent years, mainly due to their applications in paper industries for pulp treatment, improving the effectiveness of conventional bleaching. Nowadays, it is thought that virtually any cellulase-free xylanase is effective in paper industry and the attention is focused on the selection of enzymes whose characteristics, principally pH and temperature relationships are suited to pulp treatment regimes since cellulase activity adversely affects the quality of the paper pulp. Specific applications include the production of cellulose pulp and the pretreatment of pulp to boost the bleaching process (Heck *et al*., 2005). These applications are summarized in Table 2.4.

Table 2.4 Industrial applications of xylanases (Collins *et al*., 2005)

Market	Industry	Application	Function
Food	Fruit and vegetable processing	Fruit and vegetable juices, nectars and purees, oils	Improves maceration and juice clarification, reduces viscosity.
	Baking	Dough and bakery products	Improves elasticity and strength of the dough, thereby allowing easier handling, larger loaf volumes and improved bread texture.
Feed	Animal feeds	Monogastric (swine and poultry) and ruminant feeds	Decreases the content of non-starch polysaccharides.
Technical	Paper and pulp	Biobleaching of kraft pulps	Reduces chlorine consumption and toxic discharges.
		Bio-mechanical pulping	Facilitates the pulping process and reduces the use of mechanical pulping methods.
		Bio-modification of fibers	Improves fibrillation and drainage properties of pulp.
		Bio-de-inking	Facilitates the de-inking process and reduces the use of alkali.
	Starch	Starch-gluten separation	Reduces batter viscosity, improves gluten agglomeration and process efficiency.
	Textiles	Retting of flax, jute, ramie, hemp	Enzymatic retting reduces/replaces chemical retting methods.
	Bioremediation/ Bioconversion	Treatment of agricultural, municipal and food industry wastes	Treatment/recycling of wastes. Production of fermentable products.

In addition, a growing interest has been raised for xylanases due to their potential in xylooligosaccharides (XOs) production. XOs are sugar oligomers, made up of xylose units and industrially produced from lignocellulosic biomass (Vazquez *et al*., 2000). XOs are valuable products because of their health benefits like reducing cholesterol and colon cancer risk, maintaining gastrointestinal health and having beneficial effect on type 2 diabetes (Akpinar *et al*., 2009). In addition, XOs have important applications in food industry as food ingredients and novel foods. Xylanases, capable of hydrolyzing xylan, are the key enzymes in production of XOs from lignocellulosic biomass. Either by immobilization, direct addition to the reaction media or *in situ* production of by microorganisms, xylanases catalyze production of low polymerization degree XOs (Vazquez *et al*., 2000).

Nowadays, xylanases take attention for hemicellulose saccharification to increase the utilization of lignocellulosic biomasses. Therefore, most researches focused on to exploiting the saccharification and subsequent fermentation of renewable sources by using xylanases to the desired end products in order to increase the efficiency to produce bioethanol, bioenergy and other fine value-added chemicals.

2.5 Agricultural Residues in Turkey and the Importance of Corn

The geographic location of Turkey provides several advantages for extensive use of most of the renewable sources. Renewable sources in Turkey are hydro power, geothermal energy, solar energy, wind energy and biomass. Biomass, mainly wood and animal waste, is the main renewable source in Turkey and used for heating (Evrendilek and Ertekin, 2003; Kaygusuz, 2009; Karadag *et al*., 2009).

Biomass is an organic material that stores sunlight in the form of chemical energy. All biomass is produced by green plants converting sunlight into plant material through photosynthesis. Biomass appears to be an attractive feedstock for three main reasons. First, it is a renewable resource that could be sustainably

developed in the future. Second, it appears to have formidably positive environmental properties, reduced greenhouse gas (GHG) emissions, third, it appears to have significant economic potential provided that fossil fuel prices increase in the future (Demirbas, 2008).

Biomass, which is one of the earliest energy source of humanity, is used to meet a variety of energy needs such as generating electricity, heating homes, fueling vehicles and providing process heat for industrial facilities. In recent years, many attempts have been made to utilize biomass, such as wood, hazelnut shell, agricultural waste residues, waste paper and wheat straw, tea waste and olive husk, for energy production in Turkey (Balat, 2005).

Agriculture is one of the most important main activities in Turkey with the largest share of national income. Agricultural activities in Turkey are mainly focused on the production of industrial plants used to produce industrial products such as oils from olives, sunflower, cotton seeds. At the end of these production processes, a large quantity of agricultural residues is produced. In addition, a large volume of agricultural residues such as straw, shell, seed, stalk, leaves, and molasses are left on the fields during harvesting seasons (Kar and Tekeli, 2008).

Turkey has about 26.350 million ha agricultural total land (Ozturk and Bascetincelik, 2006). The total field crop production and residues in Turkey are shown in Table 2.5. According to this data, the highest amount of residues are wheat and barley straw. They are followed by corn (maize) and cotton residues. Similarly, corn is the largest grown (785 million tons) cereal in the world with doubled grain yield per unit area compared to wheat and barley (Ozcan, 2009).

Nevertheless, corn and cotton residues have four times more availability percentage as renewable source than wheat and barley straws because they are mainly used in animal feeding and animal bedding, while, the residues from corn and cotton production are remain in the field after the crops are harvested (Ozturk and Bascetincelik, 2006).

Table 2.5 The total field crop production and residues in Turkey (Ozturk and Bascetincelik, 2006)

Crops	Residues	Production (tons)	Total Residues (tons)		Available Residues (tons)	Availability (%)
			Theoretic	Actual		
Wheat	Straw	22,439,042	29,170,755	23,429,907	3,514,486	15
Barley	Straw	8,327,457	9,992,948	8,963,012	1,344,452	15
Maize	Stalks	2,209,601	5,911,902	4,970,259	2,982,155	60
	Cob		596,592	1,907,307	1,144,384	60
Cotton	Stalks	2,292,988	6,317,181	2,520,281	1,512,169	60
Sunflower	Stalks	836,269	2,341,554	2,259,121	1,355,472	60

As a widely available agricultural product in Turkey and the world, corn is used as a direct or indirect ingredient in more than 4000 different products. Main usage areas of corn are; fresh consumption, canned food, corn flour, starch, chips, appetizers, animal feed, oil, sweetener, candy, chewing gum, chocolate products, baby food, salat dressing, corn syrup, toothpaste and ethanol production, automotive, cleaning, textile and cosmetic industry. In developing countries corn is mainly used as food, whereas in developed countries the main usage area is animal feed, industrial raw material and ethanol. In USA, the biggest corn producer in the world, 23% of the corn production is utilized for ethanol production in year 2007 and a continuous increase is expected in the future (Ozcan, 2009).

With continuous growth in corn processing to ethanol, problems with the utilization of the fibrous byproducts will arise (Gaspar *et al*., 2007). Fortunately, not only the maize part of the corn, the residual fiber part is also a potential raw material for the production of various products. Therefore, use of corn and its residues has great industrial potential.

The major component of corn fiber is the pericarp that consists of hemicellulose, cellulose and remaining starch containing protein, fiber oil and lignin. Important applications for hemicellulose, the largest portion of corn, have been discovered.

The highly branched heteroxylan from corn is used as a new food gum. Xylan found another application area in bioplastics. Due to its flexibility, mechanical properties and low degradation rate by a lot of microorganisms, it proved to be an excellent additive in thermoplastic-starch composites to increase the life of the bioplastic. The beneficial effect of some xylans as an additive in papermaking was also confirmed. In recent years, great interest has been raised in hemicelluloses as polymers for chemical and pharmaceutical application, such as production of cationic biopolymers, hydrogels and long-chain alkyl ester derivatives (Gaspar *et al*., 2007).

As in the case of corn fibers, every year million tons of agricultural residues are produced and discarded as waste material. Therefore, the utilization of agricultural residues to produce renewable energy and industrially and economically value-added compounds such as biopolymers, organic acids and enzymes became very important.

2.6 Organic Acids

Organic acids are low-molecular weight C-H-O containing compounds found in all organisms and characterized by the possession of one or more carboxyl groups (Jones, 1998). Organic acids are used in industrial metal cleaning or other metal treatments and in the food and feed industry as flavor enhancers, acidifiers, stabilizers, or preservatives (Magnuson and Lasure, 2004).

Many of the commercial production processes on organic acids are excellent examples of fungal biotechnology, such as citric, gluconic, itaconic and lactic acids. *Aspergillus niger* can produce citric acid from glucose with an efficiency of 80 %, as an example (Magnuson and Lasure, 2004). Among fungi, *Aspergilli* are well known for their potential to overproduce organic acids. This ability is assumed to make them possess ecological advantage, as they can even tolerate pH values as low as 1.5 (Ruijter *et al*., 2002).

There are two arguments why these fungi overproduce ridiculous amounts of organic acids. One is they do not have regular control on organic acid metabolism, as they could not get enough free sugar within their habitats. The second one is that over producing and acidifying their habitat give them ecological competitive advantage. As an example, *Aspergillus terreus* produces itaconic acid and itaconic acid will inhibit the growth of many microorganisms because of not being primary metabolite of other organisms and acidification of the environment. Furthermore, its unusual nature may permit *A. terreus* and only a few other species to catabolize the acid (Magnuson and Lasure, 2004).

Few of the organic acids are produced on industrial scale, which are citric acid, gluconic acid, itaconic acid, oxalic acid, fumaric acid and malic acid (Ruijter *et al*., 2002). Since the market demand for oxalic, fumaric and malic acids is low, they are produced on a small-scale (Magnuson and Lasure, 2004).

Citric acid is one of the organic acid produced large amounts (over 9,000,000 tons annual global production) from fermentation process with *Aspergillus niger* or a yeast *Yarrowia lipolytica* (small amount) and increases in its consumption about 3.5-4% per year (Finegonova *et al*., 2005; Xie and West, 2009). Figure 2.9 shows the main steps of fermentation process for both organisms to produce citric acid. Its wide commercial applications in food, beverages and pharmaceutical usage are the reason for its huge market size. Furthermore, the demand for citric acid is increasing with rising in population and industrialization in developing countries (Magnuson and Lasure, 2004).

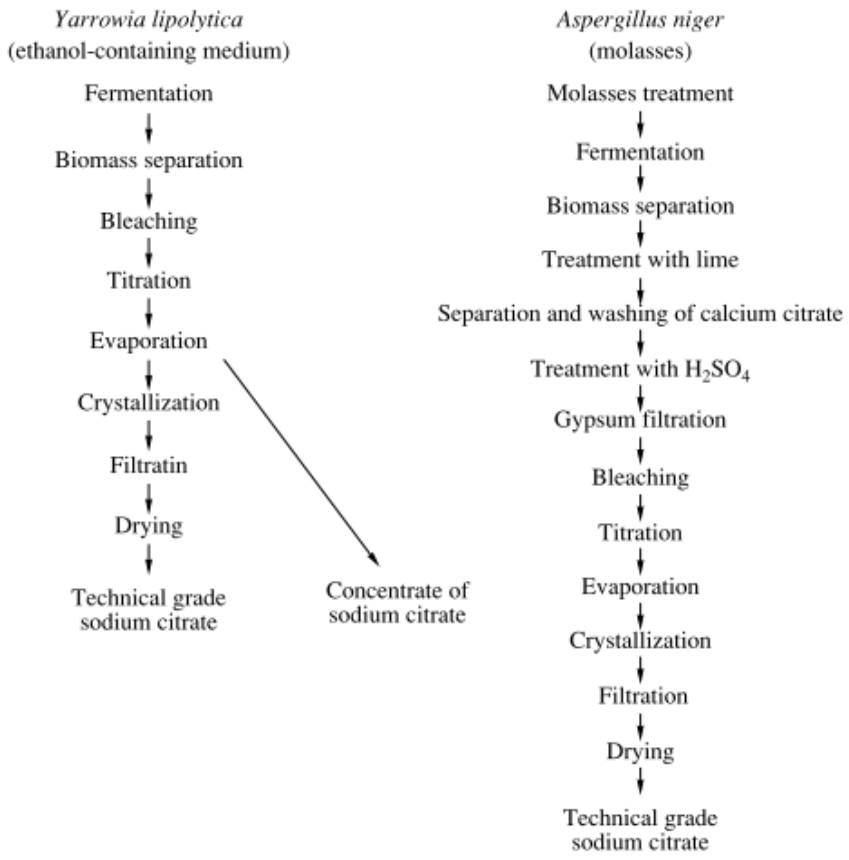


Figure 2.9 Schematic representation of citrate synthesis from *A. niger* or *Y. lipolytica* (Magnuson and Lasure, 2004)

Another organic acid produced by fermentation is gluconic acid with the commercial production of 50,000-100,000 tons per year. It is produced as by-product by extracellular activity of glucose oxidase of either *A. niger* or *Gluconobacter suboxydans* or enzymatic action of glucose oxidase and catalase obtained from *A. niger* (Ruijter *et al*., 2002; Magnuson and Lasure, 2004). Since the enzymatic production has 100% efficiency and cost-effective process, its production via fermentation process showed decreasing profile (Magnuson and Lasure, 2004).

Other organic acids having commercial applications are oxalic acid, succinic acid, lactic acid, malic acid, fumaric acid. Although they are produced by many

organisms, their microbiological productions are not economical or cannot compete with synthetic production (Zeikus *et al*., 1999; Ruijter *et al*., 2002; Magnuson and Lasure, 2004, Zhang *et al*., 2007, Engel *et al*., 2008).

2.6.1 Itaconic Acid

Itaconic acid, or methylenesuccinic acid, is an organic compound that is one of the three acids (citraconic, itaconic and mesaconic acid) obtained by the distillation of citric acid (Wikipedia, 2010). Itaconic acid is an unsaturated diprotic acid as shown in Figure 2.10.

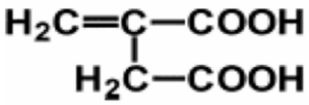


Figure 2.10 Itaconic acid structure

Itaconic acid is first discovered by Baup *et al*. after thermal decomposition of citric acid in 1837 (Willke and Vorlop, 2001). After almost one century, its biological production by *Aspergillus itaconicus* was reported (Bonnarme *et al*., 1995). After a few years Calam *et al*. reported another *Aspergillus* species (*Aspergillus terreus*) as itaconic acid producer and at those days *Aspergillus itaconicus* almost lost its ability to produce itaconic acid (Calam *et al*., 1939). In 1945, Lockwood and Reeves reported a strain of *Aspergillus terreus* (*Aspergillus terreus* NRRL 1960) yielding high amount of itaconic acid (Lockwood *et al*., 1945). From that point on, this strain is the most frequently used one for research applications and industrial production.

2.6.2 Biochemistry of Itaconic Acid Fermentation

In TCA cycle citric acid is converted to cis-aconitic acid by the enzyme aconitase and aconitase further converts cis-aconitate to isocitrate as the continuation of TCA cycle (Figure 2.11), hence; cis-aconitic acid is the intermediary product of the reaction. Decarboxylation of cis-aconitate to itaconic

acid is carried out by the cis-aconitate decarboxylase (CAD) produced in *A. terreus* (Bentley *et al*., 1955). Other organisms mentioned in literature having itaconic acid production ability were *Ustilago zeae*, *Ustilago maydis*, some *Candida* species, some *Rhodotorula* species. Highest itaconic acid production was obtained by a mutant *Aspergillus terreus* strain TN-484-M1 (82g/L) (Okabe *et al*., 2009).

Very early studies suggested three different pathways for itaconic acid production (Figure 2.12). Winskill searched for tricarboxylic acid cycle activity in relation with itaconic acid production from *A. terreus* in 1983. His study focused on the NADP and NAD amounts during acid production and ^{14}C labeling. According to his results, he claimed that itaconic acid production was related with decarboxylation of aconitic acid (Winskill, 1983). Bonnarme and coworkers was found similar result that NMR studies with a variety of ^{13}C and ^{14}C labeled substrates have confirmed the action mechanism of *cis*-aconitate decarboxylase, which interrupts the TCA cycle (Bonnarme *et al*., 1995). Lies and coworkers purified and characterized the CAD from mutant *A. terreus* in 2002. Kanamasa and friends carried out the further studies by cloning the CAD gene from *A. terreus* (Lies *et al*., 2002; Kanamasa *et al*., 2008). The enzyme CAD uses the same substrate cis-aconitic acid as aconitase (EC 4.2.1.3). There are two isozymes of aconitase in eukaryotes. One of them is found in the mitochondrial matrix and functions in the TCA cycle the other one is found in the cytoplasm as a soluble enzyme in it.

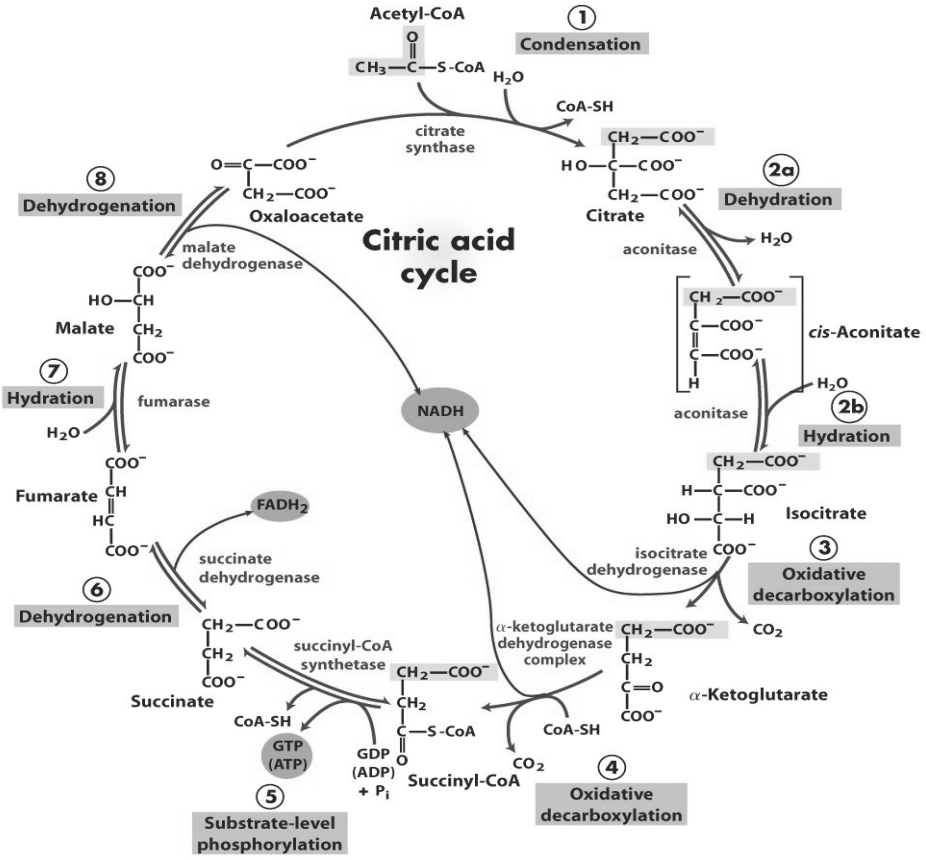


Figure 2.11 TCA cycle (Lehninger, 2004)

Itaconic acid production in *A. terreus* is very similar to citric acid production in *A. niger*, except one further step is required for the itaconic acid production. Hence, the accumulation of IA is very dependent on citrate synthase and CAD activity. cis-Aconitic Acid Decarboxylase is an enzyme that uses cis-aconitic acid as substrate and converts it into two products: itaconic acid and carbon dioxide, thus; the chemical reaction is;

$$\text{cis-aconitate} \rightleftharpoons \text{itaconate} + CO_2$$

The systematic name of this enzyme is cis-Aconitate carboxy-lyase since it belongs to the enzyme family of lyases and specifically it cleaves carbon-carbon

bonds. cis-Aconitic acid decarboxylase, CAD are other names are commonly used (2008).

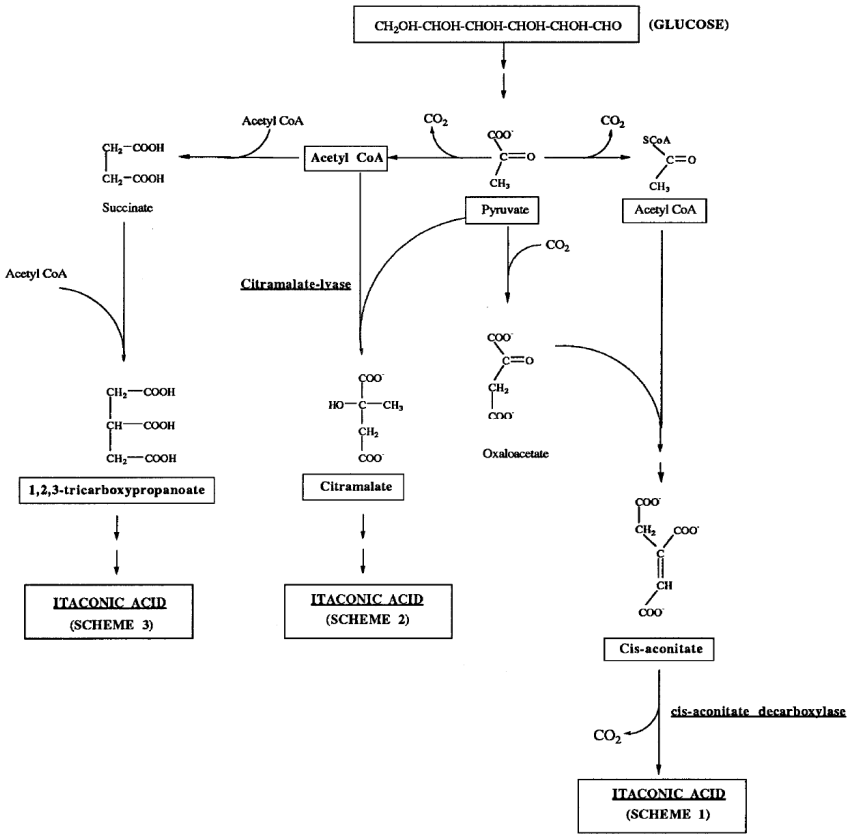


Figure 2.12 Theoretical pathways for itaconic acid production (Bonnarme *et al.*, 1995)

2.6.3 Commercial Applications of Itaconic Acid

In contrast with citric, gluconic and lactic acids, itaconic acid is used exclusively in non-food applications (Magnuson and Lasure, 2004). Table 2.6 shows the main applications of IA. Its primary application is in the synthetic polymer industry, as its methylene group is able to participate in polymerization reactions and the resulting heteropolymers have hydrophilic properties because of the acid

groups in itaconic acid (Ruijter *et al*., 2002). Moreover, itaconic acid is used in the manufacture of synthetic fibers, coatings, adhesives, thickeners and binders. The market volume has been estimated to be about 15,000 metric tons per year and is expected to grow if the selling price (estimated to be about US$4 per kg) can be reduced (Willke and Vorlop, 2001).

Itaconic acid has unsaturated double bond and active chemical properties. It is a white crystalline powder that is soluble in water, ethanol and acetone and its double bond provides a conjugated system with carbonyl groups (Okabe *et al*., 2009). Furthermore, this property makes it used as co-monomer in order to prepare acrylic fibers and rubbers, reinforced glass fiber, artificial diamonds and lens, additive in fibers and ion exchange resins in order to increases abrasion, waterproofing, physical resistance, dying affinity and better duration, water treatment system in order to prevent contamination by metallic alkali, binder and sizing agent in non-weaving fibers, paper and concrete paint, and co-polymer, plasticizers, lubricant oil, paper coating, carpets for better duration, adhesives, coatings, paints, thickener, emulsifier, surface active agents, pharmaceuticals and printing chemicals (Willke and Vorlop, 2001; Kanamasa *et al*., 2008; Okabe *et al*., 2009; Chemicalland, 2010).

Table 2.6 Applications of itaconic acid (Okabe *et al*., 2009)

Materials	Application
Vinylidene chloride containing below 2% IA	Improved adhesion to paper, cellophane
Alkali salt of poly IA	Detergent
Rubber-like resin (Copolymer of IA)	Electrical insulation
N-substituted pyrrolidones (IA with amines)	Thickeners in lubricating grease, detergents, shampoos
Imidazoline derivative	Shampoos
Polyacrylonitrile copolymer incorporating low level of IA	Efficient dying and deep shade in textile industry
Copolymer of acrylic acid and IA	Scale inhibitor in boiler
IA monoester compounds	Dental adhesives, dental fillers
Hardening agent	Contact lens
Pigmented dispersion resins containing 0.1-1.5% IA	Wet abrasion resistance
Styrene butadiene lattices containing 1-5% IA	Carpet backing or paper coating
Acrylic lattices supplemented with IA	Nonwoven fabric binder
Sulfonated poly IA	Industrial cleaner
N-vinyl 2-pyrrolidone/IA hydrogels	Antifungal drug
Poly(acrylamide-co-monomethyl itaconate) hydrogels IA	Transdermal therapy
IA	Inhibitor of fructose 2,6-bisphosphate synthesis
N-isopropylacrylamide/IA copolymeric hydrogels	Drug release
Polycarboxylic acid nanoparticles	Ophthalmic drug delivery
Poly(acrylamide-co-IA) hydrogels	Drug delivery
Poly(acrylic acid-co-IA)	Glass-ionomer cements
N-vinylcaprolactam-containing copolymer of acrylic-IA	Glass-ionomer dental cements

2.7 Aim of the Study

The major aim of this study was the production of itaconic acid fom agricultural (lignocellulosic) wastes, such as corn cobs and cotton stalks. Lignocellulosic biomass mainly contain cellulose and hemicellulose as carbohydrates and the microorganism should secrete cellulases and/or xylanases for the production of simple sugars such as glucose and xylose, to grow and to produce itaconic acid. For this purpose, a well-known itaconic acid producer, *Aspergillus terreus* NRRL 1960, was selected and its cellulase and xylanase activities were checked. Since a low cellulase (0.12FPU) but a high xylanase activities (41 IU/mL) were detected, which are consistent with the reported studies (Hrmova *et al*., 1989; Gawande and Kamat, 1999; Ghanem *et al*., 2000), the study was focused on the co-production of itaconic acid with xylanase as products. In addition, biochemical characterization of xylanase was performed.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Chemicals used in this study were analytical grade, either obtained from Sigma (N.Y., USA) or Merck Chemical Companies (Deisenhofen, Germany). Lignocellulosic by-products were provided from several local plants (corn cobs, cotton stalk, and sunflower stalk).

3.2 Microorganism and Culture Conditions

The experiments were performed with *Aspergillus terreus*, strain NRRL 1960. The organism was maintained in glycerol stock solutions (Appendix A). *A. terreus* was grown and sporulated on a solid potato dextrose agar for 4 days at 30 °C. For inoculation of liquid batch culture, stock spore solutions were prepared in 1% peptone solution. Spore concentration was determined using a haemocytometer (Appendix B). 10^7 spores/mL spores were inoculated into the 100 mL liquid culture.

Enzyme productions were carried out as shake-cultures in 250 mL flasks for a week at 35 °C at 175 rpm. 10 mL of preculture was used to inoculate 100 mL of production medium (Appendix C).

3.2.1 Carbon sources

Different lignocellulosic by-products namely birchwood xylan, corn cobs, cotton stalk, sunflower stalk, were used as carbon sources and inducers to increase the enzyme production yield. Lignocellulosic by-products were used after grinding to 2mm (Mesh No: 60)

3.2.2 Preparation of Cell Free Extract

For preparation of cell free extract (CFE), the production medium was centrifuged at 10,000 x*g* for 15 min to remove the cell debris and the supernatant was used as the crude enzyme solution.

3.3 Analytical Methods

3.3.1 Cell Dry Weight

The quantity of mycelial cells was determined based on dry weight. The cultures used for growth profile measurements were filtrated using coarse filter paper and dried at 105 °C for overnight.

3.3.2 Measurement of pH

The pH of samples was determined using a Sartorius pH meter (model PB-11) in combination with a Sartorius electrode (type PY-P11).

3.3.3 Xylanase Assay

Samples were collected from production medium after indicated time periods of incubation, filtrated and centrifuged for 1 min at 15000 x*g* at 4-6 °C to remove cells and insoluble fragments of the medium. This clarified medium solution was used as crude enzyme source for further studies.

For the assay, 1 % birchwood xylan was dissolved in 50 mM phosphate buffer at pH 7.0 and was used as substrate to measure initial reaction rate of xylanase.

Due to low solubility of xylan in aqueous solutions, 1 g of birchwood xylan was dissolved in 80 mL of buffer solution by mixing and heating up to boiling point. After boiling for 2-3 min, this solution was stirred overnight on a magnetic stirrer at room temperature. Then, the solution was diluted to 100 mL with the same buffer, mixed well and centrifuged at 5000 x*g* for 20 minutes to remove the insoluble xylan particles. This substrate solution should be stored at 4 °C for at most one week. Before the usage of the solution, it must be equilibrated to assay temperature (Bailey *et al.*, 1992).

Xylanase activity was determined by measuring the reducing sugar concentration, released during the enzymatic reaction. The reaction was carried out by mixing one volume of appropriately diluted enzyme solution with ten volumes of substrate solution, both of which were equilibrated to 40 °C and the mixture was incubated at the same temperature. Samples of 1 mL were taken with appropriate time intervals and the reaction was stopped by immediate addition of 1.5 mL of dinitrosalicylic acid (DNSA) reagent. To provide the color development, tubes are placed in a boiling water bath for 5 min, and then cooled immediately in ice bath. The optical densities of the samples were measured at 540 nm against a buffer blank after the tubes are equilibrated to room temperature (Miller, 1959).

DNSA reagent is prepared as described in Appendix D and E. In DNSA Method, reducing sugars within the samples were determined by using xylose and glucose as standard (Appendix F and G). The reaction conditions were selected according to previous studies performed in our laboratory (Yavascaoglu, 2000; Avcioglu *et al*., 2005). One unit of xylanase activity (IU) is defined as the quantity of enzyme necessary to produce 1 µmole of xylose equivalents per min at 40 °C and under the reaction conditions.

3.3.4 Enzyme activity assays

β-xylosidase, β-glucosidase, α-arabinofuranosidase, α-galactosidase enzyme assays done by using p-nitrophenol substrates. 0.4 mL of 5 mM substrates in 50 mM phosphate buffer at pH 7.0 were mixed by 0.2 mL of crude enzyme at 40 ℃ for 10 min after equilibrating both of them to 40 ℃. The reaction was stopped by addition of 1mL of 1 M Na_2CO_3 and the absorbance was measured at 410 nm (Wakiyama *et al*., 2008). One enzyme unit (U) defined as the release of 1 μmol p-nitrophenol (pNP)/min at 40 ℃.

3.3.5 Cellulase activity assay

Cellulase (EC 3.2.1.4) activity was assayed as described by Adney and Baker (1996) using 1 x 6 cm Whatman No.1 filter paper strips as substrate in 50 mM phosphate buffer at pH 7.0 and 40 ℃. Dinitrosalicylic acid (DNSA) method was used to determine reducing sugar concentration in assays by using glucose as standard (Miller, 1959). Initial reaction rates were taken into account in activity measurements. One unit of cellulase activity (U) were defined as the quantity of enzyme necessary to produce 1 μmole of glucose equivalents per min at 40℃ under the given conditions.

3.3.6 Protein Determination

Protein concentration was determined by Bradford Method using bovine serum albumin (BSA) as standard (Bradford, 1976), (Appendix H, I and J).

3.3.7 HPLC Analysis

HPLC analysis was performed using a Shimadzu LC - 20AD Prominence HPLC system with a Bio-Rad Aminex HPX-87H column at 30 ℃. Isocratic HPLC analyses were performed using 8 mM H_2SO_4 as solvent at room temperature, at a flow rate of 30 mL/h and organic acids were detected at a wavelength of 210 nm (Appendix K).

3.3.8 Characterization of the Xylanase

3.3.8.1 SDS-PAGE and Isoelectric Focusing

The homogeneity and molecular mass of xylanase were determined in the presence of sodium dodecyl sulphate (SDS-PAGE) according to the method of Laemmli (1970) using 4% stacking and 12% separating gels with Mini-Protean II Dual Slab cell system (BioRad, USA) (Appendix L). PageRuler™ Plus Prestained Protein Ladder molecular weight markers (Fermentas, Canada) were in the range of 10-250 kDa. Gel run was performed at 50 V constant voltage for 15 min, then at 100 V for 1 h. Gels were stained by silver staining method according to Blum *et al.* (1987) for molecular weight determination (Appendix M). The gel was also stained using glycoprotein detection kit (Sigma-Aldrich, Germany) to predict the possible sugar moieties of xylanase. Peroxidase with 18% carbohydrate content and BSA were used as positive and negative controls, respectively.

Isoelectric point of the protein was determined on an IEF gel containing ampholytes in a pH range of 3.5-10.0 (Sigma-Aldrich, Germany) in an Amersham Multiphor II Electrophoresis System (Amersham Biosciences, USA) in METU Molecular Biology and Biotechnology Research and Development Center. The IEF markers, in a range of 3.5-9.3 (Amersham Biosciences, USA), and the sample were run at 100 V for 15 min, at 200 V for 15 min, and at 450 V for 4 h and was then stained with Coomassie blue.

3.3.8.2 Zymogram Analysis

Zymogram analysis was performed according to the method described by Ratanakhanokchai *et al.* (1999) with some modifications. Crude enzyme sample was incubated in boiling water for only 5 sec since renaturation of the enzyme was not possible when boiled for 5 min due to its thermo-versatile nature. Then, the enzyme was electrophoresed on an SDS-polyacrylamide gel containing 0.1% birchwood xylan. After electrophoresis, the gel was incubated in isopropanol

(25% v/v) for 30 min in a 100 mM sodium phosphate buffer at pH 7.0 to remove the SDS and renature the enzyme in the gel. Then, the gel was washed two times in the same buffer without isopropanol for 30 min at 40°C. The gel was soaked in 0.1% Congo red solution for 15 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After soaking the gel into 0.5% acetic acid solution, the background turned to dark blue and clear band indicating the xylanase activity was identified.

3.3.8.3 Effect of pH and Temperature on Xylanase Activity and Stability

The activity profile of purified xylanase was determined between pH values ranging 3.0-10.0 in following buffers (50 mM): citrate (pH 3.0-5.5), sodium phosphate (pH 6.0-8.0) and sodium bicarbonate (pH 9.0-10.0) at 50 °C and temperatures between 30-80°C at 5°C intervals. For the determination of pH and temperature stability, the enzyme aliquots were preincubated at pH range of 3.0-10.0 for 4 and 48 h and temperatures 30-50°C for up to 8 h and samples were taken periodically. Residual activity was determined under standard assay conditions.

3.3.8.4 Kinetic Studies

K_m and V_{max} values were determined from both Michaelis-Menten kinetic graph by using SigmaPlot 9.0 and Lineweaver-Burk plot (Lineweaver and Burk, 1934) at different birchwood xylan concentrations ranging from 0.2 to 4.0% (w/v) under standard assay conditions.

3.3.8.5 Analysis of Xylan Degradation Products

The hydrolysis of birchwood xylan (10 g/L) was performed using 6 IU/mL crude and purified xylanase in 20 ml reaction mixture at pH 7.0 and 40°C. Samples were taken at 0, 10, 20 and 30 min, 1, 2, 3 and 48 h, boiled for 5 min and analyzed by silica gel thin layer chromatography (TLC). The substrate, xylan (Xy), was also boiled and used as reference. A 3 µl portion of each sample was

spotted onto thin layer chromatography silica gel plate (Merck, Germany). TLC was performed using a solvent system containing ethyl acetate:acetic acid:water (3:2:1, v/v) at room temperature. The plate is sprayed with a ethanol:sulphuric acid (9:1 v/v) solution heated for about 15 minutes at 130 °C until complete color development. The standards (St) used were xylose (X1), xylobiose (X2), xylotriose (X3) and xylotetraose (X4) (Megazyme, Ireland).

3.4 Pretreatment and Extraction Methods

3.4.1 Dry Heat Pretreatment

Agricultural residue was kept 16 h in an incubator at 60 °C.

3.4.2 Wet Heat Pretreatment

Agricultural residue was swelled for 16 h in an incubator at 60 °C by mixing 1:50 (w/v) with distilled water. The swollen sample was filtered and dried at room temperature.

3.4.3 Alkali Xylan Extraction

Agricultural residue was swelled for 16 h in an incubator at 60 °C by mixing 1:50 (w/v) with distilled water. The swollen residue was filtered and stirred for 3 h at room temperature in the solution of 170 mL of 24% (w/v) KOH and 1% (w/v) $NaBH_4$. After stirring, the supernatant was centrifuged at 7000 x*g* for 10 min. The supernatant was mixed with 2.5 volumes of cold ethanol:acetic acid solution (10:1). The pellet was dried at room temperature and used as extracted xylan (Zilliox and Debeire, 1998).

CHAPTER 4

RESULTS AND DISCUSSIONS

This study focused on production of xylanase and itaconic acid from *A.terreus* NRRL 1960 using renewable lignocellulosic materials as carbon sources. Corn cob, cotton stalk and sunflower stalk were used as renewable carbon sources. For this aim, two pre-defined media having inducing capacity either for xylanase or itaconic acid production were tested. Medium-1 (M1) components were derived from the media mentioned in the literature, having inducing capacity on *A. terreus* xylanase production (Ghanem *et al.,* 2000; Hrmova *et al*., 1991; Gawande and Kamat, 1999; Marques *et al*., 2003) (Appendix C). Medium-2 (M2), optimized for itaconic acid production during previous studies performed in Wageningen University (Netherlands) was used as control (modified Cros and Schneider, 1993). Within these two production media, the medium having the highest xylanase and itaconic acid production capacity was selected and further used for studies on; (i) co-production of xylanase and itaconic acid, (ii) two-step production and optimization for xylanase and itaconic acid, (iii) biochemical characterization of xylanase.

4.1 Comparison of Two Fermentation Media

To determine the medium having highest inducing capacity for co-production of xylanase and itaconic acid production, two experimental sets were carried out using two predefined media (M1 and M2) (Figure 4.1).

Production media containing 10% (w/v) glucose or 1% (w/v) xylan as sole carbon source were used as controls for itaconic acid and xylanase production,

respectively in the first set of experiments. In addition, 10% (w/v) xylose was used as carbon source instead of glucose to observe if the organism can use xylose as efficiently as glucose and 1% (w/v) xylan was used to compare xylanase production capacity of M2 with M1. The second set was designed to test the effect of initial pH adjustment to 2 on M1 and M2 (Fig. 4.1).

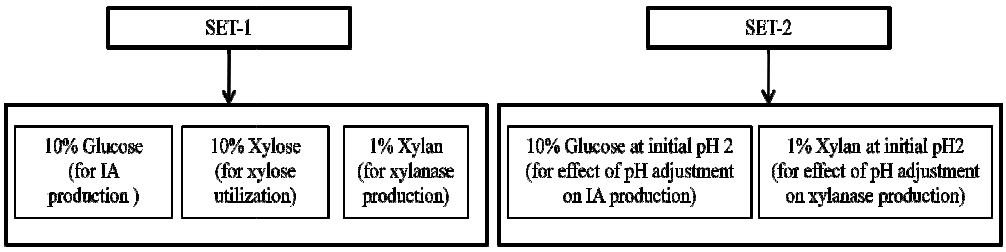


Figure 4.1 The carbon sources and concentrations used for production of xylanase and itaconic acid

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06.

It is of interest that *Aspergilli* species could not germinate at low pH values since glucose oxidase activities were very low at low pH values (Ruijter *et al*., 2002). Therefore, spores were germinated in preculture having initial pH of about 5.

4.1.1 Experimental Set 1

In Experimental Set-1, media were compared according to their itaconic acid production capacity on 10% (w/v) glucose, 10% (w/v) xylose and xylanase production capacity on 1% (w/v) xylan.

In Medium-1 (M1), it took 2–3 days for the organism to reach the stationary phase for all the cases. Furthermore, the growth profiles in M1 were almost similar to each other (Figure 4.2). But, the growth profiles in M1 and M2 were

different from each other, especially for the first days of fermentation (Figure 4.2 and 4.3). Growth in M1 was so fast in the first two days that all the cultures almost reached their maximum cellular weight. On the other hand, in M2, the growth was slower at the beginning in 1% (w/v) xylan (1Xn) containing medium and it was half of the cellular dry weight of M1 when it reached its maximum value. Indeed, the organism in M2 showed similar growth profiles for the first three days of fermentation where it grew gradually. Nonetheless, they are different during the rest of the fermentation: in M2, growth in 1Xn medium stayed constant over the rest of the fermentation. Furthermore, the growth in the 10% (w/v) glucose (10Glu) and 10% (w/v) xylose (10X) containing media continued till the 5th day of fermentation and then remained constant (Figure 4.3). If the two media were compared with respect to cellular dry weight (CDW), growth in M2 was slower and less than M1 and that was a desired feature for itaconic acid production.

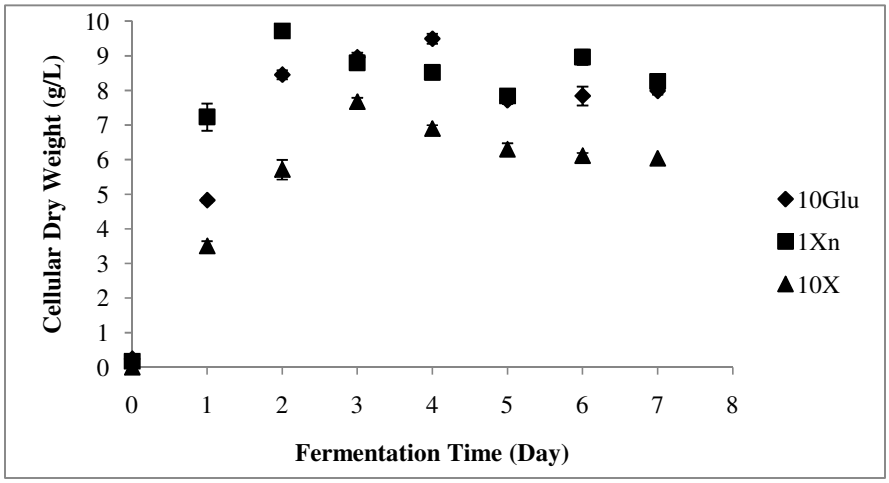


Figure 4.2 Effect of carbon source on cultivation of *A. terreus* in Medium-1
Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0, at 37 °C at 170rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

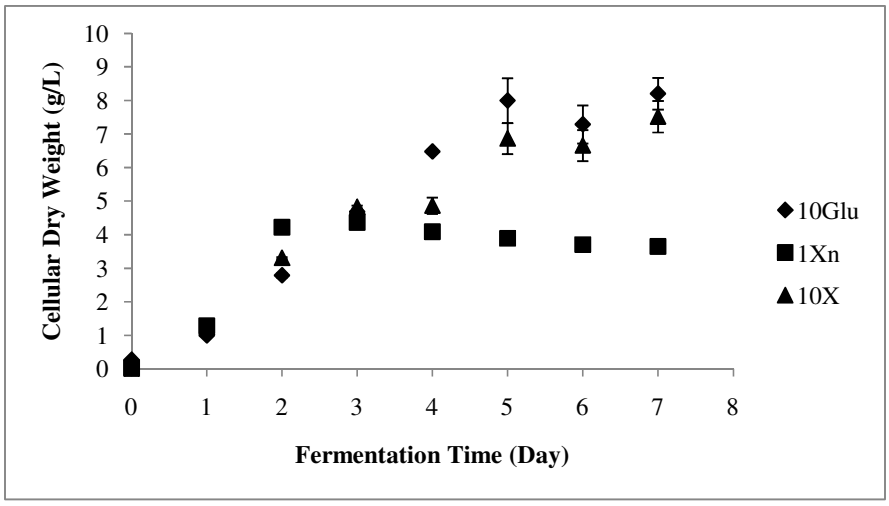


Figure 4.3 Effect of carbon source on cultivation of *A. terreus* in Medium-2
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06, at 37 ℃ at 170rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

The organism used up approximately 50% of glucose and xylose during the first four days of fermentation and the sugar concentration was almost constant for the rest of the fermentation process in M1 (Figure 4.4). In xylan containing culture, the sugar coming from preculture was consumed fast during the first two days of fermentation and reducing sugar concentration decreased to almost zero in four day. In M2, as shown in Figure 4.5, the organism in 10Glu medium used up very little amount of sugar in the first day of fermentation, then it consumed 40% of sugar in two days, after that the sugar concentration remained almost constant. On the other hand, 80% of the xylose was consumed in five days and sugar concentration stayed constant in the remaining three days in xylose containing medium. The organism used up all the sugar in three days in 1Xn medium.

Furthermore, the consumption of simple sugar was higher in xylose containing cultures than glucose containing cultures throughout the whole fermentation process in both media. There was no direct correlation with cellular dry weight and simple sugar consumption, since in M2 the usage of glucose and cellular dry weight was lower than in M1. On the other hand, xylose was consumed more in

M2 than in M1 but their cellular dry weights were almost equal to each other. Besides all, the reducing sugar concentrations were quite low in xylan containing cultures of both media.

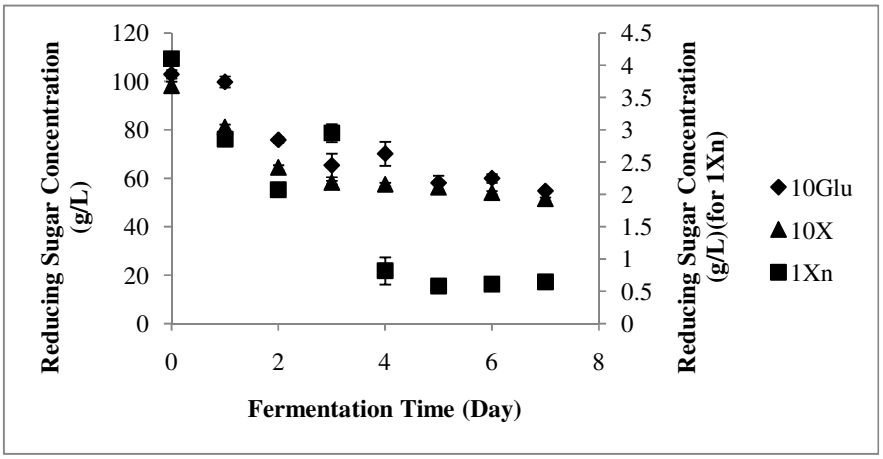


Figure 4.4 Effect of carbon source on reducing sugar concentration profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0, at 37°C at 170rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

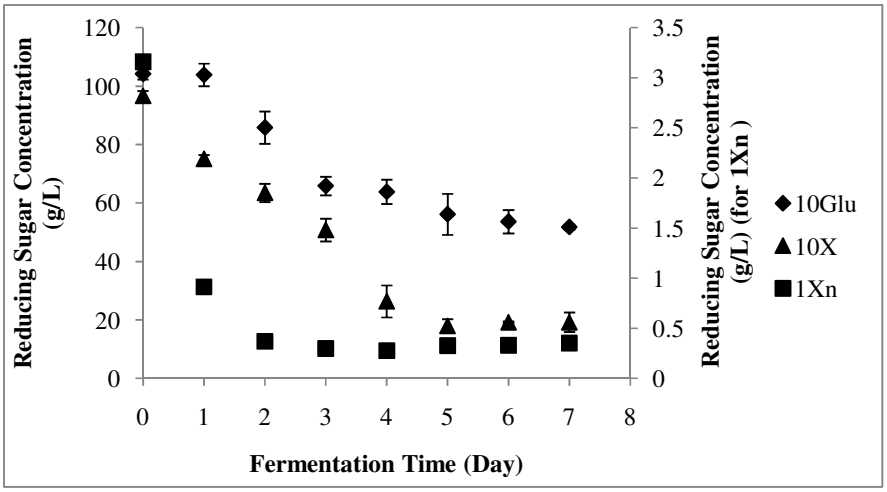


Figure 4.5 Effect of carbon source on reducing sugar concentration profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06, at 37°C at 170rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

On the contrary to growth profiles on glucose, xylose and xylan containing cultures, the extracellular protein concentration (ECP) profiles were different from each other in M1 (Figure 4.6). In glucose containing culture (10Glu), after a sharp increase in extracellular protein concentration on first day of fermentation, it stayed almost constant for the following four days. Then, it started to increase after the 5^{th} day and finally reached $70x10^{-3}$g/L extracellular protein on the 7^{th} day. On the other hand, in xylose containing culture (10X), the extracellular protein concentration gradually increased up to $80x10^{-3}$g/L throughout four days of fermentation and showed a sharp decline to $20x10^{-3}$g/L on 5^{th} day, whereas, after this decrease it started to increase again. Xylan containing (1Xn) medium extracellular protein concentration increased up to $40x10^{-3}$g/L throughout four days and then, it remained constant.

Effect of different carbon sources on extracellular protein concentration profiles in M2 is shown in Figure 4.7. Extracellular protein concentration in 10Glu medium was quite low for first two days, a gradual increase was observed throughout the following two days and on the 6^{th} day it showed a sharp increase. In 10X medium, after one day in incubator, a gradual increase in extracellular protein concentration was observed starting from the 2^{nd} day until the 5^{th} day of fermentation and then it stayed constant. On the other hand, extracellular protein concentration in 1Xn culture showed an increasing and decreasing profile. First, it had a peak on 2^{nd} day and then started to decrease till the 6^{th} day of fermentation, whereas, increase in extracellular protein concentration was observed again on 7^{th} day. As a result, 1Xn medium extracellular protein profile showed fluctuation throughout the fermentation and it reached its maximum value of $40x10^{-3}$g/L on the 2^{nd} day. Extracellular protein production was higher in M1 than M2 during overall fermentation process.

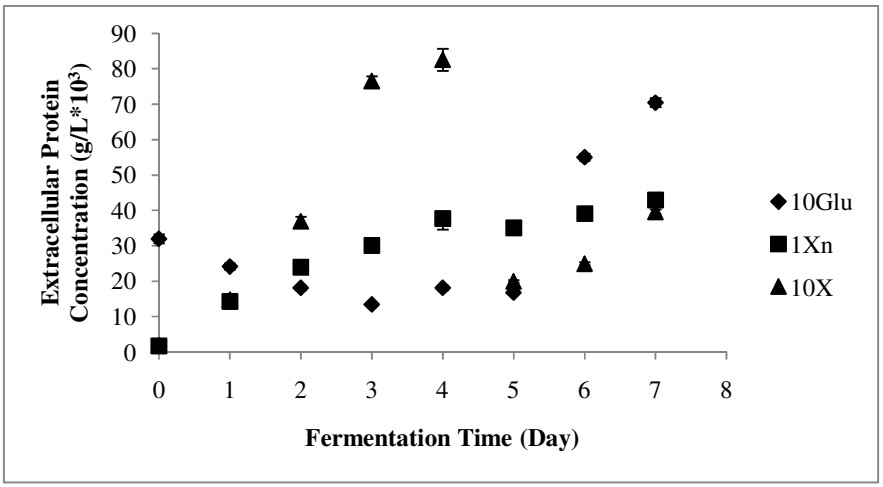


Figure 4.6 Effect of different carbon sources on extracellular protein concentration profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0, at 37°C at 170 rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

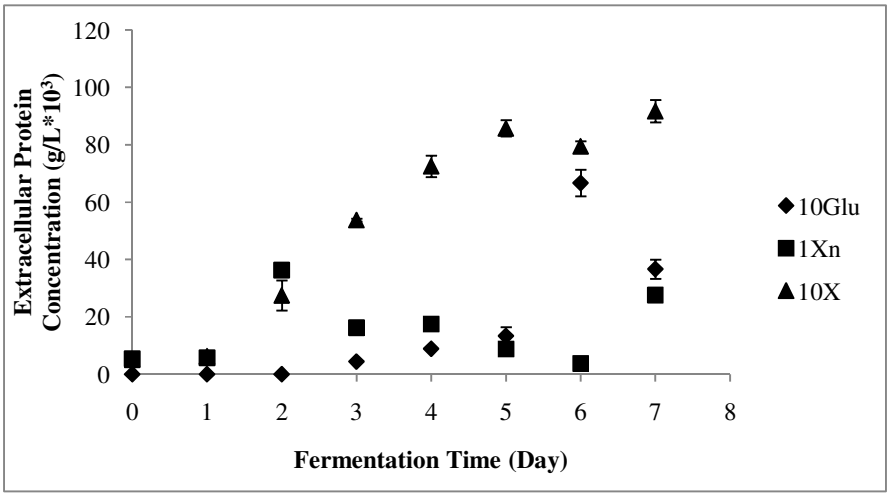


Figure 4.7 Effect of different carbon sources on extracellular protein concentration profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06, at 37°C at 170 rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

The pH profiles of simple sugar containing media; namely glucose (10Glu) and xylose (10X) were similar to each other in M1 (Figure 4.8). The pH value in 10Glu and 10X media decreased about 1.5 units through one day and then it stayed almost constant at pH 4. Nonetheless, pH in xylan containing medium (1Xn) showed a fluctuation that after a slight decrease in one day, the pH value increased to 6.5 through the next three days and then it decreased to 5.5 and stayed constant (Figure 4.8). On the other hand, pH in M2 clearly decreased for all cases (Figure 4.9). The pH of 1Xn medium decreased to 2.5 in three days and slightly increased throughout the rest of the fermentation. The pH profiles of 10Glu and 10X media differ from each other that in 10X medium the pH decreased to 2 in two days and then the decrease rate declined. Conversely, in 10Glu medium, the pH decreased to 1.6 in four days with almost constant rate and remained almost constant for the rest of the fermentation (Figure 4.9).

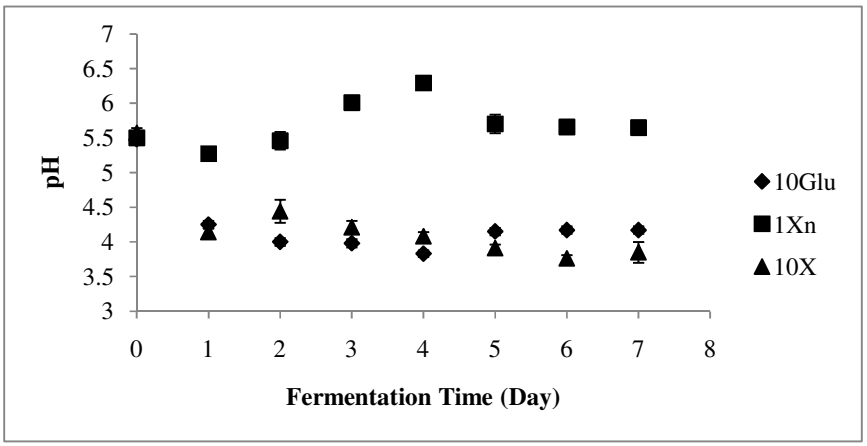


Figure 4.8 Effect of different carbon sources on pH profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0, at 37°C at 170 rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

In both media, the pH changes were almost identical for simple sugars; different profiles were observed for xylan, a polymeric carbohydrate. The different pH profiles in xylan containing media can also be related with the nitrogen sources

used since organic and inorganic nitrogen sources were used in M1 and M2, respectively (Carlile *et al*., 1994).

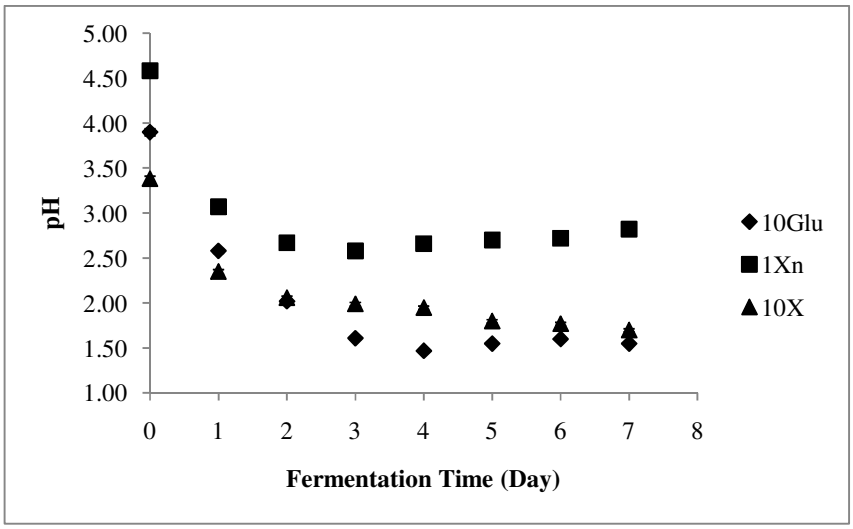


Figure 4.9 Effect of different carbon sources on pH profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06, at 37°C at 170 rpm. (10Glu: containing 10% glucose; 1Xn: containing 1% birchwood xylan; 10X: containing 10% xylose)

Ammonium nitrate and ammonium sulfate are main nitrogen sources used for organic acid production from *Aspergilli* species. Consumption of ammonium in the growth medium caused a decrease in pH, which was also another condition needed for itaconic acid production (Carlile *et al*., 1994). Low pH is a requirement for itaconic acid production in order to prevent the production of other acids (Ruijter *et al*., 2002). The excretion of organic acids such as itaconic acid or selective up-taking and the exchange of ions such as NO_3^- or NH_4^+ / H^+ help many fungi to alter their environmental pH (Walker and White, 2005). Ammonium ion take-up was followed by hydrogen ion extrusion in order to keep the ionic balance of the cell. Hence it resulted in highly acidic conditions in fermentation medium. When ammonium nitrate was used as nitrogen source, pH

increases as a result of nitrate consumption, which could follow the pH decrease because of ammonium utilization (Carlile *et al*., 1994).

M1 and M2 were also compared in terms of their inducing capacity for xylanase production. In M1, the xylanase activity became observable on 4th day and after a gradual increase, it reached 10 IU/mL (10000 IU/L) on 7th day, whereas, the profile followed different path in M2 (Figure 4.10). In M2, first xylanase production was measured on 2nd day and it was the maximum value (26000 IU/L). After reaching its maximum, the xylanase concentration decreased gradually throughout the rest of the fermentation. Xylanase production level in M2 was higher than the one in M1. Moreover, it reached its maximum level on 2nd day in M2, whereas, xylanase production was just observable after four day of incubation in M1. These findings were also consistent with the result of Ghanem *et al*. (2000) research, which claimed that for xylanase production ammonium sulfate was the best nitrogen source among the other inorganic and organic nitrogen sources.

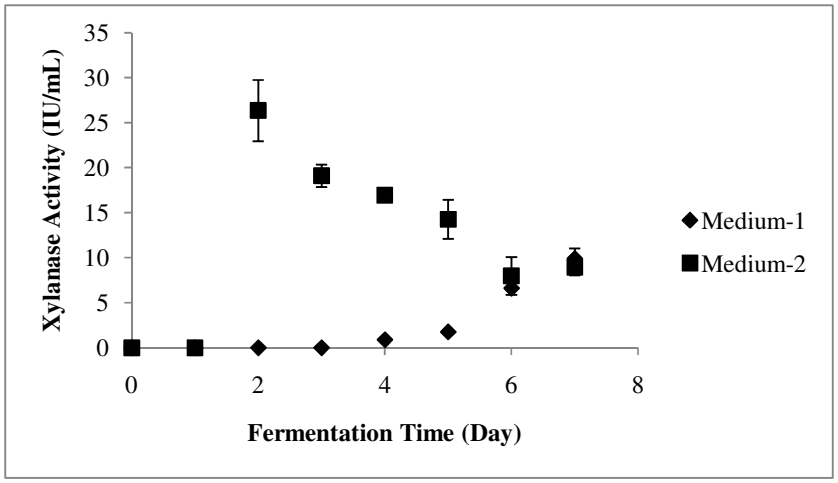


Figure 4.10 Comparison of Medium-1 and Medium-2 with respect to xylanase production levels of *A. terreus* on 1% birchwood xylan

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0, at 37°C at 170 rpm.

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06, at 37°C at 170 rpm.

It is of interest that neither the amount of growth nor the extracellular protein concentration correlated with xylanase activity. This finding is also consistent with the results of Gawande and Kamat (1999). It is especially striking that high xylanase activity was detected in the fermentation liquor in a number of cases where less growth was observed. The other important issue is limiting the growth in order to accumulate the secondary metabolites. It can therefore be said that restricting the growth would not cause any problems in xylanase production.

Itaconic acid production levels were the highest in glucose containing medium and the lowest in xylan containing medium for both medium types 1 and 2. However, as observed in Table 4.1, itaconic acid production in M2 was much better than M1 for both glucose and xylose. Thus, the results showed that M2 was much better than the M1 considering both xylanase and itaconic acid productions (Table 4.1). The results of time course studies showed that *A. terreus* could not produce itaconic acid using xylose as much as glucose. That may be due to the fact that filamentous fungi need conversion of xylose to xylulose-5-P (Figure 4.11). Further metabolism of xylulose-5-P is carried out in pentose phosphate pathway (Singh *et al*., 1992).

Table 4.1 The effects of different carbon sources in M1 and M2 on xylanase and itaconic acid productions

	Initial pH	Final pH	Maximum Dry Weight		Maximum Xylanase activity		Itaconic acid on 7th day (g/L)
			Day	gDW/L	Day	IU/mL	
10Glu-M1	5.5	4.2	4	9.5	-	-	**1.21**
10X-M1	5.6	3.8	3	7.7	-	-	0.11
1Xn-M1	5.5	5.6	2	9.7	7	**9.9**	0.002
10Glu-M2	3.9	1.5	7	8.2	-	-	**13.31**
10X-M2	3.4	1.7	7	7.5	-	-	6.95
1Xn-M2	4.6	2.8	3	4.4	2	**26**	0.001

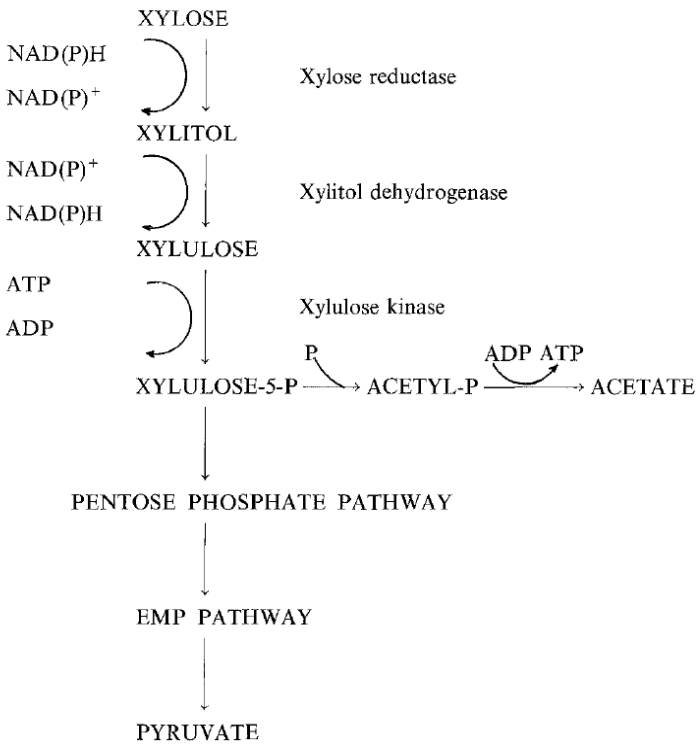


Figure 4.11 Xylose catabolism pathway in filamentous fungi (Singh *et al*., 1992)

Itaconic acid was produced gradually throughout the fermentation process. Itaconic acid production profiles of media containing glucose were shown in Figure 4.12. The organism consumed all the sugar coming from preculture in the xylan containing cultures. Furthermore, the amount of sugar in the production culture remained constant throughout the rest of the fermentation process. Therefore, the sugar coming from xylan utilization was used up continuously without leading to an increase of sugar concentration in the production medium.

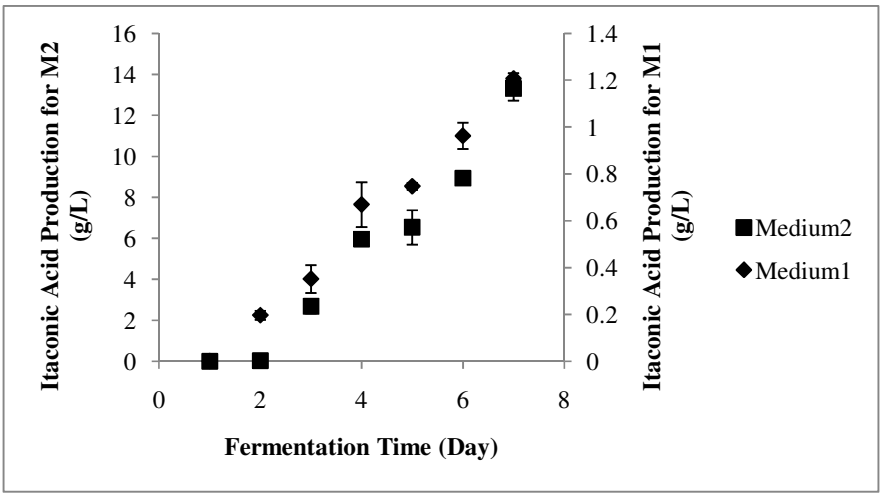


Figure 4.12 Comparison of Medium-1 and Medium-2 with respect to itaconic acid production levels of *A. terreus* on 10% glucose

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06.

It is worth mentioning that C/N ratio, which must be at least 10:1 in a balanced medium, is important in fermentation. In addition, medium having a C/N ratio greater than 10:1 can ensure accumulation of secondary metabolites (Carlile *et al*., 1994). Wilke and Varlop (2001) claimed that 100-150 g/L glucose was required for a satisfactory production rate and amount of production of itaconic acid from *A. terreus*.

As a summary, *A. terreus* NRRL 1960 is determined to utilize xylan efficiently by producing extracellular xylanase so that the microorganism can be used to produce itaconic acid on agricultural biomass. The xylanase production on 1% birchwood xylan containing medium and IA production on 10% glucose containing medium were higher in M2 than M1. The microorganism can produce itaconic acid more in glucose containing medium than xylose containing medium. However, the IA production on xylan was very low probably because of low simple sugar concentration in the medium.

4.1.2 Experimental Set 2

Second set of experiments with M1 and M2 were aimed to observe the effect of initial pH adjustment to 2 on itaconic acid and xylanase production in both media containing: 10% glucose at pH 2 (10Glu2) and 1% birchwood xylan at pH 2 (1Xn2).

Although, cellular dry weight (CDW) in 1Xn2 medium was less than in 10Glu2 medium, both culture showed similar growth profiles to each other that they reached their stationary phase on the 7th day of fermentation process by gradual increase in CDW (Figure 4.13). Adjustment of initial pH to 2 doubled the CDW in both cultures and delayed the time needed for reaching stationary phase. They grew in pellet shape for first 2 days, nonetheless, after that, the growth was in filamentous form and hence, their media became viscous. After centrifugation, 10G2 medium resulted in turbid cell free extract (CFE), while CFE of 1Xn2 medium was very clear.

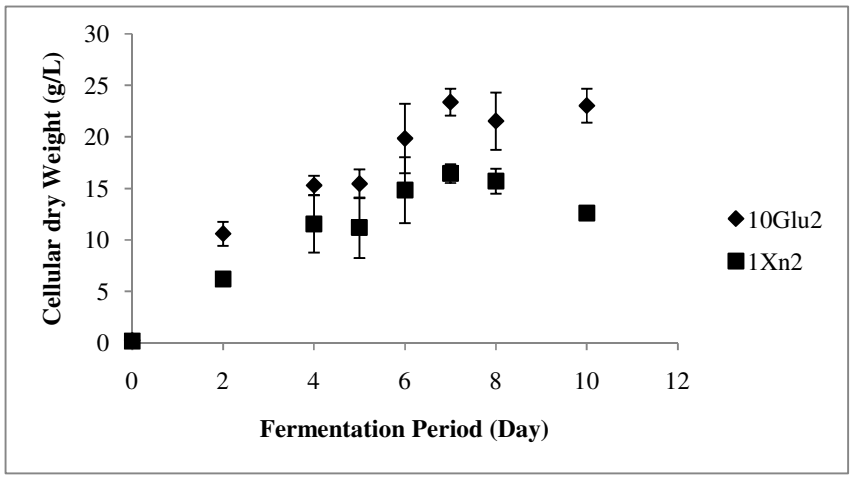


Figure 4.13 Effect of initial pH adjustment to 2 on growth profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

1Xn2 in M2 reached its stationary phase on the 3rd day after having one day lag phase and its maximum CDW was almost half of the one in the M1 (Figure 4.14). On the other hand, 10Glu2 showed completely different profile. After a sharp increase in first day, it stayed constant in following two days. This stationary phase was followed by a gradual increase in CDW and finally reached 5.5 g/L at the end of the fermentation.

By combining these experimental set-2 data (Figure 4.13 and 4.14) with the data obtained in experimental set-1 (Figure 4.2 and 4.3), it can be concluded that, xylan containing cultures showed similar profiles with each other whether their initial pH were adjusted or not. Nevertheless, cultures containing glucose followed different profiles. When initial pH of 10Glu was adjusted to 2, it showed two stationary phases and CDW was almost half of the one in the culture without pH adjustment. Therefore, limiting the growth for itaconic acid production can be achieved by using M2 with initial pH adjustment.

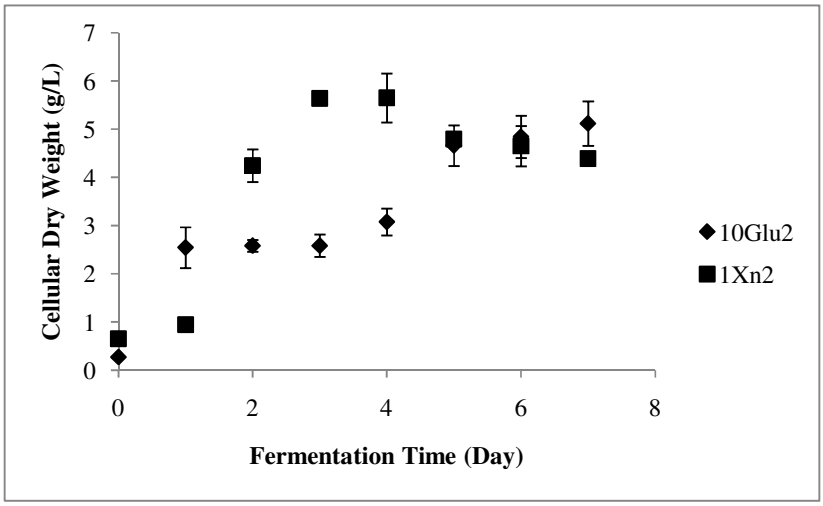


Figure 4.14 Effect of initial pH adjustment on growth profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

In M1, the organism consumed 85% sugar in 10Glu2 medium throughout 4 days and 10% in the following 3 days; similarly, sugar in 1Xn2 medium was used up in first two days of fermentation (Figure 4.15). Moreover, no increase in sugar concentration was observed in the rest of the fermentation period.

As shown in Figure 4.16, the organism in 10Glu2 medium consumed 40% of sugar throughout 3 days and 10% for following four days. The organism in 1Xn2 medium consumed all the sugar in three days. All sugar was used up in M1 and resulted in doubled growth. The results showed that the organism passed the stationary phase after depletion of sugar in M1. On the other hand, the organism consumed just 40% of sugar for the first three days of fermentation and the increase in cellular dry weight was very low compared to others.

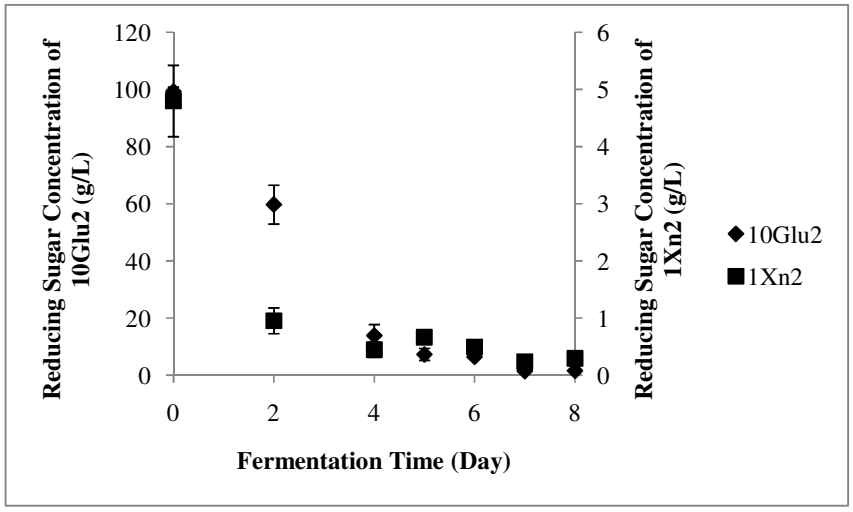


Figure 4.15 Effect of initial pH adjustment on reducing sugar concentration profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

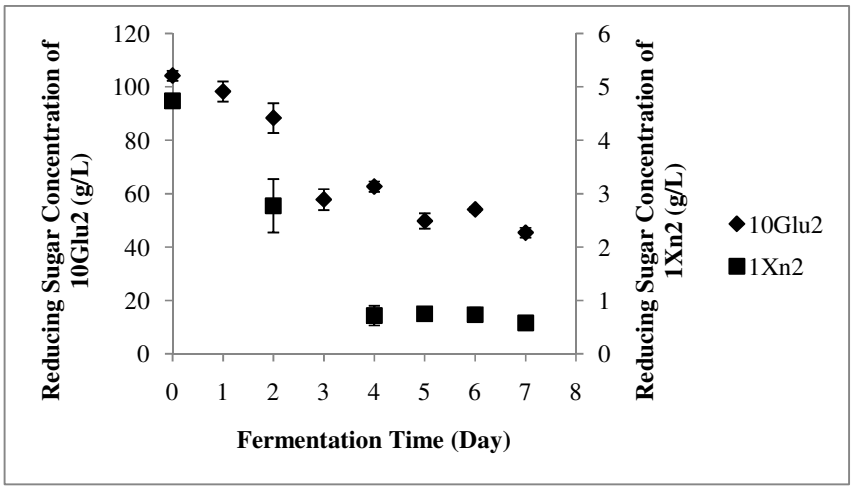


Figure 4.16 Effect of initial pH adjustment on reducing sugar concentration profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

The extracellular protein (ECP) concentration profiles of cultures in M1 were shown in Figure 4.17. The protein concentration of 1Xn2 medium gradually increased up to $120x10^{-3}$g/L on 8th day of incubation and after that it declined to $30x10^{-3}$g/L. Similarly the protein concentration in 10Glu2 medium continuously increased up to $120x10^{-3}$g/L on the 10th day of fermentation without any decrease in M1.

In M2, 10Glu2 culture showed stepwise increase in ECP concentration, whereas, 1Xn2 culture showed a curved ECP profile throughout the fermentation that it had a peak at $20x10^{-3}$g/L on 3rd day (Figure 4.18). Adjustment of initial pH caused the organism to produce high amount of extracellular protein in cultures containing glucose and it was almost ten times higher than the one in the culture without pH adjustment, whereas, it was not valid for the cultures containing xylan.

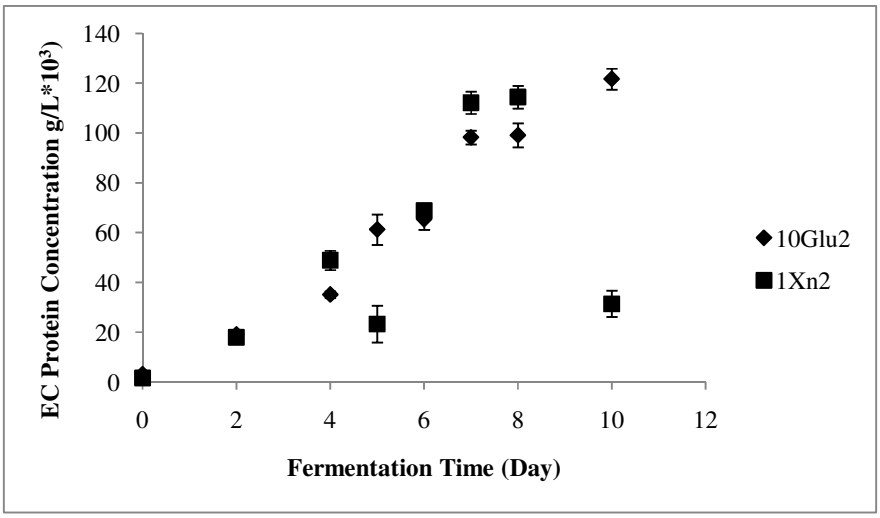


Figure 4.17 Effect of initial pH adjustment on extracellular protein concentration profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

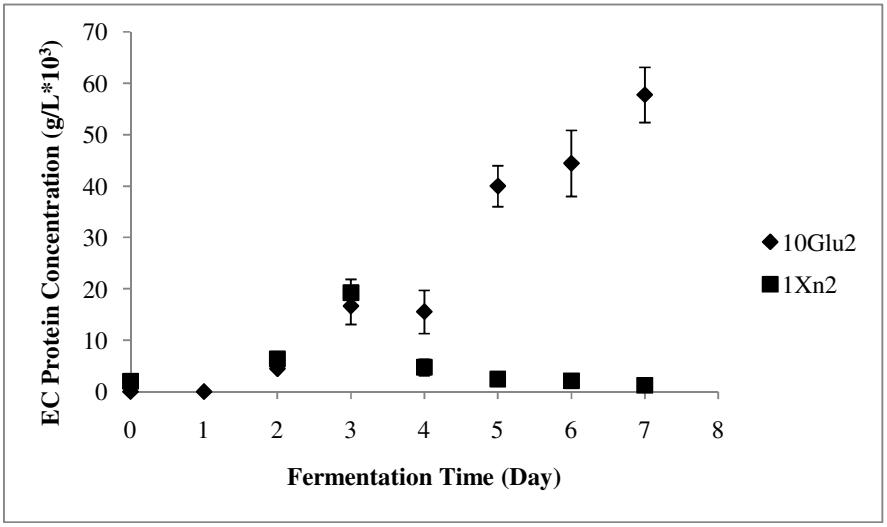


Figure 4.18 Effect of initial pH adjustment on extracellular protein concentration profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

In 10Glu2 culture of M1, pH gradually decreased from 2.5 to 1.5 throughout the fermentation process (Figure 4.19). Nevertheless, in 1Xn2, pH was almost constant through the first four days of incubation, then pH increased sharply to pH 8 in two days and it remained constant.

The pH profile of 10Glu2 culture in M2 was similar to the one in M1. In the first three days, pH of 10Glu2 of M2 was decreased to about 1.6 and remained almost constant through the rest of the fermentation (Figure 4.20). Although the pH of 1Xn2 medium decreased in the beginning of the fermentation (similar to 10Glu2 medium), after reaching pH 2, the pH increased slowly. After that, it stayed constant. Overall, except 1Xn2 in M1, the pH profiles were similar to each other between the cultures whether their pH values were adjusted or not.

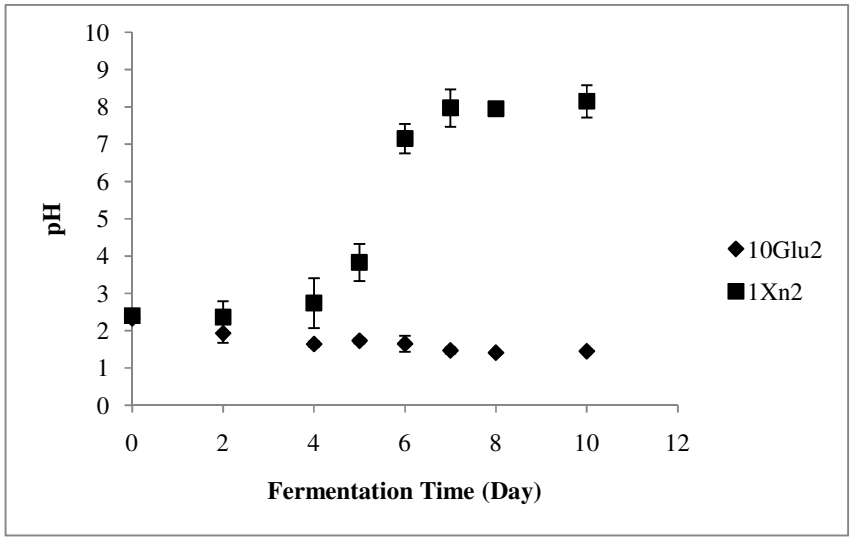


Figure 4.19 Effect of initial pH adjustment on pH profiles of *A. terreus* in Medium-1

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

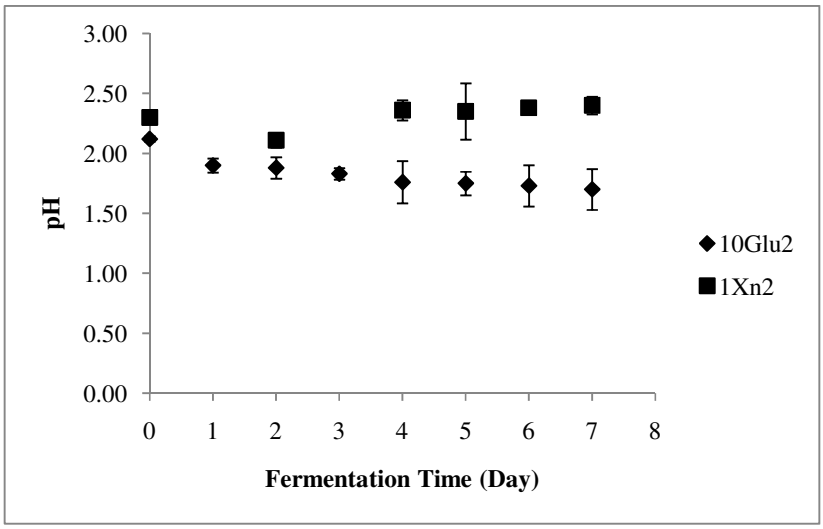


Figure 4.20 Effect of initial pH adjustment on pH profiles of *A. terreus* in Medium-2

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10Glu2: containing 10% glucose at pH 2; 1Xn2: containing 1% birchwood xylan at pH 2)

Effect of initial pH adjustment on xylanase production in M1 and M2 were also studied. The organism produced xylanase in M1 after four days of fermentation and its activity increased gradually throughout the following five days. Maximum xylanase activity in M1 was observed as 11 IU/ml (11000 IU/L) at 8th day and then started to decrease (Figure 4.21). In M2, the maximum xylanase activity of 40 IU/mL was reached after five days of fermentation by gradual increase in activity starting from the first day of fermentation. After having a peak on the 5th day, the xylanase activity showed a sharp decrease; nonetheless, it kept its level for another 3 days and started to decline again.

In general, the adjustment of initial pH increased the itaconic acid production about 33% in M2 and it also showed positive effect in M1 (Table 4.2). Although the beneficial effect of pH adjustment on itaconic acid production was observed in M1, itaconic acid production levels were much less than M2 (Figure 4.22).

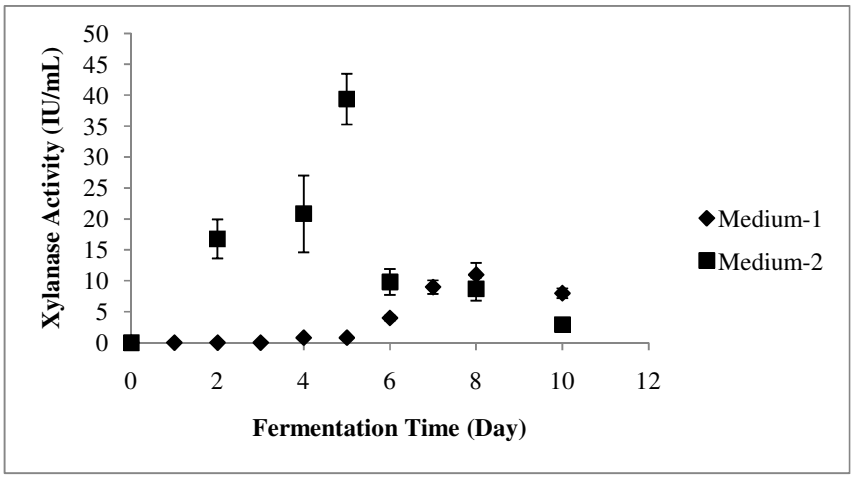


Figure 4.21 Comparison of initial pH adjusted Medium-1 and Medium-2 with respect to xylanase production levels of *A. terreus* on 1% birchwood xylan
Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (1Xn2-M1: containing 1% birchwood xylan at pH 2 in Medium-1; 1Xn2-M2: containing 1% birchwood xylan at pH 2 in Medium-2)

Table 4.2 Effect of initial pH adjustment in M1and M2 on xylanase and itaconic acid production

	Initial pH	Final pH	Maximum Dry Weight		Maximum Xylanase activity		Itaconic acid on 7th day (g/L)
			Day	gDW/L	Day	IU/mL	
10Glu2-M1	2.3	1.5	7	23.4	-	-	2.89
1Xn2-M1	2.4	8.2	7	16.4	8	11	0.003
10Glu2-M2	2.1	1.7	7	5.1	-	-	**17.67**
1Xn2-M2	2.3	2.4	4	5.7	5	**40**	1.66

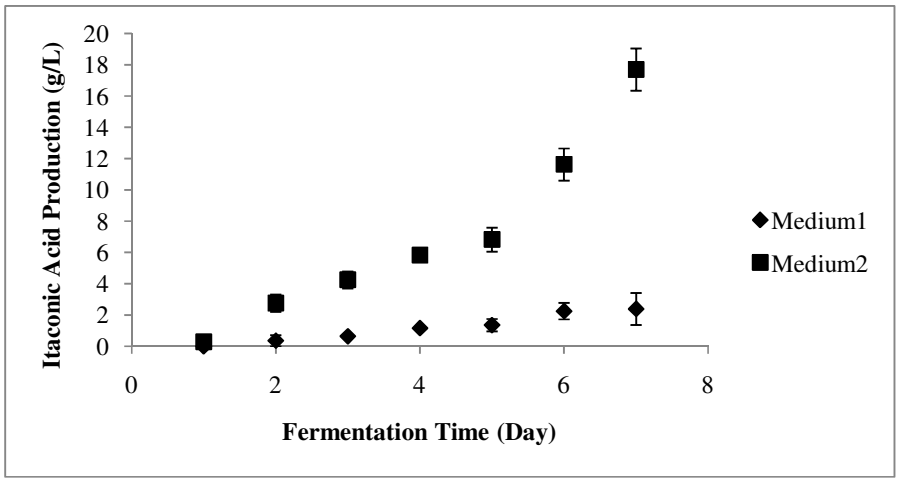


Figure 4.22 Comparison of initial pH adjusted Medium-1 and Medium-2 with respect to itaconic acid production levels of *A. terreus* on 10% glucose
Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06.

Surprisingly, the highest xylanase activity (40000 IU/L) was also observed using M2, a medium reported for itaconic acid production rather than xylanase production, with pH adjustment on 5th day of fermentation. Even though the pH adjustment showed no remarkable effect on xylanase production in M1, it changed the xylanase production trend in M2 drastically and increased xylanase production. Moreover, among xylan containing media, the highest itaconic acid production (1.66 g/L) was also observed in M2 with initial pH adjustment to 2.

Summarized results of two experimental sets on IA and xylanase production were shown on Figure 4.23 and 4.24. Since, both the highest itaconic acid and the highest xylanase production were obtained using M2 with initial pH adjustment to 2, the rest of the experiments were performed with this medium.

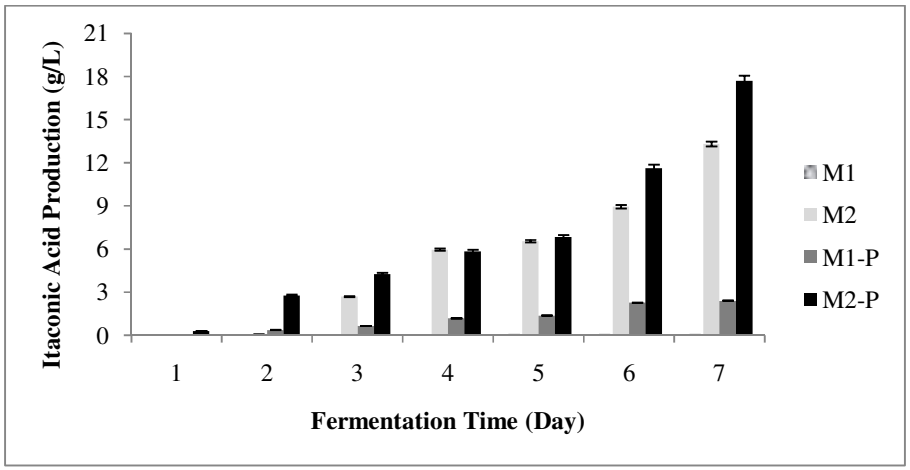


Figure 4.23 Comparison of production media and initial pH adjustment in regard to their IA production profiles on 10% glucose

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06.

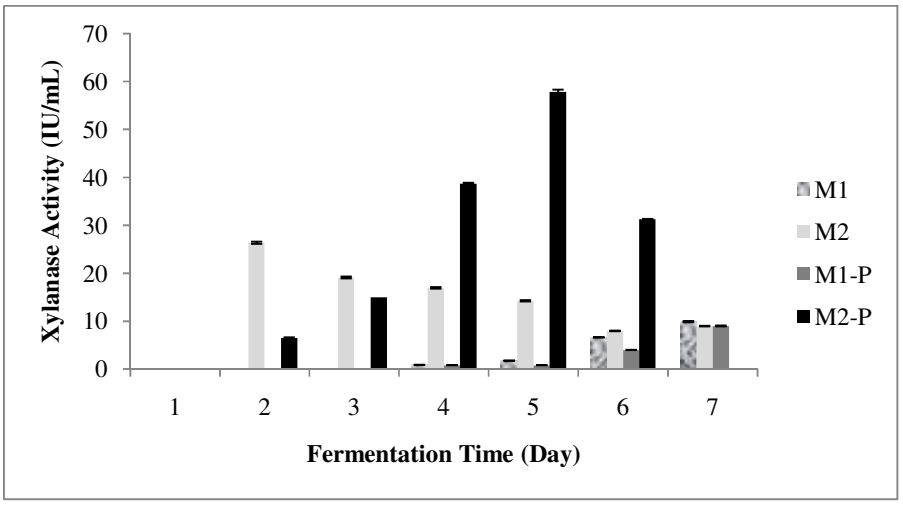


Figure 4.24 Comparison of production media and initial pH adjustment in regard to their xylanase production profiles on 1% birchwood xylan

Medium-1 (g/L): $MgSO_4.7H_2O$, 5; peptone, 1; yeast extract, 2; NaCl, 0.4; KH_2PO_4, 1; $CaCl_2.2H_2O$, 1.0.
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06.

4.2 Optimization for Co-production of Xylanase and Itaconic Acid

4.2.1 Effect of Co-utilization of Glucose and Xylan

The growth profile of *A. terreus* at an initial pH of 2 was studied when 2.5% (w/v) glucose and 1% (w/v) xylan (2.5Glu-1Xn2) were added to M2, with respect to 10% (w/v) xylose containing (10X2) medium. The highest biomass obtained was 16 g/L in 2.5Glu-1Xn2 medium (Figure 4.25). The organism showed loosely grown mycelia and the medium became more viscous throughout the fermentation. Except for the amount of the cellular dry weight and growth rates, both cultures (2.5Glu-1Xn and 10X2) showed similar CDW profiles through the six days of the fermentation. Nevertheless, it only reached the quarter of the 2.5Glu-1Xn2 cultures dry weight and half of the one in the 10X (without pH adjustment) (Figure 4.25).

The profiles of reducing sugar concentration in both cultures were similar to each other, while, the organism in 2.5Glu-1Xn2 fermentation culture used up all the simple sugars after 4 days and there was no remarkable increase in reducing sugar concentration (Figure 4.26). On the other hand, in 10X2 medium, the organism consumed 60% of xylose in the fermentation culture throughout the first five days of fermentation and then it almost stayed constant for the rest of the fermentation.

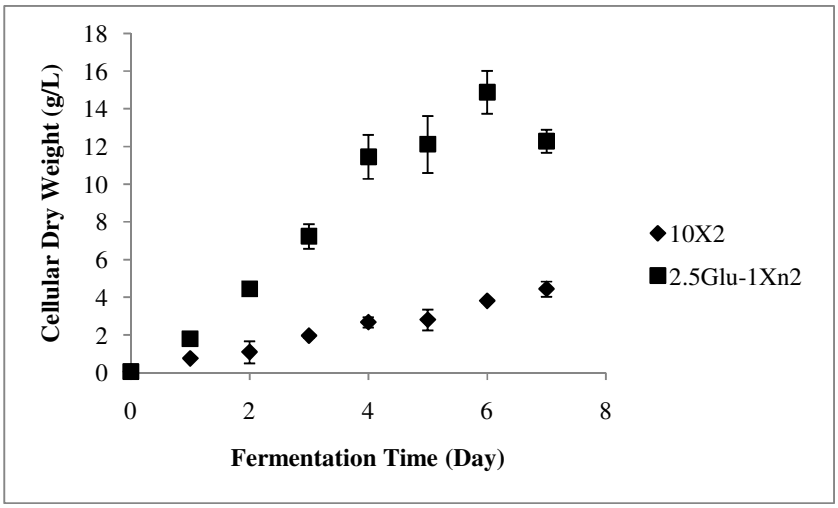


Figure 4.25 Effect of co-utilization of glucose and xylan and initial pH adjustment on xylose consumption on growth profile of *A.terreus*

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10X2: culture containing 10% xylose at pH 2; 2.5Glu-1Xn2: culture containing 2.5% glucose and 1% xylan at pH 2)

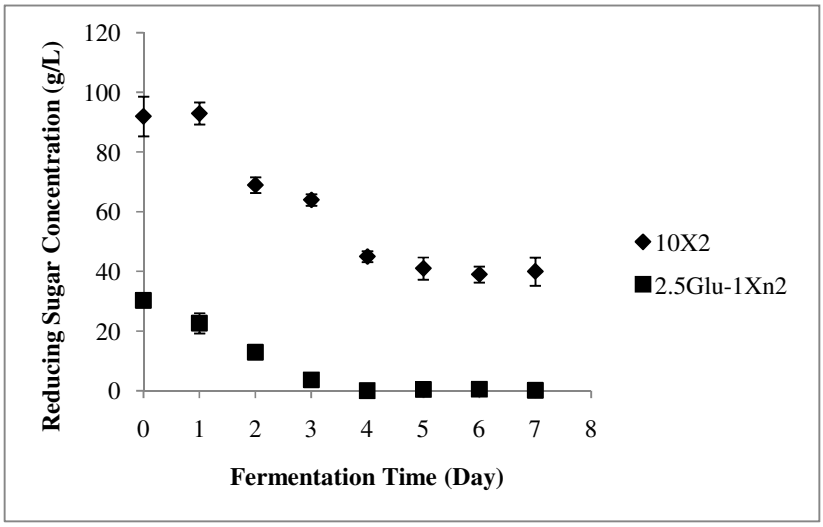


Figure 4.26 Effect of co-utilization of glucose and xylan and initial pH adjustment on xylose consumption on reducing sugar concentration profile of *A.terreus*

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10X2: culture containing 10% xylose at pH 2; 2.5Glu-1Xn2: culture containing 2.5% glucose and 1% xylan at pH 2)

The highest protein concentration ($110x10^{-3}$g/L) obtained with 2.5Glu-1Xn2 culture was observed on 6th day of fermentation (Figure 4.27). On the other hand, protein concentration in 10X2 gradually increased with a little fluctuation throughout the fermentation and reached $110x10^{-3}$g/L at the end of the fermentation. These were the highest amounts of ECP observed in M2.

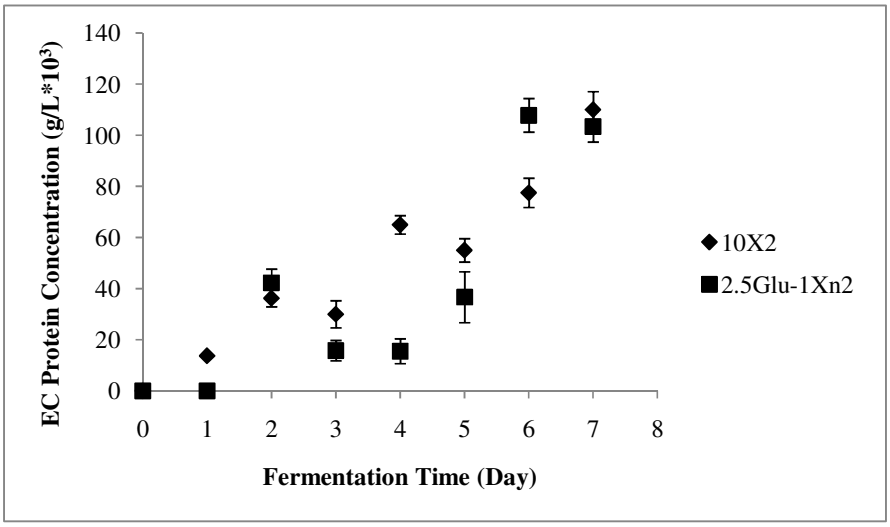


Figure 4.27 Effect of co-utilization of glucose and xylan and pH adjustment on xylose consumption on extracellular protein concentration profile of *A.terreus*
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10X2: culture containing 10% xylose at pH 2; 2.5Glu-1Xn2: culture containing 2.5% glucose and 1% xylan at pH 2)

The pH profile of 2.5Glu-1Xn2 started with a gradual decrease during the first three days, nevertheless, by a sharp increase, pH became 8 on the 6th day of fermentation and then it stayed constant (Figure 4.28). Moreover, it was similar to 1Xn2 in M1 except the decrease in first day. The pH profile of 10X2 culture showed sigmoid shape which was an outcome of continuous decrease and increase in pH. Moreover, the pH value at the end of fermentation was almost same as the initial pH.

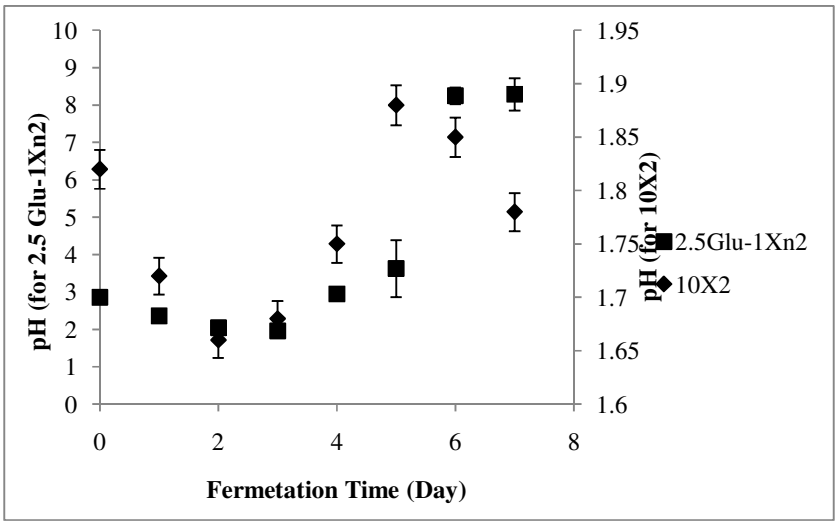


Figure 4.28 Effect of co-utilization of glucose and xylan and initial pH adjustment on xylose consumption on pH profile of *A.terreus*

Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (10X2: culture containing 10% xylose at pH 2; 2.5Glu-1Xn2: culture containing 2.5% glucose and 1% xylan at pH 2)

Xylanase activity profile of 2.5Glu-1Xn2 medium was shown Figure 4.29. Xylanase production began after depletion of simple sugar in the culture similar to the 1Xn2 in M1. Xylanase activity in 2.5Glu-1Xn2 culture was observed after four days of fermentation and reached its peak value (31000 IU/L) on the 6th day and then a sharp decrease was observed.

The results showed that the organism did not produce xylanase under high simple sugar concentration conditions. The xylanase production began immediately after the simple sugar depletion. On the other hand, the itaconic acid production was very low in both of the cultures (0.36 g/L on 7th day in 2.5Glu-1Xn2 and 4.55 g/L on 7th day in 10X2). Therefore, starting with low pH caused to a decrease in itaconic acid production when xylose used as the carbon source.

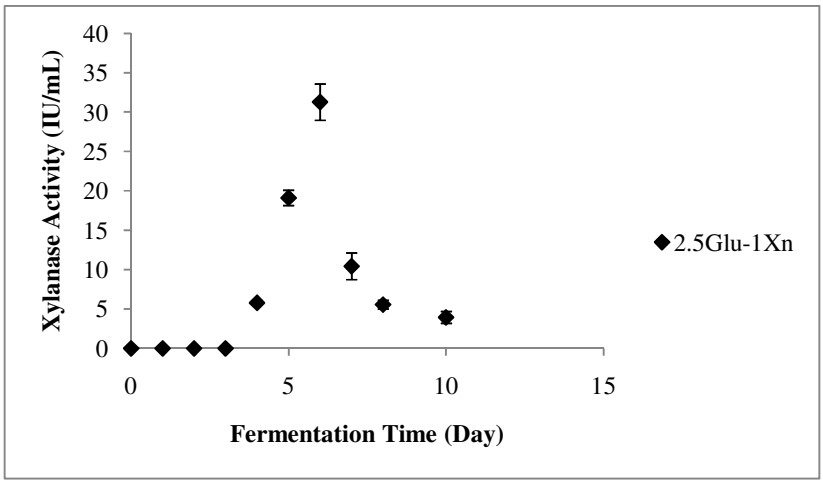


Figure 4.29 Effect of co-utilization of glucose and xylan and initial pH adjustment on xylose consumption on xylanase production of *A.terreus*
Medium-2 (g/L): CSL,0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06. (2.5Glu-1Xn: culture containing 2.5% glucose and 1% xylan at pH 2)

4.2.2 Effect of Temperature and Shaking on Xylanase and Itaconic Acid Production

In this experimental set, the effect of temperature (30 and 37 °C) and shaking on xylanase and itaconic acid production were tested. The maximum xylanase production level was observed on the 4^{th} day of fermentation, yielding approximately the same amount of xylanase production despite the differences in temperature, namely 30 and 37 °C (Figure 4.30). After reaching the maximum level, the decrease in production at 30 °C was less than in control probably because of higher stability of the enzyme at 30 °C than at 37 °C or low pH in control culture (Table 4.3). On the other hand, itaconic acid production in the culture grown at 37 °C was 5 times higher than the one at 30 °C. In contrast to the study of Nogueira *et al*. (2009), no beneficial effect of lower temperature was observed for xylanase production by *A. terreus* and nor for itaconic acid production.

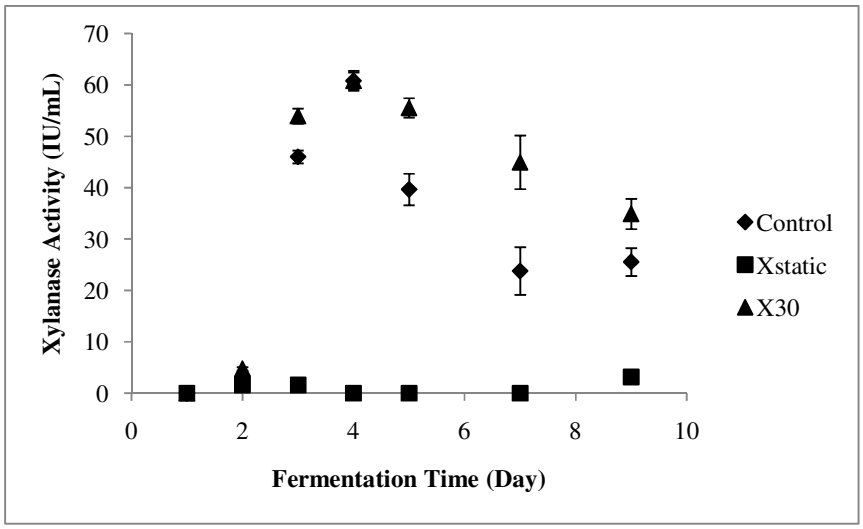


Figure 4.30 Effect of shaking and temperature on xylanase production of *A.terreus* (Medium-2 (g/L): Xylan, 10; CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; otherwise it was indicated, they were grown at 37 °C 170 rpm and in normal flask without adjusting pH) (Control: grown at 37 °C at 170 rpm; XStatic: grown at 37 °C without shaking; X30: grown at 30 °C at 170rpm)

Table 4.3 Effect of shaking and temperature on IA and xylanase production

	$pH_{Initial}$	pH_{Final}	Itaconic acid production		Xylanase production	
			Itaconic acid (g/L)	Day	Activity (IU/mL)	Day
XS	4.22	5.5	0	9	3	9
X30	4.22	3.14	0.0002	9	61	4
X37	4.22	2.44	0.001	9	61	4

(XS: Grown without shaking; X37: Grown at 37 °C and X30: Grown at 30 °C)

Nogueira and co-workers (2009) claimed that the best xylanase production was obtained at lower temperatures for some *Aspergillus* species including *A. niger* and *A. nidulans*, they also tested the static condition for growth and xylanase

production on *A. fumigatus* and *A. niveus*. The results of this experiment showed that static condition resulted in less growth but higher xylanase activity. These results made the experiment worth trying because in this way an increase in both xylanase and itaconic acid can be achieved as it was also indicated in literature that limiting the growth of *A.terreus* should increase itaconic acid production (Willke and Vorlop, 2001).

However the results showed that the xylanase production obtained under static condition was the lowest (almost negligible) among all. In addition, no itaconic acid production was observed. Moreover, under static conditions the organism showed filamentous growth and it did not produce simple sugar (Figure 4.31), which might be the reason for no itaconic acid and very low xylanase production. Gyamerah (1995a) reported that for itaconic acid production, *A.terreus* growth should be in pellet shape.

Simple sugar concentration in the culture kept at 37 °C was almost linearly increased between the 1^{st} – 3^{rd} days of fermentation and then it decreased throughout two days and finally stayed almost constant (Figure 4.31). Nonetheless, in X30 flask, simple sugar concentration showed fluctuating pattern during the fermentation and it did not exceed 1 g/L. Reducing sugars in XS was depleted very quickly and no further production of reducing sugar was observed.

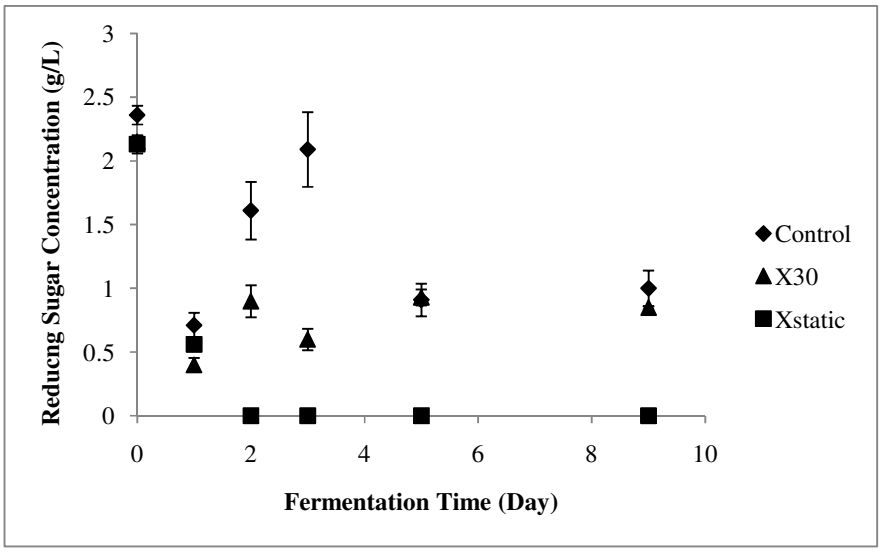


Figure 4.31 Effect of shaking and temperature on reducing sugar concentration
Medium-2 (g/L): Xylan, 10; CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; otherwise indicated, grown at 37 °C 170 rpm and in normal flask without adjusting pH) (X37: grown at 37 °C and X30: grown at 30 °C)

As a summary, performing the fermentation at 30 ℃ instead 37 ℃, and using static culture resulted in a decrease in IA production. Furthermore, the results obtained until now, showed that the xylanase production level probably was not high enough to produce high amount of simple sugar from xylan to induce itaconic acid production efficiently. Therefore, the first part of the rest of the studies was focused on the optimization for xylanase production.

4.2.3 Effect of Xylan Concentration on Xylanase Production

Concentration from 1 to 8 % of birchwood xylan was used in the production culture and their pH adjusted to 2 prior to autoclave to examine the effect of xylan concentration on xylanase production by *A. terreus* (Figure 4.32). The cultures reached their maximum xylanase activities between 3-5 days. Generally increasing xylan concentration in the medium postponed the day to reach maximum xylanase activity. Among those, 2% birchwood xylan had the best

inducing capacity through the overall fermentation and the maximum xylanase activity measured was 48 IU/mL (48000 IU/L). Although 2%, 3% and 4% xylan containing cultures reached almost the same amount of xylanase production at their maximum production (48000 IU/L), 2% xylan containing culture was more stable in xylanase production than the others. On the other hand the stability of xylanase production was almost similar in 3% and 4%. Although sharp decrease in xylanase production was observed in the one with 2%, it stayed constant longer during the rest of the fermentation. Increasing xylan concentration made the culture denser and it decreased the growth and increased the lag phase of the organism. Thus, the xylanase activities in the 6 and 8 % xylan containing cultures were very low with respect to the lower xylan containing cultures.

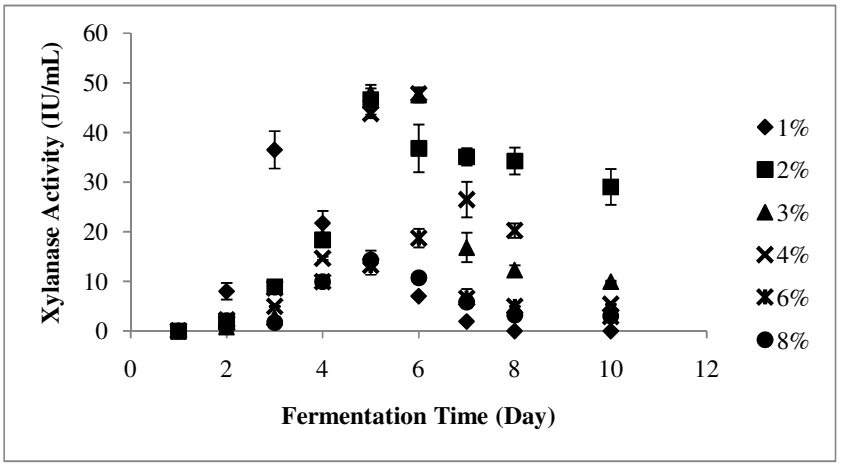


Figure 4.32 Effect of xylan concentration on xylanase production
Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm) (1%: containing 1% birchwood xylan; 2%: containing 2% birchwood xylan; 3%: containing 3% birchwood xylan; 4%: containing 4% birchwood xylan; 6%: containing 6% birchwood xylan; 8%: containing 8% birchwood xylan)

Increasing xylan concentration in the medium increased the initial reducing sugar concentration (Figure 4.33). Sharp decreases were observed in 1%, 2% and 3% xylan containing cultures on the 2nd day of fermentation. On the 6th day another sharp decrease was observed in the culture containing 1% and 2 % birchwood

xylan. After a little decrease in simple sugar concentrations in 4, 6 and 8 % containing culture the concentration stayed almost constant.

1%, 2% and 3% containing cultures first showed pH decrease in the medium (Figure 4.34). The day when the decrease observed and the sharpness of the decrease was in inversely related to the culture xylan concentration i.e. in 1% xylan containing culture, decrease was observed on the 2nd day and it is very sharp however, a minimal decrease was observed on the 3rd day for the one containing 3%. The decrease in pH was followed by an increase. However, after a little increase, the pH in 4%, 6% and 8% cultures stayed almost constant.

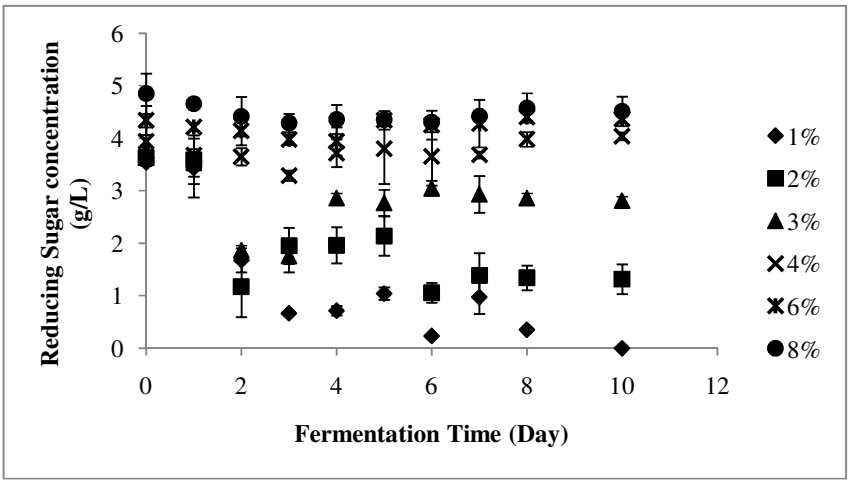


Figure 4.33 The effect of birchwood xylan concentration on reducing sugar concentration profile of fermentation cultures

Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm (Numbers indicating the w/v percentage of birchwood xylan)

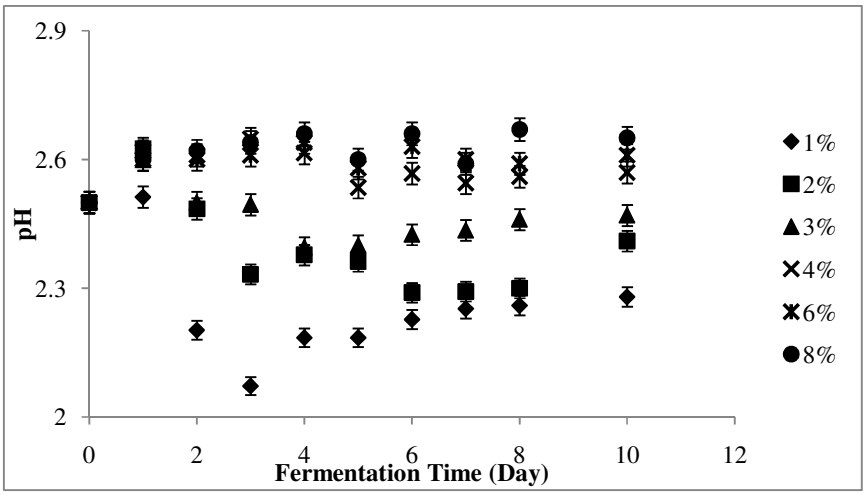


Figure 4.34 pH profile of fermentation cultures containing different concentration of birchwood xylan

Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm) (Numbers indicating the w/v percentage of birchwood xylan)

The results showed that although 2% birchwood xylan containing culture showed little decrease in activity on the next day of its maximum, its xylanase production was retained high and stable throughout the rest of the fermentation. On the other hand, 3% and 4% xylan containing culture`s xylanase production was constant on the 5^{th} and 6^{th} day, nonetheless, after that sharp decrease was observed. As regards the 1% xylan containing culture, although it reached its maximum one day earlier than 2%, 3% and 4% containing ones, its maximum level was lower than 2%. The production was not continued, thus, sharp decrease in xylanase production was observed. 6% and 8% xylan containing cultures showed very similar profiles with lower xylanase production. It was observed that on first days of the fermentation the media were denser and the density had decreased ongoing time with increasing xylanase production. The density resulted from high xylan concentration caused the organism not to grow well in the first days of fermentation. Besides all these, reducing sugar concentration profiles and the pH profiles of tested cultures similar to each other, i.e., in 1%,

2% and 3% xylan containing cultures, both simple sugar concentration and pH decreased on 2nd day of fermentation (Figure 4.33). While in 4%, 6% and 8% xylan containing cultures pH increased (Figure 4.34).

The results showed that among the tested xylan concentrations, the highest xylanase activity levels in cultures containing 2, 3 and 4% xylan were almost the same, whereas, xylanase activity in 2% xylan containing culture was more stable throughout the fermentation.

4.2.4 Effect of Direct Spore Inoculation of Production Culture, Carbon Source in the Preculture and Usage of Baffled Flask on Xylanase Production

The effect of preculture usage on decreasing lag phase was tested by direct inoculation of production culture with the same amount of spores (10^6 spores/mL). It was observed that direct inoculation did not change the xylanase production profile of the organism (Figure 4.35), while the production level decreased minimally. In the same manner, xylose was used in the preculture to decrease the lag phase of the organism in the xylan containing culture but a similar profile was observed (Figure 4.35). Nevertheless, the culture inoculated with xylose containing preculture reached its maximum xylanase activity on the 8th day and it was minimally lower than the control culture. Another variant was the usage of the baffled flask. By using baffled flask, it was aimed to increase itaconic acid production by increasing aeration as it was reported that aeration was important for itaconic acid production (Gyamerah, 1995b). Although, the xylanase activity profiles were similar (Figure 4.35), the production level obtained using baffled flask was lower than the normal one throughout the fermentation process.

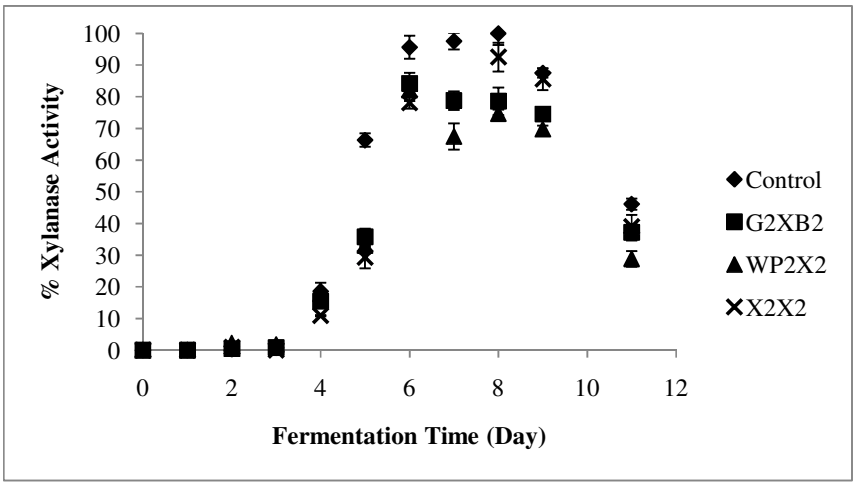


Figure 4.35 Effect of usage of baffled flask, direct spore inoculation of production culture and carbon source in the preculture
Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm) (Control: containing 2% xylan at pH 2 grown at 37 °C at170 rpm inoculated from glucose containing preculture; G2XB2: grown in baffled flask; WP2X2: inoculated directly by spores; X2X2: inoculated from xylose containing preculture)

Usage of baffled flask, direct spore inoculation and carbon source in the preculture had no significant effect on either xylanase or IA production. Thus, rest of the studies was carried out by using agricultural waste to not only to display the consumption of the biomass by the organism to produce IA but also for further optimization of xylanase production.

4.2.5 The Usage of Agricultural Biomass as Carbon Source

Corn cob, cotton stalk and sunflower stalk were used as agricultural biomass as carbon sources instead of birchwood xylan in the production culture. Birchwood xylan was used as control. Each agricultural residue was added as 1% (w/v) as a substitution of xylan in the production culture.

This experimental set-up revealed the utilization of agricultural biomass by *A. terreus*. Among the agricultural residues used in the experiment, corn cob showed the highest xylanase activity (Figure 4.36). It reached a maximum of 36 IU/ml (36000 IU/L), after continuously increasing for seven days. Xylanase

activities observed on cotton stalk and sunflower stalk were not comparable with the one of corn cob, and they were very low throughout the fermentation. This could be resulted from either high lignin content as in the case of cotton stalk or low xylan concentration as in the case of sunflower stalk (Tada *et al*., 2004; Akpinar *et al*., 2007; Gungoren *et al*., 2008) (Table 4.4).

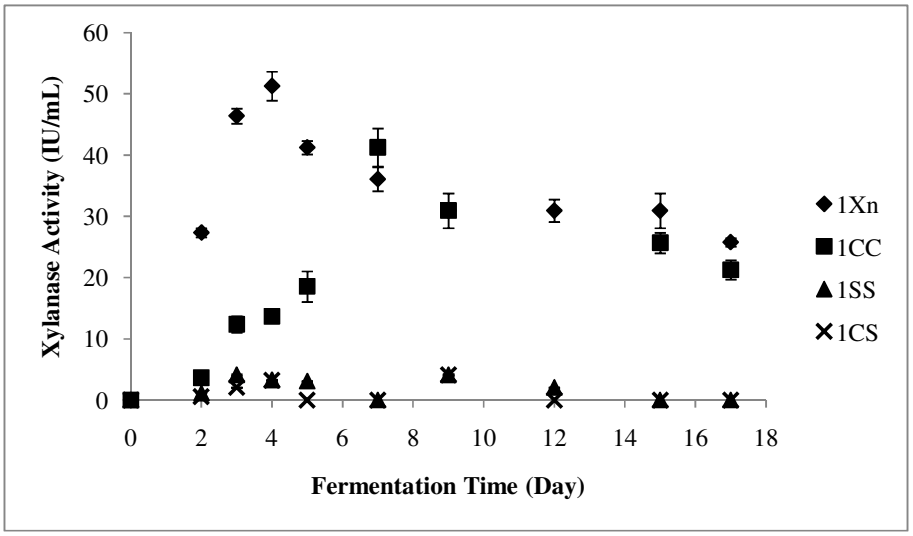


Figure 4.36 Xylanase production profiles of *A.terreus* on different agricultural wastes

(X: Xylan; CC: Corn cob; CS: Cotton stalk; SS: Sunflower stalk, 1% for each) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm and in normal flask without adjusting pH)

Table 4.4 Compositions of agricultural residues

	Lignin (%)	Cellulose (%)	Hemicellulose (%)	Reference
Corn Cob	20	32	35	Tada *et al*., 2004
Cotton Stalk	28	36	21	Akpinar *et al*., 2007
Sunflower Stalk	10	43	8	Gungoren *et al*., 2008

The agricultural residues are not sole xylan, and because their compositions differ in percentage of lignin, cellulose and hemicellulose content (Table 4.4) (Tada *et al*., 2004; Akpinar *et al*., 2007; Gungoren *et al*., 2008), none of the xylanase activities in the culture containing agricultural residues reached the activity in the control one (Figure 4.36). The maximum xylanase activity 70 IU/ml (70000 IU/L) was observed in control fermentation culture on the 4^{th} day.

The amount of produced itaconic acid was measured on HPLC. IA production was detected on birchwood xylan as 0.0001 g/L on 8^{th} day of fermentation (Table 4.5). The experiment was carried out without adjustment of pH prior to inoculation. Except for birchwood xylan and corn cob, the pH values of all other fermentation cultures increased (Table 4.5). That can explain why such a small amount of IA was produced. In the same manner, although there was a decrease in pH in corn cob culture, IA production could not be observed.

Table 4.5 Initial and final pH values of fermentation cultures

	$pH_{Initial}$	pH_{Final}	Itaconic acid production	
			Itaconic acid (g/L)	Day
1Xn	4.22	3.43	0.0001	12
1CC	4.22	2.77	0	12
1CS	4.22	6.53	0	12
1SS	4.22	5.69	0	12

(X: Birch wood xylan; CC: Corn cob; CS: Cotton stalk; CSX: Cotton stalk xylan; WBX: Wheat bran xylan; SS: Sunflower stalk, 1% each)

Cellulase activity was also measured and found to be very low on corn cob and cotton stalk (Figure 4.37 and 4.38). Both enzyme productions in corn cob containing culture began to increase on the 2nd day. Cellulase reached its maximum production level (0.12 FPU) on the 4th day of fermentation and it was very low as compared to xylanase production level which was 36 IU/mL (36000 IU/L) and reached its maximum production level on the 6th day. Both enzyme production levels in cotton stalk containing culture were very low with respect to the ones in the culture containing corn cob. The maximum production levels of xylanase and cellulase on CS were 4 IU/mL and 0.003FPU, respectively.

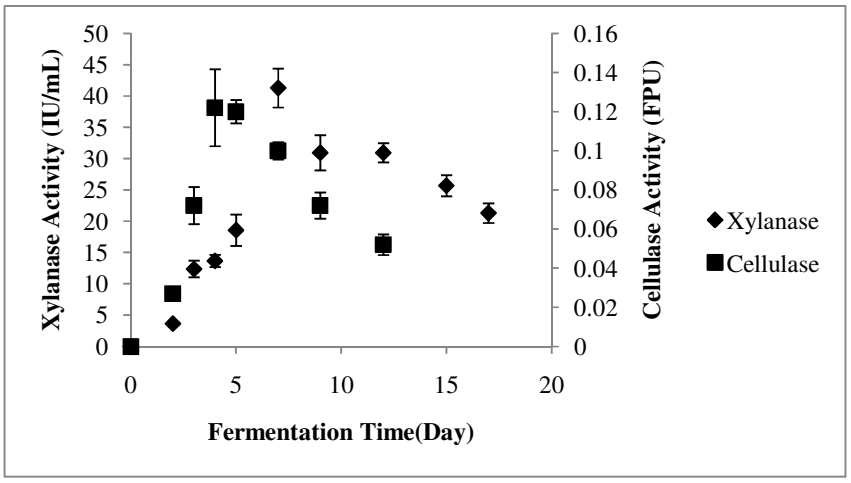


Figure 4.37 Cellulase and xylanase activity profiles of *A. terreus* on 1% corn cob
(Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 30 °C 170 rpm and in normal flask without adjusting pH)

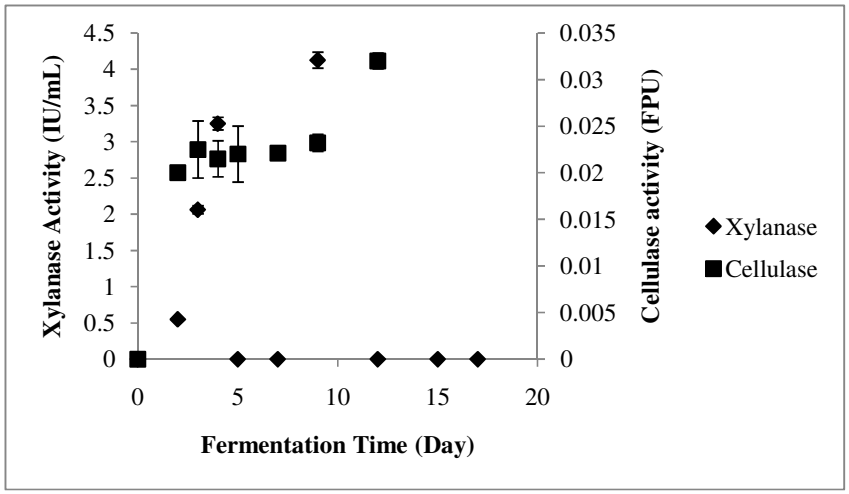


Figure 4.38 Cellulase and xylanase activity profiles of *A. terreus* on 1% cotton stalk
(Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 30 °C 170 rpm and in normal flask without adjusting pH)

As a summary, among tested agricultural residues, the organism produced the highest xylanase level on corn cob. While the cellulase activities on both corn cob and cotton stalk were very low. Because of very low amount of cellulase production, the biomass utilization mainly was based on the xylanolytic activities. Therefore, further studies were focused on optimization of xylanase production.

4.2.6 Effect of Corn Cob Concentration on Xylanase Production

To test the effect of corn cob (CC) concentration on xylanase production, 1 to 10 % corn cob was used in the production culture. The highest xylanase production was measured in the 7% corn cob containing culture and was followed by 5% containing one (Figure 4.39). Maximum xylanase activity was observed on the 6^{th} day in 7% and 10% containing cultures and it was observed in 1%, 3% and 5% containing cultures on the 7^{th} day. Although the xylanase production level increased by increasing corn cob concentration up to 7%, a decrease was observed in 10% corn cob containing culture and it was as much as in the 3% containing one. Besides, red corn cob (RCC) was used in the production culture to observe the effect of different types of agricultural residues. When 3% RCC was tried in the culture, it reached its maximum xylanase production on the 5^{th} day but it was as much as 1% CC.

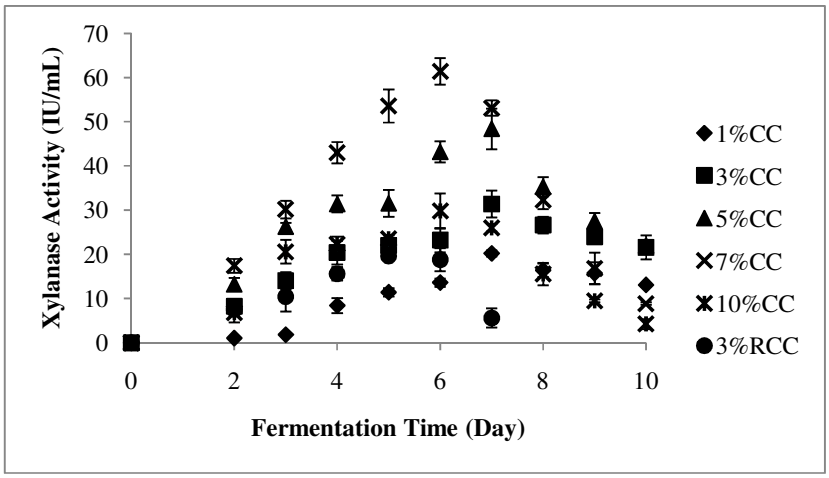


Figure 4.39 Xylanase activity profiles of *A. terreus* grown at different concentrations of corn cob

(Numbers indicating the w/v percentage of corn cob) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

Except 1% CC and 3% RCC, the reducing sugar concentration in the cultures increased throughout the fermentation period and it was proportional to corn cob concentration in the culture (Figure 4.40). The simple sugar concentration reached to 2.5 g/L in the 10% CC containing culture and it was 1.9 for 7% CC, 1.2 for 5%, 0.9 for 3% and 0 for the 1% CC and 3% RCC.

Except the 1% CC containing culture, pH decreased in the first 2 days of the fermentation process and stayed constant within the following 2 days and then started to increase (Figure 4.41). On the other hand, it increased in 1% CC containing culture step by step.

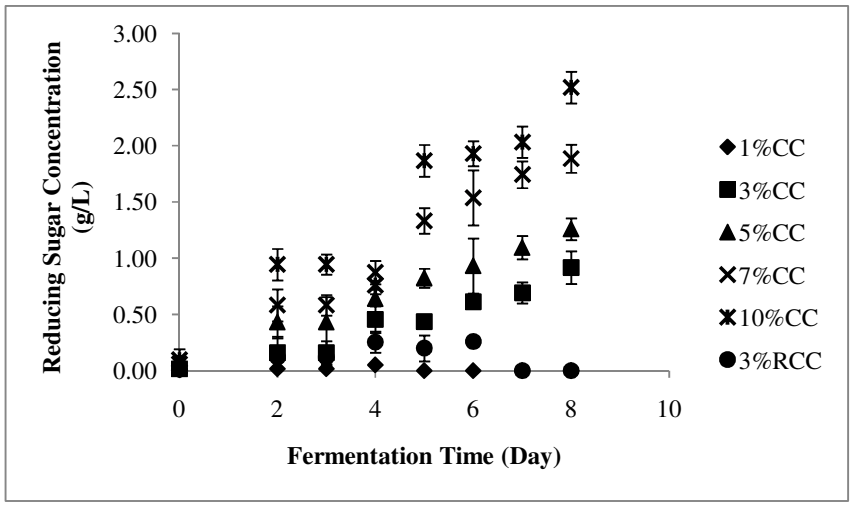


Figure 4.40 Reducing sugar concentration profile of fermentation cultures containing different concentration of corn cob

(Numbers indicating the w/v percentage of corn cob) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

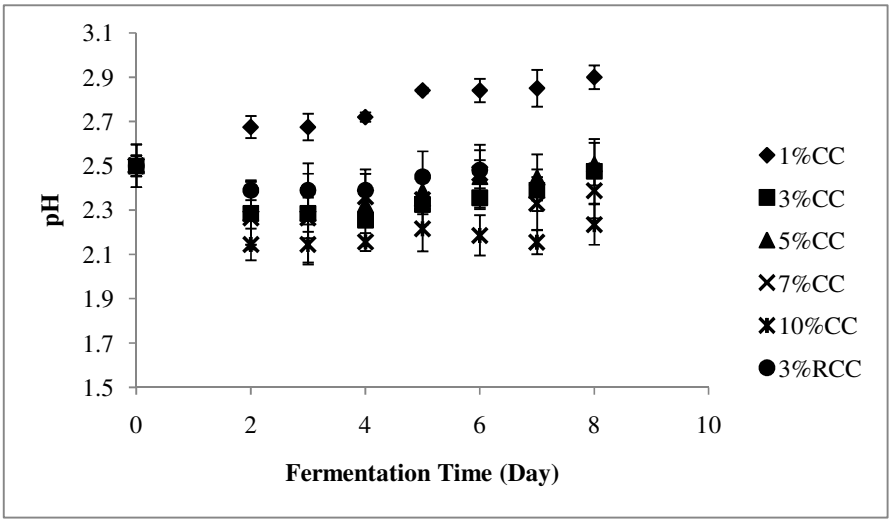


Figure 4.41 pH profile of fermentation cultures containing different concentration of corn cob

(Numbers indicating the w/v percentage of corn cob) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

As concerning xylan content of corn cob, which is 30-35% of corn cob, 7% corn cob has 2.1-2.45g xylan. Hence, it had better inducing capacity than equal amount of sole xylan. After all, as considering the maximum xylanase production day and the amount, 7% corn cob (w/v) was the best concentration for corn cob to induce xylanase production by *A.terreus*.

4.2.7 Effect of Pretreatment of Corn Cob on Xylanase Production

As a pretreatment method, the steps of alkali extraction procedure of xylan from lignocellulosic biomass were chosen. These were keeping at 60 °C (dry treatment), keeping in a ratio 1:50 with water at 60 °C (wet treatment) and the alkali extraction of xylan. Moreover, extra washing with ethanol after alkali extraction was tried. The highest xylanase activity was observed with wet pretreatment of corn cob (Figure 4.42). Furthermore, increasing the corn cob concentration to 7% in the medium increased the maximum xylanase production throughout the whole fermentation. On the other hand, dry treatment had little effect on xylanase production as they both reached almost the same maximum xylanase production but dry treated one reached one day earlier. Concerning alkali treated samples, without alcohol washing, there was no xylanase production. After alcohol washing, xylanase production was observable after 5 days and it reached a maximum of 24 IU/mL (24000 IU/L) in 1% WECC. This is thought to be due to the presence of lignin fragments and salt in the xylan precipitate, resulting from the alkali extraction procedure adapted from Zilliox and Debeire (1998). High salt and lignin concentration could inhibit the microbial growth. After washing xylan with alcohol several times, most of the inhibitory substances were removed and xylanase production was observable after 5 days. The maximum xylanase activity observed was 24 IU/mL using 1% (w/v) extracted xylan from corn cob washed with ethanol (WECC). However in 2% extracted xylan containing medium, the maximum xylanase activity was very low (6 IU/mL). Probably, even washing the extracted xylan with alcohol several times was not a complete solution for salt and lignin removal. Since the remaining salt and lignin concentration was higher in 2% xylan containing

culture, microbial growth was much more inhibited and the enzyme production decreased.

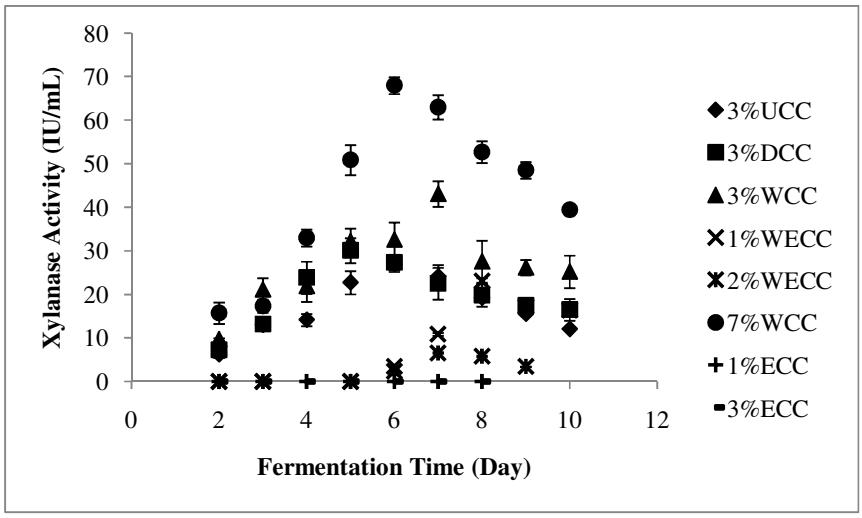


Figure 4.42 Effect of pretreatment of corn cob on xylanase production by *A.terreus*

(UCC: Untreated corn cob; DCC: Dry oven pretreated corn cob; WCC: Wet oven pretreated corn cob; 1ECC: 1% w/v extracted xylan from corn cob; 3ECC: 3% w/v extracted xylan from corn cob; 1WECC: 1% w/v extracted xylan from corn cob washed with ethanol; 2WECC: 2% w/v extracted xylan from corn cob washed with ethanol) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

Except alkali extracted xylan containing cultures, others showed similar reducing sugar profiles that increased day by day and after the 8th day reducing sugar concentration started to decrease except in the 7% WCC, in which reducing sugar concentration continued to increase (Figure 4.43). As for the non-washed alkali extracted xylan containing cultures, in the first three days, simple sugar concentration decreased, which may be due to the organism`s keeping itself alive. After that an increase was observed, this may be due to the cell burst. As for washed extracted xylan containing cultures, simple sugar concentration was almost constant on the first day of fermentations. After a little increase in the amount on 5th day, it decreased sharply in the one containing 1% but not in the

one containing 2%. On the other hand, the decrease continued in the 1% containing culture but it started to increase in the culture containing 2%.

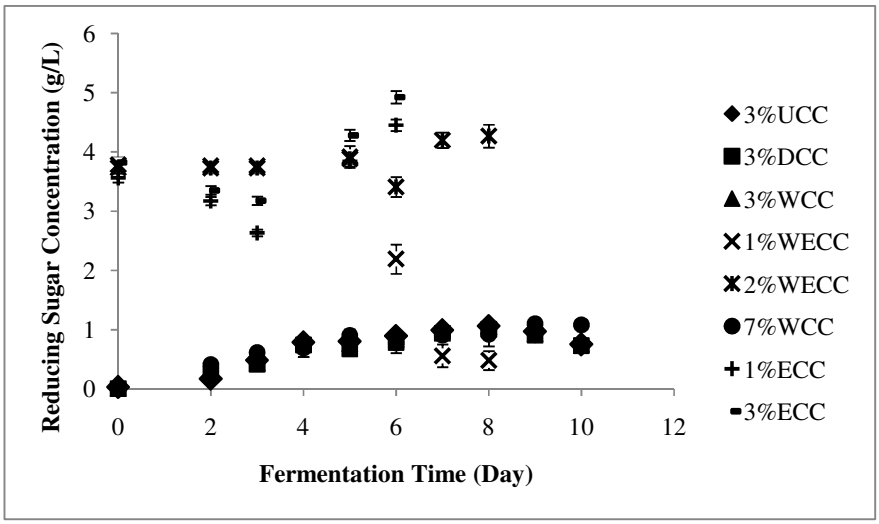


Figure 4.43 Reducing sugar concentration profile of medium containing corn cob pretreated in a different way

(UCC: Untreated corn cob; DCC: Dry oven pretreated corn cob; WCC: Wet oven pretreated corn cob; 1ECC: 1% w/v extracted xylan from corn cob; 3ECC: 3% w/v extracted xylan from corn cob; 1WECC: 1% w/v extracted xylan from corn cob washed with ethanol; 2WECC: 2% w/v extracted xylan from corn cob washed with ethanol) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

The pH profiles of untreated corn cob (UCC), dry heat (DCC) and wet heat pretreated corn cob (WCC) were almost identical, which decreased till the 4th day of fermentation and then the pH started to increase (Figure 4.44), whereas the pH of 7% WCC almost stayed constant. The profiles for non-washed xylan containing cultures were also similar to each other. The only difference was in the initial increase in pH. The increase in 3% WCC containing culture was higher than the 1% containing culture within the first three days. After that, a little decrease was observed in both cultures. The increase was also observed in washed xylan containing cultures and similar to non-washed ones, the increase

was higher in high xylan containing culture. Nonetheless, the pH stayed almost constant in 2% containing culture and a sharp decrease was observed in the culture containing 1% washed extracted xylan after 5th day.

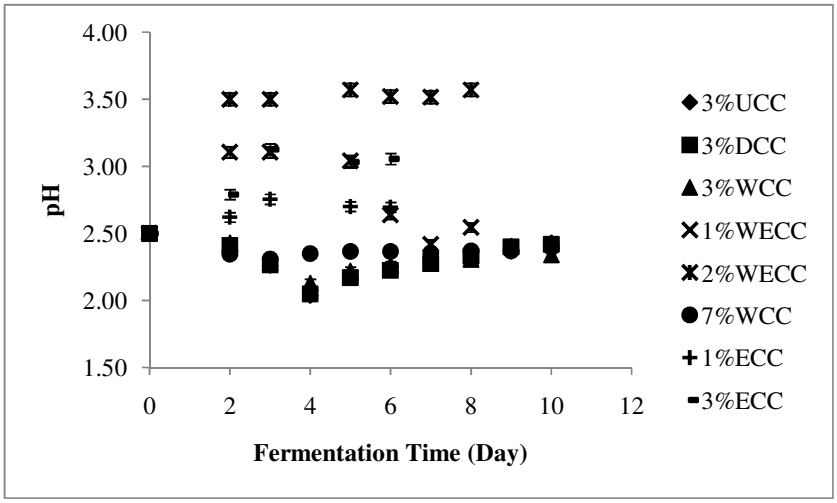


Figure 4.44 pH profile of medium containing corn cob pretreated in a different way

(UCC: Untreated corn cob; DCC: Dry oven pretreated corn cob; WCC: Wet oven pretreated corn cob; 1ECC: 1% w/v extracted xylan from corn cob; 3ECC: 3% w/v extracted xylan from corn cob; 1WECC: 1% w/v extracted xylan from corn cob washed with ethanol; 2WECC: 2% w/v extracted xylan from corn cob washed with ethanol) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

Although the dry heat pretreatment had no significant effect on xylanase production, wet heat pretreatment resulted in increase in both xylanase production level and stability throughout the fermentation process.

4.2.8 Xylanolytic System Analysis

Main xylan hydrolyzing enzymes (xylanase and xylosidase) and two accessory xylanolytic enzymes (arabinofuranosidase and galactosidase) were investigated on 7% corn cob containing culture (Figure 4.45). Furthermore, in spite of having low cellulase activity, glucosidase activity was also controlled. Except xylanase, all of them reached their maximum production level on 3rd day of fermentation.

Highest activity was observed with the glucosidase (0.014 IU/mL) and secondly with the xylosidase (0.010 IU/mL) and other three showed almost the same amount of activity at their highest production levels (about 0.009IU/mL). After having their peak levels, all showed very sharp decrease on 4th day and it was highest for the xylosidase. It was interesting that all four xylanolytic enzymes except glucosidase showed their minimum production level when xylanase reached its peak value and after that they had another peak on 6th day of fermentation. There are several studies in literature mention xylosidase and other xylanolytic enzymes from *A. terreus* and some other *Aspergillus* species on either commercial xylan or mainly on wheat bran (Hrmova et al., 1989; Manzanares et al., 1998; Kiss and Kiss, 2000; Wakiyama et al., 2008). Nevertheless, highest activity of xylosidase was 0.149 IU/mL from *A.carbonarius* (Kiss and Kiss, 2000) it was fifteen fold higher than xylosidase found in this study.

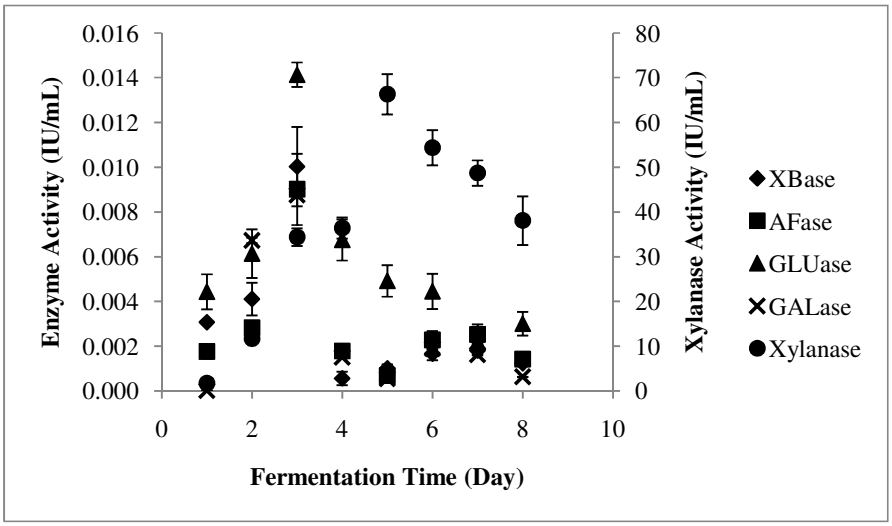


Figure 4.45 Xylanolytic system profile of *A. terreus* NRRL 1960
(Medium-2 (g/L): WCC, 70; CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

The pH and reducing sugar concentration of the fermentation medium were shown in the Figure 4.46. The decrease in pH and increase in reducing sugar concentration were much related to xylanase production level. The pH decreased till the 5th day and stayed constant and similarly the reducing sugar concentration increased till the 5th day and stayed constant rest of the fermentation. It was clearly observed from the results that reducing sugar concentration increased with the increasing xylanase production level and decreasing pH value. The interesting point was that xylanolytic enzymes reached their peak value after the day when the pH showed sharp decrease and reducing sugar concentration started to increase.

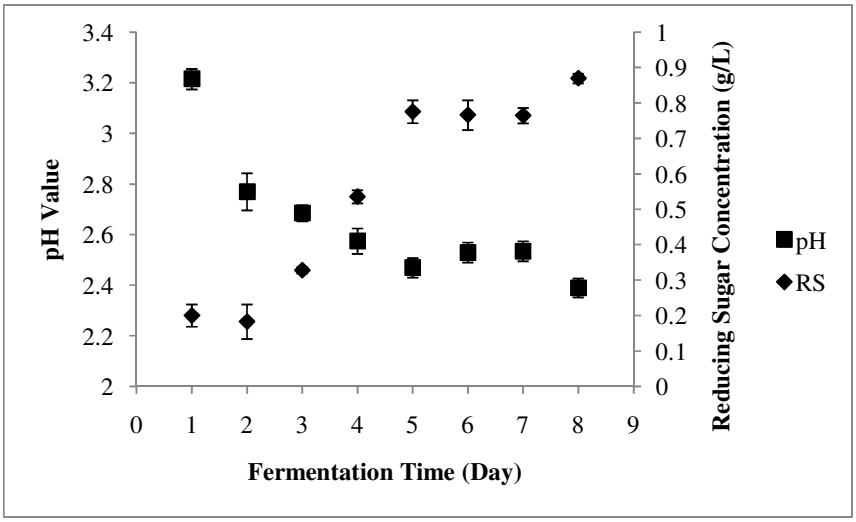


Figure 4.46 pH and reducing sugar concentration profile of *A. terreus*
(Medium-2 (g/L): WCC, 70; CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm)

As expected, xylosidase, arabinofuranosidase, galactosidase and glucosidase were also produced besides endoxylanase. Although the enzyme production levels were not so high, the presence of xylanolytic enzymes cellulase and

glucosidase other than xylanase proved that the organism can utilize agricultural residues efficiently.

4.3 Optimization of Itaconic Acid Production

4.3.1 Effect of Addition of Commercial Xylanase

From the results up to now, biomass especially xylan utilization by A. terreus and xylanase production were shown. However, since the simple sugar concentration produced mainly from xylan by the excreted enzymes of *A. terreus* was not high enough for the production itaconic acid. Hence, in this experimental set, the effect of extra xylanase addition to the culture containing different xylan concentrations to increase simple sugar concentration on xylanase and itaconic acid productions was tested. Commercial xylanase (0.5 IU/mL) was added during the inoculation to increase the release of simple sugar from xylan. This would help the organism to keep the simple sugar concentration high throughout the fermentation, thus, it could be resulted in high itaconic acid production.

First of all, commercial xylanase was characterized in terms of stability and activity against birchwood xylan and corn cob at 37 °C. Stability of the enzyme was tested by adding enzyme into the production medium without inoculation with the organism at 37 °C and its activity was measured by standard xylanase enzyme activity (Figure 4.47). As a result of stability studies, 80 % of the commercial xylanase (Shearzyme, Novazymes) activity remained after 24 hours. Additional 6 % activity loss was detected during next 72 hours. In activity tests of commercial xylanase, five different conditions were tested: 1% and 2% xylan concentrations either adjusting the pH of the production medium to 2 or do not so. Fifth one was the production medium including 3% corn cob, whose pH was adjusted to 2 (Figure 4.48). Results showed that the reducing sugar concentration reached a constant value almost after 24 hours from the beginning of reaction. Hence, the addition of commercial xylanase into the production

media may provide higher amounts of reducing sugar so that it can increase itaconic acid production. Higher concentration of xylan resulted in higher production of reducing sugar. However, the change in pH did not affect the production rate at all. The usage of 3% corn cob caused to decrease in production of reducing sugar.

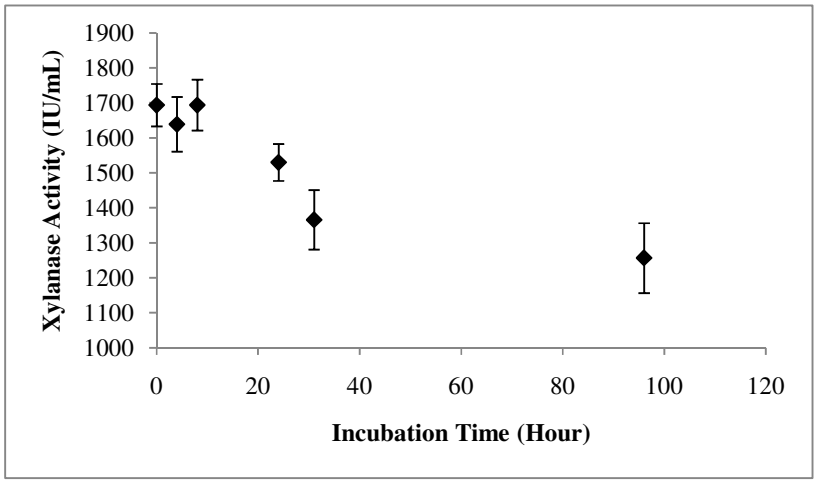


Figure 4.47 Stability of commercial xylanase at 37 °C into production medium
(Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06)

The addition of commercial xylanase during inoculation resulted in higher simple sugar at the beginning of the fermentation. Nevertheless, this could not be sufficient to induce itaconic acid production. Only 0.0004 g/L itaconic acid could be obtained. Another effect of the addition of xylanase to the medium was a decrease in xylanase production (Figure 4.49). Taking these results into consideration, to keep the reducing sugar concentration constant, the amount of commercial xylanase increased to 2.5 IU/mL.

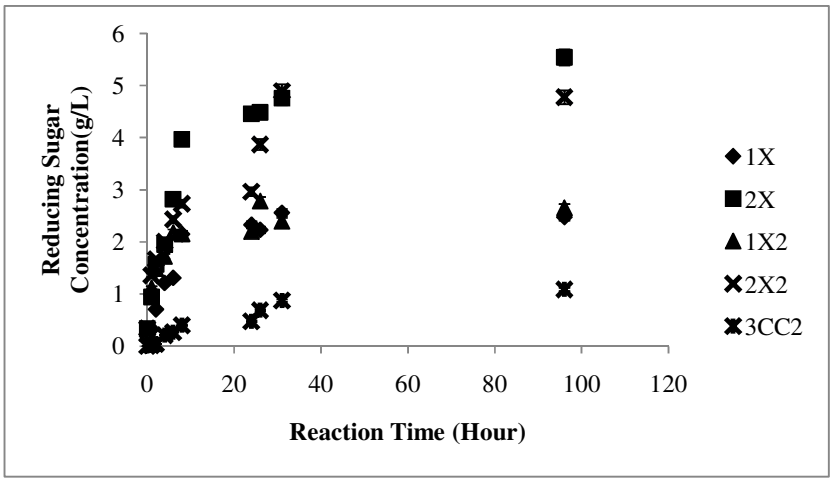


Figure 4.48 Reducing sugar production profile by using commercial xylanase enzyme at 37 °C

(1X: 1% Xylan; 2X: 2% Xylan; 1X2: 1% Xylan at pH 2; 2X2: 2% Xylan at pH 2; 3CC2: 3% Corn cob at pH 2) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; at 37 °C 170 rpm and in normal flask)

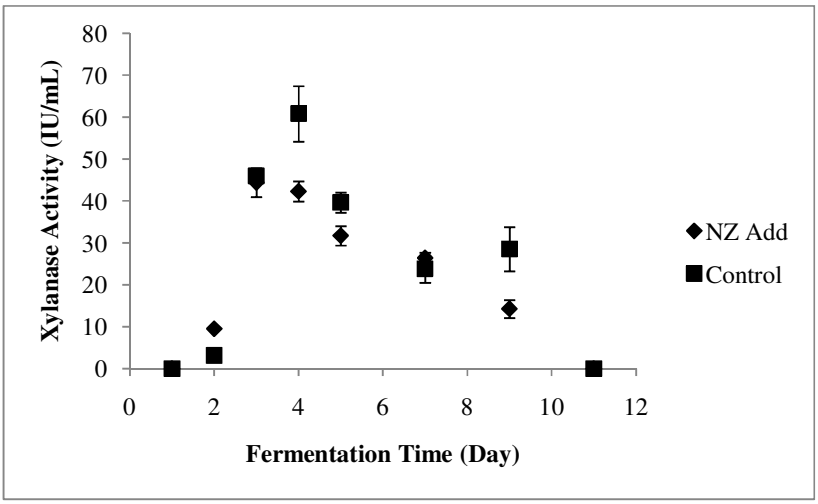


Figure 4.49 Effect of addition of commercial xylanase during inoculation

(Control: grown at 37 °C at 170 rpm without addition of commercial xylanase on 1% birchwood xylan in Medium M2; Enzyme Add.: 0.5 IU/mL commercial xylanase was added into production culture during inoculation)

Commercial xylanase (2.5 IU/ml) was added into the each fermentation cultures having 1, 2, 4 and 8% birchwood xylan, 24 hours before inoculation of production cultures. The initial reducing sugar concentrations of 1, 2, 4 and 8% were 3, 7, 12 and 14 g/L, respectively. There was a correlation with initial reducing sugar concentration and the amount of xylan used in the fermentation culture (Figure 4.50). The organism consumed most of the simple sugar in the first three days of fermentation for the cultures containing 1, 2 and 4 xylan, whereas probably, because of the high density and viscosity for 8% one, the lag phase continued more than three days.

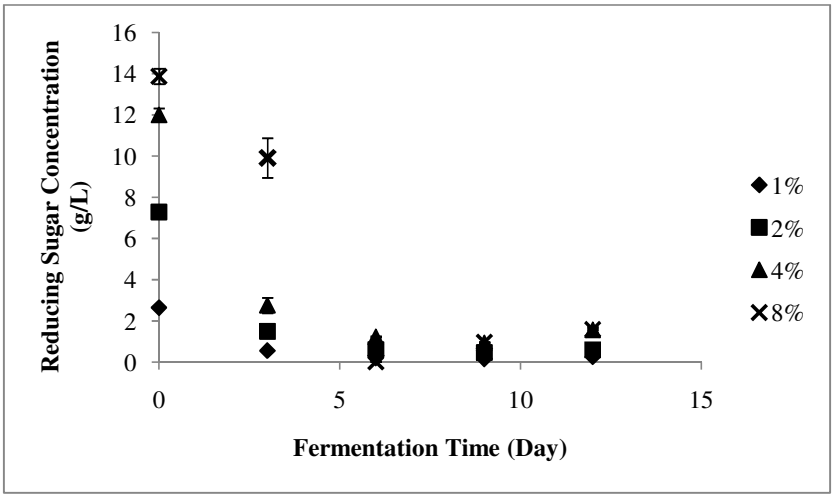


Figure 4.50 Reducing sugar concentration profiles of fermentation cultures
(1%: 1% xylan; 2%: 2% xylan; 4%: 3% xylan and 8%: 8% xylan) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm and in normal flask)

The decrease in the pH values of cultures were observed throughout the first three days of fermentations (Figure 4.51). Nonetheless, the pH values increased slightly through the rest of the fermentation. When compared to others, the increase in pH of the culture containing 8% xylan was so high that it becomes alkali from acidic condition.

The amount of produced xylanase had correlation with the used xylan concentration in the fermentation flask (Figure 4.52). That is, the highest xylanase activity was observed on the 6th day as 27 IU/ml in 8% xylan containing culture and the lowest activity was detected in 1% xylan containing culture as 6 IU/ml on the 3rd day. However, the maximum xylanase activity of the cultures was measured at different days of the fermentation process. 1% xylan containing culture reached its maximum activity value at the 3rd day, whereas 2% containing one reached at the 9th day and the other two reached at the 6th day of fermentation. Beginning with high simple sugar concentration resulted in a decrease of xylanase production level.

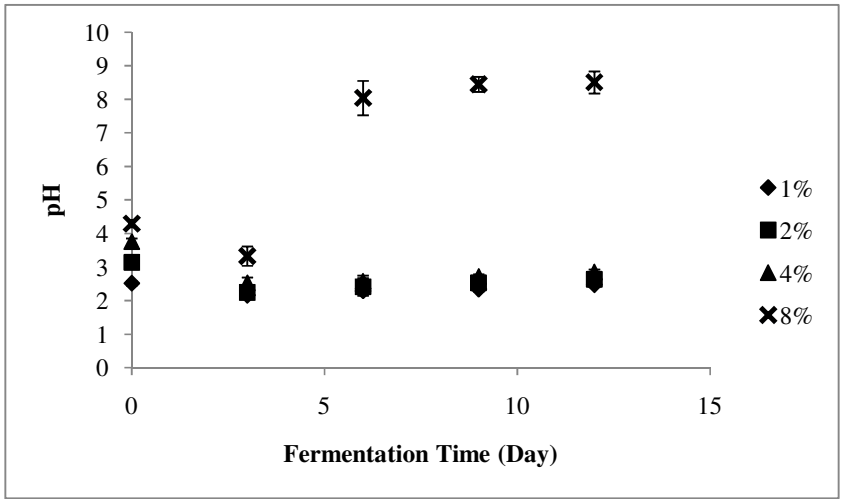


Figure 4.51 pH profiles of fermentation cultures
(1%: 1% xylan; 2%: 2% xylan; 4%: 4% xylan and 8%: 8% xylan) (Medium-2 (g/L): CSL, 0.5; $MgSO_4.7H_2O$, 5; NH_4NO_3, 3; NaCl, 0.4; $ZnSO_4.H_2O$, 0.033; KH_2PO_4, 0.5; $CaCl_2.2H_2O$, 1.0; $CuSO_4.5H_2O$, 0.06; grown at 37 °C 170 rpm and in normal flask)

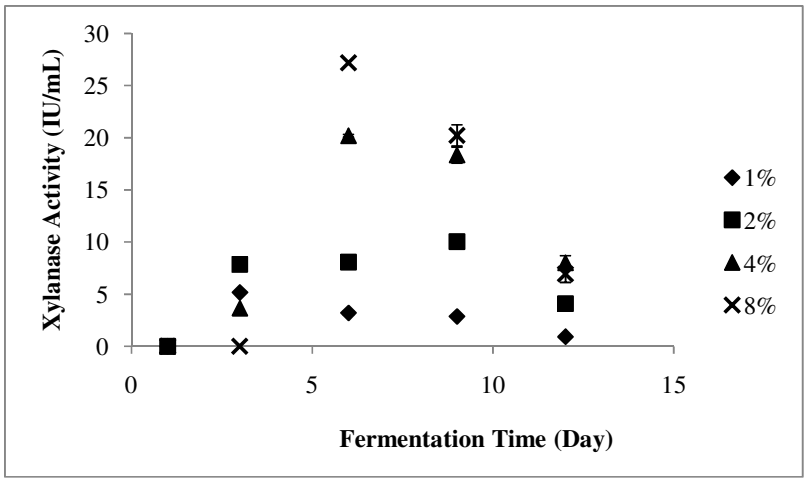


Figure 4.52 Effect of addition of commercial xylanase to different concentrations of birchwood xylan

(1%: containing 1% birchwood xylan; 2%: containing 2% birchwood xylan; 4%: containing 4% birchwood xylan; 8%: containing 8% birchwood xylan in medium M2 at 37 °C at 170 rpm; 2.5 IU/mL commercial xylanase was added 24 hours before inoculation)

Likewise, used xylan concentration showed correlation with the amount of itaconic acid production, which increased as a result of an increase in xylan concentration. In the cultures containing 1, 2, 4 and 8% xylan, the amount of itaconic acid productions after 12^{th} days were 0.0016, 0.0028, 0.0041 and 0.0432 g/L, sequentially. It was important that itaconic acid production increased almost proportional to the used xylan concentration in the production cultures from 1 to 4% xylan containing ones and suddenly its production amount increased 10 times in 8% xylan containing flask.

Based upon these data, higher initial simple sugar concentration is obtained by adding commercial xylanase to the culture. Although this resulted in a minor increase in the production of itaconic acid, xylanase production decreased. Therefore it is not a good method.

4.3.2 Effect of pH Adjustment and Commercial Xylanase and Glucose Addition

The previous experiment showed the minimal addition of extra xylanase at the beginning of the fermentation. In order to increase the IA production without reducing the xylanase production, commercial xylanase addition was decided to add at the fifth day of fermentation where the xylanase concentration was at the maximum. Besides xylanase addition, direct glucose addition to a final concentration of 50 g/L and pH adjustment to 2 were performed in parallel flasks on the 5^{th} day. Adjustment of pH, commercial xylanase (NZ) addition and glucose (Glu) addition were carried out on the 5^{th} day of fermentation when the xylanase level was at maximum. Although 100 IU/mL commercial enzyme was added into the NZ, a decrease in activity was observed in all fermentation cultures after their maximum was reached (Figure 4.53). After a sharp decrease in Glu, NZ and pH media in two days, xylanase activity level remained almost constant for pH culture and rate in activity decrease in NZ culture diminished throughout the rest of the fermentation period. On the other hand, in Glu medium, the decrease in xylanase activity level continued until the 11^{th} day of fermentation and then increased again to 30 IU/mL activity when all the glucose was depleted and the pH of the medium increased to 5.3 (Figure 4.54 and 4.55). Besides having high xylanase activity in all fermentations, itaconic acid production was observed only in the Glu medium and it was 8 g/L on 8^{th} day after 3 days of glucose addition (Figure 4.56). The other important point here is that itaconic acid concentration started to decrease after the 9^{th} day of fermentation and it was almost completely depleted at the end of the fermentation.

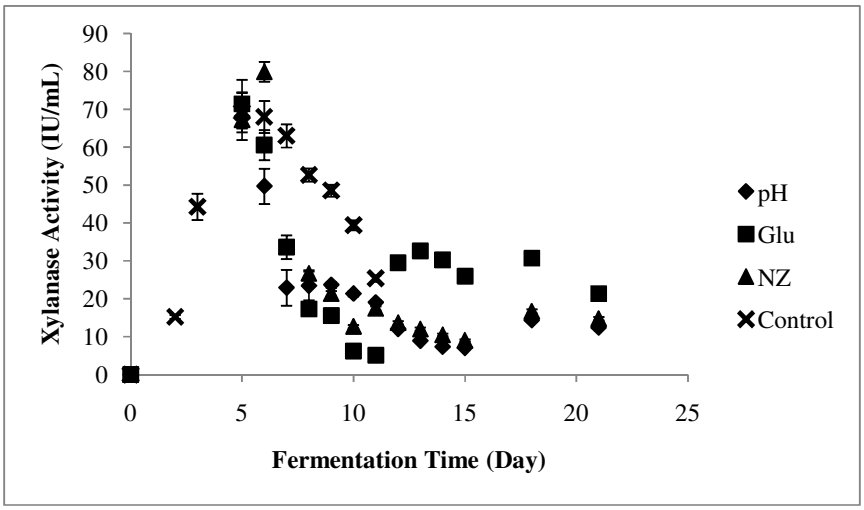


Figure 4.53 Effect of pH adjustment, glucose and commercial xylanase addition on xylanase production
(Control: grown at 37 °C at 170 rpm, adjustment and additions were performed at 5th day of fermentation)
(pH: pH adjusted to 2.0; Glu: 5g/L glucose was added; NZ: 100IU/mL commercial xylanase was added)

The pH adjustment to 2 was resulted into a decrease in xylanase activity and since there was not enough simple sugar production, itaconic acid production could not be induced. After adjustment, pH remained almost constant for 6 days and then it started to decrease, meanwhile the reducing sugar concentration started to increase, however, the xylanase activity was at its minimum level. That could be due to the starting of cell death. As concerning commercial xylanase addition, although 100 IU/mL xylanase was added into the medium, only a slight increase in xylanase activity was observed on the next day of addition (Figure 4.53). The reason can be degradation of the commercial xylanase by the organism. Similar results were already mentioned in the previous section 4.3.1. Addition of commercial enzyme into the fermentation medium throughout the fermentation did not resulted in high simple sugar production level (Figure 4.54). That was why glucose addition was carried out.

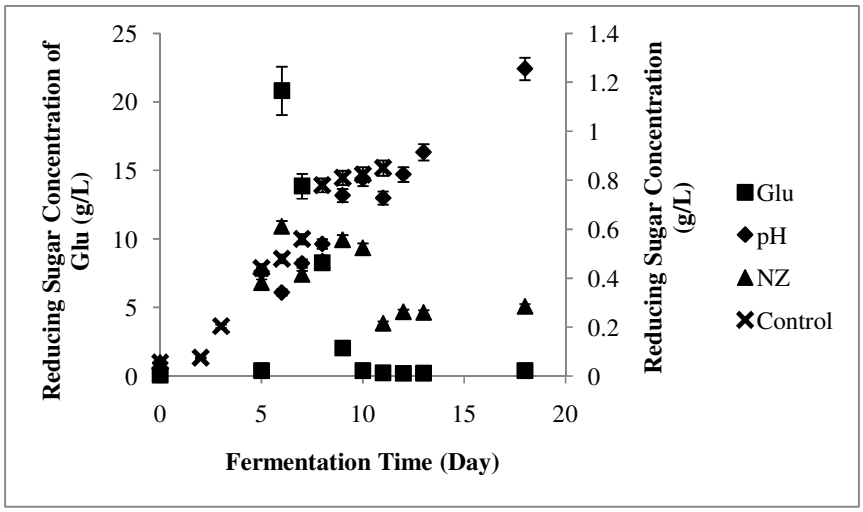


Figure 4.54 Effect of pH adjustment, glucose and commercial xylanase addition on reducing sugar concentration
(Control: grown at 37 °C at 170 rpm, adjustment and additions were performed at 5th day of fermentation)
(pH: pH adjusted to 2.0; Glu: 5 g/L glucose was added; NZ: 100 IU/mL commercial xylanase was added)

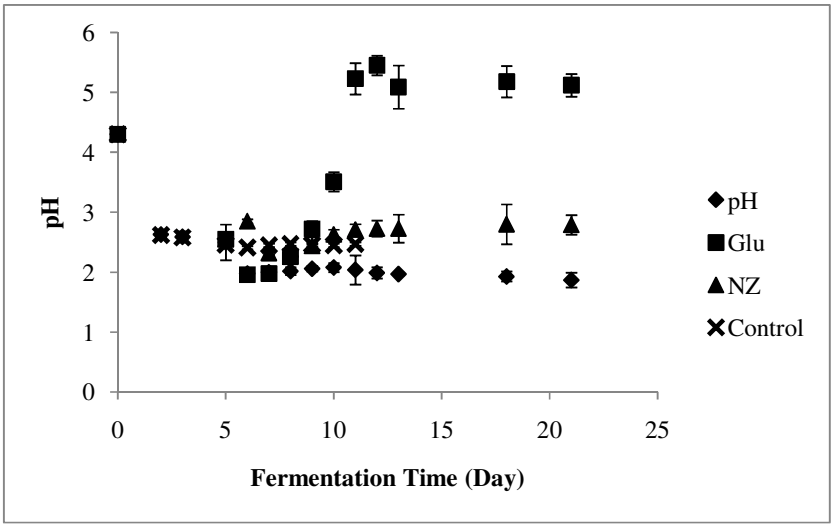


Figure 4.55 Effect of pH adjustment, glucose and commercial xylanase addition on pH
(Control: grown at 37 °C at 170 rpm, adjustment and additions were performed at 5th day of fermentation)
(pH: pH adjusted to 2.0; Glu: 5 g/L glucose was added; NZ: 100 IU/mL commercial xylanase was added)

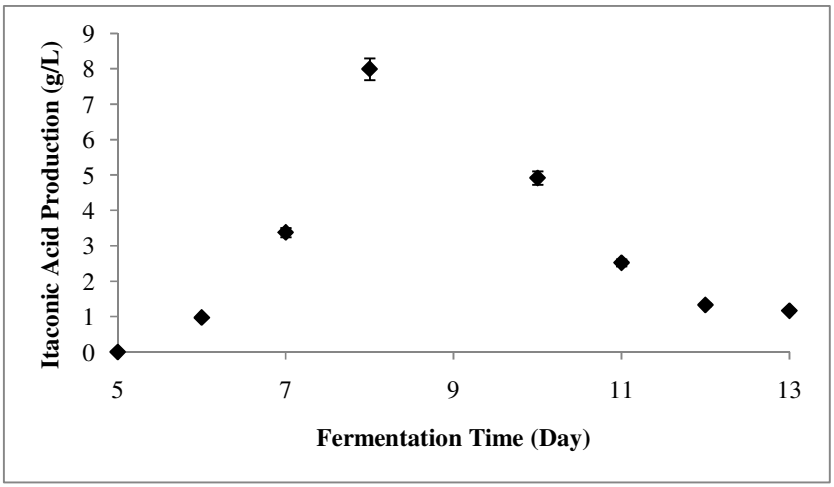


Figure 4.56 IA production profile of *A. terreus* in glucose added medium
(Control: grown at 37 °C at 170 rpm, additions were performed at 5th day of fermentation) (Glu: 50 g/L glucose was added)

It was clearly revealed that for itaconic acid production high simple sugar concentration and lower pH value (as low as 1.9) were needed. Moreover, high concentration of simple sugar should be supplied throughout the whole fermentation period; otherwise, the organism does not produce IA.

4.3.3 Comparison of Glucose Addition and Transferring into Fresh Medium

Based on previous results in 4.3.2, it was decided to increase the added glucose concentration to 10% and also transferring the organism to new M2 with 10% glucose. Glucose addition in the same fermentation medium (G medium) and transferring the organism to a new M2 containing 10% glucose (NG medium) were carried out after the organism reached the highest xylanase production on the 5th day of fermentation. The reducing sugar concentration and IA production were very consistent with each other that IA was produced during the consumption of reducing sugar in the G medium and when the reducing sugar reached its minimal concentration, IA production ceased and started to decrease (Figure 4.57). The surprising result was about the change in pH. After an initial increase on the fifth day, pH began to increase with increasing IA production till

the 13th day and then started to decrease again. That might be resulted from cell autolysis. Another surprising result was about the IA production level. The IA production reached up to 7g/L that was almost the same with the addition of 5% glucose. On the other hand, although the production of IA started lately, transferring the organism into the new medium with high glucose concentration could provide IA production (17g/L) as much high as keeping in the original medium (Figure 4.58). Moreover, the reducing sugar and pH profiles were similar to original one till the 12th day of fermentation. The organism used up to 60% of the glucose in the medium and the pH was kept about 2. After that another interesting result was observed that not only the glucose consumed sharply but also the IA concentration decreased during the following two days of fermentation. It was unclear that why the organism consumed the IA while there was enough glucose in the medium since previous results showed that the organism consumed IA when there was not enough simple sugar (less than 2g/L) in the medium.

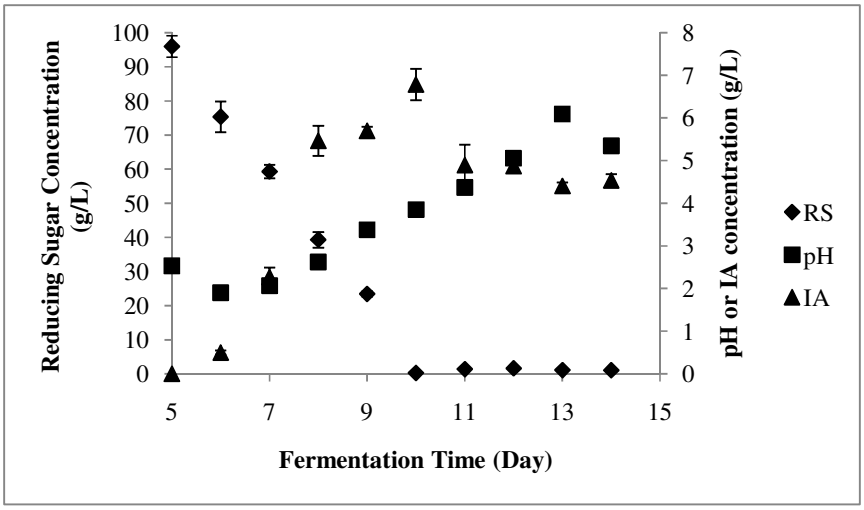


Figure 4.57 IA production profile in glucose added medium
(Control: grown at 37 °C at 170 rpm, additions were performed at the 5th day of fermentation) (Glu: 100 g/L glucose was added)

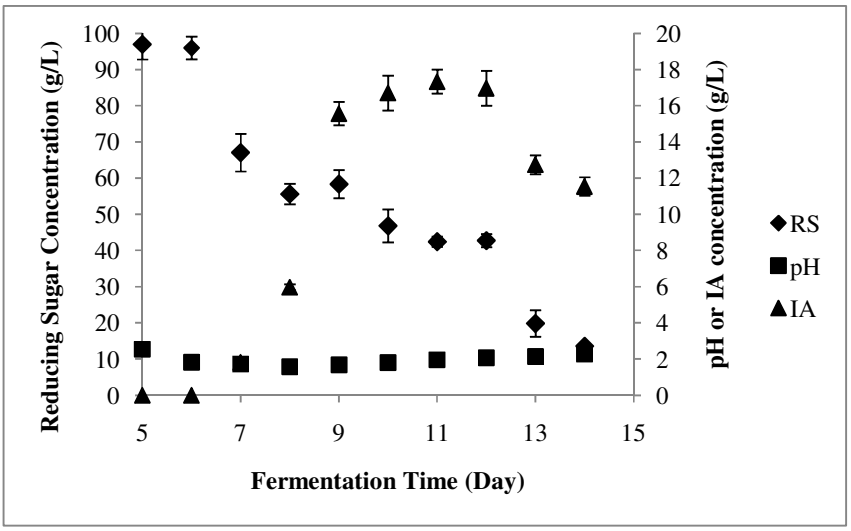


Figure 4.58 IA production profile in transferred medium
(Control: grown at 37 °C at 170 rpm, transfer were performed at 5th day of fermentation in to 100 g/L glucose containing medium)

In summary, The results showed that the best strategy for IA and xylanase production, the organism should be cultivated in M2 medium containing 7% wet heat treated corn cob for 5 days and then the microorganism should be taken to a new medium containing 100 g/L glucose for 5-6 days.

4.4 Characterization of Xylanase

Xylanase production by *A. terreus* NRRL 1960 was performed in M2 containing 2% xylan at 37 ℃ at 170 rpm for 5 days. The enzyme was purified by using one-step hydrophobic interaction chromatography technique in our laboratory and used for further biochemical characterization studies (Appendix O). Within the scope of this study, already purified xylanase was taken and examined for its molecular weight, isoelectric point, effect of pH and temperature on activity and stability, kinetic properties and xylan degradation product profile.

4.4.1 Molecular Weight and Isoelectric Point

Molecular weight of xylanase was determined as about 19 kDa by SDS-PAGE (Figure 4.59a) which was confirmed by activity staining zymogram analysis (Figure 4.59b). The isoelectric point (pI) of xylanase was determined as *c* 9.0 (Figure4.59c).

Wong et al (1988) reported a conserved relationship between molecular weights and pI values of xylanases by investigating the conservation through the evolutionary processes of xylanases purified from *Aspergillus*, *Bacillus*, *Clostridium*, *Streptomyces*, and *Trichoderma* species. Two groups are proposed: with a low molecular weight (<30 kDa) and basic pI (family-11), and with a high molecular weight (>30 kDa) and acidic pI (family-10), with some exceptions (Wong *et al*., 1988; Biely *et al*., 1997; Flatman *et al*., 2002). According to our results, *A. terreus* NRRL 1960 xylanase is a low molecular weight (19 kDa) enzyme with basic pI value (*c*. pH 9.0), hence a member of the family-11.

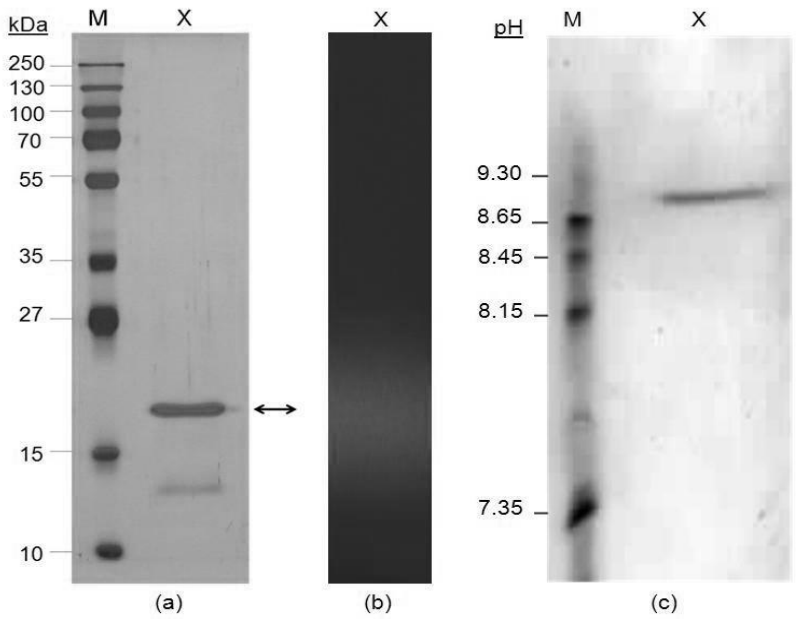


Figure 4.59 (a) SDS-PAGE (b) Zymogram (c) Isoelectric focusing analysis of *A. terreus* NRRL 1960 purified xylanase
(Lanes; M: Marker, X: Purified xylanase, 1µg/mL with 38 IU/mL)

4.4.2 Effect of pH and Temperature on Xylanase Activity and Stability

The effect of pH on the xylanase activity was studied over a pH range of 3.0-10.0. The highest activity of the enzyme was observed at pH 6.5, having a very similar activity value at pH 7.0 (Figure 4.60). Between pH values of 5.0-6.0 and at pH 7.5, about 80% of the maximum enzyme activity was detected. As moving away from neutral pH values, at pH 4.0 and pH 8.0, about 50% of the maximum enzyme activity remained. No xylanase activity was observed at pH 3.0 and 10.0.

The effect of temperature on xylanase activity was studied over a temperature range of 30-80°C at 5°C intervals. The highest xylanase activity was observed at 50°C, with relative activities at 45 and 55°C as 91 and 96%, respectively (Figure 4.61). Xylanase activity was completely lost at 75°C.

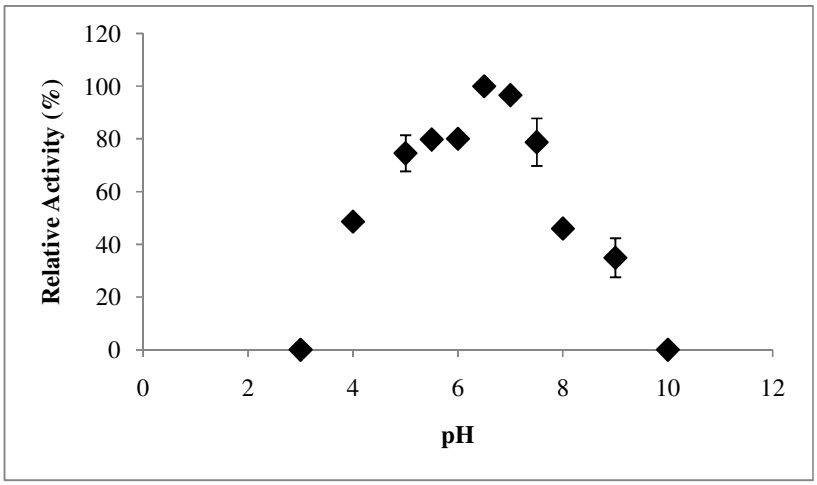


Figure 4.60 Effect of pH on *A. terreus* NRRL 1960 purified xylanase activity

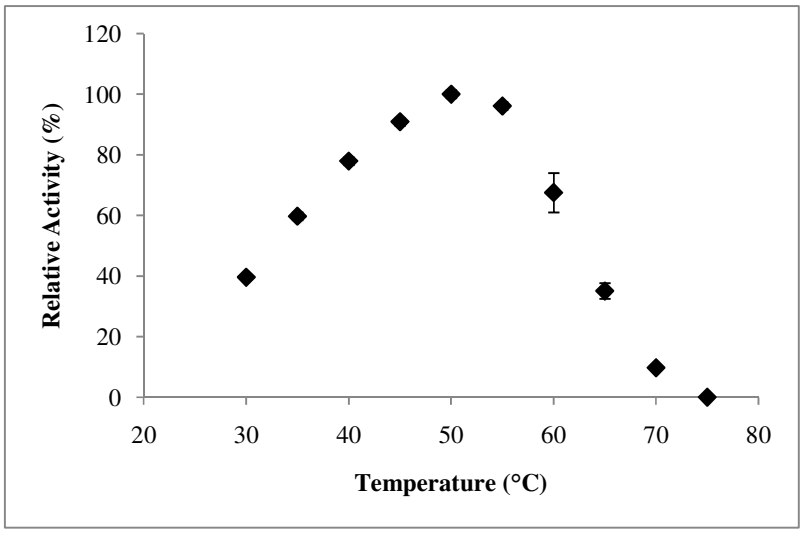


Figure 4.61 Effect of temperature on *A. terreus* NRRL 1960 purified xylanase activity

To determine the effect of pH on xylanase stability, enzyme (1µg/mL) was incubated in buffers in a pH range of 3.0-10.0 and remaining xylanase activities were determined under standard assay conditions after 4 and 48 h. After 4 h of incubation, the highest enzyme activity was identified at pH 6.0 as 82%, having similar results between 70-80% in the pH range of 3.0-11.0 (Figure 4.62). After 48 hours, the highest enzyme activity was detected at pH 6.0 and 7.0 as 76%. The enzyme retained 60-66% of its original activity in acidic pH range of 3.0 to 5.0, whereas it retained 41-58% of its original activity in basic pH range of 8.0 to 10.0. The enzyme was less stable in basic pH range than acidic pH range after 48 hours of incubation. The reason could be that in basic pH the enzyme could not preserve its tertiary structure.

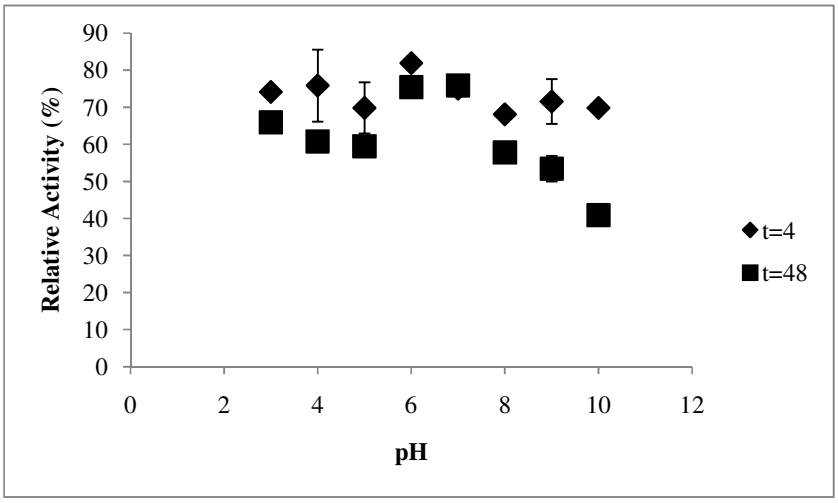


Figure 4.62 Effect of pH on *A. terreus* NRRL 1960 purified xylanase stability

To determine the thermal stability of the enzyme, purified enzyme (1µg/mL) was incubated at temperatures of 30, 40 and 50°C up to 8 h. After a specific time, an aliquot of sample was removed and assayed for its residual enzyme activity under standard conditions. It was observed that even after 8 h of incubation

about 83% of the initial xylanase activity was remained at 30 and 40°C (Figure 4.63). In contrast, at 50°C, enzyme lost 24% of its initial activity after 30 min of incubation and, after 2 h only 13% of its initial activity was remained. Total inactivation of the enzyme was observed after 6 h of incubation at 50°C.

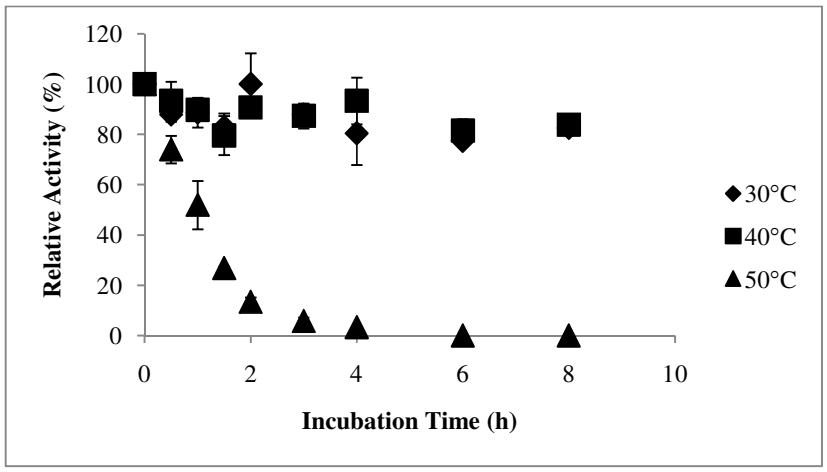


Figure 4.63 Effect of temperature on *A. terreus* NRRL 1960 purified xylanase stability

4.4.3 Kinetic Studies

Initial reaction rates of birchwood xylan hydrolysis by *A. terreus* NRRL 1960 purified xylanase were determined at different substrate concentrations ranging from 0.2 to 2.5% (w/v) and it obeyed the Michaelis-Menten kinetics (Figure 4.64). K_m as 2.17 mg xylan/mL and V_{max} value as 38.50 IU/mL were calculated from Michaelis-Menten kinetic graph by using SigmaPlot 9.0. Moreover, Lineweaver-Burk plots showed K_m and V_{max} values as 3.12±0.05 mg xylan/mL and 42.7±0.4 IU/mL, respectively (Figure 4.65). Since both kinetic calculations uses their approximations during applying either non-linear regression or linear

regression, as an average of both calculations, the K_m and V_{max} were found as 2.65 mg xylan/mL and 40.6 IU/mL, respectively.

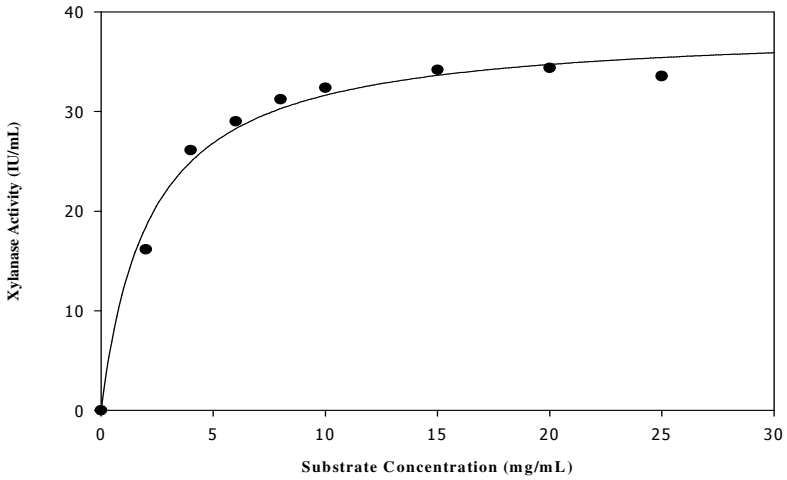


Figure 4.64 Effect of substrate concentration on *A. terreus* NRRL 1960 purified xylanase

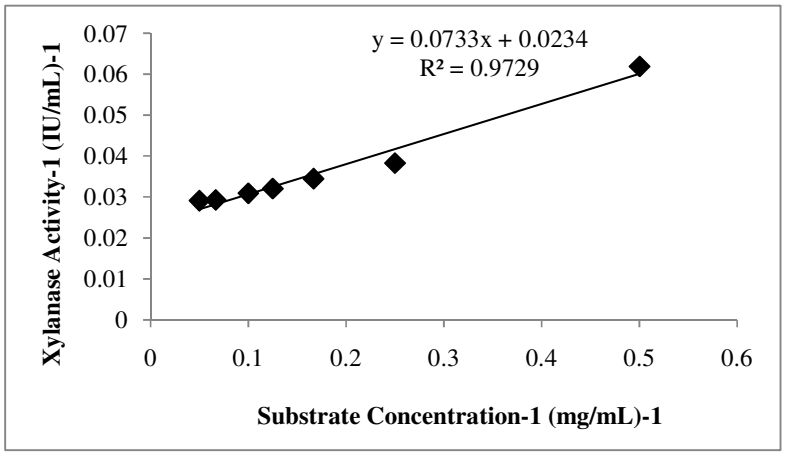


Figure 4.65 Lineweaver-Burk plot of effect of substrate concentration on *A. terreus* NRRL 1960 purified xylanase

4.4.4 Analysis of Xylan Degradation Product Profile

The thin layer chromatograms of birchwood xylan degradation by crude and purified xylanases are shown in Figure 4.66a and 4.66b, respectively. The products of xylan hydrolysis were xylobiose, xylotriose, xylotetraose and higher sugars. No xylose was detected even after 48 hr of incubation.

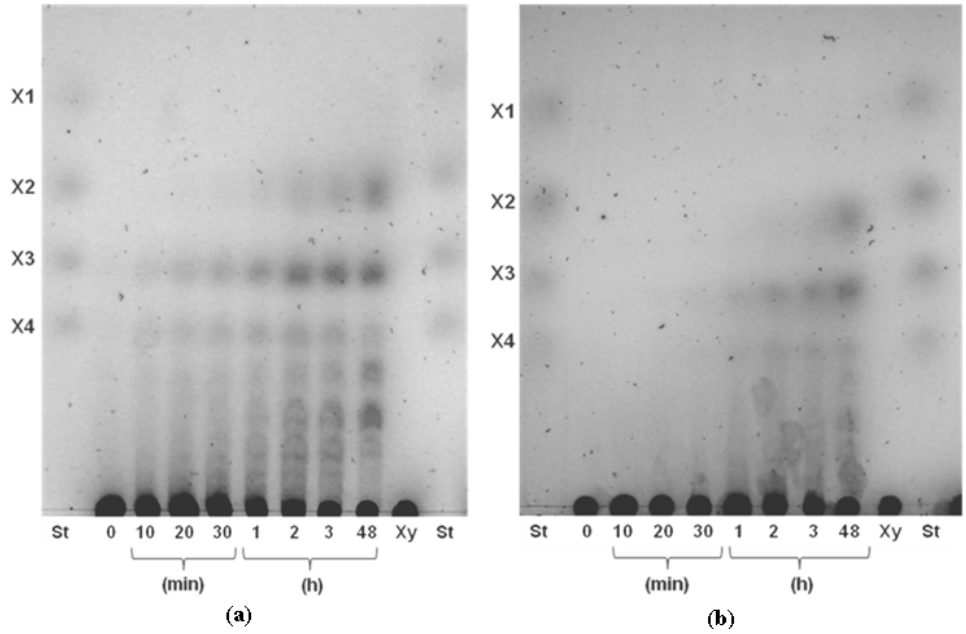


Figure 4.66 Analysis of xylan degradation product profile of *A. terreus* NRRL 1960 xylanase

(a) Crude supernatant, (b) Purified enzyme. Reaction conditions: Birchwood xylan (10 g/L) degradation using 6 IU/mL crude and purified xylanase in 20 ml reaction mixture at pH 7.0 and 40°C (X1: xylose, X2:xylobiose, X3:xylotriose, X4:xylotetrose; 2.5 µg each)

In xylan degradation studies, despite performing the reaction using the same level of enzymatic activity (6 IU/ml in reaction mixture) for both crude and purified xylanases, initial reaction products were visible only after 10 min for crude enzyme, whereas 1 h reaction time was necessary to obtain first degradation products for purified enzyme. Slightly visible products were also

detected at time zero for crude enzyme solution (Figure 4.66a), which could be due to the degradation of xylan, the carbon source used during fermentation period. The reason of the reaction rate difference between crude and pure xylanases could be the presence of other xylanolytic enzymes in the crude supernatant. Therefore, research on other xylanolytic enzymes of *A. terreus* NRRL 1960 could be another subject of further studies.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

Aspergillus terreus NRRL 1960 was cultivated on lignocellulosic biomass. The microorganism secretes xylanolytic and cellulolytic enzymes to use cellulose and xylan as carbon source. However the simple sugar concentration produced during cultivation was not enough to produce IA at high titers. Although co-production of xylanase and itaconic acid was not possible, applying a two-step fermentation determined as the optimal design to produce high xylanase and itaconic acid efficiently in medium containing 7% wet heat treated corn cob with inorganic nitrogen source. These findings indicate that agricultural wastes can be used as substrates for cultivation and xylanase production, however to produce high titers of IA, simple sugar concentration should be higher. Hence, it is possible to produce xylanase in the first step as the first product during growth of the organism and then as the second product IA is produced by transferring the organism into a high simple sugar containing medium as the second step.

Shaking, lowering temperature, direct spore inoculation and carbon source in preculture had no significant effect on either xylanase or IA production. Among the tested xylan concentrations in the range of 1-8%, 2% (w/v) was found as the best concentration and decrease was observed in xylanase production level at xylan concentrations above 4%. Likewise, higher amount of available simple sugar in the production medium caused higher IA production, whereas, higher initial reducing sugar concentration resulted in decrease in xylanase production. Besides these studies, the organism utilized corn cob more efficiently than cotton

and sunflower stalks. Furthermore, increasing the corn cob concentration and applying pretreatment increased the xylanase production level.

Addition of commercial xylanase to production culture resulted in higher initial simple sugar concentration which increased IA production slightly (with increasing xylan concentration) but decreased xylanase production.

Because of not producing sufficient amount of simple sugar from xylan, addition of high concentration of glucose or transferring the organism to a medium having high concentration of glucose were performed. Therefore, as a further study, enzymatic glucose production from cellulose part of the lignocellulosic waste can be studied and the glucose syrup obtained from enzymatic hydrolysis can be used in the fermentation medium for the production of IA.

Besides all these studies, according to its biochemical properties, the xylanase was found to be a member of family-11 xylanase group. As being capable of hydrolyzing xylan, with xylobiose and xylotriose as major products, *A. terreus* NRRL 1960 xylanase seems to be a potential enzyme for XOs production.

In addition to its novelty of being a part of the successful co-production system of xylanase and itaconic acid, the enzyme could be advantageous for use in different industrial applications due to its low molecular weight and potential to XOs production.

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APPENDIX A

PREPERATION OF STOCK SOLUTIONS AND PLATES

Preparing Glycerol Stock

- Make glycerol-peptone solution (30 % glycerol + 1 % peptone)
- Take 1 mL of spore solution and centrifuge briefly
- Take 500 µL of supernatant and add on 500 µL of glycerol-peptone solution
- Keep in -60 °C

Making Spore Plates

- Put 800 µL sterile 1% peptone solution (PS) in eppendorf tube
- Using sterile loop/needle transfer spores to PS
- After vortexing, plate 200 µL on agar and spread

APPENDIX B

PREPARATION OF SPORES SUSPENSIONS AND CULTURE INOCULATION

For plates:

- Pipette 10 mL 1% peptone solution on the spore-mat
- Scrape off the spores using a bend inoculation needle
- Transfer the spores suspension to a sterile bottle
- Prepare a 20-fold dilution (50 µL + 950 µL 1% peptone solution) and determine the concentration of spores by counting the diluted sample in a heamocytometer (see remarks).
- Calculate the concentration of spores.

Neubauer improved heamocytometer:

Depth: 0.1 mm, minimal area (A): 1/400 mm^2

Pipette spore dilution between coverslip and slide, enough liquid until the space is flooded (~20 µL) if the coverslip is chipped or in any way damaged, it must be replaced- it is no longer functional

- Count the spores (magnification 400 X) in 16 fields.
- Calculate the spores concentration in the spores suspension: spores/mL=count*d*2.5*10^5 (d=dilution) (magnification 400 x)

APPENDIX C

PRECULTURE AND PRODUCTION MEDIA

Table C.1 Preculture ingredients

	Medium 1*	**Medium 2****
Compound	**Quantity (g/L)**	**Quantity (g/L)**
Glucose/Xylose	25	25
$MgSO_4.7H_2O$	5	5
NaCl	0.4	0.4
$ZnSO_4.7H_2O$	-	0.004
KH_2PO_4	1	0.1
NH_4NO_3	-	2.0
Peptone	1	-
Yeast Extract	2	
CSL	-	0.5

*Ingredients chosen with regard to literature (Ghanem *et al.,* 2000; Hrmova *et al*., 1991; Gawande and Kamat, 1999; Marques *et al*., 2003)

**It is the modified medium used in the research of Cros and Schneider (1993)

Table C.2 Production medium ingredients

	Medium-1	**Medium-2**
Compound	**Quantity (g/L)**	**Quantity (g/L)**
Glucose/Xylose***	100	100
Xylan***	10	10
CSL	-	0.5
$MgSO_4.7H_2O$	5	5
NH_4NO_3	-	3
Peptone	1	-
Yeast Extract	2	-
NaCL	-	0.4
$ZnSO_4.H_2O$	-	0.033
KH_2PO_4	1	0.5
$CaCl.2H_2O$	-	1.0
$CuSO_4.5H_2O$	-	0.06

*** Only one is used in a medium, otherwise indicated.

APPENDIX D

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 E

PROCEDURE OF DNSA METHOD

- Enzyme and substrate solutions are mixed in a 1:10 ratio
- 1.0 mL of sample is poured into tubes containing 1.5 mL DNSA reagent
- The tubes are placed in a boiling water bath for five minutes and cooled immediately under tap water.
- The tubes are equilibrated to room temperature and read against a buffer blank at 540 nm.

APPENDIX F

PREPARATION OF REDUCING SUGAR STANDARDS

100 µg/mL of reducing sugar stock solution is prepared in buffer solution and dilutions are made for 1 ml reducing sugar sample according to the Table F.1.

Table F.1 Dilutions for reducing sugar standard curve

Reducing Sugar (µg/mL)	0	40	60	80	100
Reducing Sugar solution (µL)	0	400	600	800	1000
Buffer (µL)	1000	600	400	200	0

APPENDIX G

REDUCING SUGAR STANDARD CURVES FOR DNSA METHOD

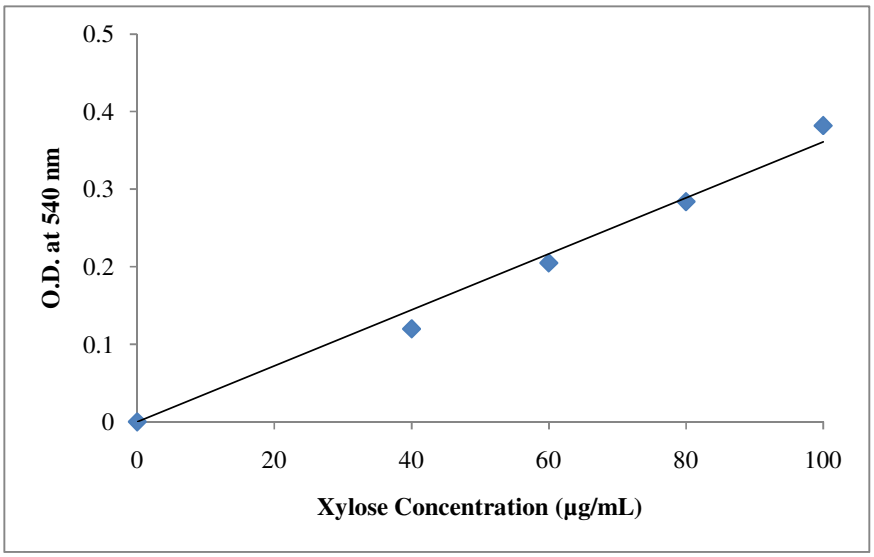

Figure G.1 Xylose standard curve for DNSA method

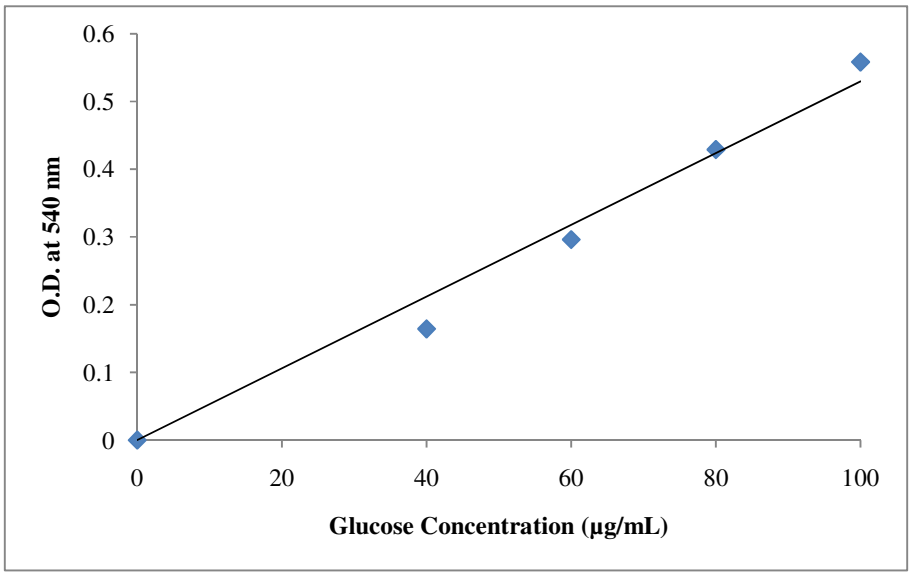

Figure G.2 Glucose standard curve for DNSA method

APPENDIX H

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 I

PREPARATION OF PROTEIN STANDARD

BSA is diluted in buffer solution to obtain a stock solution with concentration of 1000 μg/mL.

Table I.1 Dilutions for protein standard curve

Protein (μg/mL)	0	250	500	1000
BSA stock (μL)	0	125	250	500
Buffer (μL)	500	375	250	0

After preparations of the samples, 1 mL of Bradford reagent is added in the tube. After waiting 10 minutes, the optical density is measured against blank at 595 nm.

APPENDIX J

STANDARD CURVE FOR BRADFORD METHOD

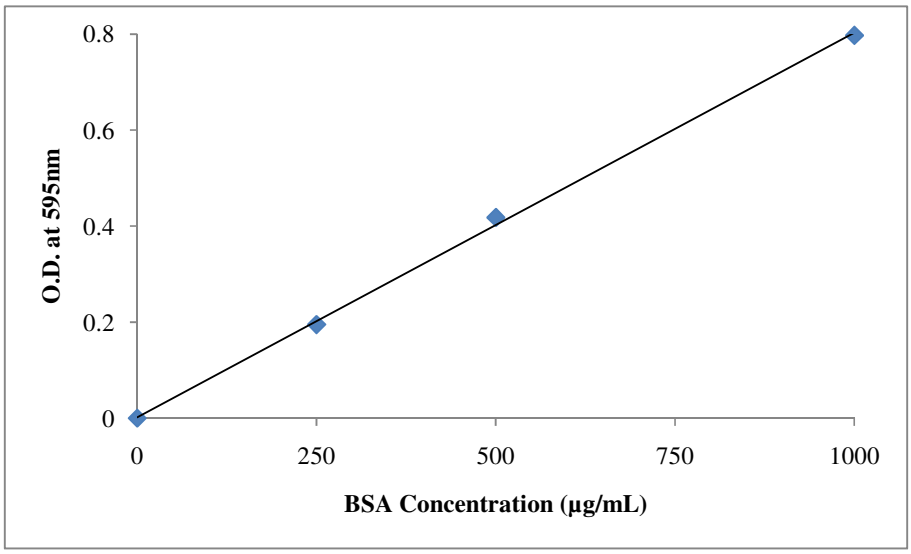

Figure J.1 Standard curve for Bradford method

APPENDIX K

STANDARD CURVE FOR ITACONIC ACID

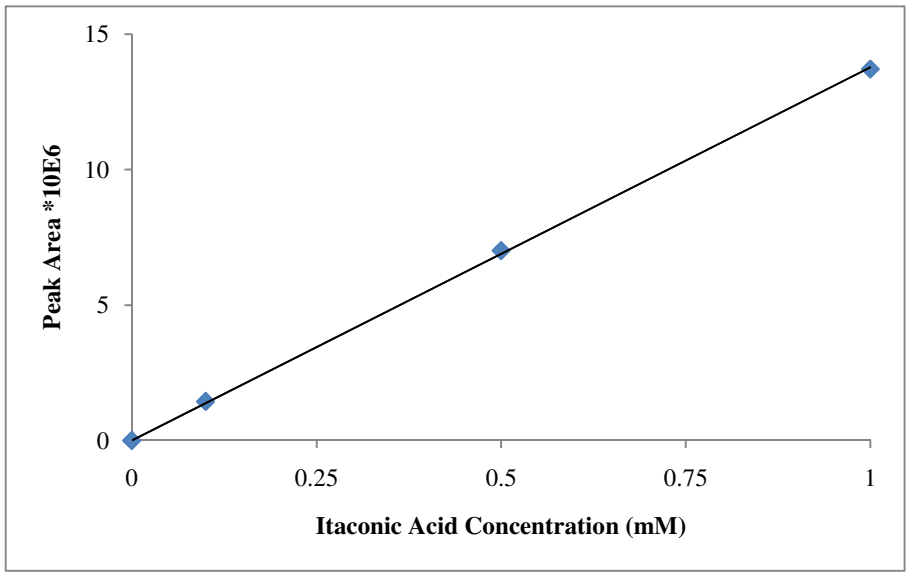

Figure K.1 Standard curve for itaconic acid

APPENDIX L

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

A discontinuous gel system according to Laemmli (1970) is used. On top of a 12 % separation gel (pH 8.8) a 0.5 cm 4 % stacking gel (pH 6.8) is prepared.

A. Gel Preparation

Compositions of the gels are given in Tables L.1. and L.2.

Table L.1 Composition of 12 % separating gel

	Solution	Volume (mL)
1	30 % acrylamide/bisacrylamide stock	4.0
2	1 M Tris/HCl pH 8.8	2.5
3	1 % (w/v) SDS	0.1
4	100 % TEMED	0.005
5	Water	3.35
6	10 % (w/v) ammonium persulphate	0.05

Table L.2 Composition of 4 % stacking gel

	Solution	Volume (mL)
1	30 % acrylamide/bisacrylamide stock	1.3
2	1 M Tris/HCl pH 6.8	2.5
3	1 % (w/v) SDS	0.1
4	100 % TEMED	0.01
5	Water	6.1
6	10 % ammonium persulphate	0.05

Procedure

- For the preparation of the separation gel, pipet the solutions 1-5 into a beaker and mix gently.
- Add the ammonium persulphate, mix gently and pour the solution between the glass plates in the casting stand.
- Cover the surface of the separation gel is with 200 µL water and allow the solution to polymerize for approximately one hour.
- Remove the water between the glass plates.
- For the preparation of the stacking gel, pipet solution 1-6 into a beaker, mix gently and pipet the solution onto the polymerized separation gel. Place the combs and allow the gel to polymerize for another hour.
- Until further use, the gels can be stored at 4 $^{\circ}$C.

B. Gel Electrophoresis

Reagents

Sample buffer

1 M Tris/HCl pH 6.8	1.0 mL
10% (w/v) SDS	1.6 mL
Glycerol	0.8 mL
β-mercaptoethanol	0.4 mL
0.05% (w/v) bromophenol blue	0.2 mL
Water till 8 mL	

Electrophoresis buffer (10 x concentrated)

Dissolve 86.4 g glycine, 18 g Tris and 6 g SDS in 600 mL water. The pH should be round 8.3. Before use dilute the buffer 10 times.

Procedure

- Mix 30 µL of sample with 10 µL sample buffer in a 1.5 mL eppendorf tube. Mix and punch the cover of the tube with a needle.
- Place the tubes in a boiling waterbath for 5 minutes and allow to cool.
- Load 25 µL of sample on the gel. (up to 5 µg per protein)
- Electrophoresis should be carried out at 120 volt until the dye front has reached the bottom of the gel.

The gels were then silver stained using the procedure of Blum *et al*. (1987).

APPENDIX M

SILVER STAINING METHOD

After electrophoresis**,** the silver staining procedure of Blum *et al*. (1987) was used.

A. Reagents

Fixer

Mix 150 mL methanol, 36 mL acetic acid and 150 µL 37% formaldehyde solution. Complete to 300 mL with distilled water. This solution can be used several times.

50% Ethanol

Mix 600 mL ethanol with 600 mL distilled water.

Pretreatment Solution

Dissolve 0.08 g sodium thiosulphate ($Na_2S_2O_3.5H_2O$) in 400 mL distilled water. Mix with a glass rod. Take 8 mL for further use in developing solution preparation.

Silver Nitrate Solution

Dissolve 0.8 g silver nitrate in 400 mL distilled water and add 300 µL 37% formaldehyde solution.

Developing Solution

Dissolve 9 g potassium carbonate in 400 mL of distilled water. Add 8 mL pretreatment solution and 300 µL 37% formaldehyde solution.

Stop Solution

Mix 200 mL methanol with 48 mL acetic acid. Complete to 400 mL with distilled water.

B. Silver Staining

Silver staining procedure is summarized in Table M.1.

Table M.1 Silver staining procedure

	Step	Treatment time	Remarks
1	Fixing	≥ 1 hr	Overnight incubation is all right
2	Washing with 50% ethanol	3 x 20 min	Fresh solution
3	Pre-treatment	1 min	Fresh solution, exact timing is necessary
4	Rinsing with distilled water	3 x 20 sec	Exact timing is necessary
5	Impregnation by silver nitrate solution	20 min	
6	Rinsing with distilled water	2 x 20 sec	Exact timing is necessary
7	Developing	~ 10 min	After a few minutes add some distilled water to slow down the reaction. Observe the color development carefully.
8	Wash with distilled water	2 x 2 min	
9	Stop	≥ 10 min	The gels can be kept in this solution overnight.

APPENDIX N

HPLC CHROMATOGRAMS

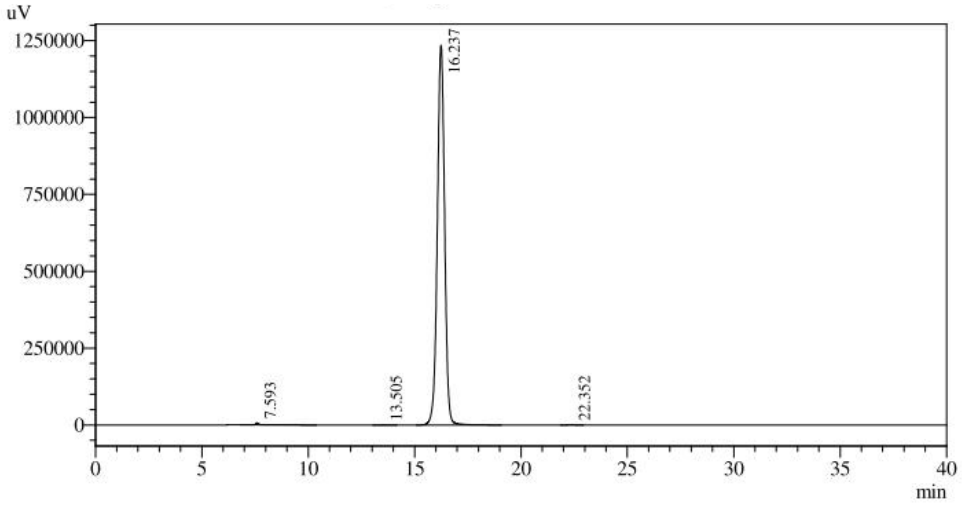

Figure N.3 HPLC chromatogram of itaconic acid as standard

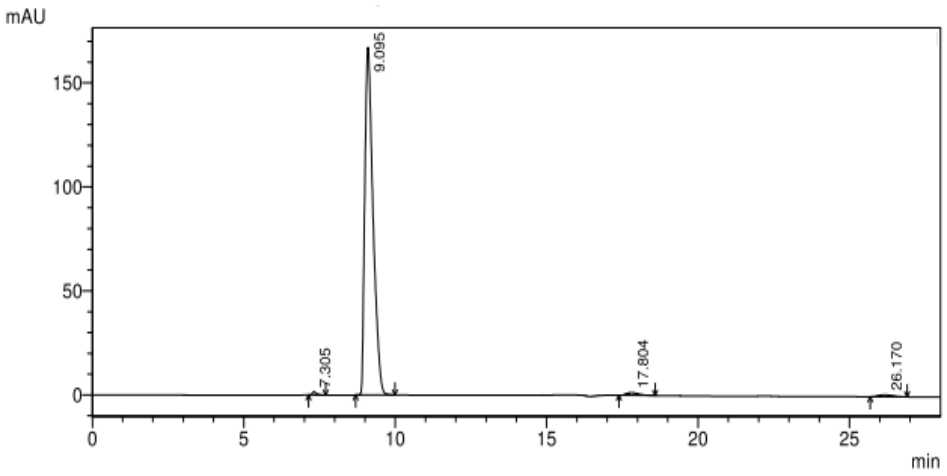

Figure N.4 HPLC chromatogram of oxalic acid as standard

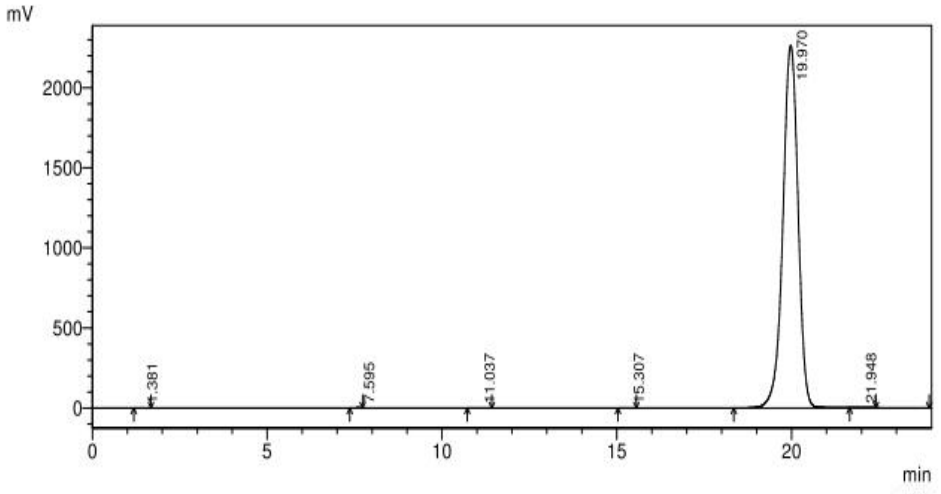

Figure N.5 HPLC chromatogram of fumaric acid as standard

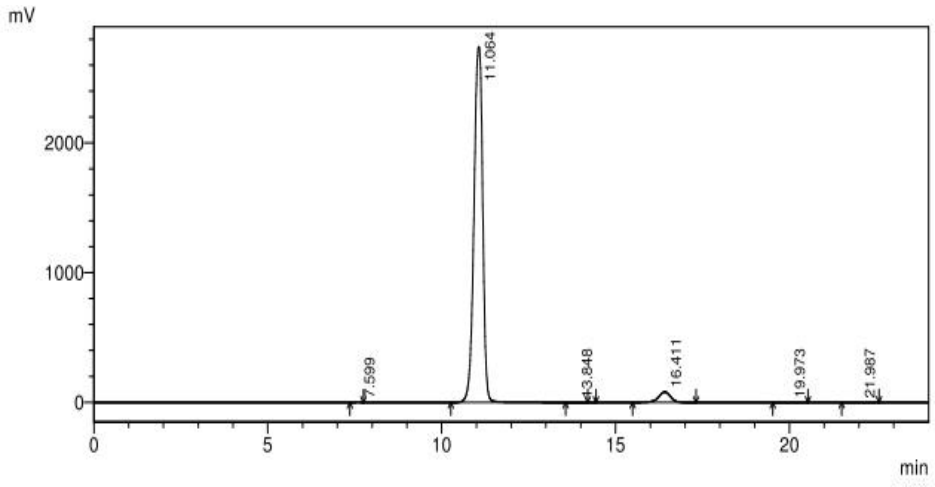

Figure N.6 HPLC chromatogram of malic acid as standard

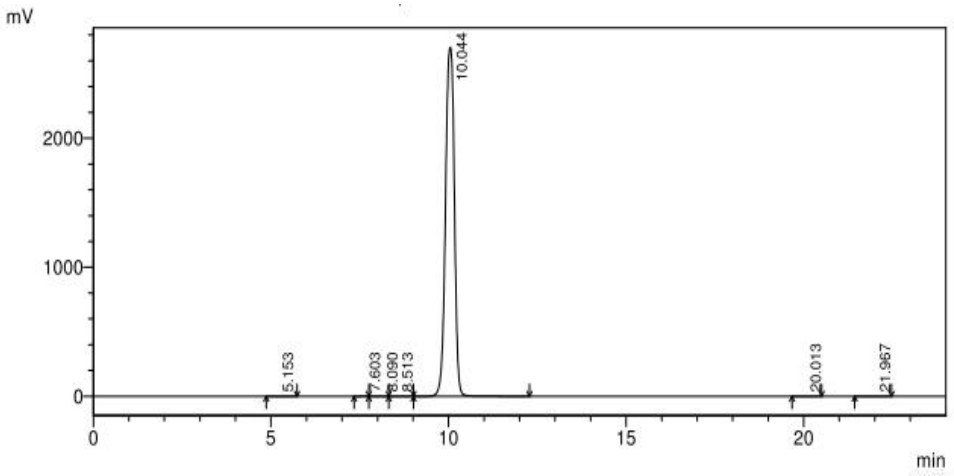

Figure N.7 HPLC chromatogram of citric acid as standard

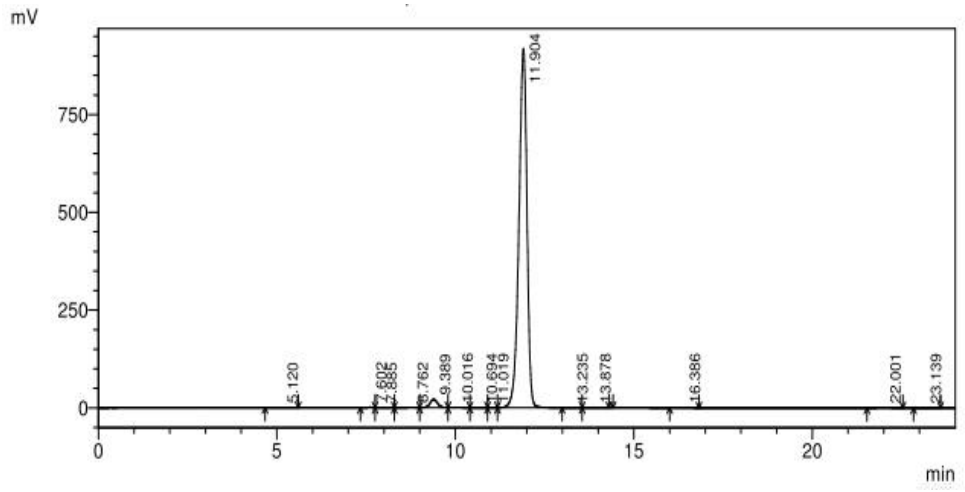

Figure N.8 HPLC chromatogram of pyruvic acid as standard

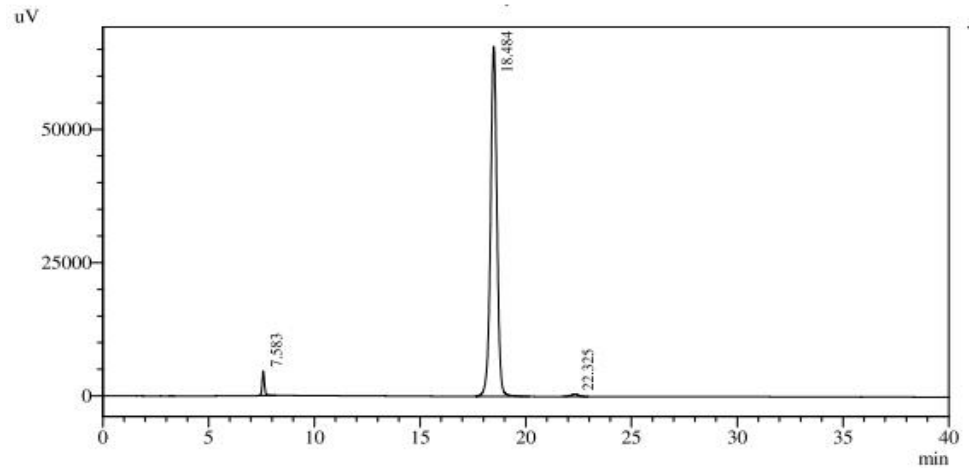

Figure N.9 HPLC chromatogram of acetic acid as standard

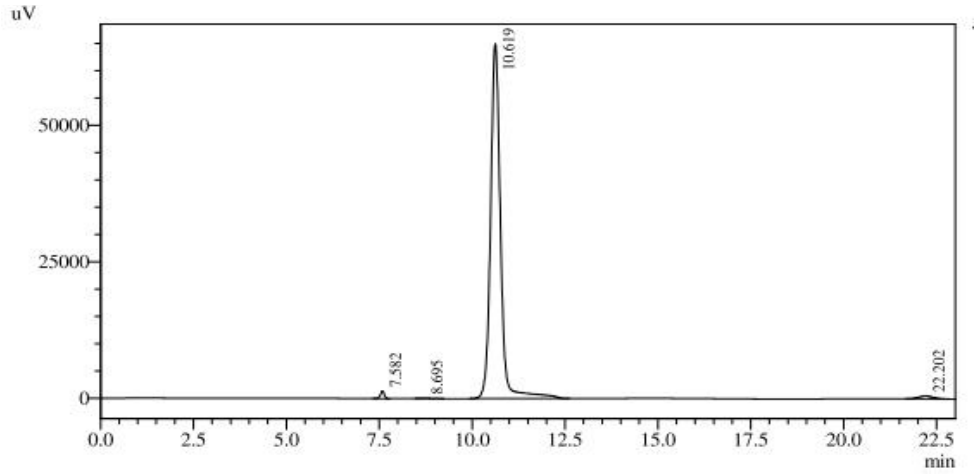

Figure N.10 HPLC chromatogram of glucuronic acid as standard

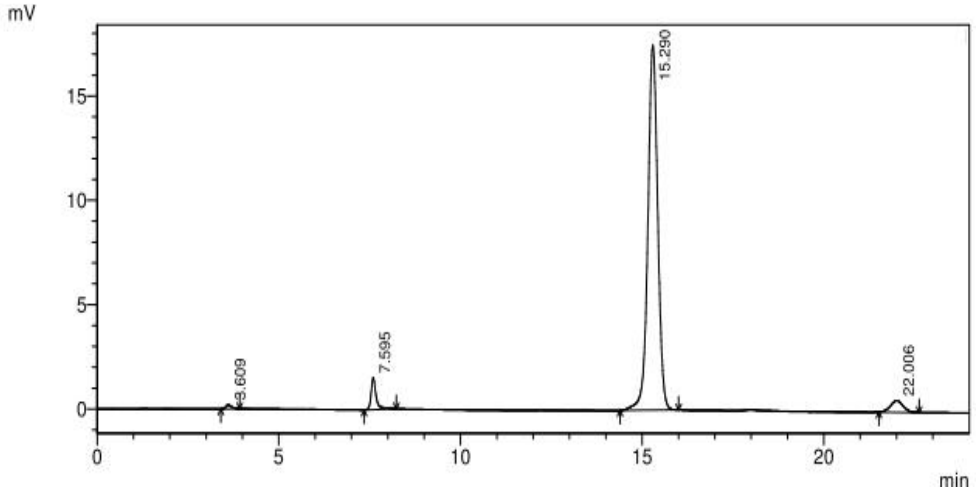

Figure N.11 HPLC chromatogram of lactic acid as standard

Table N.3 Retention times of organic acid on HPLC chromatogram

Organic Acids	Retention Time (min)
Oxalic Acid	9.15
Citric Acid	10.04
Glucoronic Acid	10.62
Malic Acid	11.06
Pyruvic Acid	11.90
Lactic Acid	15.30
Itaconic Acid	16.34
Acetic Acid	18.42
Fumaric Acid	19.97

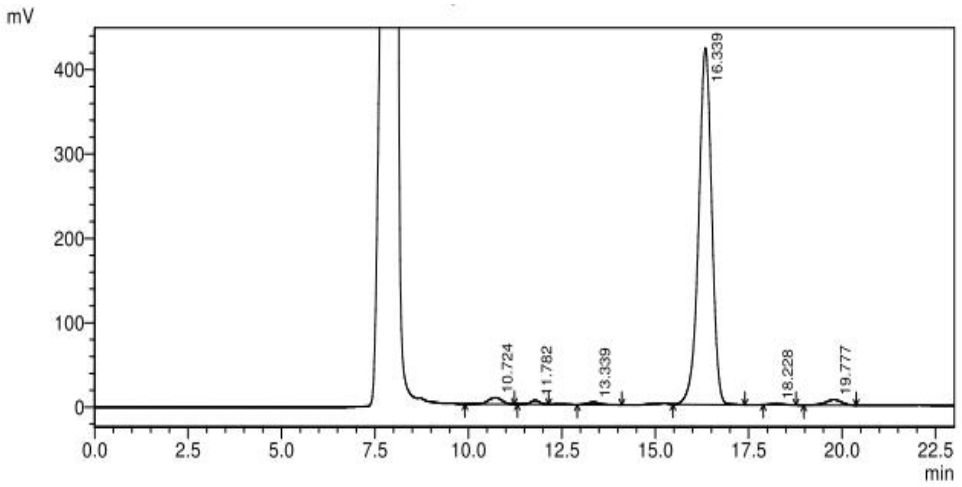

Figure N.12 HPLC chromatogram of 10% glucose containing M2-P sample at 1st day

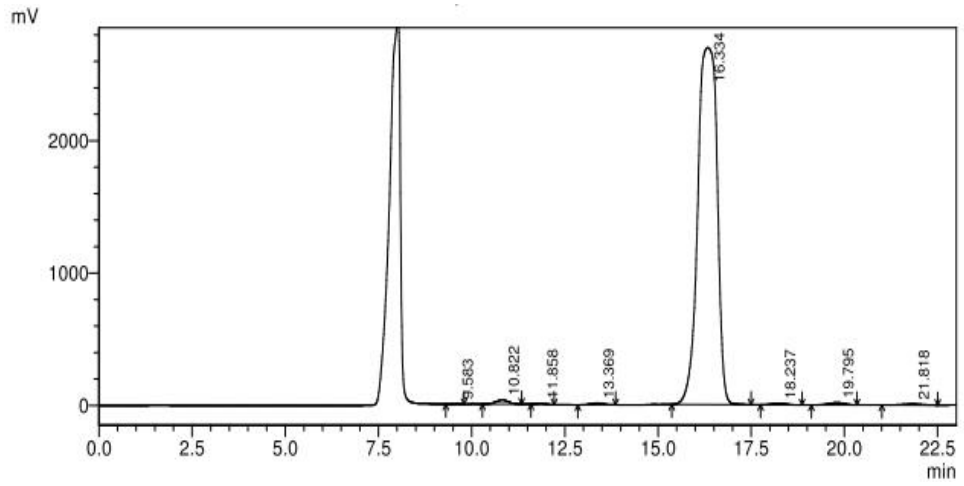

Figure N.13 HPLC chromatogram of 10% glucose containing M2-P sample at 2nd day

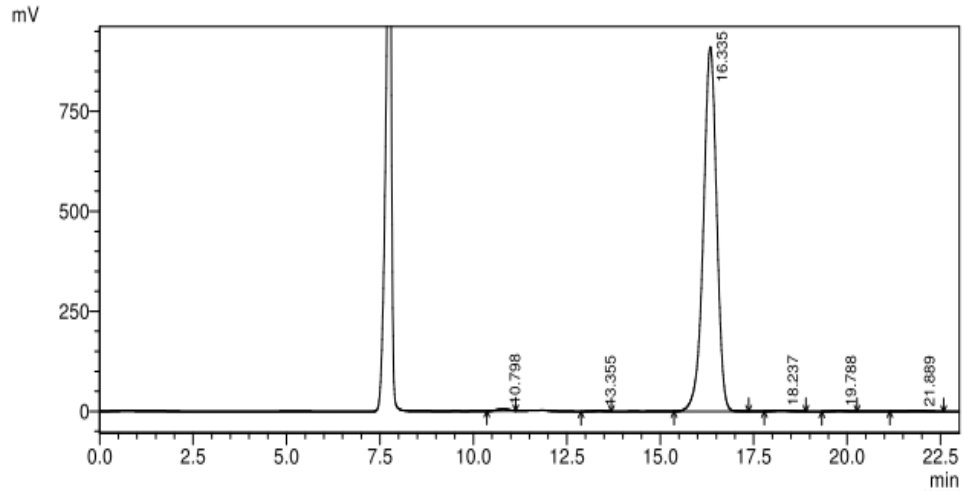

Figure N.14 HPLC chromatogram of 10% glucose containing M2-P sample at 3rd day (DF. 5)

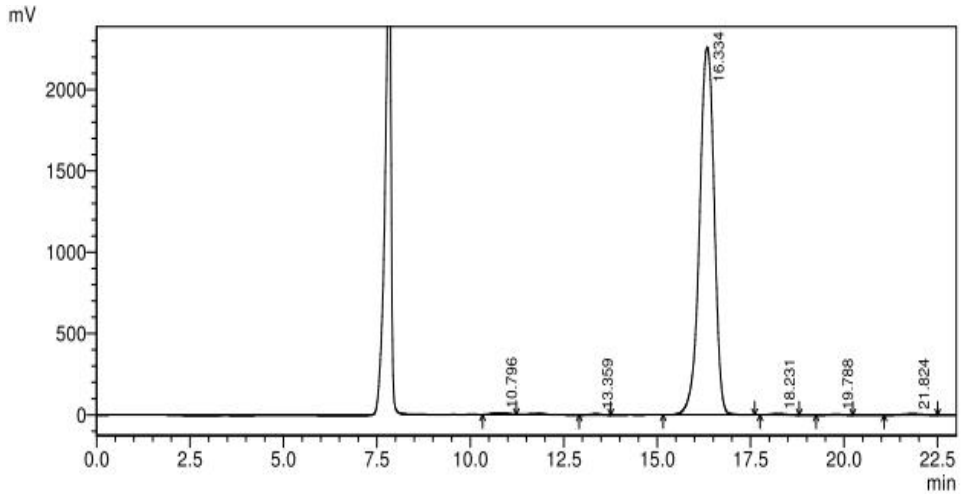

Figure N.15 HPLC chromatogram of 10% glucose containing M2-P sample at 4th day (DF. 5)

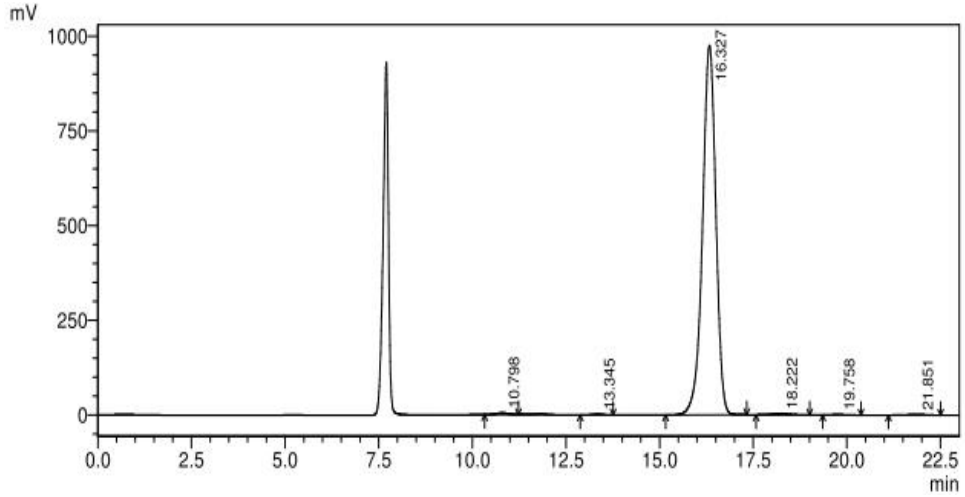

Figure N.16 HPLC chromatogram of 10% glucose containing M2-P sample at 5th day (DF. 10)

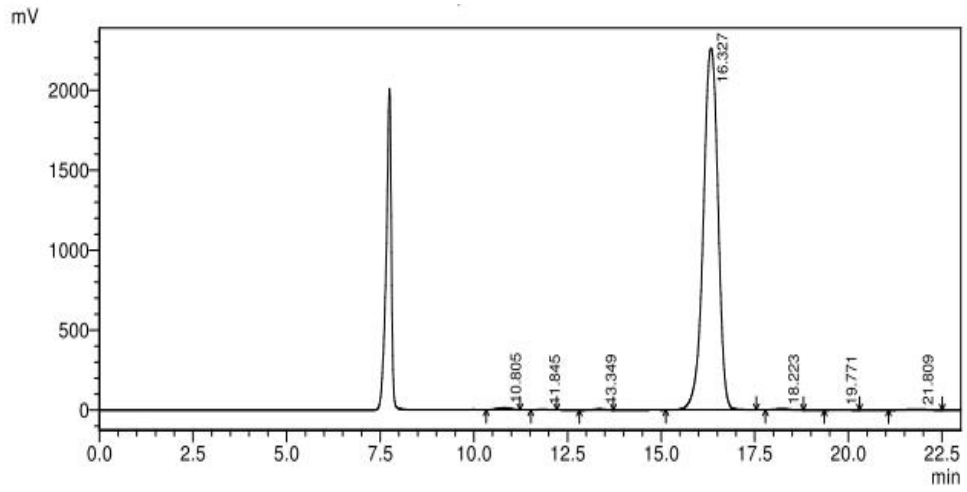

Figure N.17 HPLC chromatogram of 10% glucose containing M2-P sample at 6th day (DF. 10)

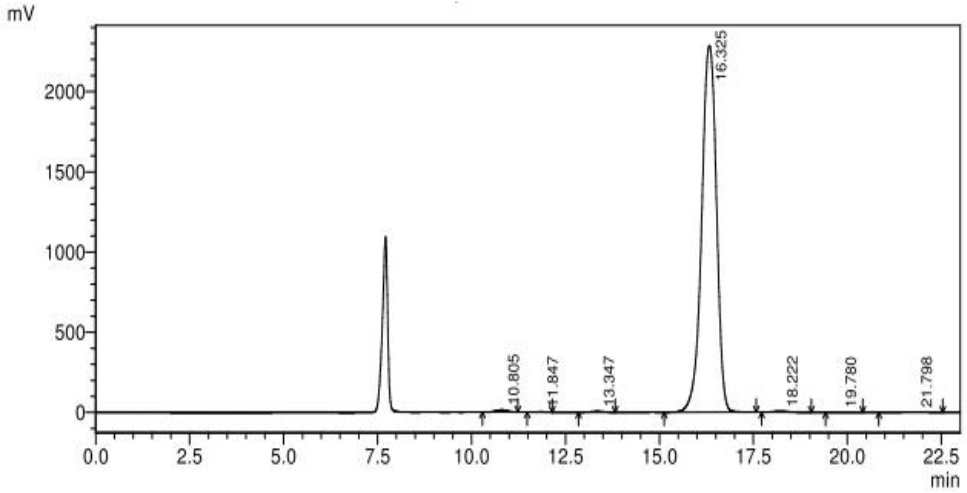

Figure N.18 HPLC chromatogram of 10% glucose containing M2-P sample at 7th day (DF.15)

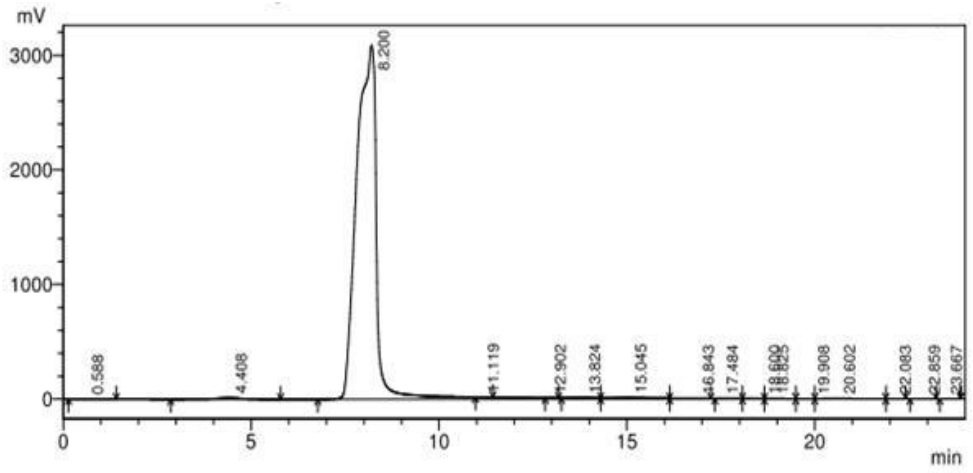

Figure N.19 HPLC chromatogram of 7% corn cob containing medium sample on 5th day

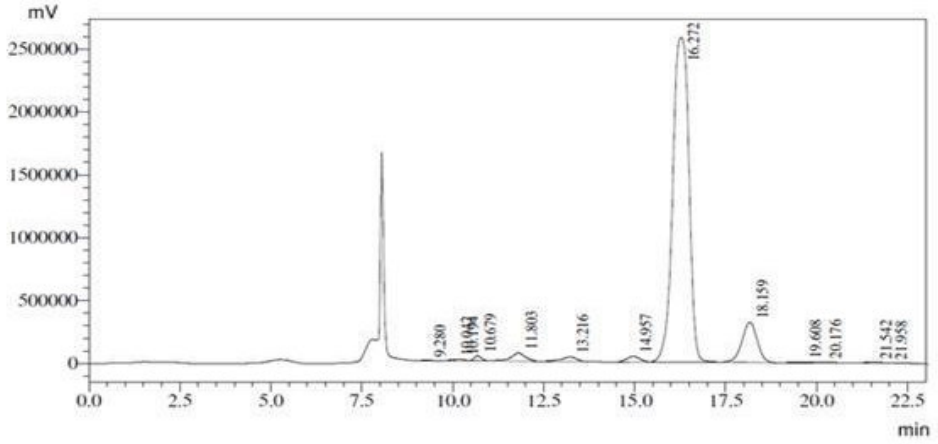

Figure N.20 HPLC Chromatogram of 10% glucose containing new medium sample at 5th day (DF. 15)

APPENDIX O

PURIFICATION OF XYLANASE

System: ÄKTA Prime FPLC system (Amersham Biosciences, USA)

Column: HiLoad 16/10 Phenyl Sepharose High Performance column (20 ml, 1.6 cm x 10 cm) (Amersham Biosciences, USA)

Operation parameters

Flow rate: 3 ml/min

Equilibration: 50 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl

Elution: Applying a linear gradient of 1.0-0 M NaCl in the same buffer

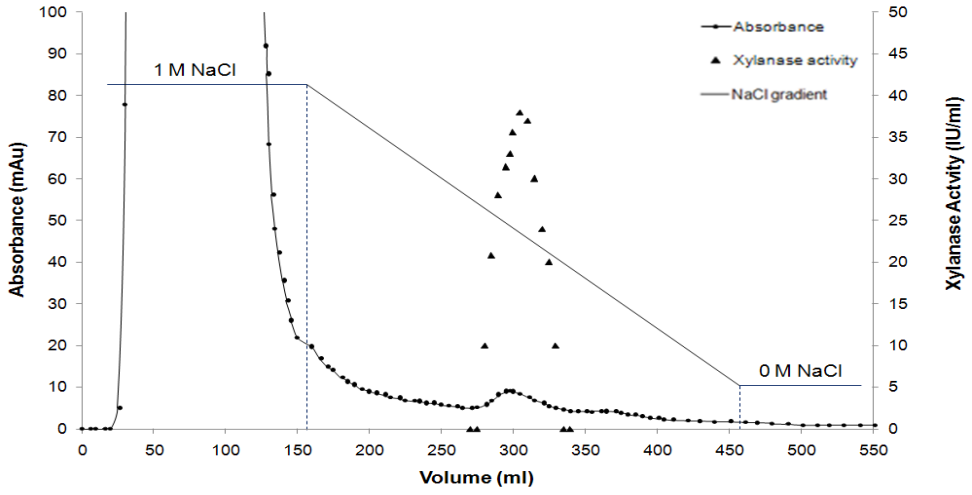

Figure O.1 *A. terreus* NRRL 1960 xylanase purification

Table O.1 Purification of xylanase from *A. terreus* NRRL 1960

Steps	Volume (ml)	Protein (µg/mL)	Total Protein (µg)	Activity (IU/mL)	Total Activity (IU)	Specific Activity (IU/µg)	Purification Fold	Yield (%)
Culture supernatant	75	14.0	1052.6	28.1	2108	2	1	100
HIC	44	0.8	35.2	29.4	1294	37	19	61

CURRICULUM VITAE

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2001	Hacettepe Oncology Institution	Summer Practice

FOREIGN LANGUAGES

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PUBLICATIONS

1. Yasinok AE, Biran S, Kocabas A, Bakir U, "Xylanase from a Soil Isolate, Bacillus pumilus: Gene Isolation, Enzyme Production, Purification, Characterization and One-Step Separation by Aqueous-Two-Phase System", World Journal of Microbiology and Biotechnology, available online

IN PREPARATIONS

1. Kocabas A, Sutay Kocabas D, Bakır U, One-step purification and characterization of a low molecular weight xylanase from *Aspergillus terreus* NRRL 1960

2. Kocabas, A., Ogel, Z.B., Bakır, U, Co-production of xylanase and itaconic acid by *Aspergillus terreus* NRRL 1960 from agricultural biomass

CONGRESSES

XIV. European Congress on Biotechnology (2009), Barcelona-Spain, Poster Presentation: "Optimization of xylanase production from *Aspergillus terreus* by using renewable agricultural lignocellulosic residues"

XVI. National Biotechnology Congress (2009), Antalya-Turkey, Poster Presentation: "Tarımsal atıklar kullanılarak *Scytalidium thermophilum* ksilanazının üretimi, saflaştırılması ve karakterizasyonu"

XV. National Biotechnology Congress (2009), Antalya-Turkey, Poster Presentation: "*Aspergillus terreus* itakonate üretiminin biyoreaktor sisteminde optimizasyonu"

VIII. National Chemical Engineering Congress (2008), Malatya-Turkey, Poster Presentation: "Toprak izolatı *Bacillus pumilus* SB-M13 kullanılarak ksilanaz üretimi için NaCl, $MgSO_4$ ve KH_2PO_4 optimizasyonu"

Nanomat (2007), Antalya-Turkey, Poster Presentation: "Preparation and characterization of surface modified magnetic γ-fe2o3 (maghemite)-silica nanocomposites used for the immobilization of invertase enzyme"

VII. National Chemical Engineering Congress (2006), Eskisehir-Turkey, Poster Presentation: "Zirai atık kullanılarak bir *Bacillus* izolatından ksilanaz üretimi"

GAP V. Engineering Congress (2006), Sanliurfa-Turkey, Oral Presentation: "Hasat sonrası pamuk bitkisinin katma değeri yüksek ürünlere biyodönüşümü"

XIV. National Biotechnology Congress (2005), Eskisehir-Turkey, Poster Presentation: "Toprak izolatı *Bacillus* M 13 kullanılarak ksilanaz üretimi için besi yeri optimizasyonu"

XII. European Congress on Biotechnology (2005), Copenhagen-Denmark, Poster Presentation: "Xylanase production, purification and characterization from a soil isolate *Bacillus M-13*"

WORKSHOPS

Biomedical Materials and Turkey, Biomaterials and Tissue Engineering Foundation, Middle East Technical University, Ankara-Turkey, May 2010

Metabolomics (Lectures and Practicals), Wageningen University, Wageningen, The Netherlands, June 2007

Safe Handling with Radioactive Materials and Sources, (Entrusted with a final exam), Training and Consultancy Van Hall Larenstein Part of Wageningen University, Wageningen, The Netherlands, 2006

One-Day Practical Bioinformatics, Harran and Suny Universities, Sanliurfa-Turkey, 2006

Microarray Training Course, Middle East Technical University, Ankara-Turkey, 2006

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