PRETREATMENT AND ENZYMATIC HYDROLYSIS OF LIGNOCELLULOSIC BIOMASS

by

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B.S., National University of Colombia,Bogotá, 2000
MS. Kansas State University, 2005

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Biological and Agricultural Engineering
College of Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

Abstract

The performance of soybean hulls and forage sorghum as feedstocks for ethanol production was studied. The main goal of this research was to increase fermentable sugars' yield through high-efficiency pretreatment technology. Soybean hulls are a potential feedstock for production of bio-ethanol due to their high carbohydrate content (≈50%) of nearly 37% cellulose. Soybean hulls could be the ideal feedstock for fuel ethanol production, because they are abundant and require no special harvesting and additional transportation costs as they are already in the plant. Dilute acid and modified steam-explosion were used as pretreatment technologies to increase fermentable sugars yields. Effects of reaction time, temperature, acid concentration and type of acid on hydrolysis of hemicellulose in soybean hulls and total sugar yields were studied. Optimum pretreatment parameters and enzymatic hydrolysis conditions for converting soybean hulls into fermentable sugars were identified. The combination of acid (H_2SO_4, 2% w/v) and steam (140 °C, 30 min) efficiently solubilized the hemicellulose, giving a pentose yield of 96%.

Sorghum is a tropical grass grown primarily in semiarid and dry parts of the world, especially in areas too dry for corn. The production of sorghum results in about 30 million tons of byproducts mainly composed of cellulose, hemicellulose, and lignin. Forage sorghum such as brown midrib (BMR) sorghum for ethanol production has generated much interest since this trait is characterized genetically by lower lignin concentrations in the plant compared with conventional types. Three varieties of forage sorghum and one variety of regular sorghum were characterized and evaluated as feedstock for fermentable sugar production. Fourier transform infrared spectroscopy (FTIR), scanning electron microscope (SEM) and X-Ray diffraction were used to determine changes in structure and chemical composition of forage sorghum before and after pretreatment and enzymatic hydrolysis process. Up to 72% of hexose yield and 94% of pentose yield were obtained using "modified" steam explosion with 2% sulfuric acid at 140°C for 30 min and enzymatic hydrolysis with cellulase (15 FPU/g cellulose) and β-glucosidase (50 CBU/g cellulose).

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Approved by:

Major Professor
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Finally, thanks to life for love, friends, science, coffee and everything…

Dedication

To the love of my life and my best friend

Juan Manuel

To my parents, my first and only heroes

Luis Antonio and Bertha

To my aunt, first PhD in my family and my inspiration

Dr. Berenice Guerrero

To my brother and his “magical realism” and my adorable nieces

Freddy, Laura Sofia and Sara Valentina

To the Republic of Colombia and its National University

CHAPTER 1 - Introduction

In 2007, about 6.5 billion gallons of fuel ethanol were produced by approximately 100 ethanol plants in the U.S. The United States needs more than 140 billion gallons of fuel for automobiles alone. At present, ethanol is primarily produced from corn. Using 100% of the 2007 corn crop (13.1 billion bushels) for ethanol production would only produce 35 billion gallons of fuel, which would only meet about 16% of our needs. Obviously, other feedstocks for ethanol production are needed. Ethanol production from lignocellulosic materials such as agricultural residues, wood, municipal solid wastes, and wastes from the pulp and paper industry is a major global task in producing liquid fuel by sustainable processes (6). These materials represent an abundant, low-cost, and largely unused source of raw materials for the production of fuel ethanol (7, 8). It is estimated that America can sustainably supply 1.3 billion tons of biomass, i.e. cellulosic material a year. That would equate to approximately 60 billion gallons of annual ethanol production (1).

Soybean hulls and forage sorghum are potential feedstocks for production of bio-ethanol because of their high carbohydrate content (≈50-60%). Soybean hulls could be the ideal feedstock for fuel ethanol production, because they are abundant and require no special harvesting and additional transportation costs as they already in the plant. About 1.8 billion bushels of soybeans are crushed for oil, protein, and soy flour production in the U.S each year. This will generate about 1 billon pounds of soybean hulls. It could be calculated that 88.1 gal of ethanol can be produced from each dry ton of soybean hulls.

Sorghum is a tropical grass grown primarily in semiarid and drier parts of the world, especially in areas too dry for corn. Sorghum cannot compete successfully with corn as a cereal in an agro-ecosystem with 900 mm or more of annual rainfall, but corn cannot replace sorghum in areas that receive less than 900 mm of rainfall. Thus, most domestic sorghum acreage is in the Southern Great Plains states with Kansas, Texas, Oklahoma and Nebraska being the leading producers. Utilization of forage sorghum for ethanol could produce ethanol yields up to 100 gal EtOH/dry ton of forage sorghum. A large opportunity exists for sorghum to contribute to our bio-energy production in sorghum-growing States. However, at present, we do not have enough

scientific information and knowledge about the use of sorghum stover, especially forage sorghum for biofuel production.

The goal of this research was to study the potential and performance of biomass products as feedstocks for ethanol production and to increase ethanol yield through increasing fermentable sugar recovery with high-efficiency pretreatment technology. Dilute acid and steam explosion as pretreatment technology were used to increase sugar recovery from soybean hulls and forage sorghum. The effect of pretreatment on enzymatic hydrolysis, final sugar yield, and ethanol fermentation were studied.

Results from this research will allow us to confirm the potential impact of using soybean hulls and forage sorghum in biofuels production and will lead to 1) capabilities to improve utilization of soybean hulls and forage sorghum as feedstock for biofuels production, and 2) improvement in biomass conversion yields from soybean hulls and forage sorghum.

General background

Use of renewable biomass, which contains a significant amount of carbohydrates such as starch, hemicellulose, and cellulose, to produce energy carriers such as transportation fuel is well recognized (9-11). Ethanol use is growing as a "clean" substitute for direct use as fuel, which can ease both natural resource limitations and environmental pollution (12). Annual fuel ethanol production in the United States was 6.5 billion gallons in 2007 (Figure 1.1). The U.S. accounts for about 35% of the world's total production (Figure 1.2) (1). The production and use of nearly 5 billion gallons of ethanol in 2006 reduced dependency on imported oil by 170 million barrels. The U.S. fuel ethanol industry is experiencing unprecedented growth. Capacity of U.S. fuel ethanol production is projected to increase to 10 billion gallons by the end of 2010 (1).

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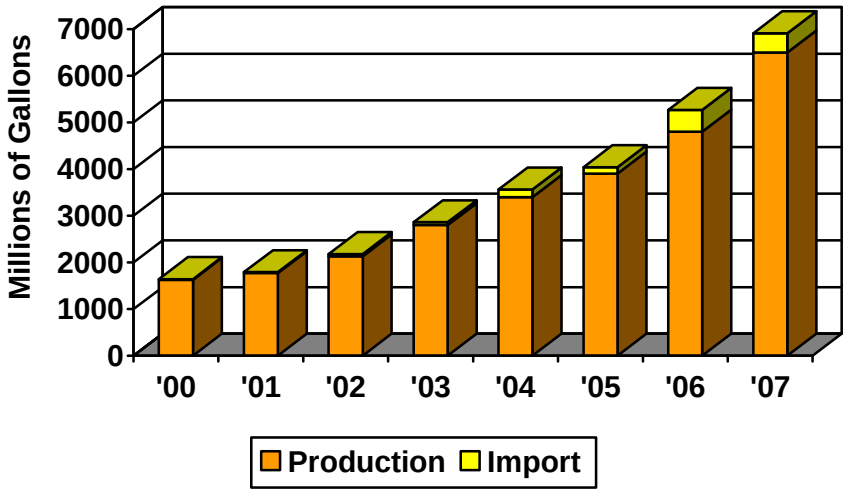


Figure 1.1 U.S fuel ethanol production (1)**.**

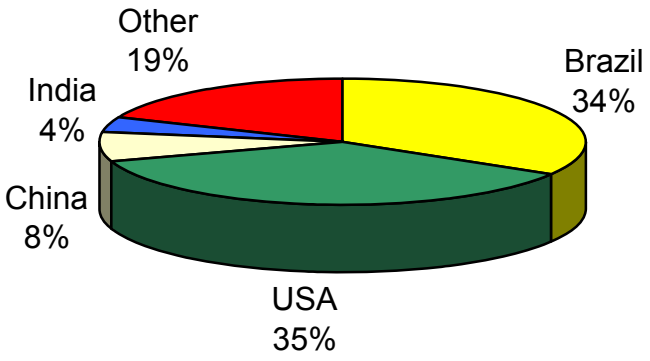


Figure 1.2 Annual world ethanol production in 2007 (1)**.**

The U. S. consumes more than 140 billion gallons of gasoline for automobiles alone. Using 100% of the 2007 corn crop for ethanol production would only produce 35 billion gallons of fuel; this would only meet about 16% of our needs. Conversion of cellulosic biomass such as agricultural residues to fuels and chemicals offers major economic, environmental, and strategic benefits, and biological processing based on cellulases offers high sugar yields vital to economic success. DOE and USDA projected that U.S. biomass resources could provide approximately 1.3 billion dry tons of feedstock for biofuels, which could meet about 40% of annual U.S. fuel

demand for transportation (13). Currently, there are 139 ethanol biorefineries operating in the United States with more than 7.88 billion gallons of annual capacity. There are 55 new refineries under construction, with seven expansions as well, with a combined total of more than 13.4 billion gallons of annual capacity (1). According to the 2005 Energy Policy Act and Renewable Fuel Standard (2005), the following is the goal for the biobased transportation fuels: increase from the current 2.5% to 4% in 2010, to 10% in 2020, and to 20% by 2030 (13).

A major problem in the hydrolysis of lignocellulosic materials is the natural resistance of the hemicellulose and cellulose toward conversion to fermentable sugars. To improve the efficiency of enzymatic hydrolysis, a pretreatment step is necessary to make the cellulose fraction accessible to cellulose enzymes (14). Delignification, removal of hemicellulose, and decreasing the crystallinity of cellulose produce more reachable surface area for cellulose enzymes to react with cellulose (7). There are no reported approaches to converting forage sorghum and soybean hulls to value-added products, especially to fermentable sugars for ethanol production. However, it would be possible to combine pretreatment procedures with enzyme hydrolysis to obtain fermentable sugars for ethanol production. The purpose of pretreatment is to alter the microscopic size and structure of the biomass, as well as its submicroscopic chemical composition and structure, so that hydrolysis of the carbohydrate fraction to monomeric sugars can be attained more rapidly and with greater yields (6). Although there are several methods available for biomass pretreatment, in general the selectivity of methods is highly restricted by the nature of the raw materials. Therefore, the goal of this proposed research is to design and evaluate selected pretreatment methods and optimize pretreatment conditions for bioconversion of soybean hulls and forage sorghum into fermentable sugars, and to identify the best option for hydrolysis of these materials using enzymes. The proposed research strongly supports our national goals for bio-energy, sustainable economic development, and especially strengthens our rural economies in agricultural areas.

Objectives

The overall objective of this research was to enhance the economic attractiveness of lignocellulosic materials for production of bio-fuels through developing an advanced method to hydrolyze soybean hulls and forage sorghum to fermentable sugars. Specific objectives of this proposed research are as follows:

Objective 1

To understand the effect of chemical composition and microstructure of soybean hulls and forage sorghum on hydrolysis process.

Objective 2

To study and identify the optimum pretreatment methods such as high-pressure injection, steam explosion and diluted acid on fermentable sugar yield from soybean hulls and forage sorghum.

Objective 3

To study the formation of inhibitors produced during pretreatment and understand their effects on enzymatic hydrolysis.

Objective 4

To study and optimize enzymatic hydrolysis of soybean hulls and forage sorghum into fermentable sugars.

Objective 5

To study the effect of the combination of pretreatment and enzymatic hydrolysis of soybean hulls and forage sorghum on preliminary ethanol fermentation.

CHAPTER 2 - Literature Review

Almost all ethanol production in the United States is based on technology that converts starch contained in agricultural crops into sugars, which are then fermented to ethanol. However, lignocellulosic materials have been also identified as potential feed-stocks, in view of their ready availability and low cost. Fermentable fractions of these feed-stocks include cellulose and hemicellulose. The structure of these materials is highly complex, and native biomass is resistant to enzymatic hydrolysis. Although it is an abundant biopolymer, cellulose is highly crystalline, water insoluble, and highly resistant to depolymerization. Utilization of cellulosic sugars faces significant technical challenges. The success of using those cellulosic sugars depends largely upon the physical and chemical properties of biomass, pretreatment methods, effective microorganisms, and optimization of processing conditions. Therefore, efficient conversion of lignocellulose to ethanol is essential (15).

Lignocellulosic biomass

Cellulose

Cellulose is a linear polymer of D-glucose units linked by β-1, 4-linked glucose. Cellulose molecules are completely linear and have a strong tendency to form intra and intermolecular hydrogen bonds (Figure 2.1a). Bundles of cellulose molecules are thus aggregated together in the form of micro-fibrils, in which highly ordered (crystalline) regions alternate with less ordered (amorphous) regions (16). The crystalline region in which the linear molecules of cellulose are bonded laterally by hydrogen bonds is characterized by the cellulose lattice which extends over the entire cross-section of the micro-fibrils. This crystalline region is bounded by a layer of cellulose molecules that exhibit various degrees of parallelism. The less ordered region is called the paracrystalline or amorphous region. The disordered region allows disintegration of the cellulose by hydrolysis into rod-like particles with aqueous, non-swelling, strong acid (17). Micro-fibrils build up fibrils and finally cellulose fibers. As a consequence of its fibrous structure and strong hydrogen bonds cellulose has a high tensile strength and is insoluble in most solvents (16). Orientation of the linkages and additional hydrogen bonding makes the polymer

rigid and difficult to break (18). The molecular arrangement of this fibrillar bundle is sufficiently regular that cellulose exhibits a crystalline X-ray diffraction pattern (17). Typically, cellulose chains in primary plant cell walls have degrees of polymerization in the range of 5,000 to 7,500 glucose monomer units, with the degree of polymerization of cellulose from wood being around 10,000 and around 15,000 from cellulose cotton. The basic repeating unit of cellulose is cellobiose. Under normal conditions, cellulose is extremely insoluble in water, which is of course necessary for it to function properly as the structural framework in plant cell walls (9).

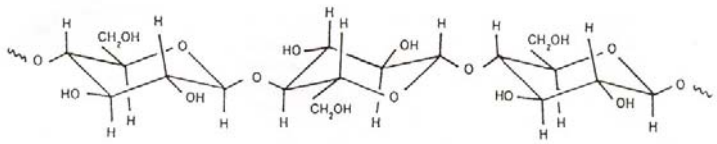
An important structural feature that affects the rate of enzymatic hydrolysis of cellulose fibers is the degree of crystallinity of cellulose (17). The crystallinity of native cellulose was experimentally determined by Segat et al. with an X-ray diffractometer using the focusing and transmission techniques (19). They measured the intensity of the 002 interference and the amorphous scatter at $2\theta = 18°$. The fraction of crystalline material in the total cellulose was expressed in terms of an X-ray crystallinity index (CrI).

Hemicellulose

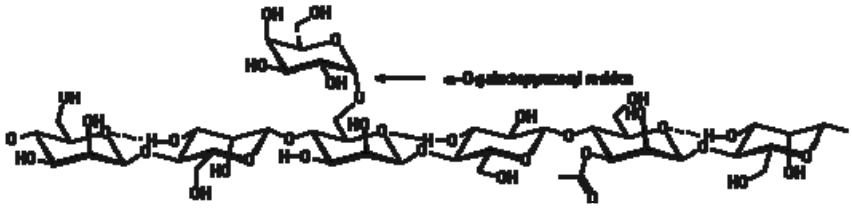
Hemicelluloses were originally believed to be intermediates in the biosynthesis of cellulose. Today it is known, however, that hemicelluloses belong to a group of heterogeneous polysaccharides which are formed through biosynthetic routes different from that of cellulose. In contrast to cellulose which is a homopolysaccharide, hemicelluloses are heteropolysaccharides (16). Hemicelluloses are heterogeneous polymers of pentoses (xylose, arabinose), hexoses (mannose, glucose, galactose), and sugar acids (Figure 2.1b). They are generally cataloged according to the main sugar residue in the backbone, e.g., xylans, mannans, and glucans, with xylans and mannans being the most common (9). Hemicellulose, because of its branched, amorphous nature, is relatively easy to hydrolyze (18). Some hemicelluloses contain mostly xylan, whereas others contain mostly glucomannans. Among softwood hemicelluloses there are galactoglucomannans, arabinoglucuronoxylan, and arabinogalactan, meanwhile hardwood hemicellulose comprises mainly glucuronoxylans and glucomannan (16). Besides xylose, xylans contain arabinose, glucuronic acid, 4-O methyleter, acetic, ferulic, and p-coumaric acids. For example, corn fiber xylan is one of the complex heteroxylans containing $\beta - (1, 4) -$ linked xylose residues. It contains 48-54% xylose, 33-35% arabinose, 5-11 % galactose, and 3-6% glucuronic acid (8).

Lignin

Lignin is a long-chain, heterogeneous polymer composed largely of phenyl propane units most commonly linked by ether bonds (8). It is presented in all lignocellulosic biomass; therefore, any ethanol production process will have lignin as a residue (18). It is a large, complex polymer of the phenylpropane and methoxy groups, a non-carbohydrate polyphenolic substance that encrusts cell walls and reinforces cells together (Figure 2.1c) (18). Lignins can be divided into several classes according to their structural elements. So-called "guaiacyl lignin" which occurs in almost all soft woods is largely a polymerization product of coniferyl alcohol. The "guaiacyl-siryngyl", typical of hardwoods, is a copolymer of coniferyl and sinapyl alcohols, the ratio varying from 4:1 to 1:2 for the two monomeric units (16). Guaiacyl-lignins have a methoxy-group in both the 3-carbon and 5-carbon positions (4). Lignin effectively protects the plant against microbial attack and only a few organisms, including rot-fungi and some bacteria, can degrade it. Lignin restricts hydrolysis by shielding cellulose surfaces or by adsorbing and inactivating enzymes. It was understood that the close union between lignin and cellulose prevented swelling of the fibers, thereby affecting enzyme accessibility to the cellulose. To solve this problem, several studies have shown that taking away lignin enhances cellulose hydrolysis (9). The conversion of cellulose and hemicellulose to fuels and chemicals generates lignin as a by-product. Such by-product can be burned to provide heat and electricity, or used to manufacture various polymeric materials (8). There are some publications on microbial breakdown of lignin; however, due to extreme complexity of the problem, a vast amount of research needs to be done (8).



(a)



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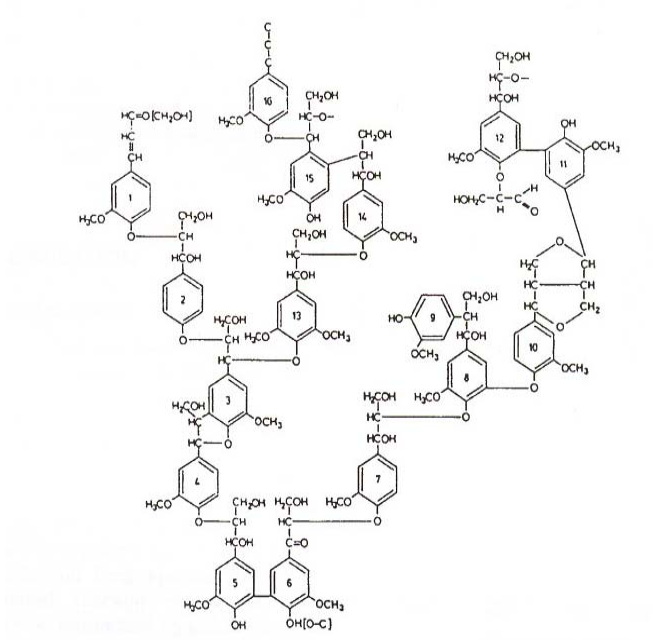


(c)

Figure 2.1 Schematic diagram of a representative section of the molecular structure of (a) cellulose, (b) hemicellulose, and (c) softwood lignin (2)

Soybean hull

Soybeans [*Glycine max* (L.) Merrill, family leguminosae, subfamily Papilionoidae] have continued as an important agricultural crop for almost every temperate-climate civilization because of their extraordinarily high content of both triglyceride oil and protein (20). The United States produces more than 38% of the world's soybean production with 3188 million bushels in 2006 (Figure 2.2 and Figure 2.3) (3) .

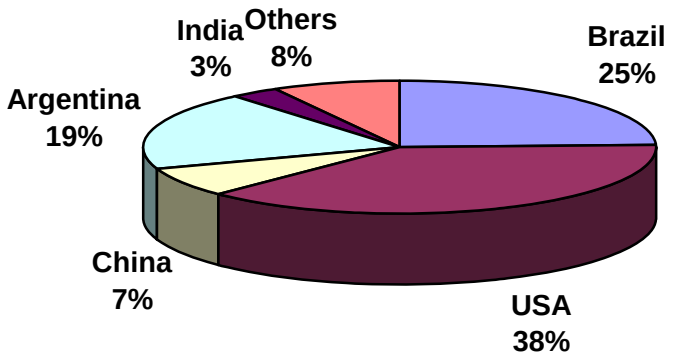


Figure 2.2 World soybean production in 2006 (3)

Soybeans are the largest single source of edible oil and account for roughly 50% of total oilseed production in the world (21). For soybean oil production, soybeans are cracked, dehulled, and rolled into flakes. This ruptures the oil cells for efficient extraction. After removal of the soybean oil, the remaining flakes can be used to produce soybean meal for animal feed. Hulls from soybeans are an important by-product of the soybean oil industry. Estimated yield of soybean hulls is approximately 10% of the original raw soybean weight (22). About 10.8 billion bushels of soybeans are crushed for oil, protein, and soy flour production in the U.S. This will generate about 1 billon pounds of soybean hulls.

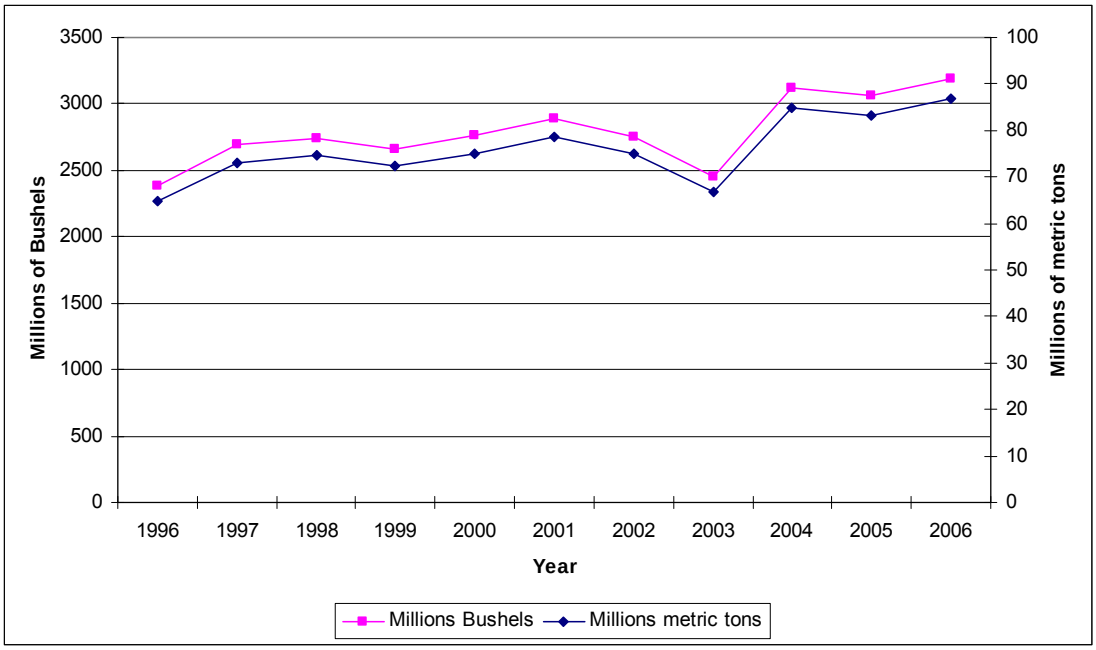


Figure 2.3 U.S. soybean production (1994-2006) (3)

The commercial value of soybean hulls has been considered much less important than soy oil and protein. As a result, soy carbohydrates have been traditionally used as animal feed and relatively few efforts have been made to study soy carbohydrates and their potential utilization (23). With rapid increase in DDGS from ethanol production using corn and grain sorghum, demand for soybean hulls for animal feed is anticipated to decrease over the next decade. An increase in biodiesel production from soybean oil would cause an oversupply of soybean hulls as a by-product. Therefore, additional use of the soybean fiber stream for the soybean industry is needed. The monomers of soy carbohydrates from soybean hulls are sugars, which can be used as substrates for bioconversion to produce chemicals and biofuels. Hulls removed from soybeans can be classified as lignocellulosic material since the major composition of carbohydrates in soybean hulls are structural components in the cell walls, including cellulose and hemicellulose. Soybean hulls are a potential feedstock for ethanol fermentation because of their high carbohydrate content (24).

Soybean hulls contain up to 50% cellulose, 10-15% hemicellulose, and 8-14% pectin (24-26). One unique aspect of soybean hulls is that lignin content is low in the fiber, which facilitates access to the hemicellulose and cellulose fraction (22). In this study, soybean hulls were found to contain about 50% fermentable sugars with approximately 12% hemicellulose, 36% cellulose,

and 2% starch. The chemical composition of soybean hulls used for this research is summarized in Table 2.1. Utilization of soybean hulls provides an economic and affordable substitute for ethanol production. Theoretical ethanol yield could be up to 88.1 gal of ethanol from each dry ton of soybean hulls.

Table 2.1 Chemical composition of soybean hulls

Component	%
Crude protein	14.2 ± 0.10
Crude fat	3.2 ± 0.03
Crude fiber	32.3 ± 0.32
NDF	49.0 ± 0.30
ADF	36.6 ± 0.11
ADL	0.2 ± 0.02
Starch	1.8 ± 0.08
Ash	4.2 ± 0.03
Hemicellulose	12.5 ± 0.32
Cellulose	36.4 ± 0.09
Total carbohydrates	50.7

Forage sorghum

Sorghum is a tropical grass grown primarily in semiarid and drier parts of the world, especially in areas too dry for corn. Sorghum cannot compete successfully with corn as a cereal in an agro-ecosystem with 900 mm or more of annual rainfall, but corn cannot replace sorghum in areas that receive less than 900 mm of rainfall. Thus, most of the domestic sorghum acreage is in the southern Great Plains states, with Kansas, Texas, Oklahoma, and Nebraska being the leading producers. Sorghum produces 33% more dry mass than corn in dry land. However, sorghum silage contains less grain and is higher in fiber than corn silage. Though the protein content of sorghum silage is similar to or slightly higher than that of corn, it is less digestible. Animal consumption of sorghum silage is also generally somewhat less than that of corn (27).

Sorghum is one of the major food crops of the world and about 14 million metric tons of sorghum grains were produced in U.S in 2007 (3). The production of sorghum also results in about 30 million tons of byproducts mainly composed of cellulose, hemicellulose, and lignin. (3) Since sorghum has the advantage that it can be grown in dry and arid climates, using sorghum residues will help to add value to the crop and develop new alternatives for biofuel production.

Forage sorghum is a member of the sorghum family and is closely related to grain sorghum, broom corn, sorghum sudan grass, and sudan grass. Forage sorghum is best adapted to warm regions and is particularly noted for its drought tolerance compared to corn. Forage sorghum (heads, leaves, and stems) grows 6 to 12 ft tall, produces more dry matter tonnage than grain sorghum, is coarse stemmed, and is traditionally produced and used for silage animal feed, which is also a viable renewable resource for ethanol production (27). In our study, forage sorghum (stems and leaves) was found to contain about 60% fermentable sugars with about 15% hemicellulose, 30% cellulose, and 15% starch, respectively as shown in Table 2.2. Utilization of forage sorghum for ethanol production would be able to obtain ethanol yields up to 100 gal EtOH/dry ton of forage sorghum.

Table 2.2 Chemical composition (%) of forage sorghum

Component	Sample			
	FS- 1	FS- 2	FS- 3	RS
Carbohydrates	66.22	62.48	59.44	59.93
Starch	8.13b	6.80c	22.91a	0.84d
Hemicellulose	22.48a	17.64c	12.32d	20.37b
Cellulose	35.51b	38.04a	24.21c	38.72a
Total amount of Lignin	13.46b	16.51a	13.58b	16.79a
Klason Lignin	14.63bc	19.14ab	11.06c	20.47a
Crude Fat	1.08b	1.07b	1.68a	1.14b
Crude Fiber	34.02b	36.87a	20.80d	29.43c
Crude Protein	5.16b	4.13c	7.46a	3.88d
Ash	9.29c	10.87a	6.93d	9.98b

Pretreatments

Pretreatment is an important tool for practical cellulose conversion processes and is crucial before enzymatic hydrolysis can take place, effectively. It is necessary in order to alter the structure of cellulosic biomass, to make cellulose more accessible to enzymes that convert carbohydrate polymers into fermentable sugars. The goal of pretreatment is to break the lignin seal, solubilize hemicellulose, and disrupt the crystalline structure of cellulose. A small amount of sugars from the hemicellulose may also be released during the pretreatment process (7). Pretreatment has been viewed as one of the most expensive processing steps in cellulosic-biomass-to-fermentable-sugars conversion, which costs as much as 30¢/of each gallon of ethanol produced (6).

Various pretreatment options are available now to fractionate, solubilize, hydrolyze, and separate cellulose, hemicellulose, and lignin components. These include steam explosion, dilute acid treatment, concentrated acid treatment, alkaline treatment, treatment with SO_2, treatment with hydrogen peroxide, ammonia fiber explosion, and organic solvent treatments.

In each option, the biomass is treated to reduce its size and open its structure. Pretreatment usually hydrolyzes hemicellulose to the sugars (xylose, L-arabinose, and others) that are water soluble (11).

Steam explosion

Steam explosion and dilute acid pretreatment are the most frequent methods for pretreatment of lignocellulosic materials, and each has been studied by many scientists and engineers. Steam explosion involves treatment of ground biomass with high-pressure saturated steam, followed by a rapid reduction of steam pressure to obtain an explosive decompression (7). Steam pretreatment effectively enhances the conversion rate of carbohydrates into fermentable sugars (28). It also improves accessibility of the cellulose and increases enzymatic hydrolysis yield (29). Residue from steam explosion contains cellulose and lignin. The lignin can be extracted with solvents such as ethanol, butanol, or formic acid (8). However, the disadvantage is loss of free sugar due to washing and purification (30). Optimum conditions of the steam explosion procedure vary with type of feedstock. Highly severe reaction conditions may result in full removal of the hemicelluloses and provide highly digestible solids, as well as partially solubilizing the cellulose fraction. However, it may also result in sugar degradation. Too mild conditions, on the other hand, may produce a low yield of oligomeric-hemicellulose-derived sugars that need further hydrolysis before fermentation, and a cellulose fraction that is still resistant to hydrolysis (31). Uncatalyzed steam explosion refers to a pretreatment technique in which lignocellulosic biomass is rapidly heated by high-pressure steam without addition of chemicals. The biomass/steam mixture is held for a period of time to promote hemicellulose hydrolysis, and terminated by an explosive decompression (6). Hemicellulose is though to be hydrolyzed by the acetic and other acids released during steam explosion pretreatment. Steam provides an effective vehicle to rapidly heat cellulosic to the target temperature without excessive dilution of the resulting sugars. Sudden pressure release rapidly reduces the temperature and reduces the reaction at the end of the pretreatment (6).

Saha, (10) reported that steam pretreatment with 1% SO_2 (w/w) at 200 – 210 ºC was superior to other forms of pretreatment of willow. By steam explosion, optimal solubilization and degradation of hemicellulose can generally be achieved by both high temperature and short residence time (270 ºC, 1 min), or lower temperature and longer residence time (190 ºC, 10 min). Varga et al., (28), studied optimization of steam pretreatment of corn stover to enhance enzymatic digestibility. They reported that corn stover treated with 2% H_2SO_4 at 200 °C for 5 min resulted in the highest enzymatic conversion rate (from cellulose to glucose), which is four times greater than untreated material. De Bari et al., (32) investigated ethanol production from mixed-sugar syrups. Hydrolyzates were prepared from enzymatic saccharification of steam–pretreated aspen chips at 215 °C for 3 min. They obtained a yield of 0.39 g xylose /L.

Dilute acid

Dilute acid process is the oldest technology for converting cellulose biomass to ethanol (first commercial plant in 1898). The main step is essentially hemicellulose hydrolysis. In simple terms, acid catalyzes the breakdown of long hemicellulose chains to form shorter chain oligomers and then to sugar monomers that the acid can degrade. However, because hemicellulose is amorphous, less severe conditions are required to release hemicellulose sugars (33). Dilute acid pretreatment (0.2 – 3.0% sulfuric acid, >160 ºC) of native lignocellulose also can be used to increase the conversion rate of cellulosic biomass. Dilute acid pretreatments function through hydrolysis of the hemicellulose components to produce a syrup of monomeric sugars, exposure of cellulose for enzymatic digestion (removal of hemicellulose and part of the lignin), and solubilization of heavy metals that may be contaminating feedstocks (34). In general, dilute sulfuric acid is mixed with biomass and held at temperatures of 160-220 °C for periods ranging from minutes to seconds to hydrolyze hemicellulose to xylose and other sugars, and then continue to break xylose down to furfural (6). The limitation of dilute sulfuric acid is corrosion, which mandates expensive materials of construction. The acid must be neutralized before the sugars proceed to fermentation (6). Acid hydrolysis releases oligomers and monosaccharides and has historically been modeled as a homogeneous reaction in which acid catalyzes breakdown of cellulose to glucose followed by breakdown of the glucose released to form HMF and other degradation compounds (6).

Lavarack et al. studied dilute acid hydrolysis of baggase hemicelluloses to produce xylose, arabinose, glucose, acid-soluble lignin, and furfural (35). They reported that hydrolysis with H_2SO_4 can be carried out at elevated temperatures (80-200 ºC) for 2 – 60 min. They also found that H_2SO_4 is more efficient as a catalyst than hydrochloric acid (HCl) for the degradation of xylose. Saha et al. described a process for the hydrolysis and conversion of rice-hull cellulose and hemicellulose to monomeric sugars (36). They used dilute acid H_2SO_4 pretreatment at varied temperatures and enzymatic saccharification. Maximum yield of monomeric sugars by dilute acid pretreatment and enzymatic saccharification using commercial cellulases was 60% based on total carbohydrate content. Chung et al. evaluated the cellulose reactivity of two lignocellulosic feedstocks, switch grass and poplar, using dilute sulfuric acid pretreatments designed for optimum xylose yield (37). Yields (percentage conversion of cellulose) were 90% and 73% of the theoretical yield for pretreated switch grass and poplar, respectively. Saha and Bothast, (38) , used dilute acid and enzymatic saccharification procedures for conversion of corn fiber to fermentable sugars. They found that corn fiber pretreated with 0.5% H_2SO_4 at 121ºC for 1 h facilitated commercial enzymes to highly hydrolyze remaining starch and hemicellulose components without generation of inhibitors such as furfural and hydroxymethyl furfural (HMF), which are generally considered inhibitors for fermentative microorganisms.

Formation of inhibitors

During pretreatment at either severe conditions or at prolonged period of pretreatment time, sugars may convert into weak acids, furan derivatives, and phenolic chemicals – typically furfural. Phenolic compounds from lignin degradation, furan derivatives (furfural and HMF) from sugar degradation, and aliphalic acids (acetic acid, formic acid and levulinic acid) are considered as fermentation inhibitors generated from pretreated lignocellulose biomass (11). Potential inhibitors are furfural, 5-hydroxymethylfurfural, levulinic acid, acetic acid, formic acid, uronic acid, vanillic acid, phenol, cinnamaldehyde, formaldehyde, etc (7). When hemicellulose is degraded, xylose, mannose, acetic acid, galactose and glucose are liberated. Cellulose is hydrolyzed to glucose (Figure 2.4). At high temperature and pressure, xylose is further degraded to furfural. Similarly, 5-hydroxymethyl furfural (HMF) is formed from hexose degradation. Formic acid is formed when furfural and HMF is broken down. Levulinic acid is formed by

HMF degradation. Phenolic compounds are generated from partial breakdown of lignin and have also been reported to be formed during carbohydrate degradation (4).

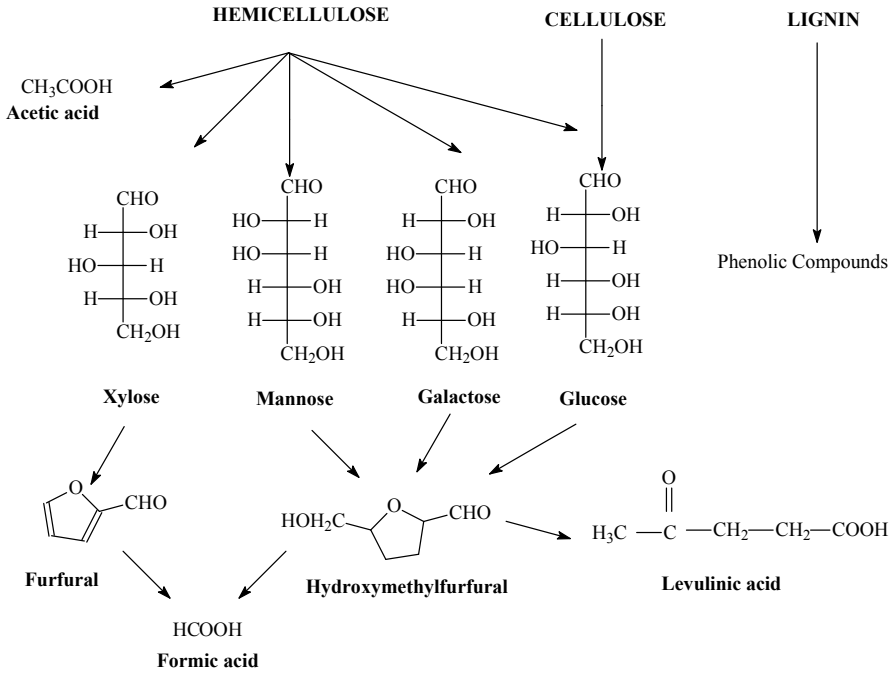


Figure 2.4 Reactions occurring during hydrolysis of lignocellulosic materials (Adapted from (4)**.**

Production of these compounds increases when hydrolysis takes place at severe conditions such as higher temperatures and higher acid concentrations (6). Sugar degradation not only reduces the sugar yield, but the degradation products such as furfural and other by-products can also inhibit the fermentation process (18). Various methods for detoxification of the hydrolyzates have been developed. These include treatment with ion-exchange resins, removal of non-volatile compounds, and/or treatment with lime or sulfite (11). Inhibitors also increase the environmental stress for the fermentative organism due to decreased water activity and increasing ethanol concentrations. Microorganisms can survive stress up to a certain limit, but cell death would occur if the stress exceeds the limit that cell can bear (39). A more detail of the inhibitory mechanism of these compounds and the effects of their interaction, as well as the influence of environmental parameters such a pH, is explained by Palmqvist et al. (4).

Enzymatic hydrolysis

Enzymatic hydrolysis is the second step in the production of ethanol from lignocellulosic materials. It involves cleaving the polymers of cellulose and hemicellulose using enzymes. The cellulose usually contains only glucans, whereas hemicellulose contains polymers of several sugars such as mannan, xylan, glucan, galactan, and arabinan. Consequently, the main hydrolysis product of cellulose is glucose, whereas the hemicellulose gives rise to several pentoses and hexoses (7). However, high lignin content blocks enzyme accessibility, causes end-product inhibition, and reduces the rate and yield of hydrolysis. In addition to lignin, cellobiose and glucose also act as strong inhibitors of cellulases (14).

Enzymatic hydrolysis of non-starch carbohydrates (cellulose and hemicellulose) has already been studied intensively. Although the structure of xylan is more complex than cellulose and requires several different specificities for complete hydrolysis, the polysaccharide does not form closely packed crystalline structures like cellulose and is, thus, more easy to get to enzymatic hydrolysis (10). Maximum cellulase and β-glucosidase activities occur at 40-60 °C and pH of 4.0 to 5.0. However, optimal conditions may change with hydrolysis residence time (7).

Enzymatic hydrolysis requires mild conditions and long periods of time. Combining pretreatment such as high temperature with dilute acid could increase the efficiency of hydrolysis of cellulosic materials. With acid, the hydrolysis can be done within a few minutes. Another problem of enzymatic hydrolysis is that the sugars released inhibit the enzyme activities during hydrolysis (7).

Palmarola et al., (40) used wheat-starch effluent to produce ethanol using the enzymatic hydrolysis method. A mixture of cellulolytic and hemicellulolytic enzymes (Celluclast 1.5 L and Ultraflo L) was used at conditions of 50 ºC and 200 rpm agitation for 48 h. Maximum sugar yield was 34.1 g per 100 g starch-free fibers, comprising 12.8 g glucose, 13.9 g xylose, and 7.4 g arabinose, corresponding to 66%, 71%, and 51% of the theoretical yields, respectively. Schimdt et al., (41), patented a method for selective hydrolysis of the hemicellulose component of a biomass material. The process is especially effective with grain fibers from corn, wheat, rice, oats, or barley. They partially solubilized hemicellulose by using acid and fully solubilized hemicellulose by using enzymes at a temperature range of 40 to 60 ºC. Varga et al., (28), studied the efficiency of cellulose conversion using a commercially available enzyme solution

(Celluclast 1.5 L and Novozyme 188) at hydrolysis conditions of 50 °C, 300 rpm, and 24 h after a steam pretreatment process. Highest overall yield of sugars was 56.1 g from 100 g of untreated material, corresponding to 73% of the theoretical, which was achieved after steam pretreatment with 2% H_2SO_4 at 190 ºC for 5 min.

Cellulose conversion

Cellulase refers to a group of enzymes that contribute to the degradation of cellulose to glucose (17). Cellulose can be degraded enzymatically to glucose by the synergistic action of three distinct classes of enzymes: the "**endo-1, 4 - β - glucanases**" (EC 3.2.1.4), which act randomly on soluble and insoluble 1, 4-β- glucan substrates; the "**exo-1, 4-β-D- glucanases**" (EC 3.2.1.91), which release D-glucose from 1, 4-β-D-glucans and hydrolyze D-cellobiose slowly and liberate D-cellobiose from 1, 4-β-glucan; and the **"β-D- glucosidases**" (EC 3.2.1.21), which release D-glucose units from cellobiose and soluble cellodextrins as well as a group of glycosides (Figure 2.5) (15). In other words, endo-glucanases act in a random manner on the regions of low crystallinity of the cellulosic fiber, whereas exoglucanases remove cellobiose (β - 1, 4 glucose dimmer) units from the non-reducing end of cellulose chains. Thus, **β - D - glucosidases** not only generate glucose from cellobiose but also reduce cellobiose inhibition, allowing the cellulolytic enzymes to function more efficiently. The cellulases and β-glucosidase are inhibited by cellobiose and glucose, respectively (4). For a complete hydrolysis of cellulose to glucose, the enzyme system must include these three enzymes in proper proportions (8).

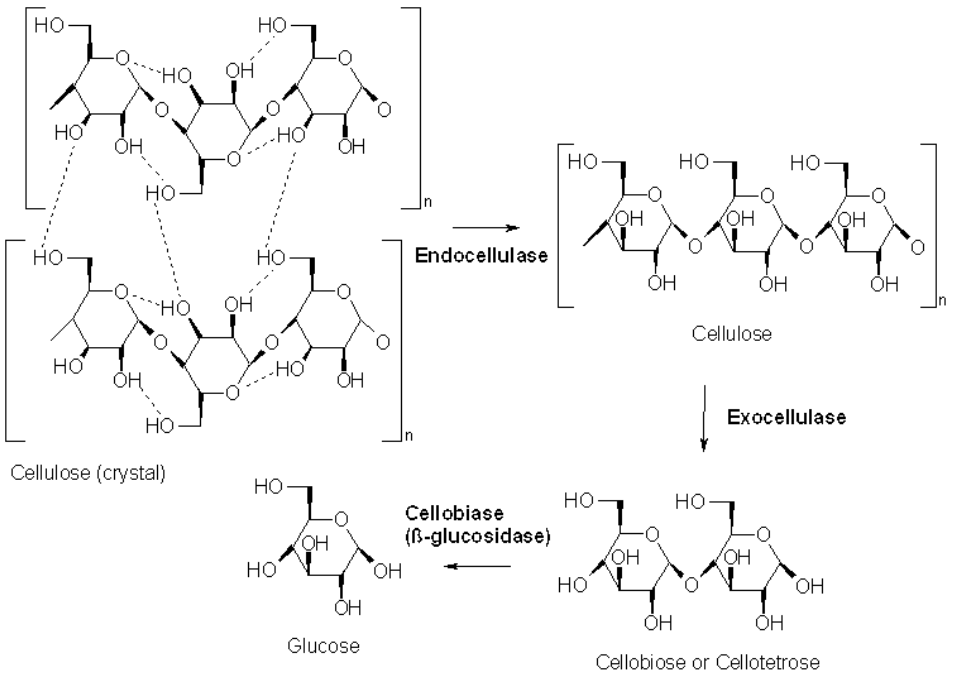


Figure 2.5 Three types of reaction catalyzed by cellulase (5)**.**

Hemicellulose conversion

Hemicelluloses are heterogeneous polymers of pentoses (xylose and L-arabinose), hexoses (mannose), and sugar acids. Xylans, major hemicelluloses of many plant materials, contain xylose, L-arabinose, and D-glucoronic acid, among others. Full hydrolysis of xylan requires **endo β - 1, 4 xylanase** (EC 3.2.1.8)**, β - xylosidase** (EC. 3.2.1.37), and several accessory enzyme activities such as **α- L- arabinosidase** (EC. 3.2.1.55) and **α - glucoronidase** (EC. 3.2.1.131). Endo-xylanase randomly attacks the main chains of xylans, and β- xylosidase hydrolyzes xylooligosacharides to xylose. The α - L - arabinosidase and α - glucoronidase take away the arabinose and 4 - O - methyl glucoronic acid substituents, respectively, from the xylan backbone (8).

Ethanol fermentation

Fermentation of lignocellulosic hydrolyzates is more difficult than the well-established processes of ethanol production e.g., from molasses and starch. Hydrolyzates contain a broader

range of inhibitory compounds, where the composition and concentration of these compounds depend on the type of lignocellulosic materials, the chemical used and nature of the pretreatment, and the hydrolysis process (7).

Theoretical ethanol yield from fiber is determined on a per-bushel basis (30).

$$Ethanol\left(\frac{gal}{bu}\right) = \frac{Fiber(lb.fiber/bu) * Carbohydrate(lb/lb.fiber) * 1.1 * 0.51(lb.ethanol/lb.freesugar)}{6.58(lb.ethanol/gal\ ethanol)}$$

where 1.1 is a conversion factor from lbs. of anhydrous sugar to lbs. of fermentable sugar.

The initial approach to enzymatically converting cellulose to ethanol involved separate operations for pretreatment of biomass to open up the structure of biomass for attack of cellulose by cellulase, addition of cellulase to pretreated biomass to release glucose, and glucose fermentation to ethanol or other products (33). Over time, the title separate hydrolysis and fermentation (SHF) emerged to designate this sequence of operations.

Today, simultaneous bioconversion of multi-sugars is one of the most ambitious challenges in the field of bio-ethanol production (32). Fermentation of lignocellulosic biomass requires either a microorganism or a scheme that can ferment the mixture of sugars derived from hemicellulose that includes glucose, xylose, arabinose, galactose, mannose, and fucose, depending on the source (42). Although traditional *S. cerevisiae* and *Zymomonas mobilis* ferment glucose to ethanol rapidly and efficiently, they cannot ferment other sugars such as xylose and arabinose to ethanol (11). Due to the relatively similar process conditions in the enzymatic hydrolysis and ethanol fermentation, the option of carrying out these two steps together in a simultaneous saccharification and fermentation (SSF) process exists (43).

Simultaneous saccharification (hydrolysis) of cellulose and hemicellulose and fermentation of sugars to ethanol improves the kinetics and economics of biomass conversion by reducing accumulation of hydrolysis products that are inhibitory to enzymes, reducing contamination risk because of the presence of ethanol, and reducing capital equipment investment. An important drawback of SSF is that the reaction has to operate at a compromised

temperature around 30 °C, instead of enzyme optimum temperature of 45-50 °C (11). Another current option is the co- fermentation/sequential scheme.

Fermentation of lignocellulosic hydrolyzates

Processing of lignocellulosic to ethanol consists of four major unit operations: pretreatment, hydrolysis, fermentation, and product separation/purification (Figure 2.6). Cellulose can be broken down by hydrolysis into glucose either enzymatically by cellulases or chemically by sulfuric or other acids. Hemicelluloses or acids hydrolyze the hemicellulose polymer to release its component sugars. Hexoses are fermented readily to ethanol by many naturally occurring organisms, but pentoses are fermented to ethanol by only a few native strains, and usually at relatively low yields (6). Ethanol fermentation can be carried out by three main steps: simultaneous saccharification and co-fermentation (SSCF), simultaneous saccharification and fermentation (SCF) and/or separate hydrolysis and fermentation (SHF)(44). Ethanol is recovered from the fermentation broth by distillation or distillation combined with adsorption. The residual lignin, unreacted cellulose and hemicellulose, ash, enzyme, microorganism and other components end up in the bottom of the distillation column (6, 45). These materials may be concentrated, and burned as fuel to power the process, or converted to various co-products (6).

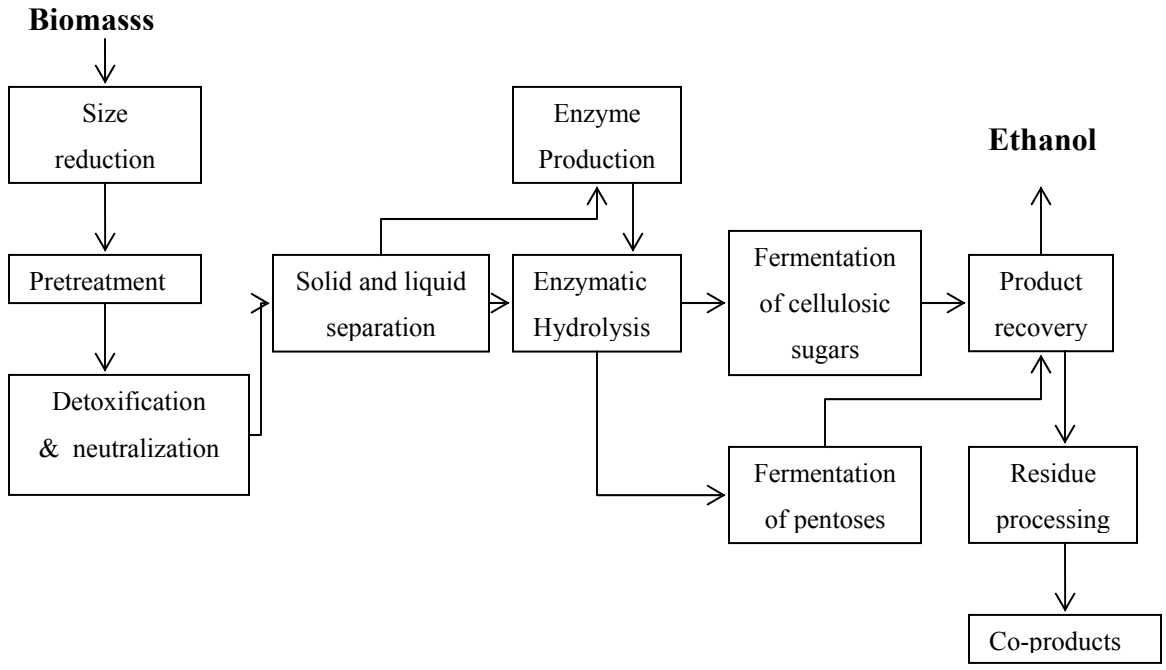


Figure 2.6 Pretreatment, enzymatic hydrolysis, and fermentation process (80).

Separate hydrolysis and fermentation (SHF)

In separate hydrolysis and fermentation (SHF), first, cellulose is hydrolyzed enzymatically into glucose and/or hemicellulose to pentose, and then sugars are fermented into ethanol. Its primary benefit is its ability to carry out each step at its optimum temperature range: 45-50°C for the enzymatic hydrolysis and around 30°C for the fermentation. Such an optimization is expected to improve the performance of each process. The major drawback of SHF is that the released sugars severely restrain cellulase and β-glucosidase during hydrolysis, which requires the use of lower solids concentrations at higher enzyme loadings to obtain reasonable ethanol yields. Low solids concentrations, however, will result in low ethanol yield, therefore, increasing the cost of fermentation and ethanol recovery (46)

Simultaneous saccharification and co-fermentation (SSCF)

The co-fermentation scheme involves the presence of a co-culture capable of converting the mixed sugars into ethanol. It is known that when the glucose level in the feedstock is much higher than xylose, co-fermentation could be a more efficient approach since the cost of separate processes would be high. Among the most common hexose– and pentose- fermenting yeasts, *Saccharomyces cerevisiae* and *Pichia stipitis* are by far the most used (32).

De Bari et al. studied the simultaneous hydrolysis of cellulose and hemicellulose, followed by detoxification through resins and co-fermentation with immobilized cells to produce ethanol. *S. cerevisiae* and *P. stipitis* immobilized in Ca- alginate beads were used as co-cultures (32). Fermentation was carried out at 30 ºC and pH 5.5. Overall yield was 0.396 $g_e/g_{s,}$ accounting for 77% of the theoretical ethanol yield.

Due to hexose sugars being easily converted by *S. cerevisiae,* and *P. stipitis* and converting pentose sugars relatively fast in a sequential scheme of the fermentation, some research on the sequential fermentation scheme was also conducted. Grootjen et al. developed a system with reactors in series for the sequential use of hexose and pentose sugars (47). Since the pentose sugars were only converted when the hexose concentration was very low, the compartment in which growth and glucose conversion occurred had to be designed to permit the glucose concentration in the other compartments to be low enough to allow xylose conversion. This is a consequence of the sequential use of substrate with two reactors and two yeasts;

however, only 20% of the xylose was converted. Wyman et al., (9), used recombinant *E. Coli* ATCC 55124 (KO11) for the simultaneous saccharification and co-fermentation (SSCF) of corn stover obtaining yields from 85 to 95 % of the theoretical depending on the pretreatment applied.

Simultaneous saccharification and fermentation (SSF)

Simultaneous saccharification and fermentation (SSF) involves the enzymatic hydrolysis of cellulose and hemicellulose to sugars, and the conversion of fermentable sugars to ethanol in the same vessel. The SSF technique provides the possibility to overcome the main difficulty of enzymatic hydrolysis i.e., decreasing the enzyme loading and therefore the production cost, making application of SSF for conversion of lignocellulosic to ethanol a more cost-effective process. The main problem of this technique is the difference among the optimum temperatures used for the enzymatic hydrolysis of cellulose and the optimum temperatures used in the ethanol fermentation (48).

Eklund et al. studied optimum conditions for SSF of SO_2-impregnated, steam-pretreated willow using commercial cellulases (Celluclast 2 L and Novozyme 188) for the hydrolysis and using both *S. cerevisiae* and *Z. mobilis* for fermentation of hexose. Optimum fermentation conditions were 37°C and pH 5.0 for 72 h. *S. cerevisiae* was superior to *Z. mobilis* concerning ethanol yield after 72 h; however, less by-product formation was observed when Z. *mobilis* was used. Results indicated that it was possible to reach more than 85% of the theoretical ethanol yield, based on the glucan available in the raw material in three days.

Kadar et al. considered use of thermo-tolerant yeast strains, which would allow higher processing temperatures, and thus increased rates of hydrolysis (43). They studied an SSF and a non-isothermal simultaneous saccharification and fermentation (NSSF) process with different temperature profiles. Ethanol production using thermo-tolerant yeast (*K.marxianus)* and ordinary baker's yeast was also investigated on different cellulose waste materials. The SSF was carried out at 40 ºC for 96 h. Results showed that *S. cerevisiae* was as good as *K. marxianus* in simultaneous saccharification and fermentation at 40º C. They also concluded, from an industrial point of view, there was no improvement by applying the NSSF operation mode because it did not increase ethanol yield; ethanol yield was lower than that from SSF. Finally, Wyman et al. used Spezyme CP and Novozyme 188 along with *S. cerevisiae* ATCC 200062 for the SSF of corn stover obtaining yields up to 95 % of the theoretical yield (9).

New recombinant microorganisms

Use of recombinant microorganisms for co-fermentation is one of the most promising approaches in the field of bio-ethanol production, though use of large-scale industrial processes still requires fine tuning of the reliability of the entire process (32). Several microorganisms have been genetically engineered to produce ethanol from mixed-sugar substrates by using two different approaches: (a) divert carbon flow from native fermentation products to ethanol in efficient mixed-sugar utilizers such as *Esherichia, Erwinia*, and *Klebsiella*; and (b) introduce the pentose-utilizing capability in the efficient ethanol producers such as *Saccharomyces* and *Zymomonas* (11).

Escherichia Coli (*E. Coli)* was genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) from the bacterium *Zymomonas mobilis* (49). Two ethanologenic strains have been used in following investigations. *E. Coli* ATCC 11303 and strain KO11. Some comparisons of yeast and bacteria using dilute acid hydrolyzates of corn cob hemicellulose as a substrate concluded that recombinant *E. Coli* strain KO11 was superior to other pentose fermenting organism in ethanol productivity, ethanol yield, and resistance to inhibitors generated during hydrolysis (50).

Padukone et al. described the characterization of recombinant *E. Coli* ATCC 11303 (pLOI 297) in the production of ethanol from cellulose and xylose (51). They examined fermentation of glucose and xylose, both individually and as a mixture, and selectivity of ethanol production under various conditions of operation. They demonstrated that xylose metabolism was strongly inhibited by the presence of glucose, and ethanol was a strong inhibitor of both glucose and xylose fermentations. They reached a high ethanol yield (84% of theoretical) using simultaneous saccharification and fermentation of cellulose with the recombinant *E. Coli*.

Hemicellulose hydrolyzates of agricultural residues such as corn stover, and corn hulls plus fibers were also fermented to ethanol by recombinant *E. Coli* strain KO11 (50). Fermentations were complete within 48h, achieving 40g ethanol L^{-1}, ethanol yields ranging from 86 to 100% of the maximum theoretical yield.

Nichols et al. constructed ethanologenic *E. Coli* strains and used it to ferment glucose, arabinose, and xylose, singly and in mixtures, to ethanol (42). They constructed strains

fermented arabinose and xylose simultaneously with glucose, rather than sequentially. They found that catabolite-repression mutants are useful for any fermentation process that could use lignocellulosic biomass as a feedstock.

Saccharomyces cerevisiae r424A, provided by the laboratory of Renewable Resources Engineering at Purdue University, has been used to ferment hydrolyzates from corn fiber hydrolysis (52). This organism was genetically engineered by cloning the xylose reductase and xilytol dehydrogenase genes from P. stipitis and the xylulokinase gene from *S. cerevisiae* into a new recombinant *S. cerevisiae*. This allowed the organism to convert xylose to ethanol. Fermentations showed that the organism can ferment the glucose and xylose from the corn fiber hydrolysates to ethanol without detoxification of the hydrolysate.

X-ray Diffraction and Fourier Transform Spectroscopy

The information concerning cristallinity of cellulose can be obtained by methods as Fourier Transform (FT) Raman, Fourier transform infrared FT-IR (IR), or solid state 13C-NMR (NMR) spectroscopy (53). The wide-angle x-ray diffraction (WAXD) and the FTIR spectra of two different crystal types of cellulose were studied in 1995. FTIR showed that cellulose have two absorption peaks in the OH stretching region. These two peaks were caused by the functions of intramolecular hydrogen bond and intermolecular hydrogen bond (54). It was also demonstrated that deconvolution of the IR spectra of cellulose and cellulose derivatives in the range of the OH stretching vibrations gives detailed evidence on cristallinity, crystal modification and degree of substitution (55).

FTIR of treated wheat straw and steam exploded wheat straw showed that polymerization degree of cellulose and hemicellulose decreased after pretreatment (56). The major FTIR absorption bands for crystalline were OH stretching vibrations at around 3352 – 3315 cm^{-1}. The absorbance of hydrogen bonded OH stretching was considerably decreased by the treatment of NaOH and carbon dioxide of cellulose (57). Another study related the effect of short-time vibratory ball milling on the shape of FTIR spectra of wood and cellulose. The most conspicuous changes in the spectra of cellulose and wood were observed at wavelength between 1034 to 2902 cm^{-1} and in the OH-stretching vibration region from 3200 to 3500 cm^{-1}. The authors suggested

that these changes are mainly associated with a decrease in the degree of crystallinity and/or a decrease in the degree of polymerization of the cellulose (58).

FT–Raman and FTIR was used in 1994 to study five different plant cell walls and their composition. Wall constituents such as pectin, proteins, aromatics and phenolics, cellulose and hemicellulose have characteristic spectral features that can be used to identify and/or fingerprint these polymers without, in most cases, the need for any physical separation. The differences in chemical composition and cross linking of the walls are strongly related to the absorption peaks and wavelength (59). Variation of lignin content of growth rings in hardwoods has been also studied by FTIR. The wavelength region of 1760-1580 cm^{-1} can be used to estimate the lignin/hemicellulose ratio of softwoods and hardwoods (60).

Techniques of FTIR and near–IR for lignocellulose analysis have been also reviewed and compared (61). FTIR of softwood during heat treatment (160 -260 °C, 2-8 h) as well as determination of chemical changes of these wood materials was studied by Kotilainen et al.(62). Micro spectroscopy in the mid-IR (IR) region has been used to gain spectra of single species of sugars and complex carbohydrates (cellulose, hemicellulose and lignin) and to identify characteristics of IR reflectance peaks and to be able to separate the species in complex media (63).

Xu et al. analyzed physical properties of pretreated soybean straw using FTIR, SEM and XRD (64). Results showed that structure of straw changed through pretreatment which is in favor of further enzymatic hydrolysis and the cristallinity of soybean straw decreased with time development of ammonia liquor pretreatment. They also identified lignin peaks at 1238 cm^{-1} (C-O of guaiacyl ring), 1315 cm^{-1} (C-O of syringyl ring), and 1504-1630 cm^{-1} (aromatic skeletal vibrations). The intensities of lignin peaks of the untreated sample were higher than those of pretreated sample, proving delignification (64).

CHAPTER 3 - Pretreatment and Enzymatic Hydrolysis of Soybean Hull

Ethanol derived from biomass such as grains, grain residues, agricultural by-products, and dedicated energy crops has great potential to be a sustainable replacement of transportation fuels and other potential applications. A dramatic increase in ethanol production using the current grain-starch-based technology may have resource limitations because grain production for ethanol will compete for the limited agricultural land available for food and feed production (65).

The United States needs more than 140 billion gallons of gasoline for automobiles alone. Currently, ethanol is primarily produced from corn. Using 100% of the 2007 corn crop would only produce 35 billion gallons of ethanol, which would only meet about 16% of the need (13). Conversion of cellulosic biomass such as agricultural residues to fuels and chemicals offers major economic, environmental, and strategic benefits, and biological processing based on cellulases offers high sugar yields vital to economic success. DOE and USDA projected that U.S. biomass resources could provide approximately 1.3 billion dry tons of feedstock per year for biofuels production, which could be enough to produce biofuels to meet more than one-third of annual U.S. fuel demand for transportation (13).

Ouhida et al. determined a chemical composition of soybean hull and its fractions by extraction with chelating, acid, and alkali agents. The overall amount of carbohydrates accounted for 63% (25). Hemicellulose, cellulose, and Klason lignin content varied from 14 to 24%, 13 to 20%, and 2.5 to 6%, respectively, when different varieties of soybean seed coat were analyzed (66). Soybean hull was also reported to contain 51% cellulose, 16% hemicellulose, and 1.4 % lignin (ADL) on a DM basis (26). Hemicellulose and cellulose content in our sample were in the mid level of the reported range. Chemical composition of soybean hull can vary extensively among sources. A large portion of this variation is partly due to occasional erroneous classification of soybean mill feed and soybean mill run as soybean hull, as well as differences in analysis techniques (26).

Nearly 1.8 billion bushels of soybeans are crushed for oil, protein, and soy flour production in the U.S. This will generate about 10.8 billon pounds of soybean hulls. Commercial value of soybean hulls has been considered much less important than soy oil and protein (67). As a result, soy carbohydrates have been traditionally used as animal feed and relatively fewer efforts have been made to study soy carbohydrates and their potential utilization (23). As a rapid increase in production of distiller's dry grain with solubles (DDGS) from ethanol production using corn and grain sorghum is expected, demand for soybean hulls for animal feed is anticipated to decrease over the next decade. An increase in biodiesel production from soybean oil would cause an oversupply of soybean hulls as animal feed. Therefore, other uses of the soybean fiber stream are needed for the soybean industry. Monomers of soy carbohydrates from soybean hulls are sugar, which can be used as substrates for bioconversion to produce chemicals and bio-fuels. The major compositions of carbohydrates in soybean hulls are structural components in the cell walls, including cellulose and hemicellulose. Ouhida et al. reported that soybean hulls contain about 16.7% DM-1 water-soluble substrates and 83.3% DM-1 water-un-extractable substrates (25). In addition to glucose, soybean hulls also contain xylose, arabinose, galactose, mannose, and uronic acid (23, 26). Development of an effective method to produce fuel ethanol from soybean hulls will help sustainable economic development and have a great impact on industrial utilization of soybean hulls for value-added products. Soybean hulls are the ideal feedstock for fuel ethanol production, because they are abundant and require no special harvesting and additional transportation costs as they already in the plant.

Production of chemicals and biobased products from renewable biomass faces significant technical challenges. Success of using biomass for biofuel production depends largely upon physical and chemical properties of the biomass, pretreatment methods, efficient microorganisms, and optimization of processing conditions. The purpose of the pretreatment is to break the lignin seal, pre-hydrolyze the hemicellulose, and disrupt the crystalline structure of the cellulose, enhancing the hydrolysis of cellulose by cellulase. Pretreatment methods such as steam explosion, dilute acid treatment, concentrated acid treatment, alkaline treatment, treatment with hydrogen peroxide, treatment with hot water, ammonia fiber explosion, and organic solvent treatments have been studied (10, 11, 36, 38, 52, 68). Among these methods, steam explosion, hot water, and dilute acid pretreatments are the most frequent options used for pretreatment of lignocellulosic materials (9, 36, 69, 70). Steam explosion efficiently enhances the conversion rate

of cellulose into fermentable sugar (6, 29, 71). Starch-free wheat fiber was efficiently hydrolyzed up to 75% yield of sugars using combined steam pretreatment and enzymatic hydrolysis, and proved that pretreatment prior to enzymatic hydrolysis could reduce enzyme loading without a reduction in sugar yield (40, 72). Rice hull was hydrolyzed up to 60% saccharification and posterior fermented to ethanol using dilute acid and enzymatic hydrolysis, confirming that diluted acid is powerful to hydrolyze hemicellulose and makes cellulose much more accessible to enzymatic hydrolysis (36). Hot water pretreatments use high pressure to maintain the water in the liquid state at elevated temperatures (200-230 °C) in which about 40 to 60% of total biomass can be dissolved in the water solution (73). We targeted sorghum fiber as a model substrate for use as lignocellulosic biomass, obtaining 75% fermentable sugars after hot water pretreatment and enzymatic hydrolysis (74).

Enayati et al. and Ouhida et al. studied enzymatic saccharification of soybean hull-based materials and demonstrated ease of biodegradability of soy hulls (25, 75). At present, there is no reported information on pretreatment, enzymatic saccharification, and fermentation of soybean hulls for biofuel. Although several methods are available for biomass pretreatment, selectivity of pretreatment methods is highly restricted by the nature of the raw materials. Objectives of this research were to study the performance of dilute acid and modified steam explosion pretreatments on degradation of soybean hulls, and to identify optimum pretreatment and enzymatic hydrolysis conditions for converting soybean hulls into fermentable sugars.

Materials and Methods

Soybean hulls with 12% moisture content and particle size <1 mm were obtained from the Cargill Company (Dayton, Ohio) and stored at 4 ºC. Chemical composition of the soybean hulls was about 36% cellulose, 12% hemicellulose, 2% starch, 18% total amount of lignin (ATL), and 14% protein (Table 3.1). Total carbohydrate composition was about 50%. Pectin content was about 6.3%.

All yields reported were normalized to the total potential glucose and xylose in the original untreated material to provide a perspective on the relative contribution of each sugar to total sugar recovery. Total theoretical yield of sugars from soybean hulls was calculated from the chemical composition as 0.54 g. sugars/g hull. Pentose yield was based on total pentose sugars

(0.14 g Pentose / g hull). Hexose yield was based on total hexose sugars (0.40 g Hexose / g hull). This, transformed into gallons of EtOH, would be 84 gallons EtOH/ton dry soybean hulls (76).

Cellulase C2605 from Sigma-Aldrich (102 FPU/ml), cell-wall degrading complex (Viscozyme L –V2010) (hemicellulase, cellulase, arabinase, and xylanase complex), and Novozyme 188 (β-glucosidase) (250 CBU/ml) from Novozyme (U.S. Office: Franklinton, N.C.) were used for enzymatic hydrolysis of soybean hulls into fermentable sugars. Sugars used for HPLC calibration were purchased from Fischer Scientific Inc (Pittsburgh, PA).

Table 3.1 Chemical composition of soybean hulls

Component	Soybean hulls (% db)
Carbohydrates	50.70
Starch	1.75 ± 0.08
Cellulose	36.43 ± 0.09
Hemicellulose	12.48 ± 0.32
Total Amount Lignin [b]	18.20 ± 0.40
Crude fat	3.20 ± 0.40
Crude fiber	32.30 ± 0.32
Crude protein	14.21 ± 0.10
Pectin	6.30 ± 0.20
Ash	4.24 ± 0.03

[a.] Means of four replicates

[b.] Calculated as AIL+ASL

Enzymatic hydrolysis

Original or pretreated soybean hulls were mixed with distilled water to obtain a solution with 10% solid content and were then treated with a mixture of enzymes. Three commercial enzymes cellulase C2605, Novozyme 188 (β-glucosidase), and cell-wall degrading complex V2010 (enzyme complex) were used for hydrolysis of cellulose and hemicellulose in soybean hulls. In these experiments, C2605 was considered as a dominant source of cellulase even other enzymes such as complex (Viscozyme L-V2010) also contain cellulase. Effects of a combination of cellulase and β-glucosidase at enzyme loading of 15 FPU/g cellulose and 50 CBU/g cellulose,

respectively, with cell-wall degrading complex added at a ratio 1:1 (v/v) cellulase/enzyme complex on hydrolysis of soybean hulls were studied. Enzymatic hydrolysis was carried out in flasks with 100 ml of slurry at 45°C and pH 4.8 for 12 to 96 h in a water-bath shaker with agitation speed of 140 rpm. Sodium azide (0.3% w/v) was used to inhibit microbial growth during the enzymatic hydrolysis. Samples were taken out each 12 h for sugar analysis. After enzymatic hydrolysis, the samples were heated at 100°C for 15 min and stored at 4°C to inactivate the enzymes. Unhydrolized soybean hulls were separated by centrifuging at 13500 *g* for 10 min at room temperature. Liquid was collected for sugar analysis.

Pretreatment with dilute acid and steam explosion

The treatment was carried out in a 1-L pressure reactor apparatus (Parr Instrument Company, Moline, IL). Soybean hulls were mixed with dilute acid to obtain 10% dry matter. The slurry (≈ 56 g soy hull/500 ml) was loaded into the reactor and treated at the desired temperature and time. After treatment, the slurry was removed from the reactor by releasing the pressure through the liquid valve to achieve "explosion" (72). To reach the desired temperature in one minute, 90 psig saturated steam was injected. The process is called "modified steam explosion", since in the "common" steam explosion treatment, steam is supplied up to a maximum pressure of 435 psi obtaining temperatures higher than 190 °C (77). Meanwhile maximum pressure obtained in the reactor after injection of steam and treatment of the material at desired temperature and time was about 100 psi; reported pressure and temperature range for the achieved 'steam explosion" was found to be between 115-435 psi and 190 to 230 °C, respectively(72, 78, 79). The effect of two levels of temperature (120 and 140 °C), two levels of reaction time (5 and 30 min), two levels of acid (1 - 2%), and two acids (hydrochloric acid and sulfuric acid) on sugar yield was studied. After treatment, the solid remaining was washed three times with 300 ml of hot deionized water (85ºC) and then freeze dried. A portion of the washed, pretreated freeze-dried solid was stored at 4°C for subsequent enzymatic hydrolysis. A liquid sample from the treatment and washing process was analyzed by HPLC to determine recovery of sugars.

Analytical methods

Cellulose and hemicellulose of soybean hulls were analyzed by Filter Bag Technology (ANKOM Technology, Macedon, N.Y.). Total amount of lignin was determined by two-stage

acid-hydrolysis procedures developed by the National Renewable Energy Laboratory (NREL) (80). The conditions of the first hydrolysis were 72% sulfuric acid, 1:10 of solid to liquid ratio, and 30°C for one hour. The conditions for the secondary hydrolysis were 4% sulfuric acid and 121°C for one hour. Starch content was determined by using commercially available kits from Megazyme (Bray, Ireland), according to AACC Approved Method 76-13 (81). Protein was determined via nitrogen combustion using a LECO FP-528 nitrogen determinator (St. Joseph, Mich.), according to AACC Approved Method 46-30. Nitrogen values were converted to protein content by multiplying by 6.25. Crude fiber, fat, and ash were determined by AOAC standard methods (82).

Concentrations of sugars were determined by HPLC using an RCM-monosaccharide column (300 x 7.8 mm; Bio-Rad, Richmond, Calif.) and refractive index detector. Samples were neutralized with $CaCO_3$, run at 85°C, and eluted at 0.6 ml/min with H_2O. Hexose yield was counted as the final amount of glucose derived from cellulose. Pentose yield was counted as the final amount of pentose sugars derived from hemicellulose.

Soybean hulls before and after pretreatments were analyzed by XRD in a Bruker AXS D-8 diffractometer settled at 40 KW, 40 mA; radiation was cupper Kα (λ= 1.54 Å), and grade range between 5 to 40° with a step size of 0.03°. Aperture, scatter, and detector slits sized were 0.3°, 0.3°, and 0.03°, respectively. Presence of crystallinity in a sample can be detected by absorption peaks. Crystallinity index (CrI) was calculated using the method of Segal et al. (19). This is determined by the ratio of the maximum intensity of the peak at the 002 lattice diffraction (in arbitrary units) or "crystalline" peak to the intensity of the "amorphous" peak in the same units at 2θ = 18°. Diffractogram was smoothened using the methodology described in Appendix Images of the surfaces of pretreated and untreated soybean hulls were taken at magnifications from 1.5 K to 3K using a Hitachi S-3500 N scanning electron microscope (SEM). The specimens to be observed were mounted on conductive adhesive tape; sputter coated with gold palladium, and observed using a voltage of 15 to 20 kV.

Analysis of variance (ANOVA) and least-significant difference (LSD) were done using SAS (SAS Institute 2005, Cary, N.C.).

Results and Discussion

Enzymatic hydrolysis of soybean hull

Sugar yields from enzymatic hydrolysis (EH) using cellulase, β-glucosidase, and hemicellulase complex without pretreatment are shown in Figure 3.1. Sugar yields increased when both a cell-wall degrading complex and cellulase enzymes were used for enzymatic hydrolysis. The highest sugar yield was 27.5%, obtained after 36 h of enzymatic hydrolysis. The addition of the cell-wall degrading complex increased sugar yield by more than 97%, compared with the enzymatic hydrolysis using cellulase alone. Total amount of sugars decreased after 12 h when the two enzymes were used. This behavior may be due to the inhibition of enzymes, sugar degradation or possible contamination during hydrolysis. A presence of non-degraded amorphous components such as hemicellulose limits the accessibility of cellulose to enzymes and diminishes the susceptibility of cellulose to hydrolysis (17). In addition, it was assumed that some yielded sugars may degrade to other compounds. The formation of chemical compounds would increase when hydrolysis takes place at severe conditions such as longer time (4). This behavior can be also attributed to consumption of sugars by other microorganisms growing during hydrolysis. Both enzyme concentration and hydrolysis time had a considerable effect on sugar yields. Sugar yields increased 24% from 12 to 36 h, proving that new sugars are continuously produced due to the action of the cell-wall enzyme complex. Thus, the addition of this complex improves hemicellulose hydrolysis, promoting easy access of cellulase enzymes to inner layers. Enzymatic hydrolysis with cellulase (15 FPU/g cellulose), β-glucosidase (50 CBU/g. cellulose), and enzyme complex, added at a ratio of 1:1 v/v, was taken as a control for further analysis.

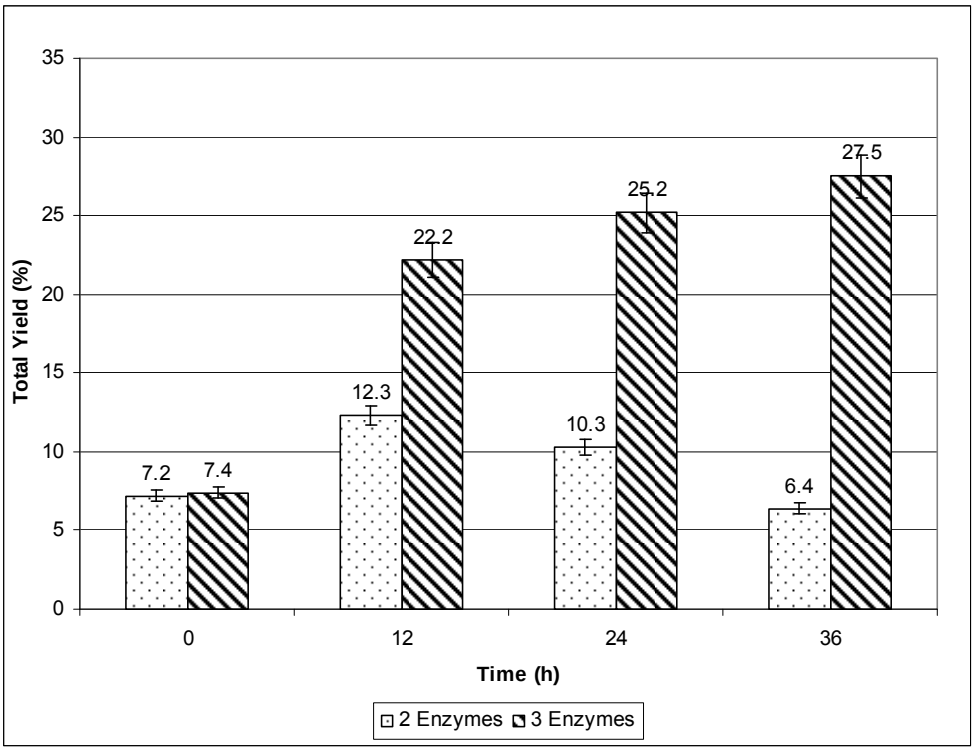


Figure 3.1 Effects of number of enzymes on sugar yields from untreated soybean hulls: two enzymes (cellulase and β-glucosidase) and three enzymes (hemicellulase, cellulase and β-glucosidase) with and enzyme loading of cellulase 15 FPU/g cellulose and 50 CBU/g cellulose

Pretreatment with Hydrochloric Acid and Steam Explosion

Soybean hulls treated with HCl and steam explosion gave a maximum pentose yield (75%) obtained at 140°C for 30 min and 1% HCl (Table 3.2). Total sugar yield based on total amount of sugars (0.54 g sugars/g hulls) was only 19.7%, because hexoses were not recovered from this pretreatment. Pentose yield increased as both reaction time and temperature increased. This suggests that longer time with higher temperature is useful to hydrolyze and degrade the hemicellulose layer. It was demonstrated that thermal expansion coefficients of hemicellulose in aqueous solutions were several times greater than that of hemicellulose in the dry samples (83, 84). The high recovery of pentose obtained using this pretreatment was also probably due to the high temperature reached by steam, which increased thermal expansion of the hulls and made the hydrogen bonds easily rupturable. Further analyses to evaluate relevance of treatment time were

carried out at four levels of time (5, 10, 30, and 50 min). However, there was no improvement with increased reaction time (data not shown).

Table 3.2 Yield (%) of sugars from soybean hulls after hydrochloric acid and steam explosion treatment[1]

Temperature (°C)	Pretreatment Time (min)	Pentose (mg/ml)	Yield (%)	
			Pentoses	Total[2]
120	5	4.0	28.9	7.6[c]
	30	8.7	61.5	16.1[b]
140	5	9.8	69.1	18.1[b]
	30	10.6	74.9	19.7[a]

[1] The concentration of acid is 1% HCl.

[2] Means of two replications. Values in the same column with the same letters are not statistically different at $p<0.05$.

Pretreatment with sulfuric acid and steam Explosion

Steam explosion with 1% H_2SO_4 at 140°C for 30 min gave a pentose yield of 90%, while using 2% H_2SO_4 at the same conditions gave a maximum pentose yield of 96% (Table 3.3). Pentose yield increased from 90 to 96% when concentration of H_2SO_4 increased from 1 to 2%. Compared with hydrochloric acid and steam explosion, this treatment increased the pentose yield from 74% up to 96% and was the most effective method for hydrolysis of hemicellulose. This suggests that both faster heating with saturated steam and use of a strong acid such as H_2SO_4 are powerful method to remove and hydrolyze hemicellulose, and could further increase the enzymatic hydrolysis of cellulose. Additionally, in Table 3.3, there is no hexose yield reported since no important amounts of hexoses after treatment were found. Treatment with 2% H_2SO_4 at 140°C for 30 min was chosen for further evaluation, with enzymatic hydrolysis using cellulase (15 FPU/g cellulose) and β-glucosidase (50 CBU/g. cellulose) without addition of the hemicellulose complex in order to compare with the control.

Table 3.3 Yield (%) of sugars from soybean hulls after sulfuric acid and steam explosion treatment [1]

Acid (%w/v)	Pentoses (mg/ml)	Yield (%)	
		Pentoses	Total[2]
1.0	12.8	90.5	23.8[b]
2.0	13.7	96.9	27.5[a]

[1] The reaction temperature was 140°C and pretreatment time was 30 min.

[2] Means of two replications. Values in the same column with the same letters are not statistically different at $p<0.05$.

Hexose yield obtained after 36 h of enzymatic hydrolysis was 72% (Table 3.4). Compared with the combination of HCl treatment and enzymatic hydrolysis, the combination of H_2SO_4 treatment with enzymatic hydrolysis increased total sugar yield from 27.5 to 79% in the same hydrolysis time of 36 h (Figure 3.2). The higher hexose yield also indicated that pretreatment with sulfuric acid and steam explosion is an effective method for improving the enzymatic hydrolysis of soybean hulls using the same enzyme loading, and allows for efficient hydrolysis even in the absence of hemicellulase complex enzymes. Additionally, in Table 3.4 there is no pentose yield reported since no detectable amounts of pentoses after enzymatic hydrolysis were found.

Table 3.4 Yield (%) of sugars from soybean hulls after sulfuric acid, steam explosion treatment, and enzymatic hydrolysis[1]

Enzymatic Hydrolysis Time (h)	Hexose (mg/ml)	Yield (%)	
		Hexoses	Total [2]
12	21.47	53.10	64.50[c]
24	28.39	70.21	77.16[b]
36	29.45	72.82	79.09[a]

[1] The pretreatment conditions are 140° C for 30 min, with 2% of H_2SO_4.

[2] Means of two replications. Values in the same column with the same letters are not statistically different at p-value<0.05.

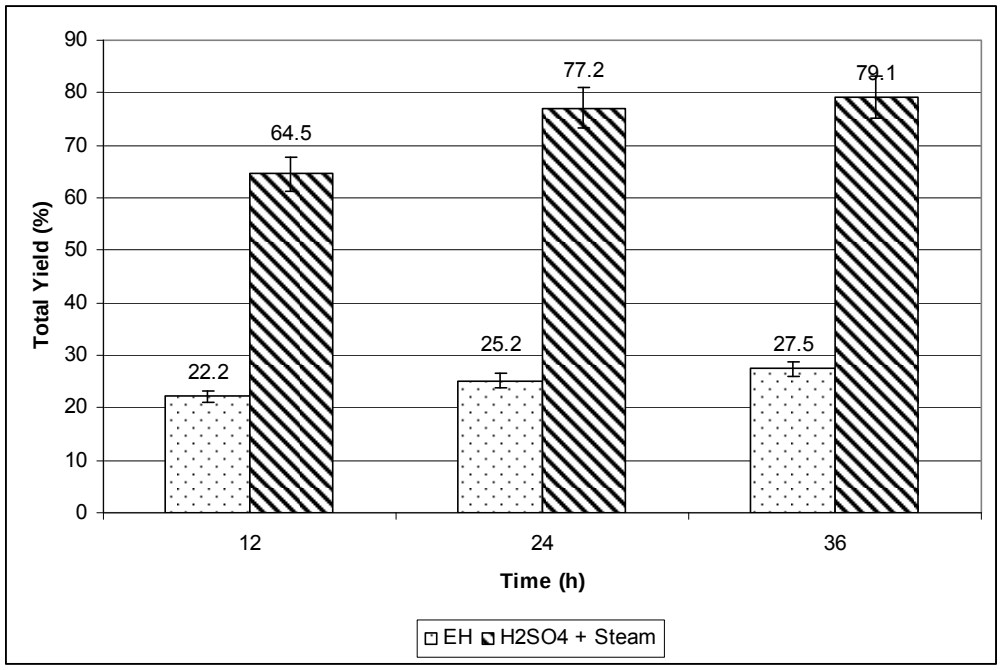


Figure 3.2 Comparison of total sugar yields (%) from enzymatic hydrolysis (3 enzymes), and combination of pretreatment (2% H_2SO_4; T: 140°C ; t: 30 min) and enzymatic hydrolysis (2 enzymes).

Effect of enzymatic hydrolysis time on sugar yields

Hydrolysis of pretreated (2% H_2SO_4, steam explosion at 140°C for 30 min) soybean hulls was carried out at a range of 12 to 96 h. The results are presented in Figure 3.3. Maximum hexose concentration was 30.53 mg/ml, which represents 75% of hexose yield and 81% of total sugar yield at a hydrolysis time of 48 h. Hexose sugar yield decreased as soon as time was prolonged. This behavior might be due to inhibition of enzymes, low cellulase loading, sugar degradation and/or contamination by other microorganisms. It is well known that during enzymatic hydrolysis of cellulose, there is transformation of cellulose into a more crystalline and structurally resistant form, which increases resistance to further hydrolysis (17). The effect of recrystallinity can be reduced if sufficient enzymes are used (6). However, our enzyme loading was relatively lower (cellulase loading of 15 FPU/g.cellulose and β-glucosidase loading of 50 CBU/g.cellulose) than some published data (>15 FPU/g.cellulase) (64, 85). In addition, sugar degradation at longer hydrolysis, and formation of furfural and other compounds, also inhibits further enzymatic hydrolysis and the fermentation process. Formation of these compounds

increase when hydrolysis takes place at severe conditions: higher temperatures, longer time, and higher acid concentrations (17). Because of less adsorption efficiency and saturation of the cellulose surface with enzymes and/or a loading that is too costly to be competitive, higher enzyme loadings were not studied in this paper. Finally, although antibiotic was used during enzymatic hydrolysis, decrease of sugar yield might be also attributed to consumption of sugars by other microorganisms.

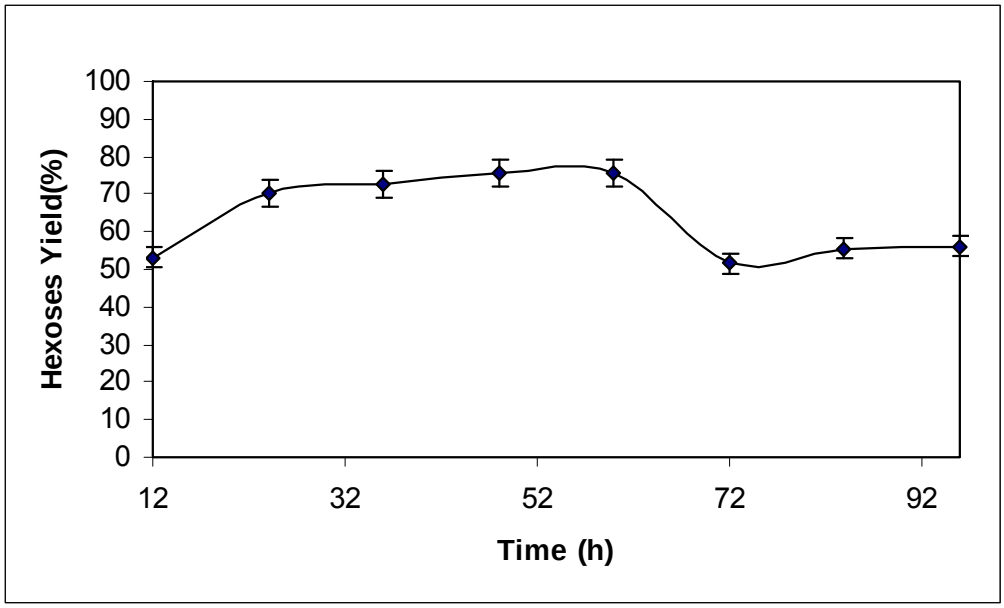


Figure 3.3 Effect of enzymatic hydrolysis time on hexose yield from soybean hulls pretreated with 2% H_2SO_4 and steam explosion (140°C, 30 min). Enzymatic hydrolysis was carried out with cellulose loading of 15 FPU/g cellulose and β-glucosidase ate temperature of 48°C and pH 4.8.

Morphological structure

Morphological features of soybean hulls before and after selected treatments are shown in Figure 3.4. A SEM image of the untreated soybean hull shows a layer covering the surface of the material. This surface layer may comprise waxes, hemicellulose, lignin, and other binding materials. This assumption needs to be validated in the future since this surface layer has been observed in corn stover, sorghum leaves and stems, and wheat straw; but no previous reports in

soybean hull and other lignocellulosic materials have been found (86-88). SEM image of the sample after enzymatic hydrolysis using a cellulase and cell-wall degrading complex shows that the compacted outer layer of the soybean hull was partially removed (Figure 3.4 B). This suggests that part of the outer layer surface can be made out of hemicellulose. A SEM image of soybean hulls treated with HCl and steam explosion shows some well-defined micro fibers (6μm of diameter), which might be evidence that cellulose fibers are agglomerates of individual cellulose micro-fibers (Figure 3.4 C). This result is in accordance with a previous report in which cellulose particles existed as aggregates of crystalline cellulose entities (89). In this case, the micro-fibers of cellulose were defined, and there was no presence of entangled layers covering the cellulose. Outer layers seemed to be hydrolyzed or degraded, exposing micro-fibers of cellulose to enzymatic attack; however, these fibers still appeared to be connected by some amorphous material, probably un-removed hemicellulose. Figure 3.4 D shows single fibers after treatment with sulfuric acid and steam explosion, resulting in fibers that have a relatively spotless and flat surface. The micro-fibers were also separated from the initial attached structure and fully uncovered, thus increasing the external surface area and porosity. No previous reports are available on the dimensions of single fibers in soybean hulls; however, we can observe that they have a length of about 40 μm and width between 5 and 6 μm.

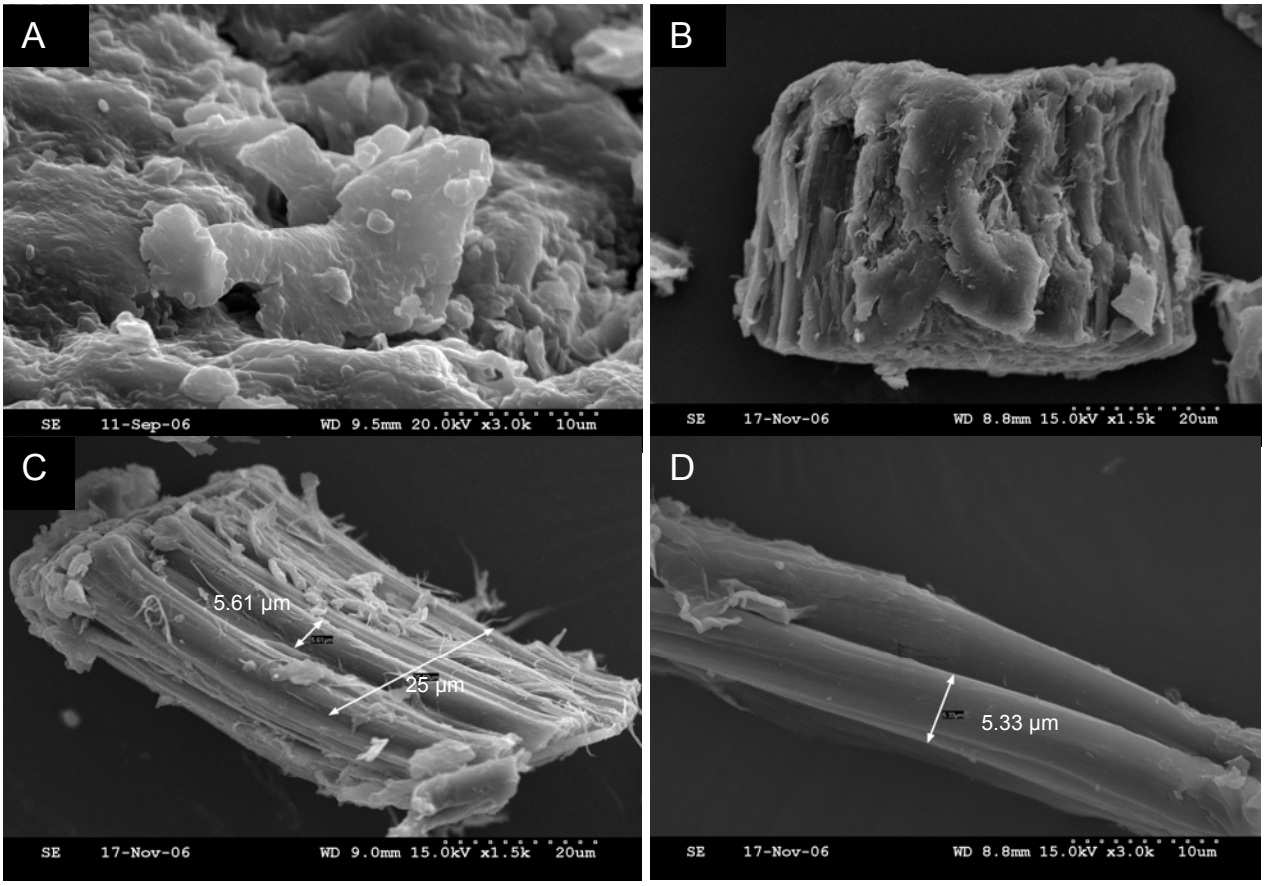


Figure 3.4 Scanning electro micrograph of a) original soybean hull ; b) after enzymatic hydrolysis using cellulose, β-glucosidase and hemicellulose enzymes for 36 h; c) after treatment 1% hydrochloric and steam explosion (140°C, 30 min) ; and d) after treatment with 2% sulfuric acid and steam explosion.

These fibrils could be evidence of the presence of micro-crystalline cellulose fibrils exposed in the remaining solid after pretreatment, suggesting that pretreatment is critical to expose cellulose to enzymatic hydrolysis. To validate this, soy hulls and solids after pretreatment were analyzed by X-ray diffraction (XRD). Figure 3.5 shows the XRD spectra of the original sample, the pretreated sample with sulfuric acid and steam explosion, and the remaining solids after pretreatment and enzymatic hydrolysis. It has been demonstrated that the ratio of intensity of crystalline and amorphous diffractions is approximately equal to the ratio of the masses of amorphous and crystalline parts of a polymer (90). Although there is, in fact, a weak crystalline peak in the XRD pattern of the untreated sample, it is not observable in Figure 3.5 when

comparing with diffraction patterns of treated samples at common scale. Analogously, this may explain why an amorphous XRD pattern of the original sample predominates the crystalline one due to the presence of a high content of amorphous materials (including hemicellulose). After pretreatment, the main peaks relative to planes 002 and 020 may be easily observed, showing that the cellulose amount increased due to the removal of lignin and hemicellulose. The profile of the diffractogram is in agreement with earlier ones reported in the literature for mycro-crystalline cellulose samples (91, 92). This suggests that pretreatment is effective in exposing cellulose to enzymatic attack. The XRD of the sample after enzymatic hydrolysis showed that the content of cellulose decreased.

After calculation of the crystallinity index of soybean hulls before and after enzymatic hydrolysis, CrI somewhat decreased from 67% to 61%. Lower crystallinity was associated with cellulose decrystallization and relatively amorphous material (85). This suggests that enzymatic hydrolysis not only degrades but also slightly facilitates decrystallization of cellulose. Even though we obtained a slight reduction of CrI after enzymatic hydrolysis, 72% hexose yield was obtained after 36 h of enzymatic treatment. This is in accordance with Lauerano-Perez et al., who concluded that cellulose crystallinity is not an important indicator for hydrolysis completion (93).

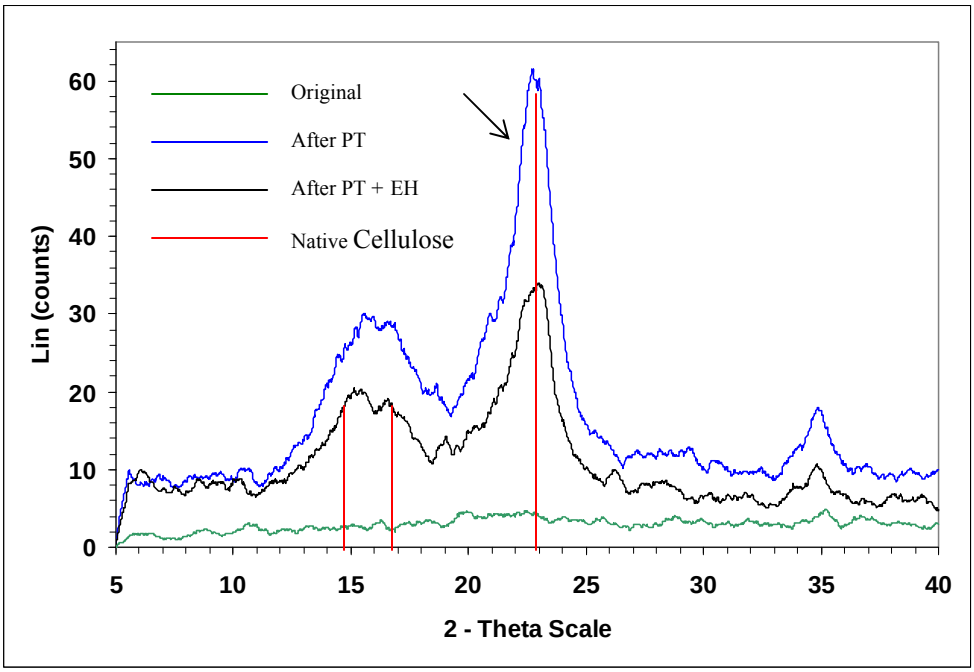


Figure 3.5 X-ray diffraction of original soybean hull, after treatment with 2% H_2SO_4 at 140°C for 30 min (PT), and after treatment and enzymatic hydrolysis with cellulose (15 FPU/g cellulose) and β-glucosidase (50 CBU/g cellulose) (PT+EH). The labeled peak is the principal 002 peak (100% intensity).

Conclusion

Soybean hulls are a potential feedstock for production of bio-ethanol because of their high carbohydrate content (≈50%) with about 37% cellulose. Pretreatment of soybean hulls could substantially improve recovery of sugars. Overall, results showed that up to 80% of total sugars in soybean hulls were recovered using pretreatment with 2% sulfuric acid and steam explosion at 140°C for 30 min, followed by enzymatic hydrolysis with cellulase (15 FPU/g. cellulose) and β-glucosidase (50 CBU/g. cellulose). This yield is much higher (>100%) than overall total sugar yields obtained by direct enzymatic hydrolysis using not only cellulase and β-glucosidase, but also hemicellulase enzymes with the same amount of enzyme loading and enzymatic hydrolysis conditions. Thus, pretreatment with sulfuric acid and modified steam explosion is crucial before enzymatic hydrolysis, and allows efficient enzymatic hydrolysis even in the absence of hemicellulase enzymes. The highest hexose yield of 72% was achieved with a combination of 2% sulfuric acid, steam explosion, and enzymatic hydrolysis. Cellulose crystallinity does not seem to be the only factor that affects enzymatic hydrolysis of soybean

hulls. Further studies concerning optimization of cellulose enzymatic hydrolysis and use of other pretreatment methods to improve hexose yields are needed.

CHAPTER 4 - Evaluation and Characterization of Forage Sorghum as Feedstock for Fermentable Sugar Production

Ethanol derived from lignocellulosic materials has great potential to be a sustainable replacement for corn grain in production of transportation fuels and energy applications. Conversion of cellulosic biomass such as agricultural residues to fuels and chemicals offers major economic, environmental, and strategic benefits, and biological processing based on cellulases offers high sugar yields vital to economic success. The U.S. DOE and USDA projected that U.S. biomass resources could provide approximately 1.3 billion dry tons/year of feedstock for biofuels production, which could produce enough biofuels to meet more than one-third of annual U.S. fuel demand for transportation (13).

Sorghum is a tropical grass grown primarily in semiarid and drier parts of the world, especially areas too dry for corn. Sorghum produces 33% more dry mass than corn in dry land. (3). About 14 million metric tons of sorghum grains (about 7.7 million acres) were produced in the United States in 2007, and more than 6 million acres of forage sorghum are planted each year, resulting in about 30 million tons of sorghum biomass (stems and leaves) composed mainly of cellulose, hemicellulose, and lignin (3). Forage sorghum, sometimes called “cane” has the potential to grow very tall (6 to 15 feet) and can produce a large amount of vegetative growth. Forage sorghums can produce as much, and in some cases more, dry matter than corn when grown with the same amount of water (94). Compared with corn, forage sorghum is cheaper to produce, has comparable yields, and has slightly lower forage quality for silage. These qualities give forage sorghum potential for use in ethanol production (95, 96). Although cellulosic biomass is receiving growing attention as a renewable feedstock, the concept is not well understood for sorghum biomass because scientific information on using forage sorghums such as brown midrib (BMR) for ethanol production is limited. In recent years, introduction of sorghum plants containing the BMR gene generated much interest because plants with this trait have lower lignin concentrations than conventional types (94). Researchers have used chemical and genetic approaches to improve forage fiber digestibility by reducing the amount of lignin or

extent of lignin cross linked with cell wall carbohydrates. BMR forage genotypes usually contain less lignin and may have altered lignin chemical composition (95, 96). Varieties with low lignin content and less lignin cross linked with cell wall carbohydrates could be easily hydrolyzed to fermentable sugars.

Pretreatment, enzymatic hydrolysis and fermentation are three major steps for ethanol production from lignocellulosic biomass. Successful use of biomass for biofuel production depends on four important factors: physical and chemical properties of the biomass, pretreatment methods, efficient microorganisms, and optimization of processing conditions. Pretreatment is crucial; it releases cellulose from the lignocellulose matrix, hydrolyzes hemicellulose, breaks and/or removes lignin, and turns crystalline cellulose into an amorphous form (10, 11). Pretreatment methods have been extensively studied (10, 11, 36, 38, 52, 97), as have efficient microorganisms and optimization of processing conditions (70, 98-104). However, at present, there are few studies about physical and chemical characterization of biomass before and after pretreatment and hydrolysis (64, 105, 106).

Cellulose and hemicellulose are the main polymers found in biomass. They are polymers of hexoses (mannose, glucose, galactose) and pentoses (xylose and arabinose), respectively. The microstructure and properties of cellulosic biomass have significant effects on bioconversion rate. Crystallinity, morphology, and surface area accessible for cellulase binding are major physical and structural factors that affect pretreatment and enzymatic hydrolysis (87, 93). We found no reported information on pretreatment, enzymatic saccharification, and fermentation of forage sorghum for biofuel. Infrared spectroscopy and X-ray diffraction could be useful tools for rapidly obtaining information about the structure of forage sorghum constituents and chemical changes occurring in various treatments. Previously, these techniques have been used to study structure and morphology of plant carbohydrates and lignocellulose (54, 62, 63, 106, 107, 107, 108). In this work, fourier transform infrared spectroscopy (FTIR) and X-ray Diffraction (XRD) were used to study changes in chemical composition and chemical structures after pretreatment and enzymatic hydrolysis. These processes were developed and optimized in previous studies to analyze the relationships among composition, microstructure, and fermentable sugars yield (74, 109). This work is part of a long-term project designed to study the feasibility of ethanol production from forage sorghum.

Materials and Methods

Materials

Four types of forage sorghum (stems and leaves) with 8% moisture content were evaluated. FS-3, BMR forage sorghum classified as a medium-early maturing hybrid, was obtained from Sharp Brothers Seed, Texas. FS-2 is a photoperiod sensitive, non-BMR sorghum/sudangrass. FS-1 is a photoperiod sensitive BMR forage sorghum (4 Evergreen BMR) from Walter Moss Seed Co. RS, obtained from Kansas State University, was used as a control; it was classified as normal forage sorghum. Sorghum biomass samples were stored at 4ºC. Chemical composition of these forage sorghums ranged from 24–38% cellulose, 12–22% hemicellulose, 17-20% lignin and 1–22% starch. Total carbohydrate composition ranged from 59 -66% (Table 4.1). All reported yields were normalized to the total potential glucose and xylose in the original untreated material to provide perspective on the relative contribution of each sugar to total sugar recovery. Cellulase (Celluclast 1.5 L, 90 FPU/ml) and Novozyme 188 (β-glucosidase) (250 CBU/ml) from Novozyme (U.S. Office: Franklinton, NC) were used for enzymatic hydrolysis of forage sorghum into fermentable sugars. Sugars used for High Performance Liquid Chromatography calibration were purchased from Fischer Scientific Inc. (Pittsburgh, PA).

Table 4.1 Chemical composition of forage sorghums.

Component [a]	Sample[b]			
	FS-1	**FS-2**	**FS-3**	**RS**
Carbohydrates	66.22	62.48	59.44	59.33
Starch	8.13b	6.80c	22.91a	0.84d
Hemicellulose	22.48a	17.64c	12.32d	20.37b
Cellulose	35.51b	38.04a	24.21c	38.72a
Total Lignin[c]	13.46b	16.51a	13.58b	16.79a
Klason Lignin	14.63bc	19.14ab	11.06c	20.47a
Crude Fat	1.08b	1.07b	1.68a	1.14b
Crude Fiber	34.02b	36.87a	20.80d	29.43c
Crude Protein	5.16b	4.13c	7.46a	3.88d
Ash	9.29c	10.87a	6.93d	9.98b

[a] Means of two replications. Values in the same row with the same letters are not statistically different at $p<0.05$
[b] FS-1, FS-2, and FS-3 are forage sorghum sample 1, 2 and 3, respectively; RS = regular sorghum.
[c] Calculated as AIL+ASL

Starch degradation

To ensure complete removal of starch before pretreatments, Liquozyme and Spyrizime (U.S. Office: Franklinton, NC) were used for starch liquefaction and saccharification, respectively. A 20-L steam jacket kettle (Model TDC/2-10, Dover Corporation, IL) with 5 L of medium containing 10% forage sorghum dry matter (DM) and 20 μL/20 g starch of Liquozyme was heated (85°C) with agitation (140 rpm) (Barnant Mixer Model 750-0230, Barrington, IL) for 1 h at pH 5.8. After decreasing the temperature to 60°C, Spyrizime (100 μL/20 g starch) was added and saccharification was allowed to proceed for another 2 h at pH 4.5 with continuous agitation at 140 rpm. After saccharification, residual forage sorghum was centrifuged (Programmable Centrifuge Model IEC PR-7000M, International Equipment Company, Needham

Heights, MA.) at 3760 *g* at room temperature for 10 min. Forage sorghum cake was freeze dried for 48 h and collected for further pretreatment and enzymatic hydrolysis.

Pretreatment with dilute acid and modified steam explosion

The treatment was carried out in a 1-L pressure reactor apparatus (Parr Instrument Company, Moline, IL). Forage sorghum was mixed with dilute acid (2% H_2SO_4) to obtain 5% dry matter. The slurry (≈27 g forage sorghum/500 ml) was loaded into the reactor and treated at 140°C for 30 min, following the "modified" steam explosion procedure described by Corredor et al. (109). After treatment, the remaining solid was washed three times with 300 ml of hot deionized water (85ºC). To avoid irreversible collapse of pores within the biomass, pretreated samples were not dried before enzymatic hydrolysis (80). A portion of the washed sample was freeze dried for 48 h, and the solid was stored at 4°C for subsequent characterization. The washed, pretreated, wet solid was stored at 4°C for subsequent enzymatic hydrolysis. A liquid sample from the treatment and washing process was analyzed by HPLC for recovery sugars.

Enzymatic hydrolysis

Pretreated forage sorghum was mixed with distilled water to obtain a solution with 10% solid content and then treated with a mixture of enzymes. Two commercial enzymes, Celluclast 1.5 L and Novozyme 188 (β-glucosidase), were used for hydrolysis of cellulose and hemicellulose in forage sorghum. Enzyme loading of cellulase and β-glucosidase was 15 FPU/g cellulose and 50 CBU/g cellulose, respectively. Enzymatic hydrolysis was carried out in flasks with 100 ml of slurry at 45°C and pH 4.8 for 12 to 96 h in a water-bath shaker with an agitation speed of 140 rpm. Sodium azide (0.3% w/v) was used to inhibit microbial growth during the enzymatic hydrolysis. Samples were taken out each 12 h for sugar analysis. After enzymatic hydrolysis, samples were heated at 100°C for 15 min and stored at 4°C to inactivate the enzymes. Unhydrolized forage sorghum was separated by centrifuging at 13500 *g* for 10 min at room temperature. Liquid was collected for sugar analysis.

Analytical methods

Cellulose and hemicellulose of forage sorghum were analyzed by Filter Bag Technology (ANKOM Technology, Macedon, NY). Total lignin was determined using laboratory procedures developed by the National Renewable Energy Laboratory (80). Starch content was determined

using commercially available kits from Megazyme (Bray, Ireland) according to AACC Approved Method 76-13 (81). Protein was determined via nitrogen combustion using a LECO FP-528 nitrogen determinator (St. Joseph, MI) according to AACC Approved Method 46-30. Nitrogen values were converted to protein content by multiplying by 6.25. Crude fiber, fat, and ash were determined by AOAC standard methods (82).

Concentrations of sugars were determined by HPLC using an RCM-monosaccharide column (300 x 7.8 mm; Bio-Rad, Richmond, CA) and refractive index detector. Samples were neutralized with $CaCO_3$, run at 85°C, and eluted at 0.6 ml/min with distilled water. Hexose yield was counted as the final amount of glucose derived from cellulose. Pentose yield was counted as the final amount of pentose sugars derived from hemicellulose.

Forage sorghum before and after treatments were analyzed by XRD in a Bruker AXS D-8 difractometer settled at 40 KW, 40 mA; radiation was cupper Kα (λ= 1.54 Å); and grade range was between 5 to 40° with a step size of 0.03°. Aperture, scatter, and detector slits were 0.3°, 0.3°, and 0.03°, respectively. Presence of crystallinity in a sample can be detected by absorption peaks. Crystallinity index (CrI) was calculated using the method of Segal et al. (19). CrI is determined by the ratio of the maximum intensity of the peak at the 002 lattice diffraction (in arbitrary units) or "crystalline" peak to the intensity of the "amorphous" peak in the same units at 2θ = 18°. Diffractogram was smoothened using the methodology described in Appendix A. Images of the surfaces of pretreated and untreated forage sorghum were taken at magnifications from 1.5K to 3K using a Hitachi S-3500 N scanning electron microscope (SEM). Specimens were mounted on conductive adhesive tape; sputter coated with gold palladium, and observed using a voltage of 15 to 20 kV.

FTIR measurement was performed in the original and treated forage sorghum using a Thermo Nicolet Nexus™ 670 FT-IR spectrophotometer equipped with a Smart Collector. Reagent KBr and samples were dried for 24 h at 50°C and then prepared by mixing 2 mg of sample with 200 mg of spectroscopy grade KBr. The analysis was carried out in the wavenumber range of 400–4000 cm-1, with detector at 4 cm-1 resolution and 32 scans per sample. OMNIC 6.1a software (Thermo-Nicolet Corporation, Madison, WC) was used to determine peak positions and intensities.

Analysis of variance (ANOVA) and least-significant difference (LSD) were done using SAS (SAS Institute 2005, Cary, NC).

Results and discussion

Fourier transform infrared spectra

Table 4.2 summarizes FTIR results for the forage sorghum samples during treatments. Figure 4.1 shows FTIR spectra of untreated samples in the wavelength region from 3800 - 900 cm^{-1}; Figure 4.2, Figure 4.3, and Figure 4.4, show FTIR spectra of untreated samples after treatment and after enzymatic hydrolysis in the fingerprint region of 1800 to 900 cm^{-1}. IR spectra of untreated forage sorghum show a strong bands associated with hydrogen bonded O-H stretching absorption around 3300 cm^{-1} and a prominent C-H stretching absorption around 2900 cm^{-1}. (Figure 4.1) (110) In the fingerprint region, between 1800-900 cm^{-1}, many absorption bands associated to various contributions from vibrations modes in carbohydrates and lignin are also present in forage sorghum (110, 111). Differences between hardwood and softwood lignin also can be observed in the fingerprint region (111). Each sample shows a distinctly different pattern of absorbance. Close inspection of the peaks shows a peculiar hemicellulose band at 1732 cm^{-1} for all original samples. In cell walls, this peak has been related to saturated alkyl esters from hemicellulose (59, 87, 110, 112). The FTIR spectrum is not discernible after treatment, which indicates that hemicellulose is almost entirely extracted by the pretreatment applied. Solubilization of pectins and some phenolics from the wall is also accompanied by changes in the 1245 cm^{-1} region and associated with changes in the 1732 cm^{-1} region (59). Changes around the 1245 cm^{-1} region have been related to C-O-H deformation and C-O stretching of phenolics plus an asymmetric C-C-O stretching of esters depending on the attached group (59). This band (1242-1247 cm^{-1}) is seen clearly in untreated samples and changes following same behavior than 1732 cm^{-1} peak. They showed a broad peak in untreated samples that fades after treatment, confirming solubilization of phenolics and removal of esters from cell wall.

Table 4.2 Assignment of the main bands in FTIR spectra for forage sorghums.

Wavenumber (cm^{-1})	Pattern in:	Assignment	Reference number
1732	Untreated samples	Alkyl esther from cell wall hemicellulose C=O; strong carbonyl groups in branched hemicellulose	(59, 87, 111, 112)
1710-1712	PT samples	C=O in phenil ester from lignin	(112)
1653 - 1549	Untreated Samples	Protein strong band of amide I and amide II, respectively.	(59)
1638-1604	PT samples	Doublet phenolics of remained lignin	(59)
1517-1516	Untreated Samples	Aromatic C-O stretching mode for lignin; guayacyl ring of lignin (softwood) .	(62, 87, 111)
1453-1456	PT samples	Syringil absorption of hardwoods (C-H methyl and methylene deformation).	(111)
1426-1429	PT samples	C-H vibrations of cellulose ; C-H deformation (asymmetric) of cellulose	(62, 111, 113)
1370-1375	Untreated Samples	C-H Stretch of cellulose	(87, 113)
1315-1317	Untreated Samples	C-O Vibration of syringil ring of lignin.	(111, 114)
1242-1247	Untreated Samples	C-O-H deformation and C-O stretching of phenolics.	(59, 113)
1159-1162	PT samples	Antysimetric stretching C-O-C glycoside; C-O-C b-1,4 glycosil linkage of cellulose.	(87, 115, 116)
1098- 1109	PT samples	C-O vibration of crystalline cellulose; glucose ring strech from cellulose	(111, 113)
1060 and 1035	PT samples	C-O vibrations of cellulose	(113)
897-900	PT samples	Amorphous cellulose vibration; glucose ring strecth	(111, 113)

Important phenolic peaks are observed as a doublet at 1604 to 1638 cm^{-1} in all samples after treatment. The band at 1638 cm^{-1} is assigned to an aromatic stretch, and the band at 1604 cm^{-1} appears associated with the α-β double bond of the propanoid side group in lignin-like structures (59). Bands at 1604 and 1638 cm^{-1} are defined after pretreatment, weaken in samples FS-2 and FS-1 after enzymatic hydrolysis, and remain in samples FS-3 and RS. This suggests that treatments in samples FS-3 and RS did not completely remove lignin but were more effective in samples FS-2 and FS-1. This also is supported by presence of peaks at 1710-1712 cm^{-1} after treatment in all forage sorghum samples, which indicate that C=O linkages of phenyl esters from remained lignin (110, 112).

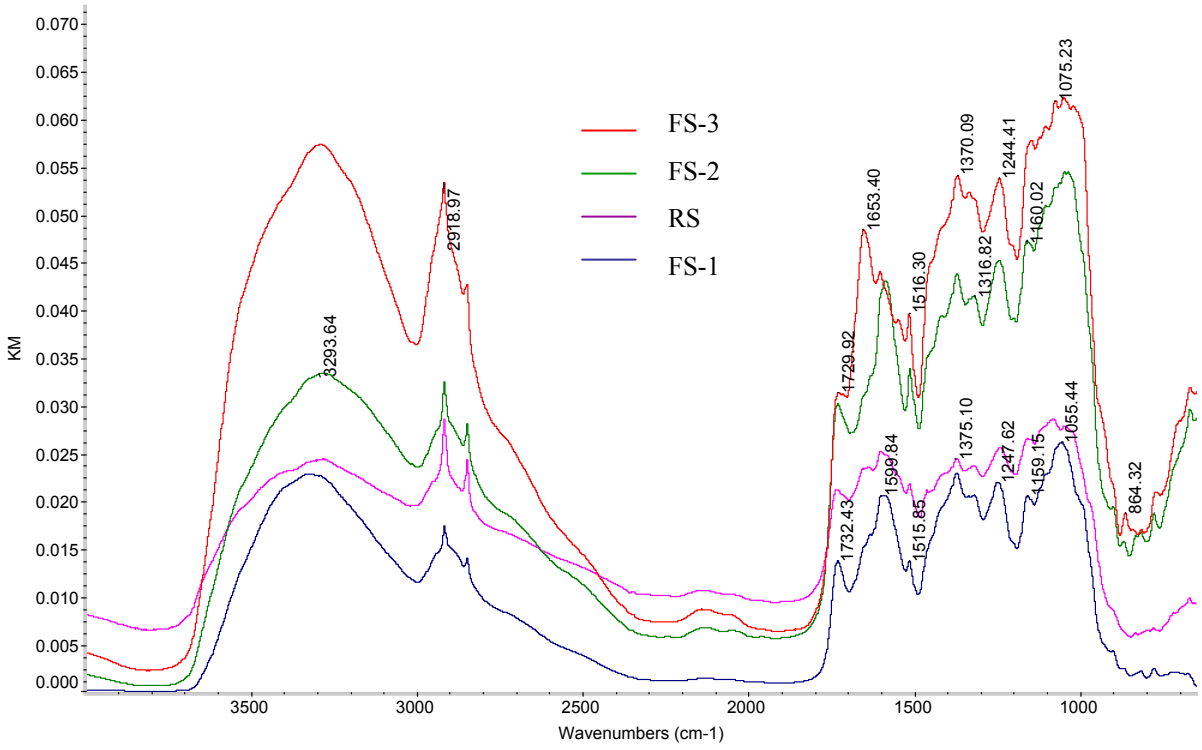


Figure 4.1 FTIR spectra of untreated forage sorghums.

Forage sorghum, a grass species, has two types of lignin (guaiacyl and syringyl rings), and softwood lignin almost exclusively contains guaiacyl rings (16, 111). These rings are seen as aromatic skeletal vibrations of the benzene ring at 1510 cm^{-1} bands (87, 110, 111, 113) and

sometimes shifted toward a higher wave number (>1510 cm^{-1}) in softwoods (111). Guaiacyl ring-related IR spectra are present in all untreated samples at 1516-1517 cm^{-1} and have a strong peak in FS-3 and FS-2. The spectra remain after treatment and are still seen after enzymatic hydrolysis with a weak band in FS-3 and RS. Bands around 1460 cm^{-1} are attributed to C-H methyl and methylene deformation common in hardwoods, and bands at 1315 cm^{-1} are attributed to C-O absorption of syringyl rings in lignin (110, 111). The presence of syringyl units in forage sorghum is evident from the bands at 1453-1456 cm^{-1}, which have a weak absorption in untreated and treated samples but after enzymatic hydrolysis remain weak in FS-R and FS-3. The same behavior is seen in the 1315-1317 cm^{-1} spectrum, which is well defined in FS-2 and RS after pretreatment; however, after enzymatic hydrolysis, these bands almost disappear. This suggests that FS-2 is composed mainly of guaiacyl rings, but RS, FS-3, and FS-1 have both syringyl and guaiacyl rings. After treatment, remotion of guaiacyl rings was more effective in FS-2 than other samples, maybe because of the strong presence of interaction among syringyl and guaiacyl rings on them.

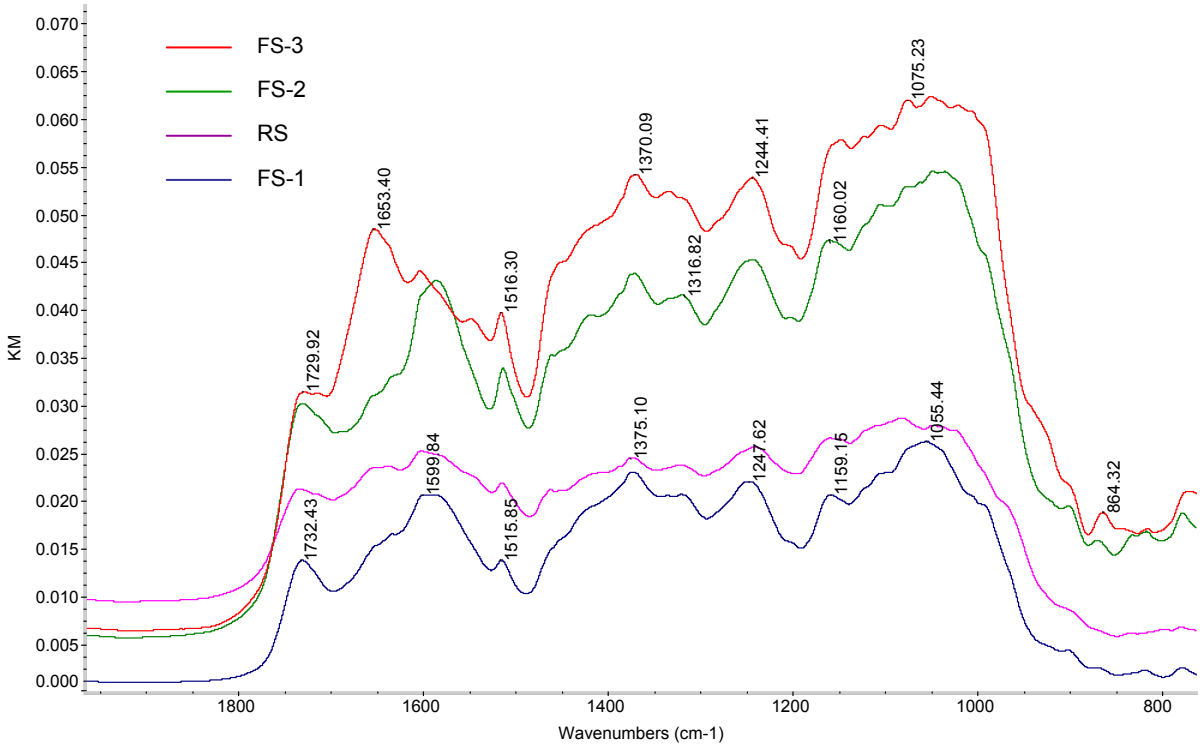


Figure 4.2 FTIR of untreated forage sorghums in the fingerprint region (900-1800 cm^{-1}).

Proteins give rise to two bands in the IR arising from the amide linkage. These bands are seen at about 1653 cm^{-1} (amide I) and 1549 cm^{-1} (amide II), often with an intensity ratio of about 2:1 (59). These bands are well defined in untreated FS-3, the sample with higher protein content (7.46%) (Table 4.1). The corresponding bands in other samples are weak but serve as confirmatory evidence of protein content in untreated samples. These bands disappear after treatment, suggesting that protein is removed with treatment.

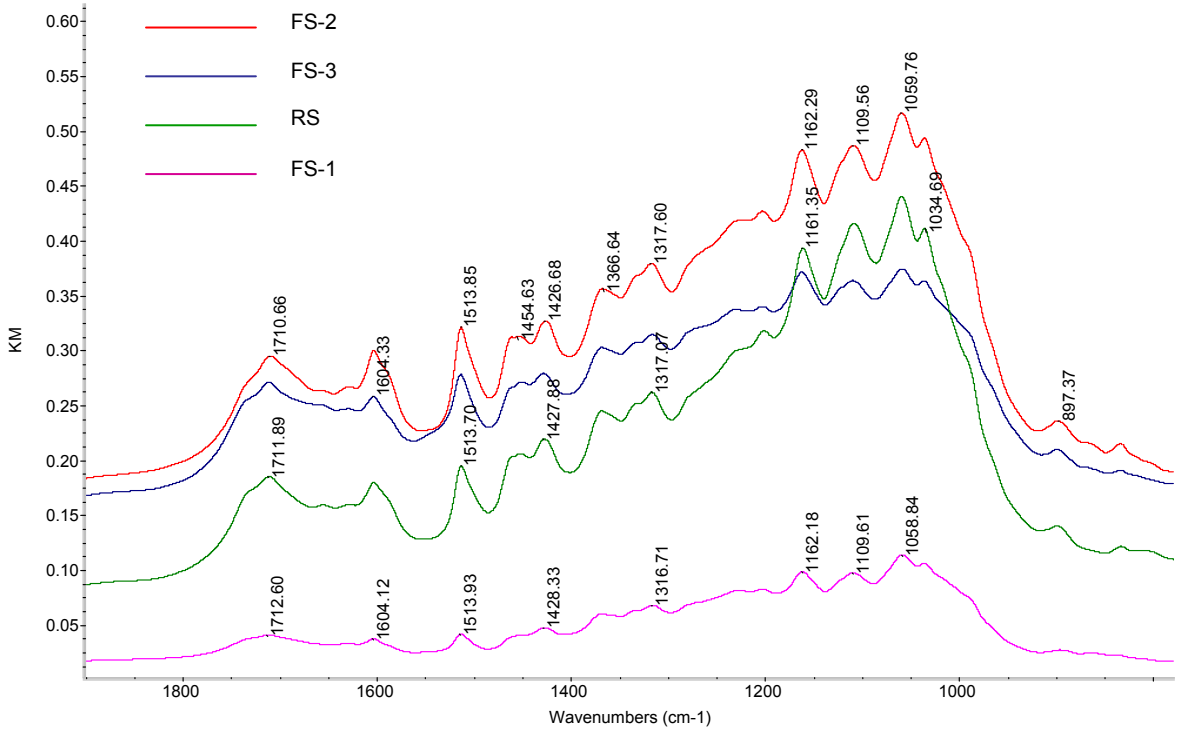


Figure 4.3 FTIR spectra of forage sorghums after dilute acid and modified steam explosion pretreatment in the fingerprint region (900-1800 cm^{-1}).

Cellulose-related bands in the FTIR spectra are seen around 1430, 1370, 1162, 1098 and 900 cm^{-1} (87, 111, 113, 115). Bands around 1430 cm^{-1} are higher in softwood and related to C-H in plane deformation (asymmetric) of cellulose (111). These bands (1426-1429 cm^{-1}) are well defined in untreated FS-3 but weak in other samples. After treatment, bands are well defined in all samples and strong in FS-3. This suggests that FS-3 is composed mainly of deformation (asymmetric) of cellulose common in softwoods. The absorbance at 900 cm^{-1} is associated with the anti-symmetric out-of-phase ring stretch of amorphous cellulose (113, 116) and the 1098 cm^{-1} band is related to C-O vibration of crystalline cellulose (113). Both the

crystalline (1098-1109 cm^{-1}) and amorphous (897-900 cm^{-1}) bands increase in intensity after pretreatment for all samples. However, bands of crystalline cellulose are more intense for FS-2 and RS, suggesting that these two samples have a higher percentage of crystalline cellulose after treatment, which is difficult to further hydrolyze with enzymes. These results indicate that treatment was more efficient at transforming crystalline cellulose to amorphous cellulose in FS-3 and FS-1 than in FS-2 and RS. The appearance of crystalline and amorphous peaks also indicates that cellulose is exposed because of the pretreatment applied. After enzymatic hydrolysis, there is still a weak peak of crystalline cellulose in FS-3 and RS; bands of amorphous cellulose appear weak in all samples, suggesting that amorphous cellulose is almost degraded with enzymes but crystalline cellulose remains in FS-3 and RS. Enzymatic hydrolysis likely degraded almost all amorphous cellulose in FS-2 and FS-1.

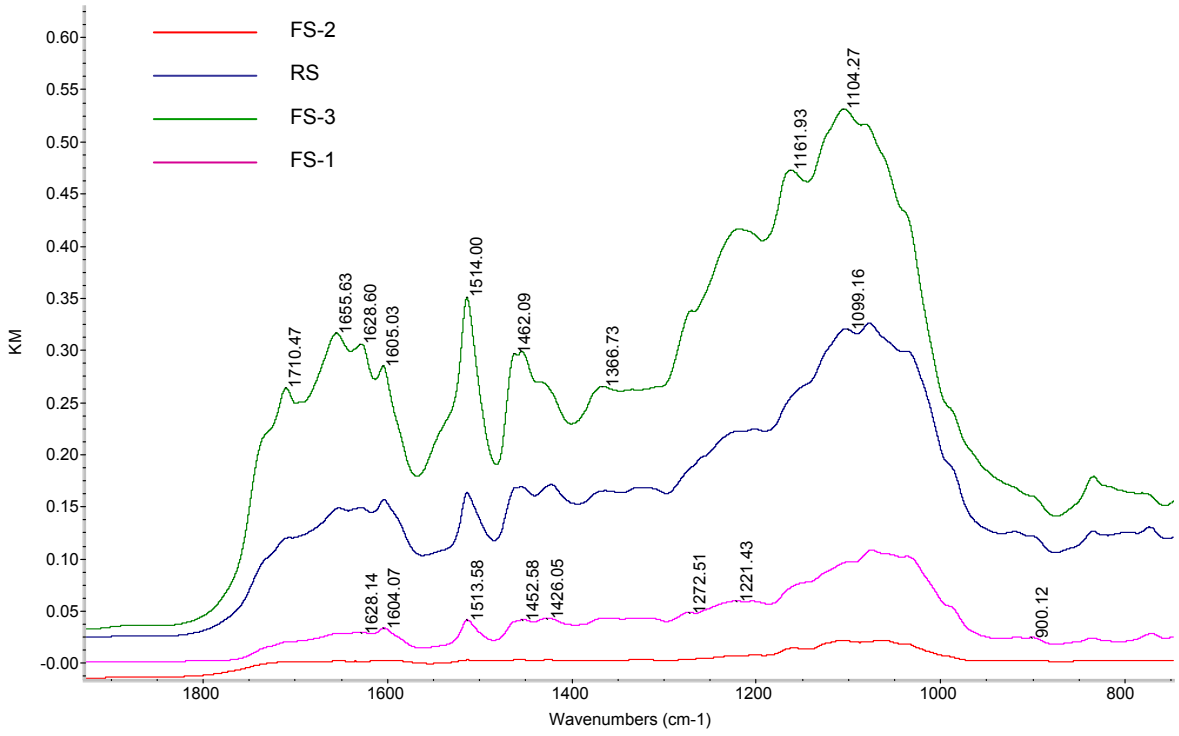


Figure 4.4 FTIR spectra of forage sorghums after enzymatic hydrolysis in the fingerprint region (900-1800 cm^{-1}).

C-H deformation (symmetric) of cellulose is indicated in bands at 1372 cm^{-1} (111, 113). This peak appears around 1370-1375 cm^{-1} in all untreated samples with a weak signal in FS-1. After treatment, the band decreases in intensity and switches to 1366 cm^{-1} in all samples;

however, it almost disappears after enzymatic hydrolysis and shows a weak band in FS-3. The decrease of this band after treatment suggests that cellulose is degraded because of the pretreatment applied and also hydrolyzed after enzymatic hydrolysis. The mainly antisymmetric stretching C-O-C glycoside in cellulose is seen around the 1162 cm^{-1} region (87, 111, 113). This antisymmetric C-O-C vibration is well defined in all treated samples (1159-1162 cm^{-1}) and turns in a flat peak after enzymatic hydrolysis. The decrease in this peak intensity could be related to degradation of β, 1-4 glycosil linkages of cellulose due to enzymatic hydrolysis. Finally, peaks around 1058 cm^{-1} and 1035 cm^{-1} seem to be well defined after treatment in all samples, but they completely disappear after enzymatic hydrolysis. Those peaks are related to C-O stretching of cellulose (111). This confirms that cellulose is fully exposed to further enzymatic hydrolysis after treatment and this procedure is efficient in degrading cellulose to its monomeric sugars.

Morphological structure

Morphological features of untreated forage sorghum samples after treatment and enzymatic hydrolysis are shown in Figure 4.5, Figure 4.6, and Figure 4.7. Untreated samples seem to have deposits on the surface (Figure 4.5a). This surface layer can include waxes, hemicellulose, lignin, and other binding materials and has also been observed in corn stover, sorghum leaves and stems, and wheat straw (86-88). We can also observe some internal plant structures such as vascular bundles and holes in the cellulose wall used for ventilation and metabolism (Figure 4.5 b and c) (117). The general particle size of untreated samples is from 50 to 100μm. The surface layer is removed during treatment, resulting in total exposure of internal structure and fibers that have a relatively clean and smooth surface as shown in Figure 4.6 b and c. We can observe some annular rings (Figure 4.6c) and macro fibrils, probably composed of single cells held together to form a fiber bundle (Figure 4.6b). These images confirm that outer layers are degraded and internal structures, including cellulose, are fully exposed after treatment. An SEM image of the sample after enzymatic hydrolysis shows that the compacted outer layer was removed (Figure 4.7b). The image also shows some well-defined micro fibers (5-16 μm of diameter), which might be evidence that cellulose fibers are agglomerates of individuals cellulose microfibers (Figure 4.7 a and c). This result is in agreement with previous reports in which cellulose particles existed as aggregates of crystalline cellulose entities (86, 117) However, these fibers appeared in some samples with serrations at the edge and are still

connected together with neighboring fibers by some amorphous material, probably unremoved hemicellulose (Figure 4.7b). No previous reports are available on the dimensions of single fibers in forage sorghum; however, we can observe that after enzymatic hydrolysis, particle size reduced notably to elements of about 60 µm length and 5 to 6 µm width. This also suggests that enzymatic hydrolysis reduced and degraded cellulose, leaving a small final solid that might need further degradation.

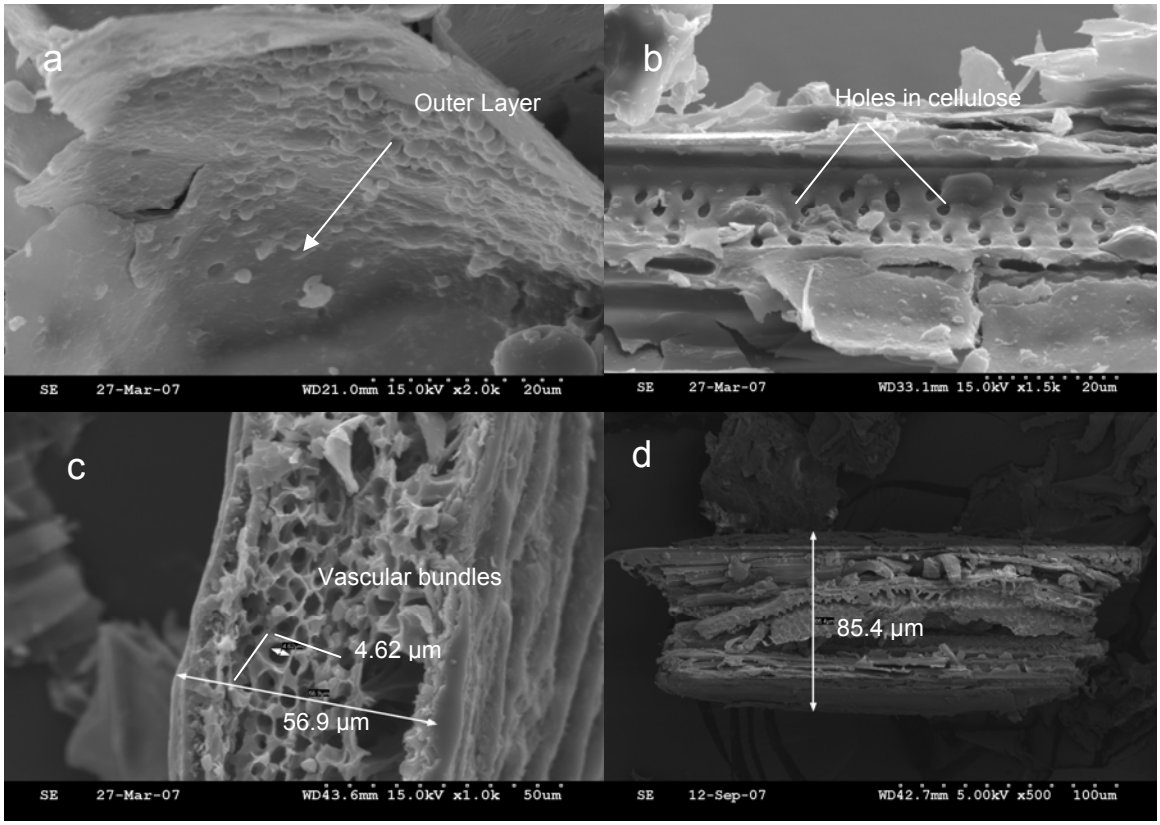


Figure 4.5 SEM images of untreated forage sorghums: a) FS-3; b) FS-2; c) FS-1; and d) RS.

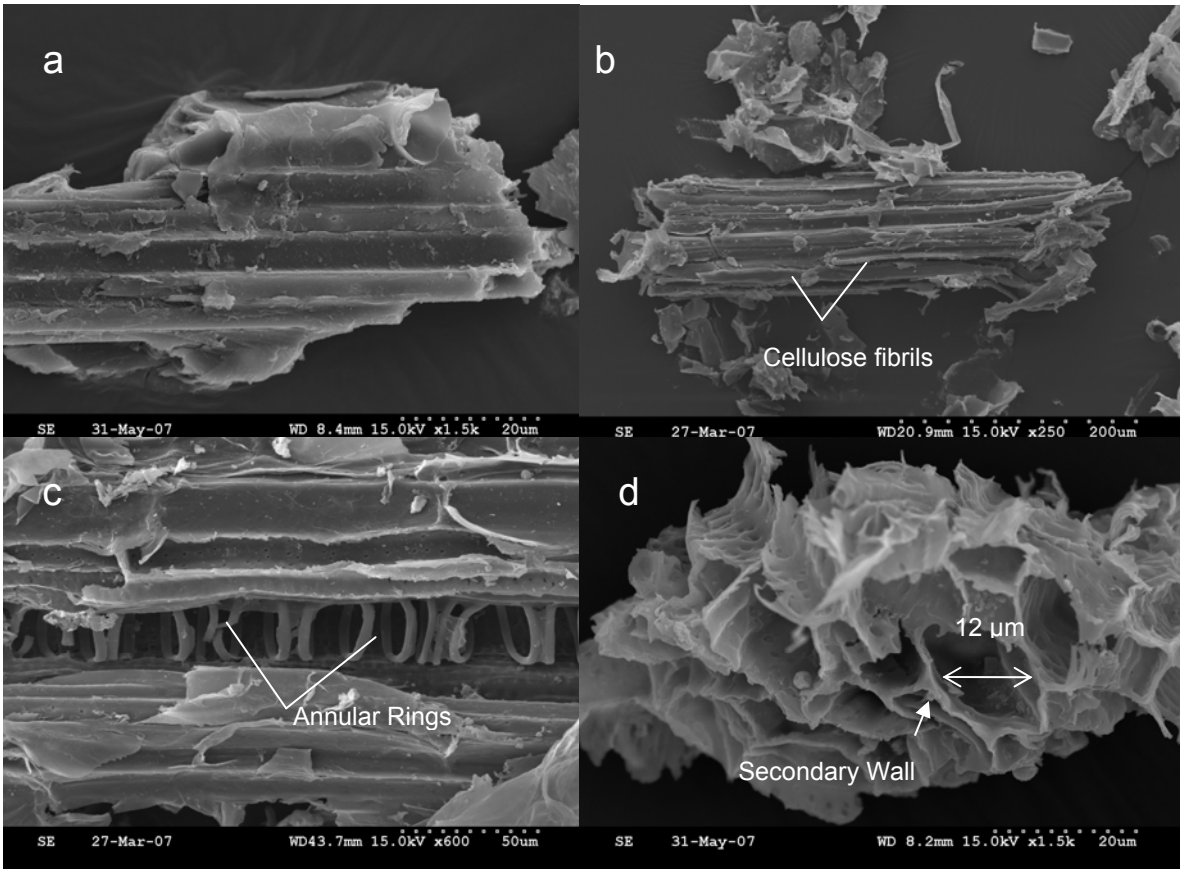


Figure 4.6 SEM images of treated forage sorghums with dilute acid and modified steam explosion pretreatment: a) FS-3; b) FS-2; c) FS-1; and d) RS.

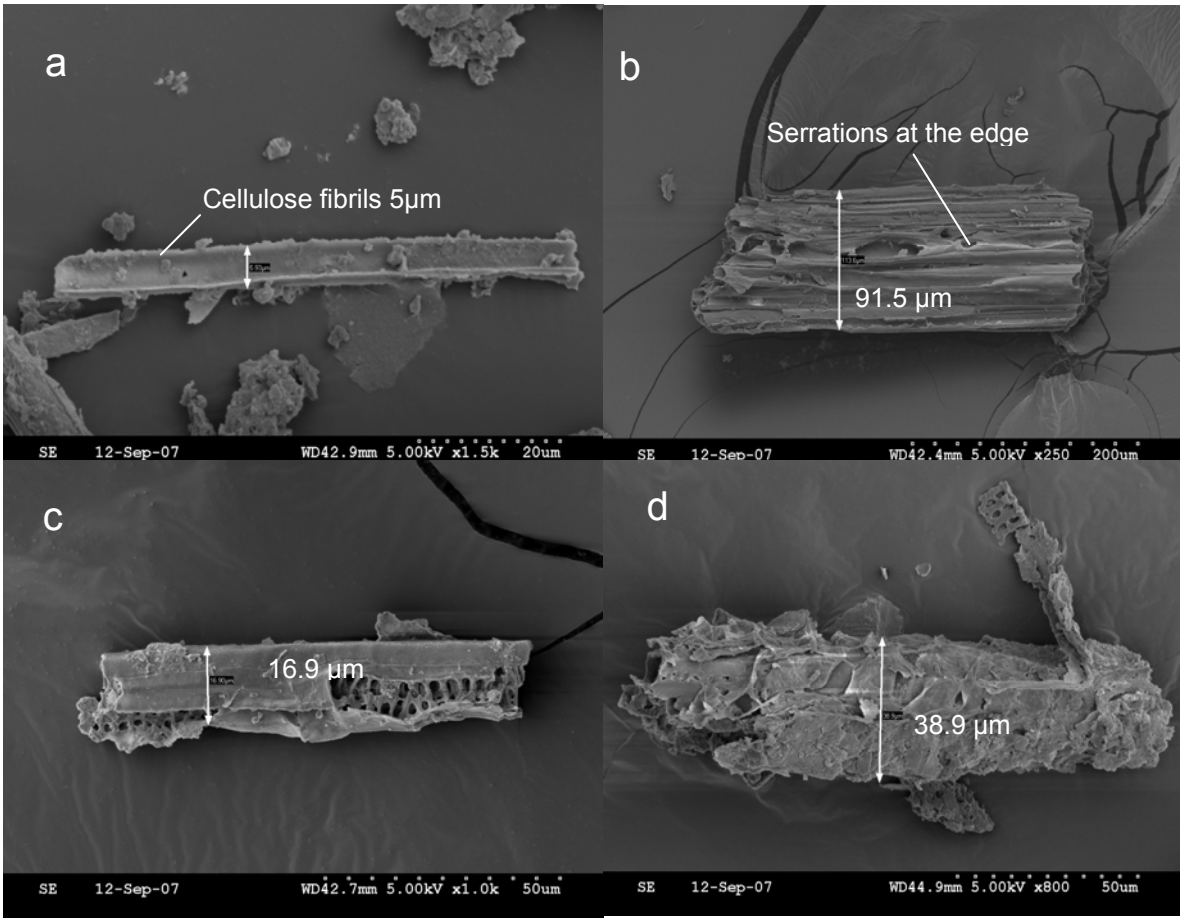


Figure 4.7 SEM of forage sorghums after pretreatment and enzymatic hydrolysis: a) FS-3; b) FS-2; c) FS-1; and d) RS.

X-Ray diffraction

Figure 4.8 shows the XRD spectra of untreated samples, the pretreated sample, and the remaining solids after enzymatic hydrolysis. Spectra show the ordered arrangement of the glucan chains that regulate the physical and chemical characteristics of cellulose. These bonds not only present a regular crystalline arrangement of the glucans molecules resulting in distinct X-ray diffraction patterns but also relate to the swelling and reactivity of cellulose (118). The ratio of intensity of crystalline and amorphous diffractions is approximately equal to the ratio of the masses of amorphous and crystalline parts of a polymer (90). In untreated FS-3 and FS-1, we observe an amorphous XRD pattern that predominates over the crystalline one, probably because of the presence of a high content of amorphous cellulose and/or amorphous materials (including hemicellulose). For untreated FS-2 and RS, the crystalline peak predominates and is well defined

at common scale. This could support the hypothesis of differences between cellulose crystallinity among samples. It seems that untreated FS-2 and RS have high crystalline cellulose content, which could be difficult for transformation to amorphous cellulose with treatments and for further hydrolysis to sugars.

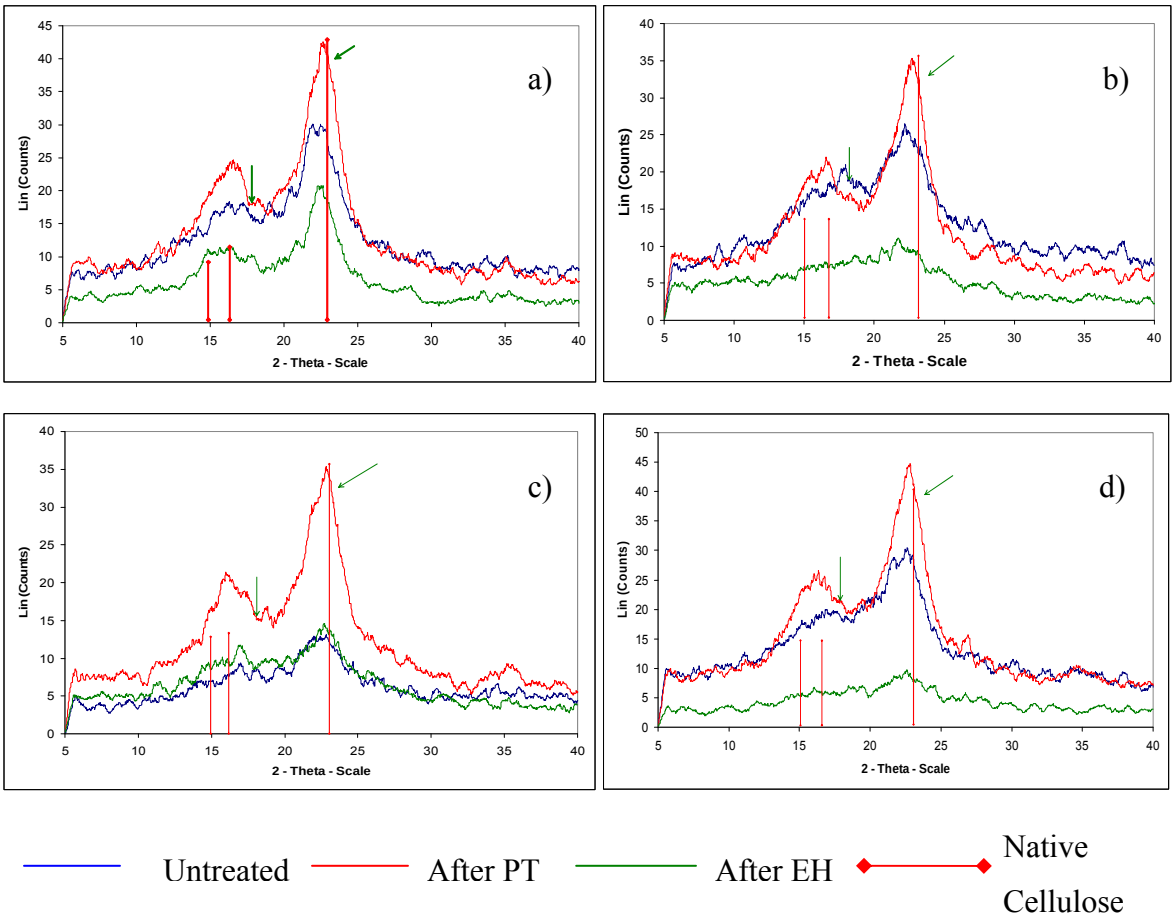


Figure 4.8 X-ray diffraction of untreated forage sorghums after pretreatment and enzymatic hydrolysis: a) FS-2; b) FS-3; c) FS-1; and d) RS. The labeled peaks are the principal 002 (100% intensity) and 101 peak of native cellulose.

After pretreatment, the main peak relative to plane 002 is easily observed in all treated samples, showing that the amount of cellulose increased because of the removal of lignin and hemicellulose. This also confirms that pretreatment is effective in exposing cellulose to enzymatic attack. Furthermore, the crystalline peak is higher in intensity for RS and FS-2, suggesting that these samples have higher content of crystalline cellulose than amorphous

cellulose after pretreatment. This provides additional confirmation of the FTIR analysis results, which showed that bands of crystalline cellulose were more intense for FS-2 and RS after treatment. Low intensity of crystalline peaks in FS-3 and FS-1 suggests that pretreatment was effective at transforming crystalline to amorphous cellulose in these samples and that enzymatic hydrolysis will be easy for these samples because they have higher amounts of amorphous cellulose. XRD of samples after enzymatic hydrolysis shows that the cellulose content decreased. The greatest change was observed in FS-3, but some well-defined crystalline peak remains in FS-2 and RS. The crystallinity pattern of FS-1 after enzymatic hydrolysis looks similar to its pattern before treatment, suggesting that enzymatic hydrolysis is more effective at hydrolyzing amorphous cellulose in FS-3 and FS-1 than in FS-2 and RS, probably because of the original type of cellulose.

We can verify these assumptions of effective hydrolysis of amorphous cellulose in samples by calculating the crystallinity index of untreated forage sorghum (CrI) using the method of Segal et al. (19) after treatment and after enzymatic hydrolysis (Table 4.3). Lower crystallinity has been associated with cellulose decrystallization as well as high value to amorphous material (86, 87). CrI values for FS-2 and RS are always higher (47-49%), even after enzymatic hydrolysis (50 – 75%), than for FS-4 and FS-1. This means that the crystalline fraction in FS-2 and RS is higher than the amorphous fraction. After pretreatment, all samples show almost the same degree of crystallinity (51-58%). However, after enzymatic hydrolysis, the crystalline peak is almost degraded for FS-3 and FS-1, as noticed from the decreased degree of crystallinity to 16 and 35%, respectively. This confirms that applied procedures easily decrystallize and degrade cellulose in FS-3 and FS-1. Profiles of the diffractograms are in agreement with previously reported results for mycro-crystalline cellulose samples (91, 92).

Table 4.3 Crystallinity Index (CrI) for forage sorghums[a]

Sample	Untreated	After PT	After EH
FS-1	38	52	35
FS-2	49	57	75
FS-3	36	51	16
RS	47	58	50

[a.] Means of two replicates

Pentoses and hexoses yield

Steam explosion with 2% H_2SO_4 at 140°C for 30 min gives a maximum pentose yield of 93% from FS-2 and a minimum pentose yield of 80% from FS-R forage sorghum. Pretreatment is more efficient at hydrolyzing hemicellulose in FS-2 and FS-3 than in RS and FS-1 (Figure 4.9). No hexose yield is reported because no significant amounts of hexoses were found after treatment. Although FS-2 has a medium content of hemicellulose (17.7%) (Table 4.1), this sample gives the maximum yield of pentose sugars followed by FS-3. However, RS and FS-1, which have high amounts of hemicellulose (20.4 and 22.4%, respectively) give low pentose yields (84 and 79%, respectively). Based on FTIR analysis, we can suggest that not only hemicellulose and lignin contents affect hydrolysis of hemicellulose but the almost exclusive presence of guaiacyl rings of lignin also affects hemicellulose degradation. The presence of these rings could facilitate effortless degradation of lignin and further hydrolysis of hemicellulose as seen in FS-2 and FS-3.

A maximum hexose yield of 79% is obtained from FS-3 after 72 h of enzymatic hydrolysis (Figure 4.10). FS-2 and RS have the lowest hexose yields (43 and 48%, respectively) after 72 h of enzymatic hydrolysis. The higher hexose yield obtained from FS-3 and FS-1 corresponds with results obtained from XRD and FTIR analysis. The ordered arrangement of the glucan chains with a dominated amorphous pattern in FS-3 and FS-1 facilitated hydrolysis of cellulose to monomeric sugars in these samples. These results also support the idea of decrystallization and hydrolysis of cellulose after enzymatic hydrolysis for FS-3 and FS-1, probably because the initial ordered arrangement controls the swelling and reactivity of cellulose.

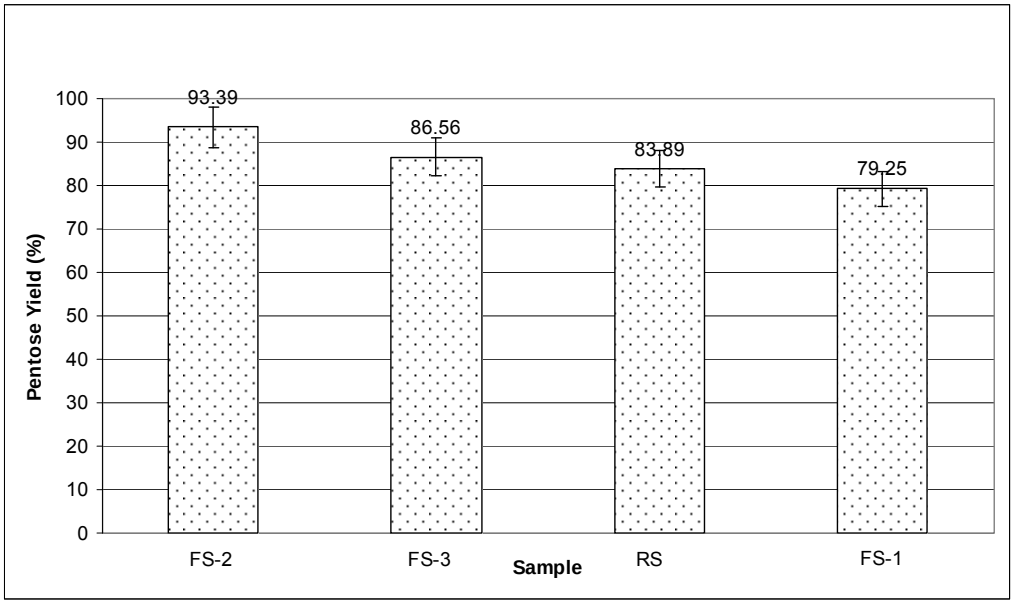


Figure 4.9 Pentose yield (%) of forage sorghums after pretreatment with 2% H_2SO_4 at 140°C for 30 min.

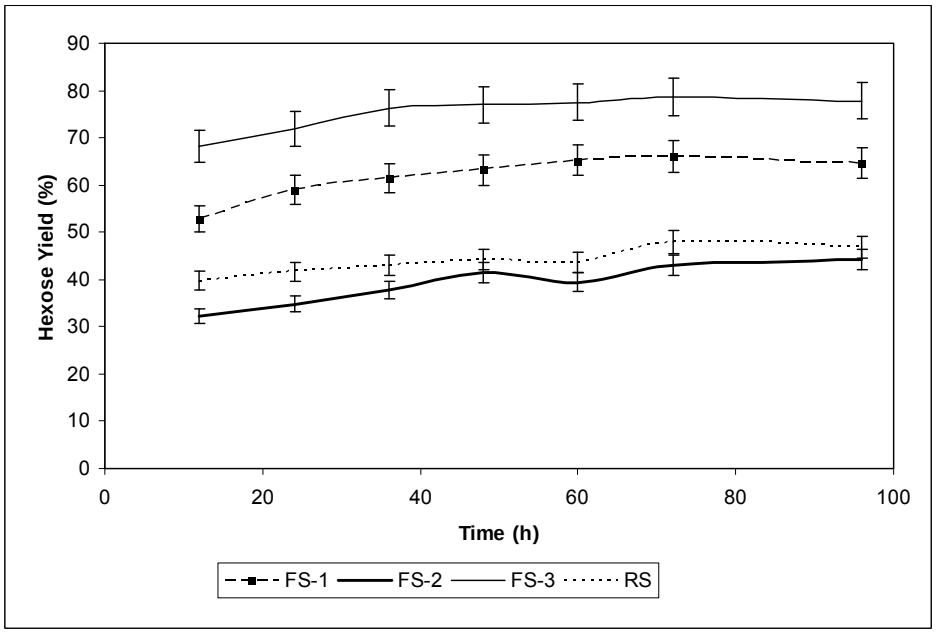


Figure 4.10 Effect of enzymatic hydrolysis time on hexose yield for pretreated forage sorghums. Enzymatic hydrolysis was carried out with cellulase loading of 15 FPU/g cellulose and β-glucosidase 50 CBU / g cellulose at 45ºC and pH: 4.8.

Conclusions

Four varieties of forage sorghum with carbohydrate content ranging from 59 to 66% and cellulose content ranging from 24 to 38% were evaluated as potential feedstocks for bio-ethanol production. FTIR, SEM, and XRD were used to characterize the physical and chemical properties of forage sorghum as affected by pretreatment and enzymatic hydrolysis. There is strong relationship among chemical structure, function, composition, and fermentable sugars yield. Up to 72% of hexose yield from FS-3 and 94% of pentose yield from FS-2 were obtained using modified steam explosion with 2% H_2SO_4 at 140°C for 30 min and enzymatic hydrolysis with cellulase (15 FPU/g. cellulose) and β-glucosidase (50 CBU/g. cellulose). Forage sorghums with a high percentage of guaiacyl rings in their lignin structure were easy to hydrolyze after pretreatment despite the initial lignin content. Pretreatment was more effective for forage sorghums with a low crystallinity index and easily transformed crystalline cellulose to amorphous cellulose, despite initial cellulose content. Additional studies on ethanol fermentation of low lignin content or modified pretreated forage sorghum are needed.

CHAPTER 5 - Fermentation of Forage Sorghum into Ethanol

Fermentation of lignocellulosic hydrolyzates is more difficult than the well-established processes of ethanol production from molasses and starch. Hydrolyzates contain a broader range of inhibitory compounds where the composition and concentration of such depend on the type of lignocellulosic materials, the chemical used and nature of the pretreatment, and the hydrolysis process (7). Ethanol fermentation can be carried out by three main steps: simultaneous saccharification and co-fermentation (SSCF), simultaneous saccharification and fermentation (SCF), and/or separate hydrolysis and fermentation (SHF) (44). For the SHF procedure, cellulose and/or hemicellulose is enzymatically hydrolyzed into glucose or pentose first, and then these sugars are fermented to ethanol (45). The SSCF involves the presence of a co-culture capable of converting the mixed sugars into ethanol (78). It is known that when the percentage of glucose in the feedstock is much higher than xylose, co-fermentation could be a more efficient approach since the cost of separation processes would be high. SSF involves the enzymatic hydrolysis of cellulose and hemicellulose to sugars, and the conversion of fermentable sugars to ethanol in the same vessel (18, 45). The SSF technique provides the possibility to overcome the main difficulty of enzymatic hydrolysis, i.e., decreasing the enzyme loading and therefore, the production cost, making application of SSF for conversion of lignocellulosic hydrolyzates to ethanol a more cost-effective process (44).

Hemicellulose hydrolyzates typically contain monomeric sugars other than D-xylose, such as D-glucose, D-mannose, D-galactose, and L-arabinose. In addition, hydrolyzates frequently contain appreciable levels of oligosaccharides as a result of incomplete hydrolysis of hemicellulose polysaccharides. In addition to mixed sugars and oligosaccharides, inhibitory compounds are usually present in pretreated material. Suspected inhibitory compounds include compounds that are hydrolyzed or solubilized during pretreatment, such as acetic acid and numerous lignin-derived aromatic compounds, as well as products of carbohydrate degradation such as furfural from xylose and hydroxymethyl furfural (HMF) from glucose (119).

One of the major problems associated with ethanol production from dilute-acid pretreated lignocellulosic biomass hydrolyzates is the inability of the fermentative microorganism to withstand inhibitory compounds formed or released during pretreatment, and

usually a detoxification step is needed to improve fermentability (4, 39). A variety of methods can be used to reduce the concentration of inhibitory compounds to non-inhibitory levels. Methods for detoxifying hydrolyzates include over-liming and heating, as well as variety of other methods such as steam stripping, roto-evaporation, ion exchange, extraction, and treatment with activated carbon and molecular sieves (119).

Use of recombinant microorganisms for co-fermentation is one of the most promising approaches in the field of bio-ethanol production, though use of large-scale industrial processes still requires fine tuning of the reliability of the entire process (32). Several microorganisms have been genetically engineered to produce ethanol from mixed-sugar substrates by using two different approaches: (a) divert carbon flow from native fermentation products to ethanol in efficient mixed-sugar utilizers such as *Esherichia, Erwinia, and Klebsiella*; and (b) introduce the pentose-utilizing capability in efficient ethanol producers such as *Saccharomyces* and *Zymomonas* (11).

Escherichia Coli was genetically engineered to produce ethanol from pentose and hexose sugars by inserting genes encoding alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*) from the bacterium *Zymomonas mobilis* (49). Some comparisons of yeast and bacteria using dilute-acid hydrolyzates of cob corn hemicellulose as a substrate concluded that recombinant *E. Coli* strain KO11 was superior to other pentose-fermenting organisms in ethanol productivity, ethanol yield, and resistance to inhibitors generated during hydrolysis (50). Its effectiveness was also demonstrated at a 150-L scale with hemicellulose syrups and at a 10,000 L scale with laboratory sugars, producing more than 40 g ethanol/L within 48 h (greater than 90% of theoretical yield) (34) . Wyman et al., (9), used recombinant *E. Coli* ATCC 55124 (KO11) for the simultaneous saccharification and co-fermentation (SSCF) of corn stover in a coordinated development of leading biomass pretreatment technologies. Hemicellulose hydrolyzates of agricultural residues bagasse, corn stover, and corn hulls plus fibers were also fermented to ethanol by recombinant *E. Coli* strain KO11 (50). Fermentations were complete within 48h, achieving 40g ethanol L^{-1}, and ethanol yields ranging form 86 to 100% of the maximum theoretical yield.

Objectives of this research were to study the fermentation properties of hydrolyzates from pretreated forage sorghum using different processing schemes such as SSF, SHF, and SSCF, and to analyze the effect of inhibitory compounds on fermentation of forage sorghum into ethanol.

Materials and Methods

Pretreatment, over liming and preparation of forage sorghum for fermentation

Forage sorghum (5% DM) was pretreated as explained in Chapter 4. Solids and syrup were separated by centrifuge at 11000 *g* for 10 min. After treatment, the remaining solids were washed three times with 300 ml of hot deionized water (85ºC). To avoid irreversible collapse of pores within the biomass, pretreated samples were not dried before SSF (80). The washed, pretreated wet solids were stored at 4°C for subsequent SSF.

Small portions of syrup were reserved for organic acids analysis. Over-liming of the hydrolyzate was carried out by adding $Ca(OH)_2$ solution to the syrup to adjust the pH to 10.5. The mixture was stirred for 30 min at 90 °C, allowed to cool slowly to room temperature, and then adjusted back to pH 6.5 with HCl. It was then centrifuged at 11,000 g for 15 min to remove any precipitate (including gypsum), and stored at 4°C before being used as substrate for fermentation with *E. Coli* (36, 50, 120, 121).

Enzymes

Cellulase (Celluclast 1.5 L) (90 FPU/ml) and Novozyme 188 (β-glucosidase) (250 CBU/ml) from Novozyme (U.S. Office: Franklinton, N.C.) were used for enzymatic hydrolysis of forage sorghum into fermentable sugars. Enzyme loading of cellulase and β-glucosidase was 15 FPU/g cellulose and 50 CBU/g cellulose, respectively. Sugars used for HPLC calibration were purchased from Fischer Scientific Inc. (Pittsburgh, PA).

Preparation of inocula

For SSF, dry yeast was activated by adding 1.0 g of dry cells into 19 mL of preculture broth (containing 20 g of glucose, 5.0 g of peptone, 3.0 g of yeast extracts, 1.0 g of KH_2PO_4, and 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter) and incubated at 38 °C for 25-30 min in an incubator operating at 200 rpm. The activated yeast culture had a cell concentration of 1×10^9 cells/mL. The dry active yeast was a gift from Lesaffre Yeast Corporation, Milwaukee, WI. For SHF/SSCF, recombinant *Escherichia Coli* ATCC ® 55124 (KO11) was used (50). This organism was grown in an LB medium (Sigma Cat. No. L-3152) that contained 1% tryptone, 0.5% yeast extract, 1% NaCl, and 40 mg/L chloramphenicol. Fresh colonies were transferred to 250-ml Erlenmeyer

flasks containing 50 ml of xylose broth and incubated for 18 h at 35°C on a rotary shaker and used as inoculum preparation.

Simultaneous saccharification and fermentation (SSF) using S. Cerevisiae

Pretreated, wet solid-forage sorghum was mixed with distilled water to obtain a solution with 10% solid content and then divided in 250-ml flasks containing 100 ml of slurry with pH 4.8. Celluclast 1.5 L and Novozyme 188 (β-glucosidase) were used for hydrolysis of cellulose. Enzyme loading of cellulase and β-glucosidase was 15 FPU/g cellulose and 50 CBU/g cellulose, respectively. The fermentation process started with the addition of 1.0 mL of the activated yeast culture, 0.30 g of yeast extract, and 0.1 g of KH_2PO_4 into the hydrolyzates in each flask. Fermentation was conducted at 38°C for 36 hours in an incubator shaker operating at 150 rpm under anaerobic conditions.

Separate hydrolysis and fermentation (SHF) using E. Coli KO11

For separate hydrolysis and fermentation (SHF) experiments, fermentation was carried out at pH 6.5 and 35°C using the liquid portion of the hydrolyzate after separation from the solids and over-liming. Aliquots (100 ml) of hydrolyzate plus 10 mL of a 10x stock solution of LB, pH 7 (100g/L Tryptone, 50g/L yeast extract) were fermented using *E. Coli KO11* in 250-ml flasks under anaerobic conditions. Inoculum size was 10% (v/v). Fermentations were sampled daily.

Simultaneous saccharification and co-fermentation (SSCF) using E. Coli KO11

Pretreated over-limed slurry of forage sorghum was added in 100-mL working volume such that the glucan content was 2% w/v. The slurry was tempered at 45°C and pH adjusted to 4.8. Enzymatic hydrolysis was carried out for 4 h before adding the microorganism. Temperature was then dropped to 35° and pH adjusted to 6.0-6.5. The inoculum size was 10% (v/v). Samples were withdrawn every 24 h to determine ethanol content and residual sugars. SSCF was carried out in the incubator shaker at 35°C with agitation speed of 150 rpm.

Analytical methods

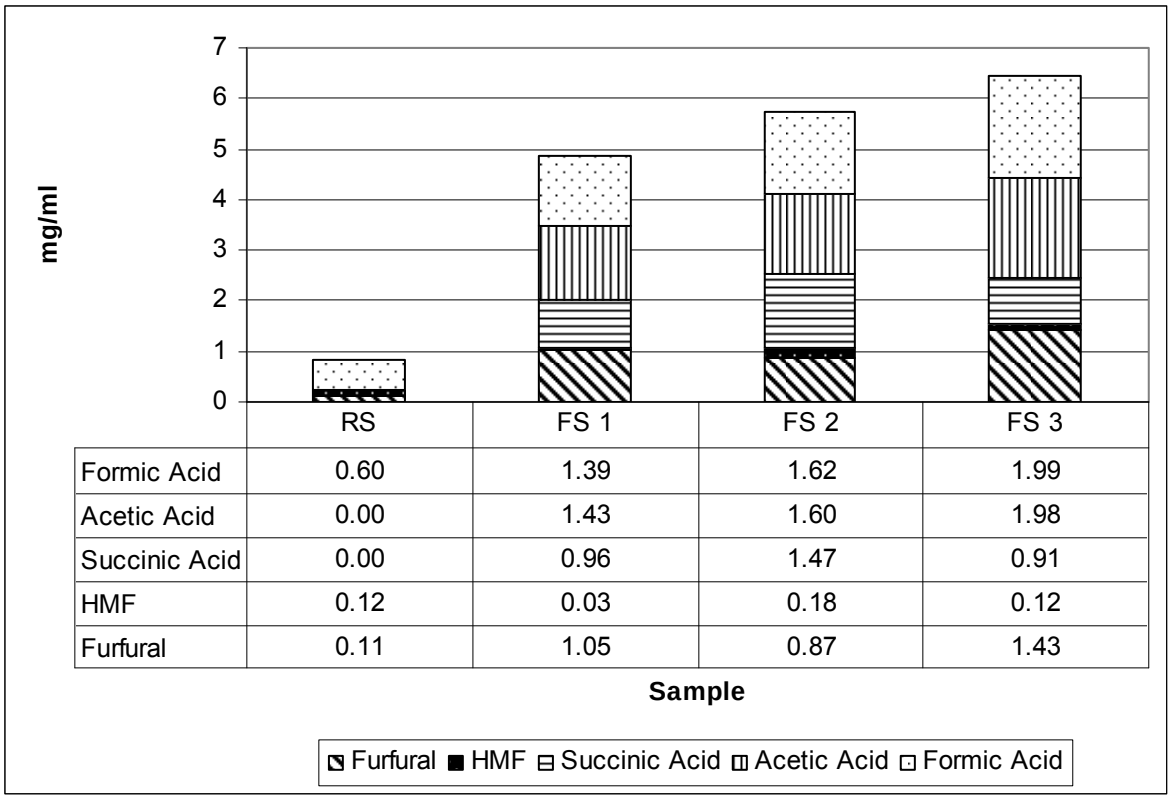
Concentrations of sugars and ethanol were determined by HPLC using an RCM-monosaccharide column (300 x 7.8 mm; Bio-Rad, Richmond, Calif.) and refractive index detector. Samples were neutralized with $CaCO_3$ when necessary, run at 85°C, and eluted at 0.6 ml/min with H_2O. Concentrations of inhibitors were determined by HPLC using an ROA-organic acid column (300 x 7.8 mm; Bio-Rad, Richmond, Calif.) and refractive index detector. Samples were run at 65°C and eluted at 0.5 ml/min with 5 mM H_2SO_4. Analysis of variance (ANOVA) and least-significant difference (LSD) were done using SAS (SAS Institute 2005, Cary, N.C.).

Results and Discussion

Formation of inhibitors

Organic acids were found in a small proportion of all samples. Formic and acetic acid, along with furfural, were the compounds with higher percentages ranging from 0.60 to 1.99 mg/ml, 1.43 to 1.98 mg/ml, and 0.11 to 1.43 mg/ml, respectively (Figure 5.1). Traces of succcinic acid and hydroxymethylfurfural were found (< 1.47 mg/ml). No traces of levulinic acid or other organic acids were found. The maximum amount of inhibitory compounds was detected in FS-3 with a total of 6 mg/ml. Results were in the range reported in the literature for steam-pretreated biomass (78).

It has been demonstrated that at higher concentrations of inhibitory compounds (> 10 mg/ml), fermentation yield decreases only slightly compared with the controls (4). It is also suggested that small amounts of inhibitory compounds will not have any effect on posterior hydrolysis and fermentation. It was expected that the FS-3 sample would developed higher amounts of inhibitory compounds since this sample released the largest amount of sugar after treatment and enzymatic hydrolysis. Unfortunately, there is little in the literature reporting quantity of inhibitory compounds after different pretreatment technologies to compare with modified steam explosion and dilute acid (36). However, we believe that the process applied produces small levels of toxic compounds, suggesting that fermentation and the enzymatic hydrolysis process will not be affected by inhibition. A better understanding of the inhibitory mechanism of individual compounds and their interaction effects will allow us better to optimize fermentation conditions.



	RS	FS 1	FS 2	FS 3
Formic Acid	0.60	1.39	1.62	1.99
Acetic Acid	0.00	1.43	1.60	1.98
Succinic Acid	0.00	0.96	1.47	0.91
HMF	0.12	0.03	0.18	0.12
Furfural	0.11	1.05	0.87	1.43

Figure 5.1 Contribution of inhibitors after pretreatment of forage sorghums.

Simultaneous saccharification and fermentation (SSF) using S. Cerevisiae

It was indicated in Chapter 4 that pretreatment removed hemicellulose and exposed cellulose to enzymatic attack. Cellulose concentration for treated solids increased to 43.9, 43.4, 33, 44.6% for FS-1, FS-2, FS-3, and RS samples, respectively (data not shown). This percentage was used to calculate theoretical and experimental yield of ethanol. Figure 5.2 showed ethanol yield from 50 to 88 % of the theoretical after SSF for all samples. These yields correspond to ethanol concentrations of 0.19, 0.12, 0.16, and 0.14 g.EtOH/g.Biomass for FS-1, FS-2, FS-3, and RS, respectively. These results are higher than the ethanol yields of 0.05 to 0.13 g.EtOH/g.Biomass from rice hulls (36). Maximum ethanol yield was found in FS-3 and FS-1, meanwhile FS-2 and RS were the lower ones. Maximum ethanol yields were expected in FS-1

and FS-3, since those two samples were the ones which developed maximum hexose yield after enzymatic hydrolysis and lower crystallinity index after pretreatment.

Results showed that SSF using *S. Cerevisiae* is an effective method to hydrolyze and ferment cellulose remaining in treated solids with high ethanol yields up to 88%.

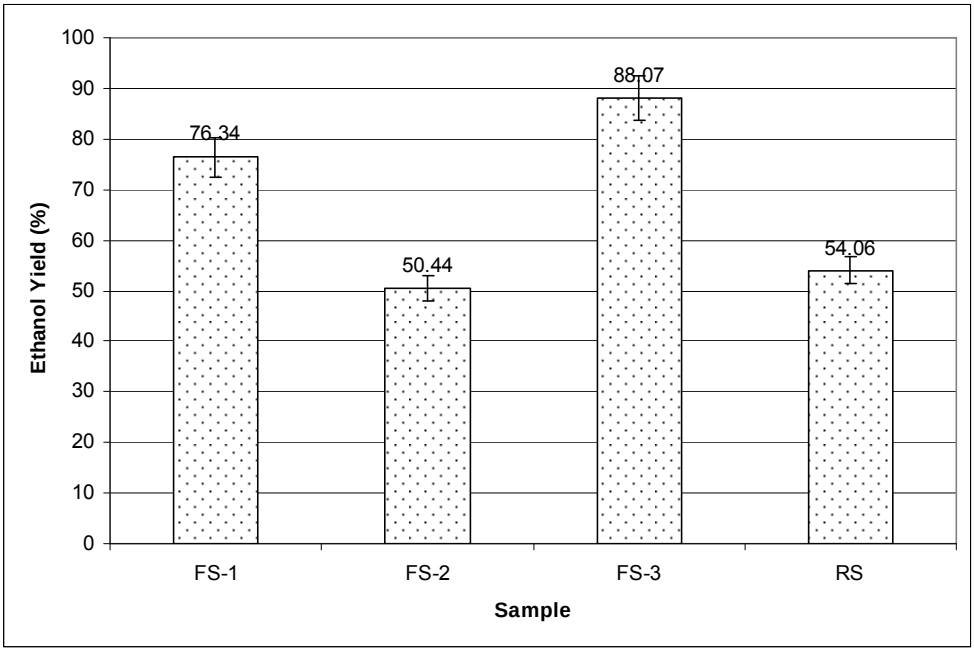


Figure 5.2 Ethanol yield (%) after SSF for 36 h of treated forage sorghums.

Separate hydrolysis and fermentation (SHF) using E. Coli KO11

Using *E. Coli* for ethanol fermentation has two drawbacks for this particular hydrolyzate from sorghum biomass. First, concentration of xylans in the hydrolyzed liquor was about 10 mg/ml after pretreatment (data not shown); thus, in 100 ml of hydrolyzate aliquot the concentration of xylan was about 1%. For *E. Coli* to grow and ferment sugars, it is necessary for sugar concentrations to be at least 4% (49). On the other hand, over-liming not only precipitated inhibitory compounds but also resulted in sugar loss, sometimes up to 5% (36). Our results showed that sugars in hydrolyzate were reduced up to 7 mg/ml after over-liming. The small amount of fermentable sugars was not enough to permit an appropriate growth and fermentation of the microorganism.

In order to demonstrate that *E. Coli* KO11 can metabolize both sugars, preliminary experiments were carried out supplementing hydrolyzate with xylose and hexose, and obtaining

final concentrations of sugars up to 4 %. After 60 h of fermentation, samples supplemented with glucose showed up to 15 to 17 g.ethanol per L; meanwhile, samples supplemented with xylose showed 11 to 15 g.ethanol per L (Table 5.1). Based on a theoretical ethanol yield of 0.51 g.Ethanol /g.sugars, this result corresponds to ethanol yields from 77 to 86% for samples supplemented with glucose and 58 to 80 % for samples supplemented with xylose. Xylose metabolism of *E. Coli* KO11 is slower than glucose metabolism, so ethanol yield was low in samples supplemented with xylose after 60 h of fermentation (50).

These results suggest that although the over-liming process reduces initial amount of sugar, *E. Coli* fermented both sugars and it is possible to use hydrolyzate liquid for fermentation without overturning the effects from inhibitors.

Table 5.1 Ethanol yield (%) of hydrolyzate supplemented up to 4 % either with xylose or glucose after 60 h of fermentation

	Ethanol Yield			
	Glucose (4 % w/v)		Xylose (4% w/v)	
Sample	mg/ml	%	mg/ml	%
FS-1	15.82	77.56	11.93	58.46
FS-2	17.22	84.42	15.75	77.20
FS-3	17.40	85.27	16.40	80.38
RS	17.56	86.09	13.46	65.97

Simultaneous saccharification and co-fermentation (SSCF) using E. Coli KO11

Although hydrolyzates from pretreated sorghum biomass were over-limed before mixing them with solids to achieve SSCF, results of these experiments were not satisfactory. After 60 h of fermentation, there was no production of ethanol. Two possible drawbacks affected the experiment. The difference of optimum temperature for enzymatic hydrolysis (45°C) and ethanol fermentation using *E. Coli* (35ºC) did not allow SSCF to obtain the best conditions for both saccharification and fermentation. In addition, one of the major problems associated with ethanol production from dilute acid-pretreated lignocellulosic biomass hydrolyzates is the inability of the fermentative microorganism to withstand inhibitory compounds formed during pretreatment

(36). Although these inhibitory compounds were precipitated during over-liming, their effects were synergetic and were not tested on enzymatic hydrolysis.

Conclusion

Pretreatment applied to lignocellulosic biomass released small amounts of inhibitors (< 10 mg/ml), which may have an effect on the downstream fermentation process. The amount of inhibitory compounds released during pretreatment increased with an increase in sugar yield after treatment. It was also demonstrated that over-liming before fermentation could reduce the effects of inhibitory compounds released during pretreatment on ethanol fermentation. Up to 86% of ethanol yield was obtained from fermentation of hydrolyzates supplemented with glucose and up to 80% of ethanol yield from hydrolyzates supplemented with xylose. These results suggest that although the over-liming process reduces initial amounts of sugar, *E. Coli* fermented both sugars and it is possible to use a hydrolyzate liquid for fermentation without overturning effects from inhibitors. More understanding of the effects of inhibitors and metabolism of xylose by *E. Coli* is necessary to achieve better results using simultaneous saccharification and the fermentation process (SSCF)

CHAPTER 6 - Summary, Conclusions and Future Work

Summary

The chart below describes the steps used for preparation, analysis, and characterization of biomass samples (Figure 6.1). It also describes the correlation among pretreatment, enzymatic hydrolysis and fermentation steps.

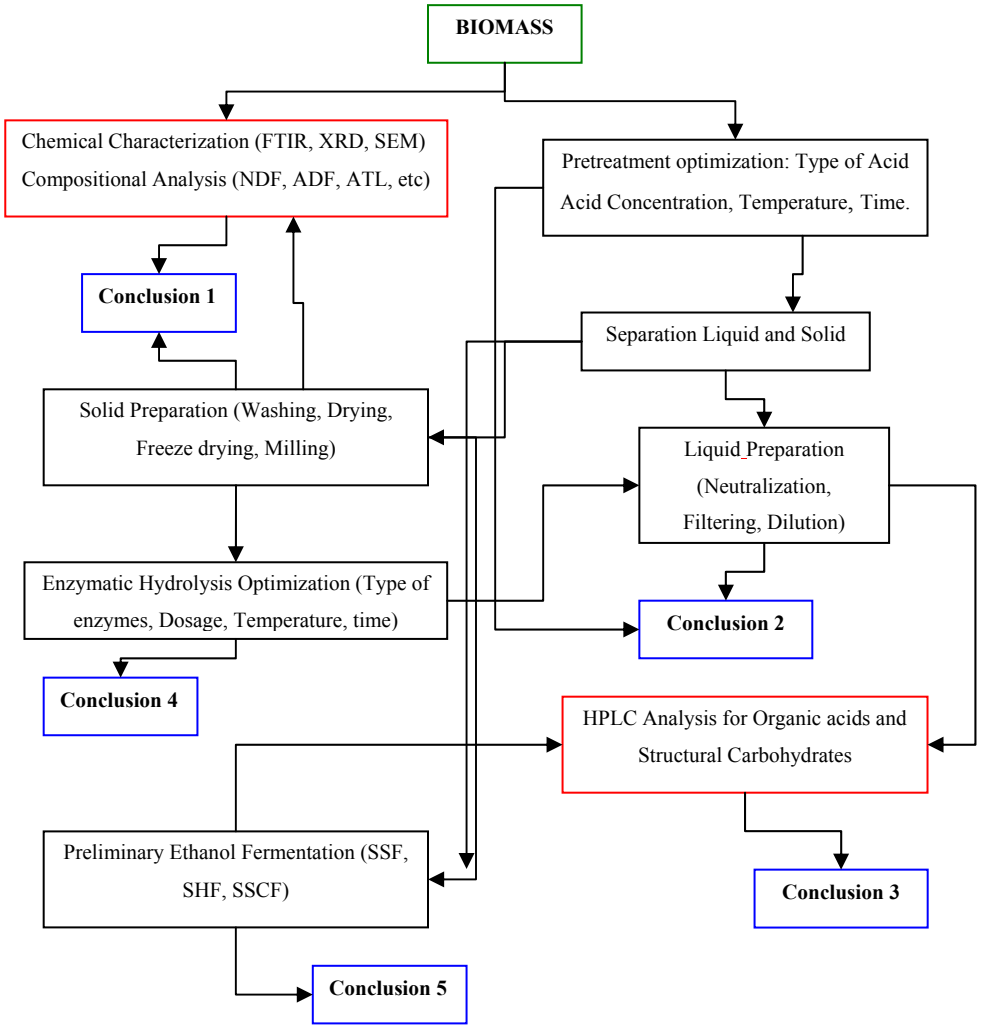


Figure 6.1 Summary of steps for characterization, pretreatment, enzymatic hydrolysis, and ethanol fermentation of lignocellulosic biomass.

Conclusions

1. Soybean hulls, three varieties of forage sorghum, and regular sorghum were tested as potential feedstock for production of bio-ethanol because of their high carbohydrate content of greater than 50% with about 24-38% cellulose. Characterization of the material using analytical tools FTIR, SEM and XRD, allowed us to better understand the internal structure, the relationship among structure-function-pretreatment-enzymatic hydrolysis-conversion rate, as well as the effect of pretreatment on the formation of inhibitors. Cellulose crystallinity does not seem to be the only factor that affects enzymatic hydrolysis of lignocellulosic material. It was demonstrated that samples with a high percentage of guaiacyl rings in their lignin structure were easy to break and hydrolyze after pretreatment despite the initial content of lignin. It was also verified that pretreatment was more effective in samples with low crystallinity index transforming their crystalline cellulose to amorphous cellulose, as well as enzymatic hydrolysis being more efficient on the same samples hydrolyzing cellulose to monomeric sugars, despite the initial cellulose content.

2. Pretreatment was optimized for soybean hulls improving recovery of sugars. Steam explosion with dilute acid was developed to obtain high sugar yields with less inhibitor compounds. Results showed that up to 80% of total sugars in soybean hulls were recovered using pretreatment with 2% H_2SO_4 and steam explosion at 140°C for 30 min, followed by enzymatic hydrolysis with cellulase (15 FPU/g. cellulose) and β-glucosidase (50 CBU/g. cellulose). This yield was much higher (>100%) than overall total sugar yields obtained by direct enzymatic hydrolysis using not only cellulase and β-glucosidase, but also hemicellulase enzymes with the same amount of enzyme loading and enzymatic hydrolysis conditions. Thus, pretreatment with sulfuric acid and modified steam explosion is crucial before enzymatic hydrolysis and allows efficient enzymatic hydrolysis even in the absence of hemicellulase enzymes. Up to 94% of pentose yield in FS-2 and 86% of pentose yield in FS-3 were obtained at optimum pretreatment and enzymatic hydrolysis conditions.

3. A combination of steam explosion with dilute acid released small amounts of organic acids (<10 mg/ml). Formic and acetic acid, along with furfural, were the compounds with higher percentages and ranging from 0.60 to 1.99 mg/ml, 1.43 to 1.98 mg/ml, and 0.11 to 1.43 mg/ml, respectively. Traces of succinic acid and hydroxymethylfurfural were found (< 1.47 mg/ml). No traces of levulinic acid or other organic acid were observed. It was believed that the amount of

inhibitory compounds formed during pretreatment was positively related to the amount of sugar yield after treatment and enzymatic hydrolysis. It was also demonstrated that small amounts of inhibitory compounds released during pretreatment did not considerably affect further fermentation.

4. A combination of enzymatic hydrolysis and pretreatment released up to 80% of total sugars in soybean hulls. This combination of enzymatic hydrolysis worked better on forage sorghum giving a maximum hexose yield of 79% for FS-3, FS-2 and RS were those with the lowest hexose yield, with 43 and 48 % hexose yield after 72 h, respectively. XRD and FTIR analysis can be used for sugar yield analysis. The results showed that the ordered arrangement of the glucan chains indicated an amorphous pattern that predominated over the crystalline, facilitating the hydrolysis of cellulose to monomeric sugars. These results also support the hypothesis of decrystallization and hydrolysis of cellulose after enzymatic hydrolysis for high sugar yield samples such as FS-3 and FS-1, probably due to the initial ordered arrangement that controls the swelling and reactivity of cellulose.

5. SSF worked better than SHF or SSCF schemes. Ethanol yield ranged from 50 to 88% of the theoretical for forage sorghum. SHF was achieved by fermentation of supplemented hydrolyzates to obtain 4% of sugars. Ethanol yields were achieved up to 86% when hydrolyzates were supplemented with glucose and 80% when samples were supplemented with xylose. These results suggest that although the over-liming process reduced the initial amount of sugar, *E. Coli* fermented both sugars and it is possible to use hydrolyzate liquid for fermentation without overturning the effects from inhibitors. The SSCF scheme was not successful, probably due to some remaining synergistic action of inhibitors in the hydrolyzates or differences in optimum conditions for enzymatic hydrolysis and fermentation using *E. Coli.*

Further research

Although forage sorghum worked better than soybean hulls for hydrolysis of fermentable sugars, optimization of pretreatment and enzymatic hydrolysis, as well as further understanding, are needed on larger sample sets. Pretreatment with steam explosion and dilute acid was effective for forage sorghum and soybean hulls. However, both materials presented drawbacks during pretreatment. Soybean hull was a difficult material to be pretreated, likely due to its high

oil content. Meanwhile, forage sorghum sometimes was so coarse and fine that it stuck in the reactor during pretreatment making steam explosion difficult. Improvement of equipment in the Biomass Process Laboratory would be necessary to overcome these difficulties.

There are well established studies of different pretreatments applied to biomass, mainly on corn stover, done by Dr. Lee at Auburn University (aqueous ammonia recycle), Dr. Wyman at Darmouth College (hot water and dilute acid hydrolysis), Dr. Dale at Michigan State University (ammonia fiber explosion), Dr. Ladisch at Purdue University (controlled pH), and Dr. Holtzapple at Texas A&M (lime pretreatment) (6, 9, 70, 122, 123). However, all of them claimed good hexose and pentose yields on corn stover and different materials. A unique method for pretreatment needs to be developed taking into account the efficiency of hydrolysis, the amount of inhibitors formed and the possible industrial scale-up implementation for different biomass. A combination of steam explosion and dilute acid is a process that could be scaled-up using facilities established in bio-refineries.

Process synthesis and modeling will be another important tool to optimize all these types of pretreatment. It could then be developed into research in optimization, scaling, and modeling of optimum conditions to develop a unified pretreatment for hydrolysis of lignocellulosic biomass.

Biotechnological transformation of lignin content and/or structure in plants could be a direction to improve use of lignocellulosic materials in bio-refineries and industrial processes. It has been demonstrated that one of the approaches for modifying lignin is to increase the amount of guaiacyl rings common in softwood samples and reduce the amount of syringyl units. The guaiacyl-to-syringyl ratio can also be estimated from the relative intensity of bands at 1270 and 1230 cm^{-1} using FTIR (111). NMR and FTIR are potential techniques to understand and quantify lignin-type content in different biomass. Another option is to determine chemical transformation of biomass "in situ" during pretreatment. Possible experiments using dilute acid and heating using FTIR could be set up in chemical engineering laboratories to better understand chemical changes during hydrolysis.

Another important research group working with lignocellulosic biomass is the one formed by Dr Dien, Dr. Qureshi, Dr. Saha, Dr. Bothast, and Dr. Cotta from the National Center for Agricultural Utilization Research, USDA. They have developed genetically engineered *E.Coli* for ethanol production from xylose and are also working with different types of biomass

(10, 36, 68, 98, 121, 124-127). A better understanding of sugar metabolism of these microorganisms could also help to develop a standard procedure to carry out the fermentation process.

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Appendix A - MATLAB code for “smoothing” of XRD spectra

Following software was used to smooth interferences found on XRD spectra of samples.

```
clc
clear
for i=1:4
A=xlsread('Results in Excel file',i,'C3:E1170');
B=xlsread('Results in Excel file',i,'B3:B1170');
windowsize=20;
b=ones(1,windowsize)/windowsize;
A1=filter(b,1,A);
xlswrite('XRD smooth',A1,i);
end
figure(1)
subplot(2,1,1)
plot(B,A)
subplot(2,1,2)
plot(B,A1)
```