UTILIZATION OF SWITCHGRASS AS A BIOFUEL FEEDSTOCK

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UTILIZATION OF SWITCHGRASS AS A BIOFUEL FEEDSTOCK

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LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two-dimensional
AGU	Anhydroglucose Unit
AIA	Acid-Insoluble Ash
As	Arsenic
ASTM	American Society for Testing and Material
B	Boron
Ba	Barium
BMGL	Ball Milled Grass Lignin
CH_2OH	Methylene Hydroxy
cm	Centimeter
CO	Carbon Monoxide
CoA	Coenzyme A
CP/MAS	Cross Polarization/Magic Angle Spinning
Crl	Crystallinity Index
CTC	Cellulose Tricarbanilate
D.I.	Deionized Water
DMSO	Dimethylsulfoxide
DP	Degree of Polymerization
DP_w	Mass-average Degree of Polymerization
EC	Enzyme Commission Number
EGU	Endoglucose Unit
FPU	Filter Paper Unit
F_{RE}	Number of Reducing End

FT-IR	Fourier Transform-Infrared Spectroscopy
FWHH	Full Width at Half-Height
GC-MS	Gas Chromatography Mass Spectrometry
GHG	Greenhouse Gass Emissions
GPC	Gel Permeation Chromatography
HHV	Higher Heating Value
HMQC	Heteronuclear Multiple Quantum Coherence
HPAEC-PAD	High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection
HPLC	High-Performance Liquid Chromatography
ICP	Inductively Coupled Plasma
IU	International Unit
kJ	KiloJoule
k	Potassium
kg ha^{-1}	Kilogram Per Hectare
KHz	Kilohertz
L	Liter
LSD	Least Significant Difference
m	Meter
M	Molar Per Liter
Mg ha^{-1}	Mega Gram Per Hectare
mg	Minigram
MJ	Megajoule
mL	Miniliter
μL	Microliter

mm	Minimeter
μm	Micrometer
Mn	Manganese
M_n	Number Average Molar Mass
ms	Microsecond
M_w	Weight Average Molar Mass
nm	Nanometer
NMR	Nuclear Magnetic Resonance
NO_3^-	Nitrate ion
od	Oven Dried
ppm	Part Per Million
Py-MBMS	Pyrolysis Molecular Beam Mass Spectrometry
s	Second
S	Sulfur
S:G	Syringyl:Guaiacyl
Si	Silicon
TAPPI	Technical Association of the Pulp and Paper Industry
tg	Trans-Gauche
THF	Tetrahydrofuran
TX	Total Halogen

SUMMARY

A complete characterization of switchgrass is essential so that the grass can be used as a resource for fuel, energy, and chemicals. This thesis research focused on biomass characterization and the hydrothermal pretreatment of switchgrass for bioethanol production.

In the first part of the thesis, chemical analyses were conducted for four populations of switchgrass, SW1-SW8. Each population consisted of 69% leaves, 27% internodes, and 4% nodes. The variations in carbohydrates, lignin, extractives content, Higher Heating Value (HHV), and syringyl:guaiacyl (S:G) ratio were determined among the populations and the morphological portions. The experimental results suggest that each population of switchgrass has a similar chemical profile, while the profiles of the morphological portions differ. The leaf portions have the highest arabinose, galactose, ash, and lignin contents and the lowest S:G ratio, while the internode portions have the highest values of these variables. The internode portions have the highest glucose content (44.3%).

In the second part of the thesis, the leaf and internode portions of switchgrass SW9 were analyzed to determine their chemical compositions and structures. The results indicate that leaves and internodes have different inorganic and organic chemical compositions. These differences include minerals, extractives, carbohydrates, and lignin content. The structure of cellulose is the same in both portions. The structure of lignin is different in terms of S:G ratio and molecular weight. The lignin S:G ratio is 0.69 and 0.74 for the leaf and internode portions, respectively. The molecular weight of acetylated

lignin is 5919.7 when obtained from the leaf portion and 4375.6 g/mol when obtained from the internode portion.

In the third part of the thesis, four populations of switchgrass, SW1-SW8, were used to study the chemistry of hydrothermal pretreatment and the ensuing effect on the digestibility of pretreated materials. The results indicate that hydrothermal pretreatment chemically modifies leaves and internodes so that they have similar chemical compositions and structures. The accessibility of switchgrass is improved by hydrothermal pretreatment as measured by Simons' Staining technique. The results also suggest that the accessibility of pretreated leaves is greater than the pretreated internodes. However, the degree of polymerization of pretreated cellulose is 23.4% greater in the internode portions than in the leaf portions. The cellulose to glucose yield is 77.4% and 44.9% for the pretreated leaf and internode portions, respectively. The lower DP_w of pretreated cellulose and greater accessibility of pretreated leaves is contributed to be a factor for the enhanced digestibility in comparison with the pretreated internodes.

In the fourth part of the thesis, hydrothermal pretreatment was performed on the extracted leaf and internode portions of switchgrass SW9 to enhance their susceptibility to cellulase. The results demonstrate that hydrothermal pretreatment increases the crystallinity of cellulose and the percentage of cellulose $I_{\alpha+\beta}$, but reduces cellulose I_{α} for both the leaf and internode portions. After hydrothermal pretreatment, the leaves and internodes have similar chemical profiles and a similar structure of cellulose. However, the DP of pretreated cellulose in the internodes is 30.5% greater than that in the leaves. Pretreated leaves have a 60.5% cellulose-to-glucose conversion yield, which is 33.8% greater than that of the pretreated internodes. The results of the enzymatic hydrolysis

studies of cellulose suggest that the reduced DP of cellulose of pretreated switchgrass was an important factor influencing the enhanced digestibility of pretreated switchgrass.

CHAPTER 1

INTRODUCTION

Problem Statement

Lignocellulosic biomass is one material that can be used to produce bioethanol. The total cost of bioethanol production from lignocellulosic biomass is higher than that of first generation bioethanol made from cornstarch and sugarcane. Several reasons contribute to the high cost of utilizing lignocellulosic biomass for bioethanol production, as described below.

The sugars in the lignocellulosic biomass are the substances used for bioethanol production. The available current technology works mainly to convert hexoses, such as glucose, mannose, and galactose, to bioethanol. Hardwood and grass are composed of approximately 30-50% hexose, [1, 2] while softwoods are made up of 60% hexoses, [3] and cornstarch contains 60-70% glucose in starch form.[4] The amount of glucose available for bioethanol production in lignocellulosic biomass is only about 10-30%.

Compared to cornstarch or sugarcane, lignocellulosic biomass contains about 15-35% lignin.[1, 5] Because of lignin, the lignocellulosic biomass is a rigid material that requires pretreatment to reduce the resistance of the biomass to enzymatic saccharification and yeast fermentation.

The saccharification of these sugars has a different process. Lignocellulosic biomass requires cellulosic enzymes, or cellulases, to hydrolyze cellulose and produce the glucose that is for ethanol production. Cornstarch, on the other hand, can be hydrolyzed with amylase for ethanol production. The hydrolysis rate of amylase is significantly faster than that of the cellulases. In other words, in order to create an equivalent amount of ethanol, 40 to 100 times more enzyme proteins are required for the

biomass hydrolysis than for the cornstarch hydrolysis. [6] In addition, the pretreatment process requires much more energy input for lignocelluloses for biofuels production. These increase the cost of bioethanol production when using lignocellulosic biomass.

In the United States, the structural and chemical variations of biomass have been well characterized in woody plants because woody plants are major bioresources for the American pulp and paper industry. However, few studies have focused on the characterization of switchgrass. For example, the structures of the major polymer, cellulose, hemicellulose, and lignin are not well studied for the switchgrass plant. The varying chemical profiles across populations and morphological portions of switchgrass were not studied extensively and understood prior to this study. Therefore, it is important for bioethanol production from switchgrass because it is able to provide basic chemical and structural plant cell wall information, which can be used for the utilization of switchgrass in bioethanol production.

Grasses such as wheat straw and corn stover have been well studied as fuels for bioethanol production. The first such project in the United States was the study of bioethanol production from corn stover. Many pretreatment methods for enhancing the susceptibility of corn stover to cellulases were generated during the investigation. However, characterization of the biomass and the chemistry of pretreatment for bioethanol production are incomplete.

Considering these advantages and disadvantages in the field of bioethanol production, research is required which focused on biomass characterization, pretreatment chemistry, and systems for saccharification and fermentation of the lignocellulose biomass. This information could reduce the cost of bioethanol production. In the course of this thesis study, research focused on biomass characterization and on the hydrothermal pretreatment of switchgrass.

Hypothesis

The recalcitrance to saccharification of biomass is a major obstacle for the conversion of lignocelluloses to ethanol. Alteration of lignin content and lignin structure could improve saccharification efficiency of switchgrass. However, other factors, such as morphology of switchgrass, may also influence the recalcitrance of lignocellulosic biomass to the saccharification for ethanol production. To be able to understand the effect of this factor for the saccharification of switchgrass, the hypothesis in thesis research is that the different morphological portions switchgrass and their structure are a contributing factor that affects the utilization of the switchgrass for fuels, chemicals, and energy. This includes the following investigations: (1) Variation of chemical profiles among populations and morphological portions of switchgrass; (2) Structure of cellulose (crystallinity and DP); (3) Lignin content and structure.

Objectives

To understand the macromolecular chemistry of switchgrass during pretreatment, the switchgrass plant was separated and studied in detail. These studies include a basic characterization of switchgrass for each morphological portion; a basic characterization of the cellulose and lignin structures of the switchgrass; and a general characterization of the chemical and structural changes in the switchgrass after hydrothermal pretreatment. To test the hypothesis on hydrothermal pretreatment, the following experiments were conducted: (1) A study of the variation in chemical profiles across switchgrass populations and morphological portions; (2) A study of hydrothermal pretreatment and the subsequent saccharification of pretreated switchgrass in populations and morphological portions individually; (3) A study of the structural changes of cellulose and lignin in each morphological portion of switchgrass by solid state CP/MAS ^{13}C-NMR/FT-IR after hydrothermal pretreatment; (4) A study of the changes in the Degree of

Polymerization (DP) of cellulose and Simons' Staining adsorbance after hydrothermal pretreatment in each morphological portion of switchgrass.

CHAPTER 2

LITERATURE REVIEW

Biofuels

History of Bioethanol

Ethanol was first used as an industrial fuel source when Otto invented the internal combustion engine. [7] Later, Henry Ford ran his first car (the 1908 Model T) on ethanol, and touted renewable resources as the key to the success of his automobiles. [5, 7] However, since World War II, the majority of transportation vehicles have become dependent on gasoline or diesel from fossil fuels. Because of the geologically uneven distribution of fossil fuels, most industrial countries have quickly become dependent on foreign oil. [7] After the oil crisis in the 1970s, biofuels began to be produced commercially, using conventional technologies, from food resources including sucrose, starch, and oil. These biofuels are called first generation biofuels. [7] Common definitions related to biofuels production are summarized in Table 2.1.

First generation biofuels include biodiesel and bioethanol. [8] Recently, second generation biofuels, made from waste vegetable oils and fats, non-food crops, and lignocellulosic resources, have started to be developed and produced commercially. [7, 8] Second generation biofuels include cellulosic ethanol, biomass to liquid (BtL), and bio-synthetic natural gas (bio-SNG). [8] Sustainable technologies to produce biofuels from renewable raw materials need to be developed due to environmental issues, the growing demand for energy, political concerns, and the medium-term depletion of petroleum. [7] As part of an effort to address the increased demand for fuels, chemicals, and energy, the integration of agro-energy crops and bio-refinery manufacturing technologies into

existing industries could lead to the sustainable utilization of biomass. [9] Bioethanol production is one example of this kind of practice.

Table 2.1 Common Definitions Related to Biofuels

term	definition
advanced biofuel [10-12]	a renewable fuel other than ethanol derived from renewable biomass instead of from corn starch or another food-based resource.
biodiesel [13]	a clean-burning alternative fuel made from domestic, renewable resources
biofuel [10-13]	any liquid fuel derived from biological material such as trees, agricultural wastes, crops, or even grass. The most common biofuels are biodiesel and bioethanol.
biopower [13]	the use of biomass to generate the electricity, heat, or steam required for the operation of a refinery.
biomass [13]	living and recently dead biological matter that can be used as fuel or for industrial production.
ethanol [13]	an alcohol-based fuel made from sugars and starch found in plants. Ethanol is the most widely used biofuel today.
first generation biofuels [10-13]	fuels made from sugar- or starch-based agricultural crops, oil crops, or animal fats using conventional technologies. The most common first-generation biofuels are biodiesel and bioethanol.
fossil fuels [13]	solid, liquid, or gaseous fuels formed in the ground after millions of years by chemical and physical changes in plant and animal residues under high temperature and pressure. Oil, natural gas, and coal are common fossil fuels.
second generation biofuels [10-12]	biofuel made from lignocellulosic resources, such as non-food crops. Second-genergation biofuel production uses new technologies to overcome the major shortcomings of the production of first-generation biofuels.

General Process of Bioethanol Production

Typically, bioethanol can be produced using either a thermo-chemical process or a biological process. [7] The thermo-chemical process uses heat and catalysis to convert bioresources into bioethanol. The biological process uses heat, chemicals, biological enzymes, and a yeast or bacterial strain to convert bioresources into bioethanol. Although the biological process takes several days to convert bioresources to bioethanol, it is a common way to produce bioethanol today. First generation biofuels are dependent on the fermentation of either sugars, which are derived from starches, or sucrose, which is derived from cornstarch and sugar cane, respectively. [7, 14] One of the major shortcomings of this production process is that these bioresources have food value and require productive agricultural lands. The current research focus has shifted toward the production of bioethanol from lignocellulosic bioresources. Second generation bioethanol

refers to bioethanol produced from lignocellulosic resources, which are non-food crops. In contrast to the first generation bioethanol process, second generation bioethanol production requires the pretreatment of the biomass in order to facilitate the deconstruction of polysaccharides into monosaccharides. [8] The chemistry and biological conversion processes of the lignocellulosic bioresources differ tremendously from that of starch- or sucrose-based bioresources (such as cornstarch and sugarcane). [7, 15] For instance, the production of starch-based first generation bioethanol requires amylase to make fermentable sugars from starch. The production of bioethanol from lignocellulosic resources, on the other hand, requires a much harsher process—pretreatment—and a more expensive enzyme—cellulase—in order to produce fermentable sugars for bioethanol production from cellulose. [7, 15] This means that second generation bioethanol tends to be more difficult and expensive to make than first generation bioethanol.

Reduce Recalcitrance of Biomass for Biofuels

The pretreatment process is a key step for bioethanol production. It changes the structure and chemistry of the lignocellulosic bioresources and improves the subsequent bioethanol yields. Reductions in recalcitrance after pretreatment have been attributed to several factors, including the alteration and/or removal of lignin and hemicelluloses; the alteration of cellulose crystallinity; an increase in cellulose reducing ends; an increased accessible surface area; and the modification of the cell wall morphology. [16, 17] Efficient pretreatment also requires minimum cellulose loss and nominal byproduct formation, since either side effect could inhibit the fermentation process. Many approaches for the pretreatment of herbaceous bioresources have been studied during the past few decades, including biological, physical, chemical, and physic-chemical pretreatments. Examples of these pretreatment approaches for switchgrass are shown in Table 2.2.

Table 2.2 Pretreatment Approaches for Bioresources

pretreatments	conditions			sugar yield[a]	ethanol yield[b]
	temperature/ time	LSR[c]	chemical or energy		
esterase (biological) [18]	27 ^{o}C, 24 h	40:1	2g esterase/g biomass, pH 5.0	22%	-
compression-milling (physical) [19]	25 ^{o}C	-	7.9 kJ/g substrate	55%	-
dilute acid (chemical) [20]	180 ^{o}C, 0.5 min	9:1	1.5% H_2SO_4 w/v	91%	~0.14
lime (chemical) [21]	100 ^{o}C, 2.0 h	9:1	0.1g $Ca(OH)_2$/g biomass	80%	-
organosolv (chemical) [22]	180 ^{o}C, 1.0 h	8:1	0.9% H_2SO_4 (w/w), 75% ethanol/water (v/v)	92%	-
ionic liquid (chemical) [23]	160 ^{o}C, 3.0 h	97:3	[C2mim] (OAc) [d]	96%	-
steam (physico-chemical) [24]	195-205 ^{o}C, 7.5-10 min	-	3% SO_2 (w/w)	93-95%	0.08-0.11
liquid hot water (physico-chemical) [25]	200 ^{o}C, 10 min	9:1		87%	0.14
ammonia fiber explosion (physico-chemical) [26]	200 ^{o}C, 5 min	-	1 g ammonia/g biomass	93%	0.20

Note: a) Glucose recovery yield after enzymatic hydrolysis; b) Ethanol yield, g ethanol/g dry biomass; c) LSR, liquid-to-solid ratio; d) [C2min] (OAc), 1-ethyl-3-methylimidazolium acetate.

These pretreatments reduce the recalcitrance of biomass. This results in a much higher monosaccharide yield from the lignocellulosic biomass after enzymatic deconstruction. Table 2.3 summarizes some of the changes that occur in the pretreated biomass, including those in lignin content, the macromolecular structure of lignin, cellulose crystallinity, the Degree of Polymerization of cellulose, and the hemicellulose content. [15-18, 27, 28] The following examples demonstrate the changes that occur after pretreatment of the lignocellulosic bioresources. Biological pretreatments rely on a microbial or enzymatic treatment that modifies the chemical composition of the biomass and improves the sugar release yield when cellulases are applied. [29] For example, Sarath et al. [18] reported that the cellulase digestibility of switchgrass improved by approximately 67% after an esterase pretreatment. This process disrupted the ester inter-linkages between phenolic acids (i.e. ferulic acid and *p*-coumaric acid) and carbohydrates. Dilute acid pretreatment is an alternative method for reducing the hemicelluloses content of the lignocellulosic biomass and improving its digestibility. Recently, Yang et al. [30] investigated a dilute acid pretreatment of switchgrass germplasms for bioethanol

production using a 1.5% sulfuric acidic solution at 121 ^{o}C for 60 min. The results demonstrated the approximately 80% of the hemicelluloses were removed using this pretreatment condition. The resulting biomass was hydrolyzed completely using cellulases. [30] Alkaline pretreatments using sodium hydroxide or lime have been reported to remove lignin and hemicellulose from switchgrass, and to enhance a subsequent enzymatic hydrolysis stage. [31] Recent studies showed that a microwave-assisted alkaline pretreatment of switchgrass at 190 ^{o}C for 30 minutes with a 0.1 g alkaline/ g biomass loading achieved a 99% total sugar release after enzymatic hydrolysis. [32] Switchgrass pretreated with a 30% aqueous ammonia solution was fermented at the pilot scale for ethanol production, providing a 72% theoretical ethanol yield. [33]

Table 2.3 Effects of Pretreatment Technologies on Bioresource Properties [15-18, 27, 28]

pretreatments	methods	solubilization lignin	solubilization hemicellulose	decrystalline cellulose	accessible surface area	DP of cellulose
biological	fungi or enzymes	●	●	ND	ND	ND
physical	milling	○	○	●	●	●
chemical	diluted acid	●	●	ND	●	●
	alkaline	●	●	●	●	●
	organic solvent	●	●	ND	●	●
	ionic liquid	●	●	ND	●	ND
	wet oxidation	●	●	ND	●	○
physico-chemical	steam	○	●	ND	●	○
	liquid hot water	○	●	ND	●	●
	ammonia fibre explosion (AFEX)	●	●	●	●	○

Note: ●, increase or positive effect; ○, minor or no effect; ND: no determine

Hydrothermal pretreatment is a promising technique for bioethanol production. [27] This pretreatment is also known as autohydrolysis, hot-water pretreatment or hot-compress water pretreatment, and uses pure water as a reaction medium to pre-treat the bioresources. [27, 34-36] This method has been studied more extensively using hardwoods and grasses than other lignocellulosic bioresources (Table 2.4). In these studies, the bioresources were generally pretreated with water at a temperature of 190-240 ^{o}C for 10-30 min. [27, 35, 37, 38] During this procedure, acids such as acetic acid were released from the

lignocellulosics, which contributed to the mild acid condition (pH 3-7). These conditions result in the removal of a large amount of the hemicelluloses, a partial removal of the lignin and cellulose, and a structural modification of the lignin and cellulose. [37, 38] These alterations significantly change the properties of the lignocellulosic bioresources making them far more susceptible to the treatment by cellulases.

Table 2.4 Conditions of Hydrothermal Pretreatment for the Various Feedstocks

biomass	hydrothermal pretreatment conditions	sugar yield[a]	ethanol yield[b]
corn stover [39]	195 ^{o}C, 15 min, liquid-to-solid ratio 94/6, particle size <2 mm, N_2	59.2%	-
green alga [40]	150 ^{o}C, 30 min, liquid-to-solid ratio 50/1	79.9%	-
Kanlow switchgrass [25, 38, 41]	200 ^{o}C, 10 min, liquid-to-solid ratio 9/1, particle size <2 mm	-	0.14
maize silage [42]	185 ^{o}C, 15 min, liquid-to-solid ratio 94/6, particle size <2 mm, N_2	-	0.14
prairie cord grass [43]	210 ^{o}C, 10 min, liquid-to-solid ratio 92/8, particle size <1 mm	97.0%	-
poplar [44]	240 ^{o}C, 4 min, liquid-to-solid ratio 10/1, particle size 2-5 mm	60.0%	0.11
rapeseed straw [45]	218 ^{o}C, 30 min, liquid-to-solid ratio 50/3, particle size <1 mm	94.9%	-
red alga [40]	200 ^{o}C, 30 min, liquid-to-solid ratio 50/1	87.9%	-

Note: a) glucose recovery yield after pretreatment; b) ethanol yield, g ethanol/dry biomass

Table 2.5 summarizes some of the reported results from recent studies in which an autohydrolysis was performed on switchgrass, as well as the subsequent yields of ethanol in each of these studies. These studies demonstrate that hydrothermal pretreatment is an attractive process and results in a high bioethanol yield (~72-92% theoretical ethanol yield of pretreated biomass). [27, 35, 37]

Table 2.5 Examples of Hydrothermal Pretreatment of Switchgrass on Ethanol Production

sample	pretreatment conditions	sugars yield[a]	fermentation condition[b]	ethanol yield[c]
Kanlow switchgrass [41]	LSR[d], 9:1, 200 ^{o}C, 10 min	glucose 3%, xylose 93%	S.Cerevisuae D5A, SSF, 72-168 h	80-92%
Kanlow switchgrass [25]	LSR, 9:1, 200 ^{o}C, 10 min	glucose 13%, xylose 94%	IMB4, SSF, 72 h	78%
Kanlow switchgrass [38]	LSR, 9:1, 190-210 ^{o}C, 10-20 min	glucose N/A, xylose 64-100%	IMB4, SSF, 72 h	22-72%

Note: a) Sugar yield is the percentages of the released sugars during pretreatment; b) Yeast strain used: S.Cerevisuae D5A and IMB4; SSF: simultaneous saccharification and fermentation; c) Theoretic ethanol yield%=$((EtOH)_t-(EtOH)_o)*100/0.511/$(glucan of dry biomass)$/1.11$; $(EtOH)_t$ is the concentration of ethanol at time t; $(EtOH)_o$ is the initial ethanol concentration; d) LSR, liquid-to-solid ratio.

Switchgrass as a Feedstock for Biofuels

Biology and Genetic Variation

Switchgrass was selected in 1991 as a promising lignocellulosic herbaceous crop for biofuel production after researchers evaluated more than 30 herbaceous crop species. [46, 47] It is a desirable lignocellulosic feedstock for biofuel production for several reasons. Switchgrass is a very productive crop, providing up to 14 tonnes dry biomass/acre. [46] The perennial nature of switchgrass leads to reduced land management and a lower level of consumption of both energy and agrochemicals. [46] In addition, switchgrass has a high tolerance for heat, cold and draught, which has enabled the plant to adapt to growing conditions throughout most of North America. [48] Generally speaking, switchgrass is spread widely across North America. It grows naturally from 55^{o} N latitude to central Mexico. [49] The physical characteristics of switchgrass were reviewed by Lewandowski et al. [49] Switchgrass is a tall perennial grass. [46] The significant mass above the ground can grow up to 3.0 meters in height. [47] Deep root systems can reach up to 3.5 meters in depth. [47] Switchgrass has inflorenscences in the form of diffuse panicles, which are about 15-55 centimeters long. [49] At the end of its long branches of inflorescence, switchgrass has

spikelets, each of which has two florets, the first one sterile and the second one fertile. [46, 49] Figure 2.1 illustrates the switchgrass plant.

Figure 2.1 Image of Switchgrass Plant [50]

The expected living period of switchgrass (root system) is about 10 or more years. [46] The diversity of switchgrass ecotypes has been attributed to three primary characteristics: the genetic diversity associated with its open-pollination reproductive mode; a very deep, well-developed root system; and efficient physiological metabolism. [47] Switchgrass is a cross-pollinated plant, able to intercross only under the same level of ploidy. [49] The basic number of chromosomes in every switchgrass cultivars is nine. The typical ploidy of the various switchgrass cultivars is tetraploid or hexaploid. [47, 49] Because it is an open pollinated species, switchgrass expresses tremendous genetic diversity, with wide variation between levels of ploidy. [47] The upland ecotypes are shorter, first-stemmed, earlier-maturing, and better adapted to drier conditions. The lowland ecotypes

are taller, coarse-stemmed, later-maturing, and better adapted to wetter field sites. The different characteristics of these two ecotypes are summarized in Table 2.6.

Table 2.6 Characteristics of Switchgrass Ecotypes [46, 49, 51]

characteristics [1]	upland	lowland
physical characteristics	0.9-1.5 meter height, first-stemmed	0.6-3.0 meter height, coarse-stemmed
growth moisture	more adapted to drier habits	more adapted to wetter sites
growth period	early-maturing	late-maturing
growth habits	most promising cultivar is Cave-in-Rock for central & northern state	the most promising cultivars are Alamo for the deep south, Kanlow for mid-latitudes
others	-	more robust and resistant to rust (*Puccinia graminis*), more bushy-type growth
types of switchgrass	Caddo, Pathfinder, Trailblazer, Forestburg, Shawnee, Shelter, Sunburst, Cave-in-Rock	Alamo, Kanlow, Carthage, and NL93

The biomass yield depends on the ecotype of the switchgrass and the latitude of the growth region, as well as on growth year, [46] soil condition, harvest time, and field management. The yield of switchgrass varies significantly across the growth field. Investigations have been conducted for the production yield of switchgrass across 13 states in the following regions: (1) mid-Atlantic (Virginia, West Virginia), (2) Southeast (Tennessee, Kentucky, North Carolina, Georgia, Alabama), (3) South-central (Texas, Arkansas, Louisiana), (4) North-central (North Dakota, South Dakota), and (5) Central (Iowa). [46] From these studies two commercial ecotypes of switchgrass, the upland and lowland varieties, were evaluated in order to determine production performance both across all regions and within each region. [46] The most promising cultivars of switchgrass for bioenergy production used in this report included lowland varieties—Alamo, Kanlow, and Carthage [52]—and one upland variety, Cave-in-Rock. These resutls demonstrated that the average biomass yield when the switchgrass was cut once per year ranged from 12-19 Mg ha^{-1} per year for Alamo switchgrass to 11.6-15.5 Mg ha^{-1} per year for Kanlow switchgrass. Lemus et al. evaluated the performance of 20 switchgrass populations in Iowa, including lowland varieties of Alamo, Knalow, Carthage, and NL93, and upland varieties such as Caddo, Pathfinder, Trailblazer, Forestburg, Shawnee, Shelter, Sunburst

et al. [49] The results showed that the average biomass yield was 2.5 Mg ha^{-1} higher per year for lowland switchgrass than for upland switchgrass. In an effort to produce higher yielding cultivars using Alamo and Kanlow switchgrass, Bouton et al. evaluated the performance of Alamo, Kanlow, and three experimental synthetics cultivars bred from Alamo and Kanlow, GA991, GA992, and GA993. These experiments were performed in Tifton and Athens, Georgia in the year 2000. [53] The resulting production yields of these cultivars in the second growth yield are shown in Table 2.7. These results indicated that Alamo and Kanlow had similar production yields. The synthetic cultivars had a significantly higher production yield than did the original Alamo and Kanlow cultivars. [53]

Table 2.7 Production Yield of Switchgrass Cultivars at Georgia in 2001 [53]

entry	yield (kg ha^{-1})		average yield (kg ha^{-1})
	Athens, GA	Tifton, GA	
GA993	12563	20791	17500
GA991	11716	21162	17384
GA992	10540	20976	16801
Kanlow	9755	15380	13130
Alamo	6313	18088	13378
LSD (P<0.05)	1620	3457	2067

Note: LSD; Least Significant Difference

Lignocellulosic Chemistry of Switchgrass

Chemical Composition and Heating Value of Switchgrass

Elemental Analysis (C, H, N, O)

Many studies have evaluated the basic elements of switchgrass for bioenergy and biofuel applications. [2, 54] These results are comparable to the elemental content of hardwoods such as hybrid poplar, though switchgrass has a lower carbon percentage and a higher oxygen percentage than do softwood (Table 2.8). The Higher Heating Value (HHV) of these bioresource components can be correlated with their chemical composition. [55, 56]

In a study of the acid-catalyzed liquefaction of bagasse in ethylene glycol, the HHV, which ranges from 11.04 to 39.59 MJ kg^{-1}, was positively correlated with the carbon and hydrogen elemental content, and negatively related to the oxygen elemental content of bagasse and its liquefaction product. [55] These results indicated that an increase in carbon content and a decrease in oxygen content led to a higher HHV. Hence, understanding combustion values and their relationship to chemical composition is an important parameter for future power applications of switchgrass. [2]

Table 2.8 Elemental Composition of Switchgrass and Other Biomass

biomass	C%	H%	O%	N%	S%	HHV, MJ/kg
Alamo [52]	48.0	5.4	41.7	0.4	-	18.2
Cave-in-Rock(<90μm) [57]	42.3	6.0	37.6	0.2	-	16.6
Cave-in-Rock(>90μm) [57]	44.3	6.0	38.2	0.3	-	17.1
corn stover [58]	46.0	5.9	41.4	0.9	0.12	18.6
hybrid poplar [58]	49.4	6.0	43.1	0.2	0.05	19.7
Kanlow [52]	48.0	5.4	41.4	0.4	-	-
pine [59]	52.8	6.1	40.5	0.5	0.09	-
switchgrass [58]	46.9	5.8	42.0	0.6	0.11	19.5
spruce [59]	53.6	6.2	40.0	0.1	0.10	-

Minerals Distribution

The quantity of ash and minerals in switchgrass ranges from 1.4% to 7.3%. Table 2.9 summarizes the inorganic compound compositions of a number of bioresources, including switchgrass. Herbaceous plants have the highest ratio of mineral content to woody biomass. In switchgrass, the leaf portion tends to have a much greater ash content than does the stem portion (Table 2.9). In general, inorganic mineral elements appear in switchgrass in the following proportion: Ca> K> P> S. Biopower generation from herbaceous plants, including switchgrass, is known to be influenced by the presence of alkali inorganic elements. These inorganic minerals contribute to the potential generation of sulfates, silicate, chlorides, and hydroxides, which can cause slogging and fouling problems during combustion. [56] Some of these process issues can be reduced using aqueous leaching of the biomass, which removes alkali inorganic elements. [56]

Table 2.9 Mineral Elements Composition of Biomass [a]

biomass	K	Ca	P	S	Mg	Na	Fe	Si	Mn	Ash	TX [b]	Al
switchgrass [60]	717	6173	494	615	542	158	113	-	41	43000	-	102
switchgrass-leaves [61]	1815	7552	676	1020	2666	322	301	15390	-	73000	6553	489
switchgrass-stem [61]	3092	1147	326	454	1096	870	85	5323	-	24500	9371	124
hybrid popular [1]	2100	5100	600	-	400	100	-	-	-	14300	-	-

Note: a) element values are presented as mg of element/kg of dry weight sample; b) TX, total halogen.

Heat of Combustion of Switchgrass

The standard measurement of the heat of combustion of biomass is the Higher Heating Value (HHV), also known as the caloric value or the heat of combustion. [56] Typical HHV is approximately 20.0 MJ kg^{-1} for wood, 17.3 MJ kg^{-1} for cellulose, and 26.7 MJ kg^{-1} for lignin. [56] The Higher Heating Values of switchgrass reported in the literature average 18.5 MJ kg^{-1} (Table 2.8). Studies showed that the HHV of wood decreased by 0.2 MJ kg^{-1} with a 1.0% increase in ash content. [56] For bioenergy and biopower applications, it is essential to determine the mineral inorganic compound content and the HHV of switchgrass.

Extractives from Switchgrass

Extractives are another minor component in switchgrass. Typically, the solvents used for the recovery of extractives include hot water, benzene/ethanol, dichloromethane, and 95% ethanol. [62] The extractives can be classified as aromatic compounds, carboxylic acids, sugars and their derivatives, alkanes, fatty acids, alcohols, and sterols. [59, 63-66] The quantity of these compounds varies among species and extraction methods. These results indicate that fatty acids are the major extractive compounds in switchgrass. Early studies on the extractive composition of switchgrass, obtained using 95% ethanol extraction, reported extractives composed of 16.4% glucan, 3.9% galactan, 0.5% arabinan, 44.3% lignin, 12.2% ash, and 0.6% protein. However, more recent investigations of switchgrass extractives, performed using hot-water extraction, suggest that the extractives contain a

greater amount of sugars than do extractives using an organic solvent extraction. Recent studies of hot-water extractions using upland switchgrass, including the St. Anthony, Forestburg, and Trailblazer varieties, are summarized in Table 2.10. [67]

Table 2.10 Hot-water Extractives from Four Cultivars of Switchgrass

extractive compounds	St. Anthony *	Forestburg *	Trailblazer2003 *	Trailblazer2004 *
total free sugars	35899	28332	29769	23628
total oligomeric sugars	6687	10585	6033	9200
total organic acids	17929	7788	13449	12936
total cations	12971	8921	11087	11378
total anions	4476	5511	3683	4858

Note: *: extractives data was represented as the weight ratio of extractive to original sample, mg/kg. The upland switchgrass samples used in this study are provided by the National Renewable Energy Laboratory (NREL), Golden, CO. St. Anthony and Forestburg cultivars are grown in South Dakota and harvested in 2004 and 2006 respectively. Trailblazer 2003 and Trailblaze 2004 are grown in Nebraska and harvested in 2003 and 2004 respectively.

The results show that sugars and organic acids are the major components in the water extractives of upland switchgrass. Other extractives include inorganic salts and an unknown red-brown fraction. Sugars compose over 50% of hot water extractives, non cell wall carbohydrates which suggests that switchgrass is a good source of fermentable sugars for ethanol production. [2] In addition, Ravindranath et al. reported that Cave-in-Rock switchgrass contained approximately 320-400 mg/kg of α-tocopherol and 89-182 mg/kg of policosanols, mainly composed of docosanol (C22), tetracosanol (C24), hexacosanol (C26), octacosanol (C28), triacontanol (C30), and dotriacontanol (C32) (Figure 2.2). [68] Other investigations indicated that antioxidant compounds, such as rutin and quercitrin, were identified using a 60% methanol extraction of switchgrass (Figure 2.2). [69] These investigations suggest that switchgrass is a potential source of value-added chemicals for the biofuel production.

A recent study on 95% ethanol extraction of stem portions of switchgrass—Alamo, GA992, GA993, and Kanlow—was reported by Yan et al. [66] The

extractive content of 95% ethanol ranges from 11% to 13% for the stem portions of four populations of switchgrass. The chemical compounds identified by GC-MS in 95% ethanol extracts contain mainly carbohydrates, fatty acids, fatty alcohols, glycerols, alkane, and sterols (Figure 2.3). Monosaccharides are the major component (57-61%) of the overall mass balance for extractives (Figure 2.3). Considered overall, the composition content of ethanol extractives is similar among the four switchgrass population, although the distribution of extractives from Alamo is slightly different from GA993, GA992, and Kanlow cultivars (Figure 2.3). Alamo contains greater amounts of acids (22%) than the other switchgrass samples (13-14%). In contrast the fatty alcohols content for Alamo (8%) is lower than the value observed for the switchgrass samples studied (i.e., 14-16%). The results also show that Alamo has the greatest amount of sterol content (9%) than GA992, GA993, and Kanlow (5-6%). The individual extractives are also similar for four switchgrass populations with the exception of trehalose, which has greater percentage in Alamo and Kanlow (20% and 19% respectively) than in two half-sib progenies, GA992 and GA993 (8% and 8% respectively). Figure 2.2 summarizes the examples of these 95% ethanol extractives determined by GC-MS analysis. [66]

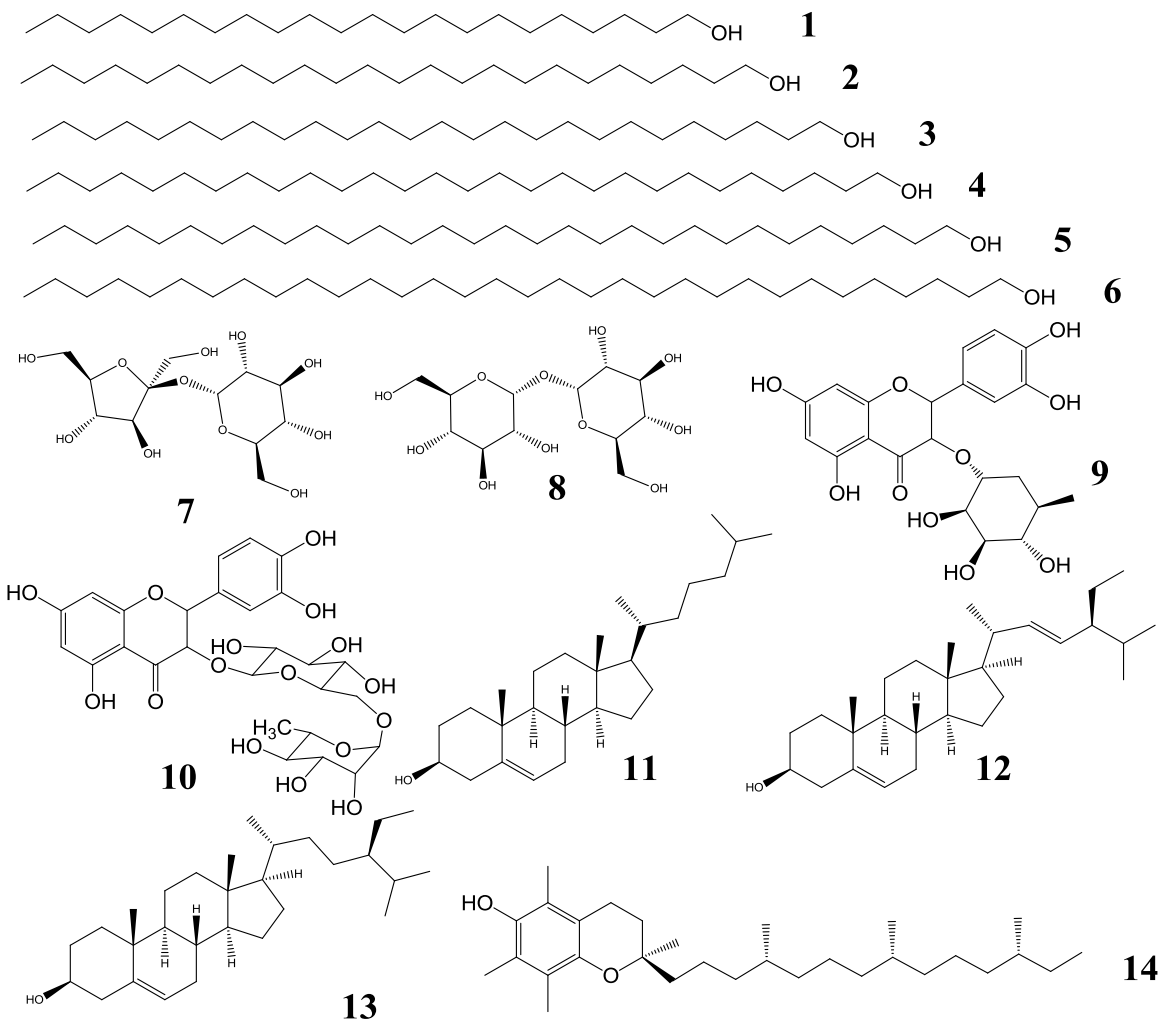

Figure 2.2 Examples of Some Compounds Extracted from Switchgrass. 1-6, Policosanols, Docosanol (C22), Tetracosanol (C24), Hexacosanol (C26), Octacosanol (C28), Triacontanol (C30), Dotriacontanol (C32); 7-8, Sugars, Sucrose, Trehalose; 9 and 10, Rutin and Quercitrin; 11-14, Sterols, Cholesterol, Stigmasterol, Beta-sitosterol, Vitamin E (α-tocopherol). [66, 68, 69]

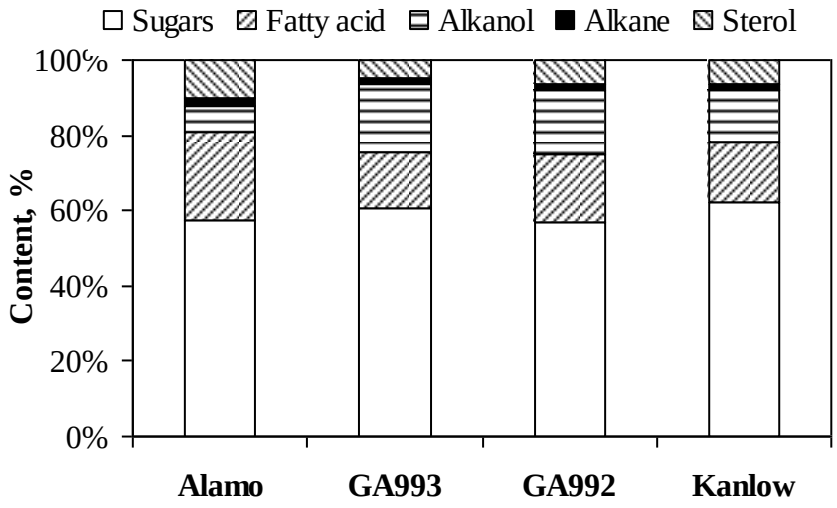

Figure 2.3 Distribution of Extractives in Stem Portion of Switchgrass [66]

Carbohydrate and Lignin Content of Switchgrass

Understanding the chemical composition of switchgrass is an important issue for the future utilization of switchgrass for the production of biofuels. Many investigations into the chemical composition of switchgrass have indicated that the chemical composition of switchgrass includes arabinose, galactose, glucose, xylose, trace mannose, and lignin as summarized in Table 2.11. These studies show that glucan, lignin, and xylan are the major components in switchgrass.

Table 2.11 Carbohydrates, Lignin, and Ash Content of Switchgrass

feedstock	dry weight%						
	arabinose	galactose	glucose	xylose	mannose	lignin	ash
switchgrass [70]	3.5	2.1	34.8	23.4	-	24.8	7.1
Kanlow switchgrass [25, 38]	3.2	1.1	40.7	24.0	0.9	18.3	5.0
Kanlow switchgrass [22]	4.4	1.4	37.3	28.1	0.1	25.6	1.7
Kanlow switchgrass [71]	3.3	1.6	43.9	25.5	-	23.5	2.2
Cave-in-Rock [72]	3.6	2.2	46.2	19.7	1.0	21.7	2.6
Alamo [72]	4.2	3.4	40.4	23.0	0.7	22.9	3.9
Kanlow [72]	4.1	3.3	40.3	23.4	0.7	20.6	3.2

Other carbohydrates, such as starch, have been measured in previous studies of Cave-in-Rock switchgrass. [73] These studies show that the starch content varies among the morphological portions of Cave-in-Rock switchgrass. The leaf portion contains about seven times the starch of the stem portion (3.9% vs. 0.5%). [73]

Cellulose

The Primary Structure of Cellulose

The term "cellulose" was first used by the French chemist Anselme Payen in 1839 to describe a purified dextrorotatory and gummy material derived from the fibrous wrap and wood of all young plant cells, seeds, cotton linters, a few mosses, and lichens. [74] Cellulose is the most abundant organic polymer in nature, accounting for about 1.5×10^{13} kg of the total biomass production on earth per year. [75] Among all cellulose-containing materials, cotton has the highest cellulose content (over 94% cellulose). [74] The typical content of cellulose is 60-80% in the bast fibers of flax, hemp, sisal, jute and ramie; 40-55% in wood; and 31-38% in switchgrass. [2, 74]

The basic building block of cellulose is β-D-glucopyranose, which links through a 1, 4-glycosidic bond. [75] The $^4C_1$ chair conformation is the preferred conformation for this anhydroglucosyl unit (AGU) (Figure 2.4). The anhydroglucosyl unit (AGU) is defined by ϕ and ψ torsion angles. [75, 76] In native cellulose, the CH_2OH side group is arranged in a *trans-gauche* (tg) position relative to the O_5-C_5 and C_4-C_5 bonds of the pyranose ring (χ angle defines the conformation of the hydroxymethyl group) (Figure 2.4). [77, 78] The repeating unit is a disaccharide glucose unit called cellobiose, because every second AGU ring is rotated 180^o.

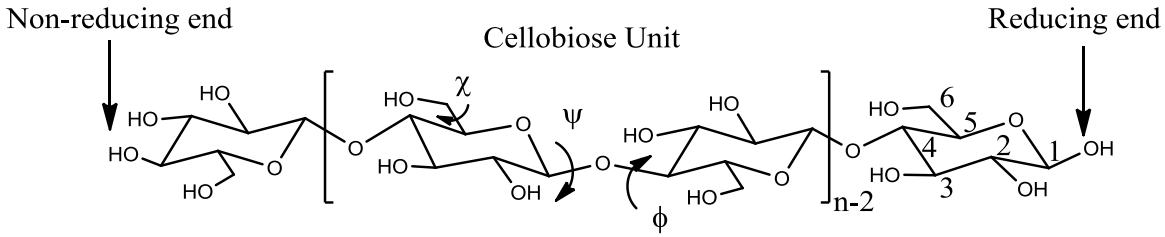

Figure 2.4 Molecular Structure of Cellulose (n=DP (Degree of Polymerization); Torsion Angles ψ and ϕ Define the Conformation of the Glycosidic Linkage; Torsion Angle χ Defines the Conformation of the Hydroxymethyl Group)

The Determination of the DP of Cellulose

The Degree of Polymerization (DP) of cellulose is determined using the number of constituent AGUs. The cellulose chain length is defined using the average Degree of Polymerization (DP_N), the average weight of the DP (DP_w), and the average viscosity of the DP (DP_v) (equation 2.1-2.3). [79, 80] The calculation of these values is based on the following equations.

$$DP_N = M_n/MW_{glu} = (\Sigma N_i M_i/\Sigma N_i)/MW_{glu} \quad \text{Equation 2.1}$$

$$DP_w = M_w/MW_{glu} = (\Sigma N_i M_i^2/\Sigma N_i M_i)/MW_{glu} \quad \text{Equation 2.2}$$

$$DP_v = M_v/MW_{glu} = (\Sigma N_i \eta/\Sigma N_i)/MW_{glu}, \text{ where } \eta = K_m M_i^{\alpha+1} \quad \text{Equation 2.3}$$

In the equations, N_i refers to the number of moles of a given fraction i; M_i refers to the molar mass of a given fraction i; M_N refers to the number average molecular weight; M_w refers to the weight average molecular weight; M_v refers to the viscosity of the average molecular weight; MW_{glu} refers to the molecular weight of the anhydroglucose (162 g/mol); η refers to the viscosity of cellulose; and K_m is a constant and the value of α for cellulose and cellulose derivatives ranging from 0.75 to 1.0.

To determine the DP of cellulose, the biopolymer must be solubilized using a solvent that disrupts the intermolecular hydrogen bonds without altering the chain length

of the cellulose. Several methods for the process of dissolving cellulose are available, including the use of metal complex solutions, such as a Cuam solution or cupriethylenediamine solution; the formation of cellulose derivatives via nitration or tricarbanilation; and the use of ionic solutions, such as N, N-Dimethylacetamide (DMAc)/LiCl. [79-81] After the successful dissolution of cellulose, the DP_N can be measured using membrane or vapor pressure osmometry, cryoscopy, or ebullioscopy; DP_w can be measured using light scattering or sedimentation equilibrium; and the DP_v can be measured using a viscosity measurement. [79] Among these methodologies, the two most commonly used techniques are viscometry for the determination of DP_v and gel-permeation chromatography (GPC) for the determination of DP_w and DP_N. [80] The polydisperse nature of cellulose means that the Degree of Polymerization is determined in a different order: $DP_w \geq DP_v \geq DP_N$.

Viscometry is a fast and convenient method for estimating the average Degree of Polymerization (DP_v) of cellulose and its derivatives. [82] There are limitations to this method of measuring DP_v, however. This method provides only the average viscosity of the molar mass (M_v) without providing any information concerning the molar mass distribution and the possible degradation effect of the inorganic complex solution. [82] An alternative way to measure the DP of cellulose is Gel Permeation Chromatography. [80] In contrast to viscometry, Gel Permeation Chromatography (GPC) provides the molar mass distribution for DP_n and DP_w. The molar mass in GPC is estimated based on a molecular weight calibration procedure, which is calibrated using well-defined polystyrene standards. [80] One common method used to determine the DP of cellulose is using GPC to measure the molecular weight of the Cellulose Tricarbanilate. Cellulose Tricarbanilate (CTC) is the derivative used most often in GPC to determine the DP of cellulose because it is characterized by the following: a complete substitution of cellulose, derivatization without depolymerization, and high solubility and stability in tetrahydrofuran. [80] Cellulose tricarbanilation is accomplished when cellulose reacts with phenyl isocyanate

in one of two solvents: dimethylsulfoxide (DMSO) or pyridine. However, cellulose oxidation and degradation have been reported to occur when cellulose is derivatized in the presence of DMSO and phenyl isocyanate. [83] Hence, pyridine is the most common solvent for the derivatization of cellulose. The dried cellulose sample is generally derivatized by adding anhydrous pyridine and phenyl isocyanate and stirring at 70 ℃ until the cellulose is completely dissolved (~48 h). The following reaction mechanism illustrates the common reaction scheme for the formation of Cellulose Tricarbanilates (Figure 2.5).

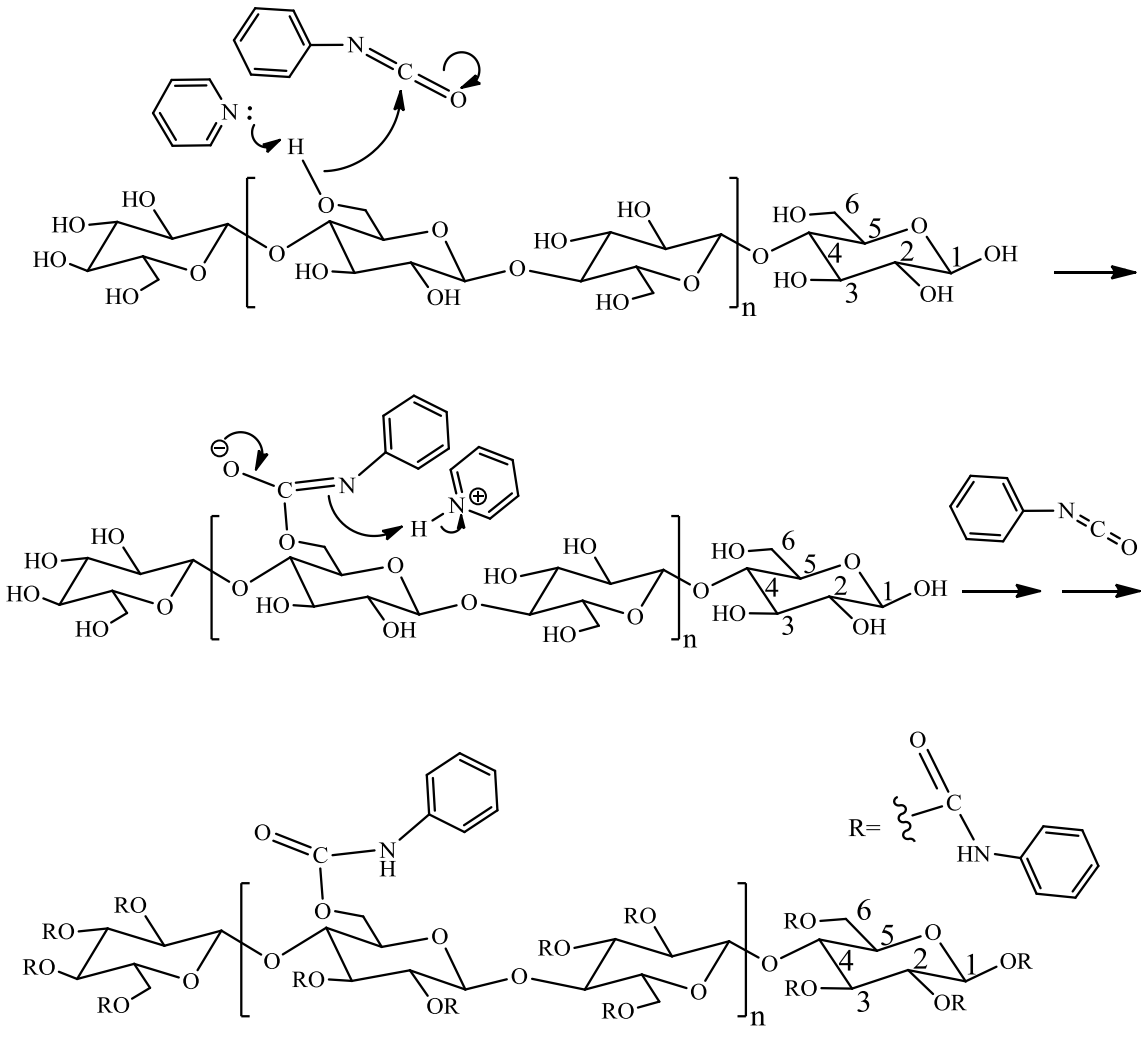

Figure 2.5 Formation of Cellulose Tricarbanilates

Table 2.12 shows the DP_w of Cellulose Tricarbanilates of various resources. For instance, cotton has a cellulose DP of 2111 AGUs, [84] while bacterial cellulose has a DP of 4244 AGUs. [85] Other preparations of cellulose, such as microcrystalline cellulose and Avicel PH-101, have a DP of ~242-480 AGUs. [86, 87] Cateto et al. recently investigated the DP_w of cellulose in Kanlow switchgrass. According to that study, Kanlow switchgrass has cellulose DP_w of 2900 AGUs. [22] Other studies have reported the DP_w of Cellulose Tricarbanilates in Alamo switchgrass as 1891 and 3300 AGUs.

Table 2.12 Degree of Polymerization of Some Selected Celluloses

cellulose resource	measuring technique	degree of polymerization (AGUs)
Alamo switchgrass [88]	CTC and GPC	1891
Avicel PH-101 [87]	CTC and GPC	242
bacterial cellulose [85]	CTC and GPC	4244
cotton cellulose [84]	CTC and GPC	2111
Kanlow switchgrass [22]	CTC and GPC	2900
microcrystalline cellulose [86]	CTC and GPC	480

Note: CTC: cellulose tricarbanlates

The Structural Characterization of Cellulose

High-resolution solid-state NMR studies have been used to investigate the structural features of cellulose since the early 1980s. Cellulose has a crystalline component and an amorphous (noncrystalline) component at the microfibril level. [75, 89] Nuclear Magnetic Resonance (NMR), infrared, and diffraction studies have shown that cellulose I_α and I_β are two polymorphs of native cellulose. [75] Atalla and Vanderhart discovered, over the course of a high resolution solid state CP/MAS-^{13}C NMR study that native cellulose I exists in two distinct crystal forms: cellulose I_α and cellulose I_β. [90, 91] Their results show that a significant chemical shift occurs between cellulose I_α and cellulose I_β for the C-1, C-6, and C-4 resonances. These results indicate that the cellulose I_α form has singlet resonances at C-1 and C-6 and a doublet resonance at C-4. The cellulose I_β form, on the other hand, has doublet resonances for C-1, C-4 and C-6. The

existence of nonequivalent chains provides a possible explanation for the observed splitting resonances in the ^{13}C CP-MAS spectra of cellulose. [78] These initial studies of the structure of cellulose I_α and cellulose I_β also suggest that cellulose I_α is the most abundant form of cellulose in bacteria and algae, while cellulose I_β is the most abundant form in the higher plants, which include cotton linter, ramie, wood et al. [75, 90-92]

In addition, these two allomorphs of native cellulose can coexist in the same resource. For instance, cellulose I_α and I_β have been observed to have overlapping NMR spectra in both Acetobacter cellulose and cotton. [91] An estimated percentage breakdown of the two allomorphs indicates that Acetobacter cellulose contains 60-70% cellulose I_α, and cotton cellulose contains 60-70% cellulose I_β. [91] Annealing experiments conducted by Yamamoto and Horii demonstrated that cellulose I_α was transformed into cellulose I_β, especially when placed in a dilute alkaline solution at a high temperature. [93] Since the initial discovery of cellulose I_α and I_β, the structure of these two distinct phases of native cellulose have also been characterized using electron diffraction [94] and infrared spectroscopy. [95] Nishiyama et al. determined the crystalline and molecular structures of deuterium-labeled cellulose I_β and cellulose I_α using a synchrotron X-ray and a neutron diffraction technique. [77, 78] The similarities and differences between cellulose I_α and I_β, as enumerated in the Nishiyama study, were summarized in Table 2.13. [77, 78]

Table 2.13 Similarities and Differences in the Characteristics of Cellulose I_α and I_β [77, 78]

cellulose I_α	cellulose I_β
a one chain triclinic unit cell	a monoclinic two chain unit cell
all glucosyl linkages are identical	two parallel chains have slightly different conformations
hydroxymethyl groups have identical configuration, tg	hydroxymethyl groups have identical configuration, tg
sheets packed in a “parallel-up” fashion	sheets packed in a “parallel-up” fashion
no inter sheet hydrogen bonds	no inter sheet hydrogen bonds
metastable and can be converted into I_β by annealing	thermodynamic stable form of native cellulose

In an early study on the structure of cellulose, Fink et al. observed light and dark areas along a cellulose microfibril using wide-angle X-ray scattering. The results suggested that these areas represented crystalline and amorphous cellulose. [96] O'Sullivan stated that "amorphous" is defined as a material which is formless or lacks a definite shape. [89] Pu et al. stated that the mobility and order of paracrystalline cellulose is between crystalline and amorphous cellulose. Ioelovich suggested that there are distorted and loosely packed surface layers called paracrystalline surface layers present on the surface of the crystallites. These layers have an average thickness of 0.4 nm and demonstrate high distortion features. [97] Paracrystalline cellulose has intermediate properties that lie between the properties of highly-ordered crystalline cellulose and those of disordered amorphous cellulose. [97] Ding et al. proposed an elementary fibril model containing 36 glucan chains and stated that elementary fibril is a heterogeneous structure. This elementary fibril contains a 6-glucan chain crystalline core which displays cellulose I_β structure. Subcrystalline (12 glucan chain) and paracrystalline (18 glucan chain) are associated with the crystal core. [98] X-rays have been used to measure paracrystalline cellulose, and have found that paracrystalline cellulose accounts for approximately 30% of the total cellulose in ramie, hamp, and jute fiber. [99] Since this observation, Lennholm et al. developed a quantitative partial least-squares (PLS) model to estimate the amorphicity index and the contents of cellulose I_α and I_β. This model uses the ^{13}C-CP/MAS NMR spectra data of various lignocellulosic materials. [100] Larsson, et al. modeled the ^{13}C-CP/MAS NMR spectrum (S (ω)) of tunicate cellulose as a superposition of Lorentzian lines (Equation 2.4). [101] They used the Levenberg-Marquardt algorithm in order to produce a χ^2-fit to the experimental data. A quantitative analysis of the relative amounts of the different allomorphs was performed using the C-4 spectral regions of tunicate cellulose.

$$S(\omega) = (i=1 \text{ to } n)\Sigma\ (a_i/\pi)\ (2\tau_i/(1+4(\omega - \omega_i)^2\tau_i^2)) \qquad \text{Equation 2.4}$$

Where, a_i represents the superposition weight of line i; ω_i gives the center of the line; and τ_i is the inverse of the Full Width at Half-Height of the line i. Each line is normalized to a_i.

Larsson and his colleagues further quantified the cellulose structure by employing the non-linear least-squares fitting of the ^{13}C-CP/MAS NMR spectra. [92] Thus a quantitative evaluation of cellulose's structure, including the structures of cellulose I_α, cellulose I_β, paracrystalline cellulose, fibril surface cellulose, and amorphous cellulose, could be obtained using this non-linear least-squares fitting of the C-4 region of the ^{13}C-CP/MAS NMR spectrum. [92] Figure 2.6 illustrates one example of a processed ^{13}C-CP/MAS experiment performed on cellulose from switchgrass.

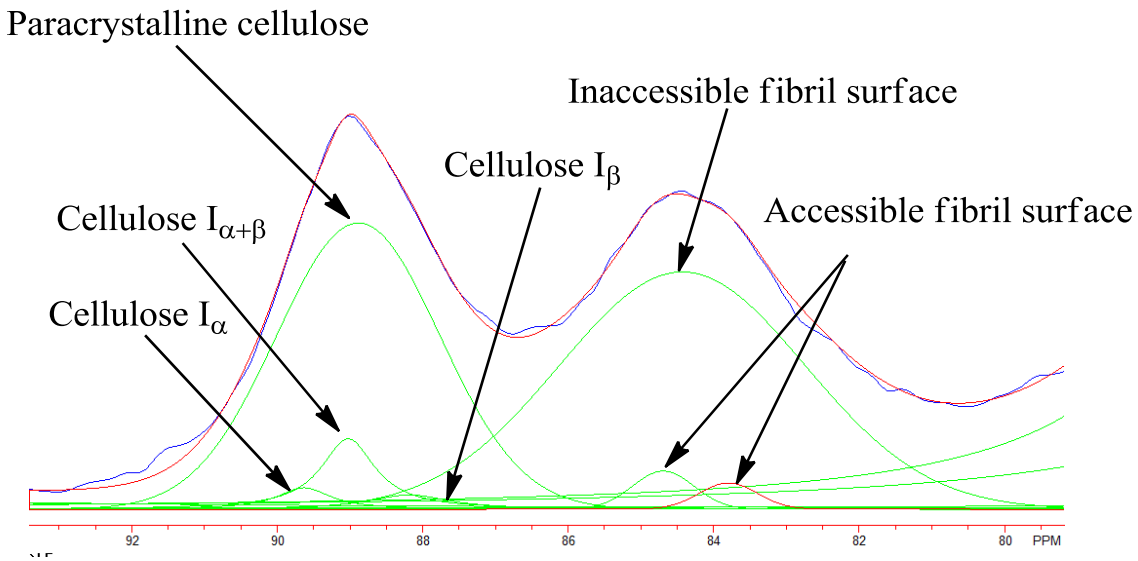

Figure 2.6 Spectral Fitting for the C-4 Region of the Spectrum of Cellulose Derived from Alamo Switchgrass

In a study of the spectral fitting of cellulose in the solid-state NMR spectra, [80, 92, 101-104] Larsson and Wickholm et al. [105, 106] proposed a model for the aggregate cellulose surface that resembles the model proposed by Preston and Cronshaw. [107] The model includes a crystalline core composed of cellulose I_α, cellulose $I_{\alpha+\beta}$, and cellulose I_β. This

core is surrounded by paracrystalline cellulose and amorphous cellulose, including two inequivalent accessible fibril surfaces and one inaccessible fibril surface. The Crystallinity Index is used to estimate the intensity percentage of the crystalline portion of cellulose. In summary, the crystalline structure of cellulose extracted from biomass can be measured using CP/MAS ^{13}C-NMR spectroscopy and a line fitting analysis at the C-4 region of the spectra.

Hemicellulose

Hemicelluloses are another abundant biopolymer on earth. [9] Their structures are more complex than that of cellulose because they are frequently branched with side chains groups, such as acetyl, galacturonic acid, glucuronic acid, and 4-*O*-methylglucuronic acid. Typically, the Degree of Polymerization of hemicellulose is around 50-300 sugar units. [9, 108] Common main chain sugars include arabinose, xylose, galactose, mannose, and glucose. These are supplemented by side chain substitutions such as acetyl, galacturonic acid, glucuronic acid, and 4-*O*-methylglucuronic acid. [108, 109]

In grasses, the possible hemicelluloses are much more varied than those in wood, and may include arabinoxylans, glucuronoxylans, arabinoglucuronoxylans, glucuronoarabinoxylans, glucomannans, xyloglucans, and mixed-linkage β-glucans. [110] Arabino (glucurono) xylans are the dominant hemicelluloses in the cell walls of the lignified supporting tissues of grasses and cereals. [110] These hemicelluloses are absent from sisal, corncobs and the straw of various wheat species. Arabino (glucurono) xylans consist of acetylated 1, 4-β-D-xylan in the main chain, and arabinoses and glucuronic acids in the side chains. These are linked with a 1, 4-glucosidic bond. [2] They are absent from sisal, corncobs and the straw of various wheat species. Arabino (glucurono) xylans consist of acetylated 1, 4-β-D-xylan in the main chain and arabinoses and glucuronic acids in the side chains. These are linked with a 1, 4-glucosidic bond. [2] Recently, Mazumder and York [111] isolated hemicellulose in the sequentially-extracted, ball-milled

alcohol-insoluble residue (AIR) of switchgrass using a 50 mM ammonium oxalate buffer, 50 mM sodium carbonate, 1 M KOH containing 1% $NaBH_4$, and 4 M KOH containing 1% $NaBH_4$. Four oligosaccharides fractions were generated after an endoxylanase treatment of the extracts. The resulting arabinose/xylose ratios were 6.7, 2.1, and 3.9 for three of the fractions, while the fourth fractions were pure xylose. A detailed structural analysis of these oligosaccharides was performed using a methylation analysis, multiple-step mass spectrometry ($ESIMS^n$), and 1D and 2D NMR spectroscopy. The results demonstrated that arabinoxylan was the most abundant component in the 1 M KOH-extracted fraction. The analytical structure showed that the arabinoxylan of switchgrass was made up of linear β-D-(1→4)-Xylp units, with α-L-Araf-(1→ and α-L-Araf-(1→2)-α-L-Araf-(1→ side chains at *O*-3 of the xylopyranosyl residues (Figure 2.7). [111] It is important to know the detailed structure of arabinoxylans in grasses because differences in the molecular features of these hemicellulosic polysaccharides (e.g. degree of branching and spatial arrangement of arabinosyl substituents along the xylan backbone) have been correlated with an alteration in cell wall properties during pretreatment. [111] The Degree of Polymerization and degree of branching or substitution of the arabinosyl in hemicellulose make hemicelluloses polymers amorphous like.

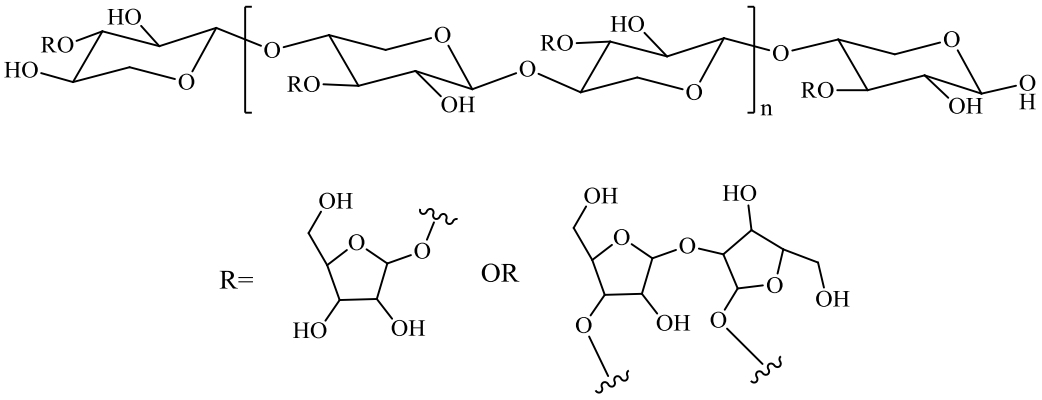

Figure 2.7 Arabinoxylans from Switchgrass [111]

Lignin

Lignin is derived from the Latin word "lignum," meaning wood. [112] The lignin macromolecule has been described as a random, three-dimensional network polymer consisting of phenylpropane units with various linkages. [113] As one of most abundant biopolymers on earth, lignin performs multiple functions that are essential to the life of the plant. [112] Lignin provides mechanical support, binding plant fibers and acting as a permanent bonding agent between cells, as well as forming a composite structure resistant to impact, compression and bending. In addition, lignin decreases cell wall permeability in the conducting xylem, which ensures the intrinsic transportation of water, nutrients and metabolites. Another primary function of lignin in the cell wall is that it prevents microorganisms from degrading the cell wall. [112]

Lignin is produced via the dehydrogenative polymerization of *p*-coumaryl, coniferyl, and sinapyl alcohol (Figure 2.8). [112, 114] Lignin can be classified into three major groups based on the type of plant resource in which it is found: angiosperm, or softwood; gymnosperm, or hardwood; and grass, or herbaceous plant. [112, 115] The lignin in angiosperms contains primarily guaiacyl units (G-units) and trace p-coumary alcohol (H-units). Gymnosperm lignin consists mainly of guaiacyl (G) and syringyl (S) units, which are present in different quantities, as well as low amounts of *p*-coumaryl alcohol (H-units). In contrast, the grass lignin in herbaceous plants contains comparable amounts of G- and S-units, and larger amounts of H-lignin than do angiosperms and gymnosperms. [115] But, grass lignin has significant levels of *p*-coumaric and ferulic acid, which is involved in crosslinking to lignin and hemicellulose. [116, 157] Table 2.14 summarizes the typical S:G: H ratios for lignin derived from common grasses.

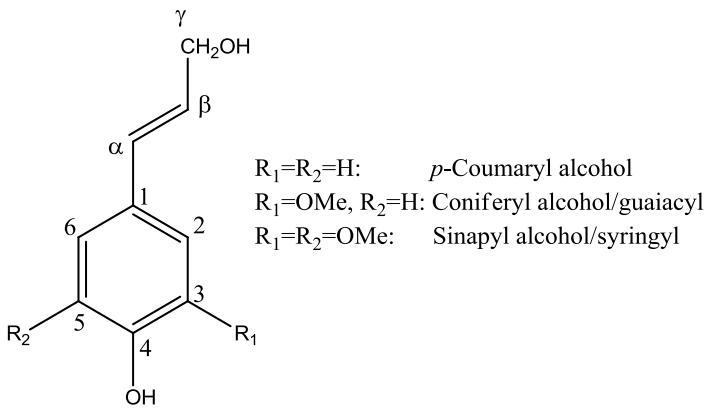

Figure 2.8 Three Building Blocks of Lignin [117]

Table 2.14 Molar Percentage of Guaiacyl (G), Syringyl (S), and *p*-Hydroxyphenyl (H) Units in Grass Lignin

origin	H%	G%	S%	analysis methodology
Alamo switchgrass [118]	8	51	41	^{13}C-NMR
Alfalfa (M. sativa) [119]	2	33	65	^{13}C-NMR
Miscanthus [120]	4	44	52	^{13}C-NMR

Biosynthesis of Lignin

Lignin is a complex crosslinking polymer synthesized in plant cell walls by the dehydrogenative coupling reaction of three major monolignols. [121] After monolignol biosynthesis, the lignin precursors are transported into the cell wall, where they are believed to polymerize through a radical coupling reaction. Monolignols are not abundant in their free form in these lignifying tissues, but are found rather as monolignol glucosides such as coniferin, which is present in gymnosperms. [122] Some evidence suggests that glucosidases attach to the cell walls during the onset of lignin biosynthesis, suggesting that monolignol glucosides are the metabolic forms in which monolignols are excreted from the cytoplasm into the lignifying zone. [123, 124] In gymnosperms and some angiosperms, monolignol 4-*O*-β-D-glucosides accumulate in high levels in the cambial tissues. In the case of gymnosperms, both coniferyl-alcohol glucosyl-transferase and

coniferin-β-D-glucosidase regulate the storage and mobilization of these monolignols. [125] The route for the transportation of monolignols in the cell wall remains under investigation.

The biosynthesis of lignin demonstrates that lignin is formed through the oxidation of the monolignols into the corresponding radicals and the polymerization of these radicals. The proposed mechanisms for this process have been reviewed. [112, 114, 124, 126-130] Early studies suggest that peroxidases and laccases are the most important enzymes for the formation of initiated radicals from monolignols. [115, 117, 125] Peroxidases are capable of creating free phenolic radicals that are resonance stabilized. [114 131] Examples of the formation of these radicals are illustrated in Figure 2.9.

β–electron spin density and steric considerations determine the reactivity of these radicals. Molecular orbital calculations of the β–electron spin densities of the lignin model compounds suggest that free electron spin densities are highest at specific sites within the phenylpropane unit. [117] These reactive sites in the phenylpropane unit include the C-1 and C-5 positions of coniferyl alcohol, phenolic hydrogen, and aliphatic β-carbon. Among these reactive sites, the C-1 and C-5 sites in coniferyl alcohol are the least reactive. Phenolic oxygen and β–carbon are therefore considered to be the most reactive species, and respond with the most abundant formation of interlinkages. [114, 117]

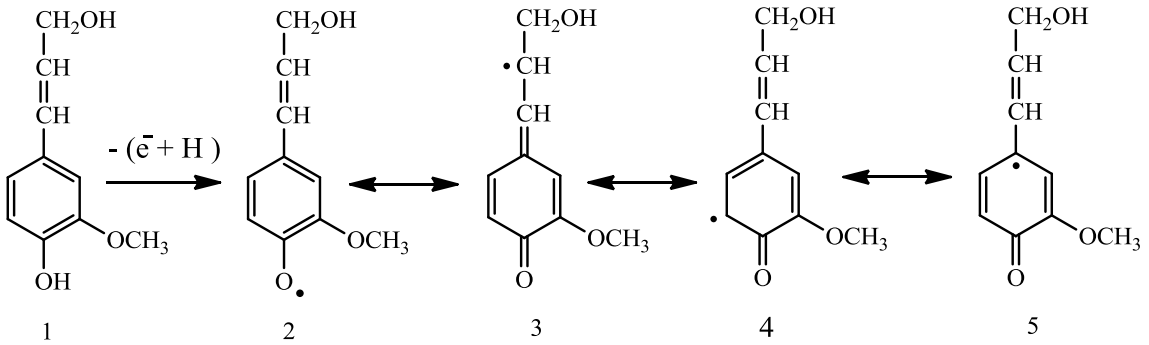

Figure 2.9 Phenolic Radicals Formed by Enzymatic Dehydrogenation of Coniferyl Alcohol 1-5 [117]

Ralph et al. [114] reviewed the results of a series of studies on the dehydrogenation of polymers (DHP) of lignin. They suggested that the dehydrodimerization of coniferyl alcohol yields β–β, β-*O*-4, and β-5 dimers, whereas sinapyl alcohol yields β-β and β-*O*-4 dimers preferentially (Figure 2.10). The β-position is the favored coupling position for monolignol dehydrodimerization. This is accomplished through cross-coupling reactions between the monolignol and the growing lignin polymer. [114] The primary sites for the cross-coupling of lignin oligomers are the 4-*O*- and 5-positions on the aromatic ring in S and G units. Figure 2.10 provides a general scheme for the coupling of preformed oligomers. This diagram suggests the following features for a cross-coupling reaction: (1) lack of evident formation of 5-5 and 5-*O*-4 linked structures between S units; (2) the formation of 5-*O*-4 linked structures between G and S units; and (3) the formation of 5-*O*-4 and 5-5 linked structures between G units. [114] These interlinkages are, however, not present in monolignol dehydrodimerization reactions. [114] These results provide the fundamental explanation that the β-aryl-ether linkage is the dominant linkage, accounting for 50% of the linkages in softwoods and 60% of those in hardwoods. [117]

This process allows the formation of the dominant interlinkage, β-aryl ether, as well the formation of lignin carbohydrate linkages and α-*O*-4 interlinkages. The addition of water to the intermediary quinonemethide leads to the formation of β-aryl ether. Figure 2.11 demonstrates that arylglycerol-β-aryl ethers form via the β-*O*-4 cross-coupling of a monolignol with an oligomer, followed by the addition of water to the quinonemethide intermediate. This process leads to the production of two isomers, because there are two reaction sites on the planar quinonemethides, at the *si* and *re* faces (Figure 2.11). The nucleophilic attack of water on the intermediate leads to the formation of erythro- and threo- isomers. The erythro: threo diastereomer ratios are approximately ~1:1 in the guaiacyl ethers and ~3:1 in the syringyl-guaiacyl ethers. [114, 132-134]

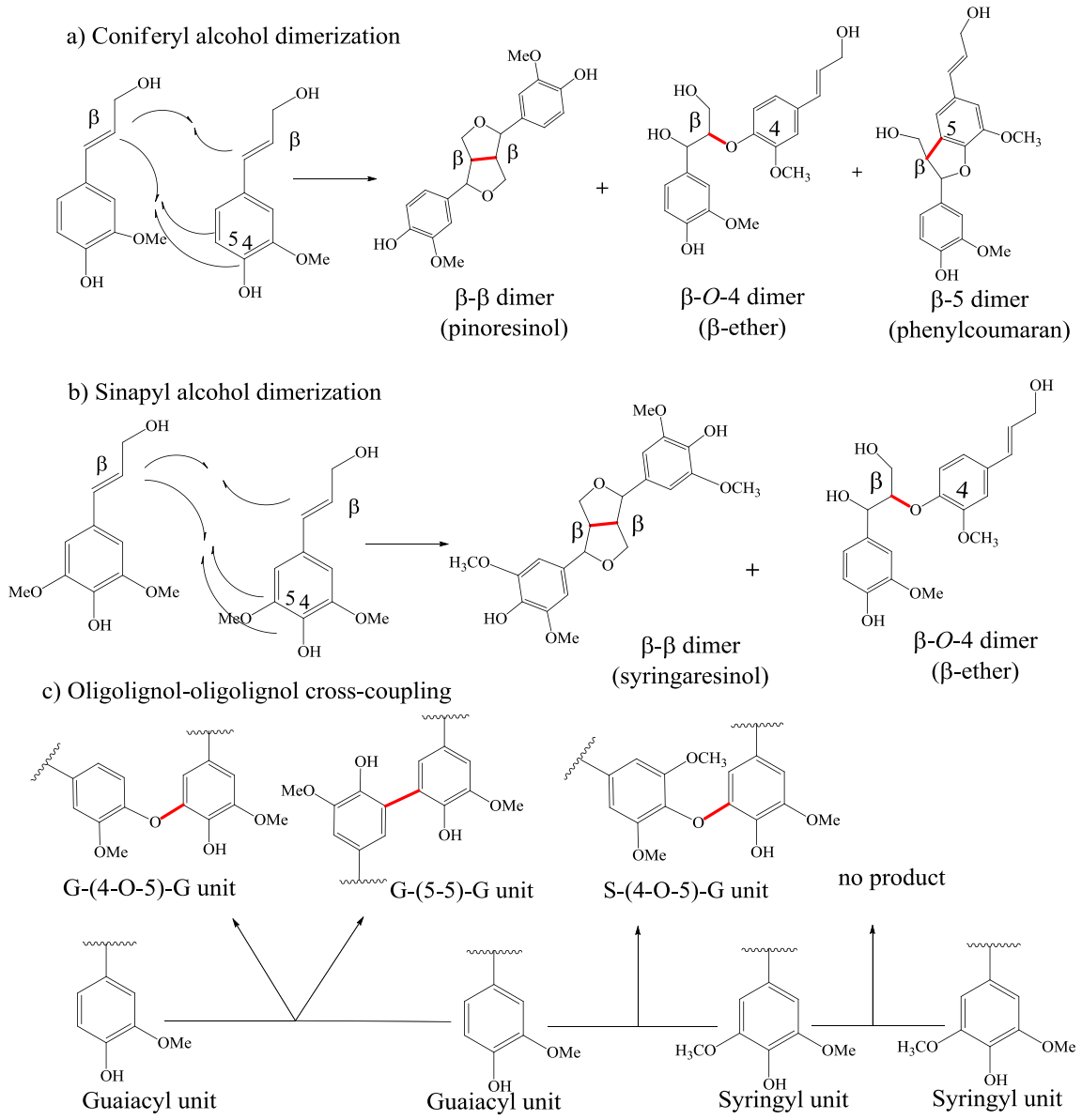

Figure 2.10 Coupling of Monolignols and Oligolignols (*a*) Coniferyl Alcohol; (*b*) Sinapyl Alcohol; (c) Oligolignol-oligolignol. (The Newly Formed Bond is Indicated in Bold) [114]

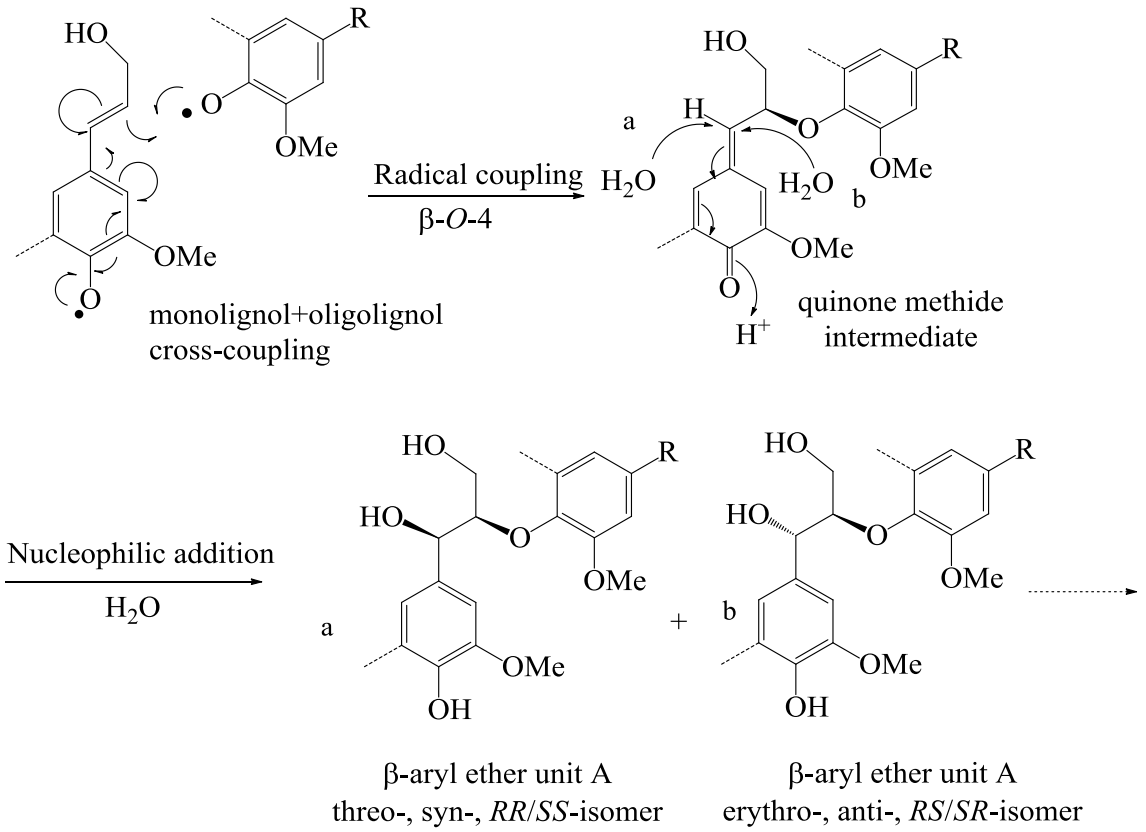

Figure 2.11 Formation of Arylglycerol-*β*-aryl Ethers through Cross-coupling of Monolignol to Oligolignols [114]

Dibenzodioxocin, a novel type of linkage in softwood lignin, was discovered by Karhunen et al. in 1995. [135-138] In their study of 2D NMR spectra on milled softwood lignin preparations, prominent correlation peaks were observed at 4.84/84.20 ppm (H_α/C_α) and 4.15/82.51 ppm (H_β/C_β) in the HMQC spectra of softwood lignin. The researchers couldn't assign these peaks to any proposed side chain lignin structure. [139] Further studies of the oxidative cross-coupling between dehydrodivanillyl alcohol and dehydrodipropyl guaiacol led to the successful formation of an 8-membered ring (Figure 2.12). This model compound of a dibenzodioxocin structure coincided exactly with the unknown correlations in the softwood lignin spectra. The formation reaction was proposed to be the oxidative coupling of a lignin precursor with a 5-5 biphenyl structure, followed by the endwise coupling to a monolignol, which ultimately would form a quinonemethide

intermediate. [114, 135] This quinonemethide would be internally trapped by the other phenol in the 5-5 moiety, producing an eight-membered ring (Figure 2.12). This linkage is extremely prevalent in high-guaiacyl lignins, as shown in Table 2.15.

β-β dehydrodimer is formed by the direct monolignol coupling. [114] Recent studies of the thioacidolysis and derivative followed by reductive cleavage (DFRC) of softwood lignin showed that most β-β products appeared to be 5-*O*-4 linked subunits (Figure 2.13). [140] Two possible pathways have been suggested for the formation of a 5-*O*-4 linked pinoresinol unit: (1) coniferyl alcohol dehyrodimerization produces pinoresinol which then cross-couple to the phenolic radical of a guaiacyl oligomer at the 5-position to form 5-*O*-4 linked pinoresinol; or (2) the cross-coupling of coniferyl alcohol with a guaiacyl oligomer directly at the C5 position produces a 4-*O*-5 structure. [140] This retained structure could further cross-couple with a new monolignol when both structures are at the β-position, thus generating the pinoresinol unit within the growing oligomer chain. [140]

Another cross-coupling interlinkage in lignin is the β-1 coupling mode. This formation mechanism was summarized by Ralph et al., [114] who suggested that the cross-coupling of a monolignol with a performed β-ether unit produces a quinonemethide intermediate (Figure 2.14). This qunionemethide may be trapped by water to form a dienone (pathway a); the intermediate may be logically internally trapped by the α-OH to form a spirodienone (pathway b); or the dienone may dehydrate to become a spirodienone (pathway d). The dienone structures may, finally, generate the conventional β-1 unit (pathway c). The spirodienone structure appears to be stable in lignins and can be detected using NMR. Further evolution of this structure results in the formation of an arylisochroman interlinkage in lignin (pathway e).

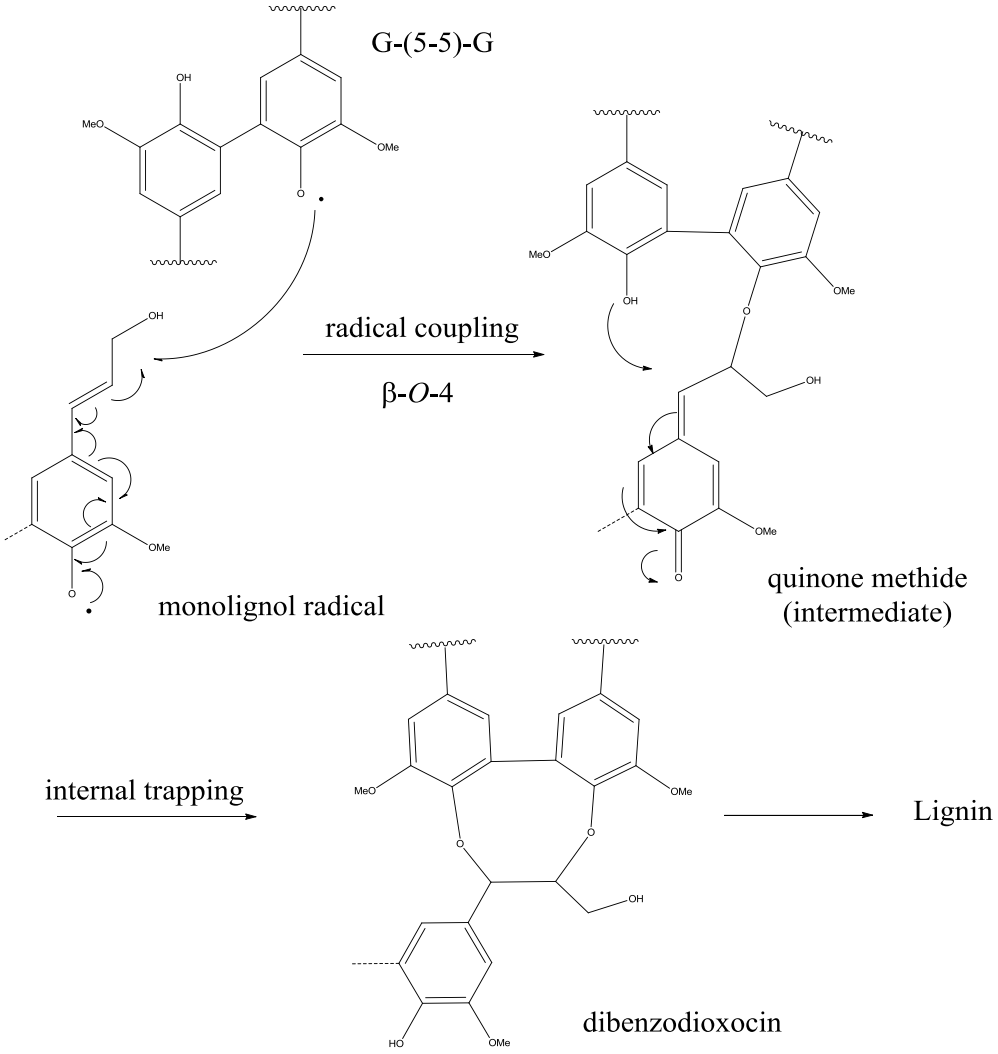

Figure 2.12 Formation of Dibenzodioxocin Unit in Lignins [114]

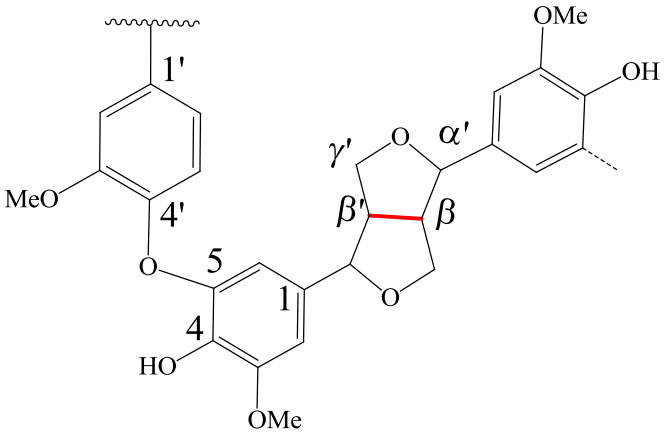

Figure 2.13 5-*O*-4 Linked Pinoresinol Unit [114]

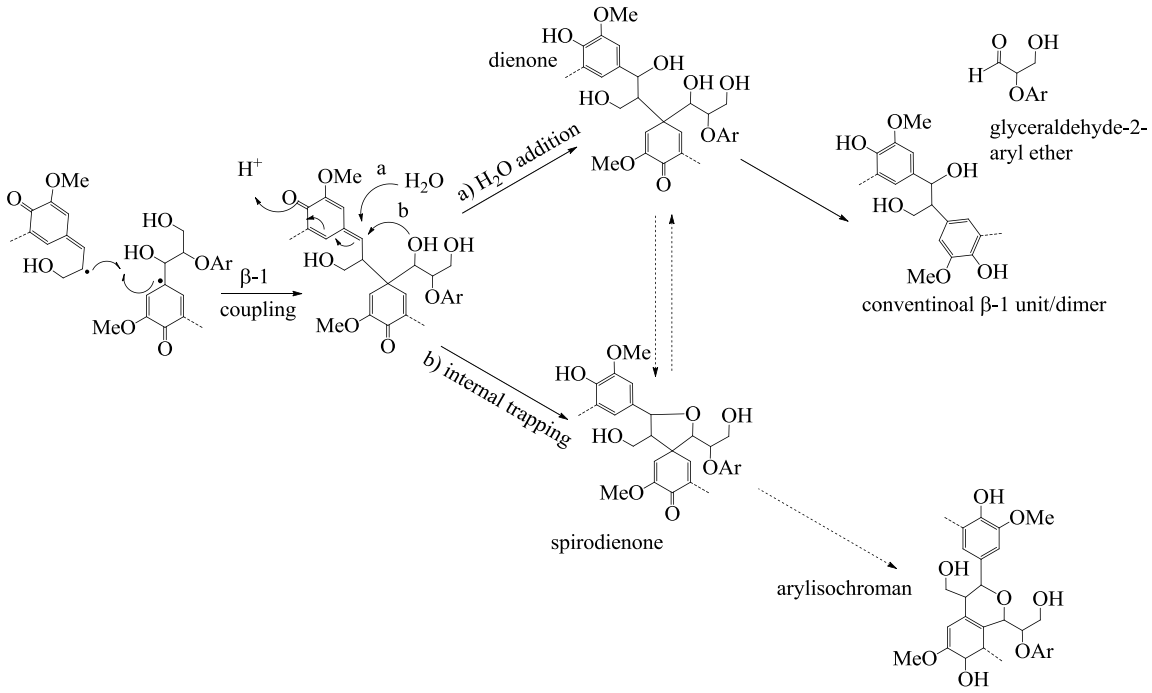

Figure 2.14 β-1 Cross-coupling Mechanisms.

Interlinkages of Lignins

A variety of structural units observed in softwood and hardwood lignin is shown in Figure 2.15, including β-aryl-ether (β-*O*-4), resinol (β-β), biphenyl (5-5), α-aryl-ether (α-*O*-4), 1, 2-diarylpropane (β-1), diphenyl ether (4-*O*-5), phenylcoumaran (β-5), and dibenzodioxocins. [114, 141]

The quantity of the primary interlinkages in the lignin polymer varies among different species. The β-aryl ether linkage, the most abundant interlinkage in lignin, accounts for approximately 45-50% of softwood lignin, 60-85% of hardwood lignin, and 39% of switchgrass lignin. [118, 142, 143] Table 2.15 elucidates the dominant interlinkages between the phenylpropane units, as well as their abundance of each of these interlinkages. The abundances of some functional groups in softwood lignin are also displayed. [143]

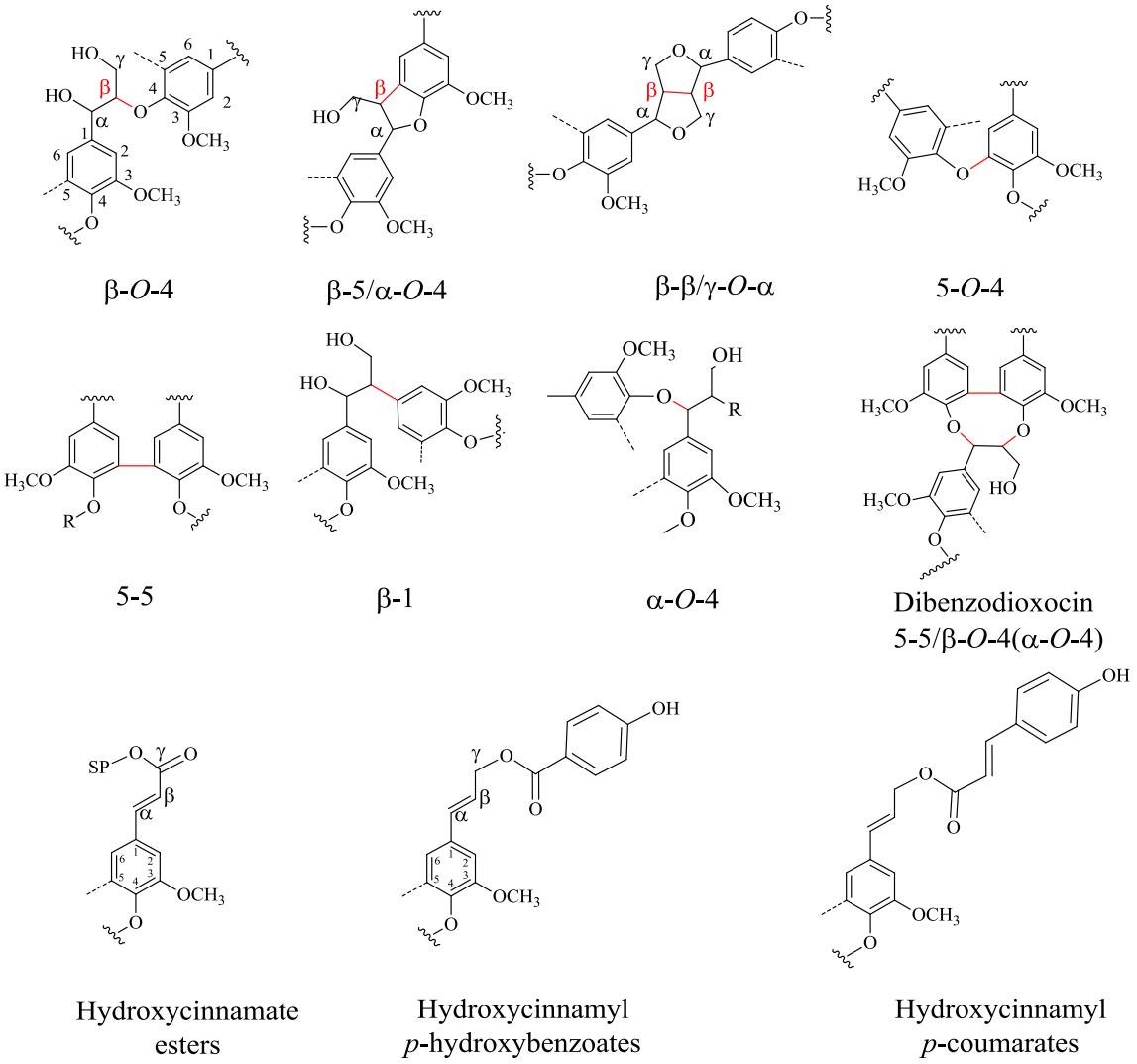

Figure 2.15 Major Interlinkages in Lignin Macromolecules[114, 141] (PS, Polysaccharides)

Table 2.15 Relative Frequencies of Linkages per 100 Phenylpropane Units in Softwood and Hardwood Lignin [144]

linkage type	name of linkages	spruce (%)	birch (%)
β-*O*-4	phenylpropane β-aryl ether	48	60
5-5	biphenyl and dibenzodioxocin	9.5-11	4.5
β-5	phenylcoumaran	9-12	6
β-1	1,2-diaryl propane	7	7
α-*O*-4	phenylpropane α-aryl ether	6-8	6-8
4-*O*-5	diaryl ether	3.5-4	6.5
β-β	β-β-linkage	2	3

The Structure of Grass Lignin

Grass lignin is composed of guaiacyl, syringyl, and *p*-hydroxyphenylpropane units. These units are connected by similar linkages to those found in hardwood and softwood lignin, but *p*-coumaric acid is esterified to the lignin at the γ-position of the propyl side chain. [115] Early studies of the products released during the alkali hydrolysis of various grasses indicated that ferulic acid and *p*-coumaric acid were released when a 1.0 M NaOH solution was applied at room temperature. [18, 145] These results provided direct evidence for the presence of ester linkages between these acids in the cell walls of grasses. [18, 145] An additional fraction of ferulic acid was released under high-temperature (170 ^{o}C) conditions, which implied that at least a fraction of the ferulic acid was contained in ether linkages. [18, 145] Such studies are some of the first to implicate the involvement of ferulate in lignification. Importantly, the involvement of polysaccharide-ferulate (or -diferulate) esters in lignification provides a mechanism for cross-linking the two disparate classes of important polymers in the cell walls of grasses: polysaccharides and lignin. Further characterization of grass lignin, performed using nitrobenzene oxidation and thioacidolysis, indicates the presence of *p*-hydroxyphenol, guaiacyl, and synapyl units in grass lignin (Table 2.14). [145, 146]

The Role of p-Coumaric Acid in Grass Lignin

One of the remarkable features of *p*-coumarates in grass lignin is that they are simply terminal pendant groups with ester linkages to the lignin [147] (Figure 2.15). These observations are consistent with the ^{13}C-NMR spectra of maize lignin, and suggest that free-phenolic *p*-coumarate esters attach exclusively to the γ-position of phenylpropane units of maize lignin. [148] Acetylated monolignols are incorporated into the lignin polymer via radical polymerization with traditional monolignols. In kenaf bast fiber, the acylation of lignin occurs frequently in syringyl units but infrequently with guaiacyl units. [149] Recent investigation has come close to obtaining the tansferase enzymes and genes

necessary for the acylation of monolignols. Hatfield et al. showed that the monolignol *p*-coumaroylation in maize occurs via *p*-coumaroyl-CoA. [150]

Sinapy alcohol is a poor substrate for peroxidases. When a 0.01 equivalent of methyl *p*-coumarate is present in the system, however, the reaction rate of the radical coupling reaction of sinapyl alcohol is accelerated. [151] *p*-Coumarate plays a central role in the transfer of radicals from *p*-coumarate to sinapyl alcohol, which rapidly accelerates dimerization. This observation also suggests that *p*-coumarates are not integrally incorporated into the polymer chains via the radical coupling reaction, but remain simply a free-phenolic pendant group with acylation on the primary γ-OH of the lignin side chain. [148, 152]

p-Coumarates are acylated at a level of about ~90% of the γ-OH of syringyl units. [153, 154] This acylation of *p*-coumarates remarkably influences the post-coupling re-aromatization reactions of the resulting qunionemethide intermediates. After γ-acetylation, the *p*-coumaroylation of the monomers results in a novel β–β coupling product, because the internal trapping pathway for the resulting quinonemethide intermediate is lost (Figure 2.16). [155]

The Role of Ferulic Acid in Grass Lignin

The actual levels of ferulate (Figure 2.16) in the cell walls of grasses are not quantified because the ferulate in most plants is not releasable by base or other treatments. Evidence implies that ferulates are involved in cross-coupling reactions with monolignols. This cross-coupling leads to the formation of interlinkages, notably β'-β, β-*O*-4', β-5', β'-*O*-4, and β'-5 linkages. [156] ^{13}C-labeling at the 9-position of ferulate aids in the NMR delineation of the various combinational coupling modes in the dehydrogenation of polymers (DHPs), notably at the β-β'-, β-*O*-4', β-5', β-*O*-4', and β-5 linkages. [156]

The generally accepted mechanism indicates that ferulates are incorporated into lignin via the radical coupling reaction. The analogous β-*O*-4' coupling of ferulate ester

is one of these examples, and is shown in Figure 2.16. In this reaction, the radical coupling of ferulate esters at the β-*O*-4' linkage forms a β-*O*-4'quinonemethide intermediate, which then preferentially re-aromatizes to form β-*O*-4' enol ether when the acidic β-proton is eliminated. Further studies show that a β-β'-coupled product between ferulate and coniferyl alcohol is identified through long-range 2D ^{13}C-^{1}H correlation experiments. [157] These results suggest that ferulates participate not only in the radical coupling reactions of monolignols, but also in cross-coupling reactions with the free-phenolic ends of growing oligomers or polymers of lignin.

Incorporation of Polysaccharide Hydroxycinnamate Esters into Lignins

p-Coumarate, ferulate, and sinapate (Figure 2.15) are acylated on polysaccharides. Grasses have relatively high levels of ferulate and lower levels of *p*-coumarate. Both acids are acylated exclusively on the primary hydroxyl (C-5) of a α-*L*-arabinofuranosyl moiety in the arabinoxylans. [147, 158-160]

The structural variety derived from the β–β-coupling of hydroxycinnamates is due to the series of purely chemical combinatorial radical coupling reactions that occur during lignification. [131] For instance, sinapate dehydrodimers and sinapate-ferulate crossed dimmers have been found predominately in wild rice. [161] A variety of ferulate dehydrotrimers and dehydrotetramers has also been reported. [116, 131, 160, 162] However, there is a little evidence for the involvement of *p*-coumarate in polysaccharide-polysaccharide cross-linking via analogous dehydrodimerization.

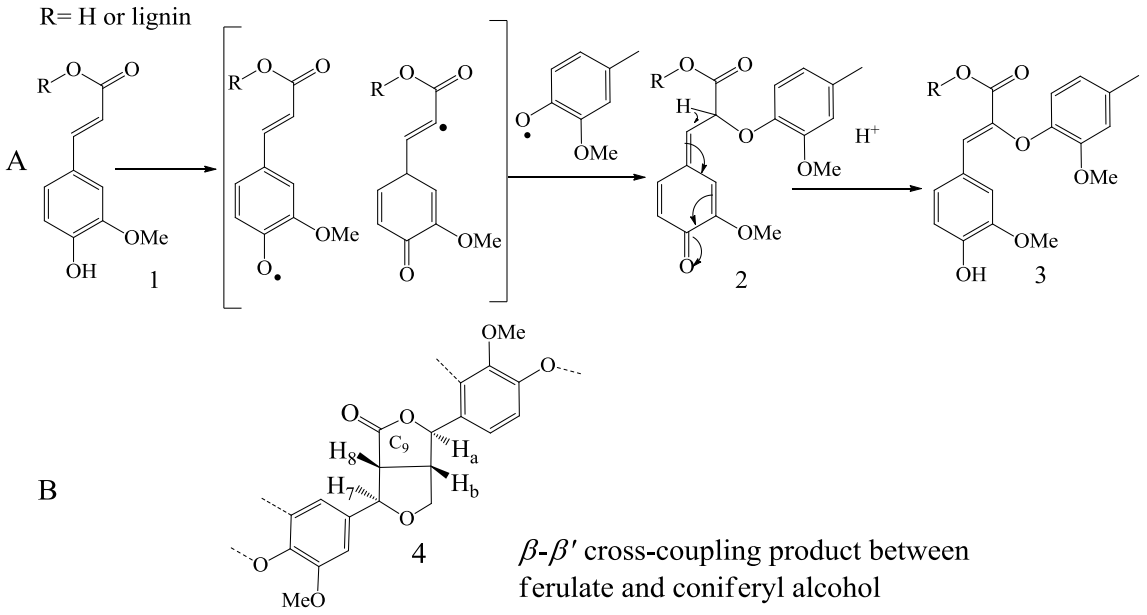

Figure 2.16 (A) An Analogous β-*O*-4' Coupling of Ferulate Ester, (1) Ferulate Esters; (2) An Intermediate Quinonemethide; (3) A Formation Product of β-*O*-4 Coupling of Ferulate Ester (B) A β-β'-coupled Product between Ferulate (1) and Coniferyl Alcohol.

Spectroscopy Analysis of Switchgrass Lignin

Ball-milling is a direct and mild method for the isolation of lignin from herbaceous plant for the structural characterization [163], and was used in the study reported by Yan et al. [66] The yield of switchgrass ball Milled Grass Lignin (BMGL) in this study was approximately 10% of the lignin content in extracted switchgrass from four populations of switchgrass, Alamo, Kanlow, GA992, and GA993. In this study, the structure of BMGL was characterized by quantitative ^{13}C-NMR analysis. Figure 2.17 provides a representative spectrum for Alamo lignin. The lignin structural assignments are summarized in Table 2.16.

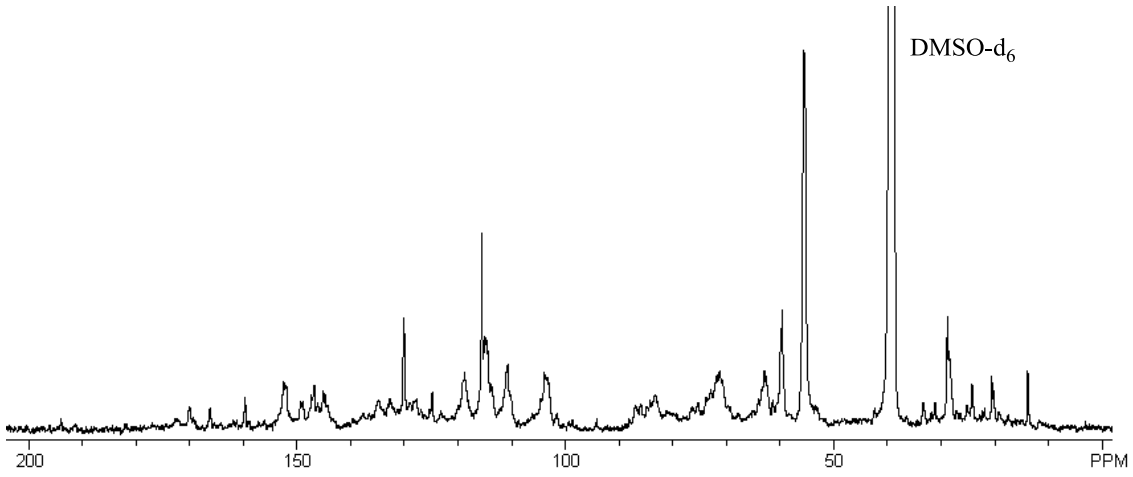

Figure 2.17 Quantitative ^{13}C-NMR Spectrum of BMGL (Reproduced with Permission) [66]

From the spectra, the chemical shifts, at δ 161- 158 ppm, δ 158-156 ppm, δ 113-110 ppm and δ 110-102.5 ppm are assigned to C-4 conjugated H-unit, unconjugated C-4 H-unit, C-2 in G-lignin (also including C-2 of ferulic acid), and C-2/6 in S-units respectively. [141] The chemical shift from δ 168-164 ppm is assigned for C_γ of conjugated acid (0.19-0.20 per aromatic ring). [18, 115] The chemical shifts at δ167, 159.7, 149.7, 146.9, 145.4, and 115.6 ppm is assigned for the carbon from *p*-coumaric acid and ferulic acid. [65, 148, 164] These results suggest that lignin from switchgrass is a HGS type complex polymer with a significant amount of *p*-coumaric acid and ferulic acid (0.18 per aromatic ring on average). The results in Table 2.16 also indicate that non-etherified *p*-coumaric structures in lignin appeared at δ158-161 ppm [65] with 0.15-0.19 per aromatic ring for the lignin of the four switchgrass populations. The C-4 carbon of unconjugated H-unit appeared at δ 158-156 ppm with a low intensity, which is attributed to a small amount of unconjugated H-unit content in switchgrass (0.05-0.09 per aromatic ring). The results also suggest that *p*-coumarate and ferulate are the major acid linked to switchgrass lignin and most of *p*-coumarate is non-etherified and esterified to lignin. From that study, the methoxy group at δ 57-54 ppm has an average integration value 0.96 per aromatic ring for the lignin from the four switchgrass populations. On average, the H: S:G ratio is 26:42:32 and the S:G

ratio is 0.75. These results demonstrate that lignin isolated from the four switchgrass populations—Alamo, Kanlow, GA992, and GA993—are similar in structure.

Table 2.16 Quantitative ^{13}C-NMR Assignments and Integration Value of BMGL

range (ppm)	assignments[a]	SW9[b]	SW11[b]	SW12[b]	SW10[b]
175-168	Unconjugated COOR	0.36	0.40	0.37	0.39
168-164	Conjugated COOR in FA and *p*-CA	0.20	0.20	0.19	0.20
161-158	C-4 in conjugated NE *p*-CA	0.17	0.15	0.19	0.19
158-156	C-4 in unconjugated H-unit	0.09	0.07	0.05	0.07
156-151	C-3 in 5-5'ET, C3/C5 in S unit	0.67	0.62	0.66	0.64
123-117	C6 in G unit and ferulic acid	0.44	0.46	0.47	0.43
117-113	C5 in G unit, C3/C5 in p-CA, C5 in FA, β-carbon in *p*-CA and FA	0.83	0.83	0.80	0.75
113-110	C2 in G unit, C2 in ferulic acid	0.41	0.44	0.40	0.40
110-102.5	C2/C6 in S unit	0.63	0.59	0.60	0.64
61-57	C-γ in β-*O*-4 (G or S) without Cα=O	0.43	0.45	0.43	0.39
57-54	OCH_3	0.99	0.98	0.97	0.91
54-52	C-β in β-β and β-5 unit	0.12	0.14	0.14	0.13
21-19	Acetyl	0.16	0.19	0.19	0.11
	H unit %	27	24	26	27
	G unit %	41	45	42	40
	S unit %	32	31	31	33
	S/G ratio	0.77	0.68	0.74	0.81

Note: [a] G: guaiacyl; H: *p*-hydroxyphenyl; S: syringyl; ET: etherified; NE: nonetherified; FA: ferulic acid; *p*-CA: *p*-coumaric acid; [b] per aromatic ring

Samuel et al. studied the lignin structure from Alamo switchgrass recently by quantitative ^{13}C-NMR, ^{31}P-NMR, and 2D-heteronuclear single quantum coherence ^{13}C-^{1}H correlation spectra analyses. Examples of the structural interlinkages of switchgrass lignin are presented in Table 2.17. This chart provides the relative abundances of the dominant linkages based on the quantitative ^{13}C-NMR analysis, as well as the abundances of some functional groups, obtained through quantitative ^{31}P-NMR analysis. These results demonstrate that the lignin of switchgrass is a HSG type with p-coumaric acid and ferulic acid linked to lignin. The analysis also indicates that the β-*O*-4 ether is the major linkage of ball milled lignin. [66, 118] Other minor linkages, such as phenylcoumarin, resinol, and spirodienone units, are also observed in these studies. [118]

Table 2.17 Proportions of Linkages and Functional Groups in Switchgrass Lignin [118]

linkage type	name of linkages	linkages, per 100 aromatic ring
β-*O*-4	phenylpropane β-aryl ether	39
β-5	phenylcoumaran	10
β-β	β-β-linked structures	
G	guaiacyl	44
S	syringyl	35
H	hydroxylphenyl	7
OMe	methoxyl	99

functional group	abundance, mmol/g lignin
aliphatic OH	3.88
condensed phenolic OH	0.20
guaiacyl phenolic OH	0.48
p-hydroxyphenyl	0.32
carboxylic OH	0.29

Hydrothermal Pretreatment of Lignocellulosic Bioresources

Hydrothermal Pretreatment Chemistry

Hemicelluloses are the most easily removed and decomposed components of bioresources under the acidic conditions of hydrothermal pretreatment. This is because hemicelluloses are branched, amorphous, and have low molecular weight polymers. [27, 37, 38] Under mild acidic conditions, the 1, 4-glycosidic ether linkage of hemicelluloses is cleaved easily to decompose hemicelluloses into oligomers and monomer sugars in an aqueous solution (Figure 2.18). Some low molecular products, such as furfural, are also formed in this process (Figure 2.19). The removal of hemicelluloses from lignocellulosic bioresources increases the rate of the enzymatic hydrolysis of cellulose by improving the accessibility of the cellulose surface to enzymes.

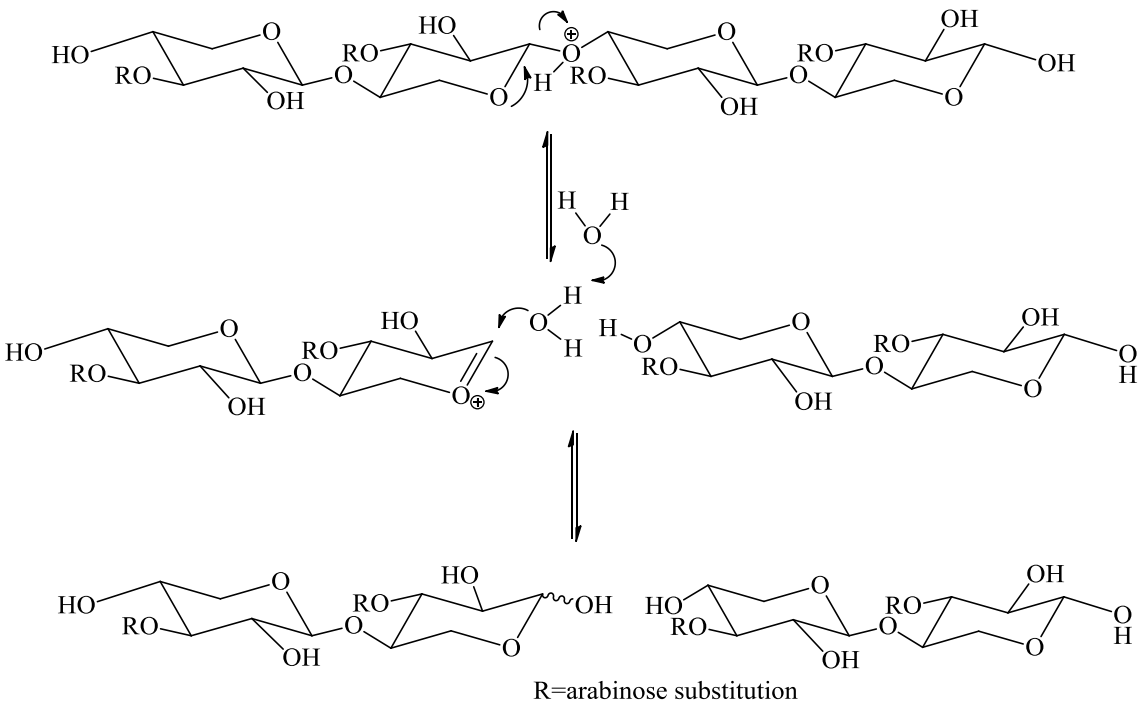

Figure 2.18 Acid Catalyzed Hydrolysis of Arabinose Substituted Xylan [111, 165, 166]

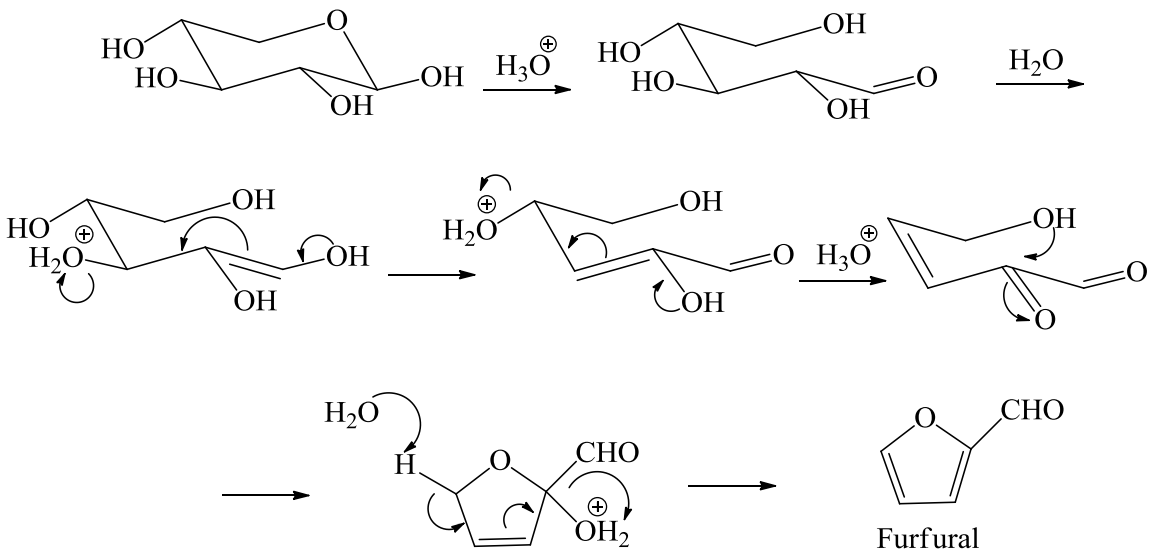

Figure 2.19 Formation of Furfural from Xylose in Acid-catalyzed Hydrolysis

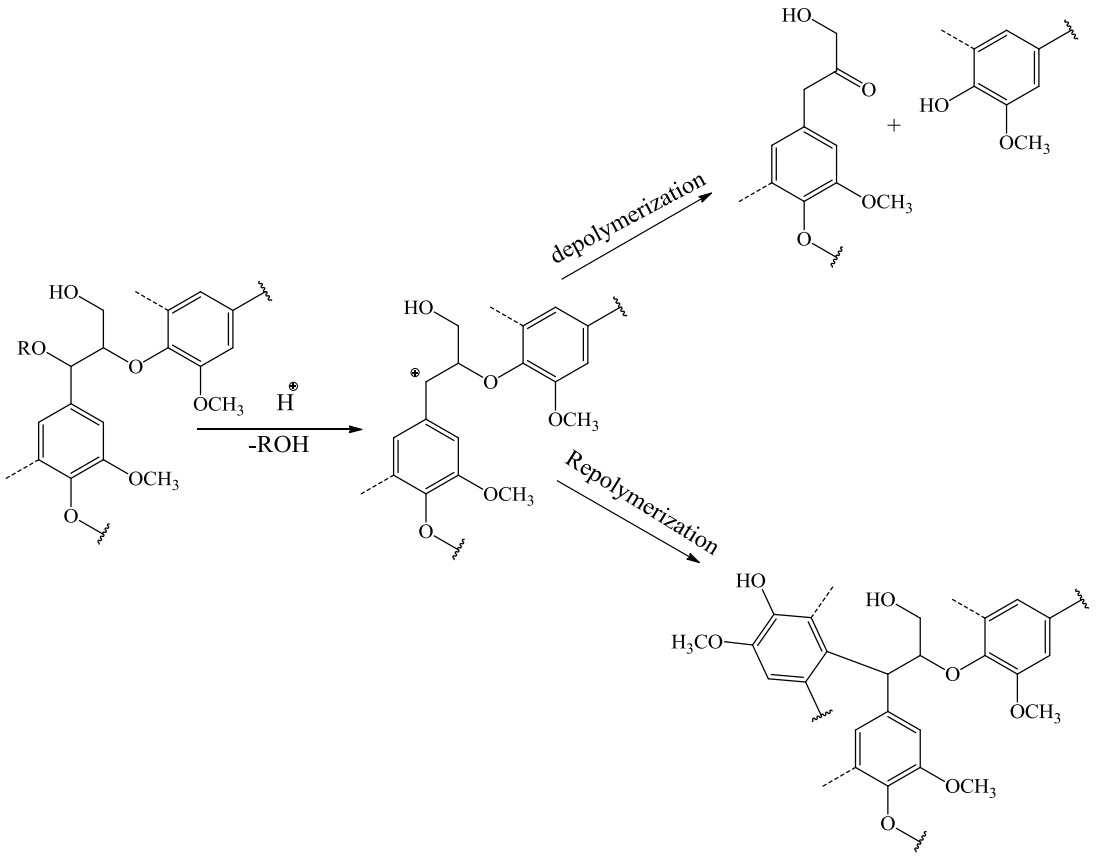

Figure 2.20 Proposed Depolymerization and Condensation of the β-*O*-4 Linkage of Lignin under Hydrothermal Pretreatment [167]

Both the depolymerization and the condensation of lignin occur under hydrothermal pretreatment. Several studies have been conducted in order to characterize the lignin structure during hydrothermal pretreatment. The results of autohydrolysis on *Eucalyptus* globulus wood showed that at 170 ^{o}C, lignin macromolecules were depolymerized by the cleavage of β-aryl ether interlinkages and were subsequently condensed during hydrothermal pretreatment (Figure 2.20). [167-169] During hydrothermal pretreatment, however, the hydrolysis rate for G-units was greater than that of S-units, according to a literature report on the hydrothermal pretreatment of Miscanthus. [170] These results also indicated that a condensation reaction and a depolymerization of lignin occurred simultaneously during hydrothermal pretreatment (Figure 2.20). [167] Figure 2.20

demonstrated the proposed mechanism of depolymerization and condensation reactions for β-*O*-4 linkage of lignin. Carbonium ion was the proposed intermediate in hydrothermal pretreatment or acidic pretreatment. In the acidic condition, this intermediate can be depolymerized through deprotonation and enolization to cleavage β-acryl ether and form beta ketone. Condensation reaction was suggested as a repolymerization of lignin hydrolyzed fraction in the acid hydrolysis of lignin. This reaction leads to the formation of a linkage between a reactive aromatic carbon and a carbonium ion at C-α of the side chain in lignin. [171] [118]

Cellulose undergoes acid-catalyzed hydrolysis during hydrothermal pretreatment. [27] Under hydrothermal pretreatment, the yield of cellulose is up to 80% at 180-240 ^{o}C. [27, 37] Studies have shown that after hydrothermal pretreatment some low molecular compounds form, such as glucose, 1, 6-glucose anhydrous, 5-hydroxymethylfurfural, etc. Figure 2.21 shows the mechanism by which cellulose is acid hydrolyzed into glucose, as well as the subsequent decomposition of glucose into hydroxymethylfurfural. Several studies have reported changes in the structure of cellulose after hydrothermal pretreatment. The results indicate that hydrothermal pretreatment starts to hydrolyze the amorphous region of cellulose at temperatures above 150 ^{o}C. [35, 36] However, for the crystalline component of cellulose, a harsher condition is required to break down the structure of cellulose. The minimum temperature for this reaction is about 180 ^{o}C. [35, 36] The changes in cellulose polymorphs after hydrothermal pretreatment were also explored. For instance, hydrothermal pretreatment increased the percentage of paracrystalline cellulose and reduced the percentage of inaccessible fibril surface cellulose. These changes were dramatically increased when the temperature of hydrothermal pretreatment went above 180 ^{o}C. [36]

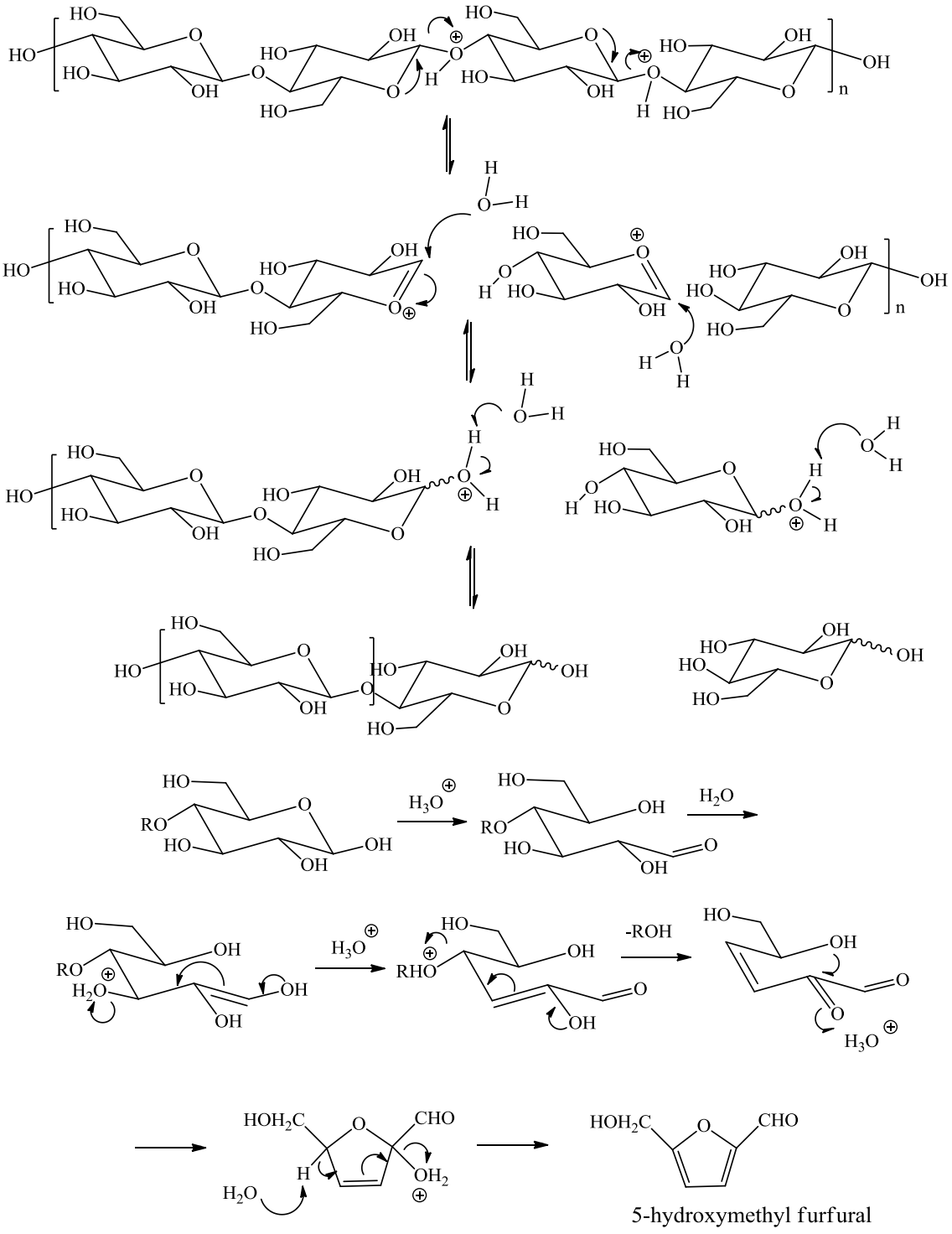

Figure 2.21 Acid Hydrolysis of Cellulose to Glucose and Hydrolysis of Glucose to 5-Hydroxymethyl Furfural[34, 35, 166]

Hallac et al reviewed the DP of cellulose for biomass in pretreatment processes recently. [80] The DP of cellulose has been suggested to decrease rapidly until it reaches the so-called "leveling-off" DP (LODP) when biomass is subjected to acid hydrolysis. Although literature has not been reported the LODP value of cellulose for hydrothermal pretreatment, typical summarized value for various pretreatment has been indicated in the range of 140 to 400 AGUs. [80]

These modifications to the structure of the hemicelluloses, cellulose, and lignin, brought on by hydrothermal pretreatment, contributed significantly to the accessibility of the lignocellulosic bioresources to cellulases during enzymatic hydrolysis.

Saccharification and Fermentation of Hydrothermally Pretreated Biomass

It is well known that cellulases include three types of enzymes, exo-glucosidase, endo-glucosidase and cellobiase. These enzymes work together to hydrolyze cellulose chain into glucose. [172, 173] These enzymes work on cellulose using specific mechanisms. Endo-glucosidase binds randomly in the middle of a cellulose chain and cleaves the cellulose chain, which reduces the DP of cellulose. Exo-glucosidase act on the glucan chain end units and releases cellobiose molecules via the cleavage of the 1, 4-glycosidic ether linkages of cellulose. Cellobiase uses a cellobiose as a substrate to produce a glucose unit. Because cellobiase only effective in the presence of endo and exo-glycosidase, it is necessary to add cellobiase to reduce the accumulation of cellobiose, and thus improve the total yield of glucose during the enzymatic hydrolysis process.

Fermentation is necessary in order to produce ethanol from lignocellulosic bioresources. Commercially, fermentation includes two types of processes, simultaneous saccharification and fermentation (SSF) and separated hydrolysis of fermentation (SHF). [15, 174] Both processes have a high conversion yield of sugars, such as glucose, into bio-ethanol. In general, SSF includes one step to produce ethanol from pretreated lignocellulosic bioresources.

In this process, the enzymatic hydrolysis of cellulose and the fermentation of glucose are integrated into a single unit to make bio-ethanol from the pretreated biomass. This process reduces the processing capital cost for the bio-ethanol conversion. SHF is another technique used to make bioethanol from biomass. In this technique, two separate processes, the saccharification of biomass and the fermentation of sugars, are necessary to produce bio-ethanol from the pretreated biomass. Because the ideal conditions for cellulosic enzymes and sugar yeast differ in temperature significantly, SHF allows more efficient bio-ethanol production. However, the capital and processing cost for the SHF process are much greater than those for the SSF process.

Suryawati et al. evaluated the effect of the hydrothermolysis pretreatment conditions on the composition of switchgrass and the ethanol yield using SSF with Kluyveromyces marxianus IMB4. [38] They utilized a two-factorial experiment with three temperatures (190, 200, and 210 ^{o}C) and hold times (10, 15, and 20 min) for treating Kanlow switchgrass. The results indicate that most xylan is removed from switchgrass when it has been treated at 200 ^{o}C for 10 min. The highest concentration of ethanol was produced from switchgrass pretreated at 210 ^{o}C for 15 min using simultaneous saccharification and fermentation (SSF) at 45 ^{o}C with the thermotolerant yeast Kluyveromyces marxianus IMB4 and 15 FPU cellulase/g glucan (72% theoretical yield). Figure 2.22 shows the changes that occur in the product under various degrees of hydrothermolysis severity. In this process, the maximum yield of furfural and HMF observed for switchgrass was 1.2% of the dry mass of the switchgrass. [38]

Effect of Cellulose Structure for Biofuel Production

Although systematic studies on the effect of cellulose structure of biomass for biofuel production has not been addressed in hydrothermal pretreatment of biomass, the cellulose crystallinity and the DP of cellulose have been studied for other pretreatment technologies.

Recent investigations have explored the structure of cellulose derived from various resources using solid-state ^{13}C-CP/MAS experiment and a line fitting process. The observed data is summarized in Table 2.18. Pu et al. used solid-state ^{13}C-CP/MAS NMR methodology to determine the structure of cellulose in bleached softwood Kraft pulp during cellulase hydrolysis. [103] The results indicate that cellulose I_α, paracrystalline cellulose, and non-crystalline cellulose, including both accessible and inaccessible fibril surfaces, are more susceptible to cellulases in the rapid initial phase of cellulose hydrolysis. During an organosolv pretreatment and the enzymatic deconstruction of *Buddleja davidii*, Hallac et al. monitored changes in the plant cell wall, and noted significant changes in the structure of cellulose. [175] These results (Table 2.18) suggest that organosolv pretreatment increases the relative proportions of paracrystalline cellulose significantly, and reduces the DP and relative proportions of crystalline allomorphs (cellulose I_α and I_β). These changes in the structure of cellulose increase the amenability of pretreated biomass to enzymatic degradation. [175] Samuel et al. investigated the ultrastructural changes in switchgrass cellulose after the grass was subjected to a dilute acid pretreatment using CP/MAS ^{13}C NMR. The results (Table 2.18**)** indicate that a dilute acid pretreatment lowers the percentage of amorphous cellulose and raises the crystallinity index of cellulose. [102] These studies suggest that the characterization of the cellulose structure is an important factor, in part, for bioethanol production.

Through a systematic study on the effect of the cellulose structure and the DP of cellulose of organosolv pretreated Buddleja davidii, Hallac et al [80] suggests that lower DP of cellulose improves enzymatic hydrolysis due to two factors: (i) increasing the number of cellulose chain reducing ends; and (ii) making cellulose more reactive to the enzymes. The Number of Reducing End (F_{RE}) is calculated from the inversed value of the DP of cellulose and has been suggested to be a factor contributing to the efficiency for cellulose hydrolysis by cellulases. [175] V äljam äe et al has addressed that the fraction of reducing ends (F_{RE}) improve the exo-glucanase activity. [176] In the enzymatic hydrolysis, the

increased reducing ends of cellulose generated by endo-glucanase accelerate the hydrolysis rate of exo-glucanase. [176]

Table 2.18 The Relative Amounts (%) of Different Cellulose Forms Estimated by Non-linear Least-squares Fitting of the C-4 Region in CP/MAS ^{13}C-NMR Spectra [65, 102, 103]

samples	crystalline cellulose I	paracrystalline	accessible fibril surface 1	accessible fibril surface 2	inaccessible fibril surfaces
Alamo switchgrass	15.1	27.3	6.2		51.3
Buddleja davidii	19.4	32.9	3.9	2.7	41.1
southern pine Kraft pulp	13.2	37.1	2.7	2.2	44.8

Effects of Severity and Bioresources for Biofuel Production

The severity factor (R_0) was developed to allow a general interpretation of the effects caused by temperature and time upon hydrothermal pretreatment. [27, 177] The equation for the calculation of the severity factor is given below.

$R_0 = t * \exp((T-100)/14.75)$ Equation 2.5

Where T is the hydrolysis temperature (^oC), t is the reaction time (min).

The hydrothermal pretreatment of other potential sources of glucose is shown in Figure 2.23. The results indicated that hydrothermal pretreatment of this biomass was conducted at a severity factor between 2.95 and 4.95. Figure 2.23 shows that the severity factors can be related to the enzymatic hydrolysis yield and theoretical production yield of ethanol from pretreated biomass. These results suggest that the yield of the enzymatic hydrolysis and the ethanol yield depend not only on the severity factors used for the pretreatment but also on the bioresources used. For instance, the theoretical ethanol yield is significantly greater for switchgrass than it is for poplar (80% vs. 60%). [41, 44]

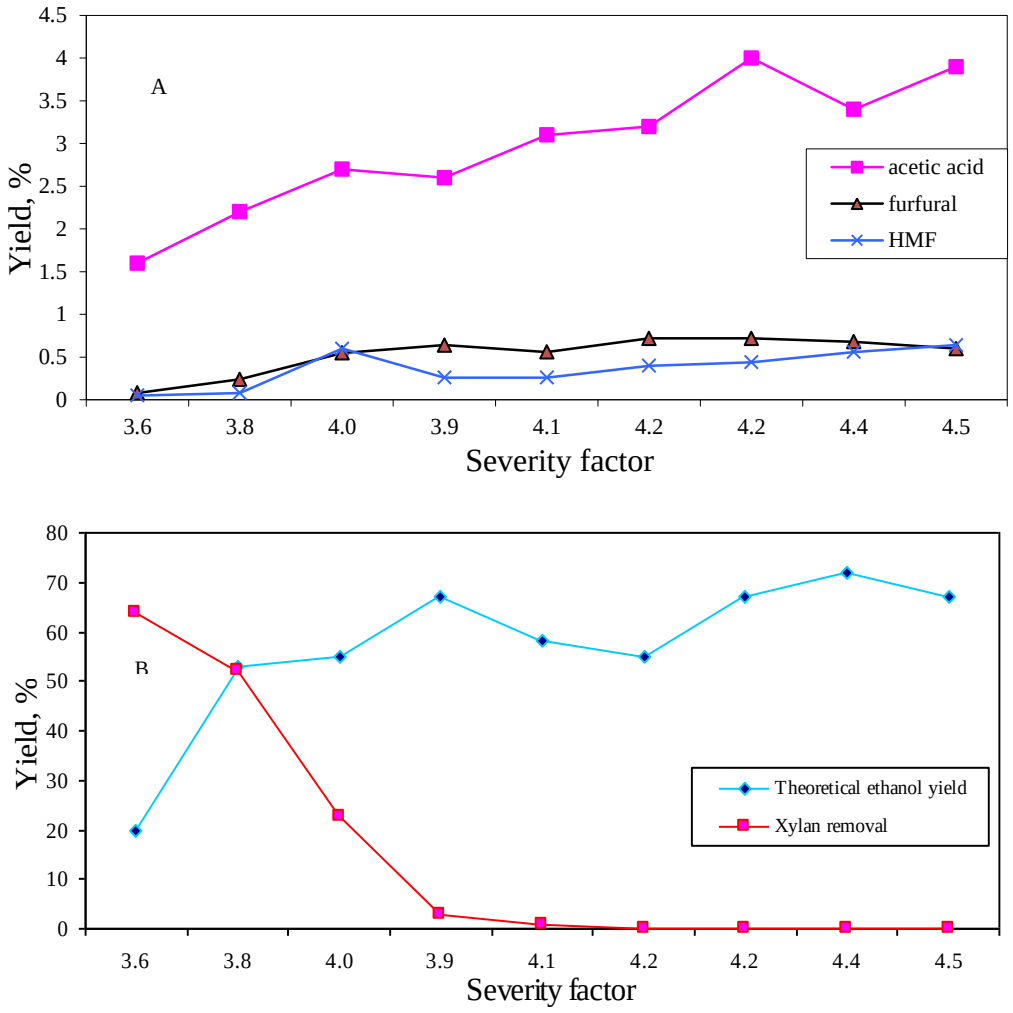

Figure 2.22 Effect of Hydrothermal Pretreatment Severity to the Yield of Byproducts (A) and the Yield of Theoretical Ethanol Production and Xylan Removal (B) [38]

Effect of Morphology on the Digestibility of Biomass

The varying degrees of the digestibility of different morphological portions of grasses (leaves, stems, or internodes and nodes etc.) have been studied in several species. For example, Dien et al. [73] studied the acid pretreatment of switchgrass with specific maturity stages and morphological portions. The results demonstrate that different growth stages and morphological portions (i.e. leaves vs. stems) have different levels of susceptibility to cellulases after a dilute acid pretreatment. The results show that the pre-

boot and post-frost stages of switchgrass performance 19% greater glucose yield by cellulases after a 2% sulfuric acid pretreatment at 121 ^{o}C for 1 hour. Another study of leaves and stems (sheath, internodes, and nodes) of switchgrass examined the response to the acid pretreatment, subsequent saccharification and fermentation. [30] The results indicate that the leaf portion takes on a more digestible form after a 1% sulfuric acid pretreatment at 121 ^{o}C in autoclave for 1 hour. [30] William et al. [178] studied the digestibility of various morphological portions of corn stover, including the leaf blade, leaf sheath, stem ring, stem pith, and corn kernel fiber. The highest dry matter loss was about 47% for the leaf sheath after 72 h hydrolysis by cellulases. These studies suggest that the morphological fractions are a factor to consider during enzymatic hydrolysis.

Shishir et al. [179] also studied the enzymatic digestibility of various morphological portions of corn stover after an ammonia fiber explosion (AFEX) pretreatment. Because the morphological portions of corn stover could be separated by particle size, the author used particle size portions to represent different morphological portions, and stated that the larger fractions were representative of the stem portions and the finer fractions were from the leaf portions. After an AFEX pretreatment and cellulase hydrolysis, the results indicated that the cellulose-to-glucose yield was comparable among different particle sizes of corn stover.

Several other studies have been conducted on the effect of silicon on the digestibility of rice straw. The results also confirm that silica content contributed negatively to the digestibility of rice straw by Holstein cow rumen. [180] According to these studies on morphological portions of biomass, the results suggest that morphological portions have different levels of digestibility after pretreatment. Stem portions are more difficult to degrade using cellulases than are other morphological portions.

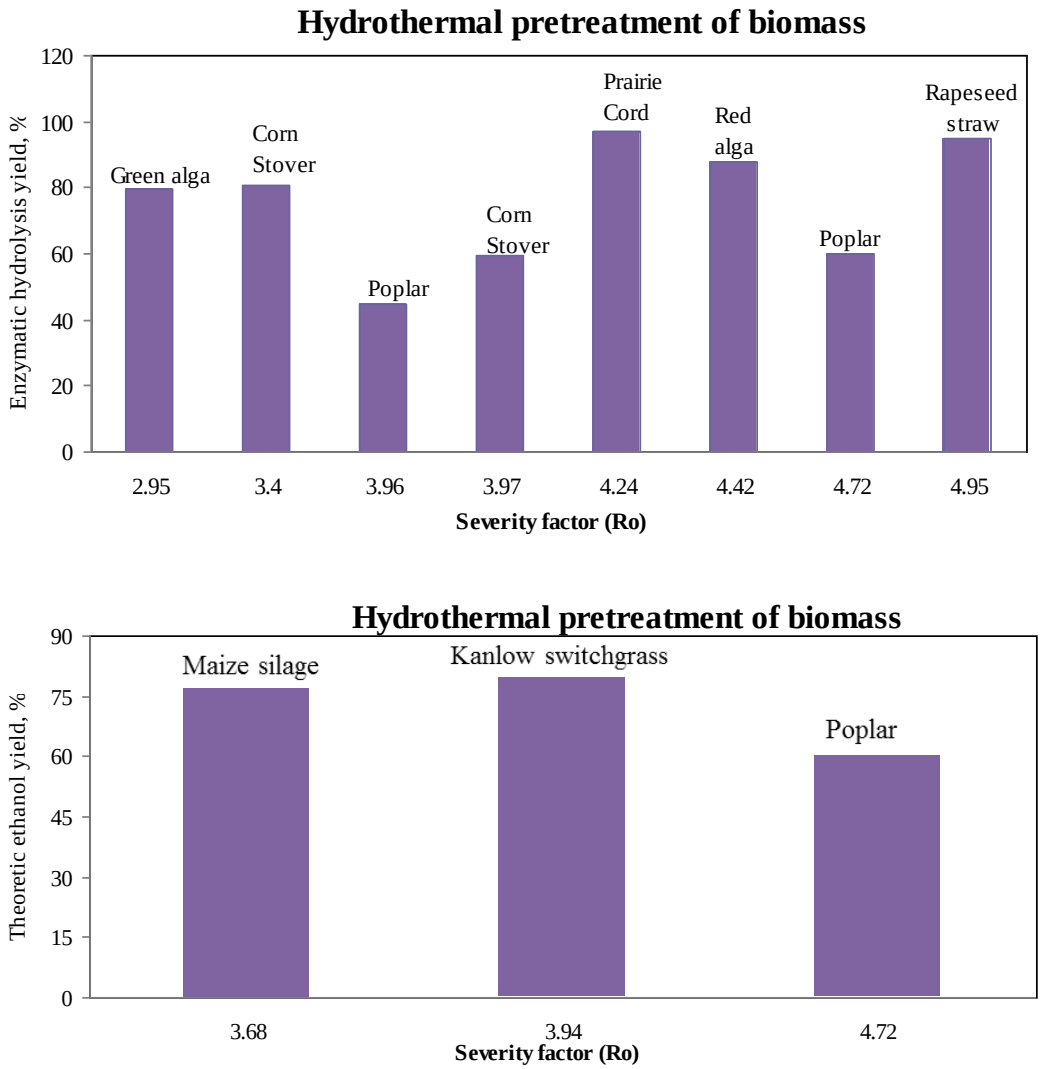

Figure 2.23 Effect of Hydrothermal Pretreatment Severity and Bioresources to the Enzymatic Hydrolysis Yield (A) [40, 43-45, 181, 182] and Theoretical Ethanol Yield (B) [41, 42, 44]

The Lignin Content and Structure Related to the Digestibility of Switchgrass

The recalcitrance to the saccharification is a major limitation for the conversion of lignocellulosic biomass to ethanol. Several studies show that alteration of lignin content and lignin structure could improve the saccharification efficiency of switchgrass. [18, 146, 183, 184]

Beyond the saccarification efficiency of switchgrass, studies on the anatomical and physiological features of switchgrass have been reported by several researchers. [146, 185] Internodes of switchgrass, which are 6 of them below the fully extended peduncle of flowering tillers, have been investigated by Sarath et al. [185] They demonstrate that the content for acid detergent lignin and cellulose changes as a function of internode development. [185] The results indicated that the content of acid detergent lignin is a steady decrease from 6.5% in internode 1 to 12.9% in internode 6 below peduncle with an increase of acid detergent cellulose from 37.0% of the dry weight in internode 1 to 431.0% of the dry weight in internode 6 along the tiller internode 1-6. [185] The 4-coumarate and ferulate are incorporated into cell-walls with significant amounts (28.2 mg/g) in the internodes close to the peduncle and the levels of them decreased with increasing lignification of the internodes (26.5 mg/g). [185] Moore et al. [186] suggested dividing the developmental stem of switchgrass into six elongation stages (E1, E2, E3, E4, E5, and E6) and three reproductive stages (R1, R2, and R3). According to this statement, Shen et al. [146] studied the lignin structure in E2, E4, and R1 stages of switchgrass. Their results indicate that the top part of the stem at E2 stage of switchgrass is more lignified significantly and has greater content of acetyl bromide lignin (~2.1 x 10^2 mg/g) compared to the bottom part of the stem (~1.6 x 10^2 mg/g). [146] The top part of the stem at the E2 stage exhibits a higher ester-linked *p*-CA content (~9.0 mg/g) than the bottom part of the stem (~6.0 mg/g), but the FA content is similar to that in the bottom part, resulting in a decrease in *p*-CA/FA ratio from the top (5.5) to the bottom (2.5) of the stem. [146] The S:G ratios of lignin are lower in the top section (0.85-0.90) than in the bottom (0.90-0.97) at both E2 and E4 stages.

Several studies have also investigated the relationships between lignin features of switchgrass and saccharification efficiency. [18, 146]

The saccharification efficiency of selected switchgrass populations has shown a negative correlation between the dry matter yield and the fiber detergent lignin content. [18]

The reduction (2.89% on average) of ester-linked phenolics by esterase improves ~67% dry weight loss after cellulase hydrolysis. [18] The lignin content and composition of switchgrass varies significantly depending on ecotype, developmental stage, and environmental factors. Although several reports on genetic variability, trait relationships, and biomass production in switchgrass are now available, there is still limited information on cell wall structure and its effects on biomass saccharification efficiency.

To assess the impact of maturity on biomass saccharification of switchgrass, lignin content, S:G ratio, and wall-bound phenolics have been investigated for the relationships between the saccharification and cell wall properties. Shen et al. [146] stated that the maturity stages of the stem inversely correlated with enzymatic hydrolysis efficiency. Through measuring anatomical, biochemical, and genetic features of switchgrass they suggested the impact of the cell wall recalcitrance to the saccharification efficiency may negatively correlate the lignin content, the amount of S and G lignin monomer, whereas positively correlate the content of ester-linked FA. [146] This gives indirect measurement of the changes of cell wall components related to the maturity of the cell wall in terms of recalcitrance to the saccharification efficiency of switchgrass. [146] Although S:G ratio is a good indicator for the cell wall maturity in this study, the results don't show a correlation to the saccharification efficiency of switchgrass sample. [146] These studies could give an initial suggest that the features of cell wall in switchgrass relate to the saccharification efficiency. Although the fundamental science of this character has not been studied for the lignin and lignin structure in switchgrass, Liu et al. [187] study the inhibition of enzymatic hydrolysis by unbound lignin and demonstrates a 15% reduction for the enzymatic digestibility of cellulose in the present of 0.1 g/L sulfonated lignin. They suggest that this is due to nonproductive adsorption of enzymes onto lignin. Formation of lignin-metal complex in the present of Cu (II) and Fe (III) could reduce the negative effect of lignin on the enzymatic digestibility of cellulose. [187]

The Correlation of S:G Ratio to the Digestibility of Biomass

The S:G ratio of biomass has been suggested to be a factor related to the sugar release of pretreated biomass, as well as the digestibility of the cell wall. Davison et al. studied the impact of the S:G ratio (1.8-2.3) and lignin content (22.7-25.8%) on the release of xylose after a dilute acid pretreatment of 8-year-old poplar wood. [188] These results indicate that poplar sample with 22.7% lignin content and 1.8 S:G ratio correlate significantly with the amount of xylose released. Gorshkova et al. reported that the S:G ratio is an indicator for the morphological portions of flax (*Linum usitatissimum* L.) stem tissues. [189] The results of that study indicate that the fiber-rich portion (S:G ratio, 2.5) has much greater S:G ratio than xylem (S:G ratio, 0.71). Other studies on the digestibility of grass indicate that there is a correlation between the S:G ratio and the digestibility of grass. Chen et al. [190] studied the lignification of tall fescue and demonstrated that the digestibility of the cell wall correlates with the S:G ratio (0.56-0.98), which has a higher value in the mature cell wall (0.98). The lower the S:G ratio and lignin content of a cell wall, the easier is digested. Gautam et al. [185] also found similar results in a study of internode structure and cell wall composition in maturing tillers of switchgrass. These studies indicate that the anatomical and physiological variations are related to maturity in the internodes of flowering tillers of switchgrass.

CHAPTER 3

EXPERIMENTAL MATERIALS AND PROCEDURES

Materials

Chemicals

All chemicals were purchased from VWR (Atlanta, GA) and used as received. Cellulase (EC 3.2.1.4. from *Trichoderma reesei*) and cellobiase (Novozyme 188 from *Aspergillus niger*) aqueous solutions were purchased from Sigma-Aldrich. Direct Blue-1 and Direct Orange-15 were purchased from Pylam products company, Inc. (Tempe, AZ 85281). Direct Blue-1 is a low molecular chemical with a well defined chemical structure (Figure 3.1). It has a molecular weight of 992.82 g/mol and a molecular area of 3.6 nm^2 (or a diameter of 1 nm). [191] Direct Orange-15 is a condensation product of 5-nitro-o-toluenesulfonic acid in aqueous alkali solution. [191] It forms an extended polymer with less defined chemical formular and structure as shown in Figure 3.1. The purified Direct Orange-15 has a molecular diameter in the range of 5-36 nm.

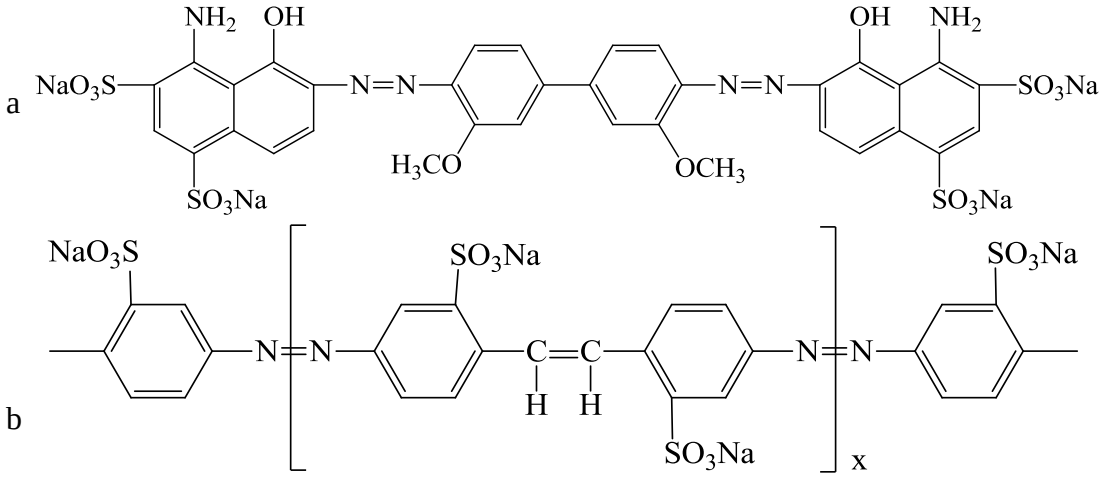

Figure 3.1 Chemical Structures of Direct Blue-1 (a) and Direct Orange-15 (b) [191]

Switchgrass Samples

This thesis study employed two sets of switchgrass samples, harvested and received from different locations and at different growth stages. Four sample populations of switchgrass—Alamo, Kanlow, GA992, and GA993—were harvested from the University of Georgia farm in August, 2008. Alamo switchgrass samples were harvested from a farm at the University of Tennessee in September, 2009. The switchgrass samples used in each chapter were summarized in Table 3.1.

Table 3.1 Summary of the Switchgrass Samples in Each Chapter

chapter\sample	switchgrass sample name	switchgrass sample code
chapter 4, 6	R3 Alamo	SW1
	R6 Alamo	SW2
	R3 Kanlow	SW3
	R6 Kanlow	SW4
	R3 GA993	SW5
	R6 GA993	SW6
	R3 GA992	SW7
	R6 GA992	SW8
chapter 5,7	Alamo	SW9

Switchgrass Samples SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8

Four populations of switchgrass—Alamo, Kanlow, GA992, and GA993—were seeded in 2000 at the University of Georgia Plant Sciences Farm near Watkinsville, GA ($33^{o}52'N$; $83^{o}32'W$) in coarse sandy loam (fine, kaolinitic, thermic typic kanhapludults). SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8 (Table 3.1) represent the switchgrass samples from two replications (R3 and R6) of these four populations of switchgrass. They were harvested and received in August, 2008. Once harvested, the switchgrass samples were air-dried until the moisture content was less than 10% of the dry weight. The leaf (including blade and sheath), stem (or internode), and node portions of the switchgrass were manually separated and ground in a Wiley mill until they passed through a 5 mm screen. Samples were then dried in a vacuum desiccator over phosphorus pentoxide for three days, which resulted in a final moisture content of 5%.

Switchgrass Samples SW9

The Department of Plant Sciences at the University of Tennessee harvested one Alamo switchgrass sample, SW9, at 4 cm above the ground in September, 2009. Once harvested, the switchgrass samples were air-dried, yielding a moisture content of ~15%. Four morphological portions of switchgrass, including a leaf portion (including blade and sheath), an internode portion, a node portion, and a seedhead portion, were manually separated and ground in a Wiley mill until they passed through a 0.841 mm screen. The leaf and internode portions of the switchgrass were then additionally sorted into three groups based on particle size: <0.297 mm, 0.297-0.707 mm, and >0.707 mm.

Biomass Constituents

Ash and Acid-Insoluble Ash Content Analyses

The ash content of the native and extracted switchgrass samples was analyzed according to TAPPI procedure T211 om-85. [192] In brief, an oven-dried switchgrass sample (0.5-1 g) was charred in a furnace heated slowly to 525 ^{o}C and held at this temperature for 8 h. After cooling to room temperature, the residue was weighed to determine the ash content of the switchgrass sample. The Acid-Insoluble Ash content was measured according to the TAPPI method T244. [193] In brief, ~50-100 mg of ash residue was treated with 6 M HCl (5.0 mL) on a heating plate until dry. This process was then repeated. An aliquot of a 6 M HCl solution (5 mL) and DI water (20 mL) were added to the dry residue, and the mixture was filtrated with Whatman® 42 filter paper. The Acid-Insoluble Ash content was determined gravimetrically after combustion at 525 ^{o}C for 8 h. Standard deviations were ≤0.5% for both ash and Acid-Insoluble Ash contents.

Trace Inorganic Elements Analyses

An Inductively Coupled Plasma (ICP) analysis was performed on the leaf and internode portions of SW9. This analysis measured trace inorganic elements using a

Perkin Elmer Optima 3000 DV Emission Spectrometer. [65] A total halogen analysis was performed by Huffman Laboratories, Inc. (Golden, CO). The standard deviation was ≤22 (mg/kg biomass) for halogen content and ≤5.0 x 10^3 (mg/kg biomass) for ash and insoluble ash content. The standard deviation for the analysis of trace inorganic elements in switchgrass was ≤5%.

Higher Heating Value of Combustion

The Higher Heating Value (HHV) of the leaf and internode portions of SW9 was measured using combustion. This process was conducted in an adiabatic oxygen bomb calorimeter according to TAPPI method T 684 om-06. [194] The standard deviation for the HHV was 0.2 MJ/kg.

Syringyl:Guaiacyl Ratios Analysis

The ground leaf, internode, and node portions of switchgrass samples SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8 were analyzed at the National Renewable Energy Laboratory (Golden, CO) to determine their syringyl:guaiacyl ratios. This analysis was performed using Pyrolysis Molecular Beam Mass Spectrometry (Py-MBMS). [195, 196] The Py-MBMS analysis employed a quartz tube pyrolysis furnace (2.5 cm inside diameter) coupled with a custom-built Extrel Model TQMS C50 molecular beam mass spectrometer for the pyrolysis vapor analysis. The ground samples (~20 mg) were pyrolyzed at 500 ^oC and injected into a 5 L/min helium stream that flowed into the mass spectrometer. A molecular beam was created during both the first vacuum stage of 10^{-3} mm of mercury and the second vacuum stage of 10^{-5} mm of mercury. These beams were then collimated using a slit in a quadruple mass spectrometer. The concentrated molecular beam then intercepted a low-energy electron beam (22.5 eV) in a quadruple mass spectrometer, yielding a positive ion mass spectrum. The mass spectra were then averaged and the background was removed using a Merlin automation data system

version 2.0. A typical Py-MBMS spectrum can be found in Agblevor's article. [197] The syringyl:guaiacyl (S:G) ratio was estimated by the sum of the syringyl peak intensities (154, 167, 168, 182, 194, 208, 210) divided by the sum of the guaiacyl peak intensities (124, 137, 138, 150, 164, 178). [196] The mass peak assignment associated with Py-MBMS spectrometry in the present study is summarized in Table 3.2. Error analysis is given in the error analysis section.

Table 3.2 Mass Spectrum Peak Assignments Associated with Py-MBMS for Switchgrass. [195] Abbreviation: m/z= Mass: Charge Ratio of Fragments Extracted. Major Lignin Peak Assignments: Syringyl (S) and Guaiacyl (G).

m/z	Mass Spectrum Peak Assignment	Lignin assignment
124	Guaiacol	G
137[a]	Ethylguaiacol, Homovanillin, Coniferyl alcohol	G
138	Methylguaiacol	G
150	Vinylguaiacol	G
152	4-ethylguaiacol, Vanillin	G
154	Syringol	S
164	Allyl-+propenyl guaiacol	G
167[a]	Ethylsyringol, Syringylacetone, Propiosyringone	S
168	4-Methyl-2, 6-dimethoxyphenol	S
178	Coniferyl aldehyde	G
180	Coniferyl alcohol, Syringylethene	S,G
182	Syringaldehyde	S
194	4-Propenylsyringol	S
208	Sinapyl aldehyde	S
210	Sinapyl alcohol	S

a. Fragment ion

Extraction Procedures for Morphological Portions of Switchgrass

Four morphological portions—leaf, internode, node, and seadhead—of the switchgrass samples, SW1-SW8, and SW9 (Table 3.1), were Soxhlet extracted. This procedure was performed using water as the solvent followed by extraction with a benzene/ethanol solution (2:1, v/v) for 8 h each at 6-10 cycles/h. The content of extractives was determined gravimetrically using standard methods described by Hallac et al. [65] The extracted biomass was air-dried for 1 day to yield a final moisture content of ~10%. The material was then dried in a vacuum oven at 40 ^{o}C overnight to yield a final

moisture content of 5%. The standard deviation for the extractives content was typically ≤1%.

Extractives Analysis of Hot-water and Benzene/ethanol Extractions

The extractives of the leaf and internode portions of SW9 were analyzed using gas chromatography-mass spectrometry. The supernatant solution was collected and concentrated at 40 ^{o}C until it reached a volume of ~30 mL. The condensed extractives solution was then diluted to a volume of 100 mL. A sample of the aqueous solution (approximately 0.3 g) was decanted into an 8 x 40 mm auto-sample vial and dried in a CentriVap concentrator equipped with a CentriVap Cold Trap (Labconco®). A sample of the benzene/ethanol solution (1 mL) was evaporated to the dryness under a nitrogen stream at room temperature for 30 min, or until dry. Heptadecanoic acid, was used as an internal standard (1 mL, 4 mg/mL in methanol), and was added to each sample vial. The mixture was evaporated under a nitrogen stream until dry. N-Methyl-N-tert-butyldimethylsilyl trifluoroacetamide (50 μL) was added as a derivative agent. This mixture (1 μL) was injected into the GC-MS system as previously described. The column used was a 6000 mm x 0.251 mm i.d., 0.25 μm, DB-5MS. The column temperature was then ramped at 20 °C/min until it reached a final temperature of 280 °C. The temperature was held constant at 280 °C for approximately 33 min. The injection temperature was 250 °C. The total ion peak area was used to quantify the individual compounds. The response factor for each individual compound was assumed as to be 1 for the purpose of the calculations. The standard deviation for the determination of the extractives compound was typically ≤5%.

Carbohydrates and Lignin Content Analyses

Morphological fractions of the switchgrass samples (160-170 mg, OD) were hydrolyzed with a 72% H_2SO_4 solution (1.5 mL) for 1 h at 30 ^{o}C. The hydrolysates were

diluted with deionized (DI) water to 4% H_2SO_4 and a second hydrolysis was carried out in an autoclave at 121 ^oC setting for 1 h. The supernatant liquid was cooled to room temperature and filtered through a porcelain crucible, and the residue was used to determine the Klason lignin content. The acid-soluble lignin content was determined using UV absorbance of the filtrate at 205 nm. [198] Hence, the total lignin content reported for each sample was the sum of the Klason and acid-soluble lignin contents. The filtrate was analyzed using Dionex chromatography, a type of high-performance anion-exchange chromatography that uses pulsed amperometric detection (HPAEC-PAD) to perform monosaccharide analysis. [199] The standard deviation for the sugars and lignin content was ≤1.8% for the leaf and internode portions of switchgrass, SW9. The standard derivations for the arabinan, galactan, glucan, xylan, Klason lignin (KL), and acid insoluble lignin (AIL) content of the leaf and internode portions of pretreated SW9 were 0.2%, 0.1%, 2.0%, 1.3%, 0.9%, and 0.1%, respectively.

Biomass Characterization of Cellulose and Lignin

Holocellulose Preparations for Leaf and Internode Portions of Switchgrass

The holocellulose portion in the leaf and internode portions of the SW1 and SW9 samples were prepared by holocellulose pulping the milled switchgrass samples (0.297 mm-0.707 mm diameter) according to the literature procedures described by Hallac et al. [65] and Hubbell et al. [81] In brief, 200 mg of the leaf and internode portions of the native and pretreated switchgrass samples, SW1 and SW9 were treated with ~4 mL of DI water, 100 mg of sodium chlorite (80%), and ~100 μL of acetic acid. This was done in a sealed glass bottle at 70 ^oC for 2 h, with three repeat oxidative treatments to reduce the Klason lignin content to about 1-2%. [65, 81] The residue was filtered after holopulping, washed with DI water, and dried.

α-Cellulose Preparation Procedure for Leaf and Internode Portions of Switchgrass

α-Cellulose preparation was carried out using an alkaline extraction of the holocellulose from both the leaf and internodes portions of the samples SW1 and SW9. This extraction was performed according to the literature procedure. [65, 81] The oven-dried holocellulose (~50 mg) was added to a sodium hydroxide solution (17.5%, ~4 mL), and left to soak at room temperature for 30 min. Then, DI water (~4 mL) was added to treat the samples for another 30 min. The residue was filtered, neutralized with acetic acid (1 M) for 5 min, and washed with DI water to yield the purified α-cellulose.

Tricarbanylation of α-Cellulose Procedure

The obtained α-cellulose (15 mg) was dried in a vacuum at 40 ^{o}C for 24 h and then treated with anhydrous pyridine (~4 mL) and phenyl isocyanate (500 μL) at 70 ^{o}C for 48 h. The reaction was then quenched with methanol. The derivative cellulose was precipitated in a methanol/water solution (7/3, v/v, ~100 mL). The precipitate was subsequently filtered through a membrane filter (pore size 0.45 mm), and washed first with a methanol/water solution (7/3, v/v, ~30 mL, 3 times) and then with DI water (~30 mL, 3 times). The α-cellulose tricarbanilates were finally air-dried for 24 h and dried in a vacuum oven at 40 ^{o}C for 24 h.

Cellulose Preparation Procedure for Extracted and Pretreated Leaf and Internode Portions of Switchgrass

The cellulose used for the structural characterization of CP/MAS ^{13}C-NMR was isolated from extracted (benzene/ethanol and hot-water) and pretreated leaf and internode portions of switchgrass sample SW9. This analysis was performed by refluxing a holocellulose sample (0.5 g of dry weight) in a 2.5 M HCl solution (~50 mL) for 4 h. The solid residue was filtrated, washed with DI water, and air-dried.

Procedure for the Degree of Polymerization of α-Cellulose

The Degree of Polymerization (DP) of α-cellulose was determined when Gel Permeation Chromatography (GPC) was performed on α-cellulose tricarbanilates. [65] The molecular weight of the α-cellulose tricarbanylates extracted from the leaf and internode portions of SW1 and SW9 was determined following a published procedure. [81] In brief, the prepared α-cellulose tricarbanilates were dissolved in tetrahydrofuran (1 mg/ml), filtered through a 0.45 μm filter, and injected in a solution form (20 μL) into a GPC system for molecular weight analysis. The system used was SECurity Agilent HPLC 1200 (a PSS-Polymer Standards Service, Warwick, RI, USA). Four 300 mm x 7.8 mm i.d. Waters Styragel columns were used (HR1, HR2, HR4, and HR6). An Agilent UV detector was used at 270 nm. Tetrahydrofuran was used as the mobile phase (1 mL/min). The data was collected and processed using WinGPC Unity software (Build 6807). Molecular weight values (M_n and M_w) were determined using a calibration curve based on six narrow polystyrene standards ranging in molecular weight from 1.5 x 10^3 to 3.6 x 10^6 g/mol. The weight-average Degree of Polymerization (DP_w) was calculated by dividing the weight-average molecular weight of α-cellulose tricarbanilates (M_w) by 519. This measurement was repeated three times per sample, and the standard deviation was calculated using this data. The standard deviations of the cellulose from the leaf and internode portions samples SW2 were 2.26 x 10^4 g/mol for M_n; 2.83 x 10^4 g/mol for M_w; 57 for DP_w; 0.06 x 10^{-2} for F_{RE}%, and 1.0 for PDI. The standard deviations of the cellulose from the leaf and internode portions of switchgrass sample SW9 were 0.11 x 10^4 g/mol for M_n; 1.20 x 10^4 g/mol for M_w; 21 for DP_w; and 0.3 for PDI.

Procedures for the Structural Analysis of Cellulose Using the Cross Polarization Magic Angle Spinning ^{13}C-NMR technique

<u>General Procedure for CP/MAS ^{13}C-NMR Measurement</u>

The ultrastructure of cellulose in the native and pretreated leaf and internode portions of SW9 was determined using a CP/MAS ^{13}C-NMR experiment and the spectral line analysis was described by Foston et al. [200] NUTS software (Acorn NMR, Inc.) was used for processing the line fitting of the C-4 region of the cellulose spectra (δ 79-92 ppm). [102] The crystallinity (%Cr1) was determined by integrating the percentage of the crystalline region (δ 86-92 ppm) into the C-4 region of the cellulose spectra (δ 79-92 ppm). [102] The standard deviation associated with this measurement was ≤2.7%.

Line-fitting Procedure for the C-4 Region of the Cellulose Spectrum

Spectra for cellulose I_α, cellulose I_β, cellulose $I_{\alpha+\beta}$, accessible fibril surface-1, and accessible fibril surface-2 were obtained by ^{13}C-NMR contact polarization magic angle spinning (CP/MAS) experiment. Analysis was done fitting the peaks in the C-4 region. An adjustment for chemical shifts using the Full Width at Half-Height (FWHH, Hz), and the intensity is shown in Table 3.3. For the inaccessible fibril surface, the FWHH used was 400 Hz. The peak intensities of the paracrystalline cellulose and the inaccessible fibril surfaces were based on the maximum fitting intensity. The FWHH of the paracrystalline cellulose was adjusted according to the final adjusted FWHH and the intensity of cellulose I_α, cellulose I_β, and cellulose $I_{\alpha+\beta}$. During the fitting process, only the intensity values for cellulose I_α, cellulose I_β, cellulose $I_{\alpha+\beta}$, accessible fibril surface-1 and accessible fibril surface-2 were adjusted.

The relative area values and parameters for all peaks that fit the C-4 region of the CP/MAS cellulose spectra were recorded individually. The Crystallinity Index (%Cr1) was determined by the ratio of the integrating of the crystalline region (δ 86-92 ppm) to the C-4 region in the cellulose spectra (δ 79-92 ppm). [118] Multiple comparisons for native and pretreated cellulose from the SW9 sample were performed using an analysis of variance (ANVOA), assuming samples as fixed effects and replicates as random effects.

A Least Significant Difference (LSD) was obtained using a 95% significant difference ($P<0.05$) among the native and pretreated switchgrass.

Table 3.3 Initial Parameters for Processing Line Fitting at C-4 Region of a CP/MAS Spectrum

assignments	chemical shift, ppm	FWHH, Hz	intensity
cellulose I_{α}	89.7	90	-
cellulose $I_{\alpha+\beta}$	89.0	91	5
paracrystalline	88.8	-	7
cellulose I_{β}	88.1	135	5
accessible fibril surface-1	84.5	100	-
inaccessible fibril surface	84.4	400	4
accessible fibril surface-2	83.6	95	4

Isolation Procedure of Lignin from Leaf and Internode Portions of Switchgrass

The isolation of lignin from the leaf and internode portions of SW9 was accomplished using a standard procedure with minor modification. In brief, extracted switchgrass (20 g o.d. leaf (<0.3 mm) and internode (<0.3 mm)) samples were dried in a vacuum at 40 ^{o}C for 24 h and milled in a 4 L porcelain jar containing 1.0 x 10^3 g of porcelain balls under A nitrogen atmospheres. The ball-milled switchgrass powder was dried in a vacuum oven at 40 ^{o}C for 24 h and extracted using a *p*-dioxane/water solution (96%, v/v, and 200 mL/20 g milled powder) for 24 h. This process was then repeated twice. The suspended *p*-dioxane/water extract was collected after 10 min of centrifugation in an 1156g relative centrifuge field (RCF). The extracts were freeze-dried to yield a crude lignin sample, which was then dissolved in an acetic acid/water solution (9/1, v/v, 20 mL/g lignin), centrifuged, precipitated into water, and recovered after 10 min of centrifugation at 1156g RCF. The lignin was washed with water (200 mL x 2), freeze dried, and then vacuum-dried at 40 ^{o}C for 24 h. This material was then dissolved in dichloroethane/ethanol (2/1, v/v and 10 mL/g lignin), centrifuged to remove insolubles, and precipitated with the addition of diethyl ether (200 mL/20 mL solution The precipitate was isolated using centrifugation and washed first with diethyl ether followed

by a wash with petroleum ether. The purified lignin was re-dissolved in an aqueous *p*-dioxane solution (50%, v/v), and freeze dried to produce the final lignin sample.

Structural Characterization of Lignin

Structural analysis of lignin was carried out using quantitative ^{13}C NMR analysis on a 400 MHz Bruker Avance/DMX NMR spectrometer using DMSO-d_6 as a solvent. The data were acquired at 50 ^{o}C using a 90^{o} pulse, 11 second pulse delay, and 10240 scans. Manual phasing and baseline correction were performed on each spectrum, along with a chemical shift calibration that used the DMSO-D_6 signal (δ 39.5 ppm) as a reference. Typically, the standard deviation for the quantitative ^{13}C-NMR analysis was $\leq$3% of the integrated values.

Hydrothermal Pretreatment

Hydrothermal Pretreatment of Leaf and Internode Portions of Switchgrass

Hydrothermal pretreatment of the native leaf and internode portions of samples SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8 were carried out in a 300 mL Parr reactor with a 4842 temperature controller and a PTFE linear (Parr series 4560, Parr Instrument Company, Moline, IL, USA). Hydrothermal pretreatment of the extracted leaf and internode portions of sample SW9 was conducted in a 300 mL bench-top Parr reactor with a 4842 temperature controller equipped with a glass liner and a cooling loop (Parr series 4560, Parr Instrument Company, Moline, IL, USA). Typically, the switchgrass samples (10 g, oven-dried) were soaked in DI water (90 mL) for 1 h. The soaked switchgrass solution was directly loaded into the Parr reactor. The solution was hydrothermally pretreated for 10 min under N_2 [25] at a maximum temperature of 200 $\pm$ 2 ^{o}C and a maximum pressure of 1.45 MPa, using a ramp temperature of 3.5 $\pm$ 0.5 ^{o}C/min. The Parr reactor was then immersed in ice water to stop the reaction. The pretreated material was filtered with Whatman ® 1 qualitative grad filter paper. The biomass

residue was washed with 1.0 x 10^3 mL of hot water (~80 ^oC) and air-dried prior to chemical analysis. The soluble lignin content of the filtrates was estimated using UV spectrophotometry at 205 nm with 110 L g^{-1} cm^{-1} as the absorptivity. [198] The filtrates were measured for pH at the beginning and end of the hydrothermal pretreatment. The biomass yield from the pretreatment was measured as a dry mass percentage of the solid residues to the original switchgrass sample. The standard deviation for the biomass yield from the hydrothermal pretreatment of the leaf and internode portions of SW9 was 0.5%.

FT-IR Analysis

Vacuum-dried leaf and internode portions of native and pretreated SW2 (~4 mg) were mixed with dry Potassium bromide (400 mg) and compacted into pellets. These pellets were analyzed using a transmittance Fourier transform infrared (FT-IR) spectroscopy (Nicolet Magna-IR spectrometer 550). All spectra were recorded between 4000 and 400 cm^{-1} using 128 scans at a resolution of 2 cm^{-1}. The ratio of amorphous cellulose to crystalline cellulose was estimated from the intensity of the amorphous cellulose peak to 900 cm^{-1} and the crystalline cellulose peak at 1098 cm^{-1}. [17]

Enzymatic Hydrolysis

Experimental Procedure for Enzymatic Hydrolysis of Pretreated Leaf and Internode Portions of Switchgrass

A mixed-enzyme system including cellulase (EC 3.2.1.4. from *Trichoderma reesei*, 957 EGU's/ml) and cellobiase (Novozyme 188 from *Aspergillus niger*, 307 EGU's /ml) was used to determine the digestibility of pretreated leaf and internode portions of samples, SW1-SW8. The enzymatic hydrolysis conditions were as follows: 2 g of pretreated switchgrass (OD) was treated with cellulase (at a loading of 49 FPU /g cellulose) and Novozyme 188 (at a loading of 40 IU/g cellulose) in a 100 mL acetate buffer solution (0.1 M, pH 4.8) at 50 ^oC for 48 h. The enzymatic hydrolysis conditions

used for the leaf and internode portions of sample SW9 were as follows: 2 g of pretreated biomass (O.D. air-dried) was treated with cellulose (at a loading of 80 U/g cellulose) and Novozyme 188 (at a loading of 40 U/g cellulose) in a 100 mL acetate buffer solution (0.1 M, pH 4.8) at 50 ^{o}C for 48 h. Other sample without air-dry was hydrolyzed by the same dosage of cellulase and cellobiase with various time, 0.5, 1, 2, 4, 8, 16, 32, 48, and 63 h. After this enzymatic treatment, the residue was filtrated through Whatman® 1 qualitative grade filter paper, washed with DI water, and air-dried. Digestibility was calculated as the dry-mass percentage of the weight lost to the glucan in the pretreated biomass (Equation 3.1). The standard deviation of the enzymatic hydrolysis yield of the biomass was 1.9% for SW9 leaves and 3.5% for SW9 internodes.

$$\text{Digestibility}\% = \frac{(Dry\ \ weight\ \ loss\ \ of\ \ enzymatic\ \ hydrolysis)\times 100\%}{(Dry\ \ weight\ \ of\ \ pretreated\ \ switchgrass\times glucan\%)} \quad \text{Equation 3.1}$$

Experimental Procedure for the Glucose Analysis of the Filtrate in the Enzymatic Hydrolysis of Pretreated Switchgrass

The glucose content of the filtrate of the enzymatic hydrolysis solution was measured using High-Performance Liquid Chromatography (HPLC). The gravimetric yield was based on the glucan content of the pretreated leaf and internode portions of switchgrass samples, SW1-SW8 and SW9. The glucose content in the aqueous solution of the enzymatic filtrate was measured using an Agilent 1200 HPLC series system, equipped with an Aminex ® HPX-42C column (300 mm x 7.8 mm) and a refractive index detector (RID). Samples (10 μL) were filtrated using a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter and eluted at 0.6 mL/min with nitric acid (10 mM). The temperatures used for the column and the RID heater were 65 and 45 ^{o}C, respectively.

The adsorbance of Direct Blue-1 and Direct Orange-15 on the Native and Pretreated Switchgrass

A modified Simons Stain's method was used to measure the adsorbance of Direct Blue-1 (DB) and Direct Orange-15 (DO) on the native and pretreated switchgrass SW9 (air-dried) according to the method described by Chandra et al. [201] DB-1 and DO-15 were prepared for a 10 mg/mL solution. DO-15 solution was fractionated using an Amicon ultrafiltration apparatus (Amicon, Beverly, MA) under a 28 psi pressure of nitrogen gas with a constant stir. The remaining solution, 20% of original volume, was collected for further preparation to obtain a 10 mg/mL solution. The extinction coefficients were obtained for DB-1 and fractionated DO-15 through a Shimazu UV-160A spectrophotometer. These values calculated in this study were $\varepsilon_{o/455}$=36.2, $\varepsilon_{B/455}$=2.72, $\varepsilon_{o/624}$=0.186, $\varepsilon_{B/624}$=14.5 $g^{-1}cm^{-1}$. A phosphate-buffered saline solution (PBS) was prepared in this experiment with sodium phosphate (monobasic) (0.3 M), sodium phosphate (dibasic) (0.3 M), and sodium chloride (1.4 mM). The adjustment of pH was applied with an HCl solution (0.1N) to obtain pH 6. The Simons' Stain solutions were prepared in a 10 mL volumetric flask. The fractionated DO-15 solution (10 mg/mL) and DB-1 solution (10 mg/mL) were added in a series of increasing volumes (0.25, 0.50, 0.75, 1.0, 1.5, 2.0 mL) to the volumetric flask. Distilled water was added to obtain final volume of the solution 10 mL. Six switchgrass samples (100 mg) were weighed into 50 mL polypropylene tube and filled with each Simons' Stain solutions (10 mL). These prepared mixtures were incubated with a shaking frequency of 200 rpm at 70 ^{o}C for 6 h. A blank solution also prepared for this experiment to adjust the concentration after the reaction. After the reaction, the mixtures were centrifuged at 8000g relative centrifuge field (RCF) for 5 min. The obtained supernatant was used to measure the absorbance at 624 and 455 nm using the UV spectrophotometer. The concentrations of DB-1 and DO-15 after the reaction for each sample were based on the equations (Equation 3.2 and 3.3) according to the Beer-Lambert Law for a binary mixture. The amount of DB-1 and DO-15 adsorbed

onto the samples was determined using the difference between the adjusted initial concentration and the concentration in the supernatant. The maximum amount of DB-1 and DO-15 adsorbed for each samples was obtained using equation 3.4 by plot 1/ [A] with 1/ [C] at equilibrium.

$$A_{455nm} = \varepsilon_{o/455} L C_O + \varepsilon_{B/455} L C_B \quad \text{Equation 3.2}$$

$$A_{624nm} = \varepsilon_{o/624} L C_O + \varepsilon_{B/624} L C_B \quad \text{Equation 3.3}$$

Where, A_{455nm} and A_{624nm} are the absorbance at 455 and 624 nm, respectively. L is the pass length, 1 cm. $\varepsilon_{O/455}$ and $\varepsilon_{O/624}$ are the extinction coefficient of DO-15 at 455 and 624 nm. $\varepsilon_{B/455}$ and $\varepsilon_{B/624}$ are the extinction coefficient of DB-1 at 455 and 624 nm. C_o and C_B are the concentration of DB-1 and DO-15 in the solution.

$$[C]/[A] = 1/K_{Ads}[A]_{max} + ([C]/[A]_{max}) \quad \text{Equation 3.4}$$

Where [C] (mg/mL) is the free DB-1 or DO-15 concentration at equilibrium, [A] (mg DB-1 or DO-15 /mg substrate) is the amount of DB-1 or DO-15 adsorbed by the substrate, [A] max is the maximum amount of DB-1 or DO-15 adsorbed onto the sample (mg/g), K_{Ads} is the adsorption equilibrium constant. The R^2 values for the estimation of adsorbance in DO-15 are 0.91, 0.82, 0.98, and 0.95 for leaves, internodes, pretreated leaves and pretreated internodes. The R^2 values for the estimation of adsorbance in DB-1 are 0.88, 0.75, 0.89, and 0.60 for leaves, internodes, pretreated leaves, and pretreated internodes.

Data Analysis

Data Analysis for Chemical Profiles of Four Populations of Switchgrass

All data for the chemical profiles of the four populations of switchgrass, including samples SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8, were reported as mean

values from two replicates. Multiple comparisons were performed using an analysis of variance (ANOVA), which assumed entries as a fixed effects and replicates as random effects. A Least Significant Difference (LSD) was obtained ($P<0.05$) among the four populations of switchgrass and the three morphological portions.

Data Analysis for Hydrothermal Pretreatment of Four Populations of Switchgrass

All results for the hydrothermal pretreatment of the four populations of switchgrass, including samples SW1, SW2, SW3, SW4, SW5, SW6, SW7, and SW8, were reported as mean values from four replicates: Alamo, Kanlow, GA993, and GA992. A student t-test was performed, which assumed entries as fixed effects and replicates as random effects. The confident interval was obtained ($P<0.05$) between the two morphological portions. The data for the DP of α-cellulose and the carbohydrate profiles were measured using one sample. This sample was measured three times, and the standard deviation was included in the results.

CHAPTER 4

CHEMICAL PROFILES OF SWITCHGRASS SW1-SW8*

Introduction

In light of insufficient long-term supply of petroleum resources on earth, increased global population, and global climate change, society has begun to develop sustainable fuels, energy and chemicals using renewable bioresources. [9] The US federal government has proposed the "20 in 10" Plan, which would reduce gasoline consumption by 20% by the year 2017. [12] In addition, the Renewable Fuel Standard (RFS) has mandated the production of 79.5 billion liters of cellulosic bioethanol by the year 2022. [10] These future demands of cellulosic biofuels will rely on cellulosic bioresources such as forests, perennial grasses, wood and agricultural residuals. [15, 108, 202] A promising feedstock for these biofuel requirements is switchgrass which is a native warm-season, C4 perennial grass with a high production yield and a wide geographical adaption in Centre and North America. [46, 203]

One of the key technologies currently required in the production of cellulosic biofuels is pretreatment, which is needed so as to increase enzyme digestibility of biomass. Pretreatment technologies reduce recalcitrance by removing lignin, hemicelluloses, and lignin-carbohydrate complexes, as well as by modifying the crystallinity of cellulose and the morphology of the cell wall. [15] Understanding the

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physical and chemical properties of switchgrass is essential for optimizing pretreatment technologies for this bioresource. Previous studies on switchgrass included cell-wall chemical composition, [204] extractive analysis, [70] and digestibility. [18, 205] Wiselogel showed that extractives loss was the major change during storage of switchgrass. [205] The variation in major components, lignin, hemicellulose, and cellulose, as reported by Sladden was low among eight varieties of switchgrass from upland and lowland ecotypes of switchgrass. [204] In a biofuel trial in Iowa, no differences between the lowland switchgrass cultivars 'Alamo' and 'Kanlow' were observed for hemicellulose, cellulose, and lignin content, nitrogen, or ash concentration; harvest timing had a much larger effect on compositions than did genotype. [52] The recalcitrance to a saccharification process is a major obstacle for the conversion of lignocellulosic biomass to ethanol. The alteration of lignin content and lignin structure could improve saccharification efficiency of switchgrass. [18, 146, 183, 184] However, other factors, such as morphology of switchgrass, may also influence the recalcitrance of lignocellulosic biomass to the saccharification for ethanol production. To be able to understand the effect of this factor for the saccharification of switchgrass, in this chapter, chemical analysis studies were conducted for four populations of switchgrass (i.e., SW1-SW2, SW3-SW4, SW5-SW6, and SW7-SW8), which were partitioned into leaves, internodes, and nodes. The variations in carbohydrate compositions, lignin and extractives content, and the syringyl:guaiacyl ratio of switchgrass were determined. Their impacts on the conversion technologies for biofuels were discussed.

Results and Discussion

Biomass Raw Materials

Law et al. [62] reported that three morphological portions of switchgrass (Cave-in-Rock), leaves, seedhead, and stem (including leaves sheath), were characterized by

different chemical and physical properties. In their study, these morphological portions of switchgrass were compared for their chemical properties including lignin, holocellulose, hot-water solubility, benzene/ethanol solubility and fibrous qualities. In brief, the leaves were distinguished from the stems by significant differences in chemical characteristics, mechanical strength, modulus and percentages of the elongation of fibers, [206] though both fractions had similar fiber length and the percentages of fines. [62] In this chapter, four populations of switchgrass with two replicates—SW1-SW2, SW3-SW4, SW5-SW6, and SW7-SW8—were studied for their chemical and physical properties. The initial and ground portions of switchgrass include leaves, internodes, and nodes. Among the examined switchgrass SW1-SW8, the percentage dry mass of three portions of switchgrass and production yield is similar (Table 4.1). On average, these four populations of switchgrass contain 27.0% internodes, 3.7% nodes, and 69.3% leaves based on dry mass.

Table 4.1 Mass Percentages of Three Morphological Portions for Four Populations of Switchgrass SW1-SW8

populations	% internodes[a]	% nodes[a]	% leaves[a]	leaves/internode ratio[a]
SW1-2	26.8	3.7	69.5	2.7
SW3-4	25.9	3.2	71.0	2.7
SW5-6	27.9	4.2	67.9	2.4
SW7-8	27.4	3.6	68.9	2.5
mean	27.0	3.7	69.3	2.6
LSD (5%)	9.3	0.7	8.9	1.2

Note: a) All data were reported as a mean value from two replicates.

Extractives Content of Four Populations of Switchgrass

The quantities and composition of switchgrass extractives vary extensively depending upon the origin of samples, the process of their preparation, and the solvents used. [62, 66, 67] Bals et al. demonstrated that the extractive content in the whole plant of switchgrass depends on the harvest time and locations and showed a broad rang of the extractives content in water and ethanol extraction (15.0-26.0%). [207] Shen et al. reported that hot-water extractives content for several populations of upland switchgrass ranges

between 11.8% and 14.9%. [146] Yan et al. also reported that the 95% ethanol extractives content was similar for four populations of switchgrass (11-13%). [66] Notably, the extractive contents are substantially different than those reported in this chapter. The exact reason for the difference is not know. However, one of the probable explanations is that the materials used by these researchers are presumably the whole plant with different populations, harvest time, and locations. Low et al. reported that the content of extractives also varied in morphological portions for Cave-in-Rock switchgrass. [62] They reported that the extractives content of Cave-in-Rock switchgrass varied in the leaves (without sheath), stem (with sheath), and seedhead for hot-water and benzene/ethanol extractions (Table 4.2). [62] Compared to Law's results on the extractives content of Cave-in-Rock switchgrass, the leaf portions of the present switchgrass contain similar amounts of hot-water extractives (19.4% vs. 20.1) and lower 6% benzene/ethanol extractives content than Cave-in-Rock (10.2% vs. 4.2% on average). [62] The extractives content of the internode portions of the present switchgrass contains slightly greater hot-water extractives and benzene/ethanol extractives content than that of Cave-in-Rock switchgrass. The materials used by these reseachers have different plant components. In Low's study, Cave-in-Rock switchgrass consists of about 5% seedhead, 30% leaf (without sheath), and 65% stem (with sheath). [62] The proportions of leaf could have a significant influence on the extractives content of the whole plant because of the greater amount of extractives content in hot water extraction than other morphological fractions. [62]

The switchgrass samples were successively extracted with hot water followed by benzene/ethanol. The extractives content of each step was shown in Table 4.2. This data indicated that these samples had significant hot-water extractives with mass yields ranging from 17.0% to 20.8%. A subsequent benzene/ethanol extraction provided gravimetric yields from 2.6% to 12.3%. In general, the extractives content were similar among the whole-plant of switchgrass samples studied (Table 4.2). However, there was a

significant difference on extractives content among the three fractions of each switchgrass sample with the leaves containing the highest amount of extractives (Table 4.2). The average percentage of hot-water extraction for internodes was 15.9% and about 4.3% greater than that of nodes. The content of hot-water extractives from leaves has almost 3.5% greater than that from internodes and 7.9% greater than that from nodes. There is no significant difference in the content of benzene/ethanol extractives between internodes and nodes. The content of benzene/ethanol extractives in leaf portions is about 6% greater than that of other portions.

Table 4.2 Extractives Content of Three Morphological Portions for Four Populations of Switchgrass SW1-SW8 and Cave-in-Rock Switchgrass

morphological portions	extraction	SW1-2[a]%	SW3-4[a]%	SW5-6[a]%	SW7-8[a]%	%LSD(5%)	Cave-in-Rock[62]%
internodes	hot-water	16.0	17.0	14.9	15.7	3.8	12.4
	benzene/ethanol	5.3	3.8	4.3	5.4	3.4	1.7
nodes	hot-water	12.0	12.5	9.3	12.4	5.2	-
	benzene/ethanol	5.1	2.6	5.4	4.0	7.8	-
leaves	hot-water	19.7	18.2	20.8	18.8	3.1	20.1
	benzene/ethanol	12.3	10.2	8.7	9.9	7.9	4.2
whole plant	hot-water	18.4	17.7	18.6	17.7	2.7	-
	benzene/ethanol	10.2	8.4	9.7	8.5	6.5	-
mean values of three morphological portions for four populations of Switchgrass	extraction	internodes	nodes	leaves	-	%LSD(5%)	
	hot-water	15.9	11.5	19.4	-	2.7	-
	benzene/ethanol	4.7	4.3	11.2	-	2.5	-

Note: a) All data were reported as a mean value from two replicates.

Chemical Compositions of Switchgrass

Comprehensive understanding of the chemical compositions of switchgrass is an important issue for future utilization of switchgrass for biofuels production. For bioethanol production, the major portions of hexoses and pentoses are converted to ethanol. [2] The lignin portion may not be directly used in this process, however, studies suggest that lignin can be converted to other types of biofuels, known as bio-oil through pyrolysis [208] and transferred into biopower in power generation plant. [2]

Table 4.3 Chemical Compositions of Three Morphological Portions for Four Populations of Switchgrass SW1-SW8

populations[a]	arabinose%[b]	galactose%[b]	glucose%[b]	xylose%[b]	lignin%[b]	ash%[b]	total%[b]
SW1-2 (s)	2.1	0.6	43.7	22.8	18.5	2.1	89.9
SW3-4 (s)	2.3	0.6	43.7	24.2	19.1	2.5	92.4
SW5-6 (s)	2.2	0.7	46.1	24.5	20.0	1.6	95.1
SW7-8 (s)	2.3	0.7	43.8	24.6	19.9	1.5	92.8
LSD (5%)	0.4	0.1	4.5	3.5	1.0	0.4	-
SW1-2 (n)	3.2	0.9	35.7	23.7	22.2	2.3	88.0
SW3-4 (n)	3.5	1.0	35.6	24.4	22.6	2.5	89.6
SW5-6 (n)	3.3	0.9	40.1	26.8	22.7	1.8	95.6
SW7-8 (n)	3.5	0.9	37.9	26.0	23.7	1.8	93.8
LSD (5%)	0.7	0.2	5.9	4.2	0.6	0.4	-
SW1-2 (l)	4.6	1.5	37.2	23.2	22.3	4.6	93.4
SW3-4 (l)	3.8	1.5	35.2	22.6	23.0	4.6	90.7
SW5-6 (l)	4.4	1.6	34.3	20.8	23.7	4.6	89.4
SW7-8 (l)	4.6	1.6	35.8	22.4	23.3	4.4	92.1
LSD (5%)	0.6	0.2	1.64	2.4	1.1	0.4	-
SW1-2 (w)	3.8	1.2	38.8	23.1	21.2	3.8	91.9
SW3-4 (w)	3.4	1.3	37.4	23.1	22.6	4.0	91.8
SW5-6 (w)	3.7	1.3	37.8	22.1	22.4	3.6	90.9
SW7-8 (w)	3.9	1.3	38.0	23.1	22.0	3.5	91.8
LSD (5%)	0.4	0.2	1.5	2.2	0.5	0.4	-

Note: [a] s: internode portions; n: node portions; l: leaf portions; w: whole plant. [b] Based on O.D. weight of switchgrass; All data were reported as a mean value from two replicates.

The chemical composition for the nodes, leaves and internodes of switchgrass were analyzed for all switchgrass samples (Table 4.3). Statistically, there is no significant difference for carbohydrate content among the four populations of switchgrass. However, SW1-SW2 and SW3-SW4 contain about 1.5% greater of lignin content than SW5-SW6 and SW7-SW8 in internode portions. The internode and node portions of SW1-SW2 and SW3-SW4 contain 0.5-1% (25%-50% coefficient of variation) greater ash content than that of SW5-SW6 and SW7-SW8. The results also showed that three portions of the four switchgrass populations contained significantly different chemical composition (Table 4.4). For example, the internode portions contain greater amounts of glucose content (8.7% more) and less hemicellulose sugars content, such as arabinose (1.1% less), galactose (0.9% less), and xylose content (1.8% less), than that from the node and leaf portions of switchgrass. The average lignin content for the leaf portions of switchgrass has the highest lignin content (i.e., 3.4% more than that of internodes) (Table 4.4).

However, the leaf portions of switchgrass contain about 2.5% lower ash content on average than the internode and node portions of switchgrass.

Table 4.4 Comparison of Average Chemical Compositions between Three Morphological Portions of Switchgrass and Other Published Results

Sample	arabinose%	galactose%	glucose%	xylose%	lignin%	ash%
internodes[a]	2.2	0.7	44.3	24.0	19.6	1.9
nodes[a]	3.4	0.9	37.3	25.2	22.7	2.1
leaves[a]	4.4	1.6	35.6	22.3	23.0	4.6
LSD (5%)	0.6	0.1	3.6	2.6	1.5	0.8
whole-plant[a]	3.7	1.3	38.0	22.8	22.1	3.7
switchgrass[197]	3.2	1.1	34.3	20.9	17.5[b]	-
corn stove [15]	5.5	2.9	36.8	22.2	23.1	-
switchgrass[70]	3.6	2.1	34.8	23.4	21.4[b]	7.1
fescue[70]	3.0	1.1	39.8	23.2	18.1[b]	6.7

Note: [a] All data were reported as a mean value from two replicates; [b] Klason lignin content

Table 4.5 S:G ratio of Three Morphological Portions for Four Populations of Switchgrass SW1-SW8 from Py-MBMS Analysis

populations	S:G ratio				S:G ratio in whole plant[a,b]
	internodes[a]	nodes [a]	leaves [a]	5%LSD	
SW1-2	0.71	0.61	0.46	0.05	0.52
SW3-4	0.67	0.62	0.46	0.03	0.52
SW5-6	0.69	0.58	0.47	0.04	0.54
SW7-8	0.67	0.60	0.46	0.05	0.52
average	0.68	0.60	0.46	0.03	0.52

Note: [a] LSD (5%): 0.04. [b] All data were reported as a mean value from two replicates.

Table 4.4 compared the chemical compositions from various bioresources against the switchgrass samples in the present study. Compared to other published results for switchgrass, the whole plant of switchgrass contains similar amount of carbohydrates and lignin content, but 3% less ash content on average. [70] In comparison to other herbaceous feedstocks, our results are similar in chemical composition. [15, 70, 197]

Py-MBMS Analysis of Switchgrass

Molecular beam mass spectroscopy was conducted for all switchgrass samples as summarized in Table 4.5. The results indicate that S:G ratio for bulk switchgrass samples

is similar. However, the S:G ratio varies widely among the node, internode and leaf portions of switchgrass. The internode portions had the highest amount of S:G ratio (average 0.68), while the leaf portions contained the lowest amount of S:G ratio (average 0.46). The S:G ratio for the internode portions (average 0.68) was very close to the literature S:G ratio (0.70) for Miscanthus lignin analyzed by NMR and thioacidolysis. [59] The observed switchgrass values differ significantly from the typical S:G ratio found for poplar which typically ranges from 1.3-2.2. [209] Chang and Sarkanen [210] demonstrated that the greater the S:G ratio the faster the delignification rate for Kraft pulping of hardwoods. The S:G ratio had also been reported to be an indicator for the morphological portion of plant. [189] The results reported by Gorshkova et al. [189] indicated that the fiber-rich portion was characterized with an elevated S:G ratio. A recent publication by Davison et al. [188] documented that both the lignin content and the S:G ratio contributed to the release of xylose from acid pretreatment. Likewise, Corredor et al. [211] reported that forage sorghums with a low syringyl:guaiacyl ratio was more readily enzymatically hydrolyzed after an acidic pretreatment. The S:G values seen in Table 4.5 suggest a potential range of switchgrass reactivity during pretreatment and subsequent enzymatic deconstruction.

Conclusion

Anderson stated that the leaf portions tend to be digested more easily than the internode portions of grass with Depol 740 ferulic acid esterase and cellulose. [178] Chen et al. [190] studied the lignification of Tall fescue and demonstrated that the ruminal degradability of cell wall was correlated with S:G ratio, which had higher values in mature cell wall. Gautam et al. [185] also suggested that the chemical composition and anatomy property was related to the maturity of the internodes cell wall along the tillers of switchgrass. The studies indicated the anatomical and physiological variation in internodes of flowering tillers of switchgrass. The varying degrees of the digestibility of different morphological portions of grasses (leaves, stems, or internodes and nodes etc.)

were studied in several species. For example, Dien et al. [73] studied the acid pretreatment of switchgrass with specific maturity stages and morphological portions. The results demonstrated that different growth stages and morphological portions (i.e. leaves vs. stems) had different levels of susceptibility to cellulases after a dilute acid pretreatment. The results showed that the pre-boot and post-frost stages of switchgrass performance 19% greater glucose yield by cellulases after a 2% sulfuric acid pretreatment at 121 ^{o}C for 1 hour. Another study of leaves and stems (sheath, internodes, and nodes) examined the response to the acid pretreatment, subsequent saccharification and fermentation. [30] The results indicated that the leaf portion took on a more digestible form after a 1% sulfuric acid pretreatment at 121 ^{o}C in autoclave for 1 hour. [30] William et al. [178] studied the digestibility of various morphological portions of corn stover, including the leaf blade, leaf sheath, stem ring, stem pith, and corn kernel fiber. The highest dry matter loss was about 47% for the leaf sheath after 72 h hydrolysis by cellulases. These studies suggest that the morphological fractions are a factor to consider during enzymatic hydrolysis.

According to the results of the chemical and structural analysis in this study, the four populations of switchgrass characterized in this chapter have similar bulk chemical properties. The most significant differences among these switchgrass are the ash and lignin content. But these differences are about 0.5-1.5% among the population. SW1-SW2 contains the lowest lignin content. However, the chemical and structural results among the three portions of switchgrass—leaves, internodes, and nodes—are significantly different. In fact, the leaves contain the highest amount of arabinose, galactose, lignin, and ash content. In addition, the leaves also have the lowest S:G ratio and glucose content. The content of the lignin and glucose among the three portions of switchgrass differs by 3.4% and 8.7%, respectively. In this study, the switchgrass samples have an average 69.3% leaf portions which could provide an opportunity for a greater yield of bioethanol production. Future development of pretreatment technologies

to convert switchgrass into biofuels will benefit from being able to tailor process chemistries to the differences noted in this report.

Thesis research has proposed that morphological fractions as a factor to influence the utilization of the switchgrass for fuels, chemicals, and energy. From this initial study, the results have strengthened that morphological fractions of switchgrass have different properties in chemical profiles. These studies provide a general database on the variations of chemical profiles for morphological portions of switchgrass. But these results in this chapter fail to answer the following questions: (1) what do these differences in chemical compositions mean in terms of the utilization of switchgrass for biofuels, chemicals, and energy? (2) How the chemical structures in morphological fractions are related to the utilization of switchgrass?

To be able to answer the questions, research in chapter 5, however, are involved to understand the fundamental chemistry in these components in different morphological fractions so that the process can be under chemical and economical control. Comparative studies between the leaf and internode portions of switchgrass are performed by compositional analysis and structural determination.

CHAPTER 5

BIOMASS CHARACTERIZATION OF MORPHOLOGICAL PORTIONS OF SWITCHGRASS SW9*

Introduction

Switchgrass, a warm season perennial C-4 grass, has been intensively studied as a potential bioenergy crop in the United States for the past decade. [46] It is a desirable lignocellulosic feedstock for biofuel production because of several features, including a high production yield reported up to 14 tonne/acre, wide adaptation, positive environmental benefits, and a renewable root system. [46, 48] There are two distinct ecotypes of switchgrass with various populations including lowland varieties (e.g. Alamo and Kanlow) and upland varieties (e.g. Trailblazer, Blackwell, Cave-in-Rock, Pathfinder, and Caddo). [46] Morphologically, switchgrass includes a root system up to 3.5 m in length, stems made up of internodes, nodes, leaf sheaths up to 3 m height, leaves, and flowers. [212]

Alamo is a variant of lowland switchgrass that originated from Texas [46] with production yields of up to 14 tonne /acre and lower lignin content in comparison to other lowland types of switchgrass. [46, 213] To be able to understand the effect of morphological fraction on the production of biofuels for switchgrass, in this chapter, the chemical structure of morphological portions of Alamo switchgrass SW9 was determined by fractionating the plant into sections and studying the plant cell wall chemistry in detail.

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The chemical constituents of switchgrass have been reported to vary according to populations, growth stage, and morphological portions sampled. [2, 52, 185, 212] For instance, elemental analysis of Cave-in-Rock populations by Lemusa et al. [52] reported the lowest amount of Cl, Mg, K, and Na with other elements being comparable to those found for Alamo and Kanlow. Typically, switchgrass has three growth stages including vegetative, boot and heading stages. [145] Jung et al. stated that the chemical constituents of switchgrass varied among the harvest period and morphological portions (leaves, stems including internodes and leaf sheath). [145] A recent investigation by Sarath et al. also found variations in the chemical constituents, especially the lignin component, along the length of tillers of switchgrass. [185] Studies on four populations of switchgrass, Alamo, Kanlow, GA992 and GA993 (derived from Alamo and Kanlow), reported only a 2% variation on bulk lignin content. [213] On the other hand, the leaf, internode, and node portions of switchgrass were shown to vary with respect to the contents of carbohydrates, lignin, ash, and extractives as well as S:G ratio. [213] Extractives from switchgrass have been reported to consist of minerals, low molecular weight and oligomeric compounds, which were Soxhlet extracted from biomass using water and neutral organic solvents. [66, 67] Many studies have shown that the quantities and composition of switchgrass extractives vary extensively depending upon the origin of samples, the process of their preparations, and the solvents used. [62, 66, 67, 213] The content of extractives also shows to vary in morphological portions. [62, 213] Low et al. reported that the extractives content of Cave-in-Rock switchgrass varied in the leaves, stem, and seedhead for hot-water and benzene/ethanol extractions. [62] In chapter 4, it also observes the differences in the extractives content of hot water and benzene/ethanol in leaf, internode, and node portions of four populations of switchgrass. [213]

Potential applications of switchgrass have been documented in the literature, including pilot-scale co-firing with coal for biopower production, [214] syngas production, [215] and bioethanol production. [48] Future improvements in the applications of switchgrass

for energy, chemicals, and liquid fuels will require a detailed knowledge of its chemical and physical properties. [65] It has been amply reported that biomass provides a sustainable, environmentally friendly means to produce biopower. [56, 214] The combustion properties of biomass are significantly correlated to the C/H/O ratios of biomass. [56, 214] In addition, biopower generation from herbaceous plants, including switchgrass, is known to be influenced by the presence of alkali metals contributing to the potential generation of sulfates, silicate, chlorides, and hydroxides which can cause slogging and fouling problems during combustion. [56] Some of these process issues can be reduced by aqueous leaching of biomass to remove alkali metals from biomass. [56] But it may be also influenced by the different ash content in the morphological fractions.

Another promising utilization of lignocellulosic feedstocks is the production of bioethanol. Practical conversion of lignocellulosic biomass into bioethanol via the biological approach requires a pretreatment step to reduce the recalcitrance of biomass to aid enzymatic deconstruction of cellulose into glucose and subsequent fermentation to ethanol. [29] Recent studies on pretreatment and saccharification of lignocellulosics indicate that the Degree of Polymerization (DP) and ultrastructure of cellulose are among the important factors that influence efficient enzymatic deconstruction of cellulose. [102, 103, 175] For instance, Samuel et al. investigated the ultrastructure changes of cellulose after dilute acid pretreatment of switchgrass using ^{13}C CP/MAS NMR. [102] These results indicated that dilute acid pretreatment reduced the percentage of amorphous cellulose and increased the Crystallinity Index of cellulose. During organosolv pretreatment and enzymatic deconstruction of *Buddleja davidii*, Hallac et al. monitored changes in plant cell wall and noted significant changes in the structure of cellulose. [175] These results suggest that organosolv pretreatment improves the yield of enzymatic hydrolysis through removal of lignin and hemicellulose, and a reduction in the DP and crystallinity of cellulose. [175] The varying degrees of the digestibility of different morphological portions of grasses (leaves, stems, or internodes and nodes etc.) have been studied in several

species. According to these studies on morphological portions of biomass, the results suggest that morphological portions have different levels of digestibility after pretreatment. Stem portions are more difficult to degrade using cellulases than are other morphological portions.

In this chapter, comparative studies between the leaf and internode portions of switchgrass were performed by compositional analysis and structural determination. GC-MS, ICP, adiabatic oxygen bomb calorimeter, and HPAEC-PAD were employed to analyze the chemical properties of the fractionated switchgrass samples. Quantitative ^{13}C NMR and CP/MAS ^{13}C NMR techniques were employed to determine the structures of lignin and cellulose, respectively.

Results and Discussion

Chemical Compositions of Switchgrass

Biomass characterization of switchgrass is an important component in the efficient utilization and conversion of switchgrass into chemicals, fuels, and energy. Populations of switchgrass including Alamo were studied for the chemical constituents of their morphological portions. [52, 213] Previous characterization studies showed that 20 switchgrass populations were comparable in their bulk chemical constituents among lowland and upland switchgrass samples. [52] The average content of acid detergent lignin, cellulose, and hemicellulose was 6.3%, 37.1%, and 32.1% for these 20 switchgrass populations, respectively. In chapter 4, studies on four populations of switchgrass SW1-SW8 demonstrated that the morphological portions of switchgrass (leaves, internodes, and nodes) differed in cellulose content, lignin and extractives content, and syringyl:guaiacyl ratio. [213] In this chapter, the morphological portions including leaves, internodes, nodes, and seedhead of switchgrass SW9 were prepared to study their chemical compositions.

The gravimetric percentages of these fractions were shown in Table 5.1. The mass percentages of these fractions included 36.4% leaves (23.1% for blade and 13.3% for sheath), 45.4% internodes, 5.0% nodes, and 13.2% seedhead. The gravimetric ratio of leaves to internodes was 0.80. These results show that the percentage of leaf portion is 32.9% lower than the previous study on the switchgrass SW1-SW8 (Table 5.1). The morphological portions of switchgrass SW9, in the present study, were shown to have significant differences in extractives content. Leaves and internodes contained 22.7% and 14.0% extractives respectively, which were 16.0% and 7.3% greater than the node portion, and 2.4% and 11.1% lower than the seedhead portion. Similar results have also been reported recently for hot-water and benzene/ethanol extractives content on other lowland and upland switchgrass varieties. [62, 67, 213]

Table 5.1 Mass Percentages and Extractives Content of Morphological Portions Switchgrass SW9

morphological portion	mass percentages%	extractives content	
		hot-water%	benzene/ethanol after hot-water%
leaf-blade	23.1	18.5	4.2
leaf-sheath	13.3		
internodes	45.4	12.4	1.6
nodes	5.0	1.8	4.9
seedhead	13.2	24.3	0.8

The chemical compounds in the extractives solution, which were identified by GC-MS analysis, are different in quality and quantity between leaves and internodes (Table 5.2). In general, switchgrass extractives can be classified as aromatic compounds, carboxylic acid, sugars, alkanes, fatty acids, alcohols, and sterols. [66, 67] To simplify subsequent analysis, the leaf and internode portions, which represented the major mass (81.8%) of the whole plant SW9 was selected for further characterization. Table 5.2 showed the extractive compounds from hot-water and benzene/ethanol extractions of leaves and internodes from switchgrass. Several biologically active compounds are found in the extractives of the present study. For example, α-tocopherol, which has antioxidant

properties, [216] is present in switchgrass leaves at a value of 85 (μg/g biomass) in the benzene/ethanol fraction. Sterols, which have broad medicinal applications, [217] are also observed in the benzene/ethanol extractives from the leaf portion (679 μg/g biomass). These biologically active compounds can be of interest as value-added products for future applications. The experimental results indicate that the internode portion has 12100 (μg/g biomass) more hot-water extractives but 3060 (μg/g biomass) less benzene/ethanol extractives than leaf portion. The hot-water extractives from leaves and internodes of switchgrass are found to have several different chemical constituents. Ribose, fructose, xylose, sucrose, malic acid, and palmitic acid are detected in hot-water extractives of the internodes but not the leaves. The leaf hot-water extractives are found to have quinic acid and galactofuranose, whereas these compounds are not detected in the internodes hot-water extractives. In addition, the leaf benzene/ethanol extractives had more extractives compounds detected by GC-MS analysis than the corresponding internodes extractives. The leaf benzene/ethanol extractives are shown to have glucose, α-tocopherol, monoglycerides, stigmasterol, and various carboxylic acids when compared to the corresponding internodes extractives.

The Higher Heating Value (HHV) of bioresource components can be correlated with their chemical composition. [65] in the study of acid catalyzed liquefaction of bagasse in ethylene glycol, the HHV ranging from 11.0 to 39.6 MJ Kg^{-1} was positively correlated to the carbon and hydrogen elemental content and negatively related to the oxygen elemental content for bagasse and its liquefaction product. These results indicate that an increase in carbon content and lower oxygen content leads to a higher HHV. Understanding combustion values and their relationship to chemical composition could be an important parameter for future biopower applications of switchgrass.

Table 5.2 Hot-water and Benzene/ethanol Extractives Compounds of Leaves and Internodes of Switchgrass SW9 by GC-MS

hot-water extractives compounds	retention time[a] (min)	leaf (μg/g biomass)	internode (μg/g biomass)
malic acid/ quinic acid	9.13/14.11	ND/138	688/ ND
C16:COOH/ C18:COOH	15.94/17.92	ND /151	34/356
D-ribose/D-fructose	12.23/13.57	ND / ND	524/1610
galactofuranose/galactose	13.48/14.45	219/163	ND/ND
glucofuranose/ glucosepyranose/glucose	13.90/14.53/15.28	111/ND/368	403/1910/1700
D-xylose/ sucrose	15.62/24.49	ND[b]/ ND	404/5620
total detected (μg/g biomass) (hot-water extractives)	-	1150	13200
benzene/ethanol extractives compounds	-	leaves (μg/g biomass)	internodes (μg/g biomass)
quinic acid/ linolenic acid	14.11/17.73	48/384	ND /31
p-hydroxyl cinnamic acid/9,12-octadecadienoic acid	14.88/17.65	32/152	10/51
C12:COOH/ C14:COOH	11.22/13.66	27/94	3/ ND
C16:COOH/ C18:COOH	15.85/17.92	332/81	ND /14
C20:COOH/ C22:COOH	20.26/23.26	116/40	ND / ND
C23:COOH/ C24:COOH	25.21/27.57	21/94	ND / ND
C25:COOH/ C26:COOH	30.45/34.03	23/73	ND / ND
C27:COOH/ C28:COOH	37.66/40.94	35/170	ND / ND
C30:COOH	48.89	276	ND
arabinose/ D-ribose	10.75/11.34	ND /94	30/3
xylose/ mannose	12.00/14.30	ND / ND	17/13
glucosepyranose/glucose	14.45/15.28	24/42/	ND/ND
cellotriose	24.07	ND	11
maltose/ inositol	24.82/14.02	ND/ ND	15/6
C24:OH/ C32:OH	32.0/53.95	44/54	11/ ND
α-tocopherol	38.62	85	ND
haptacosane/ nonacosane	24.39/29.29	70/94	ND/ ND
nonadecane/ mono palmitglyceride	36.54/22.46	39/45	ND/ ND
mono octadecanateglyceride	25.80, 26.05	71	ND
cholesterol/ stigmasterol	39.30/44.11	85/230	17/ ND
beta-sitosterol/ unidentified sterol	46.44/50.00	212/152	45/ ND
total, (μg/g biomass) (benzene/ethanol extractives)	-	3340	276

[a] The retention time of ion fragments in GC-MS

[b] ND: non detectable

Table 5.3 Mineral Inorganic Compounds, Ash Content, Acid-Insoluble Ash Content, and HHV of Leaves and Internodes of Switchgrass SW9

ICP element	leaf(mg/kg)	leaf-extracted (mg/kg)	internode (mg/kg)	internode-extracted (mg/kg)
K	9550	24	6500	12
Ca	3720	4840	460	240
Mg	2640	284	443	90
P	2170	132	1280	30
S	1020	708	318	179
Si	615	549	220	246
Mn	188	55	52	16
Na	88	14	145	14
Fe	54	70	15	12
Zn	28	22	12	4
Cu	19	16	8	4
Al	13	19	1	2
Ba	8	9	5	3
Sr	8	9	2	1
As	<3	<3	<3	<3
Pb	<2	<2	<2	<2
Sn	1	1	2	1
B	3	2	0	0
Ni	2	1	1	1
Cr	1	1	0	0
total detected(mg/kg)	20100	6760	9460	857
total halogen(mg/kg)	1670	12	606	10
ash	71000	41000	32000	16000
Acid-Insoluble Ash	27000	ND[a]	700	ND
HHV, MJ/kg	18.6	19.1	19.3	19.7

Note: [a] ND: not determined

Inorganic compounds detrimentally affect the HHV of biomass. For instance, a 1% increase of ash content results in 0.2 MJ/kg reduction of HHV. [218] For bioenergy and biopower application, it is essential to determine the mineral inorganic compounds content and the HHV of the switchgrass. The leaf and internode portions of switchgrass SW9 were measured in terms of ash content, Acid-Insoluble Ash content, and HHV as summarized in Table 5.3. These results indicated that the leaf portion of switchgrass had 39000 (mg/kg biomass) more ash content and 20000 (mg/kg biomass) more Acid-Insoluble Ash content, and its HHV was 0.7 MJ/kg less than the internode portion of switchgrass. The HHV of native leaf and internode portions of switchgrass is comparable

to the stem portion of switchgrass (18.8±0.2 MJ/kg). [213] HHV increases for post-extracted leaves and internodes presumably because of the removal of sugars and ash. This phenomenon is the reverse of previous studies on the extractives effect on HHV of softwood and hardwood, [65] which has a lower HHV after extraction. These results enhance the thesis hypothesis that morphological portions of switchgrass as a factor influence the utilization for energy resource in terms of the HHV.

The trace inorganic content for the leaf and internode portions of switchgrass was analyzed and these data were summarized in Table 5.3. The results indicate that the leaf portion has significantly greater amounts of Ca, Mg, S, Si, and Mn than the internode portion. The total halogen content was 1060 mg/kg greater in the leaf portion than in the internode portion. After hot-water and benzene/ethanol extraction, there was a significant decrease in K, Mg, P, Mn, Na, and total halogen elements contents whereas most other elements did not change significantly. This facile reduction in some inorganic elements provides an interesting opportunity to reduce the ash content of switchgrass.

Biomass composition of switchgrass SW9 was performed for the analyses of carbohydrates and lignin content. The effect of particle size, morphological portions, and extraction on the chemical composition analysis was also investigated. The results show that the composition of switchgrass SW9 varies from morphological portions: leaves, internodes, nodes, and seedhead. This analysis also shows the compositional analysis also varies slightly by particle size and extraction process (Table 5.4). In brief, the chemical composition of the leaf portion has much greater variation than the internode portion. Compared to the chemical composition of leaves, the composition of the internodes is only slightly affected by the particle size. In brief, the leaf portion is significantly different from the internodes. These results are slightly different to the previous findings on the switchgrass SW1-SW8. [213] The leaf portion is found to have comparable glucan

content and 4.4% less xylan content than the switchgrass SW1-SW8, whereas the internode portions have 2.7% and 2.4% greater glucan and lignin contents, respectively. The node fraction is found to have 6.9%, 4.6%, and 4.1% more glucan, xylan, and lignin content, respectively. Differences in growing locate/season and age of harvesting of the switchgrass could contribute to these differences in part. [145, 185, 212]

Table 5.4 Chemical Compositions of Leaves and Internodes of Switchgrass SW9

sample	particle size(mm)	ara% [a]	gal% [a]	glu% [a]	xyl% [a]	KL% [a]	ASL% [a]	Total
leaves	<0.71	2.9	1.5	30.7	15.2	19.5	3.5	73.3
internodes	<0.71	1.7	0.7	42.6	20.7	20.2	1.8	87.7
nodes	<0.71	2.8	1.0	40.5	26.8	24.7	2.1	97.9
seedhead	<0.71	2.9	1.5	36.6	19.8	23.3	4.1	88.2
leaves-extracted	<0.71	4.3	1.5	39.7	23.9	16.4	3.2	89.0
internodes-extracted	<0.71	2.0	0.6	47.2	25.5	21.1	1.6	98.0
nodes-extracted	<0.71	2.4	0.8	34.6	22.5	25.0	2.3	87.6
seedhead-extracted	<0.71	3.2	1.0	40.2	26.4	21.3	2.7	94.8
leaves-extracted	0.71-0.30	4.1	1.4	41.2	25.6	16.8	2.3	91.4
leaves-extracted	<0.30	4.0	1.5	35.0	18.7	17.9	2.1	79.2
internodes-extracted	0.71-0.30	2.1	0.6	48.0	26.1	21.8	1.5	100
internodes-extracted	<0.30	2.3	0.8	47.6	26.2	22.7	1.5	101

Note: [a] ara: arabinan; gal: galactan; glu: glucan; xyl: xylan; KL: Klason lignin; AIL: acid insoluble fraction in lignin.

Structure Characterization of Switchgrass Cellulose

The ultrastructure of cellulose is heterogeneous, made up of crystalline cellulose (I_α and I_β), paracrystalline cellulose, and cellulose at accessible and inaccessible surfaces. [105] These polymorphs can vary significantly in relative properties according to the sample origin. In the case of highly ordered cellulose originating from *Valonia*, para-crystalline and amorphous cellulose were reported in lower amounts than those typically reported for wood and cotton. [105] Pu et al. monitored the structural changes of Kraft pulp cellulose during cellulase hydrolysis and demonstrated that cellulose I_α, para-crystalline, and amorphous regions of cellulose were more susceptible to cellulase deconstruction than

cellulose I_β using solid state ^{13}C CP/MAS NMR experiment. [103] These results suggested that the characterization of the structure of cellulose is an important consideration for bioethanol production.

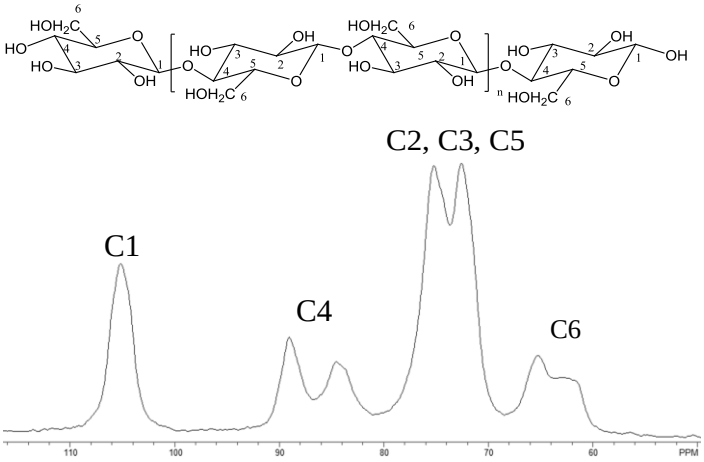

Figure 5.1 CP/MAS ^{13}C-NMR Spectrum of Leaf Cellulose of Switchgrass SW9

A pure cellulose sample was prepared from the switchgrass SW9 using holocellulose pulping followed by a mild acid treatment to remove hemicelluloses. ^{13}C CP/MAS NMR spectroscopy was used for the ultrastructure characterization of cellulose (Figure 5.1). The most informative region was the C-4 region of cellulose at 79-92 ppm. Using nonlinear least square fitting of the ^{13}C CP/MAS NMR spectra, the relative amounts of cellulose I_α, cellulose I_β, para-crystalline cellulose, celluloses at accessible and inaccessible surfaces, and cellulose Crystallinity Index were determined. The assignments and relative proportion values are shown in Table 5.5 and suggest cellulose from leaves and internodes are similar in cellulose ultrastructure.

Solid state NMR for the leaf and internode cellulose shows 30% para-crystalline cellulose and 34% inaccessible fibril surface on average. The Crystallinity Index of switchgrass for leaves and internodes are similar, with an average value of 51%, which was comparable to a recent report [102] for bulk Crystallinity Index of switchgrass SW9 (44%).

Table 5.5 Assignments of Signals in the C-4 Region of the CP/MAS ^{13}C-NMR Spectra of Isolated Cellulose from Leaves and Internodes of Switchgrass SW9

assignments	chemical shift (ppm)	FWHH(Hz)[a]	line type	relative integrated intensity%	
				leaves	internodes
cellulose I_α	89.7	90	Lorentz	1.5	1.4
cellulose $I_{\alpha+\beta}$	89.0	91	Lorentz	12.0	11.8
para-crystalline cellulose	88.8	241	Gauss	29.9	29.0
cellulose I_β	88.1	135	Lorentz	3.3	3.4
accessible fibril surface	84.5	100	Gauss	9.3	12.3
inaccessible fibril surface	84.4	400	Gauss	35.7	33.1
accessible fibril surface	83.6	95	Gauss	8.3	9.0
Crystallinity Index %	-	-	-	51.2	49.8

Note: [a] FWHH: Full Width at Half-Height

Table 5.6 Molecular Weights of Cellulose and Lignin Isolated from Leaves and Internodes of Switchgrass SW9

sample	M_w g/mol	M_n g/mol	DP_w
leaf cellulose[a]	1.54×10^6	1.35×10^5	2.97×10^3
internode cellulose[a]	1.52×10^6	1.24×10^5	2.93×10^3
leaf lignin[b]	5.92×10^3	2.30×10^3	-
internode lignin[b]	4.38×10^3	1.85×10^3	-

Note: [a] Standard derivation for cellulose M_w 2.83×10^4 g/mol, for M_n 2.69×10^4 g/mol, and for DP_w 57; [b] Standard derivation for lignin M_w 23 g/mol and M_n 17 g/mol

α-Cellulose tricarbanilates prepared from switchgrass was used to determine the Degree of Polymerization by GPC and these results were summarized in Table 5.6. Celluloses from leaves and internodes have similar weight-average molecular weight (M_w) values of 1.54×10^6 g/mol and 1.52×10^6 g/mol for leaves and internodes, respectively. The calculated DPs of cellulose are comparable, 2.97×10^3 and 2.93×10^3, for leaves and internodes, respectively.

Structure Characterization of Switchgrass Lignin

The collected spectra of ball milled lignin from leaves and internodes were shown in Figure 5.2. Quantitative ^{13}C-NMR spectroscopic data analysis was carried out by integrating the signal intensity between 162 ppm and 103 ppm and setting this value to six aromatic carbons after subtracting the integration value for the two vinyl carbons of ferulate and *p*-coumarate. [66]

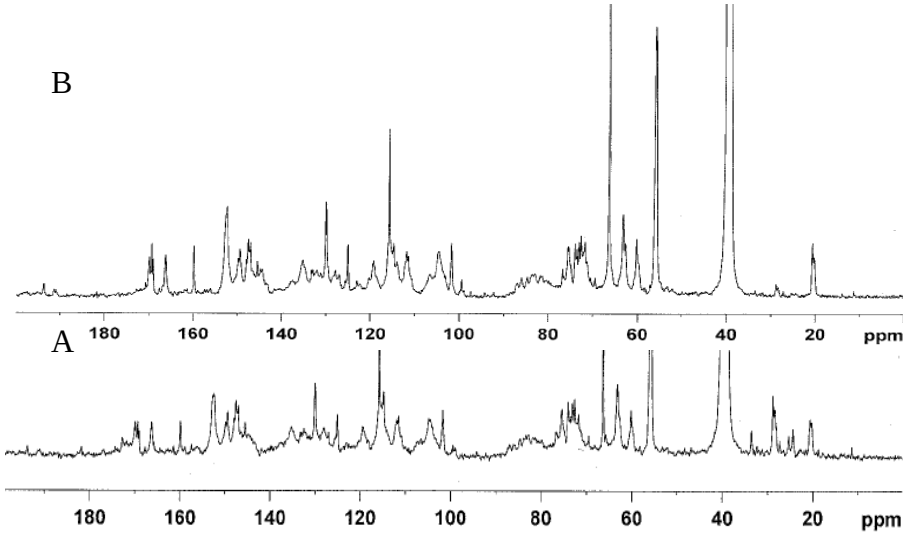

Figure 5.2 Quantitative ^{13}C-NMR Spectroscopy of Leaf (A) and Internode (B) Lignin in Switchgrass SW9

Lignin structure assignments were accomplished according to recent studies as summarized in Table 5.7. [2, 66] The methoxy group content is estimated on the basis of the relative integration range of 58-54 ppm. The results gave values of 0.95 and 0.99 per aromatic ring for leaf and internode lignins, respectively. These results are comparable to the recent investigation on the structure of lignin in switchgrass. [66] The integration of the acetyl methyl group signal (21-19 ppm) provides values of 0.18 and 0.19 per aromatic ring for the leaf and internode portions of switchgrass SW9. An unconjugated-ester signal was observed at 175-168 ppm[66, 141] and shown to be 0.48 and 0.40 per aromatic ring for leaves and internodes, respectively.

It has been suggested that the possible origin of the acetyl group in the spectra of isolated lignin is from acetylated xylan or lignin. [219] The result of sugar analysis indicates that isolated lignin contains 1.6% arabinose, 0.1% galactose, 0.9% glucose, 14.0% xylose, and 80.2% lignin. From the spectra, the C-1 xylose signal can be clearly assigned at 102 ppm. [111] The integration value for C-1 of xylose is estimated on the relative

integration range of 103-101 ppm. The results provide the value of 17 and 16 xylose units per 100 aromatic rings for leaf and internode lignins, respectively.

Table 5.7 Assignments and Integration Value of Quantitative ^{13}C-NMR Spectra of Leaf and Internode Lignins

integration range	assignments	internode(/Ar)	leaf(/Ar)
195-193	Ar-CH=CH-CHO [65]	0.03	0.02
193-191	guaiacyl or syringyl benzaldehyde [141]	0.04	0.02
175-168	unconjugated COOR [141]	0.40	0.48
168-164	conjugated COOR [220]	0.24	0.23
162-158	C_4 *p*-coumaric acid [66]	0.21	0.18
158-156	C_4 H-unit [141]	0.08	0.12
156-151	C_3 in 5-5' ET, C_3/C_5 in S unit [141]	0.68	0.59
123-117	C_6 in G unit [65]	0.51	0.52
117-114	C_5 in G unit, C_3/C_5 in *p*-coumaric acid, C_5 in ferulic acid, β-carbon in *p*-coumaric and ferulic acid [2, 65, 66]	0.81	0.82
114-108	C_2 in G unit [65]	0.46	0.47
108-103	C_2/C_6 in S unit [65, 141]	0.68	0.65
103-101	C1 in xylose [111]	0.16	0.17
90-78	α-CH in β-β' and β-1, β-CH in β-*O*-4, C2/C5 in xylose [65, 141]	0.70	0.77
61-58	C_γ in β-*O*-4 (G or S) without C_α=O [65]	0.32	0.30
58-54	methoxy [65, 141]	0.99	0.95
21-19	CH_3 in acetyl group [219](*28*)	0.19	0.18
S/G ratio	$(I_{108\text{-}103}/2)/I_{114\text{-}108}$ [65]	0.74	0.69

Note: NE: non-etherified; I: integration value

The most valuable information obtained from the quantitative analysis of the ^{13}C-NMR spectra from leaf and internode lignins is the relative amount of basic precursors present in the leaf and internode lignins. Lignin has been defined as a crosslinked complex polymer synthesized mainly through dehydrogenative polymerization of *p*-coumaryl alcohol (H), coniferyl alcohol (G), and sinapyl alcohol (S). [2] Studies on the lignin structure of C-4 perennial grasses have shown that *p*-coumaric and ferulic acid are also incorporated into lignin through ester or ether interlinkages. [66]

Table 5.7 shows that the NMR signals at 162-158 ppm are assigned for the C-4 carbon of *p*-coumaric acid (0.18 and 0.21 per aromatic ring for leaves and internodes). [66] The signal at 168-164 ppm is assigned for C-γ carbon of *p*-coumaric and ferulic acid (0.23 and 0.24 per aromatic ring for leaves and internodes). [66] These results suggest that leaf and internode lignins have similar amounts of *p*-coumaric and ferulic acid linked to

the isolated lignin. The amount of ferulic acid can be calculated by subtraction of an integration value at 162-158 ppm from 168-164 ppm. [221] These results suggest that leaf and internode lignins have 0.05 and 0.03 per aromatic ring of ferulic acid, respectively. Compared to the recent study on the structure of lignin isolated from stem portion of four populations of switchgrass, the present structure of lignin was comparable in the amount of *p*-coumaric acid on average, but slightly greater in the amount of ferulic acid (0.02 per aromatic ring on average). [66] Another study on the structure of lignin by ^{13}C-NMR indicated that dioxane lignin isolated from leaf sheath of a banana plant contained *p*-coumarate and ferulate, 0.07 and 0.05 per aromatic ring, respectively. [221] The amount of guaiacyl units for leaf and internode lignins can be calculated from the integration value at 123-117 ppm subtracting the integration value for ferulic acid. This result suggests that leaf and internode lignins have 0.52 and 0.51 per aromatic ring of guaiacyl units respectively (G unit). The amount of *p*-hydroxyphenyl unit (H unit) was calculated using the integration value at 158-156 ppm. It was found to be 0.12 and 0.08 per aromatic ring for leaf and internode lignins, respectively. The amount of syringyl unit (S unit) was calculated from half the integration value at 108-103 ppm. The values were 0.32 and 0.34 per aromatic ring for leaf and internode lignins, but these values are tentative given the presence of the C-1 xylan signal. Given these results the relative value of *p*-hydroxyphenyl/guaiacyl/syringyl unit (H/G/S) were calculated as 12.4/53.9/33.7 and 8.6/54.8/36.6 for leaf and internode lignins, respectively. The observed NMR S:G ratio including ferulic acid was 0.69 and 0.74 for leaf and internode lignins, respectively.

The major interlinkages of switchgrass, β-*O*-4, β-β', β-5', and ester interlinkages, have been observed in a previous study. [66] According to the assignments and integration values presented in Table 5.7, the relative amounts of the major interlinkage, β-*O*-4 moieties, was calculated for lignin in leaf and internode portions of switchgrass SW9. The result indicates that these inter-linkages in switchgrass lignin are comparable (0.32/Ar and 0.30/Ar) for leaf and internode lignins.

The molecular weights of the acetylated ball milled leaf and internode lignins, each containing polysaccharides, were measured by GPC. The results in Table 5.6 indicate that the molecular weight M_w of acetylated leaves ball milled lignin is 35.3% greater than that of acetylated internode sample (5920 g/mol vs. 4380 g/mol). A recent report on the molecular weight of ball milled lignin from a bulk switchgrass sample was 5000 g/mol. [2] The difference on M_w of acetylated lignin can be influenced by the present of greater amount of xylan content in the isolated leaf and internode lignins. These results also suggest that the ball milled lignins from leaves and internodes are comparable with the exception of molecular weight of their derivative form in the present investigation.

Conclusion

These results indicate that the leaves and internodes differ chemically in the amounts of inorganic elements, hot-water extractives, benzene/ethanol extractives, carbohydrates, and lignin content. However, the ultrastructure of isolated cellulose is comparable between leaves and internodes. Ball-milled lignins isolated from leaves and internodes are found to have H/G/S ratios of 12.4/53.9/33.7 and 8.6/54.8/36.6, respectively. These heterogeneous features [185] in morphological portions of switchgrass can provide potential benefits for future biofuel/biopower application.

These observations enhance the thesis hypothesis that morphological fractions of switchgrass as a factor influence the utilization of switchgrass for biofuels, chemicals, and energy. The basic chemical constituents are different between leaves and internodes of switchgrass SW9. These have been suggested to have relationship to the HHV and inorganic chemical components for biopower and bioenergy applications of switchgrass. Leaves of switchgrass have 0.7 MJ kg^{-1} lower HHV but 1060 mg/kg greater in total halogen content and 10640 (mg/kg biomass) more inorganic elemental content. These differences suggest that leaf fraction is less suitable for bioenergy production because of

greater side effect of inorganic elements and the lower HHV than internode fractions of switchgrass.

Although these basic chemical constituents are different between leaves and internodes of switchgrass SW9, the chemical structures of major components, cellulose, is comparable according to the results for the ultrastructure and Degree of Polymerization. However, the different physical structure of switchgrass in leaf and stem (including sheath) of switchgrass was reported by Reddy et al. According to their report, stem fraction had 4 degree greater microfibril angle and 5% less crystallinity than leaf fraction of switchgrass. [206] Liu et al. reported that the crystallinity of cellulose from different part of the wheat straw had little difference. The cellulose in wheat straw was identified as cellulose I allomorph with low crystallinity between 43.2% and 47.4%. [222] Regardless of these different results for cellulose structure in morphological fractions of switchgrass, the research has not been studied on the changes of cellulose structure in morphological fractions and the structure difference in morphological fractions after hydrothermal pretreatment.

Recent studies on pretreatment of switchgrass for bioethanol production have suggested that lignin content and lignin structure are important factors for the saccharification process. [146, 183, 184] Lignin has slightly different structure in H/G/S ratios between leaves and internodes of switchgrass SW9. The lignin content of extracted internodes has ~5% greater than that of extracted leaves. The different in lignin content and the structure of lignin could be a factor to influence the degradability in morphological fractions of switchgrass.

In the next chapter, switchgrass samples—SW1-SW2, SW3-SW4, SW5-SW6, and SW7-SW8—are partitioned into two morphological portions, leaves and internodes, and analyzed for chemical compositions in the previous study. These samples undergo a hydrothermal pretreatment, followed by cellulose and cellobiase treatment.

CHAPTER 6

HYDROTHERMAL PRETREATMENT OF SWITCHGRASS

SW1-SW8*

Introduction

Developing efficient conversion technologies for second generation biofuels has become a priority issue for society due to an increased demand for fuels, environmental concerns, and a decreased availability of fossil fuels. [9, 15] The development of cellulosic biofuels is predicated on the large-scale sustainable availability of lignocellulosic bioresources, such as forests, perennial grasses, wood, and agricultural residues. Switchgrass is one of the promising feedstocks for biofuels production. This C4 warm-season perennial grass is renowned for its high production yield, reaching up to 14 tons/acre per year and exhibiting wide geographical adaption in Central and North America. [2, 46] The biological technology platform for the production of bioethanol is accomplished by enzymatic hydrolysis of polysaccharides to monosaccharides, followed by fermentation to bioethanol. The practical implementation of cellulosic ethanol is dependent on the development of efficient pretreatments and saccharification.

The pretreatment process is required to increase the enzymatic digestibility of the incoming bioresource, and this is due to the natural recalcitrance of lignocellulosics. Reductions in recalcitrance after pretreatment have been attributed to several factors,

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including the removal of lignin and hemicellulose, alterations of cellulose crystallinity, an increase in cellulose reducing ends, increased accessible surface area, and the modification of cell wall morphology. [16, 17] Efficient pretreatments also require minimum cellulose loss and nominal byproduct formation that could inhibit the fermentation process.

Over the past two decades, numerous pretreatment technologies have been developed for herbaceous bioresources, including biological, dilute acid, dilute alkaline, physical, and thermal pretreatments. Biological pretreatments rely on a microbial or enzyme treatment to modify the chemical composition of the biomass and improve the sugar release yield by cellulases. [29] Sarath et al. [18] reported that the digestibility of switchgrass was improved ~67% by using an esterase pretreatment, which disrupted the ester interlinkages between phenolic acids (i.e., ferulic acid and coumaric acid) and carbohydrates. Dilute acid pretreatment is an alternative method to maximize hemicellulose removal and improve the digestibility of lignocellulosic biomass. Recently, Yang et al. [30] investigated dilute acid pretreatment of switchgrass germplasms for bioethanol production and indicated that using 1.5% sulfuric acid at 121 ^{o}C for 60 min removed approximately 80% of the hemicelluloses, facilitated complete cellulose hydrolysis by cellulases, and produced ethanol from enzymatic hydrolyzates with a 60% theoretical ethanol yield after yeast fermentation. [30]

Alkaline pretreatments using sodium hydroxide, lime, or ammonia to remove lignin and hemicellulose from switchgrass and enhance subsequent enzymatic hydrolysis of biomass [31] have been reported. Recent studies have shown that microwave-assisted alkaline pretreatment of switchgrass at 190 ^{o}C for 30 min with 0.1 g alkaline/g biomass loading achieved 99% total sugar released after enzymatic hydrolysis. [32] Aqueous ammonia (30%) pretreated switchgrass has been fermented at the pilot scale for ethanol production, providing a 72% theoretical ethanol. [33]

Hydrothermal pretreatment, so-called autohydrolysis or hot-water pretreatment, uses only water as a reaction medium with relatively high reaction temperatures (180-220 ^{o}C). [27] It is an attractive pretreatment process that leads to increased digestibility of biomass without additional chemicals required. These processes are suitable for pretreating a range of lignocellulosic substrates, including switchgrass. [16, 37] For instance, Suryawati et al. [25] reported that a hydrothermal pretreatment of Kanlow switchgrass at 200 ^{o}C for 10 min could achieve up to 70% theoretical ethanol production yield using simultaneous saccharification and fermentation. Recently, Cybulska et al. [43] investigated hydrothermal pretreatment and saccharification of Prairie cord grass and reported that under a hydrothermal pretreatment at 210 ^{o}C for 10 min, a 97% yield of glucose could be achieved after enzymatic hydrolysis of the solid residue. These investigations suggested that hydrothermal pretreatment is a promising methodology for bioethanol production from perennial grass feedstocks.

The varying degrees of the digestibility of different morphological portions of grasses (leaves, stems, or internodes and nodes etc.) have been studied in several species. Dien et al. [73] studied the acid pretreatment of switchgrass with specific maturity stages and morphological portions. The results demonstrate that different growth stages and morphological portions (i.e. leaves vs. stems) have different levels of susceptibility to cellulases after a dilute acid pretreatment. The results show that the pre-boot and post-frost stages of switchgrass performance 19% greater glucose yield by cellulases after a 2% sulfuric acid pretreatment at 121 ^{o}C for 1 hour. Another study of leaves and stems (sheath, internodes, and nodes) examines the response to the acid pretreatment, subsequent saccharification and fermentation. [30] The results indicate that the leaf portion takes on a more digestible form after a 1% sulfuric acid pretreatment at 121 ^{o}C in autoclave for 1 hour. [30] William et al. [178] studied the digestibility of various morphological portions of corn stover, including the leaf blade, leaf sheath, stem ring, stem pith, and corn kernel fiber. The highest dry matter loss was about 47% for the leaf

sheath after 72 h hydrolysis by cellulase. These studies suggest that the morphological fractions are a factor to consider during enzymatic hydrolysis. The results in Chapter 4 and 5 demonstrated that the morphological fractions of switchgrass have different properties in chemical profiles. On the other hand, the structure of cellulose and lignin is very similar in morphological fractions, leaves and internodes of switchgrass. The attributes to the degradation of morphological portions of switchgrass have not been explored. In this chapter, investigation focuses on the morphological effect on the structure of switchgrass after hydrothermal pretreatment. Four populations of switchgrass SW1-SW8 (including two morphological portions: leaves and internodes) were employed for hydrothermal pretreatment. The carbohydrate profiles, cellulose crystal structure, and Degree of Polymerization (DP) of the cellulose were analyzed for native and pretreated leaves and internodes. The digestibility of hydrothermal pretreated switchgrass SW1-SW8 and its impact on cell wall chemistry were explored for switchgrass SW2.

Materials and Methods

Sample Preparation

Switchgrass samples were seeded in 2000 at the University of Georgia plant science farm near Watkinsville, GA ($33^{o}52'$ N; $83^{o}32'$ W) on coarse, sandy loam (fine, kaolinitic, themic typic kanhapludults). Four populations of switchgrass samples—SW1-SW2, SW3-SW4, SW5-SW6, and SW7-SW8—were harvested and received in August of 2008 from the University of Georgia, Athens, GA, USA. [213] Once harvested, the switchgrass samples were air-dried until the moisture content was less than 10% of dry weight. The leaves, including blade and sheath, and internodes of switchgrass were manually separated and ground in a Wiley mill to pass through a 0.841 mm screen. Samples were then additionally sieved to achieve a final particle size between 0.297 mm and 0.707 mm screened and stored at room temperature.

Results and Discussion

Chemical Compositions of Switchgrass Feedstock

In chapter 4, the results on the chemical profile of four populations of switchgrass, SW1-SW8, indicated that these four populations had similar chemical profiles, with an exception of the lignin content of the internode portions of the switchgrass, which was shown to be 18.5%, 19.1%, 20.0%, and 19.9%, respectively. [213] These results also demonstrate that the leaf portion of switchgrass, the most abundant portion of the plant (69.0% mass on average), is chemically different from the internode portion of switchgrass. In this chapter, leaf and internode portions of the switchgrass SW1-SW8 were used for the hydrothermal pretreatment and subsequent cellulase treatments. The particle size of leaves and internodes from milled switchgrass SW1-SW8 was between 0.297 mm and 0.707 mm.

Pretreatment of Switchgrass

In this chapter, the leaf and internode portions of switchgrass were used for hydrothermal pretreatment. After hydrothermal pretreatment of the samples, the pH value of the aqueous solution decreased from near neutral to a pH of 3.5 for all the samples studied. This result indicates that acids released during hydrothermal pretreatment contribute to the pretreatment effect. [27, 37] The biomass yield from hydrothermal pretreatment among the switchgrass SW1-SW8 was comparable, ranging from 48.1-51.4% (Figure 6.1), but differed between the leaf and internode portions of switchgrass, as summarized in Table 6.1. This data shows that the average value of biomass yield for the leaf portion is about 1.9% greater than that of the internode portion (50.4% vs. 48.5%).

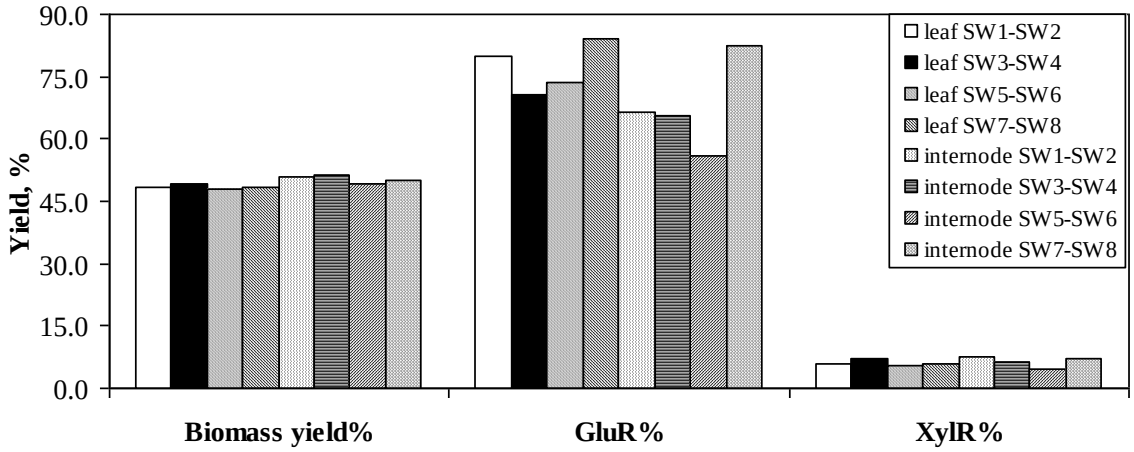

Figure 6.1 Biomass, Glucan Retention, and Xylan Retention Yield of Hydrothermal Pretreatment of Leaves and Internodes for the Switchgrass SW1-SW8. (Biomass Yield, the Dry-mass Percentage of Solid Residues to the Original Switchgrass; GluR, Glucan Retention Yield in Pretreated Solid; XylR, Xylan Retention Yield in Pretreated Solid)

Table 6.1 Chemical Compositions of Hydrothermal Pretreated Leaves and Internodes for Four Populations of Switchgrass SW1-SW8

morphology	biomass yield%	ara%[a,b]	gal%[a,b]	glu%[a,b]	xyl%[a,b]	lignin%[b]	total%	gluR%	xylR%
leaves[c]	48.5	ND[d]	0.1	49.9	2.5	39.2	91.6	77.2	6.1
internodes[c]	50.4	0.1	ND	52.1	2.8	34.3	89.2	67.3	6.5
C.I. (95%)[e]	1.4	ND	ND	11.5	0.7	4.8	-	6.3	2.5

Note: [a] ara: arabinan; gal: galactan; glu: glucan; xyl: xylan; [b] Sugars and lignin content are percentage to the pretreated switchgrass; [c] Ash for leaves: 4.6%; ash for internodes: 1.9%; AIA for leaves: 1.5%; AIA for internodes: 0.03%; [d] ND: nondetectable; [e] C. I. (95%): 95% confident interval on the differences between means of leaf and internode portion

After pretreatment, the resulting switchgrass samples were characterized for their carbohydrates and lignin content, as summarized in Figure 6.1 and Table 6.1. Using mass balance calculations, the amount of carbohydrates removed during the pretreatment is calculated as presented in Figure 6.1 and Table 6.1. These results indicate that most of the glucan is retained in the solid fraction of biomass, about 70.7-84.1% for the leaf portion and 56.0-82.3% for the internode portion. In comparison to Yang's best condition for acid pretreatment, in which they retained 58.6-66.3% of the glucan for leaf and stem (including sheath) portions of switchgrass, hydrothermal pretreatment has greater glucan

retention. [30] These results also indicate that hydrothermal pretreatment removes 92.3-95.3% xylan from internode portion and 93.0-94.6% from leaf portion. The dissolved lignin in the aqueous solution analyzed by UV was 21.1-30.4% of the lignin in the original biomass removed during the pretreatment process. This result suggests that less than 30% lignin is removed after hydrothermal pretreatment of switchgrass when compared with recent investigation on the pretreatment of Prairie cord grass, which has 88.1% lignin removal after hydrothermal pretreatment at 210 ^{o}C for 10 min. [43] The Acid-Insoluble Ash (AIA) content was used to determine the silicates and silica content in the pretreated switchgrass. [193] In this study, the AIA of the pretreated leaf portion is 1.5%, which is 1.5% greater than that of internode portions (0.03%).

FT-IR Analysis of Native and Pretreated Biomass

Bobleter [27] summarized the chemistry of hydrothermal pretreatment of lignocellulosic biomass and suggested that hydrothermal pretreatment was a hydrolysis process that was characterized by the addition of water across the glycosidic ether linkage of polysaccharides. Hence, the hydrothermal process modifies the chemical structure of the biomass. [27] Table 6.2 and Table 6.3 show FT-IR spectra data of native and pretreated switchgrass SW2. The results suggest that the pretreated leaves and internodes have similar chemical structures. In detail, lignin in leaves and internodes was characterized by the intensity ratio between 1464 cm^{-1} and 1605 cm^{-1} (1.19 and 1.25 for leaves and internodes). [212, 223, 224] The result suggests, in part, that the methoxy content of the lignin is increased for both leaf and internode portions of switchgrass after hydrothermal pretreatment at 200 ^{o}C for 10 min.

These results are consistent with the recent observation by quantitative ^{13}C-NMR on the changes in methoxy content in lignin of whole-plant Miscanthus after autohydrolysis [170]. The results show that the methoxy content of lignin is increased with increasing pretreatment temperature from 120 to 150 ^{o}C. [170] In another study, the

methoxy content of milled wood lignin after autohydrolysis of Eucalyptus globulus was unchanged in a short reaction time but decreased with increasing reaction time at 170 ^{o}C. [168, 169]

The absorbance band at 1732 cm^{-1} can be assigned for C=O stretching of hemicellulose esters. [71, 212, 225] In fact, the lower intensity in the spectra of the pretreated biomass is consistent with the loss of this functionality after pretreatment. The ratio of absorb intensity at 1732 cm^{-1} to that at 1515 cm^{-1} (aromatic ring vibration) indicates that there is a significant amount of ester linkages removed during pretreatment process. The ratio of amorphous to crystalline cellulose in native and pretreated switchgrass has been estimated by the ratio of the FT-IR signal intensity at 900 cm^{-1} and 1098 cm^{-1}. [212, 223, 225] Using this technique for native and pretreated switchgrass SW2, the data indicates that after pretreatment, the ratio of amorphous to crystalline cellulose for the leaves and internodes is decreased about 21% and 6% after pretreatment, respectively. The cellulose crystalline portion of the pretreated leaves is slightly greater than pretreated internodes. These findings are consistent with a recent study on the hydrolysis behavior of microcrystalline cellulose in hot-compressed water. In the study, Yu stated that amorphous cellulose was more susceptible to be hydrolyzed in hot-compress water with the temperature below 230 ^{o}C than was crystalline cellulose. [36]

Table 6.2 Assignments of FT-IR Spectra of Native and Pretreated Switchgrass SW2

wavenumber cm^{-1}	assignments [17, 71, 212, 223-226]	leaves[a]	internodes[a]	pretreated leaves[a]	pretreated internodes [a]
3340	OH stretching	3408	3390	3344	3348
2920	C-H stretch	2918	2916	2918	2902
1735	C=O vibration in hemicellulose and lignin	1732	1734	1732	1732
1655	conjugated C=O stretch	1651	1653	1653	1653
1603	aromatic skeletal vibrations and C=O stretch	1606	1605	1608	1605
1515	aromatic skeletal vibrations	1516	1516	1516	1516
1455	OH in plan bend	1456	1456	1456	1456
1464	CH_3 asymmetric stretch, CH_2 scissoring in lignin and carbohydrates	1464	1464	1464	1464
1427	CH_2 scissoring	1429	1427	1429	1427
1376	CH deformation vibration, CH_3 symmetric deformation in cellulose and hemicelluloses	1375	1375	1371	1371
1321	CH_2 wagging	-	-	1317	1319
1260	guaiacyl ring and C-O stretch in lignin and xylan	1255	1252	1265	1267
1206	OH in plane bending	1203	1207	1203	1205
1165	C–O–C asymmetric stretch	1163	1163	1163	1163
1108	COH in plane deformation (cellulose and hemicellulose)	1107	1109	1113	1113
900	anomeric C-group, C1-H deformation of cellulose	897	897	897	897

Note: [a] Relative absorbance value

Table 6.3 Relative Absorbances of FT-IR Spectra of Native and Pretreated Switchgrass

relative absorbance	assignments [17, 71, 212, 223-226]	leaves[a]	internodes[a]	pretreated leaves[a]	pretreated internodes [a]
A_{1732}/A_{1515}	C=O stretching (ester)/aromatic ring	1.06	1.04	0.67	0.58
A_{1653}/A_{1515}	conjugated C=O stretching/aromatic ring	1.20	0.94	0.95	0.72
A_{1464}/A_{1605}	methoxy in lignin	1.03	1.23	1.19	1.25
A_{900}/A_{1098} [b]	amorphous to crystalline ratio	0.48	0.46	0.38	0.43

Note: [a] Relative absorbance value; [b] The ratio of the peak intensity at 900 cm^{-1} to the peak intensity at 1098 cm^{-1} of the spectra.

Enzymatic Hydrolysis and DP of Cellulose

To evaluate the potential of pretreated switchgrass for bioethanol production, enzymatic hydrolysis of the pretreated switchgrass SW1-SW8 was evaluated. The enzymatic hydrolysis of switchgrass SW2 (without air-dry) was carried out with various reaction time including 0.5, 1, 2, 4, 8, 16, 32, 63 h. The results show that the percentage

of the maximum cellulose-to-glucose yield is similar, 19.9% and 13.1%, for native leaves and internodes. The percentage of the maximum cellulose-to-glucose yield is 78.4% and 37.5% for pretreated leaves and internodes with the hydrolysis for 63 h (Figure 6.2).

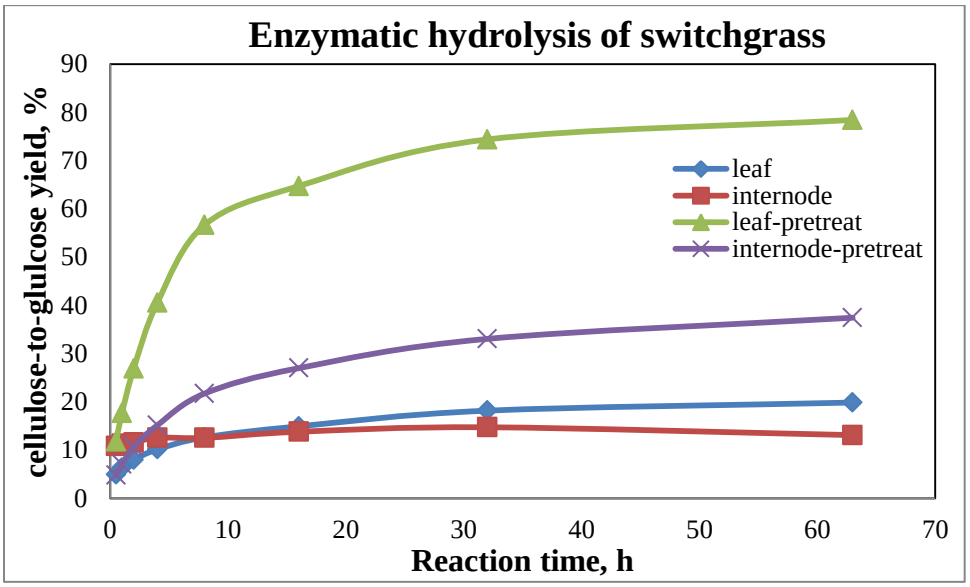

Figure 6.2 Cellulose-to-glucose Yields of Switchgrass SW2 in Enzymatic Hydrolysis with Various Reaction Time

The digestibility of the pretreated switchgrass SW1-SW8 was measured for 48 h hydrolysis using a mixed enzymatic system containing cellulase and cellobiase. The results indicate that the pretreated leaf portion has 16.1% greater dry mass digestibility and 32.5% more cellulose-to-glucose conversion yield than the pretreated internode portion (Figure 6.3 and Table 6.4). These results are coincident with the literature reported by Anderson and Akin, who found that the dry mass yield hydrolyzed by Depol 740 ferulic acid esterase and cellulase was 35.9% greater in the leaf portion than in the stem portion of corn stover. [178]

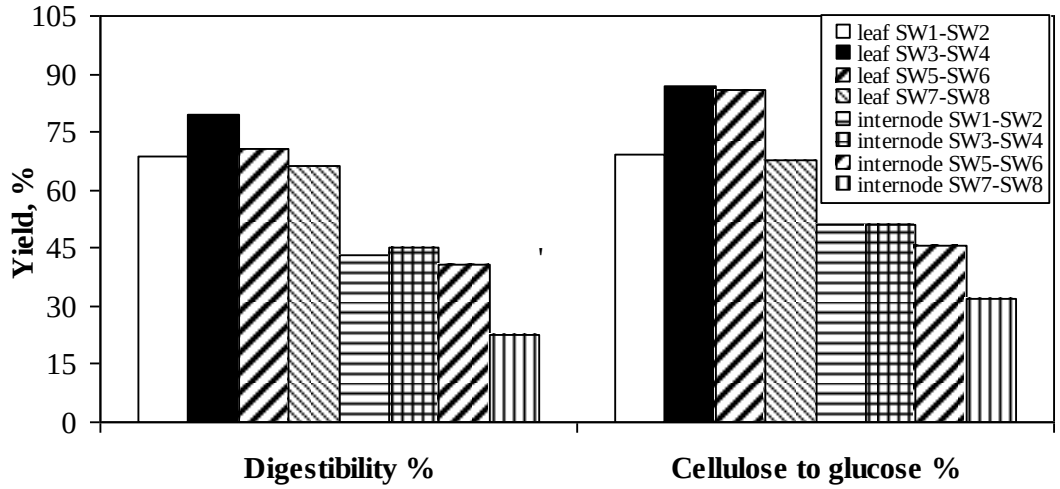

Figure 6.3 Digestibility and Cellulose-to-glucose Yield of Pretreated Leaves and Internodes for the Switchgrass SW1-SW8

Table 6.4 Chemical Compositions of Cellulase Hydrolyzed Residues in Leaves and Internodes of Switchgrass SW1-SW8

sample	ara%[a]	gal%[a]	glu%[a]	xyl%[a]	lignin%[a]	digestibility%	cellulose to glucose%[b]
leaves	0.1	0.1	31.9	2.1	61.3	70.7	77.4
internodes	0.1	ND[c]	49.8	3.1	39.1	36.9	44.9
C.I. (95%)	0.1	ND	10.3	0.7	3.8	5.0	16.7

Note: [a] Sugars and lignin content are percentage to the enzymatic hydrolyzed switchgrass; [b] Glucose yield of enzymatic hydrolysis: based on glucan content of pretreated biomass; [c] ND: nondetectable

Kumar's study [17] on the corn and poplar pretreatment indicated that the Degree of Polymerization of cellulose was an important factor for enzymatic hydrolysis of cellulose. To investigate this factor for the present hydrothermal pretreatment, switchgrass SW2, including leaf and internode portions, was analyzed for the DP_w of isolated α-cellulose (Table 6.5). The result indicates that the weight-average molecular weight of cellulose of native leaves is comparable to that of the native internodes. Hydrothermal pretreatment decreases the molecular weight of cellulose by 57% for the leaf portion and 48% for the internode portion. The DP_w of cellulose for pretreated internode portion is 23.4% greater than that of pretreated leaf portion.

In the present study, the DP_w results suggest that the differences in the DP of cellulose are a significant factor for enzymatic deconstruction of pretreated biomass. The Number of Reducing End F_{RE} calculated from the inversed value of DP_w of cellulose has been suggested to be a factor contributing to the efficiency for cellulose hydrolysis by cellulase. [27, 79] The value of F_{RE} (Table 6.5) for pretreated leaves is 23.4% greater than that for pretreated internodes. The difference in F_{RE} between the leaf and internode portions can be a factor contributing to the 33.9% greater cellulose digestibility for pretreated leaves than pretreated internodes.

Table 6.5 Molecular Weight of Cellulose for Native and Pretreated Switchgrass SW2

sample	M_n[a](10^4g/mol)	M_w[a](10^4g/mol)	DP_w[a,b]	F_{RE}%[c]	PDI[a,d]
leaves	1.81×10^5	1.68×10^6	3240	3.09×10^{-2}	9.3
pretreated leaves	1.00×10^5	7.10×10^5	1380	7.25×10^{-2}	7.2
internodes	1.59×10^5	1.72×10^6	3320	3.02×10^{-2}	10.8
pretreated internodes	1.09×10^5	8.86×10^5	1710	5.85×10^{-2}	8.1

Note: [a] Standard deviation: calculated from the measurement which was repeated three times/sample. 2.26×10^4g/mol for M_n, 2.83×10^4g/mol for M_w, 57 for DP_w, 0.06×10^{-2} for F_{RE}%, and 1.0 for PDI; [b] DP_w: weight average of Degree of Polymerization; [c] F_{RE}: Number of Reducing Ends; [d] PDI: polydispersity index

The Adsorbance of Direct Blue-1 and Direct Orange-15 for Native and Pretreated Switchgrass in Simons' Staining Measurement

Simons' Stain (SS) technique is a potentially useful semiquantitative methodology to estimate the available surface area of lignocellulosic substates. [201, 227] This methodology shows the potential to assess the effectiveness of pretreatments. Studies have employed a mixture of Direct Blue-1 and Direct Orange-15 to measure the behavior of adsorption on the pretreated biomass. Direct Blue-1 has a smaller molecular size and a weaker affinity for cellulose than Direct Orange-15. [201] When their mixture was applied to the cellulose sample, Direct Orange-15 molecules will preferentially be adsorbed on the cellulose surface than Direct Blue-1. While, the Direct Blue-1 molecules

tend to be adsorbed on the surface of small pores. This different behavior suggests the pore structure and the pore size population distribution of the cellulose samples.

The calculated results for the adsorbance of Direct Blue-1 and Direct Orange-15 for native and pretreated switchgrass (air-dried) are shown in Table 6.6 and Figure 6.4. The Simons' Stain adsorbances are 57, 41, 1.0 x 10^2, and 89 mg/g dry mass (O.D) for native leaves, native internodes, pretreated leaves, and pretreated internodes switchgrass (Figure 6.4). The results demonstrate that native leaf fractions of switchgrass adsorb 7 mg/g greater amount of DO-15 and 9 mg/g greater amount of DB-1 than internode fractions of switchgrass. Hydrothermal pretreatment improves the adsorbance of DO-15 and DB-1 significantly for both leaf and internode fractions. But the pretreated leaf fractions have 14 mg/g more DO-15 adsorbed after the pretreatment than the pretreated internode fraction. These experiments strongly suggest that pretreated leaf fractions of switchgrass have much better adsorption behavior than pretreated internode fractions of switchgrass.

Table 6.6 The adsorbance of Direct Blue-1 and Direct Orange-15 in Native and Pretreated Switchgrass SW2

sample	A_{DO} [228] mg/g	A_{DB} [228] mg/g	total mg/g	O/B
leaves SW2	34	23	57	1.5
internodes SW2	27	14	41	1.9
pre-leaves SW2	77	26	1.0 x 10^2	3.0
pre-internodes SW2	63	26	89	2.4

Note: A_{DO}, the adsorbance of Direct Orange-15; A_{DB}, the adsorbance of Direct Blue-1; O/B, ratio of the adsorbance of Direct Orange-15 to Direct Blue-1

The O/B value of pretreated switchgrass suggests that hydrothermal pretreatment increase the porosity of switchgrass. [201] This result also suggests the pretreated leaf has slightly greater porosity than pretreated internode fractions of switchgrass. Compared to the air-dried steam pretreated softwood substrate (SP) (A_{DO}, 43.4 mg/g; A_{DB}, 36.7 mg/g; total A, 88 mg/g; O/B, 1.18) , both pretreated leaf and internode have greater adsorbance. Chandra et al. demonstrated the study with Simons' staining experiment and enzymatic

saccharification of pretreated biomass. They suggested that Simons' staining technique can be a valuable diagnostic tool to estimate the available surface area of lignocellulosic substrates. [201] Studies in previous chapter suggest that pretreated leaves have about 30% greater cellulose-to-glucose yield than pretreated internodes after cellulases hydrolysis for 48 h. This could be interest to suggest that hydrothermal pretreated leaf fractions of switchgrass has greater available surface area than hydrothermal pretreated internode fractions of switchgrass because of the similarity on the chemical properties of pretreated leaf and internode fractions. These results also demonstrate that hydrothermally pretreated switchgrass has similar adsorption behavior to the never-dried steam pretreated softwood for Direct Blue-1 and Direct Orange-15.

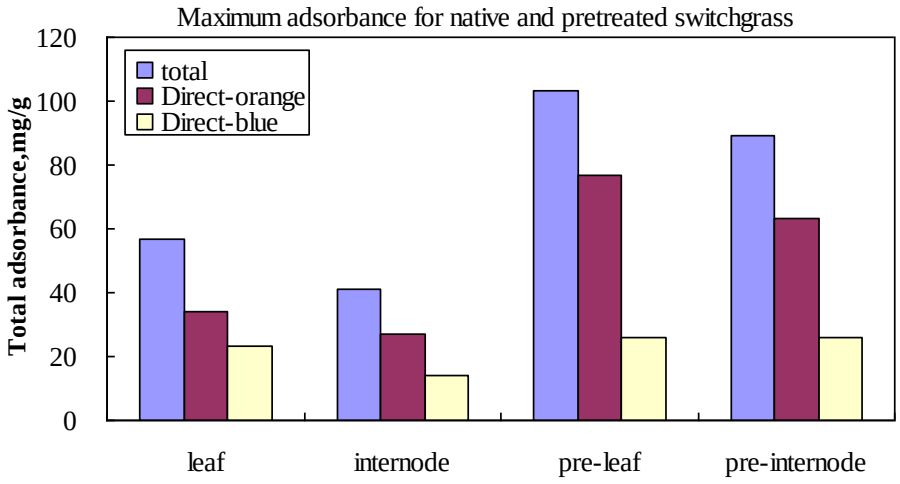

Figure 6.4 The adsorbance of Direct Blue-1 and Direct Orange-15 in Native and Pretreated Switchgrass SW2

Conclusion

Four populations of switchgrass SW1-SW8 were characterized by comparable biomass yield and digestibility after hydrothermal pretreatment. However, the results between leaves and internodes are significantly different after hydrothermal pretreatment. Hydrothermal pretreatment provides comparable gravimetric yields ranging from 48.1%

to 51.4%. The glucan retention yield is 77.2% and 67.3% for leaves and internodes of switchgrass after hydrothermal pretreatment. However, cellulose digestibility of the pretreated leaf portion of the switchgrass exhibits 32.5% greater glucose yield (77.4%) than that of the internode portion (44.0%). Hydrothermal pretreatment is characterized by large removal of hemicellulose, large retention yield of cellulose, reduction of the DP_w of cellulose, and increased digestibility of the pretreated switchgrass.

Through a systematic study on the effect of the cellulose structure and the DP of cellulose of organosolv pretreated Buddleja davidii, Hallac et al [80] suggest that lower DP of cellulose improves enzymatic hydrolysis due to two factors: (i) increasing the number of cellulose chain reducing ends; and (ii) making cellulose more reactive to the enzymes. The results demonstrate that pretreated leaves and internodes have similar chemical constituent profiles and chemical structure for cellulose and lignin but significant differences for the DP of α-cellulose. The Number of Reducing End F_{RE} calculated from the inversed value of the DP of cellulose has been suggested to be a factor contributing to the efficiency for cellulose hydrolysis by cellulases. [175] Väljamäe et al. has addressed that the fraction of reducing ends (F_{RE}) improve the exo-glucanase activity. [176] In the enzymatic hydrolysis, the increased reducing ends of cellulose generated by endo-glucanase accelerate the hydrolysis rate of exo-glucanase. [176] The value of F_{RE} for pretreated leaves is 23.4% greater than that for pretreated internodes. The difference in F_{RE} between the leaf and internode portions can be a factor contributing to the 33.9% greater cellulose digestibility for pretreated leaves than pretreated internodes. The lower DP of cellulose and greater F_{RE} for the pretreated leaf portion of the switchgrass SW2 are attributed to, in part, the enhanced cellulose digestibility in comparison with the internode portion in the present study.

The results in Simons Staining measurement of pretreated switchgrass SW2 suggest that the overall susceptibility of leaves is greater than that of internodes for the enzymatic hydrolysis. Hydrothermal pretreatment improves the adsorbance for leaves and

internodes in Simons' Stain method. Compared to the 33.9% greater cellulose-to-glucose yield for pretreated leaves, this result may provide suggestions that Simons' Stain adsorbance is one considerable factor garnered the degradation difference between pretreated leaves and internodes. Overall, the DP of cellulose and accessibility in Simons' Staining technique are suggested factors influencing the cellulose-to-glucose yield in pretreated leaves and internodes.

Although these investigations could conclude that the DP of cellulose in morphological fractions is a factor influencing the cellulose degradability in leaves and internodes after hydrothermal pretreatment, other factors may influence the degradability of cellulose in pretreated switchgrass. Cellulose structure and surface accessible area has been drawn much attention to be a factor influencing the degradability of pretreated biomass. This attribute to the degradation of morphological portions of switchgrass has been explored in the next chapter. Hydrothermal pretreatment is performed on the leaves and internodes of switchgrass SW9 to enhance the digestibility of cellulose towards cellulase. The structure of cellulose in the pretreated morphological fractions of switchgrass is explored in the next chapter.

CHAPTER 7

COMPARATIVE STUDIES ON HYDROTHERMAL PRETREATMENT AND ENZYMATIC SACCHARIFICATION OF LEAVES AND INTERNODES OF SWITCHGRASS SW9*

Introduction

Hydrothermal pretreatment, also called liquid hot water pretreatment, autohydrolysis, hot-water compression pretreatment, and hydrothermolysis, was a process to treat biomass at 160-240 ^{o}C using pure water. [229] It is an attractive process because of several advantages including: (1) use of water as a solvent; (2) significant hemicellulose removal and cellulose retention; and (3) improved digestibility of cellulose by cellulases. Typically, hydrothermal pretreatment of biomass is accomplished under mild, in-situ generated acidic conditions (i.e., pH 3-6) and it has been suggested that several reactions contribute to this effect including dissociation of water, acid-catalyzed hydrolysis of acetyl and other ester groups, and acid-catalyzed hydrolysis of ether linkages. [165] Because of the mild acidic hydrolysis conditions and heterogeneous features of biomass, hydrothermal pretreatment requires an elevated temperature to disrupt the rigid cell wall of biomass and improve subsequent enzymatic digestibility.

Hydrothermal pretreatment is suitable for a wide range of plant resources including hardwoods and grasses. [41, 230] The results of several studies suggested that the

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* This manuscript was accepted for publication in Bioresource Technology, 2011. It is entitled as "Comparative studies on hydrothermal pretreatment and enzymatic saccharification of leaves and internodes of Alamo switchgrass". The other author is Arthur J. Ragauskas from Institute of Paper Science and Technology and School of Chemistry and Biochemistry at Georgia Institute of Technology.

preferred conditions for hydrothermal pretreatment range from 160 to 240 ^{o}C with a 10-50 min reaction time to achieve maximum cellulose-to-glucose yield in subsequent enzymatic hydrolysis. [41, 45, 230] Fermentation studies reported on hydrothermal pretreated switchgrass had a 72% theoretic yield of ethanol from glucan by simultaneous saccharification and fermentation (SSF). [38] The fundamental chemistry of cellulose decomposition in hot compressed water has been reported frequently in the literature using microcrystalline cellulose as a model. [36] In these investigations, the amorphous portion of cellulose was preferentially hydrolyzed at pretreatment temperatures above 150 ^{o}C rather than the crystalline portion of cellulose which started to decompose at 180 ^{o}C. [36] In past hydrothermal investigations, it was also determined that a large portion of hemicellulose (~90%) in hardwood and grass biomass was hydrolyzed to low molecular oligomers and monomers. [41, 45, 230]

Switchgrass is a C4 perennial grass which has been studied as an energy crop in the United States for the last decade. [46] To reduce the recalcitrance of switchgrass for biofuel production, a variety of pretreatment technologies have been examined including biological, hydrothermal, dilute acid, alkaline, and ionic liquids pretreatment. [18, 38, 231, 232] These studies have been primarily focused on determining enzymatic hydrolysis and fermentation yields with optimized pretreatment conditions. The results indicated that these pretreatments significantly improve the digestibility (~60-96% cellulose-to-glucose yield) and fermentation yields (~70-80% theoretic ethanol yield) for switchgrass. Several studies have been conducted on the effect of pretreatment on the digestibility of different morphological portions of biomass. [30, 233] For example, Garlock et al. [233] studied the effect of ammonia fiber explosion pretreatment (AFEX) on the digestibility of corn stover fractions, leaf, stem, husk, and cob, through optimized conditions. Their results indicated that after AFEX pretreatment, the digestibility of the husk portion has the highest cellulose-to-glucose yield (~100%) compared to other fractions. Yang et al. [30] also conducted research on ethanol production of pre-frozen leaves and stems (including leaf

sheath) of switchgrass after a dilute sulfuric acid pretreatment. They reported that the leaf portion of switchgrass germplasms had the greatest cellulose-to-glucose yield (~ 100%) after sulfuric acid pretreatment and enzyme saccharification. Compared to the leaf portion of switchgrass germplasms, the stem portion of the sample is about 20% lower in cellulose-to-glucose yield.

To further reduce the recalcitrance of switchgrass, research has focused on the structural characterization of cellulose after diluted acid pretreatment. [102] They explored the ultrastructural changes of cellulose from switchgrass before and after dilute acid pretreatment by solid state CP/MAS ^{13}C-NMR. These results suggested that dilute acid pretreatment reduced the percentage of amorphous cellulose yielding a product with 18% more crystallinity. Although these investigations provide valuable information concerning the changes of ultrastructure and DP of cellulose during pretreatment process, the impact of these changes on enzymatic hydrolysis in morphological fractions of switchgrass has not yet been explored. In the present work, a fundamental study on hydrothermal pretreatment of two morphological portions, leaves and internodes, of switchgrass SW9 were investigated. The chemistry of hydrothermal pretreatment and its impact on the digestibility were explored in terms of DP and ultrastructure of cellulose.

Materials and Methods

Sample Preparation

Switchgrass sample SW9 harvested in the heading stage was grown at the University of Tennessee and received September, 2009. The samples SW9 were initially dried at 60 ^{o}C for 24 h followed by 48 h air dry at room temperature. The leaves (including blade and sheath) and internodes of switchgrass were manually separated and ground in a Wiley mill to pass through a 0.841 mm screen. The samples were then additionally sieved to achieve a final particle size between 0.297 mm and 0.707 mm. The

milled samples were Soxhlet-extracted with hot-water and benzene/alcohol (2/1, v/v) for 24 h, consequently. The extracted samples were initially air dried for 48 h and then vacuum-Oven Dried at 40 ^{o}C for 24 h yielding a material with less than 2% moisture.

Results and Discussion

Chemical Compositions of Switchgrass Feedstock

In chapter 5, the results on the chemical constituents of switchgrass indicate that the leaves portion of switchgrass have ~8% less cellulose content and ~3% greater lignin content than internode fractions. [213] In the present study, switchgrass samples SW9 were manually separated into leaves (36.4 wt% of whole biomass) and internodes (45.4 wt% of whole biomass). The chemical profiles of the leaf portion from switchgrass SW9 have 4.1% arabinan, 1.4% galactan, 41.2% glucan, 25.6% xylan, 19.1% lignin, 4.1% ash, and 2.8% AIA content. Compared to the chemical profile of the leaf portions, internode portion had 2.0% less arabinan, 0.8% less galactan, 6.8% more glucan and 4.2% more lignin content but 2.5% less ash and 2.4% less AIA content. These findings were in good agreement with the literature report on chemical compositions of switchgrass. [213]

Pretreatment of Switchgrass

In chapter 6, the study on hydrothermal pretreatment of boot stage switchgrass SW1-SW8 indicated that the leaf portion has greater cellulose digestibility than the internode portion after pretreatment. [234] In the prior study, boot switchgrass without Soxhlet extraction was used for hydrothermal pretreatment at 200 ^{o}C for 10 min under N_2 and resulted in 48.5% and 50.4% gravimetric yield of biomass for the leaves and internodes, respectively.

In the present study, switchgrass SW9 in the seeding stage was used for hydrothermal pretreatment with two morphological portions, leaves and internodes. The pH value of the aqueous switchgrass slurry decreased from 5.9 and 5.4 for leaves and

internodes at the start of pretreatment to mildly acidic pH values of 3.2 and 2.9 after pretreatment. The gravimetric yield of hydrothermal pretreated switchgrass was 38.2% for leaves and 56.3% for internodes portions. Table 7.1 provides the results for sugars and lignin analyses of the pretreated biomass. The percentages of removed components during hydrothermal pretreatment were calculated as the percentages of the mass of individual components in the native switchgrass and after hydrothermal pretreatment (Table 7.1). After pretreatment, most of the hemicelluloses are dissolved into the aqueous phase (i.e., 96.1% for leaves and 93.4% for internodes). This result is close to previous finding on boot switchgrass [213] and recent studies on switchgrass in the literature. [38]

Table 7.1 Chemical Composition Profiles of Hydrothermal Pretreated and Enzymatic Hydrolyzed Morphological Portions of Switchgrass SW9

sample	arabinan%	galactan%	glucan%	xylan%	lignin%
pretreated leaves	N/A[a]	0.1	65.4	3.1	29.6
removed%	N/A	97.3	39.7	95.4	41.1[b]
enzymatic treated leaves	N/A	N/A	36.4	2.4	43.8
removed%	-	-	66.0	52.7	9.6
pretreated internodes	0.2	N/A	71.2	3.2	32.6
removed%	94.6	N/A	16.5	93.1	18.8[c]
enzymatic treated internodes	0.2	N/A	64.9	3.7	32.3
removed%	-	-	23.2	1.2	19.0

Note: [a] N/A: not available; [b] Soluble lignin by UV 35.6%; [c] Soluble lignin by UV 18.5%

Macromolecular Structure Features of Switchgrass Cellulose

CP/MAS ^{13}C NMR spectroscopy was used to measure the Crystallinity Index of cellulose and the relative portions of the polymorphs for native and hydrothermal pretreated leaf and internode portions of switchgrass SW9. The spectra of the pretreated cellulose isolated from the leaf and internode fraction are comparable to the published result on switchgrass. [102] The signals can be assigned to the carbons in β-D-glucopyranosyl unit of cellulose according to the published literature values. [102] The C-4 region includes resonances attributed to crystalline/para-crystalline (δ 86-92 ppm) and

amorphous (δ 79-86 ppm) cellulose. [102] Employing line fitting methodologies, the ultrastructure forms of cellulose I_α, cellulose I_β, cellulose $I_{\alpha+\beta}$, para-crystalline cellulose, and cellulose in accessible and inaccessible surfaces were evaluated. Table 7.2 shows the results of the spectra fitting of C-4 region for native and pretreated cellulose from leaf and internode portions of switchgrass. The Crystallinity Index (CrI) of cellulose was determined by the integrated value of resonances representing the crystalline C-4 region (δ 92-86 ppm) and the entire C-4 region (δ 92-79 ppm). The CrI of cellulose from leaves and internodes showed similar results (48% vs. 46%). After hydrothermal pretreatment, the CrI of cellulose from leaves and internodes increased to 52% and 54%, respectively. These results were also consistent with other observations on CrI changes of cellulose after pH-controlled hot water pretreatment of poplar and acid pretreatment of switchgrass. [17, 102] After hydrothermal pretreatment, the structure of cellulose for both leaves and internodes changed significantly as summarized in Table 7.2. In brief, after pretreatment the cellulose is significantly increased in relative portion of paracrystalline cellulose by 25% and 9% for leaves and internodes, and decreased in relative portion of inaccessible fibril surface by 8% and 17% for leaf and internode portions of switchgrass. While the crystalline portions of cellulose in internodes and leaves are relatively unchanged after hydrothermal pretreatment, especially for leaf portion of switchgrass, a hydrolysis of amorphous cellulose and an enrichment of paracrystalline cellulose.

The Degree of Polymerization of cellulose is another factor suggested in the literature to influence the efficiency of enzymatic hydrolysis of cellulose. [175] Table 7.3 presented the results of molecular weight and Degree of Polymerization (DP) analysis of native and pretreated leaves and internodes. DP of native cellulose has been reported 2.97×10^3 and 2.93×10^3 for leaves and internodes. [235] The DP_w and the polydispersity index of cellulose are decreased for both the leaves and internodes cellulose. The DP of cellulose from the pretreated switchgrass decreases 65.7% for the leaves portion and 54.8% for the internode portion.

Table 7.2 Spectra Fitting Result of Native and Pretreated Cellulose from Leaves and Internodes of Switchgrass SW9

assignments	chemical shift (ppm)	FWHH (Hz)	relative Integrated intensity %				
			leaf	internode	pretreated leaf	pretreated internode	LSD%
cellulose I_α	89.7	90	2.1	1.2	1.4	2.2	ns[a]
cellulose I_α+I_β	89.0	91	7.9	5.2	4.7	6.1	1.7
para-crystall cellulose	88.8	241	37.6	39.9	47.3	43.6	1.8
cellulose I_β	88.1	135	1.5	1.4	2.4	2.0	ns
accessible fibril surface	84.5	100	4.7	2.0	2.8	2.6	1.0
inaccessible fibril surface	84.4	400	43.4	49.1	39.9	40.7	0.7
accessible fibril surface	83.6	95	2.8	1.2	1.4	2.8	1.1
Crystallinity Index%	-	-	48.9	46.6	52.9	54.1	1.7

Note: [a] ns: non significant

Table 7.3 Molecular Weight Distribution of Native and Pretreated Cellulose in the Leaves and Internodes of Switchgrass SW9

sample	M_n [a]	M_w [a]	DP_w [a]	PDI [a]
pretreated leaves	5.48×10^4	5.28×10^5	1020	9.6
pretreated Internodes	8.44×10^4	6.89×10^5	1330	8.2

Note: [a] Standard derivation: M_n, 0.11×10^4 g/mol; M_w, 1.20×10^4 g/mol; DP_w, 21; PDI, 0.3; DP_w, Reduction% 0.6%.

Enzymatic Hydrolysis of Hydrothermal Pretreated Switchgrass

Ethanol production from lignocellulosic biomass requires that cellulose can be converted into glucose and then fermented into ethanol. Enzymatic hydrolysis of cellulose to glucose is used to evaluate the efficiency of converting cellulose into ethanol.[236] In the present study, a mixed enzymatic system involving cellulase and cellobiase was used to test the digestibility of native and pretreated leaves and internodes. The cellulose-to-glucose yield of biomass after enzymatic hydrolysis of pretreated leaves and internodes (air-dried) is 60.5% and 26.7% for leaves and internodes with the hydrolysis for 48 h.

To explain the different digestibility of pretreated leaves and internodes, the chemical profiles of biomass (Table 7.1) and structure of pretreated cellulose was examined after enzymatic hydrolysis. Hydrothermal pretreated internodes contain 3%

more lignin content, 5.8% greater cellulose content, and 1.5% less ash content than leaves portion. The removed percentages of chemical components after enzymatic hydrolysis have also been calculated according to the yield of enzymatic hydrolysis and the content of chemical components (Table 7.1). The results indicate that 66.0% glucan of pretreated leaf portion is removed by enzymatic hydrolysis which is 42.8% greater than that in internode portions (23.2% glucan). Recent pretreatment investigations of *Buddleja davidii* indicated that the removal of lignin and hemicellulose, the reduction in DP of cellulose, and the change of ultrastructure of cellulose were among the factors that influenced the digestibility of pretreated biomass. [175] In the present study, however, both the pretreated leaf and internode portions of switchgrass have comparable lignin and hemicellulose content. The polymorphs of the pretreated cellulose in the pretreated leaf and internode portions of switchgrass are also similar whereas the DP of the cellulose in the leaf portion is 23.4% lower than that in the internode portion. Considering the similarity in ultrastructure of cellulose and chemical profiles between pretreated leaves and internodes, the change in the DP of cellulose appears to be a contributing factor, in part, influencing the digestibility of pretreated leaf and internode portions of switchgrass.

Conclusion

Hydrothermal pretreatment was performed on the leaves and internodes portions of switchgrass SW9 to enhance the digestibility of cellulose towards cellulase. Extractives free leaves portion provides 18.1% lower percentage of the pretreatment gravimetrical yield and 33.8% greater percentage of the cellulose-to-glucose yield than internodes portion.

The significant improvement of enzymatic hydrolysis yield in pretreated leaves is related to the structure of cellulose in this study. The Degree of Polymerization (DP) and

ultrastructure of cellulose were determined by gel-permeation chromatography and solid-state Cross Polarization/Magic Angle Spinning ^{13}C NMR experiments. The results suggest that hydrothermal pretreatment hydrolyzes amorphous cellulose and yields a product enriched in paracrystalline cellulose. Furthermore, the DP of cellulse is reduced to one third of the origin value after hydrothermal pretreatment. The resulting biomass after pretreatment for leaves and internodes has similar cellulose ultrastructure and chemical profiles. The results of the enzymatic hydrolysis studies of cellulose suggest that the reduced DP of cellulose of pretreated switchgrass is an important factor influencing the enhanced digestibility of pretreated switchgrass.

The results of this study indicate that hydrothermal pretreatment modifies the structure of cellulose at 200 ^{o}C for 10 min including a reduction of the DP_w of native cellulose by 65% and 54%, an increase in cellulose crystallinity by 8% and 16%, which is primarily due to an increase in the paracrystalline component for the leaf and internode portion, respectively. Although the decrease in bulk switchgrass cellulose DP after autohydrolysis has been reported, this is the first study to document differences in morphological portions of the switchgrass SW9. Furthermore, it has been extensively documented that a lower cellulose DP provides more reducing ends which enhances the overall deconstruction properties by exo-glucanase. Hence, these results suggest that the lower DP of pretreated cellulose in pretreated leaves may be a contributing factor contributed to 33.8% greater cellulose-to-glucose yield than the pretreated internode portion of the switchgrass SW9.

CHAPTER 8

OVERALL CONCLUSIONS

The thesis study started with the idea of investigating switchgrass as a potential feedstock for fuels, chemical, and energy. The chemical and physical properties of four population samples SW1-SW8 and their morphological components—leaves, internodes, and nodes—were studied in Chapter 4. The most significant differences between these switchgrass variants are in the levels of ash and lignin content. Switchgrass SW1-SW2 contains the lowest amount of lignin. The chemical and structural results for three morphological portions of switchgrass—leaves, internodes, and nodes—are significantly varied. The leaf portion contains the highest amount of arabinose, galactose, lignin, and ash. In addition, the leaves also have the lowest S:G ratio and glucose content. The lignin and glucose contents differ by 3.4% and 8.7%, respectively, among three morphological portions. These studies provide a general database on the variations of chemical profiles for morphological portions of switchgrass. Further investigations have been involved to understand the fundamental chemistry of these components in different morphological fractions so that the process of utilizing switchgrass can be under chemical and economical control.

In chapter 5, comparative studies between the leaf and internode portions of switchgrass were performed by compositional analysis and structural determination. Switchgrass SW9 was separated into morphological sections to allow for the determination of the chemical and structural properties of each morphological portion. The results indicate that the leaves and internodes differ chemically in the amounts of inorganic elements, hot-water extractives, benzene/ethanol extractives, carbohydrates, and lignin content. Leaves of switchgrass have 0.7 MJ kg^{-1} lower HHV but 1060 mg/kg greater in total halogen content and 10640 (mg/kg biomass) greater in inorganic elements

content. These results suggest that leaf fractions are less suitable for bioenergy production because of greater amounts of inorganic elements and lower HHV than internode fractions of switchgrass. Although the basic chemical constituents such as extractives, mineral inorganic elements, carbohydrates, and lignin differ in quantity and quality between the leaves and internodes of the switchgrass SW9, the ultrastructure of isolated cellulose is comparable between leaves and internodes. Ball-milled lignins isolated from leaves and internodes are found to have H/G/S ratios of 12.4/53.9/33.7 and 8.6/54.8/36.6. The lignin content of extracted internodes has ~5% more than that of extracted leaves. The difference in lignin content and the structure of lignin could be a factor influencing the degradability in morphological fractions of switchgrass. These observations enhance the thesis hypothesis that morphological fractions of switchgrass as a factor influence the utilization of switchgrass for biofuels, chemicals, and energy.

Pretreatment is one of the key technologies currently required for the production of cellulosic biofuel. In Chapter 6, four populations of switchgrass SW1-SW8 (both the leaf and internode portions) were hydrothermally pretreated without extraction to improve the digestibility of the switchgrass. The switchgrass SW1-SW8 are characterized by comparable biomass yields and levels of enzymatic digestibility after hydrothermal pretreatment. However, the switchgrass leaves and internodes perform differently after hydrothermal pretreatment. The hydrothermal pretreatment removes a large portion of the hemicellulose, retains a significant portion of the cellulose, reduces the DP_w of cellulose, and increases the reducing end of cellulose F_{RE} to improve the digestibility of the switchgrass. The lower DP of cellulose and greater F_{RE} for the pretreated leaf portion of switchgrass SW2 are attributed to, in part, the enhanced cellulose digestibility in comparison with the internode portion in the present study. The hydrothermal pretreatment improves the accessibility of leaves and internodes in Simons' Staining measurement. The results suggest that the pretreated leaves have greater accessibility to enzymes than the pretreated internodes. These results also suggest that the lower DP_w and

Simons' staining accessibility of the cellulose may have contributed to the 33.9% greater cellulose-to-glucose yield in the pretreated leaves than that in the pretreated internodes. Other factors may influence the degradability of cellulose in pretreated switchgrass. Cellulose structure has been drawn much attention as factors influencing the degradability of pretreated biomass. This attribute to the degradation of the morphological portions of switchgrass has been explored in Chapter 7.

In Chapter 7, hydrothermal pretreatment is performed on the leaves and internodes of switchgrass SW9 to enhance the digestibility of cellulose towards cellulases. The chemistry of hydrothermal pretreatment and its impact on the digestibility of switchgrass were explored for the DP_w and ultrastructure of cellulose. The results of this study indicate that hydrothermal pretreatment modifies the structure of cellulose. These modifications include a reduction of the DP_w of native cellulose by 66% in the leaves and 55% in the internodes, an increase in cellulose crystallinity by 8% in the leaves and 16% in the internodes, and an increase in the paracrystalline component for both morphological portions. These results suggest that the lower DP_w of the cellulose may contribute to the 33.8% greater cellulose-to-glucose yield in the pretreated leaves than that in the pretreated internodes.

In conclusion, switchgrass biomass is a plant resource with a wide variation among morphological portions. The lignin and cellulose have similar chemical structure. The morphological portions, leaves and internodes, are modified comparably in chemical profiles by hydrothermal pretreatment, but ultimately have different levels of DP_w cellulose, and Simons' Staining adsorbance after hydrothermal pretreatment. The heterogeneous features of the switchgrass can provide potential in that different morphological portions will be of benefit in future biofuel/biopower applications.

CHAPTER 9

RECOMMENDATIONS FOR FUTURE WORK

Several other studies might be conducted to further understand the effect of hydrothermal pretreatment technologies on the morphological portions of switchgrass as well as optimization of bioethanol production. Some particularly attractive options are as follows:

The current thesis study concludes that the differences in release in sugars after autohydrolysis could related to the cell wall structure needs to be strengthened by optical/SEM investigations of the plant cell wall structure of fractionated switchgrass before and after autohydrolysis.

Thesis studies provide initial observation of chemical profiles variation of switchgrass among population, morphology, and growth stage. However, to give a conclusion on the variation of switchgrass is required to have much greater sample size and variation region to test the hypothesis about the changes of chemical profiles related to populations, growth stages, and morphology.

APPENDIX A

SUPPLEMENTARY DATA FOR CHAPTER 5

Line Fitting Spectra of Cellulose for Native Leaves and Internodes of Switchgrass SW9

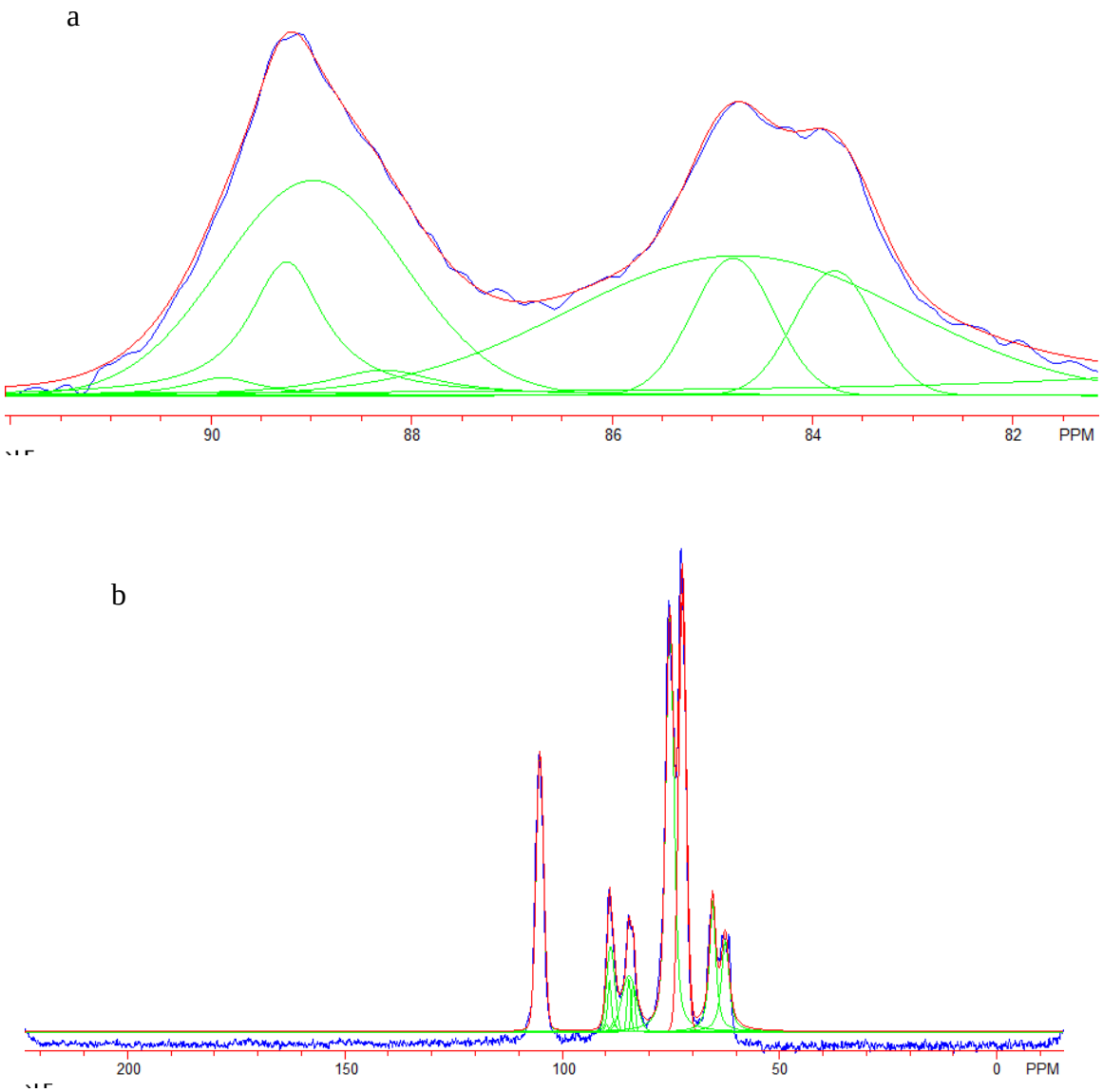

Figure A.1 Line Fitting Spectra of Cellulose for Native Leaves of Switchgrass SW9 (a) a C-4 Region of the Spectrum; (b) the Whole Spectrum

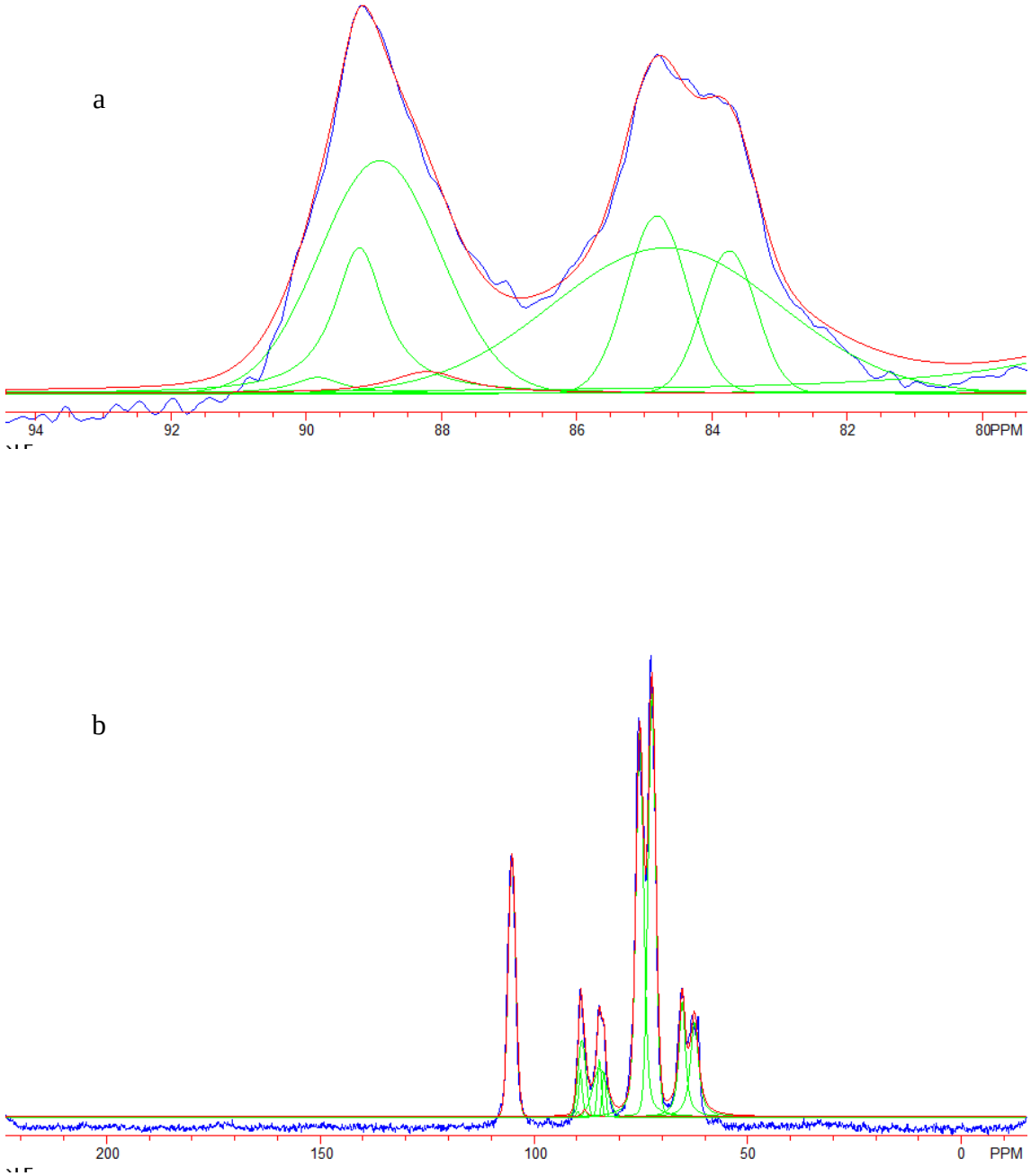

Figure A.2 Line Fitting Spectra of Cellulose for Native Internodes of Switchgrass SW9 (a) a C-4 Region of the Spectrum; (b) the Whole Spectrum

Gel Permeate Chromatography Spectra of Derivative α-Cellulose for Native Leaves and Internodes of Switchgrass SW2

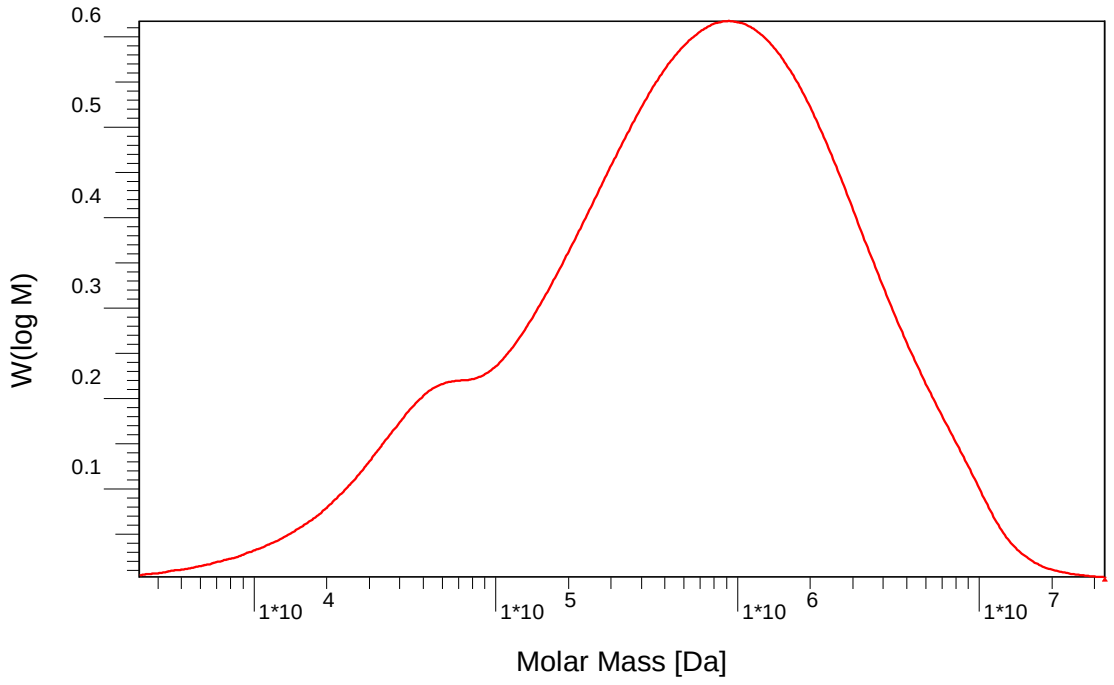

Figure A.3 Gel Permeate Chromatography Spectrum of Derivative α-Cellulose for Native Leaves of Switchgrass SW2

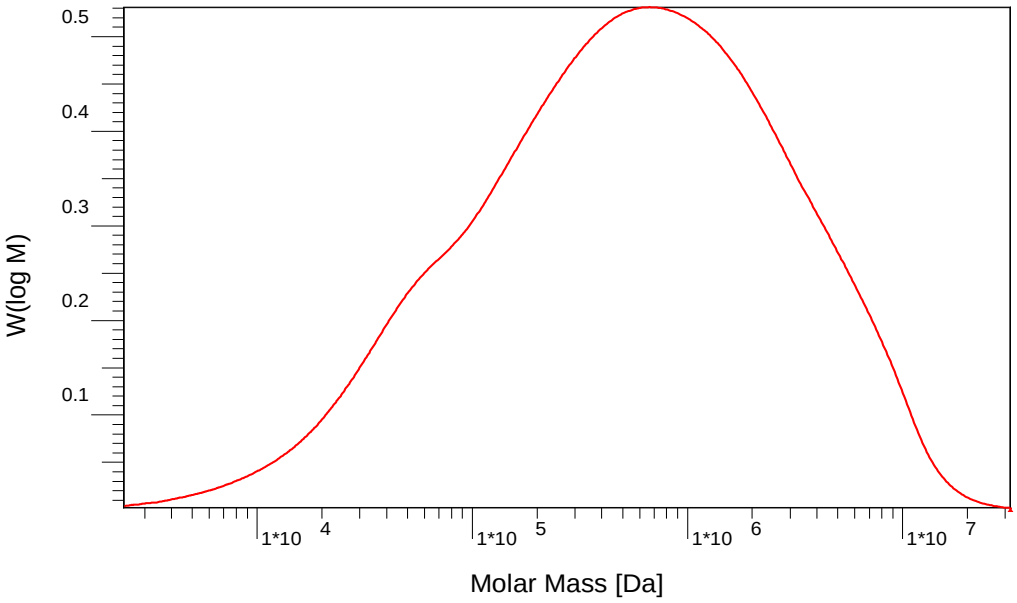

Figure A.4 Gel Permeate Chromatography Spectrum of Derivative α-Cellulose for Native Internodes of Switchgrass SW2

APPENDIX B

SUPPLEMENTARY DATA FOR CHAPTER 6

FT-IR Spectra of Native and Pretreated Leaves and Internodes of SW2

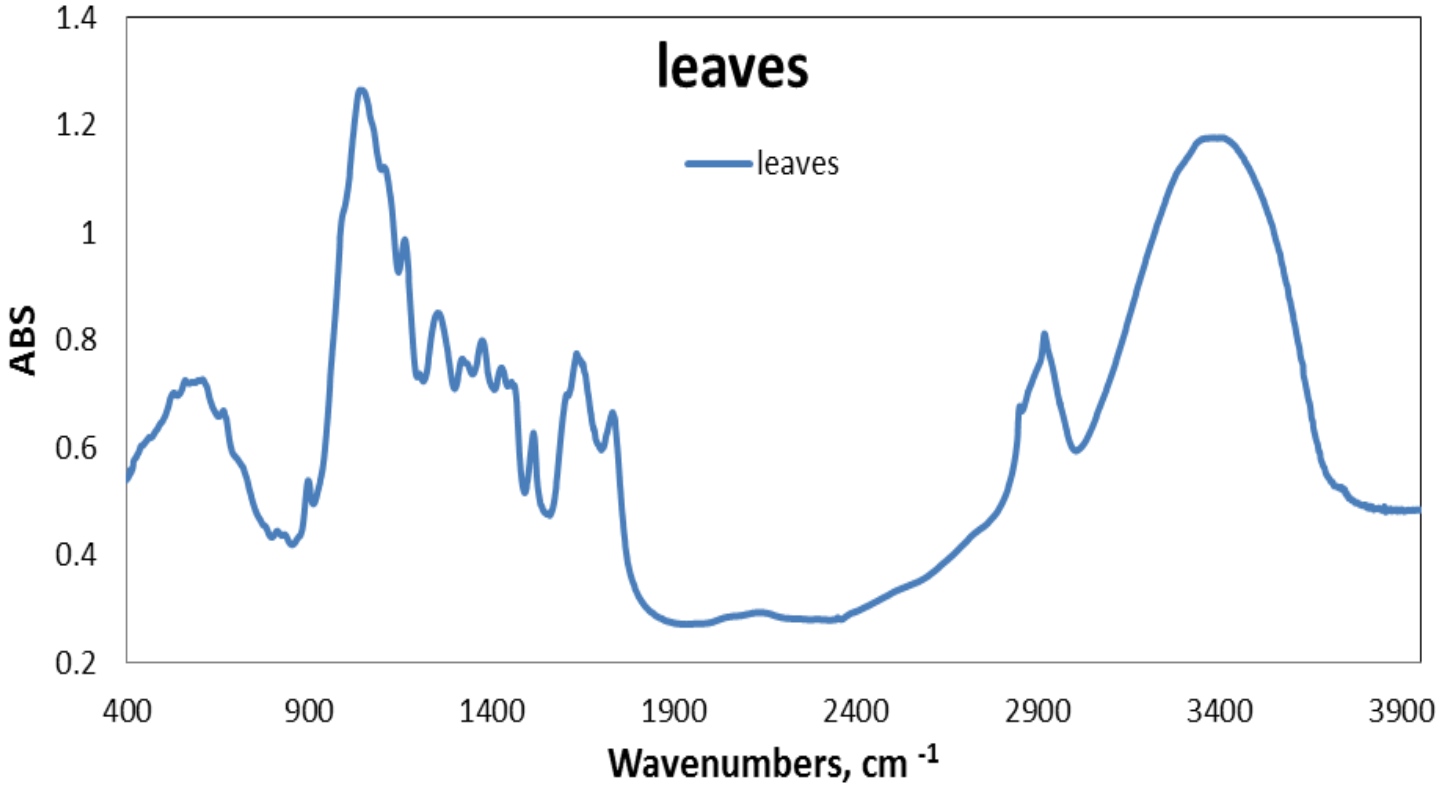

Figure B.1 FT-IR Spectrum of Native Leaves of Switchgrass SW2

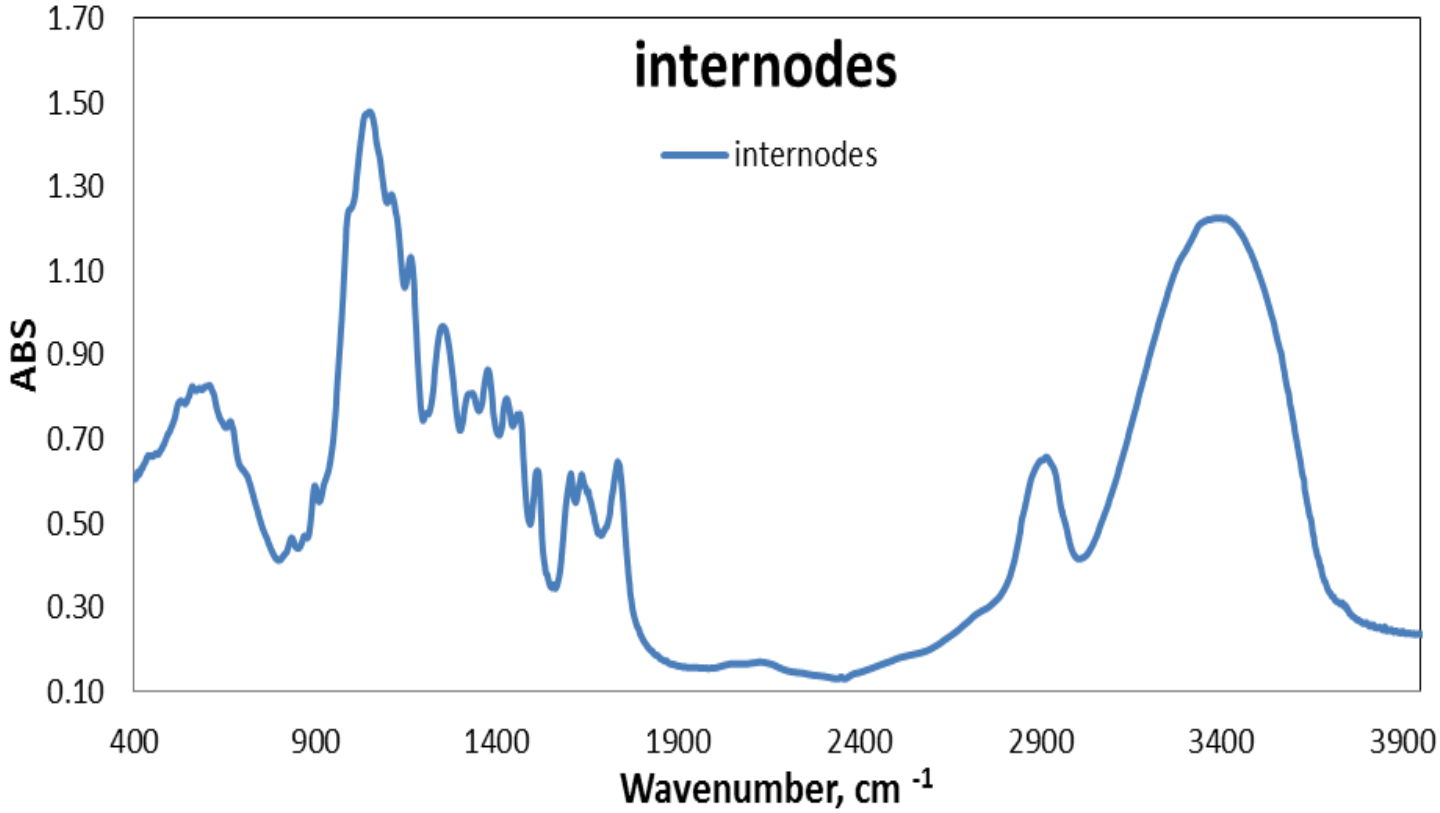

Figure B.2 FT-IR Spectrum of Native Internodes of Switchgrass SW2

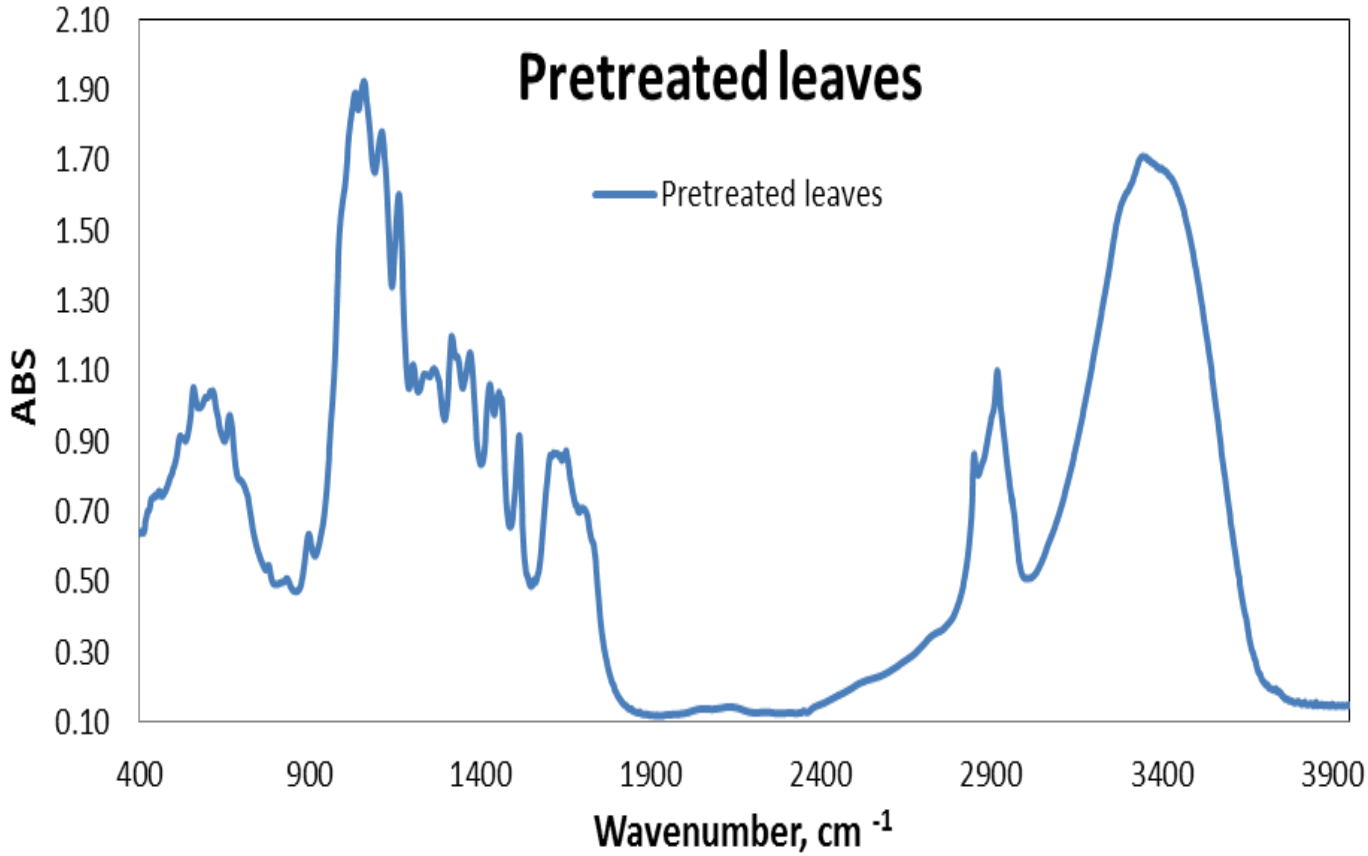

Figure B.3 FT-IR Spectrum of Leaves of Switchgrass SW2 after Hydrothermal Pretreatment

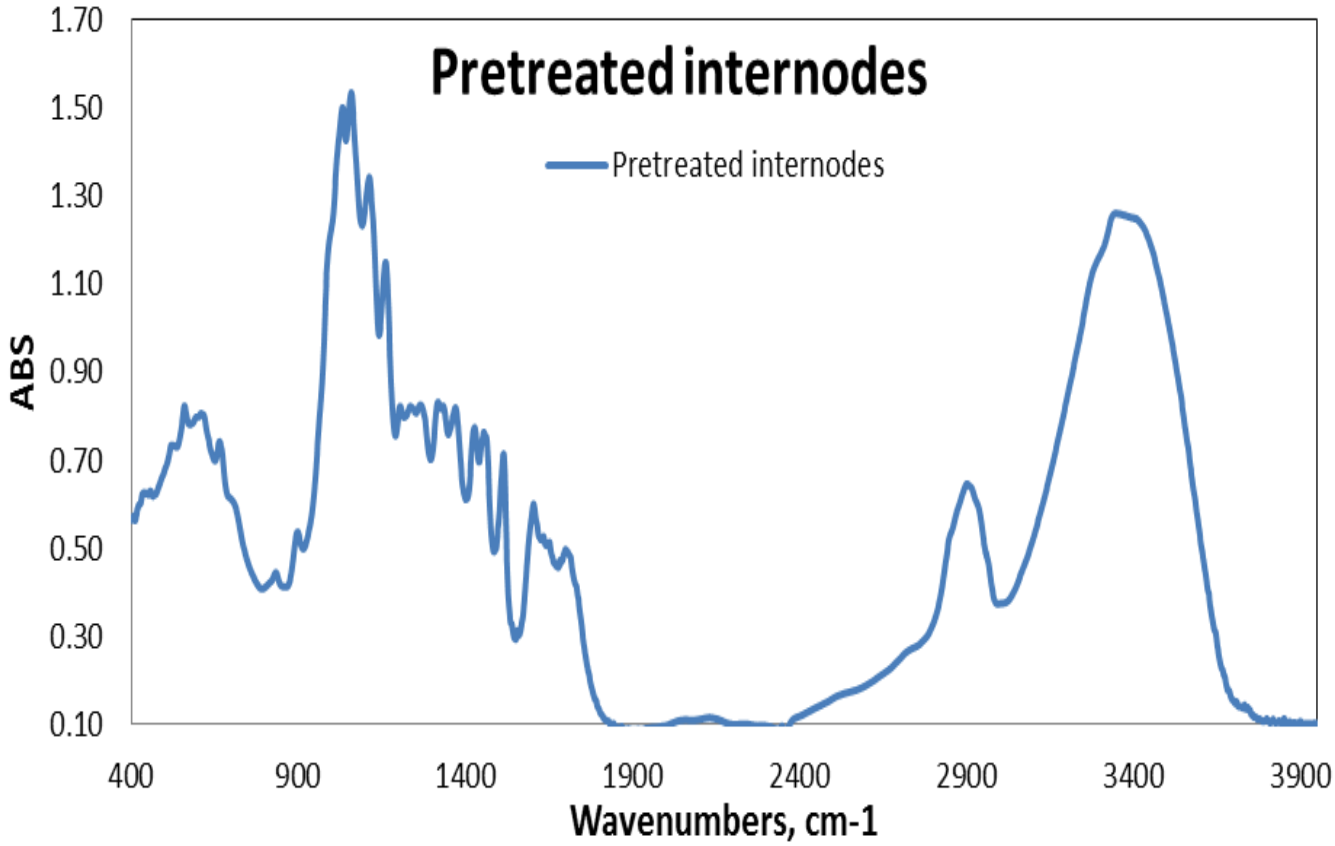

Figure B.4 FT-IR Spectrum of Internodes of Switchgrass SW2 after Hydrothermal Pretreatment

APPENDIX C

SUPPLEMENTARY DATA FOR CHAPTER 7

Line Fitting Spectra of Cellulose for Leaves and Internodes of Switchgrass SW9 after Hydrothermal Pretreatment

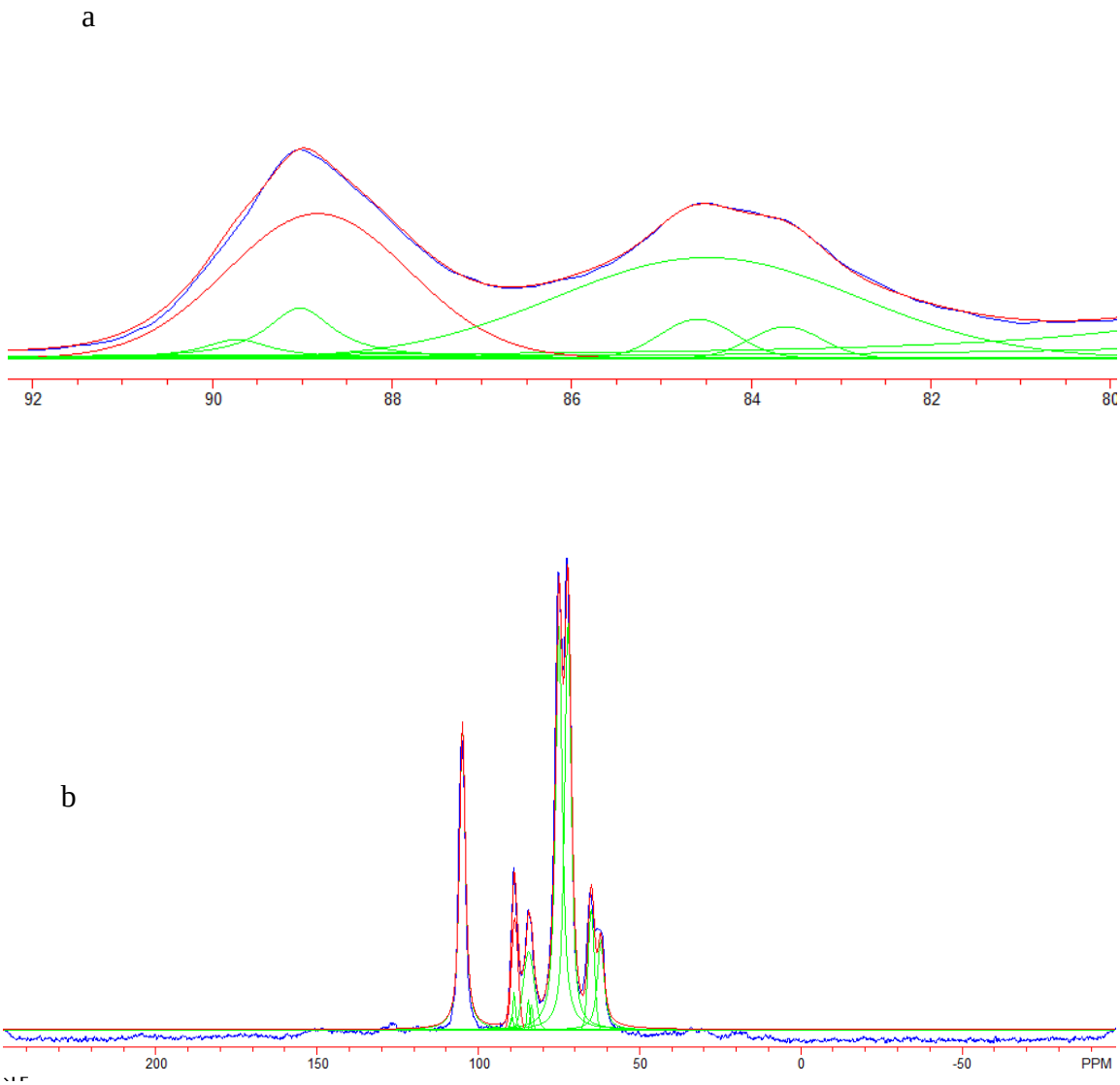

Figure C.1 Line Fitting Spectra of Cellulose for Leaves of Switchgrass SW9 (a) a C-4 Region of Spectrum; (b) the Whole Spectrum

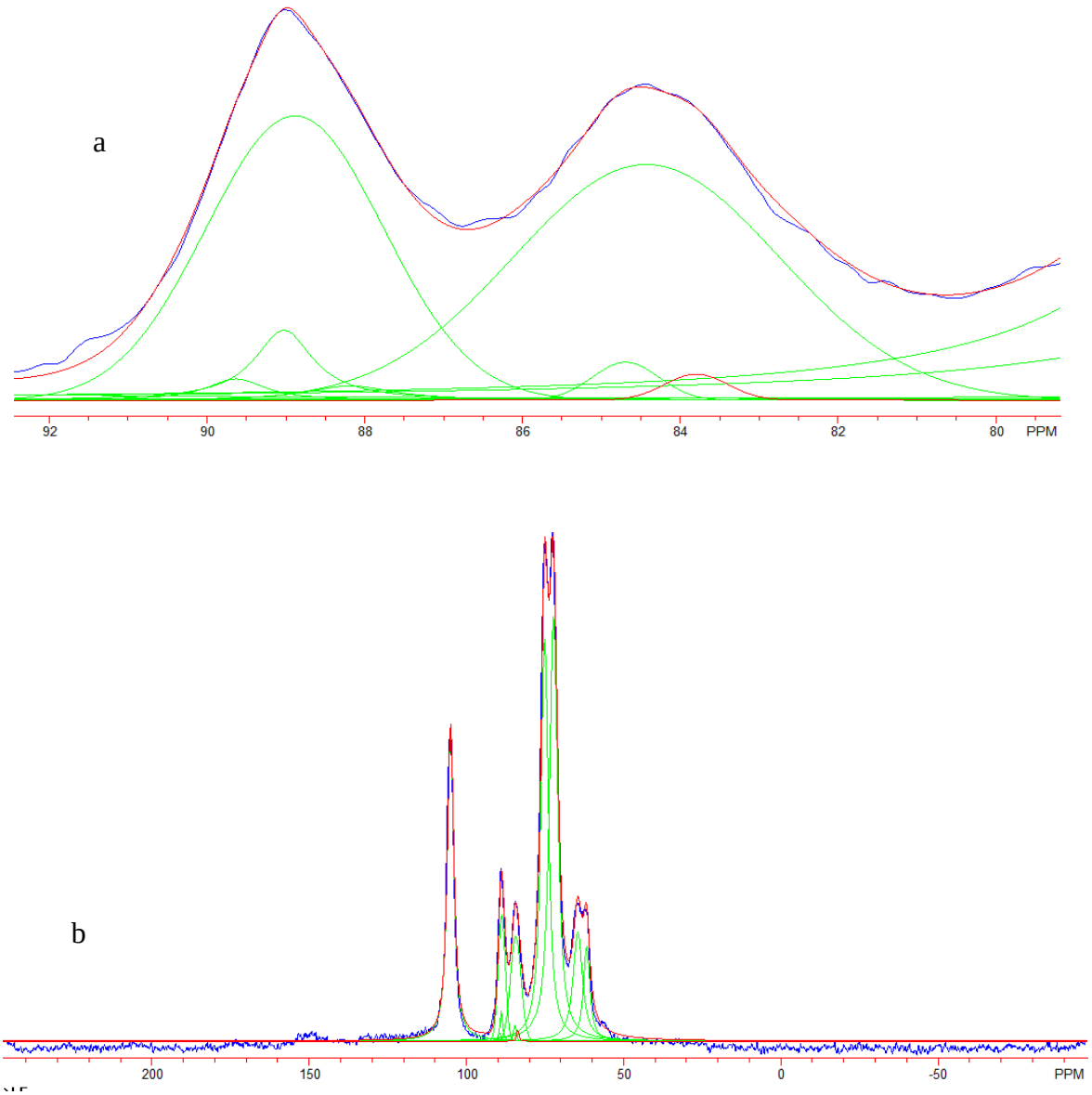

Figure C.2 Line Fitting Spectra of Cellulose for Internodes of Switchgrass SW9 (a) a C-4 Region of Spectrum; (b) the Whole Spectrum

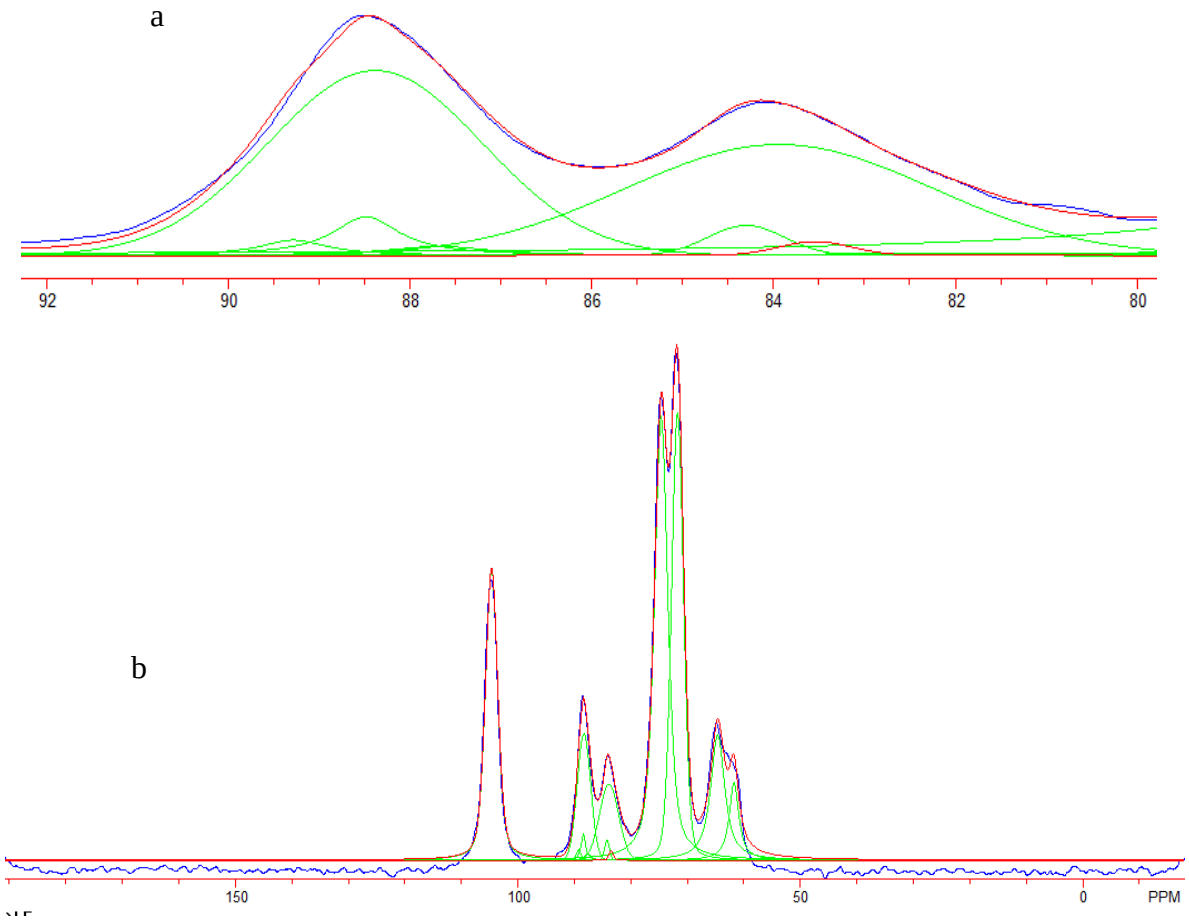

Figure C.3 Line Fitting Spectra of Cellulose for Leaves of Switchgrass SW9 after Hydrothermal Pretreatment (a) a C-4 Region of Spectrum; (b) the Whole Spectrum

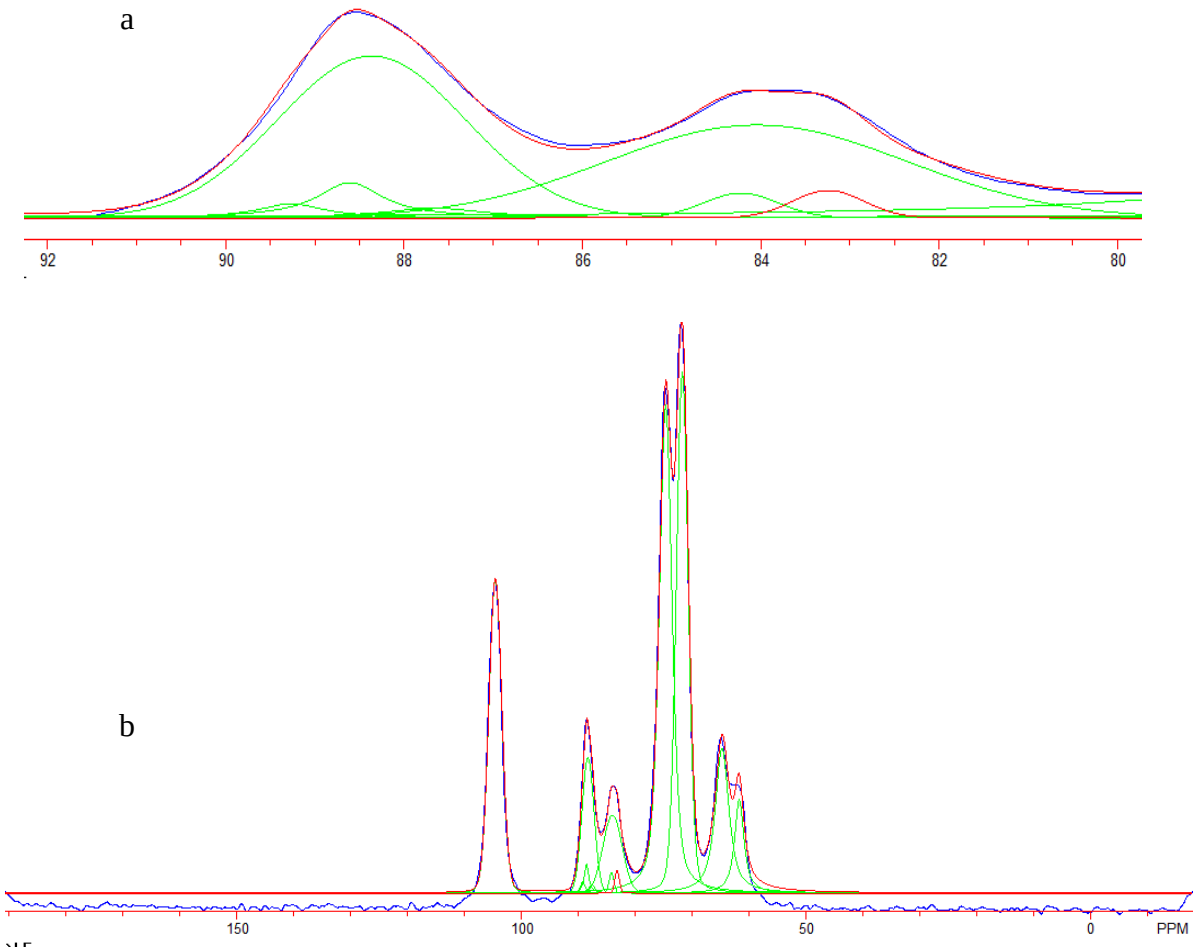

Figure C.4 Line Fitting Spectra of Cellulose for Internodes of Switchgrass SW9 after Hydrothermal Pretreatment (a) a C-4 Region of Spectrum; (b) the Whole Spectrum

Gel Permeate Chromatography Spectra of Derivative α-Cellulose for Leaves and Internodes of Switchgrass SW9 after Hydrothermal Pretreatment

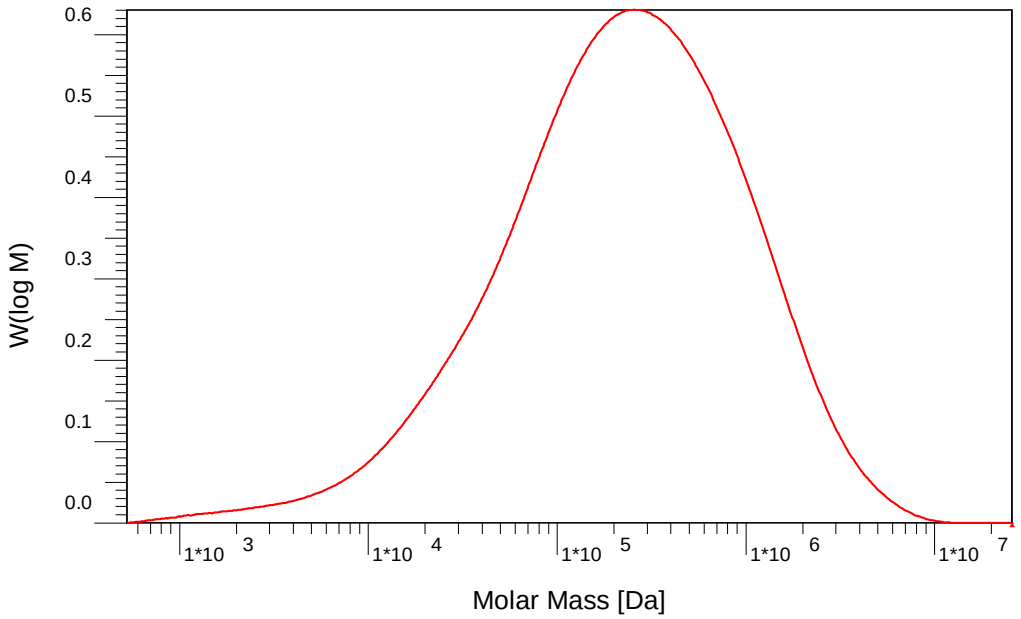

Figure C.5 Gel Permeate Chromatography Spectrum of Derivative α-Cellulose for Leaves of Switchgrass SW9 after Hydrothermal Pretreatment

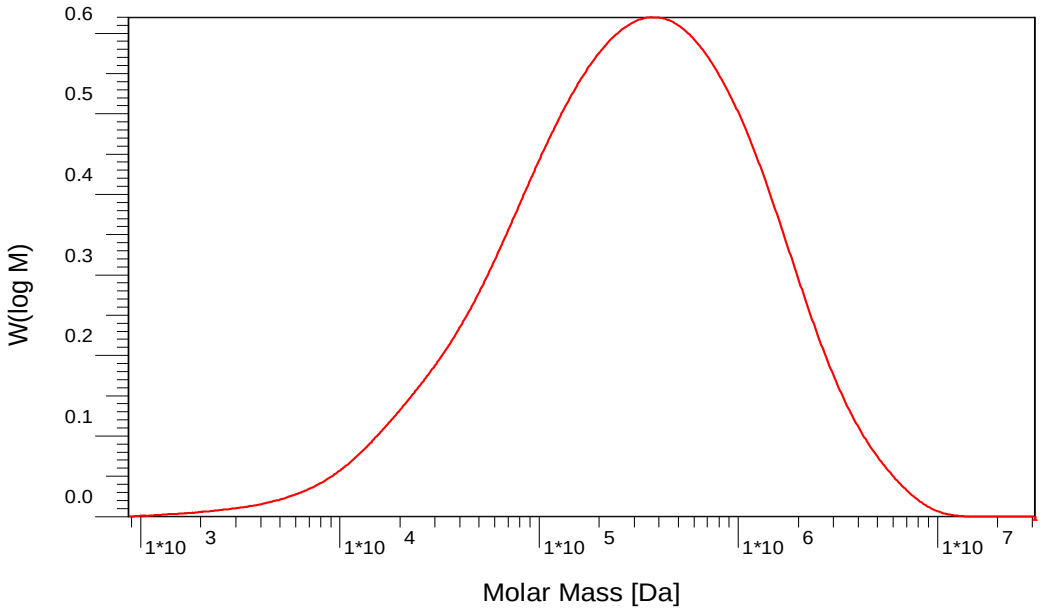

Figure C.6 Gel Permeate Chromatography Spectrum of Derivative α-Cellulose for Internodes of Switchgrass SW9 after Hydrothermal Pretreatment

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