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Enshi Liu
University of Kentucky, enshi.liu@uky.edu
Author ORCID Identifier:
http://orcid.org/0000-0002-3479-7690
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Enshi Liu, Student

Dr. Jian Shi, Major Professor

Dr. Donald G. Colliver, Director of Graduate Studies

FRACTIONATION AND CHARACTERIZATION OF LIGNIN STREAMS FROM GENETICALLY ENGINEERED SWITCHGRASS

THESIS

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science in Biosystems and Agricultural
Engineering in the College of Engineering at the University of Kentucky

By

Enshi Liu

Lexington, Kentucky

Director: Dr. Jian Shi, Assistant Professor of Biosystems and Agricultural Engineering Department

Lexington, Kentucky

2017

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ABSTRACT OF THESIS

FRACTIONATION AND CHARACTERIZATION OF LIGNIN STREAMS FROM GENETICALLY ENGINEERED SWITCHGRASS

Development of biomass feedstocks with desirable traits for cost-effective conversion is one of the main focus areas in biofuels research. As suggested by techno-economic analyses, the success of a lignocellulose-based biorefinery largely relies on the utilization of lignin to generate value-added products, i.e. fuels and chemicals. The fate of lignin and its structural/compositional changes during pretreatment have received increasing attention; however, the effect of genetic modification on the fractionation, depolymerization and catalytic upgrading of lignin from genetically engineered plants is not well understood. This study aims to fractionate and characterize the lignin streams from a wild-type and two genetically engineered switchgrass (*Panicum virgatum*) species (low lignin content with high S/G ratio and high lignin content) using three different pretreatment methods, i.e. dilute sulfuric acid, ammonia hydroxide, and aqueous ionic liquid (cholinium lysinate). The structural and compositional features and impact of lignin modification on lignin-carbohydrate complex characteristics and the deconstruction of cell-wall compounds were investigated. Moreover, a potential way to upgrade low molecular weight lignin to lipids by *Rhodococcus opacus* was evaluated. Results from this study provide a better understanding of how lignin engineering of switchgrass influences lignin fractionation and upgrading during conversion processes based on different pretreatment technologies.

KEYWORDS: lignocellulose, lignin characterization, lignin modification, genetically engineered feedstocks, pretreatment

Enshi Liu

April 4, 2017

FRACTIONATION AND CHARACTERIZATION OF LIGNIN STREAMS FROM GENETICALLY ENGINEERED SWITCHGRASS

By

Enshi Liu

Dr. Jian Shi
Director of Thesis

Dr. Donald G. Colliver
Director of Graduate Studies

April 4, 2017

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CHAPTER 1: INTRODUCTION

1.1 Research Introduction

In response to the environmental, global climate change, energy security and economic stability concerns, it is increasingly clear that the urgency of developing renewable energy cannot be ignored (Obama, 2017). Among different efforts aimed towards shifting our dependence from petroleum-based fuels and chemicals to sustainable alternatives, biorefining of lignocellulosic biomass has become one of the promising approaches (Lynd et al., 2008; Simmons et al., 2010; Tuck et al., 2012). However, the economic benefits of biomass-derived energy have not been fully achieved, partially due to the processing costs of overcoming the recalcitrance of lignocellulosic biomass. Thus, development of biomass feedstocks with desirable traits for cost-effective conversion and development of efficient pretreatment/fractionation methods are required in biofuels research.

In the current biorefinery concept, the structural carbohydrates in biomass feedstocks are used to produce renewable liquid fuels, such as ethanol and butanol; while lignin is generally considered a waste product during biomass processing (Wyman, 2007; Wyman et al., 2005). However, as suggested by techno-economic analyses, the success of a lignocellulosic biorefinery largely relies on lignin valorization to generate advanced fuels, chemicals, or materials. Lignin, as the most abundant aromatic biopolymer in nature and a major component in lignocellulosic biomass is underutilized in the current biorefining industry (Beckham et al., 2016; Ragauskas et al., 2014).

Genetic modification of plants has been demonstrated to be an efficient way to improve crop yield and stress resistance (Lawlor, 2013; Ricroch & Hénard-Damave, 2016). Extensive studies with the goal of reducing lignocellulosic biomass recalcitrance through genetic modification of the feedstocks have been conducted (Chen & Dixon, 2007; Fu et al., 2011). In particular, genetic modification of lignin biosynthetic pathways has received increasing attention. The recalcitrance of biomass feedstocks can be potentially reduced by several lignin manipulation methods, such as reduction of lignin

content, alternation of lignin subunit ratio and lignin deposition, and redesigning of lignin inter-unit linkages (Beckham et al., 2016; Hisano et al., 2011).

Switchgrass (*Panicum virgatum*) is a promising native energy crop in North America, due to its high productivity, low energy and fertilizer input requirements, and its ability to grow on marginal land (Debolt et al., 2009). Development of genetically engineered switchgrass species for better biorefining efficiency has become one of the major foci of the biofuels research community. Biomass pretreatment is currently a crucial step to overcoming the recalcitrance of lignocellulosic feedstocks thus making them more amenable to biological conversion by breaking down cell wall structure and unlocking sugars for fermentation. A number of thermochemical pretreatment methods are being developed, using solvents such as hot water, dilute acid, alkaline, organosolv, and ionic liquid (IL), to deconstruct biomass feedstocks (Dale et al., 1996; Shi et al., 2013; Wyman et al., 2011). The effect of lignin modification on fractionation, characterization, depolymerization, and upgrading of lignin in genetically engineered feedstocks is not well studied and therefore is the focus of this study.

This study aims to fractionate and characterize the lignin streams from a wild-type and two genetically engineered switchgrass species, one with low lignin content with high S/G ratio (*4CL*) and the other with high lignin content (*AtLOV1*), using three different pretreatment methods, i.e. 1% w/w sulfuric acid, 10% v/v ammonia hydroxide, and 10% w/w aqueous IL (cholinium lysinate). The visual illustration of untreated and pretreated switchgrass was obtained by scanning electron microscopy (SEM). Mass balances for different types of switchgrass were employed to determine the mass flow of major biomass components during different pretreatment processing. The molecular weight distribution of the lignin fractions recovered from both liquid and solids streams after pretreatment and enzymatic saccharification were determined by gel permeation chromatography (GPC). The abundance of lignin subunits and the cleavage of lignin inter-unit linkages were identified by ^{1}H-^{13}C HSQC NMR. The structural changes and chemical variations for wild-type and engineered switchgrass was investigated by Fourier transform infrared spectroscopy (FTIR). The thermal stability of switchgrass, before and after pretreatment, was illustrated by differential scanning calorimetry (DSC). In

addition, bioconversion of low molecular weight lignin to lipids by *Rhodococcus opacus* was preliminarily developed. Collectively, results from this study provide a better understanding of how lignin modification of switchgrass influences lignin fractionation, characterization, and upgrading during conversion processes based on different pretreatment technologies.

1.2 Research Motivations

Biofuels research has been focused on developing cost-effective processes for downstream upgrading, such as polysaccharides hydrolysis and fermentation. However, utilization of lignin to generate advanced fuels and value-added chemicals, which is considered as a critical portion in lignocellulose-based biorefinery, is not fully developed. Recently, the structural and compositional changes of lignin fractions during pretreatment have received increasing attentions. Nevertheless, how genetic modification of biomass feedstocks can affect lignin depolymerization and upgrading is still not well understood. Moreover, development of pretreatment methods for lignin fractionation and depolymerization with high efficiency and selectivity is essential for a biorefining process. Among different pretreatment methods, ionic liquid pretreatment has been considered as a highly efficient approach to fractionate lignocellulosic biomass. However, the application of ionic liquid pretreatment has been limited, partially due to the high cost of ionic liquid and its recycling process.

To bridge the gaps, we conducted studies on fractionation and characterization of lignin streams from wildtype and two genetically engineered switchgrass using three pretreatment methods to determine the effects of lignin modification on characteristics of lignin streams. Additionally, we tested a novel aqueous IL pretreatment method based on a biocompatible IL to fractionate and pretreat biomass feedstock. Utilizing dilute IL in water could reduce the viscosity, eliminate gel formation during pretreatment process, and reduce the cost of IL and its recycling. Furthermore, the biocompatibility of aqueous IL provides a potential way to upgrade lignin by biological catalysts in an aqueous phase. A side-by-side comparison was carried out on aqueous IL pretreatment, dilute acid and ammonia hydroxide pretreatment in terms of pretreatment efficiency, lignin depolymerization and characterization.

1.3 Research Hypothesis

The three hypotheses proposed for this study are as follows:

1) Biomass recalcitrance could be reduced by reducing lignin content and increasing the S/G ratio of genetically engineered switchgrass;

2) Aqueous IL could be as effective as pure IL in fractionating and depolymerizing lignin;

3) Lignin fractionation and characterization is dependent on switchgrass genotypes and pretreatment methods.

1.4 Research Objectives

The overall objective of this research is to investigate the effects of lignin manipulation on the fractionation and characterization of different lignin streams (solids and liquid) from genetically engineered switchgrass using three different pretreatment methods. The specific objectives are:

1) Fractionate lignin from wild-type and genetically engineered switchgrass species using three different pretreatment methods;

2) Characterize the lignin streams for a better understanding on how to upgrade them.

This research is helpful to address three following questions:

1) How do different biomass pretreatment methods affect lignin fractionation of genetically modified plants?

2) How does pretreatment chemistry affect the characteristics of lignin streams?

3) What does this mean to lignin upgrading?

1.5 Thesis Organization

This thesis is organized into five chapters as follows:

Chapter 1 introduces the background and motivation for this research. Research objectives and hypotheses are included in this chapter.

Chapter 2 presents a literature review on lignin chemistry, genetically engineered plants, lignin fractionation methods, lignin characterization and upgrading processes.

Chapter 3 discusses our research on fractionation and characterization of lignin streams from genetically engineered switchgrass pretreated by an aqueous IL.

Chapter 4 discusses our research on the impact of dilute acid, ammonia hydroxide, and IL pretreatment on the fractionation and characterization of genetically engineered switchgrass.

Chapter 5 introduces on-going research on bioconversion of lignin to lipids by *Rhodococcus opacus* and a summary of future directions for this research.

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

The growing concern on establishing a sustainable food, energy and water system and de-risking of our dependence on fossil fuels has stimulated the research and development of alternative energy from renewable resources (Obama, 2017; Ragauskas et al., 2006). As a requirement, production of transportation fuels and chemical products is expected to minimize the competition to food supplement. Thus, in order to address the dilemma, non-food lignocellulosic biomass feedstocks, such as energy crops, woody materials, and agricultural wastes, have become a promising resource to produce fuels and chemicals (Chundawat et al., 2011; Tuck et al., 2012). However, cost-effective conversion of lignocellulosic biomass to fuels and chemicals has not been fully reached, due to the recalcitrance of biomass, and lack of infrastructure for biomass harvesting, transportation, and storage (Hisano et al., 2011). As a consequence, research on improving the efficiency and sustainability of current technologies and development of feedstocks with desirable traits are motivated and required in the future biorefinery.

A range of chemical and/or biological processes have been developed for utilizing structural sugars, including cellulose and hemicellulose. Typically, three major processes are involved in the polysaccharides conversion, i.e. pretreatment of lignocellulosic biomass, enzymatic saccharification, and fermentation of hexose/pentose to ethanol (Carroll & Somerville, 2009; Kim & Dale, 2004; Wyman et al., 2005) (**Figure 1**). Extensive efforts have been devoted to reduce the processing cost and increase the efficiency of conversion, such as consolidated bioprocessing of lignocellulosic biomass, and reduction the costs of fungal enzymes (Lynd et al., 2008; Lynd et al., 2005). Among these efforts, however, lignin (as one of the three main components in biomass) has been considered as a waste. As compared to conversion of cellulose and hemicellulose, depolymerization and upgrading of lignin have received attentions of late. Nevertheless, lignin valorization is essential to the success of lignocellulosic biorefinery on the basis of techno-economic analyses (Beckham et al., 2016; Ragauskas et al., 2014).

**This chapter is prepared as a manuscript "The chemistry, biology and conversion technology of lignin engineered plants in the biorefinery concept"*

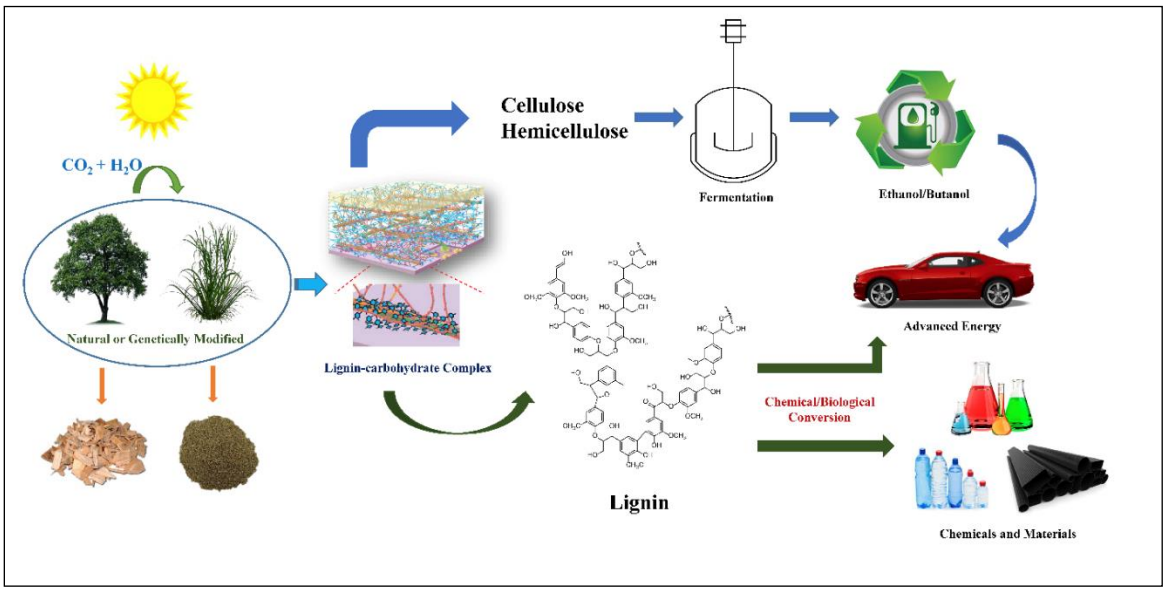


Figure 1. Schematic illustration of the production of fuels and chemicals from lignocellulosic biomass

Lignin, as the most abundant aromatic polymers in nature, provides structural strength and protection against microbial/enzymatic degradation for plants (Behling et al., 2016). Production of advanced fuels and industrial chemicals from lignin via thermal, chemical and biological conversions becomes a research frontiers in the past decades (Azadi et al., 2013; Linger et al., 2014; Xu et al., 2014) (**Figure 1**). Pretreatment is an efficient method to overcome the lignocellulosic biomass recalcitrance, and fractionate/depolymerize lignin into different streams. Moreover, characterization of different lignin streams after pretreatment is critical to meet the requirements of different lignin applications. Recently, increasing attentions have been received on the structural and compositional changes of lignin during its fractionation process (Sathitsuksanoh et al., 2014; Shi et al., 2016; Tolbert et al., 2014). The growth of citations in this area in the past decade clearly demonstrated the research momentum in these areas (**Figure 2a**, analyzed by Web of Science using the key word 'Lignin characterization').

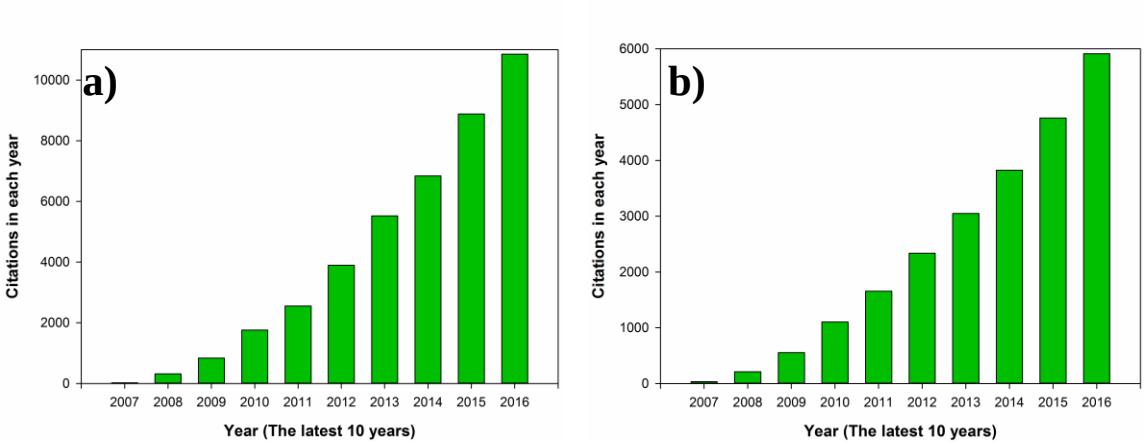


Figure 2. Citations per year in the latest ten years using the key word: a) 'Lignin characterization' and b) 'Lignin modification' (Source from ISI Web of Science)

Genetic modification is considered to be an efficient approach to enhance the plants with yield, drought and salt resistance (Cabello et al., 2014; Roy et al., 2014). Furthermore, genetically modified feedstocks have been demonstrated to be more digestible and convertible when compared to the wildtype species (Carpita & McCann, 2008; Chen & Dixon, 2007; McCann & Carpita, 2008). In particular, modification of lignin biosynthesis pathways to obtain lignocellulosic biomass with less recalcitrance has been drawn extensive attention. According to the analysis in **Figure 2b**, the increasing citations for lignin modification in each year illustrates that lignin engineering towards the goals of reducing biomass recalcitrance and improving conversion efficiency have gained wide interest to the research community developing biorefinery technologies.

As mentioned above, the characteristics of different lignin streams after pretreatment have been received extensive consideration in regarding of different lignin applications. However, the effects of genetic modification on fractionation and characterization of lignin from engineered lignocellulosic biomass feedstocks are not fully understood. In this literature review, we summarize the recent progress in lignin modification methods and current leading technologies to fractionate and characterize lignin streams. Particularly, we focus on the effects of lignin modification on the characteristics of lignin streams from engineered biomass. In addition, the outlook and challenges in the future biorefinery are briefly discussed. This review will provide

essential information on understanding of the effects of genetic modification on lignin depolymerization and characterization using current leading lignin fractionation technologies.

2.2 Biorefinery and lignocellulosic biomass

By definition, biorefinery, as an analogy to the petroleum based refinery, is a facility harboring the sustainable processing of biomass into a spectrum of marketable products and energy (De Jong & Jungmeier, 2015). From an economic perspective, the value chain of a biorefinery is determined by the market value of the products and the processing costs. In other words, the key to the success of a biorefinery is to lower the processing cost and improve the process efficiency. However, a few factors, such as biomass recalcitrance, heterogeneity of lignin, selectivity of catalysts, still hampering the thermochemical conversion technologies (like pyrolysis, gasification, etc.). From the feedstock aspect, the feedstock harvesting, transportation, preprocessing and the pretreatment to overcome the recalcitrance of lignocellulosic biomass are barriers to the current biorefinery.

2.2.1 Lignocellulosic biomass

Lignocellulosic biomass consists of three main structural components: cellulose (30-50%), hemicellulose (25-30%) and lignin (15-20%) (Menon & Rao, 2012). Compositional components in various lignocellulosic biomass feedstocks are shown in **Table 1**. Along with the three mainly components, lignocellulosic biomass has other extractable constituents, such as wax, protein, fat, phenolics and minerals (Vassilev et al., 2010). In general, lignocellulosic biomass can be grouped into four types according to its sources: 1) woody biomass and forestry wastes, e.g. poplar, Douglas fir, pine wood, and mill residuals, etc.; 2) agricultural residues, e.g. corn stover, wheat straw, rice straw, sugarcane bagasse etc.; 3) energy crops, e.g. switchgrass and miscanthus; 4) municipal cellulosic wastes, e.g. waste paper and card board etc.(Van Dyk & Pletschke, 2012). Although a lot of efforts focused on converting sugar streams in to biofuels, there are increasing consensus that all three main components, including lignin have to be further converted into biofuels and bioproducts to achieve the maximal potential of a biorefinery.

Table 1. Structural components in various lignocellulosic feedstocks

Biomass feedstocks	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Poplar	42-48	16-22	21-27	(Ragauskas et al., 2014)
Pine	30.9	19.3	29.0	(Williams et al., 2016)
Eucalyptus	24-28	39-46	19-32	(Ragauskas et al., 2014)
Corn stover	28.7-44.4	13.4-25.0	14.3-26.0	(Pordesimo et al., 2005)
Switchgrass	32.1-33.6	26.1-27.0	17.4-18.4	(Keshwani & Cheng, 2009)
Miscanthus	43.1-52.2	27.4-34.0	9.2-12.6	(Brosse et al., 2012)
Industrial hemp	43.6	15.3	21.5	(Kreuger et al., 2011)
Sweet sorghum	22.8	32.5	22.2	(Yu et al., 2012)
Wheat straw	33-40	15-20	20-25	(McKendry, 2002)
Sugarcane bagasse	50	25	25	(Pandey et al., 2000)

Cellulose is a linear polymer of around 500-1500 glucose units linked by β-1, 4-glycosidic bonds; these glucose polymers (chains) are strengthened by the strong inter-chain hydrogen bonding to form bundles of crystalline cellulose (Hendriks & Zeeman, 2009). Crystalline cellulose can be very recalcitrant to microbial/enzymatic degradation while the amorphous cellulose structure is relatively easier to degrade (Van Dyk & Pletschke, 2012). The crystalline form of cellulose is insoluble in most solvents and this property makes it difficult to be depolymerized (Kosan et al., 2007). The lignocellulosic biorefinery requires the maximum utilization of cellulose to generate fuels and chemicals. Cellulose can be decomposed into glucose molecules by enzymes (cellulases) or acids; the glucose can then be converted into biofuels and bioproducts (such as ethanol, butanol and organic acids) by microbial fermentation or catalysis (Lynd et al., 2008).

Hemicellulose is a heterogeneous polymer consists of C5 and C6 sugars, and it is more heterogeneous in structure and composition than cellulose (Dhepe & Sahu, 2010). Unlike cellulose, hemicellulose is not chemically homogeneous. The composition of hemicellulose highly depends on the cell tissue and plant species. Generally speaking, hemicellulose is made up of xylan, mannan, arabinan and galactan polymers depending on different sources. Hemicellulose may also contain xylose, arabinose, ferulic, p-coumaric acids and glucuronic acids (Saha, 2003). Xylan is the most abundant polymer of C5 sugar in nature, containing β-D-xylopyranosyl residues linked by β-1, 4-glycosidic bonds (Saha, 2003). In plants, xylan forms an important component of plant cell walls and accounts for up to 30% of the mass of the secondary cell wall in plants (Van Dyk & Pletschke, 2012). Xylose, as a kind of pentose, cannot be utilized by wild type baker's yeast, for example, *Saccharomyces cerevisiae*; however, it can be fermented into ethanol and chemicals by some other microbes (such as certain bacteria, fungi, and engineered microbes) (Xing et al., 2010). Co-fermentation of C5 and C6 sugars offers tremendous advantages in a biorefinery concept because of the improved biofuel yield, the less inhibitory effects, and the reduced downstream separation costs (Mohagheghi et al., 2015; Sedlak & Ho, 2004; Yang et al., 2007).

Lignin is most abundant aromatic biopolymer in nature. It plays important roles in plant structural support and resistance against microbial and oxidative stresses. Lignin consists of at least three different phenylpropane units, i.e. *p*-coumaryl, coniferyl and sinapyl, and these units are linked by different inter-unit linkages, such as β-O-4, β-β, β-5, 5-5, etc. (Davin & Lewis, 2005). The resistant properties and the hydrophobic nature make the biological degradation of lignin a challenging process (Hendriks & Zeeman, 2009). More details on lignin chemistry, structure and biosynthesis will be discussed in section 2.3. Lignin holds a great potential to be converted into fuels and value-added chemicals. Furthermore, as suggested by techno-economic analyses, the success of lignocellulosic biorefinery largely relies on the valorization of lignin to fuels and bioproducts (Ragauskas et al., 2014).

2.2.2 Biomass recalcitrance

Lignocellulosic biomass has evolved an efficient system to protect the structural sugars from attacks from surrounding microorganisms and enzymes. The recalcitrance of biomass is mainly driven by the cross-linked polysaccharides, glycosylated proteins, and lignin (Zhao et al., 2012). Himmel et al. summarized the natural factors contribute to the recalcitrance of lignocellulosic biomass, which includes 1) epidermal tissue of plant body (cuticle, wax), 2) structural heterogeneity and complexity of cell-wall constituents, 3) degree of lignification, 4) arrangement and density of vascular bundles, 5) hydrophobic substrates, and 6) inhibitors exist naturally or generated during conversion processes, etc. (Himmel et al., 2007). At molecular levels, the heterogeneous nature of lignin-carbohydrate complex restricts the mass and energy transfer during conversion processes. The crystalline cellulose core of cell-wall microfibrils is resistant to the access of chemicals and enzymes, due to precise arrangement of dextrins (Beckham et al., 2011). Moreover, the strong inter-chain hydrogen bonds between cellulose chains makes crystalline cellulose resistant to enzymatic digestion (Himmel et al., 2007). Collectively, all of the above mentioned features of lignocellulosic biomass limit the accessibility of chemicals, enzymes, and microbes.

In order to overcome the recalcitrance of lignocellulosic biomass, a pretreatment process is generally needed to treat the plan cell wall using physical or thermochemical methods to improve the accessibility of enzymes. Pretreatment is followed by enzymatic hydrolysis to convert cellulose and hemicelluloses to sugars. The fermentable sugars are converted to fuels or other chemicals. Extensive efforts have been made to improve the efficiency of these three steps in biorefinery (Mosier et al., 2005).

2.3 Lignin chemistry, structure, and biosynthesis

2.3.1 Lignin compositions

In nature, lignin is the second most abundant biopolymer after cellulose. Lignin plays a critically role for the structural integrity of plant biomass. In plants, lignin fills the spaces between cellulose, hemicellulose, and pectin in cell wall structure. The

compositions and structure of lignin have been extensively investigated by chemical and spectroscopic methods (Adler, 1977; Lu & Ralph, 1997; McCarthy & Islam, 2000). Generally, lignin is considered as a cross-linked amorphous polymer derived from the polymerization of *p*-coumaryl (H), coniferyl (G), and sinapyl (S) alcohols (Boerjan et al., 2003). The chemical structures of the three main phenylpropane monomers are shown in **Figure 3**. The lignin structure can be classified into two regions, one is the aromatic ring, and the other one is the aliphatic chain (Hatakeyama & Hatakeyama, 2009). Lignin content and compositions vary among plant species, cell types, and developmental stages of plants. In general, lignin in hardwood primarily consists of G and S units, while lignin in softwood is composed mostly of G units. Similar levels of G and S units plus H units are found in the compositions of lignin in grasses (Azadi et al., 2013; Hatakeyama & Hatakeyama, 2009). Determination of lignin compositions is essential to illustrate the lignin structure and to seek appropriate routes for lignin depolymerization and valorization.

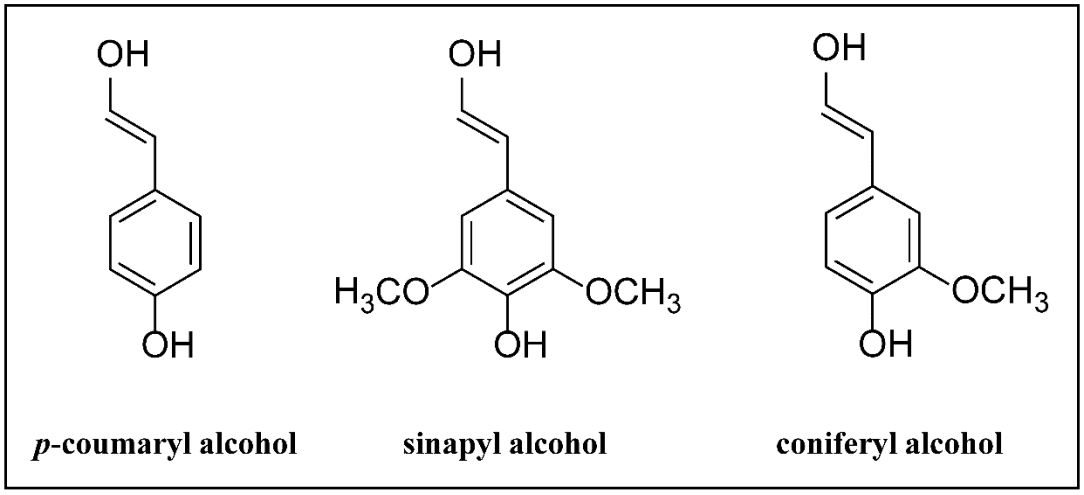


Figure 3. Three phenylpropanoid units in lignin structure

2.3.2 Lignin inter-unit linkages

As mentioned above, lignin is a highly heterogeneous polymer primarily synthesized from three monomeric precursors. The common interunit linkages and functional groups present in lignin are ether and C-C bonds (Vanholme et al., 2008). The main inter-unit linkages in lignin structure are shown in **Figure 4**. In native lignin, two

thirds or more of the total linkages are ether bonds, and the other linkages are C-C bonds. Inter-unit linkages are named by numbering the 9 C-atoms in the phenylpropanoid units, with the aromatic carbons marked from 1 to 6 and the aliphatic carbons marked as α, β, and γ (Rencoret et al., 2011). For instance, β-O-4 is the linkage between β carbon inside the aliphatic side chain and the oxygen atom attached to the C4 position of the aromatic moiety. The major linkages in the structural units of lignin are β-O-4 (β-aryl ether), β−β (resinol), and β-5 (phenylcoumaran) (Pandey & Kim, 2011). The most abundant type of inter-unit linkage is β-O-4, which comprises more than 50% of all the linkages of lignin in grass, softwood and hardwood. Other linkages include α-O-4 (α-aryl ether), 4-O-5 (diaryl ether), 5−5, α-O-γ (aliphatic ether), and β-1 (spirodienone), etc. (Chen & Dixon, 2007; Li et al., 2015). Lignin with relatively high numbers of C-C linkages compared to ether linkages is often referred to as condensed lignin, which is more rigid and less prone to degradation. The dissociation energy required to break C-C linkages such as β-5 (125–127 kcal/mol) is higher than that of ether linkages such as β-O-4 (54–72 kcal/mol) (Chung & Washburn, 2016; Vanholme et al., 2008).

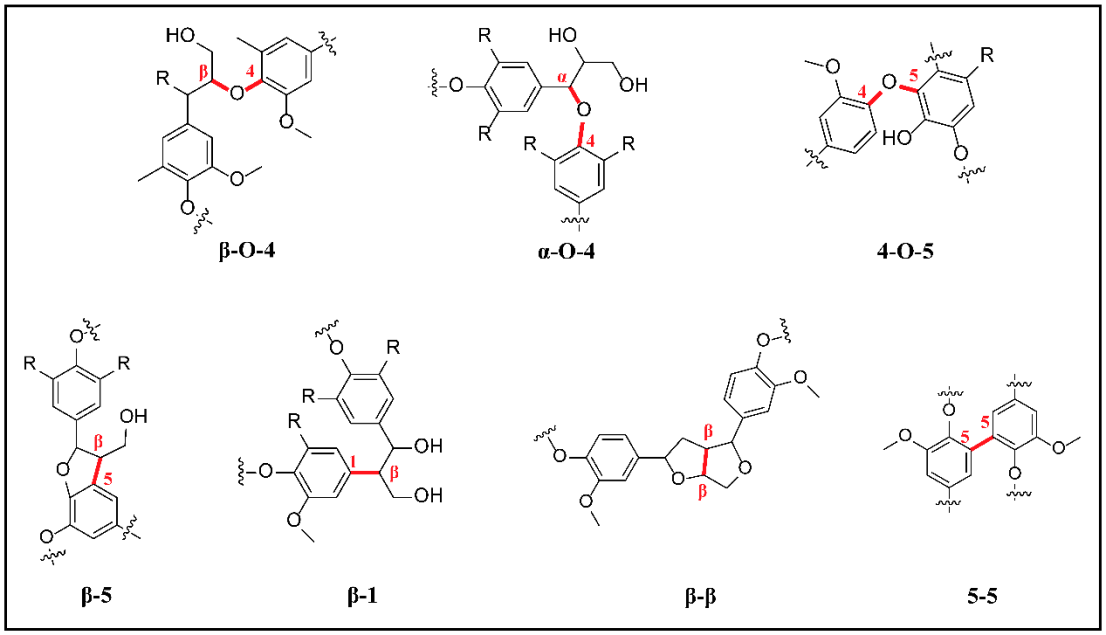


Figure 4. The main inter-unit linkages in lignin structure

2.3.3 Lignin biosynthesis

Lignin biosynthesis is a polymerization process where the three monolignols (i.e. *p*-courmaryl alcohol, sinapyl alcohol and conferyl alcohol) are linked to form *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) lignin or their mixtures. As shown in **Figure 5**, the biosynthetic pathway begins with deamination of amino acid phenylalanine to form cinnamic acid, and followed by hydroxylation reactions of aromatic ring, *O*-methylations, and different side-chain modifications (Vanholme et al., 2010). Moreover, certain types of enzymes are involved in lignin biosynthesis: phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), *p*-coumarate 3- hydroxylase (C3H), shikimate *p*-hydroxycinnamoyl transferase (HCT), caffeoyl-CoA 3-*O*-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid *O*-methyltransferase (COMT), cinnamyl alcohol dehydrogenase (CAD) (Boerjan et al., 2003; Vanholme et al., 2010).

According to the lignin biosynthetic pathway, monolignols are synthesized from phenylalanine (Phe), which is derived from shikimate biosynthetic pathway in the plastid, through the general phenylpropanoid and monolignol-specific pathways (Hisano et al., 2011). Biosynthesis of monolignols starts with the conversion from phenylalanine to cinnamic acid. Para-coumaroyl-CoA is derived from cinnamic acid by the enzymes C4H, and 4CL, then *p*-coumaroyl-CoA can be derived into *p*-coumaraldehyde and *p*-coumaroyl shikimic acid by CCR and HCT. Then, *p*-coumaroyl shikimic acid can be derived to coniferaldehyde under the function of C3H, HCT, CCoAOMT and CCR. Furthermore, coniferaldehyde can be converted to sinapaldehyde by the action of F5H and COMT. With the catalysis of CAD, *p*-coumaraldehyde, sinapaldehyde, and coniferaldehyde are able to form *p*-courmaryl alcohol, sinapyl alcohol, and conferyl alcohol, respectively. After monolignol synthesis, these monomers are transported and later oxidized by laccase or peroxidase and polymerized in the cell wall structure (Weng & Chapple, 2010). Additionally, upstream transcription factors like *NAC*, *MYB*, and *LIM* gene families are believed responsible for regulating the enzymatic expressions (Simmons et al., 2010).

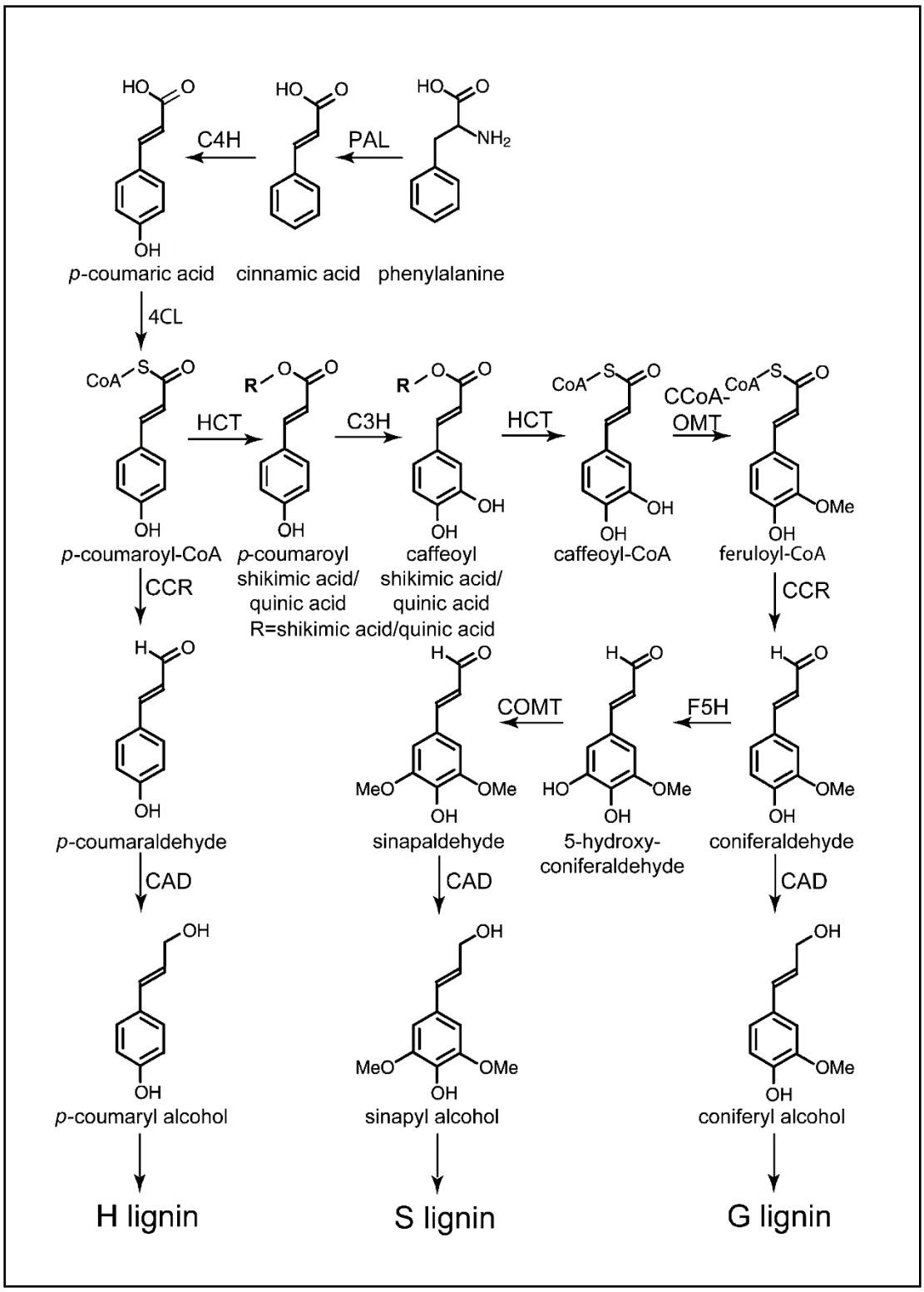


Figure 5. The main biosynthetic pathway for monolignols(Vanholme et al., 2010)

By understanding the biosynthesis of lignin, it is possible to take advantage of genetic modification approaches to modify lignin content, compositions of subunits, and the inter-unit linkages by down-regulation, overexpression, or suppression of certain genes involved in lignin biosynthesis. Despite that most revisions of lignin biosynthetic pathway and most lignin modification research are conducted in model plants, artificial design of lignocellulosic biomass feedstocks is a promising approach to develop plants with desirable characteristics for improved conversion efficiency in a biorefinery.

2.4 Engineered feedstocks

Genetic modification has been applied to various energy crops, such as switchgrass, sorghum, etc., to enhance the biomass yield, drought and pest resistance, and the convertibility to biofuels and bioproducts (McLaughlin & Kszos, 2005; Vogel & Jung, 2001). The manipulation on lignin biosynthesis or other relevant pathways is of particular interest. Several molecular biology methods have been developed (**Table 2**), including a) reduction/increase of lignin content, b) modification of lignin composition, c) alternation of lignin deposition in plant cell wall and d) alternation of interunit lignin linkages or linkage within LCC (lignin-carbohydrates complex) (Sticklen, 2007) (Hisano et al., 2009; Sticklen, 2008). Some of the efforts on lignin engineering are summarized below.

2.4.1 Modification of lignin content

Downregulation of the genes for enzymes in the lignin biosynthesis can modify the lignin structure or reduce the content of lignin in biomass. As one of the major contributor to the recalcitrance of lignocellulosic biomass, reducing lignin content is a potential way to improve the biofuels production from lignocellulose based biomass (Sticklen, 2008). Many different strategies have been developed over the past to lower the lignin content of plants. 4CL is one of the key enzymes in the lignin biosynthesis pathways, specially involved in the initial stage of monolignol biosynthesis. Lignin content was reduced through inhibiting one of the *4CL* gene *Pv4CL1* by RNA interference in switchgrass, and the production of fermentable sugar was significantly increased with this mutant (Xu et al., 2011). Baucher et al. showed that reduction of *4CL*

by over 90% resulted in 25% less of lignin in tobacco (Baucher et al., 2003). Chen and Dixon successfully reduced lignin content in Alfalfa by downregulating of *HCT* gene (Chen & Dixon, 2007). It is also reported that downregulation of CCoAOMT can cause the reduction of lignin content in alfalfa; while suppression of the ferulate 5-hydroxulase (F5H) gene can reduce the accumulation of syringyl lignin in alfalfa fibers (Nakashima et al., 2008). Furthermore, downregulating *C4H* in *Populus* led to a 30% of reduction in Klason lignin content (Bjurhager et al., 2010).

Chabannes et al. compared the downregulations of CCR and CAD, and demonstrated that both transgenic lines of tobacco exhibited a dramatic reduction of lignin content (Chabannes et al., 2001). In another study, Kawaoka et al. reported reduction of lignin content in Eucalyptus by the suppression of gene expression of the *LIM* domain transcription factor (Kawaoka et al., 2006). In transgenic aspen plant, researchers transferred an antisense *prxA3a* gene into the original aspen to suppress the expression of *prxA3a.* The results showed that the lignin content in the aspen was reduced because of downregulation gene of antionic peroxidase (Li et al., 2003). Downregulation of 4-coumarate 3-hydroxylase (C3H) in alfalfa can cause the reduction in lignin content; downregulation of CCR in transgenic tobacco resulted in a dramatic decrease in lignin content (Moura et al., 2010; Sticklen, 2008). By testing the convertibility of these engineered feedstock, it is generally believed that lowing lignin content can help to decrease pretreatment severity, improve sugar yield, and reduce enzyme usage during enzymatic hydrolysis (Biemelt et al., 2004; Hisano et al., 2009).

Table 2. Genetic modification of lignocellulosic biomass feedstocks and its effect on biomass digestibility and convertibility

Biomass feedstocks	**Gene/Enzymes regulation**	**Lignin content**	**Lignin composition**	**Digestibility**	**References**
Alfalfa	Downregulation of HCT	Reduced	Unaffected	Increased	(Chen & Dixon, 2007)
Switchgrass	Downregulation of 4CL	Reduced	Increased S/G	Increased	(Xu et al., 2011)
Arabidopsis	Disruption of MED5a and MED5b	Reduced	Almost entirely H	N/A	(Bonawitz et al., 2014)
Tobacco	Downregulation of C4H	Reduced	Increased S/G	Increased	(Sewalt et al., 1997)
Switchgrass	Overexpression of *AtLOV1*	Increased	Increased S/G	N/A	(Xu et al., 2012)
Hybrid poplar	Downregulation of C3H	Reduced	Increased S/G	Increased	(Coleman et al., 2008)
Poplar	Downregulation of 4CL	Reduced	N/A	Increased	(Simmons et al., 2010)
Hybrid poplar	Downregulation of 4CL	Reduced	Increased H	N/A	(Voelker et al., 2010)
Alfalfa	Downregulation of C3H or HCT	Reduced	Increased S/G	N/A	(Ziebell et al., 2010)
Tobacco	Downregulation of PAL	Reduced	Increased S/G	Increased	(Li et al., 2008)

N/A: Not applicable

2.4.2 Modification of lignin structural subunits (S/G/H)

In nature, the lignin in lignocellulosic biomass is composed of mainly three type of structural lignin subunits, namely *p*-coumaryl (H), coniferyl (G), and sinapyl (S) alcohols. Generally, softwood lignin mainly contains G unit while hardwood contains roughly equivalent contents of S and G unit. On the other hand, grass contains mainly G and S unit with a small present of H unit as well. From the standpoint of biological conversion, S or H lignin is preferable over G lignin, because biomass with relative high G lignin units, e.g. softwood, are more resistant to biochemical decomposition. Therefore, it is possible to modify the S/G ratio of a plant so that a less severe pretreatment is needed to converted the lignocellulosic biomass for fermentable sugars (Kishimoto et al., 2010). Indeed, modification of lignin structural units, such as S/G/H ratio is one of the main research areas of lignin engineering.

Coleman et al. used RNAi to generate transgenic hybrid poplar with the suppression in C3H expression, and obtained a significantly reduction of lignin content and an alternation of lignin monomer composition (higher H lignin, and lower G lignin) (Coleman et al., 2008). Sewalt et al. analyzed lignin content and composition in transgenic tobacco lines which were altered in the expression of C4H, and concluded that the levels of Klason lignin were reduced and syringyl to guaiacyl ratio was decreased (Sewalt et al., 1997). In another study, an antisense gene which can suppress the activity of cinnamyl alcohol dehydrogenase (CAD) was introduced into the tobacco plant. The results showed that the modification increases the cinnamaldehyde units in lignin, however, no significant change in the lignin content (Hibino et al., 2014). Reddy et al. determined the relationship between lignin and digestibility in alfalfa, and concluded that P450 enzymes C4H, C3H and F5H can lead to different changes in lignin content and composition (Reddy et al., 2005). Downregulation of F5H in *Medicago sativa* (alfalfa) can reduce the accumulation of syringyl lignin in fiber and parenchyma cells, resulting in an easily digestible material (Nakashima et al., 2008). In switchgrass, researchers overexpressed the *AtLOV1* gene, resulting in an altered lignin content and monolignol composition of cell walls; the results showed that lignin content and S/G ratio were

increased compared to wild type switchgrass (Xu et al., 2012). In a recent study, Bonawitz et al. disrupted the expression MED5a and MED5b, and produced a mutant *Arabidopsis* that contains almost entirely H lignin (Bonawitz et al., 2014).

2.4.3 Modification of lignin inter-unit linkages and lignin deposition

Modification of genes related to lignin biosynthesis can also cause changes in the lignin deposition pattern in the secondary plant cell wall, which offer another potential strategy to improve the convertibility of lignocellulosic biomass (Bonawitz & Chapple, 2013; Scullin et al., 2015). It is reported that suppression of C3'H expression in poplar can reduce the total cell wall lignin content and at the same time change the location of lignin in the secondary cell wall (Coleman et al., 2008). Rogers and Campbell summarized that the regulation of PAL gene family can provide a transcriptional regulation which may affect the timing and localization of lignification (Rogers & Campbell, 2004). Synthetic biology tool was used to rewire the secondary cell network in *Arabidopsis thaliana*, resulting in a reduction of lignin content and an increase in polysaccharide depositions in fiber cells (Yang et al., 2013).

As discussed in section 2.3.2, the dissociation energy on breaking down the ether or C-C interunit lignin linkages is different. Thus, modification of interunit lignin linkages and lignin-hemicellulose linkages offer another efficient way to alter plant to improve its convertibility. Studies on the C3'H coding gene *ref8* showed that lack of C3'H activity leads to diverse changes in phenylpropanoid metabolism (Franke et al., 2002). Modification of biomass can decrease the ferulate and *p*-coumarate cross-linking between xylan and lignin (Casler et al., 2008). Researchers used a biomimetic model system to determine how reductions in ferulate lignin cross-linking influence cell wall fermentation by rumen microbes; results showed that reducing ferulate-lignin cross-linking can improve the fiber fermentability (Grabber et al., 2009). Studies on comparing levels of p-coumarate and lignin content in corn showed that higher *p*-coumarate levels accompanied higher lignin content, indicating that *p*-coumarate aiding lignin formation in corn cell wall (Hatfield & Chaptman, 2009). Researchers introduced ester linkages into lignin polymer backbone in poplar trees by augmenting with monolignol ferulate

conjugates(Karlen et al., 2016; Wilkerson et al., 2014). Ralph summarized the recent studies on monolignol-hydroxycinnamate conjugates, and demonstrated that the compatibility of ferulate with lignification provides potential ways to prove efficiency of biomass conversion(Ralph, 2010).

2.5 Lignin fractionation

In order to upgrade lignin, the first step is to fractionate lignin from lignocellulosic biomass. However, lignin fractionation must be integrated into the other operations in a biorefinery in order to recovery the other components of the biomass feedstock. Paper and pulping industry use acidification, membrane filter, and solvent extraction methods to separate lignin. Lignin derived from Kraft pulping is called Kraft lignin. A scheme of a typical Kraft pulping is illustrated in **Figure 6**. In this process, softwood or hardwood is processed into paper through cooking, washing, bleaching, and drying processes. The lignin in black liquor can be extracted to form Kraft lignin. However, for a biorefinery focused on making biofuels, chemicals and other bioproducts from lignocellulosic biomass, it is necessary to maximize the utilization of carbohydrates and fractionate lignin to different streams.

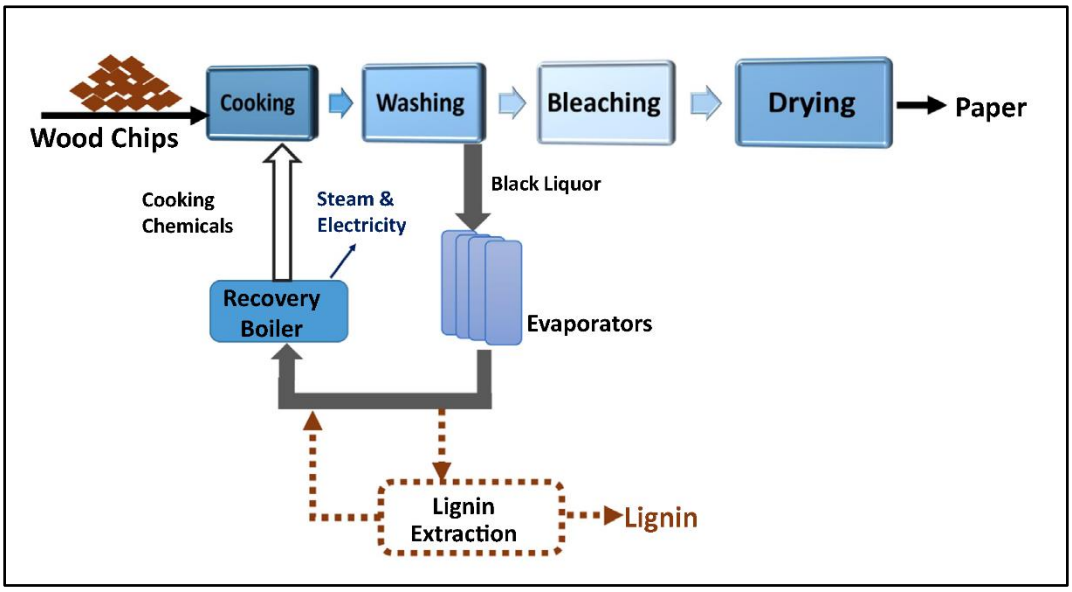


Figure 6. A typical Kraft pulping process (Haddad et al., 2017)

Lignocellulosic biomass is a potential source of renewable energy, offering a significant sustainable resource to biofuels and chemicals. The recalcitrant nature of lignocellulosic biomass makes it difficult to deconstruct and further convert this material into biofuels. In order to overcome the recalcitrance of biomass, pretreatment via physical, chemical and biological methods need to be conducted before enzymatic hydrolysis and fermentation (Wyman et al., 2011). The objective of biomass pretreatment is to make the cellulose and hemicellulose more accessible to hydrolytic enzymes without destroying the sugars and forming inhibitors to fermentation. It is the first and one of the most crucial steps for the conversion of biomass to C5 and C6 sugars (Tao et al., 2011). The pretreatment process has significant impacts on the downstream processing, such as enzymatic hydrolysis and fermentation. The typical pretreatment methods include wet oxidation, dilute acid, high pH (aqueous ammonia, lime, etc.), ammonia fiber explosion (AFEX), organosolv, steam explosion, ionic liquid, etc.

2.5.1 Physical pretreatment

Physical pretreatment rests on the principles to reduce biomass particle size through mechanical process, which is able to be done by milling, chipping and grinding, so as to improve enzyme accessibility by increase the feedstock surface area to volume ratio and reduce cellulose crystallinity and polymerization degree. Physical pretreatment is usually followed by other pretreatment processes because it is able to provide optimal particle size required by effective heat and mass transfer, though it cannot significantly enhance sugar conversion rate by itself (Alvo & Belkacemi, 1997; Fan et al., 1981; Harmsen et al., 2010; Sidiras & Koukios, 1989; Tassinari et al., 1980).

2.5.2 Physio-chemical pretreatment

2.5.2.1 Steam explosion

Steam explosion refers to a pretreatment process in which biomass is subjected to rapidly heating with high pressure saturated steam. During the heating, cellulose substrate is permeated and the hydrolysis of hemicellulose is promoted by steam. At a set time, the process will be terminated by rapidly venting the steam from reactor to atmospheric

pressure. With pressure flashed dropping, the water within cellulose substrate vaporizes and expands promptly, by which biomass is decomposed (Grous et al., 1986; Himmel et al., 1994; Wyman, 1996). Numerous studies have been examined steam explosion and subsequent enzymatic hydrolysis (Ramos et al., 1992). Hemicellulose is thought to be hydrolyzed by acids generated from hydrolysis of acetyl groups. The acetic acid released from the hemicellulose hydrolysis will further catalyze glucose or xylose degradation. Also, at high temperature, water acts as an acid (Baugh et al., 1988; Weil et al., 1997).

2.5.2.2 Ammonia fiber expansion (AFEX)

Ammonia Fiber Expansion (AFEX) is a low temperature pretreatment process catalyzed by concentrated ammonia. Similar to steam explosion pretreatment, in AFEX the biomass and ammonia mixture is saturated for a set of time under a high pressure before rapidly depressurizing to atmospheric pressure (Dale, 1986; Dale et al., 1996). The intense expansion of ammonia vapor cause depolymerization of lignin and cleavage of lignin-carbohydrate linkage, hemicellulose hydrolysis and cellulose decrystallization, all of which contribute to an enhanced enzyme accessibility (Carvalheiro et al., 2008; Chundawat et al., 2007; Lin et al., 2010). However, it is reported that AFEX pretreatment only works well on hardwood as opposed to softwood (Himmel et al., 1994; Yoon et al., 1995).

2.5.2.3 Carbon dioxide

Supercritical carbon dioxide (SCF) is in a state above the critical temperature and critical pressure, at where liquid and gas state CO_2 are able to coexist. SCF exhibits an extraordinary capability to penetrate the crystalline structure of biomass without concerning the heat and mass transfer constraint encountered by other pretreatments approaches (Sawan & Sawan, 1998). Much like steam explosion pretreatment, with a rapid depressurizing of the carbon dioxide to atmospheric pressure, the disruption of the cellulosic structure increases the accessible surface area of the cellulosic substrate to enzymatic hydrolysis. Also, important properties, such as solubility and partial coefficients, can be tuned easily. A small amount of adjustment in temperature and pressure can lead to up to 100 times of change in solubility (King & Srinivas, 2009).

However, having elevated pressure involved, economically feasibility of SCF pretreatment is a big concern when attempt to scaling up (Brodeur et al., 2011).

2.5.2.4 Hot water

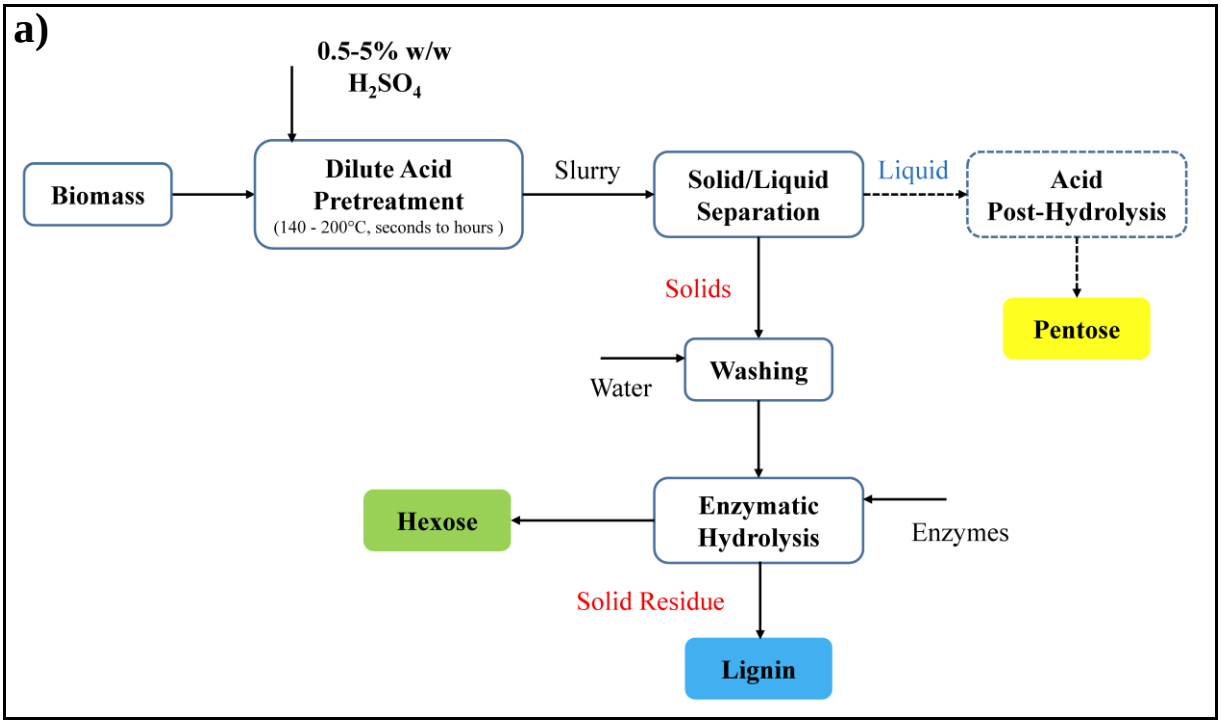
Hot water pretreatment employs high temperature water, 200-300 °C, at elevated pressure so that maintain the hot water at liquid phase. Approximately 40% - 60% of total biomass is able to be dissolved in the hot water solvolysis, of which 4 – 22 % of cellulose, 35 – 60 % of lignin and all of the hemicellulose can be dissolved (Mosier et al., 2005). Several reactor configurations, varying from co-current, counter-current, and flow-through, have been developed aiming to enhance the mass and heat transfer between the biomass and the hot water. Also, various mineral and organic acid as pretreatment catalysts, such as nitric, succinic, citric, maleic and fumaric acid, have been extensive examined (Luo et al., 2002; Mosier et al., 2002; van Walsum & Shi, 2004).

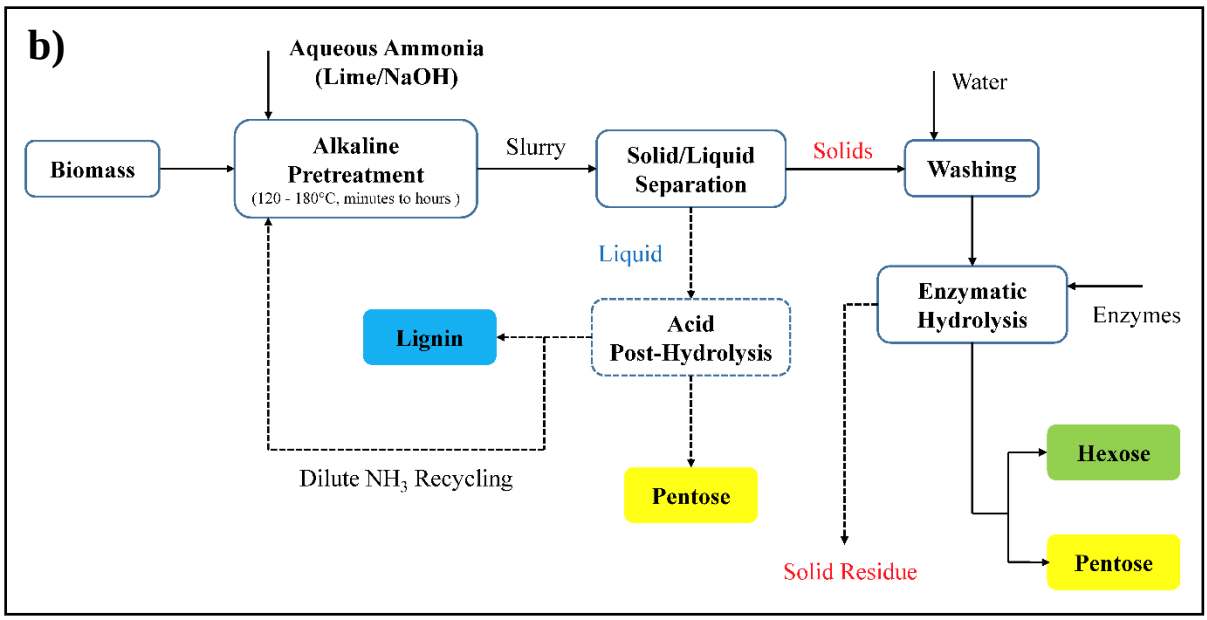
2.5.2.5 Dilute acid

Dilute acid pretreatment of lignocellulosic biomass has the mechanism of accelerating hemicellulose hydrolysis by adding acid. The low pH will make the hemicellulose solubilize in acid solution, along with lignin relocating to Avicel surface, and further increase the accessible surface for enzymes. A typical process flow of dilute acid pretreatment is illustrated in **Figure 7a**.

Dilute acid pretreatment has been extensively investigated for processing a variety of biomass feedstocks. Dien et al. conducted the dilute acid pretreatment on switchgrass, alfalfa stems and reed canarygrass under different growing stage and evaluated the sugar yields after enzymatic hydrolysis. They evaluated two sulfuric acid pretreatment methods, 121°C for 1 h and 150°C for 20 min, and got a highest glucose yield of 655 g/kg dry material from switchgrass (Dien et al., 2006). Foston and Ragauskas evaluated the effect of dilute acid pretreatment on the supramolecular and ultrastructure of switchgrass; they conducted different pretreatment at 160-180°C under different sulfuric acid concentration, and concluded that the crystallinity index increases with residence time and a reduction of molecular weight (Foston & Ragauskas, 2010). In another study,

researchers compared the transgenic and wild-type switchgrass using dilute sulfuric acid pretreatment, and concluded that the ratio of acid soluble lignin was increased in transgenic switchgrass, and led to considerably increased sugar production (Zhou et al., 2012). Jensen et al. investigated total sugar yield of enzymatic hydrolysis after dilute acid pretreatment, and they also firstly analyzed the glucose and xylose monomer and oligomer yield from aspen, balsam, and switchgrass, they achieved a highest yield of total sugar at combined severity values between 2.20 and 2.40 (Jensen et al., 2010). Dilute acid pretreatment method is commonly used in lignocellulosic pretreatment, and is considered as one of the most efficient pretreatment methods.





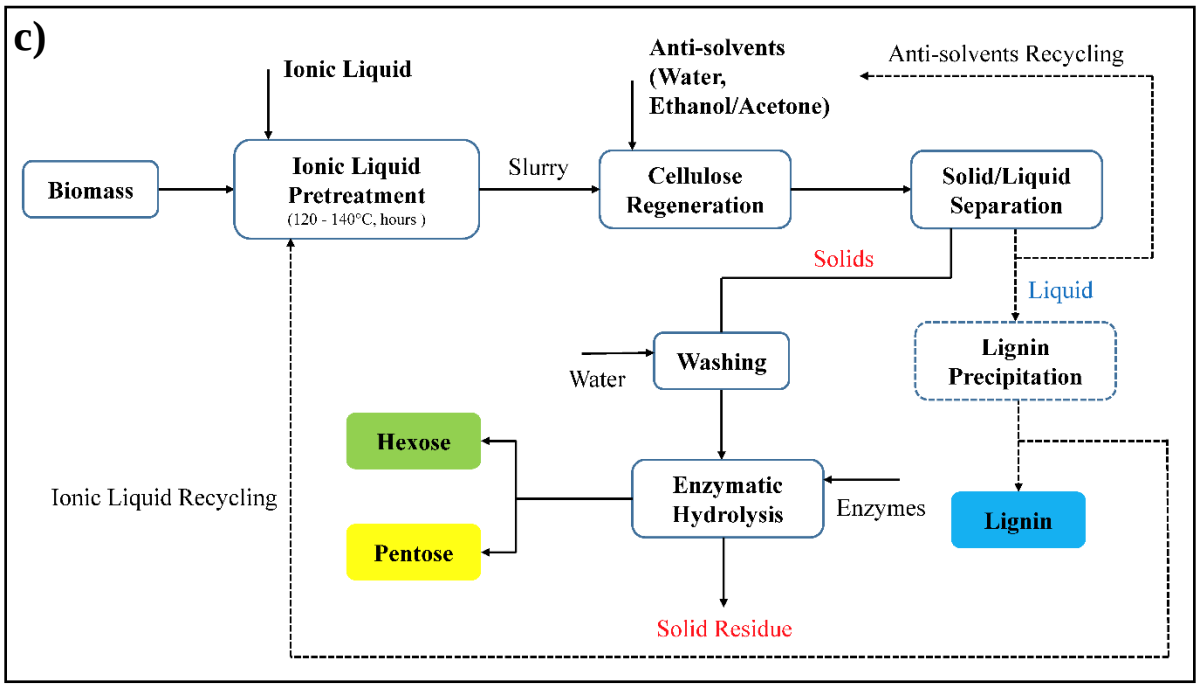


Figure 7. Pretreatment process flow chart illustrating paths of structural sugars and lignin a) Dilute acid pretreatment b) Ammonia hydroxide pretreatment c) ionic liquid pretreatment

2.5.2.6 High pH

High pH pretreatment uses alkaline to increase the pH of the reaction, like lime, sodium hydroxide, aqueous ammonia, etc. This type of pretreatment removes a large fraction of lignin and acetyl groups between lignin and hemicellulose, with the mechanism of delignification through improving lignin hydrophilicity. A typical process flow of high pH (ammonia hydroxide) pretreatment is demonstrated in **Figure 7b**.

High pH pretreatment method has been commonly used to pretreat lignocellulosic biomass and extract large fraction of lignin with high purity. Gao et al. investigated acetone, butanol and ethanol fermentation from switchgrass pretreated with 1% NaOH, resulting in a reducing sugar yield of 365 g/kg biomass and 146 g/kg ABE yield (Gao et al., 2014). Researchers developed a bench-scale continuous pretreatment system by soaking switchgrass in NaOH and thermochemical pretreatment of the biomass. At the minimum NaOH concentration and enzyme dosage, they obtained 90.8% glucose yield with the flow of 15 g/min biomass and 120 ml/min NaOH input (Cha et al., 2015). Gupta and Lee conducted the pretreatment of switchgrass using aqueous ammonia and NaOH; with the presence of H_2O_2, the authors indicated that NaOH pretreated lignin has lower aromatic content but higher guaiacyl type structure than ammonia pretreated lignin (Gupta & Lee, 2010). Xu et al. optimized the conditions of lime pretreatment of switchgrass, with best conditions of 50°C, 24 h, 0.1 g lime/g raw biomass (Xu et al., 2010). Wang et al. modified the switchgrass by having the lignin content reductions of up to 5.8%. They pretreated the switchgrass with 0.5%, 1%, 2% NaOH for 15, 30, 60 min at 121°C, resulting in a 16-18% higher sugar yield in transgenic switchgrass compared to wild types (Wang et al., 2012). Salvi et al. pretreated sorghum fibers using dilute ammonia hydroxide (sorghum: ammonia: water, 1: 0.14: 8) at 160°C for 1 h, and removed 44% lignin and 35% hemicellulose (Salvi et al., 2010). Ko et al. optimized the rice straw pretreatment conditions using aqueous ammonia, and found that 69°C, 10 h, an ammonia concentration of 21% (w/w) was the optimal reaction condition (Ko et al., 2009). Qin et al. optimized the conditions of aqueous ammonia pretreatment of corn stover, and recommended a condition of 180°C, 20% ammonia hydroxide, and 30 min. They also demonstrated that most of the lignin was degraded to soluble fragments after

ammonia hydroxide pretreatment (Qin et al., 2013). Kim et al. evaluated the pretreatment of barley hull by aqueous ammonia, and indicated that ammonia hydroxide pretreatment caused the physical changes of biomass and enhanced the enzymatic hydrolysis (Kim et al., 2008). Although there are still some issues on waste management, high pH pretreatment is still an efficient processing method for lignocellulosic biomass.

2.5.2.7 Organosolv

Organosolv pretreatment involves using organic solvent or their aqueous solution, such as alcohol, organic acids and acetone, to dissolve the lignin and part of hemicellulose (Curreli et al., 1997; Itoh et al., 2003; Pan et al., 2006; Rolz et al., 1986). Organic solvents involved in the organosolv pretreatment are always easily to be recycled by distillation. Lignin within the solvent can be isolated and extracted as feedstocks for either value added chemicals or materials, which provide a promising pathway for biorefinery from the point of view of integrated utilization of all of biomass components (Lora & Aziz, 1985). However, organic solvents are expensive. Although they can be recovered, intense energy input is required (Zhao, 2009).

2.5.2.8 Wet oxidation

In wet oxidation pretreatment, biomass is treated with water and oxygen or air above 120 °C for a set of time period (Garrote et al., 1999; Palonen et al., 2004; Varga et al., 2004). The presence of oxygen promotes the degradation of hemicellulose and possibly a part of cellulose so that enhance the generation of organic acids. With the acid catalyzing, relatively moderate temperature is available for wet oxidation reaction (Garrote et al., 1999). The attempts of combining wet oxidation and alkaline pretreatment have been reported. It is believed that the alkaline condition can significantly hinder the formation of enzyme inhibitors, such as furfural, though the solubilized hemicellulose concentration increased by alkaline agent are marginal (Ahring et al., 1996; Bjerre et al., 1996; Martin et al., 2007).

2.5.2.9 Ionic liquid

Ionic liquids are organic salts that usually melt below 100°C. Owing to their

strong solvent power, ionic liquids can solubilize whole cellulosic biomass or selectively dissolve components like lignin and cellulose. The dissolved components can be re-precipitated with anti-solvent like water, methanol and ethanol, less-crystalline reconstituted cellulose could be obtained from this pretreatment. The typical types of ionic liquids being used for biomass pretreatment include: 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$), 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$), 1-ethyl-3-methylimidazolium chloride ($[C_2C_1Im][Cl]$), cholinium lysinate, etc. The high hydrogen bonds basicity of ionic liquid breaks the intra- and inter-molecular hydrogen bonds in cellulose, making it less crystalline and easy to hydrolyze. The details of a typical ionic liquid pretreatment process are illustrated in **Figure 7c**.

Ionic liquids are considered as an efficient cellulose solvent. A lot research has been conducted on different species of biomass under relatively mild conditions. Montalbo-lomboy and Grewell utilized the ultrasonic to rapidly dissolve switchgrass in ionic liquid, 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$), sonicated at a frequency of 20 kHz assisted with heat treatment at 130°C for 24 h. The results showed that with increasing ultrasonic amplitude the carbohydrate recovery decreased, and more than 50% of the hemicellulose fraction was lost during the biomass recovery (Montalbo-Lomboy & Grewell, 2015). Perez-Pimienta et al. compared the agave bagasse and switchgrass under the pretreatment of using $[C_2C_1Im][OAc]$ at 120 and 160°C for 3 h with 15% biomass loading, and obtained higher delignification for agave bagasse (45.5%) than switchgrass (38.4%) (Perez-Pimienta et al., 2013). Researchers tested the ionic liquid pretreated biomass with commercial enzyme system; results showed that the ionic liquid pretreatment of biomass produces amorphous cellulose with little residual crystallinity and enhances its enzymatic hydrolysis (Samayam & Schall, 2010). Some research was focusing on the mechanism of biomass pretreatment with ionic liquid. Different types of biomass and the properties of untreated and pretreated biomass have been determined. Varanasi et al. developed a mechanistic understanding of biomass pretreatment with 1-ethyl-3-methylimidazolium acetate and determined the syringyl and guaiacyl ratio using pyrolysis-GC/MS and Kamlet-Taft properties of $[C_2C_1Im][OAc]$ at 120°C and 160°C. They found that there was no preferential breakdown of either S- or G-lignin in switchgrass at a lower pretreatment temperature(Varanasi et al., 2012).

Researchers employed the thermogravimetry to understand the interactions between ionic liquid and biomass components, with switchgrass pretreated by 1-butyl-3methylimidazolium acetate at temperature from 50-130°C for 6 h, resulting in an improved thermal stability after the removal of minerals by ionic liquid (Zhang et al., 2014). Ionic liquid pretreatment is a potential method of biomass pretreatment and fractionation, even though there are still some challenges (including cost of ionic liquid itself and recycling) need to be addressed. Recently, study on reducing the cost of ionic liquids and the recycling processes of ionic liquids has been extensively conducted. Researchers demonstrated that an aqueous ionic liquid, $[C_2C_1Im][OAc]$ can be as effective as pure IL in pretreating plant biomass. Furthermore, using ionic liquid and water mixtures as pretreatment agents could reduce viscosity, eliminate gel formation during pretreatment and significantly reduce the energy requirements and costs associated with ionic liquid recycling (Shi et al., 2014). In addition, the aqueous environment and biocompatibility of ionic liquids provide potential approaches to covert lignin into value-added fuels and chemicals in the liquid phase without extraction or separation.

2.5.3 Biological pretreatment

As opposed to physical and chemical pretreatment which require intense energy input, biological pretreatment takes advantage of microorganisms and their enzymatic excreta to cleavage lignin-carbohydrate linkage. White-rot fungi, such as *Pleurotus ostreatus*, *Phlebia subserialis*, *Ceriporiopsis subvermispora* and *Phanerochaete chrysosporium*, are believed the most promising microorganism to conduct biological pretreatment (Hatakka, 1983; Keller et al., 2003; Kirk & Chang, 1981; Yao & Nokes, 2014). Chinn and Nokes screened out thermophilic anaerobic bacteria for solid substrate cultivation using corn stover, sugar cane bagasse, paper pulp sludge, and wheat bran(Chinn & Nokes, 2006). Flythe et al. conducted research to determine the effect of feedstock particle size on switchgrass fermentation by *Clostridium thermocellum*(Flythe et al., 2015). Moreover, thermophilic anaerobe *Thermoanaerobacterium saccharolyticum* was investigated for production of *n*-butanol(Bhandiwad et al., 2014). However, relatively longer residence time, up to days, significantly restricts its application in large scale biorefinery. In addition, microbes' growth consumes carbohydrates, which

shrinkages the sugar yield (da Costa Sousa et al., 2009).

2.6 Lignin characterization

2.6.1 Lignin content and compositions

Lignin is a class of complex cross-linked phenolic polymers which form important structural materials to provides mechanical strength for cell wall (Martone et al., 2009). As shown in **Table 1**, the lignin content in different plant species is diverse. Numerous methods have been developed and modified to determine the amount of lignin in different plants. Early research tried to determine lignin by the stronger absorbance of lignin compounds as compared with carbohydrates in the ultraviolet region of spectrum. Also, near infrared spectroscopy was utilized to quantify lignin content in plants by comparing the intensity of several signature lignin bands representing the aromatic skeletal of lignin The other two common used methods are thioglycolate and acetyl bromide, which are based on the solubilization of lignin (Hatfield & Fukushima, 2005).

The most common used method to determine lignin content is two-step acid hydrolysis, which was originally developed by Klason and modified by NREL (National Renewable Energy Laboratory) (Sluiter et al., 2008). The concept that lignin is formed by dehydration of three monolignol, p-coumaryl (H), coniferyl (G) and sinapyl (S) alcohols was first reviewed and proposed by Freudenberg (Freudenberg, 1965). Plant species vary by the relative amount of the monolignols in lignin. Determination of lignin compositions have been developed for several decades. The lignin was broken down into low molecular weight fragments, which were identified by different technology, such as GC/MS, NMR (Lu & Ralph, 1997).

2.6.2 Lignin molecular weight distribution

The molecular weight of lignin is a fundamental property that reflects the difficulty of overcoming the recalcitrance of biomass and the valorization of lignin. The molecular weight of lignin in native biomass varies by feedstocks. And the isolation and purification procedures also matters. Milled wood lignin (MWL) (Holtman et al., 2004), cellulolytic enzyme lignin (CEL) (Chang et al., 1975) and enzymatic mild acidolysis

lignin (EMAL) (Gosselink et al., 2004) are the most commonly applied isolation approaches. Each has merits and drawbacks in respect to isolating lignin from biomass. Numerous lignin molecular weight characterization techniques are available, including Vapor Pressure Osmometry (VPO) (Gosselink et al., 2004), ultra-filtration (Jönsson et al., 2008), light scattering (static and dynamic) (Gidh et al., 2006a), mass spectrometry (Evtuguin et al., 1999) and gel permeation chromatography (GPC) (Baumberger et al., 2007; Gidh et al., 2006b), of which, the most common employed one is GPC (also known as size exclusion chromatography, SEC). El Hage et al. compared the molecular weight distribution between milled lignin and organosolv lignin from Miscanthus, and concluded that the weight average molecular weight (Mw) and number average molecular weight (Mn) of organosolv lignin were significantly lower than that of milled lignin (El Hage et al., 2009). Also, transformation of lignin during high temperature and the presence of catalyst was characterized by GPC to evaluate the conversion efficiency (Joffres et al., 2014). Salanti et al. characterized the alkaline extracted lignin from rice husk, and optimized the reaction conditions based on the GPC results (Salanti et al., 2010a). GPC analyses provided molecular weight distribution in the extracted lignin of waterlogged wood, and determined the ageing effects on the structure of archeological wood (Salanti et al., 2010b). Tejado et al. explored different physical/chemical methods to characterize Kraft pine lignin, soda-anthraquinone flax lignin, and ethanol-water wild tamarind lignin; GPC results from this research showed that the molecular distribution of three sources of lignin were different and suitable for different applications (Tejado et al., 2007). Lignin molecular weight distribution is a promising method to evaluate the efficiency of pretreatment process and to provide essential information of lignin structure/composition. Lignin streams fractionated by different pretreatment methods and the characterization of lignin are presented in **Table 3**.

Table 3. Lignin streams fractionated by different pretreatments and characterized by current biorefining technologies

Pretreatment	Biomass feedstocks	Lignin streams		Lignin molecular weight distribution (after pretreatment)		Lignin inter-unit linkages	References
		Liquid (%)	Solids (%)	Mw (g/mol)	Mn (g/mol)		
$[C_2C_1Im][OAc]$	Engineered Arabidopsis (*med5a med5b ref8*)	70.4	29.6	N/A	N/A	Cleavage of β-O-4 to a greater extent than β-β and β-5	(Shi et al., 2016)
Ethanol organosolv	Switchgrass	60.5	39.5	4200	980	Mainly break down β-O-4 linkages	(Xu et al., 2011)
Dilute acid	Poplar	~15	~85	8280	~2800	β-O-4 linkages are susceptible to dilute acid pretreatment	(Cao et al., 2012)
$[C_2mim][OAc]$	Switchgrass	40.5	59.5	N/A	N/A	Major interunit linkages are β-aryl ethers	(Shi et al., 2013)
Aqueous ammonia	Cotton stalk	~16	~84	~1700	~890	75.6% of β-O-4 linkages in cotton stalk lignin	(Kang et al., 2012)
Organosolv	Miscanthus	52	48	4690	7060	~0.41 β-O-4 linkages per aromatic ring	(El Hage et al., 2009)
$[C_2mim][OAc]$	Wheat straw	36.3	63.7	N/A	N/A	β-aryl ethers are the major linkages	(Sathitsuksanoh et al., 2014)
50% [Ch][Lys]	Rice straw	58.9	41.1	N/A	N/A	N/A	(Hou et al., 2013)
[Ch][Lys]	Switchgrass	85.1	14.9	N/A	N/A	N/A	(Sun et al., 2014)
CEL followed by hydrothermal	Hybrid poplar	~44	~56	~4800	~1700	Significant loss of β-O-4 linkages	(Trajano et al., 2013)

N/A: Not applicable

2.6.3 Lignin inter-unit linkages

Despite extensive investigation, the complex structure of lignin is not completely understood. Traditional wet chemistry analytical methods, such as degradation technique, cannot provide sufficient information to describe the entire structure of lignin, though they can be very precise (Chen, 1991). The advantage of spectroscopic techniques over wet chemistry analytical methods is that their capabilities of providing an entire lignin structure. Comparing to other spectroscopy techniques, such as Ultraviolet – visible (UV), Infrared (IR) and Raman spectroscopy, Nuclear Magnetic Resonance (NMR) spectroscopy can provide a much higher resolution (Crews et al., 1998). The development of quantitative ^{13}C NMR for lignin characterization was proposed in the early of 1980s (Robert & Gagnaire, 1981). Soon afterwards, the advancement of both software and spectra quality further improved lignin quantitative ^{13}C NMR characterization methods(Xia et al., 2001). Recent development of multi-dimensional NMR spectroscopy has allowed spectroscopy to extract various types of information about molecules (Ralph et al., 1999). NMR method was used to determine the lignin structure and composition in mutant tobacco (reduced lignin content by downregulating CCR and CAD); results illustrated that engineered tobacco contained fewer coniferyl alcohol-derived units (Ralph et al., 1998). Marita et al. reported an increase in syringyl unit content (determined by NMR) in Arabidopsis which was deficient in F5H by overexpression of *F5H* gene (Marita et al., 1999). In addition, NMR spectroscopy provided powerful diagnostic tools to elucidate lignin structure in CAD and COMT deficient poplar (Ralph et al., 2001). Pu et al. summarized the growing application of ^{31}P NMR to quantitatively characterize lignin structure and lignin/biomass derived bio-oils and biodiesels (Pu et al., 2011). Yuan et al. characterized the lignin structures and lignin-carbohydrate complex linkages in milled wood lignin and mild acidolysis lignin by ^{1}H-^{13}C HSQC NMR, and estimated the S to G ratio in two types of lignin materials (Yuan et al., 2011). Del Rio et al. compared the structural and compositional study of wheat straw lignin by DFRC (derivatization followed by reductive cleavage) and ^{1}H-^{13}C HSQC NMR, and provided information on S/G/H subunits in wheat lignin (Del Río et al., 2012). Furthermore, ^{13}C and ^{31}P NMR were developed to determine the characteristics of pyrolysis oil from softwood and

cleavage of ether bonds in lignin (Ben & Ragauskas, 2011). It is expected that 2D HSQC NMR spectroscopy will be extensively utilized to identify and quantify the lignin structure and lignin inter-unit linkages in the future biorefinery.

2.6.4 Functional groups

Fourier transform infrared spectroscopy (FTIR) is an analytical technique using infrared light to scan test materials. The resulting infrared spectrum reflects the absorption or emission of infrared from a solid, liquid or gas, by which chemical properties of organic, polymeric and in some cases, inorganic material can be identified (Griffiths & De Haseth, 2007). For many years, extensive studies associated with using FTIR to determine functionalities in lignin have been conducted and well described (Faix, 1986; Müller et al., 2008; Sarkanen & Ludwig, 1971). With the application of infrared spectroscopy technique, especially its improved techniques, such as diffuse reflectance infrared Fourier transform (DRIFT) (Freer et al., 2003), it is possible to quantify lignin in samples due to tremendous information associated with energy modes of different bonds within a single sample can be obtained. Recently, the near infrared spectroscopy (NIRS) (Shepherd et al., 2003; Yeh et al., 2004), has been developed as a lignin quantification method with the advantages of rapid, nondestructive (Hatfield & Fukushima, 2005). Additionally, extensive studies have employed FTIR to characterize the major components in lignocellulosic biomass before and after pretreatment (Kim & Lee, 2005; Li et al., 2010a). FTIR has been considered as an efficient tool to obtain the chemical fingerprinting of lignocellulosic biomass, and a reliable method to monitor the chemical changes of carbohydrates/lignin during physical/chemical processing.

2.6.5 Thermal property

Being the leftover fraction of pulp and paper industry and no better application can be found, lignin is commonly used a fuel so that recover its energy content. However, because of the large quantity, a strong interest has been manifested to valorize lignin as a potential resource of value-added chemicals, and thus, extensive investigations associated with thermal degradation behavior have been conducted (Brebu & Vasile, 2010; Caballero et al., 1997; Fierro et al., 2005; Sharma et al., 2004). As a branch of material

science, thermal analysis examines the effect of temperature change to the properties of materials. Numerous thermal analysis methods have been developed, of which, thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) are the most commonly used techniques in lignin thermal behavior determination (Erä & Mattila, 1976). TGA has been widely applied to determine the properties of material that exhibit either mass gain or loss due to oxidation or decomposition. TGA can provide a series information about physical properties, including vaporization, absorption, desorption. In addition, TGA can provide information about the chemical reactions within material under elevated temperatures, including dehydration, decomposition, and solid-gas reactions (Coats & Redfern, 1963). DSC is a thermal analysis technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Due to the capability of directly energy measuring, DSC has been widely applied to measure heat capacity, phase transition, and glass transition temperature of amorphous polymers like lignin with high precision (Wunderlich, 2005).

2.7 Lignin upgrading

2.7.1 Oxidative catalysis

Lignin depolymerization and valorization by oxidative catalysis focusing on producing aromatic compounds through the cleavage of aryl-ether bonds, carbon-carbon bonds, aromatic rings and other functional groups in lignin. Ma et al. summarized the processes of lignin oxidation and the catalysts being applied to produce chemicals, which include phenolic compounds, dicarboxylic acids and quinones (Ma et al., 2015). Organometallic catalysis, metal-free organic catalysis, acid or base catalysis are the common systems being employed to the oxidative upgrading of lignin. The most common used catalysts are nitrobenzene, metal oxides, and molecular oxygen, for instance H_2O_2/MTO, [MeRe(O2)O2], [Co(salen)], Mn(salen), Cu(salen), etc. The typical biomimetic catalysts for lignin depolymerization are synthetic metalloporphyrins, which are similar to the structure of lignin-degrading enzymes, such as LiP and MnP, and have high yield of monomer products (Li et al., 2015). Photocatalytic oxidation is method to

degrade lignin using TiO_2, ZnO_2, etc catalysts combined with UV light to save reaction time and minimize the organic pollutants (Zakzeski et al., 2010). Oxidative catalysis is the most common used method to depolymerize lignin, although the yield and selectivity still need to be improved.

2.7.2 Reductive catalysis

Reductive catalysis is a hydroprocessing involves thermal reduction of lignin by hydrogen, and an efficient process to covert lignin into low condensed lignin, phenols, and other fuels or chemicals. Reductive reaction can significantly break down carbon-carbon or carbon-heteroatom bonds, and this reaction usually takes place in exist of metal catalysis such as Pt, Ru, Ni, Pd, and Cu. In the process of producing fuels and chemicals, the reaction requires high temperature and high hydrogen pressure. Feghali et al. evaluated an efficient method of convergent reductive depolymerization of wood lignin, and successfully obtained 7-24 wt% yield from lignin and 0.5-2.4 wt% yield from woody materials (Feghali et al., 2015). Moreover, it was illustrated that the introduction of a second metal to a monometallic catalyst can enhance the catalytic performance. Parsell et al. reported a bimetallic Pd/C and Zn catalytic system to selectively cleave inter-unit linkages in lignin model compounds (Parsell et al., 2013). Due to the complex structure of native lignin, it is challenging to obtain pure products from lignin depolymerization. Reductive catalysis is a promising technology to deconstruct and further upgrade lignin.

2.7.3 Electro-catalysis

Electro-catalysis is a potential way to covert lignin into fuels and chemicals efficiently, and it displays high activity and stability than oxidative/reductive catalysts. IrO_2-based electrodes, such as Ti/RuO_2-IrO_2 and Ti/TiO_2-IrO_2 are commonly used in electro-catalysis. Milczarek et al. conducted the electro-catalysis in TRIS-HNO_3 buffers in the absence or presence of Mg2+ to determine the effect of pH on the catalytic conversion (Milczarek, 2009). The combination of ionic liquids with electro-catalysis has been demonstrated an efficient method to depolymerize and upgrade lignin. Ionic liquids can dissolve lignin effectively and can be an excellent mediator to selectively break down C-C and β-O-4 in electronic mediator systems. Reichert et al. employed a novel approach

of electro-catalysis of lignin by dissolving lignin in a special protic ionic liquid and using an anode with particular electro-catalytic activity. Results demonstrated that a wide range of aromatic fragments from lignin was identified (Reichert et al., 2012). Electro-catalysis is a promising lignin depolymerization method, however, the high cost and the electrode fouling need to be addressed before wide-spread applications.

2.7.4 Biological upgrading

2.7.4.1 Lignin degrading enzymes

Due to the bond types and heterogeneity, lignin cannot be depolymerized by hydrolytic enzymes as other natural polymers, such as starch, cellulose, protein, etc. However, it is well known that white rot fungi can produce extracellular lignin-degrading enzymes that are promising in lignin depolymerization (Wen et al., 2009). White rot fungi can secrete extracellular lignin-degrading oxidative enzymes, including lignin peroxidase (LiP, EC 1.11.1.14), manganese peroxidase (MnP, EC 1.11.1.13), and laccase (EC, 1.10.3.2) (Nousiainen et al., 2014). Lignin peroxidase is characterized by its high redox potential with hydrogen peroxide enabling oxidation of the non-phenolic aromatic compounds. Manganese peroxidase can oxide Mn^{2+} to Mn^{3+}, acting as a diffusing oxidizer (Hirai et al., 2005). Lignin peroxidase has a short-lived and highly active catalytic site on the surface of the protein that specially directs interaction with the lignin structure. However, this reaction is slow and inefficient, additional H_2O_2 should added in this enzymatic process. At the presence of H_2O_2, the reaction rate can be increased, the lignin substrate is the electron donor and the H_2O_2 is the final electron acceptor (Thanh Mai Pham et al., 2014). Unlike lignin peroxidase, laccase uses the oxygen as the electron acceptor. In a catalyzed reaction, laccase can transfer 4 electrons, and one molecule of oxygen is reduced to two molecules of H_2O. Laccase contains four copper ions, type 1 (T1), type 2 (T2) and type 3 (T3), T2 and T3 centers are close together and form a trinuclear cluster. T1 is the site where substrate oxidation takes place (Sitarz et al., 2013). Laccase has higher redox potential, which is an advantage when depolymerize lignin substrate, because it enables the enzyme to abstract electrons from the lignin subunits. Laccase uses oxygen as the final electron acceptor, no additional H_2O_2 is needed, so it

has more industrially applicable in practical lignin depolymerization.

Laccase cannot oxidize lignin alone, it is restricted to phenolic subunits, because non-phenolic subunits have higher redox potential and cannot be oxidized by enzymes alone. However, this restriction can be overcome by using a mediator, a mediator is a molecule that acts as an electron carrier between enzymes and the lignin substrate. Once the mediator is oxidized by enzymes through electron abstraction, the mediator will in turn oxidize the non-phenolic subunits of lignin (Nousiainen et al., 2014). The mediator can be classified into natural mediator and synthetic mediator. Natural mediator includes plant phenolics present in plants as secondary metabolites or fungal metabolites, like 4-hydroxybenzylic alcohol, p-cinnamic acid, sinapic acid, etc. The most common used synthetic mediator includes HBT (1-hydroxybenzotriazole), ABTS (2,2′-azinobis-3-ethylbenzthiazoline-6-sulphonate), TEMPO (2,2,6,6-tetramethylpiperidine 1-oxyl), and violuric acid (Shleev et al., 2006).

2.7.4.2 Lignin fermenting microorganisms

Lignin is a complex aromatic heteropolymer with different subunits cross-linked by different chemical bonds, thus, the nature of lignin makes microbial degradation especially difficult. However, some of microorganisms have evolved mechanisms for lignin degradation. White-rot fungi are the most widely studied lignin degrading microorganisms, however, the challenges of genetic modification in fungus limits the application of fungal lignin degradation (Xie et al., 2016; Zhu et al., 2017). Nevertheless, a variety of bacteria also contain oxidative enzymes to depolymerize lignin, such as *Rhodococcus opacus*, *Rhodococcus jostii*, *Sphingobium sp*. SYK-6, *Nocardia*, *Pseudomonas*, *Bacillus*, and *Comamonas* (Zhu et al., 2017). The environmental adaptability and availability of genetic manipulation of bacteria make it a promising approach to degrade lignin by bacterial conversion. Among various efforts on microbial degradation of lignin, bioconversion of lignin to lipids by oleaginous *Rhodococci* has been extensively studied. Kosa and Ragauskas evaluated the performance of *Rhodococcus opacus* on conversion of lignin model compounds to lipids. Results showed that can successfully grow on different lignin model substrates and can accumulate close

to 20% if their own weight lipids (Kosa & Ragauskas, 2012). Wei et al. reported the conversion of Kraft lignin to lipids by *Rhodococcus opacus*, and demonstrated that *Rhodococcus opacus* were capable to utilize oxygen-pretreated Kraft lignin as sole carbon source and accumulate significant amount of lipids (Wei et al., 2015). Le et al. developed a novel two-stage alkali-peroxide pretreatment of corn stover, and produced higher concentration of solubilized glucose and lower molecular weight lignin oligomers (Le et al., 2017). They also converted the organic substrates into lipids by *Rhodococcus opacus* with a maximal lipid production of 1.3 g/L after 48 h fermentation. Moreover, co-fermentation of *Rhodococcus opacus* and engineered *Rhodococcus jostii* RHA1 was conducted to reduce the COD of hydrothermal liquefaction aqueous waste of algae and pine (He et al., 2017b). He et al. established fundamental understanding of pathways and functional modules to enable production of lipids from lignin in dilute alkaline pretreated corn stover. The researchers demonstrated that co-fermentation of *Rhodococcus opacus* and genetically modified *Rhodococcus jostii* can produce higher lipids yield than single strain fermentation (He et al., 2017a). Although clear pathways for lignin to lipids production by *Rhodococcus opacus* needs to be identified, the bacterial conversion of lignin is a promising approach to upgrade lignin.

2.8 Summary and perspectives

Despite its great potential to a wide range of chemicals, lignin is yet an underutilized substrate, and under the current bio-refinery concept, lignin is commonly burned to generate steam and electricity. It is critical to convert lignin waste streams to high value-added chemicals to enable cost-competitive biofuels and chemicals production in a bio-refinery (Beckham et al., 2016; Mottiar et al., 2016; Ragauskas et al., 2014). Lignin's full potential as a renewable source for aromatic compounds can be unlocked only if an efficient and economic method for lignin depolymerization and valorization is developed (Prado et al., 2016). The heterogeneity of lignin (both in its varied bond chemistry and its variability between plants), however, is the primary hurdle to its targeted upgrading and reuse as a feedstock for chemicals and advanced materials (Das et al., 2012). The type and abundance of the inter-unit linkages (β-O-4, β-β, β-5, 5-5 and 5-O-4), as combinations of carbon-oxygen and carbon-carbon bonds, vary largely based on

the plant type (Zakzeski et al., 2010). Several lignin conversion methods currently under investigation are hydrolysis, hydrogenolysis, pyrolysis, catalytic oxidation, and biological depolymerization (Stärk et al., 2010).

On the other hand, improving plants characteristics for better environment resilience and more cost-effective conversion is one of the focusing areas in biomass feedstock development. Lignin, one of the main components of lignocellulosic biomass, consists of at least three different monomeric phenylpropanoid units (i.e. p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) linked by C-C and C-O bonds (Linger et al., 2014). Genetic modification of lignin showed great promise in reducing biomass recalcitrance and increasing the convertibility of biomass to fuels and chemicals (Himmel et al., 2007; Hisano et al., 2011; Xu et al., 2012). Particularly, approaches to down regulate the key enzymes involved in lignin biosynthesis have been applied in a variety of plants to reduce lignin content or alter the lignin composition (Chen & Dixon, 2007; Li et al., 2010b; Lu & Ralph, 1997; Xu et al., 2011). Moreover, modifications on the ratio of lignin subunits or lignin deposition and artificial design of lignin inter-unit/lignin-carbohydrates linkages were also showed effective in increase the accessibility of enzymes/microbes to lignocellulosic biomass (Chen & Dixon, 2007; Fu et al., 2011).

Extensive research have been conducted on how pretreatment methods affect the structural and compositional changes of biomass feedstocks and how to catalytically convert lignin to fuels and chemicals (Joffres et al., 2014; Samuel et al., 2010). However, the effects of lignin modification on lignin fractionation and characterization are not fully understood. It is necessary to investigate the fractionation and characterization of the sugar and lignin streams from wild-type and engineered plants and to build links between the biology of engineered plant, the pretreatment chemistry, and the conversion technologies. Advanced characterization methods and analytics will provide vital information about the structural and compositional changes, lignin molecular weight distribution and interunit linkages and the thermal properties of engineered biomass and fractionated lignin streams. These knowledge will help to answer the questions about 1) how do different biomass pretreatment methods affect lignin fractionation from genetically modified plants? 2) how does pretreatment chemistry affect the characteristics of lignin streams? 3) what does this mean to lignin upgrading? One would gain a better

understanding on how lignin modification impacts the fractionation and characterization of major biomass components with different pretreatment technologies. It will help to inform the plant scientists on how to create better plants and the engineers how to develop more effective and efficient conversion technologies to turn the engineered plant biomass to biofuels and other value added products.

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CHAPTER 3: FRACTIONATION AND CHARACTERIZATION OF LIGNIN STREAMS FROM GENETICALLY ENGINEERED SWITCHGRASS PRETREATED BY AN AQUEOUS IONIC LIQUID*

3.1 Abstract

As suggested by techno-economic analyses, the utilization of lignin to generate value-added products including fuels and chemicals greatly promotes the success of a lignocellulose-based biorefinery. Ionic liquids (ILs) have received increasing interest because of their high efficacy in fractionating and pretreating lignocellulosic biomass. The aim of this study was to compare the ability to fractionate the lignin streams from a wild type and two genetically engineered switchgrass species (low lignin content with high S/G ratio and high lignin content) using an aqueous bio-derived IL (i.e., 10% cholinium lysinate in water). The structural and compositional features and the impact of lignin modification on lignin-carbohydrate complex characteristics and the deconstruction of cell-wall compounds (especially lignin) were investigated. The *4CL* genotype with a lower lignin content and higher S/G ratio was demonstrated to be less recalcitrant to IL pretreatment likely due to the lower degree of lignin branching. Results further demonstrate that aqueous IL can effectively fractionate and partially depolymerize lignin from wild type and engineered switchgrass under mild conditions. Moreover, results showed over 80% of lignin dissolution from switchgrass into the liquid fraction while the remaining solids are highly digestible by cellulases. The soluble lignin underwent partial deploymerization to a molecular weight around 500-1000 Daltons. ^{1}H-^{13}C HSQC NMR results demonstrate that the variations in lignin compositions led to different modes of lignin dissolution and depolymerization during pretreatment of engineered switchgrass. This study provides a deeper understanding of how lignin engineering influences lignin fractionation and depolymerization during conversion processes based on aqueous IL pretreatment.

**This chapter has been submitted in whole for a journal publication.*

3.2 Introduction

Despite the recent fluctuation of oil and chemical markets, shifting society's dependence on petroleum based fuels and chemicals to biomass-derived products is important not only to address environmental challenges but also to increase the robustness of our energy security and economic stability (Chu & Majumdar, 2012; Obama, 2017). Biofuels derived from lignocellulosic biomass are well suited to address the challenges associated with reduced availability of petroleum liquid fuels. However, truly cost-effective production of biofuels has not yet been attained with existing technologies and processes (Cao et al., 2012; Wyman et al., 2011). Consequently, development of biomass feedstocks with desirable traits for cost-effective conversion is one of the focus areas in biofuels research (Ragauskas et al., 2014; Rinaldi et al., 2016; Simmons et al., 2010). Switchgrass (*Panicum virgatum*) is a promising bioenergy crop in North America with high productivity and low energy and nutrient requirements (Keshwani & Cheng, 2009; McLaughlin & Kszos, 2005).

Genetic modification of plants has been investigated to enhance the crop yield, drought and pest resistance, and the ease of conversion to biofuels and bioproducts (Fu et al., 2011; Xu et al., 2011; Xu et al., 2012). Particularly, manipulations of lignin pathways have drawn extensive attention. Lignin usually consists of three different phenylpropane units, i.e. *p*-coumaryl, coniferyl and sinapyl, and plays an important role in plant structural support and resistance against microbial and oxidative stresses (Davin & Lewis, 2005). In principle, reduction of lignin content, modification of lignin composition, modification of lignin deposition or modification of the lignin-carbohydrates linkages could all lead to reduced feedstock recalcitrance for the biochemical conversion (Hisano et al., 2009; Sticklen, 2008).

To overcome the recalcitrant nature of lignocellulosic biomass, a pretreatment step is commonly needed prior to the downstream saccharification and fermentation processes in a lignocellulose-based biorefinery. Compared with the other pretreatment approaches (e.g. dilute acid, ammonia, steam explosion, sodium hydroxide, etc.), ionic liquid (IL) pretreatment has received increasing interest because of ILs' high efficacy in

fractionating and pretreating lignocellulosic biomass. Imidazolium-based ILs, such as 1-ethyl-3-methylimidazolium acetate ([C_2C_1Im][OAc]), 1-butyl-3-methylimidazolium chloride ([C_4C_1Im][Cl]), and 1-ethyl-3-methylimidazolium chloride ([C_2C_1Im][Cl]), have been evaluated and proved highly effective in pretreatment of a variety of biomass feedstocks, including corn stover, switchgrass, poplar, pine wood, and municipal solid waste, etc. (Montalbo-Lomboy & Grewell, 2015; Perez-Pimienta et al., 2013; Samayam & Schall, 2010; Shi et al., 2014; Shi et al., 2013; Varanasi et al., 2012)

A group of ILs containing ions made of naturally occurring bases and acids from protein, hemicellulose, and lignin has recently emerged as a lower cost alternative to imidazolium based ILs (George et al., 2015; Liu et al., 2015; Socha et al., 2014; Sun et al., 2014). Cholinium lysinate, a bio-derived and biocompatible IL, has been demonstrated to be effective for biomass pretreatment owing to its efficacy in solubilizing lignin (Sun et al., 2016; Sun et al., 2014; Xu et al., 2016). A recent study also demonstrated that an aqueous IL ([C_2C_1Im][OAc] in water) can be as effective as pure IL in pretreating plant biomass. Using IL-water mixtures as pretreatment agents could reduce viscosity, eliminate gel formation during pretreatment and significantly reduce the energy requirements and costs associated with IL recycling (Shi et al., 2014). Furthermore, the biocompatibility of certain ILs provides a potential way to upgrade lignin allowing for the use of biological catalysts in an aqueous IL solution.

Pretreatment is a crucial step for making biomass feedstocks more amenable to biological conversion by unlocking sugars for fermentation. Nevertheless, as suggested by techno-economic analyses, the success of a lignocellulose-based biorefinery largely relies on the utilization of lignin to generate value-added products, such as fuels and chemicals(Ragauskas et al., 2014). The fate of lignin and its structural/compositional changes during pretreatment have recently received increasing attention; however, the effect of genetic modification on the fractionation and depolymerization of lignin from engineered plants is not fully understood. This study aims to fractionate and characterize the lignin streams from wild type and genetically engineered switchgrass species using an aqueous IL. The effects of lignin manipulation on the composition and enzymatic digestibility after pretreatment and enzymatic hydrolysis were investigated and compared

with lignin in untreated switchgrass. The molecular weight of the lignin fractions recovered from the liquid and solids streams after pretreatment and enzymatic hydrolysis was determined by gel permeation chromatography (GPC); while the cleavage of inter-unit lignin linkages was evaluated by ^{1}H-^{13}C HSQC NMR and compared with results from lignin in untreated switchgrass. Results from this study provide a better understanding of how lignin engineering of switchgrass influences lignin fractionation and upgrading during conversion processes based on aqueous IL pretreatment technology.

3.3 Experimental

3.3.1 Materials

Wild type and genetically engineered switchgrass (*Panicum virgatum*) were grown during the year 2015 in a greenhouse at Virginia Tech (Blacksburg, VA, USA). Transgenic RNAi-*4CL* switchgrass plants with silenced 4-coumarate:coenzyme A ligase gene (*Pv4CL1*; denoted as *4CL* thereafter) have reduced lignin content of the cell wall biomass (Xu et al., 2011). Transgenic plants with overexpression of an *Arabidopsis* transcription factor AtLOV1 (denoted as *AtLOV1* hereafter) have erected leaf phenotype and increased lignin content(Xu et al., 2012). The transgenic plants, along with the wild type controls were clonal propagated and maintained in a greenhouse with temperatures set at 22/28°C, night/day with a 12-14 h light regime. The plants were grown in Miracle-Gro Potting Mix (Miracle-Gro Lawn Products, Inc., Marysville, OH, USA) in 1.1×10^{-2} m^3 pots and watered approximately twice a week. Plant samples (the stems between the 2nd and 3rd nodes above the ground) were collected at the R3 stage(Hardin et al., 2013). Collected samples were dried at 60°C for three days and then ground by a Wiley Mill (Model 4) into a 1 mm size fraction and sieved by a Ro-Tap® testing sieve shaker (Model B, W. S. Tyler Industrial Group, Mentor, OH, US).

Cholinium lysinate (>95% purity) was synthesized following a method described elsewhere(Sun et al., 2014). The commercial enzymes including cellulase (Cellic® CTec2, 188 mg protein/ml) and hemicellulase (Cellic® HTec2, 180 mg protein/ml) were gifts from Novozymes, North America (Franklinton, NC, US).

3.3.2 Compositional analysis

Structural carbohydrates (i.e., glucan and xylan), acid-soluble and acid insoluble lignin were quantified according to a NREL laboratory analytical procedure(Sluiter et al., 2008). Briefly, an air-dried sample was mixed with 72% (w/w) sulfuric acid at a ratio of 1:10. The mixture was incubated at 30 ± 3°C for 60 ± 5 min, and stirred every 10 min. After 60 minutes of hydrolysis, deionized (DI) water was added to the mixture to reach an acid concentration of 4% (w/w), after which the mixture was autoclaved at 121°C for 1 hour. After two-stage acid hydrolysis, acid soluble lignin was measured using a spectrophotometer at 205 nm; acid insoluble lignin was obtained by subtracting the ash content from the weight of solid residues. Monomeric sugars (glucose and xylose) were determined by HPLC following a method shown in section 3.3.6.

3.3.3 Aqueous ionic liquid pretreatment

Aqueous cholinium lysinate was used for all the pretreatment experiments. Cholinium lysinate (10% w/w) and switchgrass were mixed at a ratio of 9:1 (w/w) in a 20 mL stainless steel (SS316) reactor, capped and then heated at 140 ± 2°C in a stirred oil bath for 1 h. After heating, the reactor was removed from the oil bath and quenched in ice water. The mixture was transferred from the reactor to a 50 mL centrifuge tube and centrifuged at 4000 rpm for 10 min to separate the solids and liquid. The solids were washed three times with 150 mL of hot DI water to remove the excess cholinium lysinate. The washed solids were used for further enzymatic hydrolysis. The liquid was titrated using 6 M HCl until the pH reached 1-2, and then stored at 4°C for 7 days to precipitate lignin. After precipitation, the recovered lignin was washed and freeze-dried. Monomeric sugars in the liquid phase were determined according to the NREL laboratory analytical procedure(Sluiter et al., 2006). Briefly, the residual liquid was adjusted to an acid concentration of 4% (w/w) sulfuric acid and was then autoclaved at 121°C for 1 h. Glucose and xylose in the residual liquid were determined by HPLC (see below).

3.3.4 Enzymatic hydrolysis

Enzymatic hydrolysis of the untreated and pretreated switchgrass followed the

NREL laboratory analytical procedure(Selig et al., 2008). After pretreatment, the recovered solids were mixed with 50 mM citrate buffer, 0.01 g/L sodium azide and enzymes (CTec2/HTec2, 9:1, v/v). Two enzyme loadings, 20 mg and 5.25 mg enzyme protein/g starting biomass, were tested. The saccharification was performed at 50°C for 72 h in an orbital shaker (Thermo Forma 435, Thermo Fisher Scientific Inc., Waltham, MA, US). After hydrolysis, monomeric sugar concentration was determined by HPLC. The residual solids were collected, washed, and freeze-dried for compositional analysis and lignin characterization.

3.3.5 Mass balance

Mass balances (sugars and lignin) were closed on the liquid and solid streams of fractionated switchgrass after aqueous IL pretreatment and enzymatic hydrolysis. Glucan, xylan, and lignin for mass balances were determined according to an NREL laboratory analytical procedure(Sluiter et al., 2008).

3.3.6 Analytical methods

The major monomeric sugars (glucose, xylose and arabinose) in the liquid streams from compositional analysis and enzymatic saccharification were measured by a Dionex HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) equipped with a refractive index detector and Aminex HPX-87H column and guard column assembly, using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.4 mL/min and a column temperature of 50°C. Identification and quantification of the monomeric products from lignin depolymerization were performed using an Agilent 7890B GC coupled with a 5977B MS (Agilent Technologies, Inc., Santa Clara, CA, US) and a HP-5MS (60 m × 0.32 mm) capillary column. The temperature program started at 50 °C, increased to 120°C at 10°C min^{-1} with a holding time of 5 min, then increased to 280°C at 10°C min^{-1} with a holding time of 8 min and finally increased to 300 °C at 10°C min^{-1} with a holding time of 2 min. Helium was used as the carrier gas with a flow rate of 1.2 mL min^{-1}.

3.3.7 Lignin characterization

Cellulolytic enzyme lignin (CEL) isolation: The untreated switchgrass (no

pretreatment), including wild type (WT) and two transgenic plants (*4CL* and *AtLOV1*), and their precipitated lignin-enriched solids of ionic liquid pretreatment were thoroughly extracted with a mixture of toluene-ethanol (2/1, *v/v*) in a Soxhlet for 24 h. CEL was isolated from the extracted switchgrass and the lignin-enriched solids according to a published literature procedure (**Figure 8**) (Hu et al., 2006; Yoo et al., 2016b). In brief, the extractives-free samples were loaded into a 50 mL ZrO_2 grinding jar (including 10×10 ball bearings) in a Retsch Ball Mill PM 100. The biomass was then ball milled at 580 RPM for 5 min, followed by a 5 min pause; this procedure was repeated for 1.5 h in total. The milled fine cell wall powder was then subjected to enzymatic hydrolysis with a mixture of Cellic® CTec2 and HTec2 in acetic acid/sodium acetate buffer (pH 4.8, 50°C) under continuous agitation at 200 rpm for 48 h. The residue was isolated by centrifugation and was hydrolyzed once more with freshly added enzymes. The residue obtained was rich in lignin and was washed with DI water, centrifuged, and freeze-dried. The lignin-enriched residue was extracted with dioxane-water (96% *v/v*, 10.0 mL/g biomass) for 24 h. The extracted mixture was centrifuged and the supernatant was collected. Dioxane extraction was repeated once by adding fresh dioxane-water. The extracts were combined, roto-evaporated to reduce the volume at a temperature of less than 45°C, and freeze-dried. The obtained lignin samples, designated as CEL, was used for further analysis.

Gel permeation chromatographic (GPC) analysis: The weight-average molecular weight (M_w) and number-average molecular weight (M_n) of lignin were measured by GPC after acetylation as previously described(Samuel et al., 2014). Briefly, lignin derivatization was conducted on a basis of ~3 mg lignin in 1 mL of pyridine/acetic anhydride (1:1, *v/v*) in the dark with magnetic stirring at room temperature for 24 h. The solvent/reagents were removed by co-evaporation at 45°C with ethanol, several times, using a rotatory evaporator until dry. The resulting acetylated lignin was dissolved in tetrahydrofuran (THF) and the solution was filtered through 0.45 μm membrane filter before GPC analysis. Size-exclusion separation was performed on an Agilent 1200 HPLC system (Agilent Technologies, Inc., Santa Clara, CA, US) equipped with Waters Styragel columns (HR1, HR4, and HR5; Waters Corporation, Milford, MA, US). A UV detector (270 nm) was used for detection. THF was used as the mobile phase at a flowrate of 1.0

mL/min. Polystyrene narrow standards were used for establishing the calibration curve.

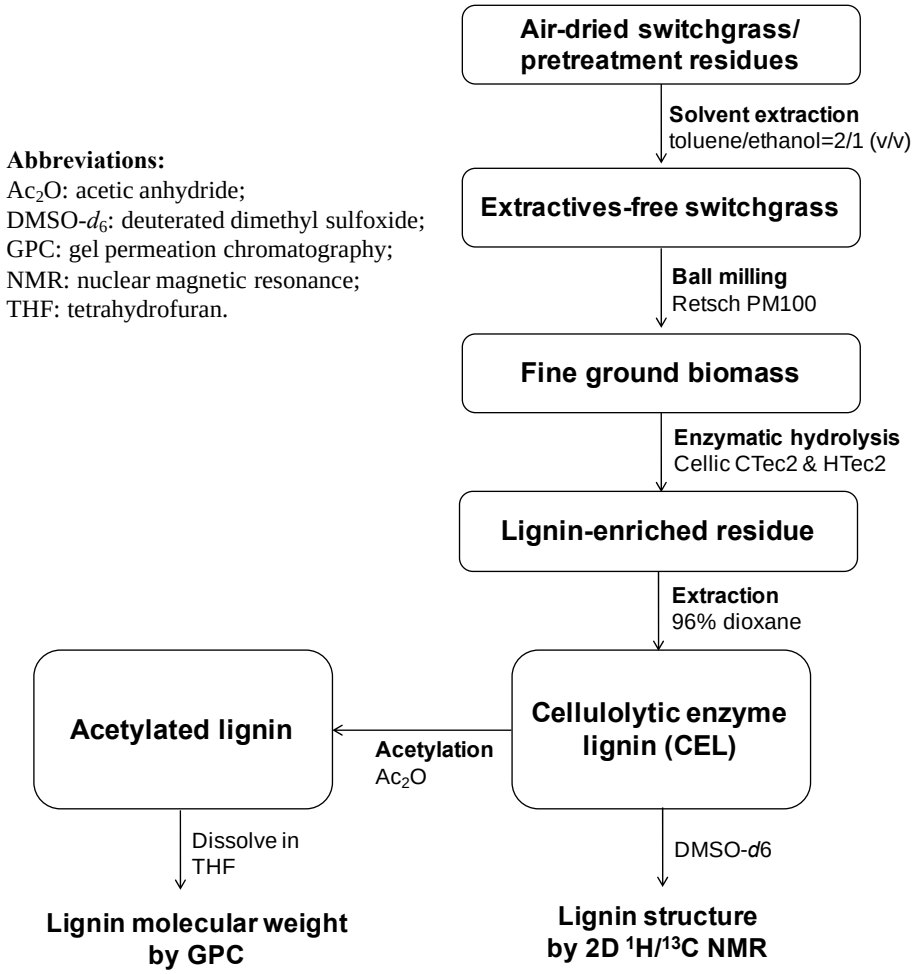


Figure 8. Cellulolytic enzyme lignin (CEL) isolation from switchgrass

NMR spectroscopic analysis: Nuclear magnetic resonance (NMR) spectra of isolated lignin samples were acquired in a Bruker Avance III 400-MHz spectrometer and spectral processing was carried out using Bruker Topspin 3.5 (Mac) software. A standard Bruker heteronuclear single quantum coherence (HSQC) pulse sequence (hsqcetgpspsi2) was used on a Bruker BBFO probe with the following acquisition parameters: spectra width 10 ppm in F2 (^{1}H) dimension with 2048 time of domain (acquisition time 256.1 ms), 210 ppm in F1 (^{13}C) dimension with 256 time of domain (acquisition time 6.1 ms), a

1.5-s delay, a $^{1}J_{C–H}$ of 145 Hz, and 32 scans. The central DMSO solvent peak (δ_C/δ_H at 39.5/2.49) was used for chemical shifts calibration. The relative abundance of lignin compositional subunits and interunit linkages was estimated using volume integration of the contours in HSQC spectra (José et al., 2015; Samuel et al., 2014; Yoo et al., 2016b). For monolignol compositions of S, G, H, *p*-coumarate (*p*CA), and ferulate (FA) measurements, the $S_{2/6}$, G_2, $H_{2/6}$, $pCA_{2/6}$, and FA_2 contours were used with G_2 and FA_2 integrals doubled. The Cα signals were used for contour integration for the estimation of interunit linkages.

3.3.8 Statistical analysis

All experiments were conducted in triplicate and the data were presented as means with standard deviations. The statistical analysis was performed by using SAS® 9.4 (SAS Institute, Cary, NC, US), with a significance level of $P<0.05$ for all the data obtained from experiments.

3.4 Results and discussion

3.4.1 Aqueous IL pretreatment of wild-type and engineered switchgrass

Due to the genetic variations in the wild type and engineered switchgrass, *4CL* and *AtLov1*, differences in the compositions of the three tested switchgrass lines are expected, both before and after aqueous IL pretreatment. According to the results shown in **Table 4**, similar levels of glucan and xylan content were observed in the three lines of switchgrass. For the WT and *4CL* switchgrass, the glucan contents were around 34%, only slightly lower than the glucan content of *AtLOV1* switchgrass. The xylan contents in the three types of switchgrass were around 21-22%. However, the lignin content in *4CL* switchgrass was significantly lower ($P<0.05$) than that of WT and *AtLOV1* switchgrass. In contrast, there was no significant difference in lignin content between WT switchgrass and *AtLOV1* switchgrass. These results align well with the corresponding gene modifications to the switchgrass plants, with reduced lignin content for *4CL* as a result of silencing the 4-coumarate:coenzyme A ligase gene(Xu et al., 2011), while the overexpression of an Arabidopsis transcription factor *AtLOV1* leads to a slight increase in

lignin content as compared with WT switchgrass(Xu et al., 2012).

Table 4. Compositional analysis of untreated and aqueous IL pretreated switchgrass*

	Raw switchgrass			Pretreated switchgrass		
	WT	*4CL*	*AtLOV1*	WT-IL	*4CL*-IL	*AtLOV1*-IL
Extractives, %	14.6 ± 0.3	18.0 ± 0.2	13.0 ± 0.1	ND	ND	ND
Glucan, %	34.5 ± 0.1[a]	33.5 ± 0.1[b]	36.0 ± 0.4[c]	49.1 ± 0.9	50.3 ± 0.8	49.4 ± 1.8
Xylan. %	21.9 ± 2.4[a]	22.7 ± 0.2[a]	21.3 ± 0.3[a]	22.1 ± 0.4	22.8 ± 0.2	20.6 ± 0.8
Lignin, %	18.6 ± 0.0[a]	16.3 ± 0.3[b]	19.1 ± 0.3[a]	12.5 ± 1.2	8.6 ± 1.2	12.6 ± 1.2
Ash, %	4.5 ± 0.1	5.3 ± 0.1	4.3 ± 0.1	ND	ND	ND
Solid recovery, %	N/A	N/A	N/A	44.0 ± 2.2	42.7 ± 3.7	51.0 ± 3.0

*Based on dry biomass; ND = not determined; N/A = Not applicable; $P<0.05$

Genetic modification to lignin pathways not only causes differences in the composition of untreated switchgrass but could also impact the dissolution and depolymerization of biomass components during a pretreatment process(Shi et al., 2016). In this study, aqueous IL, 10% (w/w) cholinium lysinate was used to pretreat the switchgrass samples. After pretreatment, the composition of the recovered solids after IL pretreatment is shown in **Table 4**. The solid recovery for the three types of switchgrass was different. After aqueous IL pretreatment, approximately 44% of the raw switchgrass was recovered for WT switchgrass; however, the solid recovery was 43% and 51%, for *4CL* and *AtLOV1* switchgrass, respectively. The high weight loss for *4CL* switchgrass is a combined effect of solubilization of lignin and xylan, and removal of extractives. Despite a reduction in lignin content for all of the three types of switchgrass, more lignin was removed from *4CL* switchgrass as compared to WT and *AtLOV1* switchgrass. These

results demonstrate that aqueous cholinium lysinate is a good solvent for lignin and that *4CL* switchgrass is more susceptible to aqueous IL pretreatment. The reduced lignin content and altered S/G ratio plausibly lead to decreased recalcitrance of the *4CL* switchgrass, thus affect the lignin dissolution during aqueous IL pretreatment.

Cholinium lysinate is a bio-derived IL and has been shown highly effective for lignin solubilization, due to the greater hydrogen bond basicity for the IL with [Lys]- anions as compared with acetate ILs(Sun et al., 2014). The solvent property of an IL is the key for biomass deconstruction and lignin depolymerization. Recent studies have demonstrated that, in some cases, an aqueous IL system can be as effective as pure IL for fractionating plant materials and extracting lignin(Shi et al., 2014). The hydrogen bond basicity (β value) representing the solvent's ability to disrupt the inter- and intra-molecular hydrogen bonding in cellulose, hemicellulose and lignin, correlates well with cellulose crystallinity as well as lignin removal and serves as a good indicator of pretreatment efficacy for $[C_2C_1Im][OAc]$-water mixtures(Shi et al., 2014). Molecular dynamics simulations provided molecular level explanations for cellulose I_β dissolution, suggesting that IL-water mixtures can be effective for solubilizing cellulose microfibrils into individual chains(Parthasarathi et al., 2015; Samuel et al., 2014).

Table 5 lists the relative abundance of lignin subunits (S, G, and H) and hydroxycinnamates (*p*CA and FA) based on 2D NMR results. In general, *4CL* switchgrass had a higher S/G ratio (0.73) than that of WT and *AtLOV1* switchgrass (0.45 and 0.55, respectively). It is generally believed that biomass with a higher S/G ratio is more digestible by cellulolytic enzymes(Li et al., 2010b; Studer et al., 2011). After pretreatment, the S/G ratios of pretreated switchgrass all increased, especially for *4CL* switchgrass for which the S/G ratio increased from 0.73 to 1.42. The increased S/G ratio can be explained by the preferential removal of G lignin as reported in a previous study which showed that certain ILs can selectively degrade G lignin(Wen et al., 2014; You et al., 2015). Ferulate (FA) and *p*-coumarate (*p*CA) are the hydroxycinnamates in lignin structure. Previous studies have suggested that switchgrass with a lower *p*CA/FA ratio is more digestible(Gabrielsen et al., 1990). Based on **Table 5**, both *p*CA and FA units were significantly reduced during aqueous IL pretreatment, especially for *4CL* switchgrass for

which FA can be barely detected in the pretreated biomass. The overall lower *p*CA/FA ratio is a plausible reason for the reduced recalcitrance of *4CL* switchgrass.

Table 5. Relative abundance of lignin subunits (S/G/H), hydroxycinnamates quantified on the basis of ^{1}H-^{13}C HSQC NMR spectra

		Raw switchgrass			Pretreated switchgrass		
		WT	*4CL*	*AtLOV1*	WT-IL	*4CL*-IL	*AtLOV1-IL*
S/G/H abundance	S%	29.6	38.3	34.8	39.3	58.7	45.2
	G%	66.0	52.8	63.5	59.4	41.3	53.5
	H%	4.3	8.9	1.7	1.3	0.0	1.2
	S/G	0.45	0.73	0.55	0.66	1.42	0.85
Hydroxycinnamates (% of Ar)	*p*CA%	59.7	85.3	57.4	21.3	16.8	24.7
	FA%	17.0	31.0	14.1	14.8	0.0	13.6
	*p*CA/FA	3.51	2.75	4.06	1.44	N/A	1.83

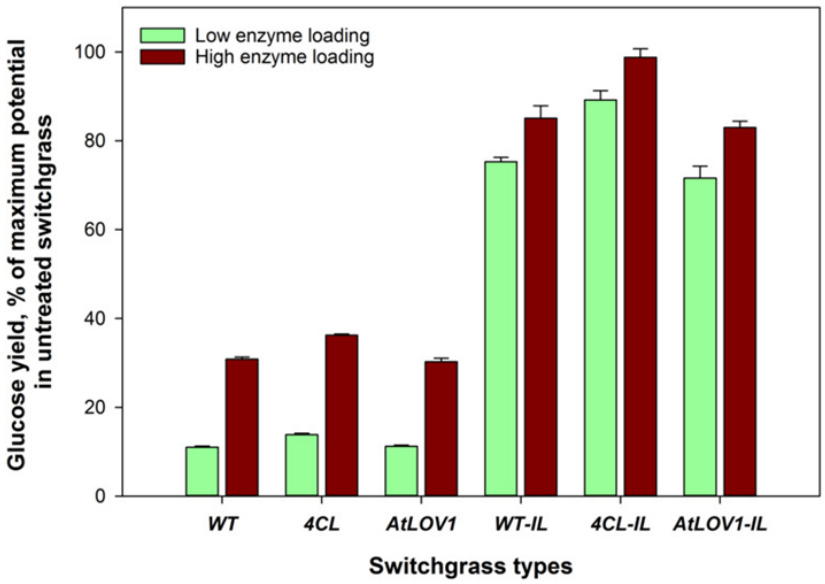
3.4.2 Saccharification of aqueous IL-pretreated switchgrass

Pretreatment with certain ILs has been shown as an efficient way to overcome the recalcitrance of biomass, unlock the lignin-carbohydrate complexes, and improve accessibility of cellulose/hemicellulose to hydrolyzing enzymes(Li et al., 2013; Shi et al., 2013). However, the effect of an aqueous IL pretreatment on the enzymatic digestibility of engineered switchgrass has not been reported. **Figure 9** shows the glucose and xylose yield under low and high enzyme loadings of 5.25 mg protein/g and 20 mg protein/g starting biomass, respectively. Aqueous IL pretreatment greatly improved the sugar yield as compared to untreated switchgrass. At both low and high enzyme loadings, pretreated *4CL* switchgrass gave the highest glucose and xylose yields among the three tested

feedstocks. After 72 h of enzymatic hydrolysis, a glucose yield of 98.8% and xylose yield of 63.2% were observed for pretreated *4CL* switchgrass under high enzyme loading. Sugar yields of pretreated WT and *AtLOV1* switchgrass were lower than that of *4CL* switchgrass. The *AtLOV1* switchgrass with upregulated lignin resulted in the lowest sugar yield among the three types of switchgrass.

Results indicate that *4CL* switchgrass was more digestible as compared to WT and *AtLOV1* switchgrass, suggesting that downregulation of lignin content and slight increase of the S/G ratio in plants can lead to increased sugar yield. This observation agrees with a few previous studies on switchgrass, poplar, *Arabidopsis* and other biomass feedstocks(Li et al., 2010b; Shi et al., 2016; Simmons et al., 2010; Studer et al., 2011). Although high enzyme loading led to ~10-15% higher glucose and xylose yields, from an economics perspective the cost of commercial enzymes is still a significant contributor to biofuel production cost(Klein-Marcuschamer et al., 2011). Thus, a low enzyme loading (5.25 mg protein/g starting biomass) was used in this study to establish a baseline for the aqueous IL fractionation process.

a)



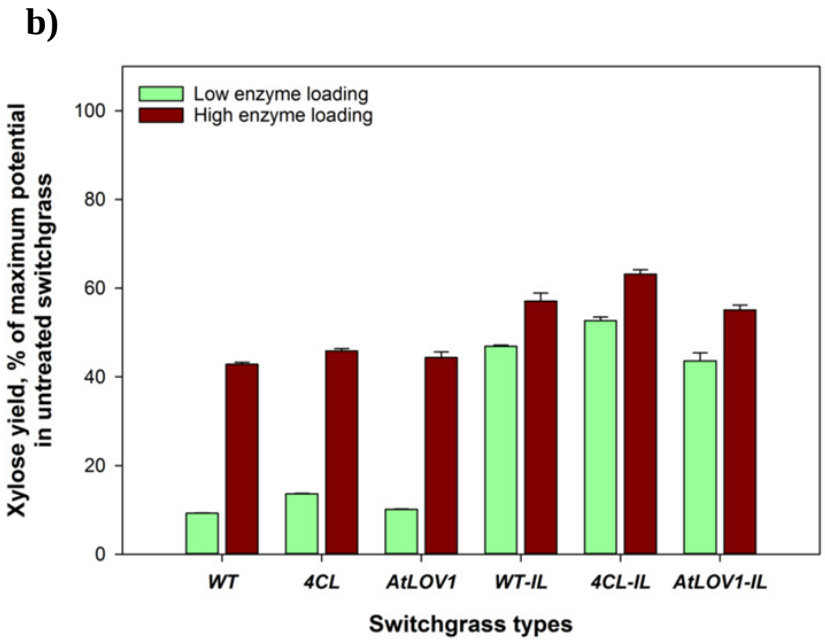
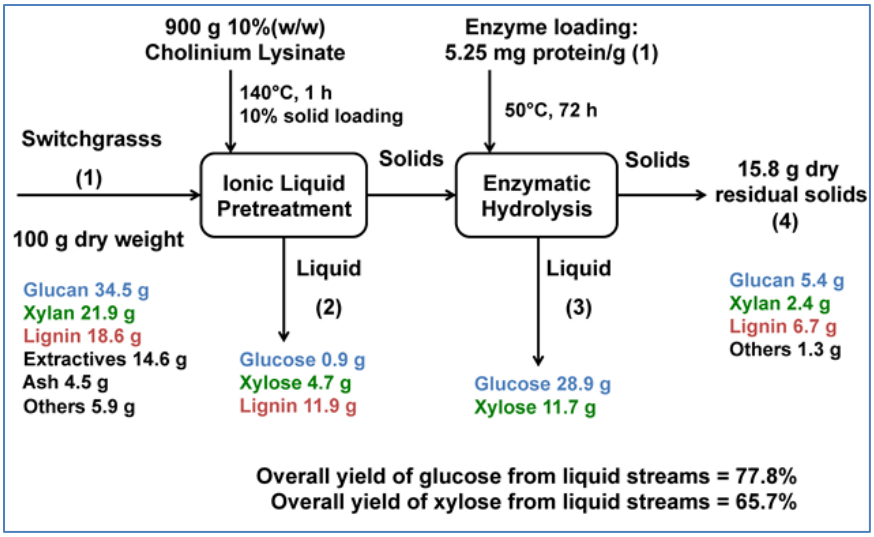


Figure 9. Glucose yield (a) and xylose yield (b) during enzymatic hydrolysis of untreated and aqueous IL pretreated switchgrass under high/low enzyme loading

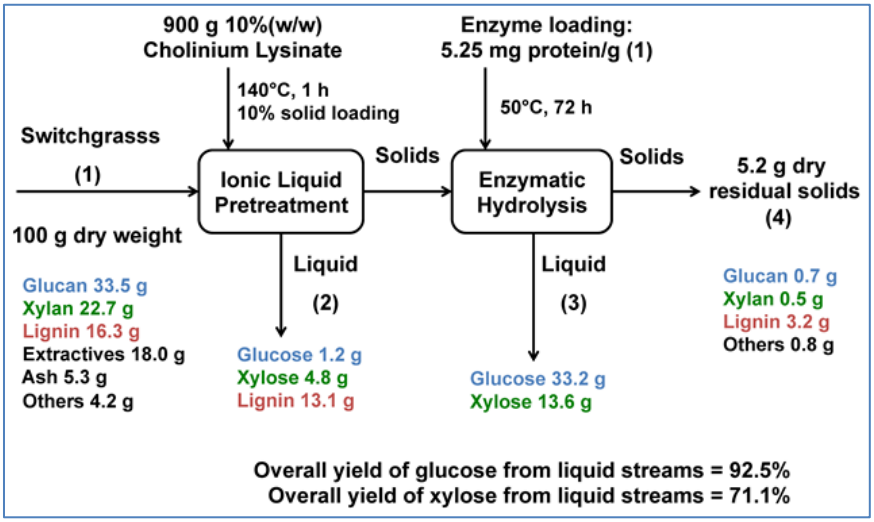
3.4.3 Fractionation of lignin streams and mass balances

In order to elucidate the mass flow of major biomass components during aqueous IL pretreatment and subsequent enzymatic hydrolysis, mass balances for glucan, xylan and lignin for the different types of switchgrass were tracked, the results being illustrated in **Figure 10**. As a general observation, starting with 100 g raw WT switchgrass, a large portion of the lignin was solubilized in the liquid stream during aqueous IL pretreatment. However, only a small portion of glucan and xylan was extracted in the pretreatment liquid, the majority being carried along with the pretreated solids and hydrolyzed to monomeric sugars during the enzymatic hydrolysis step. **Figure 10a** demonstrates that a total amount of 28.9 g glucose and 11.7 g xylose were recovered from liquid streams for WT switchgrass, which correspond to overall recoveries of 77.8% and 65.7% for glucose and xylose, respectively. For *4CL* switchgrass, the overall recoveries from liquid streams

a)



b)



c)

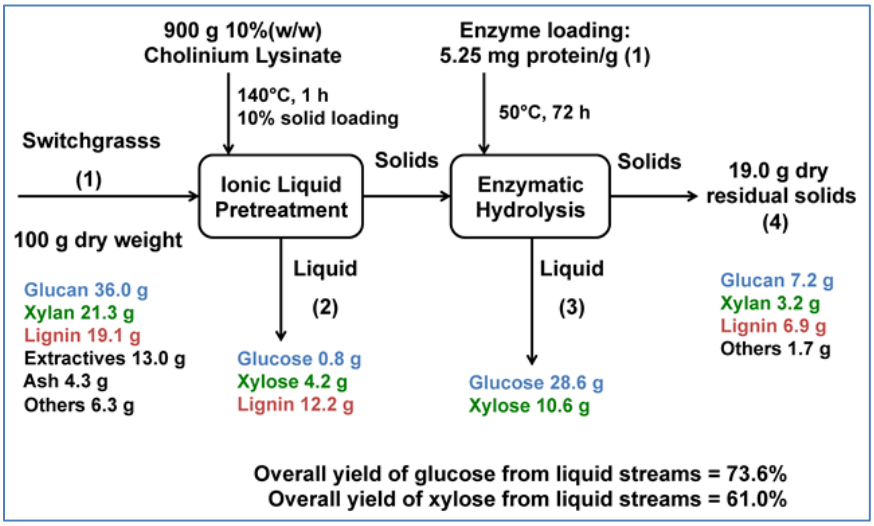


Figure 10. Mass balances for aqueous IL pretreatment and saccharification of different switchgrass genotypes: a) WT, b) *4CL* and c) *AtLOV1*

were 92.5% and 71.1% for glucose and xylose, respectively (**Figure 10b**). The lowest overall glucose (73.6%) and xylose (61.0%) recoveries from liquid streams were observed for *AtLOV1* switchgrass, mainly due to the overall lower sugar yields during enzymatic hydrolysis compared with *4CL* switchgrass. In other words, the overall glucose yield from liquid streams for *4CL* switchgrass was 15% higher than that of WT switchgrass and 19% higher than that of *AtLOV1* switchgrass. The overall xylose yield from liquid streams for *4CL* switchgrass was around 5% and 10% higher than WT and *AtLOV1* switchgrass, respectively. It can be concluded that *4CL* switchgrass is relatively more digestible due to the decreased biomass recalcitrance caused by the low lignin content and increased S/G ratio.

Clearly, the aqueous IL (cholinium lysinate) exhibited lignin solubilization capabilities, based on the compositional analysis. The fractionation of lignin into liquid and solids streams for different types of switchgrass is illustrated in **Figure 11**. Approximately 60-65% of lignin was solubilized in the pretreatment liquid stream for WT and *AtLOV1* switchgrass. However, over 80% of lignin was fractionated into the liquid stream for *4CL* switchgrass. In addition to the lower lignin content and higher S/G ratio, fewer branching lignin linkages were observed in *4CL* switchgrass when compared with WT and *AtLOV1* switchgrass from 2D NMR results. Furthermore, low *p*CA and FA content in *4CL* switchgrass can be another contributor to the reduced recalcitrance. High contents of *p*CA and FA in plants have been found to be unfavorable to the digestibility of polysaccharides(Ralph, 2010) and a recently study suggested that the monolignol ferulate bearing ester bonds are readily cleaved during alkaline pretreatment(Wilkerson et al., 2014). Therefore, the low lignin content, high S/G and low *p*CA/FA ratios of *4CL* switchgrass, in combination, make it more susceptible to aqueous IL pretreatment than the WT and *AtLOV1* switchgrass.

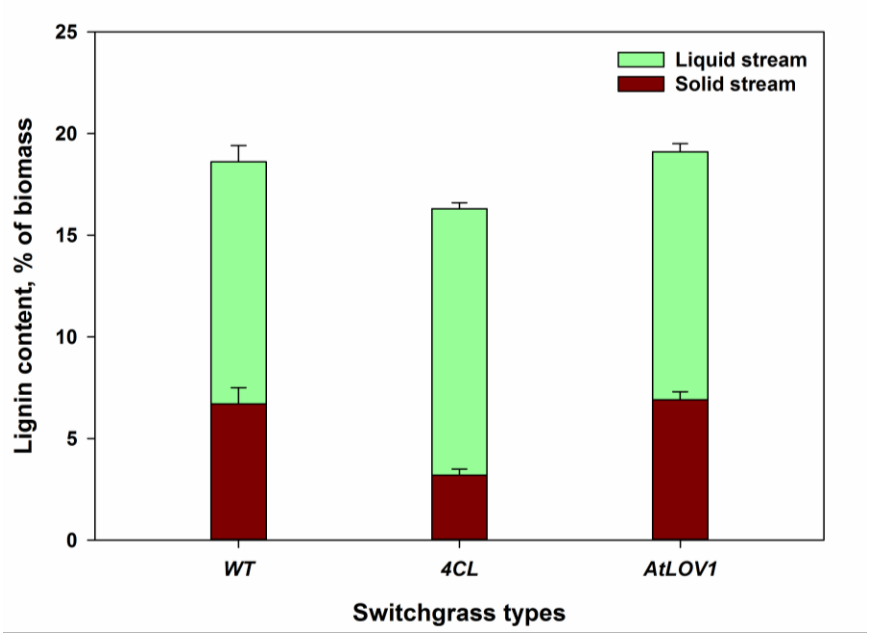


Figure 11. Fractionation of lignin streams during aqueous IL pretreatment of different switchgrass genotypes

3.4.4 Characterization of lignin streams

Gel permeation chromatographic analysis: To better understand the lignin depolymerization during aqueous IL pretreatment, GPC analysis was conducted to determine the molecular weight (MW) distribution of lignin in both untreated and pretreated (residual solids) switchgrass using a set of polystyrene molecules as standards. GPC spectra show that the lignin MWs were significantly reduced after IL pretreatment (**Figure 12**). As shown in **Figure 13**, the untreated switchgrass mainly contains relatively high molecular weight lignins. For instance, the lignin in *AtLOV1* switchgrass has a number-average molecular weight (M_n) of 5862 g/mol and a weight-average molecular weight (M_w) of 15236 g/mol, values which are slightly higher than those of WT switchgrass. In comparison, the lignin in *4CL* switchgrass has lower M_n and M_w of 4482 and 9806 g/mol, respectively. Both M_n and M_w were remarkably reduced (52-59% reduction in M_n and 37-54% reduction in M_w) for the lignin in residual solids after aqueous IL pretreatment. Furthermore, the polydispersity index (PDI) of the lignin in pretreated WT switchgrass and *4CL* switchgrass increased compared with untreated switchgrass (**Table 6**) indicating a wide span of MW after pretreatment. Taken together,

these results suggest that lignin underwent significant depolymerization during the aqueous IL pretreatment, such that the majority of lignin was depolymerized and solubilized in the liquid stream.

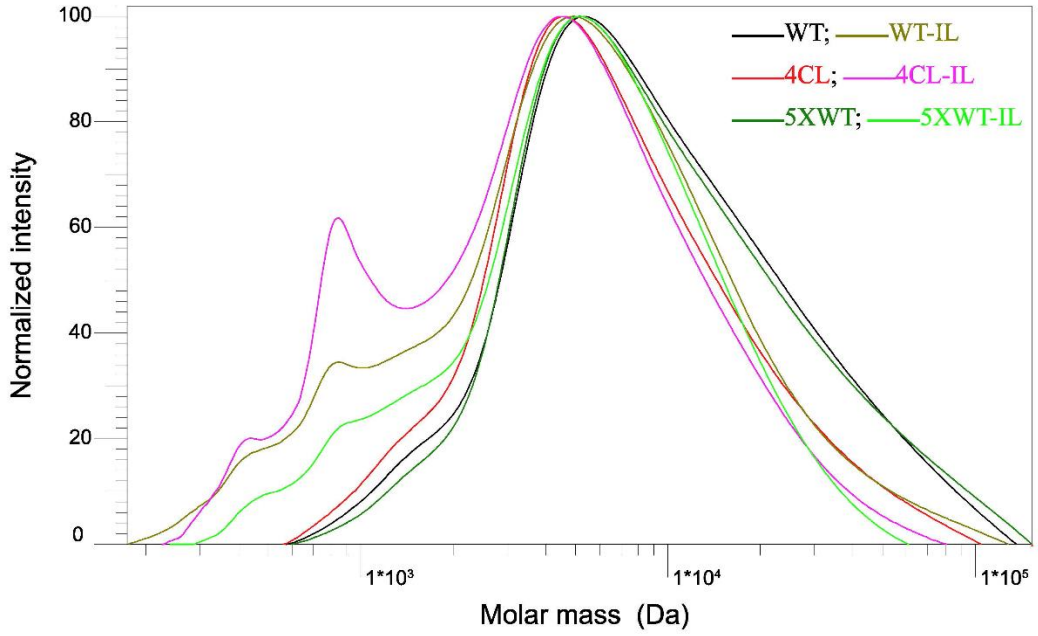


Figure 12. GPC spectra of untreated switchgrass and pretreated switchgrass

Table 6. Polydispersity index (PDI) of untreated switchgrass and pretreated switchgrass

	Raw switchgrass			Pretreated switchgrass		
	WT	*4CL*	*AtLOV1*	WT-IL	*4CL*-IL	*AtLOV1*-IL
PDI	2.57	2.19	2.60	3.71	3.12	2.47

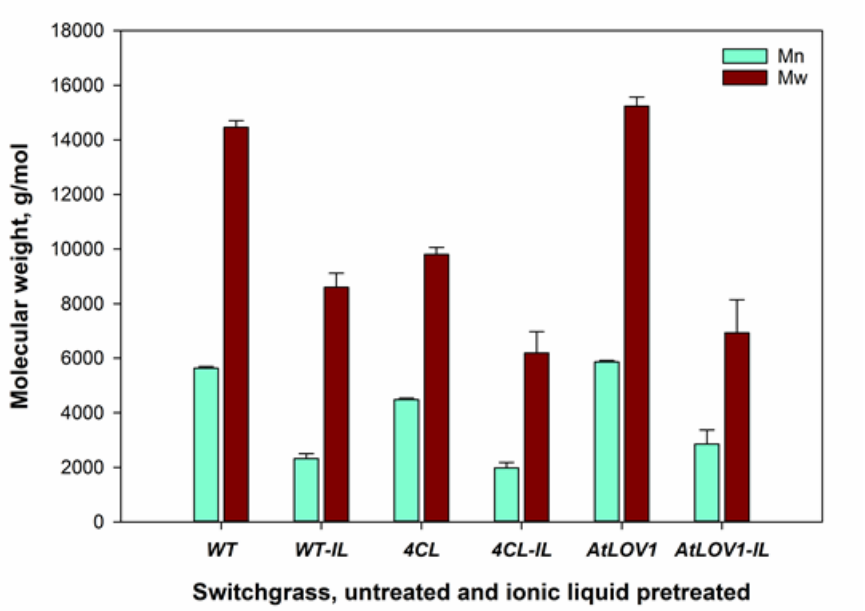


Figure 13. The number-average (Mn) and weight-average (Mw) molecular weights of lignin from untreated and aqueous IL pretreated switchgrass

Table 7 provides further details of the lignin MW distribution in the liquid streams. Soluble lignin in aqueous IL after pretreatment has an M_w of ~3300 g/mol and M_n of ~1100 g/mol for all three types of switchgrass, with slightly lower M_w and M_n observed for the *4CL* switchgrass sample. After pH adjustment to 1-2, about 30% of the lignin precipitated out from the liquid stream. The precipitated lignin has higher M_w and M_n than the soluble lignin, probably because larger MW lignin tends to precipitate readily at acidic pH values. Surprisingly, about 70% of the lignin remains dissolved in the liquid phase after pH adjustment, while the solubilized lignin had low MW (<1000 g/mol for M_w, representing lignin oligomers). The low molecular weight lignin fractions in aqueous IL were likely a result of the high extent of lignin depolymerization in the pretreatment medium as compared with the other pretreatment methods such as dilute acid or hot water pretreatment. The low molecular weight lignin oligomers that accumulated in the liquid phase are suitable for further lignin upgrading *via* catalytic or biological conversion pathways. It has been shown that certain lignin degrading enzymes or microbes can assimilate and convert low molecular weight lignin to fuel and chemical molecules (Linger et al., 2014; Ragauskas et al., 2014; Zhao et al., 2016). Thus, the results from this work point to possible pathways for developing a more selective and efficient lignin

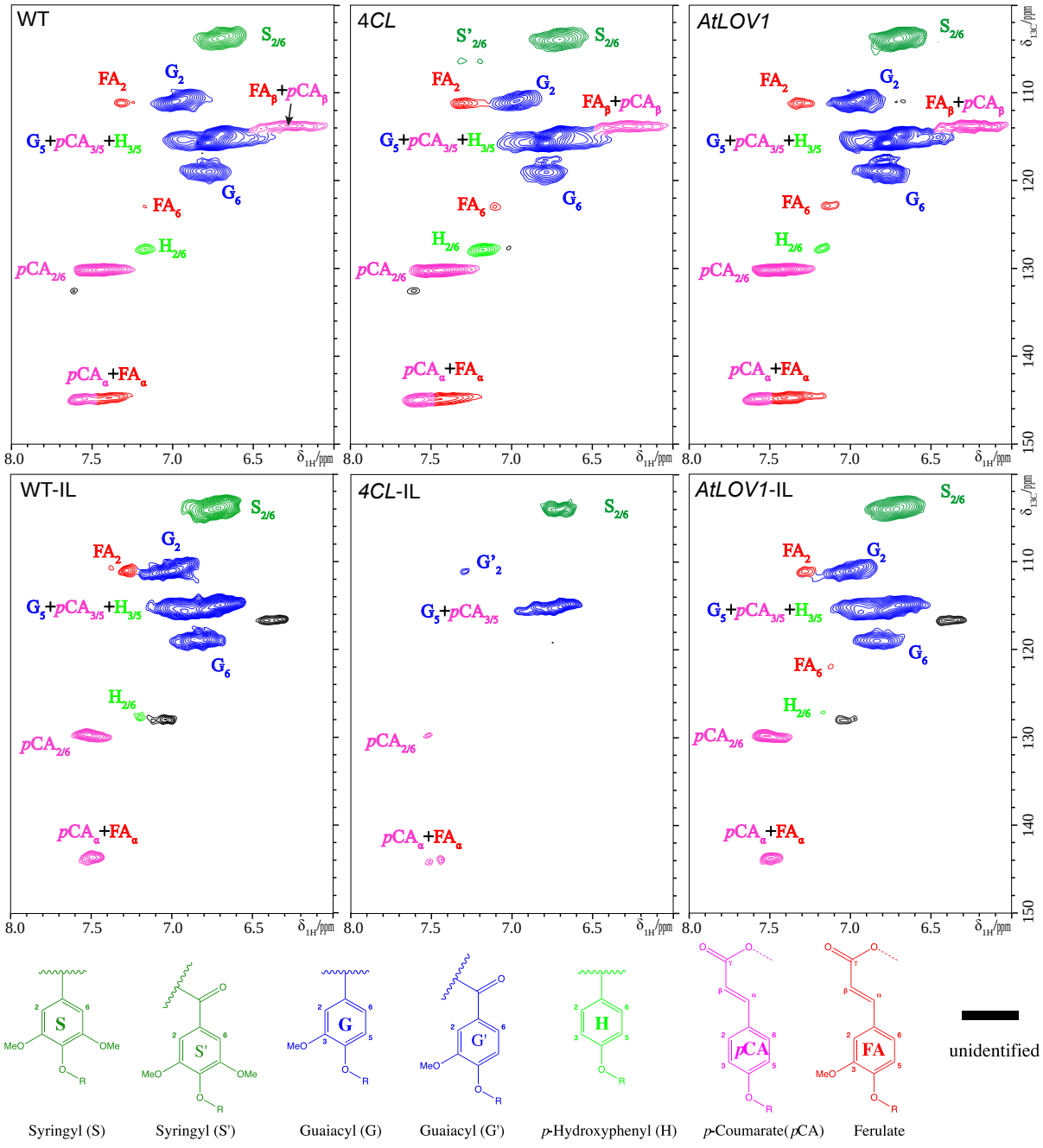
valorization process based on the aqueous IL pretreatment technology.

Table 7. Molecular weight distribution of liquid stream lignin

	Switchgrass types	Weight (% of total liquid stream lignin)	Molecular weight distribution (g/mol)	
			M_w	M_n
Liquid stream lignin (total)	WT	100	3400	1279
	4CL	100	3239	1025
	AtLOV1	100	3318	1092
Precipitated lignin	WT	29.4	4006	1780
	4CL	30.0	3852	1869
	AtLOV1	28.4	3813	1843
Soluble lignin in liquid stream	WT	70.6	783	507
	4CL	70.0	964	587
	AtLOV1	71.6	985	599

2D HSQC NMR spectroscopic analysis: To achieve a better understanding of the changes in lignin chemical structure and transformation during aqueous IL pretreatment, ^{1}H-^{13}C HSQC NMR was applied to the lignins isolated from the untreated switchgrass and IL pretreated solids. **Figure 14** illustrates the 2D NMR spectra of the aromatic regions of lignin structural subunits and the aliphatic regions of lignin inter-units and side chains for untreated and IL pretreated switchgrass. The contour size of lignin H, S, G subunits and hydroxycinnamates were used to quantify and compare the relative abundances between lignins (**Table 5**). *4CL* switchgrass had a higher S/G ratio than that

a)


WT
4CL
AtLOV1
WT-IL
4CL-IL
AtLOV1-IL
S2/6
S'2/6
G2
G'2
FA2
FAβ+pCAβ
G5+pCA3/5+H3/5
G6
FA6
H2/6
pCA2/6
pCAα+FAα
8.0
7.5
7.0
6.5
δ1H/ppm
δ13C/ppm
110
120
130
140
150
S
S'
G
G'
H
pCA
FA
MeO
OMe
R
unidentified
Syringyl (S)
Syringyl (S')
Guaiacyl (G)
Guaiacyl (G')
p-Hydroxyphenyl (H)
p-Coumarate(pCA)
Ferulate

b)

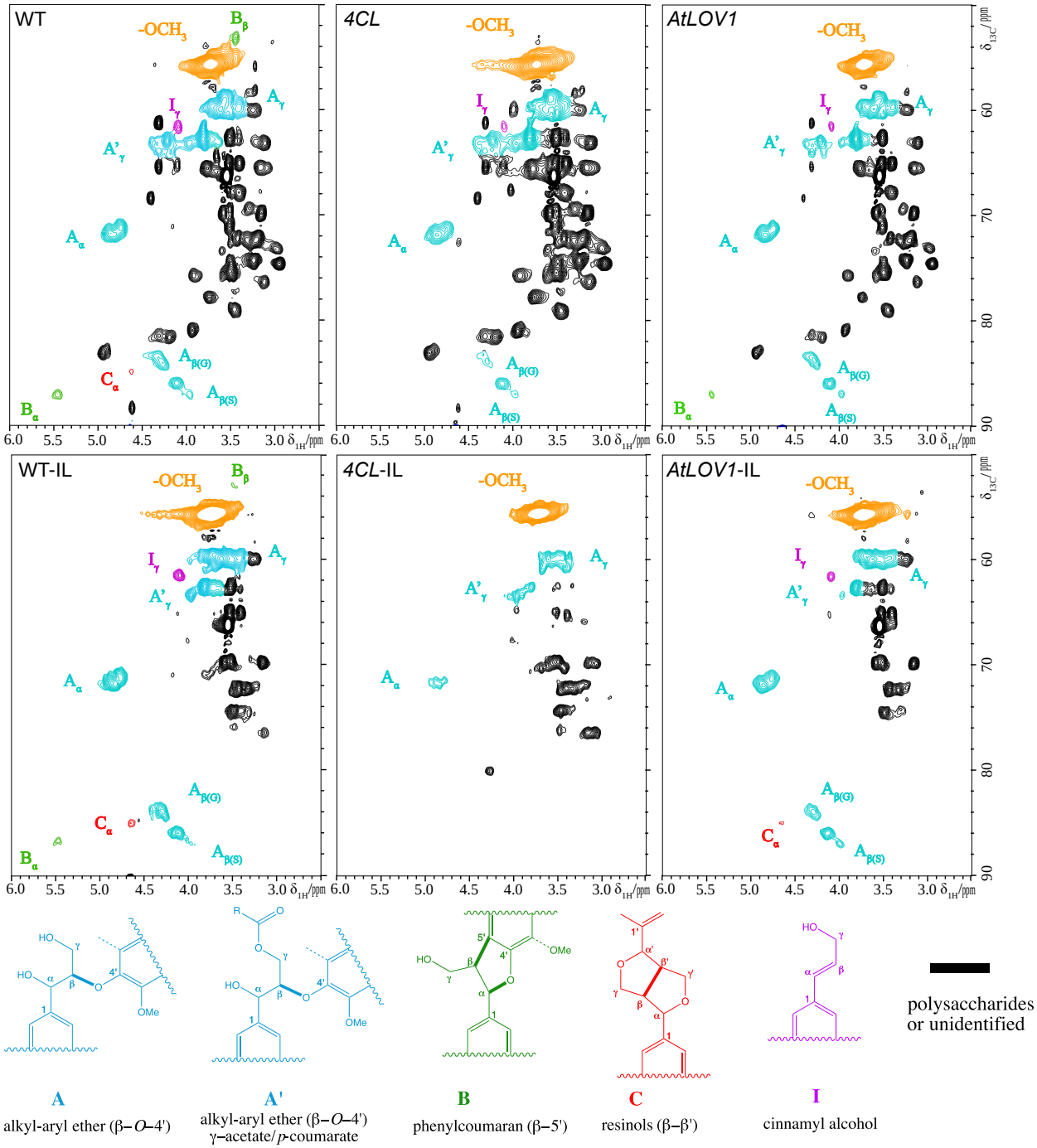


Figure 14. ^{1}H-^{13}C HSQC NMR of a) aromatic regions of lignin structural subunits and b) aliphatic regions of lignin inter-units and side chains from untreated and aqueous IL pretreated switchgrass

of WT and *AtLOV1* switchgrass. High S/G ratio in lignin has been found to positively correlate with the enzymatic digestibility of some biomass types after pretreatment, mainly due because the relatively higher concentration of labile β-*O*-4' linkages is beneficial for lignin depolymerization, migration, and removal during pretreatment(Li et al., 2016). In agreement with high S/G, the HSQC spectra of the aliphatic region revealed that lignin in *4CL* was almost completely composed of β-*O*-4' linkages, (~98%) whereas the WT and *AtLOV1* lignins had fewer lesser amounts of β-*O*-4' linkages (< 90%, **Figure 15**). WT and *AtLOV1* lignins were composed of 11% β-5' and 2% β-β', indicating more branched or condensed linkages in the lignin structure. The reduced recalcitrance of *4CL* switchgrass can be partially explained by the relatively higher S/G ratio accompanied by the higher amount of β-*O*-4' linkages, in addition to its lower lignin content in comparison to WT and mutant *AtLOV1*.

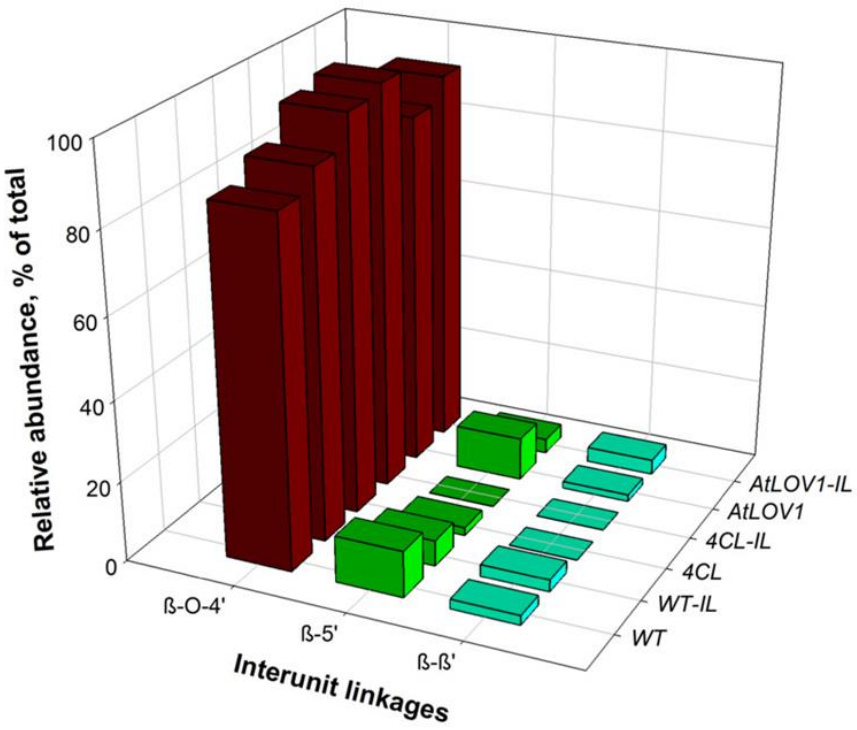


Figure 15. Relative abundance of lignin inter-unit linkages over total linkages from the untreated and aqueous IL pretreated switchgrass

After pretreatment, the lignin remaining in the biomass had a very different structure when compared to that in the raw biomass. The S/G ratio for all three genotypes of switchgrass was increased (**Table 5**), which indicates that more G lignin was removed during the aqueous IL pretreatment. Similar results of increased S/G ratio have been reported by other researchers for lignin from IL pretreated poplar (Wen et al., 2014) and *Arundo donax Linn*(You et al., 2015). Mutant *4CL* demonstrated a greater change in S/G ratio, almost double (0.73 to 1.42), compared with WT (0.45 to 0.66) and mutant *AtLOV1* (0.55 to 0.85). In addition, *p*CA and FA were removed to a significant degree during the IL pretreatment. The mutant *4CL* switchgrass exhibited a more striking removal of *p*CA and FA than the WT and mutant *AtLOV1*. For instance, the FA was completely depleted and 80% of *p*CA was removed in the mutant *4CL* switchgrass as revealed by NMR results. A recent study reported that incorporating monolignol ferulate during plant lignification resulted in reduced recalcitrance likely because the monolignol ferulate, bearing readily cleavable ester bonds, is more susceptible to alkaline pretreatment(Wilkerson et al., 2014). This is in accord with our research, given that the aqueous IL, cholinium lysinate exhibits alkaline properties. Therefore, the higher content of FA, as well as the greater extent of their removal, can be another important reason associated with the reduced recalcitrance and increased saccharification efficiency of *4CL* switchgrass.

Lignin C-C interunit linkages have been historically thought to be more suitable than alkyl-aryl ether bonds during few most pretreatment(Pu et al., 2015). Our results showed that two types of C-C bonds in switchgrass lignin, β-5’ and β-β’, exhibited different trends after ILs pretreatment. For instance, the amount of β-5’ linkages was significantly reduced for the three switchgrass types whereas the relative abundance of β-β’ linkages in WT and *AtLOV1* switchgrass was slightly increased (**Figure 15**). The decreased amount of β-5’ and increased amount of β-β’ in lignin suggests preferential cleavage of lignin fragments containing β-5’ linkages during aqueous IL pretreatment.

3.5 Conclusions

Lignin genetic engineering represents one of the main approaches for the development of biomass feedstocks with desirable traits for cost effective conversion to biofuels and chemicals. Pretreatment is an essential step to unlock sugars from cellulosic biomass for fermentation and to extract lignin for upgrading. This study investigated the fate of lignin and its structural and compositional changes during aqueous IL pretreatment of wild type and engineered switchgrass. Results indicate that switchgrass mutant *4CL* was more susceptible to aqueous IL pretreatment and more digestible during enzymatic saccharification due to its lower lignin content, higher S/G ratio, strikingly higher amount of β-*O*-4' linkages, and greater *p*CA and FA amounts as compared with the WT and mutant *AtLOV1* switchgrass. Aqueous IL (10% cholinium lysinate) was highly effective in solubilizing and depolymerizing lignin (>80% of lignin) with decreased molecular weight of lignin in residual solids and a liquid stream containing low molecular weight lignin oligomers. Results provide insights into the impact of lignin manipulation on biomass fractionation and lignin depolymerization. Furthermore, this study leads to possible directions for developing a more selective and efficient lignin valorization process based on aqueous IL pretreatment technology.

Acknowledgements

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CHAPTER 4: IMPACT OF DILUTE SULFURIC ACID, AMMONIA HYDROXIDE AND IONIC LIQUID PRETREATMENT ON THE FRACTIONATION AND CHARACTERIZATION OF GENETICALLY ENGINEERED SWITCHGRASS*

4.1 Abstract

Biofuels production from renewable resources has been motivated by the depletion of fossil fuels, and has been one of the research frontiers in the past decade. Unlike starchy feedstocks, lignocellulosic biomass does not compete with food and feed supply, thus become a promising resource for biofuels production. Pretreatment is a crucial step for making biomass feedstocks more amenable to biological conversion by unlocking sugars for fermentation. Increasing efforts have focused on elucidating the compositional and structural changes of biomass feedstocks during pretreatment and developing more efficient lignin valorization methods. However, the effects of lignin modifications on the ease of lignin fractionation and subsequent characterization are not well understood. In order to merge lignin engineering and the conversion technologies, this study focused on quantifying the ability to fractionate and subsequent characterization of lignin streams from wild-type and engineered switchgrass using three different pretreatment methods, i.e. dilute acid, ammonia hydroxide, and aqueous ionic liquid. The mass balances of sugars and lignin were calculated based on compositional analysis and enzymatic hydrolysis results. Lignin molecular weight distribution was determined by GPC. The structural and compositional changes and thermal properties of the untreated and pretreated switchgrass and the recovered lignin streams were analyzed by FTIR and DSC, respectively. Results from this study provide a better understanding on how lignin genetic modification impacts lignin fractionation and characterization using different pretreatment technologies.

4.2 Introduction

In order to address environmental concerns as well as fortify our energy security and economic robustness, it is important to mitigate the society's dependence on fossil

**This chapter is prepared as a manuscript for journal publication.*

fuels (Lynd et al., 2008; Obama, 2017; Ragauskas et al., 2006). Production of biofuels and products from renewable resources are one of the many research frontiers underway to meet the goal of shifting away from the current energy structure to sustainable alternatives (Kim et al., 2009). Lignocellulosic feedstocks become promising resources for biofuels production due to their abundant availability and inedible property (Ragauskas et al., 2014; Shi et al., 2009). However, viable economics of biofuels production have not been fully achieved, partially due to the high cost of the feedstock pretreatment process to overcome the recalcitrance of lignocellulosic biomass (Chundawat et al., 2011; Swanson et al., 2010). Thus, one promising approach is to genetically engineer biomass feedstocks with anticipated traits for cost-effective conversion into biofuels while simultaneously developing cost-effective fractionation/pretreatment technologies to prepare biomass for efficient biofuels production. Among the few energy crops under research, switchgrass (*Panicum virgatum*), a perennial C4 tall grass native in North America is one of the front runners for biofuels production (McLaughlin & Kszos, 2005; Parrish & Fike, 2005).

Improving plants characteristics for better environmental resilience and more cost-effective conversion is one of the focus areas in biomass feedstock development. Lignin, one of the main components of lignocellulosic biomass, consists of at least three different monomeric phenylpropanoid units (i.e. p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol) linked by C-C and C-O bonds (Linger et al., 2014). Genetic modification of lignin shows great promise in reducing biomass recalcitrance and increasing the convertibility of biomass to fuels and chemicals (Himmel et al., 2007; Hisano et al., 2011; Xu et al., 2012). Particularly, approaches to down regulate the key enzymes involved in lignin biosynthesis have been applied in a variety of plants to reduce lignin content or alter the lignin composition (Chen & Dixon, 2007; Li et al., 2010b; Lu & Ralph, 1997; Xu et al., 2011). Moreover, modifications of the ratio of lignin subunits or lignin deposition and artificial design of lignin inter-unit/lignin-carbohydrates linkages has been effective in increasing the accessibility of enzymes/microbes to lignocellulosic biomass (Chen & Dixon, 2007; Fu et al., 2011), thereby improving conversion efficiency.

On the other hand, different pretreatment methods have been developed to

overcome the recalcitrance of lignocellulosic biomass and subsequently improve the biofuel yield by unlocking fermentable sugars for downstream bioconversion. Dilute acid pretreatment accelerates the hemicellulose hydrolysis in lignocellulosic biomass by adding low concentration acids (Li et al., 2010a; Wyman et al., 2011). Dilute acid pretreatment has been extensively investigated for processing a variety of biomass feedstocks and has been implemented at demonstration and commercial scales (Foston & Ragauskas, 2010; Jensen et al., 2010). Ammonia hydroxide, as a solvent and weak base, is capable of dissolving a large fraction of lignin by breaking down the lignin inter-unit linkages and acetyl groups between lignin and hemicellulose (Dien et al., 2013; Gupta & Lee, 2010; Sherman et al., 2012).

Ionic liquid (IL) pretreatment recently emerged as a promising method to solubilize different components of lignocellulosic biomass. Several ILs, such as 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$) and 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$), have been demonstrated to be highly effective solvents to solubilize cellulose while the regenerated cellulose is highly digestible by cellulases (Perez-Pimienta et al., 2013; Rogers & Seddon, 2003; Shi et al., 2013; Singh et al., 2009). A range of bio-derived ILs, containing cholinium or ammonium cations and amino acid or carboxylic anions, are considered more biocompatible than the imidazolium ILs. Among those, cholinium lysinate ([Ch][Lys]) has been demonstrated highly effective on dissolving lignin (Sun et al., 2016; Sun et al., 2014; Xu et al., 2016). Moreover, aqueous ILs (mixture of IL and water) are promising solvents to solubilize lignocellulosic biomass with lower cost and viscosity comparing to pure ionic liquids (Fu & Mazza, 2011; Shi et al., 2014; Xia et al., 2014).

Lignin valorization holds the key to the success of biorefinery based on the techno-economic analyses (Beckham et al., 2016). Currently, extensive research has been conducted to determine how pretreatment methods affect the structural and compositional changes of biomass feedstocks and how to catalytically convert lignin to fuels and chemicals (Joffres et al., 2014; Samuel et al., 2010). However, the effects of lignin modification on lignin fractionation and characterization are not fully understood. In order to merge lignin engineering with conversion technologies, this study focusses on

fractionating and characterizing the sugar and lignin streams from wild-type and genetically engineered switchgrass (low lignin content with high S/G ratio and high lignin content) using three different pretreatment methods, i.e. dilute acid (1% w/w sulfuric acid), ammonia hydroxide (10% v/v), and aqueous IL (10% w/w cholinium lysinate). The effects of lignin modification on the biomass compositions (before and after pretreatments) and enzymatic hydrolysis were investigated. The mass balances of untreated switchgrass and switchgrass pretreated by different pretreatment methods were also tracked to understand how each biomass component is fractionated. Lignin molecular weight distribution was determined by gel permeation chromatography; while the structural and compositional changes were investigated by Fourier transform infrared spectroscopy. The thermal properties of untreated and pretreated switchgrass along with different lignin streams were determined by differential scanning calorimetry. Results from this study provide comparative information of how lignin modification impacts the fractionation and characterization of major biomass components with different pretreatment technologies.

4.3 Materials and methods

4.3.1 Materials

Wild-type and genetically engineered switchgrass (*Panicum virgatum*) were grown in a greenhouse at Department of Horticulture, Virginia Tech (Blacksburg, VA, USA) during year 2015. The two transgenic switchgrass plants were denoted as *4CL* (with silenced 4-coumarate: coenzyme A ligase gene) and *AtLOV1* (with overexpression of an Arabidopsis transcription factor *AtLOV1*), respectively. The *4CL* switchgrass has reduced lignin content of the cell wall biomass (Xu et al., 2011); while the *AtLOV1* line has erected leaf phenotype and increased lignin content (Xu et al., 2012). Cultivation and harvesting conditions of the wild type and transgenic plants were described elsewhere (Liu et al., Under review). Harvested materials were milled using a Wiley Mill (Model 4) into 1mm size and sieved by a Ro-Tap® testing sieve shaker (Model B, W. S. Tyler Industrial Group, Mentor, OH, US).

All chemicals are purchased from Sigma-Aldrich if not noted otherwise.

Cholinium lysinate (>95% purity) was synthesized following a method as described elsewhere (Sun et al., 2014). The commercial enzymes including cellulase (Cellic® CTec2, 188 mg protein/ml) and hemicellulase (Cellic® HTec2, 180 mg protein/ml) were gifts from Novozymes, North America (Franklinton, NC, US).

4.3.2 Compositional analysis

Glucan, xylan, acid-soluble and acid-insoluble lignin in the untreated switchgrass and pretreated switchgrass were measured using a tow-step acid hydrolysis protocol according to NREL laboratory analytical procedures (Sluiter et al., 2008). After the two-step acid hydrolysis, acid-insoluble lignin was obtained by subtracting the weight of air-dried residual solids and the ash content; while acid soluble lignin was measured by a spectrophotometer at 205 nm. Monomeric sugars (including glucose, xylose, arabinose) were determined by HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) equipped with a refractive index detector and Aminex HPX-87H column and guard column assembly, using 5mM H_2SO_4 as mobile phase at a flow rate of 0.4 ml/min and a column temperature of 50°C.

4.3.3 Pretreatment

Dilute acid (DA) pretreatment: Switchgrass were mixed with 1% (w/w) sulfuric acid at a ratio of 1:9 (w/w) in a 20 ml SS316 stainless steel reactor, capped and then heated at 160 ± 2°C in a stirred oil bath for 30 min. The pretreatment condition was selected based on a previous study (Wyman et al., 2011). After pretreatment, the reactor was removed from the oil bath and put into iced water for cooling. The pretreatment slurry was transferred to a 50 ml centrifuge tube, then centrifuged at 4000 rpm for 10 min to separate the solids and liquid. The solids were washed three times each by 50 ml of DI water and kept for further enzymatic hydrolysis. The pH of the liquid was adjust to 1-2 and stored at 4°C for 7 days to precipitate lignin. Monomeric sugars in the liquid phase was determined according to NREL laboratory analytical procedure (Sluiter et al., 2006).

Ammonia hydroxide (AH) pretreatment: Switchgrass were mixed with 10% (v/v) ammonia hydroxide at a ratio of 1:9 (w/w) in a 20 ml SS316 stainless steel reactor,

capped and then heated at 160 ± 2°C in a stirred oil bath for 40 min. The pretreatment conditions were selected based on a previous study (Wyman et al., 2011). The solids and liquid resulted from AH pretreatment were separated as described above. The liquid was titrated using 6 M HCl until the pH reaching 1-2, and then stored at 4°C for 7 days to precipitate lignin. After precipitation, the recovered lignin was washed and freeze-dried. Monomeric sugars in the liquid were determined as described above.

Ionic liquid (IL) pretreatment: Cholinium lysinate (10% w/w) and switchgrass were mixed at a ratio of 1:9 (w/w) in a stainless steel reactor, capped and then heated at 140 ± 2°C in a stirred oil bath for 1 h. The solids and liquid resulted from IL pretreatment were separated as described above. The liquid was titrated using 6 M HCl until the pH reaching 1-2, and then stored at 4°C for 7 days to precipitate lignin. After precipitation, the recovered lignin was washed and freeze-dried. Monomeric sugars were determined as described above.

4.3.4 Enzymatic saccharification and mass balance

Saccharification of untreated and pretreated switchgrass was carried out by following the NREL laboratory analytical procedure (Selig et al., 2008). The cellulase (CTec2) and hemicellulase (HTec2) enzymes were premixed at a 9:1 v/v ratio. Two enzyme loadings were tested at 5.25 mg and 20 mg protein/g starting biomass, respectively. The saccharification was performed at 50°C for 72 h in an orbital shaker (Thermo Forma 435, Thermo Fisher Scientific Inc., Waltham, MA, US). Liquid samples were taken for sugar analysis; while the residual solids were collected, washed, and freeze-dried for scale down compositional analysis and characterization.

Mass balances (sugars and lignin) for different types of switchgrass pretreated by DA, AH, and aqueous IL were tracked for both the liquid and solid streams of fractionated switchgrass. Structural carbohydrates for raw switchgrass and pretreated switchgrass were determined by compositional analysis according to NREL laboratory analytical procedure (Sluiter et al., 2008). Sugar concentration (in the liquid phase and after enzymatic hydrolysis) was determined by HPLC equipped with a Bio-Rad Aminex HPX-87H column.

4.3.5 Characterization of untreated and pretreated switchgrass

Scanning electron microscopy (SEM): Images of the untreated and pretreated switchgrass samples were obtained by SEM using a FEI Quanta 250 FEG instrument operating at SE mode under low vacuum (0.40–0.65 Torr). Samples were prepared for imaging by freeze-drying using an AdVantage 2.0 bench top lyophilizer (SP Scientific, Warminster, PA). The dried biomass samples were sputter-coated in gold and the imaging was performed at beam accelerating voltages of 2 kV.

Gel permeation chromatography (GPC) analysis: Lignin in the untreated switchgrass was isolated and extracted by CEL (cellulolytic enzyme lignin) isolation method (Hu et al., 2006; Yoo et al., 2016b). The liquid and solid stream lignin after pretreatment were prepared by following acetylation method (Lu & Ralph, 1997). In brief, 5-10mg of lignin was dissolved in a 92:8 (v/v) acetic acid and acetyl bromide mixture (2.5 ml) and stirred (every 10-15 min) at 50°C for 2 h. The acetic acid and excess AcBr were evaporated by N_2 purging. The acetylated lignin was then dissolved in THF and stored at room temperature before analysis. The molecular weight distributions, including weight-average molecular weight (M_w) and number-average molecular weight (M_n) of prepared lignin, were measured by an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA) equipped with an Ultra Violet (UV) detector. Separation was accomplished with a Mixed-D PLgel column (5 μm particle size, 300 mm x 7.5 mm i.d., linear molecular weight range of 200 to 400,000 u, Polymer Laboratories, Amherst, MA) at 80°C using a mobile phase of THF at a flow rate of 0.5 ml min^{-1}. Elution profile of materials eluting from the column was monitored at 290 nm and the chromatography was calibrated using low molecule weight polystyrene standards (Product No. 48937, Sigma-Aldrich).

Fourier transform infrared spectroscopy (FTIR): FTIR was performed by using a Thermo Nicolet Nexus 870 ESP FTIR spectrometer. Untreated and pretreated switchgrass samples, and lignin from liquid/solid stream samples (around 5 mg) were pressed to 12 psi using a spring loading jack. Sample spectra were obtained using an average of 64 scans over the wave numbers between 400 and 4000 cm^{-1} with a spectral resolution of

1.928 cm^{-1}. The raw FTIR spectra were baseline corrected and normalized using Omnic 6.1a software and compared in the range of 700-2000 cm^{-1}.

Differential scanning calorimetry (DSC): DSC was used to determine the thermal properties of untreated switchgrass, pretreated switchgrass, and lignin from liquid and solid streams. The DSC measurements represented the amount of heat consumed or released by biomass samples in regard to different temperatures. The measurement was performed using a DSC Q20 (TA Instruments.) equipped with an autosampler. DSC analysis was carried out in a temperature range of 40-500 °C at a rate of 10 °C/min with a nitrogen flow of 50 ml/min.

4.3.6 Statistical analysis

All experiments were conducted in triplicates and the data are presented with means and standard deviations. The statistical analysis was performed by SAS® 9.4 (SAS Institute, Cary, NC, US), with a significance level of $P<0.05$ for all the data obtained from experiments.

4.4 Results and discussion

4.4.1 Compositional and electron spectroscopy analysis

As shown in **Table 8**, overall, the wild type and genetically engineered switchgrass have similar glucan and xylan contents in a range of 33-36% with *AtLOV1* switchgrass showing a slightly higher glucan content than *4CL* and WT. While the xylan contents fell in a range of around 21%-23% for all three type of switchgrass. Nevertheless, lignin contents in three types of switchgrass were different. The lignin content in *4CL* switchgrass was significantly lower than that of WT and *AtLOV1* switchgrass, indicating that the lignin content was reduced by silencing 4-coumarate coenzyme A ligase gene in *4CL* switchgrass. However, the lignin content in increased lignin content switchgrass (*AtLOV1*) is not significantly higher than the wild type in this study. The relative abundance of lignin units (S, G, H) in different types of switchgrass were however altered as a result of genetic modification. *4CL* switchgrass exhibited a higher S to G (S/G) ratio of 0.73 than that of 0.45 and 0.55 for WT and *AtLOV1*

switchgrass, respectively (Liu et al., Under review). It has been reported that lignocellulosic biomass with higher S/G ratio is considered to be less recalcitrant and more digestible to enzymatic hydrolysis (Li et al., 2010b; Studer et al., 2011). Taken together, the alternation on lignin content and S/G ratio of the engineered switchgrass could affect the recalcitrance of the feedstock and lead to different fractionation patterns during pretreatment.

Genetic modification can not only alter the compositions switchgrass but also greatly impact the solubilization and deconstruction of switchgrass during a pretreatment process. As a result, the solid recovery and the composition of the pretreated switchgrass will reflect the preferential removal of sugar or lignin during pretreatment. The compositions of structural carbohydrates, lignin content, and solid recovery, before and after different pretreatment methods, were presented in **Table 8.** For DA pretreatment, almost all the xylans were removed during pretreatment, resulting in very low xylan content in the DA pretreated switchgrass; while the glucan and lignin content were increased in the pretreated switchgrass as relevant to xylan removal. However, for AH and IL pretreatment, only a portion of the xylan were removed in accompany with significant lignin removal. Aqueous IL pretreatment demonstrated the best lignin solvent power with more than 60% of lignin removed from different types of switchgrass. The resulting pretreated solids following these two pretreatment had decreased lignin content and increased glucan content as compared with untreated switchgrass. Resulting from a combined effect of removal of each components, it was noticed that the solid recovery for *4CL* switchgrass was lower than that of WT and *AtLOV1* switchgrass for all three pretreatment methods. The results suggest that *4CL* switchgrass subjected to high weight lost after pretreatment, indicating the material is highly susceptible to chemical pretreatments.

Table 8. Compositional analysis for untreated switchgrass and pretreated switchgrass[1,2]

	Wild type switchgrass (Untreated and pretreated by three pretreatments)				***4CL* switchgrass** (Untreated and pretreated by three pretreatments)				***AtLOV1* switchgrass** (Untreated and pretreated by three pretreatments)			
	WT	WT-DA	WT-AH	WT-IL	*4CL*	*4CL*-DA	*4CL*-AH	*4CL*-IL	*AtLOV1*	*AtLOV1*-DA	*AtLOV1*-AH	*AtLOV1*-IL
Extractives, %	14.6 ± 0.3	ND	ND	ND	18.0 ± 0.2	ND	ND	ND	13.0 ± 0.1	ND	ND	ND
Glucan, %	34.5 ± 0.1	51.6 ± 5.8	48.9 ± 0.3	49.1 ± 0.9	33.5 ± 0.1	53.2 ± 4.1	50.6 ± 1.0	50.3 ± 0.8	36.0 ± 0.4	56.8 ± 0.8	48.3 ± 0.8	49.4 ± 1.8
Xylan. %	21.9 ± 2.4	0.7 ± 0.0	20.0 ± 0.3	22.1 ± 0.4	22.7 ± 0.2	0.8 ± 0.1	20.3 ± 0.3	22.8 ± 0.2	21.3 ± 0.3	0.8 ± 0.1	18.2 ± 0.2	20.6 ± 0.8
Lignin, %	18.6 ± 0.0	34.4 ± 1.1	19.4 ± 2.0	12.5 ± 1.2	16.3 ± 0.3	33.3 ± 0.1	15.3 ± 2.0	8.6 ± 1.2	19.1 ± 0.3	33.7 ± 2.3	17.5 ± 0.0	12.6 ± 1.2
Ash, %	4.5 ± 0.1	ND	ND	ND	5.3 ± 0.1	ND	ND	ND	4.3 ± 0.1	ND	ND	ND
Solid recovery, %	N/A	42.0 ± 0.5	54.8 ± 4.3	44.0 ± 2.2	N/A	40.0 ± 3.5	44.7 ± 1.8	42.7 ± 3.7	N/A	45.5 ± 1.7	50.3 ± 4.6	51.0 ± 3.0

[1]Based on dry biomass; ND = not determined; N/A = Not applicable

[2]The composition for untreated switchgrass and IL pretreated switchgrass samples was adopted from a previous article (Liu et al., Under review)

Along with the solubilization of lignin or xylan during pretreatment, the cell wall structures of switchgrass underwent deconstruction by different pretreatment methods. The representative scanning electron microscopy (SEM) images were showed in **Figure 16** to provide a visual illustration of the deconstructed plant materials. The relatively smooth cell wall surfaces displayed in untreated samples have become irregular from apparent disrupted and re-localized cell wall matrix material. As compared to the intact and highly ordered structure of untreated switchgrass, all pretreated samples showed significant surface disruption and some extent of size reduction. It appeared that IL pretreatment caused the most deep etched and delineated surface as revealed by SEM. This is likely due to the removal and re-arrangement of lignin in addition to partial hemicellulose removal, all contributing to the deconstruction of cell wall structure and creating extensive new surface area by etching away cell wall matrix and leaving microfibrils exposed on cell wall structures. Delamination and increased porosity is one of the major themes for how pretreatment changes cell wall architecture to create a highly accessible surface for cellulase binding thus improving enzymatic digestibility (Donohoe et al., 2011; Wyman et al., 2011).

The compositional and structural changes correlate well with the pretreatment chemistry. Among different pretreatment methods, dilute acid pretreatment is proven to be an efficient method to pretreat lignocellulosic biomass by largely dissolving hemicellulose and partially mitigating and redepositing lignin (Donohoe et al., 2008; Pu et al., 2013). Ammonia hydroxide works as an alkaline and under high pH a high fraction of the lignin and a portion of the associated hemicelluloses are removed through improved lignin hydrophilicity by opening aromatic ring and depolymerization (Kim et al., 2008; Wyman et al., 2011; Yoo et al., 2016a). Cholinium lysinate is a bio-derived ionic liquid with high solvent power toward lignin. It has been demonstrated that cholinium lysinate is effective at lignin depolymerization and solubilization, due to the greater hydrogen bond basicity (Sun et al., 2014). Recent studies also indicated that aqueous ionic liquids system also can be as effective as pure ionic liquids on biomass pretreatment (Shi et al., 2014). By employing aqueous ionic liquids pretreatment, the cost of ionic liquids and the recycling process can be significantly reduced (Konda et al.,

2014).

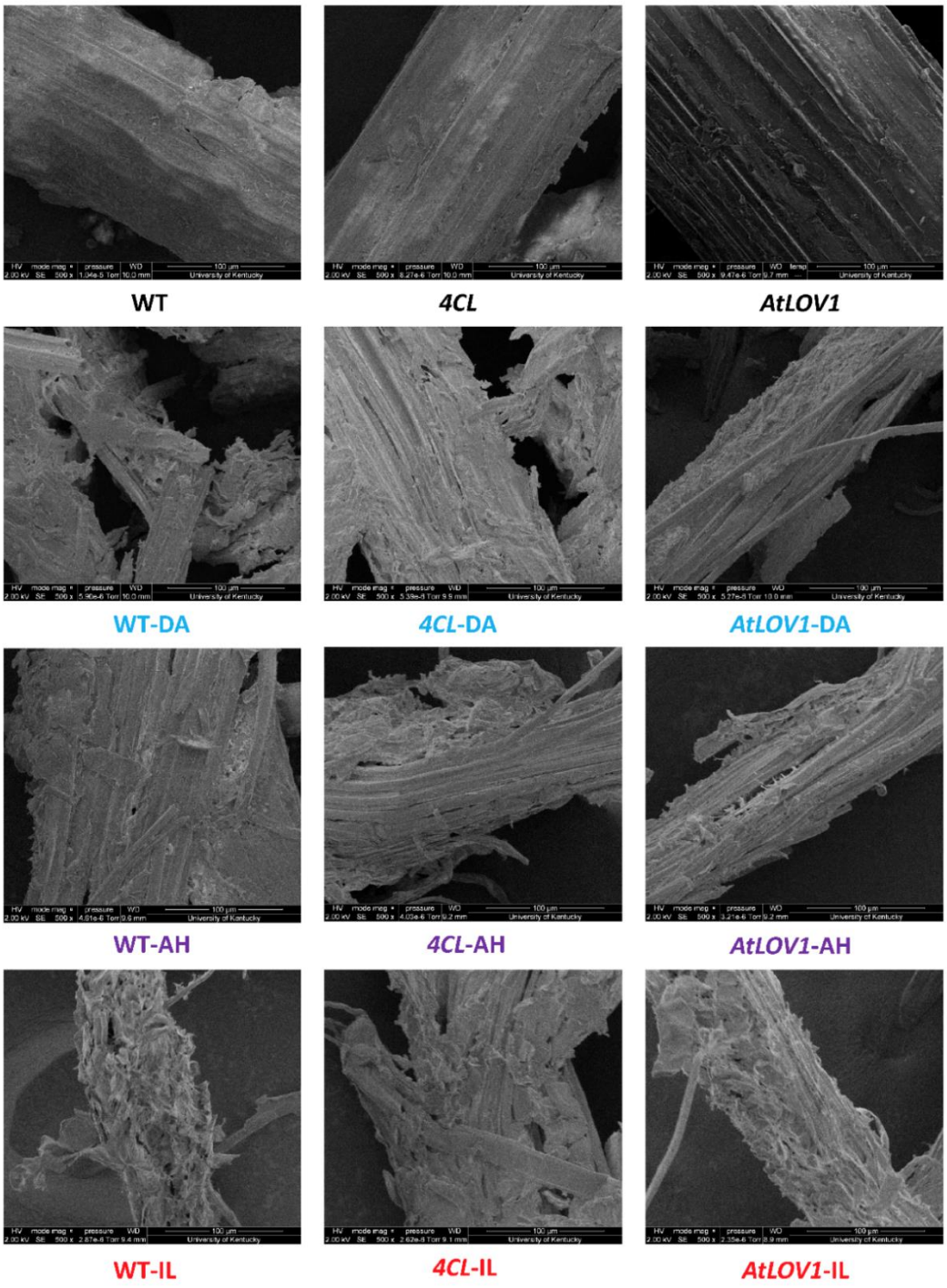


Figure 16. Scanning Electron Microscopy (SEM) of untreated switchgrass and pretreated switchgrass

4.4.2 Enzymatic saccharification of pretreated switchgrass

The effectiveness of three tested pretreatment methods was evaluated by enzymatic saccharification of the pretreated switchgrass. An effective pretreatment will unlock the polysaccharides to cellulase/hemicellulase by breaking down the lignocellulosic complex; while at the same time preserve the fermentable sugars (Shi et al., 2013). **Figure 17** indicates the glucose and xylose yields released from the resulting switchgrass solids pretreated using different pretreatment methods. As the enzyme cost still contribute a significant portion of the biofuels production cost (Klein-Marcuschamer et al., 2011), two different enzyme loadings (high enzyme loading, 20 mg protein/g starting biomass and low enzyme loading, 5.25 mg protein/g starting biomass) were compared. All the pretreatment methods led to significant improvement in the sugar yield when compared to untreated switchgrass (**Figure 18**). At 20 mg protein/g starting biomass enzyme loading, all pretreatment methods led to over 80% glucose yield across all three switchgrass feedstocks, except for AH pretreatment of *AtLOV1* switchgrass. While at low enzyme loading of 5.25 mg protein/g starting biomass, the glucose yields were 10-15% lower; while >80% glucose yield can only be obtained for the *4CL* switchgrass.

Among different pretreatment methods, aqueous IL pretreatment led to the highest glucose yield and xylose yield (over 80% for glucose yield, over 50% for xylose yield) for both high and low enzyme loadings, probably due to the high solvent power on lignin removal. Glucose yield after dilute acid pretreatment was comparable to IL pretreatment. However, due to the distinct chemistry between different pretreatment methods, xylose yield from pretreated solids varied depending on the pretreatment methods. DA pretreatment solubilizes the majority of xylan in the pretreatment liquid; while AH and IL pretreatments pretreatment only partially remove xylan was. In comparison with IL pretreatment, AH pretreatment appeared less effective especially on the recalcitrant *AtLOV1* switchgrass.

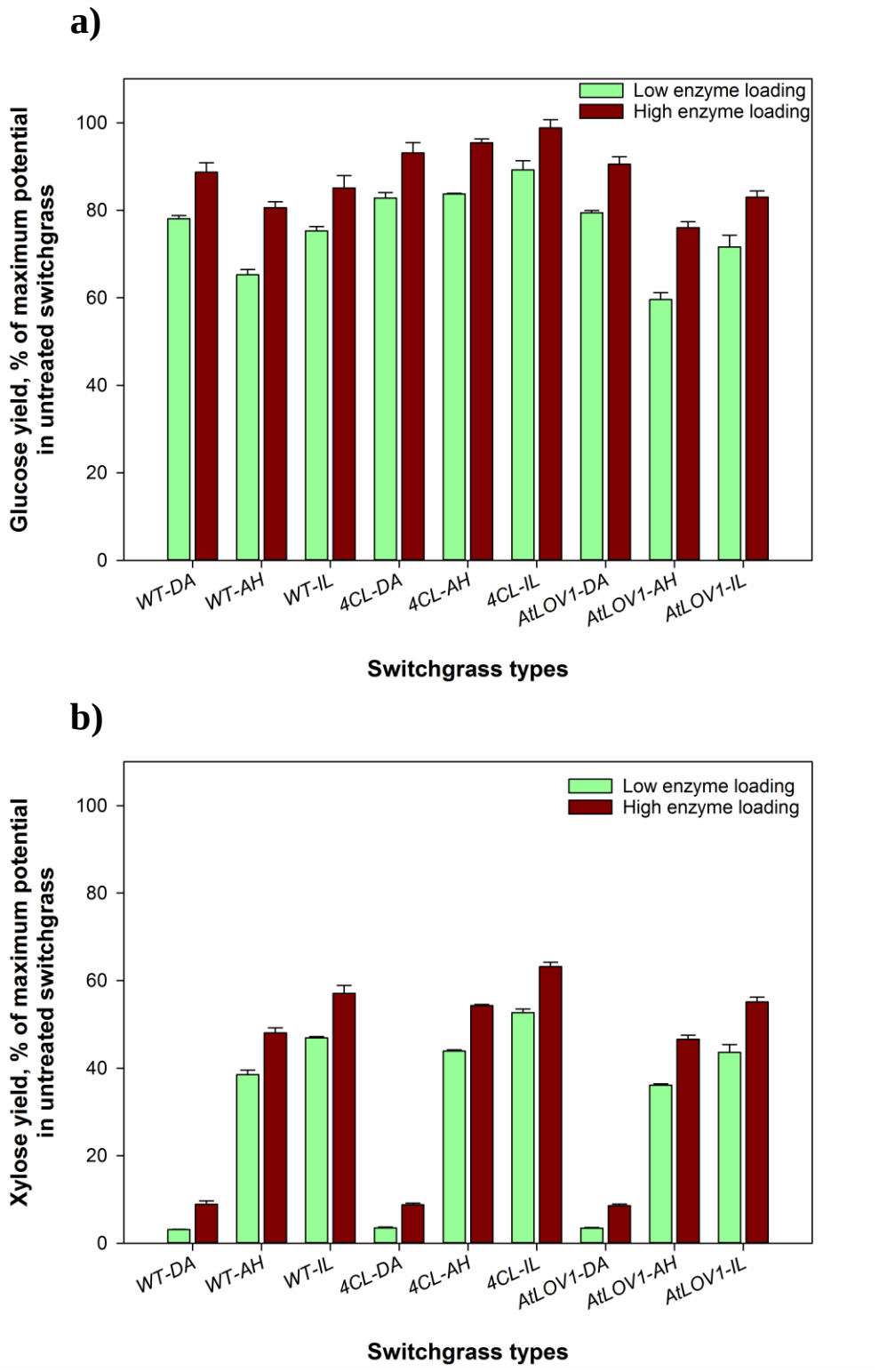


Figure 17. Glucose yield (a) and xylose yield (b) after enzymatic hydrolysis of different types of switchgrass under high/low enzyme loading

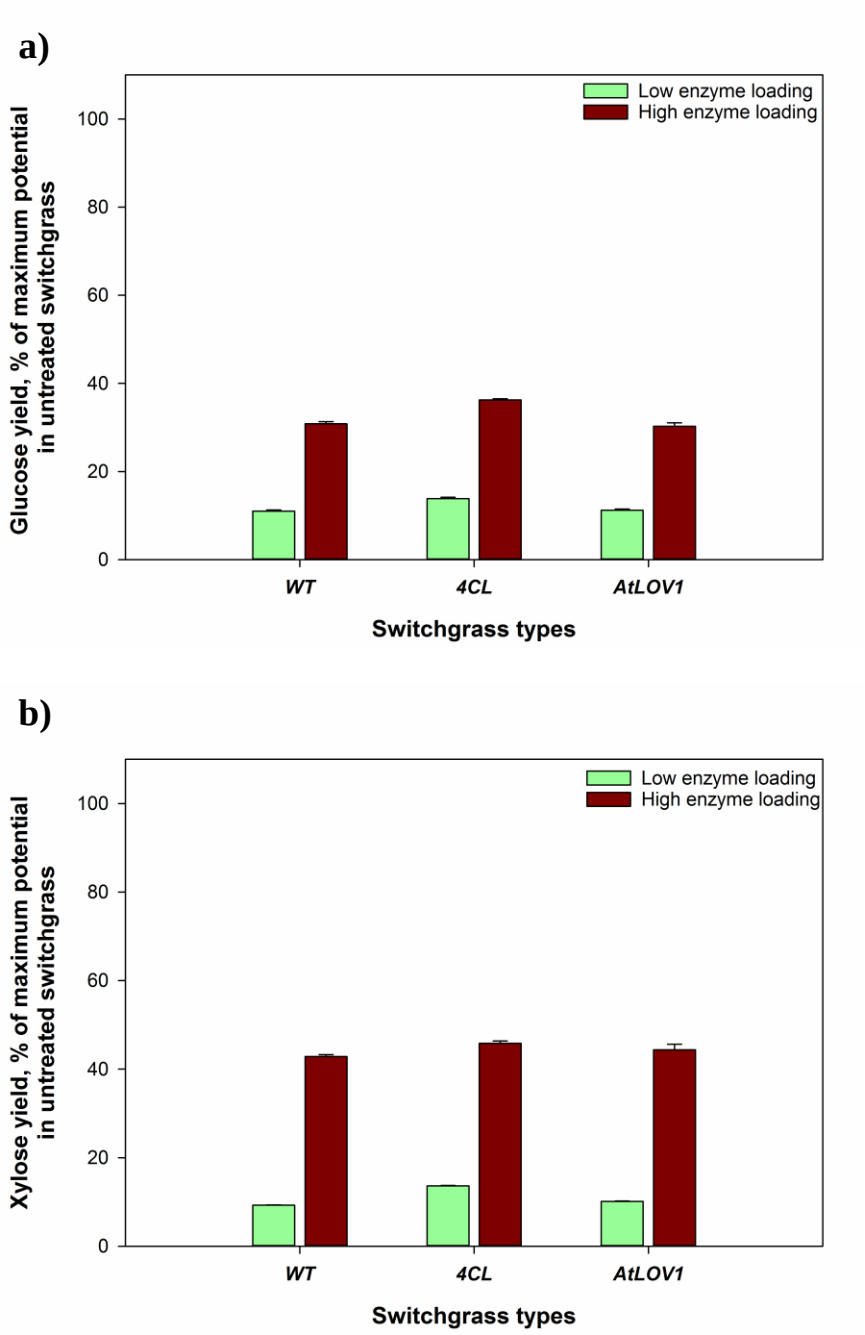


Figure 18. Glucose yield (a) and xylose yield (b) for untreated switchgrass with high/low enzyme loading

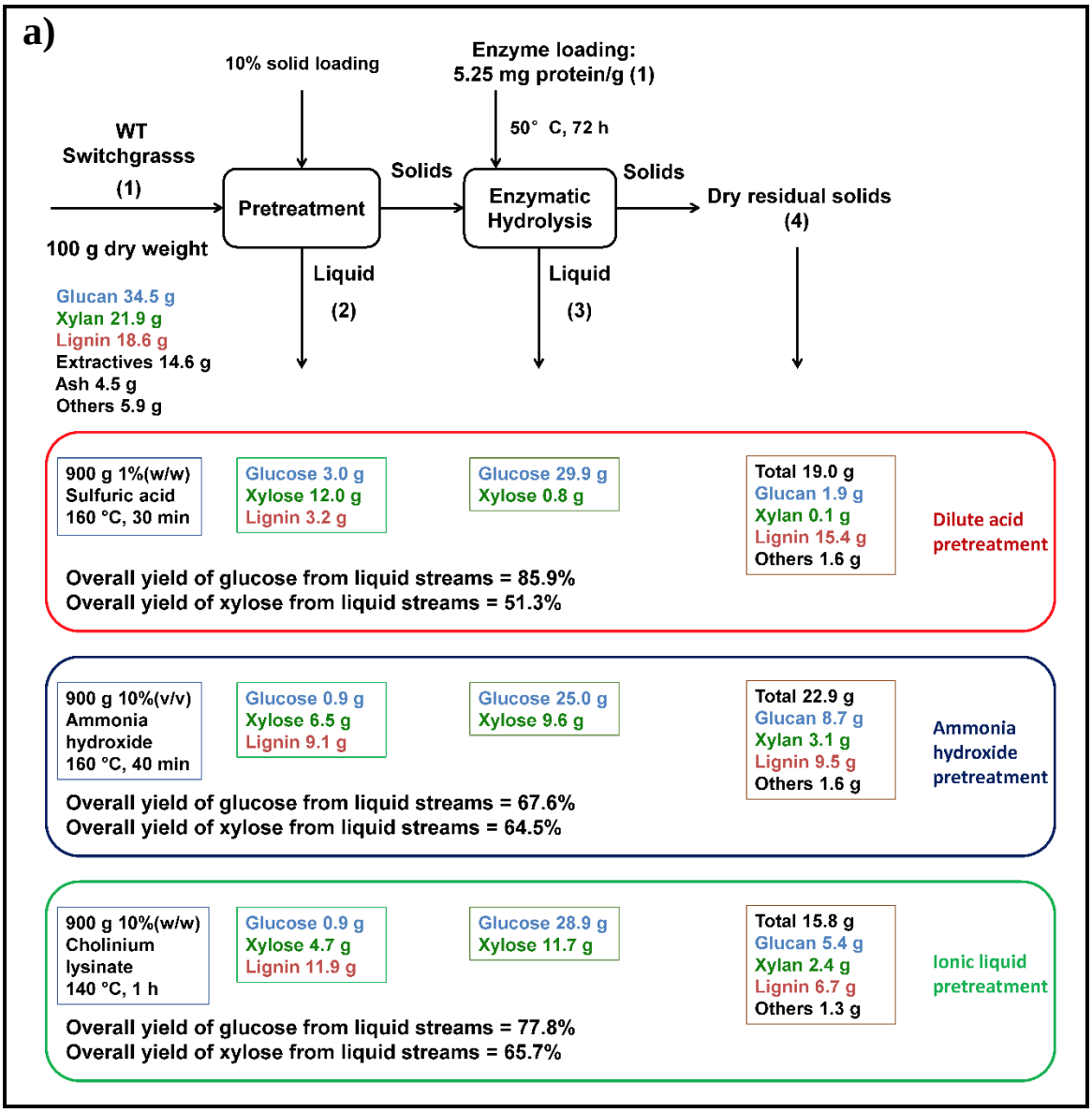
Among different switchgrass species, *4CL* switchgrass is more digestible than WT and *AtLOV1* switchgrass, indicating that downregulation of lignin and slightly increasing of S/G ratio improve the biomass feedstock's convertibility to sugars. The glucose yields for *4CL* switchgrass were over 80% for different pretreatment methods even under low enzyme loading of 5.25 mg protein/g starting biomass. *AtLOV1* switchgrass led to relative low sugar yield when compared to WT and *4CL* switchgrass, which indicates that upregulated lignin content could lead to low convertibility under the same pretreatment conditions. However, note that the pretreatment conditions selected in this study were not optimized for each individual biomass feedstock, a more severe pretreatment is likely required to *AtLOV1* switchgrass. Despite the ~10-15% increases in sugar yield at high enzyme loading, a relative low enzyme loading may offer a better trade-off between sugar yields and the profitability under a biorefinery concept. In this study, low enzyme loading (5.25 mg protein/g starting biomass) was used for subsequent investigation on mass balance and characterization.

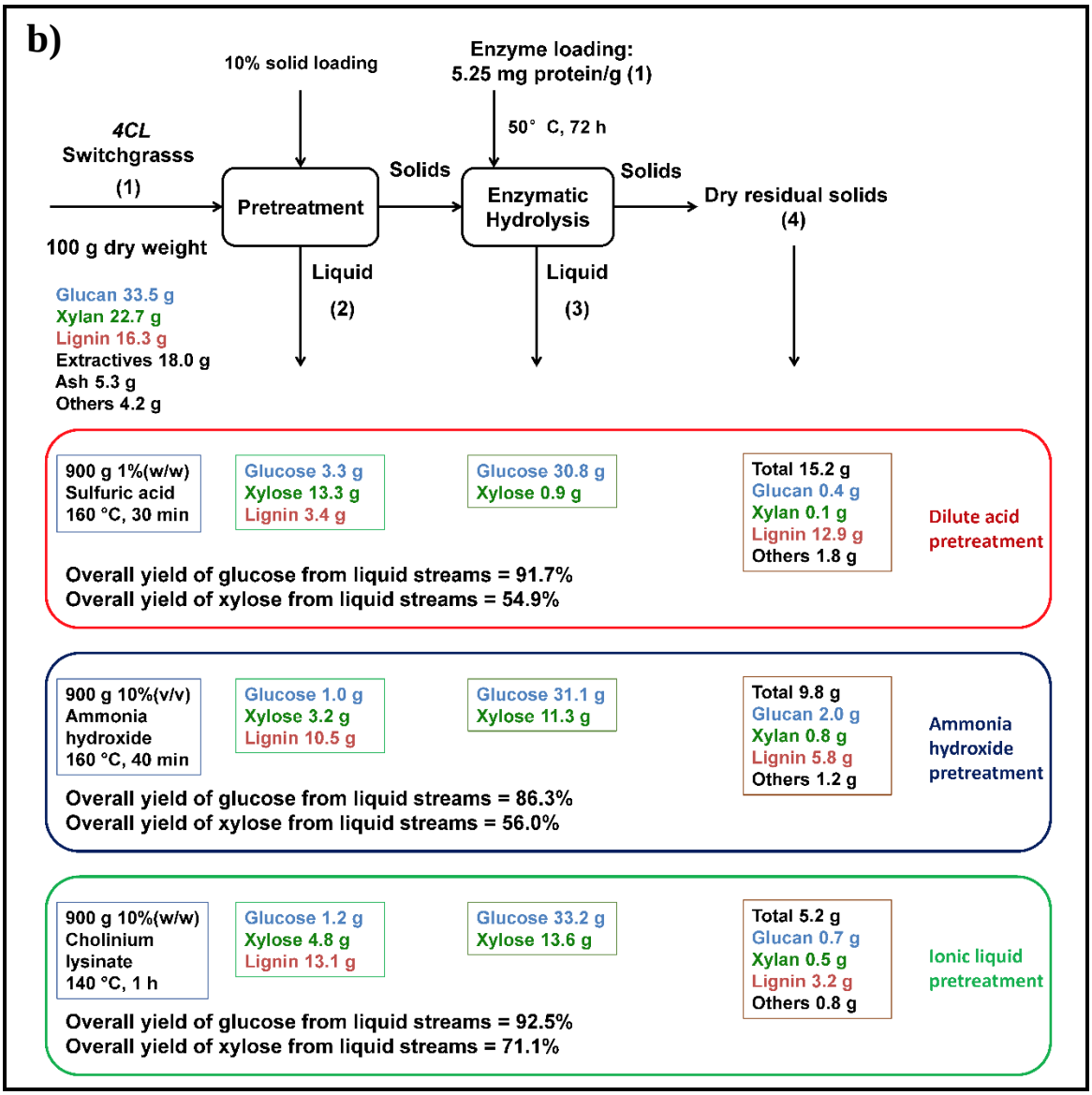
4.4.3 Mass balance and fractionation of lignin streams

The mass flow of different components in switchgrass under different pretreatment methods further illustrated the mechanisms of fractionations with respect to different pretreatment chemistry. The mass balances for glucan, xylan, and lignin on wild type and engineered switchgrass under different pretreatment methods were presented in **Figure 19**. Based on 100 g dry untreated switchgrass (starting stream #1), the solids and liquid streams tracked were #2) pretreatment liquid, #3) enzymatic hydrolysis liquid and #4) the solid residual after enzymatic hydrolysis. In general, for different pretreatment methods, the mass flow of glucan, xylan and lignin into the pretreatment liquid is different. Starting with 100 g of untreated switchgrass, DA pretreatment removed a small portion of lignin to stream #2 and the majority of lignin remained in stream #4 for all three types of switchgrass. Over 85% of glucose can be recovered from the liquid stream #3, while over 50% of xylose can be recovered from the liquid stream #2. The results demonstrate that DA pretreatment is an efficient method to solubilize xylan and generate high sugar yield. On the other hand, AH pretreatment solubilized half of the lignin and a small portion of xylan into stream #2 for different types of switchgrass. The glucose and

xylose recovered from liquid stream #3 were however lower than that of DA and IL pretreatment. For instance, around 60% of glucose can be recovered from stream #3 for WT and *AtLOV1* switchgrass, while over 85% for *4CL* switchgrass (**Figure 19b**). The pretreatment performance of ammonia hydroxide appeared not as effective as DA and IL pretreatment, thus a significant amount of glucan and xylan was still left in the solid residual (stream #4) after enzymatic hydrolysis. In contrast, aqueous IL is a highly efficient solvent to solubilize lignin. For all three types of switchgrass, a large portion of lignin and a small portion of xylan were released in stream #2; while high glucose and xylose were recovered from liquid stream #3 with low residual sugar and lignin remained in stream #4.

Comparing across different types of switchgrass, overall higher sugar recovery was obtained from liquid streams #2 & #3 for *4CL* switchgrass as compared with WT and *AtLOV1*. For instance, the overall glucose yield from liquid stream for *4CL* switchgrass pretreated by DA was 5.8% and 5.6% higher than that of WT and *AtLOV1* switchgrass, respectively, while the overall xylose yield was 3.6% and 5.5% higher. Furthermore, the overall glucose and xylose yield for *4CL* switchgrass pretreated by AH was 10% higher than that of WT and *AtLOV1* switchgrass. In particular, the highest overall glucose and xylose yield were achieved by *4CL* switchgrass pretreated by aqueous IL, which were 92.5% and 71.1% (**Figure 19b**), respectively. The overall glucose yield from liquid stream for *4CL* switchgrass pretreated by ionic liquid was 14.7% and 18.9% higher than that of WT and *AtLOV1* switchgrass, respectively. The overall xylose yield from liquid stream for *4CL* switchgrass pretreated by ionic liquid was 5.4% and 10.1% higher than that of WT and *AtLOV1* switchgrass, respectively. Taken together, *4CL* switchgrass is more digestible as compared to WT and *AtLOV1* switchgrass, which means the lower lignin content and slightly higher S/G ratio can indeed reduce the recalcitrance and improve the convertibility of engineered biomass feedstocks.


a)
10% solid loading
Enzyme loading:
5.25 mg protein/g (1)
50° C, 72 h
WT
Switchgrasss
(1)
Pretreatment
Solids
Enzymatic
Hydrolysis
Solids
Dry residual solids
(4)
100 g dry weight
Glucan 34.5 g
Xylan 21.9 g
Lignin 18.6 g
Extractives 14.6 g
Ash 4.5 g
Others 5.9 g
Liquid
(2)
Liquid
(3)
900 g 1%(w/w)
Sulfuric acid
160 °C, 30 min
Glucose 3.0 g
Xylose 12.0 g
Lignin 3.2 g
Glucose 29.9 g
Xylose 0.8 g
Total 19.0 g
Glucan 1.9 g
Xylan 0.1 g
Lignin 15.4 g
Others 1.6 g
Dilute acid
pretreatment
Overall yield of glucose from liquid streams = 85.9%
Overall yield of xylose from liquid streams = 51.3%
900 g 10%(v/v)
Ammonia
hydroxide
160 °C, 40 min
Glucose 0.9 g
Xylose 6.5 g
Lignin 9.1 g
Glucose 25.0 g
Xylose 9.6 g
Total 22.9 g
Glucan 8.7 g
Xylan 3.1 g
Lignin 9.5 g
Others 1.6 g
Ammonia
hydroxide
pretreatment
Overall yield of glucose from liquid streams = 67.6%
Overall yield of xylose from liquid streams = 64.5%
900 g 10%(w/w)
Cholinium
lysinate
140 °C, 1 h
Glucose 0.9 g
Xylose 4.7 g
Lignin 11.9 g
Glucose 28.9 g
Xylose 11.7 g
Total 15.8 g
Glucan 5.4 g
Xylan 2.4 g
Lignin 6.7 g
Others 1.3 g
Ionic liquid
pretreatment
Overall yield of glucose from liquid streams = 77.8%
Overall yield of xylose from liquid streams = 65.7%


b)
10% solid loading
Enzyme loading:
5.25 mg protein/g (1)
50° C, 72 h
4CL
Switchgrasss
(1)
Pretreatment
Solids
Enzymatic
Hydrolysis
Solids
Dry residual solids
(4)
100 g dry weight
Glucan 33.5 g
Xylan 22.7 g
Lignin 16.3 g
Extractives 18.0 g
Ash 5.3 g
Others 4.2 g
Liquid
(2)
Liquid
(3)
900 g 1%(w/w)
Sulfuric acid
160 °C, 30 min
Glucose 3.3 g
Xylose 13.3 g
Lignin 3.4 g
Glucose 30.8 g
Xylose 0.9 g
Total 15.2 g
Glucan 0.4 g
Xylan 0.1 g
Lignin 12.9 g
Others 1.8 g
Dilute acid
pretreatment
Overall yield of glucose from liquid streams = 91.7%
Overall yield of xylose from liquid streams = 54.9%
900 g 10%(v/v)
Ammonia
hydroxide
160 °C, 40 min
Glucose 1.0 g
Xylose 3.2 g
Lignin 10.5 g
Glucose 31.1 g
Xylose 11.3 g
Total 9.8 g
Glucan 2.0 g
Xylan 0.8 g
Lignin 5.8 g
Others 1.2 g
Ammonia
hydroxide
pretreatment
Overall yield of glucose from liquid streams = 86.3%
Overall yield of xylose from liquid streams = 56.0%
900 g 10%(w/w)
Cholinium
lysinate
140 °C, 1 h
Glucose 1.2 g
Xylose 4.8 g
Lignin 13.1 g
Glucose 33.2 g
Xylose 13.6 g
Total 5.2 g
Glucan 0.7 g
Xylan 0.5 g
Lignin 3.2 g
Others 0.8 g
Ionic liquid
pretreatment
Overall yield of glucose from liquid streams = 92.5%
Overall yield of xylose from liquid streams = 71.1%

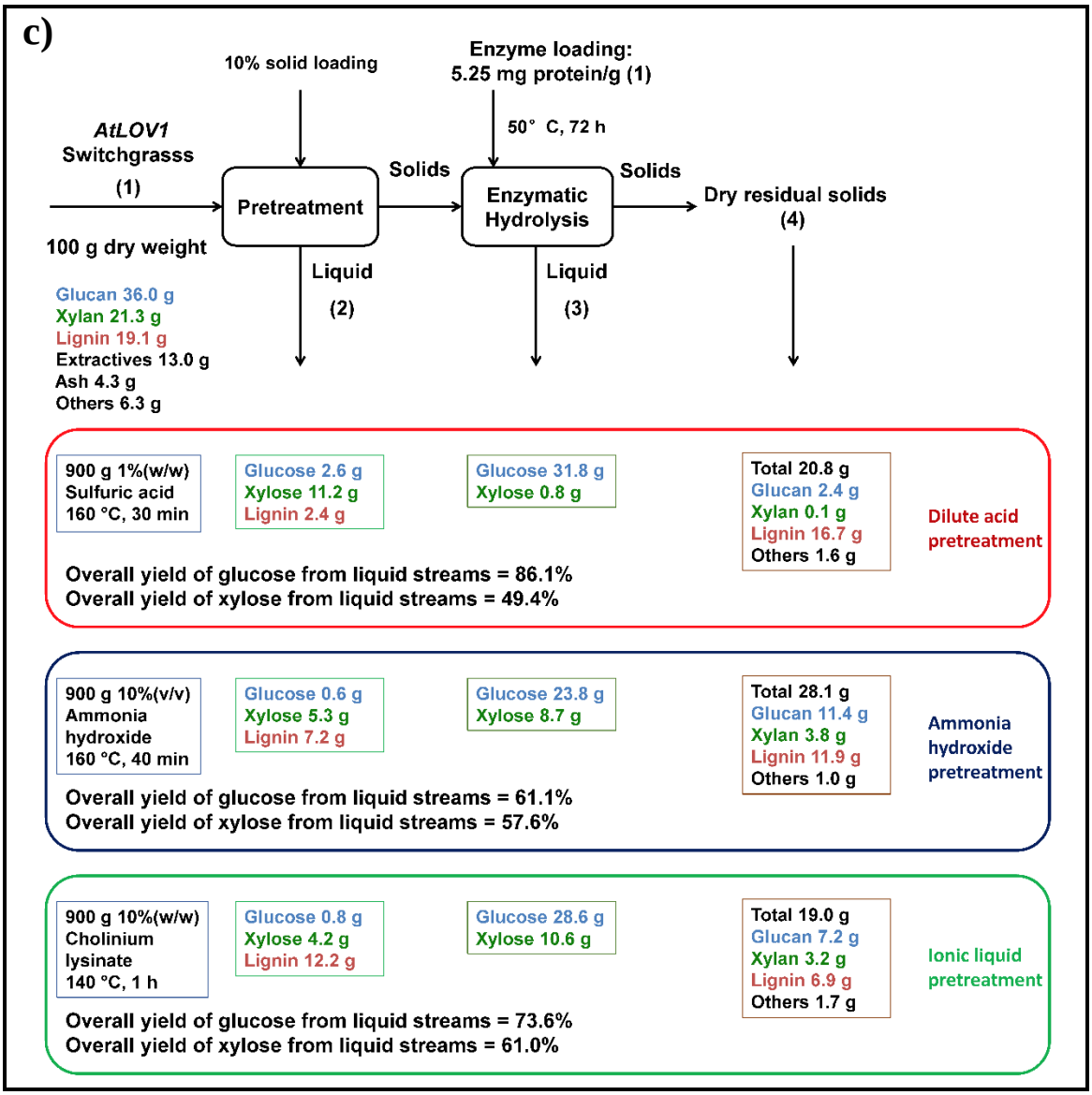


Figure 19. Mass balance after pretreatment and enzymatic saccharification a) WT switchgrass pretreated by three pretreatment methods; b) *4CL* switchgrass pretreated by three pretreatment methods; c) *AtLOV1* switchgrass pretreated by three pretreatment methods. [1]The composition for untreated switchgrass and IL pretreated switchgrass samples was adopted from a previous article (Liu et al., Under review)

To further demonstrate the solubility of lignin from different types of switchgrass by different pretreatment methods, fractionation of lignin in liquid and solid streams was presented in **Figure 20**. It can be seen that DA pretreatment dissolved only 13-21% of lignin in liquid stream #2 for different types of switchgrass, while left the rest 79-87% of lignin in the solid residual after enzymatic hydrolysis (stream #4). Nevertheless, AH pretreatment solubilized 38-65% of lignin for different types of switchgrass in the liquid stream #2, while left the rest of lignin in the solid stream #4. As for aqueous IL, 64-80% of lignin for different types of switchgrass was solubilized into the liquid stream #2, while only a small portion (20-36%) of lignin remained in solids stream #4. Results clearly demonstrate the superior solvent power of aqueous cholinium lysinate on lignin. Comparing across different types of switchgrass, lignin in *4CL* switchgrass appeared more vulnerable to pretreatment. To sum up, *4CL* switchgrass with lower lignin content and altered S/G ratio is more digestible, while the upregulated lignin content mutant, *AtLOV1*, is more resistant to different pretreatment methods. Therefore, the recalcitrance of biomass feedstocks can be reduced by lowering the lignin content and increasing the S/G ratio.

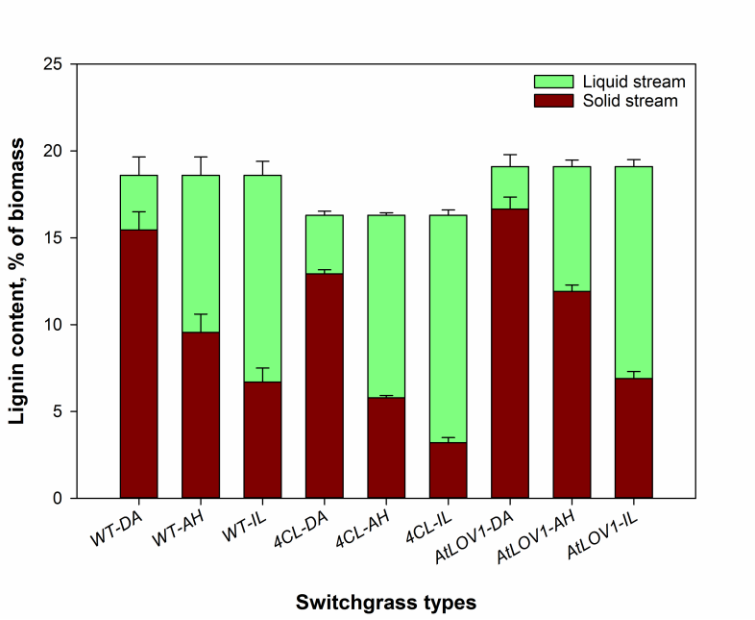


Figure 20. Fractionation of lignin streams after different pretreatment methods a) WT switchgrass; b) 4CL switchgrass; c) AtLOV1 switchgrass. [1]The data for IL pretreated switchgrass samples were adopted from a previous article (Liu et al., Under review)

4.4.4 Lignin molecular weight distribution

In order to further understand the lignin solubilization and depolymerization during pretreatment of wild type and genetically engineered switchgrass by different methods, gel permeation chromatography (GPC) was performed to determine the molecular weight distribution of lignin streams, including the untreated and pretreated solids and the precipitated lignins from pretreatment liquid. The number-average molecular weight (M_n) and weight-average molecular weight (M_w) for untreated switchgrass and different lignin streams after pretreatment was calibrated using a set of polystyrene standards (**Table 9**). The lignin extracted from untreated *4CL* switchgrass showed M_n and M_w of 4482 and 9806 g/mol, respectively, which is significantly lower than that of WT and *AtLOV1* switchgrass. In contrast, the M_n and M_w of the lignin extracted from untreated *AtLOV1* switchgrass were slightly higher than that of WT switchgrass. Furthermore, the polydispersity index (PDI) of the extracted lignin from *4CL* switchgrass is lower than that of WT and *AtLOV1* switchgrass. Collectively, results suggest that the lignin molecular weight can be reduced by downregulating lignin content and altering S/G ratio, while the lignin molecular weight can be slightly increased by overexpression of the *Arabidopsis* transcription factor *AtLOV1*.

In general, molecular weight of the lignin in the pretreated solids was significantly reduced as compared with untreated samples for all three pretreatment methods. It appeared that the lignins in pretreated *4CL* switchgrass had lower M_n and M_w when compared with WT and *AtLOV1* switchgrass, which match the low initial lignin molecule weight in untreated *4CL* switchgrass. The observation also indicates that the *4CL* switchgrass is more susceptible to chemical pretreatment. Generally speaking, ammonia hydroxide and aqueous ionic liquid pretreatment led to lower M_n and M_w across to all tested switchgrass samples than that of the resulting solids after dilute acid pretreatment, indicating these two pretreatment methods were efficient at depolymerizing lignin than dilute acid pretreatment. The precipitated lignins from the liquid streams however showed different molecular weights as compared to the corresponding solids stream lignin. It is know that the dissolved lignin oligomers repolymerized to precipitate out from the liquid stream. However, the precipitation process could be affected by the

chemistry of the pretreatment slurry as well as the composition of lignin. This, it is difficult to draw a general conclusion on what are the determining factors of the molecular weight of precipitated lignins in the liquid streams.

Table 9. The number-average (Mn) and weight-average (Mw) molecular weights of lignin in untreated (Raw) switchgrass and the liquid/solid streams for three pretreatment methods[1,2]

		Lignin in solid streams			**Precipitated lignin in liquid stream**		
		Mn (g/mol)	**Mw (g/mol)**	**PDI**	**Mn (g/mol)**	**Mw (g/mol)**	**PDI**
WT switchgrass	WT-Raw	5630	14458	2.6	N/A	N/A	N/A
	WT-DA	2547	4809	1.9	1398	3081	2.2
	WT-AH	2946	4129	1.4	1895	5044	2.7
	WT-IL	2116	2889	1.4	1780	4006	2.3
***4CL* switchgrass**	*4CL*-Raw	4482	9806	2.2	N/A	N/A	N/A
	4CL-DA	2156	3221	1.5	1739	3568	2.1
	4CL-AH	2610	2583	1.0	2147	5382	2.5
	4CL-IL	1929	2634	1.4	1869	3852	2.1
***AtLOV1* switchgrass**	*AtLOV1*-Raw	5862	15236	2.6	N/A	N/A	N/A
	AtLOV1-DA	2742	5528	2.0	1818	3804	2.1
	AtLOV1-AH	3248	4799	1.5	2232	5110	2.3
	AtLOV1-IL	2198	3416	1.6	1843	3813	2.1

[1]PDI: Polydispersity Index; [2]The data for the untreated switchgrass samples were adopted from a previous article (Liu et al., Under review)

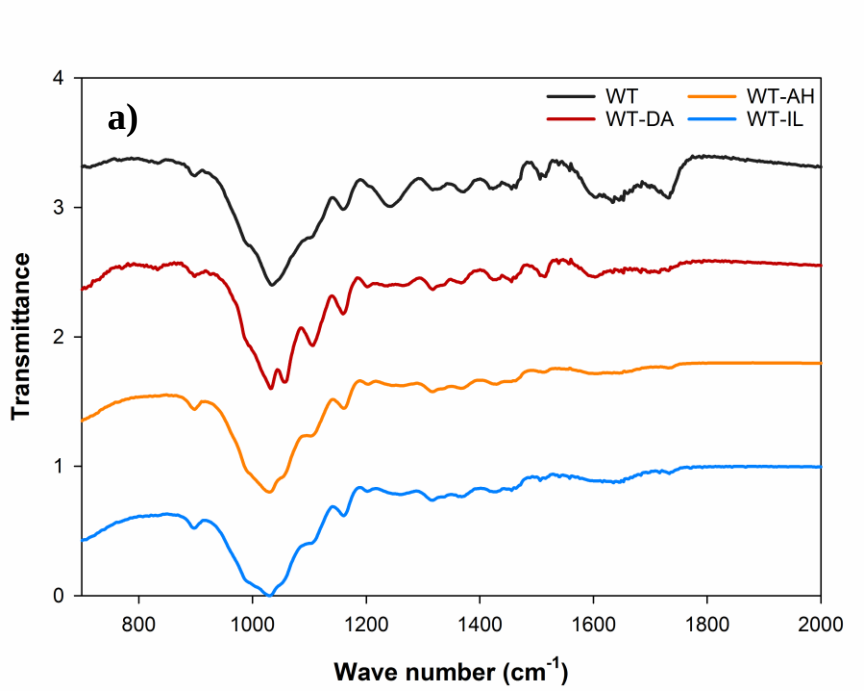
Combining the GPC results and compositional analysis, it can be seen that the aqueous IL pretreatment was superior to the DA and AH pretreatments in term of lignin solubilization and depolymerization, as indicated by the low molecular weight in the solids and the precipitated lignin. The low molecular weight lignin oligomers that accumulated in the liquid phase are suitable for further lignin upgrading *via* catalytic or biological conversion pathways. Certain lignin degrading microbes, such as *Rhodococcus* or *Bacillus* strains can assimilate and convert low molecular weight lignin to lipids or other fuel molecules (Linger et al., 2014; Ragauskas et al., 2014; Zhao et al., 2016). Thus, the results from this work point to possible pathways for developing a more selective and efficient lignin valorization process based on the aqueous IL pretreatment technology.

4.4.5 FTIR and DSC analysis

Infrared spectroscopy is a useful tool to characterize lignocellulosic biomass (Kim et al., 2003; Li et al., 2010a; Liu et al., 2009; Xu et al., 2013). In this study, FTIR was employed to investigate the structural changes and chemical variations for different types of switchgrass under different pretreatment methods. The spectra for untreated switchgrass and pretreated switchgrass was presented in **Figure 21**. To compare the chemical changes of lignin and carbohydrates, five peaks were used as references. The peaks at 900 cm^{-1} and 1098 cm^{-1} represent C-H deformation in cellulose and C-O vibrations in the crystalline region, respectively. No apparent changes at 900 cm^{-1} but significant increases at 1098 cm^{-1} peak were observed for all the DA pretreated samples as compared with untreated biomass, confirming the overall increase of crystallinity by removing nearly all amorphous hemicelluloses during DA pretreatment (Kumar et al., 2009; Xu et al., 2013). Additionally, the band at 1375 cm^{-1}, corresponding to C–H deformation in cellulose and hemicellulose, showed a significant decrease for AH and aqueous IL pretreated samples, which corroborates with the removal of hemicelluloses (Gupta & Lee, 2010; Lau et al., 2008). The peak at 1510 cm^{-1} representing the aromatic skeletal of lignin and the peak at 1329 cm^{-1} representing syringyl & guaiacyl condensed lignin, showed significant decreases for all AH and aqueous IL pretreated samples when compared to untreated and DA pretreated samples, which aligns with the removal of lignin during AH and aqueous IL pretreatment. Taken together, results clearly indicate

that DA, AH and aqueous IL pretreatment methods, via different chemistry, were all effective in disrupting the cell wall polymeric matrix by selective removal of hemicelluloses and/or lignin.

The spectra for the lignin rich samples recovered from the liquid and solids streams were shown in **Figure 22**. The signals between 1400 and 1600 cm^{-1} can be assigned to the aromatic skeletal vibrations: with the peaks at 1420 cm^{-1} and 1460 cm^{-1} representing C-H aromatic ring vibrations and C-H deformation in CH2 & CH3, respectively (Boeriu et al., 2004); whereas the peaks at 1510 cm^{-1} and 1595 cm^{-1} reflecting C=C of aromatic skeletal vibrations (El Mansouri & Salvadó, 2007). It appeared that the liquid stream lignins had similar FTIR profiles as Kraft lignin, especially for signals between 1400 and 1600 cm^{-1}. While the lignin recovered from the solids streams showed a few distinct features as compared with Kraft lignin, indicating the possible residual polysaccharides in the lignin rich materials after enzymatic hydrolysis.



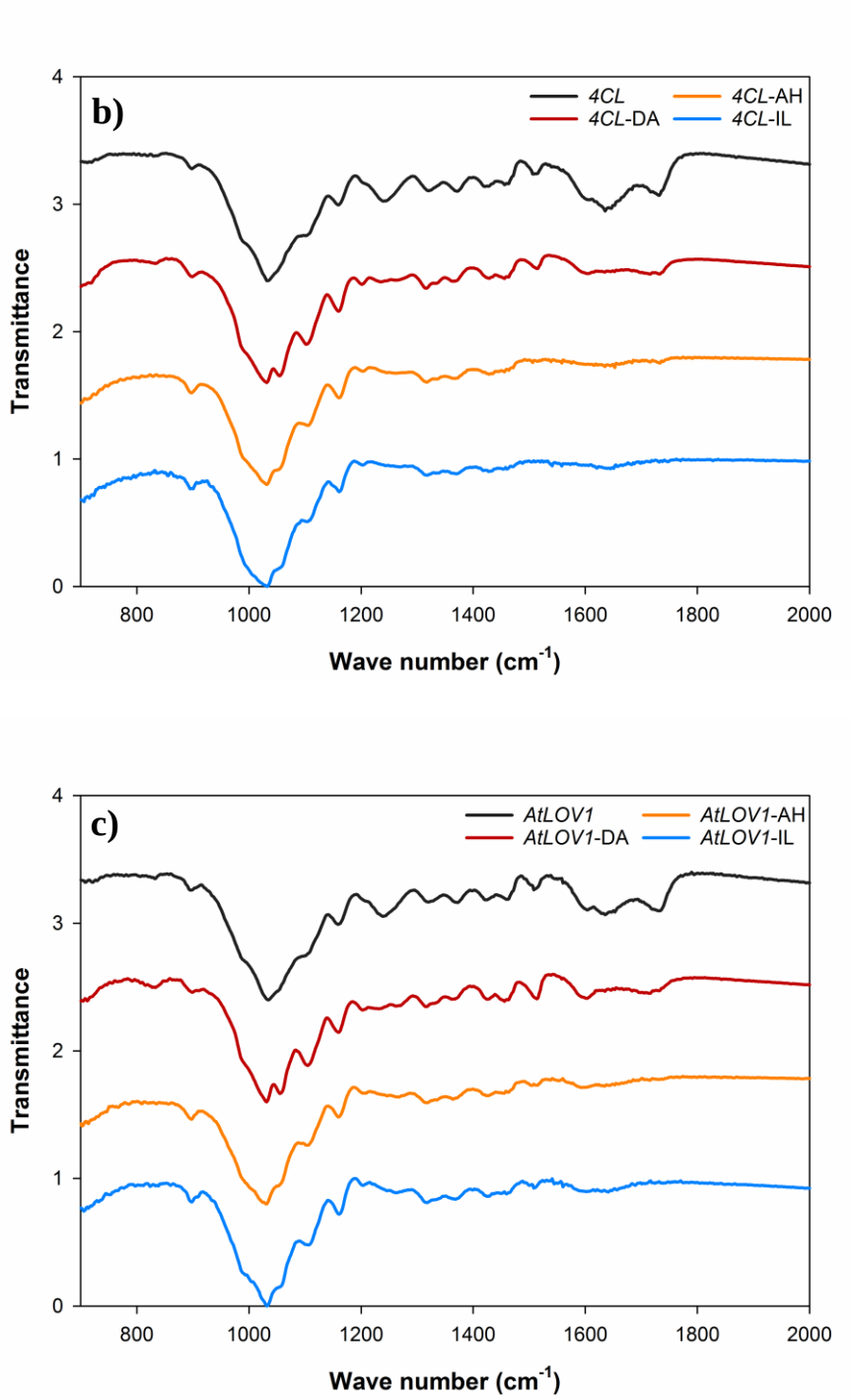
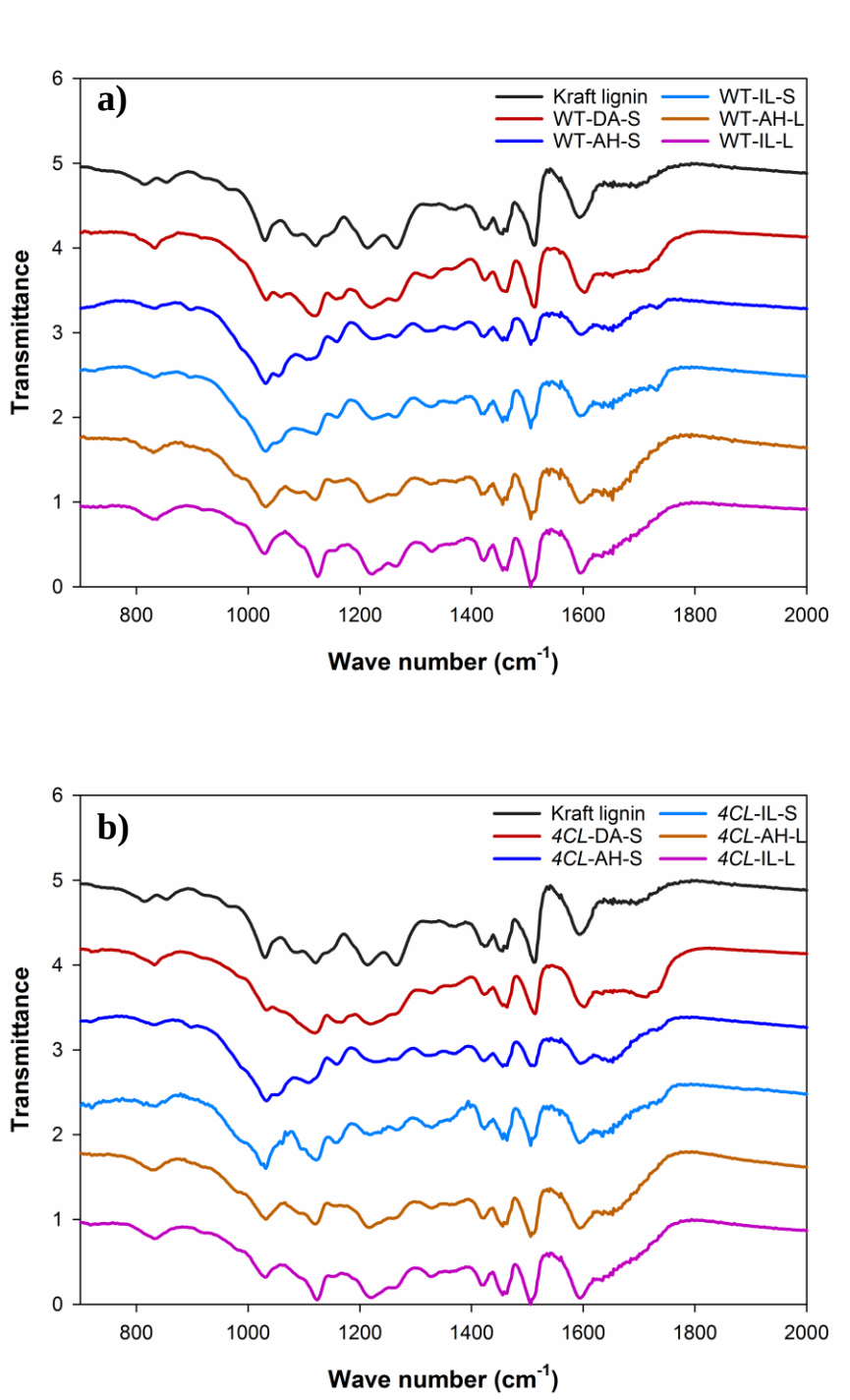


Figure 21. FTIR spectra for untreated switchgrass and pretreated switchgrass a) WT switchgrass pretreated by three pretreatment methods; b) *4CL* switchgrass pretreated by three pretreatment methods; c) *AtLOV1* switchgrass pretreated by three pretreatment methods


a)
Kraft lignin
WT-DA-S
WT-AH-S
WT-IL-S
WT-AH-L
WT-IL-L
Transmittance
Wave number (cm-1)
b)
Kraft lignin
4CL-DA-S
4CL-AH-S
4CL-IL-S
4CL-AH-L
4CL-IL-L
Transmittance
Wave number (cm-1)

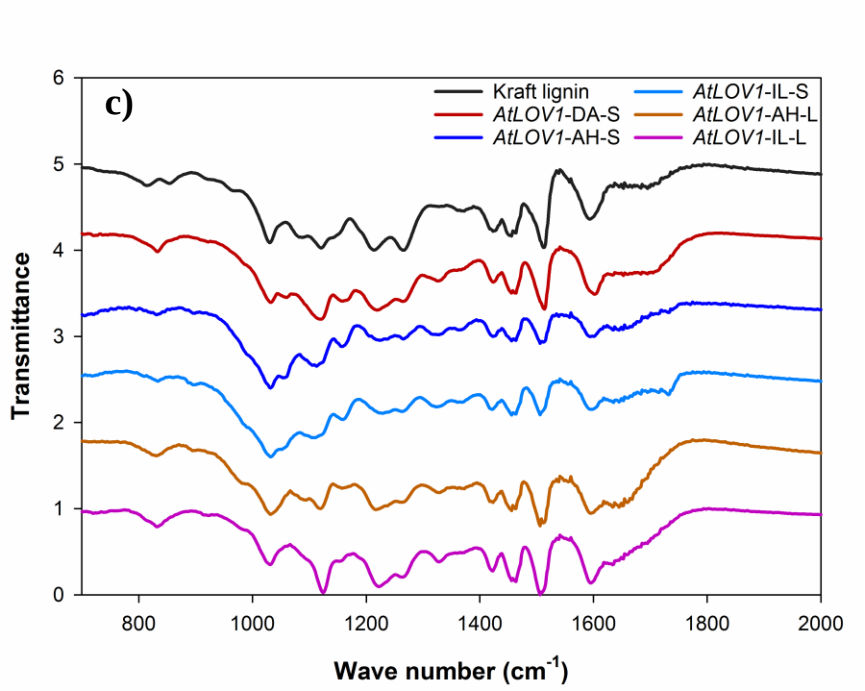
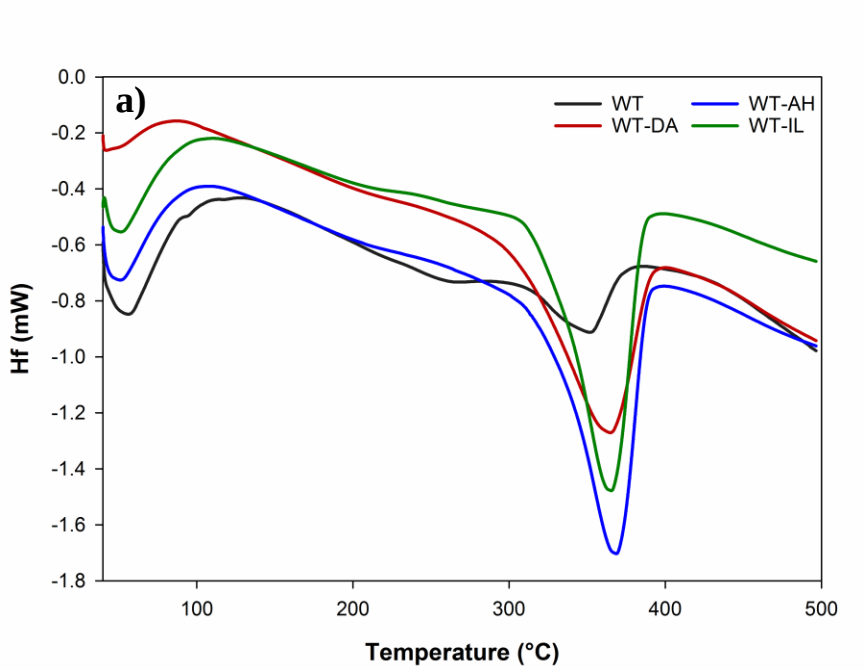
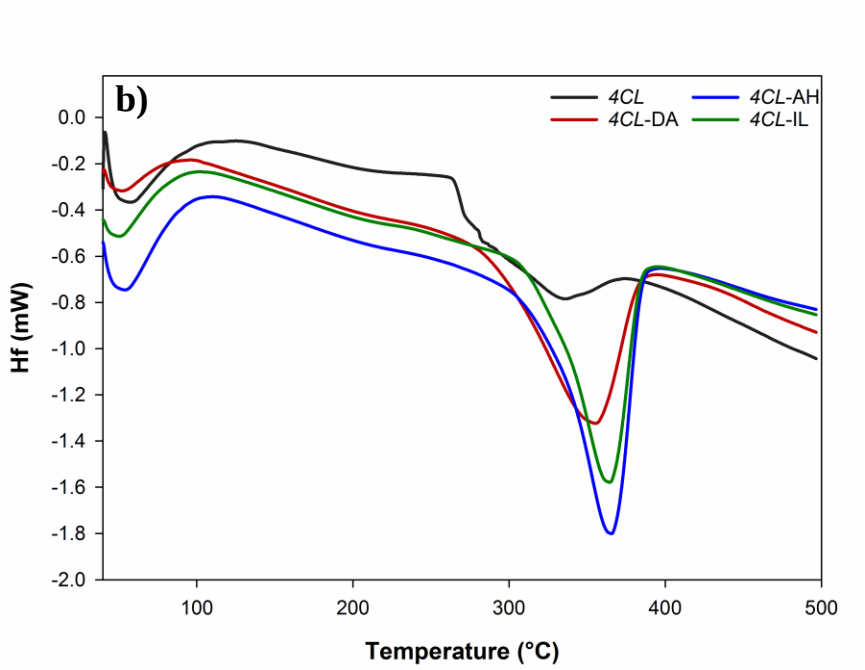


Figure 22. FTIR spectra for solid and liquid lignin streams for different types of pretreated switchgrass a) WT switchgrass pretreated by three pretreatment methods; b) *4CL* switchgrass pretreated by three pretreatment methods; c) *AtLOV1* switchgrass pretreated by three pretreatment methods

DSC has been widely used to determine the flow of heat transfer in a material at different temperature during a transformation process (Singh et al., 2013). **Figure 23** illustrates the thermograms for the pretreated and untreated samples, where the DSC curves with positive or negative H_f indicates exothermic and endothermic changes, respectively. All the untreated and pretreated samples followed two major endothermic regions at 50-100°C and 350-400°C, respectively (Kumar et al., 2010). The DSC curves of lignocellulosic material reflect the compositions and the structure of the lignin carbohydrate complex (LCC), particularly the type of bonds present between lignin and hemicelluloses (Tsujiyama & Miyamori, 2000). It seems that the genotype of switchgrass did not cause apparent differences in DSC profiles. However, the deeper endotherm minima in the range of 350-400°C for the pretreated samples when compared with the corresponding untreated biomass were probably caused by the breakdown of bonds in the LCC and the removal of amorphous lignin and hemicelluloses during pretreatment (Reh

et al., 1987). It is also noticed that the liquid stream lignins had similar DSC profiles as Kraft lignin (**Figure 24**); whereas the lignin-rich solids streams showed very different DSC profiles (as well as supported by the FTIR spectra), indicating the presence of residual polysaccharides in the lignin rich materials after enzymatic hydrolysis.





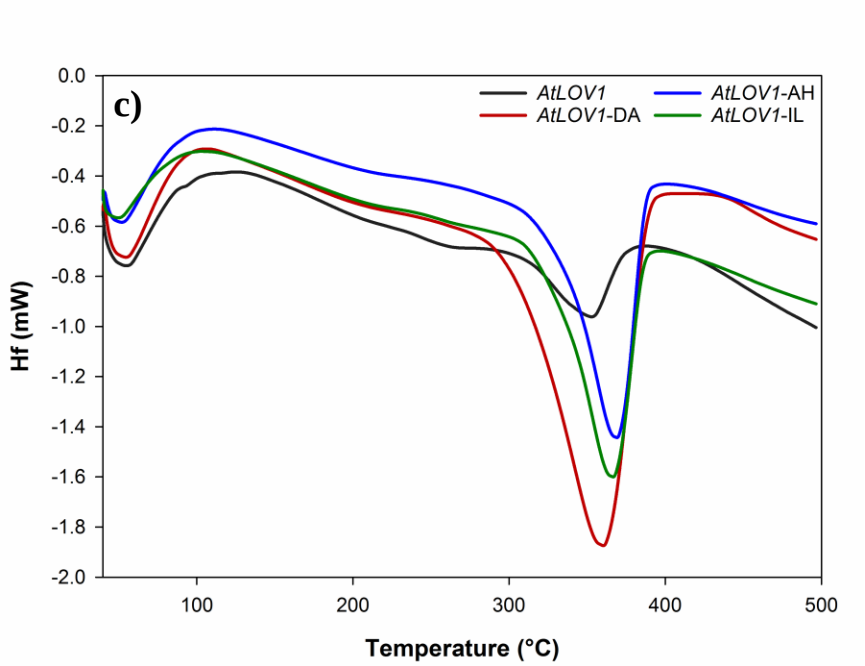
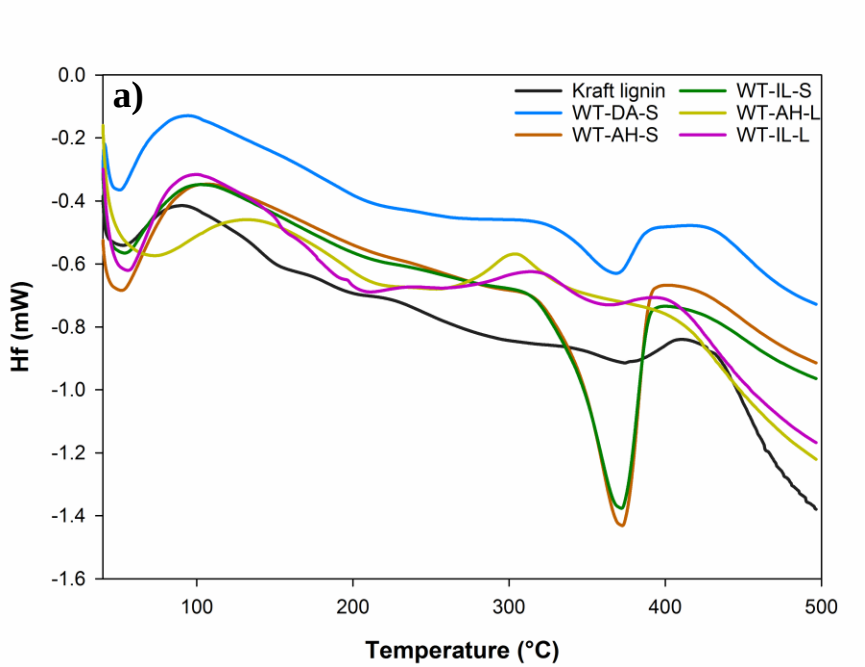


Figure 23. DSC spectra for untreated switchgrass and pretreated switchgrass a) WT switchgrass pretreated by three pretreatment methods; b) *4CL* switchgrass pretreated by three pretreatment methods; c) *AtLOV1* switchgrass pretreated by three pretreatment methods



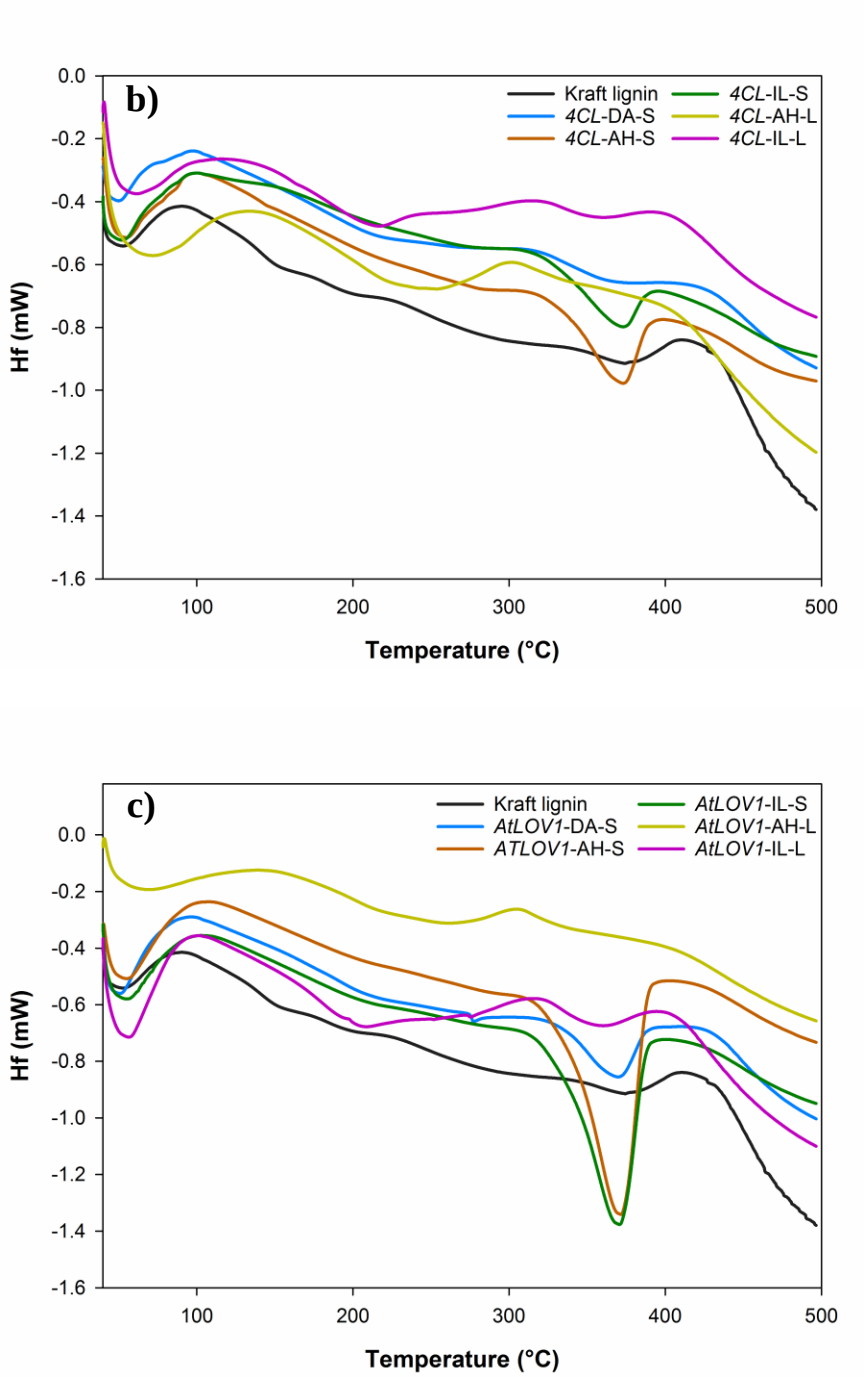


Figure 24. DSC spectra for solid and liquid lignin streams for different types of pretreated switchgrass a) WT switchgrass pretreated by three pretreatment methods; b) *4CL* switchgrass pretreated by three pretreatment methods; c) *AtLOV1* switchgrass pretreated by three pretreatment methods

4.5 Conclusions

Genetic modification is a promising route for developing biomass feedstocks with desirable traits, such as improved environment resilience, reduced biomass recalcitrance and overall increased convertibility. In this study, two genetically engineered switchgrass were compared with wild type switchgrass to understand the fractionation and characterization of these materials by different pretreatment methods. Results demonstrate that *4CL* switchgrass with lower lignin content and slightly increased S/G ratio was more susceptible to pretreatment and subsequently more digestible by enzymes as compared to wild type switchgrass and *AtLOV1* mutant. In addition, aqueous IL (cholinium lysinate) was proven to be an efficient lignin solvent, as indicated by the higher lignin solubility and lower lignin molecular weight when compared to DA and AH pretreatment. Pretreatment chemistry has significant impact on the structural and compositional changes and thermal properties of the pretreated switchgrass and recovered lignin-rich streams, as depicted by FTIR and DSC. The comparative data obtained from this work deepen our understanding on how lignin modification impacts the fractionation and characterization of biomass feedstocks. The mass flow and characteristics of the lignin streams as function of pretreatment chemistry and feedstock provide insights on developing potential ways for better utilization of lignin.

Acknowledgements

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CHAPTER 5: ON-GOING WORK AND FUTURE DIRECTIONS

5.1 Introduction

Production of fuels and chemicals from renewable lignocellulosic biomass helps to address the environmental concerns and depleting supply of fossil energy (Mosier et al., 2005; Ragauskas et al., 2006; Wyman et al., 2011). Lignin, as the most abundant aromatic heteropolymer, is generally burnt for heat and electricity in current biorefining industry. However, conversion of lignin streams to value-added products is critical to potentially increase the economic viability of a biorefinery (Ragauskas et al., 2014). Despite the recent advances in lignin chemistry (e.g. structure, composition, and biosynthesis) and catalysis, efficient and selective conversion of this complex substrate has not been fully reached (Feghali et al., 2015; Li et al., 2015; Xie et al., 2016).Several lignin conversion methods currently under investigation are hydrolysis, hydrogenolysis, pyrolysis, catalytic oxidation, and biological depolymerization (Stärk et al., 2010). Compared with the thermochemical approaches, biological conversion of lignin-derived substrates has received increasing interests owing to the advantages such as, improved selectivity using biocatalysts, mild operating conditions, less toxic chemicals and waste streams and potential low cost, etc.

The natural mechanism for lignin degradation involves two major steps, which include depolymerization of lignin into aromatic compounds and biological utilization of aromatic oligomers (Salvachúa et al., 2015; Xie et al., 2016). Several microorganisms have shown the capability of depolymerizing lignin with white-rot fungi (e.g. *Phanerochaete chrysosporium*) the most well-known lignin-degrading microbes (Beckham et al., 2016; Yao & Nokes, 2014). In addition to white-rot fungi, a large number of other microbes can efficiently utilize lignin-derived substrates, such as *Amycolatopsis* sp. 75iv2, *Rhodococcus opcus*, *Rhodococcus jostii*, *Sphingobium sp*. SYK-6, *Nocardia*, *Pseudomonas*, *Bacillus*, and *Comamonas* (Beckham et al., 2016; He et al., 2017a; Kosa & Ragauskas, 2012; Zhao et al., 2016; Zhu et al., 2017). Recently, oleaginous bacteria *Rhodococcus opacus* has received increasing attention, since it can utilize lignin as sole carbon source to produce lipids (C14 to C24) through β-ketoadipate

pathway (He et al., 2017a; Kosa & Ragauskas, 2012; Kosa & Ragauskas, 2013; Le et al., 2017; Lin et al., 2016; Wei et al., 2015). As compared to fungal lignin degradation, bacterial degradation is a more promising approach to covert lignin to value-added products, partially due to the availability and environmental adaptability of bacteria.

As introduced in the previous chapters, a large fractions of lignin with low molecular weight was accumulated in the liquid phase after aqueous ionic liquid (IL, cholinium lysinate) pretreatment. The IL, cholinium lysinate is more biocompatible than the imidazolium based ILs, such as 1-ethyl-3-methylimidazolium acetate ($[C_2C_1Im][OAc]$), 1-butyl-3-methylimidazolium chloride ($[C_4C_1Im][Cl]$), and 1-ethyl-3-methylimidazolium chloride ($[C_2C_1Im][Cl]$). In an aqueous IL pretreatment system, the majority of the lignin is extracted to the liquid phase and separation of lignin form this liquid system is however challenging. Hence, in-situ lignin valorization in aqueous IL could offer a new strategy for selective lignin depolymerization meanwhile help to tackle the challenges associated with IL recycle and product recovery, thus improving the economics of an IL-based biorefining process.

This study aims to screen the biocompatibility of different ILs and test the potential of upgrading low molecular weight lignin oligomers to lipids by *Rhodococcus opacus* (DSM 1069 and PD630). The biocompatibility of seven ILs was examined at different concentrations (1-10% w/w) using *Rhodococcus opacus*. The bioconversion of lignin model compound (vanillic acid) to lipids was carried out, and results being compared with carbohydrate substrates. Additionally, immobilization of *Rhodococcus opacus* on biochar or activated carbon was evaluated in order to improve the microbes resistance to harsh environment and for easy recovery of lipid from the fermentation broth. This study leads to possible directions for developing a more selective and efficient lignin valorization process based on aqueous IL pretreatment technology.

5.2 Materials and methods

5.2.1 Strains and materials

Rhodococcus opacus DSM 1069 and PD630 were obtained from German Collection of Microorganisms and Cell Cultures (DSMZ). All chemicals and materials are purchased from Sigma Aldrich (St. Louis, MO, US) or VWR (Radnor, PA, US). Cholinium lysinate ([Ch][Lys]) (>95% purity) was synthesized following a method as described elsewhere (Sun et al., 2014). The other ionic liquids are either synthesized in lab or purchased from a commercial source (IoLiTec, Tuscaloosa, AL). Biochar was prepared from industrial hemp at a temperature of ~450°C for 3 h under a flow of nitrogen. Activated carbon was purchased from Sigma Aldrich.

5.2.2 Media and bacteria cultivation

Media for *Rhodococcus opacus* DSM 1069 and PD630 were prepared according to DSMZ recommendations. Media for DSM 1069 contains: 4.0 g glucose, 4.0 g yeast extract, 10.0 g malt extract (plus 2.0 g $CaCO_3$ and 12.0 g agar for solid medium) per liter of distilled water. Media for PD630 contains: 30.0 g trypticase soy broth (plus 15.0 g of agar for solid medium) per liter of distilled water. The pH for media was adjusted to 7.0 to 7.2, and autoclaved or sterile filtered before use. *Rhodococcus opacus* DSM 1069 and PD630 were aerobically grown at 30°C in flasks while shaking at 150 rpm in an orbital shaker (Thermo Forma 435, Thermo Fisher Scientific Inc., Waltham, MA, US). In general, the optical density at 600nm (OD600) of DSM 1069 and PD630 reached ~1.0 after 14 h of growth. The effects of different carbon sources on the growth of DSM 1069 were investigated using glucose, xylose, or Kraft lignin as sole carbon source; while the rest ingredients of media remain the same.

5.2.3 Scanning electron microscopy

Images of the bacteria in free liquid or supported on carbon materials (biochar and activated carbon) were obtained by SEM using a FEI Quanta 250 FEG instrument operating at SE mode under low vacuum (0.40–0.65 Torr). Samples were prepared for imaging by freeze-drying using an AdVantage 2.0 bench top lyophilizer (SP Scientific,

Warminster, PA). The dried bacterial samples were sputter-coated in gold and the imaging was performed at beam accelerating voltages of 2 kV.

5.2.4 Screening of biocompatible ionic liquids

Screening of biocompatible ionic liquids was conducted using a 48-well plate and microplate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, US). Seven ILs including cholinium lysinate ([Ch][Lys]), $[C_2C_1Im][Cl]$, $[C_4C_1Im][Cl]$, $[C_2C_1Im][OAc]$, $[C_4C_1Im][OAc]$, $[DEA][HSO_4]$ (DEA), $[TEA][HSO_4]$ (TEA) were tested. The pH of IL with initial concentration of 60% (w/w) was adjusted to 7.0 using 6M HCl or NaOH. Then, the IL solution was diluted to concentrations from 1% to 10%, w/w with the media for DSM 1069 and PD630, respectively. The culture of bacteria was inoculated to the media to obtain an initial OD of 0.3-04. Working volume for each cell of the 48-well plate was 0.25 ml. After inoculation, the plate was incubated in the plate reader (shaked for 30 s before and after each reading) at 30°C and cell growth was monitored by the OD600 taken at each hour for 48 hours.

5.2.5 Lipids fermentation and extraction

Glucose or vanillic acid was added to the DSM 1069 media as sole carbon source with a concentration of 0.5%, w/v. As a comparison, activated carbon (0.5%, w/v) was introduced into the fermentation media to support the bacteria. Bacteria were fist inoculated into aerobic flasks for growing until the absorbance at 600 nm reached ~1.0. Then the cells were centrifuged at 3000 rpm for 10 min, and washed twice with physiological salt solution (0.9% NaCl). After washing, the cells were re-suspended and added to the fermentation broth at 1% v/v as inoculum. The fermentation was carried out in 40 ml vials with 10 ml working volume at 150rpm at 30°C. Samples were taken at 0 h, 12 h, 24 h, 48 h, 72 h, 96 h, and 120 h. The OD600, cell dry weight, substrate consumption, and lipids yield (for 120 h time point) were determined. After fermentation, the cells were collected by centrifuging at 4500 rpm for 10 min. The cell pellets was vortexed and re-suspended with 20 ml methanol, then incubated at 65°C for 30 min in a water bath. After incubation, 1 ml of 10M KOH was added and vortexed, then incubated at 65°C for 2 h. Samples were cooled down to room temperature and mixed with 1 ml of

concentrated sulfuric acid (98%), then incubated again at 65°C for another 2 h. To extract the lipids, 8 ml of hexane was added to the samples, mixed for 5 min followed by centrifuging at 3000 rpm for 5 min. The hexane layer was collected and put in the vacuum oven to evaporate the solvent. The amount of lipids was determined by weight.

5.2.6 Analytical methods

Cell dry weight: Cells were collected by centrifuging at 4500 rpm for 10 min and washed twice with physiological salt solution. Then all the cells were dried at 50°C till constant weight.

Substrate consumption: Glucose was determined by HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA, US) equipped with a refractive index detector and Aminex HPX-87H column and guard column assembly, using 5 mM H_2SO_4 as mobile phase at a flow rate of 0.4 ml/min and a column temperature of 50°C. Vanillic acid was determined via HPLC (Ultimate 3000, Dionex Corporation, Sunnyvale, CA) equipped with an Ultra Violet (UV) detector. Separation was achieved on a zorbax eclipse XDB-C18 column (4.6 × 150 mm, 5 µm) with column temperature maintained at 28°C and the UV detector set at 280 nm. The mobile phase at a flow rate of 1 ml/min, was a mixture of two solvents; A: water with 0.01% of acetic acid, and B: methanol. Gradient elution profile was set up as previously reported (Falconnier et al., 1994). In brief, solvent B was started at 20%, then increased to 40% at 24 min and to 100% at 27 min, and returned to 20% at 30 min.

5.2.7 Statistical analysis

All experiments were conducted in triplicates and the data was presented with means and standard deviations. The statistical analysis was performed by SAS® 9.4 (SAS Institute, Cary, NC, US), with a significance level of $P<0.05$ for all the data obtained from experiments.

5.3 Preliminary results and discussion

5.3.1 Growth of *Rhodococcus opacus*

Rhodococcus is a genus of aerobic, gram-positive bacteria found in soil, water, and plants. Several *Rhodococcus opacus* species have been identified as promising microbes to covert aromatic compounds to lipids. **Figure 25** visualizes the colonies of *R. opacus* DSM 1069 and PD630 growing on agar plates. Moreover, the SEM image of DSM 1069 and PD630 illustrates the size and visual property of the two *R. opacus* strains. The *R. opacus* is in rod shape and around 1µm of length, and tends to aggregate. **Figure 26** shows the optical density (OD600) of *R. opacus* DSM 1069 over time. According to **Figure 26**, *R. opacus* DSM 1069 grew rapidly on full media. The OD reached 1.0 in 14 h and maximum OD around 30 h.

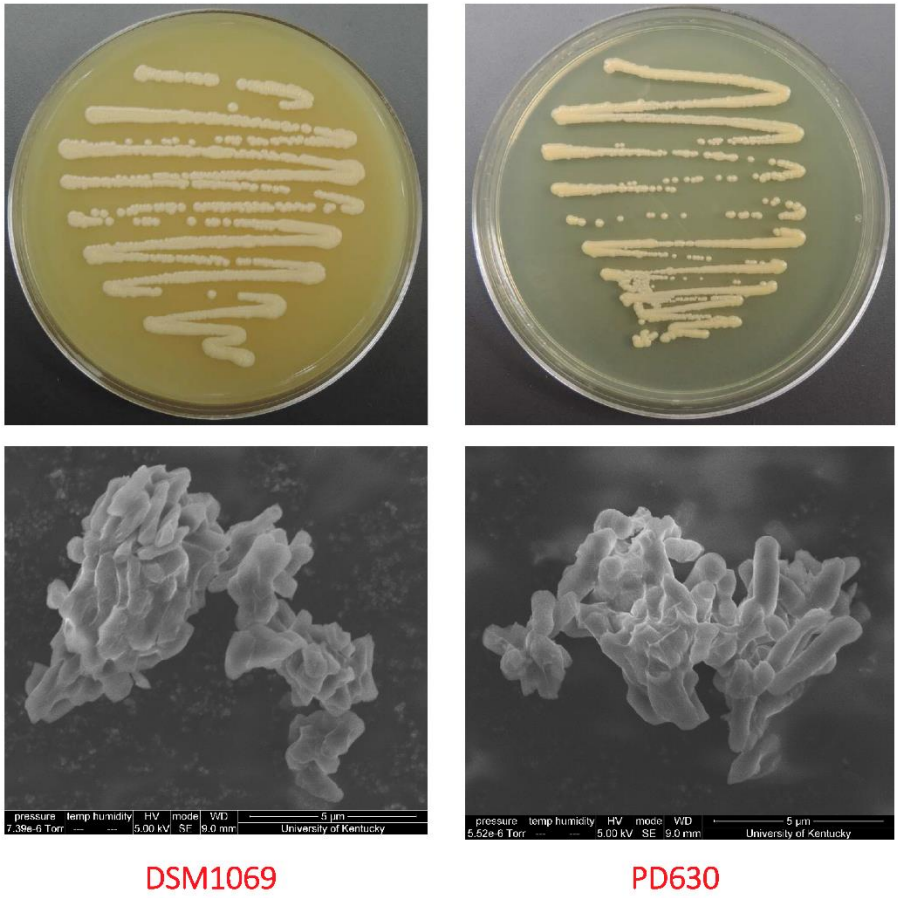


Figure 25. Agar plate growing and SEM images of DSM 1069 and PD630

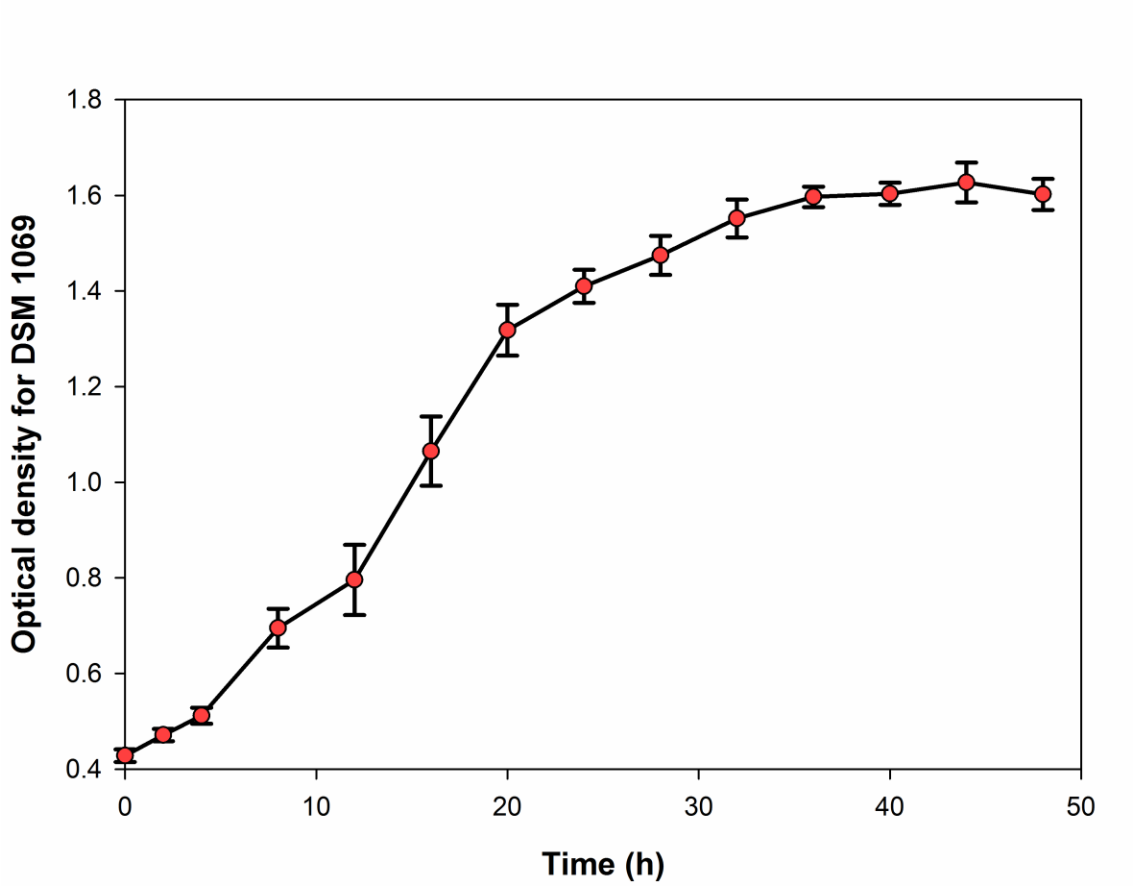


Figure 26. Optical density of DSM 1069 over growing time

5.3.2 Screening of biocompatible ILs

IL pretreatment is an efficient pretreatment method to selectively solubilize lignin or cellulose. However, it is challenging to separate lignin from IL solution and recycle the IL. In order to address these challenges, development of biocatalytic processes to upgrade lignin in aqueous ILs is highly desirable. As a consequence, screening of biocompatible ionic liquids is critical to the success of liquid phase lignin bioconversion.

The biocompatibility of different ILs, including [Ch][Lys], [C_2C_1Im][Cl], [C_4C_1Im][Cl], [C_2C_1Im][OAc], [C_4C_1Im][OAc], [DEA][HSO_4] (DEA), [TEA][HSO_4] (TEA), was examined using *R. opacus* DSM 1069 and PD630. The acidity and basicity of an IL determine its solvent property. In 60% aqueous solution, the pH for [Ch][Lys],

[C_2C_1Im][OAc], and [C_4C_1Im][OAc] was above 7.0, DEA and TEA were acidic ionic liquids, while [C_2C_1Im][Cl] and [C_4C_1Im][Cl] were close to neutral pH (**Table 10**). In order to exclude the effects of pH on microbial growth, the pH for all ILs was adjusted to 7.0 before the screening. *R. opacus* PD630 appeared a strong tendency aggregate, especially in presence of IL. The cells formed a thin film on top of the media, which influenced the reading of plate reader. Therefore, only *R. opacus* DSM 1069 was used to screen out the biocompatible ILs.

Table 10. pH of different ionic liquids used for biocompatible screening

Ionic liquids	Concentration (%, w/w)	pH
[Ch][Lys]	60	~13.1
[C_2C_1Im][Cl]	60	~6.1
[C_4C_1Im][Cl]	60	~5.6
[C_2C_1Im][OAc]	60	~9.1
[C_4C_1Im][OAc]	60	~8.1
[DEA][HSO_4]	60	~0.7
[TEA][HSO_4]	60	~1.2

The IL concentrations were tested in a range of 0-10 wt%. **Figure 27** illustrates a heatmap of the effect of the IL type and concentration on bacterial growth of *R. opacus* DSM 1069. Green means no apparent inhibition; while yellow and red mean retarded growth or complete no growth. Results show [Ch][Lys] is more biocompatible than imidazolium or ammonium based ILs. Bacteria can still grow in 10% of [Ch][Lys], a concentration proved highly effective at fractionating and depolymerizing lignin from switchgrass (Chapter 3). Bacteria can tolerate up to 5% of [C_2C_1Im][Cl], while up to 3% of [C_4C_1Im][Cl] and [C_2C_1Im][OAc]. However, 2% of [C_4C_1Im][OAc], DEA, and TEA

completely inhibited the growth of *R. opacus* DSM 1069. Results illustrated the huge variations in biocompatibility were dependent on both the cation and anion of IL. The IL, [Ch][Lys] is derived from natural bases and acids, showing better compatibility. In combination with the IL's capability in lignin fractionation and depolymerization, it is possible to use this biocompatible IL to extract lignin and biologically upgrade the low molecular weight lignin into value-added chemicals in the aqueous IL solution.

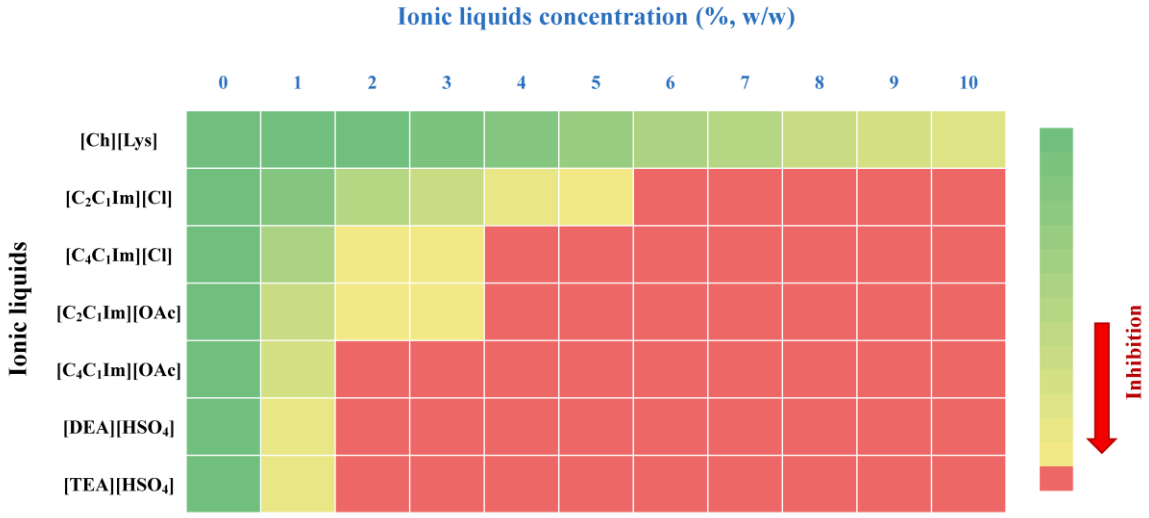


Figure 27. Heat map showing the screening of biocompatible ionic liquids

5.3.3 Bacterial growth on supports

It was observed that *R. opacus* DSM 1069 and PD630 tend to self-aggregate, especially under environmental stresses such as inhibitors and IL. The aggregation could cause problems such as poor mixing and aeration during fermentation and potential impact on lignin uptake and lipids accumulation. Taking advantage of the natural protecting mechanisms developed by the microorganism, it is possible to immobilize the bacteria on a porous support, i.e. biochar and activated carbon. Such as supported growth system could stabilize the bacteria as well facilitate the harvest. In addition, the biochar or activated carbon could absorb lignin compounds for better access to the microbial

conversion. As shown in the SEM images, both biochar and activated carbon have large surface areas and small pores on the surface of carbon (**Figure 28**). The bacteria grew well in the media with 0.5% biochar or activated carbon. Moreover, the images show that both DSM 1069 and PD630 were attached to the surface of biochar or activated carbon. In particular, bacteria filled all the pores up on the surface of activated carbon. Results demonstrated that biochar and activated carbon were good supporting material for the growth of *R. opacus* DSM 1069 and PD630. Activated carbon was selected for further test because of the high surface area and porosity.

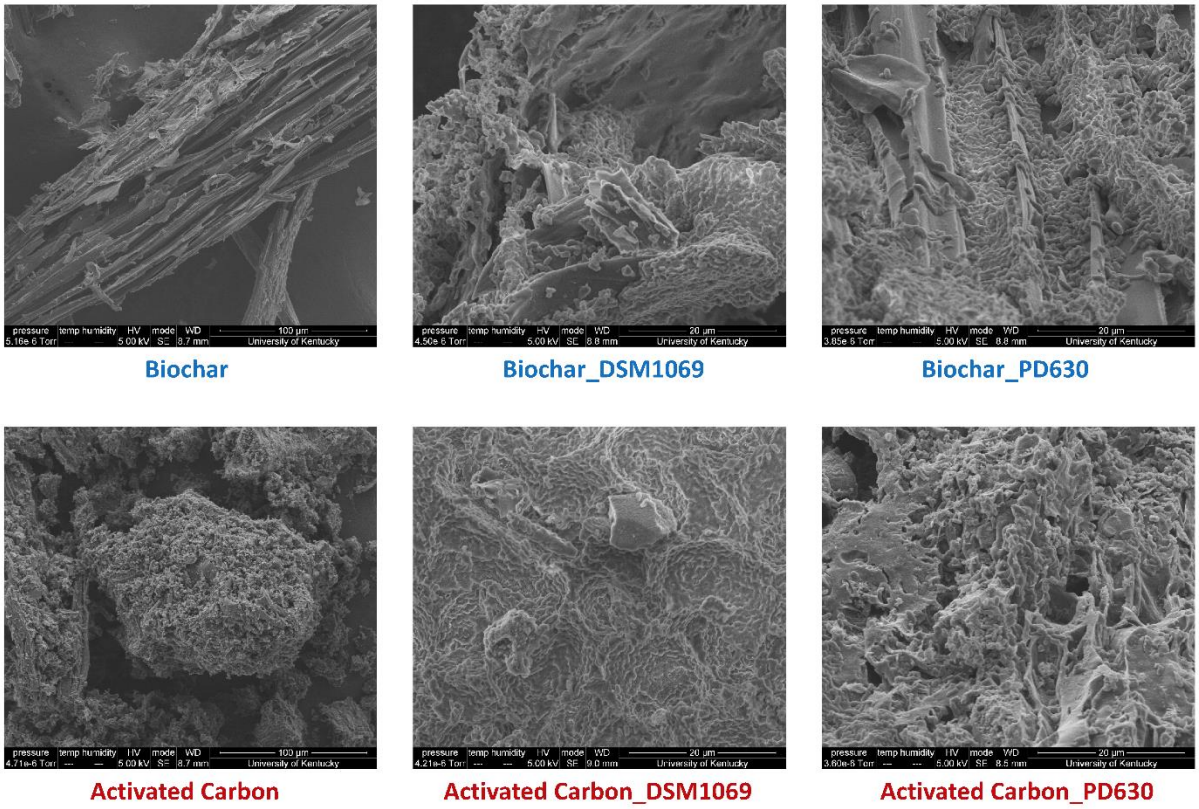


Figure 28. Bacteria growing on supports: biochar or activated carbon

5.3.4 Effects of different substrates on the growth of bacteria

The liquid phase after pretreatment contains carbohydrates (glucose and xylose), lignin oligomers (low molecular weight), inhibitors, and pretreatment chemical such as IL, etc. Therefore, the effect of different substrates on the growth of bacteria was investigated. According the growing curves, *R. opacus* DSM 1069 can utilize either glucose or xylose as sole carbon source, while prefer to glucose (**Figure 29**). Furthermore, *R. opacus* DSM 1069 was able to grow on lignin and [Ch][Lys] (1% lignin plus 5% [Ch][Lys]). However, the optical density was significantly lower than that of glucose and xylose, and longer lag time was observed. Results demonstrated that DSM 1069 did not feed on the IL, ChLys.

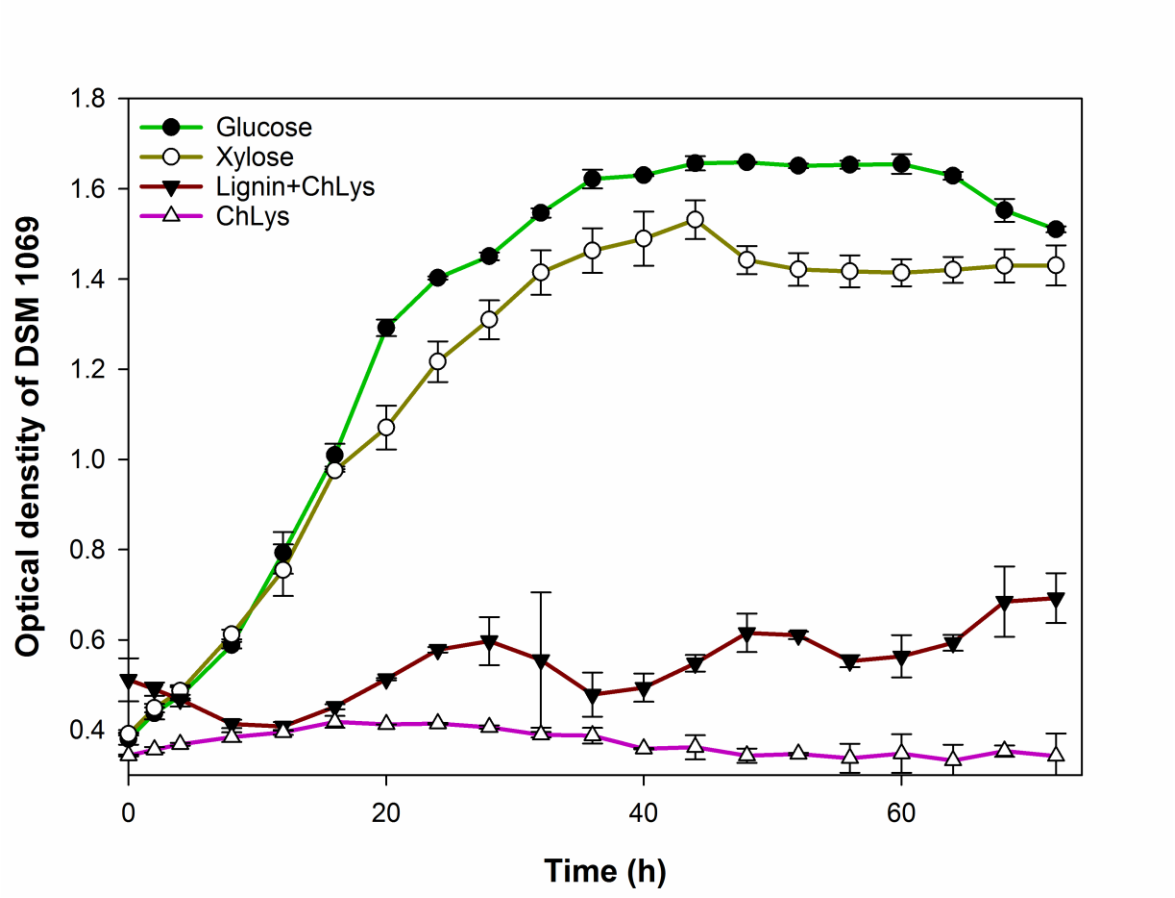


Figure 29. Effects of different substrates on the growing of DSM 1069

5.3.5 Lipids fermentation

Lipids fermentation by *R. opacus* DSM 1069 was carried out in a 500ml flask with 150 ml working volume at 30°C, 150 rpm for 120 h. The substrates consumption and dry cell accumulation were determined and presented in **Figure 30** and **Figure 31**. Glucose was quickly consumed by the bacteria. In 24 hours, the concentration of glucose was down to zero. However, there was no significant difference on glucose consumption rate between pure medium and medium supplemented with activated carbon. As for the vanillic acid substrate, the concentration of vanillic acid in media without activated carbon remained the same for the entire fermentation period. However, bacteria started to consume vanillic acid around 48 h with activated carbon added in the media. One may wonder that the decrease in vanillic acid concentration could be caused by the adsorption of vanillic acid on activated carbon. However, the adsorption usually takes much less time than 48 h. It is worth to confirm this argument by a blank run without *R. opacus* DSM 1069 inoculum. Results demonstrated that activated carbon can support better bacteria growth on vanillic acid. According to the results in **Figure 31**, for glucose, the dry cell concentration was significantly higher when activated carbon was added to the fermentation media. Glucose media led to remarkably higher dry cell concentration as compared to vanillic acid substrate. However, there was no significant difference on lipids production between different substrates (glucose/vanillic acid with or without activated carbon).

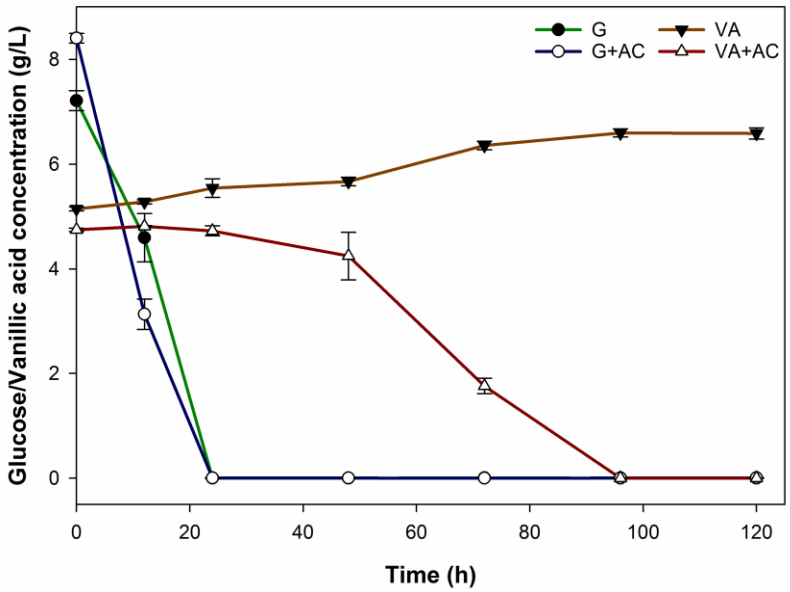


Figure 30. Substrates consumption during lipids fermentation by DSM 1069

G: Glucose, VA: Vanillic Acid, G+AC: Glucose with Activated Carbon, VA+AC: Vanillic Acid with Activated Carbon

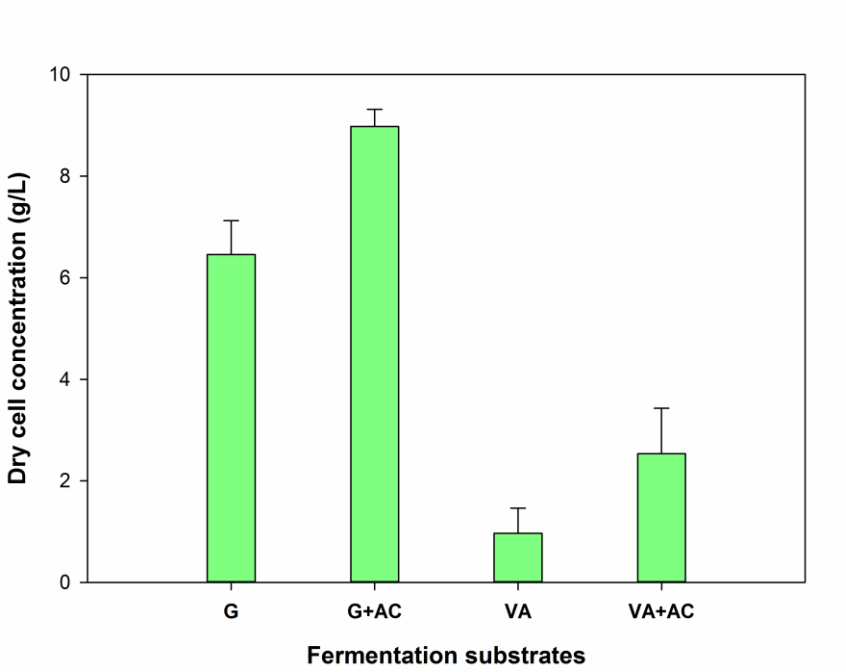


Figure 31. Dry cell accumulation during fermentation by DSM 1069

G: Glucose, VA: Vanillic Acid, G+AC: Glucose with Activated Carbon, VA+AC: Vanillic Acid with Activated Carbon

The mechanism of lipids production by *R. opacus* DSM 1069 is not well understood. However, the accumulation of lipids could be stimulated by C or N limiting media as a natural bacterial response to stressful environment. The media used in this study, contain glucose or vanillic acid, malt extract and yeast extract which is not optimized for lipids production. Future work needs to be done to optimize media composition such N source or minerals. It is necessary to move to minimal media to promote lipid production and get a better understanding of substrate utilization by *R. opacus* DSM 1069.

5.4 Summary

Development of novel processes for bioconversion of low molecular weight lignin in aqueous IL is critical to lignin valorization. Biocompatible ILs provide a potential

approach to upgrade lignin within aqueous ionic liquid solution without downstream separation. Results demonstrated that bio-derived cholinium lysinate acquired the highest level of biocompatibility. *R. opacus* DSM 1069 and PD630 are promising bacteria to convert lignin to lipids. However, the aggregation of bacteria might cause potential limitations for lignin conversion. We proposed to stabilize the bacteria by supporting on biochar or activated carbon. Preliminary results that the bacteria could easily attach to biochar or activated carbon. Moreover, effects of different substrates were investigated in this study. Results show the bacteria prefer to carbohydrate substrates, however, could utilize lignin as sole carbon source. Lipids fermentation was also preliminarily carried out in this study.

5.5 Future directions

This study investigated the effects of lignin engineering on the fractionation and characterization of lignin streams from switchgrass based on three different pretreatment methods. Results demonstrate that the recalcitrance of biomass feedstocks can be reduced by reducing lignin content and increasing S/G ratio. Moreover, aqueous IL (10% [Ch][Lys]) is an efficient solvent to solubilize and selectively depolymerize lignin as compared to dilute sulfuric acid and ammonia hydroxide. In addition, preliminary results point to a novel approach to biologically convert lignin into lipids within aqueous IL using supported *R. opacus* on porous carbon. Nevertheless, there are still several areas for the future research regarding this topic:

1) Identify lignin monomers, dimers, trimers, and oligomers solubilized in the liquid steam for better understanding of lignin fractionation in aqueous IL;

2) Optimize conditions of aqueous IL pretreatment such as solid loading, IL concentration, temperature and reaction time etc.;

3) Test other genetically modified biomass feedstocks with different ratios of lignin subunits, especially the mutants with solely S, G, or H lignin;

4) Establish a model system for supported bacteria on biochar or activated carbon to investigate the effects of substrates and inhibitors on other lignin model compounds or real lignin ;

5) Convert aqueous IL pretreatment black liquor to lipids;

6) Engineer a better *R. opacus* strain with improved IL-tolerance and conversion efficiency.

7) Study the effect of lignin degrading enzyme addition on the conversion efficiency.

5.6 Publications

Manuscripts:

1. **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass pretreated by an aqueous ionic liquid. *Submitted (Chapter 3)*
2. **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Impact of dilute sulfuric acid, ammonia hydroxide, and ionic liquid pretreatment on the fractionation and characterization of engineered switchgrass. *In preparation (Chapter 4)*
3. **Enshi Liu**, Wenqi Li, Seth DeBolt, Sue E. Nokes, Jian Shi. The chemistry, biology and conversion technology of lignin engineered plants in the biorefinery concept: A review. *In preparation (Chapter 2)*
4. Lalitendu Das, **Enshi Liu**, Areej Saeed, David W. Williams, Chenlin Li, Allison E. Ray, Jian Shi. Industrial hemp as a potential biofuels crop in comparison with kenaf, switchgrass, and biomass sorghum. *In preparation*

Conference papers/Presentations/Posters:

1. **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous ionic liquid. Poster at 2017 KY NSF Super Collider, February 24, 2017, Lexington, KY.

2. **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous bionic liquid. Flash talk and Poster at 2016 Frontiers in Biorefining Conference, November 8-11, 2016, St. Simons Island, GA.
3. **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass. Oral presentation at 2016 Annual International Meeting of American Society of Agricultural and Biological Engineers (ASABE), July 17-20, 2016, Orlando, FL.
4. Ulalo Chirwa, **Enshi Liu**, Jian Shi. Bioprocessing of soybean processing waste for bacterial biocontrol agents. Poster at 2016 Annual International Meeting of American Society of Agricultural and Biological Engineers (ASABE), July 17-20, 2016, Orlando, FL.
5. **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from pretreatment of engineered switchgrass. Oral presentation (By Dr. Jian Shi) at the 38th Symposium on Biotechnology for Fuels and Chemicals, April 25-28, Baltimore, MD.
6. **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from pretreatment of engineered switchgrass. Poster at 2016 KY NSF Super Collider, February 26, 2016, Lexington, KY.

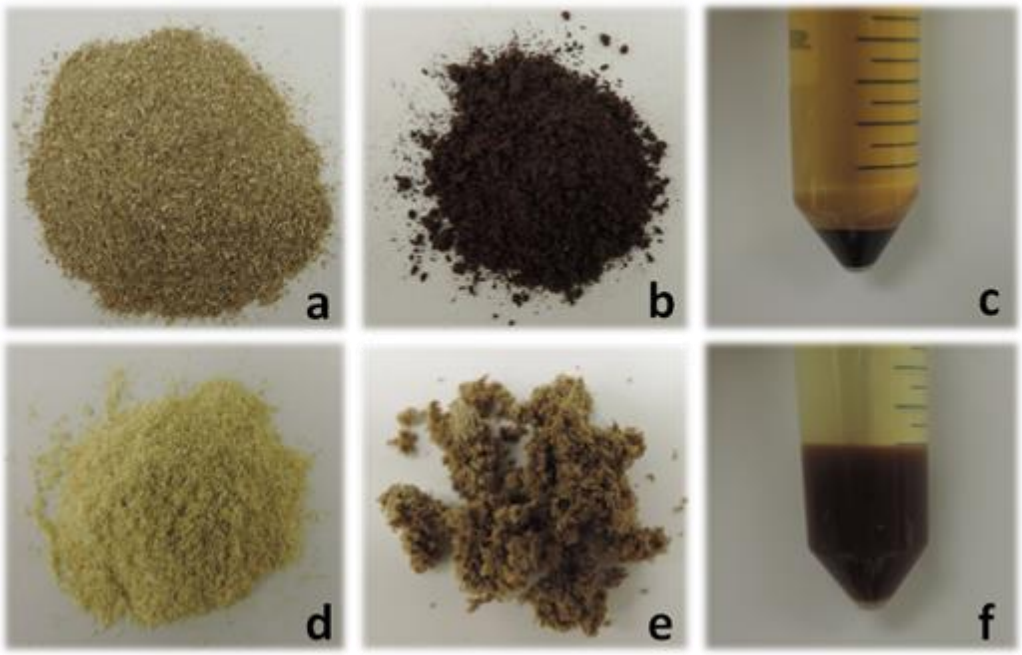
APPENDICES

Appendix A. Pretreatment of switchgrass

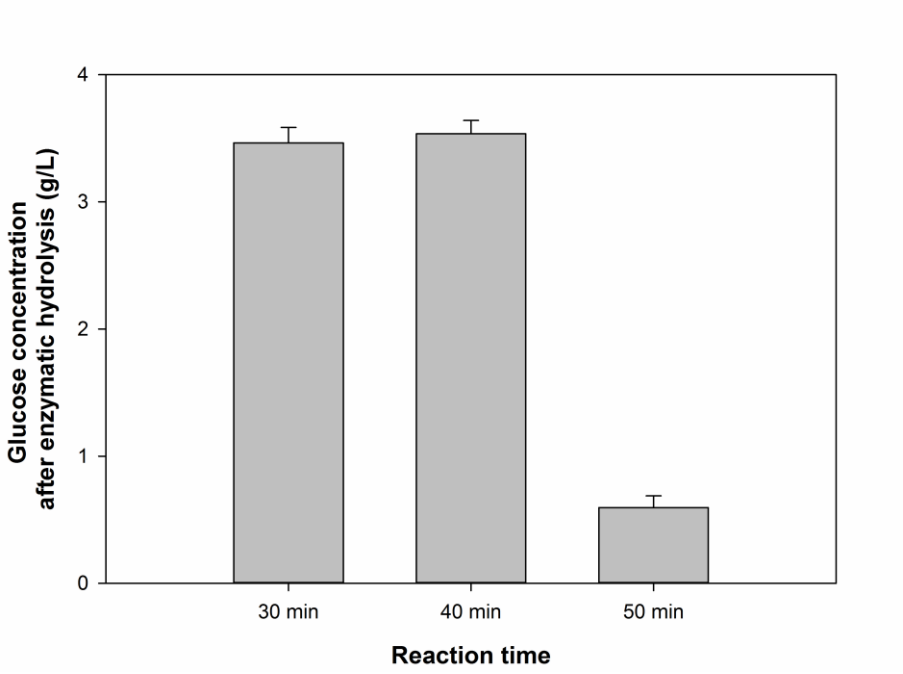
Engineered switchgrass sample

Glass and stainless steel reactor

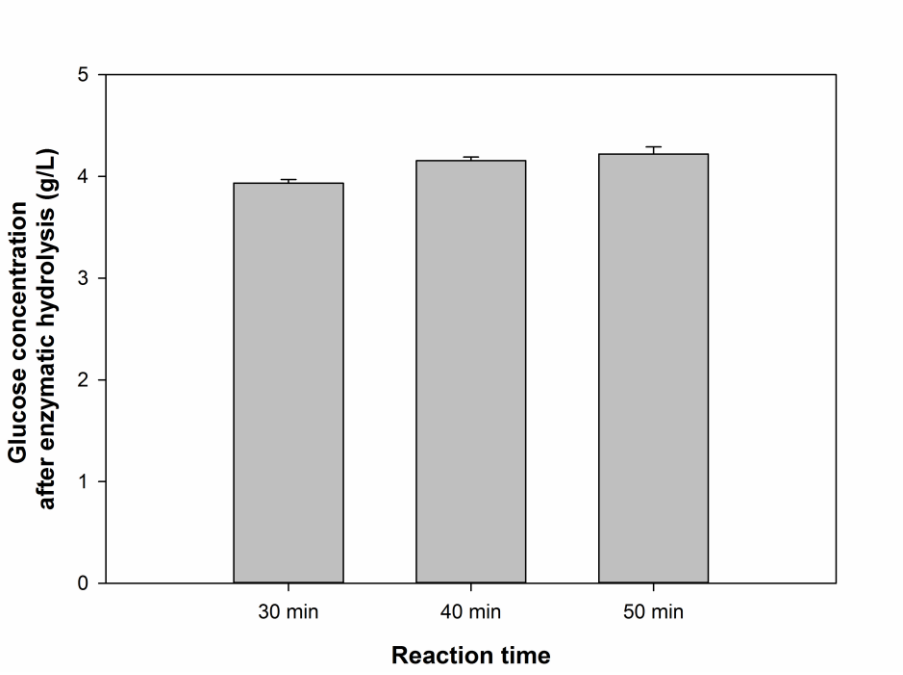
Kraft lignin (Left), lignin from liquid stream (Middle), and lignin from solid stream (Right)



a: untreated switchgrass, b: dilute acid pretreated switchgrass,
d: ammonia hydroxide pretreated switchgrass, e: ionic liquid pretreated switchgrass
c, f: precipitated lignin

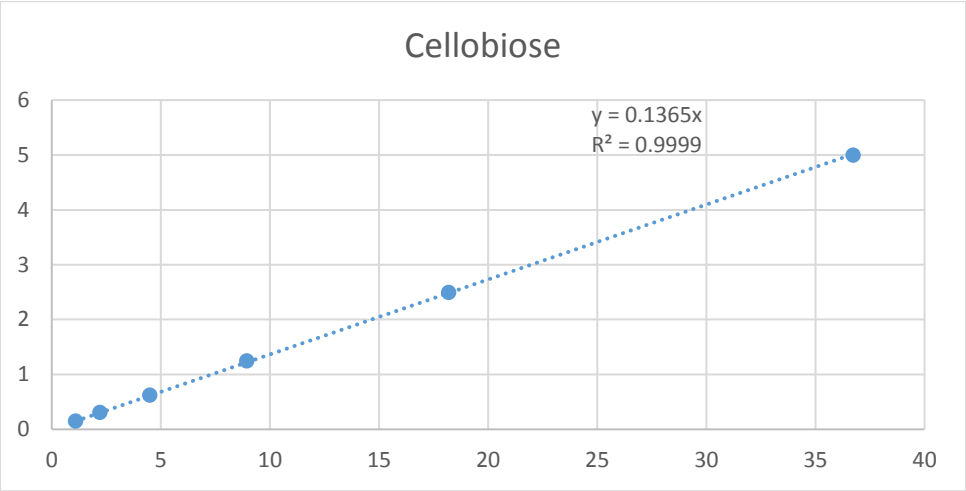


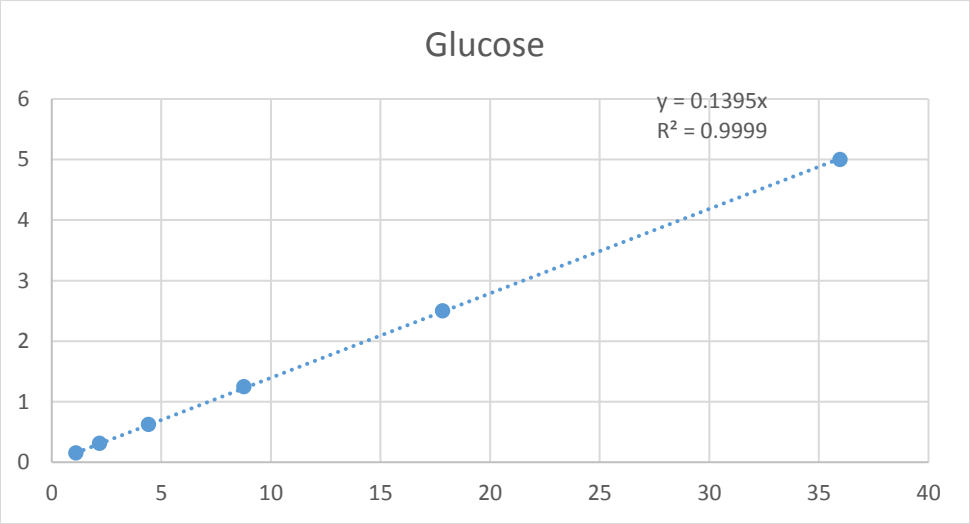
Optimization of dilute acid pretreatment

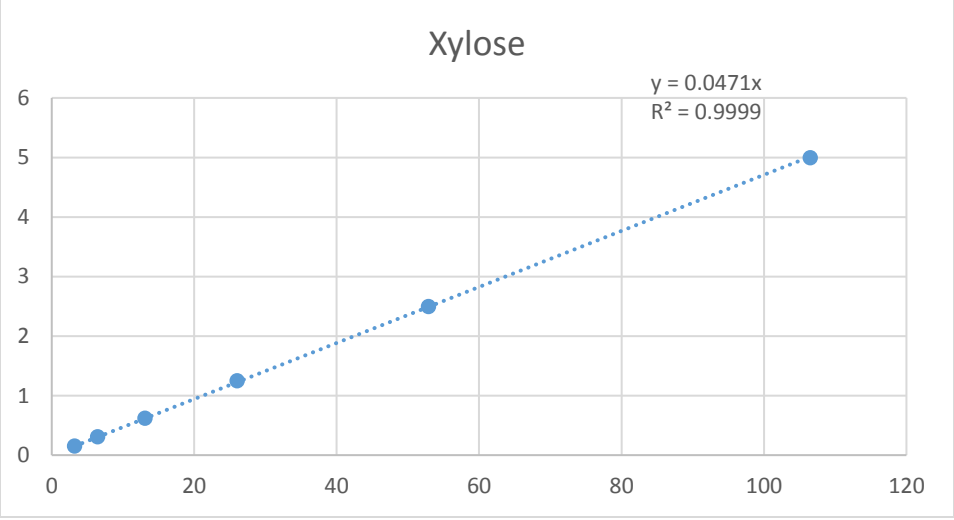


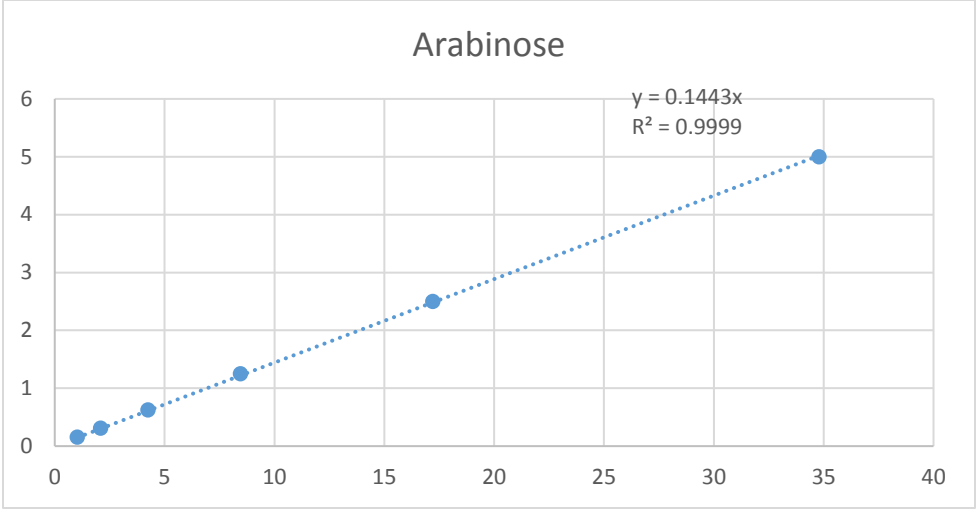
Optimization of ammonia hydroxide pretreatment

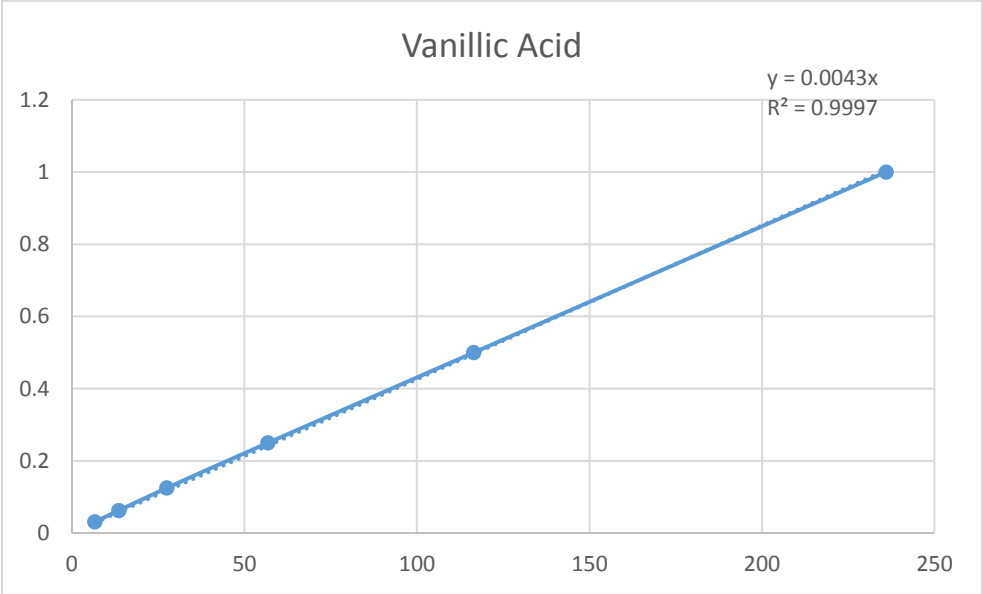
Appendix B. Standard curves and chromatogram



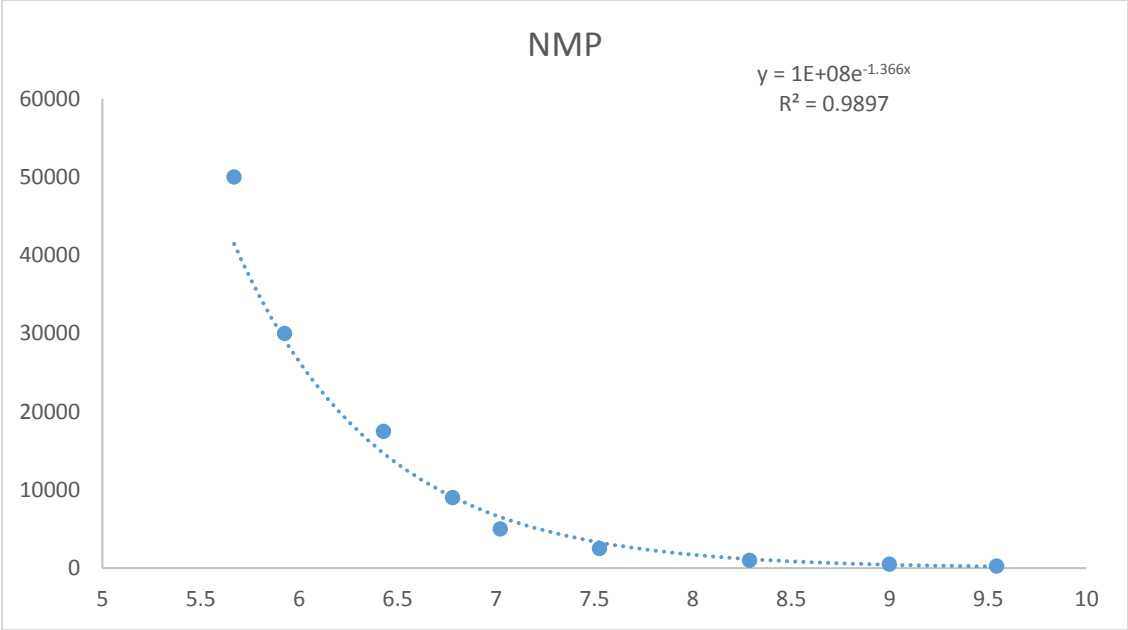


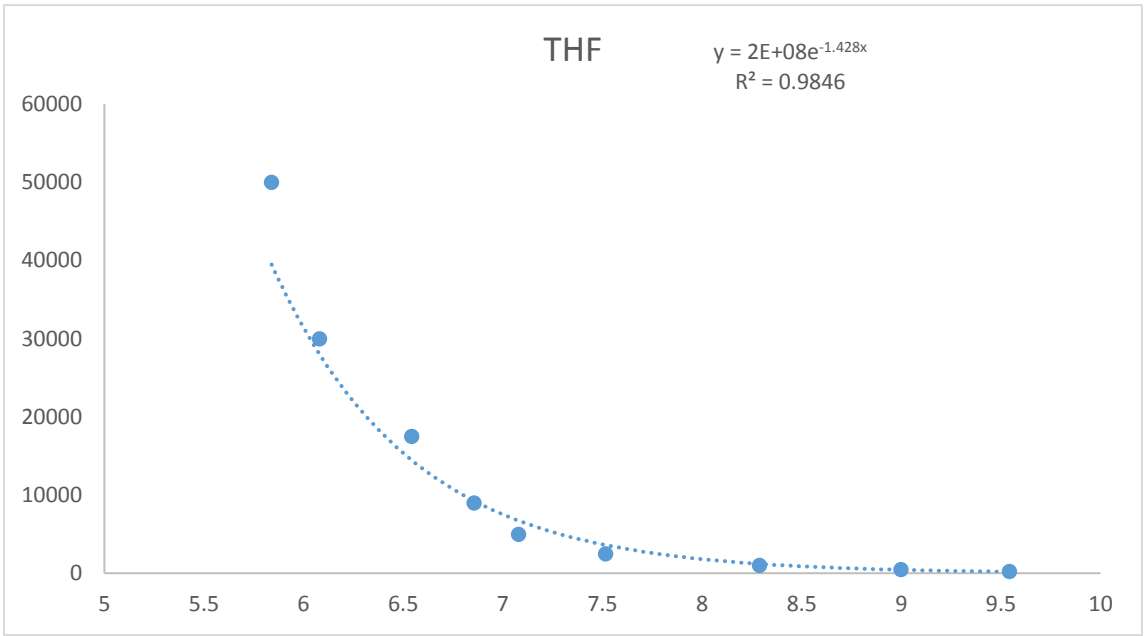




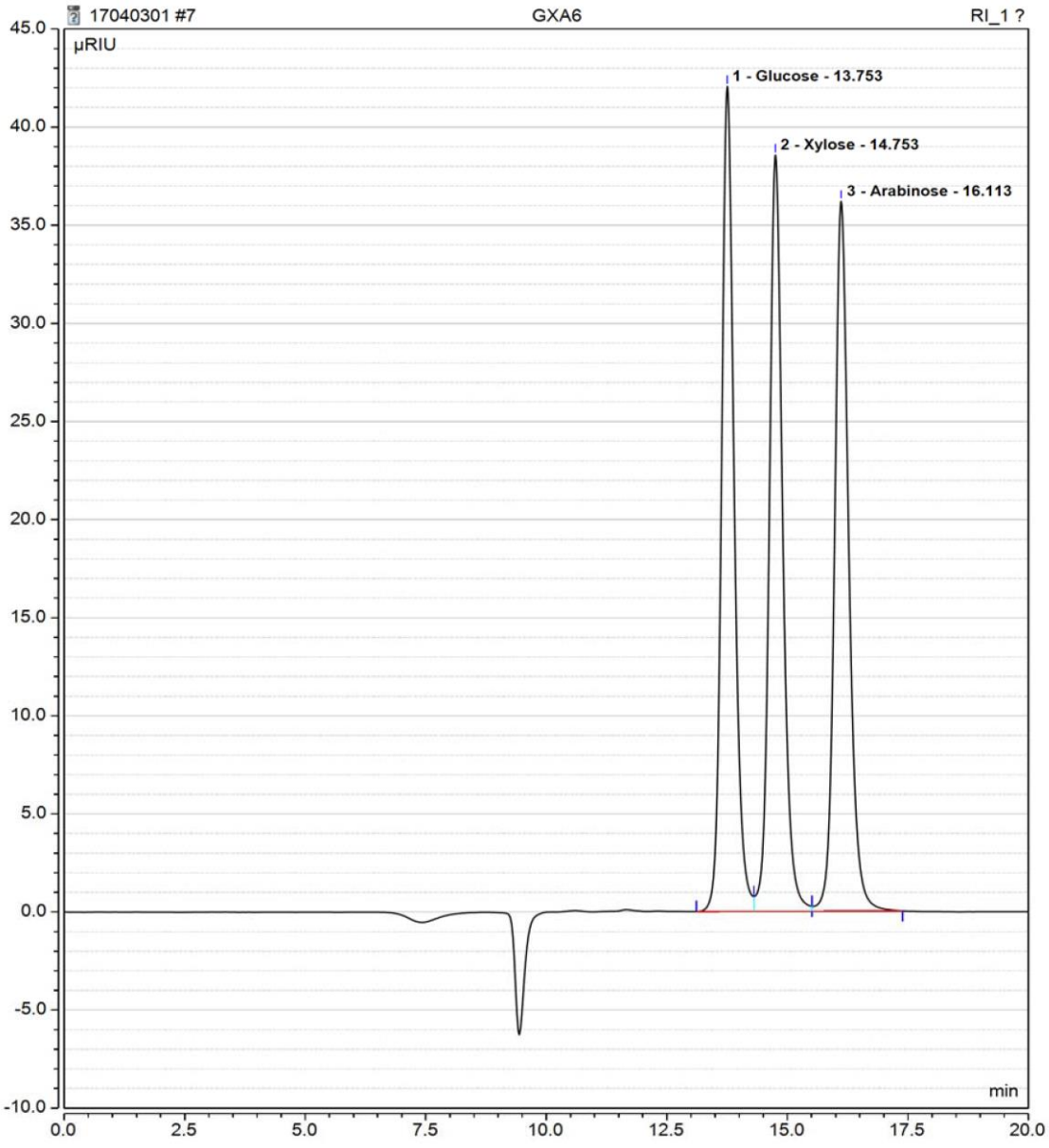


HPLC standard curves

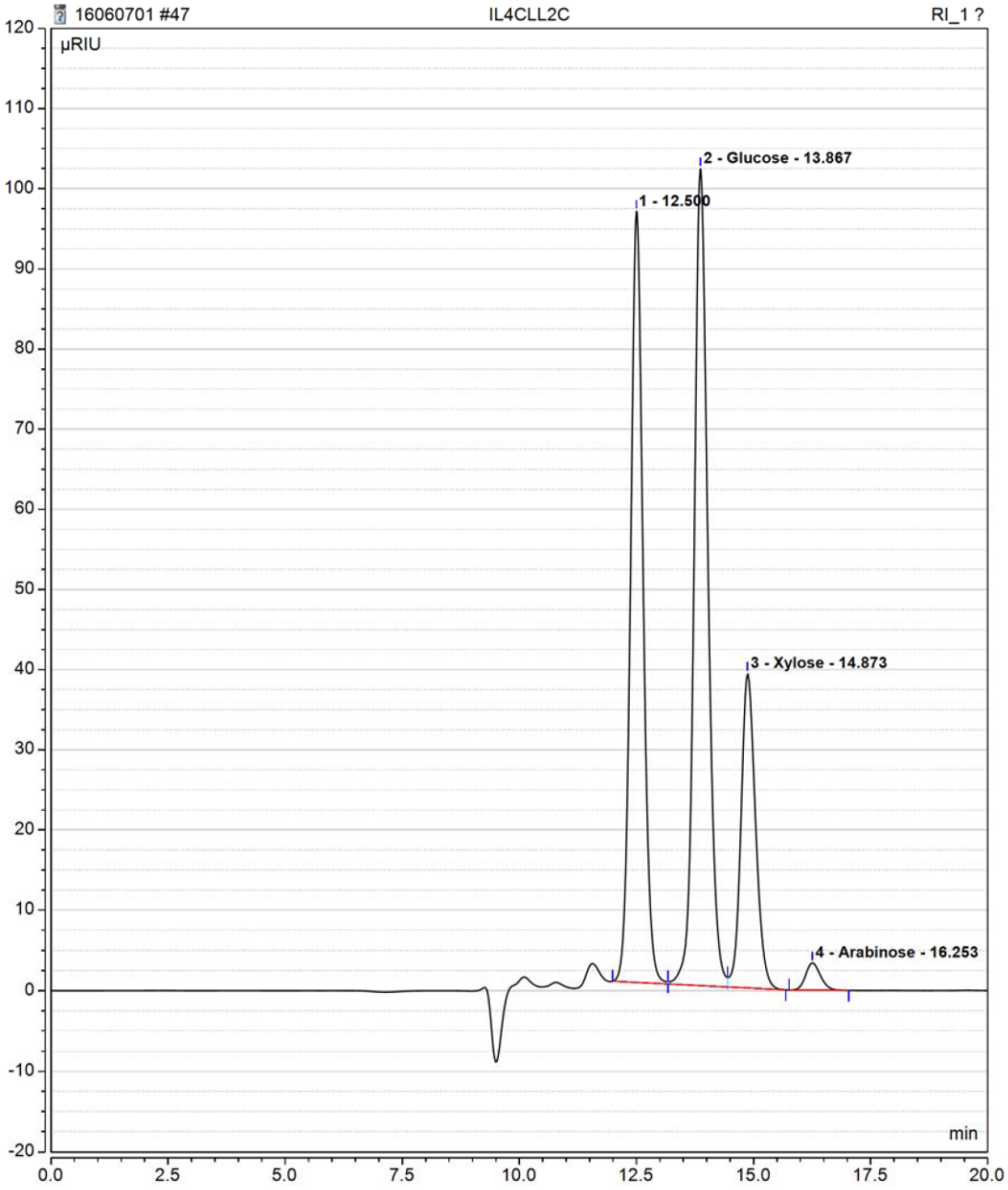




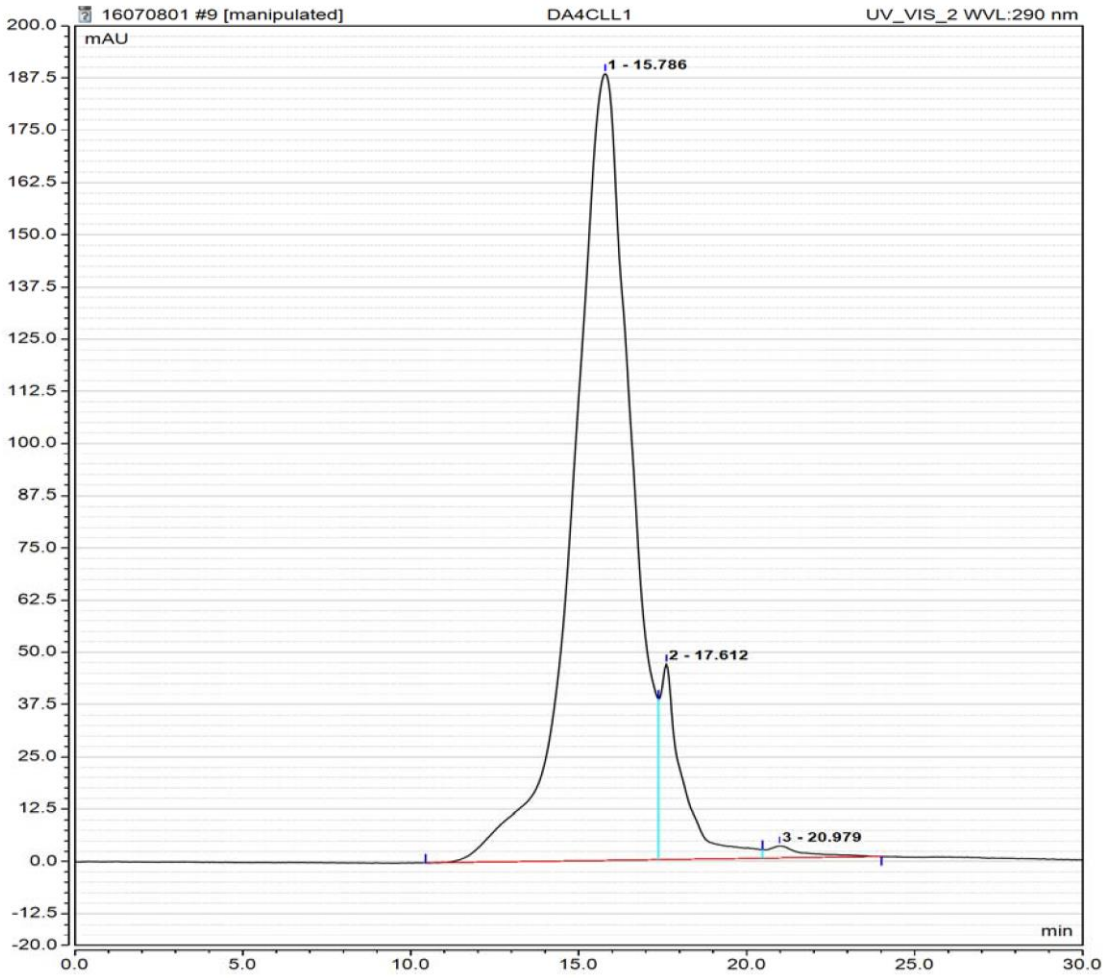
Standard curves for GPC using polystyrene with NMP or THF as mobile phase



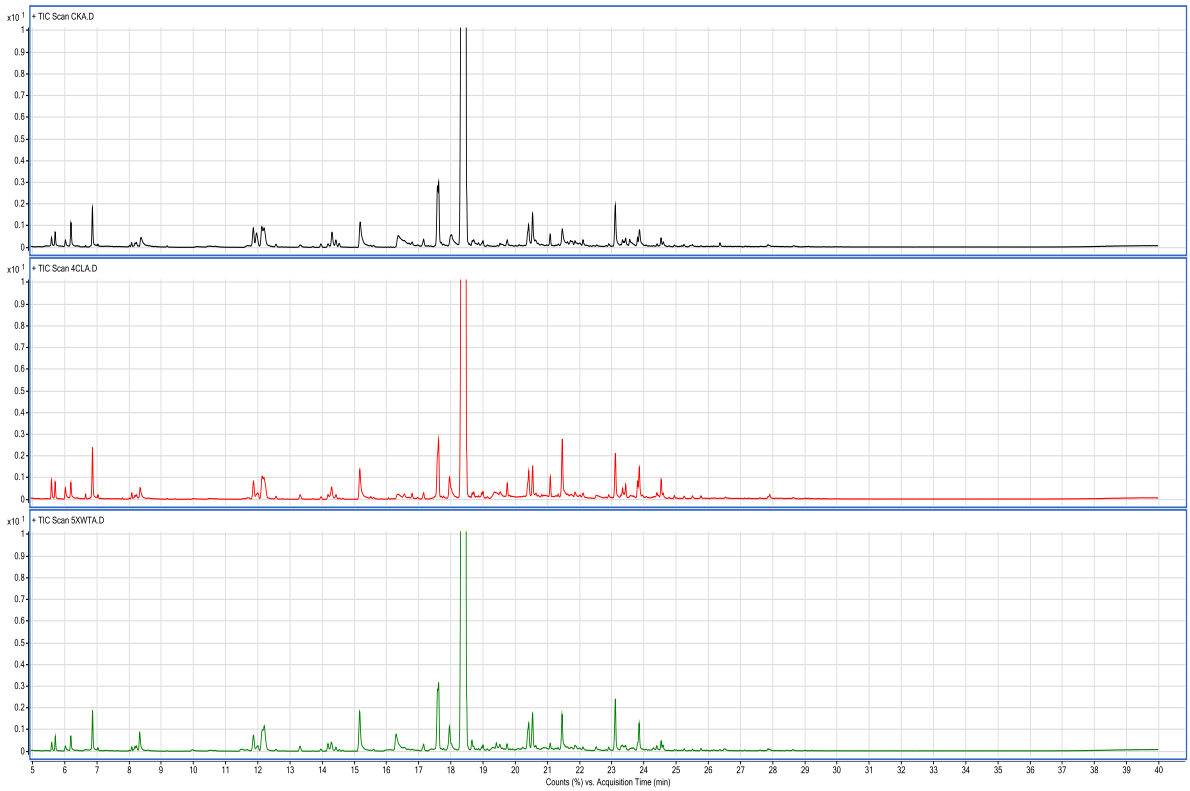
HPLC spectra for standards



HPLC spectra for carbohydrate sample



GPC spectra for liquid stream lignin



GC/MS spectra for lignin monomer detection

Appendix C. Statistical analysis for sugars yield

1	2	3	4	5	6
AH-H-4CL	AH-H-AtLOV1	AH-H-WT	AH-L-4CL	AH-L-AtLOV1	AH-L-WT
7	8	9	10	11	12
DA-H-4CL	DA-H-AtLOV1	DA-H-WT	DA-L-4CL	DA-L-AtLOV1	DA-L-WT
13	14	15	16	17	18
IL-H-4CL	IL-H-AtLOV1	IL-H-WT	IL-L-4CL	IL-L-AtLOV1	IL-L-WT
19	20	21	22	23	24
R-H-4CL	R-H-AtLOV1	R-H-WT	R-L-4CL	R-L-AtLOV1	R-L-WT

Note: AH-Ammonia hydroxide pretreatment

DA-Dilute acid pretreatment

IL-Ionic liquid pretreatment

R-Raw switchgrass

H-High enzyme loading

L-Low enzyme loading

Statistical analysis for glucose yield (α=0.05)

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1		<.0001	<.0001	<.0001	<.0001	<.0001	0.8419	0.0026	<.0001	<.0001	<.0001	<.0001	0.1257	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.0044	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.3566	0.9838	<.0001	<.0001	<.0001	<.0001	0.0369	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.0044		0.4750	<.0001	<.0001	<.0001	<.0001	<.0001	0.9481	1.0000	0.8389	<.0001	0.7077	0.0034	<.0001	<.0001	0.0041	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	<.0001	0.4750		<.0001	<.0001	<.0001	<.0001	0.0155	1.0000	0.1417	0.0064	<.0001	1.0000	0.9998	0.0091	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		0.0070	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.0070		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0010	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	0.8419	<.0001	<.0001	<.0001	<.0001	<.0001		0.7430	0.0238	<.0001	<.0001	<.0001	0.0002	<.0001	<.0001	0.1823	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
8	0.0026	<.0001	<.0001	<.0001	<.0001	<.0001	0.7430		0.9905	<.0001	<.0001	<.0001	<.0001	<.0001	0.0006	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
9	<.0001	<.0001	<.0001	0.0155	<.0001	<.0001	0.0238	0.9905		0.0011	<.0001	<.0001	<.0001	0.0002	0.1410	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
10	<.0001	<.0001	0.9481	1.0000	<.0001	<.0001	<.0001	<.0001	0.0011		0.5567	0.0594	<.0001	1.0000	0.9009	0.0007	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001	0.3566	1.0000	0.1417	<.0001	<.0001	<.0001	<.0001	<.0001	0.5567		1.0000	<.0001	0.2466	0.0008	<.0001	<.0001	0.2140	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	0.9838	0.8389	0.0064	<.0001	<.0001	<.0001	<.0001	<.0001	0.0594	1.0000		<.0001	0.0097	<.0001	<.0001	0.0009	0.8858	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	0.1257	<.0001	<.0001	<.0001	<.0001	<.0001	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	<.0001	<.0001	0.7077	1.0000	<.0001	<.0001	<.0001	<.0001	0.0002	1.0000	0.2466	0.0097	<.0001		0.8432	0.0002	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	<.0001	<.0001	0.0034	0.9998	<.0001	<.0001	<.0001	0.0006	0.1410	0.9009	0.0008	<.0001	<.0001	0.8432		0.0825	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	0.0002	<.0001	<.0001	0.0091	<.0001	<.0001	0.1823	1.0000	1.0000	0.0007	<.0001	<.0001	<.0001	0.0002	0.0825		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	0.0369	<.0001	<.0001	<.0001	0.0010	<.0001	<.0001	<.0001	<.0001	<.0001	0.0009	<.0001	<.0001	<.0001	<.0001		0.3737	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
18	<.0001	1.0000	0.0041	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.2140	0.8858	<.0001	<.0001	<.0001	<.0001	0.3737		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
19	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0029	0.0127	<.0001	<.0001	<.0001
20	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0029		1.0000	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0127	1.0000		<.0001	<.0001	<.0001
22	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.8967	0.8490
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.8967		1.0000
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.8490	1.0000	

Statistical analysis for xylose yield (α=0.05)

i/j	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9988	0.0013	0.6836	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
2	<.0001		0.6041	0.0178	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0040	1.0000	1.0000	0.1288	<.0001	<.0001	<.0001	<.0001
3	<.0001	0.6041		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9714	0.1490	<.0001	<.0001	<.0001	<.0001	<.0001
4	<.0001	0.0178	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0222	0.5224	1.0000	0.9981	<.0001	<.0001	<.0001
5	<.0001	<.0001	<.0001	<.0001		0.1756	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
6	<.0001	<.0001	<.0001	<.0001	0.1756		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
7	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9415	1.0000
8	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.7981	1.0000
9	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9594	1.0000
10	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
11	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000		1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
12	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
13	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
14	0.9988	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.1056	0.0636	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
15	0.0013	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.1056		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
16	0.6836	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0636	<.0001		<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001
17	<.0001	0.0040	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0060	0.2591	1.0000	1.0000	<.0001	<.0001	<.0001
18	<.0001	1.0000	0.9714	0.0222	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0060		0.9993	0.1276	0.0001	<.0001	<.0001	<.0001
19	<.0001	1.0000	0.1490	0.5224	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.2591	0.9993		0.9128	0.0168	<.0001	<.0001	<.0001
20	<.0001	0.1288	<.0001	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.1276	0.9128		0.8952	<.0001	<.0001	<.0001
21	<.0001	<.0001	<.0001	0.9981	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	0.0001	0.0168	0.8952		<.0001	<.0001	<.0001
22	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001		0.0028	<.0001
23	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9415	0.7981	0.9594	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.0028		0.9999
24	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	1.0000	1.0000	1.0000	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	0.9999	

Appendix D. Example for statistical analysis (SAS code and output)

```
/* Input data */
data lignin;
length treatment $12;
input treatment $ lignin;
cards;
x 18.7
x 18.6
x 18.6
y 16.6
y 15.9
y 16.3
z 18.8
z 19.1
z 19.4
;
run;

/* Check data */
proc print data=lignin;
run;

proc glm data=lignin plots=DIAGNOSTICS;
class treatment;
model lignin=treatment;
means treatment/tukey alpha=.05;
lsmeans treatment / CL pdiff adjust=Tukey;
run; quit;
```

The SAS System

Obs	treatment	lignin
1	x	18.7
2	x	18.6
3	x	18.6
4	y	16.6
5	y	15.9
6	y	16.3
7	z	18.8
8	z	19.1
9	z	19.4

The GLM Procedure

Class Level Information

Class	Levels	Values
treatment	3	x y z

Number of Observations Read	9
Number of Observations Used	9

The GLM Procedure

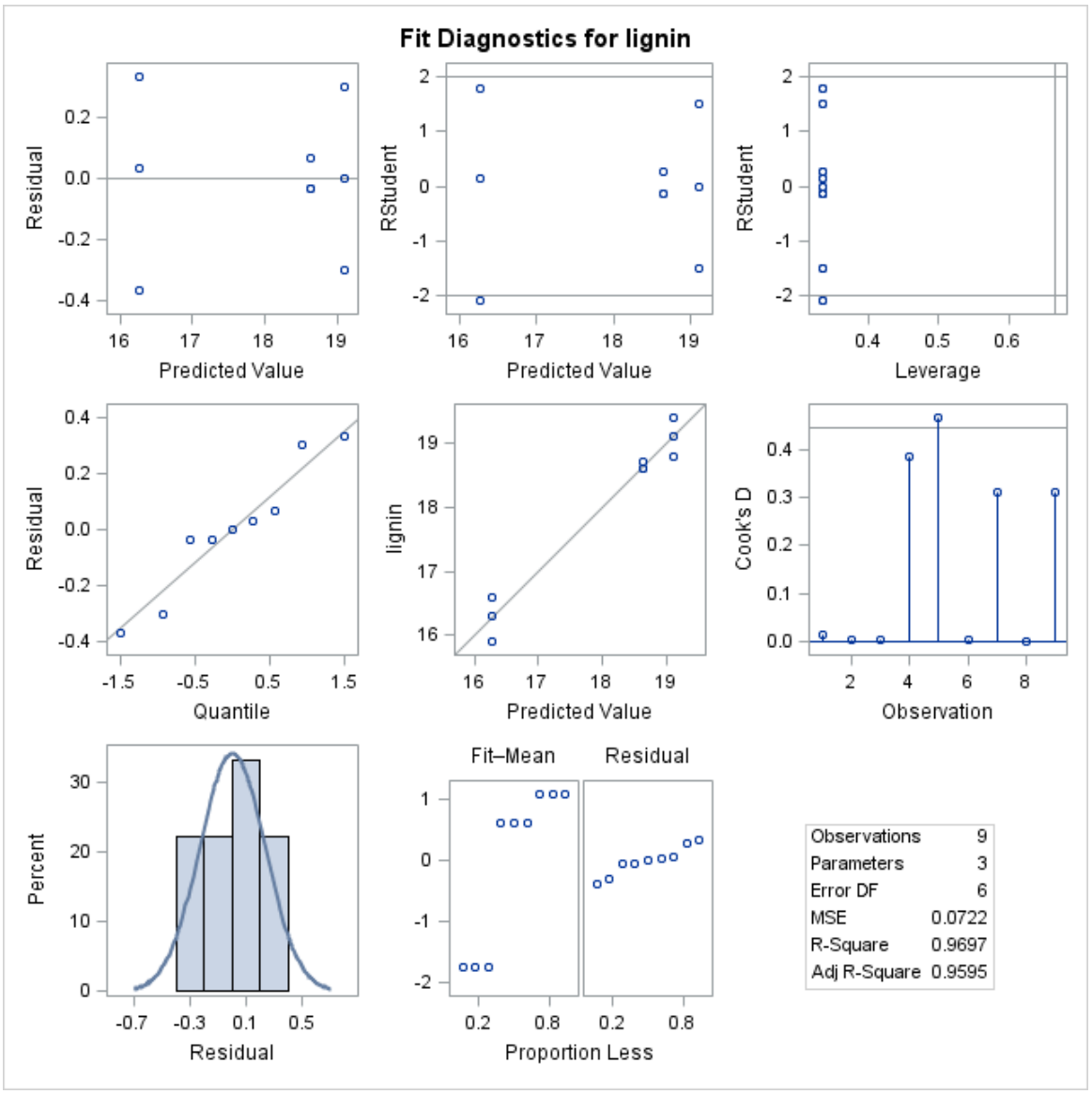
Dependent Variable: lignin

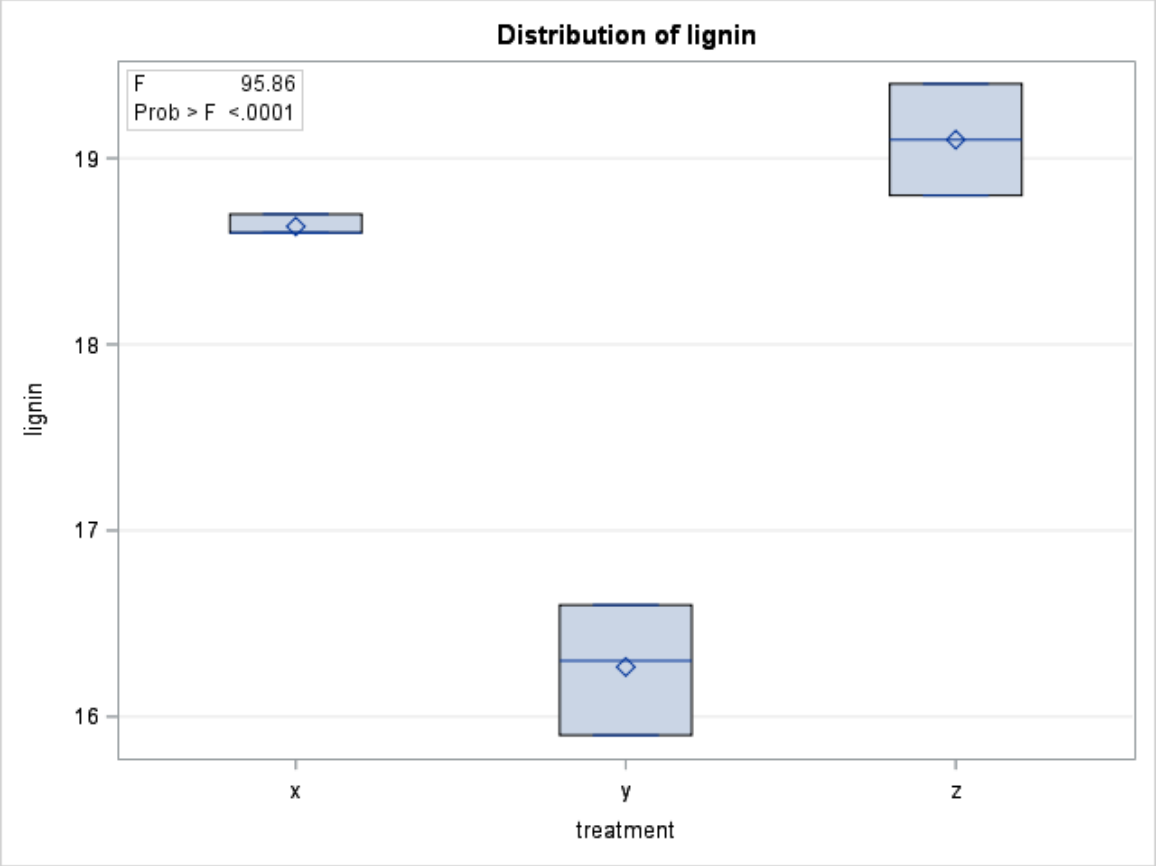
Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	2	13.84666667	6.92333333	95.86	<.0001
Error	6	0.43333333	0.07222222		
Corrected Total	8	14.28000000			

R-Square	Coeff Var	Root MSE	lignin Mean
0.969655	1.493011	0.268742	18.00000

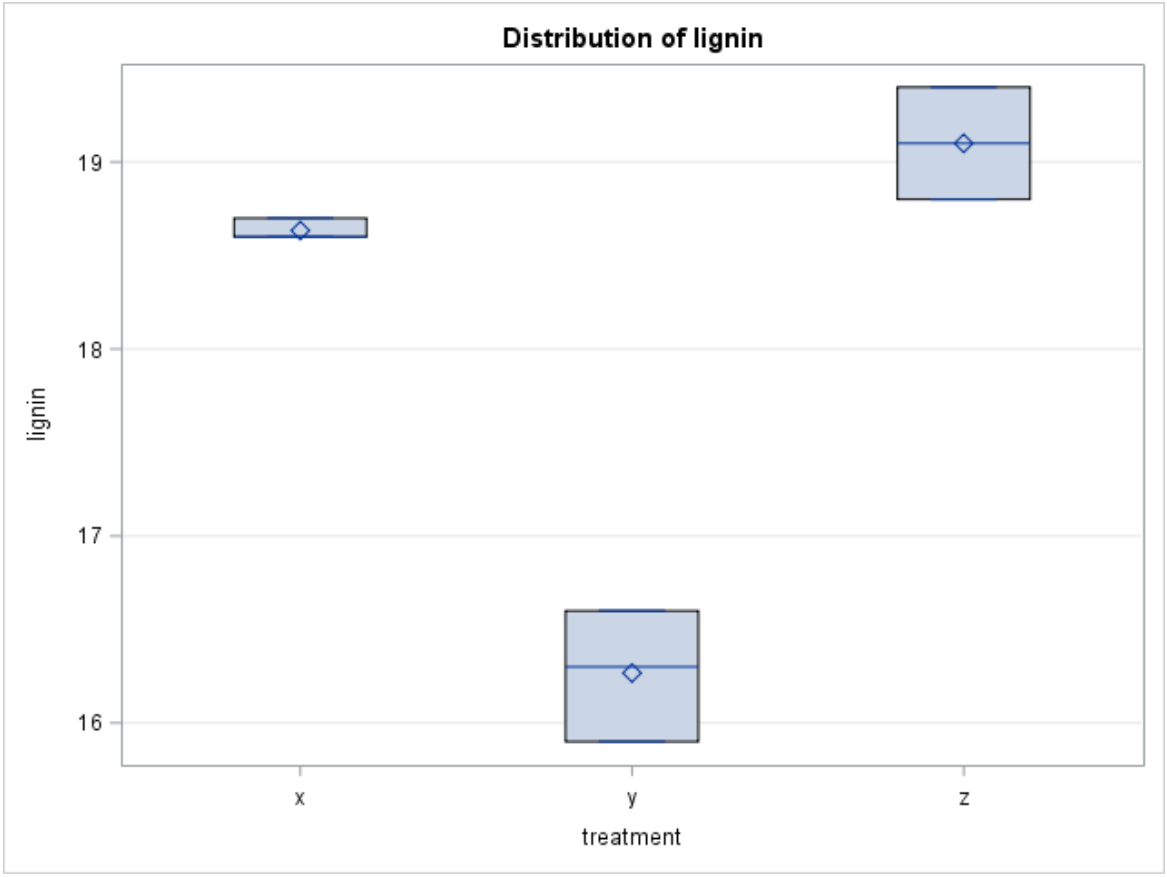
Source	DF	Type I SS	Mean Square	F Value	Pr > F
treatment	2	13.84666667	6.92333333	95.86	<.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
treatment	2	13.84666667	6.92333333	95.86	<.0001


Fit Diagnostics for lignin
Residual
Predicted Value
RStudent
Predicted Value
RStudent
Leverage
Residual
Quantile
lignin
Predicted Value
Cook's D
Observation
Percent
Residual
Fit–Mean
Residual
Proportion Less
Observations 9
Parameters 3
Error DF 6
MSE 0.0722
R-Square 0.9697
Adj R-Square 0.9595


Distribution of lignin
F 95.86
Prob > F <.0001
19
18
17
16
lignin
x
y
z
treatment

The GLM Procedure



The GLM Procedure

Tukey's Studentized Range (HSD) Test for lignin

Alpha	0.05
Error Degrees of Freedom	6
Error Mean Square	0.072222
Critical Value of Studentized Range	4.33917
Minimum Significant Difference	0.6733

Means with the same letter are not significantly different.

Tukey Grouping	Mean	N	treatment
A	19.1000	3	z
A			
A	18.6333	3	x
B	16.2667	3	y

The GLM Procedure
Least Squares Means
Adjustment for Multiple Comparisons: Tukey

treatment	lignin LSMEAN	LSMEAN Number
x	18.6333333	1
y	16.2666667	2
z	19.1000000	3

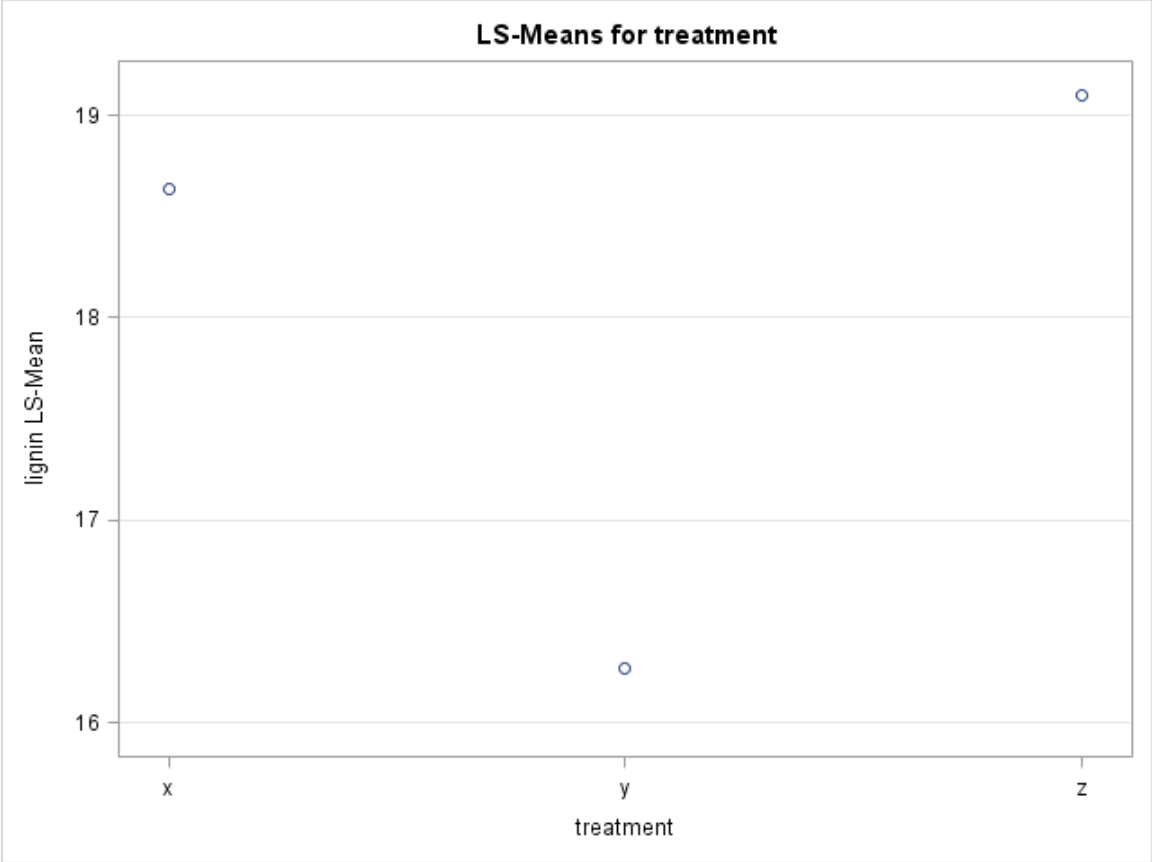
Least Squares Means for effect treatment
Pr > |t| for H0: LSMean(i)=LSMean(j)
Dependent Variable: lignin

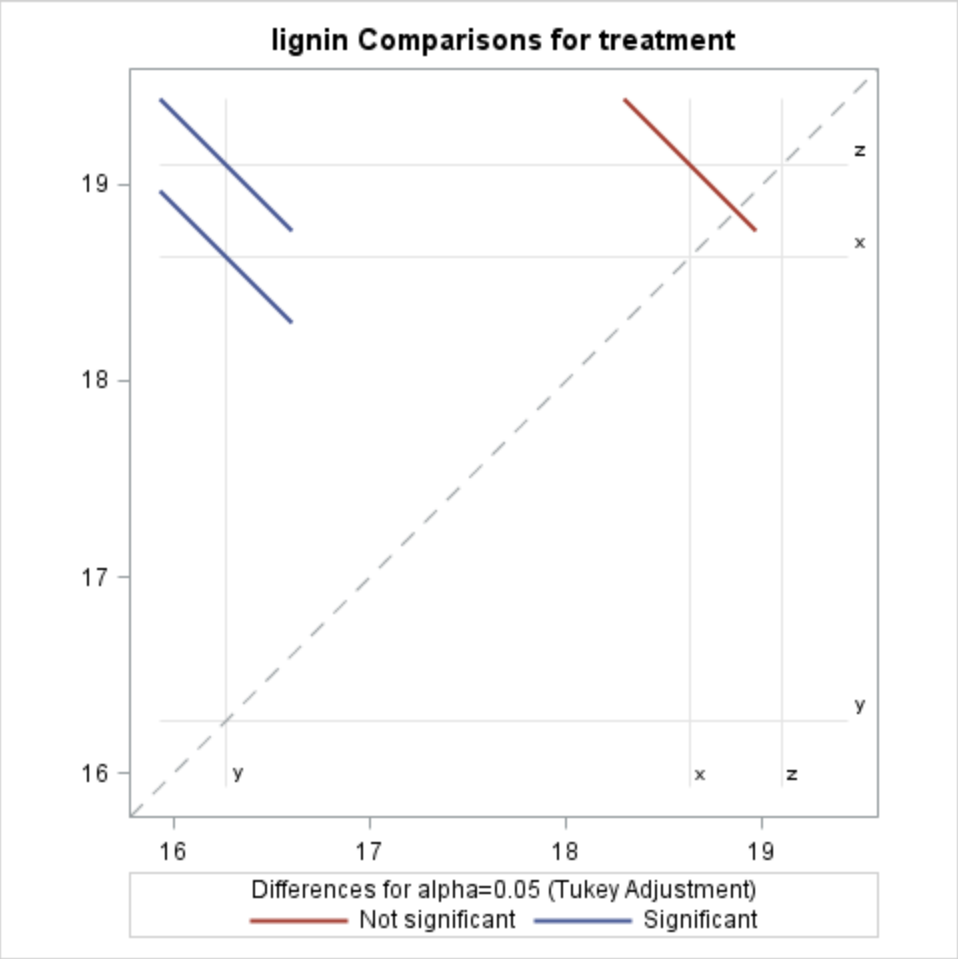
i/j	1	2	3
1		<.0001	0.1643
2	<.0001		<.0001
3	0.1643	<.0001	

treatment	lignin LSMEAN	95% Confidence Limits	
x	18.633333	18.253675	19.012992
y	16.266667	15.887008	16.646325
z	19.100000	18.720342	19.479658

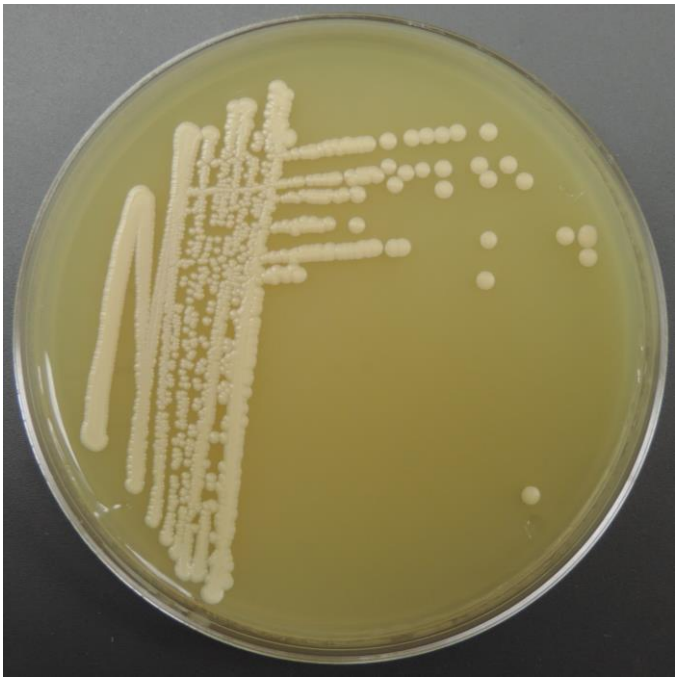
Least Squares Means for Effect treatment

i	j	Difference Between Means	Simultaneous 95% Confidence Limits for LSMean(i)-LSMean(j)	
1	**2**	2.366667	1.693409	3.039924
1	**3**	-0.466667	-1.139924	0.206591
2	**3**	-2.833333	-3.506591	-2.160076


LS-Means for treatment
19
18
17
16
lignin LS-Mean
x
y
z
treatment

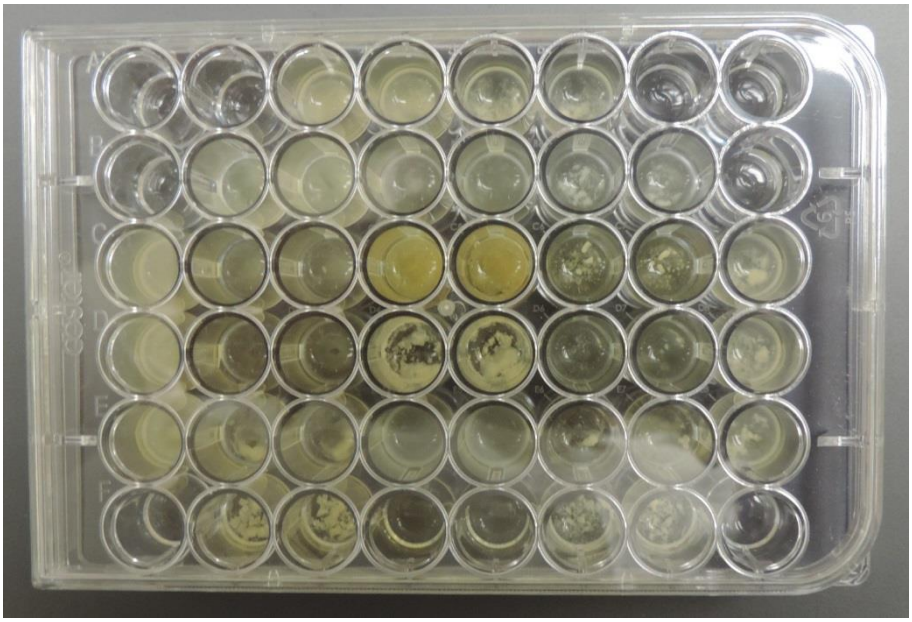

lignin Comparisons for treatment
19
18
17
16
z
x
y
y
x
z
16
17
18
19
Differences for alpha=0.05 (Tukey Adjustment)
Not significant
Significant

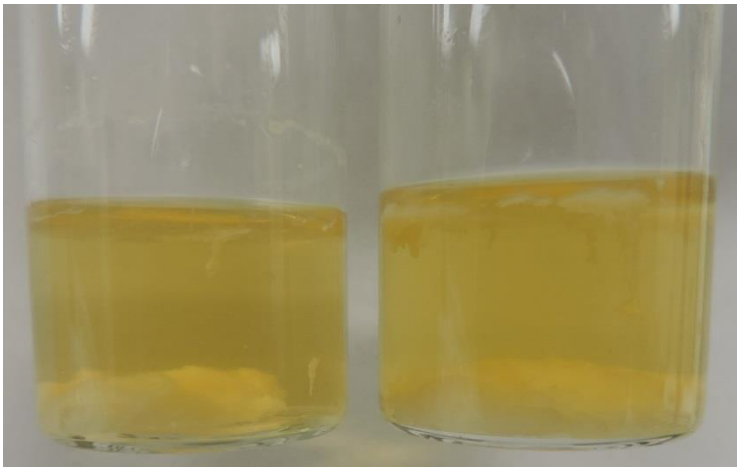
Appendix E. Bioconversion of lignin



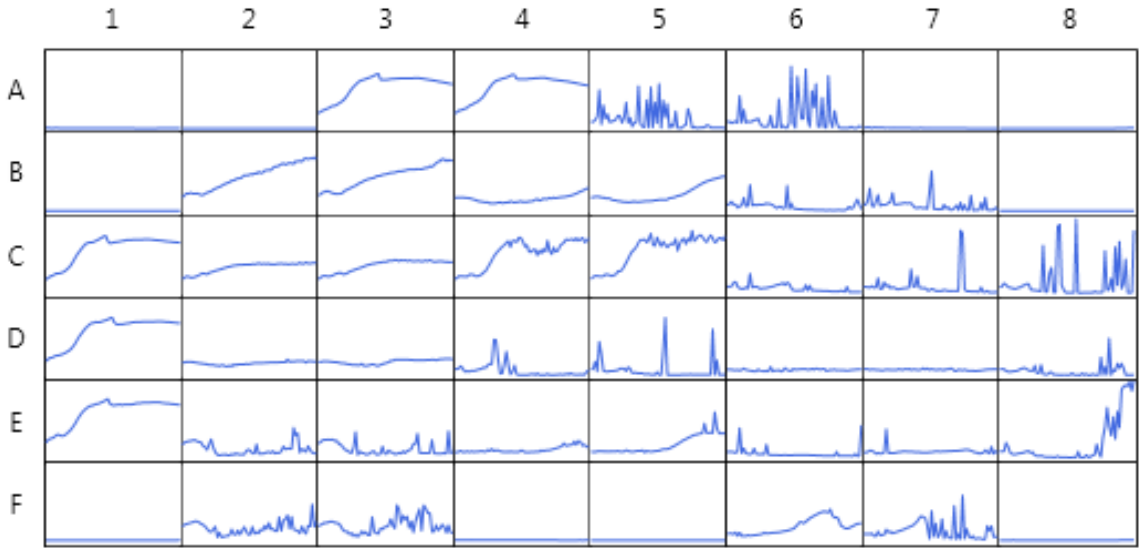
Bacterial colony of DSM 1069

Bacterial colony of PD630





Aggregation of *Rhodococcus opacus*



Screening of biocompatible ionic liquids

Lipids fermentation using glucose as carbon source (with/without adding activated carbon into fermentation media)

Lipids fermentation using vanillic acid as carbon source (with/without adding activated carbon into fermentation media)

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Figure 5. The main biosynthetic pathway for monolignols(Vanholme et al., 2010)

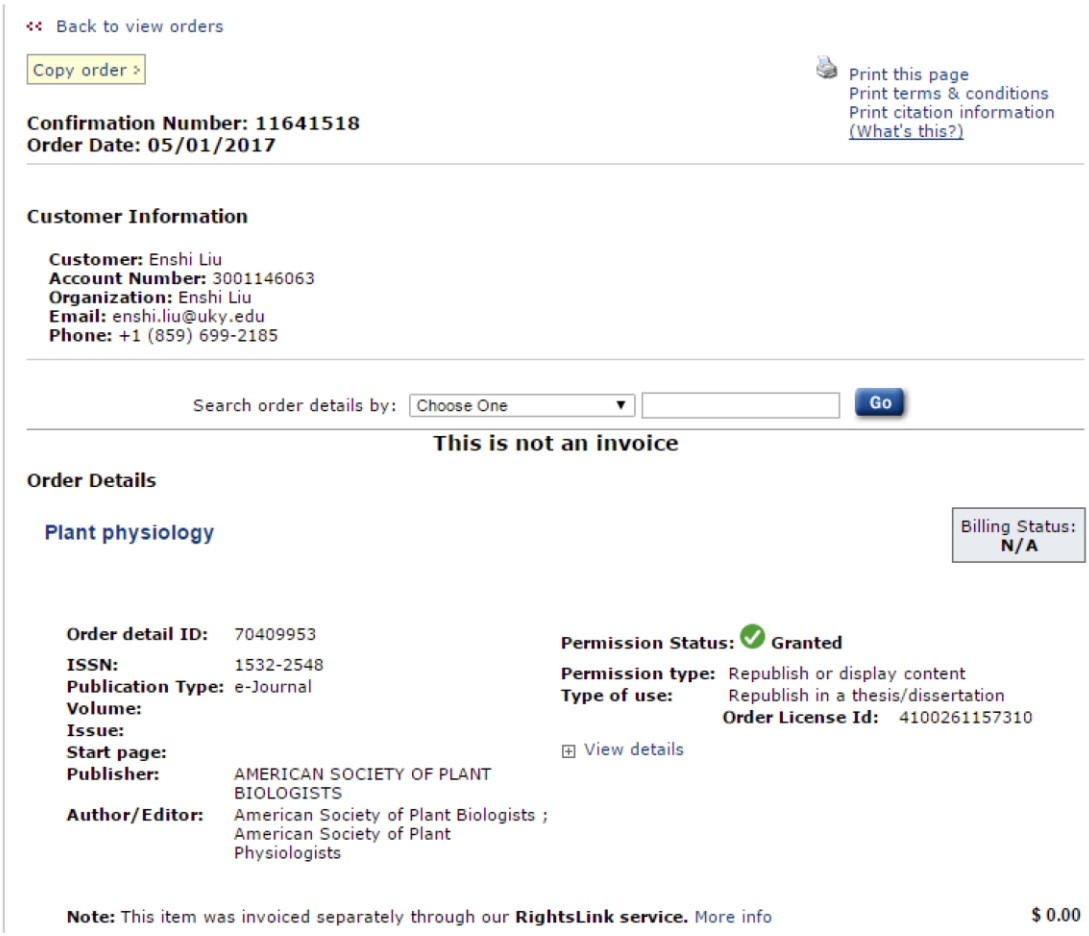

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Figure 6. A typical Kraft pulping process (Haddad et al., 2017)

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REFERENCES

Adler, E. 1977. Lignin chemistry—past, present and future. *Wood science and technology*, **11**(3), 169-218.

Ahring, B.K., Jensen, K., Nielsen, P., Bjerre, A., Schmidt, A. 1996. Pretreatment of wheat straw and conversion of xylose and xylan to ethanol by thermophilic anaerobic bacteria. *Bioresource Technology*, **58**(2), 107-113.

Alvo, P., Belkacemi, K. 1997. Enzymatic saccharification of milled timothy (Phleum pratense L.) and alfalfa (Medicago sativa L.). *Bioresource technology*, **61**(3), 185-198.

Azadi, P., Inderwildi, O.R., Farnood, R., King, D.A. 2013. Liquid fuels, hydrogen and chemicals from lignin: A critical review. *Renewable and Sustainable Energy Reviews*, **21**, 506-523.

Baucher, M., Halpin, C., Petit-Conil, M., Boerjan, W. 2003. Lignin: genetic engineering and impact on pulping. *Critical Reviews in Biochemistry and Molecular Biology*, **38**(4), 305-350.

Baugh, K.D., Levy, J.A., McCarty, P.L. 1988. Thermochemical pretreatment of lignocellulose to enhance methane fermentation: II. Evaluation and application of pretreatment model. *Biotechnology and bioengineering*, **31**(1), 62-70.

Baumberger, S., Abaecherli, A., Fasching, M., Gellerstedt, G., Gosselink, R., Hortling, B., Li, J., Saake, B., de Jong, E. 2007. Molar mass determination of lignins by size-exclusion chromatography: towards standardisation of the method. *Holzforschung*, **61**(4), 459-468.

Beckham, G.T., Johnson, C.W., Karp, E.M., Salvachúa, D., Vardon, D.R. 2016. Opportunities and challenges in biological lignin valorization. *Current Opinion in Biotechnology*, **42**, 40-53.

Beckham, G.T., Matthews, J.F., Peters, B., Bomble, Y.J., Himmel, M.E., Crowley, M.F. 2011. Molecular-level origins of biomass recalcitrance: decrystallization free energies for four common cellulose polymorphs. *The Journal of Physical Chemistry B*, **115**(14), 4118-4127.

Behling, R., Valange, S., Chatel, G. 2016. Heterogeneous catalytic oxidation for lignin valorization into valuable chemicals: what results? What limitations? What trends? *Green Chemistry*, **18**(7), 1839-1854.

Ben, H., Ragauskas, A.J. 2011. NMR characterization of pyrolysis oils from kraft lignin. *Energy & Fuels*, **25**(5), 2322-2332.

Bhandiwad, A., Shaw, A.J., Guss, A., Guseva, A., Bahl, H., Lynd, L.R. 2014. Metabolic engineering of Thermoanaerobacterium saccharolyticum for n-butanol production. *Metabolic engineering*, **21**, 17-25.

Biemelt, S., Tschiersch, H., Sonnewald, U. 2004. Impact of altered gibberellin metabolism on biomass accumulation, lignin biosynthesis, and photosynthesis in transgenic tobacco plants. *Plant Physiol*, **135**(1), 254-65.

Bjerre, A.B., Olesen, A.B., Fernqvist, T., Plöger, A., Schmidt, A.S. 1996. Pretreatment of wheat straw using combined wet oxidation and alkaline hydrolysis resulting in convertible cellulose and hemicellulose. *Biotechnology and bioengineering*, **49**(5), 568-577.

Bjurhager, I., Olsson, A.-M., Zhang, B., Gerber, L., Kumar, M., Berglund, L.A., Burgert, I., Sundberg, B.r., Salmén, L. 2010. Ultrastructure and mechanical properties of Populus wood with reduced lignin content caused by transgenic down-regulation of cinnamate 4-hydroxylase. *Biomacromolecules*, **11**(9), 2359-2365.

Boeriu, C.G., Bravo, D., Gosselink, R.J., van Dam, J.E. 2004. Characterisation of structure-dependent functional properties of lignin with infrared spectroscopy. *Industrial crops and products*, **20**(2), 205-218.

Boerjan, W., Ralph, J., Baucher, M. 2003. Lignin biosynthesis. *Annual review of plant biology*, **54**(1), 519-546.

Bonawitz, N.D., Chapple, C. 2013. Can genetic engineering of lignin deposition be accomplished without an unacceptable yield penalty? *Curr Opin Biotechnol*, **24**(2), 336-43.

Bonawitz, N.D., Im Kim, J., Tobimatsu, Y., Ciesielski, P.N., Anderson, N.A., Ximenes, E., Maeda, J., Ralph, J., Donohoe, B.S., Ladisch, M. 2014. Disruption of Mediator rescues the stunted growth of a lignin-deficient Arabidopsis mutant. *Nature*, **509**(7500), 376-380.

Brebu, M., Vasile, C. 2010. Thermal degradation of lignin—a review. *Cellulose Chemistry & Technology*, **44**(9), 353.

Brodeur, G., Yau, E., Badal, K., Collier, J., Ramachandran, K., Ramakrishnan, S. 2011. Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. *Enzyme research*, **2011**.

Brosse, N., Dufour, A., Meng, X., Sun, Q., Ragauskas, A. 2012. Miscanthus: a fast-growing crop for biofuels and chemicals production. *Biofuels, Bioproducts and Biorefining*, **6**(5), 580-598.

Caballero, J., Conesa, J., Font, R., Marcilla, A. 1997. Pyrolysis kinetics of almond shells and olive stones considering their organic fractions. *Journal of Analytical and Applied Pyrolysis*, **42**(2), 159-175.

Cabello, J.V., Lodeyro, A.F., Zurbriggen, M.D. 2014. Novel perspectives for the engineering of abiotic stress tolerance in plants. *Current Opinion in Biotechnology*, **26**, 62-70.

Cao, S., Pu, Y., Studer, M., Wyman, C., Ragauskas, A.J. 2012. Chemical transformations of Populus trichocarpa during dilute acid pretreatment. *Rsc Advances*, **2**(29), 10925-10936.

Carpita, N.C., McCann, M.C. 2008. Maize and sorghum: genetic resources for bioenergy grasses. *Trends in plant science*, **13**(8), 415-420.

Carroll, A., Somerville, C. 2009. Cellulosic biofuels. *Annual review of plant biology*, **60**, 165-182.

Carvalheiro, F., Duarte, L.C., Gírio, F.M. 2008. Hemicellulose biorefineries: a review on biomass pretreatments. *Journal of Scientific & Industrial Research*, 849-864.

Casler, M.D., Jung, H.G., Coblentz, W.K. 2008. Clonal Selection for Lignin and Etherified Ferulates in Three Perennial Grasses. *Crop Science*, **48**(2), 424.

Cha, Y.L., Yang, J., Park, Y., An, G.H., Ahn, J.W., Moon, Y.H., Yoon, Y.M., Yu, G.D., Choi, I.H. 2015. Continuous alkaline pretreatment of Miscanthus sacchariflorus using a bench-scale single screw reactor. *Bioresour Technol*, **181**, 338-44.

Chabannes, M., Barakate, A., Lapierre, C., Marita, J.M., Ralph, J., Pean, M., Danoun, S., Halpin, C., Grima-Pettenati, J., Boudet, A.M. 2001. Strong decrease in lignin

content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants. *The Plant Journal*, **28**(3), 257-270.

Chang, H.-m., Cowling, E.B., Brown, W. 1975. Comparative studies on cellulolytic enzyme lignin and milled wood lignin of sweetgum and spruce. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, **29**(5), 153-159.

Chen, C. 1991. Lignins: Occurrence in woody tissues, isolation, reactions, and structure. *Wood structure and composition*, 183-261.

Chen, F., Dixon, R.A. 2007. Lignin modification improves fermentable sugar yields for biofuel production. *Nature biotechnology*, **25**(7), 759-761.

Chinn, M.S., Nokes, S.E. 2006. Screening of thermophilic anaerobic bacteria for solid substrate cultivation on lignocellulosic substrates. *Biotechnology progress*, **22**(1), 53-59.

Chu, S., Majumdar, A. 2012. Opportunities and challenges for a sustainable energy future. *nature*, **488**(7411), 294-303.

Chundawat, S.P., Beckham, G.T., Himmel, M.E., Dale, B.E. 2011. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annual review of chemical and biomolecular engineering*, **2**, 121-145.

Chundawat, S.P., Venkatesh, B., Dale, B.E. 2007. Effect of particle size based separation of milled corn stover on AFEX pretreatment and enzymatic digestibility. *Biotechnology and bioengineering*, **96**(2), 219-231.

Chung, H., Washburn, N.R. 2016. Extraction and Types of Lignin. 13-25.

Coats, A., Redfern, J. 1963. Thermogravimetric analysis. A review. *Analyst*, **88**(1053), 906-924.

Coleman, H.D., Park, J.-Y., Nair, R., Chapple, C., Mansfield, S.D. 2008. RNAi-mediated suppression of p-coumaroyl-CoA 3′-hydroxylase in hybrid poplar impacts lignin deposition and soluble secondary metabolism. *Proceedings of the National Academy of Sciences*, **105**(11), 4501-4506.

Crews, P., Rodriquez, J., Jaspars, M., Crews, R.J. 1998. Organic structure analysis.

Curreli, N., Fadda, M.B., Rescigno, A., Rinaldi, A.C., Soddu, G., Sollai, F., Vaccargiu, S., Sanjust, E., Rinaldi, A. 1997. Mild alkaline/oxidative pretreatment of wheat straw. *Process Biochemistry*, **32**(8), 665-670.

da Costa Sousa, L., Chundawat, S.P., Balan, V., Dale, B.E. 2009. 'Cradle-to-grave'assessment of existing lignocellulose pretreatment technologies. *Current opinion in biotechnology*, **20**(3), 339-347.

Dale, B.E. 1986. Method for increasing the reactivity and digestibility of cellulose with ammonia, Google Patents.

Dale, B.E., Leong, C., Pham, T., Esquivel, V., Rios, I., Latimer, V. 1996. Hydrolysis of lignocellulosics at low enzyme levels: application of the AFEX process. *Bioresource Technology*, **56**(1), 111-116.

Das, L., Kolar, P., Sharma-Shivappa, R. 2012. Heterogeneous catalytic oxidation of lignin into value-added chemicals. *Biofuels*, **3**(2), 155-166.

Davin, L.B., Lewis, N.G. 2005. Lignin primary structures and dirigent sites. *Curr Opin Biotechnol*, **16**(4), 407-15.

De Jong, E., Jungmeier, G. 2015. Biorefinery concepts in comparison to petrochemical refineries. *Industrial Biorefineries & White Biotechnology*.

Debolt, S., Campbell, J.E., Smith Jr, R., Montross, M., Stork, J. 2009. Life cycle assessment of native plants and marginal lands for bioenergy agriculture in Kentucky as a model for south-eastern USA. *GCB Bioenergy*, **1**(4), 308-316.

Del Río, J.C., Rencoret, J., Prinsen, P., Martínez, A.n.T., Ralph, J., Gutiérrez, A. 2012. Structural characterization of wheat straw lignin as revealed by analytical pyrolysis, 2D-NMR, and reductive cleavage methods. *Journal of agricultural and food chemistry*, **60**(23), 5922-5935.

Dhepe, P.L., Sahu, R. 2010. A solid-acid-based process for the conversion of hemicellulose. *Green Chemistry*, **12**(12), 2153.

Dien, B., Jung, H., Vogel, K., Casler, M., Lamb, J., Iten, L., Mitchell, R., Sarath, G. 2006. Chemical composition and response to dilute-acid pretreatment and enzymatic saccharification of alfalfa, reed canarygrass, and switchgrass. *Biomass and Bioenergy*, **30**(10), 880-891.

Dien, B.S., O'Bryan, P.J., Hector, R.E., Iten, L.B., Mitchell, R.B., Qureshi, N., Sarath, G., Vogel, K.P., Cotta, M.A. 2013. Conversion of switchgrass to ethanol using dilute ammonium hydroxide pretreatment: influence of ecotype and harvest maturity. *Environmental technology*, **34**(13-14), 1837-1848.

Donohoe, B.S., Decker, S.R., Tucker, M.P., Himmel, M.E., Vinzant, T.B. 2008. Visualizing lignin coalescence and migration through maize cell walls following thermochemical pretreatment. *Biotechnology and Bioengineering*, **101**(5), 913-925.

Donohoe, B.S., Vinzant, T.B., Elander, R.T., Pallapolu, V.R., Lee, Y., Garlock, R.J., Balan, V., Dale, B.E., Kim, Y., Mosier, N.S. 2011. Surface and ultrastructural characterization of raw and pretreated switchgrass. *Bioresource technology*, **102**(24), 11097-11104.

El Hage, R., Brosse, N., Chrusciel, L., Sanchez, C., Sannigrahi, P., Ragauskas, A. 2009. Characterization of milled wood lignin and ethanol organosolv lignin from miscanthus. *Polymer Degradation and Stability*, **94**(10), 1632-1638.

El Mansouri, N.-E., Salvadó, J. 2007. Analytical methods for determining functional groups in various technical lignins. *Industrial Crops and Products*, **26**(2), 116-124.

Erä, V., Mattila, A. 1976. Thermal analysis of thermosetting resins. *Journal of Thermal Analysis and Calorimetry*, **10**(3), 461-469.

Evtuguin, D., Domingues, P., Amado, F., Neto, C.P., Correia, A. 1999. Electrospray ionization mass spectrometry as a tool for lignins molecular weight and structural characterisation. *Holzforschung*, **53**(5), 525-528.

Faix, O. 1986. Investigation of lignin polymer models (DHP's) by FTIR spectroscopy. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, **40**(5), 273-280.

Falconnier, B., Lapierre, C., Lesage-Meessen, L., Yonnet, G., Brunerie, P., Colonna-Ceccaldi, B., Corrieu, G., Asther, M. 1994. Vanillin as a product of ferulic acid biotransformation by the white-rot fungus Pycnoporus cinnabarinus I-937: identification of metabolic pathways. *Journal of Biotechnology*, **37**(2), 123-132.

Fan, L., Lee, Y.H., Beardmore, D. 1981. The influence of major structural features of cellulose on rate of enzymatic hydrolysis. *Biotechnology and Bioengineering*, **23**(2), 419-424.
Feghali, E., Carrot, G., Thuéry, P., Genre, C., Cantat, T. 2015. Convergent reductive depolymerization of wood lignin to isolated phenol derivatives by metal-free catalytic hydrosilylation. *Energy & Environmental Science*, **8**(9), 2734-2743.
Fierro, V., Torné-Fernández, V., Montané, D., Celzard, A. 2005. Study of the decomposition of kraft lignin impregnated with orthophosphoric acid. *Thermochimica acta*, **433**(1), 142-148.
Flythe, M.D., Elía, N.M., Schmal, M.B., Nokes, S.E. 2015. Switchgrass (Panicum virgatum) fermentation by Clostridium thermocellum and Clostridium beijerinckii sequential culture: effect of feedstock particle size on gas production. *Advances in Microbiology*, **5**(05), 311.
Foston, M., Ragauskas, A.J. 2010. Changes in lignocellulosic supramolecular and ultrastructure during dilute acid pretreatment of Populus and switchgrass. *Biomass and Bioenergy*, **34**(12), 1885-1895.
Franke, R., Hemm, M.R., Denault, J.W., Ruegger, M.O., Humphreys, J.M., Chapple, C. 2002. Changes in secondary metabolism and deposition of an unusual lignin in the ref8 mutant of Arabidopsis. *Plant J*, **30**(1), 47-59.
Freer, J., Ruiz, J., Peredo, M.A., Rodríguez, J., Baeza, J. 2003. Estimating the density and pulping yield of E. globulus wood by DRIFT-MIR spectroscopy and principal components regression (PCR). *Journal of the Chilean Chemical Society*, **48**(3), 19-22.
Freudenberg, K. 1965. Lignin: its constitution and formation from p-hydroxycinnamyl alcohols. *Science*, **148**(3670), 595-600.
Fu, C., Mielenz, J.R., Xiao, X., Ge, Y., Hamilton, C.Y., Rodriguez, M., Chen, F., Foston, M., Ragauskas, A., Bouton, J. 2011. Genetic manipulation of lignin reduces recalcitrance and improves ethanol production from switchgrass. *Proceedings of the National Academy of Sciences*, **108**(9), 3803-3808.
Fu, D., Mazza, G. 2011. Aqueous ionic liquid pretreatment of straw. *Bioresource Technology*, **102**(13), 7008-7011.
Gabrielsen, B., Vogel, K., Anderson, B., Ward, J. 1990. Alkali-labile cell-wall phenolics and forage quality in switchgrasses selected for differing digestibility. *Crop science*, **30**(6), 1313-1320.
Gao, K., Boiano, S., Marzocchella, A., Rehmann, L. 2014. Cellulosic butanol production from alkali-pretreated switchgrass (Panicum virgatum) and phragmites (Phragmites australis). *Bioresour Technol*, **174**, 176-81.
Garrote, G., Dominguez, H., Parajo, J. 1999. Hydrothermal processing of lignocellulosic materials. *European Journal of Wood and Wood Products*, **57**(3), 191-202.
George, A., Brandt, A., Tran, K., Zahari, S., Klein-Marcuschamer, D., Sun, N., Sathitsuksanoh, N., Shi, J., Stavila, V., Parthasarathi, R., Singh, S., Holmes, B.M., Welton, T., Simmons, B.A., Hallett, J.P. 2015. Design of low-cost ionic liquids for lignocellulosic biomass pretreatment. *Green Chemistry*, **17**(3), 1728-1734.
Gidh, A.V., Decker, S.R., See, C.H., Himmel, M.E., Williford, C.W. 2006a. Characterization of lignin using multi-angle laser light scattering and atomic force microscopy. *Analytica chimica acta*, **555**(2), 250-258.

Gidh, A.V., Decker, S.R., Vinzant, T.B., Himmel, M.E., Williford, C. 2006b. Determination of lignin by size exclusion chromatography using multi angle laser light scattering. *Journal of Chromatography A*, **1114**(1), 102-110.

Gosselink, R., Abächerli, A., Semke, H., Malherbe, R., Käuper, P., Nadif, A., Van Dam, J. 2004. Analytical protocols for characterisation of sulphur-free lignin. *Industrial Crops and Products*, **19**(3), 271-281.

Grabber, J.H., Mertens, D.R., Kim, H., Funk, C., Lu, F., Ralph, J. 2009. Cell wall fermentation kinetics are impacted more by lignin content and ferulate cross-linking than by lignin composition. *Journal of the Science of Food and Agriculture*, **89**(1), 122-129.

Griffiths, P.R., De Haseth, J.A. 2007. *Fourier transform infrared spectrometry*. John Wiley & Sons.

Grous, W.R., Converse, A.O., Grethlein, H.E. 1986. Effect of steam explosion pretreatment on pore size and enzymatic hydrolysis of poplar. *Enzyme and Microbial Technology*, **8**(5), 274-280.

Gupta, R., Lee, Y. 2010. Investigation of biomass degradation mechanism in pretreatment of switchgrass by aqueous ammonia and sodium hydroxide. *Bioresource technology*, **101**(21), 8185-8191.

Haddad, M., Mikhaylin, S., Bazinet, L., Savadogo, O., Paris, J. 2017. Electrochemical acidification of Kraft black liquor by electrodialysis with bipolar membrane: Ion exchange membrane fouling identification and mechanisms. *Journal of Colloid and Interface Science*, **488**, 39-47.

Hardin, C.F., Fu, C., Hisano, H., Xiao, X., Shen, H., Stewart, C.N., Parrott, W., Dixon, R.A., Wang, Z.-Y. 2013. Standardization of Switchgrass Sample Collection for Cell Wall and Biomass Trait Analysis. *BioEnergy Research*, **6**(2), 755-762.

Harmsen, P., Huijgen, W., Bermudez, L., Bakker, R. 2010. Literature review of physical and chemical pretreatment processes for lignocellulosic biomass. Wageningen UR Food & Biobased Research. 9085857570.

Hatakeyama, H., Hatakeyama, T. 2009. Lignin structure, properties, and applications. in: *Biopolymers*, Springer, pp. 1-63.

Hatakka, A.I. 1983. Pretreatment of wheat straw by white-rot fungi for enzymic saccharification of cellulose. *Applied Microbiology and Biotechnology*, **18**(6), 350-357.

Hatfield, R., Fukushima, R.S. 2005. Can lignin be accurately measured? *Crop science*, **45**(3), 832-839.

Hatfield, R.D., Chaptman, A.K. 2009. Comparing corn types for differences in cell wall characteristics and p-coumaroylation of lignin. *J Agric Food Chem*, **57**(10), 4243-9.

He, Y., Li, X., Ben, H., Xue, X., Yang, B. 2017a. Lipids Production from Dilute Alkali Corn Stover Lignin by Rhodococcus Strains. *ACS Sustainable Chemistry & Engineering*.

He, Y., Li, X., Xue, X., Swita, M.S., Schmidt, A.J., Yang, B. 2017b. Biological conversion of the aqueous wastes from hydrothermal liquefaction of algae and pine wood by Rhodococci. *Bioresource technology*, **224**, 457-464.

Hendriks, A.T., Zeeman, G. 2009. Pretreatments to enhance the digestibility of lignocellulosic biomass. *Bioresour Technol*, **100**(1), 10-8.

Hibino, T., Takabe, K., Kawazu, T., Shibata, D., Higuchi, T. 2014. Increase of Cinnamaldehyde Groups in Lignin of Transgenic Tobacco Plants Carrying an Antisense Gene for Cinnamyl Alcohol Dehydrogenase. *Bioscience, Biotechnology and Biochemistry*, **59**(5), 929-931.

Himmel, M.E., Baker, J.O., Overend, R.P. 1994. *Enzymatic conversion of biomass for fuels production*. American Chemical Society Washington, DC.

Himmel, M.E., Ding, S.-Y., Johnson, D.K., Adney, W.S., Nimlos, M.R., Brady, J.W., Foust, T.D. 2007. Biomass recalcitrance: engineering plants and enzymes for biofuels production. *science*, **315**(5813), 804-807.

Hirai, H., Sugiura, M., Kawai, S., Nishida, T. 2005. Characteristics of novel lignin peroxidases produced by white-rot fungus Phanerochaete sordida YK-624. *FEMS Microbiol Lett*, **246**(1), 19-24.

Hisano, H., Nandakumar, R., Wang, Z.-Y. 2011. Genetic modification of lignin biosynthesis for improved biofuel production. in: *Biofuels*, Springer, pp. 223-235.

Hisano, H., Nandakumar, R., Wang, Z.-Y. 2009. Genetic modification of lignin biosynthesis for improved biofuel production. *In Vitro Cellular & Developmental Biology - Plant*, **45**(3), 306-313.

Holtman, K.M., Chang, H.-m., Kadla, J.F. 2004. Solution-state nuclear magnetic resonance study of the similarities between milled wood lignin and cellulolytic enzyme lignin. *Journal of agricultural and food chemistry*, **52**(4), 720-726.

Hou, X.D., Li, N., Zong, M.H. 2013. Renewable bio ionic liquids-water mixtures-mediated selective removal of lignin from rice straw: Visualization of changes in composition and cell wall structure. *Biotechnology and bioengineering*, **110**(7), 1895-1902.

Hu, Z., Yeh, T.-F., Chang, H.-m., Matsumoto, Y., Kadla, J.F. 2006. Elucidation of the structure of cellulolytic enzyme lignin. *Holzforschung*, **60**(4), 389-397.

Itoh, H., Wada, M., Honda, Y., Kuwahara, M., Watanabe, T. 2003. Bioorganosolve pretreatments for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. *Journal of Biotechnology*, **103**(3), 273-280.

Jensen, J.R., Morinelly, J.E., Gossen, K.R., Brodeur-Campbell, M.J., Shonnard, D.R. 2010. Effects of dilute acid pretreatment conditions on enzymatic hydrolysis monomer and oligomer sugar yields for aspen, balsam, and switchgrass. *Bioresour Technol*, **101**(7), 2317-25.

Joffres, B., Lorentz, C., Vidalie, M., Laurenti, D., Quoineaud, A.-A., Charon, N., Daudin, A., Quignard, A., Geantet, C. 2014. Catalytic hydroconversion of a wheat straw soda lignin: characterization of the products and the lignin residue. *Applied Catalysis B: Environmental*, **145**, 167-176.

Jönsson, A.-S., Nordin, A.-K., Wallberg, O. 2008. Concentration and purification of lignin in hardwood kraft pulping liquor by ultrafiltration and nanofiltration. *Chemical Engineering Research and Design*, **86**(11), 1271-1280.

José, C., Lino, A.G., Colodette, J.L., Lima, C.F., Gutiérrez, A., Martínez, Á.T., Lu, F., Ralph, J., Rencoret, J. 2015. Differences in the chemical structure of the lignins from sugarcane bagasse and straw. *Biomass and Bioenergy*, **81**, 322-338.

Kang, S., Xiao, L., Meng, L., Zhang, X., Sun, R. 2012. Isolation and structural characterization of lignin from cotton stalk treated in an ammonia hydrothermal system. *International journal of molecular sciences*, **13**(11), 15209-15226.

Karlen, S.D., Zhang, C., Peck, M.L., Smith, R.A., Padmakshan, D., Helmich, K.E., Free, H.C., Lee, S., Smith, B.G., Lu, F. 2016. Monolignol ferulate conjugates are naturally incorporated into plant lignins. *Science advances*, **2**(10), e1600393.

Kawaoka, A., Nanto, K., Ishii, K., Ebinuma, H. 2006. Reduction of lignin content by suppression of expression of the LIM domain transcription factor in Eucalyptus camaldulensis. *Silvae Genetica*, **55**(6), 269-277.

Keller, F.A., Hamilton, J.E., Nguyen, Q.A. 2003. Microbial pretreatment of biomass. in: *Biotechnology for Fuels and Chemicals*, Springer, pp. 27-41.

Keshwani, D.R., Cheng, J.J. 2009. Switchgrass for bioethanol and other value-added applications: a review. *Bioresource technology*, **100**(4), 1515-1523.

Kim, H., Kim, S., Dale, B.E. 2009. Biofuels, land use change, and greenhouse gas emissions: Some unexplored variables. *Environmental science & technology*, **43**(3), 961-967.

Kim, S., Dale, B.E. 2004. Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy*, **26**(4), 361-375.

Kim, T.H., Kim, J.S., Sunwoo, C., Lee, Y. 2003. Pretreatment of corn stover by aqueous ammonia. *Bioresource Technology*, **90**(1), 39-47.

Kim, T.H., Lee, Y.Y. 2005. Pretreatment and fractionation of corn stover by ammonia recycle percolation process. *Bioresource technology*, **96**(18), 2007-2013.

Kim, T.H., Taylor, F., Hicks, K.B. 2008. Bioethanol production from barley hull using SAA (soaking in aqueous ammonia) pretreatment. *Bioresource Technology*, **99**(13), 5694-5702.

King, J.W., Srinivas, K. 2009. Multiple unit processing using sub-and supercritical fluids. *The Journal of Supercritical Fluids*, **47**(3), 598-610.

Kirk, T.K., Chang, H.-M. 1981. Potential applications of bio-ligninolytic systems. *Enzyme and Microbial Technology*, **3**(3), 189-196.

Kishimoto, T., Chiba, W., Saito, K., Fukushima, K., Uraki, Y., Ubukata, M. 2010. Influence of syringyl to guaiacyl ratio on the structure of natural and synthetic lignins. *J Agric Food Chem*, **58**(2), 895-901.

Klein-Marcuschamer, D., Simmons, B.A., Blanch, H.W. 2011. Techno-economic analysis of a lignocellulosic ethanol biorefinery with ionic liquid pre-treatment. *Biofuels, Bioproducts and Biorefining*, **5**(5), 562-569.

Ko, J.K., Bak, J.S., Jung, M.W., Lee, H.J., Choi, I.G., Kim, T.H., Kim, K.H. 2009. Ethanol production from rice straw using optimized aqueous-ammonia soaking pretreatment and simultaneous saccharification and fermentation processes. *Bioresour Technol*, **100**(19), 4374-80.

Konda, N.M., Shi, J., Singh, S., Blanch, H.W., Simmons, B.A., Klein-Marcuschamer, D. 2014. Understanding cost drivers and economic potential of two variants of ionic liquid pretreatment for cellulosic biofuel production. *Biotechnology for biofuels*, **7**(1), 86.

Kosa, M., Ragauskas, A.J. 2012. Bioconversion of lignin model compounds with oleaginous Rhodococci. *Applied microbiology and biotechnology*, **93**(2), 891-900.

Kosa, M., Ragauskas, A.J. 2013. Lignin to lipid bioconversion by oleaginous Rhodococci. *Green Chemistry*, **15**(8), 2070-2074.

Kosan, B., Michels, C., Meister, F. 2007. Dissolution and forming of cellulose with ionic liquids. *Cellulose*, **15**(1), 59-66.

Kreuger, E., Sipos, B., Zacchi, G., Svensson, S.-E., Björnsson, L. 2011. Bioconversion of industrial hemp to ethanol and methane: the benefits of steam pretreatment and co-production. *Bioresource technology*, **102**(3), 3457-3465.

Kumar, R., Mago, G., Balan, V., Wyman, C.E. 2009. Physical and chemical characterizations of corn stover and poplar solids resulting from leading pretreatment technologies. *Bioresource Technology*, **100**(17), 3948-3962.

Kumar, S., Gupta, R., Lee, Y., Gupta, R.B. 2010. Cellulose pretreatment in subcritical water: effect of temperature on molecular structure and enzymatic reactivity. *Bioresource Technology*, **101**(4), 1337-1347.

Lau, M.W., Dale, B.E., Balan, V. 2008. Ethanolic fermentation of hydrolysates from ammonia fiber expansion (AFEX) treated corn stover and distillers grain without detoxification and external nutrient supplementation. *Biotechnology and bioengineering*, **99**(3), 529-539.

Lawlor, D.W. 2013. Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. *Journal of Experimental Botany*, **64**(1), 83-108.

Le, R.K., Wells Jr, T., Das, P., Meng, X., Stoklosa, R.J., Bhalla, A., Hodge, D.B., Yuan, J.S., Ragauskas, A.J. 2017. Conversion of corn stover alkaline pre-treatment waste streams into biodiesel via Rhodococci. *RSC Advances*, **7**(7), 4108-4115.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H.V., Auer, M., Vogel, K.P., Simmons, B.A., Singh, S. 2010a. Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. *Bioresource technology*, **101**(13), 4900-4906.

Li, C., Zhao, X., Wang, A., Huber, G.W., Zhang, T. 2015. Catalytic Transformation of Lignin for the Production of Chemicals and Fuels. *Chem Rev*, **115**(21), 11559-624.

Li, C.L., Tanjore, D., He, W., Wong, J., Gardner, J.L., Sale, K.L., Simmons, B.A., Singh, S. 2013. Scale-up and evaluation of high solid ionic liquid pretreatment and enzymatic hydrolysis of switchgrass. *Biotechnology for Biofuels*, **6**.

Li, M., Pu, Y., Ragauskas, A.J. 2016. Current understanding of the correlation of lignin structure with biomass recalcitrance. *Frontiers in Chemistry*, **4**.

Li, X., Weng, J.K., Chapple, C. 2008. Improvement of biomass through lignin modification. *The Plant Journal*, **54**(4), 569-581.

Li, X., Ximenes, E., Kim, Y., Slininger, M., Meilan, R., Ladisch, M., Chapple, C. 2010b. Lignin monomer composition affects Arabidopsis cell-wall degradability after liquid hot water pretreatment. *Biotechnology for biofuels*, **3**(1), 27.

Li, Y., Kajita, S., Kawai, S., Katayama, Y., Morohoshi, N. 2003. Down-regulation of an anionic peroxidase in transgenic aspen and its effect on lignin characteristics. *J Plant Res*, **116**(3), 175-82.

Lin, L., Cheng, Y., Pu, Y., Sun, S., Li, X., Jin, M., Pierson, E.A., Gross, D.C., Dale, B.E., Dai, S.Y. 2016. Systems biology-guided biodesign of consolidated lignin conversion. *Green Chemistry*, **18**(20), 5536-5547.

Lin, L., Yan, R., Liu, Y., Jiang, W. 2010. In-depth investigation of enzymatic hydrolysis of biomass wastes based on three major components: cellulose, hemicellulose and lignin. *Bioresource Technology*, **101**(21), 8217-8223.

Linger, J.G., Vardon, D.R., Guarnieri, M.T., Karp, E.M., Hunsinger, G.B., Franden, M.A., Johnson, C.W., Chupka, G., Strathmann, T.J., Pienkos, P.T. 2014. Lignin valorization through integrated biological funneling and chemical catalysis. *Proceedings of the National Academy of Sciences*, **111**(33), 12013-12018.

Liu, E., Li, M., Das, L., Pu, P., Zhao, B., Crocker, M., Ragauskas, A.J., Shi, J. Under review. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous bionic liquid. *Green Chemistry*.

Liu, L., Sun, J., Li, M., Wang, S., Pei, H., Zhang, J. 2009. Enhanced enzymatic hydrolysis and structural features of corn stover by FeCl 3 pretreatment. *Bioresource Technology*, **100**(23), 5853-5858.

Liu, L.P., Hu, Y., Wen, P., Li, N., Zong, M.H., Ou-Yang, B.N., Wu, H. 2015. Evaluating the effects of biocompatible cholinium ionic liquids on microbial lipid production by Trichosporon fermentans. *Biotechnology for Biofuels*, **8**.

Lora, J.H., Aziz, S. 1985. Organosolv pulping: a versatile approach to wood refining. *Tappi;(United States)*, **68**(8).

Lu, F., Ralph, J. 1997. Derivatization followed by reductive cleavage (DFRC method), a new method for lignin analysis: protocol for analysis of DFRC monomers. *Journal of Agricultural and Food Chemistry*, **45**(7), 2590-2592.

Luo, C., Brink, D.L., Blanch, H.W. 2002. Identification of potential fermentation inhibitors in conversion of hybrid poplar hydrolyzate to ethanol. *Biomass and bioenergy*, **22**(2), 125-138.

Lynd, L.R., Laser, M.S., Bransby, D., Dale, B.E., Davison, B., Hamilton, R., Himmel, M., Keller, M., McMillan, J.D., Sheehan, J. 2008. How biotech can transform biofuels. *Nature biotechnology*, **26**(2), 169.

Lynd, L.R., Van Zyl, W.H., McBride, J.E., Laser, M. 2005. Consolidated bioprocessing of cellulosic biomass: an update. *Current opinion in biotechnology*, **16**(5), 577-583.

Ma, R., Xu, Y., Zhang, X. 2015. Catalytic oxidation of biorefinery lignin to value-added chemicals to support sustainable biofuel production. *ChemSusChem*, **8**(1), 24-51.

Marita, J.M., Ralph, J., Hatfield, R.D., Chapple, C. 1999. NMR characterization of lignins in Arabidopsis altered in the activity of ferulate 5-hydroxylase. *Proceedings of the National Academy of Sciences*, **96**(22), 12328-12332.

Martin, C., Klinke, H.B., Thomsen, A.B. 2007. Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. *Enzyme and Microbial Technology*, **40**(3), 426-432.

Martone, P.T., Estevez, J.M., Lu, F., Ruel, K., Denny, M.W., Somerville, C., Ralph, J. 2009. Discovery of lignin in seaweed reveals convergent evolution of cell-wall architecture. *Current Biology*, **19**(2), 169-175.

McCann, M.C., Carpita, N.C. 2008. Designing the deconstruction of plant cell walls. *Current opinion in plant biology*, **11**(3), 314-320.

McCarthy, J.L., Islam, A. 2000. Lignin chemistry, technology, and utilization: a brief history, ACS Publications.

McKendry, P. 2002. Energy production from biomass (part 1): overview of biomass. *Bioresource technology*, **83**(1), 37-46.

McLaughlin, S.B., Kszos, L.A. 2005. Development of switchgrass (Panicum virgatum) as a bioenergy feedstock in the United States. *Biomass and Bioenergy*, **28**(6), 515-535.

Menon, V., Rao, M. 2012. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. *Progress in Energy and Combustion Science*, **38**(4), 522-550.

Milczarek, G. 2009. Lignosulfonate-modified electrodes: electrochemical properties and electrocatalysis of NADH oxidation. *Langmuir*, **25**(17), 10345-10353.

Mohagheghi, A., Linger, J.G., Yang, S., Smith, H., Dowe, N., Zhang, M., Pienkos, P.T. 2015. Improving a recombinant Zymomonas mobilis strain 8b through continuous adaptation on dilute acid pretreated corn stover hydrolysate. *Biotechnol Biofuels*, **8**, 55.

Montalbo-Lomboy, M., Grewell, D. 2015. Rapid dissolution of switchgrass in 1-butyl-3-methylimidazolium chloride by ultrasonication. *Ultrason Sonochem*, **22**, 588-99.

Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., Ladisch, M. 2005. Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresource technology*, **96**(6), 673-686.

Mosier, N.S., Ladisch, C.M., Ladisch, M.R. 2002. Characterization of acid catalytic domains for cellulose hydrolysis and glucose degradation. *Biotechnology and bioengineering*, **79**(6), 610-618.

Mottiar, Y., Vanholme, R., Boerjan, W., Ralph, J., Mansfield, S.D. 2016. Designer lignins: harnessing the plasticity of lignification. *Current Opinion in Biotechnology*, **37**, 190-200.

Moura, J.C., Bonine, C.A., de Oliveira Fernandes Viana, J., Dornelas, M.C., Mazzafera, P. 2010. Abiotic and biotic stresses and changes in the lignin content and composition in plants. *J Integr Plant Biol*, **52**(4), 360-76.

Müller, G., Schöpper, C., Vos, H., Kharazipour, A., Polle, A. 2008. FTIR-ATR spectroscopic analyses of changes in wood properties during particle-and fibreboard production of hard-and softwood trees. *BioResources*, **4**(1), 49-71.

Nakashima, J., Chen, F., Jackson, L., Shadle, G., Dixon, R.A. 2008. Multi-site genetic modification of monolignol biosynthesis in alfalfa (Medicago sativa): effects on lignin composition in specific cell types. *New Phytol*, **179**(3), 738-50.

Nousiainen, P., Kontro, J., Manner, H., Hatakka, A., Sipila, J. 2014. Phenolic mediators enhance the manganese peroxidase catalyzed oxidation of recalcitrant lignin model compounds and synthetic lignin. *Fungal Genet Biol*, **72**, 137-49.

Obama, B. 2017. The irreversible momentum of clean energy. *Science*, **355**(6321), 126-129.

Palonen, H., Thomsen, A.B., Tenkanen, M., Schmidt, A.S., Viikari, L. 2004. Evaluation of wet oxidation pretreatment for enzymatic hydrolysis of softwood. *Applied biochemistry and biotechnology*, **117**(1), 1-17.

Pan, X., Gilkes, N., Kadla, J., Pye, K., Saka, S., Gregg, D., Ehara, K., Xie, D., Lam, D., Saddler, J. 2006. Bioconversion of hybrid poplar to ethanol and co-products using an organosolv fractionation process: Optimization of process yields. *Biotechnology and bioengineering*, **94**(5), 851-861.

Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T. 2000. Biotechnological potential of agro-industrial residues. I: sugarcane bagasse. *Bioresource technology*, **74**(1), 69-80.

Pandey, M.P., Kim, C.S. 2011. Lignin Depolymerization and Conversion: A Review of Thermochemical Methods. *Chemical Engineering & Technology*, **34**(1), 29-41.

Parrish, D.J., Fike, J.H. 2005. The biology and agronomy of switchgrass for biofuels. *BPTS*, **24**(5-6), 423-459.

Parsell, T.H., Owen, B.C., Klein, I., Jarrell, T.M., Marcum, C.L., Haupert, L.J., Amundson, L.M., Kenttämaa, H.I., Ribeiro, F., Miller, J.T. 2013. Cleavage and hydrodeoxygenation (HDO) of C–O bonds relevant to lignin conversion using Pd/Zn synergistic catalysis. *Chemical Science*, **4**(2), 806-813.

Parthasarathi, R., Balamurugan, K., Shi, J., Subramanian, V., Simmons, B.A., Singh, S. 2015. Theoretical insights into the role of water in the dissolution of cellulose using IL/water mixed solvent systems. *The Journal of Physical Chemistry B*, **119**(45), 14339-14349.

Perez-Pimienta, J.A., Lopez-Ortega, M.G., Varanasi, P., Stavila, V., Cheng, G., Singh, S., Simmons, B.A. 2013. Comparison of the impact of ionic liquid pretreatment on recalcitrance of agave bagasse and switchgrass. *Bioresour Technol*, **127**, 18-24.

Pordesimo, L., Hames, B., Sokhansanj, S., Edens, W. 2005. Variation in corn stover composition and energy content with crop maturity. *Biomass and Bioenergy*, **28**(4), 366-374.

Prado, R., Brandt, A., Erdocia, X., Hallet, J., Welton, T., Labidi, J. 2016. Lignin oxidation and depolymerisation in ionic liquids. *Green Chemistry*, **18**(3), 834-841.

Pu, Y., Cao, S., Ragauskas, A.J. 2011. Application of quantitative 31P NMR in biomass lignin and biofuel precursors characterization. *Energy & Environmental Science*, **4**(9), 3154-3166.

Pu, Y., Hu, F., Huang, F., Davison, B.H., Ragauskas, A.J. 2013. Assessing the molecular structure basis for biomass recalcitrance during dilute acid and hydrothermal pretreatments. *Biotechnology for biofuels*, **6**(1), 15.

Pu, Y., Hu, F., Huang, F., Ragauskas, A.J. 2015. Lignin structural alterations in thermochemical pretreatments with limited delignification. *BioEnergy Research*, **8**(3), 992-1003.

Qin, L., Liu, Z.-H., Jin, M., Li, B.-Z., Yuan, Y.-J. 2013. High temperature aqueous ammonia pretreatment and post-washing enhance the high solids enzymatic hydrolysis of corn stover. *Bioresource technology*, **146**, 504-511.

Ragauskas, A.J., Beckham, G.T., Biddy, M.J., Chandra, R., Chen, F., Davis, M.F., Davison, B.H., Dixon, R.A., Gilna, P., Keller, M. 2014. Lignin valorization: improving lignin processing in the biorefinery. *Science*, **344**(6185), 1246843.

Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J., Hallett, J.P., Leak, D.J., Liotta, C.L. 2006. The path forward for biofuels and biomaterials. *science*, **311**(5760), 484-489.

Ralph, J. 2010. Hydroxycinnamates in lignification. *Phytochemistry Reviews*, **9**(1), 65-83.

Ralph, J., Hatfield, R.D., Piquemal, J., Yahiaoui, N., Pean, M., Lapierre, C., Boudet, A.M. 1998. NMR characterization of altered lignins extracted from tobacco plants down-regulated for lignification enzymes cinnamylalcohol dehydrogenase and

cinnamoyl-CoA reductase. *Proceedings of the National Academy of Sciences*, **95**(22), 12803-12808.
Ralph, J., Lapierre, C., Marita, J.M., Kim, H., Lu, F., Hatfield, R.D., Ralph, S., Chapple, C., Franke, R., Hemm, M.R. 2001. Elucidation of new structures in lignins of CAD-and COMT-deficient plants by NMR. *Phytochemistry*, **57**(6), 993-1003.
Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F., Ede, R.M., Peng, J., Quideau, S., Helm, R.F., Grabber, J.H. 1999. Solution-state NMR of lignins. *Advances in lignocellulosics characterization*, 55-108.
Ramos, L., Breuil, C., Kushner, D., Saddler, J. 1992. Steam pretreatment conditions for effective enzymatic hydrolysis and recovery yields of Eucalyptus viminalis wood chips. *Holzforschung-International Journal of the Biology, Chemistry, Physics and Technology of Wood*, **46**(2), 149-154.
Reddy, M.S., Chen, F., Shadle, G., Jackson, L., Aljoe, H., Dixon, R.A. 2005. Targeted down-regulation of cytochrome P450 enzymes for forage quality improvement in alfalfa (Medicago sativa L.). *Proceedings of the National Academy of Sciences of the United States of America*, **102**(46), 16573-16578.
Reh, U., Kraepelin, G., Lamprecht, I. 1987. Differential scanning calorimetry as a complementary tool in wood biodegradation studies. *Thermochimica Acta*, **119**(1), 143-150.
Reichert, E., Wintringer, R., Volmer, D.A., Hempelmann, R. 2012. Electro-catalytic oxidative cleavage of lignin in a protic ionic liquid. *Physical Chemistry Chemical Physics*, **14**(15), 5214-5221.
Rencoret, J., Gutierrez, A., Nieto, L., Jimenez-Barbero, J., Faulds, C.B., Kim, H., Ralph, J., Martinez, A.T., Del Rio, J.C. 2011. Lignin composition and structure in young versus adult Eucalyptus globulus plants. *Plant Physiol*, **155**(2), 667-82.
Ricroch, A.E., Hénard-Damave, M.-C. 2016. Next biotech plants: new traits, crops, developers and technologies for addressing global challenges. *Critical reviews in biotechnology*, **36**(4), 675-690.
Rinaldi, R., Jastrzebski, R., Clough, M.T., Ralph, J., Kennema, M., Bruijnincx, P.C., Weckhuysen, B.M. 2016. Paving the way for lignin valorisation: recent advances in bioengineering, biorefining and catalysis. *Angewandte Chemie International Edition*, **55**(29), 8164-8215.
Robert, D., Gagnaire, D. 1981. Quantitative analysis of lignins by 13C NMR. *Proceedings of the International Symposium on Wood and Pulping Chemistry*. pp. 9-12.
Rogers, L.A., Campbell, M.M. 2004. The genetic control of lignin deposition during plant growth and development. *New phytologist*, **164**(1), 17-30.
Rogers, R., Seddon, K. 2003. CHEMISTRY: Ionic Liquids--Solvents of the Future? *Science*, **302**(5646), 792.
Rolz, C., de Arriola, M., Valladares, J., de Cabrera, S. 1986. Effects of some physical and chemical pretreatments on the composition and enzymatic hydrolysis and digestibility of lemon grass and citronella bagasse. *Agricultural Wastes*, **18**(2), 145-161.
Roy, S.J., Negrão, S., Tester, M. 2014. Salt resistant crop plants. *Current Opinion in Biotechnology*, **26**, 115-124.

Saha, B.C. 2003. Hemicellulose bioconversion. *J Ind Microbiol Biotechnol*, **30**(5), 279-91.
Salanti, A., Zoia, L., Orlandi, M., Zanini, F., Elegir, G. 2010a. Structural characterization and antioxidant activity evaluation of lignins from rice husk. *Journal of agricultural and food chemistry*, **58**(18), 10049-10055.
Salanti, A., Zoia, L., Tolppa, E.-L., Giachi, G., Orlandi, M. 2010b. Characterization of waterlogged wood by NMR and GPC techniques. *Microchemical journal*, **95**(2), 345-352.
Salvachúa, D., Karp, E.M., Nimlos, C.T., Vardon, D.R., Beckham, G.T. 2015. Towards lignin consolidated bioprocessing: simultaneous lignin depolymerization and product generation by bacteria. *Green Chemistry*, **17**(11), 4951-4967.
Salvi, D.A., Aita, G.M., Robert, D., Bazan, V. 2010. Dilute ammonia pretreatment of sorghum and its effectiveness on enzyme hydrolysis and ethanol fermentation. *Appl Biochem Biotechnol*, **161**(1-8), 67-74.
Samayam, I.P., Schall, C.A. 2010. Saccharification of ionic liquid pretreated biomass with commercial enzyme mixtures. *Bioresour Technol*, **101**(10), 3561-6.
Samuel, R., Pu, Y., Jiang, N., Fu, C., Wang, Z.-Y., Ragauskas, A. 2014. Structural characterization of lignin in wild-type versus COMT down-regulated switchgrass. *Front Energy Res*, **1**, 1-9.
Samuel, R., Pu, Y., Raman, B., Ragauskas, A.J. 2010. Structural characterization and comparison of switchgrass ball-milled lignin before and after dilute acid pretreatment. *Applied biochemistry and biotechnology*, **162**(1), 62-74.
Sarkanen, K.V., Ludwig, C.H. 1971. *Liguins. Occurrence, formation, structure, and reactions*. New York.; Wiley-Interscience.
Sathitsuksanoh, N., Holtman, K.M., Yelle, D.J., Morgan, T., Stavila, V., Pelton, J., Blanch, H., Simmons, B.A., George, A. 2014. Lignin fate and characterization during ionic liquid biomass pretreatment for renewable chemicals and fuels production. *Green Chemistry*, **16**(3), 1236-1247.
Sawan, S.P., Sawan, S.P. 1998. *Supercritical fluid cleaning: fundamentals, technology and applications*. Elsevier.
Scullin, C., Cruz, A.G., Chuang, Y.D., Simmons, B.A., Loque, D., Singh, S. 2015. Restricting lignin and enhancing sugar deposition in secondary cell walls enhances monomeric sugar release after low temperature ionic liquid pretreatment. *Biotechnol Biofuels*, **8**, 95.
Sedlak, M., Ho, N.W. 2004. Characterization of the effectiveness of hexose transporters for transporting xylose during glucose and xylose co-fermentation by a recombinant Saccharomyces yeast. *Yeast*, **21**(8), 671-84.
Selig, M., Weiss, N., Ji, Y. 2008. *Enzymatic Saccharification of Lignocellulosic Biomass: Laboratory Analytical Procedure (LAP): Issue Date, 3/21/2008*. National Renewable Energy Laboratory.
Sewalt, V.J., Ni, W., Blount, J.W., Jung, H.G., Masoud, S.A., Howles, P.A., Lamb, C., Dixon, R.A. 1997. Reduced lignin content and altered lignin composition in transgenic tobacco down-regulated in expression of L-phenylalanine ammonia-lyase or cinnamate 4-hydroxylase. *Plant physiology*, **115**(1), 41-50.
Sharma, R.K., Wooten, J.B., Baliga, V.L., Lin, X., Chan, W.G., Hajaligol, M.R. 2004. Characterization of chars from pyrolysis of lignin. *Fuel*, **83**(11), 1469-1482.

Shepherd, K.D., Palm, C.A., Gachengo, C.N., Vanlauwe, B. 2003. Rapid characterization of organic resource quality for soil and livestock management in tropical agroecosystems using near-infrared spectroscopy. *Agronomy Journal*, **95**(5), 1314-1322.

Sherman, S.R., Goodell, J.J., Milliken, C.E., Morris, J.A., Gorensek, M.B. 2012. A new process developed for separation of lignin from ammonium hydroxide pretreatment solutions. *Environmental Progress & Sustainable Energy*, **31**(1), 130-138.

Shi, J., Balamurugan, K., Parthasarathi, R., Sathitsuksanoh, N., Zhang, S., Stavila, V., Subramanian, V., Simmons, B.A., Singh, S. 2014. Understanding the role of water during ionic liquid pretreatment of lignocellulose: co-solvent or anti-solvent? *Green Chemistry*, **16**(8), 3830-3840.

Shi, J., Ebrik, M., Yang, B., Wyman, C.E. 2009. The potential of cellulosic ethanol production from municipal solid waste: a technical and economic evaluation. *University of California Energy Institute*.

Shi, J., Gladden, J.M., Sathitsuksanoh, N., Kambam, P., Sandoval, L., Mitra, D., Zhang, S., George, A., Singer, S.W., Simmons, B.A. 2013. One-pot ionic liquid pretreatment and saccharification of switchgrass. *Green Chemistry*, **15**(9), 2579-2589.

Shi, J., Pattathil, S., Parthasarathi, R., Anderson, N.A., Im Kim, J., Venketachalam, S., Hahn, M.G., Chapple, C., Simmons, B.A., Singh, S. 2016. Impact of engineered lignin composition on biomass recalcitrance and ionic liquid pretreatment efficiency. *Green Chemistry*, **18**(18), 4884-4895.

Shleev, S., Persson, P., Shumakovich, G., Mazhugo, Y., Yaropolov, A., Ruzgas, T., Gorton, L. 2006. Interaction of fungal laccases and laccase-mediator systems with lignin. *Enzyme and Microbial Technology*, **39**(4), 841-847.

Sidiras, D., Koukios, E. 1989. Acid saccharification of ball-milled straw. *Biomass*, **19**(4), 289-306.

Simmons, B.A., Loqué, D., Ralph, J. 2010. Advances in modifying lignin for enhanced biofuel production. *Current opinion in plant biology*, **13**(3), 312-319.

Singh, S., Simmons, B.A., Vogel, K.P. 2009. Visualization of biomass solubilization and cellulose regeneration during ionic liquid pretreatment of switchgrass. *Biotechnology and Bioengineering*, **104**(1), 68-75.

Singh, S., Varanasi, P., Singh, P., Adams, P.D., Auer, M., Simmons, B.A. 2013. Understanding the impact of ionic liquid pretreatment on cellulose and lignin via thermochemical analysis. *Biomass and Bioenergy*, **54**, 276-283.

Sitarz, A.K., Mikkelsen, J.D., Hojrup, P., Meyer, A.S. 2013. Identification of a laccase from Ganoderma lucidum CBS 229.93 having potential for enhancing cellulase catalyzed lignocellulose degradation. *Enzyme Microb Technol*, **53**(6-7), 378-85.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D. 2006. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. *Golden: National Renewable Energy Laboratory*.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D. 2008. Determination of structural carbohydrates and lignin in biomass. *Laboratory analytical procedure*, **1617**.

Socha, A.M., Parthasarathi, R., Shi, J., Pattathil, S., Whyte, D., Bergeron, M., George, A., Tran, K., Stavila, V., Venkatachalam, S., Hahn, M.G., Simmons, B.A., Singh, S. 2014. Efficient biomass pretreatment using ionic liquids derived from lignin and hemicellulose. *Proceedings of the National Academy of Sciences of the United States of America*, **111**(35), E3587-E3595.

Stärk, K., Taccardi, N., Bösmann, A., Wasserscheid, P. 2010. Oxidative depolymerization of lignin in ionic liquids. *ChemSusChem*, **3**(6), 719-723.

Sticklen, M.B. 2007. Feedstock Crop Genetic Engineering for Alcohol Fuels. *Crop Science*, **47**(6), 2238.

Sticklen, M.B. 2008. Plant genetic engineering for biofuel production: towards affordable cellulosic ethanol. *Nat Rev Genet*, **9**(6), 433-43.

Studer, M.H., DeMartini, J.D., Davis, M.F., Sykes, R.W., Davison, B., Keller, M., Tuskan, G.A., Wyman, C.E. 2011. Lignin content in natural Populus variants affects sugar release. *Proceedings of the National Academy of Sciences*, **108**(15), 6300-6305.

Sun, J., Konda, N.M., Shi, J., Parthasarathi, R., Dutta, T., Xu, F., Scown, C.D., Simmons, B.A., Singh, S. 2016. CO 2 enabled process integration for the production of cellulosic ethanol using bionic liquids. *Energy & Environmental Science*, **9**(9), 2822-2834.

Sun, N., Parthasarathi, R., Socha, A.M., Shi, J., Zhang, S., Stavila, V., Sale, K.L., Simmons, B.A., Singh, S. 2014. Understanding pretreatment efficacy of four cholinium and imidazolium ionic liquids by chemistry and computation. *Green Chemistry*, **16**(5), 2546-2557.

Swanson, R.M., Platon, A., Satrio, J.A., Brown, R.C. 2010. Techno-economic analysis of biomass-to-liquids production based on gasification. *Fuel*, **89**, S11-S19.

Tao, L., Aden, A., Elander, R.T., Pallapolu, V.R., Lee, Y.Y., Garlock, R.J., Balan, V., Dale, B.E., Kim, Y., Mosier, N.S., Ladisch, M.R., Falls, M., Holtzapple, M.T., Sierra, R., Shi, J., Ebrik, M.A., Redmond, T., Yang, B., Wyman, C.E., Hames, B., Thomas, S., Warner, R.E. 2011. Process and technoeconomic analysis of leading pretreatment technologies for lignocellulosic ethanol production using switchgrass. *Bioresour Technol*, **102**(24), 11105-14.

Tassinari, T., Macy, C., Spano, L., Ryu, D.D. 1980. Energy requirements and process design considerations in compression-milling pretreatment of cellulosic wastes for enzymatic hydrolysis. *Biotechnology and Bioengineering*, **22**(8), 1689-1705.

Tejado, A., Pena, C., Labidi, J., Echeverria, J., Mondragon, I. 2007. Physico-chemical characterization of lignins from different sources for use in phenol–formaldehyde resin synthesis. *Bioresource Technology*, **98**(8), 1655-1663.

Thanh Mai Pham, L., Eom, M.H., Kim, Y.H. 2014. Inactivating effect of phenolic unit structures on the biodegradation of lignin by lignin peroxidase from Phanerochaete chrysosporium. *Enzyme Microb Technol*, **61-62**, 48-54.

Tolbert, A., Akinosho, H., Khunsupat, R., Naskar, A.K., Ragauskas, A.J. 2014. Characterization and analysis of the molecular weight of lignin for biorefining studies. *Biofuels, Bioproducts and Biorefining*, **8**(6), 836-856.

Trajano, H.L., Engle, N.L., Foston, M., Ragauskas, A.J., Tschaplinski, T.J., Wyman, C.E. 2013. The fate of lignin during hydrothermal pretreatment. *Biotechnology for biofuels*, **6**(1), 110.

Tsujiyama, S.-i., Miyamori, A. 2000. Assignment of DSC thermograms of wood and its components. *Thermochimica Acta*, **351**(1), 177-181.
Tuck, C.O., Pérez, E., Horváth, I.T., Sheldon, R.A., Poliakoff, M. 2012. Valorization of biomass: deriving more value from waste. *Science*, **337**(6095), 695-699.
Van Dyk, J.S., Pletschke, B.I. 2012. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes--factors affecting enzymes, conversion and synergy. *Biotechnol Adv*, **30**(6), 1458-80.
van Walsum, G.P., Shi, H. 2004. Carbonic acid enhancement of hydrolysis in aqueous pretreatment of corn stover. *Bioresource technology*, **93**(3), 217-226.
Vanholme, R., Demedts, B., Morreel, K., Ralph, J., Boerjan, W. 2010. Lignin biosynthesis and structure. *Plant physiology*, **153**(3), 895-905.
Vanholme, R., Morreel, K., Ralph, J., Boerjan, W. 2008. Lignin engineering. *Curr Opin Plant Biol*, **11**(3), 278-85.
Varanasi, P., Singh, P., Arora, R., Adams, P.D., Auer, M., Simmons, B.A., Singh, S. 2012. Understanding changes in lignin of Panicum virgatum and Eucalyptus globulus as a function of ionic liquid pretreatment. *Bioresour Technol*, **126**, 156-61.
Varga, E., Klinke, H.B., Reczey, K., Thomsen, A.B. 2004. High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. *Biotechnology and bioengineering*, **88**(5), 567-574.
Vassilev, S.V., Baxter, D., Andersen, L.K., Vassileva, C.G. 2010. An overview of the chemical composition of biomass. *Fuel*, **89**(5), 913-933.
Voelker, S.L., Lachenbruch, B., Meinzer, F.C., Jourdes, M., Ki, C., Patten, A.M., Davin, L.B., Lewis, N.G., Tuskan, G.A., Gunter, L. 2010. Antisense down-regulation of 4CL expression alters lignification, tree growth, and saccharification potential of field-grown poplar. *Plant Physiology*, **154**(2), 874-886.
Vogel, K.P., Jung, H.-J.G. 2001. Genetic Modification of Herbaceous Plants for Feed and Fuel. *Critical Reviews in Plant Sciences*, **20**(1), 15-49.
Wang, Z., Li, R., Xu, J., Marita, J.M., Hatfield, R.D., Qu, R., Cheng, J.J. 2012. Sodium hydroxide pretreatment of genetically modified switchgrass for improved enzymatic release of sugars. *Bioresour Technol*, **110**, 364-70.
Wei, Z., Zeng, G., Huang, F., Kosa, M., Huang, D., Ragauskas, A.J. 2015. Bioconversion of oxygen-pretreated Kraft lignin to microbial lipid with oleaginous Rhodococcus opacus DSM 1069. *Green Chemistry*, **17**(5), 2784-2789.
Weil, J., Sarikaya, A., Rau, S.-L., Goetz, J., Ladisch, C.M., Brewer, M., Hendrickson, R., Ladisch, M.R. 1997. Pretreatment of yellow poplar sawdust by pressure cooking in water. *Applied Biochemistry and Biotechnology*, **68**(1), 21-40.
Wen, J.-L., Yuan, T.-Q., Sun, S.-L., Xu, F., Sun, R.-C. 2014. Understanding the chemical transformations of lignin during ionic liquid pretreatment. *Green Chemistry*, **16**(1), 181-190.
Wen, X., Jia, Y., Li, J. 2009. Degradation of tetracycline and oxytetracycline by crude lignin peroxidase prepared from Phanerochaete chrysosporium--a white rot fungus. *Chemosphere*, **75**(8), 1003-7.
Weng, J.K., Chapple, C. 2010. The origin and evolution of lignin biosynthesis. *New Phytologist*, **187**(2), 273-285.

Wilkerson, C., Mansfield, S., Lu, F., Withers, S., Park, J.-Y., Karlen, S., Gonzales-Vigil, E., Padmakshan, D., Unda, F., Rencoret, J. 2014. Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone. *Science*, **344**(6179), 90-93.

Williams, C.L., Westover, T.L., Emerson, R.M., Tumuluru, J.S., Li, C. 2016. Sources of biomass feedstock variability and the potential impact on biofuels production. *BioEnergy Research*, **9**(1), 1-14.

Wunderlich, B. 2005. *Thermal analysis of polymeric materials*. Springer Science & Business Media.

Wyman, C. 1996. *Handbook on bioethanol: production and utilization*. CRC press.

Wyman, C.E. 2007. What is (and is not) vital to advancing cellulosic ethanol. *Trends in Biotechnology*, **25**(4), 153-157.

Wyman, C.E., Balan, V., Dale, B.E., Elander, R.T., Falls, M., Hames, B., Holtzapple, M.T., Ladisch, M.R., Lee, Y.Y., Mosier, N., Pallapolu, V.R., Shi, J., Thomas, S.R., Warner, R.E. 2011. Comparative data on effects of leading pretreatments and enzyme loadings and formulations on sugar yields from different switchgrass sources. *Bioresour Technol*, **102**(24), 11052-62.

Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y. 2005. Coordinated development of leading biomass pretreatment technologies. *Bioresource technology*, **96**(18), 1959-1966.

Xia, S., Baker, G.A., Li, H., Ravula, S., Zhao, H. 2014. Aqueous ionic liquids and deep eutectic solvents for cellulosic biomass pretreatment and saccharification. *RSC advances*, **4**(21), 10586-10596.

Xia, Z., Akim, L.G., Argyropoulos, D.S. 2001. Quantitative 13C NMR analysis of lignins with internal standards. *Journal of agricultural and food chemistry*, **49**(8), 3573-3578.

Xie, S., Ragauskas, A.J., Yuan, J.S. 2016. Lignin conversion: Opportunities and challenges for the integrated biorefinery. *Industrial Biotechnology*, **12**(3), 161-167.

Xing, R., Subrahmanyam, A.V., Olcay, H., Qi, W., van Walsum, G.P., Pendse, H., Huber, G.W. 2010. Production of jet and diesel fuel range alkanes from waste hemicellulose-derived aqueous solutions. *Green Chemistry*, **12**(11), 1933.

Xu, B., Escamilla-Treviño, L.L., Sathitsuksanoh, N., Shen, Z., Shen, H., Percival Zhang, Y.H., Dixon, R.A., Zhao, B. 2011. Silencing of 4-coumarate: coenzyme A ligase in switchgrass leads to reduced lignin content and improved fermentable sugar yields for biofuel production. *New phytologist*, **192**(3), 611-625.

Xu, B., Sathitsuksanoh, N., Tang, Y., Udvardi, M.K., Zhang, J.-Y., Shen, Z., Balota, M., Harich, K., Zhang, P.Y.-H., Zhao, B. 2012. Overexpression of AtLOV1 in switchgrass alters plant architecture, lignin content, and flowering time. *PLoS One*, **7**(12), e47399.

Xu, C., Arancon, R.A.D., Labidi, J., Luque, R. 2014. Lignin depolymerisation strategies: towards valuable chemicals and fuels. *Chemical Society Reviews*, **43**(22), 7485-7500.

Xu, F., Sun, J., Konda, N.M., Shi, J., Dutta, T., Scown, C.D., Simmons, B.A., Singh, S. 2016. Transforming biomass conversion with ionic liquids: process intensification

and the development of a high-gravity, one-pot process for the production of cellulosic ethanol. *Energy & Environmental Science*, **9**(3), 1042-1049.
Xu, F., Yu, J., Tesso, T., Dowell, F., Wang, D. 2013. Qualitative and quantitative analysis of lignocellulosic biomass using infrared techniques: a mini-review. *Applied Energy*, **104**, 801-809.
Xu, J., Cheng, J.J., Sharma-Shivappa, R.R., Burns, J.C. 2010. Lime pretreatment of switchgrass at mild temperatures for ethanol production. *Bioresour Technol*, **101**(8), 2900-3.
Yang, F., Mitra, P., Zhang, L., Prak, L., Verhertbruggen, Y., Kim, J.S., Sun, L., Zheng, K., Tang, K., Auer, M., Scheller, H.V., Loque, D. 2013. Engineering secondary cell wall deposition in plants. *Plant Biotechnol J*, **11**(3), 325-35.
Yang, H., Yan, R., Chen, H., Lee, D.H., Zheng, C. 2007. Characteristics of hemicellulose, cellulose and lignin pyrolysis. *Fuel*, **86**(12-13), 1781-1788.
Yao, W., Nokes, S.E. 2014. Phanerochaete chrysosporium pretreatment of biomass to enhance solvent production in subsequent bacterial solid-substrate cultivation. *biomass and bioenergy*, **62**, 100-107.
Yeh, T.-F., Chang, H.-m., Kadla, J.F. 2004. Rapid prediction of solid wood lignin content using transmittance near-infrared spectroscopy. *Journal of Agricultural and food chemistry*, **52**(6), 1435-1439.
Yoo, C.G., Kim, H., Lu, F., Azarpira, A., Pan, X., Oh, K.K., Kim, J.S., Ralph, J., Kim, T.H. 2016a. Understanding the physicochemical characteristics and the improved enzymatic saccharification of corn stover pretreated with aqueous and gaseous ammonia. *BioEnergy Research*, **9**(1), 67-76.
Yoo, C.G., Pu, Y., Li, M., Ragauskas, A.J. 2016b. Elucidating Structural Characteristics of Biomass using Solution-State 2 D NMR with a Mixture of Deuterated Dimethylsulfoxide and Hexamethylphosphoramide. *ChemSusChem*, **9**(10), 1090-1095.
Yoon, H., Wu, Z., Lee, Y. 1995. Ammonia-recycled percolation process for pretreatment of biomass feedstock. *Applied Biochemistry and Biotechnology*, **51**(1), 5-19.
You, T., Zhang, L., Guo, S., Shao, L., Xu, F. 2015. Unraveling the Structural Modifications in Lignin of Arundo donax Linn. during Acid-Enhanced Ionic Liquid Pretreatment. *Journal of agricultural and food chemistry*, **63**(50), 10747-10756.
Yu, J., Zhang, T., Zhong, J., Zhang, X., Tan, T. 2012. Biorefinery of sweet sorghum stem. *Biotechnology advances*, **30**(4), 811-816.
Yuan, T.-Q., Sun, S.-N., Xu, F., Sun, R.-C. 2011. Characterization of lignin structures and lignin–carbohydrate complex (LCC) linkages by quantitative 13C and 2D HSQC NMR spectroscopy. *Journal of agricultural and food chemistry*, **59**(19), 10604-10614.
Zakzeski, J., Bruijnincx, P.C., Jongerius, A.L., Weckhuysen, B.M. 2010. The catalytic valorization of lignin for the production of renewable chemicals. *Chemical reviews*, **110**(6), 3552-3599.
Zhang, J., Feng, L., Wang, D., Zhang, R., Liu, G., Cheng, G. 2014. Thermogravimetric analysis of lignocellulosic biomass with ionic liquid pretreatment. *Bioresour Technol*, **153**, 379-82.

Zhao, C., Xie, S., Pu, Y., Zhang, R., Huang, F., Ragauskas, A.J., Yuan, J.S. 2016. Synergistic enzymatic and microbial lignin conversion. *Green Chemistry*, **18**(5), 1306-1312.

Zhao, X. 2009. Organosolv pretreatment of lignocellulosic biomass for enzymatic hydrolysis. *Applied Microbiology and Biotechnology*, **82**(5), 815.

Zhao, X., Zhang, L., Liu, D. 2012. Biomass recalcitrance. Part I: the chemical compositions and physical structures affecting the enzymatic hydrolysis of lignocellulose. *Biofuels, Bioproducts and Biorefining*, **6**(4), 465-482.

Zhou, X., Xu, J., Wang, Z., Cheng, J.J., Li, R., Qu, R. 2012. Dilute sulfuric acid pretreatment of transgenic switchgrass for sugar production. *Bioresour Technol*, **104**, 823-7.

Zhu, D., Zhang, P., Xie, C., Zhang, W., Sun, J., Qian, W.-J., Yang, B. 2017. Biodegradation of alkaline lignin by Bacillus ligniniphilus L1. *Biotechnology for Biofuels*, **10**(1), 44.

Ziebell, A., Gracom, K., Katahira, R., Chen, F., Pu, Y., Ragauskas, A., Dixon, R.A., Davis, M. 2010. Increase in 4-coumaryl alcohol units during lignification in alfalfa (Medicago sativa) alters the extractability and molecular weight of lignin. *Journal of Biological Chemistry*, **285**(50), 38961-38968.

VITA

Enshi Liu

Education/Training

2015 -	M.S.	Biosystems and Agricultural Engineering University of Kentucky	GPA: 4.00/4.00
2011 - 2015	Research Assistant	Institute of Tropical Bioscience and Biotechnology Chinese Academy of Tropical and Agricultural Sciences	
2007 - 2011	B.E.	Biological Engineering Hainan University	GPA: 3.48/4.00

Research Experience

Graduate Research Assistant, *Biosystems and Agricultural Engineering, University of Kentucky,* Lexington, KY (August 2015 to present)

Research Assistant, *Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agricultural Sciences,* Haikou, Hainan, China (August 2011 to January 2015)

Undergraduate research, *Department of Biological Engineering, Hainan University,* Haikou, Hainan, China (August 2007 to July 2011)

Publications

In progress:

1) **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous ionic liquid. *Sumitted*
2) **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Impact of dilute sulfuric acid, ammonia hydroxide, and ionic liquid pretreatment on the fractionation and characterization of engineered switchgrass.

3) **Enshi Liu**, Wenqi Li, Seth DeBolt, Sue E. Nokes, Jian Shi. The chemistry, biology and conversion technology of lignin engineered plants in the biorefinery concept: A review.

4) Lalitendu Das, **Enshi Liu**, Areej Saeed, David W. Williams, Chenlin Li, Allison E. Ray, Jian Shi. Industrial hemp as a potential biofuels crop in comparison with kenaf, switchgrass, and biomass sorghum.

5) **Enshi Liu**, Haiyan Sun, Fang Zhou, Jiuhui Li, Ming Peng. Effect of rubber seed meal on the performance of ethanol production from cassava pulp.

Published:

1) Sun Haiyan, Li Juanhua, **Liu Enshi**, Yi Xiaoping, Yang Jinghao, Peng Ming. Cloning and sequence analysis of *creA* gene from *Aspergillus niger* strain. *Food Research and Development*, 2016, 37(18): 158-161.

2) Sun Haiyan, Li Juanhua, **Liu Enshi**, Yi Xiaoping, Yang Jinghao, Peng Ming. Cloning and analysis of raw-starch-digesting-glucoamylase promoter from *Aspergillus niger* F-01 and *Aspergillus niger* G-1125. *Light Industry Science and Technology*, 2015, 2: 17-18.

3) Sun Haiyan, Li Juanhua, **Liu Enshi**, Yi Xiaoping, Yang Jinghao, Yin Yiyi, Peng Ming. Cloning and sequence analysis of gene encoded raw-starch-digesting-glucoamylase from *Aspergillus niger* G-1125. *China Brewing*, 2015, 34(2): 51-54.

4) Sun Haiyan, Li Juanhua, **Liu Enshi**, Peng Ming. Cloning and sequence analysis of gene encoding raw-starch-digesting-glucoamylase from *Aspergillus niger* F-01. *Genomics and Applied Biology*, 2015, 34(2): 308-312.

5) Zhou Fang, **Liu Enshi**, Sun Haiyan, Zhao Pingjuan, Li Juanhua, Peng Ming. Effect of drought hardening on the content of endogenous phytohormone and soluble sugar in cassava roots. *Chinese Journal of Tropical Crops*, 2013, 34(3): 486-494.

6) Zhou Fang, **Liu Enshi**, Zhao Pingjuan, Wang Wenquan, Peng Ming. Impacts of drought stress on content of endogenous phytohormones at seedling stage of cassava. *Agricultural Research in the Arid Areas*, 2013, 31(5): 238-244.

7) Zhou Fang, **Liu Enshi**, Sun Haiyan, Zhao Pingjuan, Li Juanhua, Peng Ming. Research on content changes of abscisic acid, proline, and soluble sugar in cassava leaves after drought hardening. *Southwest China Journal of Agricultural Sciences*, 2013, 26(4): 1428-1433.

8) Haiyan Sun, Pingjuan Zhao, Juanhua Li, **Enshi Liu**, Ming Peng. Selection and identification of a cellulose-producing strain. *Agricultural Biotechnology*, 2012, 1(2): 38-39, 42.

9) Sun Haiyan, **Liu Enshi**, Zhao Pingjuan, Li Juanhua, Lu Cheng, Wang Wenquan, Peng Ming. Comparison of gas chromatography and distillation-alcohol-meter method for the detection of alcohol content in cassava fermenting solution. *Liquor-Making Science and Technology*, 2012, 11: 105-107.

10) Wang Gan, **Liu Enshi**, Yu Xiaoling, Liao Wenbin, Peng Ming. Changes of endogenous ethylene in cassava leaves and abscission zone under drought stress. *Chinese Journal of Tropical Agriculture*, 2012, 32(4): 10-12.

11) Yu Xiaoling, Wang Gan, Ruan Mengbin, **Liu Enshi**, Peng Ming. Physiological and biochemical changes of leaves in different cassava varieties under water stress. *Chinese Agricultural Science Bulletin*, 2012, 28(33): 60-64.

12) Haiyan Sun, Juanhua Li, Pingjuan Zhao, **Enshi Liu**, Ming Peng. Selection and mutation of an amylase-producing strain. *Light Industry Science and Technology*, 2012, 9: 19-20.

13) Haiyan Sun, Qian Wang, **Enshi Liu**, Ming Peng. Culture medium optimization by response surface methodology for production of β-glucosidase from *Aspergillus niger*. *Light Industry Science and Technology*, 2012, 10: 8-9.

14) Haiyan Sun, Pingjuan Zhao, Juanhua Li, **Enshi Liu**, Ming Peng. Production of calcium gluconate from cassava by *Penicillium citrinum* SCG-112. *African Journal of Microbiology Research*, 2011, 5(32): 5994-5997.

Patents

1) Jianming Gao, Kexian Yi, Guangyuan He, Feng Yang, Caibo Luo, Qiang Xiao, Haiyan Sun, Chengji Jiang, **Enshi Liu**, Hui Yang, Ping Wang, Zhiwei Lu, Shiqing Zhang, Helong Chen, Jinlong Zheng, Jingen Xi, Qiaolian Liu. Gene capable of improving ethanol and glucose resistance of *Saccharomyces cerevisiae*. China Patent, No. CN103397020A, 11-20-2013.

2) Kexian Yi, Jianming Gao, Feng Yang, Haiyan Sun, Chengji Jiang, **Enshi Liu**, Guangyuan He, Qiang Xiao, Caibo Luo, Hui Yang, Shiqing Zhang, Helong Chen, Jinlong Zheng, Jingen Xi, Qiaolian Liu. Method for improving ethanol and glucose resistance of *Saccharomyces cerevisiae*. China Patent, No. CN102533842A, 7-4-2012.

Conference Papers and Presentations

1) **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous ionic liquid. Poster at 2017 KY NSF Super Collider, February 24, 2017, Lexington, KY.

2) **Enshi Liu**, Mi Li, Lalitendu Das, Yunqiao Pu, Bingyu Zhao, Mark Crocker, Arthur J. Ragauskas, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass using an aqueous bionic liquid. Flash talk and Poster at 2016 Frontiers in Biorefining Conference, November 8-11, 2016, St. Simons Island, GA.

3) **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from engineered switchgrass. Oral presentation at 2016 Annual International Meeting of American Society of Agricultural and Biological Engineers (ASABE), July 17-20, 2016, Orlando, FL.

4) Ulalo Chirwa, **Enshi Liu**, Jian Shi. Bioprocessing of soybean processing waste for bacterial biocontrol agents. Poster at 2016 Annual International Meeting of American Society of Agricultural and Biological Engineers (ASABE), July 17-20, 2016, Orlando, FL.

5) **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from pretreatment of engineered switchgrass. Oral presentation (By Dr. Jian Shi) at the 38th Symposium on Biotechnology for Fuels and Chemicals, April 25-28, Baltimore, MD.

6) **Enshi Liu**, Lalitendu Das, Bingyu Zhao, Mark Crocker, Jian Shi. Fractionation and characterization of lignin streams from pretreatment of engineered switchgrass. Poster at 2016 KY NSF Super Collider, February 26, 2016, Lexington, KY.

7) Attendee at Chinese Society for Tropical Crops Conference: Cassava Biotechnology and Functional Genomics Research, October, 2012, Sanya, Hainan, China.

8) Jin Li, Yuzhen Zhang, **Enshi Liu**. Evaluation of compatibility between aged asphalt and rejuvenator using Hansen solubility parameters. Paper (No. 223) published at ASCE Geotechnical Special Publication, GeoHunan International Conference II: Emerging Technologies for Design, Construction, Rehabilitation, and Inspections of Transportation Infrastructures, June, 2011, Changsha, Hunan, China.

Awards and Honors

- Outstanding Poster Presentation Award at 2017 KY NSF Super Collider, 2017
- Poster Competition Award (2nd Place) at 2016 Frontiers in Biorefining Conference, 2016

  (https://www.uky.edu/bae/liu-receives-2nd-place-award-poster-biorefining-conference)
- Travel Award sponsored by the National Science Foundation (NSF) for attending 2016 Frontiers in Biorefining Conference, 2016
- American Society of Agricultural and Biological Engineers (ASABE), 2016

- Association of Overseas Chinese Agricultural, Biological, and Food Engineers (AOC), Board member (*Technology Director*) at Student Activity Committee, 2016 to present
- Alpha Epsilon, the honor society of ASABE, 2016
- ASABE Future City Regional Competitions, special award judge, 2016
- Award for Excellent Thesis (*Top 10%*), June 2011
- Excellent League Member of Hainan Province, May 2011
  (*3 people in Hainan University, 100 people out of 8 million in Hainan Province*)
- National Scholarship (*Top 0.2%, almost equal to 2 years of tuition*), November 2010
- The First Prize Scholarship (*Top 3%*), November 2010
- Merit Student of Hainan University, November 2010
- College Student with Most Innovative Spirit & Practical Ability (*one person in each class*), Twice, October 2010 & October 2009
- The 1st University Creative Science & Technology Design on Environmental Protection, 3rd Prize, *Team Leader*, June 2010
- Excellent League Member of Hainan University (*one person in each class*), Twice, May 2010 & May 2008
- Award for Excellent Essay on Social Practice in Hainan Province, 2nd Prize, December 2009
- The 3rd "Longsha Cup" Chemical Engineering Design & Business Competition, 2nd Prize, *Team Leader*, October 2009
- The Third Prize Scholarship (*Top 10%*), October 2009
- Merit Award of Hainan University Career Planning Competition (*10 students*), September 2009
- Outstanding Youth Volunteer of Hainan University (*10 students*), October 2008
- Social Practice Scholarship (*one person in each class*), September 2008

Extracurricular Activities

- **Student Affairs**
  - Class President, September 2008 to June 2011
- **Volunteer**

- Visitor Service at 2010 World Expo, Shanghai, China, August 2010
- Leader of the Reception Group at Boao International Tourism Forum, Haikou, China, March 2010
- Spectator Service at the 29th Olympic Games, Beijing, China, August 2008

Internship

- Baisha Gaodi Cassava Starch Factory, March 2012
- Zhuhai Yili Gourmet Powder Co., Ltd, December 2010
- Hainan General Sanyang Pharmaceutical Co., Ltd, June 2010
- Hainan Honz Pharmaceutical Co., Ltd, June 2010
- Haikou Asia-Pacific Breweries, September 2009