Biopolymer structure analysis and saccharification of glycerol thermal processed biomass

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Abstract

Glycerol thermal processing (GTP) is studied as a novel biomass pretreatment method in this research with the purposes to facilitate biopolymer fractionation and biomass saccharification. This approach is performed by treating sweet gum particles on polymer processing equipment at high temperatures and short times in the presence of anhydrous glycerol. Nine severity conditions are studied to assess the impact of time and temperature during the processing on biopolymer structure and conversion.

The GTP pretreatment results in the disruption of cell wall networks by increasing the removal of side-chain sugars and lignin-carbohydrate linkages based on severity conditions. After pretreatment, 41% of the lignin and 68% of the xylan is recovered in a dry powdered form by subsequent extractions without additional catalysts, leaving a relatively pure cellulose fraction, 84% glucan, as found in chemical pulps.

Lignin structural analysis indicated GTP processing resulted in extensive degradation of β-aryl ether bonds through the C-γ elimination, followed by abundant phenolic hydroxyl liberation. At the same time, condensation occurred in the GTP lignin, providing relatively high molecular weight, near to that of the enzymatic mild acidolysis lignin. Better thermal stability was observed for this GTP lignin. In addition to lignin, xylan was successfully isolated as another polymer stream after GTP pretreatment. The recovered water insoluble xylan (WIX) was predominant alkali soluble fraction with a maximum purity of 84% and comparable molecular weight to xylan isolated from non-pretreated fibers. Additionally, the narrow molecular weight distribution of recovered WIX, was arisen from the pre-extraction of low molecular weight water-soluble xylan.

Additionally, a 20-fold increase of the ultimate enzymatic saccharification for GTP pretreated biomass was observed even with significant amounts of lignin and xylan remaining on the non-extracted fiber. The shear and heat processing caused a disintegrated cell wall structure with formation of biomass debris and release of cellulose fibrils, enhancing surface area and most likely porosity. These structural changes were responsible for the improved biomass digestibility. Additionally, no significant inhibitory compounds for saccharification are produced during GTP processing, even at high temperatures. While lignin extraction did not promote improvement in hydrolysis rates, further xylan extraction greatly increases the initial enzymatic hydrolysis rate and final level of saccharification.

The serial of studies fully demonstrate glycerol thermal processing as a novel pretreatment method to enhance biomass saccharification for biofuel production, as well as facilitate biopolymer fractionation. Moreover, the study shows the impact of thermally introduced structural changes to wood biopolymers when heated in anhydrous environments in the presence of hydrogen bonding solvent.

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Chapter 1 Introduction and background

1.1 Introduction and background

Lignocellulosic biomass is mainly composed of cellulose, heteropolysacccharides, and lignin [1] and is an abundant resource to produce sustainable energy, as well as a variety of precursor chemicals and biobased materials. In the past century biomass has been central to modern society as cellulose derived from plants, mainly wood, is the critical resource for the pulp and paper industry. Accompanied with its derivatives [2], cellulose has been developed in a wide range of applications since the 1800's as gun cotton, textiles, coatings, photographic film, thermostable plastics, packaging [3, 4], filtration, dialysis membranes, and pharmaceuticals [5, 6]. Heteropolysaccharides, namely hemicelluloses, represent complex structural polysaccharides that are different from cellulose in the plant cell wall. The most common hemicellulose is 4-O-methyl-glucuronoxylan, which widely occurs in hardwood species. Due to its biodegradability and film oxygen barrier property, hardwood xylan has been proposed to be a functional additive in food packaging, edible films and biomedical materials [7-9]. Also, xylan derivatives, through etherification and esterification, provide a promising approach for applications in drug delivery, paper strength additives, surfactants and antimicrobial agents [10-12]. Furthermore, lignin isolated from woody biomass provides an abundant natural resource for phenolic compounds, which have potential applications in the area of polyurethane polymers, phenolic resins, antioxidants, surfactants and biodispersants [13-18]. However, cellulose derivatives and ligninsulfonates are the only materials that have been developed for commercial markets. Part of the issue is that there has not been a consistent source of the other biopolymers at costs that are more competitive than synthetic analogs. New methods to isolate these biopolymers are critical to produce a wide array of biochemicals and bioplastics, and the earth might just well depend on it.

Earth is at a critical cross-road with the global rise of fuel demand with the depletion of fossil fuels and the dire need for the reduction of green gas emission has motivated the production and utilization of renewable energy resources such as wind, solar, nuclear and water. Additionally, biofuel produced from sustainable biomass is another major alternative. The first generation biofuels are mainly derived from corn-starch or sugarcane through batch fermentation. However,

debates and concerns [19] have arisen considering the competition between food and fuels as well as additional emission of greenhouse gas [20] from the production of first generation biofuels. As an alternative, lignocellulosic biomass has become attractive as a feedstock for biofuel production [21] due to its low cost, abundance, drought tolerance, and higher bulk density compared to crop residuals and energy grasses. Polysaccharides account for approximately 70% dry weight of lignocellulosic biomass, which can be enzymatically hydrolyzed to pentoses and hexoses, and further fermented to ethanol and/or butanol.

Although lignocellulosic biomass has attracted wide attention for biomaterials and fuel production, extensive studies are still in progress to efficiently fractionate the lignocellulosic biomass for different applications. The lignin, cellulose and hemicellulose form a recalcitrant network in plant cell walls, which restrict biopolymer fractionation and the bioconversion of polysaccharides. Thus, as the development of biorefinery industry, pretreatment has been proposed as an essential method to disrupt the protective network, facilitating enzymatic attack on polysaccharides for biofuel production and the biopolymer fractionation. Extensive research has found several pretreatment methods such as dilute acid, alkaline soaking, steam explosion, hydrothermal cooking, organosolv pulping, and ionic liquid dissolution, to enhance the biomass saccharification prior to the biofuel production. Among them, organosolv pretreatment has also been recognized as a promising method for high quality lignin production. Ionic liquid pretreatment was developed most recently, but the application is still in development considering its recovery and reduced efficiency problems when recycling the solvent. Other methods normally benefit the biomass digestibility, but would introduce severe hemicellulose degradation and lignin condensation. Until now, only steam-assisted biomass pretreatment has been reported for pilot-scale applications.

In this research, a novel biomass pretreatment method that utilizes common polymer extrusion equipment with glycerol as an additive has been developed and studied in detail to determine the impact of nine different levels of processing on properties. The specific objectives of this research are to:

- Evaluate the detailed mass balance of wood biopolymers during pretreatment and sequential extraction;

- Examine the lignin fractionation from pretreated biomass and detailed structure characterization to study the functionality of isolated lignin and how pretreatment affects lignin structure;
- Examine the effects of pretreatment on xylan fractionation and structural changes of isolated xylan;
- Determine the influence of pretreatment on biomass saccharification and explore the reason for enhanced digestibility;

To complete these objectives, this dissertation is composed of seven chapters. Chapter 1 provides the introduction and background for this entire research. Chapter 2 is the literature review on biomass pretreatment, biopolymer fractionation, novel techniques for biopolymer structure analysis as well as the recently reported extrusion and glycerol facilitated biomass pretreatment. Chapter 3 introduces and applies the novel pretreatment method on ground sweet gum fibers. A detailed mass balance of wood components after pretreatment and a series of extractions were examined. The mechanism of pretreatment induced changes of biomass composition is proposed in the chapter. Chapter 4 studies lignin fractionation from pretreated biomass with respect to the recovery yield, functionality, and detailed structural analysis. The proposed mechanism of how the pretreatment severity impacts lignin structure is presented. Chapter 5 studies xylan fractionation as another polymeric byproduct. The effects of pretreatment on xylan structure are exhibited. Chapter 6 focuses on the pretreated biomass saccharification to explore the influence of pretreatment and residual lignin and xylan on cellulose digestibility. Chapter 7 provides a summary and conclusions for this dissertation.

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Chapter 2 Literature review

2.1 Introduction to lignocellulosic biomass

Lignocellulosic biomass, including wood, herbaceous crops and agricultural residues, serves as an abundant resource for the developing biofuel and biochemical industries. The three main structural polymer components in lignocellulosic biomass are cellulose, hetero-polysaccharides known as hemicelluloses, and lignin. Among them, cellulose is the major constituent (~40-45% [1] dry weight basis), which is mainly responsible for structural support [2] lignocellulosic biomass. Hemicelluloses (~20-30% [1] dry weight basis) are the second major set of carbohydrate polymers in plant cell walls. In secondary cell wall, hemicelluloses coat the surfaces of cellulose microfibrils and form covalent bonds with lignin to contribute to cell wall organization and structural support. A recent finding [3] indicated that hemicelluloses also play a key role in regulating cell wall structure by influencing cellulose chain aggregation in biosynthetic processes. This result suggests close interaction between the polysaccharide components. Lignin is the second most abundant biopolymer (~10-30% dry weight basis) within in the secondary cell wall deposited during the death of the cell. Associated with the carbohydrate polymers, lignin serves as a thermosetting-like coating to aid in structural support water transport and provides protection towards insect and microbial attack [4]. Hence these structural biopolymers are closely associated at the molecular scale level and significant disruption to the cell wall must occur to extract individual components for utilization in a biorefinery.

2.1.1 Cellulose

Cellulose is a linear homopolysaccharide composed by β-D-glucopyranose units linked through 1,4-glycosidic bonds [2]. Cellobiose is the repeating unit of cellulose that occurs as two inverted glucose units. However, the number of glucose units in a cellulose chain is referred to as the average degree of polymerization (DP), which varies dependent upon source and location in the cell wall. Normally, the DP of a cellulose chain is in the range of 9000~10,000 [5-8]. The large abundance of hydroxyl groups along the cellulose chains renders them with a high tendency to form intra- and inter-molecular bonds. Furthermore bundles of cellulose chains are aggregated to form cellulose microfibrils, which further develope as the supporting framework of the cell wall

[1, 9]. Packing of the cellulose with a high chain regularity [10] results in highly ordered crystalline fibrils. Unusual from other polysaccharides, cellulose is a highly crystalline polymer with four principle polymorphs [11-13] dependent upon treatment. All native cellulose derived from plant tissue has the structure of cellulose I [14] with a crystallinity index of approximately 65% [7], where the cellulose chains align parallel with each other [14]. This highly-ordered crystalline region serves as a barrier towards enzymatic attack, as well as limits solubility and chemical processing; in contrast, the amorphous region is more vulnerable to chemical and enzymatic degradation and conversion [15]. Some pretreatment processes for biorefining, highlighted below, create acidic environments that give rise to partial depolymerization of cellulose chains from these less ordered regions as typical in steam-explosion and dilute acid pretreatments.

2.1.2 Hemicelluloses

Hemicelluloses, more precisely a group of heteropolysaccharides, refers to complex co-polymers of carbohydrates composed of D-xylose, 4-O-methyl-glucouronic acids, D-galactose, D-mannose and L-arabinose. Compared to cellulose, hemicellulose have a lower DP that range from 100 to 200 [1]. Structure and composition of hemicelluloses in biomass are highly dependent upon species. The primary hemicellulose in hardwood is O-acetyl-4-O-methylglucurono-β-D-xylan (glucuronoxylan, 15~30% wood dry weight) with β-(1-4) linked xylan chain as the backbone [16]. About 1 uronic acid occurs on the side group per 10 xylose units through 1-2 linkages. There are about 3.5 to 7 acetyl groups per 10 xylose units normally appearing on C2 or C3 position of xylopyranose ring, which can be easily cleaved to form acetic acid in acid-catalyzed pretreatments. In addition to xylan, the secondary hemicellulose in hardwoods is glucomannan, which accounts for 2-5% of dry wood. Compared to hardwoods, galactoglucomannan is the principle heteropolysaccharide in softwood with arabinglucoronxylan as the secondary component. Galactoglucomannan represents of 20% of dry matter of softwoods and is composed of 1-4 linked β-D-mannopyranose and β-D-glucopyranose units at a proportion ratio of 3/1 to 4/1, with galactopyranoses occuring as terminal units [17]. Normally, there is 1 O-acetyl group on the C2 or C3 position per 3 to 4 hexose rings [1]. The hemicellluloses are typically the most sensitive to biorefining operations with significant depolymerization occurring during many treatments.

2.1.3 Lignin

Lignin is a phenolpropanoid polymer widely occurring in lignocellulosic biomass. In lignocellulosic biomass, 60-70% of lignin is distributed in the secondary cell wall, where it forms covalent bonds (ester, ether or glycosidic bonds) [1] with heteropolysaccharides in a rigid structural matrix. This structure serves as a barrier for biomass fractionation and inevitable structural modification occurs on the isolated lignin, dependent upon the isolation methods.

There are three precursors for lignin biosynthesis: coniferyl, sinapyl and p-coumaryl alcohols, which are enzymatically polymerized through free radical coupling [18, 19]. Correspondingly, the three main units composed of lignin are categorized as guaiacyl (G) (from coniferyl alcohol), syringyl (S) (from sinapyl alcohol) and p-hydroxyphenyl (H) (from p-coumaryl alcohol) units, whose proportions are highly dependent upon biomass species and lignin location in woody tissues. Lignin in softwood is mainly composed of guaiacyl units and minor p-hydroxyphenyl units, whereas, lignin in hardwood and herbaceous plants is generally derived from a complex of syringyl and guaiacyl units with trace amounts of p-hydroxylphenyl units [1]. For hardwood plants, lignin in the secondary cell wall is dominated by syringyl units compared with the guaiacyl type lignin in middle lamella and cell corners [20]. Except for the complexity in structural units, a number of various linkages are formed during the lignin free radical coupling process, which can be assigned to two major categories: C-O bonds (β-Aryl ether, α-Aryl ether) and C-C bonds (β-1, β-5 and 5-5). It is generally reported that the C-O linkage is the most common bond type, which accounts for over 70% of linkages [4] in lignins. Although lignin was first documented in 1838 [21], its structure is still in controversy due to its heterogeneity and modification that arises during isolation. Earlier studies extensively proposed that lignin had a cross-linked network structure [22, 23], whereas as the application of more advanced techniques such as two-dimensional nuclear magnetic resonance and mass spectrometry, suggested a more linear oligomeric model for the isolated lignin [24].

2.2 Biomass pretreatment

Biofuel is a liquid fuel, such as ethanol and butanol, produced from biomass. The global rise of fuel demand with the depletion of fossil fuels, and the concern of greenhouse gas emission implemented in anthropogenic global warming have motivated renewable biofuel production

from sustainable biomass [9, 25, 26]. The first generation biofuels were mainly derived from corn-starch or sugarcane through batch fermentation. However, there has been much debate and concern [27] of this first generation biofuel surrounding the competition between food and fuels as well as increased greenhouse gas emission [28]. As a potential solution, lignocellulosic biomass has become more attractive as a feedstock for biofuel production [29] due to its relative low cost and large abundance. Lignocellulosic biomass contains approximately 70% dry weight of polysaccharides, and if it can be accessed then it can be enzymatically hydrolyzed to pentoses and hexoses, and further fermented to ethanol and butanol.

Figure 2-1 shows the schematic of a common microbial biofuel production platform from lignocellulosic biomass [30]. As revealed above, the lignocellulosic biomass has a complex structure that limits its enzymatic digestibility, known as its recalcitrance, based on biomass substrate structural properties, arrangement of hemicellulose and lignin, as well as low cell wall porosity (enzyme accessible area) [31]. For example, hemicelluloses and lignins in lignocellulosic biomass serve as barriers for the enzymatic action on cellulose and more importantly, lignin can adsorb cellulose, causing inefficient binding of the cellulose substrates [32, 33]. Usually, larger surface area is a prerequisite for improving enzymatic digestibility [34]. Also it is reported that a lower degree of polymerization of the biomass substrates could facilitate the accessibility of enzymes and solvents, as well as enhance the enzymatic hydrolysis rate [35]. Some studies indicated cellulose crystallinity was a dominant factor for enzymatic saccharification suggesting high crystallinity limited enzymatic hydrolysis [36-38]. Other studies disputed this idea of cellulose crystallinity limiting hydrolysis, where conversion occurred for biomass and the efficiency was related to the degree of polymerization and the accessible surface area [35].

Pretreatment by disrupting the native cell wall structure is necessary prior to the enzymatic hydrolysis step for efficient biofuel production. The pretreatment processes can have various effects on the cell wall structure as outlined below; as a result, the polysaccharides become more enzyme-accessible for the subsequent hydrolysis [39].

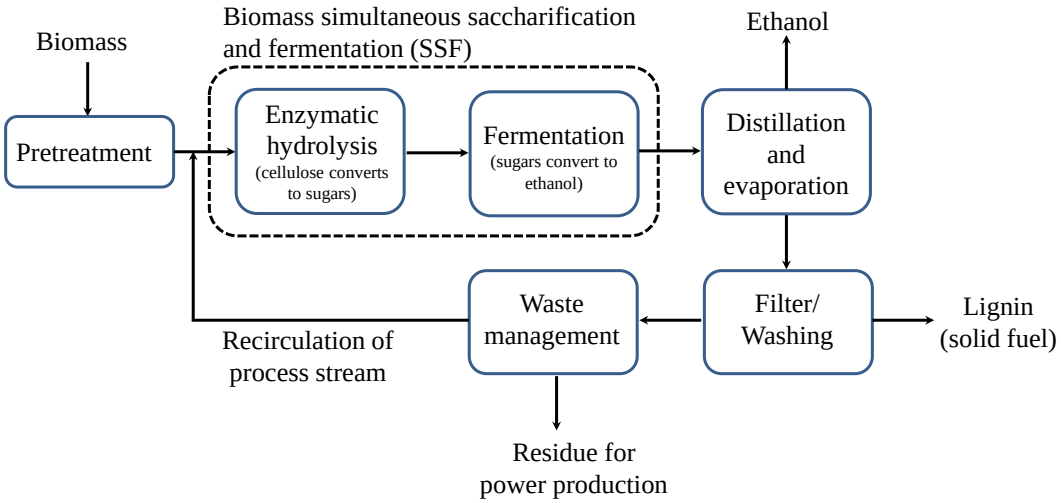

Figure 2-1 Scheme of lignocellulosic biomass microbrial conversion to biofuel. Galbe, M. and G. Zacchi, Pretreatment of lignocellulosic materials for efficient bioethanol production, in Biofuels, L. Olsson, Editor. 2007, Springer Berlin Heidelberg: New York. p. 41-65. Used under fair use, 2014.

In addition to biofuel production, pretreatment is also critical for effective fractionation of the components from lignocellulosic biomass, since it can efficiently disrupt the cell wall network composed of cellulose, hemicelluloses and lignins [40]. In one sense, a single fractionation approach is used in the pulp and paper industry with high quality cellulose isolated through delignification of woody biomass. In the biorefinery approach, hemicellulose can be isolated, especially xylan, and can be widely used in the food and pharmaceutical industries or as oxygen barrier coatings if not used as a carbon source [41-44]. Functionalized lignin isolated from woody biomass provides an abundant natural resources for phenolic units, which have the potential application in the area of polyurethane polymers, phenolic resins, antioxidants, surfactants and biodispersants [45-50]. Thus, by developing the pretreatment technique, lignocellulosic biomass is able to provide not only liquid fuels but also high-value biochemicals if the polymers can be fractionated.

During the last three decades, extensive studies have generated different pretreatment methods to overcome the recalcitrance of lignocellulosic biomass for efficient enzymatic saccharification. A successful pretreatment should meet the following criteria [27, 31, 32, 51, 52]: (1) improve the enzymatic digestibility of pretreated biomass, even with reduced enzyme loading; (2) most of the

pretreated biomass should be recovered in a useable form without severe sugar degradation into by-products; (3) the formation of inhibitory by-products are avoided, such as furfural and lignin degraded phenols, for both enzymatic hydrolysis and fermentation; (4) isolation of lignin as co-products for value-added applications; and (5) be cost effective with low capital and energy investment.

The common pretreatment methods can be generally divided into four categories [30]: physical, chemical, biological and a combination of these methods. In reality, none of these methods is viewed efficiently for all biomass species considering differences in composition and structural properties. Now, combinatorial protocols such as physicochemical methods have been accepted as the most promising and efficient pretreatment technologies, which have led to extensive research and even pilot scale tests.

2.2.1 Physical pretreatment

This method is a mechanical strategy to reduce the biomass particle size through chipping, grinding and milling. Chipping can provide a particle size of 10-30 mm while grinding and milling can reduce the size to 0.2-2 mm. With the reduction of particle size, the biomass surface area is increased with a decrease in cellulose crystallinity [53] and degree of polymerization. All of these factors contribute to an improvement in biomass digestibility. However, little improvement of digestibility was observed after particle size was smaller than 0.4 mm [54]. The mechanical pretreatment has been shown to enhance anaerobic digestibility of solid waste [55]. Ball-milling was extensively studied to improve digestibility of lignocellulosics or cellulose. Figure 2-2 showed the improved saccharification of newspaper by extending ball-milling time [56, 57]. The maximum of 72.9% cellulose-to-glucose conversion was reported for ball-milling newspaper for 7 days [58]. An advantage of mechanical comminution is that no degraded inhibitor compounds such as furfural, 5-hydroxymethylfurfural and acetate are produced for the enzymatic saccharification and fermentation [59]. However, it is not a feasible method to be adopted alone especially for woody biomass in large-scale considering the long-lasting pretreatment time and extensive energy investment [56, 60, 61]. The most common application of the physical pretreatment is serving as a pre-processing or post-pretreatment [60] to reduce biomass particle size in order to improve the efficiency of pretreatment or enzymatic digestibility.

Except for comminution, other forms of physical pretreatment were also investigated, such as ultrasonic irradiation [62] but these methods are energy intensive and the scalability is questionable.

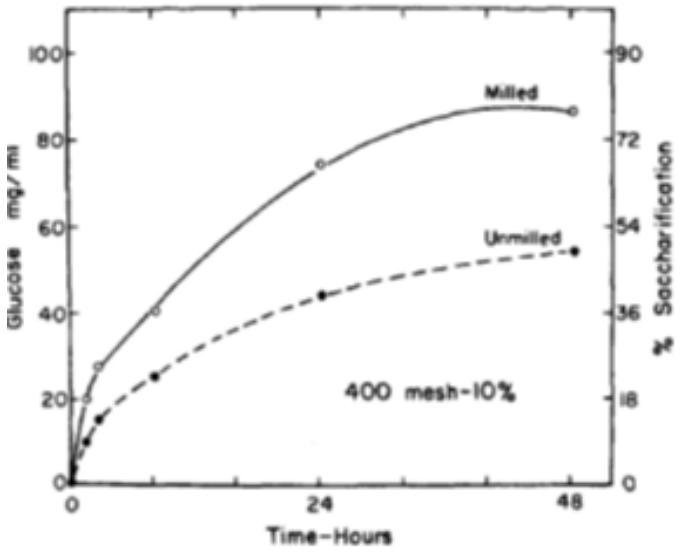

Figure 2-2 Enzymatic saccharification of ball-milled newspaper. Mandels, M., L. Hontz, and J. Nystrom, Enzymatic hydrolysis of waste cellulose. Biotechnology and Bioengineering, 1974. 16(11): p. 1471-1493. Used under fair use, 2014.

2.2.2 Chemical pretreatment

Chemical pretreatment typically involves acid or alkali catalyzed reactions in aqueous, organic, or more recently developed ionic liquid media, normally occurring at elevated temperature. Chemical pretreatment is one of the common methods used for the biorefinery to produce highly digestible biomass, and some of chemical pretreatment protocols such as organosolv are also attractive for high quality lignin isolation [63].

2.2.2.1 *Dilute acid pretreatment*

Dilute acid pretreatment is one of the most effective pretreatment methods to enhance biomass enzymatic hydrolysis [64-66]. In this method, sulfuric acid has received the most attention as the choice of acid, and other acids include hydrochloric acid, nitric acid, and phosphoric acid have also been studied [67, 68] for their effectiveness.

Dilute sulfuric acid with a concentration between 0.2%-2% is mixed with biomass at temperature ranging from 140 ℃ to 215 ℃ and processed from several minutes to one hour [69]. After pretreatment, the biomass residue has improved cellulose enzyme accessibility with almost complete removal of xylan. Thompson et al. [70] also reported enhanced specific surface area and enzymatic digestibility for the pretreated biomass as a consequence of nearly complete removal of hemicellulose for the mixed hardwood samples from dilute sulfuric acid pretreatment at 200 and 220 ℃. During this process, most of hemicelluloses especially xylans are hydrolyzed to sugars, however at elevated temperatures with the acid catalyst, hydrolyzed xylose might be further be degraded into compounds that are inhibitors for fermentation such as furfural. Kinetic studies [31, 71, 72] of xylan hydrolysis during the dilute sulfuric acid pretreatment indicated sequential first-order reactions when the pretreatment temperature was higher than 160 ℃, whereas, two parallel pseudo-first-order reactions occurred including the fast-hydrolyzing xylan reaction and the slow-hydrolyzing xylan reaction when the pretreatment temperature was lower than 160 ℃. Percentage of fast-hydrolyzing xylan in hardwood was estimated to be 70% to 80%, which was higher than herbaceous substrates [71], demonstrating that the pretreatment process must be tailored to the biomass type. During dilute acid pretreatment, β-O-4 bonds in lignin are readily cleaved accompanied with recondensation reactions due to the high temperature acid-catalyzed process [73]. Only a small amount of lignin (15% to 18% of lignin in hardwoods and 3% to 12% of lignin in grass) was solubilized during the acid pretreatment with 40% to 100% of lignin solubilization occurring in the initial heating period [74].

The dilute sulfuric acid pretreatment has been successfully performed on a wide range of biomass, from wood, agricultural residue to herbaceous crops. Torget and co-workers [66, 75] reported a highly digestible cellulose substrate (90% to 100% of cellulose digestibility for hardwoods and 85% to 90% for grass) after dilute sulfuric acid pretreatment at 140 and 160 ℃ for reaction times from 5 to 60 min for a series of short rotation hardwood and herbaceous crops. Aspen, balsam and switchgrass as examples of hardwood, softwood and herbaceous crops were studied by Jensen et al. [76] using sulfuric acid pretreatment with concentrations between 0.25% to 0.75% at a temperature range of 150 to 174 ℃. They found aspen achieved total sugar yields around 83.5% at all conditions, whereas higher acid concentrations were required for switchgrass to obtain maximum sugar yields around 96.5% considering the neutralizing effects of minerals

and ash in switchgrass. Balsam as a type of softwood remained recalcitrant with sugar yields lower than 25%. Hsu et al. [77] found 1% sulfuric acid pretreatment of rice straw at 160 or 180 ℃ for 1 to 5 minutes could effectively remove xylose and limited amounts of glucose, so that a 70% enzymatic saccharification was obtained from the pretreated biomass. Other kinds of agricultural residue such as wheat straw [78] and corn stover [79] (76% and 92.5% of total sugar yields for wheat straw and corn stover) were also successfully studied as feedstocks of dilute sulfuric acid pretreatment.

Softwood has a higher lignin content compared to hardwood and herbaceous crops and is harder to be fermented to ethanol through the common pretreatment methods for hardwood and grass. A two-stage dilute acid pretreatment was performed to enhance softwood digestibility and to avoid the production of inhibitory compounds resulting from high temperature pretreatment. Nguyen et al. [80] investigated a two-stage hydrolysis with 0.7% sulfuric acid at 190 ℃ for 3min followed by a 0.4% acid treatment at 215 ℃ for 3 min. This method was investigated to serve as a replacement for enzymatic hydrolysis by producing ethanol from the sugars in the hydrolysate. Research by Sannigrahi et al. [73] showed the major components of pretreated loblolly pine were lignin and glucan after two-stage acid pretreatment but with an increase in cellulose crystallinity due to the reduction of amorphous cellulose structure. This increase of crystallinity probably increases the barrier for cellulose enzymatic hydrolysis, where additional tailoring needs to be performed to improve the efficiency of biomass digestibility.

Dilute acid pretreatment works efficiently on a wide range of lignocellulosic biomass besides softwoods, leaving highly digestible biomass substrates with almost full removal of hemicellulose. Instead of enzymatic saccharification, acid pretreatment can also directly hydrolyze cellulose and hemicelluloses to hexoses and pentoses for future fermentation. However, one of the major concerns of dilute acid pretreatment is the formation of fermentation inhibitors. High temperature favors the pretreatment efficiency but with the risk of producing fermentation inhibitors from degradation of hemicellulose or even the hydrolyzed hexoses and pentoses [59, 81] such as furfural, 5-hydroxymethylfurfural, acetate or even further degraded levulinic acid and formic acid. Low molecular weight phenolics resulted from lignin degradation during acid pretreatment such as syringaldehyde, vanillin and vanillic acid are more inhibitory to the microbial activities [82]. Thus, additional water washing is necessary to remove the

inhibitors. In addition, neutralization after the acid hydrolysis to remove the extra acid is a prerequisite for the following enzymatic saccharification, leading to higher investment especially for two-stage acid pretreatment. Lignin resulted from dilute acid pretreated biomass is highly condensed with lower application values [73]. Other concerns including the acid corrosion on biomass handling equipment, pipes and instruments also need to be considered when evaluating a biorefinery process.

2.2.2.2 *Alkali pretreatment*

Bases such as sodium, calcium, potassium and ammonium hydroxide are widely used in alkali pretreatment to improve biomass digestibility. During pretreatment in alkali reagents, most notably NaOH, biomass is swollen with an increase in internal surface area and enzyme accessibility, whereas the degree of polymerization and crystallinity of cellulose is decreased [56]. The general mechanism [83] of dilute alkali pretreatment is described as saponification of the intermolecular ester bonds formed between xylan and other biopolymers such as lignin. In the native state, crosslinks due to the uronic ester bonds on xylan sidegroups, usually 4-O-methyl-D-glucuronic acids, limit biomass swelling. During pretreatment in alkali solution, cleavage of these ester bonds readily happens, which results in removal of the lignin-carbohydrate crosslinks and increase in biomass swelling capacity as indicated by increases in intrafbril porosity and channel size [83]. Accompanied with saponification, a major portion of lignin (60-80% [84-87] of initial lignin) is removed. Partial degradation of xylan (10-40% [88] of initial xylan dependent on the alkaline concentration) as well as removal of the acetyl [89] and uronic acid side groups from xylan [27, 30] also occur. All these effects lead to more digestible cellulose-enriched substrates. The efficacy of alkali pretreatment is highly dependent upon the lignin content of biomass [30, 31]. Agricultural residues and herbaceous crops with lower lignin contents result in better biomass digestibility after alkali pretreatment compared to wood species. For hardwoods, lignin contents decrease from 24% to 18%, the pretreated biomass digestibility increases from 10% to 40% (Figure 2-3) [90]. In contrast, there is little or no improvement on softwood species due to the relatively high lignin contents between 26% to 35% [90].

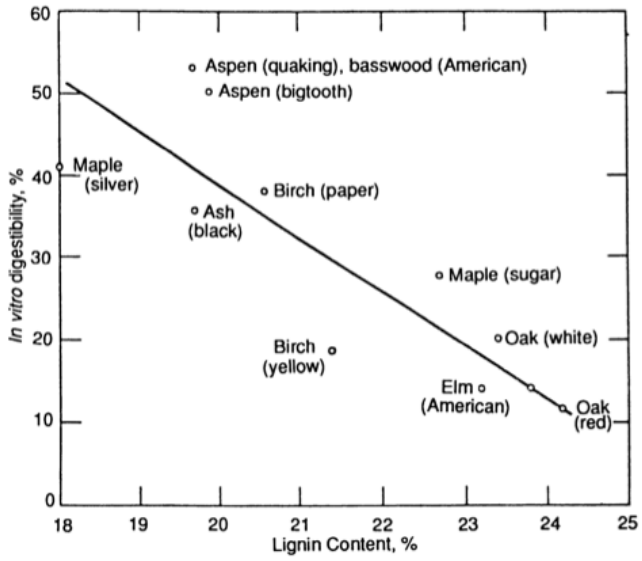

Figure 2-3 Effect of lignin content on the in vitro digestibility of NaOH-pretreated hardwood. Millett, M.A., A.J. Baker, and L.D. Satter. Physical and chemical pretreatments for enhancing cellulose saccharification. in Biotechnol. Bioeng. Symp.;(United States). 1976. Dept. of Agriculture, Madison, WI. Used under fair use, 2014.

Alkali pretreatments are now most widely studied through a combination with other techniques in order to enhance cellulose substrate digestion. A combination of wet oxidation and Na_2CO_3 alkaline hydrolysis of wheat straw was reported to reach the maximum of 65% and 50% of lignin and hemicellulose removal with 85% of cellulose-to-glucose conversion after the optimum pretreatment condition at 170 ℃, due to the combinatorial effects by wet oxidation and simultaneously solubilization of degraded lignin and hemicellulose in alkali [85]. Other techniques including wet-milling, microwave [91-93], radio frequency based dielectric heating [94] and nano-TiO_2 photocatalysis[95] assisted alkali pretreatment were studied recently on straw and grass, showing their effectiveness on lignin and hemicellulose degradation in order to produce more digestible substrates. It is unknown how some of these methods would be scaled to meet the requirements of a biofuels conversion plant.

Compared to the mostly studied alkali pretreatment using NaOH, $Ca(OH)_2$ is another important alkali reagent in this technique that is more efficient, economic (~$70/ton for hydrated lime compared to ~$325/ton for 50% NaOH liquid in 2005 [96]) and easier to recover after pretreatment. Calcium hydroxide based alkali pretreatment, normally named as lime pretreatment

(LP), is a method using lime loading of 0.1~0.5 g/g dry biomass and water loading of 5~15 g/g dry biomass to pretreat straw, grass and even hardwood under different temperature, pressure and time conditions to effectively remove lignin but without significant loss of carbohydrates. Two formats of lime pretreatment are studied mostly: the short-term pretreatment takes place at high temperature between 85~160 ℃ normally with pressure, which can be finished in 2 to 6 hours; on the other hand, the long-term pretreatment happens at ambient conditions with temperature ranging from 25 to 75 ℃ but lasts up to 8 to 12 weeks [54, 97-99]. Studies on LP pretreated herbaceous and agricultural residues such as bagasse [99], wheat straw [99], switchgrass [54], corn stover [100] indicated an enhanced substrate digestibility over 85%. For poplar with relatively high lignin content, an oxidative LP at 150 ^{o}C with 14 bar absolute oxygen was effective to remove 78% of lignin and 49% of xylan to reach as high as 97% sugar conversion at excess enzyme loading [101]. Also, Sierra et al. [98] applied the long term LP at ambient conditions on poplar showing a maximum of 7.5-fold improvement compared to the unpretreated input. Recently, an attempt [102] using the combination of 0.02 g lime/g dry biomass and 0.1 g NaOH/g dry biomass to pretreat switchgrass was conducted for more cost-efficient alkali pretreatment at ambient temperature. From their results, the optimum condition could reach a 34.5% higher total sugar conversion than the pure 0.1 g NaOH/g dry biomass pretreatment, and comparable results as using pure 0.2 g NaOH/g dry biomass. Another added value was lime pretreatment reduced the chemical cost by 43.4%.

Other alkaline pretreatments involve KOH, hydrazine and liquid ammonia are also studied [27]. A bioethanol pilot plant started by DuPont in 2010 uses mild liquid ammonium to pretreat a series of agricultural residues and grass including stalks, switchgrass, corncobs and dried leaves. Ethanol production capacity was reported about 680 and 90 gallons from every acre of switchgrass and every ton of corncobs [103]. Overall, alkali methods could avoid the formation of fermentation inhibitors such as furfural and hydroxymethyl furfural compared to acid pretreatment, since lignin removal is the main mechanism for the resultant digestible substrates. Lime pretreatment, as one of the highest potential methods in this category, has several additional benefits like lower cost, safety, ease to be recovered through carbonation [54] and application at ambient conditions, which can save the investment on high pressure reactors. However, as mentioned above, alkali pretreatment is more efficient on biomass with low lignin

content and there is no significant improvement reported for softwood that has high lignin content. Also, neutralization and water washing is necessary for the downstream processing.

2.2.2.3 *Organosolv*

Organosolv delignification has been scaled to pilot scale trials in the pulp and paper industry, and utilized as a pretreatment method, but with lower degrees of delignification degree. Organosolv pretreatment has been demonstrated as a promising method to efficiently reduce the recalcitrance of biomass and convert it to a more digestible cellulose substrate while recovering a higher quality lignin. In this method, the aqueous organic solvent mixtures are utilized to process biomass chips with or without acidic catalysts at high temperatures and pressure. Organic solvents adopted here include some low boiling point alcohols like methanol and ethanol, high boiling point alcohols like ethylene glycol and glycerol, as well as ketones, ethers, dimethyl sulfoxide, and phenols [27, 104]. Among them, ethanol based organosolv pretreatment has been most widely studied. When non-catalysts are involved, autohydrolysis functionalizes during the pretreatment since the presence of auto-ionized water at high temperature facilitates the cleavage of acetyl groups from hemicellulose especially xylan to form acetic acids, which further serve as acidic catalysts for this pretreatment [105]. A variety of extra catalysts could be added including mineral acids like H_2SO_4 and HCl, or organic acids such as acetic acid, oxalic acid, and salicylic acid [106].

Under the influence of organic solvents and catalysts, the main mechanism of organosolv pretreatment for digestible substrate production can be explained as the hydrolysis and cleavage of 1) bonds between lignin and carbohydrates, 2) ether bonds [107] in lignin, and 3) glycosidic bonds in hemicellulose. This process is followed by the dissolution of degraded lignin into the pulping liquor [106, 108]. After pretreatment, the residual biomass is a cellulose-rich substrate that could be readily hydrolyzed by enzymes; whereas, low molecular weight and higher quality lignin usually can be recovered from organic solvents. In addition, the hydrolyzed hemicellulose oligomers and monomers, or even their dehydrated products such as furfural or hydroxylmethyl furfural are left in the aqueous stream. From this point, organosolv pretreatment can not only result in digestible substrates for biofuel production, but also provide a method to successfully fractionate the three major wood polymers for individual applications.

Most of the organosolv pretreatments occur over a wide temperature range between 100 and 250 ℃ [108]. The optimum condition for ethanol organosolv pretreatment is in the temperature range between 160 to 190 ℃ for 30 to 60 minutes, with the ethanol concentration of 40-65%. Normally, a portion of 0.5 to 1.2% of sulfuric acid is added in the pretreatment as catalysts. However, it was suggested adding the acid catalysts was not necessary when the pretreatment temperature was higher than 180 ℃ since the acetic acid from lignocellulosic biomass itself could be released and further catalyze this process [104]. Saddler's group investigated the influence of ethanol organosolv pretreatment conditions on physical properties of hybrid poplar [109]. From their reports, more severe pretreatment conditions like high temperature and/or high acid concentration led to the reduced degree of polymerization, higher crystallinity and smaller fiber length of the residual cellulose substrates due to the partial hydrolysis of cellulose chains as well as removal of hemicellulose and lignin. On one hand, these results could generate a more digestible substrate; but on the other hand, the depolymerization would also reduce the sugar recovery yield for ethanol production. Thus, the condition optimization is necessary in organosolv pretreatment.

Low boiling point alcohol pretreatment is already studied on various lignocellulosic biomass to show its efficacy. Pan et al. [110] investigated 65% ethanol organosolv pretreatment on lodgepole pine killed by mountain pine beetle. Their results showed up to 75% of initial cellulose could be recovered as digestible substrates after pretreatment, which yielded 97% digestibility within 48 hours' hydrolysis. On the other hand, 79% of initial lignin was collected as ethanol organosolv lignin (EOL). Brosse et al. [111] adopted the same procedure but with a dilute acid presoaking to pretreat *miscanthus* × *giganteus* and 95% of initial cellulose was left after pretreatment with a 98% digestibility in 48 hours. Similar as ethanol, aqueous methanol pretreatment was also successfully conducted on poplar [112] and eucalyptus grandis [113]. Moreover, aqueous acetone organosolv treatment on wheat straw was studied at 205 ℃ for 1 hour to render 79% of delignification and 93% of cellulose recovery, which could yield more than 5 times glucose compared to non-pretreated wheat straw after enzymatic saccharification [114]. Additionally high boiling point glycols especially ethylene glycol [115], glycerol [116] and even glycerol carbonate [117] were also adopted recently as green solvents to pretreat biomass for enhancing the enzymatic saccharification.

Organosolv pretreatment was viewed as the one of the most promising method for bioethanol production and especially for biopolymer fractionation. It is the only method that works effectively on softwood compared to other pretreatments. Normally, most solvents can be recovered from the downstream evaporation and reused, which reduces the costs to some degree. The main disadvantage of organosolv pretreatment is attributed to the production of fermentation inhibitors as the acid pretreatment due to the existence of acid in this process. Others like environmental and health concerns relating to organic solvents also need to be considered. Recently, a North American bioenergy company, Lignol, went out of business showing the difficulty to commercialize the process in light of dynamic market forces.

2.2.2.4 *Ionic liquids (ILs)*

Ionic liquids comprised entirely of organic cations with inorganic or organic anions, were first recognized as non-derivatizing cellulose solvents [118] and then further developed their applications in wood dissolution for improving enzymatic saccharification [119, 120]. Ionic liquids have a strong solvation power [121] to dissolve cellulose, lignin or more complicated wood at temperatures normally below 150 ℃ [118, 119, 122, 123]. Most ionic liquids used in pretreatment are imidazoium salts, such as 1-allyl-3-methylimidazoium chloride ([AMIM]Cl) and 1-butyl-3-methylimidazolium chloride ([BMIM]Cl). In the ionic liquid pretreatment, wood samples with particle size of 0.1 to 2 mm are simply mixed with ionic liquids at a concentration of normally lower than 8% [119]. Then, the mixture is heated and mechanically stirred at 80 to 120 ℃ for 8 to 24 hours [119]. The complete wood dissolution is achieved by using [AMIM]Cl, [BMIM]Cl or [C2MIM][OAc], [EMIM][OAc] depending upon the wood species [119, 121, 124]. In addition, smaller wood particle size is showed to facilitate the dissolution rate, which lead to better diffusion of ionic liquids into wood cell wall [119]. The presence of water impedes the dissolution process [121]. After dissolution, cellulose-enriched materials could be regenerated through adding an anti-solvent such as acetone, water, alcohol, chloroform, dichloromethane, acetonitrile or tetrahydrofuran [125], whereas about 30% and 70% of initial lignin for wood and grass remains in the solvents dependent on the pretreatment conditions [124, 126]. Around 12% of initial hemicellulose is reported to depolymerized into oligomers during IL pretreatment at higher temperatures and longer residence time, and further washed away by water [126, 127]. The mechanism of cellulose dissolution in ionic liquids could be explained as the disruption of

hydrogen-bonding network by cations ([$EMIM^+$]) or anions ([Cl^-]) in ionic liquids [128, 129], while the mechanism of lignin dissolution has not been well resolved. The regenerated cellulose-enriched biomass typically exhibit reduced crystallinity with polymorph changing from cellulose I to II, which is further known that cellulose II has enhanced digestibility compared to cellulose I polymorphs [124, 130], and enlarged surface area [126, 131]. As a result, the regenerated cellulose-enriched residue has significantly enhanced enzymatic digestibility, with over 90% of cellulose-to-glucose conversion occurring within the first 48 hours' of enzymatic hydrolysis [120, 127, 131, 132].

Ionic liquid pretreatment as a recently developed method is widely investigated in lab scale studies using different biomass such as cotton cellulose, spruce, pine, poplar, maple, wheat straw [125, 132]. However, there are still a number of issues requiring further research, such as the higher chemical cost, recovery and reuse of ionic liquids, and yeast toxicity. For example the efficacy of an IL decreases with water content [118, 121] and residual IL has been shown to impact the cellulose enzyme activity [133, 134].

2.2.3 Physicochemical pretreatment

Physicochemical pretreatment is a combinatorial technique including a large variety of promising pretreatments, such as steam explosion, hydrothermal, and ammonia fiber/freeze explosion. Some of them have been tested in pilot scale and are near commercialization [103, 135-138].

2.2.3.1 *Steam-explosion*

Steam explosion pretreatment was first introduced and patented in 1926 [139]. It has been widely studied recently for potential commercialization since it is a relatively simple process with limited chemicals, low cost, but widely effective for a range of biomass species. In this method, biomass chips are usually pretreated at high pressure (0.7-4.8 MPa) and high temperature (160-260 ℃) in saturated steam for several seconds to a few minutes [69], followed by a rapid decompression that causes vapor to expand rapidly within the pores, "exploding" the structure. To evaluate the steam explosion conditions in general, Overend and Chornet [140] proposed the concept of severity parameter in 1987, which combined the pretreatment time (t) and

temperature (T) into a single severity factor (R_0) through equation (1). To date, all the work related to steam explosion report their results based upon the logarithm of R_0 and this has even been adjusted for treatments that include a pH change.

$$R_0 = t \times e^{(T-100)/14.75} \quad (1)$$

The main mechanism of steam explosion can be attributed to the homolytic cleavage of acetyl groups, rendering acid hydrolysis of glycosidic bonds [141] in hemicellulose accompanied with breakage of lignin-hemicellulose complex [142]. Due to the high temperatures involved in steam explosion, the auto-ionized water degrades the glycosidc bonds and acetyl groups from hemicellulose, resulting in hydromium ions from acetic acid [105]. An acid-base titration by Li et al. demonstrated that the released acids had a comparative amount as the total content of acetyl groups in aspen [141]. The acetic acid with other acids released from biomass such as formic and levulinic acids [143] further catalyze the hydrolysis of ether bonds in lignin or the linkages between lignin and hemicellulose. As a result, hydrolyzed carbohydrates including monomeric and oligomeric sugars as well as degraded compounds such as furfural and hydroxymethylfurfural would dissolve in the water portion and be further removed through downstream water washing prior to enzymatic hydrolysis. Except for a limited removal (~10-20% of original lignin [140]), most of lignin undergoes the simultaneous depolymerization and repolymerization reactions [140, 144, 145], finally redistributing on fiber surfaces as a result of softening and agglomeration due to surface tension effects [146]. In addition, the rapid pressure release used to quench the pretreatment also leads to a fragmentation of biomass cell wall into fine particles [140]. A combination effect of these above reactions greatly improves the porosity of pretreated biomass substrates, which further contributes to an increased enzyme accessibility and digestibility [146]. More recently, Langan et al. [147] reported interesting morphological changes in biomass cell wall during steam explosion through a molecular dynamics simulation. From their research, cellulose fibrils experienced an irreversible dehydration process at high temperatures during steam explosion driven by thermodynamically entropy, leading to a more ordered fibril bundle structure. This finding was in accordance with the increase in crystallinity [148, 149] after steam explosion. At the same time, a phase separation happened between the surrounding hemicellulose and lignin with a simultaneous collapse of lignin into globules accompanied by hemicellulose removal and auto-hydrolysis. Research by Langan and co-

workers revealed the increase in accessible area and water-filled voids after steam explosion pretreatment at a molecular level. To a small extent, hydrolysis of glycosidic bonds also happens in cellulose fraction, resulting in a lower degree of polymerization and inevitable glucose loss especially at high pretreatment severity [150].

Steam explosion pretreatment can be conducted with or without additional acid catalysts. As early as 1983, Dekker et al. [151] reported that pretreated sugarcane bagasse could reach more than 80% of saccharification in 24 hours after steam explosion at 200 ℃ for 4 min. Similar effectiveness was obtained for rice straw [152], wheat straw [153] and sunflower stalks [154]. Grous et al. [34] showed the steam-explosion pretreatment impact on hybrid poplar by enhancing glucose yield above 90% after 24 hours enzymatic hydrolysis; this finding was related to a dramatic increase of accessible pore volume. A recent research study [155] about steam exploded *Eucalyptus globulus* revealed that as much as 99.5% cellulose digestibility was reached after the optimal pretreatment conditions (195 ℃ and 34 min) and 91% cellulose to ethanol conversion was obtained through simultaneous saccharification and fermentation of pretreated substrates. Overall, steam explosion without catalyst exhibits its efficiency in allowing conversion especially on hardwood and agricultural residue. However, adding acid catalysts or acid pre-impregnation such as dilute sulfuric acid (H_2SO_4) [156, 157], acetic acid [158], carbon dioxide (CO_2) [153] and sulfur dioxide (SO_2) [159, 160] was adopted in most of cases to reduce the pretreatment time, enhance the enzymatic hydrolysis of pretreated substrates, increase total sugar recovery and reduce the production of inhibitors such as acetate and furfural from hemicellulose degradation [161]. More importantly, the addition of acid catalyst is essential for steam explosion of softwood. Clark et al. [160] proposed that steam explosion with 2.55% of SO_2 at 215 ℃ for 3 min reached 82% of cellulose digestibility and 57.4% of total sugar yield for pinus radiate, which rendered the softwood a comparably good candidate to hardwood for enzymatic hydrolysis. The addition of SO_2 allowed steam explosion to proceed at lower temperature, promoted cellulose and hemicellulose hydrolysis, and at the same time limited carbohydrates degradation [162].

Steam explosion is one of the most promising pretreatment techniques and has been tested at the pilot-scale in several sites around the world, such as the Italian steam explosion pilot plant at ENEA in Southern Italy, NREL pilot plant in Golden, and a demonstration-scale ethanol plant of

Iogen in Ottawa [135-137]. It works effectively on a broad range of biomass feedstock including hardwood, agricultural residue and even softwood where acid catalysts are essential for conversion. But still, some disadvantages are inevitable involved during this process, such as incomplete deconstruction of lignin-carbohydrate complex, sugar loss due to degradation at severe conditions, and production of inhibitors [163] for subsequent enzymatic hydrolysis and fermentation. Due to the simultaneous degradation and repolymerization, the lignin in steam-exploded biomass is highly condensed with broad polydispersibility, which is not attractive for applications. After steam explosion, a downstream water washing is necessary to remove the resulted acids, inhibitors, and recover the hydrolyzed hemicellulose, which will increase the economic investment.

2.2.3.2 *Hydrothermal/liquid hot water pretreatment (hydrothermolysis/LHW)*

Hydrothermal pretreatment is similar to steam explosion but uses liquid hot water instead of steam to pretreat biomass at evaluated temperature (150-230 ℃) for desired residence time (15-20 min) [51, 164]. The hot water in hydrothermal pretreatment cleaves the O-acetyl and uronic acid [51] groups to generate acids, resulting in hemicellulose hydrolysis accompanied with lignin removal to render the cellulose substrates more accessible for enzymatic saccharification [165]. Three types of reactor configurations have been utilized in hydrothermolysis, namely co-current, counter-current, and flow-through [51]. In the co-current or counter-current reactors, the biomass and liquid hot water move in the same or opposite direction; in the flow-through reactor, the hot water is passed over a stationary bed of biomass. Yang and Wyman [165] compared the flow-through and batch process for corn stover, concluding that the flow through system enhanced hemicellulose and lignin removal resulting in more digestible cellulose substrates. As a large amount of water input is flowing to the biomass, a low solid loading of 1-8% [27] is used in the process. The solubilized hemicellulose and lignin are present in low concentrations reducing the tendency of formation of fermentation inhibitors and lignin condensation on biomass surface [166].

Hardwood and agricultural residue are more favorable as feedstock for hydrothermal pretreatment than softwood due to their higher contents of acetyl groups on hemicelluloses [164]. Normally, the hydrothermolysis is performed at a pH of 5~7 to minimize sugar degradation with

the addition of KOH to maintain the pH [167], which eliminates the washing or neutralization step after pretreatment. However, the large amount of water involved in this pretreatment renders an extremely low concentration of solubilized sugars, which need more energy investment for the downstream sugar recovery.

2.2.3.3 *Ammonia fiber/freeze explosion (AFEX)*

AFEX [168-170] is similar to steam explosion but uses anhydrous liquid ammonia instead of steam to pretreat biomass at high pressure but mild temperature (usually 50-90 ℃). In AFEX process, the lignocellulosic biomass is soaked in anhydrous ammonia in a ratio of 1:1 to 1:2 for 10-60 min followed by a rapid pressure release [27], which causes evaporation of ammonia and disruption of fibrous structure. AFEX pretreatment successfully results in an improvement of enzymatic digestibility of pretreated biomass with combinatorial effects of increase in enzyme accessible area, depolymerizing and decrystallizing cellulose [168, 169]. Over 90% digestibility of AFEX pretreated coastal Bermuda grass and bagasse was reported by Holtzapple [168] with extremely less enzyme loading (5 IU/g compared to regular 80 IU/g). Unlike the case of severe hemicellulose degradation that occurs during steam explosion, AFEX does not severely solubilize the hemicellulose because of the mild pretreatment temperatures, which as following saves the total sugar yields and limits the production of inhibitors [171, 172]. Lignin distribution remains similar after AFEX but with structure alteration that enhances the water-holding capacity and digestibility [27]. In contrast, a significant degree of delignification was reported at higher pretreatment temperature (160-180 ℃) with residence time of 14 minutes when using the ammonia recycle percolation (ARP) method [173].

AFEX has demonstrated its efficiency to improve saccharification for herbaceous crops and agricultural residues that have relatively low lignin content (<20%), such as alfalfa, corn stover, wheat straw, bagasse and switchgrass, with over 90% of glucan conversion after enzymatic hydrolysis [168, 171, 174]. However, it is not attractive for higher lignin content (>25%) biomass such as newspaper, hardwood and softwood [168].

Since ammonia is volatile at ambient temperature, almost all the ammonia can be recovered for reuse after explosion. It has been reported that after pretreatment, about 0.5-1% ammonia is left in the lignocellulose chemically bound as ammonium ions [169], moreover, the remaining

ammonia serves as a nitrogen source for the following fermentation step. In addition, because of limited sugar degradation and inhibitor production during AFEX, water washing is not necessary after AFEX.

2.3 Biopolymer fractionation from biomass pretreatment and structural characterization

In addition to improving biofuel production, pretreatment is an essential step to unlock the recalcitrant structure of the secondary cell wall for efficient biopolymer fractionation. As shown previously, pretreatment results in more accessible polysaccharides for enzymatic hydrolysis to produce a stream of hexoses and/or pentoses; at the same time, lignin becomes easier to be isolated and recovered for other applications. However, it should be noted, that often the fermentation residuals from some of the pretreat processes needs to be further reacted to extract out uniform lignin streams.

2.3.1 Isolation of high quality lignin

Lignin is an abundant phenolic bioresource that can be extracted from the secondary cell wall and utilized in a variety of applications. However, the majority of the current commercial applications of lignin are limited to serving as surfactants. One reason of limited adoption in commercial applications is that lignin is embedded in a rigid cell wall structure where it forms covalent bonds with heterpolysaccharides, which actually restricts its isolation without significant degradation. Moreover, all the isolated lignin actually is in a modified state dependent upon the isolation method since the bond cleavage during lignin isolation is often accompanied with radical initiated repolymerization. The paper-making industry is the major source of isolated lignin from high quality sulfite pulp production where the lignin is sulfonated at the alpha position making it water soluble with minimal degradation [175]. Whereas the resulting lignin from kraft processes is partially depolymerized and recondensed into fractions that are commonly used as a heating resource in a paper mill [45, 176].

Several pretreatment and lignin isolation methods have been developed and studied recently for the production of more native lignin byproducts. Among them, milled wood lignin (MWL) and enzymatic mild acidolysis lignin (EMAL) are accepted as the best representatives for native lignin. As early as 1956, Björkman [177] proposed the method to isolate milled wood lignin

through ball-milling extractive free biomass in the presence of toluene in jars and balls of agate. Usually, this ball-milling lasted between 48 hours and 14 days. After that, a dioxane/water (9/1,v/v) extraction was conducted to isolate lignin from the milled wood. Although the MWL was viewed having less structural modification compared to native lignin, inevitable lignin depolymerization and oxidation occurred due to the extensive ball-milling [178]. In addition, the yield of MWL was usually less than 32%, which was not enough to fully represent the total lignin in wood. In 1975, Chang et al. [178] reported a method to isolate cellulolytic enzyme lignin (CEL) through combining ball-milling, enzymatic hydrolysis and dioxane solvent extraction, which was applicable to both hardwood (sweetgum) and softwood (Norway spruce). The CEL approach improved lignin yield by providing two lignin fractionations with small structural differences compared to MWL, but it also involved a large quantity of cellulolytic enzymes and solvents, which caused protein contamination and solvent waste. In recent studies, Argyropoulos et al. [179, 180] developed a two-step procedure to isolate lignin in higher yield and purity from ball-milled wood, through combining an initial enzymatic hydrolysis followed mild acid hydrolysis in 96% aqueous dioxane. The resulting enzymatic mild acidolysis lignin (EMAL) had a yield of 70% of initial Klason lignin both for hardwood (poplar) and softwood (black spruce), which was about 2.5 times greater than the corresponding MWLs. More importantly, the EMAL was reported of higher purity (over 87% for both poplar and spruce) with less carbohydrates and protein contamination, as well as limited marked structural differences from MWL. Lignin recovery from all these three methods was based on solvent precipitation in acidified or cold water followed by seperation of liquid and solid and drying. However, these three methods involved extensive ball-milling as a mechanical treatment in order to obtain high lignin yields [181], which was not only time-consuming but also resulted in lignin depolymerization.

2.3.1.1 *Organosolv lignin*

As an initial development for alternative to chemical pulps, organosolv pulping is widely applied to produce pulp fibers and offer a facile route to recover a highly uniform stream of lignin. This pulping technique also improves biomass digestibility and because it produces lignin with low molecular weight and possible industrial polymer applications it has been seen as an important process for the biorefinery. The most common solvent used in organosolv pretreatment

for high quality lignin isolation is aqueous ethanol combined with a small amount of acid, mainly based on "Alcell process" [21, 182]. It was reported the "Alcell process" successfully extracted lignin with high purity and low molecular weight from hardwoods utilizing 50% aqueous ethanol at 2.76 MPa and 190 ℃ in the presence of 1% sulfuric acid for 30 to 120 minutes. The "Alcell lignin" was commercialized in an industrial quantity in 1990s [45]. Recent studies to isolate organosolv lignin focused on either the optimization or modification of "Alcell process". Both neutral and acidic solvent was used for organosolv delignification. In the case of neutral organosolv delignification, autohydrolysis in the presence of water occurred as the major mechanism to render lignin depolymerization, whereas, the extra acid catalysts facilitated ether bond breakage during the delignification process [107]. It was reported that α-ether cleavage occurred to a greater extent in neutral organosolv pulping and β-ether cleavage became predominant as the solvents were more acidic [107]. Pan and Kim [183] optimized the conditions of organosolv ethanol process for extracting lignin and other saccharide-derived chemicals from hybrid poplar. Their results indicated high temperature, high acid loading and longer reaction times increased the yield of organosolv lignin. Influence of ethanol concentration on delignification was insignificant, but generally the lower ethanol concentration favored the acid-catalyzed bond cleavage that primarily happened on β-O-4 linkages [184], and higher ethanol concentration improved the dissolution of depolymerized lignin fragments. A recent study from Ragauskas et al. [185] isolated organosolv lignin from loblolly pine after 65% ethanol pretreatment at 170 ℃ for 1 hour with 1.1% H_2SO_4. Their research confirmed that lignin depolymerization happened mainly due to the acid catalyzed bond breakages of β-O-4 and ester bonds [186, 187], producing dissolved organosolv lignin. At the same time, since the benzyl carbocation was formed as a reaction intermediate [107], the acid-catalyzed condensation occurred to result in more condensed lignin structure (Figure 2-4). Thus, their results showed lignin condensation still occurred during organosolv pretreatment favored by greater acidity, higher temperature and longer pretreatment time.

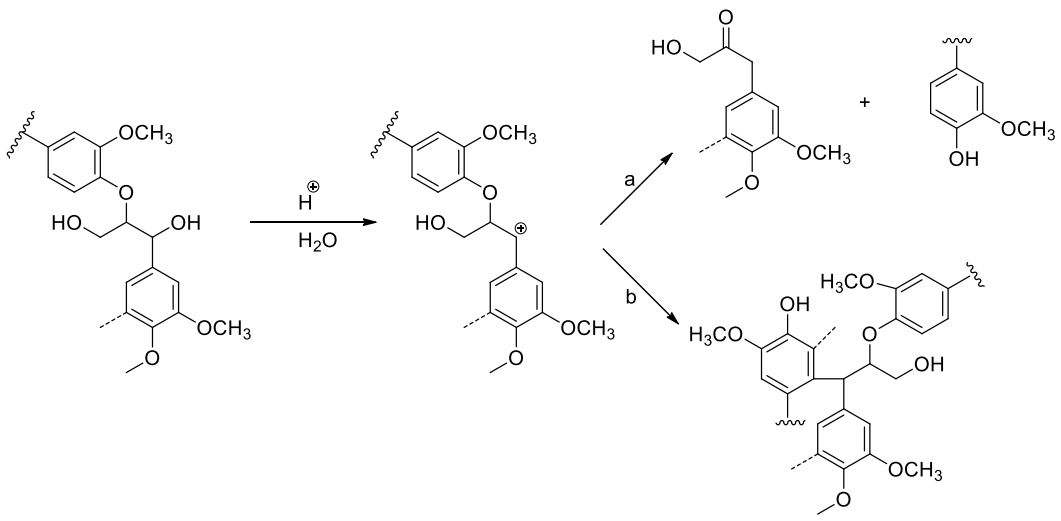

Figure 2-4 Reaction scheme in acidic organosolv delignification with carbonation as a common reaction intermediate. Sannigrahi, P., A.J. Ragauskas, and S.J. Miller, Lignin structural modifications resulting from ethanol organosolv treatment of loblolly pine. Energy & Fuels, 2009. 24(1): p. 683-689. Used under fair use, 2014.

Another study from Wildschut et al. [188] indicated 84% lignin yield from ethanol organosolv pretreated wheat straw at either 210 ℃ with 50% ethanol or 190 ℃ with 60% ethanol in the presence of 30 mM H_2SO_4 for a pretreatment time of 60 minutes. In addition, based on "Alcell process", a method of accelerated solvent extraction (ASE) [189] was developed to isolated 33% of lignin from candlenut shell, which was 4 times more than that from a standard dioxane-water acidolysis. The ASE was conducted with 60% aqueous ethanol at 100 to 195 ℃ but only took 15 minutes to isolate lignin compared the common 60 minutes used in "Alcell process". Isolated lignin from the ASE system contained more hydroxyl groups that resulted in increased reactive sites for further polymer synthesis. Mazza et al. [190] used pressurized 30% aqueous ethanol to extract lignin from flax shives in a flow reactor at 180 ℃ and 5.2 MPa that was twice the typical pressure in the "Alcell process", where they obtained a major high molecular weight and minimum low molecular weight lignin (HML and LML) in separated portions. The major HML portion exhibited the weight average molecular weight was 6 times higher than the commercial "Alcell lignin" and higher glass transition temperature, so the authors claimed that it had potential applications in the area of high thermal stability and polymer synthesis. However, the authors failed to provide more detailed results about how the high molecular weight formed

through extensive condensation or less ether bond breakage so that more work needed to be conducted for further application.

In addition to aqueous ethanol, some other solvents have been developed for biomass pretreatment followed by lignin fractionation. Huijgen et al. [114] reported the 50% aqueous acetone pretreated wheat straw at the optimum conditions of 1 hour and 205 ℃ for both sugar production and delignification. Similar to ethanol organosolv, the extracted lignin was precipitated and recovered from aqueous acetone liquor directly after the pretreatment, showing a maximum delignification of 79% based upon initial lignin content in feedstock. Ethylene glycol was first studied to pretreat woody biomass and isolate lignin in 1930 [191, 192]. According to the reports by Hibbert and Marion [191], spruce meal was pretreated by ethylene glycol with 0.05% dry hydrogen chloride as catalysts at 100 to 103 ℃ to extract lignin. The study showed 20.4% of total initial lignin in spruce feedstock was recovered through precipitation of lignin in the black liquor. The authors further proposed the resultant lignin was a compound of lignin and ethylene glycol through methoxylation and hydrolysis analysis. In 1980s, a group of researchers at the University of Sherbrook conducted a demonstration scale processing unit to treat wood flour, up to 20% by mass of wood flour dissolved in ethylene glycol [193]. The sample was transferred through a high shearing reciprocating valve assembly and then heated to 220 ℃. The authors reported that solvolysis of the wood took place where the solvent caused disruption of the wood components making the lignin extractable with good yields and favorable levels of extraction. Their paper [104] studied the recovery of dissolved "glycol lignin" through dilute acidification of spent liquor, which was proved more practical and reproducible than solvent evaporation. Up to 72% of potential lignin was recovered. The structural analysis of recovered glycol lignin suggested the low molecular weight was caused because of β-aryl ether cleavage during the solvolysis process. In addition, significant peaks from ^{13}C-NMR related to aliphatic methylene and hydroxyl groups suggested a possibility of lignin-solvent interaction formed during delignification. Glycerol as an alternative to ethylene glycol was also utilized to pretreat woody biomass for delignification. Demirbas [194] reported up to 94% delignification accompanied by extensive hemicellulose loss could be reached from aqueous glycerol treatment for 9 hours in the presence of alkaline catalysts. However, there is no detailed information about the isolated lignin recovery and further structural information.

2.3.1.2 *Steam explosion lignin*

Steam explosion pretreatment is another method facilitating lignin fractionation. After steam explosion pretreatment, the biomass residue is primarily composed of cellulose and lignin with almost all hemicellulose degraded into water-soluble monomers and oligomers. During steam explosion pretreatment, lignin undergoes a simultaneous depolymerization/repolymerization reaction [140, 144, 145, 195], and finally redistributes on fiber surfaces as a result of softening and agglomeration due to surface tension effects [146]. The redistributed lignin agglomerates/coalesce into small spheres with diameters smaller than 5 to 10 μm [140], which are easily soluble in aqueous organic solvent such as dioxane/water and hot ethanol/water, and further recoverable by solvent evaporation. Instead of organic solvents, lignin can be also isolated by subsequent alkali extraction and further precipitated through neutralization. As early as the 1980s, Chua and Wayman [145] studied lignin isolated from autohydrolyzed aspen, and proposed a lignin condensation reaction due to autohydrolysis. In 1981, Marchessault et al. [196] characterized the aspen steam-exploded lignin through methanol extraction and proposed the extensive β-O-4 bond breakages during steam explosion. Based upon previous research, Li et al. [144] concluded lignin reactions during steam explosion pretreatment, where they isolated lignin through 90% dioxane extraction from steam exploded aspen. From their results, the amount of β-O-4 linkages in isolated lignin decreased rather linearly with increasing steam explosion severity ($\log R_0$) in quantitative two-dimensional NMR, which confirmed β-O-4 bond breakage as the major reason for lignin deploymerization. On the other hand, SEC flow curves of isolated lignin and MWL models after steam explosion revealed an increase in molecular weight at higher $\log(R_0)$, since the repolymerization simultaneously occurred and became more dominant at severe steam-explosion conditions. Besides solvent extraction, Ibrahim et al. [197] isolated lignin from a series of steam-exploded agricultural residues using 20 wt% of NaOH extraction at 80 ℃ for 1 hour. Their results confirmed ether bond cleavage and condensation of lignin due to the pretreatment, but there was no more detail about recovered lignin yield and its functionality. In contrast, Li et al. [198] conducted 6 wt% of NaOH extraction at 70 ℃ for 2 hours for lignin isolation from both hardwoods (birch and aspen) and softwoods (spruce and pine) after steam explosion, where a lower alkaline concentration was applied in order to prevent further chemical structure modification. During their steam-explosion experiments, SO_2 was used to pre-

impregnate hardwood and softwood samples to facilitate high purity lignin isolation. For hardwood, 86% and 48% of delignification was reached for birch and aspen, respectively. In contrast, even with the SO_2 catalyst, alkaline lignin isolation from softwood was less efficient. SEC flow curves showed that the isolated lignin had over 50% smaller of MW_n accompanied with high molecular weight tailing because of severe lignin degradation and condensation during SO_2 pre-impregnated steam explosion. At the same time, all the isolated lignin resulted in broad molecular weight distributions, especially for hardwood (PDI of 25 for birch and 38 for aspen). This research also analyzed the lignin structure from one- and two-dimensional NMR, which revealed the isolated lignin had minimum β-O-4 remaining after SO_2 pre-impregnated steam-explosion, corresponding to the severe lignin degradation; additionally, the aliphatic OH was significantly decreased (4.57 to 1.08 mmol/g lignin for spruce and 4.85 to 0.86 mmol/g lignin for aspen) with increases of phenolic OH compared to MWL for both hardwood and softwood. An increase of carboxylic acids was also observed indicating oxidation of the lignin in this process. In addition, thioacidolysis studies of the lignin, which can chemically degrade β-O-4 bonds in lignin and further reveal the amounts of β-O-4 bonded structures, indicated significantly lower number of linkages from the isolated lignin compared to MWL (35(72) for steam-explosion lignin to 1028 (1262) for MWL of the amount of β-O-4 bonded G/S for spruce (aspen)), which further confirmed severe β-O-4 bond breakage during steam-explosion. Low degradability by thioacidolysis implied a high amount of C-C bonds resulted from lignin condensation during the pretreatment.

Based upon the previous studies, the lignin reaction mechanism during steam explosion could be concluded as follows [144, 145, 196, 199]: 1) in the acidic environment produced by steam pretreatment, where acetic acids and other woody acids were released to catalyze hemicellulose degradation and bond breakage between lignin and heteropolysaccharides, carbonium ions [145] were formed as a reaction intermediate further leading to the competition of simultaneous lignin depolymerization through acidolysis [200] and homolytical cleavage [201, 202] with the formation of α- and β-ketones [203] and 2) repolymerization with formation of new and more stable carbon-carbon bonds (Figure 2-5). The repolymerization reaction can be suppressed only at extremely mild steam explosion conditions.

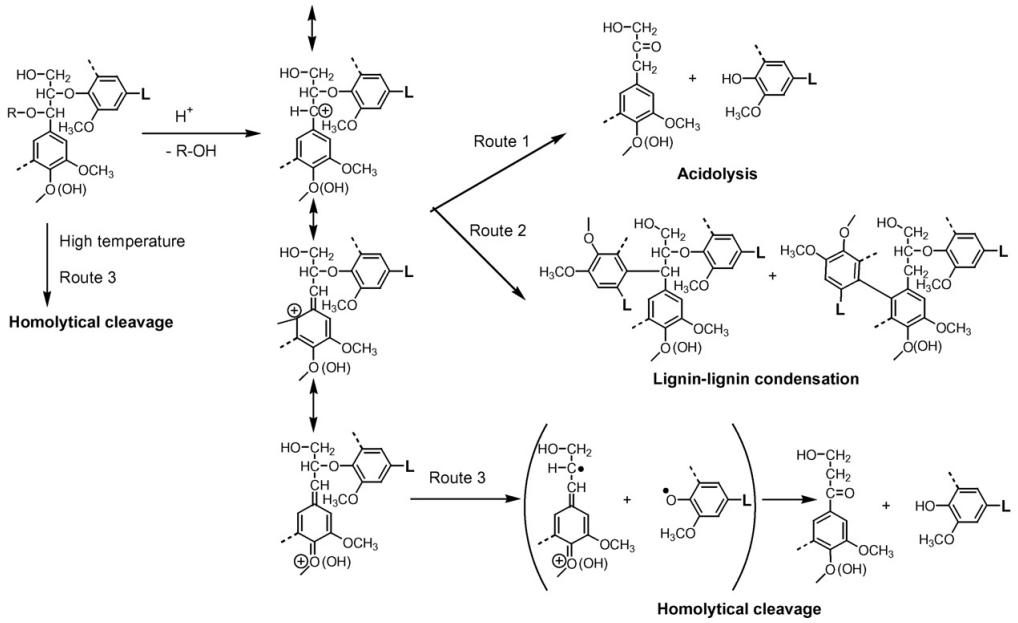

Figure 2-5 Proposed reaction scheme for lignin depolymerization and repolymerization during steam explosion. Li, J. and G. Gellerstedt, Improved lignin properties and reactivity by modifications in the autohydrolysis process of aspen wood. Industrial Crops and Products, 2008. 27(2): p. 175-181. Used under fair use, 2014.

The heterogeneity of steam-explosion lignin due to depolymerization/repolymerization actually limits its application, such as low solubility and reactivity. Comparison between ethanol organosolv and steam-explosion delignification by Saddler et al. [204] also claimed that the ethanol organosolv lignin from softwood had higher value and was more reactive than lignin isolated from steam-exploded softwood. To overcome this obstacle and minimize lignin repolymerization, efforts have been conducted to isolate lignin from steam-exploded wood without severe condensation. One way was reported to add NaOH to reduce the acidity of pretreatment further suppress the acidolysis, since the acidic conditions favored the formation of carbonium ions [199]. Furthermore, Wayman and Lora [205] found 2-naphthol was the most effective scavenger for carbonium ions to prevent its carbon-carbon repolymerization. The enhanced electron density of 2-naphthol ring by the hydroxyl group and lower activation energy compared to phenols for electrophilic reactions, resulted in 2-naphthol being more reactive to attack the carbonium ions. The C-1, C-3, C-6 and C-8 positions were viewed as reactive sites for this reaction (Figure 2-6). Consequently, 2-naphthol stabilized the carbonium ion intermediates

during steam explosion, limiting the repolymerization between neighboring lignin aromatic sites [144, 199]. Due to the suppression effect on lignin repolymerzation by applying 2-naphthol during steam explosion, the exploded aspen resulted in 91% of delignification compared to 51% without 2-naphthol through 90% dioxane extraction [144]. Moreover, the isolated lignin had more uniform structures with lower molecular weights, which was assumed to be more useful than heterogeneous one. However, since 2-naphthol was a petroleum-based product, further research needs to be performed to find a more sustainable replacement for this modification.

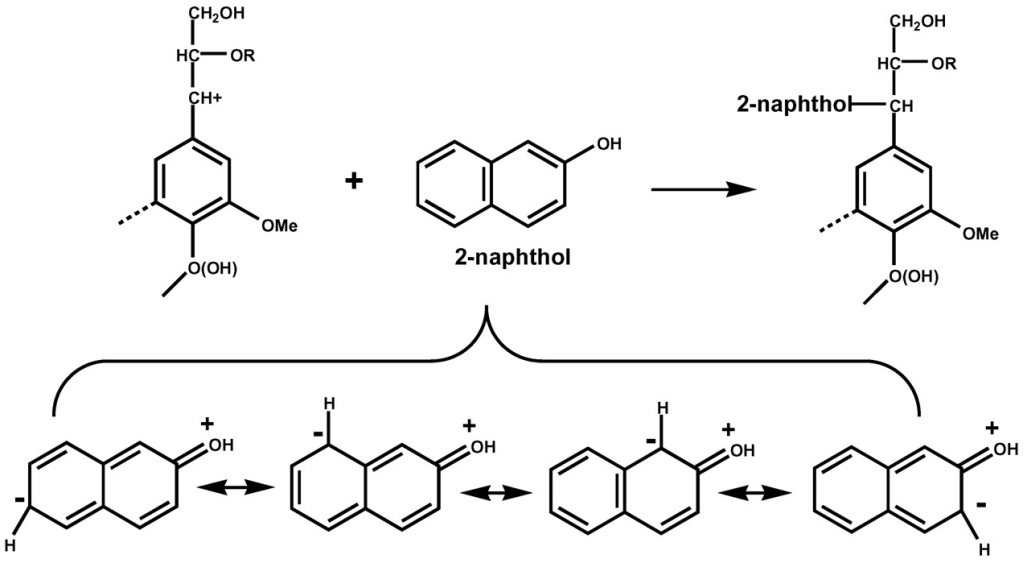

Figure 2-6 Reaction for carbonium ion and 2-naphthol during steam explosion. Li, J. and G. Gellerstedt, Improved lignin properties and reactivity by modifications in the autohydrolysis process of aspen wood. Industrial Crops and Products, 2008. 27(2): p. 175-181. Used under fair use, 2014.

In addition to the steam-exploded hardwood lignin, Saddler et al. [206] studied lignin isolated from steam-exploded and enzymatic hydrolyzed softwood (Douglas fir) since the following alkaline extraction of steam-exploded softwood was less efficient. Their results indicated after severe steam-explosion (215 ℃, 2.4 minutes with 2.4% SO_2) and enzymatic hydrolysis, lignin content in biomass residue was removed up to 94%. Similar condensation occurred on softwood lignin because of the loss of reactivity at α-C on the lignin residue. Despite of the complicated lignin structures, Saddler et al. still proposed several potential applications such as a dispersant

and a precursor for adhesive synthesis considering the high phenolic content with reactive C5 positions.

2.3.2 NMR as a powerful tool to elucidate lignin structure

One- and two-dimensional nuclear magnetic resonance (NMR) spectroscopies serve as major and powerful tools to elucidate complex lignin structures including various functional groups and intra-molecular linkages. Among them, ^{31}P-NMR has been widely utilized for the quantification of different types of hydroxyl and acid groups present in lignin (Figure 2-7), whereas ^{1}H-^{13}C correlation 2D-NMR techniques have been developed recently to afford more details about lignin intra-molecular bonding.

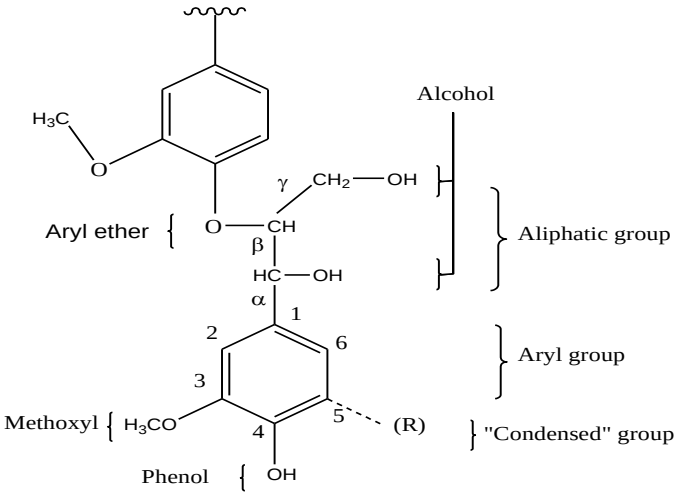

Figure 2-7 Lignin functional groups.

2.3.2.1 *<u>Quantitative ^{31}P-NMR</u>*

^{31}P with 1/2 spin has a natural abundance of almost 100%, which makes it an excellent nucleus for structure determination of phosphorus containing compounds by using NMR spectroscopy. The labile protons in various hydroxyl groups in lignin can readily be reacted with a derivatizing reagent and further produce trivalent phosphorus nuclei that are further subjected to ^{31}P-NMR analysis (Figure 2-8) [207]. In this technique, the aliphatic hydroxyl, different phenolic hydroxyls and carboxylic acids can be identified from the spectrum.

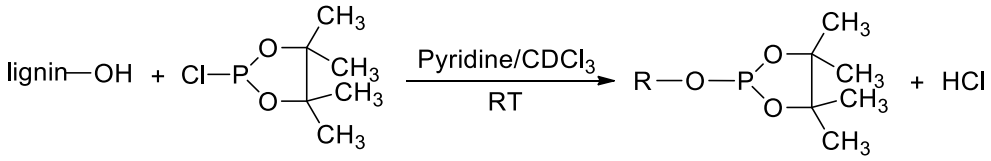

Figure 2-8 The derivatization reaction used for lignin ^{31}P-NMR (R= residues of phenols, alcohols, and carboxylic acids).

In addition, quantitative NMR allows the calculation of various functional groups, that is, the peak intensity is proportional to the abundance of corresponding functional groups. Because of the sensitivity, pulse sequence and acquisition parameters require optimization to fully quantify the functional groups.

^{31}P-NMR uses the inverse-gated decoupled pulse sequence to obtain the spectrum, which is shown in Figure 2-9 (one scan). The specific ^{31}P spin experiences during this pulse sequence are described as follows [208] . First, there are two spin activation channels used in this pulse sequence, with ^{31}P channel for signal generation, and ^{1}H and ^{13}C channel to remove signal splitting effects on ^{31}P signals. When the samples containing ^{31}P spins are introduced in an external magnetic field (B^0 is parallel to z-axis), all the random distributed spins (Figure 2-9a) are aligned along the external magnetic field (Figure 2-9b) to minimize the magnetic energy. Applying a 90^o radiofrequency pulse (rf pulse) along the x-axis on the ^{31}P channel, causes the ^{31}P spins in samples to switch from the z-axis to the x-y plane, resulting in the transverse magnetization M_x and M_y. Then the ^{31}P spins in samples are detected through the free-induction decay (FID) signal as the spins relax to the thermal equilibrium states (parallel to B^0) at the presence of external magnetic field B^0. Simultaneous with the signal collection, ^{1}H and ^{13}C decouplers are turned on to remove the splitting effects of ^{1}H and ^{13}C on ^{31}P peaks, so that it can collapse the peak multiplets caused by ^{1}H or ^{13}C J-coupling with enhancing signal-to-noise ratio (SNR) and spectrum resolution. In addition, to ensure the maximum SNR, several spectra acquisition parameters need to be considered. For example, a 90^o_x rf pulse is applied to fully excite the spins in transverse magnetization, that is, the maximum intensity is obtained in the FID. The pulse repetition time (interpulse time, T_R=data acquisition time (τ_{acq}) +relaxation delay time (d_1)) need to be sufficient to allow all spins fully relax to equilibrium states before applying the next pulse in order to obtain the quantitative spectrum. In this way, the peak intensity remains

constant after each scan, since the spin longitudinal magnetization is always M_z before each pulse, which benefits the SNR after signal averaging as well as guarantees spectrum quantification. Also, multiple scans need to be applied in order to increase SNR.

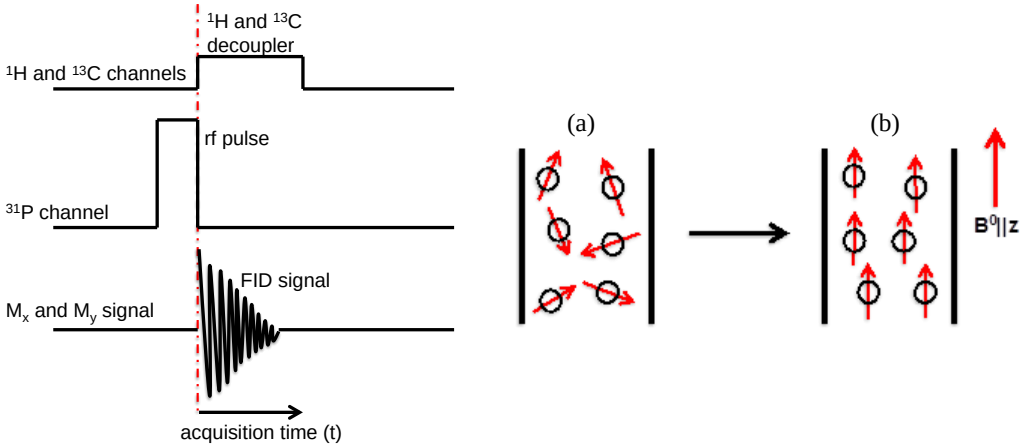

Figure 2-9 Inverse gated pulse sequence for ^{31}P-NMR (one scan) (rf: radio frequency)

Argyropoulos et al. [209-213] proposed the ^{31}P-NMR analysis of lignin in a series of paper from 1991. Their quantitative analysis of different hydroxyl groups for 6 soluble lignin samples was in satisfactory agreement with the average reports by the international wood chemistry community, which verified the validity of ^{31}P-NMR analysis [207, 214]. From their initial reports, the labile protons in lignin were derivatized using 1,3,2-dioxaphospholanyl chloride (phosphitylation reagent PR[I]) so that the structures bearing different hydroxyl groups in lignin were separated and quantitatively revealed on the ^{31}P-NMR spectra. A following paper by Granata and Argyropoulos [207] claimed that although PR[I] was useful to quantify the guaiacyl phenolic OH, carboxylic acid, and especially benefited the quantification of primary and secondary OH in erythro and threo forms of the arylglycerol β-O-4 ether structure, it rendered signal overlap between syringyl phenolic OH and those belonging to primary and condensed phenolic structures, which was extremely important for hardwood lignin structure analysis. Thus, another lignin phosphitylation reagent, 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (phosphitylation reagent PR[II]), was introduced and widely applied to better resolve the different phenolic structures in lignin especially hardwood lignin at the expense of resolution for primary and secondary aliphatic OH. In addition, the reagent II was less reactive than reagent I due to the four stable methyl groups that increased the stability of the lignin phosphitylated products [207].

For quantification through the ^{31}P-NMR analysis, the choice of internal standard was also considered, which was required as non-degradation in the solvent mixture, production of sharp and baseline-resolved peaks as well as distinct separation from sample signals. In the early studies by the Argyropoulos group [213, 214], benzoic acid was adopted as an internal standard that was identified as low reactivity after derivatizing with PR[I]. However, the NMR signal of derivatized benzoic acid fell in the carboxylic acid region, which interfered in the quantification of carboxylic acid in lignin. Cyclohexanol was utilized widely in later reports by Argyropoulos et al. [207], which exhibited a sharp peak sitting between the aliphatic and phenolic region. A concern of using cyclohexanol as internal standard was that the long relaxation time (4.6 seconds [207]) was necessary for PR[II] phosphitylated cyclohexanol even at the presence of relaxation reagent. As a result of the long relaxation time (25 seconds of delay time between pulses [207]), the total spectrum acquisition time was extended considering usually 256 scans were performed. In 2001, Zawadzki and Ragauskas [215] found N-hydroxy compounds, such as N-hydroxy-5-norborene-2,3-dicarboximide, as promising candidates for lignin ^{31}P-NMR internal standards, which was stable after derivatization, ensured reproducible results compared to previous reports and more importantly, shorten the relaxation time (0.5-2 seconds), as a result, the spectra were obtained more rapidly.

As discussed above, the spin relaxation time needs to be long enough for full relaxation of activated spins before the next pulses, in order to ensure the accurate and quantitative analysis. According to Argyropoulos et al. reports [211], the spin-lattice relaxation time (T_1) of phosphorus nuclei for a variety of lignin models was in the range of 5 to 10 seconds (around 5 s for ^{31}P of carboxylic acid derivatives and 8 to 9 s for phenols and alcohols), which resulted in a 50 to 60 seconds of pulse delay time (usually 5 times of T_1). Such a high spin relaxation time was probably due to the complicated structure of lignin and was not common in ^{31}P-NMR spectroscopy [216], and it definitely led to longer spectra acquisition times, such as a 4 h acquisition time for a total 256 scans when using 60 s pulse delay time. Thus, Argyropoulos et al. [213] utilized chromium acetylacetonate, which was capable of reducing relaxation times due to a paramagnetic metal center [217], as the ^{31}P spin relaxing reagent when collecting lignin ^{31}P-NMR spectra. As a result of addition of chromium acetylacetonate, the delay time for different ^{31}P nuclei in derivatized lignin models was reduced to 2 to 4 seconds [213].

In general, ^{31}P-NMR was widely adopted to quantitatively analyze the various hydroxyl groups as well as carboxylic acids in lignin. The phosphitylation reagent II was chosed to resolve the phenolic region for better separation of syringyl, guaiacyl and condensed phenolic structures. This was more important when analyzing hardwood lignin. N-hydroxy compounds were the more promising internal standard for quantification and the addition of nuclei relaxing reagent was necessary for stable and fast lignin ^{31}P-NMR analysis.

2.3.2.2 ^{1}H-^{13}C correlated two dimensional NMR

In addition to the various hydroxyl groups, quantitative NMR is a powerful tool to elucidate the structural characteristics as well as the amount of different bonding in lignin without degradation through wet chemistry methods. The one-dimensional ^{1}H-NMR method was developed early in 1964 [218, 219] to analyze lignin structure, however, signal overlap due to the high molecular weight and complex structure limited its quantitative application [220]. Further research has shown that inverse gated ^{1}H decoupled ^{13}C-NMR provided better resolution but resulted in lower sensitivity and lower signal-to-noise ratio, and it was difficult to determine the side chain carbons in different substructures [220]. Recently, the ^{1}H-^{13}C single bond correlated 2D-NMR (2D-HSQC), has been widely applied to elucidate lignin functional groups and inter-unit bonding patterns, which provides signal intersections generated from proton frequencies on the x-axis and their correlated carbon frequencies on the y-axis, combining the benefits of higher sensitivity ^{1}H-NMR and better resolution of ^{13}C-NMR.

Many conventional HSQC experiments were not quantitative since the various heteronuclear coupling constant caused contour volume in 2D-NMR spectra and was not proportional to the functional group abundance [220]. In contrast, Heikkinen et al. [220] first developed the quantitative 2D-HSQC (Q-HSQC) for the elucidation of lignin structure. Quantitative analysis was verified by testing a series of small molecule lignin models; these studies indicated the abundance of different inter-unit linkages determined by Q-HSQC was in good agreement with results from quantitative ^{1}H and ^{13}C-NMR. Further application of Q-HSQC on milled wood lignin identified the abundance of β-O-4, β-5, β-β inter-unit linkages as well as the dibenzodioxocin structure, which was viewed as a key lignin structural component introducing branching points, but only revealed by NMR techniques. In their research, a small molecule

lignin model compound, Apocynol, was adopted as an internal standard. However, this technique required fourfold experimental times compared to conventional HSQC. Peterson and Loening [221] improved the Q-HSQC technique and proposed a quick and quantitative HSQC (QQ-HSQC), which ensured more rapid data acquisition with the same quantitative attributes as Q-HSQC. It was reported this new methodology reduced the number of scans by a factor of four without decreasing signal intensity compared to Q-HSQC. Furthermore, Zhang and Gellerstedt [222] extensively studied the selection of suitable internal standard for quantitative measurements of polymer structures from HSQC, indicating the low-molecular-weight internal standard was not suitable to quantify the polymer structures since effects on peak quantification caused by spin-spin relaxation time (T_2) of small molecule was different from that of polymer. Instead, these effects were similar for different resonances from the same polymer structure. Thus, any signal originating from a functional group in the polymer itself was recommended as an internal standard for quantitative HSQC analysis. Based on the above studies, Crestini et al. [223] adopted QQ-HSQC methodology to quantitatively elucidate both hardwood lignin and softwood lignin structures. The lignin guaiacyl C2-H cross-peak signals were adopted as internal standards for softwood lignin, since the C2 positions were never substituted in lignin and the amount of C2-H represented the accurate amount of guaiacyl rings. In the case of hardwood lignin, the half integral of C2-H and C6-H in syringyl rings represented the abundance of syringyl units and the number of C2-H in guaiacyl rings represented the abundance of guaiacyl units. As a result, all the other structural units and inter-unit linkages were able to be quantified based upon the number of syringyl and guaiacyl rings in lignin. Studies conducted by the Ralph's group [224, 225] also used this method for quantification of G/S ratio; in addition, Ralph et al. [224] proposed the correction factors (response factors) for various inter-unit bonds when volume-integrating these peak contours. When integrating the peak contours for G/S/P units, the correction factors were neglected due to the 1:1:1 volume integral ratios resulted from a series of G/S/P dimers or trimmers of lignin models. However, the situation was different when examining the inter-unit linkages, which needed various correction factors for volume integration of contours originating from inter-unit linkages. These correction factors were determined by integrating spectra from a range of mixed trimers and tetramers of lignin models. For example, the correction factor of Cα-H in β-aryl ether was 1 compared to 0.71 in phenylcoumaran (β-5) structures. Thus, these correction factors need to be multiplied when calculating the unit ratios by

integrating the contour volumes. However, Ralph et al. [224] also mentioned these correction factors were not universally calibrated, and were dependent upon the spectrometer and acquisition conditions, and it was hard to be used by others. Furthermore, the Ralph group [226-228] conducted a series of non-degradative cell wall characterization through solution or gel state 2D HSQC, where they were able to characterize the in-situ lignin structures in plant cell wall.

2.3.3 Hemicellulose isolation

Hemicelluloses, more accurately categorized as hetero-polysaccharides, are another important polymer group from lignocellulosic biomass. Except for its role as feedstock for production of pentoses and hexoses in the biofuel industry, the isolated hemicelluloses have potential applications in the food and drug industries. However, limited methods to isolate hemicelluloses without significant degradation have required specific fractionation schemes.

The first hemicellulose extraction was traced to 1931 [229], at that time, 4% of NaOH was used to extract hemicellulose at different temperatures. Usually, successful extraction for hemicellulose could be achieved in sequential steps by increasing the alkaline concentration. A series of NaOH concentration was reported for hemicellulose extraction ranging from 0.5% to 18%. Another alkali such as KOH [230] was also common for hemicellulose isolation with a concentration range of 1% to 24% [231]. Furthermore, Yan et al. first proposed [231] a nonaqueous solvent, liquid ammonium, as an extractant for hemicellulose. In 1956, Lindberg et al. [232] explored the use of dimethyl sulphoxide as a non-aqueous solvent. Since there are covalent bonds between lignin and hemicellulose within the biomass cell wall, cleavage of this bond through acid hydrolysis or delignification to obtain the holocellulose [233] is a prerequisite for successful hemicellulose extraction, especially for softwoods due to their higher degree of lignification. In contrast, xylan extraction from milled hardwoods could be simply achieved by alkaline treatment [234].

The most common delignification method uses acetic acid and sodium chlorite to separate holocellulose as a substrate for further hemicellulose extraction, which was first performed by Wise et al. [233] in 1946. From their method, the holocellulose was successfully obtained from wood meal through reaction with sodium chlorite and acetic acid at 70 to 80 ℃ for 3 to 4 hours.

Then, sequential 5% and 24% of KOH extractions at 20 ℃ under nitrogen was conducted to isolate hemicellulose from the holocellulose, where the 5% KOH removed the large portion of uronic acid-rich hemicellulose. The alkaline extracted hemicellulose was precipitated by means of excess acetic acid and 95% ethanol. Although this method used KOH instead of NaOH because of the better solubility of potassium acetate in alcohol, there was potassium acetate contamination left in the final hemicellulose. The total hemicellulose yield was around 22% and 13% of initial biomass for aspen and pine [233]. Further studies [16, 235] by Timell were mainly conducted based upon the method of Wise to isolate a linear 4-O-methylglucuronoxylan from white birchwood. As shown from the above studies, hemicellulose could be extracted from holocellulose through alkaline extraction, however, the choice of aqueous alkali causes deacetylation [16] during the extraction process. Furthermore, this chlorite method for holocellulose separation was oxidative, resulting in inevitable cellulose [236] and hemicellulose degradation as well as formation of carbonyl and carboxyl groups on the polysaccharides. At more severe conditions, some degraded cellulose could be further extracted with hemicellulose [234]. Instead of alkaline extraction of chlorine holocellulose, Timell [237] used dimethyl sulfoxide (DMSO) to extract xylan with acetyl groups from birch holocellulose at room temperature for 3 days. About 50% of initial wood xylan was obtained, and the structure identified with 36 of O-acetyl groups per 100 xylose units, with the suggestion of the acetyal groups to be located on the C3 positions. Compared to alkaline extracted xylan from previous method, the DMSO extracted xylan had lower degree of polymerization probably because DMSO preferentially removed the lower molecular weight xylan.

Besides the conventional acidic chlorine holocellulose protocols for hemicellulose extraction, the direct hemicellulose isolation from wood was shown to minimize the chemical modification [238]. Following isolation, a sequential bleaching method using hydrogen peroxide was performed to purify the crude hemicellulose. Glasser et al. [239] compared different isolation protocols of heteropolysaccharides from barley husks and yellow poplar, where they found the alkali extraction was more efficient to obtain the polymeric xylan and a pre-delignification step resulted in better purity and lower polydispersity. From their research, a sequential hot water, 0.05N NaOH and 1N NaOH extraction was performed on Wiley-milled yellow-poplar. Each extraction step lasted for 24 hours and only the 1N NaOH extract and additional washing liquid were collected for hemicellulose recovery. The alkaline solution was neutralized by hydrochloric

acid to pH 5.6. For one step, the resulted precipitate was collected for peroxide bleaching as water-insoluble xylan. On the other hand, the supernatant was also bleached and precipitated in methanol to recover the water-soluble portion of xylan (Figure 2-10). After this sequential extraction and purification, a total of 31.4% of initial xylan in poplar feedstocks was recovered.

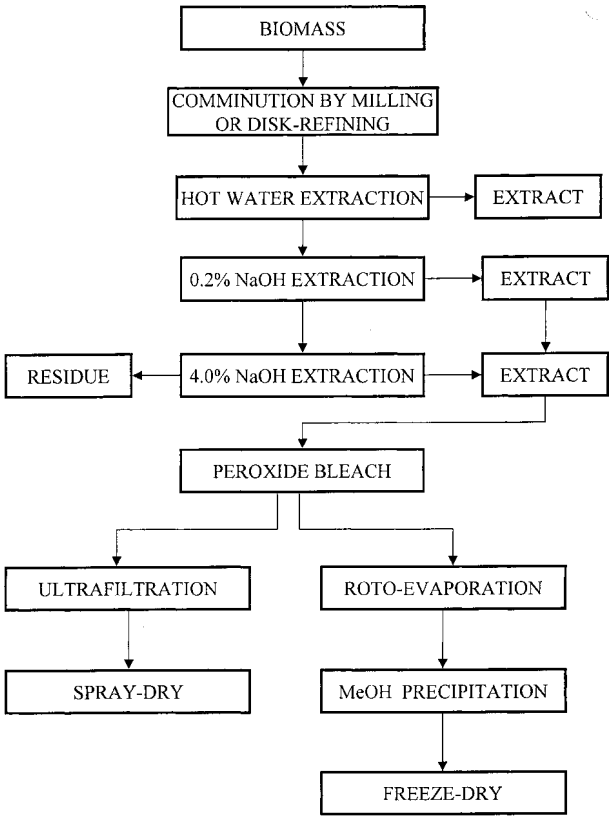

Figure 2-10 Alkali extraction protocol of xylan from poplar. Glasser, W.G., et al., Isolation options for non-cellulosic heteropolysaccharides (HetPS). Cellulose, 2000. 7(3): p. 299-317. Used under fair use, 2014.

Compared to this direct alkali extraction, an alternative method to extract and recover xylan involved pre-delignification either by acid chlorite or organosolv pretreatment. Depending upon the pretreatment severity, around 30% to 50% of initial xylan was recovered, an indication that the pre-delignification step facilitated xylan isolation. In contrast, xylan isolated from water extractions of steam-exploded biomass had low yield, low purity and a very low degree of polymerization, which was consistent with the severe hydrolytic degradation [140, 240, 241] during steam explosion.

2.4 Glycerol and extrusion to improve biomass pretreatment

2.4.1 Glycerol delignification for pulping

Glycerol is considered to be a benign solvent that has been used for industrial processes such as a plasticizing agent for melt-processed biopolymers [242] or in lab scale processes for pulping or pretreating [194]. Demirbas first reported the aqueous glycerol delignification of biomass, including hardwoods (beech, ailanthus), softwoods (spruce) and agricultural residue (wheat straw, corn stover, Hazelnut shell, etc.). In that method, biomass feedstocks were cooked in 45% to 75% (w/w) of glycerol (9/100 solid/liquid) with or without NaOH or Na_2CO_3 catalysts at temperatures ranging from 205 to 300 ℃ for 3 to 9 hours. Results showed that the maximum delignification was obtained using 72% glycerol and 1% (w/w) NaOH as catalysts after cooking at 225 ℃ for 9 hours, revealing a 91.6% delignification for Ailanthus and 89.9% for beech. The maximum delignification for spruce was 76.8%, whereas 73% to 87% for agricultural residue. Extensive degradation of hemicellulose occurred during pulping especially at temperature higher than 217 ℃. The resulting materials were cellulose-rich pulp, accounting for 80% to 94% of total cellulose in feedstocks. Similar delignification research using alkaline-glycerol was conducted by Küçük [243], which indicated the alkaline-glycerol had comparable power of delignification as n-butanol and phenol/water. A recent report by Demirbas [244] further studied wood liquefaction in glycerol. He successfully liquefied poplar meal using 75% of aqueous glycerol with or without alkali catalysts, which resulted in a maximum delignification at highest pulping temperature of 287 ℃, 82% without catalysts and 99.3% in the presence of NaOH. The high pulping temperature was ascribed as a prerequisite for the glycerol liquefaction since it reduced surface tension of glycerol pulping liquor, which promoted solvent and alkali penetration into wood cell walls. Also, the high temperature facilitated breakage of ether and glycosidic bonds that was assumed to cause lignin degradation into small fragments during glycerol pulping. As concluded by Demirbas [244], glycerol as a highly polar solvent could penetrate into wood cell walls at high temperatures and hydrogen bonded with cellulose to further swell the biomass. Under the combined influence of alkali catalysts and high temperatures, lignin degradation as well as breakage of lignin-carbohydrate bonds occurred with enhanced solubility of lignin fragments in organic solvents. However, there was no further study to identify the recovery and structures of removed lignin in aqueous glycerol. Additional study with glycerol as a pulping

solvent has been hampered by the difficulty in recycling a high boiling point solvent compared to other alcohols.

2.4.2 Glycerol facilitated biomass pretreatment

Except for biomass delignification and liquefaction, glycerol as an alternative organosolv option has been studied recently to improve biomass pretreatment either for enzymatic saccharification or biomass fractionation.

Sun and Chen [245] proposed the aqueous glycerol pretreatment to improve wheat straw enzymatic hydrolysis, where the wheat straw was immersed in 70% aqueous glycerol (solid/liquid=10 to 30/100) for 40 hours before cooking the mixture at 190 to 240 ℃ for 1 to 6 hours. This protocol was relatively simpler compared to low boiling point organic solvent pretreatment (ethanol organosolv) avoiding the need for high pressure reactors, due to the high boiling point of glycerol (290 ℃). The pretreated biomass at 220 ℃ for 3 hours showed around 98% of initial cellulose retention after separation and water-washing, whereas the loss of hemicellulose and lignin was 70% and 65% of original contents in starting biomass. A maximum of 90% enzymatic digestibility of pretreated wheat straw was obtained in 48 hours. In contrast to the commercial grade glycerol with 95% purity used in the above research, an interesting ensuing study by Sun and Chen [116] explored the possibility of applying crude glycerol from oleochemicals industry to decrease costs. Two types of crude glycerol were adopted with one from sebacic acid production containing 40% to 50% glycerol and the other from biodiesel production containing 70% glycerol. Results showed that crude glycerol led to a maximum of 75% of enzymatic digestibity after 48 hours hydrolysis, which was lower than the commercial glycerol since the lipophilic compounds in crude glycerol limited lignin removal.

Martin et al. [246] explored the aqueous glycerol pretreatment on sugarcane bagasse for biomass fractionation and enzymatic saccharification, and they also compared the effects of pretreatment with or without catalysts. The highest enzymatic digestibility was obtained as 94% when glycerol pretreatment occurred without addition of either acid or alkaline catalysts, which was in accordance with above results from wheat straw. Acid-catalyzed glycerol pretreatment played a negative role on cellulose recovery but improved xylan solubilization. Additionally, increased glycerol input facilitated lignin removal, but decreased xylan solubilization. At the same time,

Novo et al. [247] reported aqueous glycerol delignification on sugarcane bagasse without catalysts. At the optimum conditions, 80% aqueous glycerol pretreated bagasse at 190 ℃ for 240 minutes accompanied with washing by 1% sodium hydroxide solution after pretreatment to remove the hydrolyzed lignin, the resultant mixture exhibited best biomass fractionation with 80% delignification and 92% residual cellulose in pretreated substrates. Generally, the delignification behavior in this process could be explained by autohydrolysis/hydrothermal process, which means, the ionized water at high temperature degraded the glycosidc bonds and acetyl groups from hemicellulose, resulting in hydromium ions from acetic acid. The in-situ produced acid further catalyzed the hydrolysis of ether bonds in lignin or the linkages between lignin and hemicellulose [105]. Based upon this mechanism, the authors clarified that water content was very important for the non-external-catalysts involved in glycerol delignification. Another similar study [248] on Eucalyptus globulus for biomass fractionation was performed at a liquid to solid ratio of 10 to 1 using 40% to 80% glycerol for pretreatment of 40 to 90 minutes at 180 to 200 ℃. The pretreated solid residue was a cellulose-rich substrate (98% of original cellulose in feedstock) for further enzymatic hydrolysis, showing higher than 90% digestibility after 72 hours of enzymatic hydrolysis. In addition, this aqueous glycerol pretreatment led to a maximum of 77% delignification when operating at 200 ℃ for 65 minutes. The amount of xylan left in the pretreated biomass was highly dependent on water content in the pretreatment solvent mixture that was consistent with Martin's reports [246], on pretreatment time and temperature because of the hydrothermal properties, resulting in xylan solubilization in the range of 39% to 82%.

Besides agricultural residues and hardwoods, Liu et al. [249] proposed a microwave-assisted aqueous glycerol pretreatment for softwood with either organic or inorganic acids catalysts. The microwave was adopted here only for heating because of the high viscosity and low heat transfer of the pretreated mixture. The organosolvolysis of Japanese cedar chips happened with 90% glycerol in a solid/liquid ratio of 1/17 (w/w), at temperatures ranging from 160 to 210 ℃ provided by a microwave irradiator for 3 to 30 minutes. Final pretreated biomass was separated and washed, and a maximum saccharification of 53.1% was obtained after 48 hours of hydrolysis for samples pretreated at 180 ℃ for 6 minutes with 0.1% hydrochloric acid.

2.4.3 glycerol prevents biodegradation of lignocellulose

As indicated by the biodegradation of lignocellulose by brown-rot fungi, hydroxyl radical degradation via Fenton chemistry is another important mechanism to degrade lignocellulose cell wall [250]. Soluble ferric iron in plant tissues [251] could react with hydrogen peroxide that is produced via oxidation of methanol after lignin demethylation [250], to generate the highly reactive hydroxyl radicals, which could initiate the depolymerization of lignin and cellulose [252] in wood cell wall. At this point, glycerol as radical scavenger [253] is capable to prevent this degradation pathway. The existence of glycerol could consume the generated hydroxyl radical, and then minimize the degradation reaction.

2.4.4 Extrusion system for biomass pretreatment

Recently, continuous pretreatment protocols utilizing a screw-extrusion system have been studied and developed. Extruders widely applied in polymer processing industry can provide several advantages that benefit for the biomass pretreatment industry, such as excellent temperature control, allowance of high solid loading, and simultaneous heating and shearing. In 1999, a first report [254] of using extrusion processing for AFEX pretreatment was published with the aim of exploring a continuous and large-scale process. In this method, a twin-screw extruder (barrel diameter=30 mm, length/diameter=10/1) was adopted with co-rotating screws. Instead of liquid ammonia pretreatment in batch reactor, the liquid ammonia was pumped into the extruder at one-half of the barrel length to pretreat corn stover meal at 40 to 50 ℃. The die at the sample outlet actually created a high pressure and pressure drop after sample releasing. The extrusion-combined-ammonia pretreated corn stover exhibited enhanced enzymatic digestibility, with total sugar yield of 2.4 times higher than that of unpretreated samples after 24 hours hydrolysis. More interestingly, little improvement of sugar production was observed after extrusion without ammonia. Although this method facilitated continuous pretreatment and obtained comparable sugar yield as batch AFEX process, the major disadvantage was ascribed to the inevitable release of ammonia, which made it impracticable. The authors performed several modifications to prevent ammonia release such as enclosed supplemental room-ventilation units, fume hood installed directly over the extruder and low temperature for processing and die to

reduce ammonia vapor. All these efforts resulted in inadequate solutions and options to find alternative solvents for the extrusion pretreatment with ammonia.

The group of Lee conducted a line of research combining an extrusion system with biomass pretreatment starting in 2009. Their first work investigated [255] the effects of organic additives on the fibrillation of Douglas fir by a continuous extrusion. A twin-screw extruder was used with barrel diameter of 15.4 mm and length/diameter of 17. Ethylene glycol, glycerol and dimethyl sulfoxide were the three major additives studied here to decrease the operational torque. Biomass flour and additives in a mass ratio of 1/200 were loaded to the extruder at 50 rpm counter-rotating with temperature ranging from 40 to 180 ℃. The enzymatic saccharification of the resultant biomass was tested, which showed ethylene glycol was more effective as an additive for the extrusion process at 40 ℃, obtaining a maximum glucose yield of 62.4% (6 times larger than the control sample) from enzymatic hydrolysis and largest surface area of 21.32 m^2/g. The fibrillated biomass had diameters smaller than 1 μm and even 100 nm. The authors considered ethylene glycol as the best additive for biomass fibrillation through extrusion because it could hydrogen bond with cellulose microfibrils to prevent their reaggregation; however, this reason itself was not adequate enough for the poor performance of glycerol, a similar highly polar solvent as ethylene glycol. In addition, Lee et al. [256] tested the batch-type kneader to fibrilate Douglas-fir using water as additive. From their reports, 30% of ball-milled biomass and 70% of water was added to the 15 ml mixing chamber, kneading for 2 to 30 minutes at 40 ℃. After kneading pretreatment, the biomass was fibrillated with similar diameters as those from organic additive extrusion, with an increase of biomass surface area from 18 for untreated control samples to 24 m^2/g. The pretreated biomass exhibited a maximum glucose yield of 54.2% after enzymatic hydrolysis. This saccharification degree was not attractive for application; however, it provided a new direction of pretreatment to involve the extruder for biomass fibrillation. Further efforts were conducted by the group of Lee; in a recent report [257] from their research, twin-screw extruder processing was combined with hot-compressed water to pretreat Douglas fir and Eucalyptus. This new method, applying water to solid ratio of 5 to1, was conducted at temperatures from 140 to 180 ℃ and pressure afforded by nitrogen gas of 1 MPa. Since the hot-compressed water could pretreat biomass through autohydrolysis as steam and hydrothermal pretreatment, extrusion used here was found dramatically improve the enzymatic saccharification

for both wood species and to facilitate biomass fibrillation. Compared to the glucose yield from only hot-compressed water treatment, less than 5% after 48 hours enzymatic hydrolysis, the combined method resulted 5 times higher conversion for Douglas fir. Similar to results shown previously, this extrusion method caused fiber resizing down to the sub-micro or even nano scale. In general, the research by Lee et al. applied the extrusion process to biomass pretreatment with different additives, which showed the continuous extrusion system improved biomass saccharification with enlarged surface area.

Karunanithy et al. [258, 259] investigated the effects of extrusion parameters and moisture contents on pine and swichgrass in details. In the case of switch grass with moisture content of 15%, lowest screw speed of 50 rpm and highest extrusion temperature of 150 ℃ facilitated sugar recovery after enzymatic hydrolysis; whereas, the pretreated pine chips with 25% moisture extruded with a screw speed of 150 rpm at 180 ℃ obtained maximum glucose yield of 65.8% that was 6.7 times higher than the control samples.

The most recent research by Lee et al. [260] explored the combination of ionic liquids (IL) and a twin-screw extruder for the continuous pretreatment of sugarcane bagasse. Typically the major drawback of ionic liquid pretreatment was low solid loading such as 5% solid loading, however using IL with an extruder showed an increase in the solid loading in the range of 25% to 50% by mass. The ionic liquid used in this research was 1-ethyl-3-methylimidazolium acetate. As reported from this method, the composition of pretreated bagasse did not change, that is, no significant lignin and hemicellulose degradation and dissolution occurred during this pretreatment. When the solid loading was 25%, the ionic liquid extrusion process at 140 ℃ for 8 minutes led to highly digestible bagasse, with glucose yield of over 90% after 24 hours enzymatic hydrolysis. Detailed characterization of pretreated biomass showed a decrease of cellulose crystallinity and drastic increase of specific surface area (maximum of 136.4 m^2/g compared to 0.8 m^2/g for untreated samples).

The above studies suggest extrusion systems are highly attractive for developing novel pretreatment methods because they meet demands required for scalable systems. Such characteristics are related to high solid loading and continuous, and industry-preferred processing that suggests potential to develop fractionation and pretreatment into large-scale applications.

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Chapter 3 Biomass fractionation after denaturing cell walls by glycerol thermal processing

3.1 Abstract

Denaturing biopolymers allows the transformation of highly organized natural structures into industrially relevant materials such as extruded starch or gelatin. This approach was used to fractionate wood into its biopolymer constituents by treating wood particles on polymer processing equipment at high temperatures in the presence of anhydrous glycerol. Nine severities were studied to assess the impact of time and temperature during the processing. After processing, the biomass was stripped of its lignin and xylan by subsequent extractions without addition of added catalysts, leaving a relatively pure cellulose fraction, 84% glucan, as found in chemical pulps. Additionally, 41% of the lignin and 68% of the xylan was recovered in a dry powdered form. The hemicellulose side-chain carbohydrates such as arabinose and galactose were water extracted, while the majority of the mannan remained with the cellulose fiber. High temperature processing for short times in a benign solvent provides significant disruption of the cell wall, while glycerol prevents significant degradation of the biomass.

3.2 Keywords

Fractionation, Mass balance, Glycerol, Pretreatment, Lignocellulosic biomass

3.3 Introduction

Natural materials have supramolecular structures that originate from self-assembly processes that are under cellular control. For small assemblies like enzymes these proteins fold into specific architectures that impart functionality to the material. It is well known that natural architectures of the biopolymers are transformed by heat, chaotropic agents, or other solvents into non-native structures. Changes in pH or heat overwhelm secondary interactions between amino acids, carbohydrates, or other building blocks. This denaturing of structure is utilized industrially to create materials like gelatin derived from the chemical treatment of animal collagen or processing natural materials like starch on extrusion equipment to create thermo-formable bioplastics [1]. For these systems, thermal energy is applied with a plasticizer that lowers the

glass transition temperature of the material allowing polymer chains to undergo flow without degradation [2]. However, some biopolymers have specific chemical linkages, such as disulfide linkages found in keratin, which must be chemically broken before they can be denatured and melt processed into moldable bioplastics. In this case, specific reducing agents are applied to transform proteinaceous biomass such as feather during melt processing, keeping most of the polymer backbone chemically unmodified [3]. For very complex materials like woody biomass this concept of denaturing, at first glance, seems unusual to apply. The wood cell wall involves multiple structural biopolymers such as cellulose, lignin, and heteropolysaccharides like glucoronoxylan and glucomannan that have varying sensitivities to thermal treatments. Typically, a number of side-products are formed during thermal processing that self-catalyze the degradation. An example is found with steam-explosion treatment combining hydrothermal treatment that plasticizes the cell wall with explosive decompression shearing apart fibers [4]. The native structure changes significantly, however there is severe disruption of the polymeric backbones arising from the acid catalyzed reactions associated with the saponification of the hemicelluloses. This result in turn significantly reduces the molecular weight of the polysaccharide backbone, forms furfural by-products, and modifies the lignin to varying extents from depolymerization and repolymerization reactions [5-7]. While the native cell wall architecture is denatured [8], the biopolymers are significantly chemically altered [9].

Because wood and natural biomass is increasingly valued for its inherent stored energy in lignoglucose, there is burgeoning research in "clean fractionation" to separate the polymeric components of biomass into cellulose-rich, hemicellulose-rich and lignin-rich fractions [10-18]. The polymeric components serve as valuable co-products, off-setting the cost associated with bioconversion. For instance, dry distiller grains are the by-product of corn ethanol production and are usually sold as animal feed. This valuable co-product can make the difference between operating in the red or black when corn prices are high because of seasonal price fluctuations. Analogous to the grains, xylan and lignin have been assessed to have significant added value to the cellulosic biorefinery [19]. Here we report on the idea of denaturing wood to recover the biopolymer materials through thermally processing in the presence of the plasticizer glycerol. Glycols are known to protect biopolymers from high temperature dehydration reactions [20] and lower the glass transition temperature [21] with the benefit of limited acid formation occurring in anhydrous conditions. Hence, the glycerol softens the cell wall and protects it from the usual acid

reactions found in autohydrolysis research, additionally serving as a heat transfer agent, and fostering thermal homolytic reactions. This system is different from the aqueous glycerol treatments reported in the literature used as an organosolv pulping process [22-25]. Demirbas reported up to 94% delignification accompanied by extensive hemicellulose loss from aqueous glycerol treatment for 9 hours in the presence of alkaline catalysts [22]. Moreover, biomass could be liquidified with aqueous glycerol and alkali, which resulted in lignin degradation into small fragments [26]. Sun et al. and Novo et al. [24, 25] studied delignification using non-catalytic aqueous glycerol to produce biomass substrates for enzymatic saccharification from wheat straw and bagasse. Both studies indicated 65~80% delignification after processing 2 to 4 hours at temperatures of 200 to 240 ℃ [23, 24]. This present method differs from the organosolvpulping technologies that hydrolyze hemicellulose backbones and actively attack covalent linkages and create side-reactions. A series of nine different processing conditions are investigated here to track the severity of the processing for controlled denaturation and degradation with the intent to separate the biopolymers from thermally processed, glycerol-plasticized wood. The work provides a simple path to generate co-products from biomass using a benign processing solvent.

3.4 Experimental

3.4.1 Materials

Chemicals and reagents used in this research were purchased from Sigma-Aldrich, Alfa Aesar and MP Biomedicals, and used as received. Deionized water (DI-water) was produced by Millipore Direct Q3UV with a resistivity of 18.2 mΩ. A mature sweet gum (*Liquidambar styracuflua*) from Blacksburg, VA was debarked, machined to cubes, and stored in a freezer before use. Prior to pretreatment, the biomass was knife milled using a Wiley mill and sorted to a particle size between 40 to 60 mesh on a metal screen (250-420 μm). Then, the sweet gum particles were Soxhlet extracted using toluene/ethanol (427 ml/1000 ml) followed by ethanol [27, 28] to produce extractive-free wood. The resulting extractive-free sweet gum particles (SG) were air-dried at ambient temperature for 48 hours.

3.4.2 Glycerol thermal processing (GTP) pretreatment

The extractive-free biomass was pretreated with glycerol using a bench-top internal mixing head with a high intensity shear roller blade driven by a C.W. Brabender® Prep-Center® drive. The rotation speed of internal blades was 100 rpm. Biomass (~6% MC) to glycerol weight ratio was 1 to 3. Processing time was chosen as 4, 8 and 12 minutes at 200, 220 and 240 ℃. To simplify the GTP conditions, a single severity parameter (R_0) calculated from time and temperature was adopted according to the equation defined by Overend and Chornet [29].

$$R_0 = t \times e^{(T-100)/14.75} \quad (1)$$

t is the pretreatment time (min) at temperature T (degrees Celsius).

The GTP pretreatment conditions and corresponding logarithm of R_0 used in this research are shown in Table 3-1.

Table 3-1 Glycerol thermal processing conditions and corresponding severity parameter.

Sample label	T (^{o}C)	t (min)	$\log(R_0)$
SG#1	200	4	3.55
SG#2	200	8	3.85
SG#3	200	12	4.02
SG#4	220	4	4.14
SG#5	220	8	4.44
SG#6	220	12	4.61
SG#7	240	4	4.72
SG#8	240	8	5.03
SG#9	240	12	5.20

After pretreatment, the GTP pretreated sweet gum (GTPSG) was collected from the mixing head and stored at 4 ℃ until further analysis. Samples for each severity condition were run in triplicates.

3.4.3 Water extraction

The GTPSG was first DI-water extracted for 2 hours to remove the glycerol residue and any degraded components in a 40 ℃ water bath. The solid to liquid ratio was 1/10 (m/v). The water extracted biomass was collected through centrifugation and rinsed by deionized water until colorless supernatant before freeze–drying. For the accurate mass balance calculation, the water extracted GTPSG was stored in a vacuum oven (0.9 mmHg, 40 ℃) for additional 48 hours over phosphorous pentoxide (P_2O_5) after freeze-drying. Unless otherwise noted, this vacuum drying procedure was used throughout the study.

3.4.4 Solvent extraction

The water extracted GTPSG was subjected to 96% (v/v) 1,4–dioxane[30] (azeotrope) extraction at a solid to liquid ratio of 1/25 (m/v) with continuous stirring for lignin fractionation. This extraction occurred at ambient temperature for 24 hours without additional catalysts. The residual biomass was collected through filtration, and subsequently washed by 96% dioxane, deionized water, and acetone until the wash was colorless. Then, the solvent extracted GTPSG was vacuum dried thoroughly. At the same time, the dioxane extract was subjected for lignin recovery.

3.4.5 Alkaline extraction

2.5 grams of completely dried solvent extracted GTPSG were extracted by 100 ml 1 M sodium hydroxide solution for the xylan fractionaton [31]. The alkaline extraction was performed at ambient temperature with continuous stirring in nitrogen environment. After 24 hours extraction, the mixture was centrifuged to separate biomass solid residue and alkaline extract. Sequentially 100 ml 1 M sodium hydroxide solution and 100 ml deionized water was used to wash the solid residue, and the washing liquids were collected and combined with the alkaline extract. Continually, the solid residue was thoroughly washed by deionized water until pH was neutral and freeze-dried followed by vacuum dried. The combined alkaline extract and washing was used to recover xylan.

3.4.6 Compositional analysis of extracted GTPSG

Lignin and carbohydrate contents of non-pretreated SG, water, solvent and alkaline extracted GTPSG were analyzed according to the NREL laboratory analytical procedure (LAP): Determination of structural carbohydrates and lignin in biomass [32]. Each analysis was run in duplicate. The acid insoluble lignin (Klason lignin) was analyzed gravimetrically through the mass difference before and after heating the acid-hydrolyzed residue at 575 ℃. The carbohydrates in the filtrate were analyzed in duplicates using Metrohm Ion Chromatography (IC) installed with a pulsed amperometric detector (PAD), Metrohm Inc., USA. Monosaccharides in the filtrate were separated by a Hamilton RCX -30 ($250 \times 4.6\ mm$) column with DI-water as eluent. Eluent flow rate was 1 ml/min and the column temperature was 32 ℃.

350 mmol/L NaOH with a flow rate of 0.43 ml/min was introduced after column separation to aid the signal generation in PAD at 35 ℃. Five sugars including L-(+)-arabinose, D-(+)-galactose, D-(+)-glucose, D-(+)-xylose and D-(+)-mannose were quantified in the Mag IC. Net software. Cellobiose was not detected in preliminary analysis and was excluded from the analysis. Linear calibration curves were run right before every batch test with R^2 >0.9999 and relative standard error <5%. The monosaccharide concentration was converted to the relative percentage of its anhydro-form in the biomass according to the NREL standard.

3.4.7 Specific surface area of water extracted GTPSG

Nitrogen adsorption specific surface area of non-pretreated SG and water extracted GTPSG was determined using Autosorb-1C (model: AX1C-MP-LP, Quantachrome Instruments, USA). Prior to analysis, samples of SG and GTPSG were dried completely under vacuum.

About 0.8 grams of SG or GTPSG was filled in a Quantachrome brand 6 mm large bulb cell for surface area analysis. The samples were degassed at 45 ℃ until outgas pressure rise was less than 50 microns Hg per minute. The multi-point analysis was performed at a temperature of 77 K provided by liquid nitrogen. The Autosorb-1C measured the weight of the adsorbed nitrogen gas on the solid surface at different relative pressure during the test. Sample specific surface area was calculated using Brunauer-Emmett-Teller (BET) theory [27, 33, 34] for the pressure range P/P_0 of 0.05-0.3 with the linear coefficient R^2 > 0.998.

3.5 Results and discussion

Compositional analysis of the GTP pretreated extractive-free sweet gum at nine pretreatment severities encompassing a range of processing times and temperatures (Table 3-1). Compositional analysis of pretreated and water extracted biomass is shown in Figure 3-1 as a function of pretreatment severity. After GTP pretreatment and water washing, the relative content of the three predominant hardwood constituents (glucan, xylan and lignin) remained constant or slightly increased regardless of the pretreatment severity. Only the minor carbohydrates existing in the heteropolysaccharides (arabinose and galactose) were observed to decrease during processing and water washing, while mannan decreased to a lesser extent. Although GTP pretreatment occurred at comparable pretreatment severities with auto-hydrolysis

or steam-explosion treatment, it does not result in severe degradation of the xylan into water-soluble monomers and oligomers [35]. This finding suggests the glycerol thermal processing is different from the aqueous thermal treatments where acidity arising from degradation of the wood auto-catalyzes the decomposition of the backbone linkages of the polysaccharides. In fact, the water-soluble extracts consisting of glycerol and the extracted wood components had pH values that were near neutral independent of pretreatment severity (pH= 5.8 to 6.1), similar to the pH of glycerol solution at the same concentration.

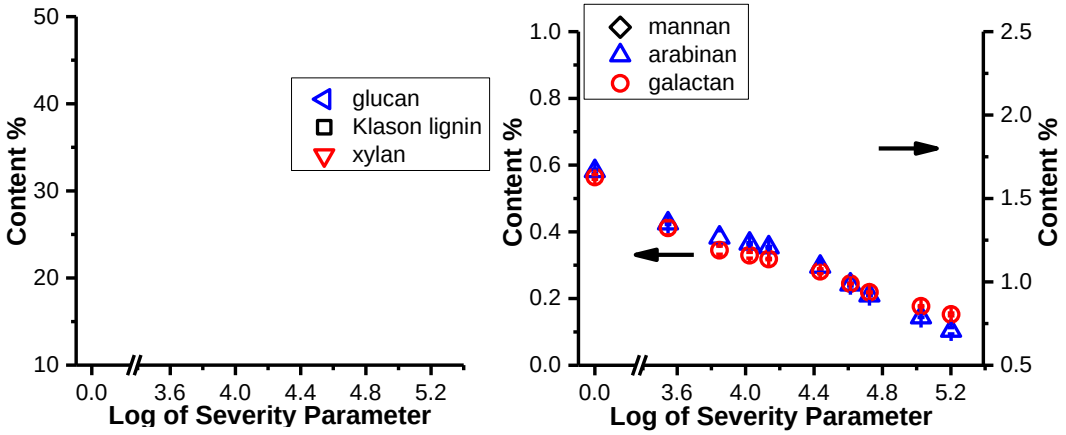

Figure 3-1 Compositional analysis of water-extracted GTPSG as a function of log(R_0).

After freeze-drying the GTP pretreated biomass, a mild solvent extraction using 96% 1,4-dioxane at ambient temperature without any acid or alkali catalysts, was adopted to isolate lignin from pretreated sweet gum. Compositional analysis of solvent extracted biomass residue revealed a strong correlation between lignin isolation with increasing GTP pretreatment severity (Figure 3-2). In comparison, no recoverable lignin could be isolated from non-pretreated sweet gum powder using this extraction procedure. The relative content of residual lignin in solvent extracted biomass decreased gradually with increasing GTP severity. Correspondingly, glucan and xylan relative concentrations were observed to increase when expressed as weight percentage of the extracted biomass. For the most severe GTP pretreatment, the residual Klason lignin accounted for 11% (w/w) of the solvent-extracted GTPSG, compared to 22% in native biomass and 25% just before solvent extraction. Up to 56% of the Klason lignin was removed after GTP pretreatment following organic solvent extraction. Lignin had the highest overall thermal stability of the biopolymers found in wood, however, close examination of the thermal

treatment of lignin revealed significant bond breakage can occur in the aryl-ether structures at temperatures below 200 ℃ [36].

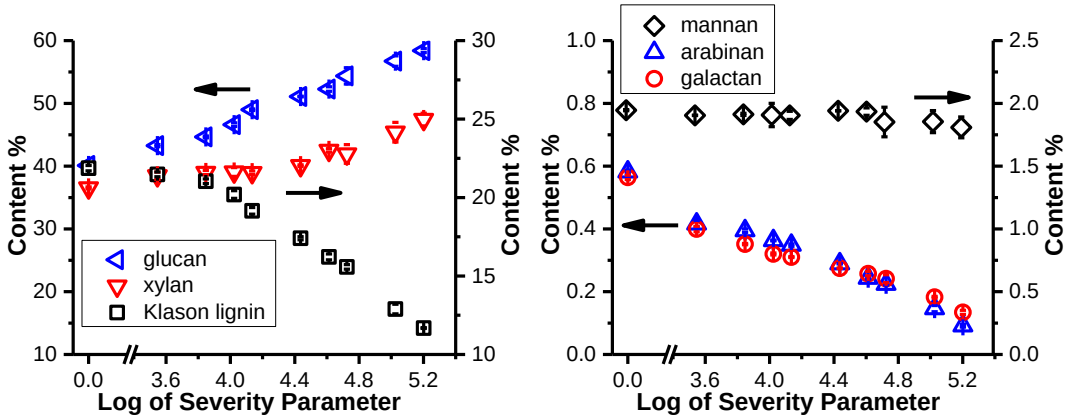

Figure 3-2 Compositional analysis of dioxane solvent-extracted GTPSG as a function of log(R_0).

After organic solvent extraction, the GTP biomass was dried and extracted with aqueous alkali. According to Figure 3-3, the relative glucan content of the extracted fiber compared to that of the non-pretreated fiber increased to 85%, while there was significant reduction of the xylan content across most of the severity treatments. The mannan content was not impacted by the alkali extraction staying relatively constant at 2.5% of the extracted fiber weight. This data showed that xylan extraction was not as sensitive to processing changes relative to the other components, as there was significant removal at low severity conditions.

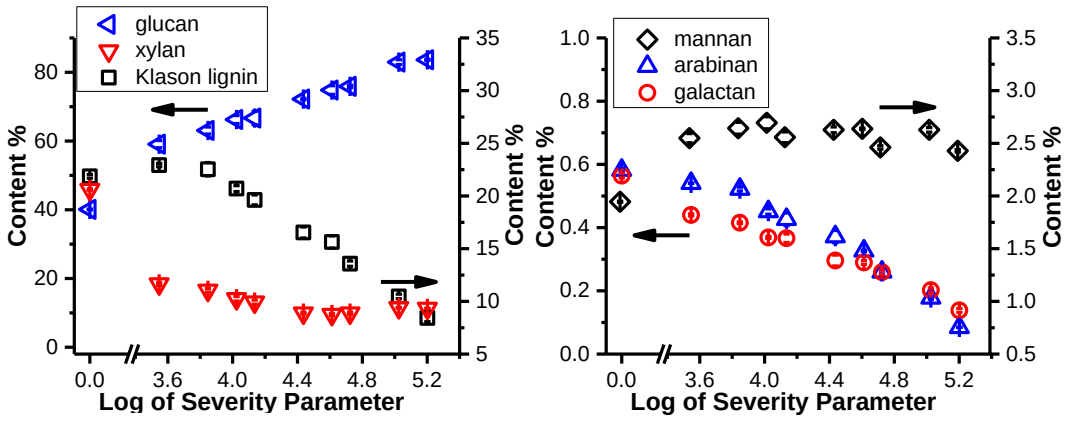

Figure 3-3 Compositional analysis of alkaline-extracted GTPSG as a function of log(R_0).

To better understand the impact of the processing conditions on the fractionation, the biopolymer concentration of the residual fiber was reported as a percent of the mass in the starting material (Figure 3-4). As indicated in Figure 3-4, cellulose content remained constant across all severity levels when disregarding fluctuations related to experimental error. At the highest severity level 98% of the cellulose remained showing that GTP pretreatment had little impact on the cellulose content. Lignin extraction was dictated by processing conditions, as there was a linear-log relationship with a R^2 factor of 0.985. The data reveals that extraction is dependent upon GTP severity removing up to 80% of the lignin without the aid of any catalyst as typically occurs with chemical pulping cooks. The main hemicellulose component in the wood, xylan, was the most extractable component across the majority of the processing severity conditions with the levels of xylan extracted reaching a maximum at the four highest severity conditions. Figure 3-4 also reveals that mannan content was reduced with processing severity but was resistant to extraction as still more than 50% of the initial mannan was left in the pretreated and extracted biomass residue at the highest severity level. However, on a total percentage basis this concentration accounted for only 2% of the mass. Additionally, processing conditions were closely linked to the extraction of side chain hemicellulose groups, galactan and arabinan, with R^2 correlations near 0.99 for both components.

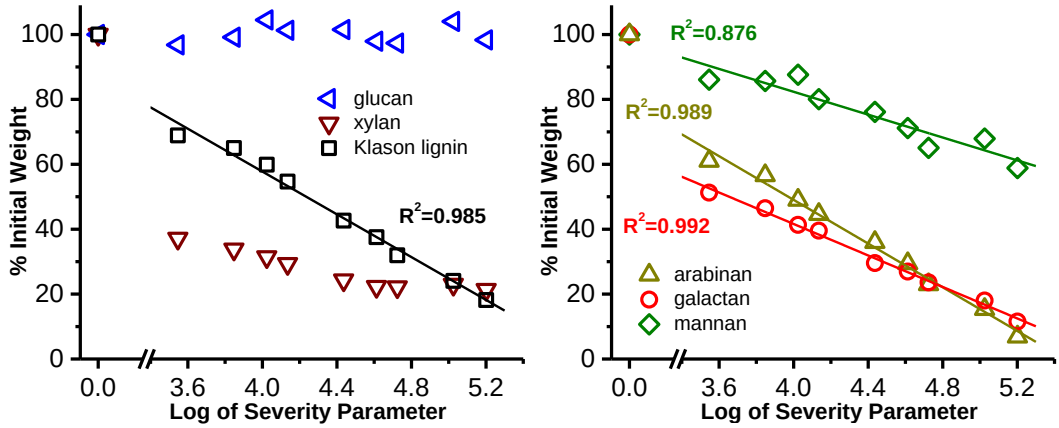

Figure 3-4 Mass balance of wood components after GTP pretreatment and extraction (calculated based on the average of duplicates with COV<10%).

The extraction of components in each step is plotted in Figure 3-5 to show the amount of biopolymer removed in each extraction stage. The majority of the arabinan and galactan residues

were removed during the water extraction stage with minor losses in the other extraction stages accounting for less than 5% of the arabinan and less than 10% of the galactan. The loss of lignin and xylan during the water extraction stage ranged from 10 to 20% dependent upon the severity and biopolymer type. While lignin itself is not water-soluble, the removal of lignin is not dictated by severity, which suggests the lignin extracted may be related to surface active lignin-carbohydrate complexes. Previous research in steam-assisted fractionation revealed 10% of the lignin was associated with the water-soluble fraction [9]. Moreover, it was clear there was preferential extraction of the lignin with the organic solvent and very little xylan was removed with the lignin during this extraction stage, yielding a high purity lignin based on simple mass balances. On the contrary, the subsequent alkali extraction revealed a near constant removal of lignin and xylan, irrespective of the processing conditions with a 1:3.5 of lignin to xylan ratio.

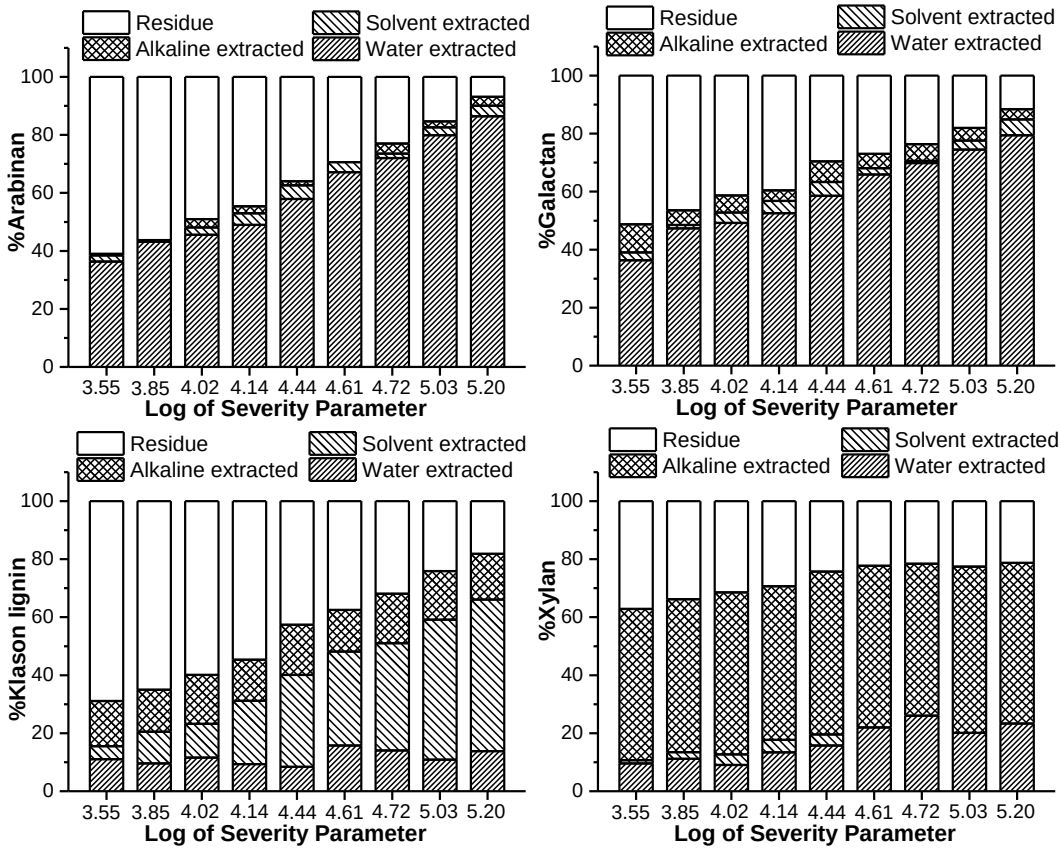

Figure 3-5 Biopolymer mass distribution (wt%) in relation to log(R_0) after GTP pretreatment and sequential extractions of water, aqueous dioxane, and 1.0N NaOH solution (calculated based on the average of duplicates with COV<10%).

Glycerol thermal processed biomass disrupted the cell wall allowing the extraction of the biopolymer constitutive components to various degrees. Processing the biomass in this manner opened the cell wall, as revealed by a change in specific surface area of the fiber (Table 3-2). For the mild and high severity conditions the surface area doubled and tripled, respectively. This data suggests the ability for enhanced access of the solvent into the cell wall structure of the biomass after disruption of the native cell wall network. The disruption is evident as the hemicellulose side chains that have been proposed to serve as connections to lignin through ether bonds [37, 38] are cleaved as a function of temperature and removed through water extraction. In one sense, this bond rupture is analogous to the breakage of disulfide linkages when processing materials like keratin with reducing agents [3] to allow polymer flow. In this case there is a strong correlation between the delignification and the loss of side chain hemicellulose components (Figure 3-6), which suggests either that there is a common activation energy for these changes to occur or key linkages that need to be broken prior to delignification.

The thermal decomposition of hemicelluloses are reported in the literature at temperatures as low as 180 to 220 ℃ [39, 40]. However, for model studies, carbohydrate structure, linkage type, degree of polymerization, and the degree of substitution impact the degradation temperatures [41, 42] and this would suggest the ability to selectively cleave carbohydrate linkages. For example, α-1,6 linkages are reported to be less stable than β-1,4 linkages, providing some insight into the selective removal of certain units. In the current work, galactose residues in hardwoods are linked along the main chain of glucomannan at the α-1,6 position [43]. Additionally, arabinose units are α-linked at the 2 or 3 position along the xylan backbone [44]. Both of these cases, based on the model study using pyrolysis, suggest a preferential mechanism in cleavage of these side groups. Currently, there is no mechanism proposed in the retention of xylans at these high temperatures when degradation occurs above 220 ℃ in an inert atmosphere [39]. Xylan has a linear repeating backbone chemistry and is known to be acetylated at a molar ratio close to 1:0.5 Xyl:Acetyl [39]. It has been reported that acetylated xylans have lower thermal stability than their deactylated counterparts [45]; however, derivatized xylan acetates are more thermally stable providing competing evidence to the impact of substitution on thermal stability [46]. Furthermore, it is reported in the literature that the predominant aryl ether linkages of lignin are sensitive to temperatures above 180 ℃ [47] and the potential breakage of these

linkages at processing temperatures in that range would contribute to the delignification of the pretreated biomass. Hence, the processing conditions that approach the thermal degradation temperature of hemicelluloses and fragmentation of lignin, give rise to thermolytic reactions for fractionation with minimal degradation of the main polysaccharide polymers in the presence of glycerol. Glycols are capable of preventing polysaccharide chain degradation at high temperature and pressure via hydrogen bond formation [20, 48]. In other words, glycerol offers protection in preventing dehydration, impacting glycosidic bond cleavage. Additionally, glycerol may serve as a source for hydrogen abstraction to prevent further radical degradation pathways. These findings suggest that thermal processing in glycerol can be used to tailor the hetero-polysaccharides, dependent upon the temperature, developing more uniform biopolymer streams from biomass.

Table 3-2 BET Specific surface area (SSA) of GTP pretreated biomass before solvent extraction.

$\log(R_0)$	SSA (m^2/g)
SG control	0.807 (0.019)
4.44	1.785 (0.064)
5.03	2.428 (0.237)

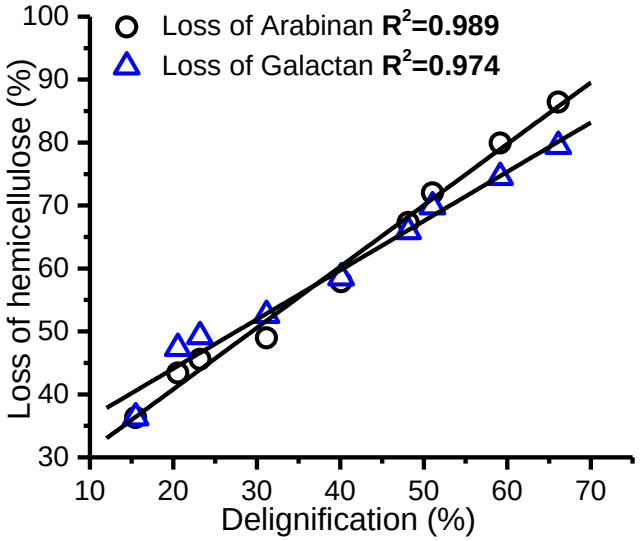

Figure 3-6 Loss of hemicellulose side-chain groups (after water extraction) as a function of delignification (after solvent extraction) (calculated based on the average of duplicates with COV<10%).

Developing a clean fractionation concept requires the recovery of components in non-modified form and in high yield. Currently, pulp and paper processes isolate a highly pure cellulose component of 80 to 95% cellulose, dependent upon pulping technologies and bleaching sequences. In the current research, glycerol thermal processing was applied as a novel pretreatment to denature the native cell wall structure facilitating biopolymer fractionation (Figure 3-7). Lignin from the highest severity treatments could be recovered at 41% dry weight from precipitation of the organic phase. This would be equivalent to 9% of the starting biomass providing an opportunity to develop added co-products from lignin. Additionally, 68% of the xylan is recovered after precipitation or 14% of the initial biomass weight. In contrast, pulping processes do not recover hemicelluloses and the main industrial source of isolated hemicellulose is processed agriculture grains. Most isolation procedures for fractionation of biomass involve acid catalysts, as used in organosolv pulping, or use dilute acid and these technologies cleave the xylan backbones to various extents. Similarly, autohydrolysis, pre-hydrolysis kraft pulp, and steam-explosion technologies significantly break the hemicellulose backbone to various degrees leaving an oligosaccharide-rich solution. An example of this is seen in the clean fractionation concept using steam-explosion [49] that achieves similar levels of lignin extraction and cellulose purity to the present study, but significant loss of the xylan into oligosaccharides occurs [9]. All

together glycerol thermal processing of biomass produces three potential sources of polymer components that equate to nearly 70% of the starting mass, which have potential values ranging from ca. $0.70/kg for glucose [50] to ca. $1.40/kg in equivalent polymer precursors, such as ethylene [51].

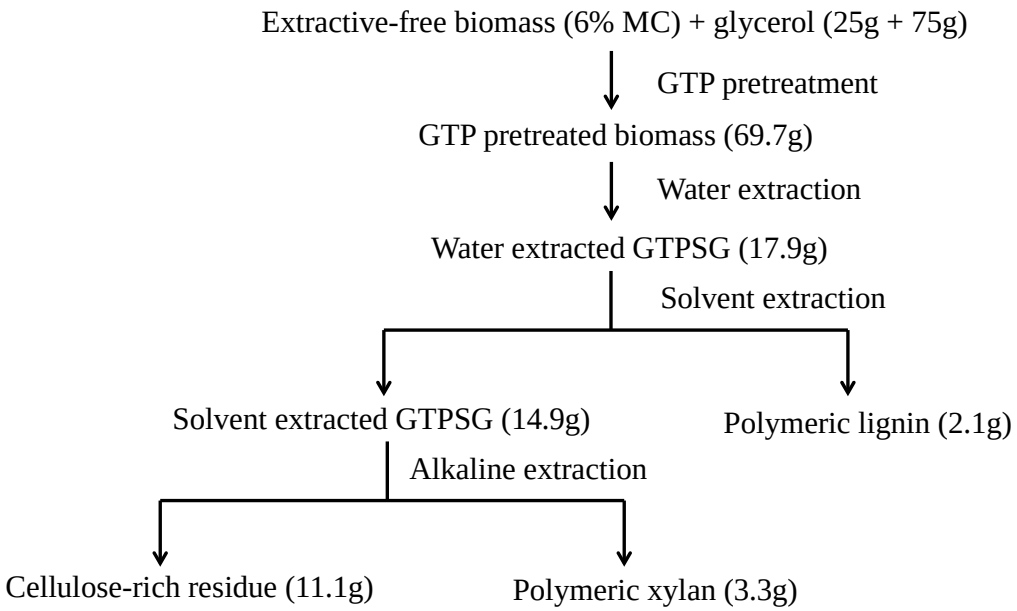

Figure 3-7 Biopolymer fractionation from GTP pretreated biomass.

3.6 Conclusions

The present study demonstrates the efficient removal of the majority of the non-cellulosic biopolymers simply through a series of extractions after treatment of the biomass using polymer processing equipment in the presence of glycerol. To evaluate this process, time and temperature variables are combined in a single severity parameter as a means of controlling the degree of cell wall disruption. Data show that delignification and removal of side-chain hemicellulose components are directly correlated to the severity of the processing condition. At the highest severity, 240 ℃ and 12 minutes, a total of greater than 80% of the initial lignin becomes extractable with more than 40% of the initial lignin recovered in a precipitated solid powder from the solvent extraction. Furthermore, greater than 65% of xylan is recovered, leaving a cellulose fiber-rich substrate that is 84% in purity. The study shows how controlled denaturing and deconstruction using thermal methods in the presence of glycerol provides for a clean

fractionation process where xylan, lignin, and cellulose are recovered in good yield, providing a source to develop value added co-products.

3.7 Acknowledgments

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Chapter 4 Qualification of lignin from glycerol thermal processing of biomass for polymeric applications

4.1 Abstract

Glycerol thermal pretreatment (GTP) was studied in this research to pretreat sweet gum for lignin isolation. The GTP process occurred at moderately severe temperatures between 200 and 240 ℃ in an anhydrous environment, providing insight into structural changes of lignin at these temperatures, common to polymer processing conditions. After GTP pretreatment, 41% of the initial Klason lignin in biomass input was recovered in a powdered form through a mild dioxane extraction. ^{31}P-NMR of the GTP lignin revealed the syringyl phenolic functionality increased linearly with the log of the severity parameter due to the alkyl aryl ether bond breakage accompanied with liberation of phenolic OH. Further structural analysis via thioacidolysis and 2D-HSQC of the isolated lignin indicated that the glycerol could also facilitate the C-γ elimination followed by the extensive β-O-4 bond decomposition. At the same time, condensation occurred on aromatic C5 to yield GTP lignin with a relatively high molecular weight, comparable to that of enzymatic mild acidolysis lignin from non-pretreated fibers. The recovered GTP lignin was more thermally stable compared to nearly all other lignins found in the literature, with a glass transition temperature that was invariant to processing severity parameters. This finding proposed a novel polymeric lignin stream recovered as byproducts from biorefinery process.

4.2 Keywords

Lignin isolation, Glycerol thermal processing, Structural analysis, NMR

4.3 Introduction

Lignin is one of the most abundant biopolymers on earth, but also one of the most perplexing to convert into an industrial feedstock for polymeric applications. There is a strong specialties market for selected lignin polymers [1-3], such as lignosulfonates, due to their self-assembling properties. However, the most abundant form of isolated lignin, kraft lignin, has not breeched the commercial market as a renewable replacement for many thermoset [4] and thermoplastic [5]

applications, although it is qualified in terms of performance [1]. Moreover, other pilot scale lignins such as steam-exploded lignin and organosolv lignin have shown enhanced performance relative to kraft lignins when used in polymeric applications [1, 6-8]. Hence, the rearrangement of lignin structure during pulping operations can negatively impact the structure of lignin. Processes to control functionality during isolation should be carefully viewed as a means to enhance the qualification of lignin for polymeric applications, if lignin will become a ubiquitous engineered polymer, similar to the abundance of it in the plant kingdom.

The paper-making industry is the major source of isolated lignin for high quality sulfate pulp production, whereas the resulting lignin is partially depolymerized and recondensed into fractions that are commonly used as a heating resource in paper mills [1, 9]. Recently, the "LignoboostTM" method [10] provides a potential consistent source of lignin for the polymeric materials market. As the development in biorefinery industry, pretreatment is an essential step utilized to unlock the biomass recalcitrant structure, which renders carbohydrates more accessible to enzymatic saccharification and fermentation, and at the same time, allows lignin isolation for novel applications. Unlike pulping, fiber quality is not important so new delignification methods to access the polysaccharides are not limited by the impacts they have on paper properties. There are several pretreatment methods that have been developed to reach efficient separation of carbohydrates and lignin, including dilute acid exposure, alkali soaks, steam explosion, hydrothermal treatment, organosolv pulping, ionic liquids, as well as mechanical and biological methods [11, 12]. Lignin isolated from steam-exploded or hydrothermal treated biomass is more heterogeneous because of the simultaneous depolymerization [13, 14] and repolymerization [13, 15] occurring to lignin during pretreatment although it is seen to have enhanced solubility relatively to kraft lignin. Acid pretreatment [11] not only results in severe lignin degradation and condensation but also causes equipment corrosion. In contrast, organosolv pretreatment is a potential candidate to produce high quality lignin with low molecular weight and high purity [1, 16], which has been utilized to develop lignin based biochemicals. The most common solvents used in organosolv pretreatment are ethanol or acetone combined with an acid catalyst based on the "Alcell" process [17]. Organosolv lignin has shown superior performance in polymeric applications to kraft lignin in practically every study where the lignins were compared [1].

Glycerol, an alternative to ethylene glycol, is considered a benign solvent that has been used for industrial processes such as a plasticizing agent for melt-processed biopolymers [18] or in lab scale processes for pulping or pretreating biomass [19]. Studies using aqueous glycerol with alkaline catalysts resulted in direct lignin removal with lignin degradation into small fragments [20, 21], considering that glycerol as a highly polar solvent could hydrogen bond with cellulose and swell wood biomass, improving penetration of alkali catalysts, which resulted in lignin degradation and solubility in solvents [21, 22].

Different from the alkaline catalyzed glycerol pretreatment, a novel method is developed to investigate the isolation of wood polymers, where non-aqueous glycerol is adopted to plasticize and swell woody biomass under the continuous treatment afforded by an internal mixing bowl at elevated temperature. This glycerol thermal processing is unique in that it is conducted at high solid loading, without acid or alkali catalysts at moderately high temperatures. Furthermore, polymeric lignin is isolated and recovered with a simple extraction process post glycerol thermal processing. The following study determines the structure of the isolated lignin recovered in this process using nuclear magnetic resonance (NMR) spectroscopy, thioacidolysis, gel permeation chromatography (GPC), and thermal analysis. The changes which occur in native lignin during processing can be directly related to processing conditions including moderately high temperatures, typically in the range of polymer processing of most thermoplastics, which have a marked effect on lignin structure.

4.4 Experimental

4.4.1 Materials

Chemicals and reagents used in this research were purchased from Sigma-Aldrich, Alfa Aesar and MP Biomedicals and used as received. A mature sweet gum (*Liquidambar styraciflua*) from Blacksburg, VA was debarked, machined to cubes, and stored in a freezer before use. Prior to pretreatment, the biomass was knife milled using a Wiley mill, and sorted to a particle size between 40 to 60 mesh on a metal screen (250-420 μm). Then the sweet gum particles were Soxhlet extracted using toluene/ethanol (427 ml/1000 ml), followed by ethanol [23] to produce extractive-free wood. The resulting materials were air-dried at room temperature for 48 hours.

4.4.2 Glycerol thermal processing (GTP) pretreatment

The extractive-free biomass was pretreated with glycerol using a bench-top internal mixing head with high intensity shear paddler driven by a C.W. Brabender® Prep-Center® drive. More details of this step were listed in Chapter 3. The GTP pretreatment severity parameter (R_0) was calculated according to the equation defined by Overend and Chornet [24] to depict the pretreatment conditions. The corresponding logarithm of R_0 in this research was shown in Table 4-1.

Table 4-1 Glycerol thermal processing conditions and corresponding severity parameters.

Sample label	T (℃)	t (min)	$\log(R_0)$
SG#1	200	4	3.55
SG#2	200	8	3.85
SG#3	200	12	4.02
SG#4	220	4	4.14
SG#5	220	8	4.44
SG#6	220	12	4.61
SG#7	240	4	4.72
SG#8	240	8	5.03
SG#9	240	12	5.20

After pretreatment, the GTP pretreated biomass was collected from the mixing head and stored at 4 ℃ until further analysis. Samples for each severity condition were run in triplicate.

4.4.3 Lignin isolation from pretreated biomass

GTP pretreated sweet gum (GTPSG) was first water extracted to remove the additional glycerol residue and any degraded components before lignin isolation. More details on water extraction were referred to Chapter 3.

Solvent extraction of thoroughly dried water-extracted GTPSG was performed using 96% (v/v) 1,4–dioxane [25] (azeotrope) at a solid to liquid ratio of 1:25 with continuous stirring at ambient temperature without catalysts. After a 24 hour extraction, the residual biomass was collected through filtration, whereas the filtrate was further passed through a 10 μm glassy-fiber filter to remove any biomass solids before concentrating to <100 ml under reduced pressure. The concentrated solvent extract was added dropwise into 1 L 0.01 M hydrochloride acid [26] (pH=2) for lignin precipitation. After settling overnight at 4℃, the precipitated lignin was collected through centrifugation and further rinsed 3 times with deionized water before freeze-drying. The recovered lignin, labeled as GTP lignin, was vacuum dried for mass balance calculations.

Compositional analysis of GTP biomass before and after solvent extraction was performed according to the NREL standard.

The GTP lignin recovery yield relative to the total initial lignin in the originial biomass was calculated as follows:

$$recovered\ lignin\% = \frac{m_{rec\ lignin}}{m_0 \times Klignin_0} \times 100\% \quad (1)$$

$m_{rec\ lignin}$: dry mass of recovered GTP lignin for specific biomass input to pretreatment;

m_0 : total dry mass of biomass input to GTP pretreatment;

$Klignin_0$: Klason lignin content in biomass imput.

A control lignin following the isolation protocol developed by Argyropoulos and co-workers [26] for enzymatic mild acidolysis lignin (EMAL) from extractive free sweet gum was used as a reference for the GTP process.

4.4.4 GTP lignin elemental analysis

Elemental analysis (carbon, hydrogen and nitrogen) of isolated GTP lignin was performed by Atlantic Microlab Inc., Georgia, USA. Contents of C, H and N were determined on automatic analyzers comprised of Perkin-Elmer Model 2400 Series II autoanalyzers, LECO CHNS-932 Analyzers; Carlo Erba Model 1108 Analyzers and Costech ECS. Results were reported as weight percentage of biomass dry weight (C%, H% and N%). Oxygen content (O%) was calculated by subtracting the composition fraction of the carbon, hydrogen, and nitrogen from unity.

4.4.5 GTP lignin acetylation and ^{1}H-NMR

Lignin acetylation was conducted according to the procedure of Glasser et al. [27] with slight modification. 100 mg dried lignin (GTP lignin or EMAL) was dissolved in 3 ml anhydrous pyridine followed by the addition of an equal volume of anhydrous acetic anhydride. The reaction was performed at room temperature for 24 hours in a nitrogen atmosphere with continuous stirring. Then, the reaction mixture was added dropwise into 200 ml 0.01 N hydrochloric acid. The acetylated lignin was collected through filtration using a 10 μm nylon filter and then rinsed using 0.01 N hydrochloric acid 3 times to remove any solvent residue, followed by a 3X deionized water wash. The resulting lignin was freeze-dried followed by vacuum drying.

20 mg of acetylated lignin was fully dissolved in 500 μl chloroform-d_6. An internal standard for NMR quantification was prepared by dissolving 20 mg 2,3,4,5,6-pentafluorobenzaldehyde (PFB) in 1.5 ml chloroform-d_6. Then, 200 μl PFB solution was added into the lignin solution before transferring to a 5 mm NMR tube. The ^{1}H-NMR was collected on a Bruker Avance II 500 MHz multinuclear spectrometer with a pulse angle of 30° and pulse delay time of 7 s. A total of 32 scans was acquired and the spectra were processed using MestReNova software. All chemical shifts were reported according to TMS internal reference at 0 ppm. Peak assignments of quantitative ^{1}H-NMR were shown in Figure A-1 according to previous reports [28-31].

4.4.6 Quantitative ^{31}P-NMR analysis of recovered polymeric lignin

Quantitative phosphorous-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR) analysis was performed on a Varian INOVA 400 MHz multinuclear spectrometer at a frequency of 162.07 MHz for ^{31}P spins. This method was adapted from reports by Argyropoulos [32-37]. Prior to analysis, all lignin samples were stored in a vacuum oven for 48 hours.

A solvent mixture composed of pyridine/chloroform-d_6 ($CDCl_3$) with a ratio of 1.6/1 (v/v) was freshly prepared for ^{31}P-NMR. Endo-N-hydroxy-5-norbornene-2,3-dicarboximide (e-HNDI) was chosen as an internal standard [38], which was prepared at a concentration of 11.32 mg/ml from the above solvent mixture, pyridine/$CDCl_3$ (1.6/1). Chromium (III) acetylacetonate ($Cr(acac)_3$) as the spin relaxation reagent [39] was prepared in $CDCl_3$ at a concentration of 10 mg/ml. 30 mg of GTP lignin or EMAL was dissolved in 500 µl of the above solvent mixture followed by adding 200 µl e-HNDI solution and 50 µl $Cr(acac)_3$ solution. The solution was thoroughly mixed using a vortex mixer. Then, 100 µl 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (PR(II)) was added as the phosphitylation reagent followed by extensive vortex mixing. The final solution was immediately transferred to a 5mm NMR tube and quantitative ^{31}P-NMR spectra were acquired immediately. Specific spectra acquisition parameters were as follows: a pulse angle of 90° and pulse delay time of 5 seconds was chosen to improve signal to noise ratio as well as full relaxation; spectra width was 33999.2 Hz; to avoid peak splitting effects, an inverse-gated decoupling on proton spins was applied. Quantitative spectra were collected without Nuclear Overhauser Effect. A total of 256 scans was collected for each sample and subsequently the spectra were processed using MestReNova software. Signal to noise ratio was improved using signal apodization: exponential 3.0 Hz with additional Gaussian 1.0 GB. Zero filling was 256 K. All chemical shifts were reported relative to the sharp peak of the phosphitylated water (residual moisture) at 132.2 ppm.

Peak assignments of quantitative ^{31}P-NMR were shown in Figure A-2, based on previous reports [34, 38]. It should be noted that syringyl phenolic groups were integrated separately from the C5 condensed units based on well-described studies in literature [34].

4.4.7 Lignin thioacidolysis-gas chromatography (GC)

Thioacidolysis of completely dried GTP lignin was performed according to the report by Rolando et al. [40]. This experiment was conducted in an anhydrous environment with dry nitrogen flow to guarantee maximum yield of lignin products, avoiding consumption of reagent by moisture.

GC analysis of trimethylsilylated thioacidolysis products was performed on an Agilent gas chromatograph (GC) equipped with a polydimethylsiloxane fused silica capillary column ($30\ m \times 320\ \mu m$, length×I.D., with $0.25\ \mu m$ film thickness). GC conditions were: Injector port 280 ℃ with a sample flow rate of 1 ml/min. The oven temp was ramped from 200 ℃ to 260 ℃ at 4 ℃/min and then increased to 300 ℃ at 30 ℃/min. Detection was performed with a flame ionization detector (FID) at 300 ℃. Hydrogen was used as the carrier gas with a flow rate of 30 ml/min. 1 µl of silylated sample was manually injected and the resulting chromatograms were analyzed using OpenLAB CDS ChemStation software.

4.4.8 2D ^{13}C-^{1}H heteronuclear single quantum coherence (HSQC) NMR spectroscopy of recovered lignin

All lignin samples were prepared using DMSO-d_6 and sonicated until homogenous in a Branson 2510 table-top cleaner (Branson Ultrasonic Corporation, Danbury, CT). The temperature of the bath was closely monitored and maintained below 55 ℃. The homogeneous solutions were transferred to NMR tubes. HSQC spectra were acquired at 25 °C using a Bruker Avance-600 MHz instrument equipped with a 5 mm inverse-gradient $^{1}H/^{13}C$ cryoprobe using a Q-HSQC ETGP pulse program (ns = 200, ds = 16, number of increments = 256, d_1 = 1.0 s) [41]. Gaussian apodization in F_2 (LB = -0.18, GB = 0.005) and squared cosine-bell in F_1 (LB = -0.10, GB = 0.001) was applied prior to 2D Fourier Transformation. Chemical shifts were referenced to the central DMSO peak at 39.5/2.5 ppm (δ_C/δ_H). Assignments of the HSQC spectra were described elsewhere [42, 43]. A semi-quantitative analysis of the volume integrals of the HSQC correlation peaks was performed using Bruker's Topspin 2.0 processing software. The C2–H2 position of the guaiacyl unit and the C2,6–H2,6 positions in the syringyl unit were considered to be stable [44] and used as internal standard that represents aromatic C9 units in the lignin. Importantly, spectra integration was performed on the same contour level.

4.4.9 Size exclusion chromatography of recovered lignin

Lignin solutions, 1% (w/v), were prepared in analytical-grade 1-methyl-2-pyrrolidinone (NMP). The polydispersity of dissolved lignin was determined using analytical techniques involving SEC UV-A_{290} as previously described [45]. An Agilent 1200 series binary LC system (G1312B) equipped with diode-array (G1315D) detector was used. Separation was achieved with a Mixed-D column (5 µm particle size, $300\ mm \times 7.5\ mm$ i.d., linear molecular mass range of 200 to 400,000 u, Agilent Technologies Inc.) at 80 ℃ using a mobile phase of NMP at a flow rate of 0.5 ml/min. Absorbance of materials eluting from the column was detected using UV-A absorbance (λ=290 nm). The SEC chromatograms were shown in Figure A-3. Spectral intensities were area-normalized and relative molecular mass were determined after calibration of the system with polystyrene standards.

4.4.10 Thermal analysis of recovered lignin

The thermal degradation temperature of dried isolated lignin was studied by thermogravimetric analysis (TGA) on a TA instruments Q100 with a heating rate of 10 ℃/min from ambient temperature to 900 ℃ for complete degradation in air environment. Initial degradation temperatures were determined at 5% weight loss.

The glass transition temperature (T_g) of lignin was determined on a TA Instruments Q100 differential scanning calorimeter (DSC). The heating rate was 5 ℃/min over a temperature range of 25 to 200 ℃ in a nitrogen atmosphere. Cycles of heat/cool/heat were performed and T_g was analyzed as the midpoint of the heat capacity transition on the first heating.

4.5 Results and discussion

Aqueous glycerol has been demonstrated to be able to swell the wood cell wall and improve solvent penetration [20, 46]. However, glycerol alone only swells wood up to 0.7% (volumetric) at room temperature but 23% with irreversible change in swelling after heating above the glass transition (T_g) [47]. In this research, glycerol was introduced at temperatures well above the glass transition of wood providing the ability to swell the wood cell wall under the influence of continuous imposed shear. Previous research demonstrated the disruption of native cell wall

network by the removal of the hemicellulose side chain components such as arabinose and galactose (see Chapter 3) during GTP processing. After pretreatment and water washing, 84% to 92% of total initial Klason lignin, dependent upon GTP severity, was preserved in the biomass. The subsequent mild solvent extraction without additional catalysts was conducted to isolate lignin from water extracted GTP biomass, with the goal of directly revealing the GTP effects on lignin isolation. The GTP lignin recovered yield and its structure changes should aid in uncovering how pretreatment severity impacted the material in order to establish the impact of GTP pretreatment on isolated lignin.

4.5.1 GTP lignin yield through mild solvent extraction

Previous reports have demonstrated that delignification from mild solvent extraction increased from 4% to 52% relative to the initial lignin mass as the GTP severity increases (see Chapter 3). After extraction, the isolated lignin was precipitated and recovered as GTP lignin in a dry powder form. Figure 4-1 showed the recovered GTP lignin yield based on the total initial lignin in biomass input. With increasing GTP pretreatment severity, the amount of recovered GTP lignin increased linearly (R^2=0.987), which indicated that GTP pretreatment facilitated lignin isolation and recovery from the cell wall. A maximum of 41% of initial lignin was successfully recovered as GTP lignin. The difference between delignification and GTP lignin yield was around 10%, which probably represented the degraded lignin fragments that were non-recoverable from the solvent. The GTP pretreatment exhibits a totally different mechanism from the delignification using alkali aqueous glycerol, which resulted in 80% to 90% degraded lignin fragments [21] dissolved in solvents. In contrary, the GTP pretreatment helps to break the connection between lignin and carbohydrates but does not fully degrade it into non-recoverable fragments.

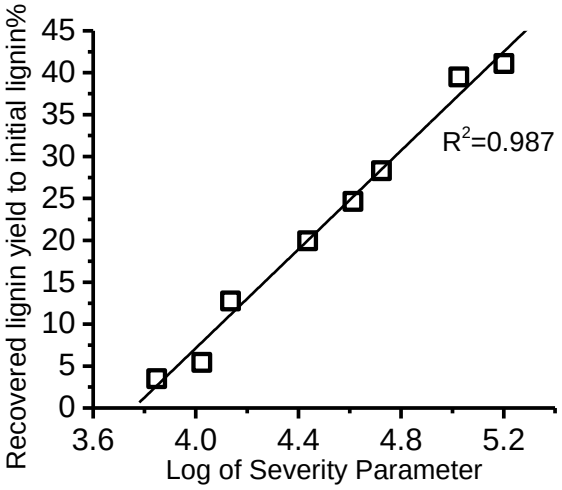


Figure 4-1 Recovered polymeric lignin yield during solvent extraction (average of duplicates).

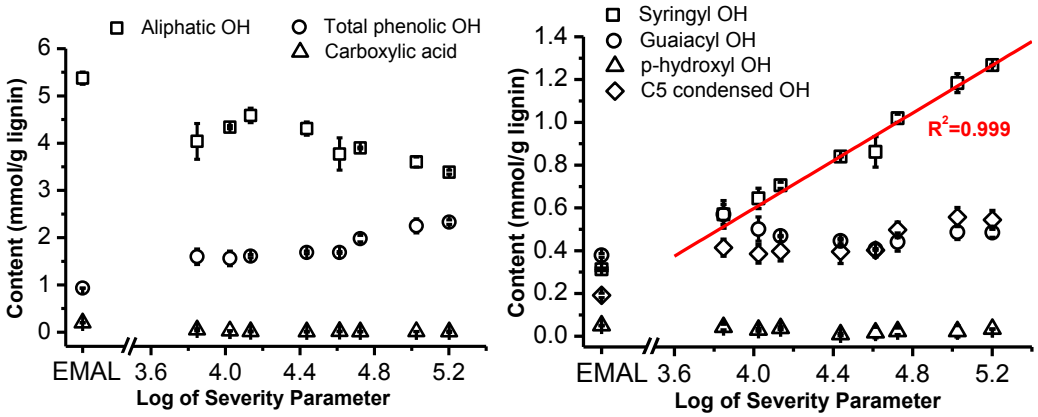
4.5.2 Quantitative ^{31}P-NMR of recovered lignin

Quantitative ^{31}P NMR [32, 48] used to calculate the content of aliphatic, phenolic hydroxyl as well as carboxylic acid groups in the recovered GTP lignin demonstrated (Figure 4-2a) total free phenolic OH increased from 0.93 mmol/g lignin in the EMAL control to 2.33 mmol/g lignin in GTP lignin at the highest severity condition. Previous work has shown that the alkyl aryl ether bond was the most vulnerable linkage to be cleaved due to thermolysis [49], acid catalyzed steam-explosion [13] or organosolv [16, 50] pretreatment. Thus, it makes sense that the GTP processing resultes in the alkyl aryl ether bond scission through solvolysis at processing temperatures ranging from 200 to 240 ℃, which further releases free phenolic OH groups. More importantly, the amount of phenolic OH groups in GTP lignin at the highest severity condition was 2.33 mmol/g lignin, which is much greater than that in common Alcell and Kraft lignins prepared at similar delignification levels (50-60%) [51]. In this case, the GTP processing is effective in producing more phenolic hydroxyls as functional groups in the isolated lignin.

In contrast, the amount of total aliphatic OH decreased from 5.38 mmol/g lignin to 3.39 mmol/g lignin as GTP severity increased. The substantial loss of aliphatic OH groups may be attributed to thermally-induced water and formaldehyde release through the cleavage of aliphatic OH groups [52]. Jakab et al. [53] reported that formaldehyde release occurred as a result of the thermal scission of γ-CH_2OH at temperatures as low as 200 ℃. It was proposed by Kawamoto

and Saka [54] that the hydrogen bonds formed between α-O and γ-OH in lignin model would ionize the γ-O, further facilitating the C-γ elimination with release of formaldehyde. Along with this mechanism, abundant hydroxyl groups from glycerol may serve as a source of hydrogen abstraction and form the hydrogen bond with γ-OH in a similar way, where the γ-elimination would proceed preferably to form the phenolic vinyl ether intermediate [55]. In addition, the α, β-dehydration [49] of lignin at temperatures over 180 ℃ was proposed as another possible reason for the decrease of aliphatic OH. However, glycerol used in the GTP pretreatment is believed to be capable to prevent dehydration reactions via hydrogen bond formation [56, 57].

The lignin C9 formula was determined using elemental analysis and proton NMR (Table A-1) and used to convert the functional group concentration into numbers per phenylpropane C9 unit. The number of aliphatic OH in EMAL was 1.2/C9, but in the isolated lignin after GTP pretreatment that number decreased from 1.0/C9 to 0.7/C9 respectively when $\log(R_0)$ increased from 4.14 to 5.20. The reduced aliphatic hydroxyl content relative to EMAL, suggests limited glycerol contamination by grafting onto lignin during processing. It was interesting to note at low severity conditions there was first a slight increase in aliphatic hydroxyl content (Figure 4-2a) prior to a consistent reduction in aliphatic hydroxyl content.



(a) (b)

Figure 4-2 (a) Content of aliphatic OH, phenolic OH and carboxylic acid in recovered lignin as a function of $\log(R_0)$; (b). Detailed phenolic OH content in recovered lignin as a function of $\log(R_0)$.

Additionally, minimalcarboxylic acid amounts in the GTP lignin (0.17-0.35/100C9) were observed in Figure 4-2a, indicating limited lignin oxidation occurred during the high temperature GTP pretreatment. Argyropoulos et al. [58] reported that EMAL was a useful model for nonoxidized lignin with low carboxylic acid content. In our research, the recovered GTP lignin had even less oxidized structure than EMAL.

Quantitative ^{31}P-NMR spectroscopy with phosphitylation reagent II is well known to resolve various phenolic structures, especially in separating syringyl and C5-condensed OH groups [34]. Figure 4-2b showed the amount changes of different phenolic hydroxyl in recovered GTP lignin. The most extraordinary change was observed for syringyl OH, which increased linearly as a function of GTP severity from 0.07 mol/C9 in EMAL to 0.3 mol/C9 in GTP lignin at highest severity. There was very little change in guaiacyl OH content with GTP severity resulting in a variation between 0.4 and 0.5 mmol/g lignin (0.08-0.09 mol/C9). As a result, the free phenolic S/G ratio increased from 0.83 for EMAL to 2.6 for GTP lignin. Similar situations [34, 59] were reported for aspen after steam-explosion pretreatment. As indicated by these results, the syringyl phenolic OH was the major product released from alkyl aryl ether bond breakage during the GTP processing, meaning that the alkyl aryl ether linkages bearing syringyl units were cleaved preferentially when exposed to high temperatures. The linear correlation between GTP lignin yield and syingyl OH yield (Figure 4-3) suggested a strong correlation between the released syringyl OH with the GTP lignin recovery.

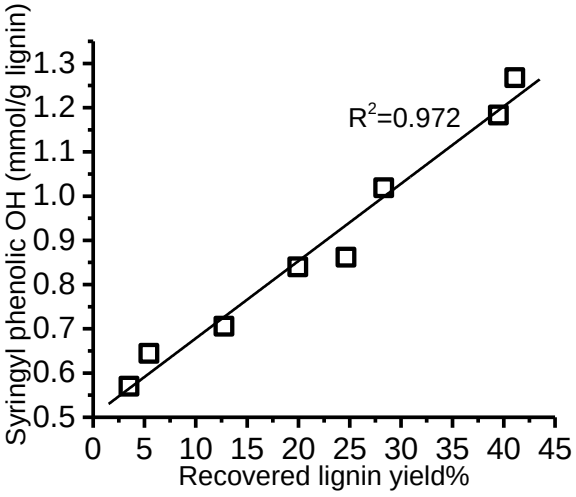


Figure 4-3 Content of syringyl phenolic OH as a function of recovered lignin yield.

C5 condensed phenolic structures are the phenolic rings with C-C or C-O-C bonds substituting for the methoxyl group at C5 position such as biphenolic units, diaryl methanes and diaryl ethers [34, 48]. These structures can be derived from the condensation of guaiacyl or p-hydroxyl phenol units with the free C5 position under the acid or alkaline delignification conditions [51]. As illustrated by Figure 4-2b, the maximum amount of C5 condensed OH groups in GTP lignin was 0.56 mmol/g lignin, which was 3 times more than that in EMAL. GTP lignin was shown to consistently have more C5 condensed OH groups than EMAL, indicating that the C5 condensation occurred during GTP processing. It has been reported that the amount of C5 condensed OH in Kraft and Alcell lignin was in the range of 0.35-0.75 mmol/g lignin at similar delignification degree [51]. Thus, GTP processing resulted in a similar C5 phenolic condensation in the recovered lignin because of the thermally induced degradation mechanism. In addition, identical and minimum production of p-hydroxylphenolic OH groups were observed in the GTP lignin.

4.5.3 Thioacidolysis-GC of GTP lignin

To investigate further the relationship of alkyl aryl ether bond breakage as a result of GTP processing, thioacidolysis was conducted and the results were shown for 3 severity conditions ($\log(R_0)$ is 4.44, 4.72 and 5.20 - analyzed individually) in Table 4-2.

Table 4-2 Contents of β-O-4 bonded G and S units (C6C3 structures) in GTP lignin and non-pretreated biomass.

$\log(R_0)$	S (mmol/g lignin)	G (mmol/g lignin)
SGC	1.2	0.45
4.44	0.77	0.14
4.72	0.59	0.13
5.20	0.31	0.087

*SGC refers to the non-pretreated sweet gum control; precision of thioacidolysis has been confirmed by the SGC samples with COV<5%.

The amounts of β-O-4 linked S and G units in non-pretreated biomass confirmed that S units were predominant in this hardwood species with a S to G ratio of 2.67. In GTP lignin isolated after pretreatment, the contents of residual β-O-4 bonded S and G units were observed to dramatically decrease. These results demonstrated that β-O-4 scission occurred during GTP pretreatment, and with increasing GTP severity more β-O-4 linkages were cleaved by the pretreatment resulting in fewer β-O-4 bonded structures. Results in Table 4-2 were converted to the number per 100 C9 rings for a clearer comparison (Table 4-3). Compared to 27/100C9 and 9.9/100C9 of β-O-4 bonded S and G units in native lignin, there were only 6.3/100 C9 and 1.8/100 C9 S and G units still existing in the β-O-4 bonded structures in the GTP lignin after the highest pretreatment severity. Table 4-3 also showed the free phenolic S/G released during GTP pretreatment as determined by ^{31}P-NMR analysis. Corresponding to the decreasing number of β-O-4 bonded structures, more S and G units were released with concurrently frees hydroxyl groups as increasing the GTP severity.

Table 4-3 Amounts/100C9 of β-O-4 bonded S/G (thioacidolysis) and free phenolic S/G (^{31}P-NMR).

log(R_0)	Thioacidolysis		^{31}P-NMR	
	S	G	S-OH	G-OH
SGC (EMAL)	27	9.9	7	8.4
4.44	16	2.9	18	9.4
4.72	12	2.7	21	9.1
5.2	6.3	1.8	26	9.8

* Molecular weight of C9 units in SGC is assumed to be the same as EMAL; S/G represents the β-O-4 bonded syringyl/guaiacyl units; S/G-OH are the syringyl/guaiacyl units bearing free phenolic OH; numbers are calculated based on the average of duplicates.

A detailed comparison of the difference in β-O-4 bonded structures and their corresponding phenolic structures was used to track the fate of S and G units during GTP processing in Table 4-4. Compared to the number of β-O-4 bonded S and S-OH units in native lignin, low severity GTP pretreatment (log(R_0)=4.44) decreased the number of β-O-4 bonded S units by 11 units per 100C9, which was the same number as the increment of S-OH groups. As increasing GTP severity, the loss in β-O-4 bonded S was about 1 unit/100C9 more than the increment in S-OH. These results indicates when the GTP condition is relatively mild (low severities), the released S units resulting from β-O-4 bond scission produce stable phenolic structures directly that do not undergo additional modification. However, at more severe GTP pretreatment levels, condensation at the 4-O position may occur. The same comparison for G units shows there is only ~14% of G released to form G-OH structures even after low severity GTP pretreatment. The G units released probably underwent condensation reactions through radical coupling, which is in accordance with the increment of C5 condensed structures revealed from ^{31}P-NMR. With increasing GTP severity, the release of G units and production of G-OH is not significant (less than 1 unit per 100 C9 rings).

Table 4-4 Amount changes/100C9 of β-O-4 bonded S/G (thioacidolysis) and free phenolic S/G (^{31}P-NMR) as increasing the GTP severity log(R_0).

delta log(R_0)	Thioacidolysis		^{31}P-NMR	
	ΔS	ΔG	ΔS-OH	ΔG-OH
SGC to 4.44	-11	-7	+11	+1
4.44 to 4.72	-4	-0.2	+3	-0.3
4.72 to 5.20	-5.7	-0.9	+5	+0.7

*(-) refers to decrease in number; (+) refers to increase in number; numbers are calculated based on the average of duplicates.

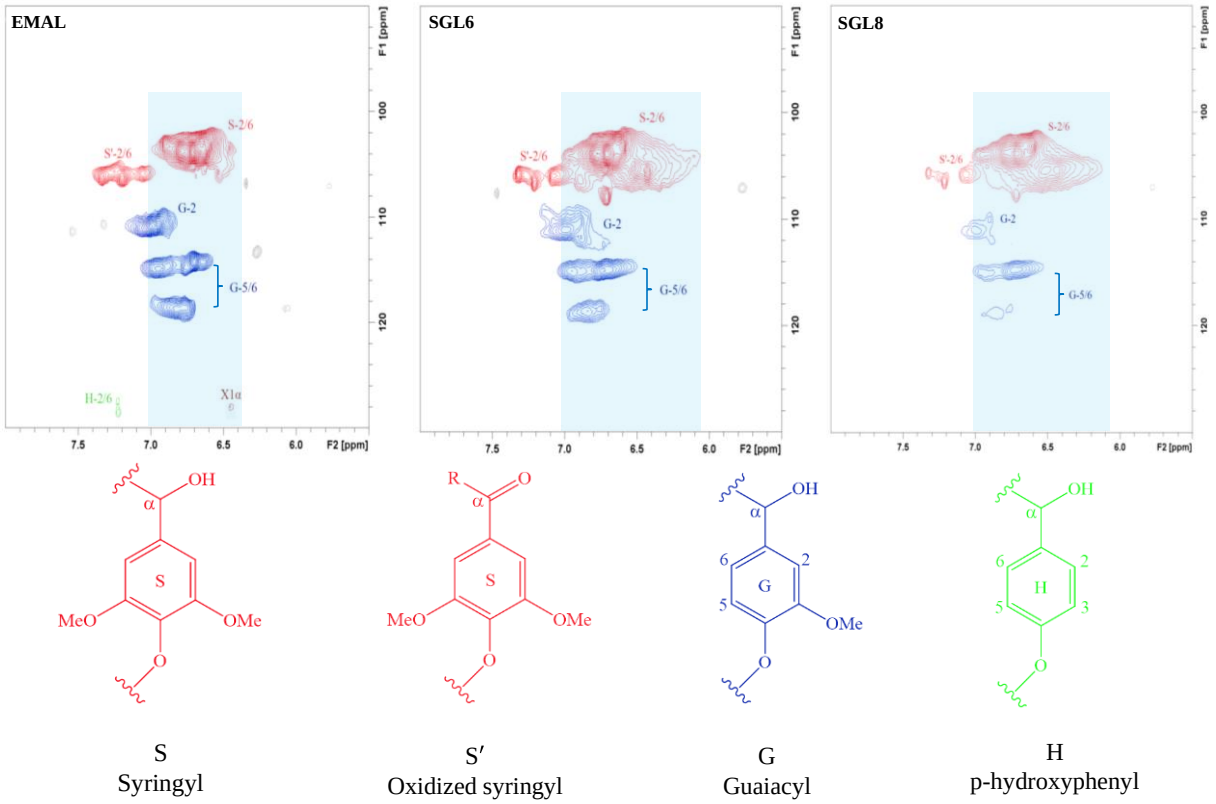
4.5.4 2D HSQC of GTP lignin

2D ^{13}C-^{1}H HSQC NMR was used to observe changes in chemical structure in EMAL and GTP lignin. Common structures in the HSQC spectra were color-coded and assigned in accordance with previously published literature [42, 43, 60-63]. Different lignin inter-units and side-chain linkages were readily distinguished in the aromatic (Figure 4-4) and aliphatic regions (Figure 4-5). Changes in lignin structure were determined based on volume integration of HSQC spectral contour correlations. All integrals displayed less than 10% error (based on the use of organosolv lignin in triplicate - data not shown), confirming the precision of the quantification from 2D HSQC spectra.

4.5.4.1 *Aromatic region ($\delta C/\delta H$ 90-130/5.5-8.0 ppm)*

Aromatic regions of EMAL and GTP lignin with their color-coded structural units were shown in Figure 4-4. The cross-signals represented different aromatic rings in lignin. As shown by Figure 4-4, only trace amounts of H units was detected in the sweet gum EMAL and its cross peak disappeared in the GTP lignin, which was consistent with the minimum presence of p-hydroxylphenol units. The calculated ratio of S/G was based on the contour volume for the C2 and C6 carbons on S and oxidized S rings, as well as the C2 carbon on G rings (Table 4-5). As

demonstrated earlier, the S/G ratio increases in GTP lignin, from 3.2 in EMAL to 4.1 at moderate, up to 7.6 at high severity. The S/G ratio for GTP lignin at high severity was strikingly larger than common values for hardwood lignin, which normally fell in the range of 1.5 to 3.3 [42, 64]. Detailed examination of aromatic region for GTP lignin suggested more information about the structure of S-type units. It has been widely reported [42-44, 63] that the S 2/6 cross peak located in the range of 6.4-7.1 ppm with a center at 6.7 ppm on ^{1}H-dimension for the native lignin present in non-derivatized cell walls. However, the cross peak of S 2/6 in GTP lignin exhibited spread contour volume compared to that in EMAL on the ^{1}H-dimension, indicating the heterogeneity between different S-type units. This spread contour has also been reported for Kraft lignin [62, 65]. Based on the chemical shift mechanism in the NMR spectrum, the more shielded protons with high electron density would shift to upfield. In this case, the electron density of protons on C2 and C6 positions on S-type units is supposed to increase, causing the contour of S 2/6 to shifts to upfield. Combined with the previous result of occurance of C5 condensation during GTP processing, the condensed aromatic units at the C5 position, such as diaryl ether, should possess a pseudo-S aromatic structure with high electron density around the C2/6 protons, which would lead to an enlarged cross peak shifting to the upfield. Thus, the C5 condensed structures may be counted as S-type aromatic units in HSQC-NMR, resulting in a much larger S/G ratio than that would be observed in hardwood lignin. Further studies need to be conducted on model compounds of highly condensed lignin structures to confirm this.



S
Syringyl

S′
Oxidized syringyl

G
Guaiacyl

H
p-hydroxyphenyl

Figure 4-4 HSQC of EMAL and isolated GTP lignin – aromatic region (same contour level).

Table 4-5 S/G ratio calculated from HSQC aromatic region for EMAL and GTP lignin.

Sample ID	log (R_0)	S/G ratio
EMAL	/	3.2
SGL 6	4.61	4.1
SGL 8	5.03	7.6

4.5.4.2 *Aliphatic side chain region (δC/δH 50-90/2.5-5.7 ppm)*

Figure 4-5 showed the aliphatic region of EMAL and GTP lignin. From these spectra, the common linkages in the lignin structure exhibited well-resolved correlations. It is widely accepted that β-O-4 linkages (substructure A) are the most abundant in lignin coupled with small

amounts of phenylcoumaran β-5 (substructure B), resinol β-β (substructure C), spirodienone β-1 (substructure D) and cinnamyl alcohol end groups (substructure X1) as revealed through HSQC [66]. Semi-quantitative characterization of the different lignin linkages and cinnamyl alcohol end groups was presented in Table 4-6 to show the changes in bonds and end-groups after GTP processing.

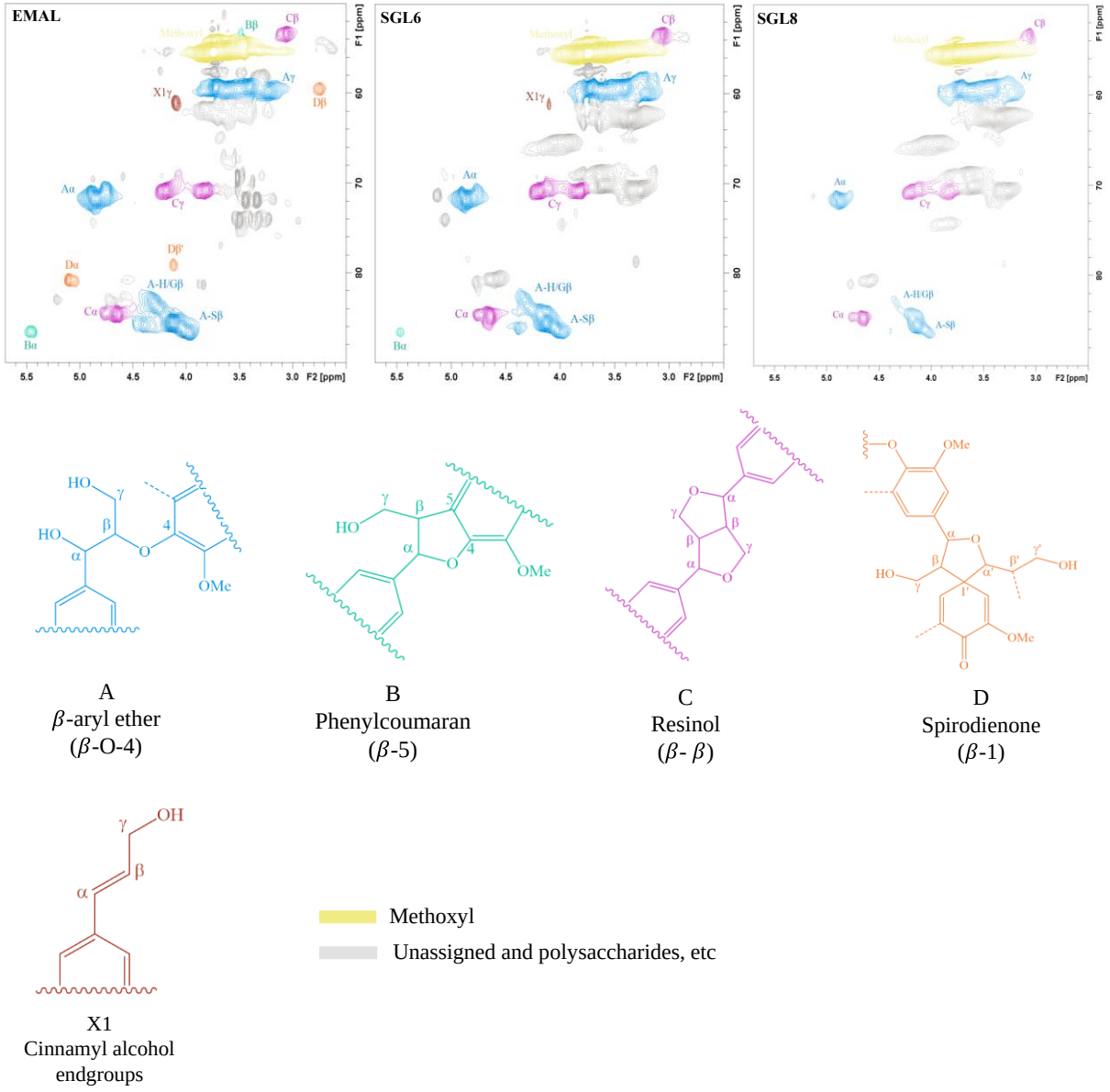


Figure 4-5 HSQC of EMAL and isolated GTP lignin – aliphatic region. A: β-aryl ether (β-O-4); B: Phenylcoumaran (β-5); C: Resionol (β-β); D: Spirodienone (β-1); X1: Cinnamyl alcohol end-groups.

Consistent with a reduction in number of β-O-4 bonded S and G units observed in the thioacidolysis data, the number of β-O-4 linkages decreased with increasing GTP severity. Compared to the abundance of β-O-4 linkages in EMAL, only 26% of these bonds remained after high severity GTP processing. Such a large decrease in β-O-4 linkages was also reported for lignin isolated from steam-exploded aspen [13]. Unlike steam-explosion pretreatments, acids such as acetic acid that were formed during steam-explosion were not responsible for the extensive scission of β-O-4 linkages in GTP processing, since the GTP pretreatment was conducted at temperatures over 200 ℃ and at ambient pressure without moisture or steam present. Instead, a direct thermal-induced decomposition of β-O-4 occurred during GTP processing in the presence of glycerol. It has been widely reported that lignin thermolysis is mainly induced by homolytic cleavage of β-O-4 through a quinone methide intermediate [67], resulting in the formation of lignin molecules with "free" vinylic units such as cinnamyl alcohol end groups [55]. However, in our case, the number of cinnamyl alcohol end-groups also decreased after GTP processing and even disappeared after high severity GTP processing. Thus, the thermal-induced homolytic cleavage is not a primary mechanism associated with the extensive β-O-4 cleavage observed. According to Kawamoto et al. [68], the side-chain hydroxyl groups, especially on the C-γ position, play a major role in the pyrolytic cleavage mechanism of the β-O-4 linkage. For the C-γ deoxyl types, the β-O-4 linkages are cleaved through a radical chain mechanism, but high temperatures in the range of 400 ℃ are necessary for thermolysis of this type [67]. However, the C-γ hydroxyl type possesses a higher reactivity, which requires a lower thermal energy for β-O-4 breakage. Haw and Schultz [69] reported that thermal decomposition of β-O-4 linkages initiated at 220 ℃ for the C-γ hydroxyl type. Additionally, the different hydrogen bonds formed by C-γ hydroxyl groups are critical in impacting the β-O-4 cleavage mechanism [54]. During GTP processing, it is possible that the C-γ OH serves as a hydrogen-donor to form hydrogen bonds with C-α oxygen [54] or even the abundant hydroxyl groups on glycerol, which facilitates C-γ elimination during GTP processing. The striking decrease of aliphatic OH revealed in the ^{31}P-NMR analysis also confirms C-γ elimination. As a result, enol ether structures are formed as a result of formaldehyde release; however the structures are not stable in the GTP processing environment, which further results in β-O-4 cleavage [54].

Hydroxyl radical induced lignin depolymerization is another important lignin degradation pathway via the reactions of soluble ferric iron in plant tissue and generated hydrogen peroxide after lignin demethylation [70]. However, glycerol is reported as a radical scavenger [71]. The abundant existence of glycerol surrounding biomass cell wall during GTP pretreatment was capable to consume the generated hydroxyl radicals, which prevented further lignin deolymerization via this pathway.

In addition to the extensive cleavage of β-O-4 linkages, the abundance of β-β and β-5 interunit bonds also decreased in the GTP lignin with increasing processing severity. The spirodienone β-1 structure was observed in sweet gum EMAL, similarly to that observed in other hardwood native lignins [72]. However, its correlation disappeared in the recovered GTP indicating the impossible formation of β-1 branched structure during processing.

Table 4-6 Molar abundance (per 100C9) of side chain linkages and end-groups in lignin.

Sample ID	log (R_0)	β-O-4	β-5	β-β	Cinnamyl alcohol
EMAL	-	58.6	2.4	17.6	3.4
SGL 6	4.61	28.8	0.9	13.2	0.7
SGL 8	5.03	15.5	0.0	10.1	0.0

4.5.5 Molecular weight of GTP lignin

Based on the destruction of native interunit linkages during GTP processing, lignin should have a much lower molecular weight compared to EMAL. The relative molecular weight of isolated GTP lignin was determined by size exclusion chromatography (SEC) using a universal calibration method. Lignin relative MW was plotted as a function of GTP severity for both the number average (M_n) and weight average (M_w) molecular weights to elucidate relative changes arising from the GTP processing, see Figure 4-6.

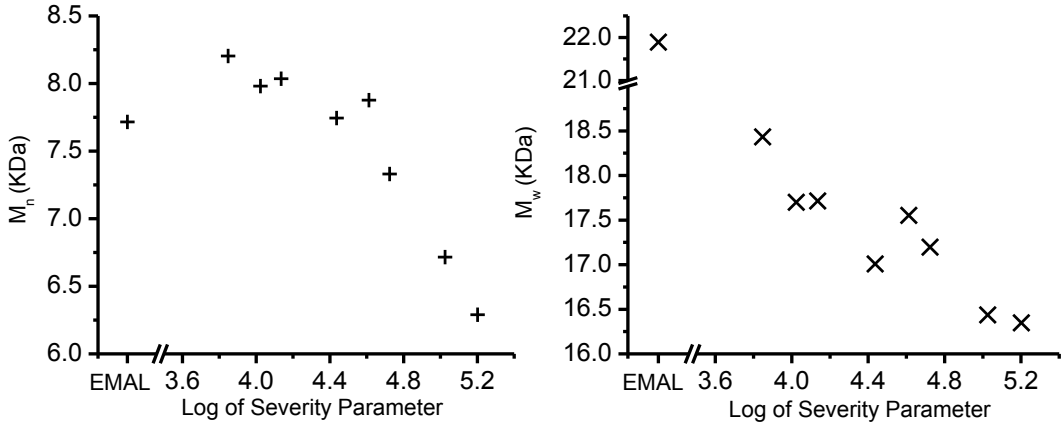


Figure 4-6 Relative molecular weight of EMAL and isolated GTP lignin as a function of log(R_0).

For the number average MW, there was an initial increase in the molecular weight relative to the EMAL control followed by a linear decrease for all remaining GTP lignin severity levels. However, a small decrease of 1500 Da in M_n was obsevered for GTP lignin at highest severity compared to EMAL relative to the extensive decomposition of native lignin linkages as revealed in the thioacidolysis and 2-D NMR data. Thus, thermally induced condensation occurred in the GTP lignin to compensate the extensive scission of bonds, along with an increase in C5 condensed units as indicated by the ^{31}P-NMR data. According to Funaoka et al. [73], a diphenylmethane (DPM) type condensation through C-α and adjacent phenolic nuclei readily occurred when wood was heated over 120 ℃. In our case, the existence of glycerol as a plasticizer during GTP processing could further accelerate this type of DPM condensation by enhancing the mobility of lignin at higher temperatures. Due to the DPM condensation, the resulting GTP lignin still showed relatively high M_n after extensive alkyl aryl ether bond breakage. However, unlike the increase in MW after steam explosion due to the extensive C-C condensation when GTP severities were above log(R_0)=3.2, there was no MW increase as GTP processing severity increased. Although GTP processing occurred at relatively high temperatures, lignin depolymerization was still predominant. Additionally, the weight average MW decreased at higher severity factors, even relative to EMAL. Interestingly, the data revealed a narrower polydispserity index for GTP lignin in the range of 2.2 to 2.6, with even a smaller variance than that of EMAL (PDI = 2.8 for EMAL). This smaller PDI was unusual for processes involving both lignin depolymerization and repolymerization processes. These results are noteworthy and

in contrast to relative changes reported in the literature for lignin isolated from steam-explosion or other steam treatments at comparable severities. Upon inspection of the correlations between molecular weight characteristics and free syringyl hydroxyl groups (Figure A-4) there seems to be a controlled depolymerization process, which suggests a pathway that leads to specific coupling reactions during the depolymerizaiton of β-O-4 and repolymerization process for this system.

4.5.6 Thermal analysis of GTP lignin

The thermal stability and the glass transition temperature of the recovered GTP lignin was determined by TGA and DSC. As shown in Table 4-7, the lignin degradation temperature (T_d) at 5% weight loss increased as a function of GTP processing severity. GTP processing of wood introduced an initial heat treatment on lignin. In other studies, Cui et al. revealed that T_d increased for isolated lignin when heated above its glass transition temperature [5]. GTP lignin displayed significantly greater thermal stability compared to other previously reported lignins [74-76] upon the first heat, even greater than fully derivatized kraft lignins [5]. This thermal stability could be a useful property in the melt processing of lignin, where commodity thermoplastics like polypropylene are processed above 170 ℃ and engineering thermoplastics are processed above 250 ℃.

On the other hand, a consistent glass transition temperature for the isolated GTP lignin (Table 4-7) was observed, which was not significantly impacted by GTP pretreatment. Prior reports indicated T_g values for several commercial lignins [77-79]: organosolv lignin had a T_g of 106 ℃, lignosulfonates had a T_g of 105 ℃ and T_g for steam-exploded and kraft lignin was around 175 and 93 ℃. Compared to these values, lignin isolated from GTP pretreated sweet gum presented a higher glass transition temperature (162-170 ℃) except for steam-exploded lignin. These results indicate the structure and functionality of lignin: including hydroxyl groups that allow significant hydrogen bonding to increase Tg [79]; while the backbones are linked through condensation products are particularly important in increasing T_g while also enhancing T_g stability. Based on the invariance of the Tg data for the 6 severities tested, it appears that the lowest severity level provides a critical modification level. The higher thermal stability of lignin isolated from GTP

pretreated biomass, with a high glass transition temperature, suggests potential application of this material in engineering thermoplastics [1, 78].

Table 4-7 Degradation temperature and glass transition temperature of GTP lignin.

log(R_0)	T_g (^oC)	Degradation temp* (^oC)
EMAL	ND	201.6
4.14	162.8 (0.2)	ND
4.44	166.4 (0.2)	276.6 (2.8)
4.61	165.9 (0.3)	282.1 (2.1)
4.72	164.5 (1.2)	288.2 (2.7)
5.03	166.2 (0.1)	292.9 (5.8)
5.20	169.5 (0.5)	296.8 (9.5)

*at 5% weight loss; ND: not determined.

4.6 Conclusions

Non-catalytic glycerol thermal pretreatment (GTP) was studied in this research as a means for isolation and recovery of lignin that would be suitable for polymeric applications. GTP is conducted at moderately severe temperatures of 200 to 240 ℃ in an anhydrous environment. Analysis of the resulting material provided insight into structural changes of lignin at these temperatures, which would be particularly useful in polymer processing operations. After GTP pretreatment, 41% of the initial Klason lignin in biomass input was recovered in a powdered form using a mild dioxane extraction. The GTP lignin phenolic functionality increased linearly with the log of the severity parameter due to alkyl aryl ether bond breakage. The amount of phenolic hydroxyl content was considerable higher than other lignins at similar levels of delignification. Further structure investigation of the isolated lignin indicated that glycerol could

facilitate the C-$\boldsymbol{\gamma}$ elimination followed by the extensive $\boldsymbol{\beta}$-O-4 bond decomposition and phenolic hydroxyl liberation. At the same time, condensation occurred, affording the GTP lignin with a relatively high molecular weight near to that of enzymatic mild acidolysis lignins. The resulted GTP lignin was more thermally stable, with a glass transition temperature that was invariant to GTP processing severity, and which possessed properties potentially suitable for polymer applications.

4.7 Acknowledgments

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Chapter 5 Polymeric xylan fractionation and structural characterization from glycerol thermal pretreated biomass

5.1 Abstract

Glycerol thermal processing (GTP) was studied as a novel pretreatment, facilitating biopolymer fractionation while preventing significant degradation of hemicellulose (xylan) into water-extractable oligosaccharides. After GTP pretreatment and sequential water and organic solvent extraction, up to 80% of the total initial xylan remained in the pretreated biomass. The majority of the polymeric xylan from GTP pretreated and water/solvent extracted biomass, could be extracted using a mild alkali extraction with separated recovery of the water insoluble portion (WIX) and water soluble portion (WSX). Results showed that xylan in WIX was in a polymeric form, whereas the WSX had a much lower degree of polymerization and more substitutions that rendered it more water soluble. After the GTP pretreatment, WIX recovery increased compared to WSX, with a maximum xylan purity of 84% for the water insoluble sample. FT-IR analysis of WIX at increasing GTP severity confirmed the high xylan purity with less glucuronic acid on the branches. In contrast, WSX contains less xylan with more lignin contamination at severe GTP conditions. Further, molecular weight analysis using GPC confirmed that the xylan in WIX isolated from GTP biomass was in a polymeric form comparable to that isolated from non-treated fibers. Additionally, the narrow molecular weight distribution with a polydispersity index of 1.1 to 1.3 was observed, most likely arising from the removal of the low molecular weight water soluble xylan during water washing.

5.2 Keywords

Glycerol thermal processing, Water insoluble xylan, Water soluble xylan, Alkali extraction

5.3 Introduction

Hemicellulose represents a complex mixture of hetero-polysaccharides, contributing to key structural components in the plant cell wall. The composition and structure of hemicellulose are highly dependent on the biomass species. For many hardwoods, the main hemicellulose is O-acetyl-4-O-methyl-glucuronoxylan (GAX), which comprises 15-30% of wood dry weight. GAX

has a β-1,4 linked xylan backbone with acetyl groups appearing on the C2/3 of xylopyranose ring (~3.5 acetyl/10 rings) [1, 2]. Additionally, 4-O-methyl glucuronic acid is α-1,2 linked to the xylan backbone as a major side-group (~1 glucuronic acid/10 rings) [2] making the structure slightly branched. Compared to cellulose, the chain length of hemicellulose is much smaller. The degree of polymerization (DP) of native hardwood xylan is in the range of 100 to 200 [3-5], but that of isolated xylan is highly dependent on the isolation method [6]. Due to its abundance, biodegradability and barrier properties when included in films, hardwood xylan has been proposed for use in food packaging materials [7], as an edible film when mixed with wheat gluten [8], and as a hydrogel with chitosan for biomedical applications [9]. Also, xylan derivatives through etherification and esterification have promise in applications such as drug delivery, paper strength additives, surfactant and antimicrobial agents [10-12]. Hence, the utility of xylans has been demonstrated and provides a potential justification for better methods permitting separation and recovery of this polymer.

Lignocellulosic biomass from hardwoods and herbaceous plants are the most abundant resources for xylan. However, the liberation of xylan from lignocellulosic biomass is limited by the recalcitrant network of cell wall [13]. One of the most common methods for xylan isolation includes a first step of delignification to produce holocellulose through acetic acid/sodium chlorite treatment, followed by a sequential alkaline extraction of xylan using sodium hydroxide or potassium hydroxide [1, 14, 15]. This produces an extensively deactylated, linear 4-O-methylglucuronoxylan from hardwood. In contrast to this method, direct xylan isolation by alkali treatment followed by sequential bleaching using hydrogen peroxide has also been studied for xylan isolation to minimize xylan structural modification [9]. Glasser et al. [6] compared different isolation protocols for heteropolysaccharides from barley husks and yellow poplar, and found that alkali extraction was most efficient for polymeric xylan isolation. Direct organosolv extraction using dimethyl sulfoxide [16] has been reported as a mild isolation method for the preservation of acetyl groups, but it preferentially isolates low molecular weight xylans. Recently, with growing development of the biorefinery industry, several pretreatment methods have been developed to effectively disrupt the cell wall network, enhancing the isolation of lignin and xylan as valuable byproducts. Pretreatments with alkali or organosolv have been proposed to pre-delignify the biomass, which will result in isolated xylan with better purity and lower polydispersity [6]. Pretreatments involving acids or autocatalyzed reactions lead to severe

hydrolytic degradation of hemicellulose during pretreatment, which are not appropriate for the isolatation of polymeric xylan [17, 18] and are therefore less desirable in this regard.

In this research, GTP as a novel pretreatment method was tested for polymeric xylan isolation from sweet gum (hardwood). Previous research (Chapter 3, 4), demonstrated that the GTP pretreatment successfully disrupted the biomass cell wall structure by extensively removing lignin-carbohydrate linkages while also enhancing surface area. As a result, lignin became more extractable via mild solvent extraction and more than 80% of the xylan was preserved. With the potential applications for polymeric xylan discussed above, the xylan extracted from GTP pretreated biomass after pre-delignification was studied in this research as an important by-product of the developing biorefinery industry. The isolated xylan was analyzed for yield and purity, and its structural properties were further correlated to GTP pretreatment conditions. The thermal stability of GTP isolated xylan when exposed to anhydrous processing conditions was also explored in this work.

5.4 Experimental

5.4.1 Materials

Chemicals and reagents used in this research were purchased from Sigma-Aldrich, Alfa Aesar and MP Biomedicals, and used as received, and deionized water (DI-water, 18.2 mΩ) was used in all cases. A mature sweet gum (*Liquidambar styracuflua*) from Blacksburg, VA was debarked, machined into cubes, and stored in a freezer before use. Prior to pretreatment, the biomass was knife – milled using a Wiley mill, and sorted to a particle size between 40 to 60 mesh on a metal screen (250-420 μm). Then, the extractive-free sweet gum particles were prepared according to ASTM standard [19].

5.4.2 Glycerol thermal processing (GTP) pretreatment

Extractive-free biomass was pretreated with glycerol using a bench-top internal mixing head with high intensity shear paddler driven by a C.W. Brabender® Prep-Center® drive as detailed previously (Chapter 3). The GTP pretreatment severity parameter (R_0) was calculated according to an equation developed by Overend and Chornet [18] to depict the steam pretreatment

conditions using temperature and residence time. The corresponding logarithm of R_0 in this research was shown in Table 5-1.

Table 5-1 Glycerol thermal processing conditions and corresponding severity parameters.

Sample label	T (^{o}C)	t (min)	$\log(R_0)$
SG#1	200	4	3.55
SG#2	200	8	3.85
SG#3	200	12	4.02
SG#4	220	4	4.14
SG#5	220	8	4.44
SG#6	220	12	4.61
SG#7	240	4	4.72
SG#8	240	8	5.03
SG#9	240	12	5.20

After pretreatment, the GTP pretreated biomass was collected from the mixing head and stored at 4 ℃ until further analysis. Samples for each severity condition were run in triplicate.

5.4.3 Water extraction and pre-delignification

The GTP pretreated biomass was first water extracted to remove the glycerol residue and any degraded components. After water extraction, the GTP biomass was freeze-dried followed by vacuum-drying, and a mild solvent extraction was then performed to isolate lignin from the GTP pretreated biomass. The solvent extracted GTP biomass was thoroughly washed by deionized water before drying. Details for these procedures are as outlined previously (Chapter 3 and 4).

5.4.4 Alkali extraction

Solvent extracted GTP biomass was alkali extracted to isolate xylan. The completely dried solvent extracted GTPSG was then further extracted at ambient temperature for 24 hours with continuous stirring using a 1 M sodium hydroxide solution at a ratio of 2.5/100 (g/ml) under nitrogen. After the extraction, the alkaline mixture was centrifuged to separate solid residues and the alkali extract. Sequentially 100 ml 1 M sodium hydroxide solution and 100 ml deionized water was used to wash the solid residue, and the washing liquids were collected and combined with the alkaline extract. The solid residue was thoroughly washed using DI water until the pH was neutral, and the material was then freeze-dried followed by vacuum drying. The combined alkaline extract and washing solution was neutralized to pH = 6.0 with acetic acid for water-insoluble and water-soluble xylan (WIX and WSX) collection. Detailed procedures are provided in Figure 5-1.

Generally, the precipitates formed during neutralization were collected through centrifugation, and supernatant was collected separately. The precipitates were then re-dispersed in methanol for purification and collected as water-insoluble xylan (WIX). The supernatant collected above was evaporated to less than 100 ml under reduced pressure and in the presence of antifoam, and then was added dropwise into 400 ml methanol with vigorous stirring. The precipitates formed during this process were also collected through centrifugation, and referred to as water-soluble xylan (WSX). The WIX and WSX were vacuum dried thoroughly for further analysis.

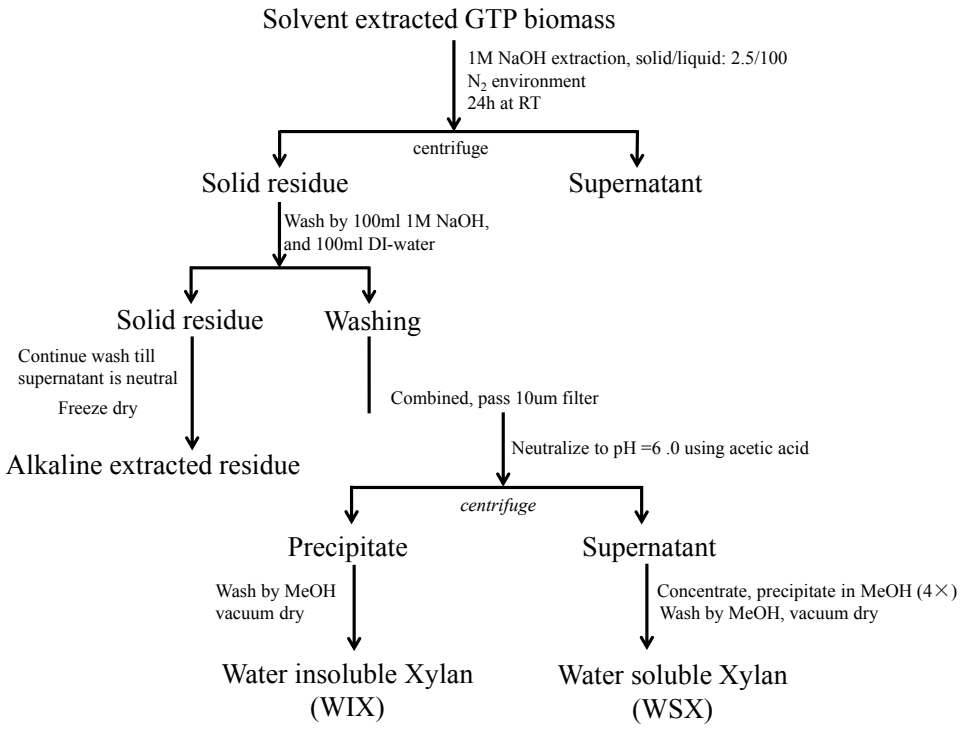

Figure 5-1 Alkali extraction schematic to recover the water-insoluble xylan and water-soluble xylan.

5.4.5 Compositional analysis of alkaline extracted biomass

The structural carbohydrates and Klason lignin in GTP biomass before and after alkaline extraction were analyzed according to the NREL laboratory analytical procedure (LAP) [20] in duplicate as reviewed previously (Chapter 3)

5.4.6 Purity analysis of WIX

A two-step acid hydrolysis (NREL standard) with slight modification was used for WIX purity analysis. Generally, 100 mg of thoroughly dried WIX was first hydrolyzed using 1 ml 72% sulfuric acid at 30 ℃ with continuous stirring for 1 hour. A 4% acid hydrolysis was then conducted for 1 hour at 121 ℃ in an autoclave before filtration. The filtrate containing hydrolyzed monosaccharides was then analyzed quantitatively using Ion Chromatography as reviewed previously (Chapter 3).The Klason lignin left in the crucible was dried in a muffle

furnace (105 ℃) for 12 hours then burned at 575 ℃ for 24 hours. The mass before and after the 575 ℃ combustion was recorded for Klason lignin calculation.

A purity analysis of WSX isolated from non-pretreated sweet gum was conducted using in the same method as above for WIX, using 50 mg WSX and 500 μl 72% sulfuric acid. The WSX isolated from the pre-delignified GTP biomass was not analyzed for purity due to the limited yield.

5.4.7 Fourier transform infrared spectroscopy (FT-IR) of WIX and WSX

FT-IR spectra of WIX and WSX were collected in transmission mode on a Thermal Nicolet 8700 spectrometer. About 5 mg of thoroughly dried WIX or WSX was mixed with 100 mg KBr to make transparent pellets. A total of 256 scans was collected per sample with a resolution of 4/cm in the range of 4000-500/cm. The spectra were baseline corrected manually using a polynomial function in Omnic software. WIX and WSX spectra were further normalized to the absorbance intensity around 1085 cm^{-1}.

5.4.8 Xylan carbanilation and molecular weight analysis

Carbanilation of WIX was conducted to improve its solubility for molecular weight analysis. Detailed procedures for xylan carbanilation were adapted from Evans et al. [21] with 50 mg dry WIX reacted with 1 ml phenylisocyanate in 10 ml dry DMSO. Dry nitrogen was used to flush the reaction vial to prevent side-reactions by moisture and the reactions were carried out in a 70 ℃ oil bath with continuous stirring. WIX was observed to fully dissolve in the reagent in 1 hour, but the reaction ws continued for 5 hours to assure full substitution. After 5 hours, 1 ml of dry methanol was added to consume any excess phenlisocyanate. The reaction mixture was then cooled in ice-water bath for 5 minutes and the WIX carbanilates were precipitated dropwise into 100 ml 70% (v%) methanol, resulting in a colloidal dispersion. Two drops of saturated aqueous sodium sulfate were added into the colloidal mixture with vigorously stirring used to break the colloidal dispersion and precipitate the WIX carbanilates. The derivatives were then collected by centrifugation, washed with aqueous methanol and DI water, and freeze-dried. A typical reaction for xylan carbanilaton was shown in Figure 5-2. The fully substituted WIX carbanilates were verified through FT-IR and ^{1}H-NMR.

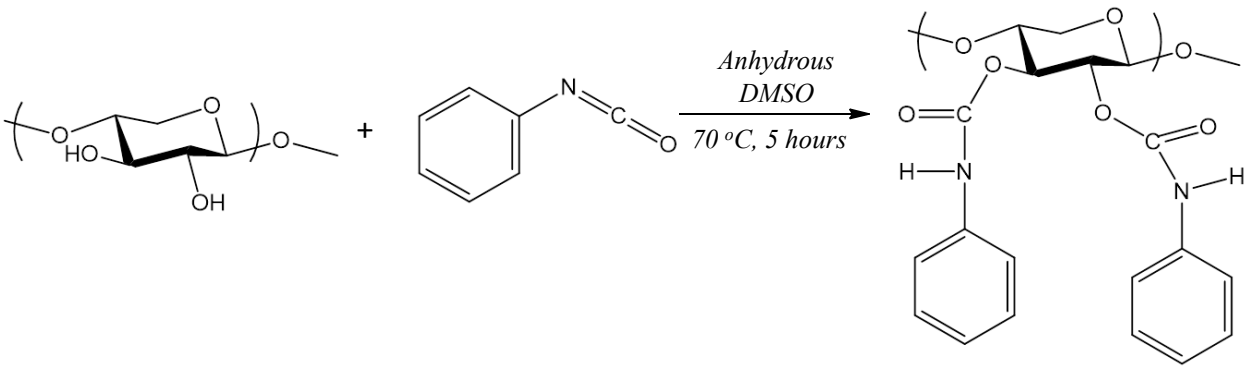

Figure 5-2 Typical xylan carbanilation.

The absolute molecular weight of thoroughly dried WIX carbanilates was obtained using an Agilent 1260 Infinity Multi-Detector SEC, installed with 3 PLgel 10 μm mixed-B 300 × 7.5 mm columns in series. NMP with 0.05 M LiBr was used as the mobile phase at 50 ℃.

5.5 Results and discussion

5.5.1 Characterization of crude xylan extracted from non-treated fiber

Alkali extraction was used to fractionate two portions of xylan that were separated upon recovery. Using this method the water insoluble xylan (WIX) precipitated when the alkali extract was neutralized, and the remaining water soluble xylan (WSX) in the filtrate after separation was isolated by precipitation with methanol. For control samples, an alkali extraction was performed on non-pretreated sweet gum 40-60 mesh fibers to yield WIX and WSX samples. This direct alkali extraction of non-pretreated biomass indicated that crude WIX was the major product with a recovery yield of 11.4% of total biomass, whereas the yield of crude WSX was 5.6% of the total biomass. A detailed compositional analysis of the isolated crude xylan is shown in Table 5-2.

Table 5-2 Composition of water insoluble xylan (WIX) and water soluble xylan (WSX) isolated from ground sweet gum extracted with 1.0M NaOH.

#	Xylan%	Lignin%	Rhamnan*%	Glucan%	Arabinan%	Mannan%
WIX SGC	76.69	4.64	0.68	0.18	0.012	0.00
	(0.06)	(0.83)	(0.00)	(0.01)	(0.006)	(0.00)
WSX SGC	54.59	9.65	2.31	0.98	0.805	1.20
	(0.31)	(1.29)	(0.01)	(0.01)	(0.007)	(0.00)

*lignin includes acid soluble and acid insoluble lignin from compositional analysis.

As shown in Table 5-2, the major component in both WIX and WSX isolated from non-treated fibers was xylan. The WIX from non-pretreated biomass had a higher xylan purity while only 55% of the WSX material recovered contained a xylan backbone. Additionally, WSX had more lignin contamination than WIX. Noteworthy was the approximate 20% to 30% total mass not accounted for in compositional analysis for the WIX and WSX samples (Table 5-2), which probably related to glucuronic acid or residual acetyl groups. The FTIR fingerprint region of these two xylan fractions was compared in Figure 5-3 with the common polysaccharide absorbance bands shown in the region between 1200 to 900 cm^{-1} [22]. In particular, the band at 1161 cm^{-1} is assigned to antisymmetric C-O-C stretches of glycosidic linkages; the band around 1085 cm^{-1} is assigned to ring C-O-C stretching; the band at 1040 cm^{-1} is assigned to the C-OH stretch of primary alcohols [23, 24]. The small band at 897 cm^{-1} represents the C-1 group frequency [22]. The absorbance band at 1720 cm^{-1} is attributed to the carbonyl group in acetyl groups or the glucuronic acid of xylan.

Observation of the spectra showed that WIX contained a clear carbonyl shoulder. Due to the extensive deacetylation that occurred during alkali extraction [9], these carbonyl groups were preferentially assigned to glucuronic acid, which indicats the WIX contained glucuronic acid side groups. The successful alkaline extraction of 4-O-methylglucuronic acid substituted xylan from hardwood has been widely reported [9, 15] in the literature and it has been proposed that

hardwood xylan isolated through alkaline extraction at ambient temperature is quite similar to native xylan except for deacetylation of the backbone [25]. However, conditions were not optimized in the current study with liquid chromatography to determine the amount of glucuronic acid in WIX samples. It is known that glucuronic acid in native xylan has an average molar ratio of 1 to 10 [2], and this would be equivalent to about 11% (w%) of the mass of WIX.

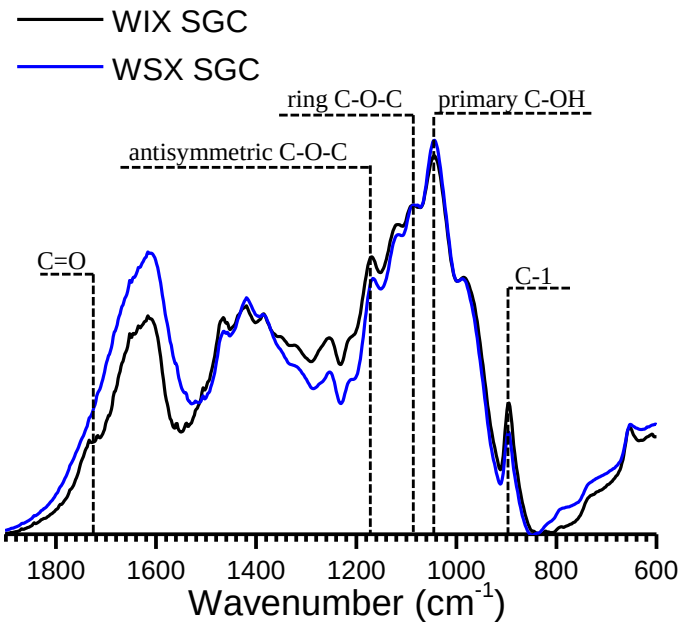

Figure 5-3 FTIR comparison of WIX and WSX isolated from non-pretreated sweet gum (spectra were normalized at 1085 cm^{-1}).

Based on the compositional analysis data, the molar ratios of lignin C9 units, as well as rhamnan units, were compared to the xylan backbone (Table 5-3). For WIX, approximately 4 lignin C9 units on average were associated with 100 xylan units. For WSX, the xylan was more heavily decorated with lignin, showing each 100 xylan units have more than 10 units of lignin associated with them. Furthermore, one anhydro-rhamnose ring previously has been proposed to be located near the reducing end of each xylan chain after alkaline extraction of hardwood [26]. Thus, in the calculations for degree of polymerization (DP) of absolute xylan in WIX and WSX from non-pretreated fibers, a single rhamnan unit was considered in each xylan chain. Based on the weight percentage of xylan to that of rhamnan (Table 5-2) and their molecular weights, DP of xylan could be calculated shown in Table 5-3. The absolute xylan in WIX had a DP of 125, corresponding with prior DP values reported for hardwood xylan [26]. Additionally, this

calculated DP was further confirmed by the number average DP from GPC analysis of the carbanalized derivative. For WSX the corresponding chain length based on the end group calculation was 27, suggesting a lower molecular weight crude xylan product.

Table 5-3 Number of lignin C9 units and rhamnan per 100 anhydro xylose ring and calculated DP.

#	lignin C9 units	anhydro rhamnose ring	DP of xylan chain
WIX SGC	3.8	0.8	125
WSX SGC	11.1	3.8	27

*The molecular weight of lignin C9 was assumed to be 210 g/mol; The DP of WIX was confirmed with GPC.

WIX isolated from non-treated fibers represented the major xylan product from mild alkaline extraction. The crude WIX had a higher xylan purity with less lignin contamination. Xylan in WIX exhibited a polymeric form with glucuronic acids side-groups and a DP of 125. In contrast to WIX, the WSX had lower xylan purity with more lignin co-precipitated. Xylan in WSX had an average chain length of 27 based on end-group analysis. The low degree of polymerization of WSX accompanied with more side-chain sugars are the likely reasons for its better water solubility.

5.5.2 Crude xylan recovery yield from GTP treated biomass

Glycerol thermal processing was conducted on the sweet gum fibers followed by two sequential extraction for biomass washing and delignification. During these steps, the fate of total initial xylan was also tracked as shown in Figure 5-4. Prior to xylan isolation through alkali extraction, 75-90% of the initial xylan was preserved in the delignified GTP fibers. With increasing GTP processing severity, more water soluble xylan was lost prior to the alkali extraction.

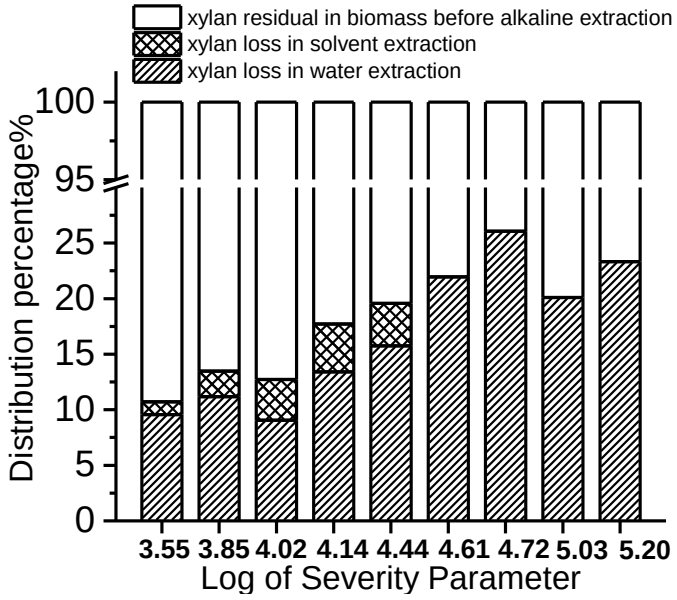

Figure 5-4 Xylan distribution percentage in sweet gum fibers after GTP, water and solvent extraction.

A mild alkaline extraction using 1% sodium hydroxide at ambient temperature was conducted to isolate both WIX and WSX xylan from the delignified GTP fibers (Figure 5-4). The yield of crude xylan was then calculated as the mass of recovered WIX/WSX over total mass of delignified fibers for alkaline extraction (Figure 5-5).

With increasing GTP processing severity, the recovery yield of crude WIX increased, while that of the crude WSX decreased. Considering a "pre-extraction" of water soluble xylan was essentially achieved by the water extraction after GTP pretreatment, it makes sense that the water soluble fraction was reduced at this recovery point. Figure 5-4 indicated that more xylan was lost during water extraction at high GTP severities, which means, there was less low MW water soluble xylan present in the GTP and pre-water/solvent extracted biomass. The hemicellulose side chains associated with LCC bonds were removed during pretreatment (Chapter 3), which likely results in the better dissolution of low molecular weight xylan in the pretreated biomass. Thus, water insoluble xylan becomes the major product from alkali extraction at the four highest severities.

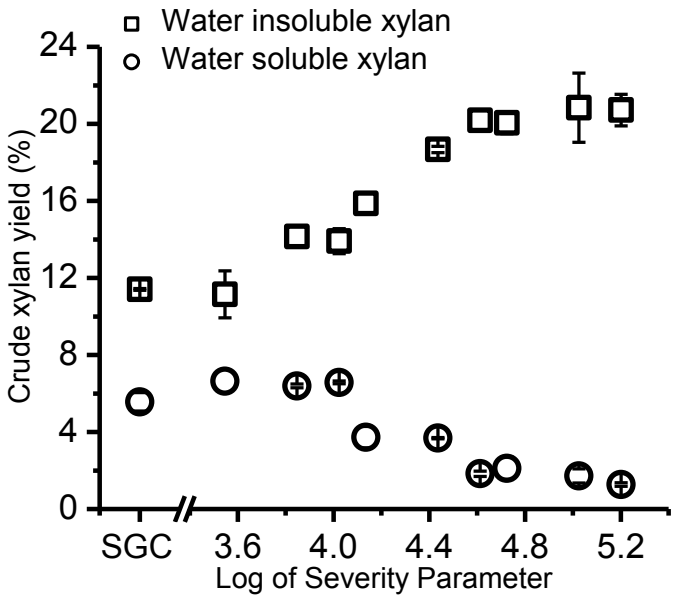

Figure 5-5 Crude xylan yield based on the total biomass weight for alkali extraction.

5.5.3 Purity of recovered WIX

Detailed compositional analysis of crude WIX was shown in Table 5-4. After low severity GTP treatment of biomass, the xylan content in the dry crude WIX was slightly lower than that of WIX isolated from non-pretreated fibers. The xylan purity increased up to 84% in WIX at high GTP severities. Also, all the isolated WIX from GTP biomass was observed to have more lignin but less rhamnan content compared to the WIX from non-pretreated fibers.

Table 5-4 Composition of crude WIX isolated from non-pretreated sweet gum, GTP pretreated and delignified fibers.

#	log(R_0)	xylan%	lignin%	rhamnan%	glucan%	mannan%	arabinan%
WIX SGC	-	76.7(0.1)	4.64(0.83)	0.682(0.004)	0.181(0.005)	0.000(0.000)	0.012(0.006)
WIX 1	3.55	75.9(0.4)	9.73(0.94)	0.589(0.010)	0.166(0.009)	0.031(0.001)	0.019(0.001)
WIX 2	3.85	73.3(0.1)	9.83(0.34)	0.534(0.009)	0.123(0.003)	0.038(0.001)	0.018(0.002)
WIX 3	4.02	75.4(0.1)	11.1(0.5)	0.518(0.003)	0.108(0.003)	0.037(0.003)	0.017(0.001)
WIX 4	4.14	73.6(0.1)	12.6(0.3)	0.499(0.010)	0.124(0.002)	0.060(0.002)	0.024(0.002)
WIX 5	4.44	75.9(0.2)	13.0(1.1)	0.476(0.008)	0.148(0.007)	0.069(0.003)	0.028(0.003)
WIX 6	4.61	78.7(0.3)	10.9(0.2)	0.479(0.009)	0.134(0.000)	0.062(0.001)	0.022(0.003)
WIX 7	4.72	77.1(0.1)	13.9(0.3)	0.349(0.003)	0.241(0.007)	0.12(0.02)	0.045(0.004)
WIX 8	5.03	82.1(0.4)	12.1(0.4)	0.392(0.005)	0.277(0.032)	0.11(0.01)	0.053(0.010)
WIX 9	5.20	83.6(0.7)	11.2(0.1)	0.355(0.007)	0.292(0.006)	0.13(0.01)	0.057(0.013)

*rhamnan content contains trace amounts of galactan; lignin includes acid soluble and acid insoluble lignin from compositional analysis.

Based on the overall biomass mass balance (Chapter 3) and xylan purity in WIX after GTP pretreatment, a detailed mass distribution of total initial xylan in the initial biomass was determined (Figure 5-6), indicating that very little xylan actually was recovered in the WSX form at higher GTP severities. The results indicated that about 19-24% of the starting xylan was removed durng the subsequent water extraction after GTP at the four most severe conditions. In these cases, only about 1-2% more xylan was then further isolated during alkaline extraction as the water soluble portion. However, when the lower GTP severity treatments were used, xylan in the final water soluble fraction during alkali extraction was in the range of 8 to 17%; whereas 10

- 20% of initia xylan was pre-extracted during the water extraction step. Thus, overall 25-28% of the starting xylan was water soluble with a minimum of the xylan removed during the solvent extraction step for the GTP pretreated samples. In comparison, a direct alkaline extraction of the non-pretreated biomass yielded 15% water soluble xylan. As indicated by this analysis, the GTP pretreatment produced almost double the amount of water soluble xylan, which was independent on the GTP severity. At the same time, a uniform maximal amount of alkali extractable xylan was obtained when the five highest GTP pretreatment severity conditions were used.

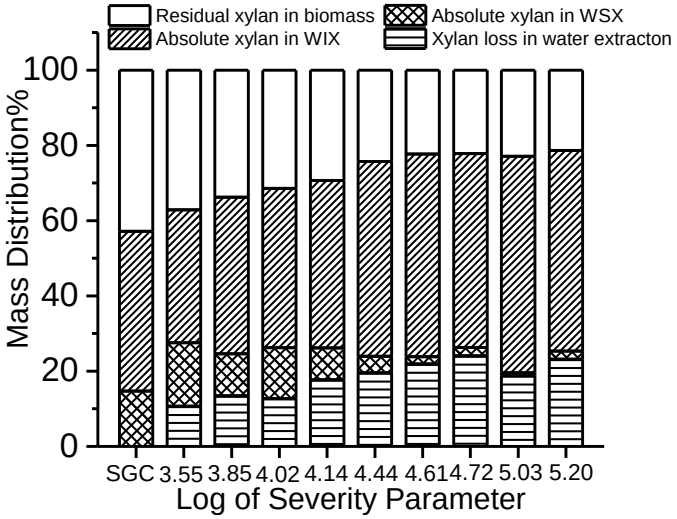

Figure 5-6 Overall xylan mass distribution based on total xylan in starting biomass for non-pretreated and GTP pretreated sweet gum fibers.

*Results are calculated through average of duplicates with a COV < 5%; total xylan in original fiber is 20.6%; SGC refers to the absolute xylan isolated from non-treated fibers through alkaline extraction.

The amounts of associated lignin and rhamnan in WIX were converted to the number of monomeric units associated with xylan backbone as shown in Table 5-5. Compared to the four lignin C9 units per 100 xylan rings in WIX from untreated fibers (reported previously), the WIX isolated from GTP treated biomass contained around ten lignin C9 units per 100 xylan rings. The relative lignin content was independent of the GTP treatment severity. This relatively higher lignin ratio is probably due to the cleavage of lignin-carbohydrate linkages, rendering lignin

more extractable after GTP pretreatment. More lignin monomers (bound by weak atomic forces) is probably co-extracted with xylan during alkaline extraction. An alternative explanation could be the degraded lignin is chemically attached to the xylan during the GTP pretreatment. As indicated by Chapter 4, extensive alkyl aryl ether bond breakage occurred on lignin, which may facilitate the formation of lignin xylan complexes from lignin monomers released by the GTP treatment. This result also indicates that further bleaching [6] of isolated WIX would be necessary if higher xylan purity is required in commercial processing. Additionally, for native hardwood xylan, one rhamnan ring normally is found near the end group of xylan chains [26], which was also confirmed in the WIX xylan isolated from the non-pretreated biomass. However, the relative content of rhamnan was inversely correlated with the GTP severity. A maximum of 50% reduction in the amount of rhamnan was observed at GTP severity increased, indicating that rhamnan units were cleaved during GTP pretreatment (Table 5-5).

Table 5-5 Number of lignin C9 units and rhamnan per 100 anhydro xylose ring for WIX isolated from non-pretreated and GTP pretreated sweet gum fibers

#	$\log(R_0)$	lignin C9 units	anhydro rhamnan ring
WIX SGC	0.00	3.8	0.80
WIX 1	3.55	8.1	0.70
WIX 2	3.85	8.4	0.66
WIX 3	4.02	9.2	0.62
WIX 4	4.14	11	0.61
WIX 5	4.44	11	0.57
WIX 6	4.61	8.7	0.55
WIX 7	4.72	11	0.41
WIX 8	5.03	9.2	0.43
WIX 9	5.20	8.4	0.38

*assume the molecular weight of lignin C9 is 210 g/mol.

5.5.4 Crude WIX and WSX FTIR analysis as a function of GTP severity

The FT-IR spectra fingerprint region for crude WIX isolated from GTP pretreated fibers showed (Figure 5-7) that the primary change in the material occurred in the resonance band at 1720 cm^{-1}, which was assigned to the carbonyl groups of glucuronic acid in xylan (deacetylation was confirmed by the ^{1}H-NMR in Figure B-1). This band existed as a shoulder in the WIX isolated from non-treated fibers; however, its intensity decreased with increased GTP severity. For WIX isolated from GTP fibers at high pretreatment severities, the carbonyl resonance disappeared. This indicates that glucuronic acid is being cleaved from the xylan backbone as GTP severity increases. At the highest severity GTP pretreatment, no carbonyls in the glucuronic acid side

groups are observed the isolated xylan. Additionally, the spectra showed similar characteristic bands in the range of 1100-900 cm^{-1} for xylan independent of GTP severity. Noteworthy was the gradual change in the 1700-1600 cm^{-1} region which is associated with the conjugated carbonyls of the lignin propyl chain and the aromatic skeleton, indicating that associated lignin structural changes are also occuring with increasing GTP severity.

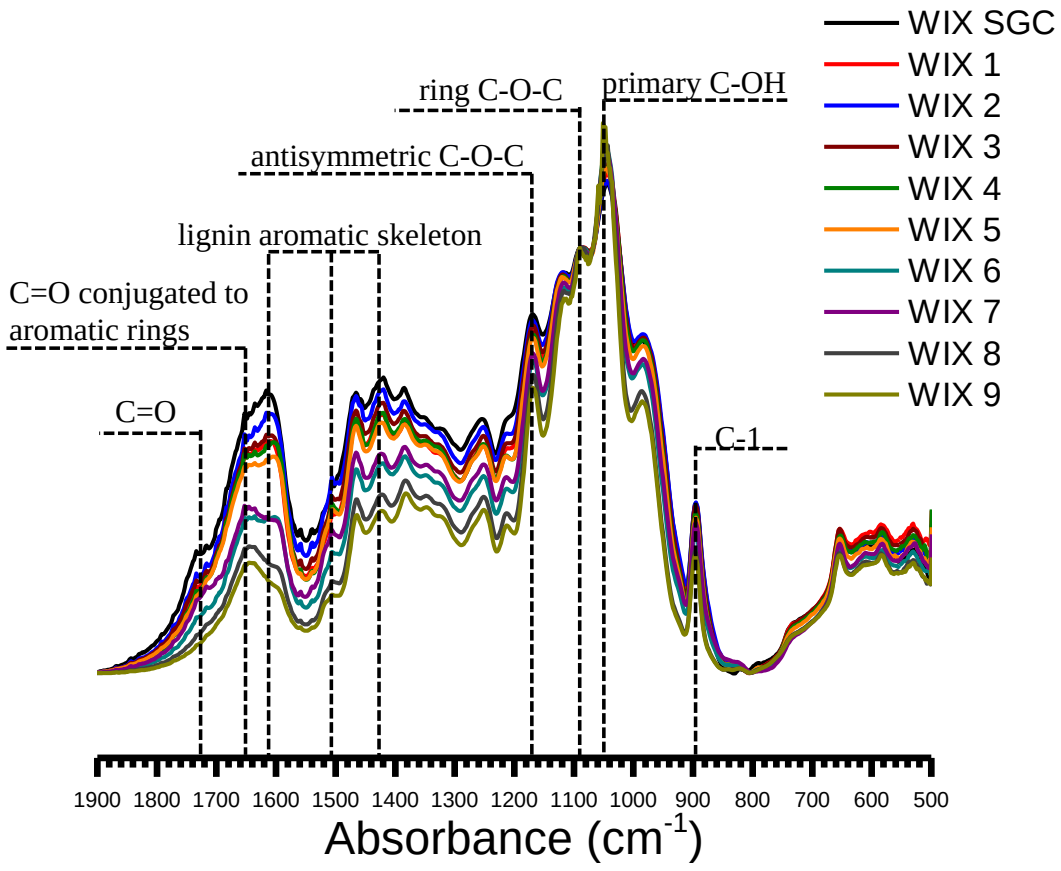

Figure 5-7 Fingerprint region of crude WIX as a function of GTP severity (Spectra were normalized at the band around 1085 cm^{-1}).

Resonance bands at 1620 cm^{-1} and 1510 cm^{-1} showed (Figure 5-8) characteristics associated with lignin [27] in the WSX samples. Although the lignin content for WSX was not quantified in this research due to the limited recovery yield, the normalized spectra of crude WSX exhibited a higher absorbance intensity for WSX isolated from GTP fibers at high pretreatment severities. These results indicate that more lignin moieties are associated with WSX as the GTP severity increases. The decreasing intensity of resonance bands at 1161 cm^{-1} suggests also that glycosidic

linkages decrease in the crude WSX with increasing GTP severity, which is in good agreement with the minimum absolute xylan content in WSX at high GTP severities.

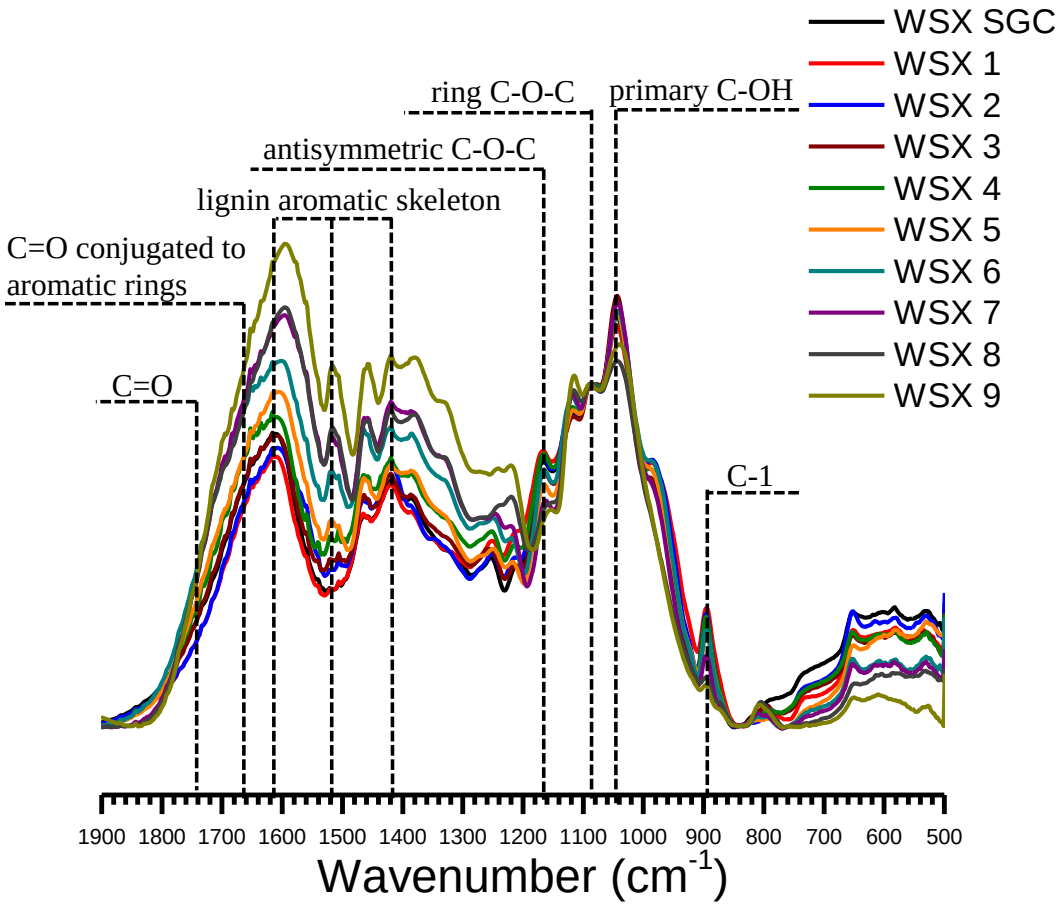

Figure 5-8 FT-IR fingerprint region of crude WSX as a function of GTP severity.

The FT-IR spectral comparisons of WIX and WSX as a function of GTP pretreatment severity on the isolated crude xylan can be summarized as follows: For the WIX, more severe GTP processing results in the cleavage of glucuronic acid, leading to a less substituted xylan backbone. It also demonstrates more lignin contamination and less absolute xylan was observed in the recovered crude WSX after GTP pretreatment compared to that in the WSX from non-pretreated fibers.

5.5.5 Degree of polymerization of the recovered xylan in WIX (GPC Analysis)

Due to the poor solubility of xylan in organic solvents, carbanilation was performed prior to GPC analysis to fully convert the hydroxyl groups for improved solubility. A typical reaction scheme

used for xylan carbanilation in this work was shown in Figure 5-2. The disappearance of hydroxyl groups in the xylan carbanilates was confirmed by FT-IR analysis (spectra can be found in Figure B-2). Absolute molecular weight results for xylan carbanilates as a function of GTP pretreatment severity are shown in Table 5-6.

Table 5-6 Absolute number average and weight average molecular weight of WIX carbanilates.

#	$\log(R_0)$	Mn(KDa)	Mw(KDa)	DP_n	PDI
WIX SGC	-	52.5	68.1	127	1.30
WIX 1	3.55	45.8 (6.2)	51.1 (6.9)	123	1.11
WIX 2	3.85	47.1 (8.3)	58.8 (16.1)	126	1.25
WIX 3	4.02	46.2 (7.9)	52.1 (9.2)	124	1.13
WIX 4	4.14	43.7 (0.8)	49.9 (0.3)	117	1.14
WIX 5	4.44	47.3 (9.4)	55.0 (11.2)	127	1.16
WIX 6	4.61	42.0 (3.6)	48.0 (3.0)	113	1.14
WIX 7	4.72	46.5 (3.2)	54.1 (4.4)	125	1.16
WIX 8	5.03	45.8 (2.4)	54.8 (0.3)	123	1.20
WIX 9	5.20	54.4 (1.4)	65.6 (0.0)	146	1.21

*DP_n: absolute number average degree of polymerization; DP_n of WIX SGC is calculated based on 1 glucuronic acid/10 xylose ring and 1 rhamnose ring per each xylan chain; DP_n of WIX isolated from GTP fibers is calculated based on no glucuronic acid and the known rhamnose amounts on xylan chain; lignin and other contamination is included in the MW and DP calculation.

The extracted WIX displayed comparable molecular weights to the WIX isolated from non-treated fibers, resulting in a DP range of 110-150 (Table 5-6). No measurable xylan chain degradation occurs during GTP processing with increasing severity. Although GTP pretreatment was conducted at similar, or even higher, severity conditions as prior steam-pretreatment studies, xylan was not hydrolyzed as the way occurs in steam-pretreatment where acetic acid release is responsible for severe xylan degradation. Instead, the molecular weight is preserved with xylan largely remaining in its polymeric form. Additionally, the derivatized xylan showed a narrow molecular weight distribution, with the PDI in the range of 1.1-1.3 for all WIX isolated after GTP pretreatment. This narrow distribution most likely arises from the selective fractionation of the low molecular weight xylan in the water extraction steps. These results indicate that the isolated water insoluble xylan after GTP pretreatment is in a polymeric form with additional lignin units, but reduced glucuronic acid side groups. These side groups usually limit assembly and thus if the lignin can be removed through mild bleaching, this could produce a highly attractive material for used in advanced renewable materials applications [6].

5.6 Conclusions

Glycerol thermal processing as a novel pretreatment was used for improved lignin fractionation and enzymatic saccharification and the resulting products analysed. As a result of GTP processing, most of xylan was preserved in the pretreated biomass. A sequential mild alkaline extraction was performed on the pretreated fibers for xylan fractionation in the form of a water soluble portion (WSX) and a water insoluble portion (WIX). The xylan in the WIX was in polymeric form whereas that in the WSX had a much lower degree of polymerization. More WIX was successfully recovered from the GTP pretreated fibers with increasing GTP severity, with a maximum xylan purity of 84%. Detailed FT-IR spectral comparisons indicated that severe GTP processing resulted in the removal of glucuronic acid side-groups from the xylan backbone in WIX. However, with increasing GTP severity, because more xylan has already been dissolved and removed by the aqueous pre-extraction, this resulted in less xylan being isolated in the WSX material. Molecular weight analysis confirmed the polymeric nature of the recovered WIX, with an especially narrow MW distribution. This research demonstrates a new method to fractionate polymeric xylan as a byproduct for the biorefinery industry.

5.7 Acknowledgments

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Chapter 6 Enhanced enzymatic saccharification through biomass glycerol thermal processing

6.1 Abstract

Glycerol thermal processing (GTP) as a novel pretreatment was applied to sweet gum biomass for enhanced biomass saccharification. Based on previous studies, extensive LCC deconstruction, with a concurrent increased surface area were resulted from GTP pretreatment. In this research, the enzymatic saccharification of GTP pretreated biomass was examined. Compared to non-pretreated sweet gum biomass, the GTP pretreatment significantly enhanced the ultimate biomass digestibility, although the biomass exhibited greater cellulose crystallinity while also retaining comparable amounts of lignin and hemicellulose. These results indicated that residual lignin and hemicellulose did not restrict cellulose enzyme attack to any greater degree after GTP pretreatment. Additionally, biomass crystallinity was not the predominant reason affecting enzymatic biomass digestibility. Saccharification of GTP biomass before and after water washing revealed that no significant inhibitory compounds were produced even at high GTP severities. The GTP pretreated biomass after solvent and alkaline extraction was also examined for enzymatic saccharification with results demonstrating that lignin was not a barrier limiting the cellulose digestibility after pretreatment, and further delignification had no positive effect on residual biomass saccharification. However, further hemicellulose removal greatly improved the cellulose hydrolysis rate, resulting in 78% of ultimate glucan digestibility. Overall, the GTP pretreatment provided a novel method for efficient biomass saccharification. Increased surface area and significant deconstruction of the LCC network due to GTP processing were the predominant characteristics responsible for enhanced biomass saccharification.

6.2 Keywords

Biomass pretreatment, Glycerol thermal processing (GTP), Enzymatic saccharification, Delignificaton, Xylan removal

6.3 Introduction

A global rise in fuel demand combined with the depletion of fossil hyrocarbons, and concern for greenhouse gas emission has motivated interest in the development of renewable biofuel and bioproducts production from sustainable biomass [1-3]. First generation biofuels were mainly derived from corn-starch or sugarcane through batch fermentation. However, debates and concern [4] about these first generation biofuels have arisen because of the competition between food and fuels as well as concern about the emission of greenhouse gas when any carbon undergoes combustion [5]. Lignocellulosic biomass has become more attractive as the feedstock for biofuel production [6] due to its low cost and large abundance. Lignocellulosic biomass contains approximately 70% dry weight polysaccharides, which can be enzymatically hydrolyzed to pentoses and hexoses, and further fermented to ethanol and/or butanol. Moreover, the abundant biopolymers such as lignin, xylan and cellulose existing in lignocellulosic biomass provide potential sustainable polymeric materials.

However, the recalcitrant structure of lignocellulosic biomass greatly impedes the efficacy of biofuel production. Several structural features have been proposed as major hurdles to both the rate and extent of biomass saccharification. Biomass surface area and pore volume are the primary physical barriers that dictate how biomass can be enzymatic deconstructed. Larger surface area and pore volume would facilitate the enzyme attack on cellulose chains, which would in-turn positively impact biomass saccharification [7-9]. A high degree of cellulose polymerization with abundant hydrogen bonds between the chains prevents enzyme access, and therefor also reduces the potential for saccharification [10]. Some studies have demonstrated that cellulose crystallinity is a dominant factor and high crystallinity limits cellulose enzyme accessibility whereas reduced crystallinity improved the initial hydrolysis rate [11-13]. Lignin [14] and hemicellulose [15] which surround cellulose in its crystalline form are also proposed to negatively impact biomass saccharification. These two components limit the efficient access of enzymes to the cellulose surface while cellulose crystallinity prevents access of enzymes internally within the cellulose crystallytes. Lytic polysaccharide monooxygenase enzymes and others in the AA9 group [16-18] can bind to, and disrupt crystalline cellulose chains at the surface, whereas other cellulase enzymes can only attack cellulose in non-crystalline forms [19]. The acetyl groups normally extensively existing on hardwood hemicellulose are also proposed to

limit the biomass digestibility [20], but specialized enzymes area also known to be involved in deacetylation [21].

Biomass pretreatment prior to the enzymatic hydrolysis is an essential step for overcoming the structural and steric barriers to enzyme access for more efficient enzymatic deconstruction in biofuel production. Over the last three decades, extensive research has explored many paths for pretreatment to try and overcome the recalcitrance of lignocellulosic biomass for efficient enzymatic saccharification. Dilute acid [22, 23], alkaline [24, 25], steam-assisted [7, 26] and organosolv [27] methods typically occur at high temperatures around 150-200 ℃ resulting in enhanced biomass surface area and significant removal of lignin and/or hemicellulose [28-36], and these pretreatments have been widely applied on hardwood, softwood and grasses [37-44], but many problems remain as undesirable side products for fermentation, such as fururals are generated when processes such as these are used [45-47]. Pretreatments with ionic liquids [48, 49] have expanded green processing technologies for efficient biomass saccharification, but more efforts are still needed to resolve the problems such as high chemical cost as well as recovery and reuse of ionic liquids [50].

Continuous pretreatment protocols involving screw-extrusion and other high-shear processing systems have been recently been developed for study. Extruders widely used in the polymer processing industry and offer several features applicable to biomass pretreatment, such as excellent temperature control, the capacity for high solids loading, and simultaneous heating and shearing during processing. The first application of extrusion-assisted pretreatment was reported in 1999 [51], where a twin-screw extruder was combined with a liquid ammonia pretreatment to enhance the digestibility of corn stover. Lee et al. [52-55] conducted a series of experiments using an extrusion system with different additives for biomass pretreatment, obtaining a maximum glucose yield of 62.4% when enzymatic saccharification was used with ethylene glycol as an additive. The resulting biomass fibers were highly fibrillated and possessed a greatly expanded surface area after extrusion. Karunanithy et al. [56] also reported a maximum digestion yield of 65.8% for pine chips when high moisture conditions were used with extrusion processes.

In this research, a continuous and scalable biomass pretreatment method was developed through the use of a batch-type mixer and glycerol additive, combined with glycerol thermal processing

in an extruder, to enhance the biomass saccharification. Additionally, a range of biomass types were examined before and after biopolymer fractionation to reveal how the biomass was deconstructed by the pretreatment.

6.4 Experimental

6.4.1 Materials

Chemicals and reagents used in this research were purchased from Sigma-Aldrich, Alfa Aesar and MP Biomedicals, and used as received. A mature sweet gum (*Liquidambar styracuflua*) from Blacksburg, VA was debarked, machined to cubes, and stored in a freezer before use. Prior to pretreatment, the biomass was knife milled using a Wiley mill, and sorted to a particle size between 40 to 60 mesh on a metal screen (250-420 μm). The extractive-free sweet gum particles were prepared according to ASTM standard [57]. Deionized water (DI-water: 18.2 mΩ) was used in all steps in this research.

6.4.2 Glycerol thermal processing (GTP) pretreatment

Extractive-free biomass was pretreated with glycerol using a bench-top internal mixing head with a high intensity shear paddler driven by a C.W. Brabender® Prep-Center® drive as detailed previously (Chapter 3). The GTP pretreatment severity parameter (R_0) was calculated according to an equation developed by Overend and Chornet [35] to define pretreatment conditions. The corresponding logarithm of R_0 in this research is shown in Table 6-1.

Table 6-1 Glycerol thermal processing conditions and corresponding severity parameters for biomass pretreatment in this research.

Sample label	T (℃)	t (min)	$\log(R_0)$
SG#1	200	4	3.55
SG#4	220	4	4.14
SG#8	240	8	5.03
SG#9	240	12	5.20

After pretreatment, the GTP pretreated biomass was collected from the mixing head and stored at 4 ℃ until further analysis. Samples for each severity condition were run in triplicate.

Pure cellulose from Whatman brand fibrous cellulose CF11 powder, was used for a reference material in this study. Only one GTP condition at 240 ℃ and 8 min was used to process the cellulose samples in triplicate. After pretreatment, the GTP pretreated cellulose was collected and stored at 4 ℃ for further analysis.

6.4.3 Water extraction

The GTP pretreated biomass was water washed to remove glycerol residues and any degraded components as detailed previously (Chapter 3). To avoid re-crystallization during drying, the water extracted GTP biomass was freeze-dried immediately after processing. The GTP pretreated CF11 cellulose was water washed and freeze-dried using the same methods, and no further extractions were conducted on these samples.

6.4.4 Solvent extraction

The water extracted GTP biomass was further solvent extracted to isolate lignin polymer as a byproduct as detailed previously (Chapter 4). The solvent extracted GTP biomass was washed using DI water until the wash water was colorless before freeze-drying the biomass.

6.4.5 Alkaline extraction

The solvent-extracted GTP biomass was alkali extracted to isolate xylan polymer as another byproduct stream as detailed previously (Chapter 5). After extraction, the biomass residue was washed using DI water until the wash water was colorless and the GTP biomass was immediately freeze-dried.

6.4.6 Biomass structural carbohydrate analysis

The carbohydrate content of the non-pretreated and GTP pretreated sweet gum were analyzed in duplicate according to NREL laboratory analytical procedures (LAP) [58] as detailed previously (Chapter 3).

6.4.7 Enzymatic saccharification

Non-pretreated and GTP pretreated sweet gum (water, solvent and alkaline extracted) biomass was enzymatic hydrolyzed to a glucan consistency of 1%. Hydrolysis using a CTec2 enzyme cocktail in a pH 5.0, 0.05 M sodium citrate buffer with 0.02% (w/v) NaN_3 to prevent the growth of microorganisms during the digestion [59], was performed in a water bath shaker at 50 ℃ and 180 rpm for 72 h. The CTec2 cocktail (protein content of 188 mg/ml) was generously donated by Novoenzymes (Franklinton, NC), and used as received. The enzyme loading for hydrolysis was based on 20 mg protein/g cellulose to avoid insufficient digestibility due to limited enzyme loading (demonstrated by enzymatic hydrolysis of Avicel, data not shown). At predetermined time intervals, well-mixed 2-ml aliquots were removed from the digest, micro-centrifuged at 13,000 rpm for 15 minutes to remove the solid biomass, and the clear supernatant was used to determine glucose released by the enzymatic treatment.

The non-pretreated and GTP pretreated CF11 cellulose was also enzymatically hydrolyzed using above method.

6.4.8 Determination of released glucose

Glucose released during enzymatic hydrolysis was determined either on a Metrohm Ion Chromatography (IC) or a Shimadzu High Performance Liquid Chromatography (HPLC). When

tested by IC, the 1 ml supernatant (pre-passed 0.2 μm filter) was diluted 50 times. The glucose in diluted aliquot was measured using a pulsed amperometric detector (PAD) after separation on a Hamilton RCX -30 ($250 \times 4.6\ mm$) column with DI-water as eluent as detailed in previous research (Chapter 3). Samples assayed by HPLC were separated using a Bio-Rad Aminex® HPX-87H column ($300 \times 7.8\ mm$) using a 5 mM sulfuric acid mobile phase with a constant flow rate of 0.5 ml/min. The sample injection volume was 15 µl and an RID-10A refractive index detector was used for glucose detection. A 6 point calibration curve using pure glucose standards was run prior to each batch of tests (R^2>0.999).

The overall enzymatic glucan digestibility (%) was calculated as follows:

$$\%\ \text{digestibility} = \frac{G_h}{(180/162) \times G_i} \times 100\% \quad (1)$$

Where G_h = the amount of soluble glucose after enzymatic hydrolysis; G_i = the initial added glucan in the biomass before enzymatic hydrolysis.

6.4.9 X-ray diffraction (XRD) of GTP pretreated biomass

The crystallinity index of the water extracted GTP biomass and cellulose was measured on a Bruker D8 Discover X-ray diffractometer with a Cu Kα radiation source (λ = 0.154 nm) generated at 40 kV and 40 mA. A 1 mm slit was used and a locked couple 2theta and theta scan was performed from 10 to 50 ° at a scan speed of 4 °/min. The biomass samples were flattened on a quartz slide with a thickness of 1~2 mm to collect the diffraction profile. Non-pretreated sweet gum and CF11 cellulose werer used as the control and reference samples.

The crystallinity index (CrI) of the different biomass samples tested was calculated according to the methods developed by Segal and coworkers [60]:

$$\text{CrI} = \frac{I_{002} - I_{AM}}{I_{002}} \times 100\% \quad (2)$$

Where I_{002} is the maximum intensity of the 002 lattice diffraction and I_{AM} is the minimum intensity between the 002 peak and the 101 peak.

6.4.10 Morphology of GTP pretreated biomass

The nano-scale morphology of the water extracted GTPSG was imaged using a NeoScope JCM-5000 Scanning Electron Microscopy (SEM). Prior to analysis, biomass samples were vacuum dried at 40 ℃ over P_2O_5 (5.4 mm Hg) for 48 hours. Sample biomass particles were then sputter-coated with 3.5-4.0 nm gold-palladium before imaging. Extractive-free sweet gum particles without GTP pretreatment were used as reference samples.

6.5 Results and discussion

Compositional analysis of non-pretreated and GTP pretreated SG used for enzymatic saccharification is shown in Table 6-2. Glucan was previously shown (Chapter 3) to be fully conserved after GTP processing and the extraction series. The water extracted GTP biomass still contains a significant amount of lignin and xylan but with a disrupted cell wall network, due to the significant removal of side-chain sugars that are responsible for lignin-carbohydrate linkages (LCC bonds) (Chapter 3). As a result of solvent extraction, the concentration of lignin in the solvent extracted GTP biomass decreased with increasing GTP severity. Alkaline extraction removed ~60% of the initial xylan and additionally ~15% of the initial lignin, resulting in a cellulose-rich substrate when the highest level of GTP processing conditions were used.

Table 6-2 Compositional analysis (wt%) of non-pretreated and GTP pretreated biomass for enzymatic saccharification.

Sample ID	Glucan%	Xylan%	Klason lignin%	Arabinan%	Galactan%	Mannan%
SG control	40.11 (0.11)	20.61 (0.07)	21.87 (0.17)	0.58 (0.01)	0.56 (0.01)	1.95 (0.00)
Water extracted GTPSG 4	47.60 (0.16)	21.25 (0.05)	23.61 (0.18)	0.35 (0.00)	0.32 (0.00)	1.87 (0.01)
Water extracted GTPSG 8	47.23 (0.54)	20.14 (0.33)	23.94 (0.33)	0.14 (0.00)	0.18 (0.00)	1.54 (0.01)
Water extracted GTPSG 9	49.00 (0.26)	20.71 (0.10)	24.72 (0.24)	0.10 (0.01)	0.15 (0.00)	1.39 (0.04)
Solvent extracted GTPSG 4	49.00 (0.12)	21.58 (0.11)	19.15 (0.21)	0.35 (0.00)	0.31 (0.00)	1.90 (0.03)
Solvent extracted GTPSG 8	56.74 (0.82)	24.16 (0.63)	12.90 (0.31)	0.15 (0.01)	0.18 (0.00)	1.85 (0.09)
Solvent extracted GTPSG 9	58.41 (0.32)	24.97 (0.41)	11.68 (0.02)	0.09 (0.00)	0.13 (0.01)	1.81 (0.08)
Alkaline extracted GTPSG 9	83.65 (0.17)	9.34 (0.01)	8.43 (0.36)	0.08 (0.00)	0.14 (0.01)	2.43 (0.01)

6.5.1 Enzymatic digestibility of biomass after GTP pretreatment and water washing

In contrast to the minimal digestibility of non-pretreated SG, the digestibility of GTP pretreated SG was greatly enhanced as the GTP processing severity increased (Figure 6-1). A maximum 20-fold increase was observed at severity level 9 [$\log(R_0)$=5.20] for the 72 hour hydrolysis corresponding to 68% digestibility of the original material as a result of the GTP pretreatment.

Prior work (Chapter 3) demonstrated that side-chain sugars were removed gradually with increasing GTP severity. The cell wall network was extensively deconstructed at severe GTP processing conditions. Although the water extracted GTP biomass still contained lignin and hemicellulose in amounts comparable to the non-pretreated SG (Table 6-2), the enzymatic digestibility was not limited by the residual lignin and hemicellulose after GTP processing. Instead, the disruption of the cell wall network by significant removal of LCC linkages facilitated access enzymes to the cellulose chains.

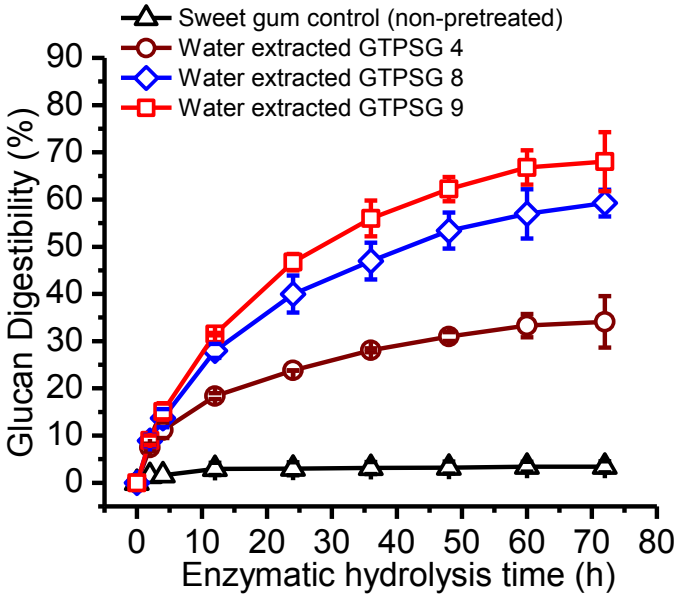

Figure 6-1 Enzymatic hydrolysis of non-pretreated and water extracted GTP biomass at different severity levels.

Unlike the GTP biomass pretreatment, the affect of GTP processing on cellulose (Figure 6-2) impacted on cellulose crystallinity and the degree of polymerization for this pure cellulose sample, which would have later impact on enzymatic saccharification. According to Figure 6-2, The GTP pretreated cellulose had only a 2-fold increase in glucan digestability after 72 hours of hydrolysis (Figure 6-2), compared to the 18-fold increase in biomass digestibility under the same GTP conditions (Figure 6-1).Thus, with the GTP pretreatment, more access of enymes to the amorphous cellulose was obtained for sweet gum biomass, which was not that prevalent in the pure cellulose samples.

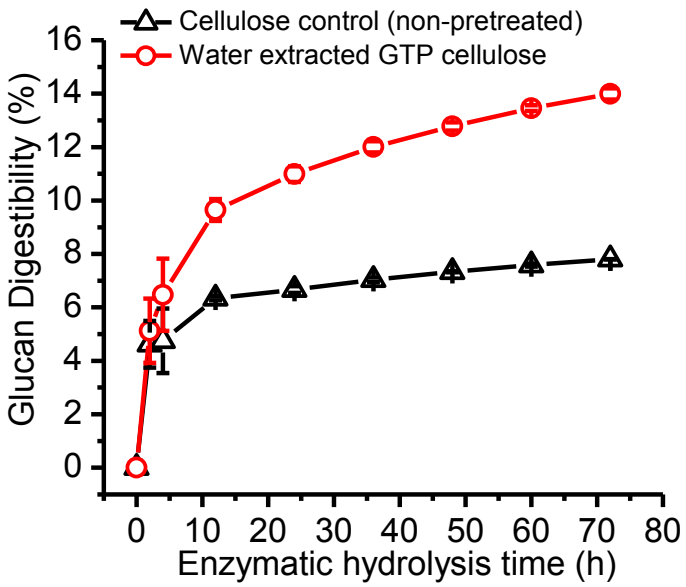

Figure 6-2 Enzymatic hydrolysis of non-pretreated and water extracted GTP cellulose.

6.5.2 XRD crystallinity of water-extracted GTP biomass

A comparison of the crystallinity index (CrI) of the GTP pretreated cellulose and biomass is shown in Table 6-3. The pure cellulose had a relatively high CrI of 72.6% for the non-pretreated cellulose, which increaseed further to 92% after GTP pretreatment. The biomass contained more amorophouse cellulose originally and a smaller increase of crystallinity is observed for the GTP pretreated biomass, from 53.7% for non-pretreated SG to ~60% after GTP pre-treatment. A similar increase in crystallinity has been reported for steam-exploded biomass [61-63], because of the degradation of amorphous cellulose and significant removal of hemicellulose, which provided greater access to the crystalline forms of cellulose for steam-induced hydrolysis. In our case, no extensive hemicellulose loss was observed during the GTP processing and water washing. As lignin and hemicellulose bonds are cleaved and the structure become more open, chemical treatment can penetrate more deeply into the cell wall structure. Crystallinity changes for both GTP pretreated cellulose and biomass would therefore most likely be attributed to the disruption of cellulose in the outer chains of the cellulose crystallytes (or perhaps the transitional chains) [64] during GTP pretreatment, resulting in biomass with greater crystallinity. These cellulose chains are less tightly bonded compared to the core and transitional chains of the cellulose crystallyte or elementary fibril [64]. The more digestible biomass substrate after GTP

pretreatment confirms that the core chain crystallinity is not a dominant reason restricting the biomass saccharification.

Table 6-3 XRD crystallinity index of water extracted biomass and pure cellulose samples after GTP pretreatment.

Sample ID	$\log(R_0)$	CrI
cellulose	-	72.6 (1.6)
GTP cellulose	5.03	92.0 (0.1)
SGC*	-	53.7 (1.8)
GTPSG 1	3.55	58.6 (2.6)
GTPSG 4	4.14	63.9 (1.5)
GTPSG 8	5.03	59.5 (0.1)
GTPSG 9	5.20	62.7 (3.1)

*non-pretreated sweet gum

6.5.3 SEM morphology structures of water-extracted GTP biomass

Particle size, porosity and surface area has been proposed as the primary properties which govern biomass saccharification [10]. Extractive-free SG particles were pre-milled and screened to a particle size of 250-420 μm for the GTP pretreatment and morphological observation of biomass using SEM before and after GTP pretreatment was done to qualitative evaluate the changes in biomass structure associated with GTP processing (Figure 6-3). The vascular elements of the cell walls were were fractured tranversely through the cross-section of the non-pretreated SG (Figure 6-3) during the milling process in sample preparation, whereas the longitudinal surface was split to reveal a relatively smooth and well-preserved surface. After a moderate severity GTP pretreatment [$\log(R_0)$=4.14], smaller wood particles were produced (low magnification images not shown here). The GTPSG 4 sample shows that the tracheal and vessel elements of the wood

particle disintegrate more readily leaving both residual ray parenchyma and tracheal (vessels and fiber) tissue remaining. Ray tissue were more susceptable to treatment and degraded more readily as would be expected since the ray parenchyma cell walls are relatively thin compared to fiber cell walls. However, with increasing GTP severity to log(R_0)=5.03 (GTPSG 8), more cellular debris with sizes around 10 μm is observed as the ray tissue was further disintegrated. At the highest levels the wood particle was severely destroyed. The LCC network, as previously reported (Chapter 3) is widely disrupted with increasing GTP severity, leading to a loose pretreated wood cell wall structure that is structurally fragile.

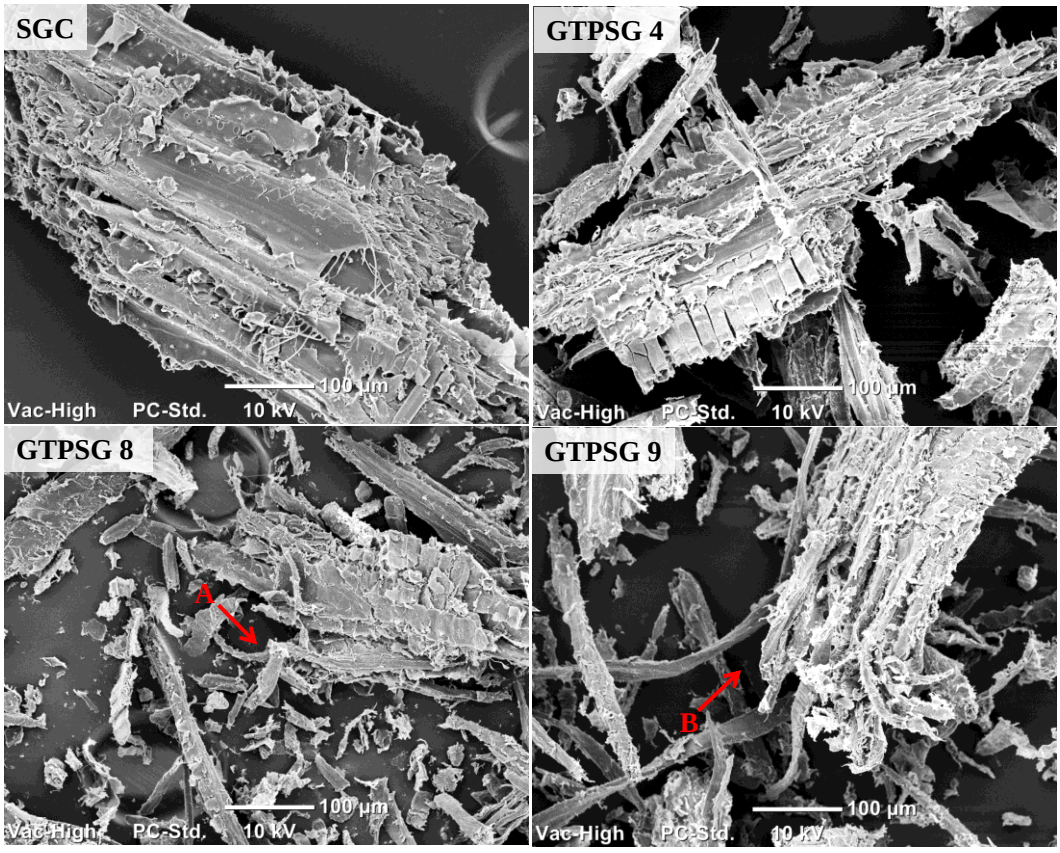

Figure 6-3 SEM images of non-pretreated and GTP pretreated SG at different severity levels

Additionally, the longitudinal cellulose fibers were observed to separate from wood bundles (Figure 6-3: see region A and B) especially for the pretreated wood at highest severity condition, due to the severe disintegration of wood surface structure. As a result, the porosity and surface area were increased, which was demonstrated by the Brunauer-Emmett-Teller (BET) surface area analysis (Chapter 3). Thus, the cellulose nanostructure becomes more exposed to enzyme

attack, rendering the both amorphous and crystalline material more susceptible in the biomass substrate.

6.5.4 Influence of water washing on biomass saccharification

Inhibitory by-products formed during pretreatment could potentially impede both the enzymatic hydrolysis and fermentation steps. Several types of inhibitors have been proposed as either toxic to organisms or which have the ability to competitively or non-competitively inhibit enzyme action (REF). These include furan derivatives associated with the degradation of pentoses or hexoses, phenolic compounds from degraded lignin, and organic acids such as acetic acid, formic acid and levulinic acid [65]. The concentration and type of inhibitors greatly depends on the pretreatment method, severity, as also on acid loading in processes which use, or generate acids. Several pretreatment methods have been demonstrated to produce these inhibitors. For instance, steam-explosion, hydrothermal and dilute acid pretreatment generates inhibitors, and water washing is needed to remove these compounds before the enzymatic [66]. Thus, an effective pretreatment for production of digestible biomass substrate should also avoid the formation of inhibitors to as great an extent as possible.

Enzymatic hydrolysis of GTPSG before and after water extraction was conducted to reveal any inhibitory effects of the GTP processing on the enzyme cocktail used. At a moderate GTP severity [GTPSG 4, $\log(R_0)$=4.14], the enzymatic hydrolysis profile of water extracted GTPSG closely tracks that of the unwashed GTP biomass during the 72 hours hydrolysis (Figure 6-4). At the most severe GTP processing conditions [GTSPSG 9, $\log(R_0)$=5.20], the final enzymatic digestibility of the unwashed GTPSG was comparable to that of the water extracted biomass at the the end of the 72 hours hydrolysis period.. Although the hydrolysis rate decreased in the unwashed GTPSG 9 sample after the first 4 hours, this lag recovered by the 72 hour period to be statistically the same as the water extracted sample. These results indicate no significant inhibitory compounds that would affect the enyzme coctail used are produced during GTP pretreatment, especially when using low and moderate GTP conditions. With increasing GTP pretreatment severity, the enzymatic hydrolysis rate decreased to a small extent but the final biomass digestibility was not influenced. Although GTP processing for digestability occurs at a similar, or even higher, severity than steam-exploded or hydrothermal pretreatments, glycerol as

additive does not behave in the same manner as autoionized water in steam pretreatment to catalyze the degradation of glycosidic bonds and acetyl groups in hemicellulose [67] resulting in degraded furan compounds. More than 80% of initial xylan was preserved after GTP processing (Chapter 3). Additionally, the amounts of glycerol left in the GTP pretreated biomass had no negative effect on the subsequent enzymatic hydrolysis. Thus, a water extraction (washing) step, which is widely conducted in steam pretreatment, is not necessary after GTP processing. The GTP pretreated biomass can be used directly for enzymatic hydrolysis, leading to more economic biorefinery processing.

No fermentation studies were conducted as part of this research, so it is unknown if any inhibitory compounds may have been generated that would reduce efficiency of organisms such as yeast or bacterial which could potentially utilize the sugars from the GTP treatment, or otherwise impact fermentation efficiencies in downstream processes.

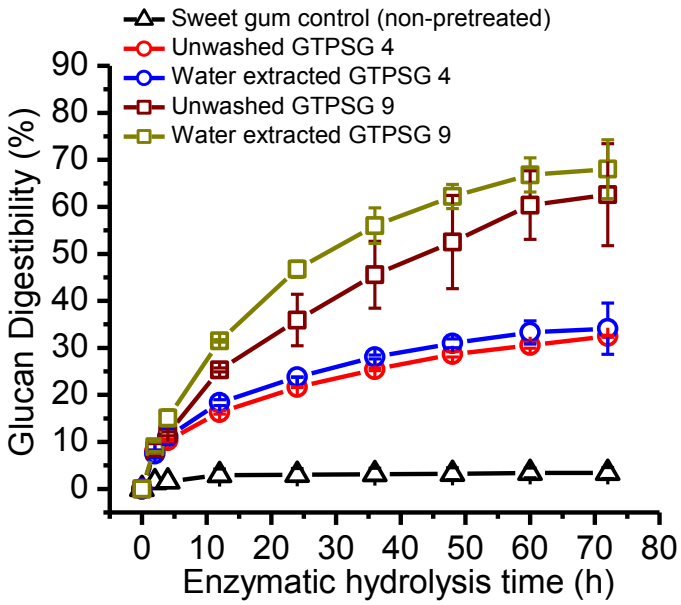

Figure 6-4 Enzymatic hydrolysis of non-pretreated and GTP pretreated SG before and after water extraction at different severity levels.

6.5.5 Effect of delignification on GTP biomass saccharification

Lignin is known to impede biomass enzymatic saccharification by interfering with cellulose enzyme binding [14]. Several studies [13, 15, 68] have demonstrated that delignification can

improve enzymatic saccharification. Althernately however, Rollin et al. [69] proposed that increased cellulose accessibility was a more important factor in enhancing saccharification than delignification. To help resolve this issue, in our research water extracted GTP biomass was solvent extracted to isolate lignin. The partially delignified, solvent-extracted GTP biomass was then studied for enzymatic saccharification. The relative content of Klason lignin and structural carbohydrate in the GTP biomass before (water extracted GTPSG) and after (solvent extracted GTPSG) extraction is shown in Table 6-2. With increasing GTP pretreatment severity, more lignin was removed during the mild solvent extraction, resulting in a decreased amount of lignin in the solvent extracted biomass residue compared to that in the water extracted GTP biomass. Additionally, no obvious hemicellulose loss was observed in this step (Chapter 3) and overall, xylan concentration increased in the pretreated biomass because of the loss of lignin.

As indicated in Figure 6-5, the hydrolysis profile of the solvent extracted GTP biomass completely overlaps with that of water extracted biomass at moderate GTP conditions [GTPSG 4, $\log(R_0)$=4.14]. The water extracted GTPSG 4 contained 23.61% of Klason lignin, whereas after solvent extraction, the lignin concentration decreased to 19.15% (Table 6.2). Although the relative percentage of lignin was reduced, the enzymatic digestibility was not further enhanced. But this may have in-part been due to the nature of the enyzme cocktail that was used as the CTec2 cocktail is more limited in crystalline cellulose binding enzymes, and after GTP pretreatment, the more crystalline elementary fibril core is what would primarily be accessible. With increasing GTP severity, the lack of effect of lignin removal on biomass digestibility is more obvious. The initial hydrolysis rate of the GTP biomass before and after solvent extraction at severe GTP processing conditions [$\log(R_0)$=5.03, 5.20] was similar. However, after 12 hours of hydrolysis, the sacchrification rate decreased for the solvent extracted GTP biomass, resulting in a lower ultimate digestibility compared to that of GTP biomass before delignification. As demonstrated previously (Chapter 3), over 60% delignification was obtained for high GTP severities after solvent extraction, leaving less than a half of the original lignin remaining in the solvent extracted biomass. Zhu et al. [13], suggested that delignification over 50% could potentially cause biomass pores to collapse, resulting in decreasing in enzyme accessibility to cellulose; however given current information about how our particularly enzyme cocktail would function with the highly crystalline cellulose, the nature of the residual cellulose in addition to the loss of lignin, and the chemical structural changes of the residual lignin in the solvent

extracted biomass also are likely reasons for this reduced hydrolysis rate. If the residual lignin bears surface chemistry favored by protein adsorption, or sterically hinders the attachment of cellulase enzymes or CMBs [69], enzymatic saccharification could also be impeded even after delignification. The results demonstrate that delignification is not a major barrier in improving biomass saccharification, although providing greater access to the cellulose substrate by removal of lignin enhances accessibility.

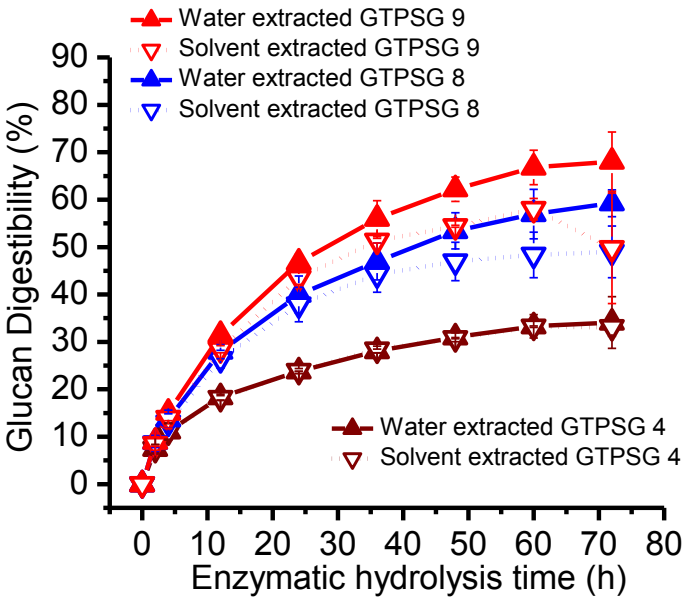

Figure 6-5 Enzymatic hydrolysis of water and solvent extracted GTP biomass at different severity levels.

6.5.6 Effect of alkaline extraction on GTP biomass saccharification

Glucuronoxylan is the major hemicellulose in hardwood, which normally arranges along the cellulose microfibrils [70] forming an abundance of hydrogen-bonds [71]. In addition to lignin, xylan is viewed as a major barrier blocking cellulase enzyme accessibility. Recent studies have demonstrated the removal of hemicellulose can enhance enzymatic hydrolysis by cellulases [15, 68, 72]. To explore this, a gentle alkali extraction was performed to isolate xylan polymer from the GTP pretreated biomass (Chapter 5). At the highest GTP severity condition ($\log(R_0)$=5.20), 56% of the initial xylan was removed during the alkali extraction. The residual alkali-extracted biomass, with 21% of initial xylan, was enzymatic hydrolyzed and results are compared with the digestibility of biomass before alkaline extraction (Figure 6-6).

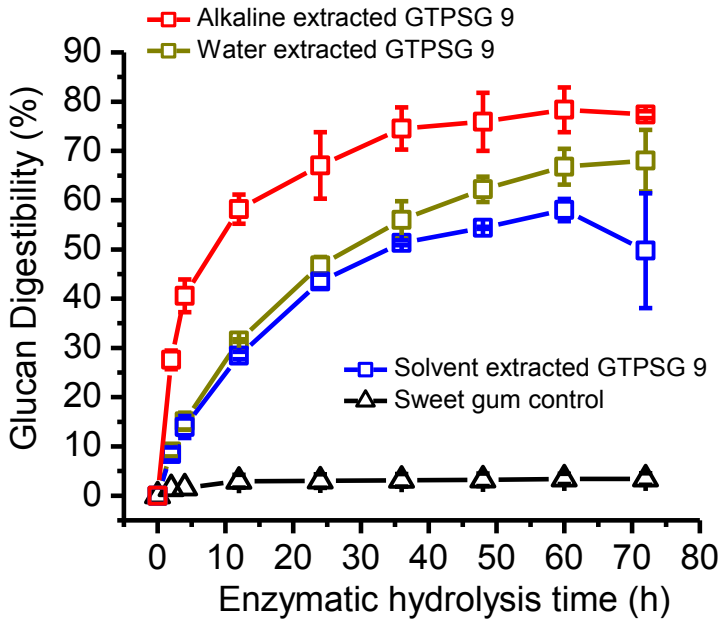

Figure 6-6 Enzymatic hydrolysis of water, solvent and alkaline extracted GTP biomass at the highest severity level.

Interestingly, a significant increase in the initial hydrolysis rate was observed for the alkali extracted GTP biomass compared to that for the water and solvent extracted GTP biomass. After an initial 2 hour hydrolysis, the digestibility was 28% for the alkali extracted biomass whereas was 9% and 8% for the water and solvent extracted biomass. Using the target cellulose to glucose conversion of 60% for comparison, for the alkaline extracted biomass this point was reached in 12 hours, but for the water extracted GTP biomass it took 48 hours. Thus, the extensive removal of xylan during the alkaline extraction process converts the biomass substrate to one which is more susceptible to cellulase enzyme attack, which greatly increases the hydrolysis rate. Ultimately, 78% of cellulose digestibility was observed after 72 hours of hydrolysis. Compared to the 68% digestibility of water extracted biomass, this number is not that impressive since biomass digestibility was already enhanced simply by GTP pretreatment. However, a significant increase in hydrolysis rate could be both a time-saving, and an economic benefit, if a biofuel industry were further developed to include xylan isolated as polymeric byproduct.

6.6 Conclusions

Enzymatic saccharification of GTP pretreated biomass was examined in this research. At the most severe GTP condition, a 20-fold increase in digestibility was observed although the biomass contained significant amounts of lignin and hemicellulose. Taken with previous results, the extensive cleavage and removal of lignin-carbohydrate linkages during GTP processing accompanied by an increase in surface area is responsible for the more susceptible biomass substrate. The GTP biomass, before and after water extraction (washing), exhibited similar digestibility, indicating no significant inhibitory compounds to the enzymes in the CTec2 enzyme cocktail used were produced during GTP processing despite this treatment being conducted at similar or higher severities than previous steam-pretreatment research. This suggests that water washing is not an essential step for enzymatic hydrolysis of biomass after GTP pretreatment. Delignification resulted in a small negative effect on biomass digestibility rather than enhancement, suggesting that residual lignin after GTP processing is not a barrier for the CTec2 enzyme cocktail action on cellulose. Further alkaline extraction extensively removes xylan, resulting in greatly increased saccharification rates.

6.7 Acknowledgments

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Chapter 7 Summary and conclusions

Glycerol thermal processing (GTP) is studied as a novel biomass pretreatment method in this research. This approach is used to facilitate biopolymer fractionation and biomass saccharification by treating sweet gum particles on polymer processing equipment at high temperatures in the presence of anhydrous glycerol. Nine severity conditions are studied to assess the impact of time and temperature during the processing.

After processing, the biomass is stripped of its lignin and xylan by subsequent extractions without additional catalysts, leaving a relatively pure cellulose fraction, 84% glucan, as found in chemical pulps. Additionally, 41% of the lignin and 68% of the xylan is recovered in a dry powdered form. The hemicellulose side-chain carbohydrates such as arabinose and galactose are water extracted at increasing GTP severities. Detailed mass balance of wood components indicates the GTP processing at high temperatures and presence of plasticizer for short times provides significant disruption of the cell wall by removing the lignin-carbohydrate linkages, while glycerol prevents significant degradation of the biomass.

As polymeric lignin is recovered as byproducts from the pretreated biomass through a mild dioxane extraction, the quantitative structural analysis is performed to understand how the GTP processing impacts lignin structure, as well as find the unique structure properties for potential applications. Quantitative ^{31}P-NMR is a major tool to reveal the various hydroxyl groups in lignin, which indicates the syringyl phenolic functionality increases linearly with the log of GTP severity parameter due to the alkyl aryl ether bond breakage. Further structure analysis through thioacidolysis and 2D-HSQC of the isolated lignin confirm the extensive breakage of **β**-aryl ether bonds during the GTP processing. Based on the structural analysis, a mechanism is proposed that the glycerol facilitates the C-$\boldsymbol{\gamma}$ elimination from lignin, followed by the extensive $\boldsymbol{\beta}$-O-4 bond decomposition and phenolic hydroxyl liberation. At the same time, inevitable condensation occurs to afford the GTP lignin with relatively high molecular weight near to that of the enzymatic mild acidolysis lignin. Thermal analysis shows the GTP lignin is more thermally stable compared to nearly all other lignins found in the literature, with a glass transition temperature that was invariant to the processing severity, which possesses potential application in polymer applications.

In addition to lignin, xylan as the major hemicellulose in hardwood species is preserved with 80% of initial after GTP processing. Followed by the GTP processing and partial delignification through the mild solvent extraction, alkaline extraction using 1 M NaOH is conducted to isolate xylan from pretreated biomass. The majority of xylan preserved in GTP biomass is extracted successfully in the format of water insoluble xylan (WIX), which has degree of polymerization comparable to that from non-pretreated fibers. In comparison, xylan recovered in water soluble form (WSX) has much lower degree of polymerization and more substitutions that render better water solubility. Additionally, WIX recovery increases compared to WSX at increasing GTP severity, with a maximum xylan purity of 84%. Pure xylan mass distribution and FT-IR analysis confirm the high xylan purity of WIX with less glucuronic acid on the branches. In contrast, less xylan but more lignin contamination is observed in WSX portion at severe GTP conditions. The comparable molecular weights of isolated xylan in WIX from GTP pretreated biomass confirm no degradation of xylan polymer occurs during this high temperature processing. Although the GTP processing occurs at comparable severities as autohydrolysis such as steam explosion, the resulted disintegration of cell wall network is not induced by the released acids that are common in steam pretreatments. The isolated xylan polymer with DP in the range of 100-150 and narrow molecular weight distribution of a polydispersity index of 1.1 to 1.3, indicates its potential applications in the degradable polymer area.

Besides the successful biopolymer fractionation from the pretreated biomass, enzymatic saccharification is studied for the GTP pretreated fibers for their applications in economic and applicable biorefinery industry. Compared to the non-pretreated sweet gum biomass, the GTP pretreatment significantly enhances the ultimate biomass digestibility with a 20-fold increment, although the biomass exhibit larger crystallinity and comparable amounts of lignin and hemicellulose. These results further indicate the residual lignin and hemicellulose do not restrict cellulose enzyme attack any more after GTP pretreatment. Saccharification of GTP biomass before and after water washing reveals no significant inhibitory compounds are produced even at such high GTP severities. Additionally further delignification of GTP biomass exhibits no positive effects on saccharifcation. In comparison, further hemicellulose removal greatly improves the initial hydrolysis rate, resulting in 78% of ultimate biomass digestibility. Overall, this GTP pretreatment provides a novel method for efficient biomass saccharification. Disrupted

biomass cell wall with formation of biomass debris and release of cellulose fibrils is more important for enhanced biomass saccharification.

The series studies of this research fully demonstrate glycerol thermal processing as a novel pretreatment to enhance the biomass saccharification for biofuel productions, as well as facilitate the biopolymer fractionation through simple post-extraction. The resulted biopolymers with unique structure properties have potential applications in polymer industry.

Appendix A Qualification of lignin from glycerol thermal processing of biomass for polymeric applications

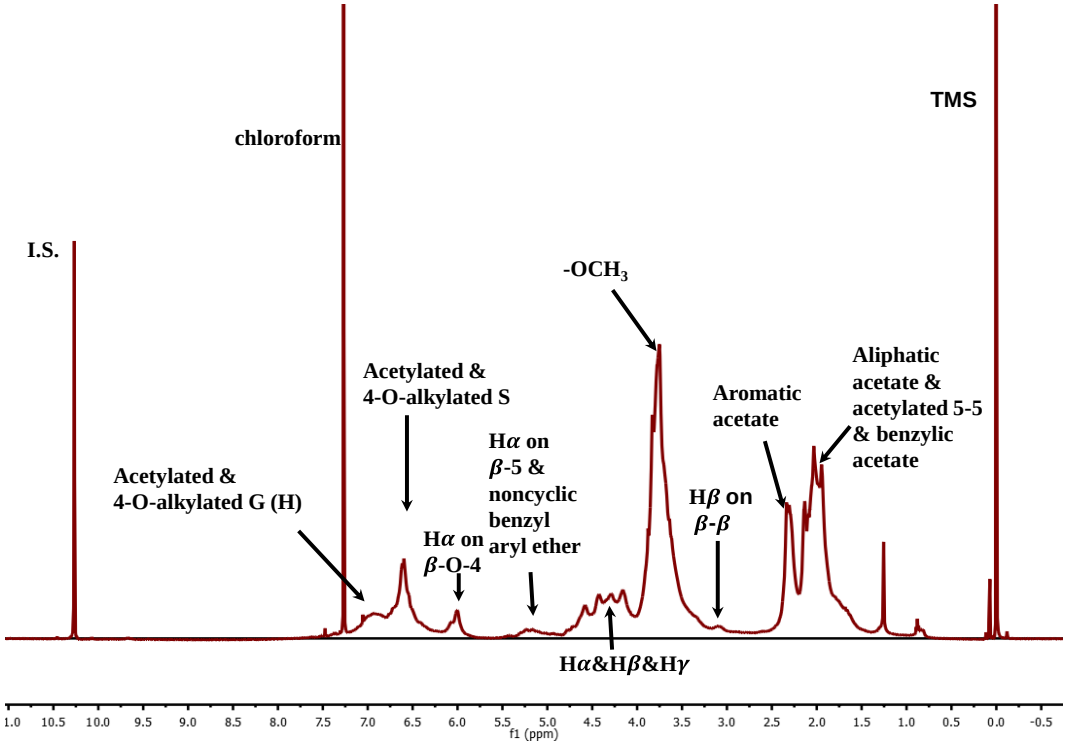

Figure A-1 ^{1}H-NMR spectra of recovered polymeric lignin with peak assignments.

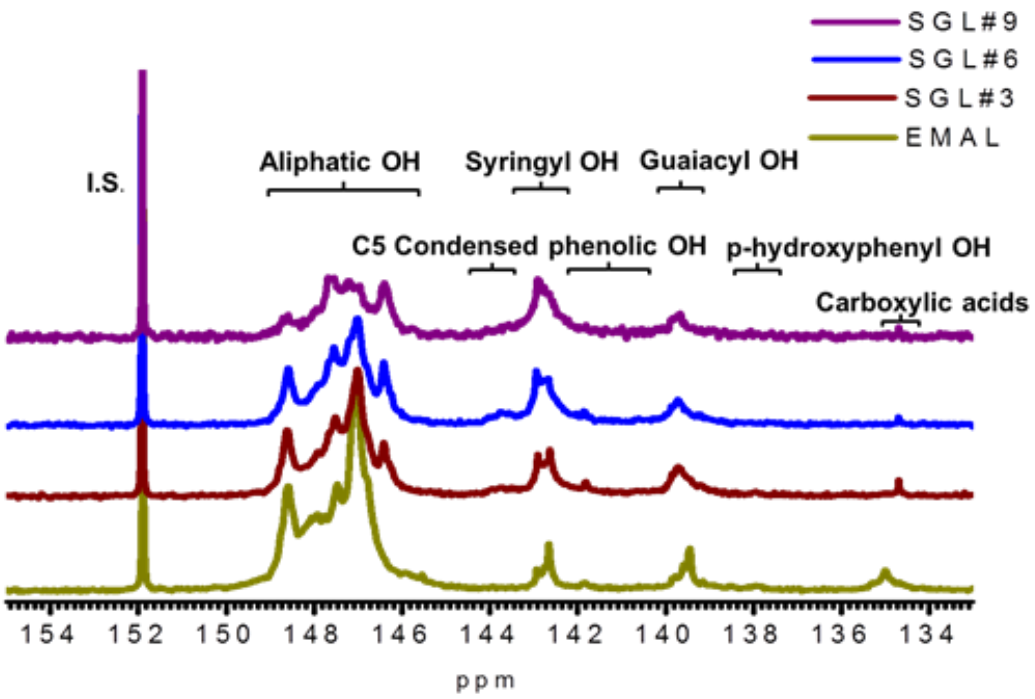

Figure A-2 ^{31}P-NMR spectra of recovered polymeric lignin with peak assignments.

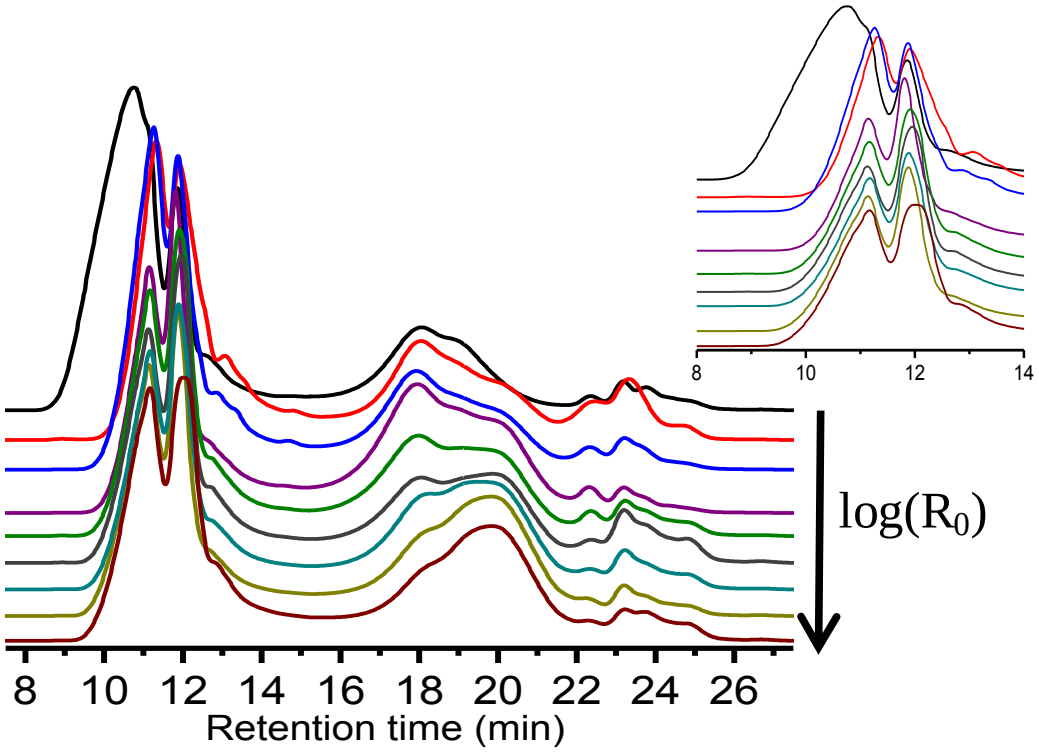

Figure A-3 SEC elution chromatograms for GTP lignin as a function of GTP severity (Black curve refers to EMAL). UV detector at 290 nm.

Table A-1 C9 formula of isolated lignin.

log(R_0)	Molecular formula of C9	MW of C9(g/mol)
EMAL	$C_9H_{7.63}O_{3.03}(OCH_3)_{1.87}$	222.1
4.14	$C_9H_{7.04}O_{2.40}(OCH_3)_{1.85}$	210.7
4.44	$C_9H_{6.79}O_{2.41}(OCH_3)_{1.85}$	210.7
4.61	$C_9H_{6.66}O_{2.27}(OCH_3)_{1.82}$	207.6
4.72	$C_9H_{6.51}O_{2.30}(OCH_3)_{1.76}$	205.8
5.03	$C_9H_{6.48}O_{2.15}(OCH_3)_{1.74}$	203.0
5.2	$C_9H_{6.51}O_{2.11}(OCH_3)_{1.74}$	202.2

*molecular weights were calculated by the average of duplicates run in elemental analysis with COV<0.2% and results from ^{1}H-NMR. Protein content for GTP isolated lignin <2%, and protein content is 5.7% for EMAL, ignored.

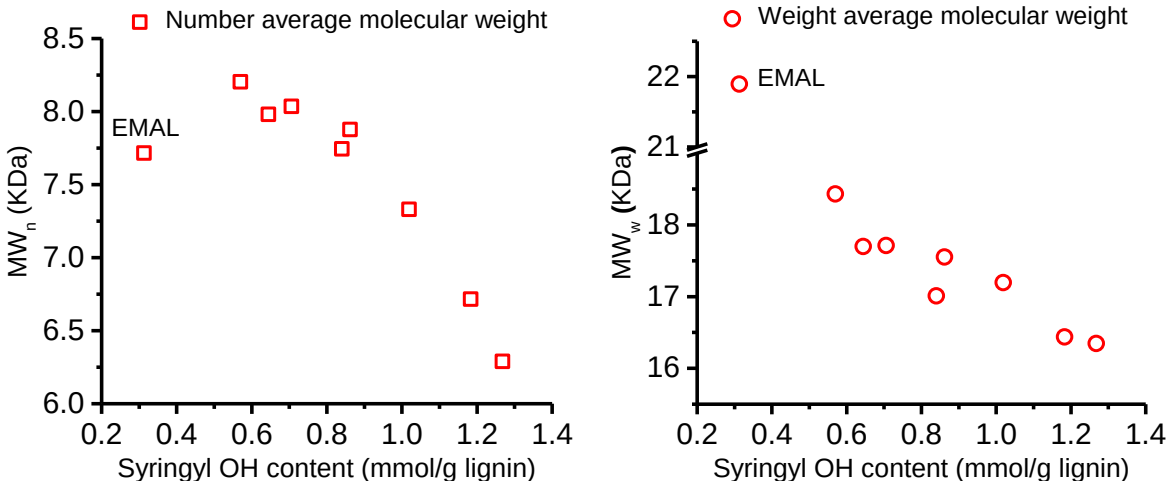

Figure A-4 Correlation between relative molecular weight and amounts of S-OH of lignin.

Appendix B Polymeric xylan fractionation and structural characterization from glycerol thermal pretreated biomass

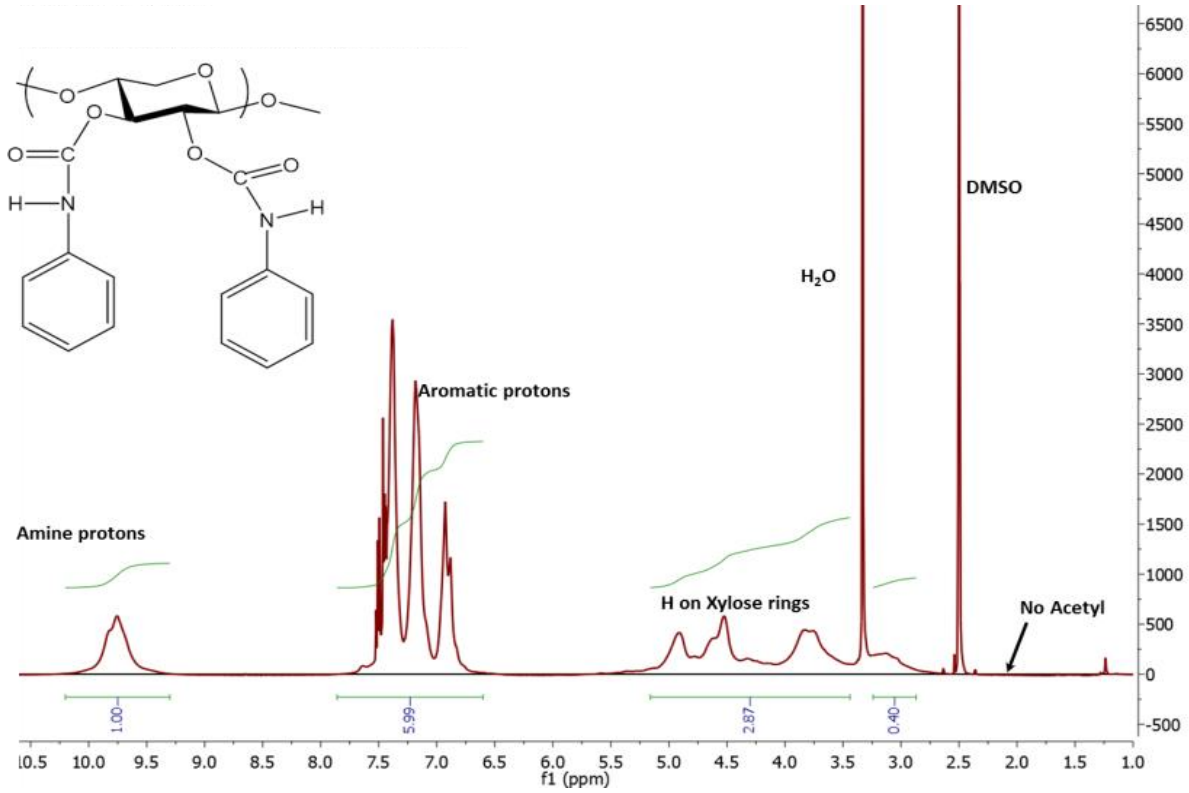

Figure B-1 Typical ^{1}H-NMR of xylan carbanilates indicating no acetyl protons at 2.2 ppm.

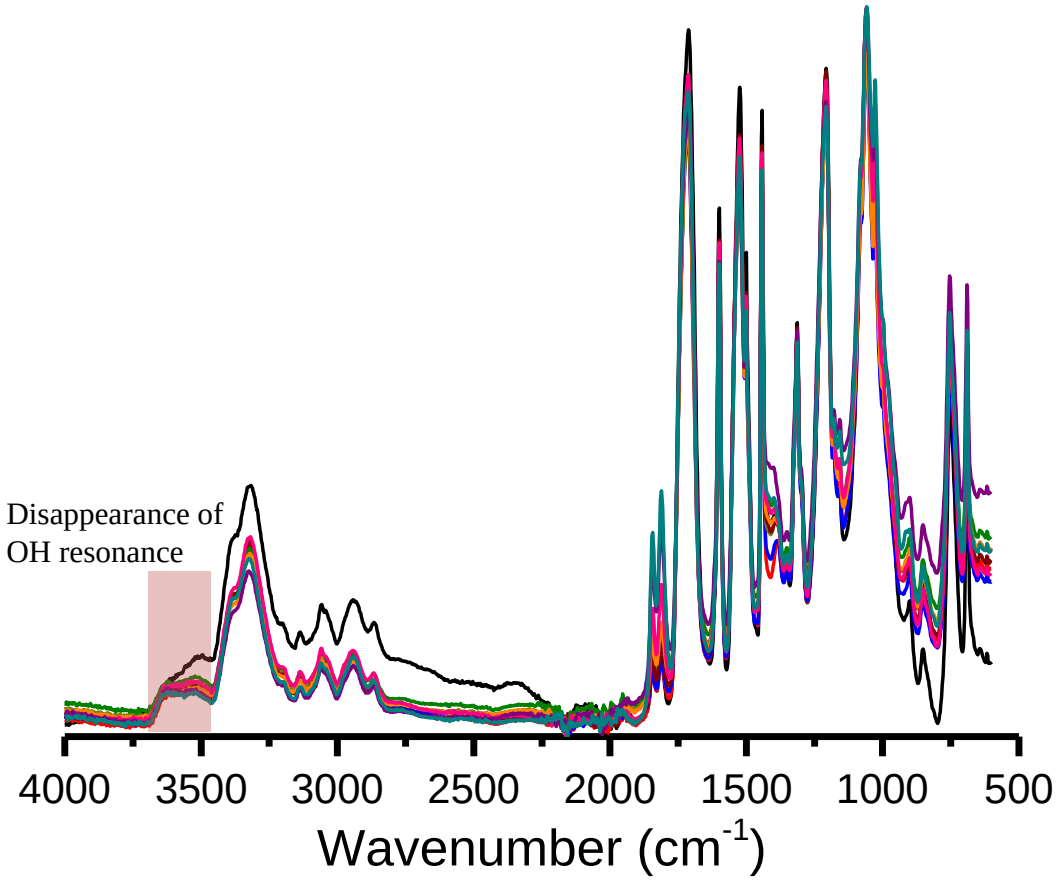

Figure B-2 FT-IR spectra of xylan carbanilates indicating fully conversion of hydroxyl groups.