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Wet explosion pretreatment of loblolly pine leads to an increase in methoxylation of the lignin

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Abstract

Background: In biorefineries, various pretreatments traditionally employ hazardous chemicals (ammonia, sulfuric acid, sulfite, etc.) for opening the softwood structure and to facilitate easy accessibility of the cellulose for further downstream processing. The resultant lignin (known as technical lignin) after extraction of the carbohydrate fraction as sugars has been either burned as fuel or used in biochemical or biofuel production. It has been observed that the technical lignin after such biomass pretreatments is often more condensed and, hence, cannot be easily used to produce fine chemicals of high value. In this study, we examine lignin after wet explosion pretreatment where the biomass in subjected to oxygen to understand how these interactions will affect lignin utilization for biochemical production.

Results: In this study, the structural transformations within the softwood lignin as a response to wet explosion (WEx) pretreatment of loblolly pine at different experimental conditions (165–175 °C, 18–30 min) were examined using GC/MS and NMR spectroscopy. The results showed that the H-type structures within the lignin molecule decreased while S-type structures increased after pretreatment. Since S-type lignin sub-units have a higher degree of methoxylation compared to H units, the potential of S-type lignin to undergo re-condensation at lower temperatures (after pretreatment), by forming bonds with other lignin sub-units, is lower due to stearic hindrance, resulting in the generation of lignin with a lower tendency to form new complex lignin bindings (high-quality biorefinery lignin).

Conclusions: The less condensed biorefinery lignin generated after WEx pretreatment was found to provide a platform for production of lignin polymer with more labile β-O-4 linkages. This type of lignin could potentially be superior for the production of high-value bio-products compared to re-condensed lignin after acidic and other types of chemical pretreatments.

Keywords: Biorefinery; Lignin; Loblolly pine; NMR; Wet explosion

Background

Analogous to petroleum refineries, the biorefineries embrace the notion of mass production of chemicals, fuels, and other products from renewable biomass sources. With the current cost and supply imbalances existing in the global energy market, renewable energy from various sources is needed to supplement or, in the long term, substitute fossil fuels to satisfy the growing energy demand in the world. Compared to fossil fuels, energy production from renewable sources will further lead to less emission of greenhouse gases (GHG) particularly CO_x and SO_x leading to global warming [1]. Lignocellulosic biomass such as agricultural and forestry residue and energy crops have been considered as a potential source of fuels and chemicals since the latter part of the twentieth century. Woody biomass especially softwood can be considered as a good candidate for generating renewable energy due to their high-carbon content in the form of sugars and lignin which can be effectively converted to energy intermediates [2].

Woody biomass usually consists of about 35–45 % cellulose enclosed within sheets containing 20–25 % hemicellulose and around 15–30 % lignin bound together through carbon-carbon linkages [3]. The lignocellulosic biomass, unlike food crops that were used for first-generation biofuels, is comparatively recalcitrant in nature due to its higher lignin content and, hence, requires thermochemical pretreatment methods before the polymers are ready to release cellulosic sugars by enzyme

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addition. In biorefinery, these cellulosic sugars will then be converted by microbes to produce platform molecules such as ethanol, butanol, or organic acids that can be used either as fuel additives or sold as specialty chemicals [4–6]. Studies have indicated that lignin possesses 1.7-fold of the energy equivalent to the energy of 1 kg of cellulose and has a higher energy density [7]. Lignin component has, therefore, been traditionally burned to provide energy. Lignin is, however, a poly-aromatic non-sugar component typically found in tissues and cell walls of vascular plants and is formed through radical coupling polymerization of p-hydroxycinnamyl, coniferyl, and sinapyl alcohol units [8].

Generally, the native lignin present in loblolly pine primarily consists of guaiacyl (G) 86–87 % and *p*-hydroxyphenyl (H) 12–13 % and a non-detectable amount of syringyl (S) monomeric units [9]. In woody biomass, lignin and carbohydrates are attached to each other by benzyl ether, benzyl ester, glycosidic-, and acetal-type bonds as revealed by wet chemistry and spectroscopy methods [10]. Apart from these ether linkages, the phenyl propane units present in the lignin are also connected forming a three-dimensional net structure that leads to biomass recalcitrance during pretreatment [11]. Hence, various pretreatments have been tested with the aim of achieving higher sugar yields including dilute acid, ammonia fiber explosion, sulfite pretreatment, etc. [12, 13]. These pretreatments, however, involve the use of chemicals that serve unique purposes during pretreatment, e.g., sulfuric acid in dilute acid pretreatment solubilizes most of the xylose [14], alkali pretreatment mainly removes lignin to improve the reactivity of remaining carbohydrates, whereas during ammonia fiber explosion pretreatment, ammonolysis of glucuronic cross-linkages is believed to be responsible for rendering the carbohydrates more accessible [15].

Studies have also indicated that such pretreatments will further affect the lignin matrix. Dilute acid pretreatment combined with steam explosion pretreatment (conducted at low pH) was shown to produce a lignin defined as pseudo-lignin (via acid-catalyzed dehydration of carbohydrates) that was deposited on the cell wall, where it reduced the cellulase activity [16]. During organosolv pretreatment, Koo et al. [17] reported that lignin droplets were formed on the surface of plant cell wall as a result of re-condensation of lignin. The study indicated that this further inhibited the action of enzymes used for sugar production through non-productive bindings with the lignin. These aforementioned studies indicated that the action of the various chemicals added during pretreatment can lead to a depolymerization of the lignin structure followed by re-distribution and re-polymerization into a much more condensed form after cooling which ends as a deposit on the outer cell wall. Such changes to the lignin fraction make it difficult to activate and convert into high-value compounds such as vanillin, syringaldehyde, and other similar products [18]. It is, hence, of interest to examine if less non-invasive pretreatment methods could provide a lignin stream which could be more suitable for further processing into fuels and chemicals.

Wet explosion (WEx) pretreatment is a combination of wet oxidation and steam explosion in which oxygen acts as an oxidizing agent in the presence of water which acts as a solvent [19, 20]. In this process, the biomass is mixed with water in a reactor and heated to set temperature with further addition of oxygen. After a set residence time, the biomass is flashed into another tank causing the disruption of fibers as a result of the sudden decompression. Developed in 2004, WEx pretreatment has been successfully used over a number of years as an effective pretreatment of lignocellulosic biomass such as sugarcane bagasse [21], wheat straw [22], and loblolly pine [23] and has been optimized for maximum sugar yields for biofuel production. These studies indicated that the WEx pretreatment was capable of producing high-sugar yields through effective degradation of hemicellulose-lignin linkages, thereby allowing easy accessibility of the cellulosic components for enzymatic action [23, 24].

In the current study, we investigate the structure of the "biorefinery" lignin (left behind after optimized pretreatment for sugar production) at the molecular level using 1D and 2D nuclear magnetic resonance (NMR) techniques. A number of studies [3, 22–25] have indicated that NMR spectroscopy is a powerful technique capable of giving compositional and structural information of biomass lignin. We used loblolly pine, the most dominant softwood indigenous to the Southern United States and constituting over one half of the standing pine volume [26], as biomass substrate for the study. The oxidation products from lignin will further be characterized using gas chromatography-mass spectrometry (GC-MS) at the optimized conditions used for sugar production from loblolly pine [16]. The primary objective of the study is to understand the structure of the biorefinery lignin with the aim of producing valuable products.

Methods

Raw material and chemicals

Loblolly pine chips were obtained from Iowa State University, Ames, IA. The chips were milled to 2-mm particle size with a Retsch cutting mill SM 200 (Retsch Inc., Newtown, PA, USA) and kept in buckets at room temperature prior to the pretreatment. Reagent grade methanol, dichloromethane, hexane, 1, 4-dioxane, acetic acid, ether, and diethyl ether were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cellulase (Cellic® Ctec2) and hemicellulase (Cellic® Htec2) were obtained from Novozymes Inc., Franklinton, NC, USA. Safety considerations and precautionary handling procedures for the chemicals were followed as per the manufacturer's provided material safety datasheets (MSDS).

WEx pretreatment

Loblolly pine chips were pretreated using WEx pretreatment in a 10-l reactor. The WEx pretreatment was performed at three different process conditions of temperature and time (175 °C, 24 min; 165 °C, 18 min; 165 °C, 30 min) adding oxygen at 5.5 bar to the headspace. The wet explosion pretreatment was done as discussed previously [16]. In brief, 1.5 kg of loblolly pine was mixed with water to make 25 % total solids and transferred to a pretreatment reactor. Oxygen was introduced in the reactor, and the reactor was then heated to the desired temperature using a hot oil heater (Chromalox, Pittsburgh, PA, USA) with a heating time of 15 min and constant stirring. At the end of the reaction time, the pretreated biomass was flashed into a 100-l flash tank.

Enzymatic hydrolysis of WEx-pretreated slurry

The enzymatic hydrolysis was performed in a 150-ml Erlenmeyer shake flask. Pretreated slurry (100 g) was adjusted with 4 M KOH to pH 5, and an appropriate amount of sodium citrate buffer was added to make 20 % total solids. The sodium citrate buffer was added to prevent the lowering of pH as a result of hydrolysis of acetyl groups that produces acetic acid [28]. The WEx-pretreated slurry was incubated for 90 h in a shaking incubator (Model IS-971; Lab Companion, Billerica, MA, USA) with digital PID microprocessor controller to maintain the temperature at 50 °C and the shaking speed at 150 rpm. The pretreated slurry was enzymatically hydrolyzed using a cocktail of cellulase and hemicellulase enzymes. Cellic® Ctec2 was added in a concentration of 60 mg enzyme protein (EP) per gram of cellulose and Cellic® Htec2 was added in a dose of 6 mg EP per gram of cellulose in the WEx-pretreated slurry.

GC-MS

The lignin-derived phenolic compounds present in the WEx-treated loblolly pine slurry were isolated using methanol as solvent. The 1-μl sample with 0.6 ml/min of He (carrier gas) was injected onto a DB-5 (30-m length × 250-μm I.D. × 0.25-μm film thickness; J&W Scientific, Folsom, CA, USA) capillary column fitted in a GC (Model: 7890A; Agilent Technologies 7890A, Agilent Technologies, Santa Clara, CA, USA) system set in a splitless mode. The GC oven was programmed such that the temperature was held at 110 °C for 2 min, was raised at the rate of 10 °C per min until the temperature was 200 °C, then held at this temperature for 1 min, and finally the temperature was raised at the rate of 5 °C until the temperature reached 280 °C and held at the final temperature for 9 min giving a total run time of just over 35 min. Eluting compounds were detected with a MS (Model: 5975C; Agilent Technologies 5975C, Agilent Technologies, Santa Clara, CA, USA) inert XL EI/CI MSD with triple axis detector and by comparison with the NIST libraries.

Lignin isolation

Loblolly pine (raw:18.4 g, WEx-pretreated: 21.25 g, and enzymatically hydrolyzed slurry: 28 g on a dry basis) was sequentially extracted with water at room temperature for 24 h, followed by methanol at room temperature for 10 h, followed by dichloromethane at room temperature for 10 h, and followed by hexane at room temperature for 10 h [42]. WEx pretreatment at 175 °C for 24 min and 5.5 bar oxygen pressure was used for this study since it showed higher lignin solubilization. The extractive-free dried cell wall residue (CWR) (raw: 15.53 g, WEx-pretreated: 15.54 g, enzymatically hydrolyzed: 15.15 g) was then ball milled (3 lbs capacity, www.pyrocreations.com) for 8 h to completely disrupt the cell wall, termed as ball milled lignin (BML). The lignin purification of the extracted BML was performed twice with 1,4-dioxane:water (9:1, 30 ml/g of biomass) for 24 h with continuous stirring at room temperature and centrifuged at 4500 rpm, and the recovered supernatant was then freeze dried. The lignin recovery of the 1,4-dioxane-extracted BML was performed using acetic acid:water (9:1). After vortex and sonication for 30 min, the supernatant was poured slowly into diethyl ether and kept at 4 °C overnight to completely precipitate the lignin isolates. The precipitated lignin isolates were centrifuged and freeze dried. Approximately 2.9 % raw, 3.0 % WEx, and 3.8 % of CWR lignin were precipitated and were used for the NMR spectroscopy as described in the next section. NMR spectra were recorded in DMSO-d_6 at 300K on a Varian NMR System 600-MHz spectrometer (Agilent Technologies, Santa Clara, CA, USA) operating at 599.69 MHz for ^{1}H and 150.8 MHz for ^{13}C.

NMR analysis

The residual solvent signal at 2.49 ppm for proton and 39.5 ppm for carbon was used for internal referencing of chemical shifts. Samples were prepared as solutions of 60 to 68 mg/0.6 ml in DMSO-d6 (Cambridge Isotope Labs, Woburn, MA, USA). Carbon spectra were acquired with a sweep width of 34,722 Hz using an acquisition time of 0.4 s and a relaxation delay of 1.6 s. A 30° pulse was used and broadband ^{1}H decoupling was used only during the acquisition time. A total of 30,000 scans were recorded for each spectrum, and the FID was apodized with 12 Hz of exponential line broadening prior to zero filling to 65K points and Fourier transformation. ^{1}H NMR spectra was recorded at the same concentration of lignin without using a sweep width of 9600 Hz. An acquisition time of 2.0 s, a relaxation delay of 3.5 s, and a 45° pulse were used to collect 64 scans for each spectrum. The free induction

decay (FID) was apodized with 0.5 Hz of exponential line broadening prior to zero filling to 65K points and Fourier Transformation. Heteronuclear singlequantum correlation spectroscopy (HSQC) spectra were acquired using pulsed field gradient coherence selection and using spectral editing to allow for discrimination of methyl and methane signals from those of methylene signals. Spectral widths of 6250 and 24,125 Hz were used for the ^{1}H and ^{13}C dimensions, respectively. An acquisition time of 0.199 s was used for the direct observe dimension, and an acquisition time of 0.0066 s was used for the indirect dimension, and 48 scans were taken per increment. A one-bond ^{1}H-^{13}C J coupling of 150 Hz was used, and a total of 2 × 160 increments in t1 were acquired using the gradient echo-antiecho selection technique for pure phase lineshape in F1. The FIDs were zero-filled once to 2048 points in t2, apodized with a Gaussian function prior to Fourier transformation. Data in t1 were extended by a factor of 2 with linear prediction followed by zero filling to 2K points, apodizing with a Gaussian function and Fourier transformation.

Heteronuclear multiple-bond correlation spectroscopy (HMBC) spectra were acquired using pulsed field gradient coherence selection and a 10-Hz long-range ^{1}H-^{13}C coupling constant. Spectral widths of 9615 and 28,653 Hz were used for the ^{1}H-^{13}C dimensions, respectively. An acquisition time of 0.128 s was used for the direct observe dimension, and an acquisition time of 0.0112 s was used for the indirect dimension, and 160 scans were taken per increment. A one-bond ^{1}H-^{13}C J coupling of 140 Hz was used for the one-bond J filter, and 320 increments in t1 were collected. The FIDs were zero-filled once to 2048 points in t2, apodized with a pseudo-echo function prior to Fourier transformation. Data in t1 were extended by a factor of 2 with linear prediction followed by zero filling to 2K points, apodizing with a sine-bell function and Fourier transformation. Data were presented in an absolute value mode in both dimensions.

Results and discussion

Compositional analysis of raw material

The raw composition of loblolly pine [16] was done to estimate the amount of each biomass constituent in loblolly pine and is shown in Table 1. It can be seen that lignin constitutes close to 30 % of the total biomass composition making it the second most abundant compound in loblolly pine.

Table 1 Compositional analysis of raw loblolly pine [16]

Components	% DM	Standard deviation
Glucan	35.97	0.46
Xylan	7.54	0.61
Galactan	2.47	0.40
Arabinan	1.57	0.16
Mannan	8.15	0.23
Lignin	30.65	0.78
Ash	0.77	0.05
Extractives	6.45	0.08

Structural characterization of raw and WEx-pretreated loblolly pine lignin

One-dimensional ^{1}H nuclear magnetic resonance

The one-dimensional ^{1}H NMR spectra from raw and WEx-treated loblolly pine lignin isolates (Fig. 1) show peaks from oxygenated aliphatic lignin side chain region from $\delta_H \sim 4$ & 5.5 ppm, aromatic resonances indicative of lignin polymeric composition from $\delta_H \sim 6.5$ & 7.5 ppm, phenolic hydroxyls from 8 & 9 ppm, and a small shoulder from carboxylic hydroxyls at $\delta_H \sim 12$ ppm.

A difference in the relative intensities at the aromatic region between the WEx-treated lignin and raw biomass lignin can be seen in Fig. 1. In this aromatic region of the ^{1}H spectra for WEx-treated lignin, chemical shift values pertinent to S-derived unit were detected as demonstrated by the existence of the resonance intensity of quaternary $S_{2/6}$ aromatic ring proton ($\delta_H \sim 6.60$ ppm). In contrast, the raw lignin isolates predominantly contained G-derived aromatic ring protons chemical shift values, typical for softwood lignin [27]. This is a clear indication that the WEx-treated lignin varies from the raw lignin in terms of monomeric composition (H, G, and/or S unit) due to lignin deconstruction and/or depolymerization after pretreatment.

One-dimensional ^{13}C nuclear magnetic resonance

The one-dimensional ^{13}C NMR spectra for the raw and WEx-treated loblolly pine lignin are shown in Fig. 2. Previous studies have been done for comparing the effect of various pretreatment methods on the loblolly pine lignin structure and are the basis for the discussions on chemical shifts for the ^{13}C NMR spectra used in this study [28–30]. The ^{13}C NMR spectra of raw biomass showed a typical softwood lignin comprising of G lignin, i.e., it largely comprised the expected prevailing G aromatic ring resonances together with characteristic methoxy (–OMe) group signals ($\delta_C \sim 55.8$ ppm). The raw lignin ^{13}C spectra also showed peaks for lignin side chain from $\delta_C \sim 60$ & 95 ppm, for $G_{2/5/6}$ from $\delta_C \sim 107$ & 124 ppm, for $H_{2/6}$ at $\delta_C \sim 130$ ppm, for G_1 from $\delta_C \sim 130$ & 140 ppm, for $G_{3/4}$ from $\delta_C \sim 150$ & 160 ppm, and for acetyl groups from $\delta_C \sim 160$ & 190 ppm.

The one-dimensional ^{13}C-NMR spectrum of the WEx-pretreated lignin isolates (Fig. 2) was comparable to that of raw loblolly pine lignin in terms of overall oxygenated aliphatic lignin carbon resonances, indicating the presence

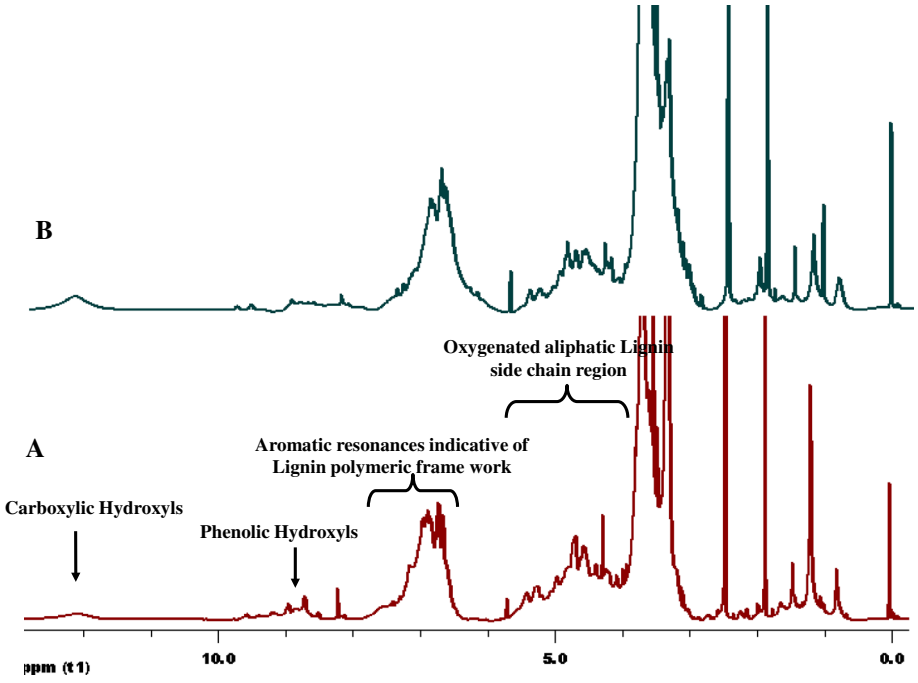


Fig. 1 One-dimensional ^{1}H NMR spectroscopic analysis of loblolly pine lignin. **a** ^{1}H NMR spectra of native loblolly pine MWL lignin; **b** ^{1}H NMR spectra of WEx-pretreated loblolly pine lignin

of the similar lignin inter-unit linkages. However, apart from those found in the spectra for the raw lignin, the ^{13}C NMR spectra of the WEx-treated lignin show new structures being formed at $\delta_C \sim 110$ ppm and $\delta_C \sim 160$ ppm and disappearance of $H_{2/6}$ signal at ~130 ppm. These signal intensities between 106 and 114 ppm and around 160 ppm usually relate to the C2, aromatic C-H bond, and the conjugated carboxyl linkages, respectively [29]. These signal intensities for the tertiary carbon 2 and 6 at δ_C 105–110 ppm are a strong indication of S-like structure within

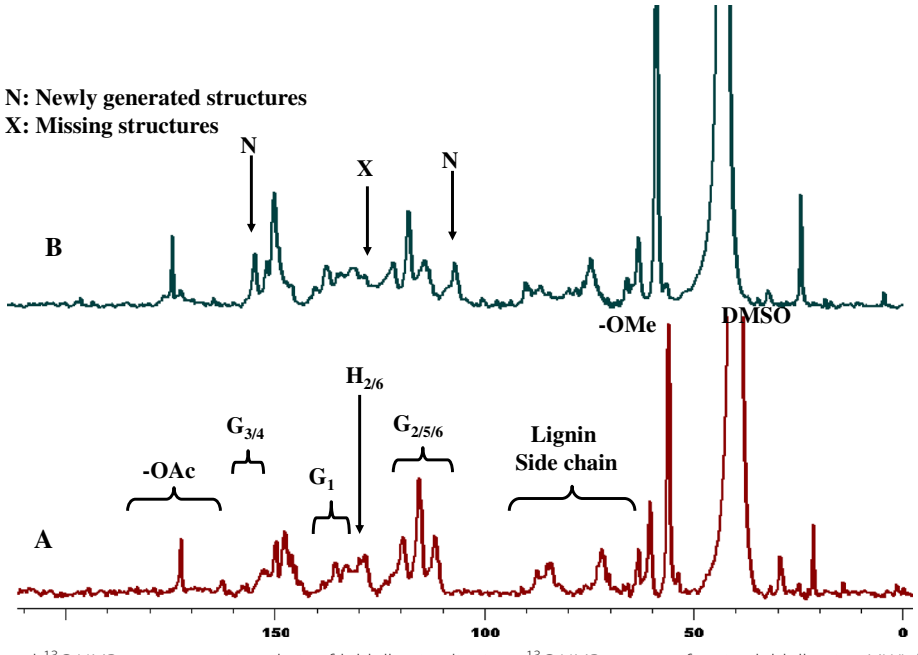


Fig. 2 One-dimensional ^{13}C NMR spectroscopic analysis of loblolly pine lignin. **a** ^{13}C NMR spectra of native loblolly pine MWL lignin; **b** ^{13}C NMR spectra of WEx-pretreated loblolly pine lignin

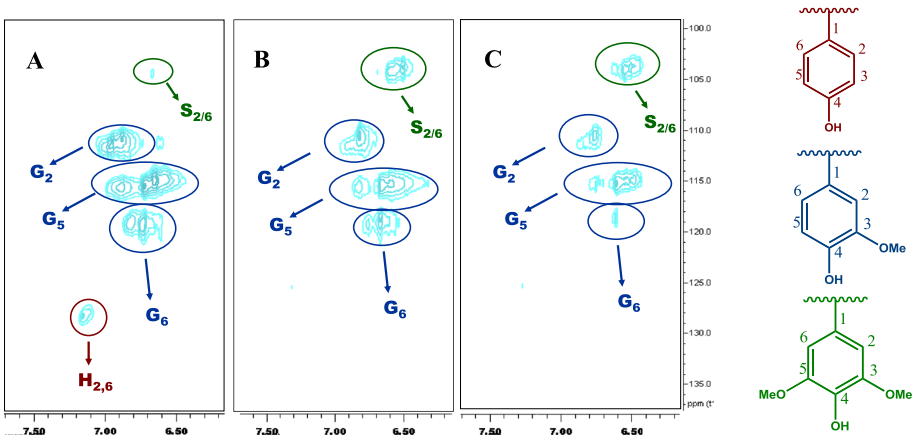


Fig. 3 Gradient-selected two-dimensional HSQC analysis of aromatic region of **a** native lignin of loblolly pine, **b** WEx-pretreated loblolly pine lignin, and **c** enzyme-hydrolyzed lignin of loblolly pine

the lignin polymeric molecule of the WEx-pretreated lignin as marked by N: newly generated structures (Fig. 2). This evidence was further reinforced through the existence of intense quaternary S aromatic ring carbon 3 and 5 (δ_C ~ 140–154 ppm) and 1 and 4 (δ_C 125–140 ppm) resonances. These results showing the formation of S-type structures in the lignin after WEx pretreatment were in agreement with the one-dimensional ^{1}H spectroscopic analysis as discussed in the previous section of this manuscript.

The peaks around 160 ppm are usually related to the conjugated carboxylic acid linkages as indicated previously, and previous studies have showed the formation of these linkages as a response to superoxide radical in lignin during wet oxidation pretreatment [31]. This study showed that the carboxylic linkages could be formed through the effect of the superoxide anion radical on the side chains in lignin. However, this study on the wet oxidation reaction on biomass reported only the cleavage of the aromatic and the aliphatic carboxyl groups from lignin. In our study, we found a significant increase in the S-type structures (as can be seen in Fig. 3) which could be obtained through interaction of these carboxyl groups with G lignin which showed a novel effect of the WEx pretreatment on the loblolly pine lignin when compared to that reported previously. It was hypothesized that this change in the S composition in the lignin molecule after WEx was through *in situ* methoxylation of the lignin's aromatic ring structure. Two-dimensional HSQC NMR was done to test the validity of this hypothesis.

Two-dimensional HSQC nuclear magnetic resonance

Since the focus of the study was to confirm the methoxylation of the aromatic ring structures in the loblolly pine lignin through WEx pretreatment, the discussion in this section has been primarily limited to the aromatic regions of the HSQC spectra. The expansion of the aromatic and unsaturated region of the two-dimensional HSQC spectrum of the lignin isolates of raw, WEx-pretreated, and enzymatic hydrolyzed loblolly pine lignin are shown in Fig. 3.

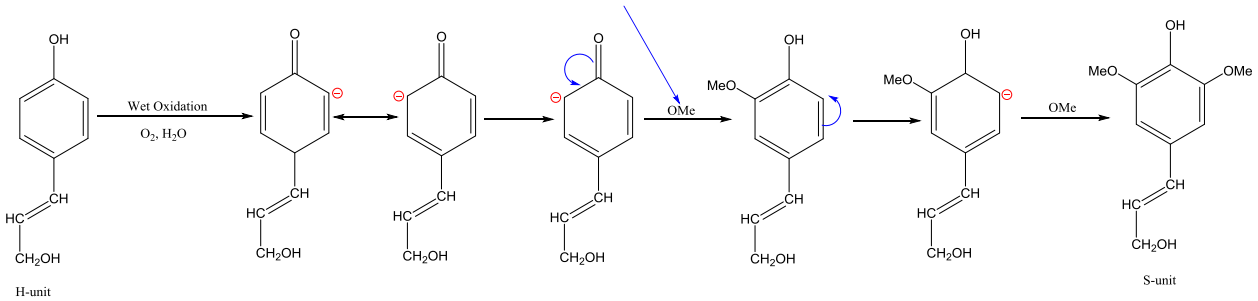


Fig. 4 Postulated reaction mechanism for the methoxylation of H lignin. The Fig. also shows the five possible resonant structures generated as a result of oxidation via the action of oxygen on H units

In the unsaturated region, the raw loblolly pine HSQC spectra showed one-bond carbon-proton correlations (δ_C/δ_H) with resonances for the $G_{2/5/6}$ carbon-proton cross-peaks at δ_C/δ_H ~ 112/6.78, 115/6.75, and 122/6.65 ppm, respectively, and $H_{2/6}$ carbon-proton cross-peaks at δ_C/δ_H ~ 128/7.18 ppm. As can be seen from Fig. 3a of raw loblolly pine, there are some peaks at δ_C/δ_H ~ 105/6.56 ppm which correspond to C-2 and C-6 aromatic ring carbon in S structures. However, the peak intensities were not as prominent as that for WEx-treated lignin shown in Fig. 3b. The HSQC spectra of WEx-pretreated lignin also showed lower intensities of G-derived aromatic carbon-proton correlations, while the H-derived cross-peaks (2, 6) completely disappeared. Similar effects of oxygen have been previously seen in eucalyptus lignin [32]. These studies confirmed an increase in oxidized S units through pyrolysis GC-MS, which showed an increased formation of oxidized lignin markers after oxygen treatment. The 2D HSQC spectra was further done on the loblolly pine lignin after enzymatic hydrolysis, and it displayed similar peak distribution of the overall lignin carbon-proton resonances as the WEX-treated lignin as shown in Fig. 3c. This suggested that enzymatic hydrolysis had no adverse effect on the observed structural changes of WEx-treated lignin and, as expected, did not induce any further modification to the S-lignin composition after enzymatic hydrolysis.

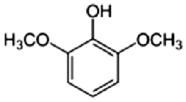
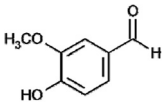
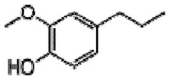
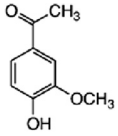
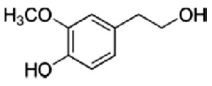
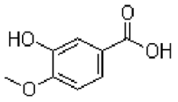
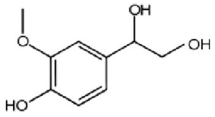
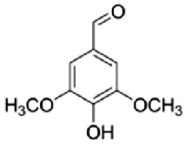
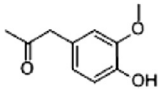
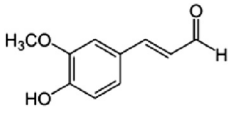
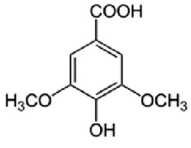
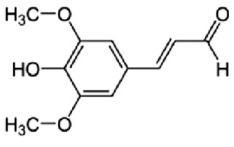
The implication of this study is significant since it is generally accepted that the action of oxygen on softwood lignin usually enriched its H units [33]. These studies have attributed this increase in H units with its high recalcitrance during oxygen delignification. However, the decrease in the H units and the resultant increase in the S units can be attributed to a selective methoxylation at the 3 and 5 position of the H-unit (and/or 5 position of the G unit, if at all) aromatic ring. Such structural modification can be assumed to occur due to the sudden explosion of the oxygen-delignified biomass material during WEx treatment followed by condensation and repolymerization of the lignin structure. Previous studies have discussed the cleavage of methyl groups from the methoxy side chains in lignocellulose during steam explosion [34]. However, these studies have been predominantly focused on the lignin oxidation during steam explosion. Studies have also shown acetic acid or acetate ions produced through C-C bond oxidative cleavage of cellulose and hemicellulose degradation products under similar experimental conditions as that discussed in this manuscript [35, 36]. These methyl or acetate released during biomass pretreatment can attack the reactive sites within the H unit of lignin which would become active during the wet oxidation phase [37]. As shown in the Fig. 4, we postulate that there are two possible reactive sites on the aromatic ring of the H unit accessible to nucleophilic attack during the wet oxidation phase. After the explosion, these reactive sites can react with the methyl and acetate linkages to form G unit and, eventually, S units. While the postulated reaction mechanism has not yet been effectively shown, it is consistent with the observed NMR results and adds valuable information on the effect of wet explosion pretreatment on the lignocellulosic biomass. GC-MS analysis of the wet exploded pine hydrolysate further supported the NMR findings as will be discussed in the next section.

It is important to understand that the S-like structures are less condensed because the 3, 5 positions on the aromatic ring are occupied by a methoxy group which provides stearic hindrance and thus restrains the carbon-carbon bond formation at those sites for condensed lignin structure [38]. On the other hand, H-like structures are more condensed because the 3, 5 positions on the benzyl ring are occupied by hydrogen which produces lesser stearic effects as compared to methoxy groups, making it amenable to further carbon-carbon bond formation via nucleophilic substitution reactions. The S-like structures are more amenable to β-O-4 cleavage for the further conversion into high-value aromatic products [38–40]. We anticipate that the generation of S-like structures within the lignin structure with predominant β-O-4 linkages will result in a greater degree of linearity or less condensation [25, 38, 41].

Lignin-derived phenolic compounds as determined by GC/MS

The phenolic compounds formed as a result of lignin depolymerization through wet explosion pretreatment have been shown in Table 2 and Fig. 5. It can be seen that these peaks listed in Table 2 cover the majority of the phenolic compounds present in the WEx-treated hydrolysate characterized using GC-MS analysis. The latter peaks referring to non-lignin-derived (X) and terpenoid (T) compounds (Fig. 5) are products obtained due to partial combustion of the oxygenated biomass during wet explosion. These compounds increased with an increase in temperature and residence time and are not the primary focus of this study. However, as expected, it was observed that phenolic compounds from lignin were either G-derived or S-derived in the WEx-pretreated slurry (Fig. 5) and no H-derived units of lignin molecule were detected. The actual peak areas for these phenolic compounds have also been shown in Table 2. It can be seen from this table that S-lignin-based compounds such as syringol, syringaldehyde, syringic acid, and sinapaldehyde increased with an increase in temperature. This can be partially observed by comparing the peak areas for these compounds at conditions (a) and (c). At the same temperature, an increase in residence time also

Table 2 Lignin compounds in aqueous phase after WEx pretreatment determined by GC-MS: (a) 165 °C, 18 min; (b) 165 °C, 30 min; and (c) 175 °C, 24 min

S. no.	Retention time (min)	Compound name	Origin	Peak area			Structure
				(a)	(b)	(c)	
1.	6.81	Syringol	S	8.18E+05	1.91E+06	1.74E+06	
2.	7.45	Vanillin	G	2.86E+07	4.10E+07	4.47E+07	
3.	8.15	4-Propyl Guaiacol	G	n/d	5.55E+05	3.56E+06	
4.	8.50	Acetovanillone	G	9.42E+05	1.41E+06	1.70E+06	
5.	9.01	Homovanillyl alcohol	G	n/d	1.63E+06	n/d	
6.	9.44	Isovanillic acid	G	4.67E+06	1.09E+07	1.05E+07	
7.	10.46	4-Hydroxy-3-methoxyphenyl glycol	G	8.34E+05	1.86E+06	n/d	
8.	10.55	Syringaldehyde	S	1.66E+07	2.42E+07	2.00E+07	
9.	11.34	Guaiacyl acetone	G	n/d	3.62E+06	8.37E+06	
10.	11.49	Coniferyl aldehyde	G	5.38E+06	1.05E+07	1.81E+07	
11.	12.40	Syringic acid	S	1.42E+06	1.96E+06	1.94E+06	
12.	14.84	Sinapaldehyde	S	2.18E+06	2.38E+06	3.76E+06	

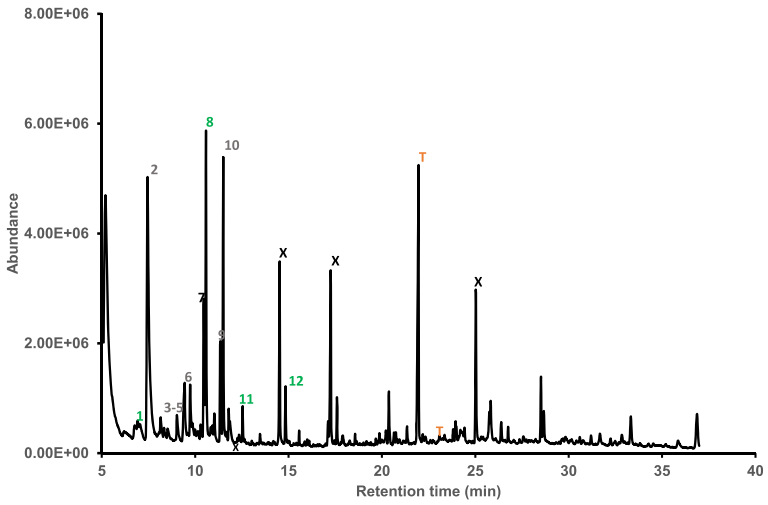


Fig. 5 GC-MS analysis of lignin-derived phenolic products from WEx-pretreated loblolly pine: **a** 165 °C, 18 min; **b** 165 °C, 30 min; and **c** 175 °C, 24 min

showed a significant increase in the amount of S-lignin-based compounds in the hydrolysate (by comparing Table 2, columns a and b). Comparing all the conditions, the significant effect of residence time on the formation of S units is clearly visible while temperature might play a minor role. This is primarily because of the small differences between the temperatures tested as a part of this study. However, our primary objective was to study the novel effect of wet explosion pretreatment on loblolly pine lignin, and further experiments are being conducted to further understand the reaction mechanism as a function of temperature and oxygen loading. Similar observations were also made for a number of G-lignin-based compounds except for some compounds such as homovanillyl alcohol and 4-hydroxy-3-methoxyphenyl glycol which were dependent on temperature and residence time. The significant observation from the reported results is that the S-type compounds increase with an increase in the severity of the wet explosion conditions. This was found to contradict the previous observations made by studying the effect of steam explosion and wet oxidation separately on lignocellulosic biomass [30–34] since these studies indicated that S units after pretreatment are cleaved to produce more G units resulting in a reduction in the S/G ratio and an increase in the lignin surface condensation. However, from both the NMR and the GC-MS analysis, wet explosion pretreatment showed an increase in the S units and decrease of H and G, which could best be explained through a selective methoxylation of H and G lignins during the pretreatment. This could also explain the absence of H-lignin-based phenolic compounds in the pretreated slurry.

Currently, we are further investigating the mechanistic aspects of *in situ* methoxylation of the lignin structure during WEx process and confirming the postulated reaction mechanism through tracing of the radical-catalyzed cleavage mechanism in the aromatic structures that are present in the biomass lignin. The results from this study can significantly revolutionize our understanding of the effect of wet explosion conditions on the lignin in the biomass.

Conclusions

Our study of loblolly pine showed that WEx pretreatment leads to structural modification within the lignin molecule. These structural modifications during WEx pretreatment were explained as a selective *in situ* methoxylation reaction of H lignin in the biomass. Analysis of extraneous resonances and cross peaks from one-dimensional and two-dimensional NMR spectra of WEx-pretreated lignin compared to the raw loblolly pine indicates that methoxylation occurred at the aromatic ring of the corresponding H and/or G units (possibly) resulting in generation of S-like structures within the lignin polymer. No further changes happen to the lignin structure during enzymatic hydrolysis. More research is underway to further understand the reactions occurring within lignin as a result of WEx pretreatment.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

DR conducted the pretreatment, enzymatic hydrolysis, and lignin isolation experiments and wrote the manuscript. DDL helped with the NMR analysis. KS worked with DDL and DR on data analysis and interpretation. BKA supervised the entire study and developed the content of the manuscript. All authors read and approved the final manuscript.

Authors' information

BKA is the Director of Bioproducts, Sciences and Engineering Laboratory (BSEL) at Washington State University. DR conducted this study as a PhD

student in chemical engineering under BKA's supervision. DDL and KS both contributed as a post doc within BSEL working on lignin applications.

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