

Effects of One-Step Alkaline and Two-step Alkaline/Dilute Acid and Alkaline/Steam Explosion Pretreatments on the Structure of Isolated Pine Lignin

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Abstract Biological valorization of biomass most often depends on the efficient reduction of plant cell wall recalcitrance and conversion of lignin – the most recalcitrant constituent – to fuels, chemicals and/or value-added substances. Lignin conversion to fuels and value-added chemicals requires a sound understanding of the structure of lignin before and after different pretreatments. In the current work, effort has been made to compare the structural differences in isolated pine lignin after one- (alkaline) and two-step (alkaline/dilute acid and alkaline/steam explosion) pretreatments. Our results indicate removal of the low molecular weight fraction of lignin after an initial alkaline pretreatment. A subsequent dilute acid pretreatment resulted in the loss of lignin inter-unit linkages such as β -O-4' aryl ethers. However, with a steam explosion pretreatment, lignin exhibited a competing condensation process leading to increased condensed lignin structures.

Keywords: Pine, Cellulolytic enzyme lignin, Alkaline pretreatment, Dilute acid pretreatment, Steam explosion pretreatment

1. Introduction

Second generation biorefineries can be defined as facilities that integrate production of biofuels alongside valorization of lignocellulosic residues. Ideally, a biorefinery utilizes all components of the biomass to make a range of products including fuels, chemicals, value-added materials and generate heat and power in proportions that maximize economic return [1, 2]. The general steps for the biological production of cellulosic ethanol are now well established and involve chemical or mechanical pretreatments, which helps reduce biomass recalcitrance and facilitating enzymatic hydrolysis of plant polysaccharides into fermentable sugars. Currently, efforts are underway to utilize lignin and other by-products from the cellulosic ethanol process to increase the profitability of biofuels production.

Development of efficient pretreatment [3] and lignin valorization [2] methods are two different approaches to accomplish economically-sustainable biorefining, but these processes are interlinked with the underlying chemistry. Pretreatment reduces biomass recalcitrance by altering several

physicochemical properties of the plant cell wall polymers including lignin. Over the years, several pretreatment processes have been developed for fractionating the targeted components from biomass. Alkaline pretreatments (AP) are effective for lignin extraction along with some hemicellulose removal [4-6]. Acid pretreatments such as dilute acid (DA) and steam explosion (SE) pretreatments are effective processes for hemicellulose extraction [7]. DA and SE pretreatments result in the enrichment of hemicelluloses in the liquid fraction leaving cellulose and lignin in the solid residue. DA pretreatment with sulfuric acid is usually performed using a low acid concentration (< 5 wt %) at a moderate temperature ranging between 120 °C – 160 °C [8, 9]. SE pretreatment is popular due to its low capital investment, moderate energy requirement, and low environmental impact [10]. This process involves heating the biomass to relatively high temperatures (160 °C – 230 °C) and pressures followed by a rapid decompression and discharge into a collecting tank, leading to mechanical disruption of the pretreated materials [11]. The structural components of lignin make it an attractive resource for value-added products including carbon fibers, resins, polymers and commodity chemicals [1, 2]. Biomass resources can be broadly categorized as hardwoods, softwoods, and herbaceous, each providing a unique lignin resource. The inherent heterogeneity in the lignin architecture makes it difficult to establish a unified biorefinery process for its valorization.

Softwoods, while often less costly than their hardwood counterparts [12] are recognized as one of the most recalcitrant biomass resources for biological conversion processes [10, 13]. Its fractionation usually requires the combination of chemical and/or mechanical pretreatments [14]. Even with the inherent hurdles of softwood processing, there are some advantages of using this type of feedstocks. Softwoods are rich in glucan, mannan, and galactan that can be converted to the hexoses which are usually preferred over pentoses by microbes that produce ethanol [15]. Alkaline pretreatments of pinewood were performed at 100-180 °C with 0-2% w/v sodium hydroxide (NaOH) for 1-5 hr and beneficial for the production of cellulosic ethanol compared to the untreated pine [16]. Acidic pretreatment of Swedish Scots pine was studied at different severities (180 °C, 2-4 wt% of sulfuric acid) by Jönsson et al. [15]. Sulfur dioxide (SO₂)-catalyzed steam explosion of pinewood was also explored at different severities by Bommarius et al. [17]. A low severity SE pretreatment removed some of the amorphous cellulose whereas a high severity SE pretreatment was capable of degrading crystalline cellulose. In addition to the above mentioned one-step processes, two-stage dilute acid pretreatment on Loblolly pine was also investigated [18]. More recently, pretreatment technologies have begun to utilize a relatively mild alkaline pretreatment followed by a more traditional DA or SE pretreatment stage [19, 20]. The proposed benefits of this approach are believed to involve an initial lignin removal stage followed by deconstruction of the plant cell wall using a traditional acidic pretreatment stage.

Depending on the severity and other pretreatment conditions, lignin undergoes physical and chemical changes contributing to a reduction in biomass recalcitrance [21]. Although many pretreatments were successfully applied on pine, there are few structural studies that determine the structure of lignin resulting from these various pretreatments. It is essential to understand the effects of the different pretreatments on lignin at a fundamental level to develop cost-effective technologies for the reduction of biomass recalcitrance and lignin valorization. To compare the lignin structure between different pretreatments and understand the changes due to the two-stage pretreatment technology, slash pinewood was alkaline pretreated followed by a dilute acid or steam explosion pretreatment. Cellulolytic enzyme lignin (CEL) was isolated from the untreated and pretreated pine and analyzed by Fourier

transform infrared (FTIR) spectroscopy, gel permeation chromatography (GPC), ^{13}C - ^1H heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) and ^{31}P NMR techniques.

2. Experimental Section

2.1 Materials.

Slash pine sawdust (residue from wood sawing) was supplied by a local sawmill (Forestal Eldorado, Misiones, Argentina). The sawdust was milled to pass through a 0.84 mm screen. All chemicals, enzymes, and materials were received from Sigma Aldrich (St. Louis, MO) and unless mentioned otherwise were used as received. The slash pine sawdust had a 30.1% Klason lignin, 39.4% glucan, 10.6% mannan, 6.4% xylan, 2.0% galactan, 1.3% arabinan and 4.6% extractives.

2.2 Pretreatments and methods.

The pine sample was subjected to an alkaline (AP) – acid (DA or SE) sequence as shown in Figure 1. After the pretreatment, the cellulolytic enzyme lignin (CEL) was isolated in each case. The following subsections describe the pretreatment conditions and the procedure for CEL isolation.

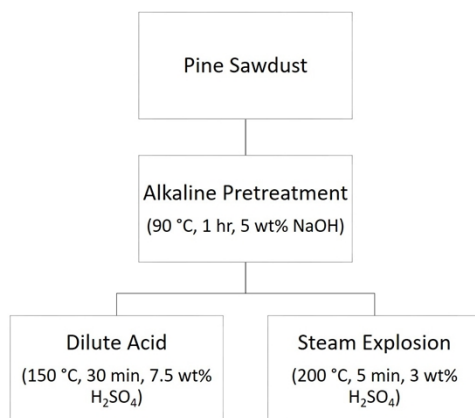


Figure 1: Description of pretreatment methods used in this study.

2.2.1 Alkaline Pretreatment. Alkaline pretreatment was carried out on 500 g oven-dried sawdust with 5 L of 0.125 M NaOH solution (liquor-to-wood ratio of 10, v/w) in a 7 L reactor (M/K Systems, Inc., Peabody, MA) with liquor circulation. Once the reaction was complete, the spent liquor was separated from the solid by centrifugation. Subsequently, the wood residue was exhaustively washed with deionized water until the effluent was pH neutral, and the solid residue was filtered and air dried. The resulting biomass was measured for moisture content (~10%), and this value was used to calculate dry weight in the subsequent usage.

2.2.2 Dilute Acid Pretreatment. This pretreatment was accomplished with 15 g of alkaline-pretreated pine (14% moisture content) added to 150 mL of 0.75 M sulfuric acid solution (liquor-to-wood ratio of 10, v/w) and the combined solution was loaded in a 200-mL stainless steel reactor. The reactor was heated to 150 °C for 30 min using a glycerin bath. Upon the completion of the reaction, the reactor was cooled

immediately using ice-water. The pretreated slurry was then filtered to isolate the solid material and washed with an excess of deionized water. The pretreated material is then processed for cellulolytic enzyme isolation (as discussed below). The material obtained after dilute acid pretreatment was rich in glucan content. While the amounts of xylan and mannan were reduced, galactans and arabinans were completely removed. The lignin content was, however, increased after this process (see supporting information).

2.2.3 Acid-catalyzed Steam Explosion Pretreatment. For the acid-catalyzed steam explosion pretreatment, alkaline-pretreated pine (280 g, moisture content of 14%) was sprayed with a 3 wt% sulfuric acid solution until it reached a moisture content of 50%. The treated pine was kept at room temperature (RT) in a sealed plastic bag overnight. The impregnated sawdust was then loaded into a reactor with automatic temperature control and coupled to a high-pressure boiler from Weco (Model GVO 10/30) for the supply of the steam. The pine sample was then heated to 200 °C for 5 min and followed by the rapid decompression into a stainless-steel container. The container was cooled to RT, and then filtered. The isolated solid residue was repeatedly washed with deionized water.

The solid residues recovered after alkaline pretreatment (AP) and SE mainly contained lignin (50%) and glucans (43%). The SE treatment was more effective than DA for hemicellulose extraction (90% and 54%, respectively). Xylans, arabinans and galactans were completely removed in the SE stage. No significant delignification took place during either acidic stage, instead an increase in lignin content was observed [19] attributed to the formation of pseudo-lignin [22]. The details of the composition analysis of all the samples are listed in the electronic supplementary information.

2.3 Isolation of Cellulolytic Enzyme Lignin (CEL). The untreated and pretreated pine biomass samples were dried and ground in a planetary micro mill (Fritsch PM 100). Planetary ball-milled wood samples were obtained according to a published procedure [23]. A 45-mL zirconium dioxide bowl with ten zirconium dioxide balls (of 1 cm diameter) were used in the micro mill. Native and pretreated pine samples were ground for 5.5 h (excluding pause time) at 10.8 Hz with 15-min pauses every 30 min to prevent the bowl from overheating. The ball-milled materials were then subjected to two consecutive rounds of enzymatic hydrolysis. An enzyme solution was prepared in 20 mM, pH 5 sodium acetate buffer containing 2 mg/mL cellulase from *Trichoderma* sp. The starting pine and pretreated substrates (1.5 g) were suspended in 100 mL enzyme solution (15 mg/mL) and then incubated in a shaking incubator set at 3.3 Hz and 35°C for 48 h. The reaction slurries were centrifuged (10 min, 67 Hz), the supernatants were removed and the residue was again suspended in acetate buffer (70 mL, pH 5) and treated with Cellulysin cellulase (2 mg/mL) for an additional 48 h at 35 °C. The residues were further collected by centrifugation, washed with distilled water (35 mL), centrifuged and freeze-dried. The freeze-dried residue was extracted (2 x 24 h) with *p*-dioxane/water (96:4, v/v; 10 mL/g biomass), at RT. After each extraction, the slurries were centrifuged (10 min, 67 Hz), and the supernatants were collected, combined and poured into a 250-mL volumetric flask. The residues from the second extraction were washed with 10 mL of 96% aqueous *p*-dioxane, centrifuged, and the supernatants added to the volumetric flask. The extracts were precipitated by dropping in water and concentrated with a rotavap (40 °C), freeze-dried, and the lignin samples were collected and weighed after vacuum drying.

2.4 Molecular Weight Analysis of CEL Before and After Various Pretreatments. The relative number average and weight average molecular weights of CEL were determined by gel permeation chromatography (GPC). Lignin samples were acetylated before gel permeation chromatography analysis.

In brief, the dried lignin (2 mg) was dissolved in a 1:1 (v/v) mixture of acetic anhydride/pyridine mixture (2 mL) and stirred at RT overnight. Anhydrous ethanol (1 mL) was then added, and after 30 min, the solvent was removed by rotary evaporation. The residue was repeatedly diluted with ethanol and evaporated under reduced pressure until all traces of acetic anhydride and pyridine were removed from the product. The acetylated lignin was then dissolved in tetrahydrofuran (0.5 mg/mL) and filtered through a 0.45- μm PTFE filter and placed in a 2 mL autosampler vial. The lignin molecular weight was analyzed by an Agilent GPC SECurity 1200 system coupled with four Waters Styragel columns (HR0.51, HR2, HR4 and HR6), an UV detector (λ 270 nm) and a RI detector. The sample injection volume was 25.0 μL and THF was used as a mobile phase with a flow rate of 1.0 mL/min. Data collection and analysis have been performed by Polymer Standards Service WinGPC Unity software (Build 6807). The molecular weight of the lignin samples was determined relative to the calibration curve generated with polystyrene standards.

2.5 FT-IR Characterization of CEL Before and After Various Pretreatments. A Perkin Elmer Spectrum 100 FTIR spectrometer with a universal attenuated total reflectance (ATR) sampling accessory (Perkin-Elmer Inc., Wellesley, MA) was used to monitor the structural changes in lignin. Lignin samples were pressed uniformly against the crystal surface via a spring-anvil, and spectra were obtained by 32 scans accumulation from 4,000 to 500 cm^{-1} at 4 cm^{-1} resolution. The ATR correction and the baseline correction were carried out orderly by PerkinElmer Spectrum software (Perkin-Elmer Inc., Norwalk, CT) provided with the equipment. Background correction of the obtained spectra has been performed using the Spectrum program from Perkin Elmer.

2.6 HSQC Analysis of CEL Before and After Various Pretreatments. HSQC experiments were carried out with a Bruker Avance III 400 MHz NMR spectrometer fitted with a Broadband Observe probe. Samples were prepared for the HSQC experiments as follows: 50 mg of lignin sample was added to 0.5 mL of DMSO- d_6 , and stirred at 45 °C for 4 h. The samples were characterized employing a standard Bruker pulse sequence with 13 ppm spectra width in F2 (^1H) dimension with 1024 data points (95.9 ms acquisition time), 210 ppm spectra width in F1 (^{13}C) dimension with 256 data points (6.1 ms acquisition time), a 90° pulse, 0.23 s acquisition time, 0.5 s pulse delay, $^1J_{\text{C-H}}$ of 145 Hz and 64 scans. NMR data were processed using MestreNova (Mestre Laboratories, Spain) software packages.

2.7 ^{31}P NMR Analysis of CEL Before and After Various Pretreatments. Typically, 20-40 mg of vacuum-dried lignin was dissolved in CDCl_3 /pyridine (1/1.6) and to this solution was added 50 μL (11.4 mg/mL) of chromium(III) acetylacetonate (relaxation agent). A 0.10 mL of 0.12 M internal standard – endo-N-hydroxyl-5-norbornene-2,3-dicarboximide – solution in CDCl_3 /pyridine was also added to aid in the quantification of lignin subunits. Phosphitylation has been performed with an excess (100 μL) of phosphitylating agent 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) according to the standard literature procedure [24, 25]. ^{31}P NMR data have been collected using a Varian 500 NMR spectrometer fitted with OneNMR_W036 probe and using a 90° pulse and a 25 s delay. NMR spectra were processed using Mestrenova software suite.

3. Results and Discussions

Pine sawdust samples were extracted with a mixture of toluene and ethanol (2:1, v/v), air-dried and subjected to alkaline pretreatment (in a first step), followed by either a dilute acid or steam explosion pretreatment. This step was followed by two rounds of enzymatic hydrolysis to remove cellulose and

hemicellulose from the pretreated pine samples. The cellulosytic enzyme lignin (CEL)s was then isolated according to the literature [23]. The yields of CEL are ~15-20% (based on the initial Klason lignin of extracted pinewood), except for DA pretreatment (6%).

3.1 ATR FT-IR spectroscopy

Attenuated total reflection (ATR) FT-IR spectroscopy was performed on all the pine lignin samples before and after pretreatments. The baseline corrected FT-IR spectra, shown in Figure 2A, exhibited prominent peaks at 3375 cm^{-1} and 2924 cm^{-1} , which were assigned to O–H stretching and C–H stretching vibrations, respectively [26]. The width of the O–H stretching frequency became narrower after alkaline, dilute acid and steam explosion pretreatments suggesting a possible disruption of intermolecular hydrogen bonds in lignin [27]. The bands at 1720 and 1660 cm^{-1} were assigned to the unconjugated and conjugated C=O stretch of the carbonyls in lignin subunits, respectively. The band at 1720 cm^{-1} was observed to be decreased after alkaline and dilute acid pretreatments. This decrease in intensity after alkaline pretreatment was followed by an intensity enhancement after the subsequent steam explosion pretreatment, which could have happened due to the oxidation of alcohols to carbonyls at the lignin linkage units (Scheme 1). A reduction at the 1595 and 1509 cm^{-1} bands – assigned to lignin aromatic vibration and stretching – after alkaline pretreatment is attributed to the removal of lignin inter-linkages (Figure 2B) [26]. The aromatic ring vibrations (1595, 1509, 1463, 1453 and 1420) are also prominent in the FT-IR spectra highlighting the abundance of phenolics in the lignin structure. Also, the bands linked particularly to guaiacyl unit, 1265 cm^{-1} (aromatic ring vibration) and 1138 cm^{-1} (C–H in-plane deformation), 870 and 810 cm^{-1} (C–H out-of-plane vibration at 2, 5 and 6–positions at guaiacyl ring) were also observed underscoring the abundance of guaiacyl units in these lignin samples. The band at 1030 cm^{-1} is assigned to primary alcohol units. The literature assignments for the observed IR bands are summarized in Table 1.

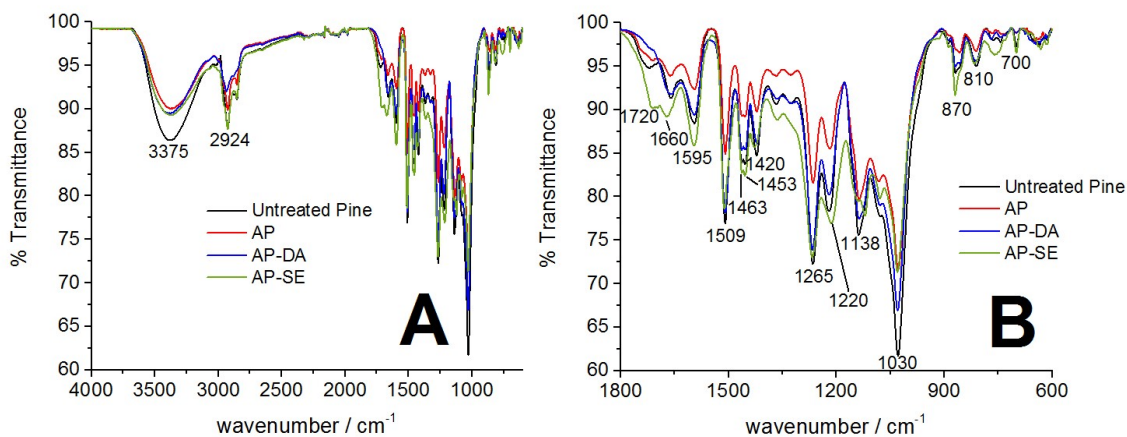


Figure 2: FT-IR spectra of the CEL samples isolated from untreated and pretreated pine samples: full spectrum (A), zoomed-in fingerprint region (B). Each spectrum is an average of at least three spectra recorded for each sample. AP stands for alkaline pretreatment, while AP-DA and AP-SE represent two-step pretreatments with dilute acid and steam explosion.

Table 1: Assignment of IR bands to the corresponding chemical stretches

| Wavenumber/ cm^{-1} | Band assignments | Ref |
|------------------------------|------------------|-----|
|------------------------------|------------------|-----|

| | | |
|----------|--|-------------|
| 3375 | O–H stretching | [26] |
| 2924 | C–H stretching | [26] |
| 1720 | unconjugated C=O stretch | [27] |
| 1660 | conjugated C=O stretch | [28, 29] |
| 1595 | aromatic skeletal vibrations | [26, 28-32] |
| 1509 | aromatic skeletal vibration | [26] |
| 1463 | C–H deformation | [26] |
| 1453 | O–H in-plane bending | [26] |
| 1420 | C–H in-plane deformation | [26] |
| 1265 | vibration of the guaiacyl ring together with C=O stretch | [31, 32] |
| 1220 | combined C–C and C–O stretching | [26] |
| 1138 | C–H in-plane deformation of the guaiacyl ring | [33] |
| 1030 | C–O stretch of primary alcohol | [26] |
| 870, 810 | C–H out-of-plane vibrations in 2, 5, and 6-position of the guaiacyl ring | [33] |

3.2 Gel permeation chromatography

GPC is frequently employed to measure the molecular weight of lignocellulosic polymers, and previous studies have identified it as a valuable tool in monitoring the structural changes of lignin such as the relative extent of depolymerization and repolymerization [34-37]. GPC analysis was performed on acetylated lignin samples to identify the effects of one- and two-step applied pretreatments on the pine lignin samples. It was found that the alkaline pretreated residual lignin exhibited an increased weight-average molecular weight (M_w) compared to the untreated pine lignin sample (i.e., M_w for pine lignin changed from 16000 in the extracted lignin from untreated pine to 21000 g/mol in AP lignin), as presented in Figure 3. On the other hand, both lignins from AP-DA and AP-SE treatments showed a drastic decrease in molecular weight (AP-DA lignin M_w 7100 g/mol and AP-SE M_w was 7700 g/mol). GPC analysis also revealed that PDI (polydispersity index) increased in the AP-DA sample whereas it remained almost unchanged for the untreated and alkaline-pretreated sample.

The increase in the weight-average (M_w) and number-average (M_n) molecular weight of CEL isolated from the alkaline pretreated sample can be explained by molecular weight distribution (MWD) of the CEL samples (Figure 4). While there is not a major shift in the peak maximum, the alkaline pretreated (AP) sample shows a loss of the low molecular weight fraction and a gain in the high molecular weight fraction, attributed to the removal of some labile low weight lignin fractions. The same trend has been observed in the average molecular size of dissolved lignin during the Kraft pulping process, in which lignin is dissolved early in the Kraft cook and has a fairly low molecular weight [38]. On the other hand, the subsequent steam explosion (SE) process slightly shifts the maxima toward the low molecular weight end, since SE process generates low molecular weight lignin from high molecular weight units [39]. This counteracts the effect of AP, so the polydispersity index (PDI) remains similar to that of the untreated CEL sample. The most severe effect on the CEL has been observed after the dilute acid pretreatment (DA), since the peak maxima shifted further towards the low molecular weight end, showing an significant

increase in the low molecular weight lignin fractions. The elimination of high molecular weight lignin could be due to the cleavage of the aryl ether linkage in β -O-4' moieties (Scheme 1).

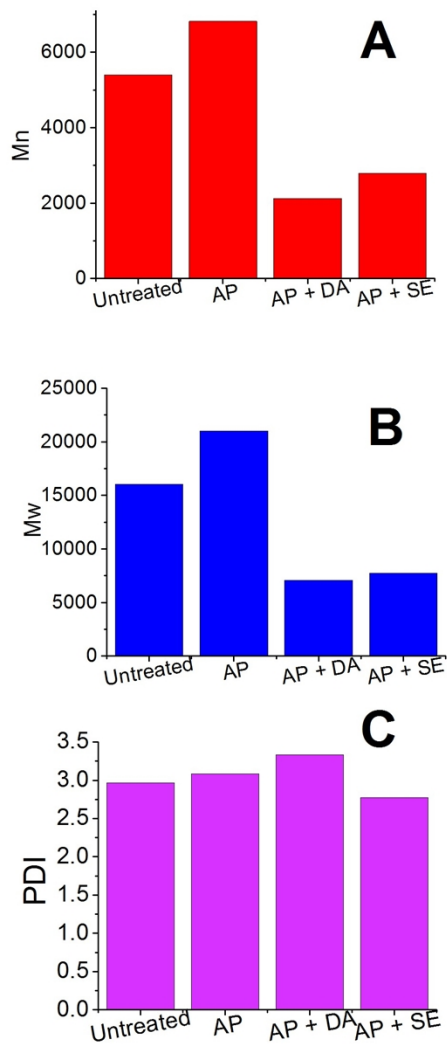


Figure 3: Change in molecular weight of CEL from pine biomass after different pretreatments. (A) Number-average molar mass, (B) Weight-average molar mass and (C) polydispersity index (PDI).

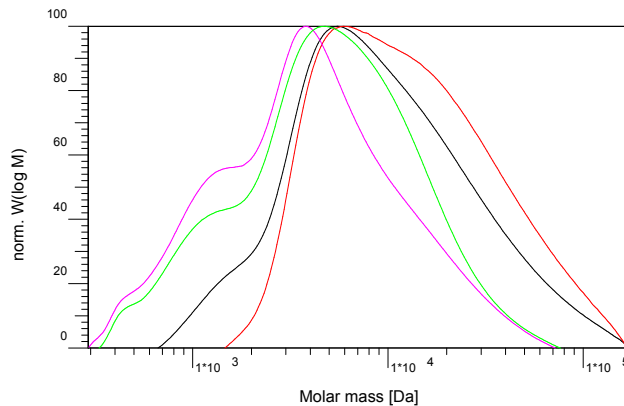
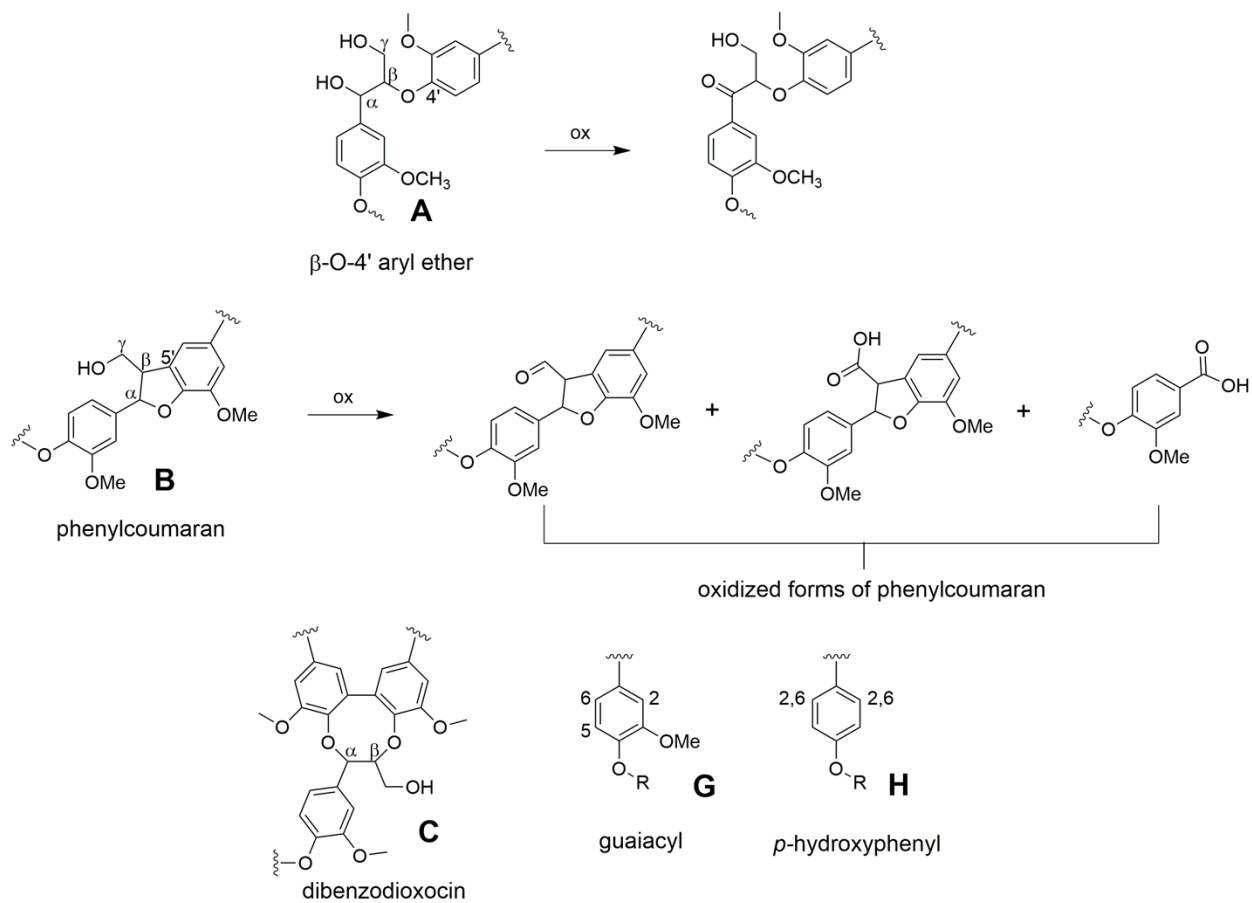


Figure 4: Molecular weight distribution of cellulolytic enzyme lignin (CEL) of pine before and after pretreatments. Untreated pine (black), AP (red), AP-DA (pink) and AP-SE (green).

3.3 HSQC NMR studies

2D ^1H - ^{13}C heteronuclear single quantum coherence (HSQC) correlation has been shown to be useful in elucidating the structure of biomass and its components including lignin [34, 40-43]. HSQC studies were carried out on the lignin samples isolated from untreated and pretreated pine samples and the spectra were assigned according to the literature [35, 42]. Figure 5 depicts both the aromatic and aliphatic regions of the HSQC spectra of the slash pinewood CEL. Pine lignin primarily contains G-type unit (guaiacyl units), which has been confirmed by the FTIR and is again well reflected in the HSQC spectrum. The aromatic region of the CEL isolated from the untreated pine sample in Figure 5A shows a C–H correlation of the aromatic ring of G units and trace amounts of $\text{H}_{2/6}$ units. $\delta_{\text{C}}/\delta_{\text{H}}$ peaks at 118.6/6.8, 114.7/6.8 and 110.7/7.0 ppm are all assigned to G-type lignin (G_6 , G_5 , and G_2 , respectively), whereas the cross-peak at 128.5/7.2 ppm stands for the C_2/H_2 and C_6/H_6 correlation of H-type lignin. On the aliphatic portion of the spectrum (Figure 5B) prominent cross-peaks can be observed for methoxyl units (55/3.5 ppm) and the β -O-4' (71.5/4.8 ppm for $\text{C}_\alpha/\text{H}_\alpha$ and 85.2/4.2 ppm for $\text{C}_\beta/\text{H}_\beta$) and β -5' phenylcoumaran (86.4/5.5 ppm for $\text{C}_\alpha/\text{H}_\alpha$ and 62.3/3.7 ppm for $\text{C}_\gamma/\text{H}_\gamma$) subunits. Table 1 summarizes the HSQC cross peaks of lignin samples. Interestingly, weak-intensity cross peaks of dibenzodioxocin [44, 45] (C in Scheme 1) at $\text{C}_\alpha/\text{H}_\alpha$ (83.4/4.8) were observed in the untreated (shown in electronic supplementary information for clarity) [46]. These peaks completely disappeared after alkaline, dilute acid and steam explosion pretreatments.

Scheme 1: Subunits of pine lignin and its proposed oxidized form after dilute acid and steam explosion pretreatments [47].



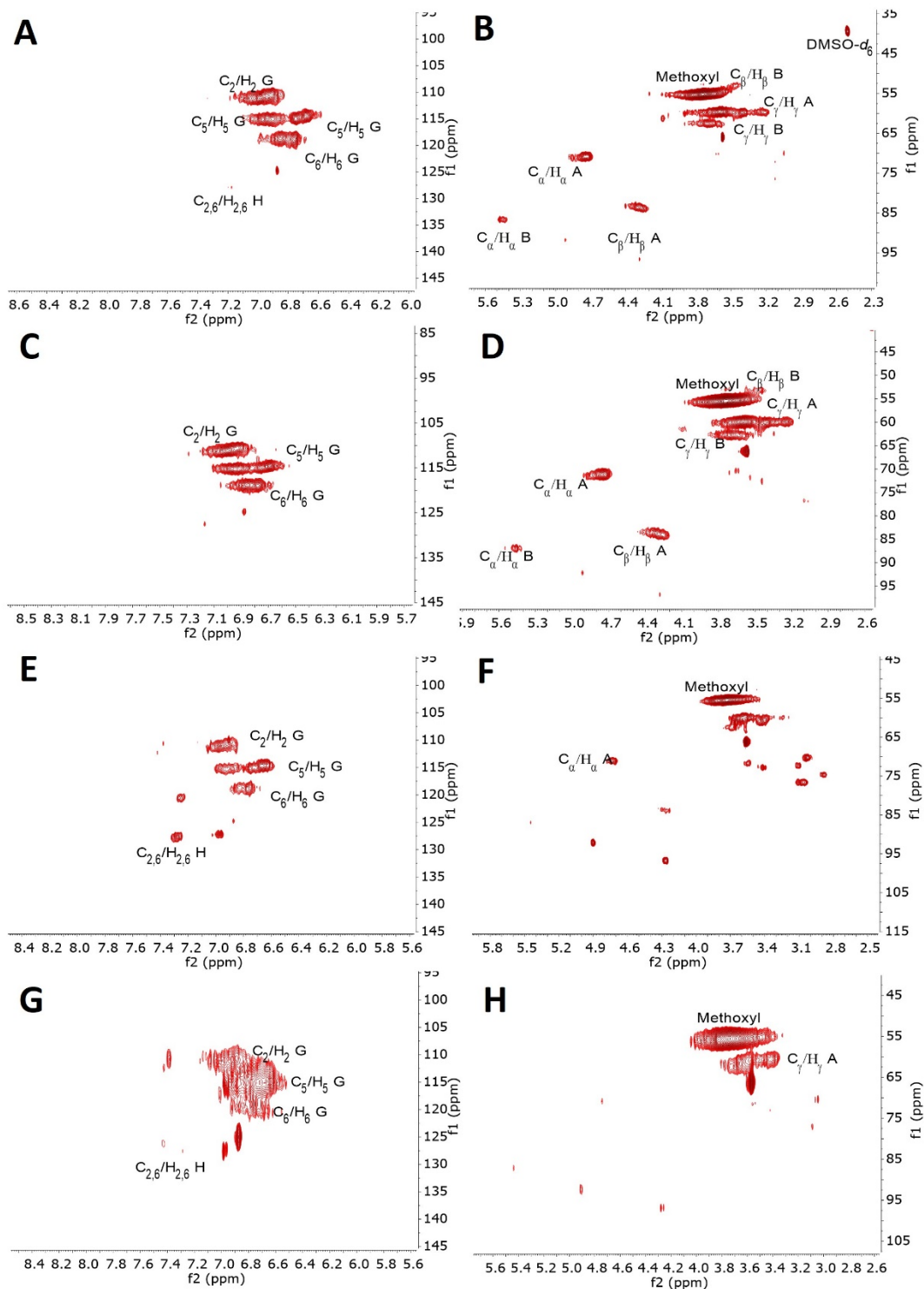


Figure 5: 2D HSQC NMR data of CEL from untreated (A, B), one-step alkaline- (C, D), two-step alkaline and dilute acid- (E, F) and two-step alkaline and steam explosion- (G, H) treated pinewood samples (G is observed at a noise level). Aromatic regions (A, C, E, G) and aliphatic regions (B, D, F, H) are shown separately.

The CEL in alkaline pretreated pine, on the other hand, shows a similar HSQC spectra to the untreated HSQC spectra of pine (i.e., Figure 5AB and 5CD). The aliphatic region of the HSQC spectra of the CEL after alkaline pretreatment shows an enhanced intensity of β -O-4' (A in Scheme 1) and phenylcoumaran (B in Scheme 1) as compared with the untreated sample. On the contrary, DA and SE pretreatments were found to have rather diminished β -O-4' and β -5' linkages (Figure 5F and 5H). In the aromatic region, the intensity of G- and H-type lignin decreased upon dilute acid pretreatment. The effect of steam explosion pretreatment is more severe as it removes H-type lignin and parts of the G-type lignin and produces some of their oxidized components (Scheme 1) as shown by the overlapping cross-peaks. The removal of β -O-4', phenylcoumaran and parts of G-type and H-type lignin by both DA and SE processes is consistent with the observed decrease in the molecular weight, as shown by the GPC results. The disappearance of dibenzodioxocin linkages is reflective of the severity of this pretreatment and changes in the structure of lignin.

Table 2: Assignment of correlation in HSQC spectra of pine cellulolytic enzyme lignin

| δ_C/δ_H (ppm) | Assignment |
|---------------------------|---|
| 53.2/3.5 | C_β/H_β in phenylcoumarin subunit (B) |
| 55.0/3.5 | C/H in methoxyl group |
| 59.7/3.4 | C_γ/H_γ in β -O-4' structure (A) |
| 62.3/3.7 | C_γ/H_γ in phenylcoumarin (B) |
| 71.5/4.8 | C_α/H_α in β -O-4' structure (A) |
| 85.2/4.25 | C_β/H_β in β -O-4' structure (A) |
| 83.4/4.8 | C_α/H_α in dibenzodioxocin (C) |
| 86.4/5.5 | C_α/H_α in phenylcoumarin subunit (B) |
| 110.7/7.0 | C_2/H_2 in guaiacyl unit (G) |
| 114.7/6.8 | C_5/H_5 in guaiacyl unit (G) |
| 118.6/6.8 | C_6/H_6 in guaiacyl unit (G) |
| 128.5/7.2 | $C_{2,6}/H_{2,6}$ in <i>p</i> -hydroxyphenyl unit (H) |

3.4 ^{31}P NMR

This technique has proved to be a useful tool in deciphering lignin structure by identifying its hydroxyl groups. The basis of this study resides with the successful phosphorylation of various OH groups of lignin. To ensure complete phosphorylation, an excess of phosphorylating agent – 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) – was added to the NMR sample as per the standard literature procedure [24, 25, 48].

^{31}P NMR spectra of lignins from the untreated and pretreated pine lignin samples exhibited the presence of aliphatic OH, carboxylic acid and lignin phenolic OH (predominantly guaiacyl and minor amounts of *p*-hydroxyphenyl). Peak identification and quantitation were accomplished following literature procedure [24, 25] and details of this analysis are shown in Figure 6.

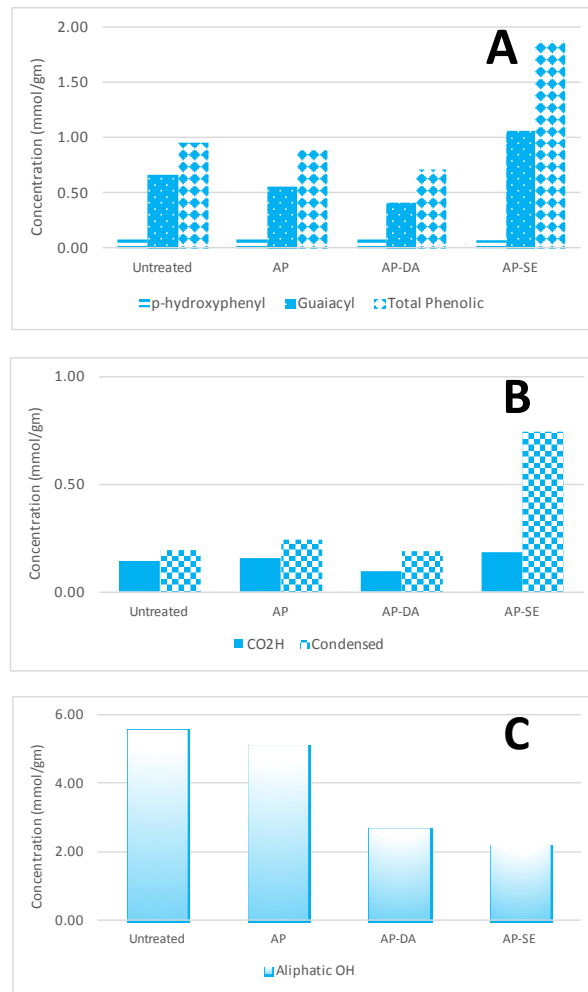


Figure 6: ^{31}P NMR quantification of hydroxyl groups (phenolics OH (A), carboxylic acid and condensed OH (B) and aliphatic OH (C)) of lignin in the untreated and pretreated pine lignin.

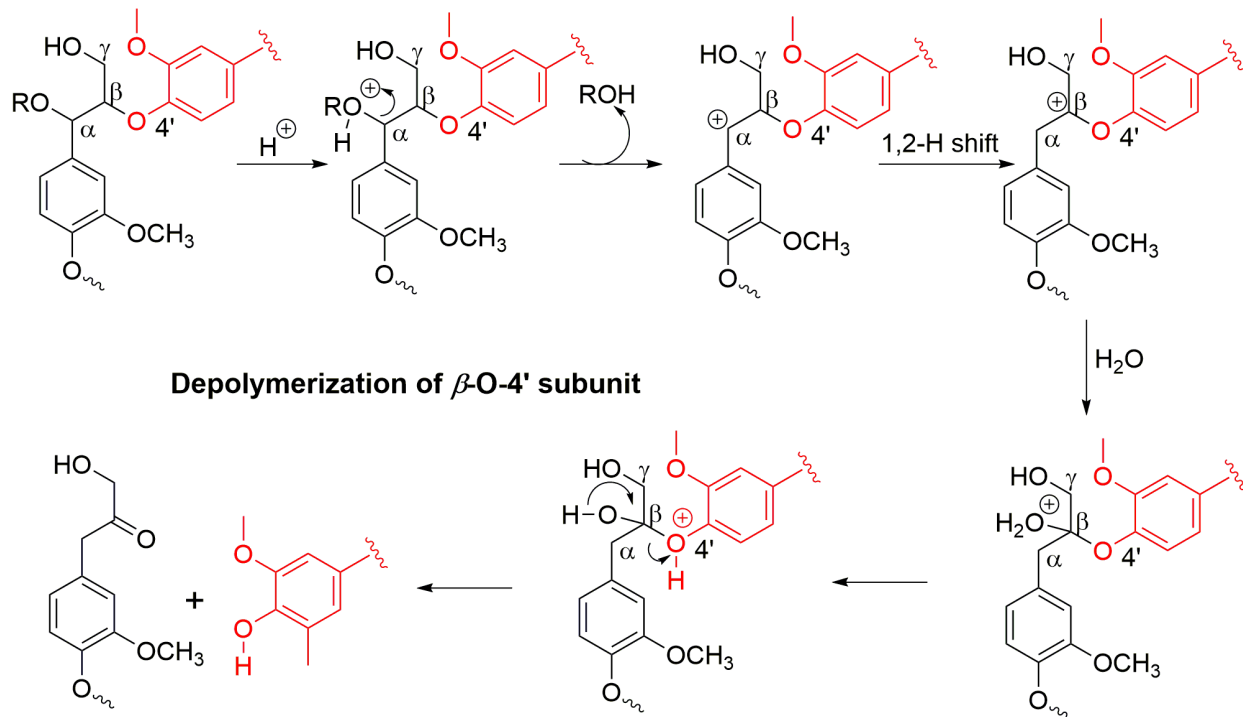
Being a softwood, pine lignin's ^{31}P NMR spectrum shows clear signals for phenolic guaiacyl lignin (G). Total phenolic content is determined by summing condensed, G- and H-type phenolic lignin units (Figure 6A). After alkaline pretreatment, the amount of mainly G- and some H-type phenolics decreased. Dilute acid pretreatment (AP-DA) further reduced the amount of guaiacyl OH content from 0.56 to 0.41 mmol/g. The amount of aliphatic hydroxyl group content also decreased during both pretreatments (Figure 6C). ^{31}P NMR spectra of the steam explosion-treated sample also showed an increased amount of condensed phenolic OH groups (Figure 6B), which is believed to be generated by condensation reactions which have been previously reported [39, 49]. The carboxylic acid content also remains unchanged during the alkaline pretreatment process, while it is only slightly reduced in the case of acid pretreatment. Such a reduction in the carboxylic acid content has also been observed for spruce wood [50].

Steam explosion, on the other hand, is a more established and widely employed mechano-chemical pretreatment and is used to disrupt cellulose crystallinity [17], delignification [10], and easy hydrolysis of

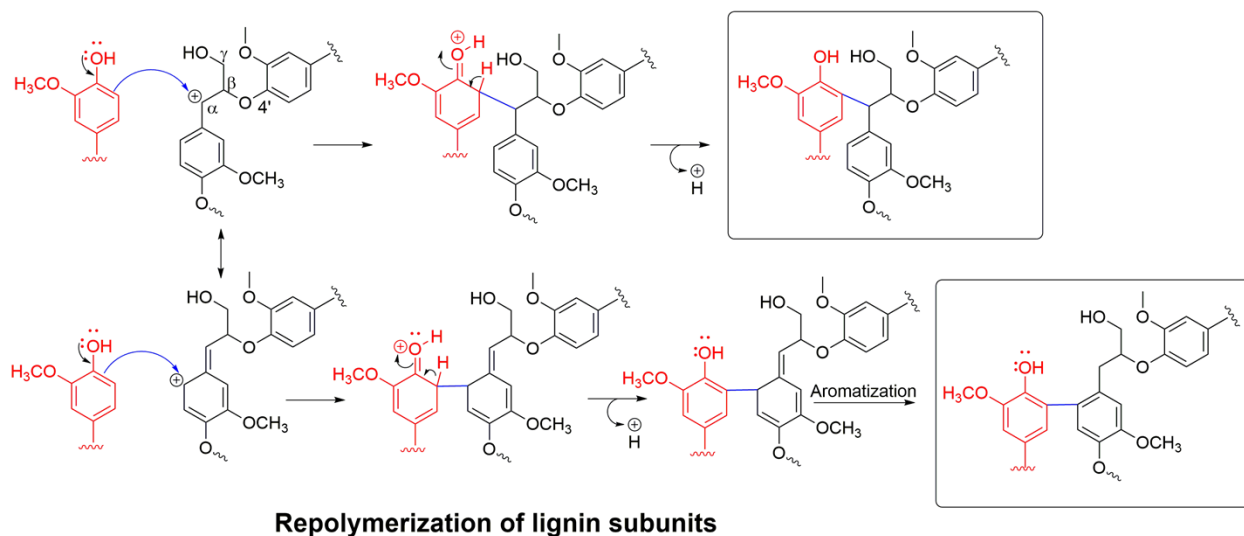
hemicellulose.³² The acidic condition, under which steam explosion is performed in this study, can lead to the formation of a carbonium ion at the benzylic position from a β -O-4' subunit, with the elimination of a molecule of water or alcohol (ROH in

Scheme 2). The prevalence of G-type lignin in the steam-treated (AP-SE) sample can be explained by the formation of a guaiacyl moiety from the cleavage of β -O-4' units (of type G-O-G or G-O-H). However, smaller lignin fractions can be recombined to produce guaiacyl-based condensed structures (Figure 6B) and more stable fractions involving formation of C-C bonds [49]. Such a lignin depolymerization and repolymerization scheme have previously been observed in the steam explosion pretreatment of aspen wood. A plausible mechanism for such repolymerization is shown in Scheme 3. Finally, SE lignin has less aliphatic OH groups and more phenolic hydroxyl groups than untreated lignin. This effect has also been observed after two-stage DA pretreatment of loblolly pine [18]. The origin of the higher condensation of SE lignin compared to DA lignin is probably due to the severity of the applied SE pretreatment [34].

Scheme 2: Plausible mechanistic pathway for depolymerization of cellulolytic enzyme lignin from two-step pretreatment of pine sawdust



Scheme 3: Plausible mechanistic pathway for the repolymerization of cellulolytic enzyme lignin from two-step pretreatment of pine sawdust



4. Conclusion

Cellulolytic enzyme lignin – from pine sawdust – was isolated after one-step alkaline and two-step alkaline/dilute acid and alkaline/steam explosion pretreatments. Alkaline pretreatment altered the lignin structure in a way that increased the amounts of high molecular weight components, observed by an increase in M_w using gel permeation chromatography. The subsequent pretreatments under acidic condition (DA and SE) led to the rupture of β -O-4' as observed by a decrease in intensity of the ^1H - ^{13}C HSQC spectra. Under acidic conditions, the β -O-4' linkage unit got cleaved leading to the formation predominantly G-type monolignol units. However, in case of steam explosion, the depolymerization process is also accompanied by a competing repolymerization leading to the formation of condensed structures as observed from solution state ^{31}P NMR spectroscopy. Such a depolymerization by fragmentation of β -O-4' structures and polymerization by acid-catalyzed condensation between the aromatic C_6 or C_5 and a carbonium ion were observed during two-stage dilute acid pretreatment [18] and acid-catalyzed steam explosion [49]. This study highlights depending on the severity of the pretreatment lignin structure can be altered by depolymerization and repolymerization of its subunits. A molecular-level understanding of structural changes in lignin – after the applied pretreatments – is crucial for subsequent application and valorization.

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Appendix A. Supplementary Data

Supplementary data related to this article can be found online.

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