Condensed Lignin Structures and Re-Localization Achieved at High Severities in Autohydrolysis of Eucalyptus globulus Wood and Their Relationship With Cellulose Accessibility

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ABSTRACT: Eucalyptus globulus wood was subjected to autohydrolysis pretreatment at different severity factors. The pretreated materials were enzymatically saccharified at a substrate load of 10% (w/v) using a cellulase enzyme complex. Around 82–95% of original glucans were retained in the pretreated material, and the enzymatic hydrolysis yields ranged from 58% to 90%. The chemical and structural changes in the pretreated materials were investigated by microscopic (SEM, LSCM) and spectroscopic (2D-HSQC NMR and FT-IR) techniques. 2D-NMR results showed a reduction in the amounts of β-O-4 aryl–ether linkages and suggested the presence of newly condensed structures of lignin in the biomass pretreated at the more severe conditions. Furthermore, the microscopic analysis showed that lignin migrates out of the cell wall and re-deposits in certain regions of the fibers at the more severe conditions to form droplet-like structures and expose the cellulose surface. These changes improved the glucose yield up to 69%, on dry wood basis. Biotechnol. Bioeng. 2015;9999: 1–1.
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Introduction
Lignocellulosic biomass (LCB) has long been recognized as a potential sustainable source of sugars for biofuels and other bio-based materials. Due to its complex structure, a pretreatment step is usually conducted to reduce the recalcitrance of LCB to increase the accessibility of the cellulose fibers to the enzymatic hydrolysis. Different pretreatment methods have been applied to fractionate the biomass, including physical (milling and grinding), physicochemical (steam explosion, autohydrolysis, hydrothermolysis, and wet oxidation), chemical (alkali, acid, oxidizing agent, organic solvents), biological (fungi and enzymes), and combinations of these methods (Castro et al., 2013; FitzPatrick et al., 2010; Monroy et al., 2012; Pu et al., 2013; Santos et al., 2013). The hydrothermal pretreatment, which uses hot water as a hydrolysis medium (autohydrolysis), is used to selectively separate hemicellulose from lignin and cellulose in hardwoods (Castro et al., 2013; Garrote et al., 1999; Leschinsky et al., 2009; Romaní et al., 2010). After pretreatment, the material is separated into two fractions, one containing the water-soluble components and the other containing the insoluble residual solids. Most of the hemicelluloses are in the soluble fraction together with a soluble fraction of lignin, whose amount depends on the severity of the process. The solid residue consists mainly of cellulose and insoluble lignin, which may be used as the substrate for further enzymatic saccharification and fermentation to bioethanol. During the pretreatment process, the structures of carbohydrates and lignin change due to the high temperatures, pressures, and pH of the reactions. Hydronium ions are generated from both water (Akiya and Savage, 2002) and organic acids (predominantly formic and acetic acids, formed from the hydrolysis of formyl and acetyl groups in hemicelluloses) that catalyze the hemicelluloses de-polymerization to water-soluble sugars and oligosaccharides (Garrote et al., 1999). The extended hemicellulose de-polymerization and degradation during autohydrolysis depends on the LCB nature and the severity of the pretreatment.

Different de-polymerization and re-condensation reactions take place during the hydrothermal pretreatment of lignin (Garrote et al., 1999; Li et al., 2007). The fragmentation of lignin usually results in a slight delignification, and the lignin removal depends on the severity of the pretreatment. The main reaction is the cleavage of
the β-O-4 aryl–ether bonds, which increases the content of phenolic hydroxyl groups and may form condensed lignin products from the different fragments (Leshinsky et al., 2008; Santos et al., 2013). Non-productive enzyme adsorption into the lignin rich material is a major inhibitory mechanism, preventing efficient enzymatic hydrolysis of carbohydrates. Even though mainly the hydrophobic interactions were considered, also electrostatic interactions are important, as demonstrated by Rahikainen (2013). In this study, the enzyme–lignin interactions were evaluated using isolated lignins from steam explosion, non-treated spruce and wheat straw, and hydrothermally pretreated wheat straw. The enzyme–lignin interaction were proven to be dependent on cellulases modular structures, temperature, and pH. Considering that the lignin and the enzymes have ionizables groups (with different pKₐs), the strong inverse correlation between pH and the enzymes binding to lignin is an evidence of electrostatic interactions.

The importance of these interactions depends on the structures of both, the enzyme model and the origin of the lignin.

The formation of lignin-like compounds (also called pseudo-lignin) has been demonstrated at low pH values, especially at more severe conditions. According to some authors, these compounds are generated from hemicelluloses and lignin degradation products (Kristensen et al., 2008). Sannigrahi et al. (2011) demonstrated the formation of pseudo-lignin from carbohydrates without a significant contribution from lignin during dilute acid pretreatment, particularly at high severities, but insignificant formation was observed at low severities. Hu et al. (2012) isolated and characterized the pseudo-lignin produced from dilute acid pretreated poplar holocellulose and α-cellulose, demonstrating that the pseudo-lignin, a lignin-like aromatic material, contained carbonyl, carboxylic, aromatic, and aliphatic structures. Pseudo-lignin is hydrophobic as lignin (Roman-Leshkov et al., 2006) and its features of the residual lignin on the pretreated material. The distribution of lignin over the fibers, and the main structural features of the residual lignin on the pretreated material. The influence of the severity conditions of the hydrothermal pretreatment on the glucose yield during the enzymatic saccharification was also evaluated.

Materials and Methods

Raw Material

E. globulus wood chips were obtained from a commercial plantation of a Chilean forest company located in the Biobío Region (Southern Chile). The wood chips (2.5 × 1.5 × 0.2 cm) were thoroughly mixed to obtain a single uniform sample, air dried to ~10% moisture, and stored in dry conditions before use.

Autohydrolysis Process

Industrial size wood chips were cooked to perform the hydrothermal pretreatment. The samples were treated in a 1000-mL Parr reactor (Moline, IL) loaded with 100 g of wood chips (dry basis) and a water–wood ratio of 4:1 (v/w). The reactor was heated at 3.5°C/min, and the cooking was performed at maximum temperatures (175, 185, 195, and 205°C) for 30 min, which corresponded to severities (Sₙ) of 3.89, 4.20, 4.48, and 4.78, respectively. Sₙ was calculated according to Sixta (2006). After cooking, the pretreated material (pulp) was disintegrated in a TAPPI laboratory blender, thoroughly washed with tap water, and centrifuged. The total pulp yield was determined, based on the weight of the pulp divided by the weight of the wood chips (both on a dry basis), multiplied by 100. The pretreated samples were stored in plastic bags at 4°C for further use.

Chemical Characterization of Wood and Pulp Samples

Milled wood samples (40/60 mesh) were extracted with ethanol/toluene according to the TAPPI method 204 cm–97 (2001). The extracted wood and pulp samples were characterized based on their carbohydrate content using the methodology described by Ferraz
et al. (2000). In a test tube, 300 mg of extractive-free milled wood or pulp was weighed, and 3 mL of 72% (w/w) H2SO4 was added. The hydrolysis was carried out in a water bath at 30°C for 1 h and stirred every 10 min. Subsequently, the acid was diluted to 4% (w/w) with 84 mL of distilled water, and the mixture was transferred to a 250-mL Erlenmeyer flask and autoclaved for 1 h at 121°C. The residual material was cooled, filtered through a number 4 sintered glass filter, and rinsed with water. The solid fraction, which consisted of insoluble lignin (Klason lignin) was dried at 105°C and weighed. The concentration of monomeric sugars in the soluble fraction was determined by measuring the absorbance at 205 nm using a spectrometer (LaChrom-Merck-Hitachi (Tokyo, Japan)) equipped with a refractive index detector and Aminex HTX-87H column (BioRad, Hercules, CA) at 45°C, a mobile phase of 5 mM H2SO4 and a flow rate of 0.6 mL min⁻¹. Glucose, xylose, arabinose, and acetic acid were used as external calibration standards. The glucans content was calculated by multiplying the acetic acid content by 0.9; the xylans content obtained from xylose was multiplied by 0.88; and the acetyl content was calculated by multiplying the acetic acid content by 0.7. The acid-soluble lignin in the aqueous fraction was determined by measuring the absorbance at 205 nm using 110 L g⁻¹ cm⁻¹ as the absorbivity. The total lignin was the sum of the soluble and insoluble lignin. All samples were analyzed in triplicate.

Scanning Electronic Microscopy

The surfaces of fibers were imaged before and after pretreatment with a scanning electron microscope (SEM) using a Jeol JSM-6380LV instrument under a high vacuum, operating with a secondary electron detector. The samples were dried at room temperature and coated with conductive gold paint with a 500 Å particle size in a S150 Edwards Sputter Coater. Imaging was performed at a beam accelerating voltage of 20 kV with tungsten filament as the electron source.

Laser Scanning Confocal Fluorescence Microscopy (LSCM)

A LSM710 confocal microscope (Axio Imager.Z1, Jena, Germany) with a ZEN 2008 that uses an excitation laser at 488/27 over an emission range of 490–560 nm and a 20× EC Plan Neofluar objective (N.A. 0.5) zoom 1.7. Software v. 5.0 (Zeiss) were used to acquire multichannel fluorescence images of the pulps obtained at the different severities. The pulp samples were suspended in water prior to the analysis.

FT-IR Analysis

The direct transmittance spectra of milled wood without pretreatment and pulp samples were measured using the KBr pellet technique. To prepare the discs, 1.5 mg of dried sample was mixed with 200 mg KBr (spectroscopic grade-Merck) in an agate mortar. The resulting mixture was successively pressed at 5000 psi for 2 min. The IR-spectra were recorded using a Perkin Elmer Spectrum System 2000 FT-IR spectrometer with a DTGS detector (PerkinElmer, Inc. Waltham, MA). The spectra were measured against air at a spectral resolution of 4 cm⁻¹, and 64 scans were taken per sample. The average spectrum for each sample was used for the evaluation.

2D-NMR Spectroscopy

For the 2D-NMR analysis of the whole cell walls, the dry pretreated materials were ball-milled using a Retsch PM100 vibratory ball mill at 600 rpm and zirconium dioxide (ZrO2) vessels (50 mL) containing ZrO2 ball bearings (10 × 10 mm). Each ~200 mg of sample was ground for 1 h (in 10 min on/5 min off interval cycles). The ball-milled pretreated material (approximately 50 mg) was transferred into 5 mm NMR tubes. The sample was distributed along the sides and bottom of the tube. DMSO-d6 Pyridine-d5 (4:1, v/v) was carefully added down the side of the NMR tube. The NMR tubes were sonicated for 30 min until the turbid gel began to clear and appeared to be homogeneous, according to the method previously developed (Kim et al., 2008; Kim and Ralph, 2014; Velle et al., 2009). The 2D-NMR heteronuclear single quantum coherence HSQC spectra were recorded at 25°C on a Bruker AVANCE 700 MHz spectrometer fitted with a cryogenically cooled 5 mm gradient probe with inverse geometry using Bruker's standard pulse sequence.

Enzymatic Saccharification

The autohydrolysis pretreated pulps were enzymatically hydrolyzed in 250 mL Erlenmeyer flasks at a substrate concentration of 10% (w/v) in sodium citrate buffer (pH 4.8, 0.05 M) using a commercial cellulase enzyme complex (NS-22128 CCN03128; 71 FPU mL⁻¹) supplemented with β-glucosidase (NS-22128 DCN00216; 265 CB mL⁻¹) at 50°C in a shaker at 150 rpm. The enzyme dosages used were 20 FPU and 20 CBU of cellulase and β-glucosidase, respectively, per gram of dry material. The enzymatic hydrolysis of each sample was performed for 72 h. The content of glucose released was analyzed by HPLC. The yield is expressed as the percentage of glucose released in the enzymatic hydrolysis divided by the potential glucose available in the pretreated material. All measurements were performed in triplicate.

Results and Discussion

Chemical Composition

The chemical composition of the wood and the pulps obtained from autohydrolysis pretreatment at different severity conditions (S₀) are shown in Table I. As expected from hydrothermal pretreatments, the main effect on the composition of the wood is the substantial removal of hemicelluloses. The content of xylans in the pulp obtained at S₀ = 3.80 was 3.6% (approximately 17% of the original amount in wood), and xylans were not detected in pulps generated at higher severities. The molecular weight and the presence of side chain substituents influence the solubility of xylans. Acetyl groups, arabinose, and uronic acid positively affect the solubility of xylans. Increasing the severity favors the removal of xylans due to the change in pH, which results from the release of the acetic acid that catalyzes the hydrolysis reaction. At the beginning, the easily
removed xylans are lignin-free with a low degree of polymerization (DP). As the hydrolysis proceeds, the low-DP fractions increased and were solubilized. The xylans bound to lignin required more drastic conditions for cleavage, and these are likely the xylans that remained in the pulp depending on the reaction severity. The acidity of the pretreatment facilitates the cleavage of linkages between lignin and oligosaccharides (Pu et al., 2013). The content of glucans in the solid fraction varied between 58% and 90%, depending on the severity. The amount of glucans in the pulps obtained at \( S_o \) values of 3.89, 4.20, 4.48, and 4.78 represented 93%, 89%, 87%, and 82% of the original content in the wood sample, respectively. The lignin content in the pretreated material (sum of the solid residue in the hydrolysis in two steps with sulfuric acid and soluble lignin) varied between 25% and 37%, representing 75%, 63%, 76%, and 98% of the value determined in wood for \( S_o = 3.89, 4.20, 4.48, \) and 4.78, respectively. Due to the high value determined for Klason lignin at \( S_o = 4.78 \), it is possible that not only lignin but also pseudolignin was quantified. Hydrothermal pretreatment can fragment the lignin, which generally results in a slight delignification of the biomass depending on the severity of the pretreatment. The relative increase in the content of lignin in the pretreated materials at the higher severity conditions is partly due to the concomitant loss of polysaccharides and the formation of condensed lignin products (Leschinsky et al., 2008; Santos et al., 2013). This increase could also be due to the formation of lignin-like compounds, as reported in previous studies (Hu et al., 2012; Kumar et al., 2013; Sannigrahi et al., 2011). At high pretreatment temperatures, the polysaccharides suffered acid-catalyzed dehydration, yielding structures that are referred to as pseudo-lignin. Pseudo-lignin, which is not derived from native lignin, is acid insoluble and, therefore, contributes to relatively higher values in the determination of Klason lignin.

### Lignin Distribution

The SEM images of the hydrothermally pretreated samples obtained at different severity conditions are shown in Figure 1. The surface of the pretreated material was strongly disrupted, mainly in the samples obtained at higher pretreatment severities. The formation of discrete droplets on the cell wall surface was observed, and these droplets are characteristics of lignin deposits, as reported by Kristensen et al. (2008). These droplets have also been attributed to pseudo-lignin, which usually is spherical in structure (Hu et al., 2012; Pu et al., 2013; Sannigrahi et al., 2011). The distribution, size, and abundance of these droplets over the fiber vary, depending on the pretreatment severity, and are more evident in the pulps obtained at higher severities (\( S_o = 4.78 \)).

Donohoe et al. (2008) reported that lignin mobilizes and expands within the cell wall matrix at pretreatment temperatures exceeding its melting temperature. In an aqueous environment, the lignin settles as droplets on the cell walls to reduce its hydrophobic surface in contact with water. This evidence was also supported by Kristensen et al. (2008) and Selig et al. (2007). The regions of the cell wall adjacent to the coalesced lignin appear to open, to improve the glucose yield during enzymatic hydrolysis.

### Table I. Chemical composition of wood and pulps obtained from autohydrolysis pretreatment at different severities.

<table>
<thead>
<tr>
<th>Severity ( S_o ) (temperature, °C)</th>
<th>Solid fraction (%)</th>
<th>Glucans (%)</th>
<th>Xylans (%)</th>
<th>Arabinosyl groups (%)</th>
<th>Acetyl groups (%)</th>
<th>Total lignin (%)</th>
<th>Glucose yield E.H. at 72 h (DPB) (%)</th>
<th>Glucose yield E.H. at 72 h (DWB) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood chips</td>
<td>46.9 ± 0.2</td>
<td>14.4 ± 0.2</td>
<td>0.7 ± 0.1</td>
<td>3.4 ± 0.3</td>
<td>nd</td>
<td>24.9 ± 0.4</td>
<td>58.4 ± 0.7</td>
<td>49.1 ± 0.6</td>
</tr>
<tr>
<td>3.89 (175)</td>
<td>69.2</td>
<td>63.3 ± 0.3</td>
<td>3.55 ± 0.02</td>
<td>nd</td>
<td>nd</td>
<td>26.9 ± 0.3</td>
<td>81.2 ± 1.1</td>
<td>64.9 ± 0.9</td>
</tr>
<tr>
<td>4.20 (185)</td>
<td>61.3</td>
<td>68.0 ± 0.3</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>30.3 ± 0.1</td>
<td>89.3 ± 0.8</td>
<td>66.5 ± 0.6</td>
</tr>
<tr>
<td>4.48 (195)</td>
<td>62.5</td>
<td>64.9 ± 0.2</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>37.0 ± 0.1</td>
<td>90.2 ± 1.1</td>
<td>69.6 ± 0.8</td>
</tr>
<tr>
<td>4.78 (205)</td>
<td>66.1</td>
<td>58.1 ± 0.1</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

Time at maximum temperature 30 min; water, wood ratio 4:1; nd, not detected; all concentrations are on a dry basis. E.H., enzymatic hydrolysis; DPB, dry pretreated material basis; DWB, dry wood basis.

### Figure 1

![SEM images of fibers: (a) untreated E. globulus wood and (b) pretreated materials obtained by autohydrolysis at higher severity \( S_o = 4.78 \), with droplets associated with lignin coalescence over the fiber.](image-url)
the accessibility of cellulose to enzymes (Kristensen et al., 2008). However, Selig et al. (2007) noted that the re-deposited lignin droplets on the biomass surface detrimentally impact enzymatic hydrolysis.

**Laser Scanning Confocal Microscopy (LSCM)**

The intrinsic autofluorescence of lignin (emission at 530 nm) can be used to assess its localization on the cell wall. Figure 2 shows the LSCM image of the pulp obtained at a severity of \( S_o = 4.78 \). Small spheres associated with lignin condensation were observed along the fiber, as previously observed by Romani et al. (2010). The droplets were present not only on the cell wall surface, but they also coalesced inside the fiber. The migration and relocation in the concentrated distribution of lignin during autohydrolysis pretreatment could increase the accessibility to the cellulose microfibrils.

**FT-IR Analysis**

The FT-IR spectra of the *E. globulus* wood and pretreated samples are shown in Figure 3. The wood and pulps obtained at low severity showed a band at 1743 cm\(^{-1}\), which was assigned to the C═O stretching from acetyl groups in hemicelluloses (Schwanninger et al., 2004). This band disappears in the most severely pretreated samples, as the pretreatment removed hemicelluloses. The intensity of the band at 1595 cm\(^{-1}\), which corresponds to aromatic ring stretching (Schwanninger et al., 2004), negatively correlated with the severity, and a new band (near 1614 nm) emerged. The reduction or shift in this position is attributed to a condensation reaction and/or splitting of the lignin aliphatic side chains (Kumar et al., 2009). At 1502 cm\(^{-1}\), the wood yielded a band assigned to aromatic skeletal vibrations (Faix, 1991), which was reduced in the pretreated samples as the severity increased. A new band at 1517 cm\(^{-1}\) was observed, which was strongly enhanced in the sample obtained at \( S_o = 4.78 \), indicating a possible modification in the lignin structure (Schwanninger et al., 2004). The band near 1239 cm\(^{-1}\), which corresponds to the syringyl ring and C—O stretching in lignin and xylans (Popescu et al., 2007), decreased in the pretreated material. The sample obtained at \( S_o = 4.78 \) showed a signal at 1221 cm\(^{-1}\), which was related to the condensation of G-type units (Faix, 1991). The results showed that the structure of wood components, especially lignin, changed due to hydrothermal pretreatment. Some of the changes could be corroborated with the subsequent NMR analysis.

**NMR 2D-HSQC**

Solution-state 2D-NMR gives an interpretable structural fingerprint of the lignin and carbohydrate components of the cell wall, without structural modification beyond that applied during the ball milling and ultrasonication steps. This approach constitutes a rapid method to compare the structural characteristics of lignin and oligosaccharides (Kim et al., 2008; Yelle et al., 2008). The HSQC NMR spectra of *E. globulus* wood, and pulps obtained via autohydrolysis pretreatment, revealed the effect of the severity on the relative degradation of the lignin side chain and aromatic units. Figures 4 and 5 show the HSQC (1-bond \(^{13}\)C—H correlation) spectra from *E. globulus* wood and pretreated materials obtained at different severities. The peaks were assigned using a published database (Ralph et al., 2004). The spectra in Figure 4 show the aliphatic region (polysaccharide and lignin side chain). The \( \beta \)-aryl ether \( A_n \) contour (light blue) in the sample obtained at \( S_o = 4.20 \) is smaller than that obtained at \( S_o = 3.89 \) and disappears in the samples obtained at \( S_o = 4.48 \) and \( S_o = 4.78 \), providing qualitative evidence of the cleavage of \( \beta \)-aryl ether bonds. Many studies indicated that the cleavage of \( \beta \)-O-4’ units is the most important reaction during the acidic pretreatment of hardwoods, forming new phenolic lignin units and Hibbert’s ketone type substructures (Pu et al., 2013; Samuel et al., 2013; Santos et al., 2013). The formation of phenolic units is an important change to open the lignin structure to improve the cellulose substrate for further enzymatic hydrolysis (Moxley et al., 2012). Furthermore, \( \beta \)-aryl ether cleavage can result in new and more stable bonding, such as the C—C in the lignin structure (Cao et al., 2012; Li et al., 2007). This leads to further delignification and the formation of free hydroxyl groups and a decrease of aliphatic hydroxyl groups, according to Kangas et al. (2014) who also reported the formation of methylated quinones as intermediaries of the homolytic breaking of \( \beta \)-aryl ether bonds.
Additionally, the formed free radicals can also promote lignin condensation reactions (Holopainen-Mantila et al., 2013). During the pretreatment at high severities, the condensation increased the Klason lignin contents and the lignin-droplet deposition over the cell walls, as shown in the SEM images. Substructures such as resinol (purple) are fairly stable (Cao et al., 2012; Pu et al., 2013; Ralph et al., 2004) and withstand the hydrothermal pretreatment, even at elevated severities. The amount of acetylated xylans (orange) were reduced during the autohydrolysis pretreatment, and thus the intensity of these signals decreased until they disappeared in the samples obtained at \( S_0 = 4.48 \) and \( S_0 = 4.78 \). This change is expected for the progressive removal and deacetylation of hemicelluloses, which was confirmed based on the decrease and disappearance of the acetyl cross peaks as the severity increased at \( \delta_C/\delta_H = 20.9/2.01 \) (not shown). Acetyl groups in xylans were removed at low pretreatment severities, and then xylans are removed at intermediate severity conditions, while at the highest severity conditions, they were completely removed and only signals from cellulose in concordance with signals reported by Kim and Ralph (2014) were observed.

The aromatic/unsaturated regions of the HSQC spectra are shown in Figure 5. The aromatic rings of lignin units are the main cross-signals. \( C_2,6-H_2,6 \) correlation signals for syringyl units (S, dark green) were observed at \( \delta_C/\delta_H = 104.2/6.8 \) ppm in all samples, independent of the severity at which they were obtained. The \( C_2-H_2, C_5-H_5, \) and \( C_6-H_6 \) correlations for guaiacyl units (G, red) were observed at \( \delta_C/\delta_H = 111/7.0, 115/6.7-7.0, \) and \( 119/6.8 \) ppm, respectively, in the \( E. \) globulus wood without pretreatment, but disappeared in the pulp samples, indicating a decrease in the protonated aromatic carbon and the condensation of lignin in the hydrothermal pretreatment. New signals were observed in the spectra at \( \delta_C/\delta_H = 105.7/6.5, 112.5/6.7, \) and \( 115.05/6.8 \) ppm, which are similar to those reported by Kangas et al. (2014), which positively correlated with the pretreatment severity and could be associated with the re-condensation of lignin, and ring aromatic modification. Also a new signal was observed at \( \delta_C/\delta_H = 55.9/4.54 \), which could be associated with changes in resinol subunits due to \( \beta-\beta \) linkage modification produced at higher severity of the pretreatment, as previously observed by Tran et al. (2015) in Kraft lignin experiments.

Figure 3. Infrared spectra of 1850–800 cm\(^{-1}\) region from untreated \( E. \) globulus wood, and pretreated materials obtained by autohydrolysis at different severities.
Figure 4. Expanded side chain region of HSQC spectra of untreated *E. globulus* wood and pretreated materials obtained by autohydrolysis at different severities.

Figure 5. Expanded aromatic/unsaturated region of HSQC spectra of untreated *E. globulus* wood and pretreated materials obtained by autohydrolysis at different severities.
However, more research is still needed to extend the knowledge of the modification of lignin structure.

Enzymatic Saccharification

As indicated in Table I, the glucan content in the spent solids that were employed as substrate for enzymatic saccharification increased from 63.3% ($S_o = 3.89$) up to 68.0% ($S_o = 4.20$) and then decreased to 64.9% ($S_o = 4.48$) and 58.1% ($S_o = 4.78$). Figure 6 shows the experimental results expressed in terms of the glucan-to-glucose conversion. After 72 h, the conversion increased 58.4% for the solids obtained at $T_{max} = 175^\circ C$ ($S_o = 3.89$) to 81% for the solids obtained at $T_{max} = 205^\circ C$ ($S_o = 4.20–4.78$), achieving cellulose conversions in the range of 81-90% (on dry pulp base, dpb), and about 40–70% (on dry wood base, DWB), where the yield decreases by degradation of carbohydrates at higher severities. Table I shows the glucose yields of enzymatic hydrolysis from the pretreated material at different severities. The results indicate an increase in the glucose yields as the severity of the pretreatment increased, reaching, at $S_o = 4.78$, a glucose yield up to 90.2% (DPB) and 69.6% (DWB). Several studies had indicated that the presence of pseudo-lignin and lignin coalescence affected the enzymatic saccharification, forming inhibitory hydrophobic bonds with the cellulases (Donohoe et al., 2008; Kumar et al., 2013; Mussatto et al., 2008). However, in this work, the re-distribution of lignin-like droplets above the fiber does not affect negatively the enzymatic saccharification. Indeed, this lignin redistribution, together with xylan removal, improved considerably the enzymatic hydrolysis.

Conclusions

Spectroscopic and morphological analyses allowed the identification of lignin migration and re-localization to a more concentrated distribution, as well as changes in lignin structure in hydrothermally pretreated $E. globulus$ wood, which was more evident at high severities. During autohydrolysis, the lignin was deposited as droplets on the surface of the cell-wall, and produced more exposed cellulose areas. Autohydrolysis did not affect the lignin content, but the accessibility to cellulose increased due to structural alterations and their redistribution over the fiber. This resulted in a high glucans-to-glucose conversion during the enzymatic saccharification, which was more relevant than non-productive adsorption of lignin with enzymes.

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References


Useful Method 204 cm⁻³ / TAPPi Useful Methods, Technical Association of the Pulp and Paper Industry, Atlanta, GA.