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CHEMICAL AND MICROSTRUCTURAL CHANGES IN *EUCALYPTUS GLOBULUS* FIBERS SUBJECTED TO FOUR DIFFERENT PRETREATMENTS AND THEIR INFLUENCE ON THE ENZYMATIC HYDROLYSIS

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ABSTRACT

In order to understand the relation between chemical composition, microscopic structure and enzymatic digestibility, different *Eucalyptus globulus* wood pretreated samples were examined. Pretreated materials obtained by steam explosion and autohydrolysis were compared with those obtained by organosolv and kraft processes. Chemical analyses of pretreated materials showed a decrease in the content of xylans, except in the kraft pulp. FT-IR spectra showed that the residual lignin in autohydrolysis pulp had experienced greater changes compared to those in steam explosion and organosolv pulps, whereas minor changes in lignin kraft pulp were observed. The fiber morphology indicated that autohydrolysis pretreatment was the most aggressive treatment. Reduction in the content of lignin and its redistribution on the fiber wall were confirmed through confocal laser microscopy. The formation of discrete lignin droplets deposited on the surface of the fibers was observed in all pretreatments, with a higher frequency in organosolv followed by steam explosion. A significant increase in enzymatic accessibility was achieved in organosolv, autohydrolysis and steam explosion pulps, due to xylans removal combined with lignin redistribution. Homogeneous lignin distribution and higher xylan content may be related to the low enzymatic hydrolysis efficiency in kraft pulp.

***Keywords*:** Enzymatic hydrolysis, lignin micro-droplets, organosolv, kraft process, steam explosion, autohydrolysis, *Eucalyptus globulus*.

INTRODUCTION

Lignin plays an important role in enzymatic hydrolysis, functioning as a physical barrier for the enzymatic accessibility to cellulose. On the other hand, lignin increases the recalcitrance of the pretreated substrate via nonspecific binding and irreversible adsorption of cellulases, decreasing glucose yields [1-3]. The partial removal, modification and redistribution of lignin on cellulose fibers are very important to increasing the superficial area and improving enzymatic hydrolysis yield [4-5]. Currently, there are controversies with regard to the redistribution of lignin fragments in pretreated fibers. The lignin fragments can appear as spherical structures and/or amorphous lignin, which may occur due to high severity conditions in pretreatments that exceed the melting temperature of lignin. Hence, they cause their migration through the cell wall via coalescence and the eventual progressive collapse of microfibrils. Furthermore, the lignin droplets formed during pretreatment become mobile particles in the matrix of the biomass, and due to the aqueous medium, the particles return to be deposited on the fibers during cooling [4]. Donohoe et al. (2008) proposed that droplets of lignin may occlude porous structures in the cell wall, causing a blockade of enzymatic accessibility, and that this effect was not limited to the outermost surface of the wall. Importantly, the relative homogeneous distribution of lignin was modified by fusion and migration in more concentrated and localized particles. Probably, this process increased the accessibility to individual microfibrils within the cell wall. Furthermore, Kristensen et al. (2008) [6] arrived at a similar conclusion after performing analyses of wheat straw with hydrothermal and steam explosion pretreatments. Their findings indicated that the partial removal of hemicelluloses and lignin relocation were important factors in increasing digestibility. This was more important than disrupting the cell wall structure and crystallinity of cellulose.

Selig et al. (2007) [7] demonstrated that droplets of lignin generated under dilute acid conditions can negatively affect the saccharification of corn stover. However, these authors also proposed that the observed phenomenon could be due to the additional cellulose surface blockade that resulted in an increase in non-specific binding of enzymes to a larger surface area presented by smaller droplets. Recently, Li et al. (2014) [8] proposed a potential mechanism for the inhibition of enzymatic hydrolysis caused by lignin droplets deposited on fiber. These results showed that non-specific binding of enzymes to lignin is not the primary mechanism of inhibition but rather an obstruction of the surface of cellulose by these lignin droplets. However, both factors are potentially responsible depending on the chemical nature and size of the lignin polymer.

Different types of pretreatment can modify the biomass in different ways, thereby affecting the accessibility of the materials. Pretreatment can be classified into physical, chemical, physicochemical biological and combined pretreatments [9]. Autohydrolysis and steam explosion pretreatments are attractive for hardwoods because they can be used without the addition of chemical reagents to destructuralize biomass (autocatalysis) [10, 11]. These pretreatments are efficient in hemicellulose removal, though not in lignin removal. However, after homolytic disruption of the β-O-4 linkage from lignin, the formed radicals undergo rearrangement and radical coupling (condensation), resulting in a variety of phenolic subproducts derived from lignin [12]. Kraft process, currently used for pulp production, has also been reported as a pretreatment for the production of bioethanol via high efficiency delignification [13]. This is due to the presence of OH- and SH- ions in the pretreatment liquor that facilitate the formation of smaller and hydrophilic lignin molecules [14, 15]. The other delignification process is organosolv pretreatment that uses solvents to remove the lignin via depolymerization reaction that leave more exposed cellulose fibers and allowing the recovery of very little modified lignin, resulting in the production of a new quality product [16-18]. Moreover, several studies have shown that small spherical clusters of lignin and pseudo-lignin (from carbohydrate degradation)[19] were formed on the surface of the fiber at high severity pretreatment. These studies examined the role of pseudo lignin on enzymatic digestibility [7, 20-22].

An understanding of the effects of pretreatments on the raw material, pretreated material characteristics and barriers for enzymatic hydrolysis represented by lignin micro-droplets formation are essential for the development of economic and competitive processes for bioethanol production In this study, Eucalyptus globulus wood was pretreated by different methods. The effects produced on the material by steam explosion or autohydrolysis pretreatments (both of low delignification), were compared with those obtained by organosolv or kraft processes (both of high delignification). Chemical composition and micro-structures of the pretreated material and how the wood components were distributed (which mainly consisted of lignin distribution) and how the differences in the structure of the pretreated materials affected enzymatic accessibility, was studied. The pretreated materials were characterized by infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), confocal laser microscopy (CLM) and compositional analysis.

EXPERIMENTAL

Chemical characterization of raw and pretreated materials

E. globulus wood chips were provided by a pulp mill from the Biobío Region, Chile. The bark-free particulate size utilized was between 2.5 x 1.5 x 0.3 cm. Wood chips were milled in a knife mill and sieved through a 45/60 mesh. The milled wood was extracted with acetone:water (90:10 v/v) for 16 h to determine the amount of extractive material. The milled wood samples (300 mg) were hydrolyzed with 72% sulfuric acid (3 mL) at 30ºC for 1 h. Then, the acid was diluted to 4% (by the addition of 84 mL of water) and

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the mixture was heated at 121ºC, 1.1 atm, for 1 h. The resulting material was cooled and filtered through a porous glass filter (number 4). Solids were then dried to constant weight at 105ºC and determined as insoluble lignin. The soluble lignin concentration in the filtrate was determined by measuring the absorbance at 205 nm, using the value of 110 $Lg^{-1}cm^{-1}$ as the absorptivity. The concentration of monomeric sugars and acetic acid in the soluble fraction was determined by high-performance liquid chromatography (HPLC) (LaChrom-Merck-Hitachi (Tokyo, Japan)) equipped with a refractive index detector and Aminex HTX-87H column (Bio-Rad, Hercules, CA) at 45°C, a mobile phase of $5x10^{-3}$ mol L^{-1} H_2SO_4 and a flow rate of 0.6 mL min^{-1}. Glucose and xylose were used as external calibration standards. The glucans content was calculated by multiplying the glucose content by 0.9; the xylans content obtained form the xylose content multiplied by 0.88; and the acetyl groups content was calculated by multiplying the acetic acid content by 0.7. [23]

Pretreatments

Autohydrolysis was performed in a 1 gal Parr reactor (Parr Insstruments, Moline, IL) loaded with 100 g of wood chips (dry basis) and water-wood ratio of 6:1 (v/w). The reactor was heated at 3 ºC min^{-1}, and the cooking was conducted at 194ºC and residence time of 1 min.

Organosolv was performed in the same reactor, charged with 100 g of wood chips (dry basis) and ethanol:water (ethanol 37 %v/v): wood 6:1 (v/w), the reactor was heated at 3 ºC min^{-1}, and the cooking was carried at 206ºC for 6 min.

Kraft pulping was performed in a rotary digester equipped with 4 independent 1.5 L vessels (Regmed, Brazil). Each vessel was loaded with 100 g of wood chips (dry basis) and 400 mL of liquor with 10% active alkalis (AA), and 30% sulfidity (both calculated on a dry wood basis and expressed as NaOH equivalents). The reactor heating rate was 2.1ºC/min, and the cooking temperature was 160ºC for 30 min.

Steam explotion pretreatment was performed at 200ºC for 9.5 min in an equipment with a 5 L reactor vessel.

After each pretreatment reaction, the liquors were drained and the pulps were washed with abundant tap water. All pretreatment conditions were obtained in a previous work using experimental designs to maximize the glucose yields (data not shown).

FT-IR Analysis

FT-IR spectra of the pretreated materials were obtained in a Perkin Elmer Spectrum System 2000 FT-IR equipped with a triglycine sulfate detector and beam splitter of KBr. For the spectrum acquisition the KBr pellet technique was used. To prepare the discs, 2 mg of the sample (dried at 40ºC for 12 h) were mixed with 200 mg of KBr (spectroscopic grade-Merck) in an agate mortar. The resulting mixture was successively pressed at 5000 psi for 2 min. IR spectra were measured in the range 4000 a 400 cm^{-1} in a transmittance mode with 64 scans and 4 cm^{-1} of resolution. All of the spectra were adjusted at a baseline and normalized for comparison.

Scanning Electronic Microscopy (SEM)

Electronic microcopy images were obtained using a Jeol JSM-6380LV Scanning Electron Microscope with a secondary electron detector. Samples were dried at room temperature and then covered with a layer of gold. Images were obtained with voltage acceleration between 15 and 20 kV.

Confocal Laser Microscopy (CLM)

A confocal spectral microscope Zeiss model LSM780 with a laser line Ar488 nm and objective Plan-Neofluar 20x/0.50 was used. Fibers were suspended in nanopure quality water for 15 min. Two drops of a suspension of each sample were placed on a slide and analyzed.

Enzymatic hydrolysis (EH)

For enzymatic hydrolysis, cellulase NS-22128 (71 FPU/mL) supplemented with β-glucosidase NS-22118 (370 CBU/mL) provided by Novozymes (Denmark) was used. The enzyme dosage utilized was 20 FPU and 20 CBU per gram of pretreated material. Enzymatic hydrolysis was performed in 125-mL Erlenmeyer flasks at substrate concentration of 10% (w/v) in 50 mL sodium citrate buffer (pH 4.8, 0.05 mol L^{-1}) in an orbital shaking incubator at 50ºC and 150 rpm for 72 h. The glucose content 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 raw material and pretreated materials is shown in Table 1. The content of lignin observed in the kraft and organosolv pretreated materials was lower than those in autohydrolysis and steam explosion pretreated materials. A high and similar (~85%) removal of xylan by autohydrolysis, steam explosion and organosolv pretreatments were observed, but by kraft the removal was only of 32%. Moreover, the conservation of glucans in the pretreated materials was higher than 90% of the content in the raw material for the four pretreatments. In kraft and autohydrolysis pretreatments more glucans were degraded, removing 8.4% and 6.6%, respectively (Table 1). However, in the kraft process only achieved a xylan removal of 32%. In autohydrolysis and steam explosion pretreatments, a high removal of xylan is expected because the acidic conditions generated by the release of organic acid from biomass components and a decrease of the pK_w of water at the elevated temperature, facilitating the solubilization of hemicellulose. In contrast, the material obtained by the kraft process has a high content of xylan, this process was designed to preserve hemicellulose to produce a greater strength of cellulose fibers. In relationship to the content of lignin, organosolv pretreatment was the most efficient in terms of removing lignin, reaching 51.1%, followed by 39.0% for the kraft process. The autohydrolysis and steam explosion pretreatments achieved a slight delignification, with a removed of 12 %.

Table 1: Pulp yield, chemical characterization of raw material and pretreated materials after different pretreatments (expressed in dry %wood).

	Pulp yield [a]	Glucan[b]	Xylan[b]	Acetyl groups	Lignin[b]
Raw material	--	43.6	16.2	3.7	26.4
Kraft	75.6	39.9 ± 1.9 (8.4)	11.0 ± 0.6 (32)	ND[c]	16.1 ± 0.6 (39)
Organosolv	60.9	43.4 ± 1.0 (0.5)	2.4 ± 0.0 (85)	0.9± 0.0	12.9 ± 0.4 (51)
Autohydrolysis	71.1	40.7 ± 1.2 (6.6)	2.4 ± 0.1 (85)	1.1± 0.1	23.2 ± 1.6 (12)
Steam explosion	72.0	42.1 ± 0.6 (3.4)	2.7 ± 0.0 (83)	1.1± 0.1	23.2 ± 0.2 (12)

[a] Pulp yield determined for each pretreatment (%), [b] removal percentage in parentheses, [c] Non determined

FT-IR analysis

The FT-IR spectra of the *E. globulus* wood (used as control) and pretreated materials are shown in Figure 1. All the samples showed a band at 1740 cm^{-1}, assigned to the C=O stretching from acetyl groups in hemicelluloses. [20] The intensity of this band decreased in all the pretreated materials, indicating a high removal of acetyl groups. In autohydrolysis, organosolv and steam explosion pretreatments this decrease in the band intensity is due mainly to hemicellulose solubilization. However, the kraft pretreated material presented high xylan content, although it was highly deacetilated. Both findings are supported by chemical characterization (Table 1) where a significant decrease in acetyl and xylan groups was observed for the material pretreated by autohydrolysis, organosolv or steam explosion. However, the kraft pretreated material presented high xylan content, and a low acetyl groups percent.

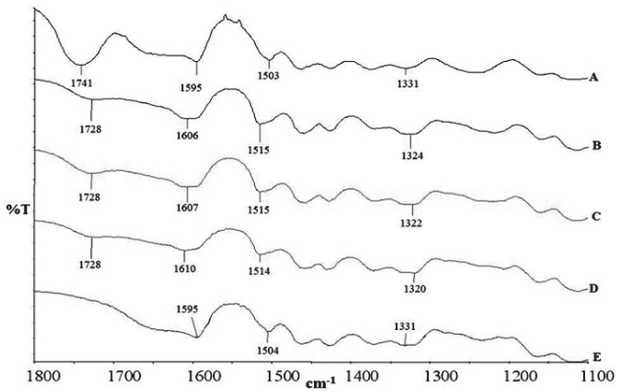

Figure 1: Infra-red spectrum of raw and pretreated material between 1800 and 1100 cm^{-1}. A: *E. globulus* as the control; B: Autohydrolysis pulps; C: Steam explosion pulp; D: Organosolv pulps and E: kraft pulp.

The intensities of the bands at 1595 cm^{-1}, corresponding to aromatic ring stretching, and 1505 cm^{-1}, assigned to aromatic skeletal vibrations, associated with lignin [24], were greater in the material pretreated by autohydrolysis. This could be due to the incorporation of new aromatic systems, added to the splitting of the lignin aliphatic side chains and the formation of new covalent bonds by condensation reactions [25]. This was also confirmed by comparing the lignin/carbohydrate ratio [26] of the pretreated materials contrasted to wood (not pretreated) using the band of 1505 cm^{-1} for lignin and bands of (a)1375 cm^{-1}, (b) 1158 cm^{-1} and (c) 895 cm^{-1}, which corresponded to: (a) bending of the C-H bond of cellulose I, cellulose II and hemicellulose; (b) the C-O-C asymmetric stretching link of cellulose I and cellulose II; and (c) the symmetric stretch C-O-C, O-C-C and C-C-H links of amorphous cellulose, respectively. For the material obtained by autohydrolysis, higher or similar values of these ratios (compared with those obtained for the raw material) were determined, because the slight delignification and high carbohydrates conservation during pretreatment. Similar effect was observed in the steam explosion pretreated material (Table 2). In the kraft and organosolv pretreated materials, a decrease in the lignin/carbohydrate ratio was observed due to the decrease in lignin content in both pretreated materials (Table 1).

Table 2: Lignin/carbohydrate ratio calculated from absorption (A) in the FT-IR spectrum.

	A 1505/ A 1375	A 1505/ A 1158	A 1505/ A 895
Raw material	0.74	0.63	2.1
Kraft	0.62	0.41	1.7
Organosolv	0.56	0.38	2.0
Autohydrolysis	0.86	0.60	3.4
Steam explosion	0.73	0.50	2.5

In autohydrolysis and steam explosion pretreated materials, the appearance of a band at 1221 cm^{-1} indicated a higher content of guaiacyl lignin condensate compared to etherified guaiacyl [24], where the majority of ether linkages are derived from the β-O-4 structure (50 links per100 C9 units) [27], indicating a breakdown of these structures and a potential increase in C-C bonds, such as β-β, 5-5 or β-5, generating more stable structures under pretreatment conditions. Another observed effect was the displacement of bands (in relation to the position of the bands in the non-pretreated material) at 1595cm^{-1} and 1505 cm^{-1} to 1610 cm^{-1} and 1515 cm^{-1}, respectively. This finding was due to the disruption of the propyl group and β-O-4 structures [28]. This was also related to the band at 1330 cm^{-1}, producing an increase in intensity compared to the non-pretreated feedstock. This band also corresponds to the stretching of the CH bond of the aromatic ring, particularly in syringyl lignin [29]. However, this band has been observed when guaiacyls were condensed in the 5-position [30]; this effect demonstrates the greater intensity in steam explosion and autohydrolysis pulps. Moreover, the band shift (1330 cm^{-1}) in kraft pretreated materials was not observed, indicating that the kraft lignin is not modified at the level that occurs in the other three pretreatments and retains some of its original structure.

Scanning electron microscopy (SEM)

The effect on the physical characteristics of the pretreated *Eucalyptus* fibers was observed through SEM. The morphological characteristics of the pretreated samples obtained by autohydrolysis and steam explosions (Fig. 2H and 2J) showed a high fiber deterioration compared to the kraft and organosolv pretreated materials (Fig. 2B and 2D). The mechanism of the kraft delignification process aims to maintain the highest integrity of the fiber without removing large amounts of hemicellulose, which is required to bring good qualities to the paper. A similar effect is observed by organosolv process, but it is more severe compared to the kraft process.

It was possible to observe particles on the fibers in the four evaluated pretreatments. Some of the particles are spherical and others are irregular (Fig. 2). The particles are characteristics of lignin deposits, as a consequence of the lignin solubilization and subsequent coalescence into molten bodies that migrate within and out of the cell walls, and deposited as droplets on the cell walls. [4, 7] However, it is possible that these globules were pseudo-lignin resulting from degradation of carbohydrates fraction or some combination of both, lignin and pseudo-lignin. In the pretreatments, mainly under severe conditions, some molecules of pentoses and hexoses, released from hemicellulose and cellulose, generate through side reactions soluble furans and insoluble degradation products, termed pseudo-lignin (Qian 2010; Kumar 2013). The phenomenon of pseudo-lignin formation has been reported for a wide variety of pretreated substrates [4, 20, 31], but its mechanism has not been clearly defined.

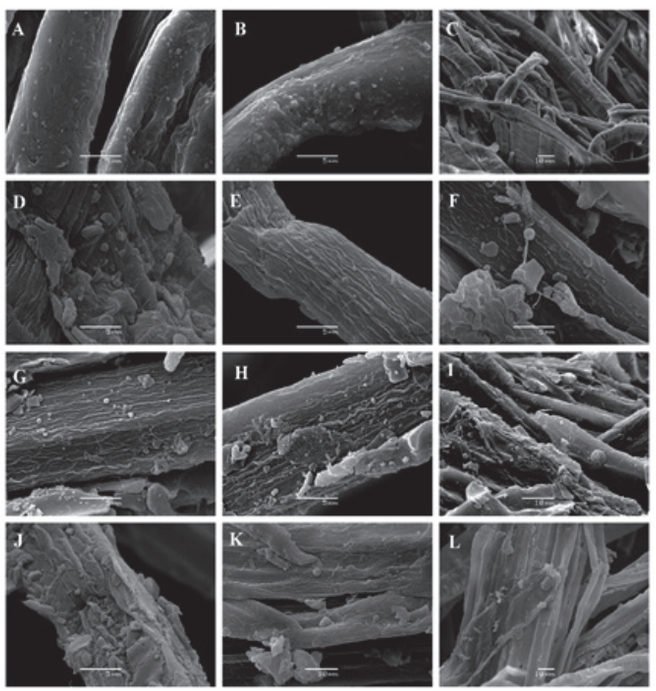

Figure 2: SEM images of fibers of pretreated materials obtained by kraft (A, B and C); organosolv (D, E and F); autohydrolysis (G, H and I) and steam explosion pretreatments (J, K and L).

Confocal laser microscopy (CLM)

Lignin is a phenolic macromolecule formed from the dehydrogenation of phenylpropanes followed by radical coupling, where the precursor molecules are the trans-coniferyl alcohol and sinapyl alcohol, guaiacyl and syringyl precursors, respectively [27]. It is known that different types of phenolic compounds, such as hydroxycinnamic acids, stilbenes and coumarins, are strongly fluorescent when irradiated with UV or blue light [32]. This enables the observation of the presence and redistribution of lignin on or along the pretreated fibers using CLM [33]. The distribution of fluorescence produced by the presence of lignin was found along the fibers in the four evaluated pretreatments, but it was mainly distributed in the fiber wall (Fig. 3). However, fluorescent droplets that corresponded to lignin micro-droplets deposited on the surface of the fibers were also found. The formation of these droplets was previously observed through SEM (Fig. 2D, 2I, 2K). Analysis of lignin remaining in the wall fibers in contrast to lignin micro-droplets formation may

allow for a better understanding of the effect of pretreatment on lignin and the cellulose accessibility to cellulolytic enzymes.

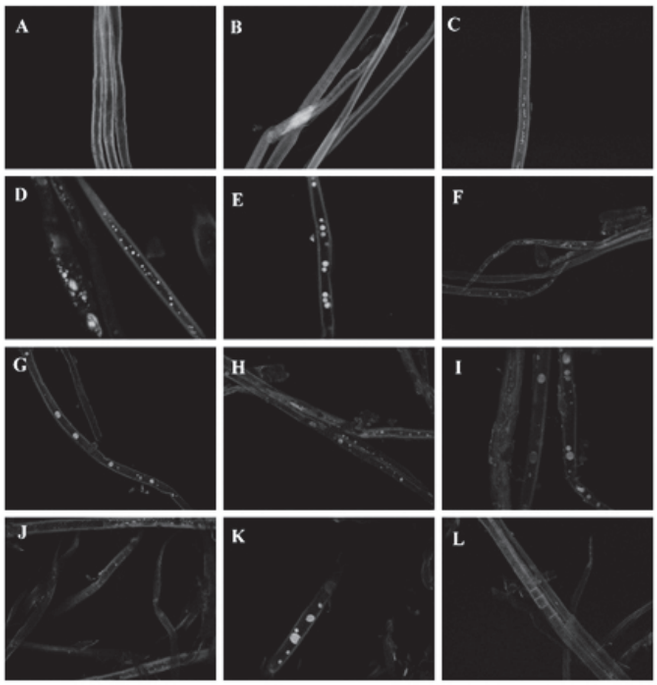

Figure 3: Lignin distribution in kraft (A, B and C); organosolv (D, E and F); autohydrolysis (G, H and I) and steam explosion pretreated materials (J, K and L) using CLM.

In organosolv, steam explosion and autohydrolysis processes, lignin is widely redistributed in superficial micro droplets and amorphous particles, whereas in the kraft process, the lignin is homogeneously distributed. However, it is also possible that in this new form of distribution, the lignin is accumulated in pores (pit, corners), since these regions are important for the diffusion of the enzymes, generating a new barrier to access cellulose [4]. Moreover, results of the present study are consistent with previous studies published by our research group. Thus, it this form of lignin is identified as an important factor for the high conversion of cellulose to glucose, the heterogeneous redistribution of lignin, resulting in, a significant decrease in the recalcitrance of pretreated materials [22, 34, 35].

In the pretreatment, when hemicellulose is removed, the lignin is more exposed to changes that promote its redistribution in the form of micro-droplets, generating random areas on the fibers with lower lignin content. The formation of lignin micro-droplets can be explained by a process of solubilization and subsequent precipitation of lignin on the fibers. At low temperature, due to the water high dielectric constant (78.54 a 25°C), the lignin is insoluble in water, but at the extreme conditions of temperature and pressure used in the pretreatments, the dielectric constant of water dramatically decrease, generating a favorable environmental for the solubilization of lignin. Subsequently during the cooling process, the dielectric constant of water is restoring causing the precipitation of lignin on the fibers.

Enzymatic hydrolysis (EH)

EH of kraft and organosolv pretreated materials showed 46% differences in glucose conversion, 54% and 99.5% (on dry wood base), respecively, despite having a similar content of lignin (Fig. 4, Table 1). This could be due to two factors: the first factor is related to the content of hemicelluloses in the pretreated material. The kraft material showed 14.5%, and the lignin can be chemically linked to the carbohydrate in the carbohydrate-lignin complex. This prevents access to cellulose. While, the content of hemicelluloses in the organosolv pretreated material was only 3.9%. The second factor is related to the distribution of the remaining lignin in the fibers, as it was observed through confocal laser microscopy (Fig. 3D, 3E and 3F). When the lignin is distributed in micro-droplets and amorphous particles on the surface of the pretreated fibers, leaves areas with lower lignin content and thus more cellulose accessible. In contrast, in the fibers of kraft pulp, lignin was distributed uniformly across the fiber wall (Fig. 3A, 3B and 3C), contributing to an increase in the recalcitrance of the substrate.

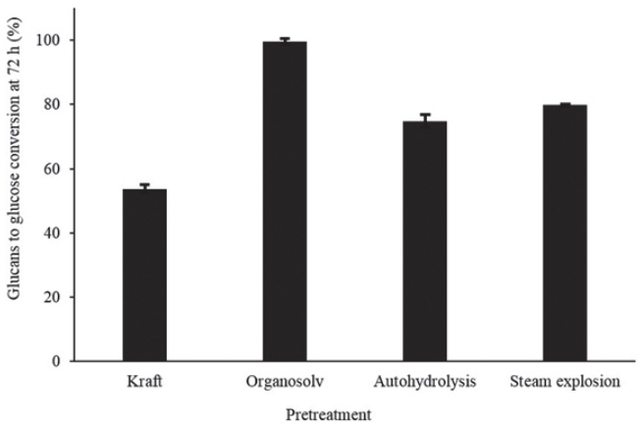

Figure 4: Enzymatic hydrolysis of glucan contained in *E. globulus* pulps from different pretreatment after 72 h on dry wood bases.

Regarding the pretreatments of steam explosion and autohydrolysis, the difference in enzymatic hydrolysis yield was 5% (Fig. 4), 80% and 75% on dry wood bases, respectively. Both pretreatments are similar and primarily based on acid autocatalysis. The acetic acid generated from the hydrolysis of acetyl groups in hemicelluloses catalyzes the partial hydrolysis of carbohydrates. It is likely that the difference in performance between the pretreatments is given by the chemical transformations that lignin undergoes when subjected to the severities of these pretreatments. These changes were observed using infrared spectroscopy, indicating a high probability that the lignin from autohydrolysis pretreatment was more condensed than the lignin from fibers pretreated by steam explosion (Fig. 1).

CONCLUSION

A low removal of hemicelluloses and homogeneous distribution of lignin on the fiber of kraft pulp were the main barriers to enzymatic hydrolysis resulting in a low yield of glucose. Exceptionally, lignin micro-droplets were observed in a single fiber by kraft process, this is not representative of this pretreatment. During autohydrolysis and steam explosion pretreatments, the lignin was deposited as droplets on the surface of the cell-wall, and produced more exposed cellulose areas. Both pretreatments affected no lignin content and a high quantity of hemicelluloses were removed. The accessibility to cellulose increased due to structural modification and the lignin relocalization over the fiber. These resulted in a high glucans to glucose conversion during enzymatic hydrolysis. Whereas the organosolv pretreatment was that with the greatest effect on the lignin content, a major presence of lignin droplets on the surface of cell-wall was observed. Highest conversion of glucans to glucose was obtained; product to high removal of xylan followed a heterogeneous redistribution of lignin, decreasing significantly the recalcitrance of *E. globulus* fibers.

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