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A Comprehensive Approach of *Eucalyptus globulus* Acid Sulfite Pretreatment for Enzymatic Hydrolysis

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Abstract: The effect of different acid sulfite pretreatment conditions on released components in the hydrolysates and the pretreated solid residues' response to enzymatic hydrolysis for *Eucalyptus globulus* chips was investigated. Sodium bisulfite (0–15%), and sulfuric acid (0–5%) were used to pretreat chips at 170 °C and 190 °C, for as long as 30 min. The hydrolysates were analyzed through high-performance liquid chromatography (HPLC) and spectrophotometry. Overall porosity and pores larger than 2.65 nm (size of a typical cellulase) on the solid residues were estimated using glucose and two dextrans with different hydrodynamic radii as probes. The external specific surface area was analyzed by dynamic light scattering. The solid residues underwent enzymatic hydrolysis with an enzymatic cocktail. Very high (84–95%) carbohydrate conversion was achieved for either an extensively delignified biomass or a biomass with very high content of sulfonated residual lignin (23.4%), since internal porosity enables enzymes accessibility. At least 5% sodium bisulfite and 1% sulfuric acid was required to attain a carbohydrate release above 90% in the enzymatic hydrolysis. Results suggest that the presence of sulfonated lignin does not impair the enzymatic hydrolysis rate and extent. The increase of pretreatment temperature had a positive effect mainly on the initial rate of carbohydrates release in the enzymatic hydrolysis. The increase of the wood material dimensions from pins to conventional chips significantly decreased the hemicellulose removal in acid sulfite pretreatment but had a small effect on the enzymatic yield.

Keywords: acid sulfite pretreatment; *Eucalyptus globulus*; enzymatic hydrolysis; carbohydrates yield; internal porosity; lignin

1. Introduction

The development of second-generation biofuels has been lately receiving a lot of attention due to the limitations of using crops for producing first-generation bioethanol [1,2]. Alternative feedstocks, such as lignocellulosic biomass, have been used to substitute crop feedstocks [2–5].

The difficulty of producing bioethanol from lignocellulosic biomass lies mainly in its recalcitrance and this is a major barrier to the economic development of bio-based fuels and products [6,7]. Although cellulose and hemicelluloses from lignocellulosic biomass are hydrolysable polysaccharides that give way to carbohydrates whose fermentation results in ethanol, the cell walls of lignocellulosic materials form a barrier that has a natural resistance to microbial and enzymatic deconstruction [6–9].

Several factors such as lignin content, lignin distribution and its structure, cellulose crystallinity and degree of polymerization, pore size and internal porosity, end-product inhibition, need for synergism, and non-productive enzyme adsorption on lignin have been suggested to account for the recalcitrance of lignocellulosic materials to pretreatment and notably to enzymatic hydrolysis [3,6,10–14]. Several different approaches are being developed to pretreat lignocellulosic materials so that its

complex architecture can be disrupted, thereby making its cellulosic components more accessible to enzymes [6,7,9,10,15].

Apart from delignification, lignocellulosic biomass pretreatments can cause structural changes in the fiber cell wall in the form of delamination and the development of pores of different sizes [16]. While mechanical treatments of the fibers contribute to an increase in the pores size in the cell wall due to the repeated bending, cracking, and compressing, chemical treatments, on the other hand, create new pores through the removal of lignin and polysaccharides [16,17]. Regarding lignin, apart from the physical barrier, the non-productive binding of enzymes on lignin is the critical issue [6,18]. The results from SPORL (Sulfite Pretreatment to Overcome Recalcitrance of Lignocelluloses) pretreatment of lignocellulosic biomasses [6] suggest that sulfonated lignin can remain in the solid residue without drastically impairing the enzymatic hydrolysis. Another prerequisite for an effective enzymatic hydrolysis is enzyme accessibility [19–21]. Grönqvist et al. reported that the formation of micro- and macropores in the pulp fibers during mechanical shredding correlates with the susceptibility of the fibers to enzymatic hydrolysis [19]. Moreover, the formation of pores in the pulp fibers with a minimum size similar to that of the enzyme's hydrodynamic radii during mechanical and chemical treatments correlates with the susceptibility of the fibers to enzymatic hydrolysis [17,19]. Other authors [20,21] reported on the role of the cellulose accessibility, using more specific methodologies.

In a previous work, using high sulfite charges, we observed a very good enzymatic hydrolysis of pretreated *E. globulus* biomass, even with reduced enzymes charges [22]. In the present work, we have widened the range of the sulfite charges to lower values aiming to reduce the pretreatment cost. The effect of different pretreatment conditions on the carbohydrates and byproducts released into the pretreatment hydrolysates and the enzymatic digestibility of the corresponding solid residues was studied. The effect of the global pore volume and volumetric fraction of the solid residues' pores correspondent to the hydrodynamic radius of a catalytic unit of a typical cellulase enzyme on the enzymatic hydrolysis performance was also studied.

2. Materials and Methods

2.1. Raw Material Preparation

For the present study *Eucalyptus globulus* wood chips were milled in a knife mill (Retsch Mühle, Haan, Germany), coupled with an output sieve of 10 mm × 10 mm; the fine material was separated using an 18-mesh screen. The resulting material measured around 1–2 mm by 10 mm (pin chips). Normal sized chips (25 mm × 15 mm × 4 mm) were also used (conventional wood chips). The content of dry matter in the wood chips and the solid residues from pretreatment was determined according to the ISO 638:2008. The chemical composition of *E. globulus* wood is as follow: glucan: 49.8%; xylan: 15.0%; mannan: 1.2%; galactan: 2.2% acetic acid: 3.8%; total lignin: 23.1%; extractives: 1.0% [22].

2.2. Pretreatments

The pin chips as well as normal sized chips were pretreated using sodium bisulfite and/or sulfuric acid, as described in Costa et al. [22]. The conditions included sodium bisulfite charges that ranged between 0 and 15% and sulfuric acid charges that ranged from 0 to 5%; pretreatment temperatures of 170 °C and 190 °C were tested. The time at maximum temperature ranged from 0 to 30 min. Particular attention was devoted to small low charges of both chemicals aiming to make the process more sustainable. After the pretreatments, the reactors' content was diluted and thereafter fragmented in a laboratory blender for 1 min (Snijders Analysers, Tilburg, Holland). To prevent evaporation of volatile compounds, a sample of the hydrolysate from each reactor were collected through gravity aided filtration, prior to washing, and their chemical composition was analyzed by high-performance liquid chromatography (Thermo Scientific, Autosampler, Accela 600 pump, Accela PDA, Waltham, MA, USA) and UV-visible spectrophotometry (Thermo Scientific Helios Omega, Waltham, MA, USA), as described below. The solid residues were collected, after extensive washing with water by vacuum filtration, using

a filter paper as filtration medium. The weight and dry matter content of the solids and filter paper were determined. The solid residue yield (SRY) was determined as described elsewhere [22]. The contents of insoluble lignin, acid soluble lignin, and total carbohydrate in the wood chips and pretreated solid residues were determined. For this, a laboratory analytical procedure for the determination of structural carbohydrates and lignin in biomass, from the National Renewable Energy Laboratory (NREL) was used [23]. The content of lignin in the solid residues was also estimated through the ISO 302:2015 standard procedure for the determination of the kappa number, using the multiplying factor of 0.125 to convert kappa number in percentage of lignin. All the pretreated biomasses were preserved wet at 4 °C until the enzymatic hydrolysis stage. The sulfur content of the dry samples was estimated with a HITACHI S 2700 scanning electron microscope (Tokyo, Japan). Images were formed through secondary electrons. The high voltage used was 20 kV. An EDS (Energy dispersive X-ray spectroscopy) attachment was used to ascertain the sulfur presence on the solid residue biomasses.

2.3. Analysis of Pretreatment Hydrolysates

Carbohydrates, organic acids (acetic and formic acids) and carbohydrate byproducts (furfural and hydroxymethylfurfural, (HMF)) in the pretreatment hydrolysates were analyzed by a HPLC system coupled with an Aminex® column (HPX-87H, 300 × 7.8 mm) (Bio-Rad), a refractive index detector, and a UV-visible detector, as described elsewhere [22]. Samples were filtered with a 0.45 µm nitrocellulose syringe filter membrane, prior to the HPLC quantification. Carbohydrates and by-product yields, concerning the initial wood weight, were determined considering the compounds concentrations (arithmetic average of two measurements, at least) in the pretreatment hydrolysates and the final liquid-to-wood ratio (as described in the following for the soluble lignin yield (SLY)). Carbohydrates are expressed as anhydrous units. pH was measured at 20 °C, before and after pretreatment.

The soluble lignin and related compounds' quantification in the pretreatment hydrolysates was performed through UV-visible spectrophotometry using a Thermo Scientific Helios Omega UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), according to the aforementioned NREL's-laboratory analytical procedure [23]. Readings were performed in duplicate at a wavelength of 205 nm. Prior to the readings, appropriate dilutions were performed so that the read absorbances were in the range of 0.2 to 0.7. A sulfuric acid solution 3% (*w/w*) was used to dilute the samples and as a blank. An absorptivity coefficient of 110 $Lg^{-1}cm^{-1}$ was used for the lignin [24]. Therefore, the Beer-Lambert law was used to determine the soluble lignin and related compounds concentration (SL) in the hydrolysates, taking into account the dilution factor [24]. The SLY in hydrolysates, regarding the initial wood weight, was calculated through Equation (1):

$$SLY = (SL \times Vt/W) \times 100 \quad (1)$$

where SLY is the soluble lignin yield, (%); SL is the soluble lignin concentration, (gL^{-1}); Vt is the total volume of the hydrolysate (L) and W is the initial weight of the oven-dried (o.d.) wood (g).

2.4. Solid Residues Porosity

The global porosity of the pretreated solid residues was estimated by the measurement of the water retention value (WRV) according to the ISO 23714:2014 standard procedure or calculated based on the mass removal, assuming a material density of 1.5 g/mL.

To discriminate between the global porosity and the effective porosity for the enzymes to be applied later in the process, an attempt was done to determine the volumetric fraction of the solid residues' pores correspondent to a catalytic unit's hydrodynamic radius of a typical cellulase enzyme [18]. Dextrans and glucose were used as molecule probes: Dextran D11000 (Sigma-Aldrich, St. Louis, MO, USA) has a molecular weight of 9000–11,000 and a hydrodynamic radius of 2.65 nm, which is in the same order of magnitude of the catalytic unit of a typical cellulase enzyme [3]. Dextran D2000000 (Sigma-Aldrich, St. Louis, MO, USA) has a molecular weight of 1,500,000–2,000,000 and

a hydrodynamic radius of 35.06 nm. Glucose (Sigma-Aldrich, St. Louis, MO, USA) was also used as a probe molecule, to estimate the global porosity, assuming that its hydrodynamic radius (0.4 nm) is small enough to enter every pore.

The probes hydrodynamic radii were calculated according to Equation (2) [25].

$$r = [0.53(M)^{0.5}]/2 \quad (2)$$

where r is the hydrodynamic radius of the molecule (nm) and M is the molecular weight (g/mol).

Solutions with a concentration of 4% (*w/v*) were prepared for each probe molecule. A given amount of solid residue, after centrifugation under the same conditions of the WRV procedure, was added to the probe solution to attain a solid content of 6% in the falcon tubes. The tubes were vigorously agitated to ensure a good dispersion of the solutions and the probes were left in contact with the biomasses for over 24 h. A blank was performed for each biomass, adding water to the tubes, instead of the probe solutions. All essays were performed in duplicate. Afterwards, the supernatant was recovered by centrifugation at 3000× *g* for 15 min, covering the centrifuge tubes with parafilm to prevent water evaporation. The concentration of the probe in the supernatant was determined by measuring the rotation angle of polarized light in an ADP 220 Polarimeter (Bellingham & Stanley Ltd., Tunbridge Wells, UK), using water as baseline. The same procedure was followed for the blanks (to identify any carbohydrate possibly released from the pulp). The initial probe solutions were also tested. The decrease of the rotation angle in the samples, regarding the corresponding value for the initial probe solutions, which was attributed to the dilution water in the sample accessible to each probe, was calculated. It was assumed that the Dextran D2000000 does not penetrate the water inside the fiber wall and consequently the dilution effect observed corresponds to the free water. The added dilution observed for the Dextran D11000, regarding the D2000000 was assumed as the water in the pores larger than 2.65 nm. The values are normalized by the precise number of solid residues, determined by drying at 105 °C.

The external specific surface area (SSA) of the solid residues was determined using a LS Particle Analyzer (Beckman Coulter, LS 200, CA, USA).

2.5. Enzymatic Hydrolysis

A cellulosic ethanol enzyme kit from NOVOZYMES® (Denmark) composed of 6 different enzyme solutions was used to evaluate the enzymatic hydrolysis performance of the solid residues. An enzymatic cocktail was prepared from all 6 enzyme solutions, in order to achieve a synergic effect, adjusting the charge of each enzyme, according to Costa et al. [22], which represent a low enzyme charge (for cellulase 3.3 FPU/g of biomass). Subsequently, the cocktail was added to the previously extensively washed solid residues and they were subjected to enzymatic hydrolysis at a 1% solid content, a pH of 5.5 and at 50 °C, under permanent agitation. The enzymatic hydrolysis was maintained for as long as 144 h. Samples of the enzymatic hydrolysates were taken in duplicate at 2, 7, 15, and 24 h and then every 24 h after that. The hydrolysates' carbohydrate content was monitored by HPLC, as previously described.

2.6. Experimental Errors

At least two measurements were carried out for all variables and the mean values were reported. Experimental errors that were calculated as the standard deviation were shown by the error bars in the figures. For Tables 1 and 2 the standard deviations are as follows: pH ± 0.01; glucose ± 0.2; xylose, mannose, and galactose (XMG) ± 0.2; HMF ± 0.005; Furfural ± 0.005; acetic acid ± 0.1; formic acid ± 0.1; SLY ± 0.3; total removal ± 0.4; SRY ± 0.5; lignin ± 0.5; WRV ± 0.03; ε (SRY) ± 0.5; ε (>2.65 nm) ± 1; SSA ± 10. Excel® software was used to calculate the standard deviations and plot the error bars in the graphs.

3. Results and Discussion

Table 1 shows the pretreatment hydrolysates' pH, the percentage of compounds that were released, and the characteristics of the solid residues for the pretreatment experiments carried out at maximum temperature during 30 min, for the pin wood chips. The results for the reaction times of 0 and 15 min at 170 °C maximum temperature, as well as, those for the corresponding wood chips with conventional size and replications are available in Appendix A (Table A1).

The percentages of glucose, combined XMG, products of carbohydrate degradation (furfural and HMF), formic and acetic acids and also the combined soluble lignin and all other degradation products (pseudo-lignin) absorbing at 205 nm (SLY) that were released into the hydrolysates, concerning the initial biomass weight, were calculated following the HPLC determination of the corresponding compounds' concentrations in the pretreatment hydrolysates.

As it can be perceived in Table 1, the formation of acidic components (acetic and formic acids) during the pretreatments led, in general, to a decrease of the hydrolysates pH (initial liquor pH vs. final pH). The increase in the severity of the pretreatment conditions (namely acid charge and maximum temperature) has led to higher extraction of hemicelluloses, as expected. The increase of temperature from 170 °C to 190 °C, even during a short period of time (30 min), had an important impact on carbohydrates extraction; for mild chemical reaction conditions (e.g., 2s_1ac) the XMG removal increased from 14.5% to 18.5% on wood, which practically is the XMG content in the wood.

Interestingly, very high percentages of carbohydrates can also be released from the wood by delignification (without acid addition), as revealed by the experiments 15s_0ac (at 170 °C and 190 °C), highlighting the positive role of delignification, associated with the decrease in pH due to the acetic acid release from the xylan, on the hemicellulose extraction. As we can see in Table 1, the lignin content in the solid residue can be higher than that of the raw material. This can be due to the conjugation effect of two factors: the increase of lignin content because of hemicelluloses extraction and/or the formation of pseudo-lignin. In fact, when the SLY and the amount of residual lignin present the solid residue (as Klason lignin, determined directly through the aforementioned NREL procedure, or estimated using the kappa number; please see Figure A1 for the relationship between the lignin content determined through the two methodologies) are counted together, taking in account the SRY, the result is higher than the initial lignin content of the wood (23.1%), particularly for harsher hydrolysis pretreatment conditions (e.g., 15s_5ac and 2s_2.5ac). This increase is in good agreement with the degradation of carbohydrates in aromatic compounds and its precipitation on the solid residue, as was reported [21,26,27]. To improve the confidence in the reported values, the global mass balance was also done (column named "total" in Table 1) and the values are close to 100%.

With the purpose of evaluating the efficiency of the enzymatic hydrolysis stage, the lignin content in each solid residue was determined (Table 1) and the calculations of the total carbohydrate enzymatic yield were based on the lignin-free material. Using the previously mentioned NREL procedure, the carbohydrates composition of the solid residue was also evaluated for several samples. Hemicelluloses are practically absent in those carried out under the more acidic conditions (such as 0s_2.5ac, 5s_2.5ac and 15s_5ac), in accordance with their high concentration in the corresponding hydrolysates (Table 1).

Table 1. Solid residue yields and dissolved components in the pretreatment hydrolysates, for the different pretreatment conditions of pin wood chips (time at maximum temperature: 30 min).

Pretreatment Hydrolysates (% on Initial Biomass Weight)													
				Carbohydrates Yield (%)		Byproducts Yield (%)							
Pretreat Temp (°C)	Pretreat Liquor [a]	Initial Liquor pH	Final pH	Glucose	XMG	Furfural	HMF	Acetic Acid	Formic Acid	SLY [b] (%)	Total Removal (%)	SRY (%) (Lignin (%))	Total (%)
170	0s_0ac	6.91	3.09	0.4	11.2	0.007	0.006	1.5	0.5	5.3	18.9	77.4 (25.5)	98.5
	0s_1ac	1.60	1.41	3.5	11.5	0.024	0.008	5.1	1.0	10.6	31.7	64.7 (23.1)	96.4
	0s_2.5ac	1.29	1.33	2.7	14.5	0.010	0.009	4.6	1.2	7.5	30.5	67.3 (19.7)	97.8
	2s_1ac	1.57	1.69	2.4	14.5	0.030	0.014	4.8	1.3	22.4	45.4	51.7 (20.6)	97.1
	2s_2.5ac	1.58	1.33	4.1	16.7	0.031	0.014	3.9	1.2	17.4	43.3	54.7 (26.6)	98.0
	5s_1ac	2.33	1.95	2.2	17.1	0.145	0.011	4.4	0.8	21.5	46.2	52.1 (23.4)	98.3
	5s_2.5ac	1.75	1.24	5.4	16.9	0.042	0.017	4.2	1.4	20.2	48.2	50.1 (27.3)	98.2
	15s_0ac	4.26	2.71	2.8	17.1	0.092	0.053	3.4	1.0	21.3	45.7	53.6 (8.67)	99.3
	15s_5ac	1.44	1.32	2.6	16.9	0.362	0.056	4.6	1.5	21.6	47.6	49.5 (26.4)	97.1
190	0s_0ac	6.85	3.02	2.4	7.00	0.028	0.014	5.7	2.7	7.3	25.1	71.8 (26.3)	96.9
	0s_1ac	1.65	1.83	4.8	12.7	0.035	0.017	4.5	1.7	14.5	38.3	60.4 (18.6)	98.7
	2s_1ac	1.85	1.63	6.0	18.5	0.031	0.017	6.5	1.6	17.1	49.7	49.6 (20.7)	99.3
	15s_0ac	4.33	2.41	4.9	19.4	0.174	0.095	6.8	1.4	22.9	55.7	42.4 (7.35)	98.1
	15s_5ac	1.84	1.34	6.5	19.2	0.199	0.104	7.2	1.7	21.3	56.2	39.8 (27.0)	96.0

[a] 5s: bisulfite charge = 5%; 2.5ac = sulfuric acid charge = 2.5%; [b] includes all the components that absorbed at 205 nm; (XMG: combined xylose, mannose, and galactose; SLY: soluble lignin yield; SRY: solid residue yield).

Figure 1 shows the effect of the enzymatic hydrolysis reaction time on the total carbohydrate (includes glucose and XMG) release of the solid residues that were subjected to different pretreatment liquor compositions, at 170 °C.

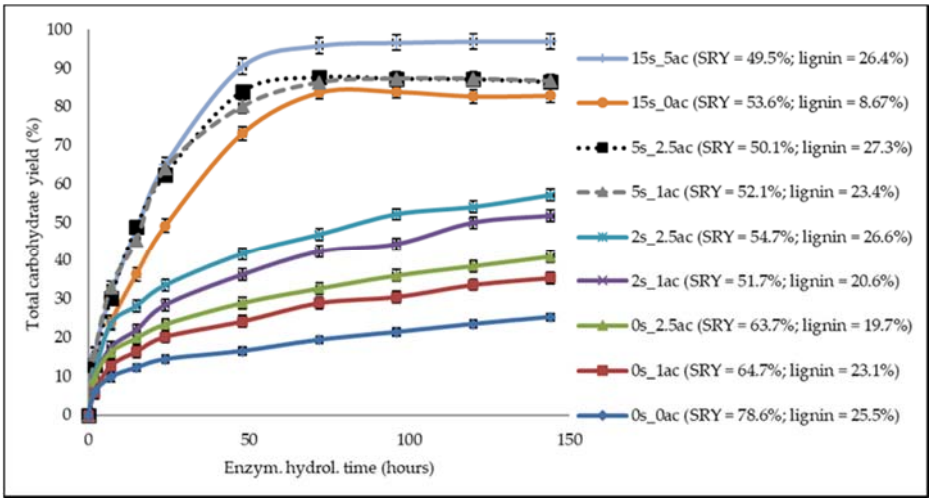

Figure 1. Effect of the enzymatic hydrolysis time on the total carbohydrates yield for biomasses pretreated with different liquor compositions at 170 °C for 30 min.

The carbohydrates released by the enzymatic cocktail clearly increases with the increase of acid charge in the pretreatment stage: the total carbohydrate yield increased from 25.2 to 35.4 and 41% when the charge of acid increased from 0 to 1 and 2.5%, with no bisulfite added. Similar behavior was observed for the sodium bisulfite charges of 2 and 5% and the maximum carbohydrates yield was attained at 15s_5ac (96.9%). However, at low bisulfite charge (2%) it was not possible to attain an acceptable carbohydrate yield in the enzymatic process (51.7 and 57.1% for 2s_1ac and 2s_2.5ac, respectively); the S content estimated by SEM-EDX is close 0 and 0.14%, respectively. A drastic increase in enzymatic hydrolysis was observed when the bisulfite charge increased from 2% to 5%, regardless of solid residues yields being of the same order of magnitude (51.7 and 54.7% for 2s_1ac and 2s_2.5ac vs. 52.1 and 50.1% for 5s_1ac and 5s_2.5ac, respectively). The S content of the 5s_2.5ac sample determined by SEM-EDX was 0.36%, proving the higher sulfonation of the residual lignin in the solid residue. Figure 2 presents the enzymatic yield as a function of the global porosity (increase) of the solid residue, because of the mass removal.

In this figure the uppermost cluster is associated with sulfite charge of 5% or higher, suggesting the role of lignin sulfonation, reported by several authors [28,29]. In addition, the enzymatic yield in the range 86.6–88.7%, obtained for 5s_1ac and 5s_2.5ac, is higher than that obtained for the 15s_0ac solid residue (82.9%), despite the low lignin content of the 15s_0ac solid residue (6.4.% of lignin vs. 19.5–21.1%; Table 1, SRY column). It should be mentioned, however, that the enzyme charges regarding the carbohydrates in the solid residue 15s_0ac are slightly lower than the charges for the 5s_2.5ac sample, due to the higher lignin content of the last one. These results suggest that the presence of sulfonated lignin in the solid residue, even in very high amounts, does not impair the enzymatic rate and extent, probably because sulfonation decreases the non-productive adsorption of enzymes on lignin [28,29]. Lignin extraction from the solid residue significantly enhances the process, but it seems that lignin extraction is not a prerequisite for extensive enzymatic hydrolysis, even for relatively low enzymatic charges, as is the case in the present work.

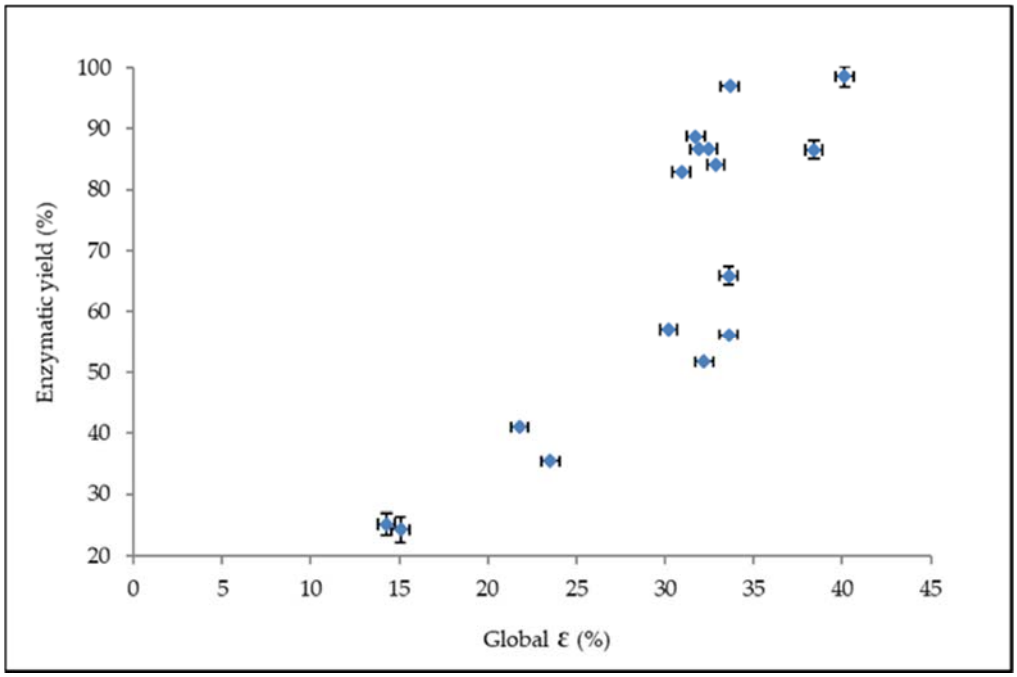

Figure 2. Effect of global porosity (ε) on the enzymatic yield.

To study the influence of temperature, some pretreatments were carried out at 190 °C. Figure 3 shows the effect of pretreatment temperature on enzymatic hydrolysis yield, for wood pin chips treated with 2% and 15% sulfite and 0 and 5% sulfuric acid.

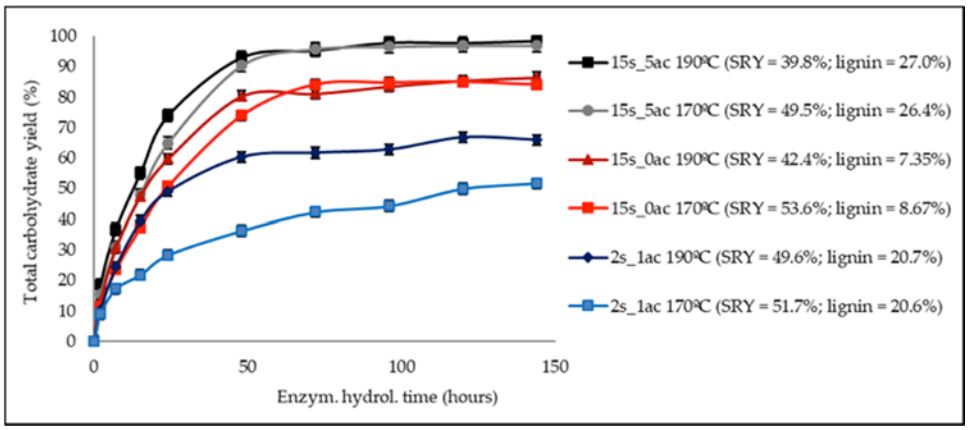

Figure 3. Effect of pretreatment temperature on total carbohydrates yield throughout the enzymatic hydrolysis.

As it can be seen in Figure 3, increasing the pretreatment temperature from 170 to 190 °C increased the initial rate of carbohydrates release during the enzymatic hydrolysis for all tested solid residues, even when the enzymatic hydrolysis extent remained unaffected (15s_0ac and 15s_5ac). Both effects are particularly notorious in the solid residue pretreated with 2s_1ac, where the extent of the reaction was also positively affected (from 51.7 to 65.9% for 170 and 190 °C, respectively), in addition to the initial reaction rate. These results are in good agreement with the increase of the WRV of the solid residues from 1.31 g/g to 1.35 g/g. The corresponding increase in WRV for the 15s_0ac was from 1.39 g/g to 1.51 g/g.

Regarding the effect of the wood chip size on the acid sulfite pretreatment there is a very notorious decrease of mass removal with the increase of chip size, from the wood pin chips to the conventional chips size (Table A1), which evidence the effect of the mass transfer rate on the acid sulfite pretreatment. The effect on the enzymatic hydrolysis yield is much smaller, around 2%, which indicates that reducing the size of the chips in the pretreatment is not worth it.

Table 2 presents the experimental data respecting WRV, global porosity estimated from the WRV (ε (WRV)) and the increment in porosity due to material removal (Δε(SRY)), porosity concerning pores with hydrodynamic radii higher than 2.65 nm (ε (>2.65 nm)), the external SSA and the maximum extent of polysaccharides enzymatic hydrolysis for some selected solid residues.

The enzymatic yield correlates positively with the WRV, which includes the water in the fiber wall but also the water between fibers. Because of this, WRV is not directly correlated with the internal porosity of the fiber wall. In addition, the increase in WRV can also be due to the lignin sulfonation, which increases its hydrophilicity.

Table 2. Physical properties of the pretreated solid residues and their correspondent enzymatic hydrolysis extent.

Pretreatment (170 °C)	WRV (g/g)	ε (WRV) (%)	Δε (SRY) (%)	ε (>2.65 nm) (%)	SSA, (cm^2g^{-1})	Enzymatic Hydrolysis Extent (%)
0s_0ac	1.10	62.7	15.1	28	1653	25.2
0s_2.5ac	1.25	65.6	21.8			41.0
2s_1ac	1.31	66.7	32.2	35	5678	51.7
5s_1ac	1.45	69.0	31.9			86.6
5s_2.5ac	1.53	70.1	33.3		5578	88.7
15s_0ac	1.39	67.9	30.9		1389	84.1
15s_5ac	1.43	68.6	33.7	38		96.9

The porosity concerning pores with hydrodynamic radii higher than 2.65 nm (ε (>2.65)) increased from 28% (0s_0ac, autohydrolysis) to 35% (2s_1ac), for moderate acid sulfite treatment conditions, but just a little more to 38% even for the harshest conditions (15s_5ac). This small increase can result from the balance between the created and collapsed pores. The collapse of the small pores was previously reported by Pihlajaniemi et al. [20]. Although the magnitude of the values presented is in good accordance with those reported by Grönqvist et al. [19], the increase of internal porosity (ε (>2.65)) from 35% (2s_1ac) to 38% (15_5ac) (around 9%) cannot justify the huge increase in the enzymatic yield from 51.7% to 96.9%. The WRV also increased about 9%. The reason behind these results can be related with others factors, such as the increase of cellulose accessibility, the decrease of cellulose's crystallinity index [20] and the increase in lignin hydrophilicity, as a consequence of sulfonation. The SEM-EDX results support this increase in sulfonation of the residual lignin in the solid residue.

The external SSA does certainly play a role on the initial enzymatic hydrolysis rate, as reported by other authors [12], but it does not seem to be the key parameter for the maximum enzymatic extent; similar external SSA (5678 vs. 5578) resulted in very different enzymatic yields (51.7% vs. 88.7%) and the 15s_0ac solid residue, which is mainly made of delignified individualized fibers, exhibits a very high enzymatic yield despite the low external SSA.

Close to 90% of enzymatic yield was obtained in the present study after a pretreatment with a 5% charge of sodium sulfite and 2.5% of sulfuric acid, at a cellulase charge of 3.3 FPU/g of solid residue. These results are better than previously reported [22], mainly in terms of chemical consumption.

4. Conclusions

The acid sulfite pretreatment of *Eucalyptus globulus* seems to be a technical viable step in the global process of carbohydrates release from the biomass. Almost all the hemicelluloses in the *E. globulus* can be extracted in the pretreatment step, using moderate reaction conditions (5s_1ac or 15s_0ac, 170 °C) and the cellulose-rich solid residue performs very well in the enzymatic process. Extensive delignification in the pretreatment, accompanied with moderate acidic conditions (16s_0ac, 170 °C), has a very positive

role in the enzymatic hydrolysis of the solid residue (83–84%). Global and small sized porosity are key parameters in the enzymatic performance, but extensive delignification seems not to be a prerequisite for extensive enzymatic hydrolysis, if the residual lignin was sulfonated. Actually, a charge of 5% sodium bisulfite and 1 to 2% of sulfuric acid was enough to also achieve a very good performance in the enzymatic hydrolysis stage (86–89%), despite the high lignin content (23.4%), suggesting that the presence of even high amounts of sulfonated lignin do not impair the enzymatic hydrolysis rate and extent, since internal pores are available. The increase of the pretreatment temperature (from 170 °C to 190 °C) had a positive effect mainly on the initial rate of carbohydrate release in the enzymatic hydrolysis, which correlates positively with the WRV obtained for the respective biomasses. The decrease in size of the chips has a positive, but marginal effect on the enzymatic process, regarding the huge effect of lignin sulfonation.

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Appendix A

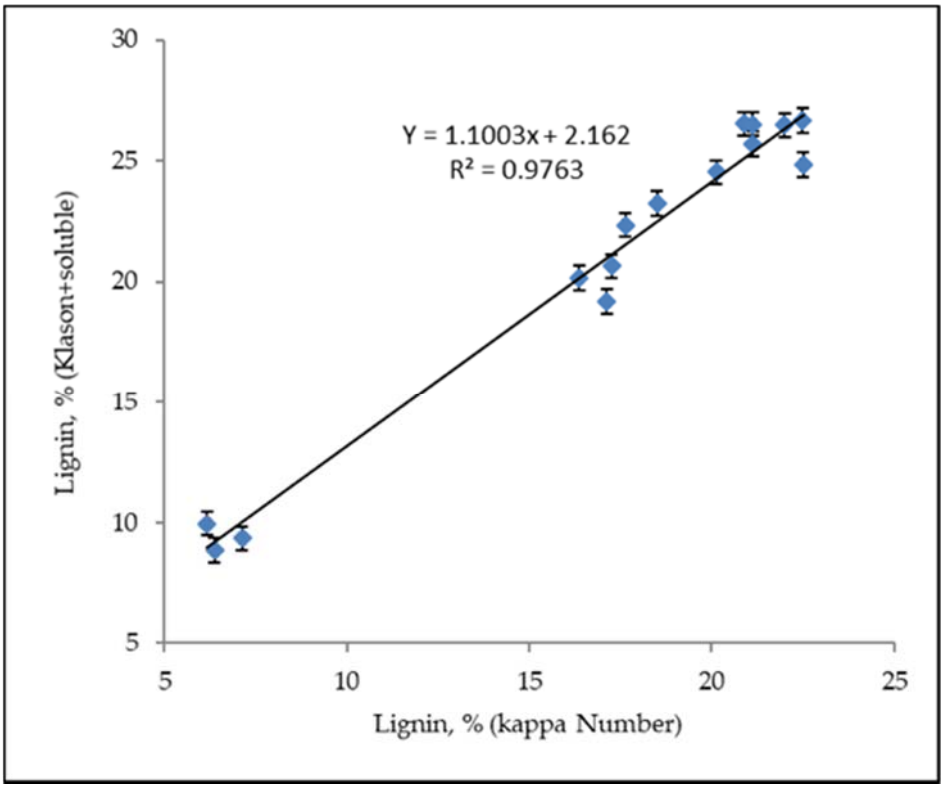

Figure A1. Relationship between the Klason lignin (included acid soluble lignin) and lignin estimated by the kappa number, using the conversion factor of 0.125.

Table A1. Dissolved components in the pretreatment hydrolysates and solid residue yields, for the different pretreatment conditions, using pin wood chips and conventional ones.

Pretreatment Hydrolysates (% on Initial Biomass Weight)														
					Carbohydrates Yield (%)		Byproducts Yield (%)				SLY [b] (%)	Total Removal (%)	SRY (%) (Lignin (%))	Total (%)
Pretreat Temp (°C)	Time (min)	Pretreat Liquor [a]	Initial Liquor pH	Final pH	Glucose	XMG	Furfural	HMF	Acetic Acid	Formic Acid				
170	0	0s_0ac	6.87	2.99	0.2	10.6	0.005	0.004	1.1	0.3	4.8	17.0	80.0 (22.4)	97.0
	15	0s_0ac	6.87	2,90	0.5	12.8	0.007	0.006	1.2	0.4	5.1	20.0	78.6 (22.5)	98.6
	0	0s_1ac	1.60	1.63	3.2	9.7	0.018	0.005	5.1	1.0	7.8	31.7	71.4 (18.5)	97.3
	15	0s_1ac	1.60	1.47	3.4	9.9	0.020	0.006	4.2	1.0	9.9	25.9	67.7 (18.9)	96.6
	0	0s_2.5ac	1.29	1.37	1.5	11.9	0.008	0.006	2.0	0.7	6.4	22.5	75.4 (15.3)	97.9
	15	0s_2.5ac	1.29	1.34	2.2	13.7	0.009	0.007	3.7	0.9	7.0	27.5	69.4 (15.9)	96.9
	0	2s_1ac	1.57	1.56	2.1	12.1	0.027	0.013	4.1	0.9	17.9	37.0	61.1 (15.8)	98.1
	15	2s_1ac	1.57	1.62	2.3	13.7	0.030	0.015	4.3	0.8	19.8	41.0	56.0 (16.3)	97.0
	0	2s_2.5ac	1.26	1.70	3.6	15.4	0.019	0.008	4.2	0.8	17.0	41.0	55.9 (19.9)	96.9
	15	2s_2.5ac	1.26	1.62	4.2	16.0	0.023	0.011	4.0	0.8	19.6	44.6	52.2 (20.3)	96.8
	0	15s_0ac	3.82	2.49	0.6	1.1	0.013	0.006	1.2	0.0	21.8	24.7	74.0 (8.9)	98.7
	15	15s_0ac	3.82	2.24	1.7	9.7	0.228	0.036	3.8	0.0	22.2	37.7	53.6 (7.9)	91.3
	0	15s_5ac	1.44	1.39	1.7	9.3	0.012	0.003	2.5	0.0	18.2	31.7	67.9 (18.9)	97.8
	15	15s_5ac	1.44	1.35	2.3	15.3	0.321	0.038	4.0	0.0	20.3	42.3	55.5 (20.5)	97.8
Conventional wood chips														
	30	5s_1ac	2.55	2.01	1.4	9.9	0.070	0.045	2.4	0.2	17.4	31.4	66.7 (29.7)	98.1
	30	5s_2.5ac	2.12	1.80	2.9	9.8	0.064	0.070	3.4	0.1	18.8	35.1	59.5 (31.2)	94.6
	0	15s_0ac	3.82	2.49	0.3	0.7	0.015	0.008	1.0	0.0	7.6	9.6	86.1 (17.1)	95.7
	15	15s_0ac	3.82	2.24	1.0	7.5	0.042	0.007	2.3	0.0	9.9	20-7	76.1 (19.1)	96.8
	30	15s_0ac	3.82	2.14	2.0	13.0	0.320	0.071	3.6	0.0	12.2	31.2	66.9 (21.8)	98.1
	0	15s_5ac	1.44	1.39	1.1	5.9	0.048	0.007	2.2	0.0	6.8	16.1	79.3 (17.7)	95.4
	15	15s_5ac	1.44	1.35	1.9	12.9	0.153	0.027	3.5	0.0	10.8	29.3	67.3 (21.1)	96.6
	30	15s_5ac	1.44	1.32	2.2	14.5	0.680	0.102	4.1	0.0	11.2	32.8	63.8 (22.8)	96.6

[a] 5s: bisulfite charge = 5%; 2.5ac = sulfuric acid charge = 2.5%; [b] includes all components absorbing at 205 nm; (XMG: xylose + mannose + galactose; SLY: soluble lignin yield; SRY: solid residue yield).

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