Industrial Crops & Products 130 (2019) 528–536

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop

Physical and chemical characteristics of pretreated slash pine sawdust influence its enzymatic hydrolysis

Julia Kruyeniski[a], Paulo J.T. Ferreira[b], Maria da Graça Videira Sousa Carvalho[b], María E. Vallejos[a], Fernando E. Felissia[a], María C. Area[a,*]

[a] Programa de Celulosa y Papel - Instituto de Materiales de Misiones, IMAM (UNaM-CONICET), Posadas, Argentina
[b] CIEPQPF, Department of Chemical Engineering, University of Coimbra, R. Sílvio Lima, Polo II, 3030-790, Coimbra, Portugal

ARTICLE INFO

Keywords:
Pretreatments
Delignification
Enzymatic hydrolysis
Pine sawdust
Crystallinity
Porosity

ABSTRACT

The aim of this work was to evaluate the effect of different pretreatments applied to industrial slash pine sawdust (*Pinus elliottii*) on its enzymatic hydrolysis. NREL laboratory analytical procedures for standard biomass analysis were used to characterize the thirty one fibrous materials produced. Eight pretreated materials were selected considering both the chemical composition and the enzymatic hydrolysis yield to further evaluate crystallinity and porosity, determined by X-ray diffraction and thermoporosimetry, respectively. Diluted acid, steam explosion, and slight alkali treatments revealed to be not effective to enhance enzymatic hydrolysis of resinous pines since a maximum yield value of only 25% was achieved. On the contrary, strong alkali treatments promoted the lignin removal, thus increasing the accessibility of the material for enzymatic hydrolysis. In accordance, the highest values of the cellulose crystallinity (about 75–76%) were also obtained. No correlation was found between the porosity and the enzymatic hydrolysis yield. It was concluded that to enhance enzymatic hydrolysis the lignin content of the pretreated material should below. As for the hemicelluloses content, only its interaction with the lignin content is significant, as revealed by the regression model obtained.

1. Introduction

The global interest in the use and production of biofuels has grown significantly in the last decade focused on the reduction of greenhouse gases emissions. National and international policies with subsidies and tax exemptions as well as biofuel blending directives have influenced the biofuel production (Cremonez et al., 2015; Laaksonen-Craig, 2008; Willem van Gelder et al., 2012).

The high volumes of processed wood in South American countries together with the objective of increasing the use of renewable energy make the second-generation bioethanol production a viable option to valorize the residues of the forest industry. In Argentina there is approximately 1.4 million hectares of planted forest, 60% of which located in the Northeast region of the country, with the predominance of softwoods(*ca.* 700,000 ha), mainly *Pinus elliottii* and *Pinus taeda* (Beale and Ortiz, 2013; SENASA, 2014).In addition, industrial wood processing generates nearly 50% of waste (1.5 million of dry ton per year) not properly availed (FAO, 2009; PROBIOMASA, 2012; Uasuf and Hilber, 2012),being pine sawdust one of the main residues. This is an attractive low-cost lignocellulosic biomass, due to its high hexoses content and broad availability, but nevertheless, it has not been exhaustively studied, mainly because of the high crystallinity of cellulose and the high lignin and extractives content. A typical chemical composition of *Pinus elliottii* (Misiones, Argentina) is about 41–44% of cellulose, 28–31% of lignin, 27–33% of hemicelluloses, and 2–4% of extractives (Stoffel et al., 2014).

The second generation bioethanol obtained from cellulose involves three stages: biomass pretreatment (fractionation), saccharification of cellulose, and fermentation. The cell wall polysaccharides are embedded in a lignin matrix that hinders their conversion to sugars, which can be further fermented to ethanol. The pretreatments are selected according to the physical and chemical characteristics of the raw material and aim to extract the hemicelluloses and lignin, opening void spaces (pores) in the fibres to increase the accessibility of the remaining components for the subsequent processing steps (Kafle et al., 2015; Lloyd et al., 2017). Endoglucanases, exoglucanases, and β-glucosidases or cellobiases form an enzymatic complex that acts synergistically to degrade cellulose to glucose (Kumar et al., 2012; Cameron et al., 2015). The pretreatments should avoid the formation of inhibitors for enzymatic hydrolysis and/or fermentation (Luo et al., 2010).

* Corresponding author.
E-mail address: cristinaarea@gmail.com (M.C. Area).

https://doi.org/10.1016/j.indcrop.2018.12.075
Received 22 July 2018; Received in revised form 20 December 2018; Accepted 22 December 2018

Different pretreatments may be used, namely with diluted acid, alkali or steam explosion. Both the dilute acid and steam explosion treatments remove hemicelluloses increasing the porosity of the fiber (Kumar et al., 2010; Newman et al., 2013; Stoffel et al., 2017, 2014). Nevertheless, lignin significantly affects cellulose swelling and accessibility, producing low enzymatic hydrolysis yields (Kumar et al., 2012).

Alkali is usually used for delignification, but it also degrades the polysaccharides, through the alkali-driven peeling reaction of the reducing end, and the chain scission caused by the oxidation of the glycosidic linkage between glucose residues (Park et al., 2015). This drawback may be overcome by the use of anthraquinone (AQ) as an additive since it promotes delignification and preserves the carbohydrates (Grace and Malcolm, 1989). However, although still used in Argentina at industrial scale, anthraquinone is not allowed in some countries and therefore new delignification processes must be tested to improve the yield of enzymatic hydrolysis (Kruyeniski et al., 2016; Meier, 2015).

The changes in the structure of the fiber wall, due to the hemicelluloses and lignin removal (and also relocation), increase its porosity and the surface area available for the contact of the enzymes with cellulose (Meng and Ragauskas, 2014; Donaldson and Vaidya, 2017). This is why porosity is usually measured to indicate the accessible surface area to cellulose enzymes (Meng et al., 2013). However, it has been reported that the increase of accessible pore volume when lignin contents are lower than 15% makes no significant difference for enzymatic hydrolysis yield (Stoffel et al., 2014; Vaidya et al., 2016; Yu et al., 2011). Some authors also claim that there is a reduction of the cellulose crystallinity resulting from the pretreatments, which also improves the conversion to glucose (Milagres et al., 2011), whereas others found the opposite (Kumar et al., 2013; Park et al., 2010; Yoshida et al., 2008).

Softwoods are highly recalcitrant to enzymatic hydrolysis (Morales et al., 2017; Stoffel et al., 2014; Suckling et al., 2017) and the more recent studies and reviews refer mostly to spruce and fir as a source for bioethanol production and just a few include softwoods or mixed softwoods (Alvarez-Vasco and Zhang, 2017; Inoue et al., 2016; Pan et al., 2005; Wingren et al., 2003).

In this context, the aim of this work was to evaluate the effect of different pretreatments applied to pine sawdust (*Pinus elliottii*) on the fibers chemical composition, crystallinity and porosity, and their consequences on the extent of the enzymatic hydrolysis. Although the influence of the content of hemicelluloses and lignin on enzymatic hydrolysis has been reported by other authors (Bansal et al., 2009; Draude et al., 2001; Pielhop et al., 2015; Zhu et al., 2010), it is the first time that a mathematical model, comprising simultaneously distinct fractionation processes aiming at extracting both compounds from the same raw material is presented.

2. Materials and methods

2.1. Materials

Slash pine sawdust (*Pinus elliottii*) was provided by a local sawmill (Forestal Eldorado and Forestal AM, Misiones). The sawdust was air-dried, screened and maintained in closed plastic bags. The fraction retained in a 3 mm square opening was used for the steam explosion pretreatments, whereas the fraction passing the 3 mm square openings and retained in a 0.177 mm square opening was used for the others pretreatments. Fines passing the 0.177 mm square openings were discarded. The chemical composition of pine sawdust was determined in a previous work.

Different treatments were performed and the corresponding details are presented elsewhere (Imlauer et al., 2011; Kruyeniski et al., 2015; Stoffel et al., 2017, 2014). A simplified experimental scheme of the treatments applied to the raw material (pine sawdust) and the range of treatment conditions are shown in Fig. 1.

An alkaline deresination was used as the first step for extractives removal, followed by a diluted acid hydrolysis as a second step for hemicelluloses extraction. Steam explosion (SE) was also used in this second step, with or without the application of the first step. A post alkaline washing was applied to some of the steam exploded materials. For another fraction of the raw-material, different treatments focused on lignin extraction were applied to the pine sawdust without previous desresination: kraft-anthraquinone, soda-anthraquinone, and organosolv. One of the soda-anthraquinone materials was further delignified by an oxygen step. Thirty-one treated materials were obtained and characterized to evaluate the relevance of their chemical composition.

2.2. Methods

2.2.1. Characterization of the fibrous materials

The chemical composition of the pretreated solids including structural carbohydrate components (glucose, mannose, galactose, xylose, arabinose) and both acid-insoluble and acid-soluble lignin (NREL/TP 510-42618),was determined by duplicate using the Laboratory Analytical Procedures (LAP) of the National Renewable Energy Laboratory (NREL).Carbohydrates were determined by HPLC (Waters chromatograph), equipped with refractive index detector, using a SHODEX SP810 column operating at 85 °C with ultrapure water as the mobile phase at a flow rate of 0.6 mL/min. The system was equipped with Deashing Refill Cartridges (Bio-Rad) and a Carbo-P Refill Cartridge (Bio-Rad) prior to the column. The quantification of acetic acid content in hydrolyzates (acetyl groups) was carried out by the same HPLC equipment but using an AMINEX-HPX97H (BIO-RAD) column operating at 35 °C, 4 mmolL^{-1} H_2SO_4 as eluent, 0.6 mL/min, quantifying by UV spectrophotometry using a diode array detector.

The concentration of the polymeric sugars was calculated from the concentration of the corresponding monomeric sugars, using an anhydrous correction factor of 0.88 (132/150) for C-5 sugars (xylose and arabinose) and of 0.90 (162/180) for C-6 sugars (glucose, galactose, and mannose).

In this work, the hemicelluloses content was calculated as the sum of hexosanes (mannans plus galactans) with pentosanes (xylans, acetyl groups, plus arabinans).

2.2.2. Crystallinity index (CrI)

The crystallinity of the original and treated solids was determined by X-ray diffraction, no duplicates were made. The sawdust was milled, dried (48 h at 40 °C), and the fraction passing a 0.177 mm square opening was used for analysis. Spectra were obtained using a Philips X' Pert-MDP diffractometer. The diffraction patterns were recorded using a cobalt X-ray source and data were collected at 0.025° (2θ) resolution, from 5° to 45° (2θ). Crystallinity index (CrI), was calculated from the height ratio between the intensity of the crystalline peak ($I_{002} - I_{am}$) and total intensity (I_{002}) and based on the Segal equation (Eq. 1) (French and Cintrón, 2013). The peaks I_{002} and I_{am} correspond to 2θ = 26° and 21.5°, respectively.

$$CrI = \left(\frac{I_{002} - I_{am}}{I_{002}}\right)x100 \quad (1)$$

2.2.3. Pore size distribution

The pore volume distribution of the studied materials was evaluated by thermoporometry based on differential scanning calorimetry (TP-DSC). Thermoporosimetry is considered an appropriated method to evaluated porosity in lignocellulosic materials. This technique is considered an appropriated method to evaluate porosity in lignocellulosic materials (Hill and Papadopoulos, 2001; Park et al., 2006), since other methods like nitrogen adsorption and desorption make the assumption that the structure of the pore is rigid (Sing, 2001). The trials were not conducted in duplicate.

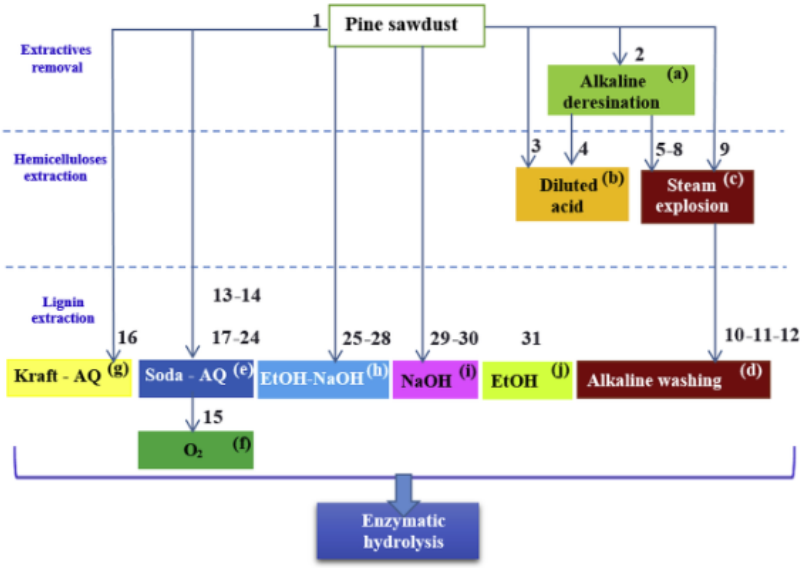

Fig. 1. Simplified scheme of the treatments applied to the pine sawdust. 31 treated samples were obtained. Operating conditions: a) 5:100 NaOH o.d.m. LSR 10/1, 90 °C, 60 min; b) 7.5 g L^{-1} H_2SO_4, LSR 10/1, 150 °C, 30 min; c) SE: 180–200 °C, 5–7.5 min, 0.75:100-3:100 H_2SO_4 o.d.m.; d) 60 °C, 60 min, 0.4:100 NaOH o.d.m., LSR 10/1; e) 19.8–55.2 g L^{-1}NaOH, 0.11:100 AQ o.d.m., 110–185 min, 170 °C, LSR 5–10/1; f) 5:100 NaOH o.d.m., 120 °C, 60 min, 4413 mmHg O_2; g) Kraft-AQ, 25 g L^{-1}NaOH, 0.11:100 AQ o.d.m., 170 °C, 170 min, LSR 10/1; h) 35/65 EtOH/H_2O, 30–50 g L^{-1}NaOH, 60–90 min, 170 °C, LSR 5/1; i) 30–50 g L^{-1}NaOH, 170 °C, 90 min, LSR 5/1; j) 35/65 EtOH/H_2O, 170 °C, 90 min, LSR 5/1; o.d.m.: on mass of dry wood. AQ: anthraquinone. LSR: Liquid to solid ratio.

TP-DSC was performed in a TA-Q100 instrument with a TA Refrigerated Cooling System 90. Never-dried samples (0.5–2 mg on mass of dry wood) were prepared inside T-zero aluminum pans, which were then sealed with a hermetic lid.

TP-DSC was performed according to procedures detailed elsewhere (Driemeier et al., 2012; Park et al., 2006). Samples were initially frozen at -70 °C and then stepwise heated to 5 °C, which melts in a controlled way the formed ice. Ice melting was calorimetrically detected and converted to ice mass. Ice melting below 0 °C is due to melting of ice in nanometric pores, with pore diameters θ estimated from melting temperature depression ΔT using the Gibbs-Thomson equation (Park et al., 2006):

$$\theta = -\frac{2K_c}{\Delta T} \quad (2)$$

K_c depends on the enthalpy and temperature of the bulk phase transition, liquid density, and ice-water interface energy. For TP-DSC of celluloses, a value of *Kc* of 19.8 nm °K was used (Park et al., 2006). The result of TP-DSC is a cumulative mass of confined ice (named freezing bound water) given as a function of θ. To obtain the distribution of pore sizes (*ΔPore volume* (mL/g)/*ΔPore Diameter* (nm)), the difference between neighboring values of the cumulative distribution is divided by the difference of neighboring values of the pore size (Park et al., 2006).

2.2.4. Enzymatic hydrolysis

The measurement of cellulose activity was done in terms of "filter paper units" according to the NREL/TP-510-42628 standard. The activity of β-glucosidases was determined by their capacity to hydrolyze 4-nitrophenol β-D-glucopyranoside (p-NPG) to 4-nitrophenol (p-NP). This method consists in adding 0.5 mL of different enzyme dilutions to 2 mL of 1 mmol L^{-1} of a p-NPG solution, incubating 30 min at 50 °C, and then stopping the reaction with 2.5 mL Na_2CO_3.Finally, the measure of absorbance is made at 400 nm (Matsuura et al., 1995).

The never dried solid material resulting from the pretreatment stage was submitted to saccharification with cellulases from *Trichoderma Reesei* and cellobiases from *Aspergillus niger* (trade names of commercial enzymes provided by Sigma-Aldrich), according to NREL-LAP standards (NREL/TP- 510-42629), with some modifications. This stage was made by duplicate. Cellulases is an enzymatic complex that includes: endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91), and β-glucosidases (EC 3.2.1.21).

The hydrolysis reaction was performed by shaking the material at 130 rpm for 72 h at 50 °C and pH 4.8 adjusted with 0.05 M sodium citrate buffer. The enzyme dose was 20 FPU per gram of glucans for cellulases whereas for cellobiases 40 IU per grams of glucans was used (Stephen et al., 2012). The glucose content in the resulting enzymatic hydrolysates was determinate by HPLC using an AMINEX-HPX97H (BIO-RAD) column operating at 35 °C with H_2SO_4 4mM as eluent at a flow rate of 0.6 mL/min.

Enzymatic hydrolysis yield (digestibility) was calculated according to Eq. 3.

$$\textit{Enzymatic hydrolysis yield } (EHY,\ \%) = \frac{\textit{glucose x } 0.9}{\textit{glucans in the material}}\ x\ 100 \quad (3)$$

2.2.5. Statistical analysis

The statistical analysis was performed using Statgraphics software, for a confidence level of 95%.

3. Results and discussion

3.1. Effect of the chemical composition of the material on enzymatic hydrolysis

The chemical composition of sawdust was 39.9% glucans, 20.5% hemicelluloses (6.5% xylans, 2.0% galactans, 1.3% arabinans, 10.7% mannans, and 1.8% acetyl group), 3.5% total extractives, and 31.4% lignin (all on mass of dry wood), (Stoffel, 2016). The enzymatic hydrolysis yield (EHY), as a function of the carbohydrates and lignin contents, is depicted in Figs. 2 and 3, respectively, for the fibrous materials obtained by the different pine sawdust pretreatments.

All treatments caused a reduction of the hemicelluloses content in the fibrous material which should improve the accessibility of cellulose to enzymes (Zhu and Pan, 2010). The effect of the steam explosion (SE) was the most significant, followed by that of dilute acid hydrolysis. The alkaline treatments preserved partially the hemicelluloses. However, no correlation between the extent of hemicelluloses extraction and the EHY was observed (Fig. 2). The diluted acid treatment slightly enhanced the EHY (about 7.5% in trials 3 and 4, respectively) with respect to the untreated material (trial 1:3.8%); whereas the steam explosion treated sawdust reached higher values. On the other hand, treatments with high alkaline charge (treatments 14, 16, 19, 21, 23, 24, 26, and 28) led to a higher EHY (about 90% and higher).

Due to the high hemicelluloses extraction, steam explosion led to a fictitious increase of lignin content in the mass balance, as depicted in Fig. 3. On the contrary, EtOH–NaOH and NaOH–AQ treatments

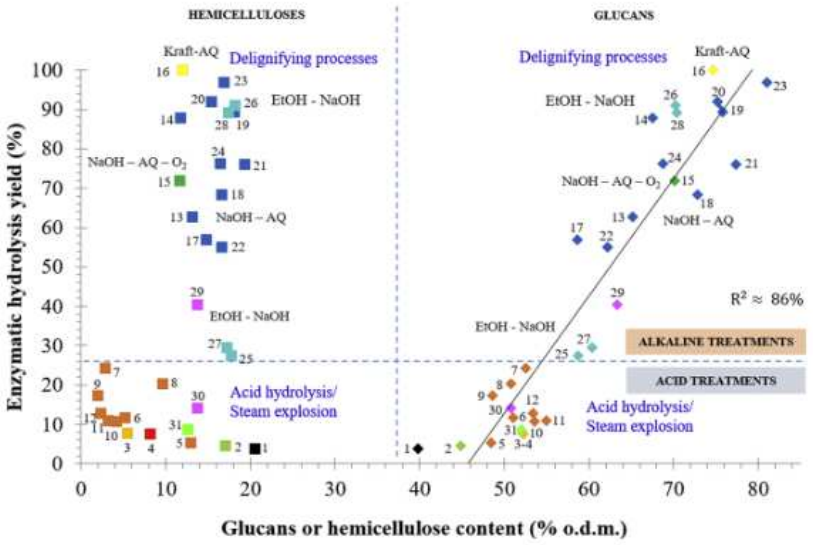

Fig. 2. Enzymatic hydrolysis yield at 72 h in function of glucans and hemicelluloses content in the treated fibrous materials.

removed lignin and partially preserved the hemicelluloses, increasing the glucans content in the mass balance (Fig. 2). Therefore, the alkaline delignification treatments produced fibrous materials with the lowest lignin and the highest glucans contents. No significant differences ($p < 0.05\%$) were observed in the glucans final content in materials treated by NaOH–AQ and kraft–AQ processes.

Contrary to the hemicelluloses, a positive correlation between enzymatic hydrolysis yield and glucans content in all treated materials is visible, for glucans content between 45 and 80%, regardless the treatment to which the material was subjected (Fig. 2). This behavior can be approximately described by the empirical model of Eq. 4.

$$EHY = -128.3 + 2.86\,x\ glucans\ content\ R^2 \approx 86\% \quad (4)$$

As for lignin content, two different tendencies can be found regarding its impact on EHY (Fig. 3). The EHY decreases with the increase of the lignin content for values below 20% (alkaline treatments 13–29), and the model of Eq. 5 can be satisfactory assigned to this trend.

$$EHY = 112.3 - 4.34\,x\ lignin\ content\ R^2 \approx 95\% \quad (5)$$

Besides the benefit of removing lignin, which is an inhibitor of the EH, alkaline treatments swell the lignocellulosic material and thus increase the internal surface area. In addition, this treatment also breaks the linkage between lignin and the carbohydrate fractions, making the material more accessible to enzymes (Yu et al., 2011). As a consequence, the EHY is enhanced. This is in agreement with the fact that alkali is widely known as one of the most effective agents for biomass swelling and that the degree of swelling affects the enzymatic hydrolysis (Aditiya et al., 2016; Galbe and Zacchi, 2002).

As for values of the lignin content superior to 20% (steam explosion experiments), the opposite occurs, *i.e.*, the slope is positive, but it does not mean that EHY increases with the increase of the relative amount of lignin, because the real lignin content is barely affected by the process. This apparent contradiction arises from the calculation of the pulp composition, since lignin percentage increases exclusively due to hemicelluloses decrease by the extraction processes, as already mentioned. In fact, it is the removal of hemicelluloses (Fig. 2), as well as the concomitant disturbance of the lignin-carbohydrates matrix, that improve the fibers porosity and the area available for the EH. Moreover, the lignin structure was probably modified (Stoffel, 2016) since in spite of the hemicelluloses extraction (Fig. 2) the EHY was almost not affected.

Wong et al. (1988) reported the influence of fiber porosity to cellulose digestion in *Pinus radiata* treated by steam explosion. They observed that the increase in pore volume and surface area improved the enzymatic hydrolysis, achieving 24.1% of EHY. This value is similar to EHY obtained with the material from treatment 7.

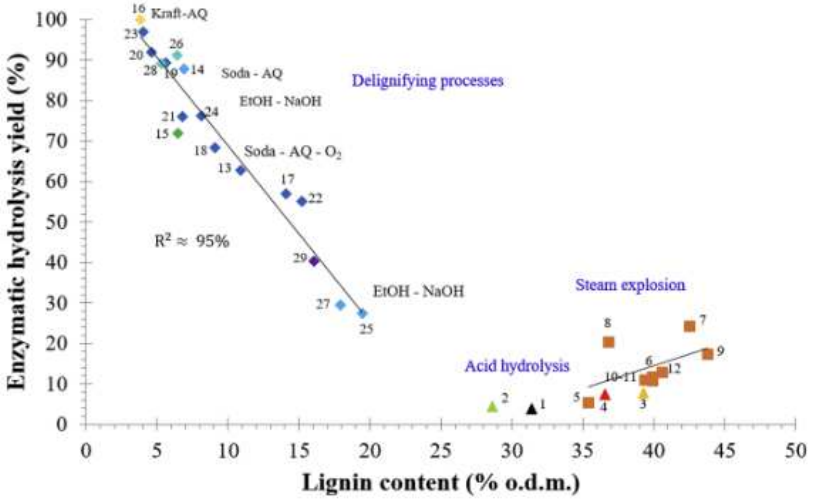

Fig. 3. Enzymatic hydrolysis yield at 72 h in function of lignin content in the treated fibrous materials.

Table 1
Treatment conditions of all studied samples.

Nº	Pretreatment	Conditions	Ref.
1	Untreated	–	[1]
2	Alkaline desresination	90 °C, 60 min, 5 % NaOH*	
3	Dilute acid hydrolysis	150 °C, 30 min, 7.5 g/L H_2SO_4	
4	Alkaline desresination + dilute acid hydrolysis	90 °C, 60 min, 5 % NaOH* and 150 °C + 30 min, 7.5 g/L H_2SO_4	
5	Steam explosion (SE) [(a)]	190 °C; 7.5 min; 0.75 % H_2SO_4*	[2]
6	Steam explosion [(a)]	180 °C, 5 min, 3 % H_2SO_4*	
7	Steam explosion [(a)]	200 °C, 5 min, 3 % H_2SO_4*	
8	Steam explosion [(a)]	200 °C, 5 min, 3 % H_2SO_4 (wet material)*	
9	Steam explosion	190 °C; 7.5 min; 0.75 % H_2SO_4*	
10	Steam explosion [(a) (b)]	180 °C, 5 min, 3 % H_2SO_4*	
11	Steam explosion [(a)(b)]	200 °C, 5 min, 3 % H_2SO_4*	
12	Steam explosion [(b)]	190 °C, 7.5 min; 0.75 % H_2SO_4*	
13	NaOH - AQ [(c)]	25 g/L NaOH, 110 min, 170 °C, LSR: 10/1	[3]
14	NaOH - AQ [(c)]	25 g/L NaOH, 170 min, 170 °C, LSR: 10/1	
15	NaOH - AQ - O_2 [(c)]	25 g/L NaOH, 170 min, 170 °C, LSR: 10/1	
16	Kraft –AQ [(c)]	25 g/L NaOH, 170 min, 170 °C, LSR: 10/1	[4]
17	NaOH - AQ [(c)]	25 g/L NaOH, 110 min, 170 °C, LSR: 10/1	
18	NaOH - AQ [(c)]	25 g/L NaOH, 170 min, 170 °C, LSR: 10/1	
19	NaOH - AQ [(c)]	50 g/L NaOH, 108 min, 170 °C, LSR: 5/1	
20	NaOH - AQ [(c)]	50 g/L NaOH, 170 min, 170 °C, LSR: 5/1	
21	NaOH - AQ [(c)]	37.5 g/L NaOH, 185 min, 170 °C, LSR: 5/1	
22	NaOH - AQ [(c)]	19.82 g/L NaOH, 140 min, 170 °C, LSR: 10/1	
23	NaOH - AQ [(c)]	55.17 g/L NaOH, 140 min, 170 °C, LSR: 5/1	
24	NaOH - AQ [(c)]	37.5 g/L NaOH, 140 min, 170 °C, LSR: 5/1	
25	EtOH – NaOH[(d)]	35/65 ethanol/agua, 30 g/L NaOH, 60 min, 170 °C	[5]
26	EtOH – NaOH[(d)]	35/65 ethanol/agua, 50 g/L NaOH, 60 min, 170 °C	
27	EtOH – NaOH[(d)]	35/65 ethanol/agua, 30 g/L NaOH, 90 min, 170 °C	
28	EtOH – NaOH[(d)]	35/65 ethanol/agua, 50 g/L NaOH, 90 min, 170 °C	
29	NaOH[(d)]	50 g/L NaOH, 90 min, 170 °C	
30	NaOH[(d)]	30 g/L NaOH, 90 min, 170 °C	
31	EtOH[(d)]	35/65 ethanol/agua, 90 min, 170 °C	

*o.d.m.: on mass of dry material; (a) subjected to a previous alkaline deresination (60 min, 5% NaOH o.d.m., 90 °C); (b) subjected to a post alkaline washing (60 °C, 60 min, 4% NaOH o.d.m.); (c) 0.11% AQ o.d.m.; (d) LSR: 5/1.
[1] Stoffel et al., 2014; [2] Stoffel et al., 2017; [3] Imlauer et al., 2011; [4] Imlauer et al., 2014; [5] Kruyeniski et al., 2015.

Table 2
Chemical composition (% on mass of dry wood) of all evaluated materials; crystallinity index (CrI), *Δ Pore volume/Δ Pore Diameter* (for D = 0.792 nm), and enzymatic hydrolysis yield (EHY) of the solid materials from the selected treatments.

N°	Treatment	Glucans (%)	Hemicellulose (%)	Lignin (%)	CrI (%)	ΔV/ΔD *100 (D = 0.792 nm)	EHY %
1	Untreated	39.85	20.51	31.41	56.4	2.084	5.4
2	Alkaline desresination	44.88	17.1	28.65			4.5
3	Dilute acid hydrolysis	52.2	5.47	39.25			7.7
4	Alkaline desresination + dilute acid hydrolysis	52.25	8.18	36.56			7.5
5	Steam explosion [(a)]	48.4	13	35.4			5.4
6	Steam explosion [(a)]	51.01	5.23	39.9			11.7
7	Steam explosion [(a)]	52.53	2.82	42.5	65.7	6.791	24.1
8	Steam explosion [(a)]	42.6	1.3	49.86			20.3
9	Steam explosion	48.6	2	43.81			17.3
10	Steam explosion [(a) (b)]	53.6	4.2	39.9			10.7
11	Steam explosion [(a)(b)]	55,00	3.2	39.4			11
12	Steam explosion (b)	53.4	2.3	40.6	67.2	6.788	12.9
13	NaOH - AQ [(c)]	65.13	13.17	10.9			62.7
14	NaOH - AQ [(c)]	67.55	11.71	6.9			87.8
15	NaOH - AQ - O_2 [(c)]	70.1	11.68	6.5	76.1	26.85	72
16	Kraft –AQ [(c)]	74.68	11.97	3.8	76.3	4.899	100
17	NaOH - AQ [(c)]	58.6	14.81	14.1			56.9
18	NaOH - AQ [(c)]	72.8	16.63	9.1			68.3
19	NaOH - AQ [(c)]	75.8	18.09	5.6			89.3
20	NaOH - AQ [(c)]	75.2	15.4	4.6			92
21	NaOH - AQ [(c)]	77.4	19.36	6.8			76.1
22	NaOH - AQ [(c)]	62.2	16.61	15.2			55.1
23	NaOH - AQ [(c)]	81,00	16.92	4,00	75.5	8.190	96.9
24	NaOH - AQ [(c)]	68.7	16.41	8.1			76.2
25	Ethanol and NaOH	58.75	17.79	19.49	65.0	15.81	27.4
26	Ethanol and NaOH	70.25	18.23	6.41	70.4	13.07	91.1
27	Ethanol and NaOH	60.41	17.22	17.9			29.5
28	Ethanol and NaOH	70.41	17.4	5.28			89.2
29	NaOH	63.3	13.78	16.05			40.4
30	NaOH	50.75	13.72	27.64			14.2
31	Ethanol	51.99	12.65	30.4			8.7

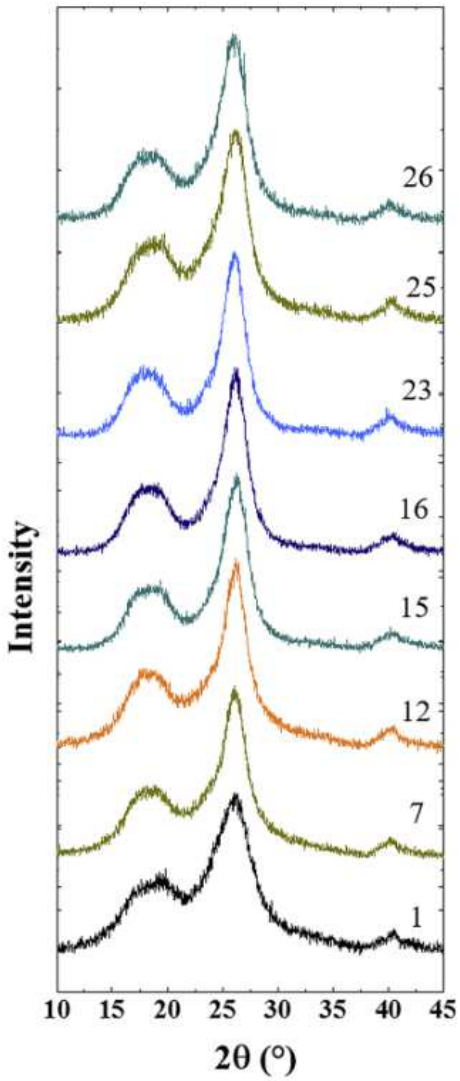

Fig. 4. X-ray diffractograms of selected samples.

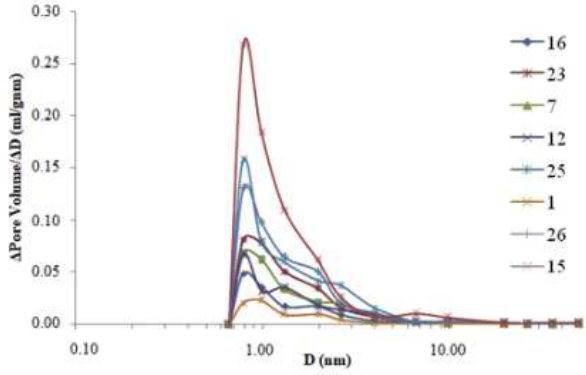

Fig. 5. Pore diameter distribution in selected treated materials.

To quantify the effect of both the contents of lignin and hemicelluloses, the data of the 31 experiences were used to build the model shown in Eq. 7.

$$EHY = 102 - 1.81\,x\,lignin\ content - 0.09\,lignin\ content\ x\ hemicelluloses\ content \quad R^2 \approx 95\% \tag{6}$$

This equation is valid for lignin content between 3% and 45% and hemicelluloses content between 2.5% and 20%. The model reveals that both the lignin content and the lignin - hemicelluloses content interaction are detrimental for the enzymatic hydrolysis.

To better understand the effect of hemicelluloses and/or lignin extraction on the EHY, eight samples of pine sawdust (untreated and pretreated) were selected to analyze their crystallinity and porosity. The samples were selected because they had similar enzymatic yield but different lignin content or the opposite. The specific conditions of the corresponding pretreatments are detailed in Table 1.

3.2. Effect of the crystallinity and porosity on the enzymatic hydrolysis yield

The chemical composition, crystallinity index (CrI), *ΔPore volume/ΔPore Diameter*, and EHY of the selected pretreated materials are shown in Table 2.

CrI was calculated according to the Segal method used to determine the crystallinity in lignocellulosic materials: the amount of crystalline material is represented by the height of the highest diffraction peak, whereas the amount of amorphous material is represented by the height of the minimum intensity between the major peaks (French and Cintrón, 2013). The X-ray diffractograms of the selected samples are shown in Fig. 4.

The CrI of untreated pine sawdust was 56.4%, similar to other works with pine (Andersson et al., 2004). Alkaline delignification treatments 15, 16 and 23 produced the most delignifyed materials (lignin < 7% on mass of dry material) and the highest CrI values (about 76%), whereas the CrI of the materials from the steam explosion and EtOH-NaOH treatments were lower (about 66% in treatments 7, 12 and 25). A strong and significant positive linear correlation ($r = 0.94$, $p < 0.05$) was found between the CrI (varying from 56.4% to 76%) and the glucans content (varying from 40% to 81% on mass of dry material). On the contrary, a weak though significant negative correlation with lignin was detected ($r = -0.72$, $p < 0.05$). The increase of crystallinity with the delignifying treatments is due to the removal of amorphous materials, such as lignin, hemicelluloses, and amorphous cellulose is in agreement with the findings of other authors (Kumar et al., 2013; Park et al., 2010; Yoshida et al., 2008). The EHY of the material correlates positively with CrI ($r = 0.85$, $p < 0.05$), as expected since both the EHY and CrI correlate with the glucans content, as discussed before (pure material generates higher EH yield), showing the difficulty of decoupling CrI from changes in other properties (Park et al., 2006).

The increase in CrI produced by the steam explosion treatment is originated by hemicelluloses extraction, as shown in data included in Fig.6.

Pores are already present in cellulose fibers in nature and can be generated during chemical and mechanical treatments, being the evolution of porosity useful to know the accessibility of the cell walls. The effect of the different treatments of this study on the pore size distribution of the lignocellulosic materials is plotted in Fig. 5.

The results reveal that regardless the treatments, all distributions have a defined mode in the range 0.66–1.32 nm, and when comparing to the original sawdust raw material there is an increase of the number of pores but not of their diameters. This effect has been described by other authors. For example, in a study of structural changes of photoperiod-sensitive sorghum after pretreatment with sulfuric acid, they found that after the pretreatment the microvoid volume of the material increased in length but not in average diameter (Xu et al., 2013).

The increase in the number of pores in the range 0.66–1.32 nm is consistent with the findings of Frey-Wyssling (Frey-Wyssling, 1938), who concluded that the regions occupied by non-cellulosic components within the microfibrils of the cell wall are 1 nm wide.

The removal of lignin generally produces more pores than the removal of hemicelluloses by steam explosion treatments. This could be related to the proposed model of Salmén and Olsson (Salmén and Olsson, 1998) of the ultrastructural arrangement of wood polymers in the fiber wall, in which the xylans are trapped in a lignin matrix and the glucomannans are bound to cellulose. Therefore, the removal of lignin

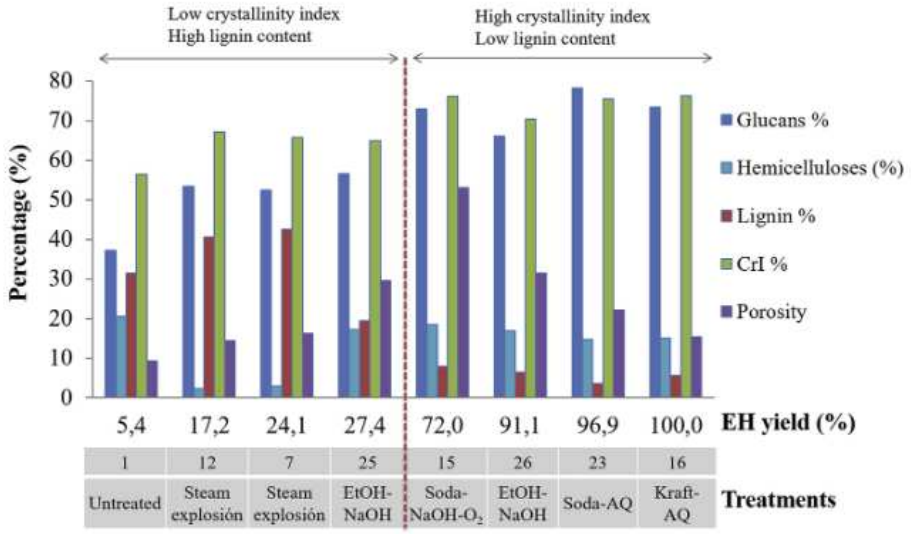

Fig. 6. Influence of glucans, hemicelluloses, lignin, crystallinity index, and cumulative porosity on the enzymatic hydrolysis of pretreated pine sawdust.

opens the structure much more than if only the hemicelluloses are extracted.

Stone et al (Stone et al., 1969), using the solute exclusion technique, found a correlation between digestibility and the accessibility of 30 Å (3 nm) molecules, suggesting that the diameter of the enzyme molecule would be 30 Å. Grethlein and Converse (Grethlein and Converse, 1991) also using solute exclusion, put as a first approximation a size for the cellulases of *T. reesei* of 51 Å (5.1 nm). Comparing with the range of maximum porosity found for the studied materials (0.66–1.32 nm); it can be assumed that there is no direct relationship between the obtained pore distribution and digestibility since the diameter of the enzyme it would be between 3–5 times greater than the pore diameter of the material. However, taking into account that different techniques are used to perform the measurements, it is possible that the figures are not coincident.

Processes like pulping and bleaching increase porosity by lignin and hemicelluloses removal from the fibers (Park et al., 2006). The impact of the treatments on the porosity (untreated < < Kraft-AQ < SE < NaOH-AQ < < EtOH-NaOH < < NaOH-AQ-O_2), reveals that oxygen delignification greatly increases the number of pores.

The influence of glucans, hemicelluloses, lignin, crystallinity index, and cumulative porosity on the enzymatic hydrolysis of the pretreated pine sawdust is summarized in Fig. 6.

The increase in CrI produced by the steam explosion treatment is due to the hemicelluloses extraction, as shown in data included in Fig. 6. Nevertheless, the enzymatic hydrolysis yield does not increase significantly. Similarly, despite the abovementioned positive effect of the steam explosion on porosity, there is not a relevant increment of the enzymatic hydrolysis yield. This behavior can be explained by the highly lignified structure of these pines and the nature of their lignin (more guayacil units), which would limit the swelling of the material. Yu et al (Yu et al., 2011) found, for the same lignin content, greater digestibility for hardwoods than for conifers, confirming again that conifers are more recalcitrant. The disparity on the yield of SE samples 7 and 12 (24.3 and 12.9%), can be attributed to alkali washing after SE, which partially redistributes the dissolved lignin on fibers, thus limiting the enzymes access (Donaldson et al., 1988; Soccol et al., 2011; Wong et al., 1988). The delignification increase did not enhance porosity in a noticeable way, possibly because of the redistribution of the residual lignin.

4. Conclusions

The results of this extensive and methodical work performed on the same raw material show that all applied treatments to slash pine sawdust increased the enzymatic hydrolysis yield but lignin content and location has shown to have the dominant effect on biomass recalcitrance to enzymatic digestibility. Hemicelluloses extraction up to 89% (referred to the initial content) played a minor role, with enzymatic hydrolysis yields less than 25%.

To enhance the enzymatic hydrolysis, the lignin content of the pretreated material should be low. As for the hemicelluloses content, only its interaction with the lignin content is significant, as revealed by the regression model obtained. Strong alkali treatments produced the highest cellulose crystallinity index (about 75–76%) and also the highest enzymatic hydrolysis yield, although the effect of crystallinity may be confused with that of the increased glucans content. A comprehensive study of the effect of crystallinity on enzymatic hydrolysis is then required.

All treatments increased the number of pores but not their diameter. Porosity did not show a clear influence on EHY: it improves the enzymatic hydrolysis when it results from delignification but has a minor effect when it is due to the extraction of hemicelluloses. The relationship between the pore size, the size of the enzymes, and their relation to the efficiency of the HE process is not clear and it needs more systematic studies to be revealed.

Acknowledgments

The authors acknowledge the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) for the doctoral scholarship; PROCYP, IMAM (Argentina) for the financial support and the CIEPQPF, at the Department of Chemical Engineering, University of Coimbra (Portugal) for the access to some of the experimental facilities.

References

Aditiya, H.B., Mahlia, T.M.I., Chong, W.T., Nur, H., Sebayang, A.H., 2016. Second generation bioethanol production: a critical review. Renew. Sustain. Energy Rev. 66, 631–653. https://doi.org/10.1016/j.rser.2016.07.015.

Alvarez-Vasco, C., Zhang, X., 2017. Alkaline hydrogen peroxide (AHP) pretreatment of softwood: enhanced enzymatic hydrolysability at low peroxide loadings. Biomass Bioenergy 96, 96–102. https://doi.org/10.1016/j.biombioe.2016.11.005.

Andersson, S., Wikberg, H., Pesonen, E., Maunu, S.L., Serimaa, R., 2004. Studies of crystallinity of Scots pine and Norway spruce cellulose. Trees - Struct. Funct. 18, 346–353. https://doi.org/10.1007/s00468-003-0312-9.

Bansal, P., Hall, M., Realff, M.J., Lee, J.H., Bommarius, A.S., 2009. Modeling cellulase kinetics on lignocellulosic substrates. Biotechnol. Adv. 27, 833–848. https://doi.org/10.1016/j.biotechadv.2009.06.005.

Beale, I., Ortiz, E.C., 2013. El sector forestal argentino - Bosques implantados. Rev. Divulg. Técnica 1–6.

Cameron, H., Campion, S.H., Singh, T., Vaidya, A.A., 2015. Improved saccharification of

steam-exploded *Pinus radiata* on supplementing crude extract of Penicillium sp. 3 Biotech 5, 221–225. https://doi.org/10.1007/s13205-014-0212-2.
Cremonez, P.A., Feroldi, M., Feiden, A., Gustavo Teleken, J., José Gris, D., Dieter, J., de Rossi, E., Antonelli, J., 2015. Current scenario and prospects of use of liquid biofuels in South America. Renew. Sustain. Energy Rev. 43, 352–362. https://doi.org/10.1016/j.rser.2014.11.064.
Donaldson, L., Vaidya, A., 2017. Visualising recalcitrance by colocalisation of cellulase, lignin and cellulose in pretreated pine biomass using fluorescence microscopy. Sci. Rep. 7, 44386. https://doi.org/10.1038/srep44386.
Donaldson, L.A., Wong, K.K.Y., Mackie, K.L., 1988. Ultrastructure of steam-exploded wood. Wood Sci. Technol. 22, 103–114. https://doi.org/10.1007/BF00355846.
Draude, K.M., Kurniawan, C.B., Duff, S.J.B., 2001. Effect of oxygen delignification on the rate and extent of enzymatic hydrolysis of lignocellulosic material. Bioresour. Technol. 79, 113–120. https://doi.org/10.1016/S0960-8524(01)00055-4.
Driemeier, C., Mendes, F.M., Oliveira, M.M., 2012. Dynamic vapor sorption and thermoporometry to probe water in celluloses. Cellulose 19, 1051–1063. https://doi.org/10.1007/s10570-012-9727-z.
FAO, 2009. Análisis del Balance de Energía derivada de Biomasa en Argentina. WISDOM Argentina. Final Report.
French, A.D., Cintrón, M.S., 2013. Cellulose polymorphy, crystallite size, and the Segal Crystallinity Index. Cellulose 20, 583–588. https://doi.org/10.1007/s10570-012-9833-y.
Frey-Wyssling, A., 1938. Submicroscopic structure and maceration pictures of native cellulose fibers. Pap. Fabr. 36, 212–217.
Galbe, M., Zacchi, G., 2002. A review of the production of ethanol from softwood. Appl. Microbiol. Biotechnol. 59, 618–628. https://doi.org/10.1007/s00253-002-1058-9.
Grace, T.M., Malcolm, W.E., 1989. Alkaline Pulping in Joint Executive Committee of the Vocational Educational Committees of the Pulp and Paper Industry, Pulp and Paper Manufacture. TAPPI Press.
Grethlein, H.E., Converse, A.O., 1991. Common aspects of acid prehydrolysis and steam explosion for pretreating wood. Bioresour. Technol. 36, 77–82. https://doi.org/10.1016/0960-8524(91)90101-O.
Hill, C.A.S., Papadopoulos, A., 2001. A review of methods used to determine the size of the cell wall microvoids of wood. J. Inst. Wood Sci. 15, 337–345.
Imlauer, C.M., Felissia, F.E., Stoffel, R., Area, M.C., 2011. In: Misiones, U.N.de (Ed.), Tratamiento alcalino e hidrólisis ácida del aserrín de pino para la biorrefinería forestal. Jornadas Científico Tecnológicas, Posadas, Misiones.
Imlauer, C.M., Kruyeniski, J., Area, M.C., Felissia, F.E., 2014. Fraccionamiento a la soda-AQ de aserrín de pino para la biorrefinería forestal. In: Bolivariana, U.P. (Ed.), VIII Congreso Iberoamericano de Investigación en Celulosa y. Papel. Medellìn, Colombia.
Inoue, H., Fujimoto, S., Sakaki, T., 2016. Two-step hot-compressed water treatment of douglas fir for efficient total sugar recovery by enzymatic hydrolysis. BioResources 11, 5124–5137.
Kafle, K., Lee, C.M., Shin, H., Zoppe, J., Johnson, D.K., Kim, S.H., Park, S., 2015. Effects of delignification on crystalline cellulose in lignocellulose biomass characterized by vibrational sum frequency generation spectroscopy and X-ray diffraction. Bioenergy Res. 8, 1750–1758. https://doi.org/10.1007/s12155-015-9627-9.
Kruyeniski, J., Felissia, F.E., Area, M.C., 2015. Enzymatic hydrolysis of pine pretreated with ethanol and sodium hydroxide. Concepción, Ude (Ed.), 3er Congreso Iberoamericano Sobre Biorrefinerías (CIAB), 4 to Congreso Latinoamericano Sobre Biorrefinerías, y 2do Simposio Internacional Sobre Materiales Lignocelulósicos.
Kruyeniski, J., Ferreira, P.J., Carvalho, M.G., Felissia, F.E., Area, M.C., 2016. Physical and chemical characteristics of pretreated pine sawdust and its enzymatic hydrolysis. Iberoamerican Congress on Pulp and Paper Research (CIADICYP).
Kumar, L., Chandra, R., Chung, P.A., Saddler, J., 2010. Can the same steam pretreatment conditions be used for most softwoods to achieve good, enzymatic hydrolysis and sugar yields? Bioresour. Technol. 101, 7827–7833. https://doi.org/10.1016/j.biortech.2010.05.023.
Kumar, L., Arantes, V., Chandra, R., Saddler, J., 2012. The lignin present in steam pretreated softwood binds enzymes and limits cellulose accessibility. Bioresour. Technol. 103, 201–208. https://doi.org/10.1016/j.biortech.2011.09.091.
Kumar, R., Hu, F., Hubbell, C.A., Ragauskas, A.J., Wyman, C.E., 2013. Comparison of laboratory delignification methods, their selectivity, and impacts on physiochemical characteristics of cellulosic biomass. Bioresour. Technol. 130, 372–381. https://doi.org/10.1016/j.biortech.2012.12.028.
Laaksonen-Craig, S., 2008. The determinants of foreign direct investments in Latin American forestry and forest industry. J. Sustain. For. 27, 172–188. https://doi.org/10.1080/10549810802225275.
Lloyd, J.A., Murton, K.D., Newman, R.H., Suckling, I.D., Vaidya, A.A., 2017. Careful selection of steaming and attrition conditions during thermo-mechanical pretreatment can increase enzymatic conversion of softwood. J. Chem. Technol. Biotechnol. 92, 238–244. https://doi.org/10.1002/jctb.4975.
Luo, L., van der Voet, E., Huppes, G., 2010. Biorefining of lignocellulosic feedstock - Technical, economic and environmental considerations. Bioresour. Technol. 101, 5023–5032. https://doi.org/10.1016/j.biortech.2009.12.109.
Matsuura, M., Sasaki, J., Murao, S., 1995. Studies on β -Glucosidases from soybeans that Hydrolyze Daidzin and genistin: isolation and characterization of an isozyme. Biosci. Biotechnol. Biochem. 59, 1623–1627. https://doi.org/10.1271/bbb.59.1623.
Meier, E., 2015. WOOD! Identifying and Using Hundreds of Woods Worldwide. Wood Database.
Meng, X., Ragauskas, A.J., 2014. Recent advances in understanding the role of cellulose accessibility in enzymatic hydrolysis of lignocellulosic substrates. Curr. Opin. Biotechnol. 27, 150–158. https://doi.org/10.1016/j.copbio.2014.01.014.
Meng, X., Foston, M., Leisen, J., DeMartini, J., Wyman, C.E., Ragauskas, A.J., 2013. Determination of porosity of lignocellulosic biomass before and after pretreatment by using Simons' stain and NMR techniques. Bioresour. Technol. 144, 467–476. https://doi.org/10.1016/j.biortech.2013.06.091.
Milagres, A.M.F., Carvalho, W., Ferraz, A., 2011. Topochemistry, Porosity and Chemical Composition Affecting Enzymatic Hydrolysis of Lignocellulosic Materials, Routes to Cellulosic Ethanol. Springer New York, New York, NY, pp. 53–72. https://doi.org/10.1007/978-0-387-92740-4_5.
Morales, M., Pielhop, T., Saliba, P., Hungerbühler, K., Rudolf von Rohr, P., Papadokonstantakis, S., 2017. Sustainability assessment of glucose production technologies from highly recalcitrant softwood including scavengers. Biofuels Bioprod. Biorefining 11, 441–453. https://doi.org/10.1002/bbb.1756.
Newman, R.H., Vaidya, A.A., Imroz Sohel, M., Jack, M.W., 2013. Optimizing the enzyme loading and incubation time in enzymatic hydrolysis of lignocellulosic substrates. Bioresour. Technol. 129, 33–38. https://doi.org/10.1016/j.biortech.2012.11.028.
Pan, X., Arato, C., Gilkes, N., Gregg, D., Mabee, W., Pye, K., Xiao, Z., Zhang, X., Saddler, J., 2005. Biorefining of softwoods using ethanol organosolv pulping: preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products. Biotechnol. Bioeng. 90, 473–481. https://doi.org/10.1002/bit.20453.
Park, S., Venditti, R., Jameel, H., Pawlak, J., 2006. Changes in pore size distribution during the drying of cellulose fibers as measured by differential scanning calorimetry. Carbohydr. Polym. 66, 97–103. https://doi.org/10.1016/j.carbpol.2006.02.026.
Park, S., Baker, J.O., Himmel, M.E., Parilla, P.A., Johnson, D.K., 2010. Cellulose crystallinity index: measurement techniques and their impact on interpreting cellulase performance. Biotechnol. Biofuels 3, 10. https://doi.org/10.1186/1754-6834-3-10.
Park, J., Shin, H., Yoo, S., Zoppe, J.O., Park, S., 2015. Delignification of lignocellulosic biomass and its effect on subsequent enzymatic hydrolysis. BioResources 10, 2732–2743. https://doi.org/10.15376/biores.10.2.2732-2743.
Pielhop, T., Larrazábal, G.O., Studer, M.H., Brethauer, S., Seidel, C.-M., Rudolf von Rohr, P., 2015. Lignin repolymerisation in spruce autohydrolysis pretreatment increases cellulase deactivation. Green Chem. 17, 3521–3532. https://doi.org/10.1039/C4GC02381A.
PROBIOMASA, 2012. Utilizacion de biomasa con fines energeticos en la Provincia de Misiones, Reunión de presentación. Proyecto para la promoción de la energía derivada de biomasa PROBIOMASA. Consejo ejecutivo y consultivo de energías renovables, biocombustibles e hidrógeno.
Salmén, L., Olsson, A., 1998. Interaction between hemicelluloses, lignin and cellulose: structure-property relationships. J. Pulp Pap. Sci. 2, 99–102.
SENASA, 2014. Bosques argentinos, actividad forestal y economías regionales. [WWW Document]. .
Sing, K., 2001. The use of nitrogen adsorption for the characterisation of porous materials. Colloids Surf. A Physicochem. Eng. Asp. 187–188, 3–9. https://doi.org/10.1016/S0927-7757(01)00612-4.
Soccol, R.C., Faraco, V., Karp, S., Vandenberghe, L.P.S., Thomaz-Soccol, V., Woiciechowski, A., Pandey, A., 2011. Lignocellulosic Bioethanol, Biofuels. Elsevier, pp. 101–122. https://doi.org/10.1016/B978-0-12-385099-7.00005-X.
Stephen, J.D., Mabee, W.E., Saddler, J.N., 2012. Will second-generation ethanol be able to compete with first-generation ethanol? Opportunities for cost reduction. Biofuels, Bioprod. Biorefining 6, 159–176. https://doi.org/10.1002/bbb.331.
Stoffel, R.B., 2016. Fraccionamiento de aserrín de pino destinado a una biorrefinería forestal. Doctoral dissertation. Universidad Nacional de La Plata, Facultad de Ingeniería. http://sedici.unlp.edu.ar/handle/10915/52523.
Stoffel, R.B., Felissia, F.E., Silva Curvelo, A.A., Gassa, L.M., Area, M.C., 2014. Optimization of sequential alkaline–acid fractionation of pine sawdust for a biorefinery. Ind. Crops Prod. 61, 160–168. https://doi.org/10.1016/j.indcrop.2014.06.047.
Stoffel, R.B., Neves, P.V., Felissia, F.E., Ramos, L.P., Gassa, L.M., Area, M.C., 2017. Hemicellulose extraction from slash pine sawdust by steam explosion with sulfuric acid. Biomass Bioenergy 107. https://doi.org/10.1016/j.biombioe.2017.09.019.
Stone, J.E., Scallan, A.M., Donefer, E., Ahlgren, E., 1969. Digestibility as a simple function of a molecule of similar size to a cellulase enzyme. In: Hajny, G.J., Reese, E.T. (Eds.), Cellulases and Their Applications. American Chemical Society, Washington, DC, pp. 219–241.
Suckling, I.D., Jack, M.W., Lloyd, J.A., Murton, K.D., Newman, R.H., Stuthridge, T.R., Torr, K.M., Vaidya, A.A., 2017. Biotechnology for Biofuels A mild thermomechanical process for the enzymatic conversion of radiata pine into fermentable sugars and lignin. Biotechnol. Biofuels 1–13. https://doi.org/10.1186/s13068-017-0748-6.
Uasuf, A., Hilber, J., 2012. El uso de la biomasa de Origen Forestal con destino a bioenergía en la Argentina. Buenos Aires. .
Vaidya, A.A., Donaldson, L.A., Newman, R.H., Suckling, I.D., Campion, S.H., Lloyd, J.A., Murton, K.D., 2016. Micromorphological changes and mechanism associated with wet ball milling of *Pinus radiata* substrate and consequences for saccharification at low enzyme loading. Bioresour. Technol. 214, 132–137. https://doi.org/10.1016/j.biortech.2016.04.084.
Willem van Gelder, J., German, L., Bailis, R., 2012. Biofuels investments in tropical forest-rich countries: implications for responsible finance. Sustain. Account. Manag. Policy J. 3, 134–160. https://doi.org/10.1108/20408021211282296.
Wingren, A., Galbe, M., Zacchi, G., 2003. Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. Biotechnol. Prog. 19, 1109–1117. https://doi.org/10.1021/bp0340180.
Wong, K.K.Y., Deverell, K.F., Mackie, K.L., Clark, T.A., Donaldson, L.A., 1988. The relationship between fiber-porosity and cellulose digestibility in steam-exploded *Pinus radiata*. Biotechnol. Bioeng. 31, 447–456. https://doi.org/10.1002/bit.260310509.

Xu, F., Shi, Y., Wang, D., 2013. Bioresource Technology towards understanding structural changes of photoperiod-sensitive sorghum biomass during sulfuric acid pretreatment. Bioresour. Technol. 135, 704–709. https://doi.org/10.1016/j.biortech.2012.08.141.

Yoshida, M., Liu, Y., Uchida, S., Kawarada, K., Ukagmi, Y., Ichinose, H., Kaneko, S., Fukuda, K., 2008. Effects of cellulose crystallinity, Hemicellulose, and lignin on the enzymatic hydrolysis of Miscanthus sinensis to Monosaccharides. Biosci. Biotechnol. Biochem. 72, 805–810. https://doi.org/10.1271/bbb.70689.

Yu, Z., Jameel, H., Chang, H., Park, S., 2011. The effect of delignification of forest biomass on enzymatic hydrolysis. Bioresour. Technol. 102, 9083–9089. https://doi.org/10.1016/j.biortech.2011.07.001.

Zhu, J.Y., Pan, X.J., 2010. Woody biomass pretreatment for cellulosic ethanol production: technology and energy consumption evaluation. Bioresour. Technol. 101, 4992–5002. https://doi.org/10.1016/j.biortech.2009.11.007.

Zhu, J.Y., Pan, X., Zalesny Jr., R.S., 2010. Pretreatment of woody biomass for biofuel production: energy efficiency, technologies, and recalcitrance. Appl. Microbiol. Biotechnol. 847–857. https://doi.org/10.1007/s00253-010-2654-8.