Optimization of the Pretreatment of *Prosopis nigra* Sawdust for the Production of Fermentable Sugars

Eliana P. Dagnino,[a,b,]* Ester R. Chamorro,[a] Silvia D. Romano,[b,c] Fernando E. Felissia,[d] and María C. Area [c,d]

The black carob tree (*Prosopis nigra*) is a particularly valued species in Chaco, Argentina on account of its hard wood. Finding a use for the sawdust, the main industrial residue of wood, could be useful within the furniture industry of any country in the tropics seeking to apply the biorefinery concept. For the conversion of wood carbohydrates to bioethanol, a pretreatment stage is necessary. The objective of this work was to find the acid pretreatment conditions that maximize the extraction of xylose with minimum degradation, while maximizing the concentration of glucans in the pretreated solid to obtain better enzymatic accessibility, using black carob tree sawdust as the raw material. The optimization was carried out by use of a central composite design (CCD) with two independent variables: the concentration of the sulfuric acid solution and the heating time. Optimal enzymatic hydrolysis occurred at the mean values of the tested acid solution concentration (1.2%) and after shorter heating times (10.2 min). The concentration of sugars after the enzymatic hydrolysis of the pretreated solid over a time period of 72 h was three times higher than the untreated solid.

Keywords: Optimization; Acid pretreatment; Black carob tree sawdust; Bioethanol

*Contact information: a: Grupo UTN de Investigación en Química Orgánica Biológica Facultad Regional Resistencia, Universidad Tecnológica Nacional, French 414, 3500, Resistencia, Argentina; b: Grupo de Energías Renovables, Facultad de Ingeniería, Universidad de Buenos Aires, Paseo Colón 850, C1063ACV, Capital Federal, Argentina; c: Consejo Nacional de Investigaciones Científicas y Técnicas, Av. Rivadavia 1917, 1033, Capital Federal, Argentina; d: Programa de Celulosa y Papel, Facultad de Ciencias Exactas, Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, 3300, Posadas, Argentina; *Corresponding author: pdagnino@frre.utn.edu.ar*

INTRODUCTION

The biotechnological industrial processes used to obtain biofuel are aimed to be environmentally friendly, self-sustainable, and to have a competitive cost compared to their petrochemical equivalents. Bioethanol is one of the main examples of how biotechnology has improved significantly. This liquid biofuel, produced from raw materials rich in saccharose, starch, or complex carbohydrates, can be used as a partial or complete replacement for gasoline (Duff and Murray 2006; Otero *et al.* 2007; Sassner *et al.* 2008; Wang and Sun 2010; Zheng and Zhang 2009).

Some investigations have shown that wood waste has the potential to be used as a raw material for the production of bioethanol (Chin *et al.* 2011; Kim *et al.* 2011; Monavari *et al.* 2009). The black carob tree (*Prosopis nigra*) from the Chaco province in Argentina could be one of these raw materials. The genus *Prosopis (*Leguminosae, Mimosoideae*)* includes approximately 44 species of trees distributed in the arid and semi-arid regions of America, Africa, and Asia. The world centre of biodiversity of this genus is in Argentina, where 28 species are found, 13 of which are endemic (Burkart 1976).

The black carob is rich in extractives with a high density wood, and it is widely used in the furniture industry. According to the Argentinian Ministry of Production and Environment, its manufacture and industrialization (approximately 8000 tons/year of black carob furniture is produced), produce shavings and sawdust in sawmills and carpentries (around 1500 tons/year of sawdust) (Martina *et al.* 2008; Zorrilla 2005). This accumulation poses certain dangers, such as the risk of fire, air pollution, and the proliferation of vermin. At present, this waste is burnt, buried, or stored for a period of time. However, it has been shown that the wood waste produced by industrialization has potential to be used as a raw material for the production of bioethanol (Duff and Murray 1996). This waste could be used as raw material rich in complex carbohydrates for improving environmental impact and safety. Additionally, sawdust has the ideal size for processing in the initial stage of bioethanol production. Thus, operations with high energy requirements and costs can be avoided (Galbe and Zacchi 2007; Otero *et al.* 2007; Sassner *et al.* 2008; Zhu and Pan 2010).

Lignocellulosic materials such as black carob tree sawdust are made up of three structural polymers: cellulose, hemicelluloses, and lignin. They also have several water-soluble composites of low molecular weight (water-soluble fraction), composites that are soluble in organic solvents (so-called extractives), and a small content of proteins and mineral salts (Fengel and Wegener 1984; Sjöström 1993).

In the cellulose structure, crystalline and amorphous areas alternate. Highly crystallinity areas, which represent 55 to 75% of the cellulose fiber, are more stable and are more difficult to be penetrated with solvents and reagents. On the other hand, the more disorganized areas are more accessible and susceptible to chemical attack; they enable the swelling, lengthening, and flexibility of the fiber. The crystalline and amorphous areas do not have well-defined boundaries.

Hemicelluloses have a lower molecular weight than cellulose. They have a branched structure with short side chains, they readily absorb water, and it is relatively easy to hydrolyze hemicelluloses to simple sugars. Xylans are the main components of angiosperms, monocots (grasses), and dicots (hardwoods) (Fengel and Wegener 1984; Sjöström 1993).

The process of producing bioethanol from lignocellulosic materials based on enzymatic hydrolysis and fermentation is considered to be the most promising method of creating bioethanol (Otero *et al.* 2007; Duff and Murray 2006; Sassner *et al.* 2008; Wang and Sun 2010; Zheng and Zhang 2009). The aim of a pretreatment stage is to increase the effective surface area in order to expose the cellulose to enzymatic attack (Duff and Murray 2006).

Pretreatments such as organosolv, steam explosion, and dilute acid processes have been widely studied, as they have potential for industrial application (Agbor *et al.* 2011; Duff and Murray 1996; Galbe and Zacchi 2007; Monavari *et al.* 2009; Park *et al.* 2010; Sannigrahi *et al.* 2010; Sassner *et al.* 2008; Sidiras *et al.* 2011; Wang and Sun 2010; Zheng and Zhang 2009). Most references on wood waste involve one or two stages of dilute acid pretreatment, generally sulfuric acid, in concentrations lower than 4% (Galbe and Zacchi 2007), whereas others have used a mixture of steam with sulphur dioxide and oxalic acid as a catalyst (Chin *et al.* 2011; Dagnino *et al.* 2013; Kim *et al.* 2011; Monavari *et al.* 2009; Sassner *et al.* 2008; Sidiras *et al.* 2011; Silva *et al.* 1998; Sreenatha and Jeffries 2000). The acid pretreatment in one stage followed by enzymatic hydrolysis of the cellulose produces good yields of fermentable sugars. In two-stage pretreatment, the first and second stages are performed at low and high severity, respectively, to hydrolyze sequentially the hemicelluloses and the cellulose. This strategy has the drawback of its highest energy and chemicals consumption.

By using one stage of pretreatment with acid, the hemicelluloses are mostly hydrolyzed, generating monomeric sugars dissolved in the liquid from the treatment (Fengel and Wegener 1984; Kim *et al.* 2011; Monavari *et al.* 2009; Park *et al.* 2010; Sannigrahi *et al.* 2010; Sassner *et al.* 2008; Sidiras *et al.* 2011; Zhu and Pan 2010). No effect would result from a second stage of dilute acid treatment, and due to the high temperatures and acidic conditions of the pretreatment, these sugars can degrade, producing furfural, which results from the degradation of the pentoses (xylose and arabinose) and 5-hydroxymethylfurfural (HMF), made by the degradation of the hexoses (glucose, mannose, and galactose). In turn, the furfural can degrade into formic acid or it can polymerize, while HMF gives rise to large equimolecular quantities of formic and levulinic acids. The acetyl groups of the hemicelluloses can also decompose, making acetic acid, which acts as a catalyst (autocatalized pretreatments of autohydrolysis) (Kumar *et al.* 2009; Megawati *et al.* 2010; Mosier *et al.* 2005).

The objective of this work was to find the best conditions for pretreatment using sulfuric acid and heat of the black carob tree sawdust, in order to maximize the extraction of xylose with minimum degradation, and to maximize the concentration of glucans in the pre-treated solid for improved enzymatic accessibility.

EXPERIMENTAL

Materials

Black carob tree sawdust was used as the raw material. It was supplied by sawmills in the city of Resistencia, Chaco, Argentina. The sample consisted of material that passed through a 40-mesh screen but was retained on a 100-mesh screen. Afterwards, it was washed thoroughly with boiling distilled water, stirring for two minutes. The washed sample was filtered, dried at 60 °C until it reached a constant weight, and finally it was stored in a closed vessel at room temperature until it was used.

The cellulase enzymes were obtained from the Enzyme and Biomass Laboratory, Institute of Biotechnology, University of Caxias do Sul (Dillon *et al.* 2011). The enzyme system was composed of β-glucosidases (0.6 U/ml), endoglucanases (16.5 U/ml), and xylanases (14.2 U/mL), with a cellulase activity of 3.2 FPU/mL.

Pretreatments

The pretreatments were carried out following a Central Composite design of experiments (CCD) of two factors with four center point replicates (Test Nº 9 to 12). CCDs are very efficient optimization designs, and useful in response surface methodology, since they provide much information about linear effects, interactions, curvature effects, and overall experimental error, in a minimum number of required runs. These designs have the advantage of being rotatable, which means that the variance of the predicted response at any point depends only on the distance of the point from the design center point. Rotatable CCD uses alpha values to describe the external circular design geometry, *i.e.*, all points at the same radial distance from the center point have the same magnitude of prediction error. The considered variables were the sulfuric acid solution concentration and the heating time (Table 1).

The second and third lines of Table 1 represent the coded values (or the converted variables) that arise from converting the real values (lines 4 and 5). Coding the factor level is a way to transform the scale of measurement for a factor so that the high value becomes +1 and the low value becomes -1, such that the design matrix has all orthogonal columns.

Table 1. Conditions of the Pretreatment of the Black Carob Tree Sawdust with Dilute Sulphuric Acid (Design CCD)

Test Nº	1	2	3	4	5	6	7	8	9	10	11	12
H_2SO_4	-1	1	-1	1	-α	+α	0	0	0	0	0	0
Time	-1	-1	1	1	0	0	-α	+α	0	0	0	0
H_2SO_4 (% w/v)	0.3	1.7	0.3	1.7	0.0	2.0	1.0	1.0	1.0	1.0	1.0	1.0
Time (min)	16	16	44	44	30	30	10	50	30	30	30	30

The raw material was treated with solutions of sulfuric acid of different concentrations (0.3 to 2.0% w/v of H_2SO_4). The design included an autohydrolysis point with distilled water (Table 1, Test 5). A scheme of the diluted acid pretreatment procedure of black carob tree sawdust is shown in Fig. 1.

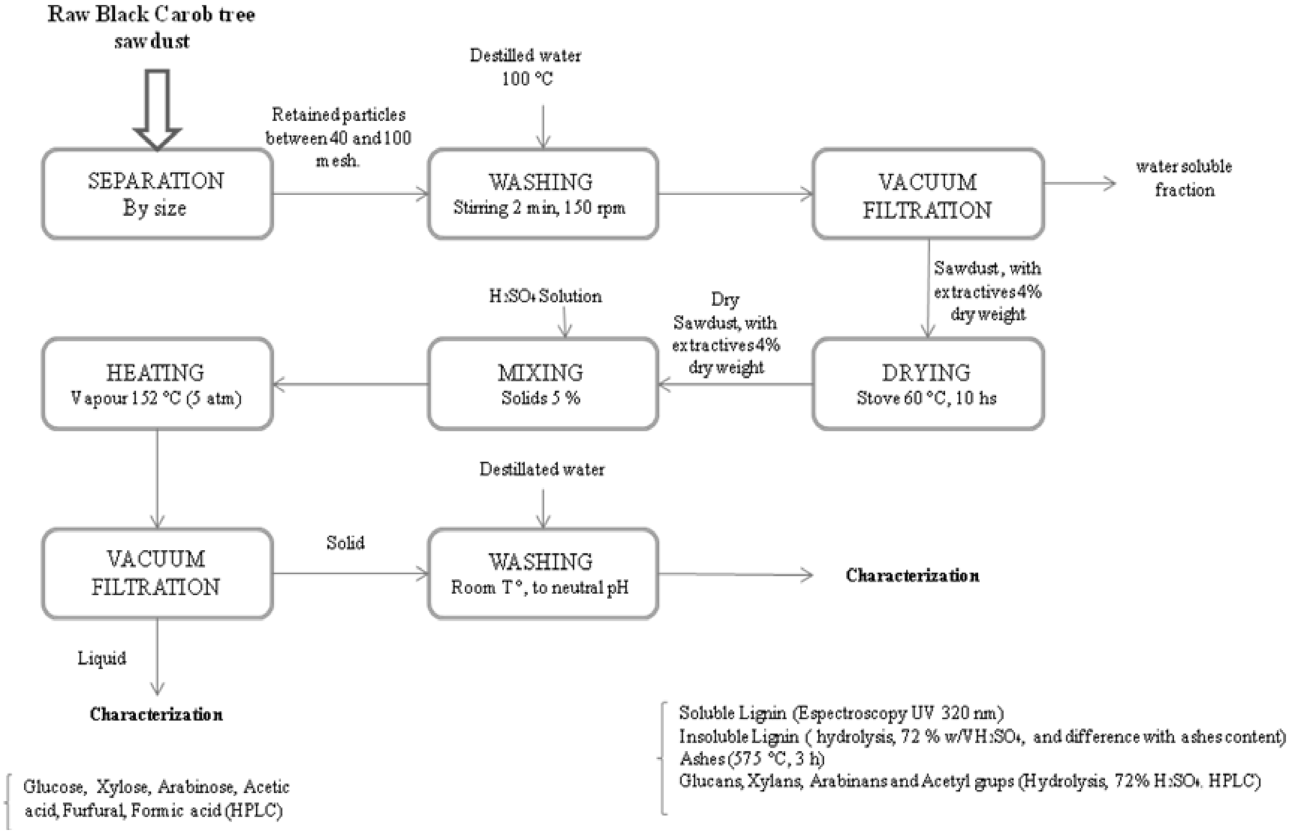

Fig. 1. Scheme of the diluted acid pretreatment procedure of black carob tree sawdust

The sawdust was mixed in a proportion of 5% solids, using 5 g of dried solid and 100 mL of the acid solution of variable concentration, according to the test. Then, the mixtures were placed in an autoclave with a vapor pressure of 5 absolute atmospheres (152 °C), with a residence time of 10 to 50 min, according to the design. Each test required an initial period of 20 min to reach the required pressure. A quick decompression was carried out when the heating time was completed. After this, the liquid was separated from the solid by means of hot vacuum filtration. In all cases, the separated liquid was refrigerated at 4 °C, while the pretreated solid was washed several times with distilled water to remove the residual acid solution. The solid was then dried in an oven at 60 °C for 10 hours and stored in desiccators for subsequent characterization.

Characterization Tests

The characterization tests of the raw material were carried out according to NREL-LAP (National Renewable Energy Laboratory-Laboratory Analytical Procedure) standards. They included: total solids and moisture (NREL/TP-510-42621), extractives in water and ethyl alcohol (NREL/TP-510-42619), structural carbohydrates: glucans, xylans, and arabinans, acetyl groups, lignin soluble and insoluble in acid (NREL/TP-510-42618), and ash (NREL/TP-510-42622).

The pretreated solid was characterized in the same way, excluding the determination of extractive substances in water and alcohol. The quantification of sugars, organic acids, and degradation products, was carried out by means of liquid chromatography HPLC (Waters chromatograph), using the column AMINEX-HPX87H (BIO-RAD) with the following chromatographic conditions: eluent: H_2SO_4 4 mM, flow: 0.6 mL/min, temperature: 35 °C, detector: refraction index and diode array.

The quantification of the homopolymers (glucans, xylans, arabinans) in the composition of the solid matter was carried out using the hydrolysis stoichiometric factors of 0.88 (or 132/150) for sugars with five carbons (xylose and arabinose) and 0.90 (or 162/180) for sugars with six carbons (glucose). The concentration of acetyl groups was defined as the product of the acetic acid concentration, evaluated by HPLC, and the stoichiometric factor 0.717.

The pretreatment liquid was characterized by the determination of the sugar content (glucose, xylose, arabinose) and breakdown products: organic acids (formic and acetic), furfural, and 5-hydroxymetylfurfural (HMF). The concentrations of degraded sugars were calculated as the product of the HMF concentration and the stoichiometric conversion factor 1.4286 (degraded hexoses) and the product of the furfural concentration and the stoichiometric factor 1.5625 (degraded pentoses).

The measurement of cellulase activity was done in terms of “filter paper units” (FPU/mL) and was carried out according to Ghose (1987). This method measures the reducing sugars formed in the first 60 min of the reaction of a mixture of diluted enzyme solution (0.5 mL) with 50 mg Whatman Nº 1 filter paper (substrate). The pH value was controlled at 4.8 with 0.05 M citrate buffer. The mixtures were incubated at 50 °C, and the reducing sugars produced after 60 min were analyzed by the DNS method (Miller 1959).

The results were analyzed by a multifactorial analysis of variance (ANOVA and optimization), with the concentration of the sulfuric acid solution and the time of treatment as independent variables. The statistical software Statgraphics was used, and the applied significance level was 5%. The equations were expressed in converted variables.

Enzymatic Hydrolysis

The solid fraction resulting from the pretreatment of *Prosopis nigra* sawdust at optimum conditions (1.2% acid and 10.2 min), without drying, was saccharified with the cellulase enzymes, according to NREL-LAP standards (NREL/TP-510-42629) with some modifications. The hydrolysis reaction was performed by shaking the material at 200 rpm for 72 h at 50 °C and pH 4.8, adjusted with a 0.05 M sodium citrate buffer. The enzyme dose was 40 FPU per gram of cellulose. An excess of enzymes was used in order to avoid any limitation of this variable. The samples (1 mL) were kept at -16 °C before HPLC analysis.

RESULTS AND DISCUSSION

Raw Material Characterization

The sawdust was washed with boiling distilled water for 2 min. The results of the chemical characterization of the sawdust of black carob tree (*Prosopis nigra*), untreated and thoroughly washed, can be seen in Table 2.

Table 2. Characterization of the Sawdust of Black Carob Tree (*Prosopis nigra*), Before and After Washing

Components	Raw black carob tree sawdust*	Washed black carob tree sawdust*
Total structural carbohydrates	47.5	54.0
Glucans	34.1	39.6
Xylans	12.6	13.6
Arabans	0.7	0.7
Total lignin	33.2	39.4
Insoluble acid lignin	31.2	37.2
Soluble acid lignin	2.0	2.2
Acetyl groups	1.9	2.2
Ash	0.5	0.5
Extractives	14.6	4.0
Others	2.2	-
*All values are expressed as percentages on a dry sawdust basis		

The objective of washing was to eliminate as much of the removable substances in hot water as possible, because they can interfere with the chemical reactions (reagent consumption) and can also be inhibitors to the production of fermentable sugars by enzymatic hydrolysis (Taherzadeh and Karimi 2007). Washing the raw material reduced the proportion of extractive substances from 14.61% to 4.00% on a dry basis. According to this, approximately 4% of the extractives are insoluble in water and remained in the sawdust. The approximated content of extractive substances (% on anhydrous weight of sawdust) of the black carob tree is 9.11% organic extractives, 5.17% aqueous extractives, 2.70% tannins, 5.28% non-tannic substances and 10.22 mg GAE/g d.m. (mg of gallic acid equivalent per gram of dry mass) of phenolic compounds, and 0.08 mg CE/g d.m. (mg of catechin equivalent per gram dry mass) of flavonoids (Pizzo *et al.* 2011).

The composition of structural carbohydrates, lignin, ash, and acetyl groups of the raw material used in this work is similar to other hardwoods (Table 2). For example, other authors have reported that *Prosopis juliflora* has approximately 66.20% holocellulose (47.50% α-cellulose and 18.70% pentosans), 29.10% Klason lignin, and 2.02% ash (Gupta *et al.* 2009). *Salix* sp. has presented 41.4% glucans, 15.0% xylans, 24.2% Klason lignin, 0.9% ash, and 2.9% acetyl groups (Sassner *et al.* 2008). Additionally, *Eucalyptus* g*randis* has 44.65% cellulose, 15.33% xylans, 25.77% lignin, 3.25% extractive substances, and 11% unidentified compounds (4-O-methylglucuronic acid and acetyl groups) (Emmel *et al.* 2003).

Characterization of the Pretreated Solid

The results of the analytical determinations, made on the pretreated solid with different concentrations of sulfuric acid solution and heating time, according to the experimental design (Table 1), can be seen in Table 3.

Arabinans and acetyl groups were also determined. Arabinans (0.12%) were found only in the pretreated solid of Test 5 (without acid, 30 min). The acetyl groups were found in Tests 1 (0.3% w/v H_2SO_4, 30 min), 5 (without acid, 30 min), and 7 (1% w/v H_2SO_4, 10 min), with values of 0.74%, 1.69%, and 0.36% respectively. The raw material had low concentrations of arabinans and acetyl groups (Table 2), and due to the hydrolysis caused by the acid pretreatment, these were solubilized in the pretreatment liquid as arabinose and acetic acid.

Due to the mostly crystalline structure of the cellulose, the proportion of glucans presented only a few differences with the application of different pretreatments. The analysis of variance indicated three significant effects ($p < 0.05$) with respect to the variability of the glucan concentration. The increase in the response with the concentration of the sulfuric acid solution (C) and the heating time (t) and C with a quadratic exponent ($C2$) indicates the existence of a maximum. The regression equation (Eq. 1) that fits this data is shown in Table 5. The results were as expected, because high acid concentration and heat exposure time produce extensive hydrolysis of xylans. Consequently, the remaining glucans in the pretreated solid (referred to as a percentage on a dry basis) increase.

Table 3. Pretreated Solid Characterization

Test N°	Glucans*	Xylans*	Lignin*	Recovered solids
1	38.30	4.37	41.33	87.88
2	40.47	0.14	46.16	75.53
3	42.49	0.06	46.06	68.41
4	46.98	0.03	48.02	68.19
5	39.01	10.92	38.80	97.00
6	45.60	0.00	43.44	61.03
7	41.50	3.12	42.73	80.98
8	44.37	0.06	51.93	66.07
9	44.49	0.09	46.36	75.86
10	44.13	0.10	45.44	76.20
11	44.83	0.05	45.15	75.42
12	44.85	0.11	48.78	74.97

*Values are percentages on a dry basis of pretreated solid.

In the regression equation for the percentage of glucans in the pretreated solid (Table 5, Eq. 1), the existence of a quadratic term with a negative regression coefficient indicates a maximum. The maximum values that can be reached in the tested conditions are between 47.0% and 48.0%, obtained with high acid concentrations and long heating times (Fig. 2a).

The optimum value to maximize the glucan concentration in the pretreated solid was achieved at 152 °C, using a solution of sulfuric acid of 1.69% w/v (coded variable +0.92) for 49.8 min (coded variable +1.41). Other authors have found that the optimum conditions for *Saccharum* sp. (sugarcane) were 158 ºC, 6.79% oxalic acid, for 16 min (Scordia *et al*. 2010). For *Eucalyptus grandis* wastes, the optimum conditions were 157 ºC, 0.65% sulfuric acid, for 20 min (Canettieri *et al.* 2007) and for *Liriodendron tulipifera* (yellow poplar) they were 151 ºC, 0.042 g/g oxalic acid, for 13 min (Kim *et al.* 2011).

Xylans are the main components of the hemicelluloses of hardwood. These polymers of amorphous structure are hydrolyzed in the presence of acid and heat. In this study, the xylans were hydrolyzed in almost all tests, except for the trials carried out at a low acid concentration and for a short time (Table 3), such as 1 (0.3% w/v H_2SO_4, 16 min), 5 (without acid, 30 min), and 7 (1% w/v H_2SO_4, 10 min).

The analysis of variance indicated that both studied variables had significant influence on the proportion of xylans that remained in the pretreated solid, according to Equation 2 observed in Table 5.

The graphic of contours estimated for xylans in the pretreated solid can be seen in Fig. 2b. The proportion of xylans in the pretreated solid reached minimum values (0.0 to 0.2) within a relatively wide area, which was comprised of several combinations of the two variables.

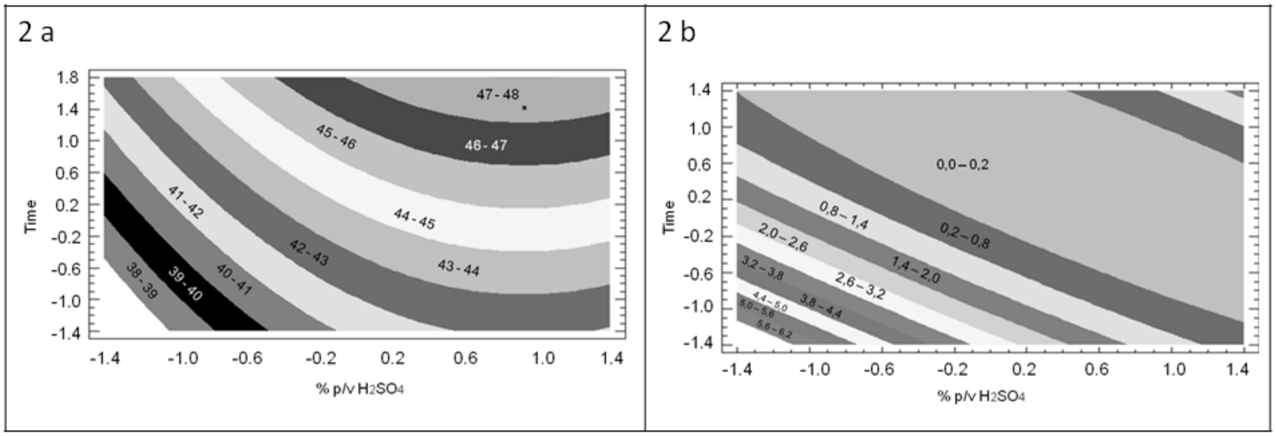

Fig. 2. Graphic of estimated contours for a) glucans in the pretreated solid and b) xylans in the pretreated solid

The statistical analysis was carried out, excluding trial 5 (autohydrolysis, without acid, 30 min). This was considered to be a special case, as these conditions produced low hydrolysis, compared to the rest of the tests. The effect of the interaction indicates that at relatively high times corresponds to the highest extraction of xylans at any acid concentration, whereas at a higher acid concentration, the extraction of xylans is higher at any heating time.

The acid pretreatment in the studied conditions did not have any observable effect on the lignin in the pretreated solid. The variation of 13.2% between the maximum and minimum value is attributable to the modifications in the composition of the pretreated solid (dry basis). The extraction of hemicelluloses causes a relative increase in the other components (glucans and lignin). The maximum value (51.93%) occurred in trial 8 (1%, 50 min), which produced extensive extraction of xylans, whereas the minimum value (38.80%) was obtained in trial 5 (autohydrolysis, 30 min).

Finally, the recovered solids (mostly cellulose) were influenced negatively by both variables in the study: the concentration of the sulfuric acid solution (p = 0.0024) and the time (p = 0.0115). In the case of the autohydrolysis process, the recovery of the solids after pretreatment was almost complete. The regression equation that fits the data is Eq. 3, located in Table 5.

The proportion of recovered solids decreased with both variables, reaching a minimum value at the point where the concentration of the acid solution and the heating time had maximum values.

Characterization of the Liquid from Pretreatment

The characterization of the liquids in each trial can be observed in Table 4. Results are expressed as a percentage on a dry basis of the treated sawdust.

Table 4. Characterization of Liquid from the Pretreatment

Test Nº	Glucose*	Xylose*	Arabinose*	Formic acid*	Acetic acid*	Furfural*	HMF*
1	0.78	11.91	1.33	0.29	2.20	0.14	0.01
2	3.50	13.61	1.24	0.73	2.99	1.39	0.06
3	6.42	9.06	0.98	0.95	3.13	2.66	0.13
4	6.61	8.91	0.97	0.96	3.08	2.74	0.13
5	0.22	4.33	1.28	0.22	0.74	0.02	-
6	6.50	9.20	1.20	0.83	3.10	2.17	0.20
7	1.20	13.16	1.24	0.21	2.38	0.16	0.02
8	5.47	10.75	1.31	0.86	2.95	1.76	0.12
9	3.67	14.20	1.29	0.99	3.08	1.39	0.08
10	3.33	13.06	1.20	0.89	2.93	1.59	0.07
11	3.57	13.85	1.28	0.95	2.98	1.29	0.07
12	3.50	13.44	1.22	1.15	2.92	1.22	0.07
*Values are percentages on dry basis of untreated solid.							

The proportion of xylose in the liquid was high, whereas the glucose was found in low quantities (Table 4). These results are attributed to the high reactivity of the hemicelluloses (amorphous and more accessible) in comparison with the cellulose (crystalline and packed in a microfibrillar array).

The glucose in the liquid has two origins: hemicelluloses and amorphous cellulose. The ANOVA analysis indicated that the percentage of glucose in the liquid was linearly influenced by both variables, according to Eq. 4 (Table 5). The graphic of estimated contours (Fig. 3a) shows straight areas, indicating the linear influence of both independent variables on the concentration of glucose, reaching its maximum value (8.0 to 8.8%) at high values of them.

According to the statistical analysis (Eq. 5 in Table 5), the proportion of xylose in the liquid increased with acid concentrations (p = 0.0002) but the quadratic effect made it decrease, since the negative sign involves a concave form. The heating time had a similar quadratic behavior (p = 0.0030), resulting in concentric circles (with a maximum in the center), lightly deformed by effect of the interaction.

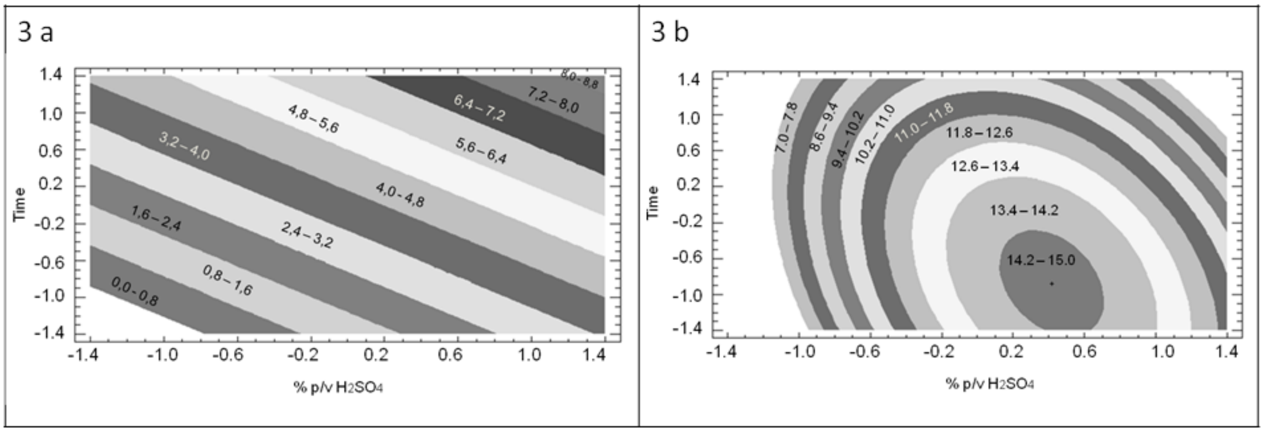

Fig. 3. Graphic of estimated contours for a) glucose in the liquid and b) xylose in the liquid

The proportion of xylans and the xylose concentration show the extent to which the hemicelluloses were hydrolyzed in each case. In the liquids of trials 1 and 7 (low acid concentrations and short times) and in trial 5 (autohydrolysis), 16.7% and 96.0% (respectively) of the total xylose was present as oligomers.

Figure 3b shows the variation of xylose content in the liquids from pretreatment. The minimum values correspond to extreme conditions. Even though higher concentrations of the acid solution and longer treatment times produced the most extensive hydrolysis of xylans, the dissolved sugars were immediately degraded as a consequence of the severe conditions. This explains the existence of the maximum value of xylose at the mean acid concentration (1.3%) and at a short heating time (17.7 min). The optimum found through ANOVA analysis was 14.46% xylose. Kim *et al.* (2010) reported a maximum extraction of xylose by applying a pretreatment with 0.042 g/g of oxalic acid at 158 °C for 13 min.

The concentration of the products of sugar decomposition (furfural, 5-hydroxymetylfurfural, and formic acid) increased at high levels of the studied variables. This fact is related to the results shown for the proportion of xylose. That is, when the concentration of xylose decreases in the liquid, the proportion of the decomposition products increases. The statistical significance of the heating time (p = 0.0016) is higher than that of the acid concentration (p = 0.0169). The equations of the fitted models can be observed in Table 5 (Eq. 6 to 8).

The concentration of glucose in the liquid from the pretreatment was much lower than the concentration of xylose. The same behavior can be observed in their respective degradation products, furfural and 5-hydroxymetylfurfural.

The regression equation for acetic acid (Eq. 9 in Table 5), resulting from the hydrolysis of acetyl groups, shows that the heating time had a more significant influence than the acid concentration (p = 0.0002 *vs.* p = 0.0035). The analysis of the interaction effect shows that high concentrations of sulfuric acid produce immediate deacetylation, which does not happen at low concentrations.

Table 5. Regression Equations and Their Coefficients

% Components	Regression Equations*	Regression Coefficient	Equation Number
Pretreated Solid			
% Glucans	$43.81 + 2.00 * C + 1.85 * t - 1.08 * C^2$	$R^2 = 0.82$	1
% Xylans	$0.09 – 0.97 * C - 1.10 * t + 0.51 * C^2 + 1.05 * C * t + 0.69 * t^2$	$R^2 = 0.99$	2
Performance	$76.63 – 7.93 * C – 5.99 * t$	$R^2 = 0.75$	3
Pretreatment Liquid			
% Glucose	$3.71 + 1.51 * C + 1.81 * t$	$R^2 = 0.85$	4
% Xylose	$13.64 + 1.84 * C – 1.01 * t – 3.46 * C^2 - 1.19 * C * t – 0.86 * t^2$	$R^2 = 0.93$	5
% Furfural	$1.38 + 0.55 * C + 0.77 * t$	$R^2 = 0.77$	6
% HMF	$0.09 + 0.04 * C + 0.04 * t$	$R^2 = 0.77$	7
% Formic acid	$0.99 + 0.16 * C + 0.27 * t - 0.18 * C^2 – 0.18 * t^2$	$R^2 = 0.85$	8
% Acetic acid	$2.97 + 0.16 * C + 0.23 * t - 0.21 * C * t - 0.14 * t^2$	$R^2 = 0.96$	9
* Expressed as converted variables.			

Optimization

The objective of pretreating lignocellulosic residues is to improve the enzymatic accessibility, increasing the fermentable sugar production for maximization bioethanol production. Pretreatment with diluted acid solutions generates two products: 1) a porous solid with a large surface area, a high concentration of glucans, and the remaining lignin and ashes, and 2) a liquid which contains dilute sugars (mostly xylose), as a result of the hydrolysis of hemicelluloses, as well as the degradation products of sugars, acetyl, and glucuronic groups. The pretreated solid is susceptible to hydrolysis by cellulase enzymes, producing glucose, which is subsequently fermented for bioethanol production, whereas the degradation products in the liquid can act as inhibitors of the fermentation process. On the other hand, the extracted xylose can be suitable for diverse uses, such as the production of bioethanol, sweeteners, food, and pharmaceuticals. Consequently, an efficient pretreatment is one that maximizes the concentration of glucans in the solid and the concentration of xylose in the liquid, while minimizing the main degradation product (furfural).

The desirability function is the most popular solution for the multi-response optimization problem. This approach to simultaneously optimize multiple equations, translates the functions to a common scale ([0, 1]) and combines them using the geometric mean and optimizing the overall metric. The graphic of the estimated contours of the desirability function shown in Fig. 4 was obtained using the Optimization of Multiple Answers tool of Statgraphics software.

It can be observed from Fig. 4 that the optimum value does not appear among the tested conditions (the desired function does not reach value 1), but it seems to be located somewhere near the point -1.4 of time and 0.1 of acid (as transformed variables). The best desirability in the graphic (0.7 to 0.8) could be obtained with the mean value of the acid concentration (1.2%, coded value 0.31) and a short heating time (10.2 min, coded value -1.41).

Results of similar pretreatments applied to hardwoods by other authors are presented in Table 6. The severity factor (*log* R_0) is an index used in several works to compare the conditions of the pretreatment and their effects. This parameter can be used for estimation because it does not provide an exact measure of the effect of the process (Ahring *et al*. 2007; Pedersen and Meyer 2010).

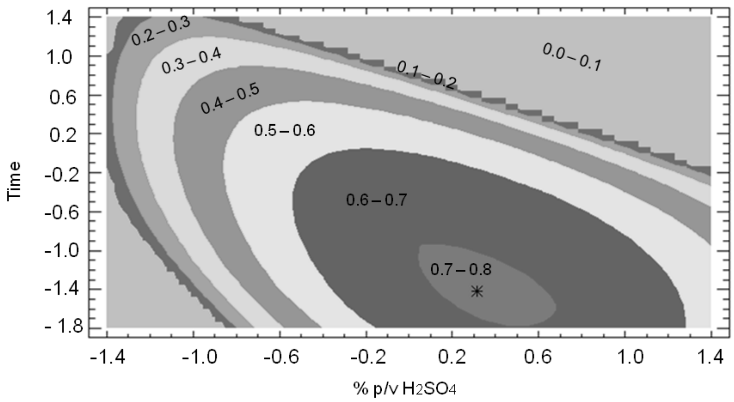

Fig. 4. Optimization of multiple answers (maximization of glucans in the solid and xylose and minimization of furfural in the liquid)

The correlation (Eq. 10) indicates the relevance of the pretreatment as a function of the heating time t (min) and the temperature T (°C), T_{ref} = 100 °C.

$$log\ (R_0) = log\ (t * exp\ (T - T_{ref}) / 14.75) \qquad (10)$$

When the pretreatment is carried out in acidic conditions, the effect of the pH is calculated according to the Eq. 11.

$$Combined\ severity\ (CS) = log\ (R_0) - pH \qquad (11)$$

The combined severity factor calculated for black carob tree sawdust using an acid pretreatment at optimum conditions was 1.86, which was slightly lower than those found by other authors for hardwoods and other lignocellulosic wastes (Table 6).

Table 6. Results of Acid Pretreatments on Hardwoods and Other Lignocellulosic Wastes

Raw Material	Conditions	CS	References
Yellow poplar	15 °C; 0.042 g/g oxalic acid (weight/dry matter); 13 min	2.62	Kim *et al*. (2011)
Salix	200 °C; 0.5% sulphuric acid; 4 or 8 min	2.56-2.86	Sassner *et al.* (2008)
Acacia dealbata	180 °C; 0.8% sulphuric acid; 15 min	2.74	Ferreira *et al.* (2011)
Eucalyptus grandis	210 °C; 0.175% sulphuric acid; 2 min	2.09	Emmel *et al.* (2003)
Populus	160 °C; 0.1 mol/m^3 sulphuric acid; 2.5 to 5 min	2.16-2.47	Foston and Ragauskas (2010)
Rice husk^a	152 ºC; 0.3% sulphuric acid; 33 min	1.82	Dagnino *et al.* (2013)
Black carob tree	152 ºC; 1.0% sulphuric acid; 12 min	1.87	This work

Although the optimum conditions of the acid pretreatment obtained by the same methodology for rice husk were different (0.33% w/v, 33 min), the severity factor was very similar. The difference could be caused by the rubbers and waxes present in the husk, which makes it resistant to the pretreatment, requiring a different combination of variables.

An additional set of diluted acid pretreatments of black carob tree at optimal conditions was performed to validate the predicted values. The experimental value for glucan concentration in the solid was 51.2%, whereas the predicted value was 41.7%. Moreover, the experimental value for xylose extracted and solubilized in the liquid was 13.8% and the predicted value was 14.1%. Meanwhile, the experimental value for furfural concentration in the liquid was 0.9% and the predicted value was 0.5%. A reason for the differences between the experimental and the predicted values for glucans in the solid fraction may be that the fit of the regression equation for yield (solid recovery), even if significant, was not as good as the others. This affects the percentage of the carbohydrates obtained in the solid fraction, which is referred to dry basis solids. In the liquid face, the correlation between the experimental and predicted value of xylose was excellent, and in the case of furfural, the differences could be consequence of measurable errors due its low concentration (<1). Nevertheless, it was minimized in these experimental conditions, considering the values in Table 4.

Enzymatic Saccharification of Pretreated Sawdust

The solid fraction resulting from the pretreatment of sawdust with 1.2% acid for 10.2 min was enzymatically saccharified, and it was compared with the saccharification of untreated black carob tree sawdust. The performance of enzymatic hydrolysis was determined by measuring the yield of glucose from cellulose conversion, expressed as the percentage of glucose released in relation to the total amount of glucose in the solid. As the experimental enzymes gave low conversions of glucose, another trial was performed using the enzyme from Novozymes S.A. The yields of glucose from the pretreated (at optimum conditions) and non-treated sawdust were 70% and 23.3%, respectively. Thus, the performance of the hydrolysis of the pretreated solid was three times higher than the performance of untreated sawdust, in the same conditions after 72 h of reaction.

The enzymatic hydrolysis of hardwoods is enhanced by a pretreatment with dilute acid (Sassner *et al.* 2008; Wyman *et al.* 2009), whereas it seems that the enzymatic conversion of cellulose after an acid pretreatment is unsatisfactory for softwood (Zhu and Pan 2010). The difference in reactivity between softwoods and hardwoods, even at the similar lignin contents may be due to the structure and distribution of lignin and to lignin-carbohydrate complex (Yu *et al.* 2011).

CONCLUSIONS

1. The optimum pretreatment process that maximizes glucans in the pretreated solid and xylans in the liquid, while minimizing furfural in the liquid was achieved with the mean value of the tested acid solution concentration (1.2%) after a short reaction time (10.2 min).
2. The optimum value for the maximization of glucan concentration in the pretreated solid was achieved using a solution of sulfuric acid of 1.7% w/v for 49.8 min. There was an increase of the percentage of glucans in the solid because of the loss of other components at the highest studied levels of acid concentration and heating time.

3. The xylans in the pretreated solid were almost completely hydrolyzed in most of the trials. The most extensive hydrolysis, that is, the lowest level of remaining xylans, was obtained in the treatments using 1.7% and 2.0% w/v of sulfuric acid.
4. The concentration of xylose in the liquid from pretreatment increased with the acid concentration and decreased with the heating time, due to its degradation to furfural. The pretreatment that maximized xyloses in the liquid was the one that used a 1.3% w/v solution of sulfuric acid for 19.7 min.
5. The performance of the enzymatic hydrolysis of the pretreated black carob tree sawdust was three times higher than the performance of untreated sawdust, in the same conditions after a reaction time of 72 h.
6. Washing the black carob tree sawdust with distilled water extracted approximately 70% of the substances soluble in water.

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