Fractionation of Broom (*Cytisus striatus*) Biomass Components *via* Mild Sulfite Pretreatment and Enzymatic Hydrolysis

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The potential of broom biomass to produce oligo- and monosaccharides was investigated using mild sulfite pretreatment conditions followed by enzymatic hydrolysis. Both treatments were analyzed *via* response surface methodology using an experimental central composite rotatable design 2^4 + star, which explored the following variables: sulfuric acid charge (0% to 3%), sodium sulfite charge (0% to 4%, maximum temperature (150 °C to 190 °C), and time at maximum temperature (0 min to 30 min). Oligo- and monosaccharides in the pretreatment hydrolysates were determined using high performance liquid chromatography. The amount of total extracted xylose, mannose, and galactose ranged from 3.5% to 15.8% of the initial biomass, while the model estimated optimal reaction conditions enabled the extraction of practically all hemicellulose in the raw material. However, the mildest pretreatment reaction conditions, with low temperature and low sulfuric acid charges, provided a hydrolysate where a major part of the extracted polysaccharides remained in oligomer form, enabling their separation by filtration. The cellulose-rich solid residue was submitted to enzymatic hydrolysis using a Novozymes® enzymatic cocktail. The enzymatic hydrolysis was successful, but some polysaccharides remained in the solid residue, mainly composed of lignin. An enzymatic yield of 60% was attained with no added sulfite in the pretreatment at 190 °C, despite the confirmed positive role of sulfur content in the solid residues.

Keywords: Cytisus striatus; Sulfite pretreatment; Enzymatic hydrolysis; Response surface methodology

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INTRODUCTION

To move from an oil-based economy to the sustainable exploitation of natural resources, it is essential to fully utilize the renewable raw materials potential, *e.g.*, lignocellulosic materials. These materials are available all over the world, they can grow in relatively poor soils, and their annual increment is approximately 3 x 10^{11} tons (Belgacem and Gandini 2008), having the potential to replace oil consumption. In simple terms, the biggest difference between lignocellulosic materials and oil is that oil components are relatively easily separated by distillation, whereas lignocellulosic components are chemically bonded and strongly interpenetrated in a complex composite. Therefore, the fractionation of vegetal biomass polymeric components, *e.g.*, cellulose, hemicelluloses, and lignins, remains a major challenge. Several approaches have been followed, that the authors can sum up in two global approaches: dissolution (with or without derivatization) and depolymerization (total or partial) of the lignocellulosic components.

One of the possibilities with the highest potential to accomplish the second approach is *via* polysaccharide depolymerization, *i.e.*, releasing sugars and leaving the lignins, using enzymes, due to the mild reaction conditions that are required and the environmentally friendly nature of the processes. However, the non-productive adsorption of the polysaccharide depolymerization enzymes by lignins, the high cellulose crystallinity, and the low accessibility of enzymes into the cell wall, to mention just some factors, impairs a cost-effective deconstruction of the cell wall with the corresponding sugar release (Hamelinck *et al.* 2005; Zhu *et al.* 2009; Alvira *et al.* 2010; Zheng *et al.* 2016; Yoo *et al.* 2020). To overcome some of these limitations, a biomass pretreatment before enzymatic hydrolysis is the current conventional strategy. The pretreatment intends to create enzymatic accessibility and remove and/or modify the lignins to minimize their non-productive enzyme adsorption (Pihlajaniemi *et al*. 2016; Yang and Pan 2016; Yoo *et al.* 2020). Concerning accessible surface area and pore volume related issues, Grönqvist *et al*. (2014) reported that the formation of micro- and macropores in pulp fibers during mechanical shredding correlates with fibers susceptibility to enzymatic hydrolysis. Cellulose accessibility has also been studied (Pihlajaniemi *et al*. 2016).

Zheng *et al.* (2016) studied the adsorption of cellulase and β-glucosidase on lignocellulosic substrates and confirmed the irreversible and high affinity of both enzymes for lignins. Moreover, several studies have confirmed that the chemical composition and the lignin structure affect the cellulase affinity and therefore the cellulase availability, in terms of an effective cellulose depolymerization process (Yu *et al.* 2014; Li *et al.* 2016; Yang and Pan 2016). Several authors demonstrated that hydrophobicity particularly potentiates the adsorption of cellulase by lignins (Eriksson *et al*. 2002; Yang and Pan 2016), although cellulase can be adsorbed by lignins through other mechanisms, *e.g.*, hydrogen bonding and electrostatic interactions (Lou *et al*. 2013). In this case, the enzyme surface charge, which is dependent on the enzyme isoelectric pH and on the pH of the medium, can play an important role as well. As a result of this phenomenon, some enzymes are preferentially adsorbed by lignins, but other combinations of lignin/enzymes are less favorable, making enzymes more available for cellulose adsorption and hydrolysis. In this respect, it is important to refer that the sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) was proposed some years ago, and its efficiency has been demonstrated (Zhu *et al*. 2009; Liu *et al.* 2011). The introduction of sulfonic groups to the lignins was demonstrated (Lou *et al*. 2013).

Another issue is the required delignification extent to attain good enzymatic hydrolysis. Yoo *et al.* (2015), who worked with aqueous and gaseous ammonia and corn stover, suggested that the removal of ligins was not a prerequisite for effective enzyme hydrolysis. More recently, Kellock *et al*. (2019), who worked with spruce and wheat straw under hydrothermal acid conditions in the pretreatment stage, have shown that "inhibition was mainly caused by the effect of the pretreatment and was not an inherent characteristic of the lignin". Moreover, saccharification can be improved by modifying the lignin without removing its major part (Chen *et al.* 2007; Yoo *et al*. 2015).

Hydrothermal treatment is one of the most used pretreatments methods to enhance enzymatic hydrolysis, but recently Kellock *et al.* (2019) concluded that the severity of the treatment correlates well with the inhibitory effect of the lignin. Therefore, in the present work, a mild sulfite pretreatment was tested, *i.e.* low sulfite and acid charges.

The current work is devoted to a shrub, *Cytisus striatus*, also known as broom, that is abundant in the Mediterranean region despite being poorly exploited. Only a few papers have been published which explore this shrub (Ferreira *et al.* 2009; Gil *et al.* 2012; Costa

et al. 2016). One of them (Ferreira *et al.* 2009) was focused on enzymatic hydrolysis optimization of the substrate after a dilute acid pretreatment, using relatively high enzymes charges. Another study (Gil *et al.* 2012) optimized the dilute acid pretreatment. In a previous work of the authors, an acid sulfite pretreatment was tested, and the corresponding response in terms of enzymatic performance was studied (Costa *et al.* 2016). Using relatively high sulfite (5 to 15%) and acid (0 to 5%) charges in the pretreatment, hemicelluloses are mostly recovered as monomers and the enzymatic hydrolysis was effective even using a low enzymatic charge. Considering the need to reduce the overall cost to make this kind of process cost-effective, as well as attending to the possibility of recovering the hemicelluloses as oligomers in the pretreatment stage, in the current work, lower charges of sulfite and acid were explored.

Thus, the aim of the present investigation was to evaluate the response of *Cytisus striatus* to the sulfite pretreatment under mild reaction conditions, with the goal of maximizing hemicelluloses release, as oligosaccharides and/or monosaccharides, while minimizing cellulose degradation in the first stage, and maximizing glucose release during enzymatic hydrolysis, leaving a lignin-rich residue as a third stream. A central composite rotatable design 2^4 + star was used to achieve these goals. The optimization was performed according to the correlations obtained for each experimental variable in terms of the design factors ($NaHSO_3$, H_2SO_4, time, and temperature).

EXPERIMENTAL

Materials and Methods

Raw material preparation

For the present study, *Cytisus striatus* (broom) branches were milled in a knife mill (Retsch Mühle, Haan, Germany) with an output sieve size of 10 mm x 10 mm; the fines were removed with an 18-mesh screen, which resulted in a material with a 1 mm to 2 mm width and thickness and a 10 mm length (pin chips). The dry matter content of the pin chips and the pretreatment solid residues were determined according to ISO standard 638 (2008). The chemical composition of the raw material was as follows: glucan: 41.6%; xylan: 17.9%; mannan: 3.0%; galactan: 3.4%; acetic acid: 4.1%; total lignin: 23.3%; and extractives: 4.1% (Costa *et al.* 2016).

Pretreatments

The broom pin chips were pretreated using sodium bisulfite (reagent grade, Sigma-Aldrich) and sulfuric acid (reagent grade, Sigma-Aldrich) at a liquid to wood ratio of 5/1, as described in Costa *et al.* (2016). The tested conditions included the following variables: sodium bisulfite charges (ranging between 0% and 4%), sulfuric acid charges (ranging from 0% to 3%), pretreatment maximum temperatures (ranging between 150 °C and 190 °C), and reaction times at maximum temperature (ranging between 0 min and 30 min). The current work was devoted to low charges for both chemicals, aiming to make the process more sustainable and enable the recovery of oligosaccharides. The effects of the variables were explored according to an experimental arrangement in the form of a central composite design 2^4 + star, as explained later.

After the pretreatments, the content of the reactor was diluted and thereafter disintegrated in a laboratory blender for 1 min (Snijders Analysers, Holland, Netherlands). In order to minimize the loss of volatile compounds, samples of the hydrolysates were

collected *via* filtration at atmospheric pressure, prior to extensive washing of the solid residues with distillated water. The resulting solids were weighed, and their dry matter content was determined. The solid residue yield (SRY) was determined as a percentage of the initial raw-material mass (oven dry base). The residual lignin content of the solid residue was estimated using the Kappa number analysis (an oxidative analytical procedure usually followed in the pulp sector to estimate the residual lignin content in pulps), according to ISO standard 302 (2015). All pretreated washed solid residues were preserved wet at 4 °C until undergoing enzymatic treatment. The sulfur content (S) of the corresponding dry samples was estimated with a HITACHI S 2700 scanning electron microscope (using a high voltage of 20 kV). Images were formed through secondary electrons. An Energy-dispersive X-ray spectroscopy (EDS) attachment was used to ascertain the sulfur, carbon, and oxygen presence on the pretreatment solid residue biomasses.

Analysis of pretreatment hydrolysates

The pretreatment hydrolysates chemical composition was analyzed, directly and after additional hydrolysis, to ensure the complete depolymerization of the oligosaccharides remaining in the sulfite hydrolysate, *via* high-performance liquid chromatography (HPLC) and UV-visible spectrophotometry. The samples were filtered with a 0.45 μm nitrocellulose syringe filter membrane, prior to the HPLC quantification. Therefore, the oligosaccharides present in the original sulfite hydrolysates would be retained in the 0.45 μm filter membrane syringe and consequently were not detected by the HPLC system. After additional hydrolysis, carried out with analytical purposes, all oligosaccharides were converted into the corresponding monomers and were consequently detected by the HPLC system. The difference between the sugar composition after and before the additional hydrolysis allowed for an estimate of the material in its oligomeric form to be determined.

Sugars, organic acids (acetic and formic acids), and sugar degradation products (furfural and hydroxymethylfurfural (HMF)) present in the hydrolysates were analyzed *via* a HPLC system provided with an Aminex® HPX-87H (300 mm ×7.8 mm) column (Bio-Rad), refractive index, and UV-visible detectors, as previously described in Costa *et al.* (2016). The sugars and by-product yields, regarding the initial wood weight, were determined considering the concentrations of the compounds (arithmetic average of at least two measurements) in the hydrolysates determined by HPLC and the final liquid-to-wood ratio. The sugars were expressed as anhydrous units.

The soluble lignin content in the hydrolysates was determined *via* UV-visible spectrophotometry, following the previously reported procedures (Costa *et al*. 2016).

Enzymatic treatment

An enzyme kit for the hydrolysis of lignocellulosic materials from Novozymes® composed of 6 different enzyme solutions was used to evaluate the enzymatic hydrolysis performance of the solid residues subjected to pretreatment. After appropriate dilution, the cocktail was added to the wet solid residues, and the biomass was subjected to enzymatic hydrolysis at a 1% solid content, a pH of 5.5, and a temperature of 50 °C, under constant agitation. Both the enzyme charges and the operating conditions were reported elsewhere (Costa *et al*. 2016). The enzymatic hydrolysis process was maintained for as long as 96 h. Samples of the enzymatic hydrolysates were taken in duplicate at 2 h, 7 h, 15 h, and 24 h and then every 24 h after that. The glucose and xylose contents in the enzymatic

hydrolysates were monitored *via* HPLC, as previously described for the sugars in the pretreatment hydrolysates.

Experimental design and data analysis

Response surface methodology (RSM) is widely used to optimize manufacturing processes and product designs, usually involving several response variables (Shah *et al.* 2004). The experimenter fits a model to each response using ordinary least squares (OLS), based on observed data from a designed experiment.

In a typical RSM study, the experimenter will build an empirical model such as the second-order model, as shown in Eq. 1,

$$y_r = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<} \sum_{j=2}^{k} \beta_{ij} X_i X_j + \epsilon \quad (1)$$

where y_r is the r^{th} response factor, X_i is the i^{th} independent factor, β_0 is the intercept, β_i is the first-order model coefficient, β_{ii} is the quadratic coefficient for the factor *i*, and β_{ij} is the linear model coefficient for the interaction between factors *i* and *j*. These models determine the *X*'s settings that produce the optimal or at least acceptable values for the responses y_1, y_2,…, y_n. The independent variables X_i are coded as shown in Eq. 2,

$$X_i = \frac{x_i - x_{i,mid}}{\Delta x_i} \quad (2)$$

where $x_{i,mid}$, x_i, and X_i represent the uncoded value at the center point, uncoded value, and coded value for the i^{th} independent variable, respectively. The response surface is typically driven by some unknown physical mechanism; the model represents an empirical model of this system. Optimization techniques are then usually applied, as shown in studies by Kunamneni and Singh (2005); Ferreira *et al*. (2009); Jeong *et al.* (2009); Gil *et al.* (2012); and Chi *et al*. (2019).

An experimental central composite rotatable design 2^4 + star, consisting of a central two-level factorial plus additional star points was adopted, generated using Statgraphics® Centurion XVII. The design consisted of a five-level-four-factors CCRD, requiring 25 experiments made in random order. Table 1 shows the coded and uncoded independent factors (X_i), levels, and experimental design for the four independent factors: $NaHSO_3$ charge (%), H_2SO_4 charge (%), temperature (°C) and time (min). The experimental design range was based on previous experimental results with the same raw material (Costa *et al*,, 2016), exploring low chemical charges for process sustainability.

Table 1. Factors and Their Levels for Central Composite Design 2^4 + Star

Variable	Symbol	Coded Factor Levels				
		−2	−1	0	1	2
$NaHSO_3$ Charge (%)	X_1	0	1	2	3	4
H_2SO_4 Charge (%)	X_2	0	0.75	1.5	2.25	3
Temperature (°C)	X_3	150	160	170	180	190
Time (min)	X_4	0	7.5	15	22.5	30

RESULTS AND DISCUSSION

Acid Sulfite Pretreatment

Experimental results and models statistical analysis

The experiments were carried out as per the CCRD 2^4 + star design, expressed in Table 1, and the experimental results are shown in Table 2. This table also includes the results from the enzymatic hydrolysis of the solid residue after sulfite hydrolysis for convenience. For the sulfite pretreatment samples, the percentages of glucose (including cellobiose), XMG (combined xylose, mannose, and galactose), oligomers, degradation products (furfural, HMF, and formic acid), and acetic acid released in the aqueous medium, as well as the final pH (pH_F) and solid residues yield (and the corresponding lignin content) were evaluated. Table 2 includes all design points plus seven complementary points.

The results for the most important response variables of the second order response surface model, in the form of analysis of variance (ANOVA), are given in Table 3. For convenience, the statistical analysis of the pretreatment effects on the enzymatic yield response are also shown in this section. All models, except the SRY and degradation products, had p-values less than 0.05, which indicated that those models fit with a statistical significance level of 5.0%. The suitability of the fit of the models was also checked using the determination coefficient (R^2), which shows the percentage of variation in the response that has been explained by the fitted model. The variations not explained by the models vary from 9.2 to 20.3; for enzymatic hydrolysis only 4.6% of the total variation was not explained by the models. The SRY model p-value and R^2 showed that the four factors did not adequately explain its evolution. Table A1 shows the ANOVA values for each degradation product and acetic acid.

The application of the response surface methodology yielded coefficients for the four factors, for its second order and correspondent interactions, listed in Table A2 with the correspondent standard error and p-values. The smaller the p-value, the more significant the corresponding coefficient is. Only the main factors are all listed, while just the statistically significant second order and interaction factors (p-value is less than 0.05) are shown. Concerning the different factors, it was observed that factor X_1 ($NaHSO_3$ charge) was statistically non-significant for all pretreatment response factors (glucose, XMG, oligomers, and degradation products), but it was significant for the enzymatic hydrolysis of the solid residues, demonstrating the role of the delignification/lignin sulfonation on the enzymes performance. Factors X_2 (H_2SO_4 charge) and X_3 (temperature) were significant for all response variables, except for SRY, furfural, and formic acid. Factor X_4 (time) was only significant for HMF, residual lignin in the solid residue, and enzymatic hydrolysis. Concerning the interactions, the most relevant were X_2 (H_2SO_4) and X_3 (temperature), which were significant for XMG, acetic acid, and HMF.

From the results expressed in Table A2, the following regression equations were established as empirical relationships between the experimental response values and the tested variables in coded units, as shown in Eq. 3 through Eq. 13,

$$y_{Gluc.} = 1.34 + 0.02X_1 + 0.67X_2 - 0.48X_3 + 0.04X_4 \quad (3)$$

$$y_{XMG} = 13.5 + 0.4X_1 + 3.1X_2 + 1.5X_3 + 0.6X_4 - 5.4X_2X_3 - 2.4X_2X_4 - 1.6X_3^2 - 2.3X_3X_4 \quad (4)$$

Table 2. Experimental Results

Controlled Factors				Experimental Results										
$NaHSO_3$ (%)	H_2SO_4 (%)	*T* (ºC)	*t* (min)	pH_F	Gluc. (%) *	XMG (%)	Olig. (%)	Degr. Prod. (%)	Furfural (%)	HMF (%)	Formic A. (%)	Acetic A. (%)	SRY (%) [Lignin %]	Enz. Yield (%)
2	1.5	150	0	1.77	0.22	3.28	3.28	0.137	0.021	0.000	0.12	1.164	83.3 [7.88]	7.7
			15	1.81	1.30	7.57	7.57	0.179	0.071	0.004	0.10	1.986	78.2 [11.38]	
			30	1.75	2.04	13.77	13.77	0.523	0.120	0.009	0.39	3.818	76.1 [12.00]	17.8
4	3	150	0	1.5	1.72	10.23	10.23	0.325	0.068	0.004	0.25	2.925	73.4 [9.00]	14.3
			15	1.43	2.02	12.25	12.25	0.443	0.131	0.006	0.31	3.402	68.2 [12.00]	
			30	1.46	1.99	13.57	13.57	0.347	0.139	0.001	0.21	3.735	64.4 [15.50]	35.4
3	0.75	160	7.5	2.43	0.84	5.25	5.25	0.067	0.005	0.005	0.06	1.345	84.8 [11.25]	16.2
			22.5	2.39	2.40	14.14	14.14	0.327	0.124	0.009	0.19	3.815	70.0 [13.13]	21.0
3	2.25	160	7.5	1.53	1.48	12.02	12.02	0.399	0.149	0.014	0.24	3.530	68.8 [14.13]	23.9
			22.5	1.56	1.90	14.13	14.13	0.440	0.175	0.014	0.25	3.813	64.8 [15.00]	38.6
1	2.25	160	7.5	1.63	2.13	13.19	13.19	0.724	0.161	0.015	0.55	3.504	63.9 [12.88]	21.1
			22.5	1.59	2.18	17.87	17.87	0.787	0.183	0.024	0.58	4.849	63.4 [14.13]	31.8
1	0.75	160	7.5	2.46	1.24	3.98	3.98	0.278	0.051	0.001	0.23	1.764	83.5 [12.00]	10.7
			22.5	2.48	1.51	8.80	8.80	0.349	0.128	0.008	0.21	2.483	78.1 [13.50]	17.2
2	1.5	170	0	1.86	1.28	10.40	10.40	0.333	0.157	0.011	0.17	2.762	66.6 [13.13]	27.2
			15	1.97	0.69	11.29	11.29	2.016	1.738	0.027	0.25	2.876	62.2 [14.00]	
			30	2.04	0.79	12.51	12.51	0.421	0.219	0.021	0.18	3.660	62.1 [14.38]	37.8
0	1.5	170	15	2.12	0.67	11.28	11.28	0.395	0.187	0.017	0.19	3.113	63.7 [16.75]	34.3
2	0	170	15	3.48	0.55	10.53	10.53	0.292	0.175	0.011	0.11	2.791	68.8 [14.75]	26.6
2	3	170	15	1.56	1.76	15.20	15.20	0.619	0.264	0.034	0.32	5.315	57.3 [16.50]	55.5
4	1.5	170	15	1.89	0.63	10.87	10.87	0.894	0.332	0.023	0.54	2.833	56.0 [12.75]	50.6
3	0.75	180	7.5	2.33	0.92	15.56	15.56	0.831	0.599	0.020	0.21	4.765	72.6 [16.75]	
			22.5	1.94	0.85	12.54	12.54	1.065	0.792	0.053	0.22	4.816	64.9 [18.75]	51.4
3	2.25	180	7.5	1.57	1.01	13.81	13.81	0.998	0.585	0.047	0.37	3.989	58.6 [18.13]	53.6
			22.5	1.56	1.55	13.14	13.14	1.130	0.827	0.100	0.20	5.110	60.9 [21.00]	63.6

1	2.25	180	7.5	1.58	1.40	14.49	14.49	1.348	0.705	0.025	0.62	4.704	64.9 [17.25]	49.0
			22.5	1.61	1.67	11.23	11.23	1.024	0.825	0.039	0.16	3.923	59.7 [17.75]	51.4
1	0.75	180	7.5	2.33	0.87	15.61	15.61	0.737	0.482	0.026	0.23	4.575	72.1 [16.88]	
			22.5	2.24	1.00	15.80	15.80	1.109	0.777	0.042	0.29	6.184	66.8 [20.88]	52.5
2	1.5	190	0	1.92	1.01	15.22	15.22	1.150	0.766	0.072	0.31	4.259	64.1 [14.88]	50.5
			15	2.02	1.41	12.57	12.57	1.148	0.793	0.091	0.26	5.623	60.9 [22.00]	
			30	2.14	0.97	6.18	6.18	1.237	0.980	0.152	0.11	4.521	58.0 [22.00]	68.9
0	0.75	190	0	2.98	0.73	13.25	13.25	0.531	0.217	0.021	0.29	4.005	66.4 [18.00]	43.8
			15	3.04	0.73	10.55	10.55	0.504	0.224	0.007	0.27	4.629	64.8 [20.38]	
			30	3.05	0.72	7.03	7.03	0.631	0.169	0.126	0.34	4.961	63.2 [20.50]	60.0
*Cellobiose is included														

Table 3. ANOVA of Models

Model	Gluc.	XMG	Olig.	Degr. Prod.	SRY	Lignin	Enzym. Hydrol. 96h
Model d. f.	5	8	8	4	5	7	5
p-value	0.0000	0.0001	0.0000	0.1148	0.1995	0.0000	0.0000
Error d. f.	21	18	18	18	20	19	21
Standard Error	0.147397	1.88185	1.59266	0.51003	9.32106	1.24521	3.72577
R^2	88.39	79.72	85.26	17.67	28.87	90.81	95.42
Adj. R^2	85.62	70.71	78.70	14.58	11.09	87.42	94.32

$$y_{Olig.} = 0.6 - 0.2X_1 - 4.8X_2 - 2.1X_3 + 0.6X_4 + 2.4X_1^2 + 1.4X_3^2 - 3.6X_3X_4 + 1.4X_3^2 \quad (5)$$

$$y_{Degr.} = 0.84 + 0.05X_1 + 0.29X_2 + 0.51X_3 + 0.14X_4 \quad (6)$$

$$y_{Furf.} = 0.33 + 0.00X_1 + 0.08X_2 + 0.42X_3 + 0.09X_4 \quad (7)$$

$$y_{HMF} = 0.018 + 0.001X_1 + 0.006X_2 + 0.027X_3 + 0.009X_4 - 0.008X_2X_3 + 0.010X_3^2 + 0.008X_3X_4 \quad (8)$$

$$y_{Form.} = 0.25 - 0.10X_1 + 0.14X_2 + 0.01X_3 - 0.01X_4 \quad (9)$$

$$y_{Ac.} = 3.90 + 0.10X_1 + 0.98X_2 + 1.42X_3 + 0.38X_4 - 1.35X_2X_3 - 0.74X_2X_4 \quad (10)$$

$$y_{SRY} = 68.5 - 1.3X_1 - 5.5X_2 - 3.9X_3 - 4.7X_4 + 8.5X_2X_3 \quad (11)$$

$$y_{Lignin} = 17.3 + 0.51X_1 + 1.06X_2 + 6.26X_3 + 1.74X_4 + 1.49X_1X_2 + 1.03X_3^2 \quad (12)$$

$$y_{Enz.Hyd.} = 39.5 + 6.9\,X_1 + 11.8X_2 + 27.0X_3 + 8.7X_4 - 4.4X_4^2 \quad (13)$$

Discussion

As can be seen in Table 2, the final pH of the reaction medium changed significantly as the pretreatment conditions were changed, which was primarily due to the sulfuric acid charge, but also due to the acetic acid release from the biomass. The released acetic acid into the hydrolysate increased as the total reaction time at maximum temperature (X_4) was increased.

As previously stated, the authors' objective in the pretreatment stage was hemicellulose extraction, as either monomers or oligomers. The authors started the analysis with glucose, since this monomer can be originated from glucans, galactoglucomannans, and from cellulose itself. Considering the raw material chemical composition, where mannose and galactose represent 3.0% and 3.4%, respectively, and taking into account a conservative proportion of 3:1:0.25 (Man:Glu:Gal) reported in literature for the galactoglucomannans in softwood (Fengel and Wegener 1983), the authors can speculate that some of the glucose (around 1%) detected in the acid sulfite hydrolysate comes from these hemicelluloses present in the broom shrub. Considering the data in Fig. 1, it is clear that the degradation of cellulose into glucose in the pretreatment was very low.

Figure 1 also shows the effect of the operating variables in the acid sulfite stage on the removed glucose (cellobiose included). The increase in acid charge favors the removal of glucose (see also Eq. 3), but the values remain low. The negative effect of temperature will be analyzed later in the paper.

Figure 2 illustrates the magnitude of hemicellulose removal (XMG) and the effect of two variables; the sulfuric acid charge, which had a largely positive effect, and the $NaHSO_3$ charge, which had a small positive effect, likely due to the increasing accessibility of the acid to the hemicelluloses in the biomass through delignification. Equation 4 takes into consideration the effect of all variables. The magnitude of the hemicellulose extraction was one order of magnitude higher than that of glucose, which clearly demonstrates the fractionation efficiency of the acid sulfite pretreatment. Considering that a small amount of the hemicellulose monomers are further transformed in degradation products, the removed hemicellulose could reach values of approximately 18%, which represented 75% of the maximum value of 24% in the raw material. Gil *et al.* (2012), who worked with the

same species with dilute acid treatment, reported a value of 28.4% for the maximum total reducing sugars, which included glucose and hemicellulose monomers. However, the raw material composition was different, and both the stalks and leaves were used, whereas in the present work only the stalks were used. Romaní *et al.* (2011), who worked with *Eucalyptus globulus*, reported xylan removal percentages in the range of 72% to 83%, for autohydrolysis severity factors of 3.79 and 3.94. From a fractionation point of view, it was also of great importance to evaluate the amount of removed hemicelluloses in the form of oligomers.

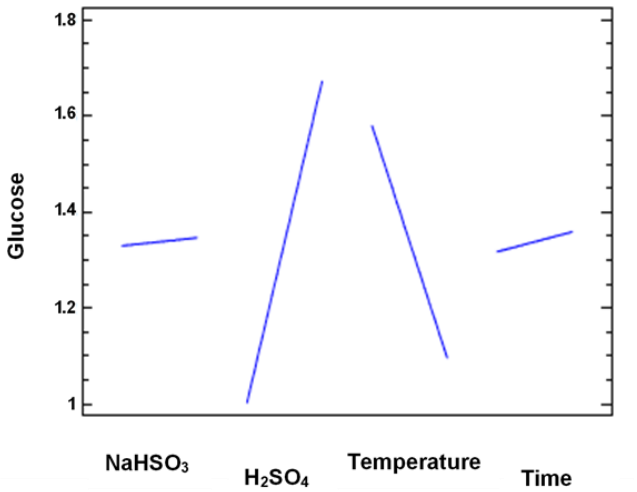

Fig. 1. Sensibility analysis of the effect of the operating variables on glucose (cellobiose included) in the hydrolysates

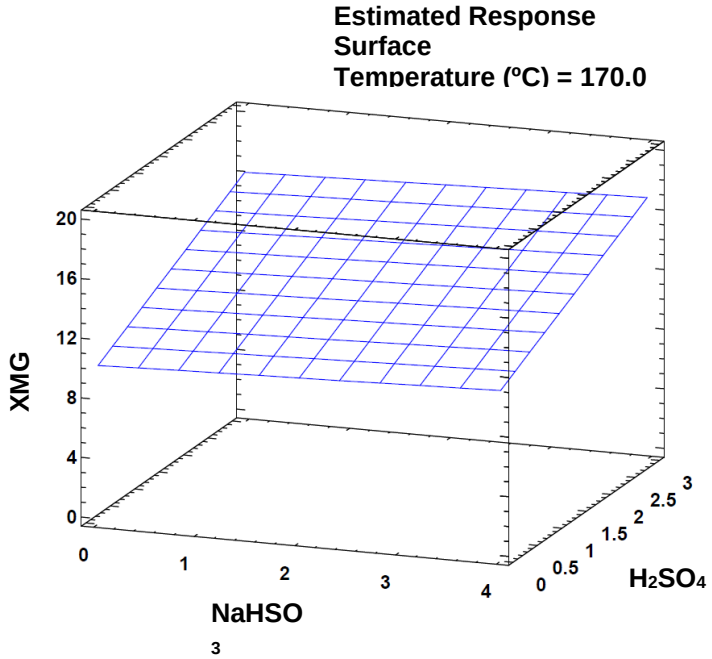

Fig. 2. Effect of $NaHSO_3$ and H_2SO_4 charges on the amount of XMG (monomer + oligomers) released into the hydrolysates

Figure 3 shows the effect of the same variables on the XMG in oligomeric form, and point out that most of the hemicelluloses released into the reaction medium were in

oligomeric form when the sulfuric acid charge was moderate or null, *i.e.*, autohydrolysis conditions (Fig 3 vs Fig. 2). These results revealed an additional possibility of fractionation in the sulfite hydrolysate stream; this result is discussed later in the paper, *i.e.*, the required operating conditions to maximize oligomer recovery. The characterization of the degree of polymerization of these oligomers deserves additional study.

The sulfite hydrolysate stream also contained furfural and HMF, from the degradation of C5 and C6 sugars, respectively, acetic acid from the acetyl group in the hemicelluloses, and formic acid from the sugar degradation products. As can be observed in Table 2, the amount of furfural is much higher than the amount of HMF as a natural consequence of the higher concentration of C5 (mainly xylose) sugars in the reaction medium. Figure 4 shows the relative effect of the different operating variables on the degradation products (including furfural, HMF, and formic acid); it is clear the increment of degradation products with sulfuric acid charge, temperature, and time (see Eq. 6).

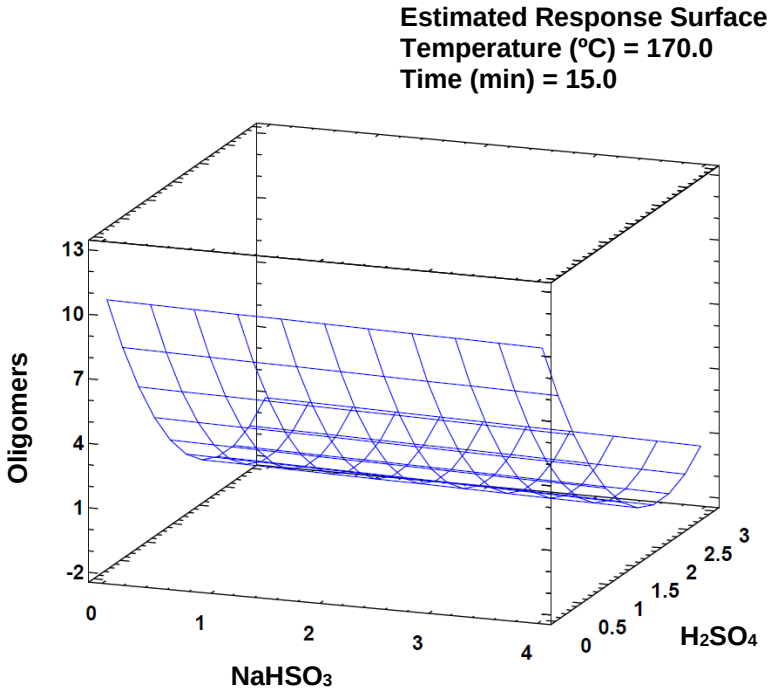

Fig. 3. Effect of the $NaHSO_3$ and H_2SO_4 charges on the amount of oligomers released into the hydrolysates

Moreover, Eqs. 7 and 8 clearly show evidence of the strong positive effect of temperature on the furfural and HMF production through the degradation of sugars; the coefficients for the reaction temperature (X_3) were much higher than those of the other factors. The amount of these products was practically not affected as the sodium sulfite charge (X_1) was increased, since it acts on lignins, primarily promoting lignin sulfonation. Concomitantly with the increase of furfural and HMF as the temperature increased, the glucose content decreased (as shown in Fig. 1) and XMG (as shown in Fig. 5) exhibits a strong negative interaction between the acid charge and temperature, which indicated that both glucose and XMG were degraded after being released into the reaction medium. These findings are in strong agreement with the kinetic data available for the degradation reactions (Blechschmidt and Heinemann 2006).

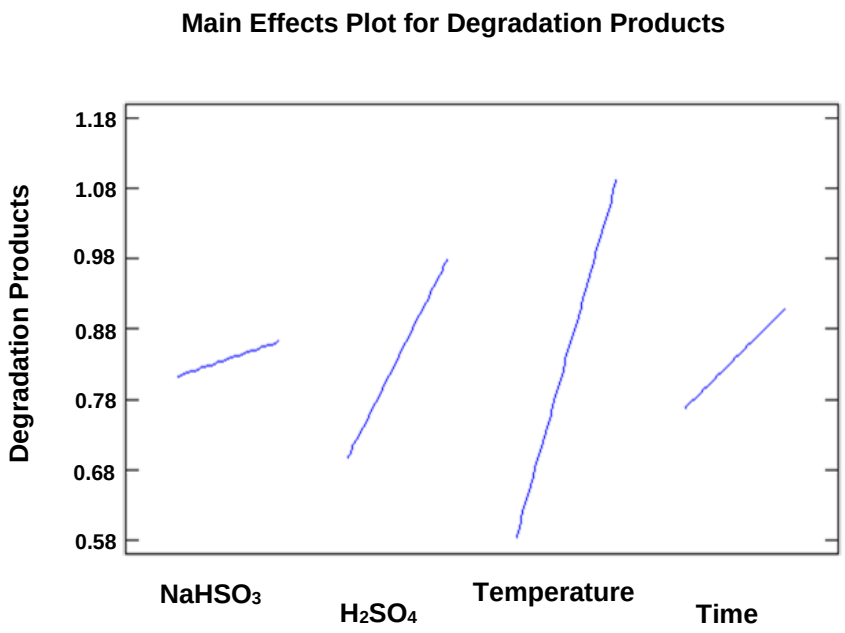

Fig. 4. Sensibility analysis of the effect of the operating variables on the degradation products in the hydrolysates

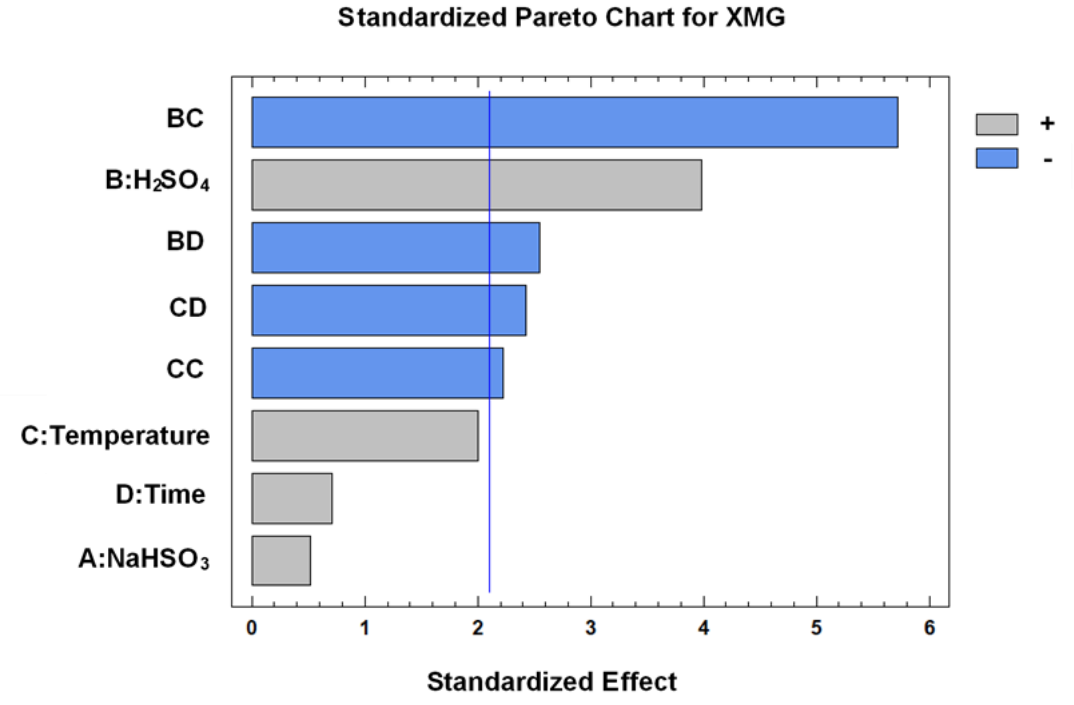

Fig. 5. Standardized Pareto chart for XMG

The effect of the operating variables can also be observed by the amount of solid residue (as shown in Eq. 11). The SRY decreased with an increase of each variable; for the selected ranges, the sulfite charge (X_1) had the smallest effect, and the acid charge (X_2) had

the largest effect. Interestingly, a strong interaction between the acid charge (X_2) and the temperature (X_3) was revealed, which indicated a strong retreat in the dissolution of the biomass components. These results were in accordance with the lignin and sugar degradation products precipitation on the solid for harsh reaction conditions (Meng and Ragauskas 2014). Figure 6 shows the relationship between the SRY and a modified severity factor that integrates temperature over time and pH. This data revealed that another factor, likely the presence of sulfite in the reaction medium, affected the SRY (as shown in Eq. 11); Eq. 4 also supports the effect of sulfite on the amount of sugar released to the medium.

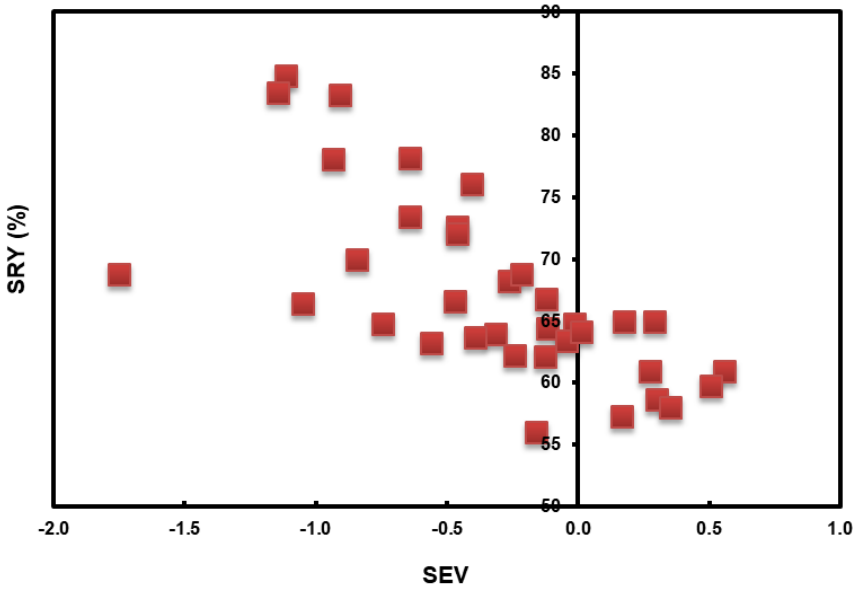

Fig. 6. Relationship between the solid residue yield (SRY) and the severity factor that integrates temperature over time and pH

Optimization of Experimental Models in the Pretreatment

The optimization strategy was defined as maximizing the amount of XMG and oligomers and minimizing the amount of glucose and degradation products (furfural, HMF, and formic acid), as shown in Table 4.

To maximize the amount of XMG, as monomers, $NaHSO_3$ charge, H_2SO_4 charge, and time should be at their maximum, while temperature should remain at its minimum. The estimated attained value (23.3%) was close to the amount of XMG identified in the raw material, which provided evidence of the potential of this process. The XMG oligomers attained their maximum value at minimum levels of all factors, except for time at maximum temperature. Degradation products were minimized at minimum levels of H_2SO_4, temperature, and time, being $NaHSO_3$ at levels over 2.6%, except for furfural (0.4%). Lignins minimization in the solid residue required high $NaHSO_3$ charges, simultaneously requiring minimum levels of H_2SO_4 charge, time, and temperature. A low level of lignins in the solid residue favored the next phase of enzymatic hydrolysis, but from a fractionation point of view, it would be better if the amount of lignins was as high as possible in the solid residue, in order for it to be separated later during the global process. Minimizing the SRY required maximum $NaHSO_3$ charges, H_2SO_4 charges, and time, while the temperature was at its minimum.

Table 4. Optimization

Factor	Gluc. (%)	XMG (%)	Olig. (%)	Deg. Prod. (%)	Furf. (%)	HMF (%)	Acetic A. (%)	Formic A. (%)	SRY (%)	Lignin (%)
Optimization	*Min.*	*Max.*	*Max.*	*Min.*	*Min.*	*Min.*	*Min.*	*Min.*	*Min.*	*Min.*
Attained Value	0.02	23.3	25.9	-0.1	-0.27	-0.01	-3.0	-0.01	43.8	10.6
$NaHSO_3$ (%)	4.0	4.0	0.0	2.5	0.41	2.6	2.8	4.0	4.0	3.9
H_2SO_4 (%)	0.76	3.0	0.0	0.0	0	0	0	0	3.0	0
Temperature (°C)	150.0	150.0	150.0	150.0	150	150	150	152	150.0	150
Time at maximum temperature (min)	0.0	30.0	30.0	0.3	0.1	22.3	0.0	30	30.0	0.4

Enzymatic Hydrolysis

To evaluate the efficiency of the enzymatic hydrolysis process, the amount of carbohydrates in each solid residue was estimated, by subtracting the estimated amount of lignins from the SRY, since the total carbohydrate enzymatic yield calculations were based on a lignin-free material.

Figure 7 illustrates the effect of the enzymatic reaction time on the total amount of sugar (xylose and glucose) released from the solid residues subjected to different pretreatment conditions (at constant temperature of 170 °C).

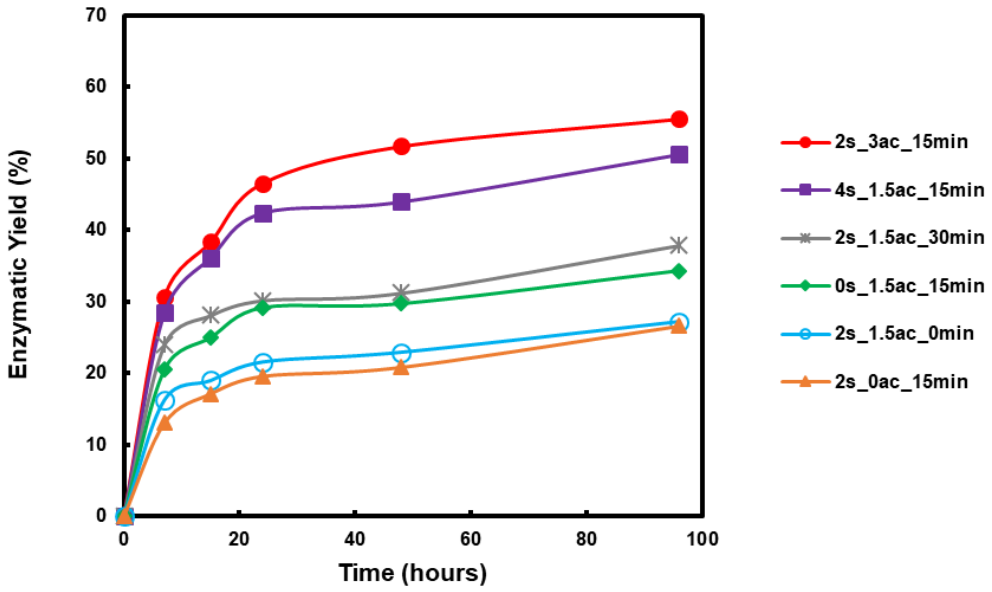

Fig. 7. Effect of the enzymatic hydrolysis time on the total carbohydrates yield for biomasses pretreated under different reaction conditions (at constant temperature of 170 °C)

The values of the enzymatic yield were clearly lower than those reported by the same research group (Costa *et al*. 2016) for the same biomass when pretreated under higher sulfite and acid charges, which indicated the need for higher levels of sulfite and acid to attain a good performance in terms of enzymatic hydrolysis. In addition, the more recalcitrant nature of this biomass regarding *E. globulus* was also confirmed; the enzymatic yield of broom was approximately 50% with 4% sulfite and 1.5% sulfuric acid (SRY = 56%), whereas *E. globulus* reached an enzymatic yield of approximately 80% with 5%

sulfite and 0.9% sulfuric acid (SRY = 62.4%) (Costa *et al*. 2016). The relative sensitivity of the enzymatic yield regarding lignin sulfonation remains unclear, but the comparison of the data for the 2s_1.5ac_30 min and 4s_1.5ac_15 min assays (as shown in Fig. 7), pretreated at 170 °C, strongly suggests a positive effect due to increased sulfonation (a higher $NaHSO_3$ charge), because the enzymatic yield increased from approximately 37.8% (SRY = 62.1%) to 50.6% (SRY = 56%). However, it should be noted that the corresponding SRY decrease from 62.1% to 56% does not seem to justify the observed increase in enzymatic yield. In fact, for the assay 3s_0.75ac_22.5 min (SRY = 64.9%), carried out at 180 °C, the enzymatic yield was 51.4%, *i.e*., slightly higher than the result of the assay 4s_1.5ac_15 min (performed at a temperature of 170 °C) despite the high SRY, which suggested that the SRY was not determinant in this SRY range.

To better analyze the role of the pretreatment sulfite charge on the enzymatic yield, the sulfur (S) content, and the carbon to oxygen (C/O) ratio in the solid residue was analyzed *via* SEM-EDX. Table A3 presents the results for the representative samples and Eq. 14 and Eq. 15 summarize the influence of the pretreatment parameters (normalized) on the S content and C/O ratio.

$$\frac{C}{O} = 1.506 - 0.0337\,X_1 + 0.118X_2 + 0.0598X_3 - 0.222X_4 \quad (14)$$

$$S(\%) = 0.09637 + 0.0913\,X_1 + 0.0148X_2 - 0.0096X_3 + 0.066X_4 \quad (15)$$

The S content in the solid residue primarily increased as the $NaHSO_3$ charge was increased, but other parameters also played a role. Regarding the C/O ratio, this parameter increased with the increase in acid charge, reflecting the expected role of the lignin condensation reaction when subjected to an acid medium. The presence of HSO_3^- (a nucleophilic agent) reduced the occurrence of the C-C condensation reaction. As mentioned above, at a given pretreatment temperature, an increase in $NaHSO_3$ charge (accompanied with an increase in the S content in the solid residue) increased the enzymatic yield. These results could be due to the decrease of unproductive adsorption of enzymes by the sulfonated lignins. Several authors have suggested that the enzymatic hydrolysis process can be improved by modifying the lignins instead of removing a major portion of the lignins (Chen *et al*. 2007; Yoo *et al.* 2015). However, when the enzymatic yield is represented as a function of S content in the solid residue, including the experiments with different pretreatment temperatures, the results (as shown in Fig. 8) suggest that at high pretreatment temperatures, the positive effect of the S content in the solid residue is strongly diminished. In accordance with the results in Fig. 8, Eq. 13 also reveals that the acid charge and temperature had a stronger influence on the enzymatic yield than the $NaHSO_3$ charge. According to the statistical analysis, the enzymatic yield can be slightly slowed down for too long pretreatments (as shown in Eq. 13). This could be due to the cellulose enrichment of the solid residue (the amount of hemicellulose removed increases with pretreatment time), with a higher crystallinity index regarding hemicelluloses, which decreased the extent of enzymatic hydrolysis; the collapse of some internal porous structures with the removal of hemicelluloses was also referred to in the literature (Pihlajaniemi *et al*. 2016).

When the first-order model coefficients from Eq. 13 (sugar release by enzymatic hydrolysis) and Eq. 11 (SRY) are compared, it is apparent that the relative importance of the factors are different. For the sugars released by the enzymatic process, the sulfite charge and the temperature gain relative importance against the acid charge, in comparison with influence of the SRY factors, highlighting the roles of both temperature and sulfite charge in pretreatment on enzymatic performance.

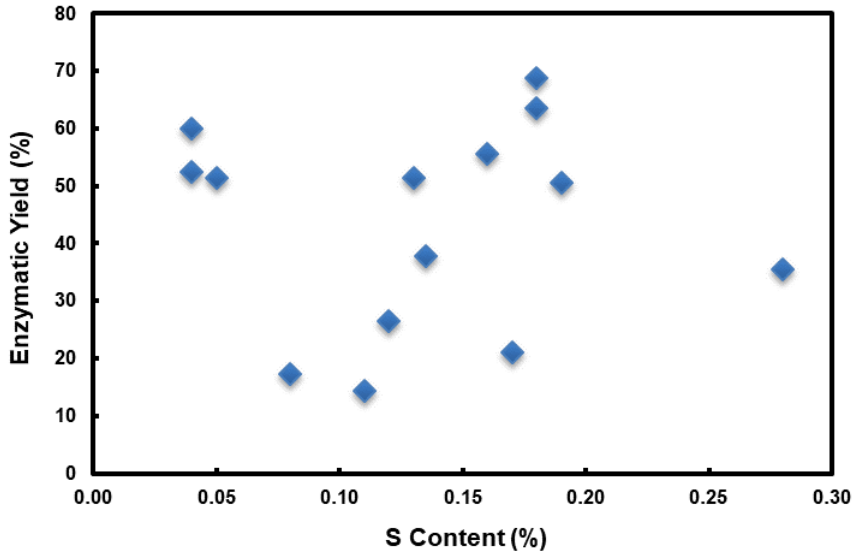

Fig. 8. Correlation between the sulfur content of the solid residues after enzymatic hydrolysis and the correspondent enzymatic yield

The results in Fig. 9 clearly show that the global amount of mass removal (complementary to the SRY) was not linearly correlated with the enzymatic yield.

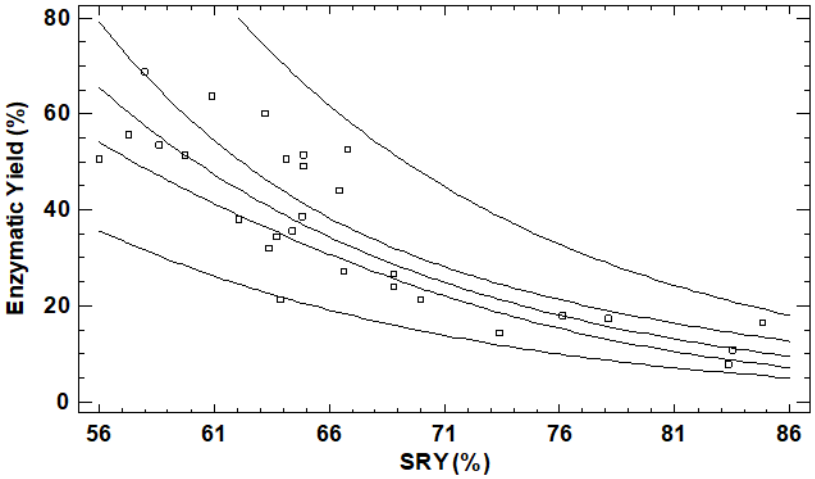

Fig. 9. Effect of solid residue yield (SRY) on the enzymatic hydrolysis yield for all the tested pretreatment conditions

Until approximately 70% SRY (removal of 30% of the solids), the amount of sugars released by the enzymatic process gradually increased as the SRY decreased, but it remained low; for lower SRY values, the enzymatic yield exponentially increased. In addition, in a SRY range from 62.5% to 67.5%, drastically different enzymatic yield values were obtained. A close analysis indicated that the uppermost values in this range were associated with a higher temperature (180 °C to 190 °C) in the pretreatment and sulfonation processes.

Regarding optimization of the enzymatic hydrolysis process, the attained estimated maximum was 87.2% and corresponds to maximum values for the $NaHSO_3$ charge (4%),

H_2SO_4 charge (3%), and temperature (190 °C), while the time remained at a high level (22.5 min), which indicated the high recalcitrant nature of broom shrubs, particularly requiring high sulfuric acid charges, namely in comparison with *E. globulus*.

The solid residue after a successive acid sulfite pretreatment and enzymatic hydrolysis was primarily composed of lignins.

In summary, it was demonstrated that the required conditions to maximize cellulose hydrolysis in the solid residue by the enzymatic system are apparently contradictory with the very mild reaction conditions required to recover the hemicellulose in the oligomer form in the pretreatment stage. Therefore, the topic deserves additional research, including using multifunction optimization, although the present work suggests moderate temperature, and both high sulfite charge and reaction time in the pretreatment.

CONCLUSIONS

1. In the present work broom (*Cytisus striatus*) was successively subjected to a mild acid sulfite pretreatment and enzymatic hydrolysis. The experimental data obtained from experimental design enabled the identification of the optimum reaction conditions (which are maximum time at minimum temperature (150 °C), under autohydrolysis conditions) that maximized the release of hemicelluloses in the first stage as oligomers.
2. If the objective is the complete removal of hemicelluloses in the form of monomers, 3% sulfuric acid and 4% sulfite should be used.
3. In the second stage, the experimental results for the release of glucose by the enzymatic hydrolysis were lower than that obtained in a previous work with the same raw material, because in the present work, a lower sulfite and acid charges were applied. The optimization results confirmed the need to increase both the sulfite and sulfuric acid charges.
4. The comparison of the results from the present work with a previous one, where *E. globulus* was assayed, confirmed the more recalcitrant nature of broom, in comparison to *E. globulus*.
5. Although the positive role of the S content in the enzymatic yield of the solid residue was confirmed, an enzymatic yield of 60% was attained without the addition of sulfite in the pretreatment when the temperature was raised to 190 °C, putting in evidence the positive role of high temperature in the pretreatment.
6. To maximize the hemicelluloses recovery, as oligomers, in the pretreatment stage without compromising the enzymatic hydrolysis of cellulose in the solid residue, both high sulfite charge and reaction time should be explored.

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APPENDIX

Supplementary Materials

Table A1. Models ANOVA for degradation products

Model	Furfural	HMF	Formic A.	Acetic A.
Model d.f.	4	7	4	6
P-value	0.0001	0.0000	0.0210	0.0000
Error d.f.	22	19	22	20
Stnd. Error	0.172682	0.00635569	0.110535	0.538426
R^2	63.91	89.44	39.56	82.96
Adj. R^2	57.34	85.55	28.57	77.84

Table A2. Effects ± Standard Errors (P-value)

Effect ± S.E. (P-value)	Gluc. (%)	XMG (%)	Olig. (%)	Degr. Prod. (%)	Furfural (%)	HMF (%)	Formic A. (%)	Acetic A. (%)	Lignin (%)	SRY (%)	Enzym. Hydrol. (%)
Average	1.34±0.04	13.50±0.49	0.65±0.69	0.84±0.13	0.33±0.03	0.018±0.001	0.25±0.02	3.90±0.10	17.3±0.41	68.49±1.83	39.57±0.96
X_1: $NaHSO_3$	0.02±0.06 (0.6204)	0.40±0.77 (0.6102)	-0.19±0.65 (0.7764)	0.05±0.09 (0.6017)	0.00±0.07 (0.9515)	0.001±0.002 (0.7042)	-0.10±0.04 (0.0430)	0.10±0.22 (0.6581)	0.51±0.51 (.3260)	-1.32±3.81 (0.7313)	6.92±1.52 (0.0002)
X_2:H_2SO_4	0.67±0.06 (0.0000)	3.06±0.77 (0.0009)	-4.84±0.65 (0.0000)	0.29±0.09 (0.0062)	0.08±0.07 (0.2855)	0.006±0.002 (0.0164)	0.14±0.04 (0.0053)	0.98±0.22 (0.0002)	1.06±0.51 (0.0503)	-5.49±3.81 (0.1645)	11.77±1.52 (0.0000)
X_3: Temperature	-0.48±0.06 (0.0000)	1.54±0.77 (0.0606)	-2.15±0.65 (0.0039)	0.51±0.09 (0.0000)	0.42±0.07 (0.0000)	0.027±0.002 (0.0000)	0.01±0.04 (0.7741)	1.42±0.22 (0.0000)	6.26±0.51 (0.0000)	-3.88±3.81 (0.3207)	26.97±1.52 (0.0000)
X_4: Time	0.04±0.06 (0.0134)	0.55±0.77 (0.4855)	0.63±0.65 (0.3436)	0.14±0.09 (0.1392)	0.09±0.07 (0.1930)	0.009±0.002 (0.0019)	-0.01±0.04 (0.7441)	0.38±0.21 (0.1021)	1.74±0.51 (0.0029)	-4.72±3.81 (0.2287)	8.69±1.52 (0.0000)
X_1^2			2.45±0.65 (0.0014)							-	-
X_1 X_2									1.49±0.49 (0.0507)		
X_2^2									1.03±0.49 (0.0031)		
X_2 X_3	-	-5.38±0.94 (0.0000)	-			- 0.008±0.003 (0.0247)		-1.35±0.25 (0.0001)		8.51±4.66 (0.0827)	-
X_2 X_4	-	-2.40±0.94 (0.0200)	-					-0.74±0.27 (0.0114)			-
X_3^2	-	-1.62±0.73 (0.0391)	1.37±0.65 (0.0498)			0.011±0.002 (0.0003)			1.66±0.49 (0.0031)	-	-
X_3 X_4	-	-2.29±0.94 (0.0257)	-3.58±0.80 (0.0003)			0.008±0.003 (0.0247)				-	-
X_4^2	-		1.35±0.65 (0.0528)							-	-4.45±1.44 (0.0056)

Table A3. Elemental Analysis of Sulfur and Carbon /Oxygen Ratio for the Acid Sulfite Pretreatment Solid Residues and Ground Raw Material

$NaHSO_3$ (%)	H_2SO_4 (%)	Temp. (ºC)	Time (min)	C/O ratio [error, 2- sigma]	S (wt%) [error, 2-sigma]
Ground raw material				1.25 [0.3]	0.04 [0.06]
4	3	150	0	2.00 [0.4]	0.11 [0.06]
			30	1.34 [0.3]	0.28 [0.07]
3	0.75	160	22.5	1.22 [0.2]	0.17 [0.07]
1				1.33 [0.3]	0.08 [0.06]
2	0	170	15	1.32 [0.3]	0.12 [0.06]
2	3		15	1.41 [0.3]	0.16 [0.07]
2	1.5		30	1.35 [0.3]	0.14 [0.06]
4	1.5		15	1.42 [0.3]	0.19 [0.07]
3	0.75	180	22.5	1.32 [0.3]	0.13 [0.06]
3	2.25			1,45 [0.3]	0.18 [0.07]
1	2.25			1.44 [0.3]	0.05 [0.06]
1	0.75			1.55 [0.3]	0.04 [0.06]
2	1.5	190	30	1.45 [0.3]	0.18 [0.07]
0	0.75			1.43 [0.3]	0.04 [0.06]