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Fractionation of *Eucalyptus globulus* Wood by Glycerol–Water Pretreatment: Optimization and Modeling

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ABSTRACT: A glycerol-organosolv process can be a good alternative for *Eucalyptus* wood fractionation into its main compounds, improving the enzymatic saccharification of the cellulose. A study of process variables - glycerol–water percent content, temperature, and process time - was carried out using a Box-Behnken experimental design. The cellulose obtained from pretreated solids was recovered almost quantitatively, leading to a solid with a high percentage of cellulose (77 g/100 g of pretreated solid), low lignin content (9 g/100 g of pretreated solid), and 18% of residual hemicellulose in the solid at 200 °C, 56% of glycerol–water and 69 min. The enzymatic saccharification was enhanced achieving 98% cellulose-to-glucose conversion (under conditions: liquid to solid ratio 20 g/g and enzyme loading 20 FPU/g of solid). This study contributes to the improvement of biomass fractionation by exploring an eco-friendly treatment which allows for almost complete wood fractionation into constituents and high levels of glucose recovery available for subsequent yeast fermentation to bioethanol.

1. INTRODUCTION

Nowadays, the increasing fossil fuel prices and the concern for environmental sustainability represent an important worldwide challenge. Therefore, the search and use of alternative energy sources are essential in order to minimize fossil fuel related issues. One of the possible responses is the bioethanol production from lignocellulosic materials (LCMs), so-called second Generation Ethanol (2G).[1]

LCMs are widely available, and they do not compete directly with food crops.[2] These raw materials contain primarily structural components - cellulose, hemicellulose, and lignin - which form a complex three-dimensional structure.[3] *Eucalyptus globulus* wood (EGW) is a fast growing LCM.[4,5] Large amounts of EGW residues, such as bark, cross-cut ends, and out-of-specification wood chips resulting from kraft pulping processing are currently burned for electricity or heat production.[6] These residues could be alternatively as feedstock for 2G ethanol and added-value coproducts production.[7] Significant changes in the structure of EGW are required in order to enable a techno-economically viable 2G ethanol process. A typical 2G ethanol process includes at least 5 stages: i) size reduction (milling) ii) pretreatment, iii) saccharification, iv) fermentation, and v) distillation of fermentation broth. The first two stages are meant to overcome enzyme accessibility limitations. These steps can be reduced since the saccharification and fermentation can be carried out simultaneously (process also called simultaneous saccharification and fermentation, SSF). The SSF presents more advantages than separate hydrolysis and fermentation (SHF) derived from limited cost, decreased product inhibition, and contamination risks.

The literature describes a wide spectrum of pretreatments to modify the structure of LCMs.[8−10] An efficient pretreatment of LCMs that generates a high fractionation is of most importance for the economic use of this renewable resource.[11] The criteria for an effective pretreatment have been reported by several authors,[10,12] and they include i) avoiding size reduction, ii) preserving hemicellulose fraction, iii) limiting formation of inhibitors, iv) minimizing energy input, and v) being cost-effective. More recently, organosolv pulping has been used in pulp and paper production as an environmental alternative to conventional kraft or sulfite pulping processes,[13] and its potential in LCMs utilization has been demonstrated.[14] The organosolv pretreatment is conducted in an organic solvent or in a solution or mix of solvent and water and generally leads to solubilization of the lignin of LCMs lignins.[15] Moreover, organosolv pretreatment can be considered as a good strategy for a feasible biorefinery of LCM[16−19] presenting remarkable advantages: i) the organic solvents can be recovered and recycled and ii) the recovered lignin has desirable properties that are of interest for the production of several coproducts.[20,21] Organosolv pretreatment has also been used to improve enzymatic saccharification, in combination with catalysts such as sulfuric acid, alkali, and others[22−24] or without catalysts.[18,25] Furthermore, the lignin removal from LCM can be improved in comparison with other pretreatments since the delignified solid is highly susceptible to cellulose saccharification (higher than 90% of the theoretical maximum), helping to reduce the enzyme loading in the saccharification process.[26]

In addition, glycerol is considered a high boiling point alcohol and it is a good wood lignin solvent if the appropriate wood fractionation conditions are selected.[27] However, the high energy consumption for solvent recovery is a disadvantage of this kind of solvents. The use of membrane filtration can be an effective tool due to reduce the energy demand and reduce the chemical consumption.[28] Thus, the use of glycerol in

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organosolv pretreatment of EGW can be considered a promising and sustainable alternative for the direct utilization of this industrial byproduct contributing to the commercial success of bioethanol and biodiesel production.[29]

In this context, the aim of this work is to study the influence of operational conditions (temperature, time, and glycerol−water percentage) on the pretreatment of EGW using glycerol as solvent for biomass delignification and improvement of enzymatic accessibility of pretreated wood. For this reason, the fractionation of EGW into its main components (glucan, hemicellulose-derived compounds and lignin) and the susceptibility of delignified solid to enzymatic saccharification were evaluated.

2. MATERIALS AND METHODS

2.1. Raw Material. In this work, *Eucalyptus globulus* wood (EGW) was used as raw material. The feedstock was obtained from a local pulp mill (ENCE, Pontevedra, Spain). EGW samples were air-dried, homogenized and milled to pass a 2 mm screen. Finally, the conditioned EGW was stored in a dark and dry place until use.

2.2. Analysis of Raw Material. The chemical composition of raw material was analyzed by TAPPI standards for extractives and moisture content (T-264-cm-07), ash content (T-211-cm-93), and quantitative acid hydrolysis with 72% w/w sulfuric acid (T-249-em-85). The liquid from acid hydrolysis was analyzed for sugars (glucose, xylose, arabinose) and acetic acid by HPLC using a Jasco 830-IR intelligent refractive-index detector (Jasco, Tokyo, Japan) and a Metacarb 87H column (300 × 7.8 mm, Varian, USA), under the following conditions: mobile phase 0.005 M H_2SO_4, flow rate 0.7 mL min^{-1}, and column temperature of 60 °C. The monosaccharide concentrations were employed for the glucan, xylan, arabinan, and acetyl groups determination, using the following stoichiometric factors: 180/162, 150/132, 150/132, and 60/43, respectively. The Klason lignin was determined gravimetrically from the insoluble acid residue of quantitative acid hydrolysis. The acid soluble lignin content was measured spectrophotometrically using the method of Maekawa et al.[30] Uronic acids were determined spectrophotometrically[31] using galacturonic acid as a standard for quantification. Protein was determined from the nitrogen content of EGW, which was measured by elemental analysis (Flash 1112 Series, Termo Finningan).

2.3. Organosolv Pretreatment. EGW was submitted to organosolv delignification with glycerol−water solutions (in a liquid solid relation, LSR_D = 10:1 g glycerol−water solutions/g EGW) at desired temperature and time. The assays were carried out in a 160 mL total volume batch cylinder reactor fabricated from 316 stainless steel (previously described by Ruiz et al.[26]). The reactor was submerged in an oil bath with temperature control, previously heated at the desired temperature (heating time of 5 min). When the experiment was finished, the reactor was removed from the oil bath and cooled down in an ice−water bath for 5 min). The delignified solids and liquors were separated and recovered by vacuum filtration. Aliquots of liquors were subjected to quantitative posthydrolysis with H_2SO_4 (4% w/w) at 121 °C for 20 min. The samples were analyzed by HPLC for sugars determination as mentioned above.

The delignified solids were washed with 1% (w/w) sodium hydroxide solution to remove the adsorbed lignin and others compounds from solid surface. Straightaway, the delignified solids were washed with abundant distilled water until reaching neutral pH. The washed solids were used to calculate the solid yield (*SY*), gravimetrically. The percentage of delignification was calculated as

$$\%\ \text{Delignification}\ (D) = 100 \cdot \frac{KL_{rm} - KL_{DS} \cdot \frac{SY}{100}}{KL_{rm}} \quad (1)$$

where KL_{rm} is the percentage of Klason lignin present in raw material, KL_{DS} is the percentage of Klason lignin in delignified solids, and *SY* is the solid yield of pretreatment.

The recovered solids were analyzed using the same methodology explained in the section 2.2.

2.4. Enzymatic Saccharification of Delignified Solids. Enzymatic saccharifications of pretreated solids were carried out in 100 mL Erlenmeyer flasks with an orbital shaker (150 rpm) at 50 °C and pH = 4.85 (using 0.05 N citrate acid-sodium buffer). Cellulase (Celluclast 1.5 L) and β-glucosidase (Novozyme 188) were kindly provided by Novozymes (Bagsvaerd, Denmark) and used during all enzymatic saccharification trials. The activity of cellulase was measured by the Filter Paper assay.[32] The β-glucosidase activity was determined as International Units.[33] The initial enzyme activities were 44.7 FPU/mL and 509.3 UI/mL for Celluclast 1.5 L and Novozyme 188, respectively.

The enzymatic saccharification assays were performed at liquids-to-solids ratio (LSR_{HE}) = 20 g/g, enzyme to substrate ratio (ESR) = 20 FPU/g, and β-glucosidase/cellulase ratio (Cb/Cl) = 5 UI/FPU. The enzymatic saccharification trials started when the enzymes were added. The samples were withdrawn at times (0, 2, 9, 21, 45, and 72 h) and then centrifuged (600 rpm, 10 min). The supernatants were separated and analyzed by HPLC for glucose, xylose, and acetic acid.

The cellulose-to-glucose conversion data was extrapolated to the following equation[34]

$$CGC_t = CGC_{max} \cdot \frac{t}{t + t_{1/2}} \quad (2)$$

where CGC_t is the cellulose-to-glucose conversion obtained at time *t*, CGC_{max} is the cellulose-to-glucose conversion predicted for an infinite reaction time, *t* is the enzymatic saccharification time (h), $t_{1/2}$ (h) is the time needed to reach $CGC = CGC_{max}/2$.

CGC_t is calculated as

$$CGC_t = 100 \cdot \frac{G_t}{G_{pot}} \quad (3)$$

where G_t is the concentration of glucose obtained at time *t*, and G_{pot} is the potential glucose calculated as

$$G_{pot} = \frac{Gn}{100} \cdot \frac{180}{162} \frac{\rho}{LSR_{EH} + 1 - \frac{KL}{100}} \quad (4)$$

where *Gn* is the glucan content of delignified solids (g of glucan/100 g of delignified solid dry basis), 180/162 is the stoichiometric factor, ρ is the density of the hydrolysis enzymatic medium (average value, 1005 g/L), LSR_{EH} is the liquid to solid ratio (20 g/g), and *KL* is the Klason lignin of delignified solid (g of Klason lignin/100 g of delignified solid dry basis).

2.5. Experimental Plan: Box-Behnken Design. The effects of operating conditions such as temperature (T_D, °C), time (t_D, min), and ratio glycerol−water solutions (*GW*, %) on organosolv-delignification pretreatment were evaluated (see

Table 1). For this purpose the experimental conditions of the three above-mentioned variables were designed, based on an

Table 1. Experimental Variables Involved in This Work

variable	definition and units	nomenclature	value or range
Independent			
	temp of delignification (°C)	T_D	180−200 °C
	time of delignification (min)	t_D	40−90 min
	glycerol−water solutions w/w (%)	GW	40−80%
Dependent			
	solid yield (%)	SY or y_1	
	glucan (%)	Gn or y_2	
	xylan (%)	Xn or y_3	
	delignification (%)	D or y_4	
	acetyl groups (%)	AcH or y_5	
	posthydrolyzed liquor: xylose (g/L)	X or y_6	
	cellulose to glucose conversion (%)	CGC_{max} or y_7	
	time to achieve $CGC_{max}/2$ in enzymatic saccharification (h)	$t_{1/2}$ or y_8	
	glucose at 72 h in enzymatic saccharification	G_{72h} or y_9	
Fixed			
	liquid to solid ratio of delignification (g/g)	LSR_D	10 g/g
	liquid to solid ratio of enzymatic hydrolysis (g/g)	LSR_{EH}	20 g/g
	enzymatic to substrate ratio (FPU/g)	ESR	20 FPU/g
	β-glucosidase/cellulase ratio (UI/FPU)	Cb/Cl	5 UI/FPU
	pH of enzymatic saccharification	pH	4.5
	temp of enzymatic saccharification (°C)	T	50 °C

experimental Box-Behnken design with three replicates in the central point (total: 15 experiments). The trials were performed as described in section 2.3 and were summarized in Table 2. The operating variables were expressed in terms of normalized variables (x_1, x_2, and x_3), with variation ranges (−1, 1), which are related to corresponding independent variables (T_D, t_D, and GW) as follows:

$$x_1 = 2\cdot\frac{T_{Di} - T_{Dme}}{T_{Dmax} - T_{Dmin}} \quad (5)$$

$$x_2 = 2\cdot\frac{t_{Di} - t_{Dme}}{t_{Dmax} - t_{Dmin}} \quad (6)$$

$$x_3 = 2\cdot\frac{GW_{Di} - GW_{Dme}}{GW_{Dmax} - GW_{Dmin}} \quad (7)$$

where the subscript i is the considered experiment, me is the average value of the variable min and max, and min and max mean minimum and maximum values of the respective variation ranges, respectively.

Empirical models were used for the interrelationship of dependent and independent variables

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij}x_i + \sum_{i=1}^{2}\sum_{k\geq i}^{2} b_{ikj}x_i x_k \quad (8)$$

where y_j (j = 1 to 4) is the dependent variable; x_i or x_k (i or k: 1 to 2, $k\geq i$) are the normalized, independent variables (defined in Table 1), and $b_{0j}...b_{ikj}$ are regression coefficients calculated from experimental data by multiple regression using the least-squares method. The experimental data were fitted to the proposed models using commercial software (Microsoft Excel, Microsoft, USA).

3. RESULTS AND DISCUSSION

3.1. Composition of Raw Material. The chemical composition of raw material, expressed in g/100 g of EGW in oven-dry basis, was determined to be the following: glucan 44.99 ± 0.4; xylan 16 ± 0.7; Klason lignin 24.7 ± 0.23, acid soluble lignin 2.95 ± 0.9, acetyl groups 2.96 ± 0.07; extractives 2.5 ± 0.17, ashes 0.22 ± 0.02, uronic acids 5.1 ± 0.09, and protein 1.25 ± 0.03. These values are in agreement with the literature.[35]

3.2. Chemical Composition of Delignified *Eucalyptus globulus* Wood. On the basis of reported data,[29,36] the EGW was submitted to an organosolv-delignification pretreatment using glycerol as solvent, under selected operating conditions. Table 2 summarizes all the experimental results, specifically the solid yield (SY), chemical composition of delignified solid glucan (Gn), xylan (Xn), acetyl groups (AcH), (expressed as g compound/100 g of delignified solid), xylose (X) concentration in the liquor (g/L) after the pretreatment, and the delignification percentage.

For the evaluation of the pretreatment, the solid yield (SY) of the experiments was measured varying in the ranges of 51 to 81% (Table 2). These extreme values were obtained in the experiments 2 and 5, respectively. The lowest SY (51%) corresponds to a delignification percentage of 72% with respect to raw material (Table 2). The glucan content (Gn) of delignified solids was in the range of 54 to 83 g glucan/100 g of delignified solid (corresponding to experiments 5 and 8, respectively). The maximal value of glucan (83%) in the solid implied a recovery of glucan of 98% in respect to raw material. This result demonstrates the selectivity of each pretreatment to preserve the cellulose in the solid. Taking into account the recovery of glucan after the pretreatment step, we obtained an average value of 94% (calculated as percentage of glucan in delignified solid in respect to glucan in raw material), with the glucan losses representing a value lower than 9%. These results can be compared with reported data on glycerol−water.[36,37] Martín et al.[36] achieved 72 g of cellulose/100 g of pretreated sugar cane (190 °C, 80% of glycerol−water, 1 h and 0.94% of H_2SO_4). These authors concluded that the use of acid (as reaction catalyst) solubilized part of the cellulose, being the highest overall cellulose convertibility (94%) obtained without other chemicals.[36] On the other hand, Sun and Chen[37] yielded a cellulose recovery of 94 to 96% from wheat straw at 220 °C for 3 h and 70% of different-type aqueous glycerol liquor.

In relation to the hemicellulosic fraction, the xylan content in the solids varied in the range of 4 to 13 g of xylan/100 g of delignified solid (experiments 8 and 3). The values of xylan recovery (expressed as percentage of xylan in the delignified solid with respect to xylan in the raw material) were in the range of 12 to 61% (experiment 8 and 10) resulting in an average recovery of 32%. The xylan solubilization can be more

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Table 2. Operational Conditions (Expressed in Terms of Dimensionless and Dimension Independent Variables) and Experimental Results Obtained for Dependent Variables y_1 to y_9, Concerning Solid Yield, Composition of Solid and Liquid Phase, and Enzymatic Hydrolysis

run	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Independent Variables															
T_D (°C) *or* x_1	180 (−1)	200 (1)	180 (−1)	200 (1)	180 (−1)	200 (1)	180 (−1)	200 (1)	190 (0)	190 (0)	190 (0)	190 (0)	190 (0)	190 (0)	190 (0)
GW (%) *or* x_2	40 (−1)	40 (−1)	80 (1)	80(1)	60 (0)	60 (0)	60 (0)	60 (0)	40 (−1)	80 (1)	40 (−1)	80 (1)	60 (0)	60 (0)	60 (0)
t_D (min) *or* x_3	65 (0)	65 (0)	65 (0)	65 (0)	40 (−1)	40 (−1)	90 (1)	90 (1)	40 (−1)	40 (−1)	90 (1)	90 (1)	65 (0)	65 (0)	65 (0)
Dependent Variables															
SY (%) *or* y_1	66.5	51.3	76.0	55.1	81.0	57.0	62.9	53.2	63.9	78.1	58.9	59.8	59.1	58.0	57.0
	a) solid phase composition (%)														
Gn or y_2	67.6	81.1	53.9	75.1	51.9	71.6	68.0	83.0	64.6	54.1	72.5	72.4	73.1	74.0	74.5
Xn or y_3	7.0	4.0	12.8	8.6	11.2	7.0	7.9	3.5	7.8	12.5	4.8	9.3	7.4	7.3	7.3
KLSD	20.9	13.3	19.0	10.2	20.2	13.5	16.7	12.9	18.7	18.9	18.3	11.1	15.1	15.3	14.9
D or y_4	43.7	72.5	41.6	77.3	33.7	68.9	57.5	72.2	51.6	40.2	56.4	73.1	63.8	64.0	65.7
AcH or y_5	0.8	0.4	1.4	0.7	1.5	0.3	0.9	0.2	0.6	1.7	0.6	0.9	0.4	0.4	0.5
	b) liquid phase composition (g/L)														
X *or* y_6	11.3	10.6	4.5	11.1	3.7	12.3	11.4	12.4	12.8	6.5	14.7	11.0	13.6	13.4	13.5
glucose	1.03	1.37	0.97	0.96	1.01	1.00	0.81	1.24	1.19	1.03	1.31	0.89	1.26	1.34	1.20
arabinose	0.16	0.15	0.24	0.09	0.27	0.11	0.19	0.03	0.16	0.13	0.21	0.12	0.31	0.35	0.31
acetic acid	3.06	4.05	4.76	4.91	5.20	3.59	4.05	4.58	3.23	3.56	4.10	5.27	5.49	4.76	5.05
furfural	0.48	1.88	0.21	0.75	0.12	0.93	0.50	1.34	0.46	0.27	1.29	0.96	1.10	1.12	1.14
	c) enzymatic hydrolysis of solid phase														
CGC_{max} (%) *or* y_7	100.0	100.0	72.2	100.0	39.7	100.0	100.0	100.0	100.0	95.0	100.0	100.0	100.0	100.0	100.0
$t_{1/2}$ (h) *or* y_8	11.6	4.0	16.2	4.9	15.2	6.1	9.5	3.5	11.3	18.8	6.3	5.9	5.1	5.0	4.5
G_{72h} (g/L) *or* y_9	32.8	43.2	17.5	38.3	9.4	38.0	36.7	44.4	32.5	22.6	38.6	36.5	39.2	39.5	39.4

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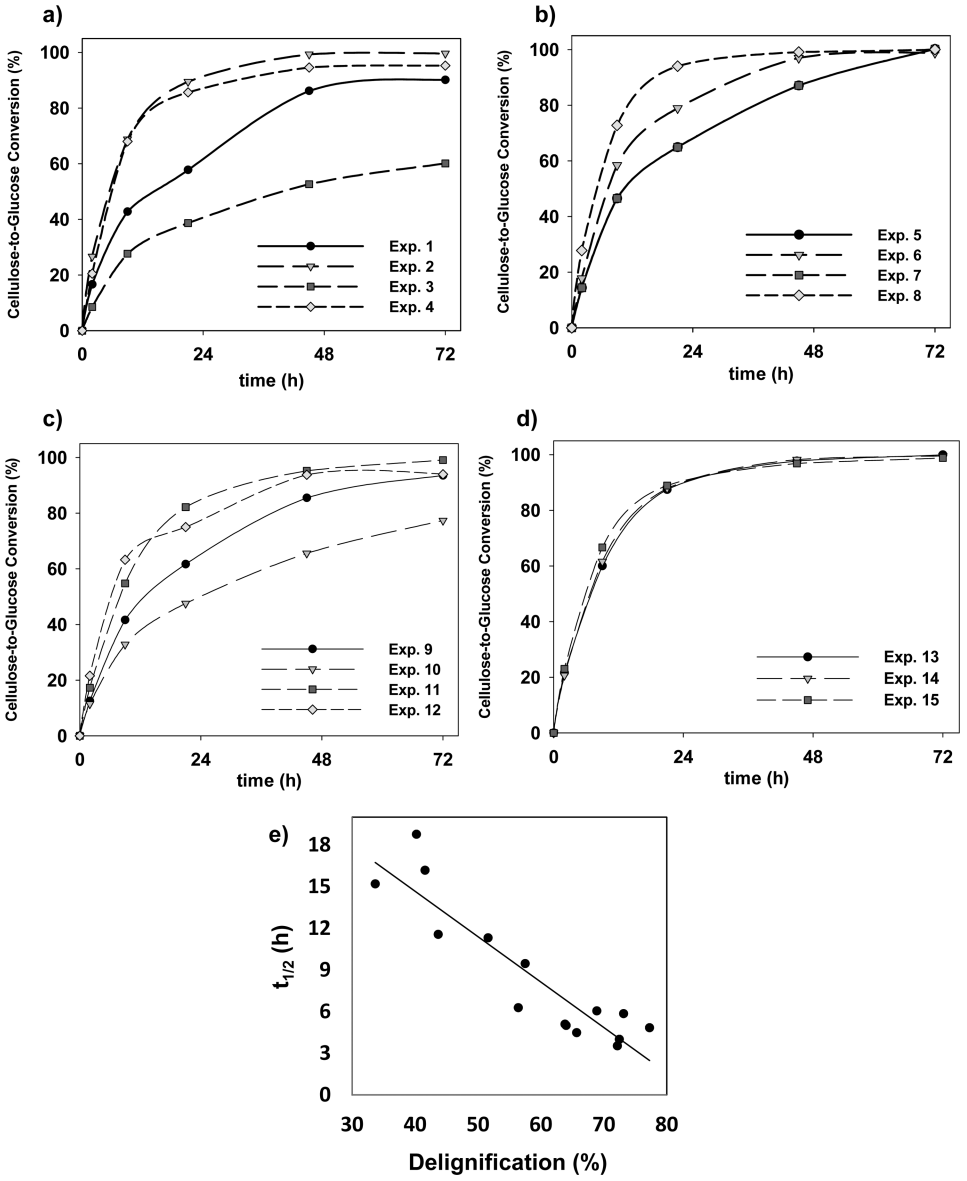

Figure 1. a-d) Time course of cellulose-to-glucose conversion (%) of enzymatic saccharification experiments, under the following conditions: LSR_{EH} = 20 g/g and ESR = 20 FPU/g. **e)** Dependence of kinetic parameter ($t_{1/2}$, h) on delignification percentage (D, %) of EGW.

influenced by high water content,[29] being this behavior typical of the hydrothermal process.[38] As it can be seen in Table 2, the minimum xylan solubilization during this pretreatment was 39%, corresponding to experiments 3 and 10 (using an 80% of *GW*, T_D = 180 and 190 °C, respectively). Nevertheless, assays 4 (200 °C, 65 min, 80% *GW*) and 12 (190 °C, 90 min, 80% *GW*) showed a xylan solubilization of 70% and 65%, respectively. These higher values of xylan solubilization could be due to the additional effect of temperature and time of the pretreatment. The effect of alkaline polyol pretreatment on softwood pulping and, mainly, the influence of time and temperature (using the severity factor, R_0) was recently studied.[39] These authors concluded that the hemicellulose and lignin were considerably dissolved with increasing of R_0.[39]

Considering the EGW biomass delignification process, the minimum (10%) and maximum (21%) values of lignin in the pretreated solid were reached under conditions of T_D = 200 °C, *GW* = 80%, t_D = 65 min and T_D = 180 °C, *GW* = 40%, t_D = 65 min, respectively. The removal of lignin (measured as percentage of delignification) varied in the range of 34 to 77% (corresponding to experiments 5 and 4), with an average value of 59%. Interestingly, values higher than 68% of delignification were obtained when the working temperature was 200 °C. Novo et al.[29] reported that the highest delignification percentage (79.9%) of wheat straw was attained at 190 °C with 80% of glycerol−water during 240 min. In this work, a similar delignification percentage (77%) was obtained using EGW and operating at 200 °C, 80% of *GW*, and 65 min. The delignification temperature was higher than reported by Novo et al.,[29] nevertheless the employed time in our pretreatment was 3.7-fold lower.

After the pretreatment step, the posthydrolyzate aliquot from liquid phase was analyzed for the quantification of hemicellulose-derived compounds, mainly sugars (glucose, xylose, and arabinose), acetic acid, and furfural (see Table 2). In relation to the sugars, xylose was the most abundant sugar in the liquor and represented about 64% (measured as average percentage of xylose in respect to the total of compounds

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Table 3. Regression Coefficients and Statistical Parameters Measuring the Correlation and Significance of the Models

parameter	y_1	y_2	y_3	y_4	y_5	y_6	y_7	y_8	y_9
b_0	58.03[a]	73.84[a]	7.3[a]	64.51[a]	0.45[a]	13.5[a]	100.00[a]	4.9[b]	39.4[a]
b_1	−8.73[a]	8.67[a]	−2.0[a]	14.29[a]	−0.37[a]	1.9[a]	11.01[b]	−5.0[a]	8.5[a]
b_2	3.55[a]	−3.77[a]	2.5[a]	1.01	0.29[a]	−2.0[a]	−4.09	0.8	−4.0[a]
b_3	−5.65[b]	6.72[a]	−1.6[a]	8.10[a]	−0.19[b]	1.7[a]	8.16[c]	−3.3[b]	6.7[a]
b_{11}	1.28[a]	−0.83	−0.2	−1.52	0.05	−2.7[a]	−10.38	1.9	−3.4[c]
b_{22}	2.92[a]	−3.57[a]	1.0[a]	−4.24[b]	0.31[b]	−1.4[b]	3.44	3.9[b]	−3.0[c]
b_{33}	4.23[a]	−4.38[a]	0.3	−4.93[b]	0.22[c]	−0.8	−4.69	1.8	−3.8[b]
b_{12}	−1.43[b]	1.94[c]	−0.3[c]	1.72	−0.07	1.8[a]	6.94	0.5	2.6
b_{13}	3.57[a]	−1.17	−0.1	−5.14[b]	0.14	−1.9[a]	−15.08[b]	0.8	−5.2[b]
b_{23}	−3.32[a]	2.59[b]	−0.1	7.03[a]	−0.21[c]	0.7	1.25	−2.0	2.0
R^2	0.997	0.991	0.995	0.984	0.951	0.977	0.850	0.926	0.974
F	177.83	58.68	113.89	33.45	10.82	23.44	3.14	6.96	20.96
significance level	99.999	99.985	99.997	99.248	99.133	99.857	88.963	97.708	99.813

[a]Coefficients significant at the 99% confidence level. [b]Coefficients significant at the 95% confidence level. [c]Coefficients significant at the 90%

solubilized in the liquid phase). The xylose concentration varied in the range of 3.7 to 14.7 g/L (experiments 5 and 11), corresponding to 21 and 84% of xylan conversion into xylose sugar monomers, respectively. The maximal xylose concentration (14.7 g/L) was obtained under the operating conditions of 190 °C, 40% *GW*, and 90 min. In most of the experiments, the xylose concentration was higher than 10 g/L, except in the experiments 3, 5, and 10 with a xylan recovery in the solid higher than 56% meaning that the process conditions were not suitable for a good solubilization on of the hemicellulose fraction. The concentration of others hemicellulose-derived compounds was analyzed and revealed reduced amounts of these (see Table 2). The concentration of glucose and arabinose varied in the range of 0.7−1.4 and 0.08−0.35 g/L, respectively. As result of acetyl groups degradation during extreme conditions of biomass pretreatment, the acetic acid concentration reached a maximum under the intermediate conditions of pretreatment (190 °C, 65 min, and 60% of *GW*) varying in the interval of 3.1 to 5.5 g/L. Furthermore, as a result of sugars degradation (mainly pentose sugars), the furfural concentration varied in the range of 0.1−1.9 g/L (Table 2). This behavior is in good agreement with the known pattern of sugar and acetyl groups degradation in high temperature acid pretreatment.[38] Martín et al.[36] obtained the maximal xylose concentration (5 g/L) for 1 h of process at 190 °C and 60% of glycerol−water.

3.3. Enzymatic Saccharification of Delignified Solids.

The pretreated and delignified solids were used after as substrate to evaluate their suitability for enzymatic saccharification process. All saccharification assays were carried out under favorable conditions listed in Table 1 (LSR_{EH} = 20 g/g and the ESR = 20 FPU/g). Figure 1 shows the enzymatic hydrolysis profiles of pretreated EGW with glycerol under the fifteen different pretreatment conditions (see Table 2). The displayed experimental data are expressed in terms of cellulose to glucose conversion (%), calculated by eq 2.

The different profiles illustrated in Figure 1a-d show the effect of pretreatment operating conditions on cellulose to glucose conversion. A percentage of cellulose to glucose conversion of 80% (*CGC*) was obtained in the experiments carried out at T_D = 200 °C and $t_D \geq$ 65 min at 21 h of saccharification process. On the other hand, at T_D = 180 °C the *CGC* was lower than 60%. After 41 h of saccharification, a *CGC* higher than 95% was achieved in the experiments carried out at T_D = 200 °C or T_D = 190 °C and $t_D \geq$ 65 min. The lowest *CGC* at 72 h (*GGC* = 60%) was obtained in experiment 3 (T_D = 180 °C, GW = 80%, and t_D = 65 min). In most of experiments (except experiments 3 and 10) at 72 h, the *CGC* was higher than 90%, possibly meaning an alteration of native structure and an improvement of enzyme accessibility to the cellulose. These data are in agreement with the reports by Martín et al.[36] and Sun and Chen[37] on enzymatic saccharification of sugar cane bagasse and wheat straw pretreated solids, respectively. These authors concluded that glycerol pretreatment was effective in enhancing the saccharification of cellulose being the highest overall glucose achieved in the glycerol−water mixture without catalyst (e.g., NaOH and H_2SO_4).

The experimental data of enzymatic kinetics followed a typical pattern and was well adjusted by the empirical equation (see eq 2) with a very good regression (R^2 = 0.97−0.99). Table 2 shows the values of CGC_{max} and $t_{1/2}$ variables obtained from eq 2. In most of the experiments the values of CGC_{max} were higher than 95%. Interestingly, the values of $t_{1/2}$ were lower than 7 h when the percentage of delignification was higher than 60%, which is expected since there is a fairly linear interrelationship for $t_{1/2}$ and percentage of delignification (Figure 1e). Moreover, the variation range determined for $t_{1/2}$ was 3.5−18.8 h (experiments 8 and 10, respectively) showing the high differences among the saccharification kinetic trials.

Organosolv pretreatment using solvents (e.g., methanol or ethanol) dissolves the lignin and part of hemicellulose leaving the solid enriched in cellulose.[40] In that work, Zhao and coworkers verified that the enzymatic saccharification of pretreated solid is significantly enhanced when the accessible surface area is increased.

In this context, the improvement of the enzymatic saccharification process (conversion percentage of cellulose) promoted by the glycerol organosolv pretreatment can be compared with the results reported in the literature using ethanol-organosolv. Using hybrid poplar pretreated with ethanol = 60%, time = 60 min, T = 180 °C, and 1.25% H_2SO_4, 85% of cellulose was recovered as monomeric glucose after 48 h of saccharification with 20 FPU/g cellulose.[41] Also, Brudecki et al.[42] reported a saccharification yield higher than 90% using switchgrass as raw material, with methyl isobutyl ketone−ethanol−water = 28%, time = 40 min, T = 136 °C, and H_2SO_4 as catalyst 0.75%. On the other hand, Wildschut et al.[43] showed high susceptibility to saccharification (86% of glucose yield) at 72 h and 3% of solids using wheat straw as raw

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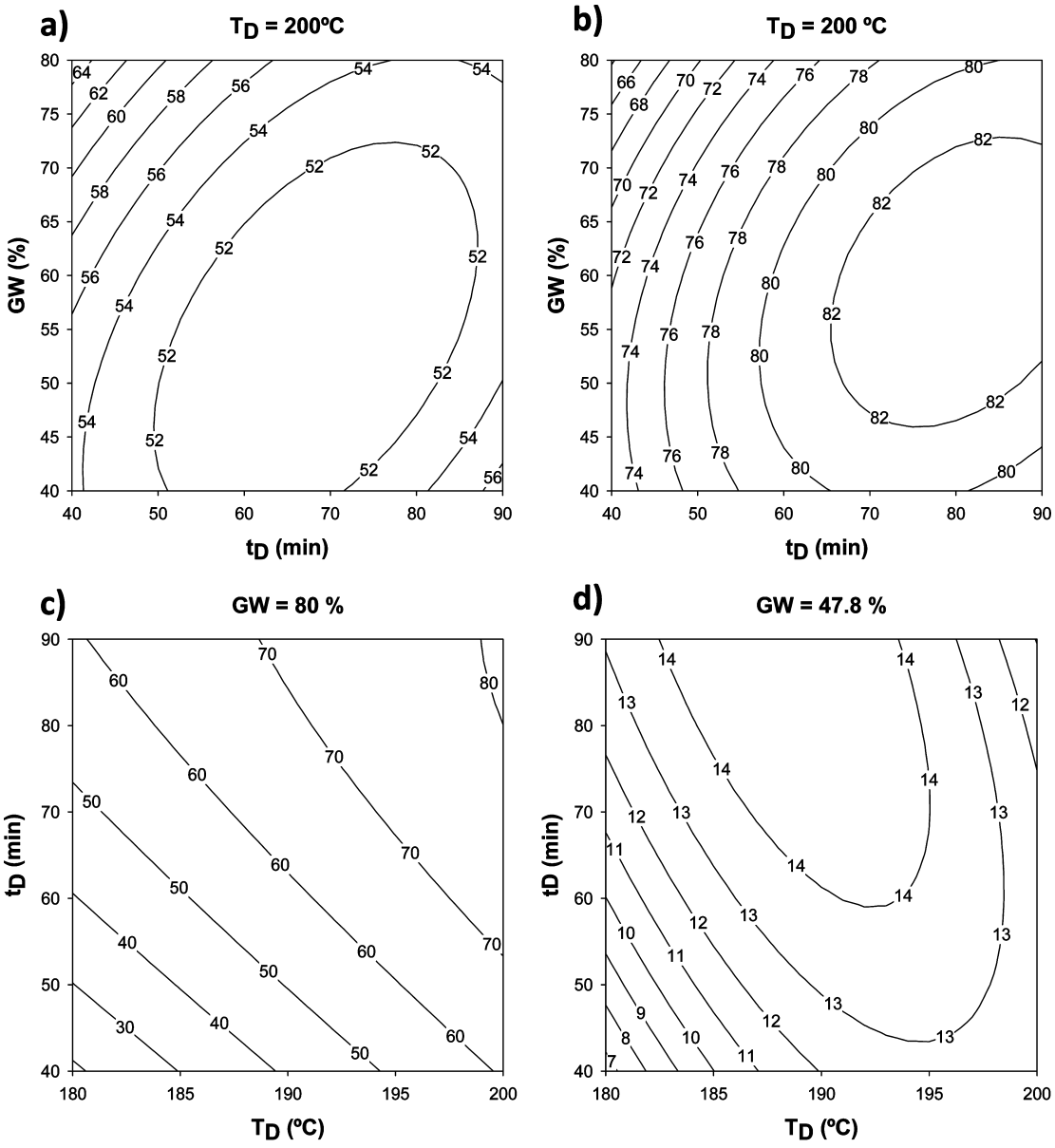

Figure 2. Response surface of **a)** Solid Yield (*SY*, g of delignified solid/100 g of raw material) on time of delignification (t_D, min) and percentage of glycerol−water (*GW*, %). **b)** Glucan (*Gn*, g glucan/100 g delignified solid) on time of delignification (t_D, min) and percentage of glycerol−water (*GW*, %). **c)** Delignification (D, g of removal lignin/100 g of lignin in raw material) on time of delignification (t_D, min) and temperature of delignification (T_D, °C), fixed glycerol−water (*GW*) = 80%. **d)** Xylose (X, g of xylose from hydrolyzed liquor/L)) on time of delignification (t_D, min) and temperature of delignification (T_D, °C), fixed glycerol−water (*GW*) = 47.8%.

material and ethanol−water (50%) at 210 °C. Hallac et al.[44] reported a 85% of cellulose to glucose conversion using 1% of pretreated *Buddleja devidii* with ethanol−water (50%) at 180 °C during 60 min and 1.25% H_2SO_4.

Moreover, the results with organosolv can be also compared with other pretreatments such as hydrothermal treatments (autohydrolysis). Romaní et al.[35] reported a cellulose to glucose conversion of 98% using autohydrolyzed *Eucalyptus* wood (T_{max} = 230 °C or logR_0 = 4.67) at 72 h. On the other hand, Min et al.[45] studied the effect of lignin on the enzymatic saccharification of hardwood using alkali treatment and acid treatment. They concluded that the substrates pretreated by alkaline solutions give higher sugar production and higher sugar conversion than substrates treated by acid solutions due to greater lignin removal.

Thus, using the glycerol−water organosolv treatment, that is an eco-friendly and economic pretreatment strategy, similar results were obtained regarding literature, specifically a high susceptibility of cellulose toward saccharification (above of 90%). Moreover, this innovative pretreatment proposal has the ability to break the structure of the EGW and generate a delignified solid with increased cellulose percentage, which is important to attain a high ethanol concentration in subsequent yeast fermentation step.

3.4. Response Surface Methodology Assessment. The Response Surface Methodology (RSM) is a useful tool for optimization and interpretation of operating conditions on dependence variables.[35] Table 2 shows a summary of results for the response variables (*Gn, Xn, Dm AcH, X,* CGC_{max}, $t_{1/2}$, *and* G_{72h}) studied in this work. The referred dependent variables

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were well interpreted by the empirical model, as it can be verified from fitting parameters (see Table 3). Table 3 lists the value determined for regression coefficients b_{0j} to b_{23j}, the statistical significance (based in the Student́s *t test*) and the statistical significance of model (Fischeŕs *F* parameter). The Student́s *t* test shows that linear and quadratic terms were highly significant ($P < 0.05$). The average coefficient (R^2) of the model (except for CGC_{max}) was 0.97 for all variables, which indicates that the model is adequate to represent the real relationships among the selected variables. On the other hand, the high values of *F* confirm a good fit of data.

Figure 2 a;b) represents the predicted values of variables *SY* and glucan percentage in delignified solid (*Gn*) as function of t_D and *GW* (fixing the temperature at 200 °C). Figure 2a shows the modeling of *SY*. The minimum value of *SY* (50%) was obtained at 200 °C, *GW* = 54%, and t_D = 68 min. In a wide range of t_D and *GW* values, the *SY* was ≤55%, when the T_D = 200 °C. The most influential variable on *SY* was T_D (see Table 3). At high processing temperatures it is believed that the organic acids released from the wood act as catalysts for rupture of the lignin-carbohydrate complex.[46] Glucan content in the pretreated solid is represented in Figure 2b. The highest glucan content (84%) was achieved at 200 °C, *GW* = 59%, and t_D = 81 min, being that the temperature is the most influential variable. Under the fixed conditions of T_D = 200 °C (Figure 2b), the *Gn* was higher than 80% operating at t_D > 65 min, independently of *GW* percentage.

On the basis of results determined for model coefficients (Table 3), Figure 2 c;d) shows the influence of operating conditions on percentage of delignification (*D*) and xylose concentration (*X*) in the liquid phase. In Figure 2c, the variable *D* increases as long as temperature and time increased fixing the *GW* variable at 80%. The highest value of delignification was 80%, which was obtained at 200 °C, $t_D \geq 58$ min and *GW* = 80%. The plot of xylose concentration (see Figure 2d) shows that the concentration increases for all values of t_D and for T_D 188 °C. Maximum content of xylose (14.84 g/L) in the liquid phase was achieved at the following operating conditions: T_D =188 °C, t_D = 90 min, and *GW* = 48%. In Figure 2d, a decreasing of xylose concentration can be observed, which can be explained by the degradation process of this monomeric sugar at extreme conditions (high temperature and time).

Figure 3 shows the surface plot of the interactive effects of temperature and time on cellulose to glucose conversion maximal (CGC_{max}) and $t_{1/2}$, fixing the percentage of glycerol−water at 60%. The maximum value predicted of CGC_{max} (%) by the model was reached between 195 and 200 °C and t_D > 50 min (see Figure 3a). As a general pattern, the highest CGC_{max} correspond to samples pretreated at high T_D and t_D, with a limited influence of the *GW*. Figure 3b represents the three-dimensional response plot of $t_{1/2}$ (fixed condition *GW* = 60%) to investigate the effect of t_D and T_D. The $t_{1/2}$ decreases when the t_D and T_D increase. Concerning the dependence of $t_{1/2}$ on experimental variables, its value was 4 h when the operating conditions were $T_D \geq 190$ °C and $t_D \geq 60$ min. For the same conditions the dependent variable CGC_{max} achieved 100% of saccharification. Taken together, these results revealed that the pretreated solid by glycerol-organosolv were highly suitable substrates for glucose production in relatively short times of saccharification.

3.5. Optimization of Glucose: Selection of Operational Conditions and Model Validation. On the basis of these reported data, the model was used to choose an optimal

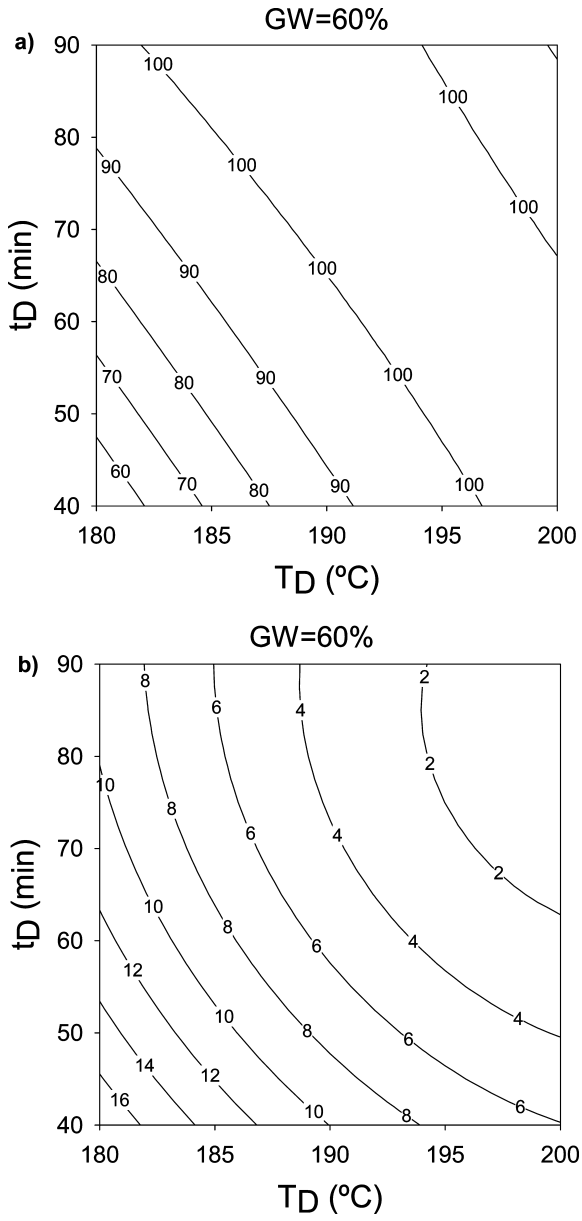

Figure 3. a) Contour lines calculated for variable Cellulose to Glucose Conversion maxima (CGC_{max}, %) on time of delignification (t_D, min) and temperature of delignification (T_D, °C). **b)** Contour lines calculated for variable time needed for achieved $CGC_{max}/2$ ($t_{1/2}$, h) on time of delignification (t_D, min) and temperature of delignification (T_D, °C). Results calculated from glycerol−water (GW) fixed to 60%.

condition to attaining a high concentration of glucose from a delignified solid. For this purpose, the effect of *GW* and t_D variables on glucose concentration at 72 h (G_{72h}) was represented in Figure 4. A predicted glucose concentration higher than 44 g/L was achieved working at T_D = 200 °C, *GW* between 48 and 64%, and t_D between 63 and 70 min. The model was used for prediction of the maximum concentration of glucose employing the following operational conditions: T_D = 200 °C, *GW* = 56.2%, and t_D = 68.8 min. Thus, an additional assay was performed for model validation. The experimental results of validation assay for studied variables were as follows: y_1 (%) = 55.01; y_2 (%) = 76.92; y_3 (%) = 4.93; y_4 (%) = 63.24; y_5 (%) = 0.45; y_6 (g/L) = 12.8; y_7 (%) = 98.78; y_8 (h) = 3.51; and y_9 (g/L) = 41.78. These data confirm the sustainability of

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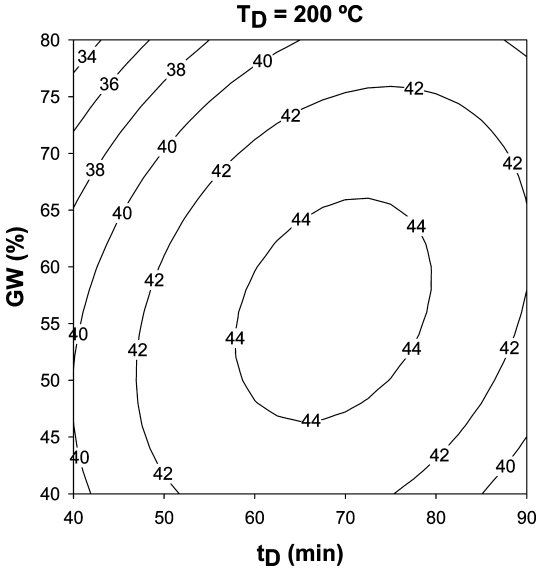

Figure 4. Response surface of glucose after 72 h of enzymatic saccharification (G_{72h}, g/L) on time of delignification (t_D, min) and glycerol–water (GW, %). Results calculated from temperatue of delignification (T_D) fixed to 200 °C.

the models for reproducing and predicting the experimental results. For mentioned data, the calculated relative error was ≤10% for the majority of variables.

In order to highlight the optimization results, Figure 5 shows the mass balance of the optimal condition. The EGW was fractionated by glycerol–water pretreatment, obtaining in separated streams: a delignified solid suitable to enzymatic saccharification and solubilized lignin and hemicellulose-derived compounds (mainly xylose). After the pretreatment followed by enzymatic saccharification, per 100 kg of EGW 46.0 kg of glucose was obtained with an overall yield glucose of 92%. On the other hand, in the liquid phase per 100 kg of EGW: 15.9 kg of soluble lignin and 13.2 kg of xylose were obtained, removing 64.3% of lignin and 82% of xylan.

4. CONCLUSIONS

The results presented in this work showed that the delignification process using a mixture of glycerol–water is an efficient pretreatment for fractionation of *Eucalyptus globulus* wood improving the enzymatic saccharification process. Pretreatment in glycerol–water 56.2% during 68.8 min at 200 °C resulted in 82% of hemicellulose hydrolysis (as xylose), 64.3% delignification, and 92% cellulose recovery. The empirical models allowed a generalized interpretation of data and optimization of variables for a maximum concentration of glucose of 42 g/L, corresponding to a conversion yield of cellulose to glucose higher than 98%. Moreover, this study contributes to the improvement of biomass pretreatment technologies by exploring an innovative, sustainable, and eco-friendly operational strategy that allows attaining high levels of glucose available for subsequent yeast fermentation to bioethanol.

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Notes

The authors declare no competing financial interest.

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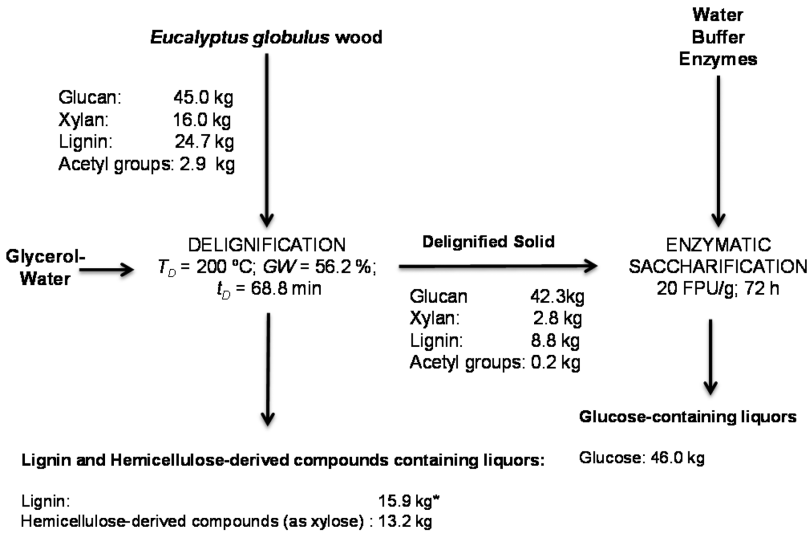

Figure 5. Mass balances for optimal conditions of glycerol–water pretreatment.

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