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Valorization of Eucalyptus wood by glycerol-organosolv pretreatment within the biorefinery concept: An integrated and intensified approach

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ABSTRACT

The efficient utilization of lignocellulosic biomass and the reduction of production cost are mandatory to attain a cost-effective lignocellulose-to-ethanol process. The selection of suitable pretreatment that allows an effective fractionation of biomass and the use of pretreated material at high-solid loadings on saccharification and fermentation (SSF) processes are considered promising strategies for that purpose. *Eucalyptus globulus* wood was fractionated by organosolv process at 200 °C for 69 min using 56% of glycerol-water. A 99% of cellulose remained in pretreated biomass and 65% of lignin was solubilized. Precipitated lignin was characterized for chemical composition and thermal behavior, showing similar features to commercial lignin. In order to produce lignocellulosic ethanol at high-gravity, a full factory design was carried to assess the liquid to solid ratio (3–9 g/g) and enzyme to solid ratio (8–16 FPU/g) on SSF of delignified Eucalyptus. High ethanol concentration (94 g/L) corresponding to 77% of conversion at 16FPU/g and LSR = 3 g/g using an industrial and thermotolerant *Saccharomyces cerevisiae* strain was successfully produced from pretreated biomass. Process integration of a suitable pretreatment, which allows for whole biomass valorization, with intensified saccharification-fermentation stages was shown to be feasible strategy for the co-production of high ethanol titers, oligosaccharides and lignin paving the way for cost-effective Eucalyptus biorefinery.

1. Introduction

Lignocellulosic biomass conversion into biofuels (as bioethanol) is considered a promising alternative to replace fossil fuels, being one of investment priorities of European Union to attain a sustainable growth within Horizon 2020 [1,2]. In this sense, global demand of ethanol production could grow to exceed 125 billion liters [3]. Short-rotation plantations (as *Eucalyptus*) are forest lignocellulosic biomass considered one of the major renewable energy sources with a potential ethanol production of 7000 L/ha which could satisfy great part of the energetic needs [4]. In recent years, the research devoted to biomass bioconversion into bioethanol is gaining significant prominence, which is reflected in an increase of publishing in this field [5–7]. Although the investigation in lignocellulosic ethanol has allowed the improvement of the technology the reduction of production costs is still mandatory for the industrial establishment of these processes. The intensification of the process can be the path to follow to attain an economic feasible process. The use of high solid loads and the integral use of all biomass fractions are key strategies for this purpose. Furthermore, for a proper evaluation of these strategies the integration of all stages of the process has to be considered.

For the integral use of all biomass fractions, the selection of a suitable pretreatment is determinant [8]. Organosolv pretreatment is considered a feasible process to enhance the enzymatic saccharification since it allows an effective high delignification and disruption of its recalcitrant structure. Moreover, organosolv processing is suitable for a biorefinery approach in which the obtained lignin has desired properties and the organic compound can be recovered easily [9]. Additionally, the use of glycerol (cheap industrial by-product from the biodiesel sector) on organosolv process has been lately suggested as a valuable green solvent, being an

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attractive approach for the treatment of biomass [10–14].

For further cost reduction, the intensification of the ethanol production process must be considered namely by using high-solid loads leading to high-gravity fermentations. In this context, the high-gravity fermentation, used in brewing and starch-based fermentation industries, produces a 10–15% (v/v) and improves the overall productivity as well as reduces capital cost and energy input comparing to normal gravity [15,16]. Therefore, the application of high-gravity technology in lignocellulosic ethanol plant could be an interesting strategy to achieve a cost-competitive process since the water economy of the process would be improved and lower costs of distillation would be involved. However, the implantation of this technology in the case of lignocellulosic feedstock implies the use of high biomass loadings in all process stages [17] which shows several operational limitations and challenges due to a lack of available water, the difficulty to mix and handle and the poor mass and heat transfer [18]. In consequence, the ethanol yields are usually low and the required enzyme loads are high [18]. In this context, only recently have few works reported efficient high concentration of cellulosic ethanol >4% (w/v) [19,20]. Moreover, high-gravity fermentation is related with stress responses in yeast, being essential for the use of industrial strains able to rapidly adjust their metabolism to harsh industrial conditions [16]. In this regard, industrial distillery environments as "cachaça" (Brazilian distilled beverage) are a good example of robust yeasts source for efficient high-gravity ethanol fermentation [21] and with higher thermotolerance, both features of great interest for the industry. The use of yeasts able to ferment at temperatures above 35 °C allows overcoming the main drawback of simultaneous saccharification and fermentation (SSF) process that is the difference between the optimal temperature of saccharification and fermentation [22]. SSF strategy implies the reduction of capital costs, reduces the enzyme loadings and increases the productivity [23–25]. Other alternative that could be considered is to carry out a pre-saccharification before the SSF process, also known as Pre-saccharification and Simultaneous Saccharification and Fermentation (PSSF) [26]. PSSF has been employed to reduce the viscosity of slurry at high solid loadings [27–29].

The strategy followed in this work shows a feasible process using glycerol as green solvent for the fractionation of *Eucalyptus globulus* wood (EGW) in order to obtain, within a biorefinery context, a pretreated biomass susceptible to be used as substrate at high-solid loadings (>30%) on saccharification and fermentation processes, as well as, a recovered lignin with similar features to commercial lignin. The effect of glycerol-organosolv pretreatment on pretreated EGW and organosolv lignin was evaluated by SEM, FT-IR, TGA and X-Ray. In addition, following a PSSF strategy, high-gravity ethanol production using a robust industrial and thermotolerant *Saccharomyces cerevisiae* strain was optimized under intensified conditions of low enzyme and high solid loadings by a full factorial design. Overall, the work carried out in this study opens new paths for cost-efficient lignocellulosic bioethanol production processes from a biorefinery approach by bridging and intensifying pretreatment, saccharification and fermentation stages.

2. Materials and methods

2.1. Raw material

Eucalyptus globulus wood (EGW) was collected from a paper mill (ENCE, Pontevedra, Spain), milled and stored in a dry place until to be used. The raw material was previously analyzed by Pereira et al. [30] following standard procedures for structural carbohydrates and lignin determination (NREL/TP-510-42618). The chemical composition, expressed in g/100 g of raw material on dry basis, was: 44.70% of glucan; 16.01% of xylan; 1.09% of arabinan; 2.96% of acetyl groups; 27.70% of Klason lignin; 0.2% of ashes and 2.0% of extractives (see Table 1).

2.2. Pretreatment: glycerol-organosolv of EGW

Fig. 1 shows a scheme of the process followed in this work in which a biorefinery approach of EGW is proposed using organosolv process with glycerol-water as solvent in order to obtain in separated streams: cellulose for bioethanol production, hemicellulose as xylooligosaccharides and solubilized lignin. For that, the EGW was submitted to organosolv pretreatment in a 160 mL total volume batch cylinder stainless reactor and submerged in an oil bath with PID temperature controller, previously heated at 200 °C for 69 min for the pretreatment, the heating up and cooling were not taken into consideration. The EGW was mixed with 56% of glycerol-water at Liquid to Solid Ratio (LSR) = 10 g of glycerol-water/1 g of EWG on dry basis. The conditions of operation were chosen on basis of previous work [11]. After treatment, the delignified EGW was separated from liquid phase (black liquor) by vacuum filtration and washed with 10 g of NaOH (1%, w/w)/g of delignified EGW at 20 °C and two washes with approximately 1 L of distilled water/g of delignified EGW at 60 °C and 20 °C until pH = 7 (according to Dominguez et al. [14]) in order to remove adsorbed lignin from the pretreated solid (see Fig. 1). Washed delignified EGW was air-dried and quantified for solid yield (SY) determination (Table 1). The amount of liquid phase recovered (1046 g/100 g of EGW) was quantified considering the solubilized fraction of EGW (calculated as 100-SY). One aliquot of liquid phase (black liquor) was analyzed for derived-hemicellulose compounds (acetic acid, furfural and mono- and oligo-saccharides) concentration by acid post-hydrolysis treatment (121 °C, 4% w/w H_2SO_4 and 20 min) and quantified by HPLC. The solubilized lignin was precipitated adding 2 g of 0.3 M HCl/g of black liquor, recovered by centrifugation and kept overnight at 4 °C. The precipitated lignin (organosolv lignin) was dried at 50 °C to constant weight (15.6 g of lignin/100 g of raw material). In addition, acid soluble lignin in the liquor was measured (0.1%) by UV–vis spectroscopy (NREL/TP-510-42618).

2.3. Organosolv lignin characterization

2.3.1. Fourier-transform infrared (FT-IR)

The organosolv lignin and standard lignin (alkali lignin with low sulfonate content purchased in Sigma-Aldrich) were analyzed by a

Table 1
Solid Yield and composition data of EWG, Delignified EGW and liquid phase after organosolv pretreatment.

	EGW (g/100 g raw material, oven dry basis)	Delignified EGW (g/100 g of pretreated EGW, oven dry basis)
Solid Yield (SY)		54
a) Solid phase Composition		
Glucan	44.7	82.5
Hemicellulose		
Xylan	16.01	1.02
Arabinan	1.09	–
Acetyl groups	2.96	1.18
Klason Lignin	27.7	17.86
b) Liquid phase composition (g of monomer equivalent/L)		
Glucooligosaccharides		0.96
Xylooligosaccharides		11.08
Arabinoologosaccharides		0.09
Acetyl groups		4.91
Furfural		0.75

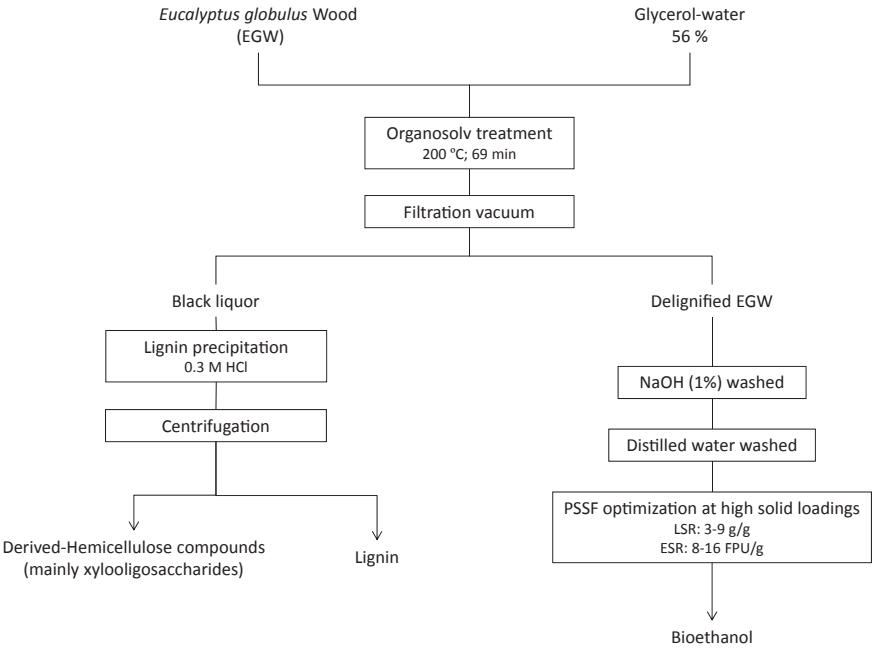

Fig. 1. Scheme of biorefinery approach followed in this study for co-production of ethanol, xylooligosaccharides and lignin from glycerol-organosolv pretreated *E. globulus* wood.

FT-IR spectrometer (FTLA 2000 series, ABB Bomem Inc., Quebec, Canada) for their characterization. The FT-IR spectra were obtained operating with a resolution of 4 cm^{-1}, 20 scans, and frequency range of 4000–400 cm^{-1} according to Gonçalves et al. [31]. FTIR bands were identified by comparison with reported data from literature [32–36].

2.3.2. Thermal analysis of organosolv lignin

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of organosolv lignin and standard lignin samples were carried out in Shimadzu TGA-50 and DSC-50 equipment, respectively in order to evaluate their thermal behavior. The experiments were conducted, under N_2 atmosphere and the measurements were carried out in the range of 25–600 °C with a linear increase of 10 °C/min [37].

2.4. Delignified EGW characterization

2.4.1. Chemical composition

The pretreated EGW was analyzed in triplicate for content of glucan, xylan, Klason lignin and acetyl groups following the same method used for raw material characterization and listed in Table 1.

2.4.2. Scanning electron microscopy (SEM) analysis

Micrographs of raw material (EGW) and pretreated solid (delignified EGW) samples were obtained using a scanning electron microscope (Nova NanoSEM 200, Netherlands). The images were obtained using a voltage of 10 kV at 500–fold magnifications.

2.4.3. X-ray diffraction analysis and crystallinity

The crystallinity index (CrI) was measured in X-ray diffractometer (Bruker D8 Discover, USA) using 40 kV and 40 mA. CrI was calculated as follow [30]:

$$CrI = \frac{I_{002} - I_{am}}{I_{002}} \cdot 100 \quad (1)$$

where, I_{002} = maximum intensity (2θ, 22.6°) of the (002) lattice diffraction; I_{am} = intensity of the amorphous diffraction (2θ, 18.7°).

2.5. Pre-saccharification and simultaneous saccharification and fermentation (PSSF) of delignified EGW

2.5.1. Microorganism and inoculum preparation

The strain used in this work was *Saccharomyces cerevisiae* CA1185, isolated from Brazilian "cachaça" fermentation processes [16,21]. Stock cultures were maintained on agar YPD plates at 4 °C. Cells were inoculated in 250 mL Erlenmeyer flasks filled with 100 mL YPD medium (50 g/L of glucose, 20 g/L of peptone and 10 g/L of yeast extract) at 30 °C and 150 rpm for 20 h. The cells were aseptically recovered by centrifugation for 10 min at 6000 rpm and 4 °C and suspended in 0.9% NaCl, obtaining a concentration of 200 mg fresh yeast/mL. The saccharification and fermentation assays were inoculated with 5 mg fresh yeast/mL.

2.5.2. PSSF experiments for evaluation of high-gravity ethanol fermentation from delignified EGW

The evaluation of enzyme to substrate ratio (ESR) and liquid to solid ratio (LSR) on ethanol production using delignified EGW as substrate was carried out by full factorial design (3^2) with three replicates at the central point (total: 11 experiments). The experimental data were fitted to the proposed equations using commercial software (Microsoft Excel, Redmon, Washington, USA). The conditions of experimental plan were listed in Table 2.

The assays (Table 2) were carried out in 100 mL Erlenmeyer flasks placed in an orbital shaker at 150 rpm and pH = 5 (adjusted by NaOH or HCl). PSSF media were prepared mixing amounts of delignified EGW, water, nutrients, enzymes and yeast needed to achieve the conditions listed in Table 2. The nutrients (20 g/L of peptone and 10 g/L of yeast extract) were autoclaved (121 °C, 20 min) separately from delignified EGW and water. The cellulase enzyme (Cellic CTec2) used was kindly provided by Novozymes (Bagsvaerd, Denmark). The initial activity of cellulase and β-glucosidase (120 FPU/mL and 780 UI/mL, corresponding to 63 and 69 mg of protein/mL quantified by Bradford method [38], respectively) were measured following procedures described in Ghose

Table 2
Experimental design used and results obtained in the evaluation of the pre-saccharification and simultaneous saccharification and fermentation of delignified *Eucalyptus globulus* wood: Ethanol concentration obtained at 120 h of fermentation (E_{120}), Cellulose to Ethanol Conversion (CEC) and productivity (Qp_{48}).

	Independent variables: real and normalized				Dependent variables		
Run	ESR (FPU/g)	LSR (g/g)	x_1	x_2	$y_1 = E_{120}$ (g/L)	$y_2 = CEC_{120}$ (%)	$y_3 = Qp_{48}$ (g/L·h)
1	8	3	−1	−1	80.76	65.68	1.33
2	12	3	0	−1	89.63	72.90	1.65
3	16	3	1	−1	94.29	76.69	1.95
4	8	6	−1	0	58.58	85.05	1.09
5	12	6	0	0	57.43	83.38	1.20
6	12	6	0	0	56.76	82.41	1.22
7	12	6	0	0	56.95	82.68	1.19
8	16	6	1	0	63.45	92.13	1.29
9	8	9	−1	1	42.05	87.89	0.81
10	12	9	0	1	43.00	89.89	0.86
11	16	9	1	1	43.96	91.89	0.90

[39] and Paquot and Thonart [40], respectively. The PSSF assays were carried out at 48.5 °C during 24 h, favorable conditions for enzyme action. After saccharification, the temperature was decreased until 37 °C and the inoculum was added. Samples were taken at 0, 9, 24, 28, 32, 48, 80, 90 and 120 h, centrifuged (10 min, 6000 rpm) and analyzed by HPLC for glucose and ethanol concentrations with a Varian MetaCarb 87H column, 0.005 M H_2SO_4 mobile phase at 60 °C at a flow rate of 0.7 mL/min using a refractive index detector.

Cellulose to Ethanol conversion (CEC_{120}) at 120 h was calculated as follow:

$$CEC_{120}\ (\%) = 100 \cdot \frac{E_{120}}{Gpot \cdot \left(\frac{90}{180}\right)} \quad (2)$$

where E_{120} is the ethanol concentration obtained at 120 h of fermentation, 92/180 is the stoichiometric factor of glucose conversion into ethanol, *Gpot* is the potential glucose concentration (corresponding to total conversion of the glucan present in the delignified EGW into glucose). *Gpot* was calculated as:

$$Gpot = \frac{Gn}{100} \cdot \frac{180}{162} \cdot \frac{\rho}{LSR + 1 - \frac{KL}{100}} \quad (3)$$

where, *Gn* is the amount of glucan present in delignified EGW (g of glucan/100 g of delignified EGW), 180/162 is the stoichiometric factor of glucan into glucose, ρ is the density of the reaction medium (average value, 1005 g/L), *LSR* is the liquid to solid ratio of each experiment (g/g), *KL* is the Klason lignin content of delignified EGW (g of Klason lignin/100 g of delignified EGW). Klason lignin was used to calculate the solid solubilization during saccharification and to correct the variation of the liquid volume at the end of the experiments [41].

3. Results and discussion

3.1. Eucalyptus globulus wood (EGW) processing: organosolv-glycerol pretreatment

Chemical composition (cellulose, hemicellulose and lignin), physical features (re-distribution of main components, surface area and average size) and supramolecular structure of processed lignocellulosic biomass were used to understand the effect of pretreatment on the improvement of enzymatic saccharification [5,42].

In this sense, EGW was submitted to glycerol-organosolv treatment under described conditions mentioned above and chosen on basis of previous work [11] in which the optimization of biomass fractionation and sugar production from cellulosic fraction were carried out. The chemical composition of delignified EGW (expressed in g/100 g of pretreated solid on dry basis) was: 82.5 ± 0.32 of glucan, 1.02 ± 0.06 of xylan, 17.86 ± 0.1 of Klason lignin and 1.18 ± 0.01 of acetyl groups (see Table 1). Based on the results obtained, the glucan was almost totally recovered in the solid phase (99.7%) and 65.2% of lignin was removed (lignin in NaOH washes was measured and represented a 2.4 g of lignin/100 g of raw material) and 96.6% of xylan was solubilized. These results can be compared with reported data with glycerol-water treatment [43] in which 94% of cellulose was recovered and 64% of lignin was removed using a delignified wheat straw at 220 °C for 4 h. Comparing to other solvents as ethanol, similar results were obtained. The processing of wheat straw by 65% of ethanol-water yielded a pretreated pulp with 98% of glucan recovery and 46% of delignification at 220 °C for 20 min [44]. On the other hand, in this study the 94.2% of hemicellulose fraction (considering xylan, acetyl groups and arabinan) was solubilized in the liquid phase obtaining mainly 11.08 g/L of xylo-oligosaccharides (measured as xylose after acid post-hydrolysis) corresponding to xylan solubilization of 63.7% which could be recovered by membrane technology [45]. Moreover xylose concentration as monomer (3.2 g/L) and products of degradation from dehydration of pentoses as furfural were quantified which corresponded to an 18.4% and 6.3% of xylan present in the raw material, respectively. Under harsh conditions, it is possible that reactions of condensation of sugars with furfural occur arising decomposition products like humins that can represent part of the solubilized xylan. [46]. A previous evaluation of enzymatic susceptibility [11] displayed the cellulose to glucose conversion of 98% using low solid and high enzyme loadings (5% and 25 FPU/g, respectively). The glucose concentration obtained was 45 g/L (or 23 g/L of potential ethanol) considered not competitive to achieve a cost-effective process of bioethanol. In this sense, the evaluation of saccharification and fermentation processes at high-solid and lower enzymes loadings are necessary to attain competitive ethanol concentrations (as can be seen in Section 3.3).

3.2. Structural changes of pretreated EGW

The chemical treatments cause important structural and physical changes in the biomass which allow an enhancement of enzymatic saccharification of cellulose [47]. In order to study the disruption of lignocellulosic structure, the determination of crystallinity index (CrI) of the cellulose and scanning electronic micrograph (SEM) of delignified EGW were carried out.

Fig. 2 shows the SEM images of EGW (a) and pretreated EGW (b). In Fig. 2b, the disaggregation of fibers is clearly noticeable and the

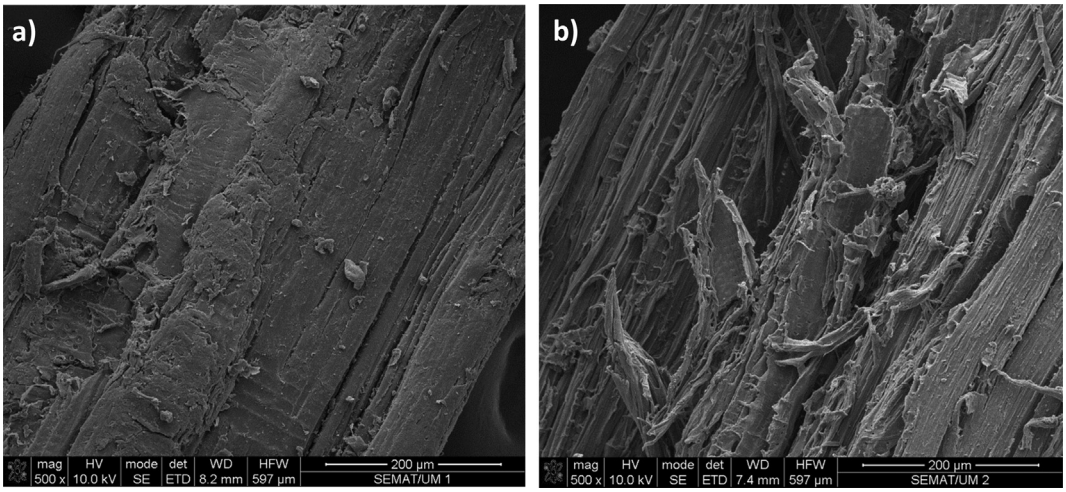

Fig. 2. Scanning Electron Microscopy images: a) *Eucalyptus globulus* wood (EGW), b) delignified EGW from glycerol-organosolv treatment.

size reduction is significant compared to the raw material (Fig. 2a) that displays a higher ordered surface. Recent works on aqueous glycerol pretreated lignocellulosic materials have reported similar observations [48–50]. During organosolv pretreatment, part of the lignin and hemicellulose are solubilized and depolymerized increasing the surface area and allowing an open structure [50]. These features enhance the enzymatic saccharification of cellulose [10]. Moreover, this fact was also reported by Sun and Cheng [51], who compared micrographs of pretreated wheat straw fibers by atmospheric aqueous glycerol and by steam explosion. These authors observed fibers more severely damaged by organosolv process that could explain why the enzymatic hydrolysis obtained with glycerol treatment was higher than the achieved with steam explosion.

Fig. 3 shows X-ray diffraction patterns of EGW and delignified EGW samples in order to compare the crystallinity of cellulose from untreated raw material and pretreated biomass. The peaks around 15–16 and 21–22° (2θ) are specific of lignocellulosic materials and characteristic of cellulose I. The crystalline region of pretreated biomass was higher than that of raw material (78.1% and 61.8%, respectively). This increase of crystallinity is probably due to the removal of amorphous materials such as hemicellulose and lignin during treatment, remaining the ordered cellulose. Sun et al. [48] also reported an increase in CrI of wheat straw after an aqueous glycerol treatment. Moreover, this feature was also observed by Cui et al. [52] which evaluated α-cellulose from different pretreatments (glycerol, ionic liquids, sodium hydroxide and ethylinediamide) and observed a higher crystallinity of sample treated with glycerol than with other treatments. The CrI is considered as important factor related to enzymatic hydrolysis of cellulosic substrates [5]. Nevertheless, this index alone does not elucidate the complex and recalcitrant structure of lignocellulosic biomass [5]. Delignified EGW was previously shown to be more prone to enzymatic saccharification than untreated EGW [11]. The micrographs images of delignified EGW (Fig. 2) and the CrI (Fig. 3) corroborate these previous findings as changes of structure are visible (Fig. 2) and reflected in CrI (Fig. 3).

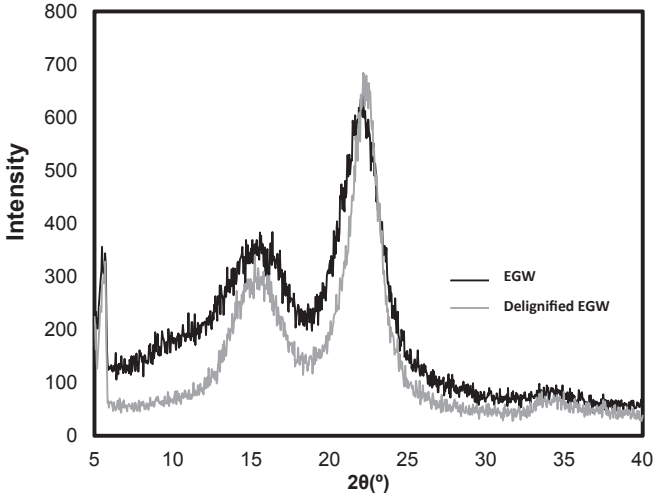

Fig. 3. X-ray Diffraction curves of untreated *Eucalyptus globulus* wood (EGW) and delignified EGW.

3.3. Organosolv lignin structure

The bioethanol obtained in biorefinery platforms generates large amounts of lignin, traditionally burnt for internal energy use [33]. To determine the feasibility and possible alternative applications of lignin, an analysis of its composition and structure is necessary since the processing conditions shape the purity and chemical characteristics of the extracted lignin. Thus, the characterization of lignin obtained from organosolv pretreatment of EGW was carried out using a Fourier transform infrared spectroscopy (FT-IR). In addition, a thermogravimetric analysis was conducted to evaluate its thermal behavior.

Fig. 4 shows the spectra of isolated lignin from organosolv process and standard lignin (extracted from alkali process) in the range of 4000 to 400 cm^{-1}. FT-IR spectra displayed typical patterns of lignocellulosic functional groups: carbonyl and carboxyl groups in the region of 1700 cm^{-1}, aromatic ring in the band of 1500 cm^{-1}, hydroxyl groups and phenol compounds between 3600 and 3200 cm^{-1}, C–O and C–O–H bonds related with residual carbohydrates in the samples appear typically between 1135 and 952 cm^{-1}. On the other hand, FT-IR spectrum of lignin from organosolv treatment shows a higher intensity of signals assigned to syringyl units (1329 cm^{-1}) than to guaiacyl units (1265–1130 cm^{-1} band), typically of hardwood species [36]. The differences in the intensity of FT-IR spectra of organosolv lignin and standard lignin were observed in signal correspondent to syringyl and guaiacyl units. Based on the analysis of FT-IR spectra (Fig. 4), organosolv

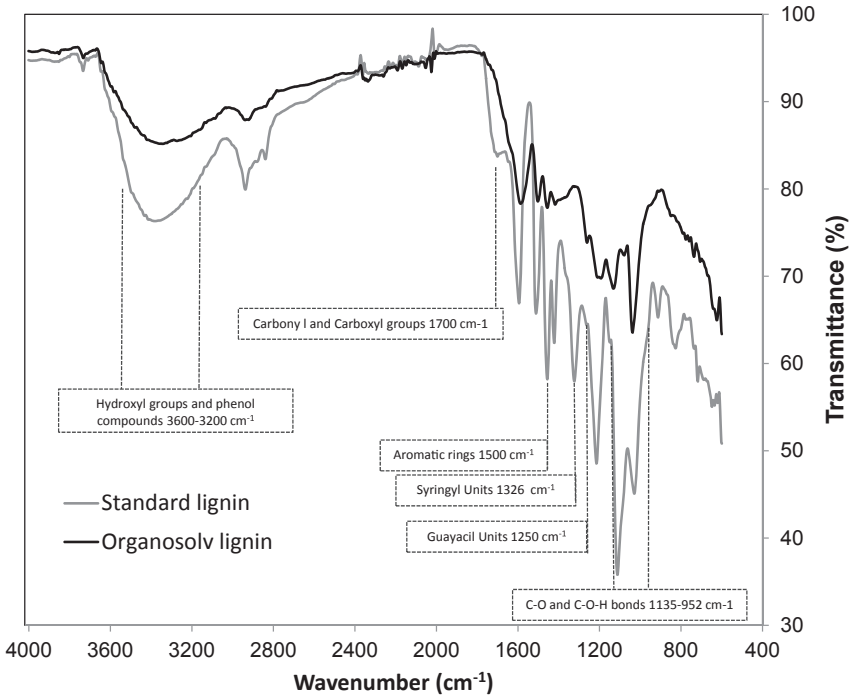

Fig. 4. FT-IR spectra of organosolv lignin recovered by precipitation and standard lignin.

lignin shows similar chemical composition to standard lignin (commercialized and extracted from alkali process).

Fig. 5 shows a TGA and DSC patterns of standard lignin and organosolv lignin. The samples were degraded between 25 and 600 °C. Based on TGA-analysis, the slight differences observed around 100 °C between the two samples can be due to moisture removal [51]. In the range of 100–260 °C, the weight loss of lignin samples is almost constant with a degradation of 10 and 5% for standard and organosolv lignin, respectively. Therefore, both lignin samples are thermally stable until about 260 °C. These results are in agreement with previously reported work in which several lignin samples obtained from ethanol-water treatment of sugarcane bagasse were characterized [35]. The lignin samples were similarly degraded between 250 and 350 °C with a weight loss <30%. Nevertheless, at 350 °C the organosolv lignin lost weight faster than standard lignin. A 20% of standard lignin was degraded comparing to 80% of organosolv lignin between 350 and 600 °C. On the other hand, DSC curve of organosolv lignin showed two thermal events: an endothermic event defined by peak at 69.2 °C with an enthalpy change of 213.9 J/g, which was attributed to the loss of moisture and a second exothermic event at 358 °C associated enthalpy change of 98.42 J/g. The first transition is related with a vaporization of moisture in the sample and the second one could correspond to generation of phenol compounds produced for the breaking of the bonds between monomeric units and lignin. Similar results were also observed in the thermal analysis of lignin samples from bagasse obtained by ethanol-water process [35].

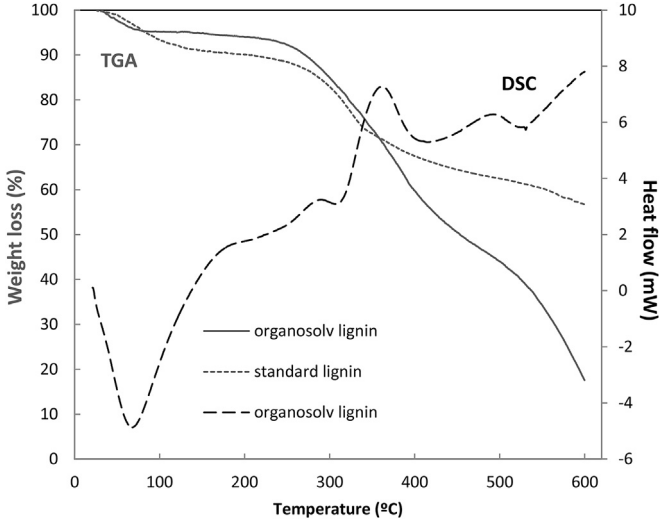

Fig. 5. Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) curves obtained for organosolv lignin and standard lignin.

3.4. Pre-saccharification and simultaneous saccharification and fermentation of delignified EGW at high solid loadings

EGW processed by glycerol-organosolv treatment and containing 83% of glucan was used as substrate for ethanol production at high-gravity conditions (LSR = 3–9 g/g or 33–11% of solid). In order to improve the mass transfer and operational conditions, enzymatic pre-saccharification was performed for 24 h. After that, *S. cerevisiae* CA1185 strain was inoculated. In Fig. 6, the time course of ethanol fermentation is shown. High glucose concentration (54–84 g/L) was accumulated within 24 h of saccharification (see Fig. 6). These results displayed the effectiveness of pretreatment to obtain a lignocellulosic biomass highly susceptible to enzymes. As can be observed in Fig. 6, the glucose was rapidly consumed within 8 h after inoculation. In most of the experiments, the stationary phase was achieved at 48 h (Fig. 6). Therefore, the volumetric productivity Qp_{48} was calculated at 48 h (or 24 h of fermentation) and collected in Table 2. The values of Qp_{48} varied in the range 0.81–1.95 g/(Lh), corresponding to extreme conditions (run 9 and 3, respectively). These results are comparable with the literature data in which productivities of 2.02 and 1.8 kg/m^3 at 6 h were obtained using

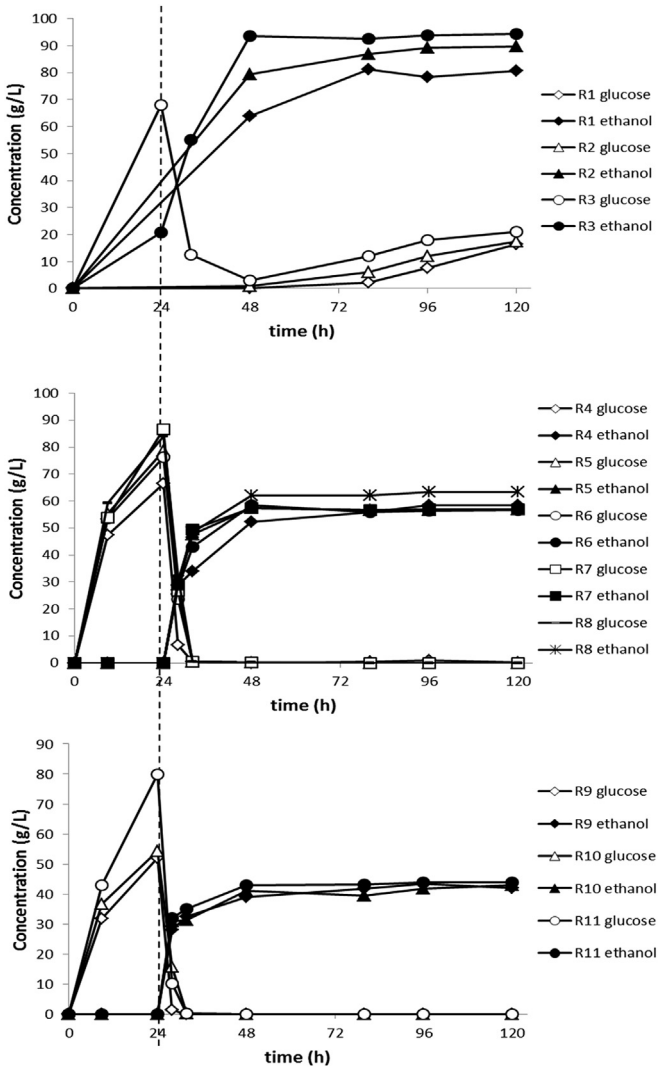

Fig. 6. Kinetics of ethanol and glucose concentration in PSSF assays (listed in Table 2), the vertical dotted line shows the time of inoculation. The relative error of data was ≤2%.

delignified wheat straw by ethanol-acid sulphuric treatment at high-solid loading [53].

On the other hand, when operating with LSR = 3 g/g and ESR = 16 FPU/g, maximal ethanol concentration (94.3 g/L) corresponding to 76.7% of ethanol conversion was achieved at 120 h. The lowest ethanol concentration (42.1 g/L) corresponding to 87.9% of ethanol conversion was obtained with 11.1% of dry matter loading or LSR = 9 g/g. The results reveal more pronounced effect of enzyme loading on ethanol concentration when operating at LSR <6 g/g. To attain effective lignocellulosic ethanol process the ethanol titer should be >4% (w/v) [17]. The set of experiments studied in this work showed higher concentration of ethanol >4% (w/v) even with the lowest enzyme loading (ESR = 8 FPU/g) in which 80 g/L of ethanol was obtained with a 65% of conversion. Cellulose to ethanol conversion (CEC_{120}) at 120 h of fermentation was calculated by Equation (2) and listed in Table 2. As expected, higher conversion of ethanol was obtained using low solid loadings (LSR = 9 g/g) for different enzyme to substrate ratios (8, 12 and 16 FPU/g). Furthermore, the effect of enzyme amount was more pronounced at LSR = 3 g/g (run 1–3) in which the addition of 16 FPU/g increased the ethanol conversion by 16.8% when comparing to 8 FPU/g of cellulase loading.

Finally, the variables listed in Table 2 were used for the modeling of ethanol production and cellulose to ethanol conversion. For that, mathematical relationship of independent (LSR and ESR) and dependent variables (E_{120}, EC and Qp_{48}) were carried out by a second order polynomial equation, expressed as:

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij}x_i + \sum_{i=1}^{2}\sum_{k\geq i}^{2} b_{ikj}x_i x_k \quad (4)$$

where y_j ($j = 1$ to 3) 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 2), and b_{0j} … b_{ikj} are regression coefficients calculated from experimental data. The regression coefficients of experimental model, the significance (based on the Student's *t-test*), the correlation coefficient (R^2) and the significance model design (based on the Ficher's *F-test*) are listed in Table 3. The results of Table 3 show a good adjustment of model and the significant effect of studied variables on ethanol concentration, ethanol conversion and ethanol productivity. The models obtained from results listed in Table 2 allowed the optimization of ethanol production by considering as optimal criteria: maximal ethanol concentration and maximal ethanol conversion. For that, a multiple response regression using coefficients of Table 3 was carried out. The optimized calculated variables were as follows: LSR 5.09 g/g and ESR 16 FPU/g to produce 83.3 g/L of ethanol with ethanol conversion of 82.7%.

So far, few works have reported high-gravity ethanol fermentation from lignocellulosic biomass using a solid loading higher than 20% [17,20,54–57]. It is important to highlight that an ethanol concentration of 80 g/L was obtained by Cannella and Jorgensen [29] using 30% of hydrothermally treated wheat straw and low enzyme loading (7.5 FPU/g of pretreated biomass). Nevertheless, a system based on horizontal rotation of reactor (roller bottle reactor) was used to improve the material mixing up to 30% of dry matter solids. The reactor design was studied by several authors as a strategy to overcome the mixing difficulties observed during SSF in shake flasks. Caspeta et al. [55] employed a mini-reactor with peg-mixer and a compact overhead stirrer to obtain 64 g/L of ethanol from delignified agave bagasse (ethanolov pretreatment) by separate hydrolysis and fermentation (20 FPU/g and 10% of solids). Moreover, a rotary drum reactor simple to scale up was used to produce 47 g/L of cellulosic ethanol (corresponding to 66% of conversion) from sugarcane processed by NaOH treatment using 20% of solids and 7.5 FPU/g [56]. In addition, helical stirring bioreactor was suitable for the ethanol production of 76 g/L with a 59% of conversion from steam exploded corn stover using 30% of solids and 15 FPU/g [57]. Nevertheless, the direct comparison in this type

Table 3
Values and significance of regression coefficients and statistical parameters measuring the correlation and significance of models for the pre-saccharification and simultaneous saccharification and fermentation assays of delignified EGW.

Coefficients	E_{120}	CEC_{120}	Qp_{48}
b_{0j}	58.10[a]	84.22[a]	1.20[a]
b_{1j}	3.39[a]	3.68[b]	0.15[a]
b_{2j}	−22.61[a]	9.07[a]	−0.40[a]
b_{11j}	1.33	2.28	−0.01
b_{22j}	6.62[a]	−4.91[b]	0.06[c]
b_{12j}	−2.91[b]	−1.75	−0.13[a]
R^2	162.75	22.30	150.29
Fexp	0.99	0.96	0.99
Significance level (%)	>99	>99	>99

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

of processes is not straightforward since the choice of a suitable reactor is defined by rheological properties of pretreated lignocellulosic which are determined by the type of raw material and pretreatment conditions [15]. All this has influence on ethanol concentration and conversion. On the other hand, fed-batch SSF strategy was also used to increase ethanol concentration. Kim et al. [58] produced 39.9 g/L of ethanol from pretreated poplar sawdust by three batch of biomass.

In comparison with other pretreatments (steam explosion, hot water, alkali treatment) discussed above, organosolv process was originally considered an expensive method. Nevertheless, organic solvents are suitable for the pure lignin and co-products (as hemicellulose derived compounds) recovery with great potential in the chemical industry [59]. In general, techno-economic evaluation carried out to compare different pretreatments takes into account the combustion of lignin. The suitable use of lignin and co-products obtained in organosolv process significantly affects the production costs indicating that an appropriated revalorization of these products allows the profitability of the process [59,60].

Overall, the highest ethanol concentration (94 g/L) was obtained in this work when comparing to reported values in literature for cellulosic ethanol (discussed above) [55–58]. This ethanol concentration corresponds to 25 L of ethanol/100 kg of EGW dry basis being compared favorably with data obtained by other authors such as Cuevas and co-workers who obtained 13.1 kg or 16 L of ethanol/100 kg of pretreated olive stones with 50% of ethanol (v/v) [53]. Although in some case the enzyme loading used was lower [29], the reduction of added cellulase could be improved by the recycling of these enzymes (as was reviewed by Gomes et al. [61]) which could be used in successive SSF batch.

4. Conclusions

The processing technology described in this work (Fig. 1) provides an efficient fractionation of EGW using a green solvent (as glycerol), obtaining per 100 kg of EGW: 45 kg of glucan in a solid phase and 18 kg of solubilized lignin and 8 kg of hemicellulose-derived compounds (as xylooligosaccharides) in another stream separately. The lignin solubilized by organosolv pretreatment was characterized for chemical composition and for its thermal behavior, showing interesting features compared to commercial lignin. Moreover, delignified EGW was successfully converted to ethanol by PSSF at high solid loading (LSR = 3 g/g) and moderate enzyme loadings (16 FPU/g) using an industrial and thermotolerant *S. cerevisiae* strain. As consequence, 94 g/L of ethanol with a 77% conversion of cellulose-to-ethanol were obtained. As far as we know, these results show the highest ethanol concentration from lignocellulosic biomass reported in literature. Overall, we demonstrate the feasibility of the intensification of EGW-to-ethanol processes following a valorization approach that integrates a suitable and sustainable pre-treatment with intensified saccharification-fermentation stages that consider high solid and low enzyme loads together with a robust and thermotolerant yeast strain.

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