Integrated approach for effective bioethanol production using whole slurry from autohydrolyzed *Eucalyptus globulus* wood at high-solid loadings

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HIGHLIGHTS

- Integrated process optimization was evaluated for 2G bioethanol production.
- Enzymatic saccharification of whole-slurry from pretreatment was optimized.
- Two strategies with/without presaccharification to ethanol production were compared.
- Optimized conditions lead ethanol titer >6% using industrial yeast strain by SSF.
- Effective bioethanol production from whole slurry *E. globulus* was achieved.

ARTICLE INFO

ABSTRACT

One of the most important targets and challenges in the second generation bioethanol is the development of a cost-effective process on large-scale. In this context, the high solid loading on saccharification and fermentation and the use of whole-slurry from pretreatment could be promising alternatives to obtain high ethanol concentrations and to decrease operational costs and wastewater. In this work, *Eucalyptus globulus* wood was submitted to non-isothermal autohydrolysis treatment (T_{max} = 210 °C) and the whole-slurry obtained was assayed for the optimization of enzymatic saccharification at different solid and enzymes (CTec2 and HTec2) loadings using a Box–Behnken experimental design. Under the optimized conditions (liquid solid ratio 6.4 g/g, cellulase to substrate ratio 22.5 FPU/g and hemicellulase to substrate ratio 500 UI/g), two strategies were evaluated for ethanol production (Simultaneous Saccharification and Fermentation, SSF and Presaccharification and Simultaneous Saccharification and Fermentation, PSSF), using an industrial and robust *Saccharomyces cerevisiae* strain. High concentrations of ethanol (50.2 and 48.8 g/L) and productivities (0.63 and 0.55 g/L h) were obtained by SSF and PSSF, respectively. The SSF process proved to be an advantageous strategy to obtain concentrations >6% (v/v) of ethanol with elevated conversion (95%) even employing high solid loading and non-detoxified hydrolysate. Following an integrated optimization process, cost-effective bioethanol production conditions from whole-slurry *E. globulus* wood were determined and validated experimentally, representing a step-forward towards its industrial implementation.

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1. Introduction

The abundant amount of biomass and its availability allow its processing, which could become a commercial opportunity. Lignocellulosic materials (LCMs), as *Eucalyptus globulus* wood (EGW), represent a renewable and widespread biomass source that does not compete with crops used in feed (such amylaceous materials and sugar biomass). The biorefinery approach consists in the fractionation of lignocellulose biomass as alternative to petroleum refinery [1,2] and its sustainable development allows the possibility to obtain commercial products such as chemical products, materials, energy and fuels [3].

The processing or pretreatment of LCMs is considered as first step in a biorefinery [4,5], being the main barrier its recalcitrant complex structure that is formed by its three main components

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(hemicellulose, lignin and cellulose). A wide number of technologies have been proposed for fractionation of LCMs in aqueous alkali and acid media, or mechanical pretreatments to improve the enzymatic accessibility [6–8]. In this context, hydrothermal treatment or alternative also known autohydrolysis or liquid hot compressed water is a possible pretreatment to biomass processing that yields a liquid phase (hydrolysate) composed by hemicellulose derived-compounds (mainly oligosaccharides and monosaccharides) and solid phase enriched in cellulose and lignin [9].

The bioethanol is one of the most important liquid fuels obtained from the processing of LCM [10]. In this context, the Policy Energy Act (PEA) and the Energy Independence and Security Act (EISA) have agreed to reach 36 billion gallons of bioethanol in 2022 [11]. However, bioethanol from LCM is not widely implemented on large-scale due to the high initial investment and high production operational costs. One alternative to reduce capital-operational cost can be to use the whole-slurry (liquid + solid) or slurries after treatment without washing (avoiding water consumption) and/or operating with high solid loadings (to obtain elevated final ethanol concentration and so decrease the distillation cost) [12,13]. Besides, the incorporation of hydrolysate is preferable since it increases the sugar concentration availability for yeast subsequent fermentation [14].

Nevertheless, some soluble derived compounds of pretreatment (weak acids, furan and phenolic compounds) present in the whole-slurry are effective inhibitory compounds of the saccharification and fermentation processes [15,16]. Taking into account that detoxification step of liquid phase is not desirable due to the high cost (that can represent up to 22% of total cost of production of bioethanol) [17], one alternative to physic-chemical methods of detoxification could be the biological treatments. Biological detoxification involves the use of enzymes and microorganism that are able to act on the toxic compounds present in the hydrolysate [18].

The cellulolytic enzymes are inhibited by compounds released during the pretreatment (mainly phenolics compounds and oligosaccharides) [19,20], indicating the need of robust enzymes able to maintain their activity in presence of these compounds [21]. In this sense, several researches have recently been carried out on improving the properties of *Trichoderma reesei* cellulases [22,23]. Moreover, the use of cocktail hemicellulases can improve the saccharification since it acts on hemicellulose enhancing the cellulose accessibility [24].

On the other hand, the furan (furfural and hydroxymethyl furfural) and phenolic compounds clearly affect the growth and metabolism of yeast [25,26]. Thus, one of the major advances in bioethanol production could be the selection of robust microorganism able to ferment sugars in presence of inhibitor compounds and attain high ethanol titers at the end of fermentation process [27]. A recent published work showed that the industrial distillery environments as "Brazilian bioethanol plants" are a remarkable source of efficient yeast strains for lignocellulosic fermentation processes, able to degrade furans compounds and obtain an efficient ethanol performance [28].

Regarding strategies of operation, the bioethanol production can be performed using separate hydrolysis and fermentation (SHF), and Simultaneous Saccharification and Fermentation (SSF) or to conduct a Pre-saccharification followed by Simultaneous Saccharification and Fermentation (PSSF) [29]. In this context, the study of all involved stages in the bioethanol production (including: the operational conditions of LCM fractionation, the use of robust enzymes and yeast able to remain their activity in presence of inhibitors) is the main objective to obtain a sustainable and integrated process.

This work studies the use of whole-slurry from autohydrolyzed *E. globulus* wood in order to obtain high concentration and conversion of glucose in the enzymatic saccharification assay using novel cellulolytic enzymes (CTec2 and HTec2). In the optimized conditions of enzymatic hydrolysis, PSSF and SSF experiments were carried out to evaluate the maximum ethanol yields obtained by a robust industrial strain of *Saccharomyces cerevisiae*.

2. Materials and methods

2.1. Raw material

E. globulus wood (EGW) was kindly provided by ENCE (local pulp factory; Galicia NW Spain). The samples were air-dried, homogenized and milled to pass an 8 mm screen. Finally, the lot was stored in a dark and dry place until use. The EGW composition was previously analyzed by Pereira et al. [28], containing (expressed in g/100 g of raw material on dry basis, average values of three replicate ± standard deviation): glucan 44.70% ± 0.81; xylan 16.01% ± 0.35; arabinan 1.09 ± 0.05; acetyl groups 2.96% ± 0.28; Klason lignin 27.70% ± 0.61; ashes 0.2% ± 0.03 and extractives 2% ± 0.17.

2.2. Pretreatment: autohydrolysis

EGW was submitted to autohydrolysis pretreatment. EGW and distilled water were mixed in a Liquid to Solid Ratio (LSR) = 8 kg of water/kg of oven-dried raw material in a pressurized reactor at T_{max} = 210 °C (non-isothermal regime). The reaction media was heated and cooled following the standard temperature profiles reported by Romaní et al. [30]. When the treatment was finished the solid was recovered for solid yield determination (SY) and separated of hydrolysate by filtration. The liquid and solid phases were analyzed for chemical composition (as described below) and listed in Table 1.

2.3. Enzymes

The enzymes employed in this work, cellulases (Cellic Ctec2) and hemicellulases (Cellic Htec2) were kindly supplied by Novozymes, (Bagsvaerd, Denmark). The Celluclast 1.5 and Novozyme 188 were also used for comparative reason. The Cellic CTec2 was used for saccharification of cellulose into glucose. The β-glucosidase enzyme supplementation was not necessary due to the high activity of β-glucosidase present in Cellic CTec2. The hemicellulase enzyme Cellic HTec2 was added to hydrolyse the xylooligosaccharides and improve the cellulose saccharification. The cellulase

Table 1
Chemical composition of pretreated solid and hydrolysate.

g Of compound/100 g of raw material oven dry basis	
Solid Yield (SY)	71.66
Pretreated solid	
Glucan	42.47 ± 0.47
Xylan	1.40 ± 0.10
Arabinan	0.0 ± 0.00
Acetyl groups	0.21 ± 0.06
Klason lignin	24.08 ± 0.50
Hydrolysate g of compound/100 g of raw material oven dry basis	
Glucose	0.53 ± 0.05
Xylose	7.33 ± 0.15
Arabinose	0.15 ± 0.03
Acetic acid	2.58 ± 0.07
HMF	0.27 ± 0.01
Furfural	1.38 ± 0.12
Glucooligosaccharides	0.95 ± 0.24
Xylooligosaccharides	7.43 ± 0.74
Arabinooligosaccharides	0.00 ± 0.0
Acetyl groups	2.11 ± 0.35

activity of Cellic Ctec2 was measured by the Filter Paper assay [31] and was expressed as Filter Paper Unit (FPU). The β-glucosidase activity of Ctec2 was determined as International Unit (IU), using ρ-nitrophenyl-β-D-glucopyranoside as substrate [32]. The xylanase activity of Cellic Htec2 was assayed as described in Bailey et al. [33], using birchwood xylan. The activities were 120 ± 10 FPU/mL and 779.8 ± 20.3 UI/mL for CTec2 and 1690 ± 211.6 UI/mL for HTec2.

2.4. Enzymatic hydrolysis of whole slurry: experimental plan and statistical analysis

A Box–Behnken, second-order design based on the three-level incomplete factorial designs, experimental plan was carried out (3 factors with three replicates of the central point, total experiments 15) for the optimization of whole-slurry saccharification (liquid + solid). The number of experiments (N) was calculated as follows [34]:

$$N = k^2 + k + c_p \tag{1}$$

where k is the factor number ($k = 3$) and c_p is the replica number of the central point.

The structure of the experimental plan is summarized in Table 2. The independent variables were: Liquid to Solid Ratio (LSR) = x_1, Cellulase to Substrate Ratio (CSR) = x_2 and Hemicellulase to Substrate Ratio (HSR) = x_3. The substrate of variable x_2 is referred to pretreated solid and the substrate of x_3 is corresponded with the concentration of xylooligosaccharides in the hydrolysate. The dimensionless ones of independent variables were calculated (with variation range from −1 to 1) following the equation:

$$x_1 = 2 \cdot \frac{LSR_i - LSR_{mean}}{LSR_{max} - LSR_{min}} \tag{2}$$

$$x_2 = 2 \cdot \frac{CSR_i - CSR_{mean}}{CSR_{max} - CSR_{min}} \tag{3}$$

$$x_3 = 2 \cdot \frac{HSR_i - HSR_{mean}}{HSR_{max} - HSR_{min}} \tag{4}$$

where the subscripts i is the considered experiment, mean 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.

The independent variables were correlated with dependent variables by a second-polynomial equation following the generalized expression:

$$y_j = b_{0j} + \sum_{i=1}^{2} b_{ij}x_i + \sum_{i=1}^{2}\sum_{k \geq i}^{2} b_{ikj}x_ix_k \tag{5}$$

where y_j ($j = 1$ to 4) is the dependent variable; x_i or x_k (i or k: 1 to 3, $k \geq i$) are the normalized, independent variables (defined in Table 2), and $b_{0j} \ldots b_{ikj}$ 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 (Statgraphics, Plus 5.1, Warrenton, VA, USA). The regression coefficients ($b_{0j} \ldots b_{ikj}$) of experimental, the significance (based on the Student's t-test), the correlation coefficient (R^2) and the significance of model design (based on the Fisher's F-test) are shown in Table 3.

The enzymatic hydrolysis assays were carried out in a 100 mL Erlenmeyer flasks (50 mL of medium) at 48.5 °C and pH = 4.85 (0.05 N citric acid-sodium citrate buffer) in an orbital shaker (150 rpm). The assays were prepared with pretreated solid, hydrolysate and enzymes at desired amounts (see Table 2). Representative samples were withdrawn (approximately, a volume of 0.8 mL) from experiments at times 0, 2, 4, 9, 24, 48, 72 and 96 h, centrifuged (6000 rpm for 10 min). The samples were withdrawn with cut tips (proportional amount of solid and liquid) to avoid variations in the liquid to solid ratio, not affecting the subsequent analysis of glucose concentration (analyzed by HPLC).

The cellulose to glucose conversion (CGC) was determined as follow:

$$CGC_t = 100 \cdot \frac{G_t}{G_{pot}} \tag{6}$$

where CGC_t is the cellulose to glucose conversion (%) at time t, G_t is the concentration of glucose obtained at time t and G_{pot} is the potential glucose calculated as:

$$G_{pot} = \frac{Gn}{100} \cdot \frac{180}{162} \frac{\rho}{LSR + 1 - \frac{KL}{100}} \tag{7}$$

where Gn is the glucan content of pretreated solids (g of glucan/100 g of autohydrolyzed solid dry basis), 180/162 is the stoichiometric factor, ρ is the density of the hydrolysis enzymatic medium (average value, 1005 g/L), LSR is the liquid to solid ratio in the considered experiment and KL is the Klason lignin content of substrate (g of Klason lignin/100 g of oven-dry autohydrolyzed solid).

Table 2
Operational conditions of Box–Behnken experimental design (dimensional and dimensionless independent variables) and experimental results obtained for dependent variables.

Run	Independent variables						Dependent variables			
	LSR (g/g) x_1	CSR (FPU/g) x_2	HSR (UI/g) x_3	x_1	x_2	x_3	G_{96h} (g/L) y_1	CGC_{96h} (%) y_2	CGC_{max} (%) y_3	$t_{1/2}$ (h) y_4
1	4	8	300	−1	−1	0	63.4	37.8	43.9	14.6
2	25	8	300	1	−1	0	18.5	64.8	74.9	10.4
3	4	30	300	−1	1	0	102	61.0	71.4	17.6
4	25	30	300	1	1	0	28.1	98.8	100	5.40
5	4	19	100	−1	0	−1	93.8	56.0	68.6	18.5
6	25	19	100	1	0	−1	27.6	97.0	100	7.57
7	4	19	500	−1	0	1	108	64.7	74.6	16.2
8	25	19	500	1	0	1	27.1	95.1	100	5.41
9	14.5	8	100	0	−1	−1	23.2	48.8	53.0	8.95
10	14.5	30	100	0	1	−1	45.2	94.8	100	12.6
11	14.5	8	500	0	−1	1	40.6	85.3	100	13.0
12	14.5	30	500	0	1	1	47.7	100	100	10.3
13	14.5	19	300	0	0	0	45.9	96.2	100	12.4
14	14.5	19	300	0	0	0	45.8	96.2	100	13.5
15	14.5	19	300	0	0	0	45.9	96.2	100	12.6

Table 3
Regression coefficients and statistical parameters measuring the correlation and significance of the models.

Parameters	G_{96h} (g/L) $y1$	CGC_{96h} (%) $y2$	CGC_{max} (%) $y3$	$t_{1/2}$ (h) $y4$
b_{0j}	45.84[a]	96.23[a]	100.00[a]	11.77[a]
b_{1j}	−33.30[a]	17.01[a]	14.55[a]	−4.76[a]
b_{2j}	9.68[a]	14.75[a]	12.45[a]	−0.12
b_{3j}	4.26[c]	6.11[b]	6.62[b]	−0.34
b_{11j}	16.09[a]	−17.35[a]	−14.95[a]	0.46
b_{22j}	−8.93[b]	−13.28[a]	−12.51[b]	−0.25
b_{33}	2.28	−0.67	0.76	−0.31
b_{12j}	−7.27[b]	2.70	−0.60	−2.01[c]
b_{13j}	−3.78	−2.64	−1.49	0.02
b_{23j}	−3.70	−7.77[b]	−11.76[b]	−1.58
R^2	0.987	0.975	0.960	0.943
F_{exp}	41.33	21.78	13.36	9.22
Significance level	>99	>99	>99	>98

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

2.5. Microorganism, growth conditions and inoculum

S. cerevisiae PE-2, isolated from Brazilian bioethanol production plant, was used in this work [35]. Stock culture was maintained on YPD (1% (w/v) of yeast extract, 2% (w/v) of bacto-peptone and 2% (w/v) of glucose) in agar plates at 4 °C. Yeast cells was grown in a medium containing 50 g/L of glucose, 20 g/L peptone and 10 g/L of yeast extract. The inoculum was incubated at 30 °C in an orbital shaker (200 rpm) for 24 h (optical density at 600 nm of 3–4). The biomass was aseptically recovered by centrifugation and resuspended in 0.9% (w/v) NaCl to a concentration of 200 g fresh yeast/L. The final concentration of inoculum in the experiments (PSSF and SSF) was 8 g wet yeast/L or 1.8 g/L dry weight.

2.6. Simultaneous Saccharification and Fermentation (SSF) and Presacchaarification and Simultaneous Saccharification and Fermentation (PSSF)

SSF and PSSF experiments were carried out in 100 mL Erlenmeyer flasks fitted with perforated rubber stoppers enclosing glycerol-locks (to allow CO2 releasing and to avoid O2 input) and placed in orbital shaker at 150 rpm. The SSF media were prepared under the optimal conditions calculated from experimental design (LSR = 6.4 g/g; CSR = 22.4 FPU/g; HSR = 500 UI/g). The SSF experiments (pretreated solid, water and buffer) were autoclaved separately from the nutrients and the hydrolysate. The nutrients were prepared to obtain in the experiments the following concentration: 20 g/L of peptone, 10 g/L yeast extract. The hydrolysate was sterilized by filtration and added in the amount necessary for each experiment. In PSSF experiment, the flasks were incubated for enzymatic hydrolysis during 24 h in optimal conditions of enzymes (48.5 °C). After the pre-saccharification stage the temperature was decreased to 35 °C and the yeast was added. Samples of SSF and PSSF were withdrawn, centrifuged (6000 rpm for 10 min) and analyzed for glucose, xylose, arabinose, acetic acid and ethanol concentration. The Ethanol Conversion (EC) was calculated by the following equation:

$$EC = \frac{E_{max}}{G_{pot} \cdot 0.511} \cdot 100 \qquad (8)$$

where E_{max}, is the maximal ethanol concentration (g/L) at time t, 0.511 is the stoichiometric factor for glucose conversion into ethanol and G_{pot} is the potential glucose.

2.7. Analytical methods

The raw material and autohydrolyzed EGW were analyzed by TAPPI standards: T-264-cm-07 for extractives and moisture, T-211-cm-93 for ash and T-249-em-85 for quantitative acid hydrolysis (QAH). The hydrolysate from pretreatment was analyzed for monosaccharides, oligosaccharides and inhibitor compounds. One aliquot of hydrolysate was subjected to quantitative post-hydrolysis (4% H2SO4, 121 °C for 20 min) for oligosaccharides determination and analyzed by HPLC. The increase in the concentration of sugars and acetic acid provided a measure of oligomers and linked acetyl groups. Second aliquot of hydrolysate was directly analyzed by HPLC for monosaccharides (glucose, xylose and arabinose), hydroxymethylfurfural (HMF), furfural (F) and acetic acid quantification. The samples from enzymatic hydrolysis and fermentation were analyzed for glucose, xylose, arabinose, acetic acid and ethanol concentration by HPLC. The analyses were carried out by HPLC using a Jasco 830-IR intelligent refractive-index detector and a Metacarb 87H column. Operation was performed at 60 °C using mobile phase 0.005 M H2SO4 and flow rate 0.7 mL/min.

3. Results and discussion

3.1. LCM processing: autohydrolysis pretreatment

The use of whole-slurry (liquid + solid) coming from EGW autohydrolysis was studied to optimize the enzymatic hydrolysis of cellulose and glucose fermentation yields. Fig. 1 shows the experimental scheme carried out in this work, integrating the evaluated stages: autohydrolysis treatment, enzymatic saccharification and fermentation.

The autohydrolysis conditions were chosen on the basis of previous research [36] in which the overall balance showed 93.8% of polysaccharide recovery in liquid and solid phase under the selected conditions (T_{max} = 210 °C). As can be observed in Table 1, in this work the glucan was almost all retained in the solid phase with a 95% of recovery (expressed as the percentage of glucan present in autohydrolyzed EGW respect to the glucan present in the corresponding amount of oven-dry raw material). The 83.9% of Klason lignin remained in the pretreated solid (expressed as percentage of lignin in pretreated solid respect to the lignin present in oven-dry raw material). It was observed that part of lignin was solubilized. On the other hand, the percentage of xylan was present in the pretreated solid in low amount (1.4 g of xylan in pretreated solid/100 g in oven-dry raw material). The composition of hydrolysate was also reported (see Table 1). The hydrolysate was composed mainly by mono- and oligo-saccharides that represent 72.1% with respect to the total identified compounds in the hydrolysate. The main sugars present in hydrolysate were xylose and xylooligosaccharides (7.4 and 7.43 g/100 g of raw material, respectively). It is important to highlight that the concentration of inhibitor compounds (acetic acid, HMF and furfural) were 2.6, 0.3 and 1.4 g/100 g in oven-dry raw material, respectively. The presence of degradation compounds causes a substantial inhibition effect on cell growth and ethanol production at concentrations typical for lignocellulose hydrolysate, as have been reported by several authors (reviewed by Almeida et al. [37]). In general, the concentrations of inhibitor compounds obtained in this work are in the range of hydrolysate from hardwoods [25].

3.2. Enzymatic hydrolysis of whole slurry from autohydrolysis pretreatment: enzymes employed and time course of cellulose to glucose conversion

The pretreated EGW was mixed with hydrolysate and used as substrate in enzymatic hydrolysis experiments. The enzymes
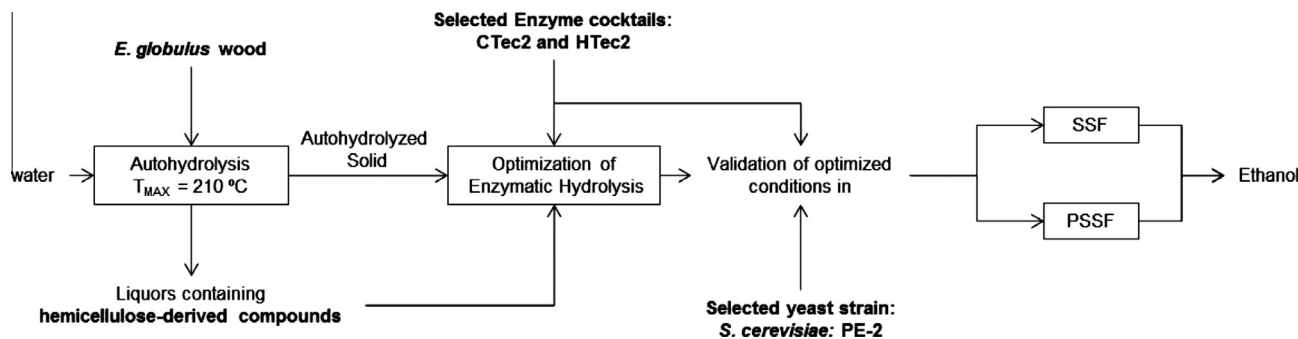
Fig. 1. Integrated approach followed in this study for ethanol production from whole slurry of autohydrolyzed *E. globulus* wood.

Celluclast 1.5L and Cellic CTec2 were assayed for the whole-slurry saccharification under conditions LSR = 6 g/g and Enzyme to Solid Ratio = 16 FPU/g. The activity of Celluclast 1.5L was 44 ± 3.55 FPU/mL and was measured as described in Section 2.3. Overall, the results obtained using Cellic CTec2 were more satisfactory than the results achieved by Celluclast (supplemented with Novozyme 188). The glucose concentration using Cellic CTec2 was 80.6 g/L (corresponding to 76.4% of conversion) and the glucose achieved by Celluclast 1.5L was 50.2 g/L (conversion of 51.6%) using the whole-slurry. This behavior was also observed by Cannella et al. [38] that obtained a cellulose conversion 25% lower from hydrothermally pretreated wheat straw at 30% of solids using Celluclast/Novozyme 188 compared to Cellic CTec2. Additionally, the results obtained in this work are in agreement with previous study [39] in which the inhibitory effect of hemicellulosic liquid phase (HLP) on enzymatic hydrolysis was studied using Celluclast 1.5L and Novozyme 188. This study showed that the use of 100% HLP decreased the enzymatic conversion of cellulose from 88% to 55% at LSR = 6 g/g and enzyme loading of 25 FPU/g. Moreover, the addition of xylanases had a positive effect with an increase of 35% of glucose concentration (operating 4 UI/FPU at LSR = 6 g/g and ESR = 16 FPU/g). Based on the results achieved in these preliminary experiments, the selected enzymes employed for enzymatic optimization study were the novel enzymes Cellic Ctec2 and Cellic HTec2. The hemicellulase was used to avoid the inhibition of cellulase produced by the presence of xylooligosaccharides. This fact has been documented in several studies [20,40]. The inhibition could be related with the binding of xylooligosaccharides to cellobiohydrolases (CBHI) from *T. reesei* and with a competitive mechanism for xylooligosaccharides of CBHI [41].

The enzymatic hydrolysis experiments were carried out under the operational conditions listed in Table 2. Different solid and enzyme loadings were studied for the optimization of enzymatic operational conditions with the target to obtain high concentration of glucose and increase the cellulose conversion yield. The glucose concentration at 96 h for each experiment (G_{96h} or y_1) is indicated in Table 2. As can be observed, the glucose concentration varied in the range of 18.5–108.4 g/L (experiments 2 and 7, respectively) corresponding to the highest and the lowest LSR (25 and 4 g/g, respectively). Glucose concentrations >45 g/L were obtained with LSR = 14.5 g/g for enzyme loadings of 19 and 30 FPU/g. Nevertheless, minor concentration of glucose (23.4 g/L) was achieved with low amount of enzyme (8 FPU/g). Interestingly, the addition of 500 UI/FPU of hemicellulase allowed a higher glucose concentration (40 g/L) with 8 FPU/g. The CGC$_{96h}$ at 96 h (or y_2) was reported in Table 2 and varied in the range 37.8–100%, obtained in experiments 1 and 12, respectively. The CGC$_{96h}$ = 37.8% was achieved for the lowest studied conditions of LSR and ESR (4 g/g and 8 FPU/g) and intermediate amounts of HSR (300 UI/FPU). On the other hand, the highest conversion of cellulose was reached at

intermediate LSR (14.4 g/g) and higher enzyme loadings (30 FPU/ g). Remarkably, for 8 FPU/g of cellulase loading CGC$_{96h}$ was improved when the hemicellulases addition was 500 UI/g comparing with 100 UI/g (achieving 85.3% and 48.8% of conversion respectively, run 11 and 9). Nevertheless, this improvement was not significant when the cellulase addition was higher (30 FPU/g) obtaining 94% and 100% of conversion for 100 and 500 UI/g (run 10 and 12). The xylose was also measured and was present in concentrations of 13.6–21.4 g/L at the end of saccharification (data no shown). The increase of xylose is the result of saccharification of xylooligosaccharides from hydrolysate and the hydrolysis of xylan left in the autohydrolyzed EGW. The conversion of xylan and xylooligosaccharides into xylose was in the range of 81.5–100%.

In general, the results achieved in this study can be compared favorably with the literature data using the whole-slurry strategy. Arvaniti et al. [42] reported a glucose yield of 45% using rape straw treated by wet oxidation (195 °C, 15 min and 15 bar) with 17% solid loading and 15 FPU/g. On the other hand, Hodge et al. [43] obtained a concentration of glucose <80 g/L employing pretreated corn stover by dilute sulfuric acid, (corresponding to 70% of conversion) when the enzyme loading was 17 mg/g. Further, in this same study, when the addition of enzyme was 52 mg/g, 100 g/L of glucose were produced with a 90% of conversion. Moreover, Alvira et al. [24] used wheat straw processed by steam explosion (210 °C during 2.5 min) as substrate in enzymatic hydrolysis at 10% of solids adding cellulase, xylanase and arabinofuranosidase and the authors obtained a glucose concentration of 16 g/L with a conversion <50%.

Fig. 2 shows the time course of cellulose to glucose conversion (CGC) of enzymatic hydrolysis for each experiment. The experiments carried out at LSR 25 g/g achieved a maximal conversion yield of 85% at 24 h of saccharification. As can be observed in Fig. 2, the kinetic showed a typical pattern. In this sense, the experimental data were fitted to the following equation [44]:

$$CGC_t = CGC_{max} \cdot \frac{t}{t + t_{1/2}} \qquad (9)$$

where CGC_t is the cellulose to glucose conversion (%) 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 $CGC = CGC_{max}/2$.

Table 2 shows the results calculated by Eq. (9) for the variable CGC_{max} and $t_{1/2}$ (or y_3 and y_4). For most of experiments, CGC_{max} was >70%, except in the experiments 1 and 9 in which the CGC_{max} was <55%. The value of $t_{1/2}$ for enzymatic saccharification was in the range 5.4–18.5 h corresponding to extremes values of LSR 25 and 4 g/g, respectively. High solid loading may hinder the enzymatic saccharification as a result of insufficient mass transfer (as water is not available) [21].
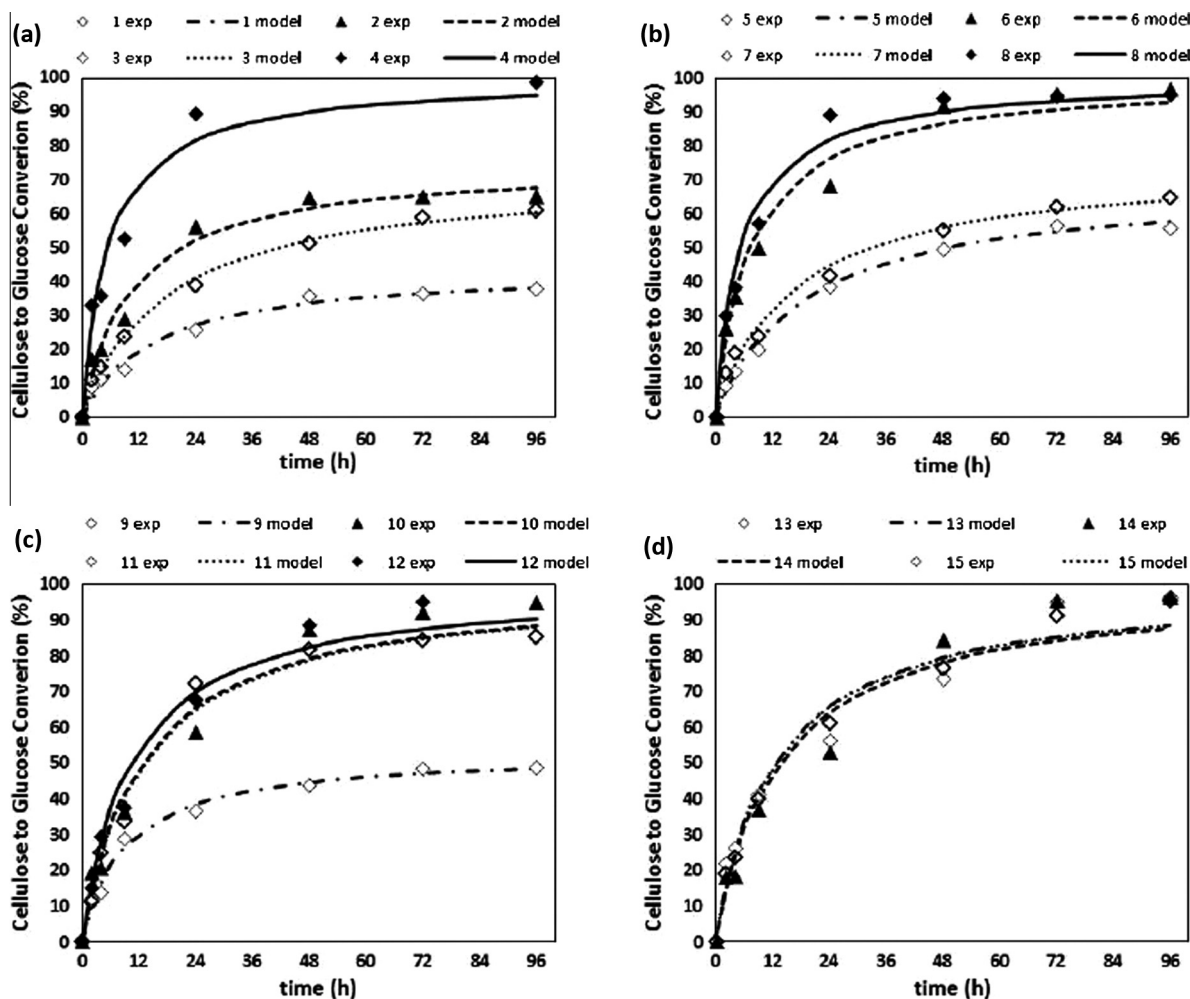
Fig. 2. Time course of cellulose to glucose conversion (%) in enzymatic hydrolysis experiments (see Table 2) carried out using the whole-slurry (liquid + solid) from autohydrolyzed *E. globulus* wood: experimental points (symbols) and model prediction (lines): (a) run 1 to 4; (b) run 5 to 8; (c) run 9 to 12; (d) run 13 to 15.

3.3. Response surface methodology assessment

For a better interpretation and evaluation of results, the independent variables (LSR, CSR and HSR) and dependent variables (G_{96h}, CGC_{96h}, CGC_{max} and $t_{1/2}$) listed in Table 2 were correlated using the empirical polynomial described previously (see, Eq. (5). Table 3 shows the good fitting of results (0.94–0.99). The high R^2 with an average value of 0.97 displays a good correlation of data. In addition, the elevated values of F also confirmed a good adjustment of data.

Fig. 3a and b shows the predicted dependence of G_{96h} and CGC_{96h} values as function of LSR and CSR. The variable Hemicellulase to Substrate Ratio (HSR) was the least influential variable. Therefore, the HSR was fixed at 500 UI/FPU in Fig. 3a and b for its representation. The most statistically significant variable on glucose concentration was the LSR. As can be observed in Fig. 3a, the glucose concentration increased with a decrease of LSR. On the other hand, it is important to highlight that the increase of cellulase loading above 19 FPU/g was not necessary for a significant rise of glucose concentration. The maximal predicted concentration of glucose (101 g/L) was obtained under the follow conditions: LSR = 4 g/g, CSR = 30 FPU/g and HSR = 300 UI/FPU. Fig. 3b shows the influence of LSR and CSR on CGC_{96h}. Conversions above 90% were obtained for values of CSR in the range 19–23.4 FPU/g and conditions of LSR > 9 g/g. The surface of Fig. 3b shows an optimal condition (100% of CGC_{96h}) corresponding to LSR = 19 g/g,

CSR = 21.9 FPU/g and HSR = 100 UI/FPU. As can be observed, the maximal enzyme loading was not necessary to achieve the maximal conversion of cellulose to glucose.

Fig. 3c and d shows the dependence of predicted values of CGC_{max} and $t_{1/2}$ with independent variables. In a wide range of the studied variables the CGC_{max} achieved values of 100% (LSR > 12.5 g/g and CSR > 20 FPU/g, see Fig. 3c). For LSR = 6.1 g/g (equivalent to 16.4% of percentage solid) the CGC_{max} was ⩾80% when the cellulase addition was above of 24.5 FPU/g. Fig. 3d represents the influence of LSR and HSR on the $t_{1/2}$. Values of $t_{1/2}$ < 5 h were obtained for HSR > 380 UI/FPU and LSR > 23 g/g. The most influent variable on $t_{1/2}$ was the LSR (see Table 3) and the other variables did not have a significant influence.

3.3.1. Optimization and model validation

In second generation bioethanol, the obtainment of high glucose concentration during enzymatic hydrolysis step is important to maximize the final ethanol titers and consequently decrease the cost involved in the distillation stage [21]. In this context, these results show that the highest LSR (25 g/g) achieved elevated conversion (98.8%), however the glucose concentration under these conditions was very low (28.1 g/L) for industrial interests. In order to optimize the whole-slurry saccharification process, an optimal condition was calculated on basis to obtain the maximal concentration and conversion of glucose. Thus, the variables G_{96h} and CGC_{96h} were used as response variable for the multiple response
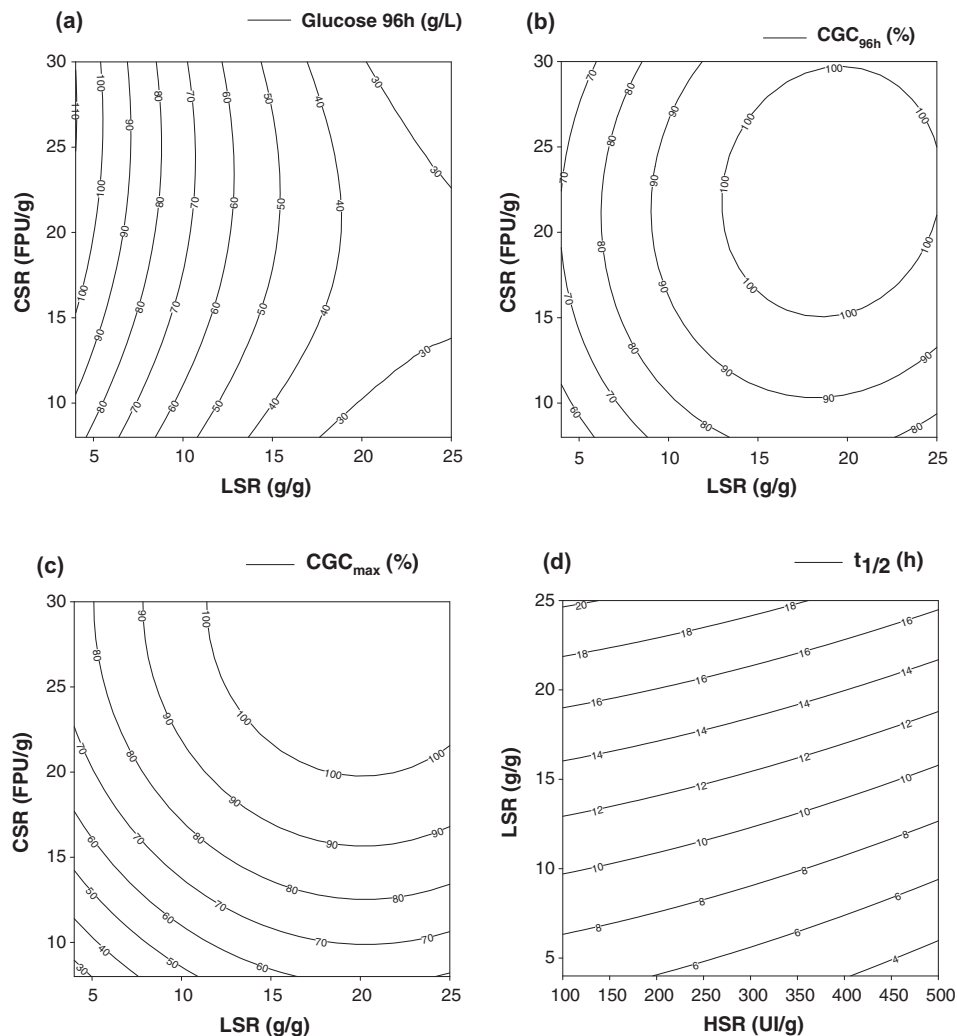
Fig. 3. (a) Dependence of glucose at 96 h (g/L) on Liquid to Solid Ratio (LSR (g/g)) and Cellulase to Substrate Ratio (CSR (FPU/g)), fixed Hemicellulase Substrate Ratio (HSR) = 500 UI/FPU. (b) Dependence of cellulose to glucose conversion at 96 h (CGC$_{96h}$ (%)) on Liquid to Solid Ratio (LSR (g/g)) and Cellulase to Substrate Ratio (CSR (FPU/g)), fixed Hemicellulase Substrate Ratio (HSR) = 500 UI/FPU. (c) Dependence of cellulose to glucose obtained at infinite time (CGC$_{max}$ (%)) on Liquid to Solid Ratio (LSR (g/g)) and Cellulase to Substrate Ratio (CSR (FPU/g)), fixed Hemicellulase Substrate Ratio (HSR) = 500 UI/FPU. (d) Dependence of the time needed for achieving CGC$_{max}$/2 ($t_{1/2}$ (h)) on Liquid to Solid Ratio (LSR (g/g)) and Hemicellulase Substrate Ratio (HSR (UI/FPU)), fixed cellulase substrate ratio (CSR) = 30 FPU/g.

optimization of model. The maximal predicted conversion and concentration were obtained employing the following operational conditions: LSR = 6.4 g/g, CSR = 22.5 FPU/g and HSR = 500 UI/FPU. The predicted glucose concentration and cellulose conversion were 93.5 g/L and 86.3%, respectively. For the validation of model, an additional assay was carried out under the mentioned conditions. The results of validation assay were 85.5 g/L of glucose and 81.3% of cellulose to glucose conversion, the relative error was 8.5% and 5.8%, respectively. These data verify the sustainability of the model for reproducing and predicting the experimental results.

3.4. Simultaneous saccharification and fermentation of optimal condition

On the basis of reported data, saccharification and fermentation experiments of whole-slurry were carried out under optimized conditions (LSR = 6.4 g/g, CSR = 22.4 FPU/g and HSR = 500 UI/FPU). The use of hydrolysate has an inhibitory effect on the saccharification and fermentation and this inhibition will depend on the compounds concentration [45,46]. Therefore, the selection of suitable microorganism for the fermentation in presence of inhibitor

compounds is determinant for a good fermentation performance [27,47]. The strain used in this work was selected in previous study in which the *S. cerevisiae* PE-2 showed the best capacity of fermentation in presence of hydrolysate from autohydrolyzed *E. globulus* in comparison with several industrial and laboratory background strains [28]. Considering the xylose present in the hydrolysate, one of the main challenges in the bioethanol from LCM is the engineering of robust microorganisms able to rapidly ferment all sugars present in the hydrolysate [27]. In this context, the selected strain may be used as host for engineer desired physiological characteristics as xylose fermentation, following the physiological engineering concept [48]. The use of whole-slurry strategy poses some challenges including the hydrolysate inhibitory effect on yeast growth and fermentation [26] and moreover, the different optimal temperature for saccharification and fermentation processes [29]. For these reasons, different percentages of hydrolysate (65%, 70%, 75% and 100%) were assayed at 30, 35 and 37 °C in separate hydrolysis and fermentation (data not shown). These results showed a strong inhibition of fermentation with the increase of the hydrolysate percentage and temperature. On basis of these preliminary results, the experimental conditions chosen for sequent
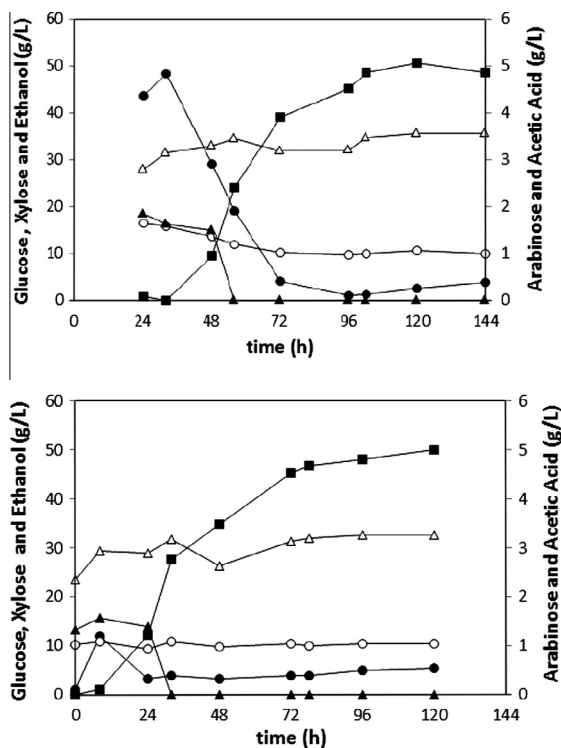
Fig. 4. Time course of glucose (●), xylose (○), arabinose (▲), acetic acid (△) and ethanol (■) concentration in optimized conditions (LSR = 6.4 g/g; ESR = 22.5 FPU/g and HSR = 500 UI/FPU) (a) by Presaccharification of 24 h and Simultaneous Saccharification and Fermentation (PSSF). (b) By Simultaneous Saccharification and Fermentation (SSF).

saccharification and fermentation assays were the 70% of hydrolysate and an operational temperature of 35 °C. Under these conditions, Simultaneous Saccharification and Fermentation (SSF) was compared with Presaccharification and Simultaneous Saccharification and Fermentation (PSSF) for maximum ethanol production. The 70% of hydrolysate was added in the liquid solid ratio defined in the previous section. The operational conditions were described in Section 2.6.

Fig. 4a and b shows the time course of PSSF and SSF fermentation experiments, respectively. The glucose accumulation during the presaccharification can be seen in Fig. 4a. The glucose concentration achieved (43.8 g/L) corresponding to a 41.5% of cellulose conversion at 24 h of saccharification. The accumulated glucose was consumed within 48 h after the yeast was added. In the SSF the glucose was lower (10.9 g/L) at 8 h of fermentation (see Fig. 4b) since the saccharification and fermentation was carried out simultaneously. The main results (ethanol conversion, maximal ethanol concentration and productivity at 72 h) were listed in Table 4. Ethanol conversion of 94.7% and 92.1% (corresponding to an ethanol concentration of 50.2 and 48.8 g/L or 6.3% and 6.1% (v/v)) were obtained for SSF and PSSF, respectively. As can be observed in Fig. 4, the step of presaccharification did not show a favorable effect on ethanol fermentation. Moreover, the time needed to achieve the stationary phase was higher. Thus, using these experimental conditions the SSF configuration was the strategy more recommendable to maximize the ethanol yields. Once again, the results obtained in these fermentation tests can be compared favorably with the literature. Zhou et al. [49] obtained similar results (47.1 g/L of ethanol, corresponding to 72% of theoretical yield) using pretreated pine beetle killed lodgepole pine wood by SPORL at 18% of solids loading and *S. cerevisiae* YRH400 (engineered yeast strain for xylose fermentation). Moreover, Öhgren

Table 4
Operational conditions used and results obtained in PSSF and SSF experiments using whole-slurry (70% liquid + solid).

Experiment	Operational conditions			Results		
	LSR (g/g)	ESR (FPU/g)	HSR (UI/g)	Qp72 (g/L h)	Emax (g/L)	EC (%)
SSF	6.4	22.5	500	0.63	50.2	94.7
PSSF	6.4	22.5	500	0.55	48.8	92.1

Q_{p72}: productivity obtained at 72 h (g/L h).
E_{max}: maximal concentration of ethanol (g/L).
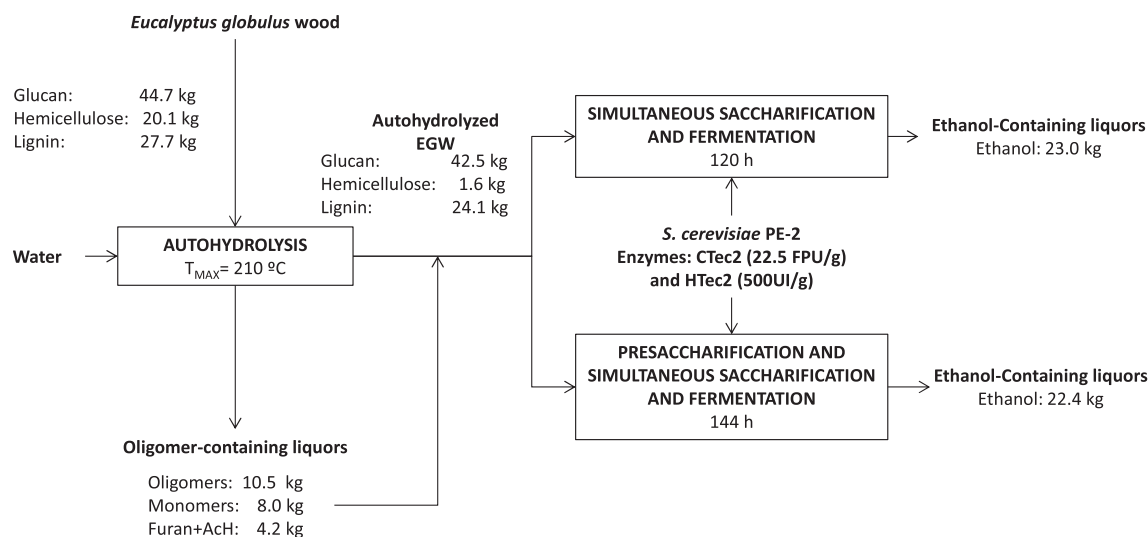EC: Ethanol Conversion (%).

Fig. 5. Mass balances for SSF and PSSF experiments.

et al. [50] reported an 81.5% of ethanol yield and 21.4 g/L of ethanol using whole slurry from steam-pretreated corn stover with additional xylanases supplementation by SSF. On the other hand, Varga et al. [51] achieved 52 g/L of ethanol (6% v/v) corresponding to 78% ethanol yield by SSF from wet oxidized corn stover dry matter (17% of solid and enzyme loading of 43.5 FPU/g cellulose). In other study where SSF was carried out with whole steam-pretreated spruce slurry at 13.7% of solid, very low ethanol yield was obtained, however the yield increased from 3.9% to 62.1% when a prehydrolysis (48 °C for 24 h) was realized [52].

The results obtained in this work confirmed the good performance of fermentation with high productivity ($Q_{p72} > 0.6$ g/L h) and elevated ethanol concentration (higher than 6%, v/v), even comparing to literature. The use of an industrial robust strain, naturally more resistant to overall stress conditions (including stress induced by inhibitors), could provide an essential key for the attainment of an efficient fermentation step that allow the ethanol process from lignocellulosic materials. Considering the overall balance of the process, the potential ethanol from cellulose presents in EGW is 26.98 kg/100 kg wood (see Fig. 5), therefore the ethanol yield of whole-slurry SSF and PSSF processes obtained in this work is 85.3% and 82.9%, respectively.

4. Conclusion

This work provides results for the development of ethanol production process using the whole slurry from autohydrolyzed *E. globulus* wood at high solid loadings that could help to establish a cost-effective process. New advanced cellulolytic enzymes preparation (CTec2 and HTec2) showed better results for saccharification of whole-slurry from autohydrolyzed EGW than Celluclast 1.5L/Novozyme 188. The glucose concentration was optimized by experimental design and 85.5 g/L was obtained corresponding to a conversion of 81.5% operating under the following conditions: LSR = 6.4 g/g, CSR = 22.5 FPU/g and HSR = 500 IU. The saccharification and fermentation of pretreated solid was carried out with 70% of hydrolysate using an industrial yeast strain. The SSF strategy was more advantageous than PSSF. The highest ethanol concentration (50 g/L or 6.3% (v/v)) was obtained using high solid loadings and no-detoxified hydrolysate with elevated conversion (95%) contributing to obtain a robust process in the field of second generation bioethanol.

Acknowledgements

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