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Co-production of biofuels and value-added compounds from industrial *Eucalyptus globulus* bark residues using hydrothermal treatment

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ABSTRACT

In this work, hydrothermal treatment was assessed for the fractionation of industrial *Eucalyptus globulus* bark residue (EBR) to obtain biofuels and value-added compounds (such as oligosaccharides and phenolic compounds) in separated streams. Hydrothermal treatment was evaluated under non-isothermal regimen in the range of maximum temperature (T_{max}) of 177–228 °C or severities (S_0) between 2.76 and 4.25. The highest oligosaccharides concentration (17.5 g/L) was achieved at S_0 of 3.69, corresponding to hemicellulose recovery of 77.30%. Under all severities evaluated in this work, over 90.94% and 84.17% of cellulose and lignin remained in the solid phase, respectively. The increase of S_0 improved 4.38-fold the enzymatic saccharification of cellulose, the highest glucose yield (84%) being achieved at S_0 of 4.04. Considering the maximal recovery of polysaccharides as glucose and oligosaccharides from the liquid and solid phases, S_0 of 4.04 was selected for bioethanol production using high solid loadings and following different strategies (simultaneous saccharification and fermentation – SSF and pre-saccharification and simultaneous saccharification and fermentation – PSSF). The utilization of 15% hydrothermally pretreated EBR without nutrient supplementation resulted in 26 g/L of ethanol, independently of the strategy used. An increase up to 17.5% solids and employing nutrient supplementation enabled the production of 38 g/L (or 4.8% v/v) of ethanol by PSSF.

1. Introduction

Over the last decades, a significant growth on world population brought to our daily lives new problems of resources depletion and, even more important, an environmental crisis that already affects specific regions of the globe such as India or China [1]. Indeed, the rising demand for energy and other chemical commodities and materials, usually based on the petrochemical industry, is producing alarming levels of GHG emissions [2]. Accordingly, significant efforts have been made, mostly by academia, aiming cleaner energy sources and alternative routes to the chemical synthesis of some of these components.

Lignocellulosic materials are the only renewable raw material in sufficient amount to partially replace fossil sources, which are cheaper and usually do not compete with food crops [3]. However, and because they present a very recalcitrant structure for most of the cases, their conversion also involves more complex operations, such as the application of an initial pretreatment [4]. Lignocellulosic pretreatments aim to disrupt the lignocellulosic structure, generally associated with the presence of lignin and to the crystallinity of cellulose, thus improving enzymes accessibility to cellulose [5]. Furthermore, they can also have a fractionation function since each component (cellulose, hemicellulose and lignin) can be selectively recovered in separated streams depending on the selected pretreatment, following a biorefinery approach. In this context, different options have been used so far, ranging from simple mechanical pretreatments to physical, chemical, physico-chemical, biological treatments and/or a combination of thereof [6,7]. Aiming the application of environmentally-friendly processes, higher attention has been given to the utilization of hydrothermal pretreatments, namely autohydrolysis (or liquid hot water) and steam-explosion, as they do not require the utilization of harmful chemicals [8] or expensive anti-corrosion materials [9]; water acts here as a sole external catalyst through its ionization products (H_3O^+ and OH^-), being complemented with acetic acid originated from acetyl groups from the raw-material. In a biorefinery scheme, the hydrothermal treatment represents one of the best options for the initial biomass processing, as it allows selective solubilization of hemicelluloses, originating a final solid mostly composed by cellulose and lignin [4,10].

Despite all the research conducted so far on converting

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lignocellulosic materials, these processes generally still cannot compete with their counterparts from the petrochemical industry [11]. Aiming to reduce the cost associated to the lignocellulosic material, new and less expensive options have been studied, ranging from industrial [12], agro [13] or forest residues [14], to municipal wastes [15].

In the particular context of the Portuguese economy, the pulp and paper industry, which is one of the largest in this sector, is mostly based on *Eucalyptus globulus* wood [16]. On this industry, large amounts of eucalyptus bark are annually generated as a residue from manufacturing processes [17]. According to Santos et al. [18], for each tonne of bleached Kraft pulp produced, a pulp mill can generate approximately 0.2 tons of bark residue. In Portugal, the generation of *Eucalyptus globulus* bark was estimated at 0.5 Mton in 2017 [16]. This residue can, however, still present interesting levels of polysaccharides – 61%, but also extractives (9.86%) and lignin (21.86%) [19]. Recent studies on these residues already showed their potential in the production/recovery of polyphenols [17,20], lignin [21], glucose [16], as well as solid fuels [19].

On the other hand, the valorization of eucalyptus barks for bioethanol production has barely been studied with very few works conducted so far. The organosolv delignification of *E. nitens* bark allowed the production of 33 g/L of ethanol [22]. On the other hand, 0.14 g/g_{bark} of ethanol was obtained from *Eucalyptus dunnii* bark after an ionic liquid pretreatment [23]. In the context of bioethanol production, the most widely implemented strategies include; i) separate hydrolysis and fermentation (SHF), ii) simultaneous saccharification and fermentation (SSF) or iii) a combination of both, known as pre-saccharification and simultaneous saccharification and fermentation (PS-SSF) [24]. Moreover, intending to increase final ethanol concentrations, reducing distillation costs of the lignocellulose-to-ethanol process, the use of high solid loadings in the saccharification and fermentation processes is becoming more evaluated [25–29].

Therefore, the objective of this work was to evaluate hydrothermal treatment as an environmentally-friendly pretreatment approach for the fractionation of *E. globulus* bark aiming to maximize the recovery of all fractions, with especial emphasis in the production of cellulosic ethanol. After assessing the most suitable conditions of hydrothermal treatment for hemicellulose-derived compounds recovery as oligosaccharides and enzymatic saccharification of cellulose into glucose, distinct integration approaches for ethanol production at high solid loadings, concerning hydrolysis and fermentation, were also evaluated and compared.

2. Materials and methods

2.1. Raw material

Industrial eucalyptus bark residues (EBR) from *Eucalyptus globulus* were kindly provided by RAIZ (Forest and Paper Research Institute), being generated during the process of pulp and paper manufacturing by The Navigator Company, Portugal. As received material was initially air-dried (until a humidity inferior to 10%), milled through a 6 mm screen and finally sieved (to remove particles smaller than 0.1 mm) and homogenized on a single lot. EBR were then stored in a dark and dry place until further use.

2.2. Analysis of the raw material

EBR were assessed for their composition on the main lignocellulosic constituents, namely polysaccharides (cellulose and hemicellulose), lignin, extractives, humidity and ash. Humidity was determined by dry weight measurement, and the extractives were quantified following NREL procedures (NREL/TP-510-42619). The extracted solid was then subjected to quantitative acid hydrolysis with 72% (w/w) sulphuric acid following NREL/TP-510-42618 [30]. Klason lignin was determined gravimetrically from the final solid obtained after filtering the acid hydrolysate. All analyses were conducted in triplicate.

2.3. Hydrothermal treatment of eucalyptus bark residues

To evaluate the efficiency of hydrothermal treatment for the fractionation of EBR, several experiments were conducted under the non-isothermal regimen, and the obtained products (hydrothermal liquors and pretreated solids) were subsequently analyzed. Therefore, EBR (previously air dried and milled) were initially mixed with water at a liquid to solid ratio (LSR) of 10 g (oven-dry basis)/g in a Parr reactor (Model 4848). This consisted of a 1.9 L pressurized vessel complemented with two internal impellers and a cooling loop for tap water circulation, heated by an external fabric mantle. For each treatment, the lignocellulose mixture was first heated until a desired maximum temperature (T_{max}), which ranged between 177 °C and 228 °C, and immediately after that, the heating source was removed enabling the reactor content to cool down rapidly. This process was conducted with an agitation of 150 rpm and following the heating–cooling profiles, as shown in Fig. 1. The intensity of each pretreatment was estimated through its severity (S_0) according to Eq. (1) [31], which considers the combined effect of temperature and time over the heating and cooling stages and it is defined as the logarithm of the reaction ordinate (R_0) [32].

$$S_0 = \log\left[\int_0^{t_{MAX}} \exp\left(\frac{T(t) - T_{REF}}{\omega}\right)\cdot dt + \int_{t_{MAX}}^{t_F} \exp\left(\frac{T'(t) - T_{REF}}{\omega}\right)\cdot dt\right] \quad (1)$$

where, t_{MAX} and t_F refer to the time requested for T_{max} to be reached and to the total time of the heating-cooling profiles (limited by T_{REF}), respectively, while T(t) and T'(t) correspond to the temperature profiles for the heating and cooling stages. T_{REF} is the reference temperature (373.15 K) and ω is an empirical parameter related to the activation energy, set to 14.75 K for this raw material.

2.4. Analysis of pretreated solids and hydrothermal liquors

After the hydrothermal pretreatment, and once the pressurized vessel reached the atmospheric pressure and a temperature below 80 °C, the reactor was opened and the slurry was filtered with a vacuum pump to separate the hydrothermal liquor from the pretreated solids (spent solids). One aliquot of the hydrothermal liquor was collected and stored for the quantification of glucose, xylose, arabinose, acetic acid, hydroxymethylfurfural (HMF) and furfural by HPLC. Another sample was subjected to a quantitative post-hydrolysis (4% (w/w) sulfuric acid; 121 °C; 20 min; in triplicate) and analyzed by HPLC to further determine the content of oligomers and linked acetyl groups in the liquor. A sample was also stored for posterior quantification of total phenolic compounds (TPC).

Pretreated solids were washed with distilled water, air-dried and then their weight and humidity quantified to estimate the Solid Yield (SY) of the hydrothermal pretreatment (g solid recovered after autohydrolysis/100 g raw material). Afterwards, a sample was collected, milled to particle size <0.5 mm and analyzed for its composition (in triplicate) employing the same protocol as previously used for the raw material. The remaining solid was stored on a dry and dark place until further use.

2.5. Susceptibility of the pretreated solids towards enzymatic hydrolysis

To evaluate the efficiency of the hydrothermal treatment on the disruption of the lignocellulosic structure, and the consequent increase of enzymes accessibility to the substrate, pretreated solids were subjected to enzymatic hydrolysis under favourable conditions. The pretreated solids were initially suspended in 0.1 M acetic acid/sodium acetate buffer (pH 4.8) at a solid loading of 5% in a 250 mL Erlenmeyer flask. After sterilization (121 °C; 20 min) and cooling to room temperature, 20 FPU/g_{solid} of Cellic Ctec2 were added to the solids, being then incubated at 40 °C and 150 rpm for 70 h (in duplicate). The activity of Cellic Ctec2 (120 FPU/mL) was determined following the method

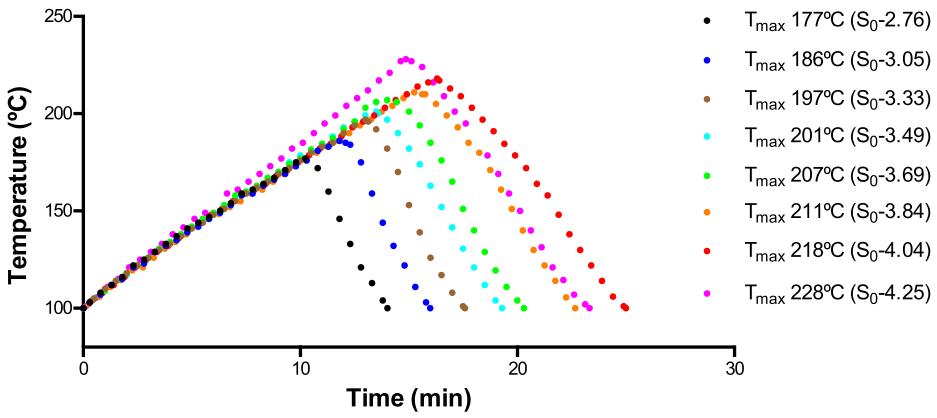


Fig. 1. Heating-cooling profiles for the pretreatments conducted at different severities.

described by Ghose [33]. Glucose yield (GY), measured as g of glucose per 100 g of potential glucose in pretreated biomass, was determined as follows:

$$Glucose\ \ Yield(GY,\%) = \frac{G_t}{G_{POT}} \cdot 100 \tag{2}$$

where, G_t is the glucose concentration at time *t* from enzymatic hydrolysis of pretreated EBR, and G_{POT} is the glucose potential and it was calculated as follows:

$$G_{POT} = \frac{Gn}{100} \cdot \frac{180}{162} \cdot [solid\ \ loading] \tag{3}$$

where, *Gn* is the glucan content of the pretreated EBR (g glucan/100 g pretreated EBR), 180/162 is the stoichiometric factor for cellulose hydration upon hydrolysis, *[solid loading]* is the concentration of pretreated EBR (in g/L).

2.6. Inoculum preparation

The strain employed in this study was the industrial *Saccharomyces cerevisiae* Ethanol-Red®, commonly reported for its efficiency at high temperatures [34,35]. For inoculum preparation, 2–3 colonies (from stock cultures kept on YPD agar plates) were transferred into 250 mL Erlenmeyer flask, containing 100 mL of YPD medium (50 g/L glucose, 20 g/L peptone and 10 g/L yeast extract) and then incubated at 30 °C and 150 rpm for 18 h. The cells were aseptically collected by centrifugation (10 min; 4000 g) and resuspended in 0.9% NaCl to a final concentration of 200 mg fresh yeast/mL. This suspension was then used on the simultaneous saccharification and fermentation (SSF) and the pre-saccharification and simultaneous saccharification and fermentation (PS-SSF) experiments in a concentration of 8 g/L (fresh yeast).

2.7. Pre-saccharification and simultaneous saccharification and fermentation (PS-SSF) and simultaneous saccharification and fermentation (SSF) of pretreated EBR

In the scope of EBR utilization for bioethanol production, the solid obtained from the pretreatment selected was subjected to enzymatic hydrolysis and fermentation under different configurations as described as follows. The enzymatic hydrolysis and subsequent fermentation of EBR suspensions were conducted following two distinct main approaches according to the time of adding the cells: on a first approach cells and cellulases were added at the same time, corresponding to the typical simultaneous saccharification and fermentation (SSF); opposing to that, cells were added only after a pre-saccharification period of 24 or 48 h (*t*) (in which the enzymatic hydrolysis was carried out at 50 °C and 200 rpm), followed by a period of SSF (PS_t-SSF). For enzymatic hydrolysis, similar procedures to those employed in the previous tests of the susceptibility of solid hydrolysis were used, with some modifications. Intending process intensification, solids suspensions were prepared with a superior consistency (high solid loadings), ranging between 15 and 17.5% solids (w/v). Excepting for the pre-saccharification periods, incubations were conducted at 35 °C and 180 rpm. Also, supplementation with 20 g/L peptone and 10 g/L yeast extract was used in some experiments. All tests were conducted on duplicate in 250 mL Erlenmeyer flasks with a working volume of 50 mL of liquid.

The performance of SSF and PS-SSF was quantified through the ethanol yield (EY), as follows:

$$Ethanol\ \ Yield(EY,\%) = \frac{[EtOH]_t - [EtOH]_0}{0.51 \cdot f \cdot [EBR] \cdot 1.11} \tag{4}$$

where, [EtOH] *t* or *0* is the ethanol concentration (g/L) at given time *t*, or *0* at the beginning of the fermentation; 0.51 is the stoichiometric coefficient of glucose to ethanol conversion; f is the cellulose fraction of dry EBR (g/g); [EBR] is the concentration of pretreated lignocellulosic biomass at the beginning of SSF or PSSF (g/L); 1.11 is the conversion factor of cellulose into glucose.

Ethanol production was mathematically modelled following the equations described by Rodrigues et al. [36]:

$$r_P = \frac{dP}{dt} = P_r\left(1 - \frac{P}{P_{max}}\right)P \tag{5}$$

where, r_P is the volumetric rate of product formation, *t* is the time (h), *P* is the ethanol concentration (g/L), P_{max} is the maximum concentration of ethanol (g/L), and P'_r is the ratio between the initial volumetric rate of ethanol formation (r_{P0}) and the initial concentration of ethanol P_0 (g/L). Integration of Equation (5) results in the following expression for ethanol concentration:

$$P = \frac{P_0 P_{max} e^{P_r t}}{P_{max} - P_0 + P_0 e^{P_r t}} \tag{6}$$

The model was adjusted to the experimental data, and the kinetic parameters (P_0, P_{max} *and* P_r) were calculated applying the least-squares method using commercial software (Solver, Microsoft Excel 2016).

2.8. Analytical procedures

2.8.1. Quantification of sugar monomers, acetic acid, furan compounds and ethanol

HPLC (High-Performance Liquid Chromatography) was used to quantify the content of sugar monomers (glucose, xylose and arabinose), acetic acid, furan compounds (HMF and furfural) and ethanol over the different parts of this study. After diluted (when applies) and filtered, samples were eluted on a Varian MetaCarb 87H column at 60 °C, with 0.005 M H_2SO_4 at a flow rate of 0.6 mL/min, coupled with refractive-

index and UV detectors.

2.8.2. Quantification of phenolic compounds

The total content of phenolic compounds present on the hydrothermal liquors was estimated by Folin-Ciocalteu method [37]. For each liquor, 100 μL of sample (previously filtered and diluted) were added on a test tube to 2 mL of 7.5% (w/v) sodium carbonate and 500 μL of Folin reagent, finally completing with distiller water to a total volume of 10 mL (in triplicate). After properly mixed, test tubes were incubated for 5 min at 50 °C and then allowed to cool to room temperature. Final absorbance was then measured at 700 nm using a spectrophotometric microplate reader (Bio-TeK Synergy HT). A calibration curve was elaborated with gallic acid in concentrations ranging from 0.2 to 2.5 g/L; total phenolics were hence expressed as g of gallic acid equivalent (GAE)/L.

3. Results and discussion

3.1. Eucalyptus bark chemical characterization

Opposing to eucalyptus wood, literature data on the fractionation of eucalyptus bark is rather scarce, with most of the existing results focusing on its characterization and/or in the extraction of bioactive compounds and the effect of pretreatment on its enzymatic saccharification [16,17,21,38,39]. As for eucalyptus wood, eucalyptus bark also presents an interesting potential, mostly due to a considerable amount of polysaccharides (67.17%) and, to a lower extent, lignin (21.86% Klason lignin) and a minimal content of ash (2.63%), as shown in Table 1. Indeed, cellulose and xylan accounts alone for nearly 63% of EBR total composition (Table 1), rendering an interesting potential for the production of both chemicals and energy [22,40]. This composition can be compared with previous characterizations of barks from several species of *Eucalyptus,* such as *E. globulus* and *E. nitens* [16,22,41]. On the other hand, Lima and co-authors [17] studied the bark of 11 species of eucalyptus, observing that they presented diverse compositions. For example, while *E. resinifera* showed a similar composition to the reported in the current work, i.e. 65.6% of polysaccharides and 24.3% of Klason lignin, *E. globulus* presented 66.6% of polysaccharides and 14.4% of Klason lignin, showing that other factors may contribute to the chemical composition, such as genetic variability between different sources, plant age, climatic and soil conditions [42].

3.2. Hydrothermal treatment for eucalyptus globulus bark fractionation

Few works in the literature report the valorization of eucalyptus barks with energy purposes, namely liquid or solid biofuels [19,40]. In this work, hydrothermal treatment was evaluated as the first step for the valorization of industrial EBR within a biorefinery concept.

Similar to what has been commonly reported for eucalyptus wood, the hydrothermal treatment showed to be quite efficient in the solubilization of mainly hemicellulose from the raw material, as shown in Table 2 and Fig. 2. Fig. 2 shows the chemical characterization of EBR after autohydrolysis at several severities (expressed as g of component/100 g of pretreated EBR) and the levels of recovery for the main lignocellulosic fractions (glucan, xylan and Klason lignin). The pretreatment conducted under the mildest condition (S_0 = 2.76) already enabled a solubilization of approximately 14% of the initial raw material (EBR) and a relative increase on glucan content of nearly 8%. However, the pretreatment effects were more pronounced only when T_{max} reached 197 °C (S_0 = 3.33) with solid solubilization of 24.8%, further increasing until it stabilized around 30% for a T_{max} of 211 °C (S_0 of 3.84). Accordingly, there was a clear concentration of the glucan fraction on the pretreated solid, increasing nearly 30% to 62 g of glucan/100 g of pretreated solid (Fig. 2A), which represents a recovery of 92% of the initial glucan in raw material (Fig. 2B). This glucan content in the hydrothermally pretreated EBR was slightly superior to the glucan concentration obtained by autohydrolysis treatment using the same raw material [16]. The average glucan recovery achieved in this work was 92.34%, which can be compared to results obtained in the literature for autohydrolysis treatment with hardwoods such as *Eucalyptus globulus* wood or *Paulownia tomentosa* wood [43,44], showing the selectivity of this treatment limiting the glucan losses.

On the other hand, xylan content on the pretreated solid was reduced from 15 to 6 g of xylan/100 g pretreated solid, resulting from its solubilization into the hydrothermal liquor, which steadily increased until a value of 71% for a S_0 of 3.69. Regarding the solubilization of xylan using the hydrothermal treatment, similar results were already obtained using several raw materials (such as vine shoots, corn cob, eucalyptus wood, agave bagasse) [43,45,46]. Finally, and regarding Klason lignin, similar to what has been observed for glucan there was also a slight concentration effect for this component on the pretreated solid, even with some level of degradation observed at higher severities, which possibly resulted in the formation of some phenolic compounds (Fig. 2A). Nevertheless, Klason lignin recovery reached a minimum of 84% for a S_0 of 3.69 and interestingly, started to increase for higher values of severity, reaching a recovery rate around 92% (Fig. 2B). This could be explained by re-polymerization of lignin, which may occur for higher temperatures [47].

As for the pretreated solids, hydrothermal liquors showed a close relationship with the severity of the pretreatment, either by the presence of hemicellulose solubilized as oligomers, but also by the presence of sugar degradation compounds (namely, furfural and hydroxymethylfurfural). Oligomers were the most abundant class of compounds (achieving a maximal concentration of 17.5 g/L), composed mainly by xylooligosaccharides (XOS). This result can be positively compared to the obtained by an autohydrolysis treatment at S_0 of 4.4, which reported 7.7 g of oligomers per 100 g of eucalyptus bark [16]. In this work, XOS concentration steadily increased until a maximum at a S_0 of 3.69, achieving 65.7% of xylan recovery as XOS, after which they started to decline possibly due to its degradation into xylose, which attained its maximal concentration (3.30 g/L) at a S_0 of 4.25. Similar behaviour was observed for glucooligosaccharides (GOS), though on a lower extent, as their concentrations decreased from 3.12 g/L to 2.30 g/L over the entire range of severities tested. Similar to what was observed for XOS, it was also possible to observe the maximum concentrations for arabinooligosaccharides (AOS) and acetyl groups (AcG), which were reached for a S_0 of 3.33: 0.83 g/L and 3.50 g/L, respectively.

The monomer sugars (i.e. glucose and xylose), on the other hand, evidenced a different behaviour. As they mostly result from the hydrolysis of oligosaccharides, they tend to increase as harsher pretreatment conditions are employed. Indeed, glucose concentrations on the hydrothermal liquors raised from 0.48 g/L, for a S_0 of 2.76, to 1.30 g/L for a S_0 of 4.04; above this point a decline was observed, possibly due to a more intense degradation into hydroxymethylfurfural (HMF). This compound, which is formed from the degradation of hexoses, was mainly found when T_{max} reached 218 °C (S_0 = 4.04), achieving a maximum of 0.65 g/L for the harshest pretreatment (S_0 = 4.25). Similarly, the contents of xylose and arabinose also increased with the severity, reaching a

Table 1
Composition of eucalyptus bark residues (expressed in g per 100 g raw material on an oven-dry basis ± standard deviation).

Component	g/100 g dry solid
Cellulose (measured as Glucan)	47.51 ± 1.02
Hemicellulose	
Xylan	15.32 ± 0.33
Arabinan	0.99 ± 0.08
Acetyl groups	3.35 ± 0.15
Acid-insoluble lignin (Klason lignin)	21.86 ± 0.59
Acid-soluble lignin	2.56 ± 0.05
Extractives	2.04 ± 0.01
Ashes	2.63 ± 0.23

Table 2
Chemical composition of liquid phase after hydrothermal treatment of *Eucalyptus globulus* bark.

T_{MAX} (°C)	177	186	197	201	207	211	218	228
S_0	2.76	3.05	3.33	3.49	3.69	3.84	4.04	4.25
g of pretreated EBR/100 g raw material – oven dry basis								
Solid Yield	86.21 ± 0.72	81.64 ± 1.33	75.23 ± 0.51	73.10 ± 0.92	70.18 ± 0.39	69.87 ± 0.66	69.53 ± 0.21	68.92 ± 0.35
g or (*) g monomer equivalent/L								
Glucose	0.48 ± 0.02	0.69 ± 0.01	0.96 ± 0.05	1.08 ± 0.02	1.08 ± 0.07	1.21 ± 0.05	1.30 ± 0.07	1.06 ± 0.04
Xylose	0.21 ± 0.02	0.39 ± 0.01	0.63 ± 0.00	1.17 ± 0.09	1.25 ± 0.07	2.12 ± 0.06	2.60 ± 0.11	3.30 ± 0.10
Arabinose	0.13 ± 0.00	0.23 ± 0.03	0.42 ± 0.01	0.81 ± 0.05	0.91 ± 0.00	0.99 ± 0.05	0.86 ± 0.02	0.75 ± 0.04
Acetic acid	0.43 ± 0.01	0.72 ± 0.02	0.90 ± 0.02	1.05 ± 0.05	1.18 ± 0.03	2.02 ± 0.07	2.40 ± 0.05	4.44 ± 0.12
Hydroxymethylfurfural	0.003 ± 0.000	0.006 ± 0.000	0.015 ± 0.001	0.025 ± 0.003	0.044 ± 0.001	0.079 ± 0.006	0.369 ± 0.007	0.654 ± 0.068
Furfural	0.003 ± 0.000	0.014 ± 0.000	0.052 ± 0.004	0.25 ± 0.00	0.47 ± 0.02	0.82 ± 0.05	1.04 ± 0.06	1.60 ± 0.05
Gluco-oligosaccharides*	3.12 ± 0.09	3.42 ± 0.18	2.84 ± 0.21	2.73 ± 0.04	2.61 ± 0.05	2.59 ± 0.11	2.47 ± 0.06	2.30 ± 0.04
Xylo-oligosaccharides*	3.20 ± 0.05	5.95 ± 0.02	9.36 ± 0.67	9.58 ± 0.35	11.11 ± 0.29	9.53 ± 0.17	9.24 ± 0.21	6.91 ± 0.25
Arabino-oligosaccharides*	0.77 ± 0.03	0.80 ± 0.05	0.83 ± 0.07	0.42 ± 0.01	0.39 ± 0.02	0.22 ± 0.01	–	–
Acetyl groups*	3.10 ± 0.23	3.11 ± 0.06	3.50 ± 0.14	3.46 ± 0.28	3.35 ± 0.04	2.57 ± 0.11	2.11 ± 0.03	0.36 ± 0.04
Total phenolics (GAE)	0.79 ± 0.01	0.91 ± 0.08	1.00 ± 0.05	1.09 ± 0.08	1.39 ± 0.04	1.51 ± 0.12	1.86 ± 0.06	2.21 ± 0.14

*Reported as equivalent of monomers.

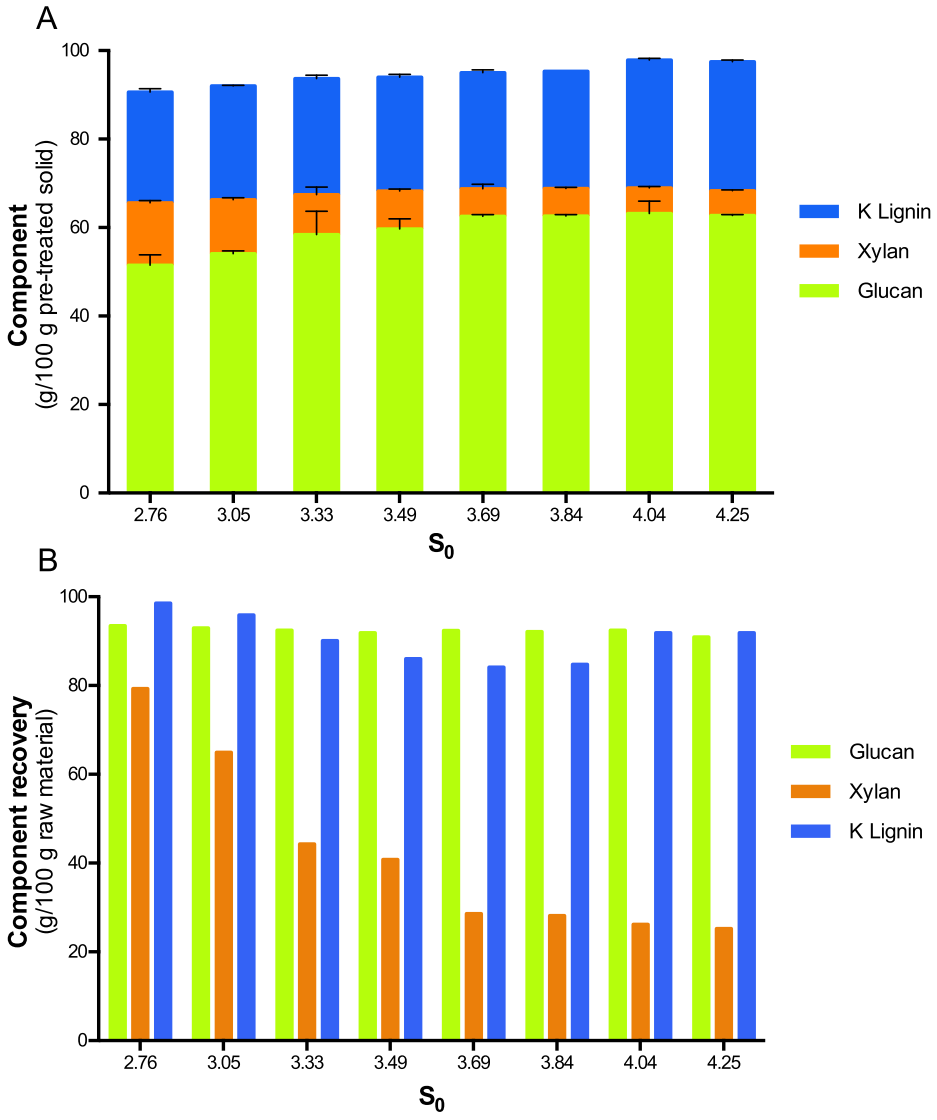


Fig. 2. Effect of autohydrolysis severity on (A) the composition of the pretreated solid and (B) the recovery of its main components.

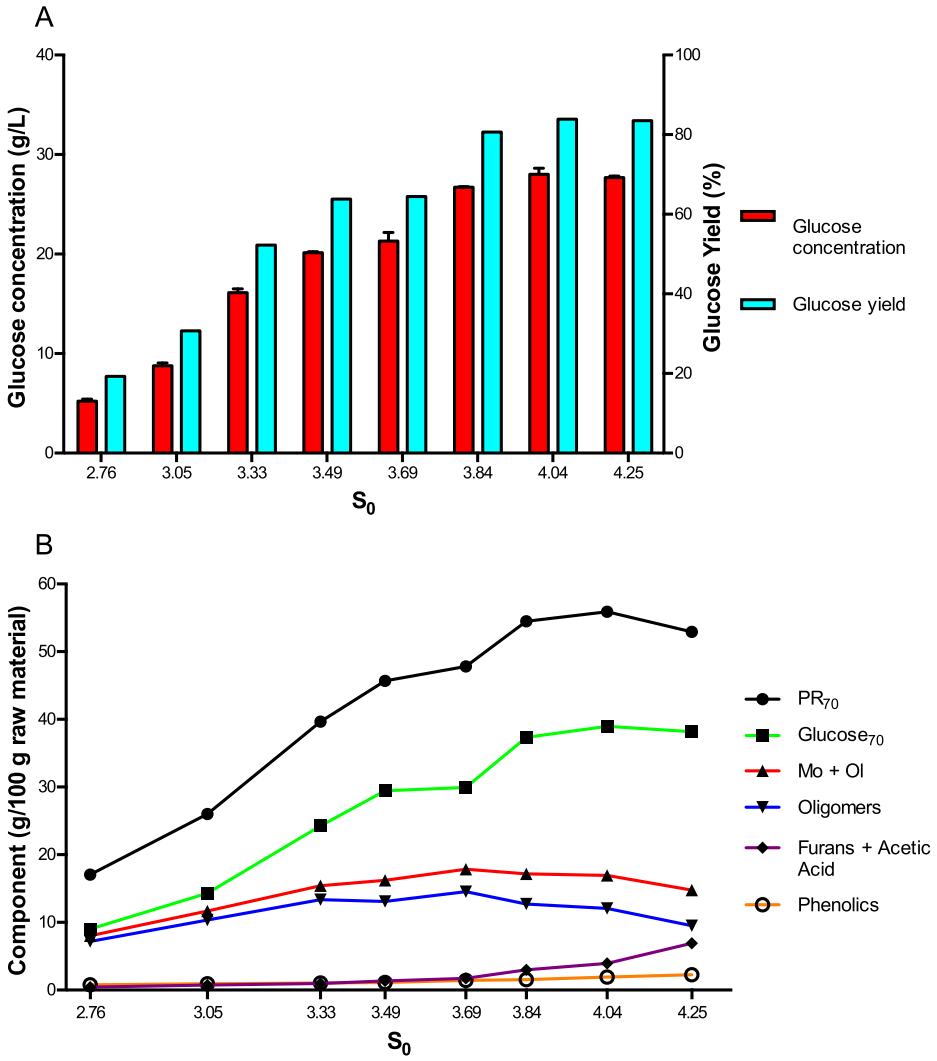
maximum of 3.30 and 0.99 g/L, respectively. As for what was observed for glucose, partial degradation of pentose sugars also occurred, suggested by a constant increase of furfural, especially when T_{max} reached 201 °C (S_0 = 3.49), achieving a maximum of 1.60 g/L. This was especially visible from the values of arabinose concentration as they reached a maximum of 0.99 g/L, for a S_0 of 3.84, decreasing after that. Adding to the furan compounds, it is also very relevant to refer the presence of acetic acid, which steadily increased up to a concentration of

4.44 g/L in the harshest pretreatment ($S_0 = 4.25$); this is still below the critical inhibitory concentrations towards industrial yeast cells [48]. Finally, it is worth noting the presence of a still considerable amount of phenolic compounds, increasing with the severity to a maximum of 2.21 g/L (GAE); these were possibly originated from the degradation of lignin, which especially occurs for higher temperatures. These values are comparable to those obtained in literature for hydrolysates from agro-industrial residues (namely vine pruning, eucalyptus wood, apple pomace and corn cob) using autohydrolysis treatment [13,49].

3.3. Enzymatic hydrolysis of the pretreated solids and overall recovery of different compounds from both fractions

From an economic standpoint and in the context of multiple streams valorization, an adequate balance must be found between the conversion of the pretreated solid by enzymatic hydrolysis and the recovery of added-value products from the hydrothermal liquor (such as oligosaccharides and phenolic compounds). Under a wide range of severities evaluated in this work, distinct pretreated EBR were produced, which would differ not only on their glucan content but also on its digestibility, resulting from varying levels of modifications on the recalcitrance of the raw material.

To evaluate the combined action of these two factors, promoting the effect of different recalcitrance levels and, on the other hand, attenuating mass transfer limitations, the enzymatic hydrolysis of the pretreated solids was assessed employing a solids consistency of 5% (w/v) and an enzyme dosage of 20 FPU/g_{solid}, both considered very favourable from an operational standpoint. As we can observe from Fig. 3A, harsher pretreatments generally produced solids resulting in higher concentrations of glucose. For the solid obtained from the pretreatment with a S_0 of 2.76, the mildest condition, only 5.2 g/L of glucose were produced after 70 h of hydrolysis; opposing to that, the solid produced from the pretreatment with a S_0 of 4.04 was hydrolyzed into approximately 28 g/L of glucose, a 4.38-fold increase. Even though harsher conditions generally led to an increase of glucan content on the pretreated solids (Fig. 2A), hence their glucose potential, the differences on glucose production in this case were most likely due to different recalcitrance levels of the pretreated solids. Indeed, the application of higher temperatures during hydrothermal pretreatments is generally more effective in disrupting lignocellulosic structures [4], leading to increased



Mo + OI *- glucose, xylose, arabinose and their correspondent oligomers on the hydrothermal liquor*
Glucose$_{70}$ *- glucose produced after 70 h of enzymatic hydrolysis*
PR$_{70}$ *- sum of monomers and oligomers in the hydrothermal liquor and glucose from a 70 h enzymatic hydrolysis*

Fig. 3. Effect of pretreatment severity on (A) glucose production after 70 h of enzymatic hydrolysis (5% solids; 20 FPU/g_{solid}) and (B) on the overall mass balance of hemicellulose-derived and phenolic compounds contained on the hydrothermal liquor or released from the pretreated EBR after enzymatic hydrolysis.

accessibility of enzymes to the substrate. This is especially evident from the values of glucose yield (Fig. 3A), which also increased with the level of severity. As an example, while the solid from the mildest pretreatment was only hydrolyzed to the extent of 19% (after 70 h), this value increased to 83% when the harshest conditions were employed. Similar results have previously been reported by Araya et al. [50] for the autohydrolysis of *E. globulus* wood, in which the glucan-to-glucose conversion yield increased from 58 to 90% when the severity increased from 3.89 to 4.78. These results can also be compared to those obtained from autohydrolyzed eucalyptus bark at S_0 of 3.6 and 4.0 (45 FPU/g of commercial enzymes Sacyme Yield and Ultimase BWL) that reported 83 and 89% of glucose yield, respectively [16]. Similar glucose yields (80%) were also obtained from eucalyptus bark using hydrothermal treatment with carbon dioxide [51] and a pretreatment with the ionic liquid $[Et_4N][Me_2NC_4SO_3]$ [21]. In another study, the combination of a protonic and an aprotic ionic liquid also resulted in an improvement of the enzymatic saccharification of eucalyptus bark, achieving 90% of cellulose conversion [38]. Finally, it may also be relevant to refer that in the current work, when S_0 increased from 3.84 to 4.25 (the highest severity), there was no significant variation in glucose production, suggesting that an increase in pretreatment severity in this range would no longer be advantageous.

From an economic point of view, the results presented above suggest an interval of pretreatment severities that might be highly interesting as they enable a good compromise between the recovery of added-value compounds from the hydrothermal liquors (such as oligosaccharides and phenolic compounds) and the production of fermentable sugars from the solid. Fig. 3B presents the variation of the concentration (expressed as g of component/100 g of EBR) of hemicellulose-derived and phenolic compounds on the hydrothermal liquors and the glucose produced from enzymatic hydrolysis, for different pretreatment severity. Indeed, the pretreatment becomes particularly promising when S_0 reaches a value of 3.69, which also corresponds to the point of maximum oligosaccharides production. For superior values, and although occurring a gradual decrease of oligosaccharides on the liquors, the production of fermentable sugars from the enzymatic hydrolysis increased significantly, resulting on an equal increase in the overall polysaccharides recovery (encompasses the sum of monomer and oligomer sugars from the liquor and glucose produced from enzymatic hydrolysis of the solid), which reached a maximum of 55.9 g/100 g of raw material for a S_0 of 4.04. Adding to this, the economic value that can be retrieved from phenolic compounds is superior as their presence increases with the severity. A further increase on the severity will no longer be advantageous as the recovery of polysaccharides starts to decrease, most likely due to a reduction of overall glucan recovery, which is observed at S_0 of 4.25 (T_{max} = 228 °C) (Fig. 2B). Similar behaviour was reported by Romaní et al. [43] for *E. globulus* wood; the authors observed that total polysaccharides recovery (for a 72 h hydrolysis) reached a maximum of approximately 54 g/100 of raw material at T_{max} of 220 °C, decreasing afterwards for superior levels of severity. In a previous work from Araya et al. [50], the levels of glucan in the solids obtained from autohydrolysis of wood chips were reported to increase until a maximum of 68% for a severity of 4.20; when pretreatment severity further increased to 4.48 and 4.78, the levels of glucan decreased to 65 and 58%, respectively.

3.4. Potential of bioethanol production from EBR

In the scope of biomass residue valorization for biofuels production, the hydrolysis and fermentation of autohydrolyzed EBR were assessed under different conditions. Based on the previous results, both the profiles of enzymatic hydrolysis and the composition of the different autohydrolysis fractions, the solid from the pretreatment carried out at a S_0 of 4.04 was selected; this represents, simultaneously, the point of maximum polysaccharides recovery and maximum glucose production from enzymatic hydrolysis (Fig. 3B). To evaluate either the possible occurrence of end-product inhibition of cellulases by glucose or, on the other hand, a lack of nutrients in the beginning of fermentation, cells addition was tested at different times of hydrolysis, namely at the beginning of fermentation (SSF1, Table 3) or after 24 h of pre-saccharification at the optimal conditions of cellulase activity (PS_{24}-SSF1, Table 3). A pre-saccharification (or liquefaction) step has been used to improve mass transfer and to reduce mixing issues during the SSF [52]. This liquefaction can be carried out during short times (e.g. 6 h), as described by Castro and co-workers [53], who reported a 24.3 g/L of ethanol production from *Eucalyptus benthamii* treated by a dilute-phosphoric-acid steam pretreatment. Nevertheless, operating under high solid loadings and/or with highly viscous suspensions would probably require a longer duration of this step.

As we can observe from Fig. 4, the maximum concentrations of ethanol produced from the two strategies were very similar (25.81 and 25.72 g/L for SSF1 and PS_{24}-SSF1, respectively). Nevertheless, for the experiment SSF1 the peak of ethanol concentration was achieved at 72 h, requiring a considerably inferior amount of time, hence resulting on higher ethanol productivity (0.36 vs 0.16 g/L h; Table 3). This may have resulted from an attenuated end-product inhibition of cellulases by glucose and, to a lower extent, an enhancement of solids liquefaction due to a superior initial volume. Indeed, since these tests were already conducted with a solids consistency of 15% (w/v), some level of end-product inhibition can be expected; also, given the high water-retention capacity of this material, the suspensions employed on these tests presented a high viscosity, with a very small volume of free liquid (*data not shown*). The addition of cells at an early moment (SSF strategy), when there is a minimal availability of sugars, hence showed no visible adverse effects on yeast performance. On the other hand, conducting a pre-saccharification period (under optimal conditions) for 24 h showed, in a similar way, no significant benefits for process efficiency, suggesting that the possible gains from carrying out an hydrolysis step at the optimal conditions (for the hydrolytic activity of cellulases) were surpassed by those coming from a reduced end-product inhibition of cellulases. Overall, even though these ethanol levels are inferior to others reported on studies of lignocellulosic ethanol production [54], they are already comparable to what has been achieved so far using eucalyptus bark residues [22]; further improvements may be achieved if particular limitations could be addressed.

It is worth noting that yeast cells may have performed under sub-optimal conditions, as suggested by the profiles of glucose concentration. In fact, the values of ethanol conversion yield for both cases were around 55% of its theoretical value (Table 3). For the case following a PS-SSF approach (PS_{24}-SSF1), the peak of glucose was achieved at 72 h of hydrolysis, only 48 h after cells addition; also, after 144 h of fermentation, there was still a glucose residual of 12 g/L. This seems to suggest that cells consumed glucose at a very small rate, which opposes to the well-known high-performance of this strain [34]. Additionally, even though glucose consumption seemed to occur much faster in the SSF strategy, after a specific time cells stopped to consume glucose, leaving a residual of approximately 11 g/L (Fig. 4). These facts may suggest a possible lack of nutrients required by cells, either for growth or for the fermentation routes. It is most relevant to refer that, apart from the sugars released from the hydrolysis of the lignocellulosic solids, no other nutrients were added to the solid suspensions. In a previous work by Kelbert et al. [55] on the hydrolysis and fermentation of autohydrolyzed *E. globulus* wood, the authors observed that by supplementing the medium with different nutrients (e.g. corn steep liquor, urea, cheese whey and different salts), the ethanol levels achieved by the industrial *S. cerevisiae* PE-2 strain had a 7-fold increase.

Taking into account these results, and in the overall aim of process intensification, a second set of experiments (SSF2 and SSF3 and PS_{48}-SSF2 and PS_{48}-SSF3) was conducted encompassing the nutritional supplementation of the solids mixture but also the utilization of a superior solids consistency (17.5%, opposing to 15% solids in the previous tests) and an extended pre-saccharification period (48 h), to obtain higher

Table 3
Experimental results and kinetic parameters of several strategies of SSF and PS-SSF with and without nutritional supplementation for ethanol production from EBR pretreated at S_0 of 4.04.

Experiment	Operational conditions	Experimental results				Kinetic parameters			
		Solid load (%)	$[Ethanol]_{max}$ (g/L)	$Yield_{ethanol}$ ($\%_{theoretical}$)	Q_{P-72} (g/L h)	P_r (h^{-1})	P_{max} (g/L)	F	R^2
SSF1	No nutrients addition	*15*	25.81 ± 0.83	55.26	0.36	0.246	23.90	220.7	0.969
PS_{24}-SSF1		*15*	25.72 ± 1.77	55.06	0.16	0.033	26.93	520.6	0.936
SSF2	Supplementation with peptone + yeast extract	*15*	27.13 ± 1.01	59.59	0.38	3.474	25.32	5.78	0.982
PS_{48}-SSF2		*15*	35.55 ± 0.69	78.10	0.49	0.262	35.74	22.55	0.799
SSF3		*17.5*	33.43 ± 1.82	64.28	0.46	3.474	31.26	15.67	0.933
PS_{48}-SSF3		*17.5*	38.03 ± 0.33	73.14	0.52	0.271	38.19	49.87	0.851

Q_{P-72} – Ethanol productivity for a process time of 72 h.

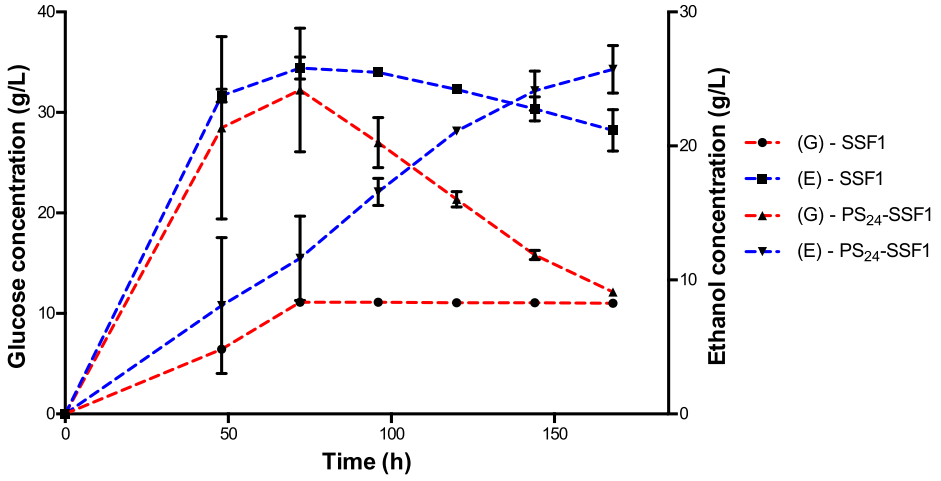


Fig. 4. SSF and PS_{24}-SSF experiments without nutritional supplementation of EBR pretreated at S_0 of 4.04, using 15% of solids and 20 FPU/g_{solid}.

ethanol concentrations. As Fig. 5 suggests, the addition of specific concentrations of peptone (20 g/L) and yeast extract (10 g/L) showed to be very efficient on tackling some of the cells nutrient limitations. This was particularly visible from the profiles of glucose concentration for the PS-SSF strategy (Fig. 5B) where glucose is reduced to nearly 0 g/L after only 24 h of cells addition. Additionally, there was a global increase in the values of ethanol production yield, ranging now between 60 and 78% (Table 3), possibly reflecting a general improvement on cells metabolism. It is also most interesting to observe that after 72 h, the ethanol concentration started to gradually decrease, which may be largely explained by ethanol being consumed by yeast cells as result of glucose depletion (diauxic shift).

A slight increase in solids loading to 17.5% (w/v) showed to be feasible despite the increased mechanical constraints from a higher viscosity. Interestingly, and opposing to what would be expected for a higher solids loading [56], the ethanol production yield increased from 60 to 64% in the case of an SSF approach (SSF2 and SSF3, Table 3). We hypothesize that a superior initial glucose production (enabled by a higher solids loading) could have represented a slight advantage for cells growth and posteriorly for ethanol production. Opposing to that, comparing the PS_{48}-SSF2 and PS_{48}-SSF3 strategies, the ethanol production yield decreased from 78 to 73% when solids consistency increased, as it would be expected. Since glucose concentration stabilized after 72 h of hydrolysis for both solid loadings (Fig. 5B), this reduction on ethanol yield did probably not result from kinetic constraints of the process, such as those one could expect from a superior end-product inhibition (from glucose) or higher mixing issues [57]. On the other hand, a superior solids loading possibly led to an increase of non-productive binding of cellulases onto lignin [58], which accounts for approx. 29% of EBR composition, hence affecting final cellulose hydrolysis to a greater extent.

In what regards the extension of the pre-saccharification period, clear improvements were also observed on process efficiency (Table 3). Specifically, by deferring cells addition to 48 h (PS_{48}-SSF experiments), maximum ethanol concentrations increased from 27 to 36 g/L for a solids consistency of 15% (SSF2 vs PS_{48}-SSF2) and 33 to 38 g/L for a solids consistency of 17.5% (SSF3; PS_{48}-SSF3). This is particularly interesting since for a pre-saccharification period of 24 h no clear benefits were observed, adding to the fact that pre-saccharification periods as small as 6 h have already been successfully reported [53]. Although a direct comparison can not be established between the two pre-saccharification periods, this result combined with the fact that solids suspensions only reach liquefaction after 24 h may suggest that a pre-saccharification of 24 h is not sufficient to obtain significant improvements. Before this point, solids suspensions present a very high viscosity, meaning a very small amount of free liquid available for cellulases mobility, therefore hampering their action towards the solid.

Finally, the ethanol profiles discussed above were fitted to the model expressed in equation (6), and different kinetic parameters were estimated (Table 3). The coefficient of determination R^2 was higher than 0.9 for all assays, except for experiments PS_{48}-SSF2 and 3, showing a good relationship between experimental and calculated data; additionally, the statistical significance of the models is supported by Fischer's F parameter. As a general trend, the fermentation rates of ethanol (P_r) for SSF experiments were 7 and 13 fold higher compared to those obtained for PS-SSF experiments conducted without and with nutrients added, respectively, which is probably related to the time of yeast cells addition. The fermentation rate was clearly improved with the addition of nutrient supplements, increasing from 0.246 to 3.474 h^{-1} for SSF experiments at 15% solids. Nevertheless, PS-SSF was more suitable to achieve a higher P_{max} of ethanol (38 g/L). David and co-workers [24] have also evaluated the kinetic parameters of ethanol production from

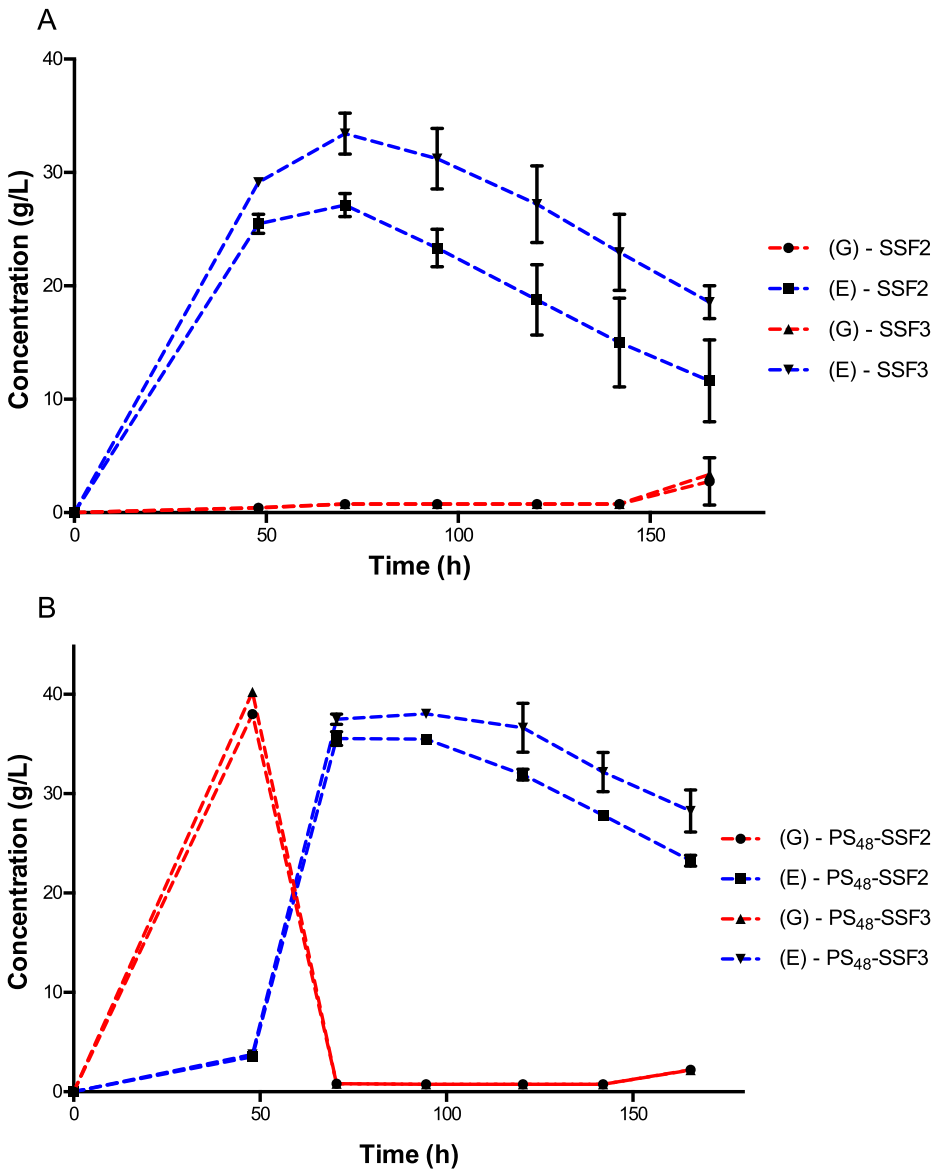


Fig. 5. SSF (A) and PS_{48}-SSF experiments (B) with EBR pretreated at S_0 of 4.04, using 15% (SSF2 and PS_{48}-SSF2) and 17.5% (SSF3 and PS_{48}-SSF3) of solid loading, with nutritional supplementation.

pretreated corn cob using several strategies (SHF, SSF and PSSF) and employing the laboratory strain *S. cerevisiae* BY4743. The authors observed that, while the PSSF process enabled the highest maximum ethanol production rate (3.08 g/L/h), the SHF process achieved the highest value of maximum ethanol production (P_{max} of 26.82 g/L). The best strategy for ethanol production hence seems to be case-specific, being dependent of numerous factors such as the raw material, the pretreatment, the solid and enzyme loadings (with great influence in the viscosity and rheology of the system), among others [59–61].

Overall, interesting ethanol concentrations were obtained in this study, which can still be improved through further process intensification. Nevertheless, it is most relevant to refer that they already correspond to the highest levels of ethanol reported so far for this specific raw material; while most of the available studies report the utilization of eucalyptus wood, only 33 g/L were reported by Romaní et al. [22] using organosolv pretreated barks from *Eucalyptus nitens*.

3.5. Overall mass balance of EBR valorization

In the scope of the biorefinery concept, all fractions from a lignocellulosic material should be valorized. Considering the previous strategies for the hydrolysis and fermentation of EBR resulting on the highest ethanol productions, the potential amounts of relevant compounds were estimated for a specific quantity of raw material (Fig. 6). From an initial amount of 100 kg of EBR, an autohydrolysis pretreatment at S_0 of 4.04 results in 69.5 kg of pretreated solid. Most of the economic value of the liquor would come from oligomers (14.2 kg), especially *xylo*-oligomers, used as a functional food ingredient. Regarding the monomer sugars, their utilization by fermentation will not be very feasible as they are present in very small concentrations (1.30 g/L glucose; 2.59 g/L xylose); in this context, a stage of post-hydrolysis could be an interesting option to convert the oligomers fraction into higher levels of monomers (e.g. xylose can be further converted into xylitol or furfural).

The pretreated solid can be hydrolyzed, using a commercial cellulases cocktail (Cellic Ctec2), and the released sugars further fermented, namely into ethanol. Depending on how these two processes are conducted, the process requirements for energy and chemicals and the amounts of ethanol produced will be different. Overall, the utilization of a PS-SSF strategy enables the production of the highest amounts of

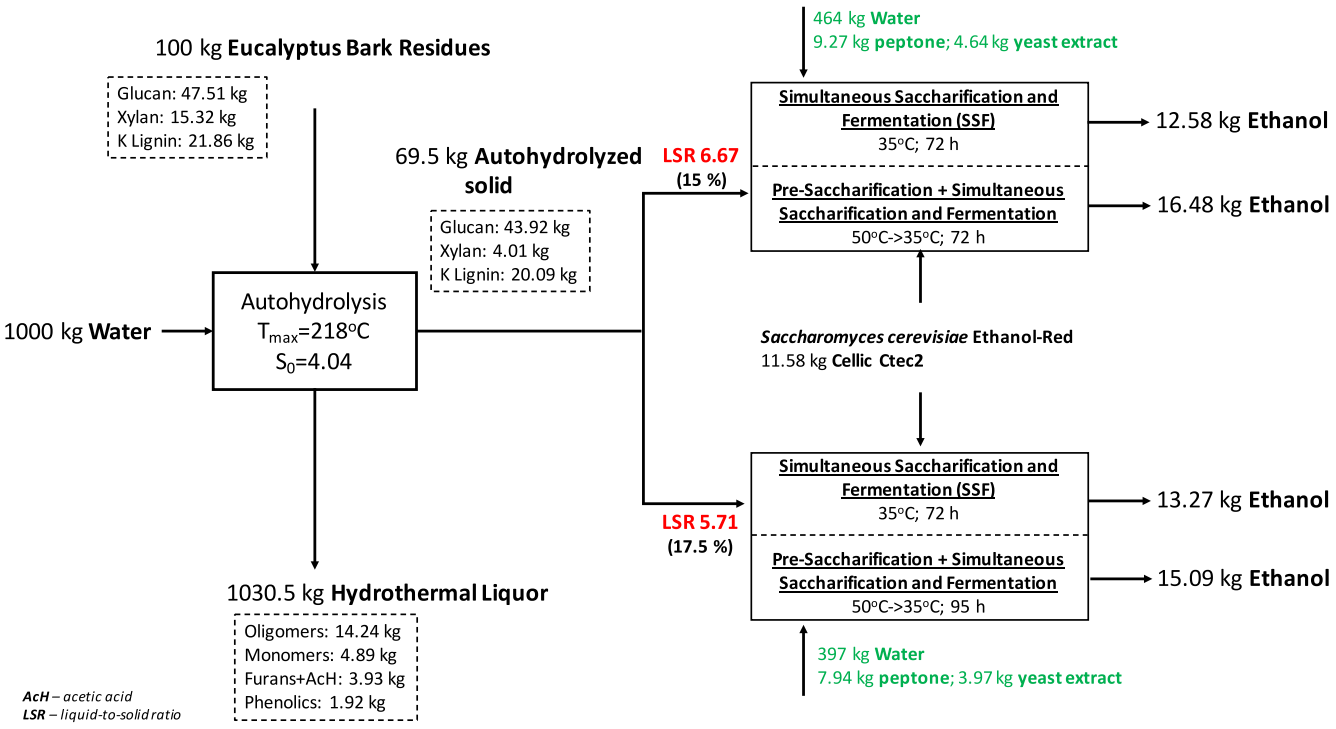


Fig. 6. Overall mass balance for distinct scenarios of hydrolysis and fermentation of pretreated EBR.

ethanol: for a solids consistency of 15%, 16.48 kg of ethanol can be produced per 100 kg of raw material, decreasing to 15.09 kg for a consistency of 17.5%. It worth noting, however, that the working volume for the latter case is inferior, which will not only imply lower water requirements and equipment costs, but even more important, reduced distillation costs.

4. Conclusion

The integral valorization of *Eucalyptus globulus* bark using autohydrolysis was here assessed following a biorefinery scheme. A pretreatment S_0 ranging 3.69–4.25 enabled the production of interesting levels of oligosaccharides (95–145 kg/ton$_{bark}$) and phenolic compounds (14.3–22.8 kg/ton$_{bark}$), while also resulting on a highly suitable solid to be further converted into chemicals or energy. Under selected conditions of pretreatment severity and applying a Pre-Saccharification-SSF strategy, 151–165 kg$_{ethanol}$/ton$_{bark}$ were produced, corresponding to the highest levels reported so far for this raw-material. This work hence showed the significant economic potential of eucalyptus bark residues, which can be efficiently accessed through an autohydrolysis pretreatment.

CRediT authorship contribution statement

Daniel G. Gomes: Conceptualization, Writing - original draft, Validation, Formal analysis. **Michele Michelin:** Conceptualization, Writing - original draft, Validation, Funding acquisition. **Aloia Romaní:** Conceptualization, Writing - original draft, Validation, Funding acquisition. **Lucília Domingues:** Validation, Funding acquisition. **José A. Teixeira:** Validation, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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