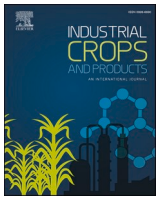
Industrial Crops & Products 175 (2022) 114253

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Co-production of bioethanol and xylosaccharides from steam-exploded eucalyptus sawdust using high solid loads in enzymatic hydrolysis: Effect of alkaline impregnation

Eloísa Rochón [a,*], María Noel Cabrera [b], Valentina Scutari [a,c], Matías Cagno [c], Abigail Guibaud [b,c], Santiago Martínez [c], Silvia Böthig [c], Nikolai Guchin [d], Mario Daniel Ferrari [a], Claudia Lareo [a]

[a] Departamento de Bioingeniería, Facultad de Ingeniería, Universidad de la República, 11300 Montevideo, Uruguay
[b] Grupo de Ingeniería de Procesos Forestales, Facultad de Ingeniería, Universidad de la República, 11300 Montevideo, Uruguay
[c] Centro de Investigaciones en Biocombustibles 2G, ANCAP/Latitud – Fundación LATU, Edificio Los Abetos, 11500 Montevideo, Uruguay
[d] ANCAP, Desarrollo de Energías Renovables, Paysandú s/n y Avenida del Libertador, 11100 Montevideo, Uruguay

ARTICLE INFO

Keywords:
Alkaline impregnation
Bioethanol
Eucalyptus grandis
Steam explosion
Xylosaccharides

ABSTRACT

In this work, *Eucalyptus grandis* sawdust was investigated as raw matter for the co-production of xylosaccharides (XS) and bioethanol by steam explosion pretreatment with and without a previous NaOH impregnation stage. The acetyl groups were completely removed in the NaOH impregnation step, which inhibited the autohydrolysis during the steam explosion pretreatment. The best results, in terms of XS recovery and glucan content in the pretreated substrate, were obtained when *E. grandis* was subjected to steam explosion at 200 °C for 10 min without alkaline impregnation. 76 kg of XS per ton of sawdust were obtained from the hemicellulosic hydrolysate and were mostly xylose or short xylo-oligosaccharides (XOS) (83% and 17%, respectively). High hydrolysis efficiencies were obtained using high solids loading (27%) with the steam pretreated material. High ethanol concentrations (75.6 g/L) and yields (259 L per ton of dry raw sawdust) were obtained by simultaneous saccharification and fermentation (SSF), representing a suitable strategy for ethanol production from eucalyptus sawdust.

1. Introduction

Recently, interest in the production of chemicals and fuels from renewable feedstocks, such as lignocellulosic materials, within a bio-based and low-carbon economy approach has increased to reduce climate change and global warming (Mussatto and Dragone, 2016; Romaní et al., 2013). Among these materials is eucalyptus, which has high cellulose (40.6–48.4%) and hemicellulose (13.7–16% of xylan) content and compositional uniformity (Cebreiros et al., 2021; Guigou et al., 2019; Romaní et al., 2012). Furthermore, eucalyptus wood is one of the most abundant wood species in Uruguay, and its use in producing cellulose pulp is rapidly increasing (Guigou et al., 2019). In 2015, Uruguay produced approximately 2,600,000 tons of eucalyptus pulp, and it is expected to almost double its production by 2022 (Uruguay XXI, 2021). Therefore, the amount of sawdust produced, a by-product or waste product from local pulp and paper industries, will also increase. Currently, three sawmills process 365,000 m^3 of eucalyptus wood per year, generating a residue of almost 40% of the processed wood. These residues are mainly used as fuels to generate steam and energy for the processes and also to be sold to the national electric grid (Uruguay XXI, 2021).

Bioethanol production from lignocellulosic materials has been well studied in recent years. Different pretreatments are used to deconstruct the internal structure of lignocellulosic materials and enable access to the sugars in the cellulosic fraction. Alkali, acid, hydrothermal, and steam explosion pretreatments are some of the technologies currently used. Among them, steam explosion is one of the most utilized because it presents advantages such as low environmental impact, high energy efficiency, less hazardous process chemicals, high sugar recovery, and good effectiveness in removing hemicellulose (Alvira et al., 2010; Bonfiglio et al., 2019; Cebreiros et al., 2021; Chiarello et al., 2016; Martín-Sampedro et al., 2012; Molaverdi et al., 2021; Risso et al., 2020).

* Corresponding author.
E-mail address: merochon@fing.edu.uy (E. Rochón).

https://doi.org/10.1016/j.indcrop.2021.114253
Received 28 July 2021; Received in revised form 7 October 2021; Accepted 5 November 2021
Available online 16 November 2021

After the explosion, a solid and a liquid phase are obtained. The solid phase is composed mainly of cellulose and lignin. The liquid phase (hydrolysate), depending on the treatment severity, is mainly composed of xylose and xylo-oligosaccharides (XOS), with a low degree of polymerization (DP). Also, acetic acid, furfural, and other degradation products with commercial interest could be formed. Even though xylose and XOS are raw materials for xylitol and furfural production processes (Bonfiglio et al., 2021; Clauser et al., 2016), XOS are gaining interest as prebiotics for human and animal feed (Carvalho et al., 2016; Mäkeläinen et al., 2010; Míguez et al., 2021). In addition, XOS with a low degree of polymerization have a higher market price than xylose. Therefore, designing the process to optimize XOS production can make second-generation ethanol production economically viable. On the other hand, alkaline pretreatments are frequently used to enhance the enzymatic cellulose hydrolysis of several lignocellulosic materials, being efficiently used with hardwoods (Kim et al., 2019; Singh et al., 2015). Besides of alkaline pretreatment, there are various pretreatment strategies that can be applied to enhance the enzymatic hydrolysis (Shen et al., 2021; Tian et al., 2017). In alkaline pretreatments, the hemicelluloses are removed into liquor in polymeric form, allowing them to be used to produce new materials such as biofilm or biogels, which require polymers with high DP (Carvalho et al., 2016). Sodium hydroxide, as an alkaline catalyst, offers a good delignification capacity and a low inhibitor production (McIntosh and Vancov, 2010; Singh et al., 2015). Also, it has been reported that solid recovery after steam explosion treatment is high when the material is previously impregnated with NaOH (Park et al., 2012). Thus, a combination of alkaline and steam explosion pretreatments could combine the advantages of both methods. In this work, the steam explosion of eucalyptus sawdust impregnated with alkali was studied as a pretreatment method in order to recover the hemicelluloses in the liquor in polymeric form, remove the lignin and increase the cellulose swelling to enhance the enzymatic hydrolysis.

The enzymatic hydrolysis of the cellulosic fraction (pretreated solid) is an important step in the production of bioproducts obtained from the fermentable sugars of these lignocellulosic materials. The use of high solid loadings ($\geq$ 15%) presents many advantages compared with lower solid loadings (Modenbach and Nokes, 2012). It improves the overall productivity, decreases the energy consumption in product purification, and decreases both the production and capital costs (Chiarello et al., 2016; Larnaudie et al., 2019a; Modenbach and Nokes, 2012). By using high solid loadings in enzymatic hydrolysis, high sugar concentration is obtained, which is necessary to obtain a high ethanol concentration in the fermentation. However, it is also a challenge because of mass transfer limitations, inhibition effects from products derived from the pretreatment on both hydrolysis and fermentation steps, hydrolysis inhibition due to high sugar concentration, and process handling problems because of high viscosity (Larnaudie et al., 2019b; Pinheiro et al., 2019).

This work studies the co-production of xylosaccharides (XS) and bioethanol from *Eucalyptus grandis* sawdust pretreated by steam explosion with and without a previous alkaline impregnation stage. XS obtained from the hemicellulosic hydrolysates from steam explosion pretreatments were evaluated in terms of yields and their DP distribution. The solid fraction was then subjected to enzymatic hydrolysis in which the effects of solid loading and enzyme dosage were studied. After the pretreatment and enzymatic hydrolysis conditions had been defined, separate hydrolysis and fermentation (SHF) and pre-saccharification followed by simultaneous saccharification and fermentation (PSSF) and simultaneous saccharification and fermentation (SSF) process configurations were evaluated.

2. Materials and methods

2.1. Preparation and characterization of raw material

E. grandis sawdust, provided by a local sawmill, was used as the raw material (Urufor, Rivera, Uruguay). The sawdust was kiln-dried at 40 °C until reaching a humidity of approximately 15% and stored at room temperature. The sawdust particle size was 19% > 2 mm, 33% between 1 mm and 2 mm, 27% between 1 mm and 0.5 mm, and 21% under 0.5 mm.

2.2. Steam explosion pretreatment without and with alkaline impregnation

Before the steam explosion pretreatment, eucalyptus sawdust was mixed with a NaOH solution in a solid-liquid ratio of 1:4 using a helicoidal stirrer for 30 min and kept in a conditioning chamber at 23 °C for 20 h. After impregnation, the solid fraction was separated by press filter processing (20 MPa) and dried in a solar oven for two days until reaching a humidity of approximately 25% before entering into the steam explosion equipment. Different dosages of NaOH were tested using solutions with concentrations of 0, 10, and 20% (w/w), respectively. For the assays that were not alkaline impregnated, the sawdust was water-impregnated to reach approximately 25% moisture content and left overnight.

A semi-continuous pre-pilot equipment (Advance Bio Systems LLC, model S1401-D2011) installed at the Pilot Plant of Latitud in the Technological Laboratory of Uruguay (LATU, Montevideo) was used for steam explosion pretreatment. The steam explosion was carried out at different temperatures (180, 190, and 200 °C) for 10 min. After the pretreatment, the solid and liquid fractions (pretreated eucalyptus sawdust and extracted liquor, respectively) were separated by filter pressing (20 MPa). The pretreated solid was washed with water three times (5:1 ratio of water to pretreated solid) and separated from the washing waters by filter pressing. The washed pretreated eucalyptus solid was then characterized for total solids, carbohydrates, and lignin according to NREL protocols and stored at 4 °C until its use in enzymatic hydrolysis experiments. The liquid fractions collected referred to as hemicellulosic hydrolysate (the extracted liquor, the washing waters, and the condensed liquid from the released steam) were kept for the later analysis of products derived from hemicellulose. Selected extracted liquors were used to separate XS and their subsequent chemical characterization.

The pretreatment severity factor (S_0) was calculated as follows:

$$S_0 = log\left(t \quad e^{\frac{T-100}{14.75}}\right) \tag{1}$$

with t the residence time (min), T the reaction temperature (ºC), and 14.75 a fitted value (Overend et al., 1987). A combined severity factor (CS_0) was used to take into account the effect of alkaline conditions (Park et al., 2012):

$$CS_0 = log\left(t \quad e^{\frac{T-100}{14.75}}\right) - pOH = log\left(t \quad e^{\frac{T-100}{14.75}}\right) + log[OH^-] \tag{2}$$

2.3. XS precipitation

The XS fraction was separated from selected extracted liquors from the steam explosion pretreatment by ethanol precipitation according with the conditions optimized elsewhere (Cabrera, 2021). The characteristics of the precipitates obtained were studied for their further use as a xylan source. For this purpose, 100 mL of the liquor were used. The pH was adjusted to 7.0 ± 0.1 with 6 M HCl, and the precipitated fraction basically composed of lignin fragments was separated by centrifugation at 3000 *g* for 20 min. 80 mL of the resulting liquid were mixed with ethanol (95.5%) in a 1:1 ratio (by volume) with magnetic stirring for 30 min. After 48 h at 4 °C, the solution was centrifuged at 3000 *g* for 20 min, and the precipitated obtained was washed with fresh ethanol and dried at 40 °C until achieving a constant weight.

2.4. Enzymatic hydrolysis of pretreated eucalyptus

2.4.1. Enzymatic hydrolysis of the pretreated eucalyptus without and with alkaline impregnation: Preliminary tests

The pretreated eucalyptus was enzymatically hydrolyzed using Cellic CTec 2 (Novozymes) purchased from Sigma Aldrich® (cellulase activity 160 and 125 FPU/mL for solids without and with alkaline impregnation, respectively). The solid loading and enzyme dosage were 15% (w/w) and 30 FPU/gglucan, respectively. The enzymatic hydrolysis was performed in 250-mL Erlenmeyer flasks containing 100 mL of the suspension at 50 °C and pH 4.85 (using 0.05 M citric acid-sodium citrate buffer) with orbital agitation at 150 rpm (Infors HT Ecotron, Switzerland). Samples were taken every 24 h until 96 h and at 168 h. For glucose concentration analysis, the supernatants (cellulosic enzymatic hydrolysate) were heated at 95 °C to inactivate the enzymes and centrifuged at 7100 *g* for 30 min to remove the solid wastes. The tests were conducted in duplicate.

The enzymatic hydrolysis efficiency was calculated as:

$$\text{Cellulose hydrolysis } efficiency(\%) = \frac{(G - G_0)}{\frac{Gn}{100}\bullet 1.11\bullet}\frac{[solid]}{100}\bullet\rho\bullet 100 \quad (3)$$

where G and G_0 are the final and initial glucose concentration (g/L), respectively, in the enzymatic hydrolysis assays, V is the liquid volume (L), Gn is the glucan content of the pretreated solid (g/100 g dry pretreated solid), 1.11 is the factor to convert glucan to glucose, [solid] is the dry solid concentration at the beginning of the enzymatic hydrolysis (g dry pretreated solids per 100 g of liquid), and ρ is the density of the liquid fraction (g/L).

The pretreated solid with alkaline impregnation, which achieved the best performance in the enzymatic hydrolysis preliminary tests, was enzymatically hydrolyzed using Cellic CTec2 (cellulase activity 200 FPU/mL) at different solid loadings (18–20%), with and without the addition of polyethylene glycol 6000 (PEG 6000) at a rate of 0.05 $g/g_{dry\ pretreated\ solid}$ as an enhancer of the hydrolysis efficiency cellulose (Camesasca et al., 2015). The enzymatic hydrolysis was performed as previously described for 168 h. Samples were withdrawn every 24 h until 96 h and at 168 h. The tests were conducted in duplicate.

2.4.2. Enzymatic hydrolysis of pretreated eucalyptus without alkaline impregnation: Solid loading and enzyme dosage evaluation

The effect of solid loading and enzyme dosage on the glucose concentration reached and enzymatic hydrolysis performance were evaluated using a rotational central composite design (RCCD) ($\alpha = 1.414$) with four repetitions of the central point and including axial points. The pretreated solid without alkaline impregnation, which achieved the best results in the preliminary enzymatic hydrolysis tests (2.4.1), was enzymatically hydrolyzed using Cellic CTec2 (cellulase activity 160 FPU/mL) at different solid loadings (15–25%) and enzyme dosages (10–25 FPU/g_{glucan}) to maximize glucose concentration with a high hydrolysis efficiency. The enzymatic hydrolysis was performed as previously described (2.4.1) with 50 mL of the suspension for 96 h. Samples were taken at 48, 72, and 96 h for analysis. For the hydrolysis assays with a solid content $\geq$ 25%, an extra sacrificed flask was used in order to have a representative sample due to high solid content.

An analysis of variance (ANOVA) was utilized to statistically evaluate the effects of the enzyme dosage (ED) and the solid loading (SL) on glucose concentration (G). The ED, SL, and G were expressed in FPU/g_{glucan}, % (w/w), and g/L, respectively. Effects were considered significant when $p < 0.05$. The variables ED and SL were normalized and coded as x_1 and x_2, respectively:

$$x_1 = \frac{ED - 17.5}{7.5} \quad (4)$$

$$x_2 = \frac{SL - 20}{5} \quad (5)$$

2.5. Microorganism and inoculum preparation

Dry *Saccharomyces cerevisiae* (Fleischmann, Uruguay) was used in all the fermentations assays. It was reactivated using a medium with 60 g/L of glucose, 3 g/L yeast extract, 3 g/L malt extract, and 5 g/L peptone in 500-mL Erlenmeyer flasks with 250 mL of medium. The pH was adjusted to 4.5, and it was sterilized at 121 °C over 15 min. It was incubated in an orbital shaker at 30 °C and 150 rpm for 14–16 h.

2.6. Fermentation of pretreated eucalyptus without alkaline impregnation

2.6.1. SHF configuration

The pretreated eucalyptus was enzymatically hydrolyzed using Cellic CTec 2 (cellulase activity 125 FPU/mL). It was performed for 48 h as previously described in 2.4.1. The solid loading was 27% (w/w) and enzyme dosage 25 FPU/g_{glucan}.To remove the solid wastes, the supernatants were centrifuged and collected for analysis. The experiments were performed in duplicate.

Ethanol fermentation by SHF configuration was performed in 250-mL Erlenmeyer flasks with 100 mL of enzymatic hydrolysate containing 143.1 g/L glucose, 6.6 g/L cellobiose, and 0.3 g/L xylose. The pH was adjusted to 6.0 ± 0.1 with 2 M NaOH, and it was sterilized at 121 °C, 15 min. Then, 5% (v/v) of the sterilized nutrient solution (prepared 20X stock) was added. The nutrient solution contained 30 g/L yeast extract, 30 g/L malt extract, and 50 g/L peptone. It was then inoculated with a *Saccharomyces cerevisiae* suspension to reach 1×10^8 cells/mL. Experiments were performed in duplicate at 30 °C with orbital agitation at 100 rpm for 24 h. Routinely, samples were taken during fermentation for sugar and product analysis. The ethanol yield (EY) ($L_{ethanol}/ton_{dry\ sawdust}$) and the ethanol conversion (EC) ($g_{ethanol}/100\ g_{theoretical\ ethanol}$) were calculated as follows:

$$EY = \frac{E_t\bullet V}{\rho\bullet M} \quad (6)$$

$$EC = \frac{E_t}{0.511\bullet\frac{Gn}{100}\bullet}1.11\bullet\frac{M}{V}\bullet 100, \quad (7)$$

where E_t is the ethanol concentration at time t (g/L), V is the fermentation volume (L), ρ is the density of ethanol at 20 °C (789 g/L), M is the eucalyptus dry mass (tonne), 0.511 is the stoichiometric glucose into ethanol conversion factor, Gn is the glucan content of the pretreated solid (g/100 g dry pretreated solid), and 1.11 is the factor to convert glucan to glucose.

2.6.2. SSF and PSSF configurations

Fermentations were performed using the SSF and PSSF configurations in 250-mL flasks with 100 mL of 27% (w/w) of pretreated solid suspension supplemented with 5% (v/v) nutrient solution, citrate buffer solution at pH 4.85, and the same enzyme solution as used for SHF configuration at the same enzyme dosage (25 FPU/g_{glucan}) before inoculation with a *Saccharomyces cerevisiae* suspension to reach 1×10^8 cells/mL. For the PSSF experiments, a 24 h of pre-hydrolysis at 150 rpm and 50 °C was performed before inoculation took place. All experiments were performed in duplicate at 30 °C in an orbital shaker at 100 rpm for 24 and 48 h for PSSF and SSF, respectively. Routinely, samples were taken during fermentation for sugar and product analysis.

2.7. Analytical methods

The chemical composition of the raw and pretreated material was determined following NREL protocols (Sluiter et al., 2008a, 2008b, 2008d, 2008e).

The chemical composition and total solids content of the liquid fraction from steam explosion pretreatments were determined following NREL protocols (Sluiter et al., 2008a, 2008c, 2008d). Glucose,

cellobiose, ethanol, furfural, glycerol, HMF and organic acids (formic and acetic acids), were quantified by HPLC (Shimadzu, Kyoto, Japan) using RI and SP detectors and an Aminex HPX-87 H column (Bio-Rad Laboratories Ltd., USA) at 35 °C. H_2SO_4 (5 mM) was used as mobile phase at 0.6 mL/min. Glucose and xylose corresponded to the enzymatic hydrolysates of the NaOH-impregnated solids and were quantified with an Aminex HPX-87 P column operating at 80 °C with type I deionized water as eluent at 0.6 mL/min. The molecular weight distribution of XS in the non-impregnated liquid fractions was determined by HPLC-GPC (Shimadzu, Kyoto, Japan) using RI detector and an Aminex HPX-42A column (Bio-Rad Laboratories Ltd., USA) at 60 °C with type I deionized water as eluent at 0.6 mL/min. Standards of xylobiose (DP = 2), xylotriose (DP = 3), xylotetraose (DP = 4), and xylopentaose (DP = 5) were obtained from Megazyme Ltd. (Ireland), and xylose standard (DP = 1) was provided by Sigma-Aldrich.

The precipitates obtained after the ethanol addition were analyzed by FTIR-ATR (Shimadzu IR Affinity 1-S with an ATR Pike MIRacle accessory). The spectra were performed between the wavenumbers 4000–600 cm^{-1} with a resolution of 4 cm^{-1}; each spectrum is the result of 32 scans. A diamond ATR crystal with a diameter of 1.8 mm and ZnSe optical surface was used. Then, the precipitates were dissolved in NaOH 0.1 M, and the carbohydrate, lignin, and ash content were determined according to the above procedures. The molecular weight distributions were determined by HPLC-GPC (Shimadzu, Kyoto, Japan) with a set of 3 PSS® MCX 1000 Å columns in series with 2 PSS MCX 100 Å columns and an RI detector. Poly (styrene sulfonate) sodium salts (PSS®) were used as standards with the following molecular weights: 4230, 7930, 10600, 14900, 20700, 29100, and 64200 Da. A buffer solution of pH 12.0 with NaOH and NaH_2PO_4. H_2O was used as the mobile phase.

Scanning electron microscopy (SEM) was utilized to compare the morphology of the raw and pre-treated materials and evaluate the changes in the external structure caused by the pretreatments. A JEOL JSM5900 SEM operated with an accelerating voltage of 20 kV and 12 mm of working distance was utilized. Small sized samples were previously sputtered with a thin layer of gold.

The Cellic CTec 2 cellulase activity was determined following the NREL protocol for each new enzymatic extract vial (Adney and Baker, 2008).

The cellular concentrations (total and viable) were determined by microscope direct counting in a Neubauer chamber. Living and dead cells were quantified using methylene blue as a dye.

2.8. Statistical analysis

The ANOVA of the experimental data using Tukey's test by InfoStat software (student version 2013, Universidad Nacional de Córdoba, Argentina, http://www.infostat.com.ar) was performed to determine statistical differences ($p \leq 0.05$).

For the RCCD design assays, Statistica® software was used to perform ANOVA to determine statistical differences ($p \leq 0.05$). A linear regression was implemented after eliminating nonsignificant regression parameters. A good fit was considered when the model presented a significant regression coefficient (R^2) and nonsignificant lack of fit.

3. Results and discussion

3.1. Pretreatment of E. grandis sawdust

The *E. grandis* sawdust was composed mainly of carbohydrates (43.6 ± 2.1% glucan and 11.1 ± 0.5% xylan), followed by total (acid soluble and insoluble) lignin (30.5 ± 0.5%). The remaining constituents were acetyl groups (3.0 ± 0.2%), water-ethanol extractives (3.6 ± 0.8%), and ash (0.3 ± 0.1%). The composition was similar to that reported by other works on eucalyptus sawdust and eucalyptus wood (Cebreiros et al., 2021; Guigou et al., 2019; Romaní et al., 2012) and presented some minor variations with data reported for different eucalyptus species (Martín-Sampedro et al., 2012).

The chemical composition of liquor hydrolysates and pretreated solids is presented in Table 1. As expected, the solid fraction was enriched in glucan and lignin content by the steam explosion pretreatment compared to the untreated *E. grandis* sawdust (Table 1) due to a high hemicellulosic fraction (mainly xylan and acetyl groups) removal. The principal constituents of the pretreated solids were glucan (59–63% w/w) and lignin (32–46% w/w). No acetyl groups were detected in any of the pretreated solids, probably due to their extensive solubilization in the pretreatment stages. Xylan was not detected in the pretreated solids without NaOH impregnation. However, it was hardly removed in the solids that were impregnated with NaOH, reaching xylan contents of 7–9% w/w in the pretreated solids. This could be due to the low content of hydronium ions present during the steam explosion, which can be attributed to the high removal of acetyl groups achieved (87%) during the previous NaOH impregnation step. This was consistent with the pH values found in the hemicellulosic hydrolysates, which were in the range 5–6 and 2.5–3 in the solids impregnated and not impregnated with NaOH, respectively.

Fig. 1 shows the SEM images of the raw fibers and steam-exploded fibers with and without NaOH impregnation. A low level of damage can be observed in the structure of the impregnated solids, which is consistent with the low hemicellulose solubilization achieved in this pretreatment, as previously mentioned.

The hemicellulosic hydrolysates from the non-impregnated solids contained xylose, xylo-oligosaccahrides, acetic acid, glucose, glucosaccharides, furfural, and lignin fragments (Table 1). As expected, the furfural concentration increased with the temperature in detriment of the XS concentration. A small number of glucosaccharides was also found. Fig. 2 shows the relative molecular weight distribution of the XS in the extracted liquors. At 180 °C, xylose accounted for 60% of the XS, which increased to almost 70% at 200 °C. At this temperature, less than 3% of the XS had a DP of 5 or higher. The three wash waters of each condition present the same distribution profile as the corresponding extracted liquor.

The components found in the extracted liquor and in the washing liquors revealed that three washing stages to the solid fraction were necessary to remove possible inhibitors (i.e., acetic acid, lignin fragments, furfural) and to recover the solubilized components. In the liquid fractions from the non-impregnated solids, at least 75% of the XS, xylose, and XOS were recovered in the extracted liquor and the washing water from the first washing stage. The acetyl groups were found mostly in the extracted liquor as acetic acid and in the first washing water (at least 60% of the total acetyl groups); however, an important fraction was found in the condensates (more than 15%). Furfural was mainly found in the condensate stream, which accounts for at least 70% of the total recovered furfural. The volatile compounds were swept by the steam released from steam explosion and partially recovered in the condensates in the condensation chamber, which was not completely closed. According to the mass balance, 20–70% of the furfural produced was not recovered. Hydrolysates obtained by steam explosion with NaOH impregnation contained XS with molecular weights greater than 2000 Da.

During the processing of the NaOH-impregnated solids, an additional liquid stream is obtained from the impregnation step. This stream was composed mainly by acetic acid and XS. Deacetylation has been reported as one of the main reactions taking place during the alkaline treatment at ambient temperature for bioethanol production (Chang and Holtzapple, 2000), also in alkaline or) alkaline chemi-mechanical pretreatment of wood in the pulp industry (Inalbon et al., 2009, Zanuttini and Marzocchi, 1997), and more recently the effect of a previous deacetylation was reported as beneficial for bioethanol production due to the deacetylated solid being more susceptible to enzymatic hydrolysis (Castro et al., 2017). The XS content in the impregnated liquors was 2.6% of dry sawdust (20% of xylan content of the raw material) at the most concentrated alkaline conditions used, which was comparable with

Table 1
Chemical composition of steam exploded-pretreated *Eucalyptus grandis* sawdust (10 min residence time) without and with previous NaOH impregnation (solid-liquid ratio of 1:4 at 23 °C for 20 h): solid and liquid fraction.

		Without NaOH impregnation		With NaOH impregnation[a]		
Components	Unit	180 °C	200 °C	180 °C, 20% w/w	190 °C, 10% w/w	200 °C, 20% w/w
Liquid fraction						
Gluco-oligomers	g glucose equivalent/100 g sawdust	0.34 ± 0.02	0.25 ± 0.02	0.12 ± 0.02 (0.18 ± 0.02)	0.03 ± 0.01 (0.18 ± 0.02)	0.06 ± 0.02 (0.16 ± 0.02)
Xylo-oligomers	g xylose equivalent/100 g sawdust	3.9 ± 0.4	1.3 ± 0.2	2.0 ± 0.3 (2.7 ± 0.4)	1.5 ± 0.2 (1.3 ± 0.2)	2.4 ± 0.3 (2.6 ± 0.3)
Glucose	g/100 g sawdust	0.38 ± 0.02	0.80 ± 0.03	0.04 ± 0.01 (nd)	nd	0.15 ± 0.03 (nd)
Xylose	g/100 g sawdust	6.9 ± 0.3	6.3 ± 0.2	0.48 ± 0.05 (nd)	0.14 ± 0.03 (nd)	0.70 ± 0.05 (nd)
Acetic acid	g/100 g sawdust	4.0 ± 0.2	4.0 ± 0.2	0.63 ± 0.08 (2.6 ± 0.2)	0.76 ± 0.10 (2.6 ± 0.2)	1.4 ± 0.2 (2.6 ± 0.2)
Formic acid	g/100 g sawdust	0.73 ± 0.09	0.90 ± 0.09	0.98 ± 0.10 (nd)	0.21 ± 0.03 (nd)	1.6 ± 0.1 (nd)
5-HMF	g/100 g sawdust	0.16 ± 0.04	0.38 ± 0.07	n.d.	n.d.	n.d.
Furfural	g/100 g sawdust	0.38 ± 0.06	3.0 ± 0.1	0.05 ± 0.01 (nd)	n.d.	n.d.
Acid insoluble + soluble lignin[b]	g/100 g sawdust	3.1 ± 0.2	2.7 ± 0.2	0.46 ± 0.03	6.3 ± 0.3	5.5 ± 0.3
Solid fraction						
Glucan	g/100 g treated solid	59.4 ± 0.6	60.1 ± 0.2	61.0 ± 3.1	62.8 ± 1.1	59.7 ± 3.2
Xylan	g/100 g treated solid	nd	nd	9.4 ± 1.2	8.3 ± 1.1	7.4 ± 2.1
Acetyl groups	g/100 g treated solid	nd	nd	nd	nd	nd
Acid insoluble lignin	g/100 g treated solid	34.5 ± 1.5	44.5 ± 0.0	29.4 ± 2.0	29.4 ± 1.4	31.0 ± 1.8
Acid soluble lignin	g/100 g treated solid	2.1 ± 0.2	1.9 ± 0.0	3.3 ± 0.1	2.9 ± 0.1	2.5 ± 0.5

nd: not detected
[a] Hydrolysates value includes extracted liquor, wash waters and condensate fraction. Values in brackets are from the impregnation stage liquor.
[b] Calculated as the difference between total lignin (acid soluble and insoluble) in the raw material and the total lignin in treated solid, both in raw material base.

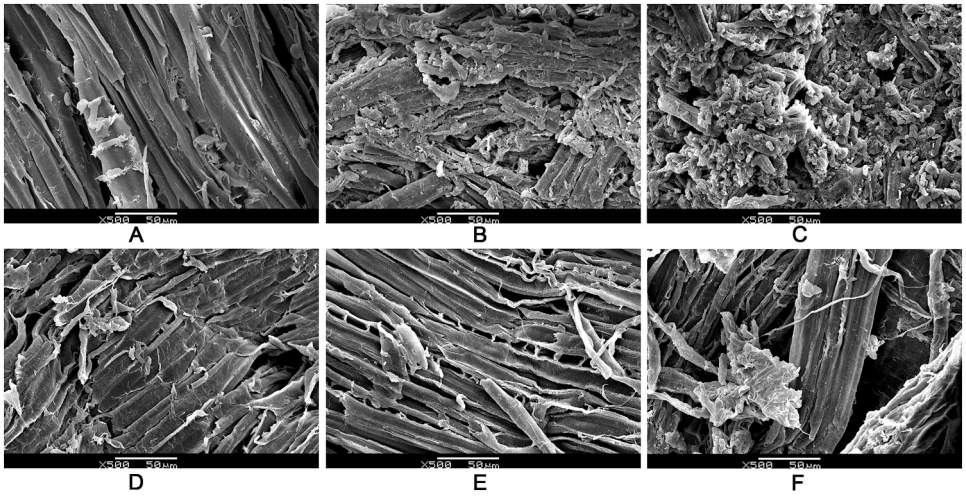


Fig. 1. SEM images of raw fibers (A) and steam-exploded pretreated fibers without alkaline impregnation (B: 180 °C, and C: 200 °C) and with alkaline impregnation (D: 20% NaOH, 200 °C, E: 10% NaOH, 190 °C and F: 20% NaOH, 180 °C).

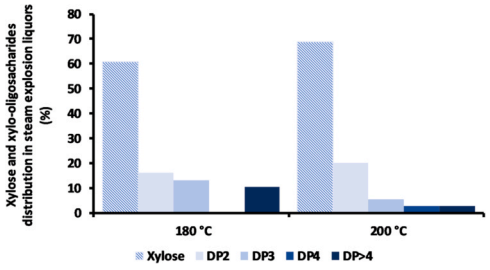


Fig. 2. Relative composition of different degree of polymerization of the carbohydrates presents in the steam explosion hydrolysates obtained by steam explosion of sawdust without NaOH impregnation. DP: degree of polymerization considering anhydro-xylose as the monomeric unit.

the values reported elsewhere (Al-Dajani and Tschirner, 2008). The solubilized xylan was found in a polymeric or oligomeric form as no xylose was found in the liquors. The precipitate obtained from the impregnation stage comprised 47 ± 3% of XS, 1.8 ± 0.3% of acetyl groups, 17.0 ± 4% of acid insoluble compounds (probably phenolics and extractive compounds), and 31 ± 1% of ash, among other components not identified. The precipitate obtained from the steam explosion liquor comprised 49 ± 3% of XS and 26 ± 4% of acid soluble and insoluble lignin, among other components not identified. The average molecular weight of the latter was 48900 ± 600 Da.

The ATR-FTIR spectrum of the ethanol precipitate from the steam-exploded extracted liquor after the NaOH impregnation stage (Fig. 3A) showed a wide and intense peak, with a maximum at 1036 cm^{-1}, the typical peak of the (1−4)-β-xylans, which corresponds to the stretching vibrations of C–C and C–O bonds and C–OH rotation from ring and side groups (Buslov et al., 2009; Kacurakova et al., 1998; Kacuráková et al., 2000). The bands at 1160–1100 cm^{-1} correspond to the glycosidic bond

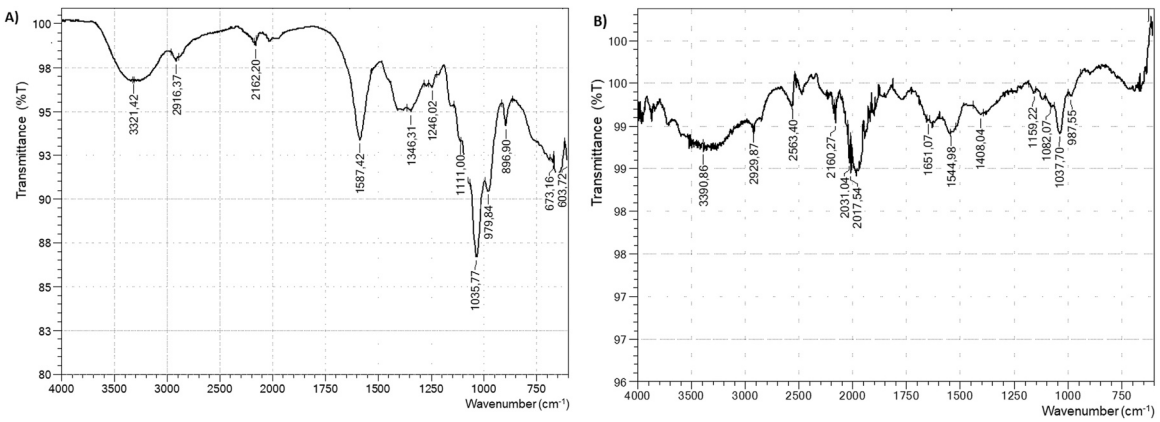


Fig. 3. ATR-FTIR spectrum of the precipitates obtained from A) steam exploded hydrolysate after the NaOH impregnation stage and B) the impregnation liquor.

(C-O-C) (Kacuráková et al., 2000). A very wide band at 980 cm^{-1} is assigned to the scissor-like vibrations of the CH_3 and C-OH groups (Chen et al., 2015; Kacurakova et al., 1998). The band at 897 cm^{-1} is typical of the β-glycosidic bond (Chen et al., 2015). The broad and intense regions in the spectrum at 3600–3000 cm^{-1} are assigned to the intra- and intermolecular vibrations of hydroxyl groups as well as the presence of water from hydration (Buslov et al., 2009; Kacurakova et al., 1998). It should be noted that the peak corresponding to free water that is characteristic of the region was not observed. The peak at 2916 cm^{-1} could be assigned to the stretching vibrations of the CH_3, CH_2, and CH groups that appear in this region. This may be due to the presence of acetyl groups or 4-O-methyl-D-glucuronic acid (Buslov et al., 2009). These bands confirmed the presence of a structure of (1−4)-β-xylans with acetyl and glucuronic side groups, typical for hardwood hemicelluloses. However, the dark colour of the precipitates and the signal in the range of 1800–1500 cm^{-1}, which are assigned to the stretching vibrations of the C = C and C-H groups, confirmed the presence of lignin fragments in the sample (Buslov et al., 2009).

The ethanol precipitate from the impregnation liquor (Fig. 3B) had the same bands reported for the preceding precipitate but at a lower intensity. This bands confirmed the existence of XS in a polymeric state in the precipitate and the presence of lignin fragments. However, the presence of a strong band, in the region of 2100–2000 cm^{-1}, which could not be assigned to any compound and not corresponding to any xylan or lignin signal, decreased the other bands' intensity.

3.2. Enzymatic hydrolysis of pretreated eucalyptus

3.2.1. Enzymatic hydrolysis of the pretreated eucalyptus without and with alkaline impregnation: Preliminary tests

Fig. 4A and B show the glucose concentration and hydrolysis efficiency profiles for the non-impregnated solids (180 and 200 °C) and solids impregnated with 20% NaOH and pretreated at 200 °C. Table 2 shows the chemical composition of the enzymatic hydrolysates of all the solids pretreated for 168 h.

Although the degree of delignification of the pretreated solids was low (Table 1), the enzymatic digestibility of the pretreated solids without NaOH impregnation increased with temperature (Table 2). A hydrolysis efficiency of 96% was obtained for the solid pretreated at 200 °C, while only 69% was obtained for that pretreated at 180 °C. This result agrees with that reported by Park et al. (2012) for *E. grandis*, who found that an increase in steam explosion temperature enhanced the likelihood of enzymes to access cellulose due to the xylan and lignin removal, which act as a barrier for the enzymes to access to cellulose. Depending on its severity, pretreatment can also induce changes in the lignin such as depolymerization, condensation, and reallocation, which can facilitate the accessibility of the enzyme to cellulose (Ramos, 2003). The enzymatic hydrolysates reached a glucose concentration of 105 g/L for the pretreated solid at 200 °C. Cellobiose was also found in the enzymatic hydrolysates, 5 and 7 g/L for solids pretreated at 180 and 200 °C, respectively. Xylose was not detected as there was no xylan in the pretreated solid.

Concerning the alkaline-impregnated pretreated solids, a clear correlation between hydrolysis efficiency and steam explosion temperature (or severity factor) was not observed. No significant differences were observed for hydrolysis efficiency for the different conditions studied

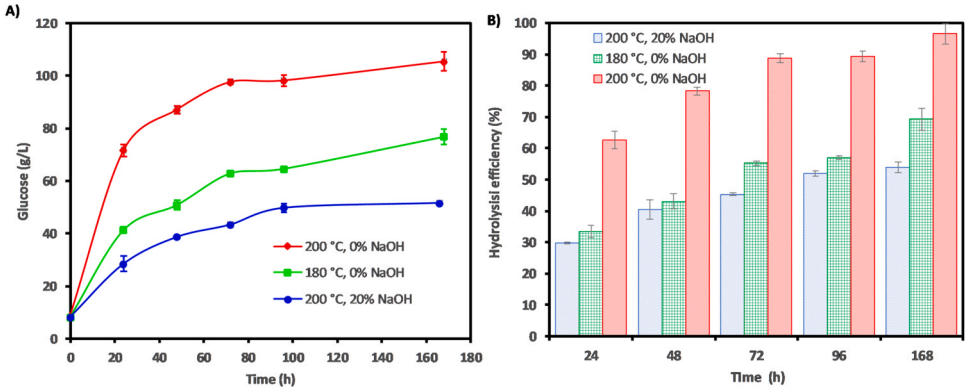


Fig. 4. Glucose concentration (A) and hydrolysis efficiency (B) profiles during enzymatic hydrolysis of the non-impregnated solids (180 and 200 °C) and solids impregnated with 20% NaOH and pretreated at 200 °C. Solid loading and enzyme dosage were 15% (w/w) and 30 FPU/g_{glucan}, respectively.

Table 2
Chemical composition of enzymatic eucalyptus hydrolysates and hydrolysis efficiency for tests performed at 15% solid loading and 30 FPU/g_{glucan} after 168 h.

Severity factor/combined severity factor	Conditions		Chemical composition (g/L)			Enzymatic hydrolysis efficiency (%)
	Temperature (°C)	NaOH (% w/w)	Glucose	Cellobiose	Xylose	
3.36	180	–	76.8 ± 2.9	4.9 ± 0.1	nd	69.3 ± 3.5
3.94	200	–	105.4 ± 3.5	7.3 ± 0.0	nd	96.5 ± 3.1
4.05	180	20	48.7 ± 2.7	5.9 ± 0.1	14.9 ± 1.0	47.2 ± 2.6
4.05	190	10	54.8 ± 0.8	6.3 ± 0.0	18.7 ± 0.2	52.7 ± 0.8
4.64	200	20	51.7 ± 1.6	7.0 ± 1.2	11.8 ± 0.4	54.0 ± 1.6

nd: not detected

($p > 0.05$), whose values were in the range of 47–54% (Table 2). Glucose and cellobiose concentrations of 49–55 and 6–7 g/L, respectively, were achieved. Xylose was also detected in the hydrolysates (12–19 g/L) because the xylan, which was not completely removed, was hydrolyzed by xylanases present in the commercial enzyme preparation used (Cellic CTec 2), as reported by Larnaudie et al. (2019a).

The better performance of steam-explosion without alkaline impregnation compared to the pretreatment with impregnation could be due to the action of acetyl groups during pretreatment. The best hydrolysis performance was obtained for the solid that was not NaOH-impregnated and steam exploded at 200 °C. This can be attributed to the fact that the hemicellulose was almost completely removed during the pretreatment (based on the xylan and acetyl groups' components), which could improve cellulase access to the cellulose surface (Bura et al., 2009). As can be observed in the SEM images in Fig. 1, the 200 °C steam-exploded solid presented a more damaged structure compared to the solid obtained after steam explosion at 180 °C, having a high superficial area that may favor the enzymatic attack. Moreover, as an almost complete solubilization of acetyl groups occurred during the NaOH impregnation step (Table 1), the steam explosion pretreatment was not as effective in breaking the material structure (no acetic acid formation) at high temperatures, which could explain the worse enzymatic hydrolysis performance compared to the non-impregnated solids. According to the SEM images (Fig. 1), the fibers of the NaOH-impregnated solids did not appear to be damaged, which highlights the hypothesis that the OH groups neutralized the acidic components, reducing the extent of hemicellulose autohydrolysis. These results are not in accordance with the data reported by Park et al. (2012), who obtained a higher enzymatic hydrolysis performance when a NaOH impregnation stage was performed before the steam explosion of *E. grandis* wood. They reported that the better enzymatic hydrolysis results of the impregnated solids were due to the high lignin removal reached and the increase in crystallinity, which allowed the enzymes to have easy access to the cellulose. Although the authors used a lower solid loading (5% w/w) than that in this work, the enzymatic hydrolysis efficiencies of the NaOH-impregnated solids were comparable (10–67% vs. 47–54%, respectively).

In order to improve the cellulose hydrolysis of NaOH-impregnated solids, the addition of PEG 6000 was evaluated since it has been reported that the presence of a surfactant (as well as some proteins) could reduce the negative lignin-enzyme effect by hydrophobic interactions with lignin residues (Ázar et al., 2019; Camesasca et al., 2015). High solid loadings (18% and 20%) were used. fig. 5 shows the glucose concentration and hydrolysis efficiency profiles obtained during the enzymatic hydrolysis. Cellulose to glucose conversions of 45% and 42% were achieved with 18% and 20% solid loadings, respectively. Although the addition of PEG 6000 improved the hydrolysis efficiency for both solid loadings at 28% and 34%, respectively, the values achieved were low. A glucose concentration of 76 g/L was effectively obtained at 20% (w/w) solid loading when PEG 6000 was added (hydrolysis efficiency of 59%). Xylose was also detected in the hydrolysates (4–10 g/L) due to the fact that the xylan was not completely removed during pretreatment as explained above.

Compounds such as furfural, acetic acid, formic acid, and HMF, which could be formed during pretreatment and act as possible fermentation inhibitors, were not detected in any of the enzymatic hydrolysates, both for solids impregnated and non-impregnated with NaOH. In the non-NaOH-impregnated treatment, the furfural volatilizes as it is formed and is mainly found in the condensate, so it is hardly in contact with the solid. In the NaOH-impregnated treatment, furfural and HMF were not formed during the steam explosion despite severe treatment conditions.

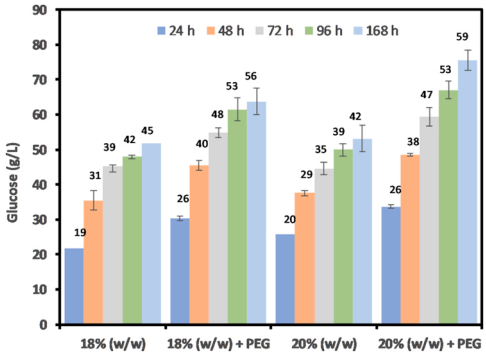


Fig. 5. Glucose concentration (in left axis) and hydrolysis efficiency (values on bars) profiles during enzymatic hydrolysis of 20% NaOH and 200 °C steam explosion-pretreated eucalyptus sawdust, at solids loading of 18 and 20% (w/w) without and with PEG 6000 addition.

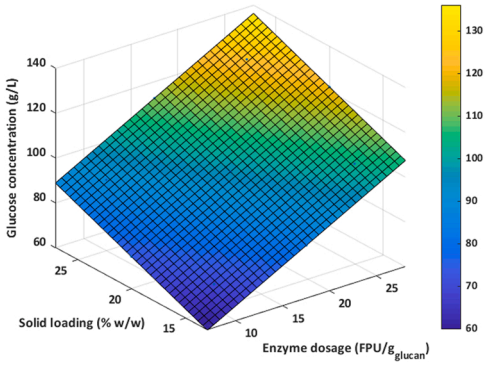


Fig. 6. Glucose concentration as a function of solid loading and enzyme dosage of the RCCD design for steam exploded solids without alkaline impregnation at 200 °C.

3.2.2. *Enzymatic hydrolysis of pretreated sawdust without alkaline impregnation: Solid and enzyme dosage evaluation*

The production of enzymatic hydrolysates with a high sugar concentration and low enzyme dosages is important in terms of the process economics of the bio-based product industry as it can reduce equipment size and facilitate downstream processing. To produce bioethanol at a high concentration, it is necessary to perform the fermentation process with a high sugar concentration (Kim et al., 2019). To achieve high

Table 3
Glucose concentration of the enzymatic eucalyptus hydrolysates and hydrolysis efficiency obtained in the RCCD tests at 48 h.

Run	x_1	x_2	Enzyme dosage (FPU/g_{glucan})	Solid loading (%, w/w)	Glucose concentration (g/L)	Hydrolysis efficiency (%)
1	-1	-1	10	15	80	78
2	1	-1	25	15	96	92
3	-1	1	10	25	104	59
4	1	1	25	25	125	72
5	0	0	17	20	108	78
6	0	0	17	20	95	67
7	0	0	17	20	101	74
8	0	0	17	20	108	78
9	0	-1.41	17	13	71	80
10	0	1.41	17	27	120	63
11	-1.41	0	7	20	69	50
12	1.41	0	28	20	106	76

sugar concentrations with low enzyme dosages, an experimental design was carried out to evaluate enzyme dosage and solid loading.

Table 3 shows the experimental results of the RCCD for enzyme dosage (ED) and solid loading (SL) using the pretreated solid at 200 °C for 10 min without alkaline impregnation. The model obtained for the glucose concentration (G) as a function of enzyme dosage and solid loading was as follows:

$$G\ (g/L) = 98.833 + 11.16574\ x_1 + 15.28706\ x_2 \quad (R^2 = 0.84) \tag{8}$$

The response surface obtained is shown in Fig. 6. Both enzyme dosage and solid loading showed a significant effect on glucose concentration ($p < 0.05$). As expected, higher hydrolysis efficiencies were observed for lower solid loadings and higher enzyme dosages. However, a relatively high hydrolysis efficiency (72%) was obtained for a high solid loading (25%) when a high enzyme dosage was used (25 FPU/g_{glucan}) (run 4). As the solid loading increased, the glucose concentration increased but the hydrolysis efficiency decreased (Table 3). This could be due to mass transfer limitations because high amounts of soluble molecules in the hydrolysate affect the cellulases diffusion mechanism to reaction sites within the substrate due to increased viscosity (Roberts et al., 2011). It has also been reported that the reduction of available water affects the hydrolysis efficiency under high solid loadings (Selig et al., 2012). As the enzyme dosage increased, higher glucose concentrations and hydrolysis efficiencies were obtained for the same solid loading. The increase in enzyme dosage also compensated for the lower hydrolysis efficiencies observed when a very high solid loading was used (25%) (runs 3 and 4), which is in agreement with the data reported by Modenbach and Nokes (2012). A high sugar concentration was obtained (125–120 g/L) for the assays with a high solid loading (25–27%) (runs 4 and 10, respectively). Concerning those assays, a higher hydrolysis efficiency was obtained with 25 FPU/g_{glucan} compared to 17 FPU/g_{glucan} (72 and 63%, respectively), which confirms that high enzyme dosages are needed when a high solid loading is used. However, increasing the enzyme dosage between 17 and 28 FPU/g_{glucan} showed not to have a significant effect on hydrolysis efficiency (74% as the average of the central point and 76%, respectively) when a solid loading of 20% was used. This was probably due to the moderately low solid content. Thus, the highest solid loading evaluated (27%) and a relatively high enzyme dosage (25 FPU/g_{glucan}) were chosen to obtain a high glucose concentration with a hydrolysis efficiency higher than 63% (run 10; Fig. 6).

Eq. (8) predicts a glucose concentration of 129 ± 6 g/L at 48 h for an enzymatic hydrolysis with 27% (w/w) solid loading and 25 FPU/g_{glucan} enzyme dosage. To confirm this value, an assay was performed under these experimental conditions. Fig. 7 shows the sugar concentration and hydrolysis efficiency profiles during 96 h of hydrolysis. As expected, 134 ± 5 g/L of glucose was achieved at 48 h, which was within the range predicted by the model. As expected, the hydrolysis efficiency

Fig. 7. Glucose concentration and hydrolysis efficiency profiles during enzymatic hydrolysis of pretreated eucalyptus sawdust by steam explosion at 200 °C, at solid loading and enzyme dosage of 27% (w/w) and 25 FPU/g_{glucan}, respectively.

obtained was 74 ± 3%, similar to that obtained for 25% and 25 FPU/g_{glucan} (72%) in the RCCD.

3.3. SHF, SSF, and PSSF of E. grandis pretreated by steam explosion without alkaline impregnation

Fig. 8 and Table 4 show the glucose and ethanol profiles and fermentation results, respectively, for the configurations evaluated. For the SSF configuration, an ethanol concentration of 75.0 g/L was reached at 36 h with a total productivity (hydrolysis + fermentation) of 2.1 g/Lh. Similar ethanol concentration results ($p < 0.05$) were reached in both SHF and PSSF configuration processes (ethanol concentration of 71.8 and 70.2 g/L, respectively). The total productivity reached in the PSSF configuration process (2.0 g/Lh) was also similar to that obtained in SSF at 36 h. However, the SHF process showed a worse performance in terms of total productivity (1.0 g/Lh) because of the long pre-saccharification time (48 h). In the SSF process, the sugar concentration in the medium was low (16 g/L) when the microorganism was inoculated. As the hydrolysis and fermentation continued, the microorganism consumed the sugars at a similar rate as they were released by the enzyme, and therefore the glucose concentration was maintained at approximately 0 g/L during the entire process. Since the last sample of the fermentation was taken at 48 h, the enzymatic hydrolysis was not expected to be completed.

Based on the model developed (8), a maximum of 129 ± 6 g/L of glucose was expected to be released in the fermentation broth at 48 h, and, considering the amount of sugar present in the enzyme extract (16 g/L at the start of the fermentation), the stoichiometric ethanol

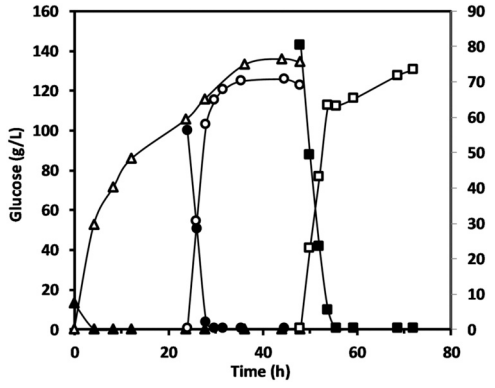


Fig. 8. Glucose (filled symbols) and ethanol (white symbols) concentration profiles for steam-exploded eucalyptus sawdust (200 °C, 10 min) without alkaline impregnation during fermentation: SHF (square), PSSF (circle) and SSF (triangle).

Table 4
Fermentation parameters of ethanol production from steam explosion-pretreated eucalyptus at 200 °C for 10 min without alkaline impregnation by SHF, SSF and PSSF configurations using *S. cerevisiae*.

Fermentation configuration	SHF	PSSF	SSF
Time (h)	68 (48 + 20)[a]	35 (24 + 11)[b]	48
Initial sugars (g/L)	150 ± 6	106 ± 0.1	16 ± 1
Glycerol (g/L)	4.5 ± 0.4	4.8 ± 0.1	2.5 ± 0.0
Ethanol (g/L)	71.8 ± 1.0	70.2 ± 0.6	75.6 ± 1.5
Ethanol conversion (%)	78 ± 1	82 ± 8	83 ± 2
Fermentation productivity (g/Lh)	3.5 ± 0.0	6.2 ± 0.1	1.6 ± 0.0
Total productivity (g/Lh)	1.0 ± 0.0	2.0 ± 0.0	

[a] Time (pre-saccharification time + fermentation time).
[b] Time (pre-saccharification time + saccharification and fermentation time).

concentration expected was in the range 71–77 g/L, similar to that achieved (75.6 g/L).

To obtain a high ethanol conversion, longer fermentation times are needed (72–96 h), which would result in a slow progressive increase in ethanol production. Although the fermentation was not completed, the ethanol concentration achieved was, to the author's knowledge, higher than that reported in the literature from steam-exploded eucalyptus. Romaní et al. (2013) reported a maximum of 51 g/L of ethanol by SSF of steam-exploded *Eucalyptus globulus* wood at 210 °C for 10 min (liquid to solid ratio (LSR) of enzymatic hydrolysis of 6 and enzyme dosage (ED) of 10 FPU/g). Higher ethanol concentrations have been reported from eucalyptus pretreated by autohydrolysis. Guigou et al. (2019) reported an ethanol concentration of 58 g/L via the PSSF strategy from *E. grandis* pretreated by autohydrolysis and soda pulping (LSR of 7.5, ED of 25 FPU/g_{glucan}). Romaní et al. (2012) obtained an ethanol concentration of 67.4 g/L via the SSF strategy from *E. globulus* pretreated by autohydrolysis at 230 °C (S_0 = 4.67) under similar enzymatic hydrolysis experimental conditions as those utilized in this work (LSR and ED of 4 g/g and 16 FPU/$g_{substrate}$ and 3.7 g/g and 15 FPU/$g_{substrate}$, respectively), those results being the highest ethanol concentrations reported from eucalyptus to the author's knowledge. More recently, Cunha et al. (2018) obtained 93 g/L of ethanol from *E. globulus* wood and cheese whey powder using a modified *Saccharomyces cerevisiae* for β-galactosidase production under SSF.

In this work, yields of 246, 241, and 259 L ethanol per ton of raw material for the SHF, PSSF, and SSF processes, respectively, were obtained. The SSF process reached the highest yield, which is similar to that found in the literature. Guigou et al. (2019) reached an ethanol yield of 254 L per ton of *E. grandis* sawdust pretreated by autohydrolysis and soda pulping. Chiarello et al. (2016), who used steam explosion for the pretreatment of *E. urograndis* chips obtained 219 L ethanol per ton under the SHF configuration process, which was lower than that obtained in this work for a similar pretreatment and fermentation configuration (246 L per ton). Recently, Trevorah et al. (2018) reported an ethanol yield of 181 L per ton of *E. obliqua* sawdust pretreated by organosolv using gamma-valerolactone.

Even though PSSF is commonly used when high solid loadings are used during the enzymatic hydrolysis, the microorganism did not show problems in the presence of insoluble solids and presented a comparable performance during both the SSF and PSSF strategies, which could mean an advantage at the industrial scale due to its easier characteristics of use.

4. Conclusions

The steam explosion pretreatment of *E. grandis* sawdust at 200 °C for 10 min without NaOH impregnation was a suitable method for the recovery of XS in the hydrolysate and glucan in the pretreated solid. 76 kg of XS per ton of eucalyptus sawdust were obtained, which were mostly as xylose or XS with a DP of 4 or lower. The steam pretreated solid was efficiently enzymatically hydrolyzed at a high solid loading (27%) with an enzyme dosage of 25 FPU/g_{glucan}, which allowed the acquisition of enzymatic hydrolysates with a high glucose concentration (134 g/L). High ethanol concentrations (75.6 g/L) and yields (259 L per ton of dry raw sawdust) were obtained by the SSF configuration process, representing an attractive strategy compared to the others evaluated (SHF, PSSF) for ethanol production from eucalyptus sawdust. The NaOH-impregnation step did not show good results both in terms of XS recovery and enzymatic hydrolysis, probably due to the autohydrolysis inhibition caused by the complete solubilization of the acetyl groups during NaOH impregnation.

CRediT authorship contribution statement

Eloísa Rochón: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Visualization, Supervision. **María Noel Cabrera:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Co-writing-original draft, Visualization, Supervision, Project administration, Funding acquisition. **Valentina Scutari:** Methodology, Validation, Formal analysis, Investigation. **Matías Cagno:** Methodology, Validation, Investigation. **Abigail Guibaud:** Methodology, Validation, Formal analysis, Investigation. **Santiago Martínez:** Methodology, Investigation. **Silvia Böthig:** Project administration, Visualization. **Nikolai Guchin:** Project administration, Visualization. **Daniel Ferrari:** Conceptualization, Validation, Visualization, Writing – review & editing. **Claudia Lareo:** Conceptualization, Validation, Resources, Visualization, Writing – review & editing.

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.

Acknowledgements

The financial support was provided by CIDEB (Centro de Biocombustibles de Segunda Generación, Fundación Latitud - ANCAP, Uruguay). The authors would like to thank Urufor for providing the sawdust used in this work.

References

Adney, B., Baker, J., 2008. Measurement of Cellulase Activities. Lab. Anal. Proced. (NREL) Natl. Renew. Energy Lab. Golden, CO, USA.
Al-Dajani, W.W., Tschirner, U.W., 2008. Pre-extraction of hemicelluloses and subsequent kraft pulping Part I: alkaline extraction. Tappi J. 7, 3–8.
Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., 2010. Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: a review. Bioresour. Technol. 101, 4851–4861. https://doi.org/10.1016/j.biortech.2009.11.093.
Ázar, R.I.S.L., Morgan, T., Barbosa, M.H.P., Guimarães, V.M., Ximenes, E., Ladisch, M., 2019. Impact of protein blocking on enzymatic saccharification of bagasse from sugarcane clones. Biotechnol. Bioeng. 116, 1584–1593. https://doi.org/10.1002/bit.26962.
Bonfiglio, F., Cagno, M., Rey, F., Torres, M., Böthig, S., Menéndez, P., Mussatto, S.I., 2019. Pretreatment of switchgrass by steam explosion in a semi-continuous pre-pilot reactor. Biomass Bioenergy 121, 41–47. https://doi.org/10.1016/j.biombioe.2018.12.013.
Bonfiglio, F., Cagno, M., Yamakawa, C.K., Mussatto, S.I., 2021. Production of xylitol and carotenoids from switchgrass and *Eucalyptus globulus* hydrolysates obtained by intensified steam explosion pretreatment. Ind. Crop. Prod. 170, 113800 https://doi.org/10.1016/j.indcrop.2021.113800.
Bura, R., Chandra, R., Saddler, J., 2009. Influence of xylan on the enzymatic hydrolysis of steam-pretreated corn stover and hybrid poplar. Biotechnol. Prog. 25, 315–322. https://doi.org/10.1002/btpr.98.
Buslov, D.K., Kaputski, F.N., Sushko, N.I., Torgashev, V.I., Solov'eva, L.V., Tsarenkov, V. M., Zubets, O.V., Larchenko, L.V., 2009. Infrared spectroscopic analysis of the structure of xylans. J. Appl. Spectrosc. 76, 801–805. https://doi.org/10.1007/s10812-010-9282-z.
Cabrera, M.N., 2021. Extracción y purificación de hemicelulosas provenientes de madera de eucalipto. PhD. Thesis, Engineering School. Universidad de la República, Montevideo, Uruguay. ISSN 1688-2776.

Camesasca, L., Ramírez, M.B., Guigou, M., Ferrari, M.D., Lareo, C., 2015. Evaluation of dilute acid and alkaline pretreatments, enzymatic hydrolysis and fermentation of napiergrass for fuel ethanol production. Biomass Bioenergy 74, 193–201. https://doi.org/10.1016/j.biombioe.2015.01.017.

Carvalho, D.M., de, Queiroz, J.H., de, Colodette, J.L., 2016. Assessment of alkaline pretreatment for the production of bioethanol from eucalyptus, sugarcane bagasse and sugarcane straw. Ind. Crop. Prod. 94, 932–941. https://doi.org/10.1016/j.indcrop.2016.09.069.

Castro, R.C., de, A., Fonseca, B.G., dos Santos, H.T.L., Ferreira, I.S., Mussatto, S.I., Roberto, I.C., 2017. Alkaline deacetylation as a strategy to improve sugars recovery and ethanol production from rice straw hemicellulose and cellulose. Ind. Crop. Prod. 106, 65–73. https://doi.org/10.1016/j.indcrop.2016.08.053.

Cebreiros, F., Risso, F., Cagno, M., Cabrera, M.N., Rochón, E., Jauregui, G., Boix, E., Bothig, S., Ferrari, M.D., Lareo, C., 2021. Enhanced production of butanol and xylosaccharides from *Eucalyptus grandis* wood using steam explosion in a semi-continuous pre-pilot reactor. Fuel 290, 119818. https://doi.org/10.1016/j.fuel.2020.119818.

Chang, V.S., Holtzapple, M.T., 2000. Fundamental factors affecting biomass enzymatic reactivity. In: Finkelstein, M., Davison, B.H. (Eds.), Twenty-First Symposium on Biotechnology for Fuels and Chemicals. Applied Biochemistry and Biotechnology. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-4612-1392-5_1.

Chen, Z., Hu, T.Q., Jang, H.F., Grant, E., 2015. Modification of xylan in alkaline treated bleached hardwood kraft pulps as classified by attenuated total-internal-reflection (ATR) FTIR spectroscopy. Carbohydr. Polym. 127, 418–426. https://doi.org/10.1016/j.carbpol.2015.03.084.

Chiarello, L.M., Ramos, C.E.A., Neves, P.V., Ramos, L.P., 2016. Production of cellulosic ethanol from steam-exploded *Eucalyptus urograndis* and sugarcane bagasse at high total solids and low enzyme loadings. Sustain. Chem. Process. 4, 1–9. https://doi.org/10.1186/s40508-016-0059-4.

Clauser, N.M., Gutiérrez, S., Area, M.C., Felissia, F.E., Vallejos, M.E., 2016. Small-sized biorefineries as strategy to add value to sugarcane bagasse. Chem. Eng. Res. Des. 107, 137–146. https://doi.org/10.1016/j.cherd.2015.10.050.

Cunha, M., Romaní, A., Carvalho, M., Domingues, L., 2018. Boosting bioethanol production from Eucalyptus wood by whey incorporation. Bioresour. Technol. 250, 256–264. https://doi.org/10.1016/j.biortech.2017.11.023.

Guigou, M., Cabrera, M.N., Vique, M., Bariani, M., Guarino, J., Ferrari, M.D., Lareo, C., 2019. Combined pretreatments of eucalyptus sawdust for ethanol production within a biorefinery approach. Biomass Convers. Biorefin. 9, 293–304. https://doi.org/10.1007/s13399-018-0353-3.

Inalbon, M.C., Mocchiutti, P., Zanuttini, M., 2009. The deacetylation reaction in *Eucalyptus wood*: kinetics and effects on the effective diffusion. Bioresour. Technol. 100, 2254–2258. https://doi.org/10.1016/j.biortech.2008.10.054.

Kacurakova, M., Belton, P.S., Wilson, R.H., Ebringerova, A., 1998. Hydration properties of xylan-type structures: an FTIR study of xylooligosaccharides. J. Sci. Food Agric. 77, 38–44.

Kacuráková, M., Capek, P., Sasinková, V., Wellner, N., Ebringerová, A., 2000. FT-IR study of plant cell wall model compounds: pectic polysaccharides and hemicelluloses. Carbohydr. Polym. 43, 195–203. https://doi.org/10.1016/S0144-8617(00)00151-X.

Kim, D.H., Park, H.M., Jung, Y.H., Sukyai, P., Kim, K.H., 2019. Pretreatment and enzymatic saccharification of oak at high solids loadings to obtain high titers and high yields of sugars. Bioresour. Technol. 284, 391–397. https://doi.org/10.1016/j.biortech.2019.03.134.

Larnaudie, V., Ferrari, M.D., Lareo, C., 2019a. Techno-economic analysis of a liquid hot water pretreated switchgrass biorefinery: effect of solids loading and enzyme dosage on enzymatic hydrolysis. Biomass Bioenergy 130, 105394. https://doi.org/10.1016/j.biombioe.2019.105394.

Larnaudie, V., Ferrari, M.D., Lareo, C., 2019b. Enzymatic hydrolysis of liquid hot water-pretreated switchgrass at high solid content. Energy Fuels 33, 4361–4368. https://doi.org/10.1021/acs.energyfuels.9b00513.

Mäkeläinen, H., Forssten, S., Saarinen, M., Stowell, J., Rautonen, N., Ouwehand, A.C., 2010. Xylo-oligosaccharides enhance the growth of bifidobacteria and *Bifidobacterium lactis* in a simulated colon model. Benef. Microbes 1, 81–91. https://doi.org/10.3920/BM2009.0025.

Martín-Sampedro, R., Eugenio, M.E., García, J.C., Lopez, F., Villar, J.C., Diaz, M.J., 2012. Steam explosion and enzymatic pre-treatments as an approach to improve the enzymatic hydrolysis of *Eucalyptus globulus*. Biomass Bioenergy 42, 97–106. https://doi.org/10.1016/j.biombioe.2012.03.032.

McIntosh, S., Vancov, T., 2010. Enhanced enzyme saccharification of *Sorghum bicolor* straw using dilute alkali pretreatment. Bioresour. Technol. 101, 6718–6727. https://doi.org/10.1016/j.biortech.2010.03.116.

Míguez, B., Gullón, P., Cotos-Yáñez, T., Massot-Cladera, M., Pérez-Cano, F.J., Vila, C., Alonso, J.L., 2021. Manufacture and prebiotic potential of xylooligosaccharides derived from *Eucalyptus nitens* wood. Front. Chem. Eng. https://doi.org/10.3389/fceng.2021.670440.

Modenbach, A.A., Nokes, S.E., 2012. The use of high-solids loadings in biomass pretreatment-a review. Biotechnol. Bioeng. 109, 1430–1442. https://doi.org/10.1002/bit.24464.

Molaverdi, M., Karimi, K., Mirmohamadsadeghi, S., Galbe, M., 2021. High efficient ethanol production from corn stover by modified mild alkaline pretreatment. Renew. Energy 170, 714–723. https://doi.org/10.1016/j.renene.2021.02.002.

Mussatto, S.I., Dragone, G.M., 2016. Biomass pretreatment, biorefineries, and potential products for a bioeconomy development. Biomass Fractionation Technologies for a Lignocellulosic Feedstock Based Biorefinery. Elsevier Inc. https://doi.org/10.1016/B978-0-12-802323-5.00001-3.

Overend, R.P., Chornet, E., Gascoigne, J.A., 1987. Fractionation of lignocellulosics by steam-aqueous pretreatments. Philos. Trans. R. Soc. London. Ser. A Math. Phys. Soc. 321, 523–536.

Park, J.Y., Kang, M., Kim, J.S., Lee, J.P., Choi, W., Il, Lee, J.S., 2012. Enhancement of enzymatic digestibility of *Eucalyptus grandis* pretreated by NaOH catalyzed steam explosion. Bioresour. Technol. 123, 707–712. https://doi.org/10.1016/j.biortech.2012.07.091.

Pinheiro, T., Coelho, E., Romaní, A., Domingues, L., 2019. Intensifying ethanol production from brewer's spent grain waste: Use of whole slurry at high solid loadings. New Biotechnol. 53, 1–8. https://doi.org/10.1016/j.nbt.2019.06.005.

Ramos, L.P., 2003. The chemistry involved in the steam treatment of lignocellulosic materials. Química Nova 26 (6), 863–871. https://doi.org/10.1590/s0100-40422003000600015.

Risso, F., Rochón, E., Cebreiros, F., Ferrari, M.D., Lareo, C., 2020. Effect of corn steep liquor on butanol fermentation of eucalyptus cellulose enzymatic hydrolysate. Ind. Biotechnol. 16 (2), 99–106. https://doi.org/10.1089/ind.2019.0036.

Roberts, K.M., Lavenson, D.M., Tozzi, E.J., McCarthy, M.J., Jeoh, T., 2011. The effects of water interactions in cellulose suspensions on mass transfer and saccharification efficiency at high solids loadings. Cellulose 18, 759–773. https://doi.org/10.1007/s10570-011-9509-z.

Romaní, A., Garrote, G., Ballesteros, I., Ballesteros, M., 2013. Second generation bioethanol from steam exploded Eucalyptus globulus wood. Fuel 111, 66–74. https://doi.org/10.1016/j.fuel.2013.04.076.

Romaní, A., Garrote, G., Parajó, J.C., 2012. Bioethanol production from autohydrolyzed Eucalyptus globulus by Simultaneous Saccharification and Fermentation operating at high solids loading. Fuel 94, 305–312. https://doi.org/10.1016/j.fuel.2011.12.013.

Selig, M.J., Hsieh, C.W.C., Thygesen, L.G., Himmel, M.E., Felby, C., Decker, S.R., 2012. Considering water availability and the effect of solute concentration on high solids saccharification of lignocellulosic biomass. Biotechnol. Prog. 28, 1478–1490. https://doi.org/10.1002/btpr.1617.

Shen, B., Hou, S., Jia, Y., Yang, C., Su, Y., Ling, Z., Huang, C., Lai, C., Yong, Q., 2021. Synergistic effects of hydrothermal and deep eutectic solvent pretreatment on co-production of xylo-oligosaccharides and enzymatic hydrolysis of poplar. Bioresour. Technol. 341, 125787.

Singh, J., Suhag, M., Dhaka, A., 2015. Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: a review. Carbohydr. Polym. 117, 624–631. https://doi.org/10.1016/j.carbpol.2014.10.012.

Sluiter A, Hames B, Hyman D, Payne C, Ruiz R, Scarlata C, Sluiter J, Templeton D, Wolfe J., 2008a. Determination of total solids in biomass and total dissolved solids in liquid process samples. NREL/TP-510-42621. Golden, CO, USA.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008b. Determination of ash in biomass. NREL/TP-510-42622. Golden, CO, USA.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008c. Determination of sugars, byproducts, and degradation products in liquid fraction process samples. NREL/TP-510-42623. Golden, CO, USA.

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2008d. Determination of structural carbohydrates and lignin in biomass. NREL/TP-510-42618. Golden, CO, USA.

Sluiter, A., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008e. Determination of extractives in biomass. NREL/TP-510-42619. Golden, CO, USA.

Tian, D., Chandra, R.P., Lee, J.-S., Lu, C., Saddler, J.N., 2017. A comparison of various lignin-extraction methods to enhance the accessibility and ease of enzymatic hydrolysis of the cellulosic component of steam-pretreated poplar. Biotechnol. Biofuels 10, 157.

Trevorah, R.M., Huynh, T., Vancov, T., Othman, M.Z., 2018. Bioethanol potential of *Eucalyptus obliqua* sawdust using gamma-valerolactone fractionation. Bioresour. Technol. 250, 673–682. https://doi.org/10.1016/j.biortech.2017.11.084.

Uruguay XXI Forestry sector in Uruguay 2021 2021.Accessed July 13⟨https://www.uruguayxxi.gub.uy/uploads/informacion/4e52d8c6a598944eb1ddc97bbf85233df5c290ba.pdf⟩.

Zanuttini, M., Marzocchi, V., 1997. Kinetics of alkaline deacetylation of poplar wood. Holzforschung 51, 251–256. https://doi.org/10.1515/hfsg.1997.51.3.251.