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Enhanced cellulose enzymatic hydrolysis of pilot-scale steam-exploded *Eucalyptus grandis* chips with previous acid impregnation for bioethanol production

Camila Bacquerié [a], Mairan Guigou [a], Florencia Cebreiros [a], Valeria Larnaudie [a], María E. Roman [b], Matías Cagno [b], Facundo Rodríguez [a], Fernando Bonfiglio [b], Mario D. Ferrari [a], Claudia Lareo [a,*]

[a] Biochemical Engineering and Bioprocess Department, Chemical Engineering Institute, Faculty of Engineering, Universidad de la República, Julio Herrera y Reissig 565, 11300 Montevideo, Uruguay
[b] Latitud - LATU Foundation, Avda. Italia, 6201 Montevideo, Uruguay

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ABSTRACT

The enzymatic hydrolysis of cellulose from *Eucalyptus grandis,* pretreated through diluted acid impregnation and steam explosion in a continuous pilot-scale reactor, was evaluated for bioethanol production. The best pretreatment conditions were achieved at 180 °C and 0.5 % sulfuric acid, resulting in an enzymatic hydrolysis efficiency of 61.4 %. The liquid fraction contained up to 89 g/L of xylosaccharides, with a xylan recovery (expressed as xylose) of 50.6 %. The effects of solid content (16–24 % w/w), enzyme dose (15–25 FPU/g_{glucan}), and pH (4.8–6) on enzymatic hydrolysis were assessed using a modified Box-Behnken experimental design. All factors, as well as the interaction between solid content and pH, were significant for achieving high glucose concentrations with high enzymatic hydrolysis efficiency. Higher pH values consistently enhanced hydrolysis efficiency. Multi-objective optimization identified the optimal conditions as pH 6, 24 % solid content, and an enzyme dose of 25 FPU/g_{glucan}, maximizing glucose concentration (122 g/L), enzymatic hydrolysis efficiency (68 %), and glucose yield (49 %). Separate enzymatic hydrolysis and fermentation produced 52 g/L of ethanol, with an overall glucan-to-ethanol conversion efficiency of 46 %. In contrast, when pre-saccharification for 24 h was followed by simultaneous saccharification and fermentation, the performance was lower. Only minimal additional hydrolysis occurred during fermentation. These results contribute to the development of more efficient biomass-to-bioethanol conversion processes, supporting the development of sustainable energy technologies based on lignocellulosic resources.

1. Introduction

In the context of increasing energy demand and under the paradigm of sustainable development, the production of biofuels that are both economically viable and environmentally sustainable is essential. Among these, lignocellulosic bioethanol has emerged as a promising alternative due to its lower greenhouse gas emissions and fossil energy consumption compared to gasoline [1–3], and its potential for conversion into sustainable aviation fuels [4,5].

To address concerns related to food security and land use, advanced biofuels produced from agricultural and forestry residues or non-food energy crops are gaining importance. These feedstocks enable diversification, promote rural development, and improve energy independence, especially in countries without oil production. Despite the expansion of electric vehicles, global biofuel demand is expected to rise significantly by 2050 [6].

Abbreviations: CC, condensate; CIDEB, Centro de Investigación y Desarrollo en Biocombustible 2G; CSF, combined severity factor; FPU, filter paper units; GOS, gluco-oligosaccharides; HMF, 5-hydroxymethylfurfural; HPLC, High performance liquid chromatography; NREL, National Renewable Energy Laboratory; PSSF, pre-hydrolysis-simultaneous saccharification and fermentation; SEM, scanning electron microscope; SHF, separate hydrolysis and fermentation; STEX, steam explosion; T, temperature; t, time; WW1, first washing water; WW2, second washing water; XOS, xylo-oligosaccharides; XS, xylosaccharides.

* Corresponding author.
E-mail address: clareo@fing.edu.uy (C. Lareo).

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Table 1
Chemical composition of steam-exploded *E. grandis* wood, component recovery in pretreated solid fractions and enzymatic hydrolysis efficiency of steam-pretreated substrates at 96 h.

CSF[a]	Conditions		Chemical composition (%)			Recovery in pretreated solids[b] (%)				Enzymatic hydrolysis[c]	
	Temperature (°C)	Acid concentration (% w/w)	Glucan	Xylan	Lignin	Solids	Glucan	Xylan	Lignin	Glucose (g/L)	Efficiency (%)
0.38	160	0.25	57.2 ± 3.2	7.8 ± 1.1	33.5 ± 0.1	ND	ND	ND	ND	24.7 ± 1.5	15.2 ± 0.8
0.84	160	0.50	61.6 ± 2.8	3.7 ± 0.6	36.4 ± 0.2	57.5 ± 3.3	72.6 ± 7.3	14.9 ± 3.2	72.1 ± 3.6	49.4 ± 5.5	32.8 ± 3.9
0.50	180	0	63.0 ± 1.0	4.9 ± 0.6	35.2 ± 0.3	69.6 ± 3.1	88.8 ± 5.4	23.8 ± 4.2	84.3 ± 3.2	44.3 ± 1.6	30.8 ± 2.9
0.98	180	0.25	64.1 ± 2.1	2.7 ± 0.6	36.9 ± 0.2	64.9 ± 0.9	85.1 ± 3.9	12.1 ± 2.3	82.4 ± 0.8	68.9 ± 0.8	46.3 ± 0.5
1.27	180	0.50	59.4 ± 7.1	1.8 ± 0.1	40.2 ± 0.2	61.2 ± 0.5	74.3 ± 8.2	7.5 ± 0.3	84.6 ± 1.1	85.7 ± 4.2	61.4 ± 3.5

[a]Combined severity factor.
[b]Calculated as g of component in the solid fraction per 100 g of component in the raw material.
[c]Enzymatic hydrolysis performed at 16 % solid and 25 FPU/g_{glucan}.
Acetyl groups were not detected.
ND: Not determined. For operational reasons, the solid recovery could not be calculated under these conditions. However, since these values do not affect hydrolysis efficiency and glucose concentration, the chemical characterization results of the solid and liquid phases after pretreatment and enzymatic hydrolysis are reported.

Currently, cellulosic ethanol production represents only a small fraction of global ethanol production [4], and its large-scale implementation faces challenges such as feedstock cost and supply, conversion efficiency, and technological reliability. While agricultural residues have been widely studied, their logistics often limit industrial scalability. Dedicated lignocellulosic crops like *Eucalyptus grandis*, a fast-growing species prevalent in Uruguay, offer a promising alternative due to their availability and promising chemical composition [7,8].

Pretreatment is a critical step in bioethanol production, as it breaks down the complex lignocellulosic matrix, enhancing the accessibility of carbohydrates for enzymatic hydrolysis and fermentation, while also recovering other valuable components for diverse applications [9,10]. Among various techniques, steam explosion (STEX) is widely studied for its cost-effectiveness and scalability. It involves exposing biomass to high-pressure steam followed by rapid decompression, which disrupts the fiber structure and improves enzyme accessibility [11–15]. When combined with acid impregnation, STEX can achieve efficient hemicellulose solubilization at milder conditions, reducing inhibitor formation and improving downstream processing [9,14,16–18].

Cellulose hydrolysis is typically carried out using cellulases. However, achieving the high sugar concentrations required for efficient ethanol distillation (>80–100 g/L), demands high solids loading, which introduces challenges such as mass transfer limitations, enzyme inhibition, and non-productive binding of enzymes to lignin. In addition to solids content, key factors that strongly influence the efficiency of enzymatic hydrolysis are enzyme dosage and pH, which affect both the reaction kinetics and enzyme stability [19–25]. Therefore, a systematic evaluation of these parameters is crucial to identify optimal operating conditions that maximize glucose release while ensuring process efficiency and economic viability.

Various fermentation strategies have been explored to improve bioethanol production. Separate hydrolysis and fermentation (SHF) allows independent optimization of each step, while pre-saccharification followed by simultaneous saccharification and fermentation (PSSF) can reduce overall processing time and enzyme inhibition, though it requires compromises in process conditions [24,26–28].

The aim of this work was to evaluate the enzymatic digestibility and fermentability of *Eucalyptus grandis* that had been previously pretreated by acid impregnation and STEX in a continuous pilot-scale reactor, for the production of bioethanol fuel. The effects of pH, solid content, and enzyme dose on cellulose hydrolysis efficiency and glucose concentration were studied. In addition, SHF and PSSF strategies were evaluated for the conversion of steam-exploded *E. grandis* into ethanol.

2. Materials and methods

2.1. Raw material

Eucalyptus grandis was provided by LUMIN plywood plant, located in Tacuarembó, Uruguay. The wood received was cut, chipped (in two steps) and dried at 40 °C in a pilot-scale wood drying kiln to a moisture content of approximately 10 %. The chemical composition, expressed as g of component per 100 g of raw material on dry basis, was: (48.9 ± 1.3)% glucan; (14.5 ± 1.5)% xylan; (1.8 ± 0.4)% acetyl groups; (26.4 ± 0.1)% and (2.7 ± 0.1)% acid-insoluble and acid-soluble lignin, respectively; (0.2 ± 0.1)% ashes and (3.3 ± 0.1)% extractives. Arabinan was not detected. The particle size was as follows: 1.4 % higher than 3350 µm, 6.5 % between 2380 and 3350 µm, 22.0 % between 1400 and 2380 µm, 33.7 % between 840 and 1400 µm, 21.7 % between 500 and 840 µm, and 14.8 % below 500 µm. The fines (material fraction below 500 µm) were discarded.

2.2. Acid impregnated steam-explosion pretreatment

E. grandis chips were soaked overnight in dilute sulfuric acid solutions at different concentrations, with a moisture content of 30 %, at 21 °C in a controlled temperature chamber. STEX of the acid-soaked material was carried out in a continuous pilot equipment (Advance Bio Systems LLC, model S1401-D2011). Description of the equipment has been detailed by Bonfiglio et al. [29]. The experimental runs were carried out according to the experimental design detailed in Table 1, to evaluate the effect of temperature (160 °C and 180 °C) and sulfuric acid concentration (0–0.5 %, expressed as g of acid per 100 g of dry material) on the enzymatic digestibility of steam-exploded *E. grandis*. The residence time in the reactor was 10 min. Each condition was evaluated by duplicate.

After STEX pretreatment, the steam-exploded slurry was filtered using a fabric by filter pressing (20 MPa) to separate the solid and liquid (hemicellulosic liquor) fractions. The solid fractions were water-washed three times at room temperature using a liquid-to-solid ratio of 5:1 (5 g of tap water per g of dried pretreated solid). The washed pretreated eucalyptus solids were characterized according to the National Renewable Energy Laboratory (NREL) protocols and stored at 4 °C for enzymatic hydrolysis assays, while liquid fractions and washing waters were stored at – 18 °C for chemical analysis.

A combined severity factor (CSF) developed by Chum et al. [30], which integrates the three main parameters that significantly influence the process —temperature, time, and acid concentration— has been

used to compare different dilute acid pretreatments [11,13,14,31,32]. The CSF was calculated using Eq. (1), where T is the temperature (°C), t is time (min) and pH refers to the pH of the liquid fraction (liquor).

$$CSF = \log\left(texp\left(\frac{(T-100)}{14.75}\right)\right) - pH \quad (1)$$

2.3. Enzymatic hydrolysis of steam-exploded E. grandis

The effect of pretreatment conditions on the enzymatic digestibility of steam-exploded *E. grandis* was first evaluated. For this, enzymatic hydrolysis assays were carried out in 250-mL Erlenmeyer flasks containing 100 mL of suspension in an orbital shaker (Infors HT Ecotron, Switzerland) at 150 rpm and 50 °C. The solid content was 16 % (w/w), the enzyme dose of Cellic CTec2 (Sigma Aldrich®) was 25 FPU/g_{glucan} and the pH 4.8 (0.05 M acetate buffer). The Cellic CTec2 preparation had a cellulase activity of 144 FPU/mL. Samples were taken routinely for sugar analysis, which were treated at 95 °C for 10 min to deactivate the enzyme, and then centrifuged to separate the supernatants, which were analyzed by HPLC for sugar quantification.

Once the pretreatment conditions in terms of temperature and sulfuric acid concentration were selected, a three-level Box-Behnken experimental design with a central point, five replicates, and eight additional points, was used to evaluate the effects of initial pH (4.8, 5.4, 6), solid content (16 %, 20 %, 24 %), and enzyme dose (15 FPU/g_{glucan}, 20 FPU/g_{glucan}, 25 FPU/g_{glucan}) on enzymatic hydrolysis. The experiments were conducted in 250-mL Erlenmeyer flasks containing 100 mL of suspension at 50 °C and 150 rpm, using Cellic CTec2 preparation with a cellulase activity of 174 FPU/mL. The pH was adjusted to the desired values using acetate buffer solutions, and chloramphenicol 20 mg/L was added. The responses evaluated were final glucose concentration and hydrolysis efficiency of glucan.

2.4. Ethanol production

The pretreatment conditions of the raw material (temperature and acid concentration) and the enzymatic hydrolysis conditions (enzyme dosage, initial pH and solids content) were selected based on experiences described in the previous sections. Fermentation was performed using the *Saccharomyces cerevisiae* strain Thermosacc®. Duplicate experiments were conducted under two configurations: a) SHF (Separate Hydrolysis and Fermentation): enzymatic hydrolysis was performed for 72 h at 50 °C, followed by fermentation of the resulting hydrolysate at 37 °C; b) PSSF (Pre-saccharification and Subsequent Simultaneous Saccharification and Fermentation): enzymatic hydrolysis was carried out for 24 h at 50 °C prior to simultaneous saccharification and fermentation, conducted at 37 °C after inoculation with the yeast strain.

The experiments were carried out in 250-mL Erlenmeyer flasks with orbital shaking (150 rpm). The pH was adjusted to 6 using an acetate buffer solution (0.050 M), chloramphenicol 20 mg/L was added, and the medium was supplemented with 5 mL of a nutrient solution containing 100 g/L peptone, 60 g/L yeast extract, and 60 g/L malt extract. The final volume was 100 mL. The Cellic CTec2 preparation with a cellulase activity of 244 FPU/mL was used. The initial yeast cell concentration was $1.5x10^8$ cells/mL. Samples were taken routinely for sugar and ethanol analysis and cell counts.

2.5. Analytical methods

The composition of the raw material and pretreated raw material were determined according to NREL analytical procedures [33–36]. For extractives determination, an 8-h extraction with water followed by a 16-h extraction with ethanol were used. The chemical composition of the liquid fractions from STEX pretreatments (hemicellulosic liquor, washing waters, and condensates) were also determined following NREL protocols [35,36]. Glucose, cellobiose, furfural, HMF, organic acids (formic and acetic), ethanol, and glycerol, were quantified by HPLC (Shimadzu, Kyoto, Japan) equipped with a RID-10A detector and a Biorad Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) at 45 °C. Sulfuric acid (0.005 M) at 0.6 mL/min was used as a mobile phase. Xylo-oligosaccharides (XOS) and *gluco*-oligosaccharides (GOS) contents in the liquors and washing waters were determined as the difference between the xylose or glucose content before and after hydrolysis by diluting sulfuric acid. Xylosaccharides (XS) were also

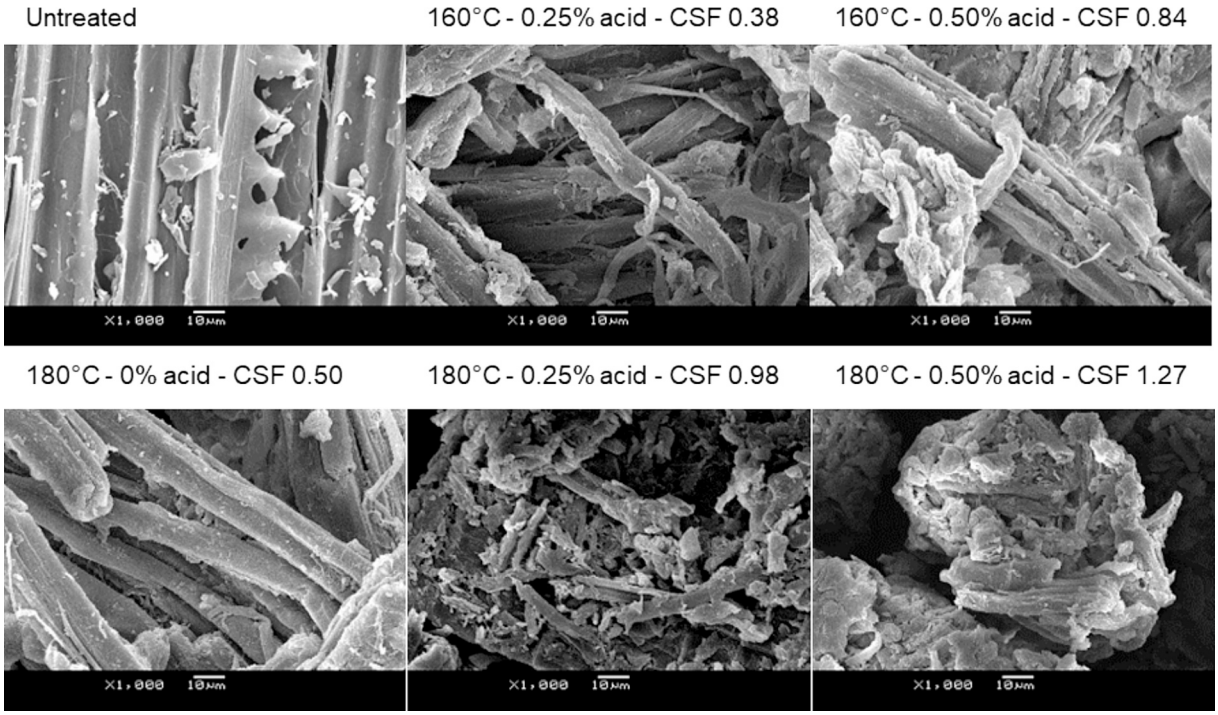

Fig. 1. SEM images of untreated and steam-exploded *E. grandis* under different conditions, at 1000x magnification.

Table 2
Composition of the liquid fractions resulting from the pretreatment: liquid or liquor fraction, first washing water (WW1), second washing water (WW2) and condensate (CC).

CSF	Conditions		Liquid fraction	Concentration (g/L)										
				Glucose			Xylose			Arabinose	Acetic acid	Formic acid	Furfural	HMF
	T (°C)	H_2SO_4 (% w/w)		Monomeric	Oligomeric	**Total**	Monomeric	Oligomeric	**Total**					
0.38	160	0.25	Liquor	2.4 ± 1.4	4.1 ± 0.2	**5.6 ± 0.3**	15.5 ± 5.3	37.2 ± 3.3	**52.8 ± 8.7**	3.2 ± 0.6	9.2 ± 1	1.4 ± 0.1	0.2 ± 0.1	0.2 ± 0
			WW1	0.5 ± 0.2	<0.1	**0.5 ± 0.2**	3.4 ± 0.5	7.4 ± 0.1	**10.7 ± 0.6**	0.5 ± 0.2	2.0 ± 0.1	0.2 ± 0.1	<0.1	<0.1
			WW2	0.1 ± 0.1	0.5 ± 0.3	**0.6 ± 0.4**	0.6 ± 0.1	1.1 ± 0.1	**1.7 ± 0.2**	0.1 ± 0	0.5 ± 0.1	0.2 ± 0.1	<0.1	<0.1
			CC	N/D	N/D	**N/D**	N/D	N/D	**N/D**	N/D	N/D	0.5 ± 0.1	N/D	N/D
0.84	160	0.50	Liquor	6.3 ± 0.1	2.1 ± 0.3	**8.6 ± 0.2**	51.5 ± 0.2	29.5 ± 0.8	**81 ± 0.6**	5.0 ± 0.1	12.1 ± 0.5	2.2 ± 0.1	0.4 ± 0.1	<0.1
			WW1	1.5 ± 0.1	1.2 ± 1.0	**2.8 ± 1.0**	9.3 ± 0.3	5.7 ± 0.2	**15 ± 0.5**	1.1 ± 0.1	2.6 ± 0.6	0.9 ± 0.6	0.3 ± 0.2	<0.1
			WW2	0.2 ± 0.1	<0.1	**0.2 ± 0.1**	1.6 ± 0.1	0.5 ± 0.2	**2.1 ± 0.3**	0.2 ± 0.1	0.5 ± 0.1	<0.1	<0.1	<0.1
			CC	N/D	N/D	**N/D**	N/D	N/D	**N/D**	N/D	N/D	1.5 ± 0.1	0.4 ± 0.1	N/D
0.50	180	0	Liquor	2.1 ± 0.5	1.7 ± 0.7	**3.5 ± 0.9**	18.9 ± 2.1	58.8 ± 0.9	**77.7 ± 1.2**	4.4 ± 0.1	14.7 ± 0.9	2.3 ± 0.4	0.3 ± 0.2	0.3 ± 0.2
			WW1	0.3 ± 0.1	0.1 ± 0.1	**0.4 ± 0.1**	2.9 ± 0.4	7.8 ± 0.9	**10.8 ± 1.3**	0.7 ± 0.2	2.3 ± 0.1	0.3 ± 0.1	<0.1	<0.1
			WW2	<0.1	<0.1	**<0.1**	0.4 ± 0.2	1 ± 0	**1.3 ± 0.4**	0.2 ± 0.2	0.4 ± 0.1	0.2 ± 0.1	<0.1	<0.1
			CC	N/D	N/D	**N/D**	N/D	N/D	**N/D**	N/D	N/D	0.9 ± 0.1	0.7 ± 0.1	N/D
0.98	180	0.25	Liquor	8.9 ± 0.7	4.1 ± 1.1	**12.9 ± 2**	59.7 ± 5.8	29.1 ± 8.6	**88.8 ± 14.6**	5.3 ± 1.7	14.7 ± 0.8	3.9 ± 0.3	0.5 ± 0.2	0.4 ± 0.3
			WW1	1.3 ± 0.2	0.2 ± 0.1	**1.5 ± 0.2**	8.9 ± 0.1	2.9 ± 0.6	**11.8 ± 0.5**	0.7 ± 0.5	2.5 ± 0.4	1.2 ± 0.5	0.2 ± 0.1	0.2 ± 0.1
			WW2	0.3 ± 0.1	<0.1	**0.3 ± 0.1**	2.0 ± 0.5	0.3 ± 0.2	**2.1 ± 0.8**	0.2 ± 0.1	0.7 ± 0.1	0.2 ± 0.1	<0.1	<0.1
			CC	N/D	N/D	**N/D**	N/D	N/D	**N/D**	N/D	N/D	1.2 ± 0.1	1.0 ± 0.1	N/D
1.27	180	0.50	Liquor	20.2 ± 2.0	4.5 ± 1.2	**24.9 ± 3.5**	67.9 ± 7.9	15 ± 4.6	**82.9 ± 12.5**	5.2 ± 1.1	11.4 ± 1.8	5.0 ± 0.5	0.9 ± 0.1	0.2 ± 0
			WW1	2.6 ± 0.1	0.5 ± 0.1	**3.0 ± 0.2**	8.8 ± 0.9	1.5 ± 0.3	**10.3 ± 1.2**	0.8 ± 0.2	2.0 ± 0.1	1.2 ± 0.5	<0.1	0.2 ± 0.1
			WW2	0.8 ± 0.2	<0.1	**0.8 ± 0.2**	2.6 ± 0.7	0.1 ± 0.1	**2.7 ± 0.7**	0.2 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	<0.1
			CC	N/D	N/D	**N/D**	N/D	N/D	**N/D**	N/D	N/D	3.5 ± 0.1	3.2 ± 0.1	0.1 ± 0.1

CSF: combined severity factor. Oligomeric glucose and oligomeric xylose are expressed in terms of the equivalent monomer (glucose or xylose). HMF: 5-hydroxymethylfurfural. N/D: not detected.

reported as xylose measured after acid hydrolysis. All analyses were done in triplicate.

The cellulase activity of Cellic CTec 2 was determined following the NREL protocol for each new enzymatic extract vial [37].

Scanning Electron Microscope (SEM) images were acquired using a JEOL JSM-5900LV electron microscope. Dried samples were covered with a thin gold film and then exposed to an acceleration voltage of 20 kV. Images were captured at magnifications of 1000x.

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.

2.6. Calculation and statistical analysis

Results were analyzed using analysis of variance (ANOVA) to determine statistical differences ($p \leq 0.05$) and response surface methodology by the statistical software Design Expert 7.0.0 (Stat-Ease Inc., Minneapolis, USA).

3. Results and discussion

3.1. Steam explosion pretreatment of E. grandis

Chemical characterization of steam-pretreated *E. grandis* wood is presented in Table 1. The pretreatment resulted in the complete removal of extractives and acetyl groups from the solid, leaving a composition of glucan, xylan, and lignin ranging from 59.4 % to 64.1 %, 1.8 % to 7.8 %, and 35.2 % to 40.2 %, respectively. Most of the lignin remained in the solid fractions (recovery higher than 85 %). Similar values were reported by McIntosh et al. [26] for *Eucalyptus grandis* pretreated in a STEX system after being impregnated with sulfuric acid. The process was carried out at temperatures ranging from 170 °C to 190 °C for 15 min, with acid concentrations of 0.6 % and 4.8 % (w/w). They reported glucan contents of 39.8 %–52.4 %, xylan contents of 0 %–9.7 %, and lignin contents of 26.3 %–38.0 %. At 180 °C and 0.6 % acid, the contents of these components were 42.1 %, 7.3 %, and 28.8 %, respectively.

No clear trend was observed regarding glucan content in the solid fraction as sulfuric acid concentration increased. However, higher

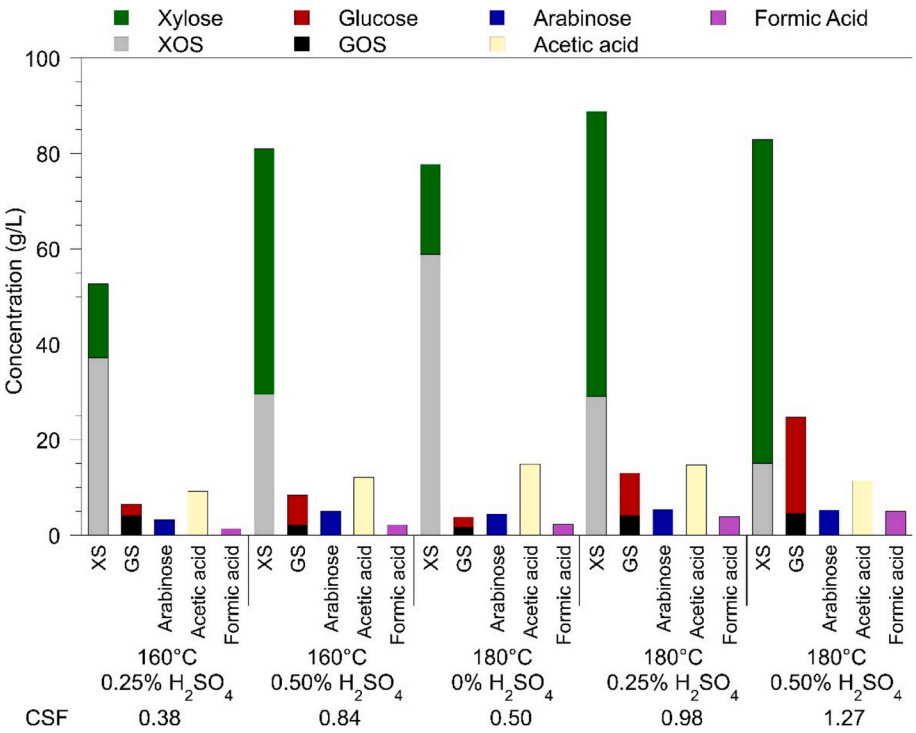

Fig. 2. Concentration of different components in the liquor (hemicellulosic hydrolysate) after pretreatment. XS: xylosaccharides; GS: glucosaccharides; GOS: oligomeric glucose; XOS: oligomeric xylose; 5-HMF: 5-hydroxymethylfurfural.

sulfuric acid concentrations led to a decrease in xylan content and an increase in lignin content, which is an expected behavior with increasing pretreatment severity. Additionally, in some cases, the total composition values exceeded 100 % (Table 1), possibly due to pseudo-lignin formation. During pretreatment, changes occur in biomass chemistry, particularly through acid hydrolysis and the partial dissolution of hemicellulose and lignin, which can lead to undesired secondary reactions such as carbohydrate dehydration and lignin condensation [38]. Furans are partially lost as volatiles during pretreatment or may undergo condensation reactions, resulting in the formation of acid-insoluble humins and/or pseudo-lignin. This apparent overestimation of lignin content is attributed to the accumulation of these compounds, which are subsequently quantified as acid-insoluble lignin [38], a limitation that the NREL protocols are unable to distinguish [39].

The SEM images of untreated and pretreated solids are shown in Fig. 1. For the untreated raw material, the compact structure of the lignocellulosic biomass, with the fibers tightly bound together exhibiting the typical morphology of long plant cells with well-defined cell walls, was observed.

It can be seen that the most severe pretreatment conditions (180 °C and 0.5 % of acid) resulted in the highest deconstruction of the lignocellulosic matrix, as expected. Under this condition, the biomass exhibited significant fiber breakdown, which may also have led to the formation of pseudo-lignin, as previously discussed. In contrast, the STEX condition at 180 °C and 0 % acid showed substantial deconstruction, but with a greater presence of mostly intact fibers.

Interestingly, although the CSF clearly differs between the conditions 160 °C with 0.25 % acid and 160 °C with 0.5 % acid, the SEM images do not show evident differences. However, the increased presence of debris under the 160 °C and 0.5 % acid condition suggests a greater degree of lignocellulosic matrix deconstruction, which is consistent with the chemical composition of the solids. In the case of 180 °C with 0.25 % acid, a higher degree of deconstruction is observed compared to both 160 °C conditions, with fewer unaffected fibers present.

The composition of the liquid fractions (liquor obtained from the pretreatment and separated using a filter press, two washing waters from the first two washing stages of the pretreated material, and the condensate) is presented in Table 2, while Fig. 2 shows the total concentration of the main components. The total xylose concentrations (oligomer + monomer) in the liquors varied between 53 g/L and 89 g/L, with the highest concentration obtained at 180 °C with 0.25 % acid impregnation. In contrast, total glucose concentrations ranged from 3 to 27 g/L. In the first washing waters, high xylose concentrations were recovered (ranging from 9 to 15 g/L total xylose), along with up to 3 g/L total glucose, corresponding a total xylose recovered between 25 and 45 %, and until 2.3 % of total glucose. In the second washing waters analyzed, lower concentrations were observed, ranging from 2 to 4 g/L total xylose, which corresponds to a total xylose recovered of 3 to 9 %.

Concerning the relationship between the monomeric and oligomeric xylose, Fig. 2 shows that as the acid concentration increased, the xylose concentration in the liquor after pretreatment increased at both temperatures, while the XOS concentration decreased, as expected.

The recovery of glucan (89 % to 78 %) in solid and xylan as xylose (64 % to 50.6 %) in the liquid decreased with acid concentration from 0 to 0.50 % for 180 °C. Similarly, for an acid concentration of 0.50 %, the recovery of xylan as xylose was higher at 160 °C (68.0 % vs 50.6 %).

Regarding other components analyzed in the liquor, acetic acid concentrations ranged from 10 to 15 g/L, and formic acid concentrations ranged from 1 to 5 g/L. Furfural concentrations were generally below the detection limit in liquors from milder pretreatments, reaching levels of 0.9–1.0 g/L at 180 °C and 0.5 % sulfuric acid in the liquor, and up to 3 g/L in the condensate. HMF showed a similar trend, with concentrations of up to 0.4 g/L in the liquor at 180 °C and 0.25 % sulfuric acid, and 0.5 g/L in the condensate at 180 °C and 0.5 % sulfuric acid.

Similar values were reported by Emmel et al. [40], who found that the best condition for hemicellulose recovery in the liquid fraction (mostly as xylose) from eucalyptus chips impregnated with 0.175 % (w/w) sulfuric acid and pretreated by STEX was 210 °C for 2 min, achieving approximately 70 % of the total theoretical xylose yield. McIntosh et al. [26] reported glucan recovery values ranging from 69.9 % to 93.4 % for *Eucalyptus grandis* pretreated in a STEX system, previously impregnated with sulfuric acid at temperatures between 170 °C and 190 °C for 15

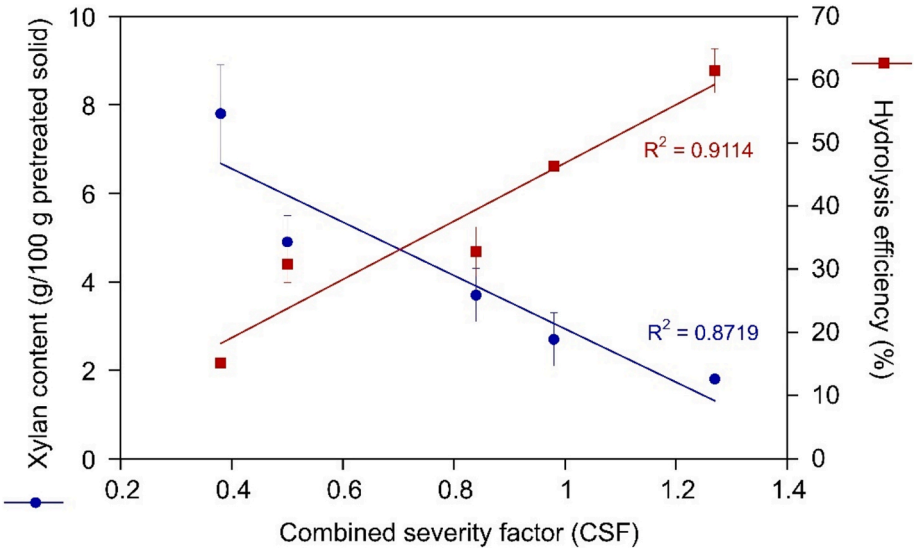

Fig. 3. Hydrolysis efficiency and xylan content of pretreated solid vs the combined severity factor (CSF).

Table 3
Experimental design and enzymatic hydrolysis parameters for *E. grandis* wood pretreated after 72 h of hydrolysis.

Run	Solid content (%)	Enzyme dose (FPU/g_{glucan})	Initial pH	Final pH	Glucose[a] (g/L)	Hydrolysis efficiency[b] (%)	Overall glucose production yield[c] (%)
18	14.6	25	4.8	4.8	70.8 ± 1.0	65.0 ± 1.1	46.9 ± 0.8
22	16	15	4.8	4.8	60.5 ± 0.9	52.1 ± 0.9	37.6 ± 0.6
8	16	20	4.8	4.8	65.9 ± 1.0	58.7 ± 1.0	42.3 ± 0.7
10	16	15	5.4	5.1	66.2 ± 1.0	60.6 ± 1.0	43.7 ± 0.7
13	16	25	5.4	5.1	76.0 ± 1.1	63.5 ± 1.1	45.8 ± 0.8
23	16	15	6.0	5.2	72.5 ± 1.1	63.3 ± 1.0	45.7 ± 0.7
25	16	15	6.0	5.2	71.5 ± 1.0	62.5 ± 1.0	45.1 ± 0.7
3	16	20	6.0	5.2	78.4 ± 1.1	67.4 ± 1.1	48.6 ± 0.8
11	20	15	4.8	4.7	75.5 ± 1.1	52.0 ± 0.9	37.5 ± 0.6
17	20	25	4.8	4.7	90.0 ± 1.3	59.8 ± 1.0	43.2 ± 0.7
21	20	20	4.6	4.5	75.2 ± 1.1	50.3 ± 0.8	36.3 ± 0.6
2	20	20	5.4	5.0	89.9 ± 1.3	61.2 ± 1.0	44.2 ± 0.7
4	20	20	5.4	5.0	89.9 ± 1.3	61.3 ± 1.0	44.2 ± 0.7
9	20	20	5.4	5.0	88.2 ± 1.3	59.9 ± 1.0	43.2 ± 0.7
12	20	20	5.4	5.0	91.6 ± 1.3	62.6 ± 1.0	45.1 ± 0.7
16	20	20	5.4	4.7	88.9 ± 1.3	60.5 ± 1.0	43.6 ± 0.7
7	20	15	6.0	5.1	87.3 ± 1.3	60.8 ± 1.0	43.9 ± 0.7
1	20	25	6.0	5.1	104.9 ± 1.5	71.0 ± 1.2	51.2 ± 0.9
20	20	20	6.3	5.1	102.1 ± 1.5	70.5 ± 1.2	50.8 ± 0.9
15	24	20	4.8	4.6	93.0 ± 1.4	52.0 ± 0.9	37.5 ± 0.6
5	24	15	5.4	4.9	94.7 ± 1.4	54.6 ± 0.9	39.4 ± 0.7
6	24	25	5.4	4.9	112.5 ± 1.6	62.7 ± 1.0	45.2 ± 0.7
14	24	20	6.0	5.0	116.3 ± 1.7	66.6 ± 1.1	48.0 ± 0.8
24	24	25	6.0	4.9	121.7 ± 1.8	68.4 ± 1.1	49.3 ± 0.8
19	25.4	25	4.8	4.6	112.9 ± 1.6	59.0 ± 1.0	42.6 ± 0.7

[a] Glucose concentration obtained after 72 h.
[b] Hydrolysis efficiency calculated considering the glucan composition of pretreated material.
[c] Overall glucose production yield calculated based on the glucan content of the raw material.
Runs 18 to 25 correspond to additional points in the experimental design.

min, with acid concentrations of 0.6 % and 4.8 % (w/w). At 180 °C and 0.6 % acid, glucan recovery was 92.1 %.

3.2. Enzymatic hydrolysis of steam-exploded E. grandis

The results for final glucose concentration and hydrolysis efficiency, obtained with a solids content of 16 % (w/w) and an enzyme loading of 25 FPU/g_{glucan} after 96 h, are presented in Table 1. It was observed that increasing the CSF enhanced enzymatic hydrolysis efficiency. Both enzymatic digestibility and xylan removal increased with pretreatment severity in a linear manner, with good linear regression coefficients (R^2), as shown in Fig. 3. The best enzymatic hydrolysis condition was achieved in the pretreatment conducted at 180 °C with 0.5 % acid, resulting in a hydrolysis efficiency of 61.4 %. In contrast, the least effective condition corresponded to the pretreatment at 160 °C with 0.25 % acid. Consequently, the pretreatment at 180 °C with 0.5 % acid was selected for further studies aimed at optimizing enzymatic hydrolysis.

Emmel et al. (2003) reported higher cellulose conversion (90 %) for eucalyptus chips impregnated with 0.175 % (w/w) sulfuric acid and pretreated in a STEX system at a higher temperature of 200 °C. McIntosh et al. [26] reported glucan digestibility values ranging from 9.4 % to 68.0 % for *Eucalyptus grandis* pretreated in a STEX system after impregnation with sulfuric acid at temperatures between 170 °C and

Table 4
ANOVA for final glucose concentration (a), hydrolysis efficiency (b), and overall glucose production yield (c).

(a)

Source	Sum of squares	Df	Mean square	F value	p-value	Significance at $p < 0.05$
Model	6751.09	6	1125.18	274.14	< 0.0001	S
A – Solids content	3365.67	1	3365.67	820.03	< 0.0001	S
B – Enzyme dose	732.61	1	732.61	178.50	< 0.0001	S
C – pH	1035.04	1	1035.04	252.18	< 0.0001	S
AB	21.26	1	21.26	5.18	0.0353	S
AC	41.85	1	41.85	10.20	0.0050	S
BC	2.86	1	2.86	0.70	0.4146	NS
Residual	73.88	18	4.10	–	–	–
Lack of Fit	66.59	13	5.12	3.51	0.0867	NS
Pure Error	7.29	5	1.46	–	–	–

S: significative. NS: no significative

(b)

Source	Sum of squares	Df	Mean square	F value	p-value	Significance at $p < 0.05$
Model	729.12	6	121.52	46.37	< 0.0001	S
A – Solids content	30.75	1	30.75	11.73	0.0030	S
B – Enzyme dose	222.15	1	222.15	84.77	< 0.0001	S
C – pH	543.32	1	543.32	207.31	< 0.0001	S
AB	1.98	1	1.98	0.76	0.3960	NS
AC	11.29	1	11.29	4.31	0.0525	NS
BC	2.69	1	2.69	1.03	0.3243	NS
Residual	47.17	18	2.62	–	–	–
Lack of Fit	42.70	13	3.28	3.67	0.0798	NS
Pure Error	4.47	5	0.89	–	–	–

S: significative. NS: no significative

(c)

Source	Sum of squares	Df	Mean square	F value	p-value	Significance at $p < 0.05$
Model	369.76	6	61.63	43.09	< 0.0001	S
A – Solids content	16.94	1	16.94	11.84	0.0029	S
B – Enzyme dose	111.99	1	111.99	78.30	< 0.0001	S
C – pH	276.05	1	276.05	193.00	< 0.0001	S
AB	0.63	1	0.63	0.44	0.5164	NS
AC	4.87	1	4.87	3.41	0.0814	NS
BC	1.69	1	1.69	1.18	0.2909	NS
Residual	25.74	18	1.43	–	–	–
Lack of Fit	23.41	13	1.80	3.86	0.0725	NS
Pure Error	2.33	5	0.47	–	–	–

S: significative. NS: no significative

190 °C for 15 min, with acid concentrations from 0.6 % to 4.8 % (w/w). The highest digestibility was observed at 190 °C, 15 min, and 4.8 % sulfuric acid. At 180 °C for 15 min, increasing the acid loading from 0.6 % to 2.4 % resulted in an increase in digestibility from 15.5 % to 32.8 %, respectively, at the laboratory scale. However, at the pilot scale under the same conditions (180 °C, 15 min, and 2.4 % acid), the glucan digestibility was higher (71.8 %), with a total glucose conversion of 63.6 %.

Additional experiments were conducted using *E. grandis* pretreated at 180 °C with 0.5 % (w/w) sulfuric acid to enhance hydrolysis efficiency and evaluate the effects of initial pH, solids content, and enzyme dosage. A three-level Box-Behnken experimental design was employed, incorporating five replicates of the central point, and eight additional runs. The analyzed responses included final glucose concentration, glucan hydrolysis efficiency, and overall glucose production yield. Results are summarized in Table 3, with glucose concentrations ranging from 60.5 to 121.7 g/L, hydrolysis efficiencies from 50.3 % to 71.0 %, and overall glucose production yield from 36.3 % to 51.2 %.

A statistical analysis was performed with a significance level of $p < 0.05$. Table 4 presents the Analysis of Variance (ANOVA) results for glucose concentration, hydrolysis efficiency, and overall glucose production yield. The results followed a linear model with interaction between the factors for final glucose concentration and a linear model for glucan hydrolysis efficiency and overall glucose production yield. For all responses studied, the analysis indicated that solid content, enzyme dose, and initial pH were significant factors. In the case of final glucose concentration, the interaction between solid content and pH, and solid content and enzyme dose, were also significant. Eqs. (2) to (4) show the mathematical models found in terms of coded values. The coefficients of determination (R^2) for final glucose concentration, hydrolysis efficiency, and overall glucose production yield were 0.9892, 0.9244 and 0.9222, respectively.

$$Glucose(g/L) = 88.85 + 16.6A + 7.85B + 7.74C + 1.52A.B + 2.26A.C - 0.61B.C \quad (2)$$

$$Hydrolysisefficiency(g/L) = 60.95 - 1.72A + 4.47B + 5.55C \quad (3)$$

$$Overallglucoseproductionyield(g/L) = 43.92 - 1.28A + 3.17B + 3.95C \quad (4)$$

where *A* is solids content, *B* is the enzyme dose, and *C* is pH.

Regarding the final glucose concentration, increasing the values of all factors had a positive effect. Solid content was the most significant factor, followed by enzyme dose. For hydrolysis efficiency and overall glucose production yield, solid content had a negative effect, while initial pH and enzyme dose had positive effects. The negative impact of solid content could be attributed to enzyme inhibition caused by high glucose concentrations or limitations in mass transfer. The most significant factor influencing hydrolysis efficiency and overall glucose production was pH, followed by enzyme dose. The optimized hydrolysis conditions obtained by maximizing the three responses (final glucose concentration, hydrolysis efficiency and overall glucose production yield) were pH 6.0, 24 % solid content, and enzyme dose of 25 FPU/g_{glucan}. The maximum enzyme dosage used in the experiments (25 FPU/g_{glucan}) is considered an upper limit due to the high cost and environmental impact of enzymes. While higher enzyme doses may enhance hydrolysis efficiency, it is essential to balance efficiency gains against these costs.

After 72 h of hydrolysis, despite the use of buffering solutions, the pH was lower than the initial value in most cases, except for some experiments where the initial pH was 4.8, in which the pH remained unchanged (Table 3). The final pH under optimal conditions was similar to the pH recommended by the enzyme supplier (4.8).

Fig. 4 shows the effect of pH on hydrolysis efficiency for the different experimental conditions studied. Increasing the pH, the hydrolysis efficiency increased in all cases. Similar values were found by Larnaudie et al. [25] for switchgrass pretreated with autohydrolysis at 200 °C for 5 min, who found that by increasing the solids content from 15 % to 25 %, the optimal pH increased from 4.8 to 6. Lan et al. [20] and Romero et al. [22] also reported an increase in the optimal pH (value close to pH 6) of enzymatic hydrolysis by increasing the solids content for different biomasses.

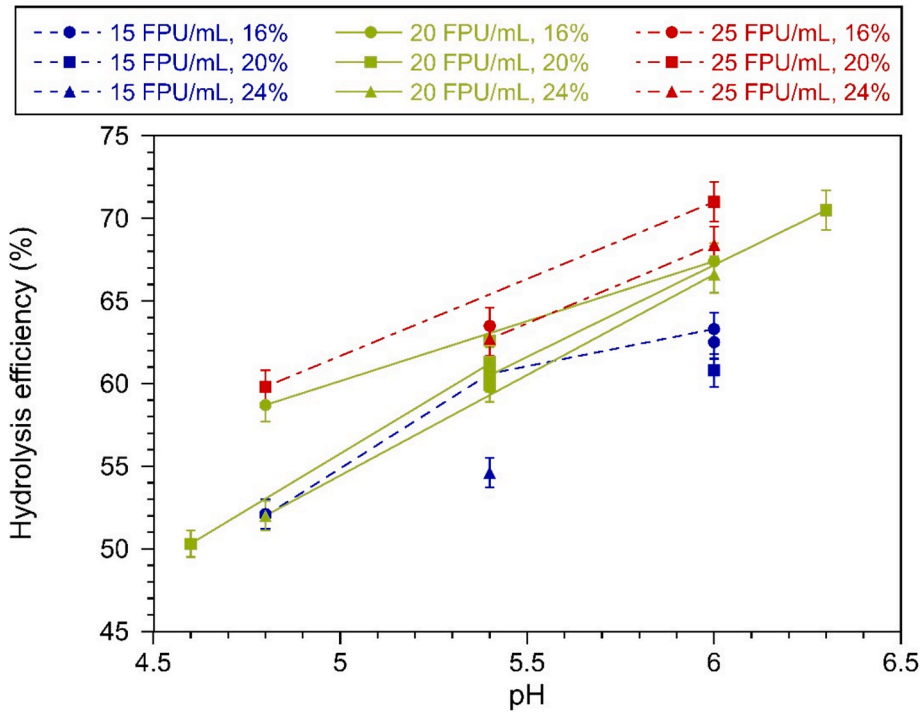

Fig. 4. Hydrolysis efficiency vs initial pH for all experimental conditions studied.

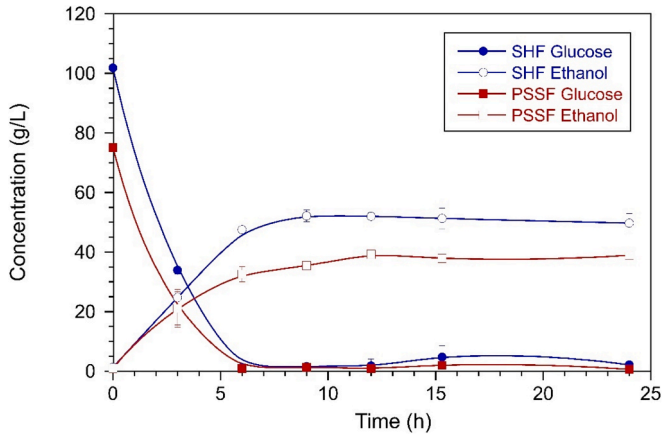

Fig. 5. Glucose and ethanol concentration profiles over time for the SHF and PSSF experiments. Saccharification conditions: pH 6.0, 24% (w/w) solid content, and an enzyme dose of 25 FPU/g_{glucan}.

3.3. Fermentation of eucalyptus hydrolysates under selected pretreatment conditions and enzymatic hydrolysis

The fermentation profiles obtained for *E. grandis* pretreated with STEX and enzymatically hydrolyzed under the conditions selected in the previous section are presented in Fig. 5, for the two process configurations evaluated, SHF and PSSF. The values of the evaluated parameters are presented in Table 5.

In the SHF experiments with pretreated solids, a final ethanol concentration of 52 g/L was achieved after 9 h of fermentation. An overall efficiency of 46 % was obtained for the conversion of glucan from raw eucalyptus into ethanol. The PSSF experiments showed limited ethanol production once the glucose primarily derived from the 24 h pre-hydrolysis was depleted, highlighting the limited extent of hydrolysis. Although a low hydrolysis rate was expected, particularly due to the temperature used, the efficiency did not reach previously reported levels. After 12 h of fermentation, an ethanol concentration (39 g/L) corresponding to an overall conversion efficiency of 34 % was achieved. Extending the incubation time to 72 h showed no additional hydrolysis or fermentation (hence, only data up to 24 h is presented in Fig. 5). This unexpected behavior requires further investigation. In both experiments, final cellobiose and glycerol concentrations of 2.6–4.0 g/L and 3–5 g/L, respectively, were observed. Xylose concentrations remained between 2–4 g/L.

McIntosh et al. [26] reported similar results with *Eucalyptus grandis* pretreated by STEX at 180 °C for 15 min with 2.4 % sulfuric acid at a pilot scale. Fermentations were conducted with 15 % (w/w) solid content in PSSF mode using 20 FPU/g_{glucan} at 30 °C, both at laboratory scale (300 g of pretreated solid) and pilot scale (15 kg of pretreated solid). Final ethanol concentrations of 25.0 g/L and 17.8 g/L were achieved at laboratory and pilot scales, respectively, corresponding to ethanol conversions—calculated based on the theoretical maximum glucose in the pretreated solid—of 54.1 % and 42.0 % at 48 h. They concluded that cellulose digestion under high substrate loading in the PSSF configuration was inefficient. To improve this outcome, they suggested developing improved bioreactor designs optimized for high solid content, alongside yeast and enzymes capable of narrowing the gap between the optimal temperatures for enzymatic activity and fermentation, to facilitate commercially viable processes.

In another work, McIntosh et al. [41] evaluated SSF fermentations (20 % solids, 96 h, 36 °C) of eucalyptus pretreated by STEX with acid impregnation (180 °C, 15 min, 2.4 % (w/w) sulfuric acid) and hydrolyzed using 40 FPU/$g_{biomass}$ of Cellic CTec2 and 30 mg/$g_{biomass}$ of PEG 6000. They reported final ethanol concentrations higher than those in the present study (60 g/L ethanol) and an ethanol yield of 85.8 % based on glucose from the original biomass, equivalent to 187 kg of ethanol per ton of original biomass.

Regarding different fermentation strategies, Neves et al. [27] found results similar to those of the present study using sugarcane bagasse pretreated by STEX, with and without the addition of sulfuric and phosphoric acids. The SHF mode yielded higher ethanol production than SSF and PSSF. However, when considering the total process time, PSSF resulted in higher productivity values.

Table 5
Hydrolysis and fermentation parameters for the SHF and PSSF experiments. Saccharification conditions: pH 6.0, 24% solid content, and enzyme dose of 25 FPU/g_{glucan}.

Run	Final time (h)	Ethanol (g/L)	Hydrolysis efficiency[a] (%)	Overall glucose conversion efficiency[a,b] (%)	Overall ethanol conversion efficiency		Productivity ($g_{ethanol}$/Lh)
					Based on pretreated solids[c] ($g_{ethanol}$/100 $g_{theoretical\ ethanol}$)	Based on raw material[b] ($g_{ethanol}$/100 $g_{theoretical\ ethanol}$)	
SHF	81	52.1 ± 2.0	70.1 ± 1.4	50.5 ± 1.0	63.6 ± 2.5	45.9 ± 1.8	0.64 ± 0.02
PSSF	36	39.2 ± 0.5	49.4 ± 2.9	35.6 ± 2.1	47.9 ± 0.6	34.5 ± 0.4	1.09 ± 0.01

[a] At 72 h for SHF assays and 24 h for PSSF assays.
[b] Calculated based on the glucan content of raw material.
[c] Calculated based on the glucan content of pretreated solid.

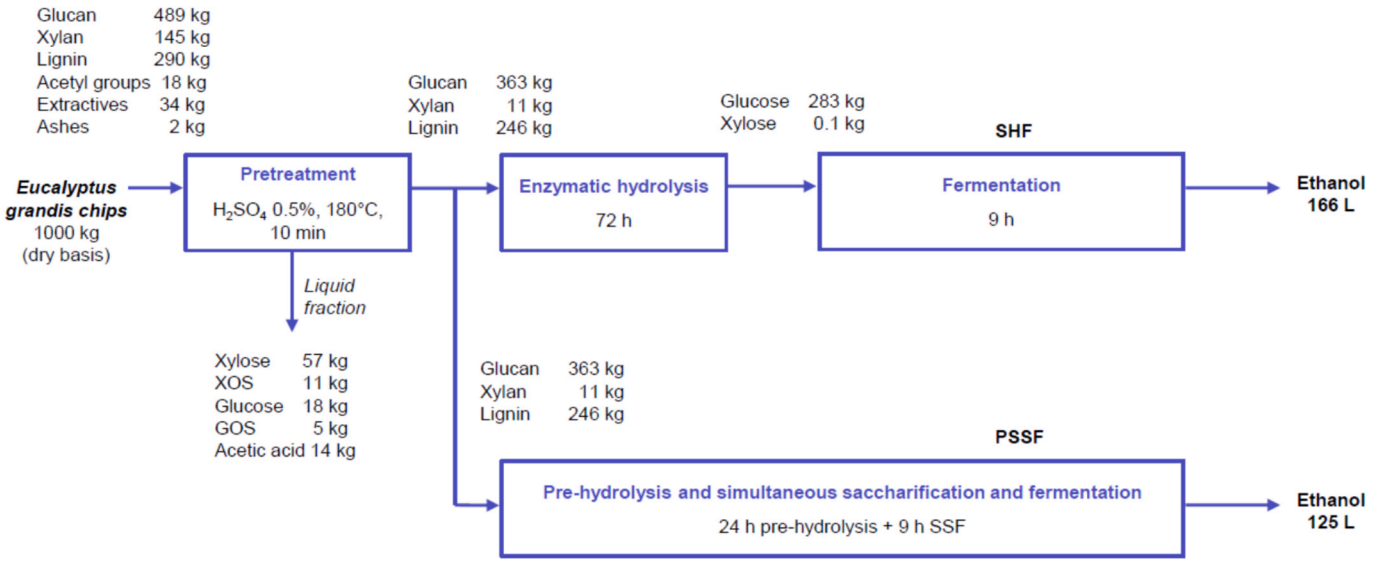

Fig. 6. Overall mass balance of the two enzymatic hydrolysis and fermentation strategies (SHF and PSSF) for production of ethanol from *E. grandis*. Enzymatic hydrolysis was carried out using a 24 % (w/w) solid loading, with the pH adjusted to 6 and an enzyme loading of 25 FPU/g_{glucan}. Fermentations were performed at 37 °C.

3.4. Mass balance

Fig. 6 shows a mass balance for the main components of *E. grandis* wood chips, based on the experimental results obtained and considering the two evaluated configuration for saccharification and fermentation. The diluted acid STEX pretreatment (180 °C, 10 min, 0.5 % acid) enabled the solubilization of 45 % of the xylan as xylose and XOS, and 82 % of the acetyl groups as acetic acid. These results indicate that the diluted acid STEX pretreatment is effective at fractionating hemicellulose into a liquid stream (rich in xylan-derived products and acetic acid, which can be recovered as value-added chemicals) while cellulose and lignin remain in the solid fraction. This solid fraction also became susceptible to enzymatic hydrolysis.

According to the chemical composition of *E. grandis* wood, 1000 kg of oven-dried material contains 489 kg of glucan, 290 kg of lignin, and 145 kg of xylan. After STEX, 620 kg of pretreated *E. grandis* (62 % of the raw material) were recovered as a solid fraction, containing 363 kg of glucan, 246 kg of lignin, and 11 kg of xylan (Fig. 6). The remaining material (105 kg) was solubilized into the hemicellulosic hydrolysate and washing waters, comprising 11 kg of XOS, 57 kg of xylose, 18 kg of glucose, 14 kg of acetic acid, and a minor amount of GOS (5 kg).

To produce ethanol from this steam pretreated *E. grandis,* an enzymatic hydrolysis step is required to convert carbohydrates (mainly glucan) into fermentable sugars. The residual xylan in the pretreated solid, representing 8 % of the original xylan content of the raw material, could also be valorized in subsequent stages by supplementing xylanases and using microorganisms capable of fermenting xylose. Although the exploitation of these additional fractions lies beyond the scope of this work, the yields and composition data presented here serve as valuable inputs for further studies.

Two strategies were evaluated for ethanol production: SHF and PSSF. In the SHF strategy, the solid fraction underwent enzymatic hydrolysis (24 % solid loading, 25 FPU/g_{glucan} enzyme loading, 72 h), yielding a hydrolysate containing 283 kg of glucose and 100 g of xylose as fermentable sugars, which was then fermented (37 °C, 9 h, pH 6) to ethanol. In the PSSF strategy, the solid fraction first underwent a pre-hydrolysis step under optimal conditions (24 % solid loading, 25 FPU/g_{glucan} enzyme loading, 50 °C, 24 h), followed by fermentation through the addition of the commercial yeast Thermosacc® (37 °C, 9 h, pH 6). The SHF and PSSF strategies achieved ethanol yields of 162 and 138 L per ton of raw material, respectively.

4. Conclusions

The pretreatment of *Eucalyptus grandis* using diluted acid impregnation and STEX in a continuous pilot-scale reactor proved effective for bioethanol production. The best enzymatic hydrolysis conditions were achieved with solids pretreated at 180 °C and 0.5 % sulfuric acid. The hemicellulosic liquor obtained under these conditions contained high xylosaccharides concentrations (up to 89 g/L), corresponding to a xylan recovery of 50.6 %. The experimental design identified solid content, enzyme dose, and pH as significant factors, with optimal conditions yielding 122 g/L glucose, 68 % enzymatic efficiency, and 49 % of overall glucose production yield. It was observed that higher pH values consistently enhanced hydrolysis efficiency. SHF produced 52 g/L ethanol with an overall conversion efficiency of 46 %, demonstrating

higher performance compared to PSSF, where limited additional hydrolysis occurred under fermentation conditions. These findings highlight the potential of this process to efficiently fractionate lignocellulosic biomass and optimize glucan conversion to bioethanol, supporting its future industrial-scale applications.

CRediT authorship contribution statement

Camila Bacquerié: Investigation, Methodology, Writing – review & editing, Formal analysis, Validation, Conceptualization. **Mairan Guigou:** Validation, Conceptualization, Visualization, Formal analysis, Methodology, Writing – review & editing, Investigation. **Florencia Cebreiros:** Visualization, Formal analysis, Writing – review & editing, Investigation, Validation, Conceptualization, Methodology. **Valeria Larnaudie:** Writing – original draft, Investigation, Writing – review & editing, Methodology, Visualization, Formal analysis, Validation, Conceptualization. **María E. Roman:** Methodology, Validation, Investigation, Formal analysis. **Matías Cagno:** Methodology, Validation, Investigation, Writing – review & editing, Conceptualization. **Facundo Rodríguez:** Methodology, Investigation. **Fernando Bonfiglio:** Writing – review & editing, Investigation, Validation, Methodology. **Mario D. Ferrari:** Visualization, Writing – review & editing, Conceptualization, Validation, Funding acquisition. **Claudia Lareo:** Writing – original draft, Project administration, Validation, Funding acquisition, Visualization, Methodology, Writing – review & editing, Resources, Formal analysis, Conceptualization.

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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Data availability

Data will be made available on request.

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