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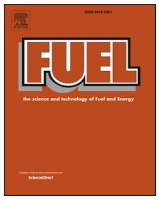

Full Length Article

Enhanced production of butanol and xylosaccharides from *Eucalyptus grandis* wood using steam explosion in a semi-continuous pre-pilot reactor

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ABSTRACT

In this work, the co-production of xylosaccharides and butanol from *Eucalyptus grandis* wood using steam explosion pretreatment in a biorefinery platform was investigated. The effect of different temperature conditions on xylosaccharide production and selectivity as well as enzymatic hydrolysis of pretreated *E. grandis* substrates was studied using a pre-pilot steam explosion reactor. Under selected conditions (200 °C, 1.5 MPa, 10 min), steam explosion pretreatment led to about 50% xylan recovery as xylooligosaccharides (XOS) and xylose and 80% glucan enzymatic hydrolysis even at high solid loading. A xylosaccharide-rich hydrolysate was effectively separated by ion-exchange and resin treatment, containing mostly xylose and xylobiose as XOS. The fermentation performance of four native butanol-producing strains (*Clostridium acetobutylicum* DSM 1731, *Clostridium beijerinckii* DSM 6422, *Clostridium beijerinckii* DSM 6423 and *Clostridium saccaroperbutylacetonicum* DSM 14923) were evaluated using semi-synthetic media and enzymatic hydrolysates for butanol production. Results showed that ABE strain *Clostridium beijerinckii* DSM 6422 and IBE strain *Clostridium beijerinckii* DSM 6423 are robust native strains for butanol production from lignocellulosic materials. This work highlights a potential integrated biorefinery process for the efficient utilization of *E. grandis* wood to produce biofuels and biochemicals.

1. Introduction

Lignocellulosic biomass represents an attractive resource for use in the production of biofuels and/or value-added chemicals due to its low cost, abundance and carbohydrate-rich composition. However, several important challenges should be overcome in order to develop cost-effective and large-scale processes using lignocellulosic biomass as raw material. The valorization of lignocellulosic biomass involves the use of the chemical components of the material such as carbohydrate (cellulose and hemicellulose) and non-carbohydrate (mainly lignin, a polyphenolic macromolecule) fractions to produce commercially value-added products. However, these main components are interconnected with one another in the biomass matrix, providing recalcitrance to pretreatment. To selectively fractionate the lignocellulosic biomass and separate its main chemicals components for further valorization, an effective and adequate pretreatment is necessary.

Among the different pretreatment methods reported in literature, steam explosion represents a low-cost and environmentally more acceptable pretreatment process in industry with minimal waste generation and environmental impacts, since no chemicals are required except for water. Extensive research and development have been carried out towards steam explosion pretreatment for a wide range of lignocellulosic materials [1–5]. Its efficiency for hemicellulose solubilization in its monomeric and oligomeric constituents was recently demonstrated [4]. During steam explosion pretreatment, the more labile hemicellulose fraction from lignocellulosic biomass is hydrolyzed by hydronium ions or by acetic acid released from the hemicellulose acetyl groups. The main hydrolysis products are oligomers and monomers derived from the hemicellulosic fraction, which can be recovered in a separated stream commonly known as hemicellulosic hydrolysate. However, depending on the severity of the pretreatment, sugar-degradation products can also be produced along with oligomers and monomers in the hemicellulosic hydrolysate [6].

The production of oligosaccharides from lignocellulosic materials has gained increasing attention after demonstrating the potential of using biomass-derived oligomers as biodegradable oxygen barrier films,

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emulsifiers, food thickeners, reinforcing agent in cellulose pulp and adhesives, and prebiotics [4,7–8]. Among the different oligosaccharides, xylooligosaccharides (XOS) are carbohydrates composed of β-xylose backbone, which can be found in nature in fruits, vegetables and honey, but can also be produced by hydrolysis of the polysaccharide xylan found in the hemicellulosic component of plant cell walls of lignocellulosic materials. Due to the abundance of xylan in different biomass sources, especially hardwood and grasses, interest in the production of xylan-derived products, and particularly XOS, has greatly increased in the past few years. Moreover, xylose also represent a valuable xylan-derived product which can be used as feedstock for the production of biofuels and other sugar-derived products. For instance, both XOS and xylose can be used in the xylitol production, which has gained increasing attention nowadays and represent an attractive product [9–10]. XOS and xylose can be produced by steam explosion pretreatment through extraction of the xylan from the biomass matrix and hydrolysis of this high molecular weight polysaccharide to obtain xylan-derived products of lower degree of depolymerization. Adsorption and ion-exchange resins have been already evaluated for detoxification of sugar-rich streams prior to their microbial fermentation for the production of value-added products such as bioethanol or xylitol [11–14]. Some work was also reported on the use of these resins for the purification of XOS-rich streams either to produce XOS or other applications [15–17]. Adsorption resins (e.g. Amberlite® XAD series resins) have been used in the removal of furfural and phenolic compounds given their selectivity towards these compounds, allowing the XOS to be recovered in the eluted liquor. Likewise, anionic resins (whether strong or weak anionic resins) have been used to remove aliphatic and uronic acids present in hemicellulosic hydrolysates [11].

Butanol has received considerable attention as a biofuel because it is more compatible with combustion engines than ethanol. Moreover, it has been recognized as a chemical and solvent used in various chemical industries [18]. Biobutanol can be produced by microbial fermentation using *Clostridium* sp. through the so-called acetone-butanol-ethanol (ABE) fermentation process. Some natural *C. beijerinckii* strains can produce isopropanol instead of acetone, through the isopropanol-butanol-ethanol (IBE) fermentation, producing a non-corrosive alcohol mixture which could be directly used as a biofuel. These microorganisms can use a wide variety of monomeric sugars as carbon source such as glucose, xylose and cellobiose, which make them suitable to be used with lignocellulosic feedstocks [19–20]. However, byproducts formed during pretreatment of lignocellulosic biomass due to hemicellulose hydrolysis, sugars and lignin degradation, can inhibit *Clostridium* sp. and affect butanol production. Weak acids (e.g. acetic, formic and levulinic), furan derivatives (e.g. hydroxymethylfurfural (HMF) and furfural) and phenolic compounds (e.g. ferulic acid, vanillic acid, hydroxybenzoic acid, syringaldehyde and *p*-coumaric acid) are mainly formed. The formation of the different byproducts greatly depends on the pretreatment method and operational conditions such as temperature and time. Moreover, it was previously reported that coexistence of these compounds can severely affect butanol yield due to synergistic effects of these compounds [21–22].

Eucalyptus grandis is one of fast-growing eucalyptus species widely used in local pulp industries. Its availability and physicochemical composition (mainly carbohydrate content) make it a promising lignocellulosic biomass for the production of value-added products, which represents an attractive way to diversify the existing agro-industrial chain contributing its sustainability. The aim of this work was to evaluate the suitability of steam explosion pretreatment using a semi-continuous pre-pilot reactor scale unit for an effective valorization of *E. grandis* wood to produce butanol and xylosaccharides. Although there has been extensive research on hemicellulose solubilization for XOS recovery using hydrothermal pretreatments [23–26], reported data on xylan extraction and recovery as xylose and XOS from biomass using steam explosion pretreatment is limited. Several pretreatment temperature conditions and their effect on sugar recovery and formation of inhibitors in hemicellulosic hydrolysates were studied. Ion-exchange and adsorption resins treatments were investigated for xylosaccharides purification in hemicellulosic hydrolysates. Enzymatic hydrolysates were prepared for butanol fermentation from steam exploded *E. grandis* under optimized conditions to achieve high glucose yields. The performance of four native *Clostridium* strains, three ABE producers (*C. acetobutylicum*, *C. beijerinckii,* and *C. saccharoperbutylacetonicum*) and one IBE producer (*C. beijerinckii*), were first evaluated using glucose and/or xylose as carbon source in semi-synthetic and enzymatic hydrolysate media. The most promising strain, which was selected based on butanol yields and productivities achieved as well as the ability to ferment the enzymatic hydrolysate, was selected for further investigations. This work highlights a potential integrated biorefinery process for the efficient utilization of *E. grandis* wood to produce value-added products.

2. Materials and methods

2.1. Raw material preparation and characterization

Eucalyptus grandis wood was used as raw material, which was prepared by first chipping and then milling *E. grandis* wood logs with a cutting mill to pass a 4-mm screen. It was kiln-dried at 40 °C until 10% w/w moisture content, screened and stored at room temperature until use. The particle size distribution was: 2.3% between 3.35 mm and 4 mm, 29.4% between 1.40 mm and 3.35 mm, 52.7% between 1.19 mm and 1.40 mm, 11.8% between 0.50 mm and 1.19 mm, and the rest below 0.5 mm. The fines, which was considered the material fraction below 0.5 mm, was discarded.

2.2. Steam explosion pretreatment

Steam explosion pretreatment was performed in a semi-continuous pre-pilot equipment (AdvanceBio Systems LLC, model S1401-D2011) installed at the pilot plant of Latitud, Fundación LATU located in the Technological Laboratory of Uruguay with a processing capacity of 10 kg/h, which was previously described [5]. The *E. grandis* wood was impregnated with water to reach 20% moisture content and left overnight. The steam explosion experiments were performed by processing approximately 2.5 kg of *E. grandis* wood at temperatures of 180 °C (0.9 MPa), 190 °C (1.1 MPa) and 200 °C (1.5 MPa) at a residence time of 10 min (severity factor of 3.36, 3.65 and 3.94, respectively). The severity factor (S_0) was calculated according to Eq. (1), where t is the residence time (min), T is the reaction temperature (°C), and 14.75 is a fitted value [27].

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

After each pretreatment, the steam exploded slurry was removed and filtered using a fabric by filter pressing (20 MPa) to separate the solid (unwashed pretreated *E. grandis* wood) and liquid (hemicellulosic hydrolysate) fractions. The solid fractions were water-washed 3 times at room temperature by mixing tap water with the solid fraction at a ratio of 5:1 (5 g of water per g of oven dried pretreated solid) in a portable concrete mixer during 10 min, followed by filter pressing to separate the solid fraction (washed pretreated *E. grandis* wood) and washing waters. Solid fractions were stored at 4 °C for enzymatic hydrolysis assays while liquid fractions and washing waters were stored at −20 °C for chemical analysis. To evaluate the effect of performing the washing step on subsequent enzymatic hydrolysis and fermentation, some unwashed pretreated *E. grandis* wood was separated and stored at 4 °C for further experiments.

2.3. Enzymatic hydrolysis of steam exploded E. grandis wood

Enzymatic hydrolysis was performed at 50 °C and pH 4.85 (using

0.05 M citrate buffer) in 250-mL Erlenmeyer flasks with orbital agitation (Infors HT Ecotron, Switzerland) at 150 rpm. The solid loading was 16% (w/w) with an enzyme loading of 30 FPU/g_{glucan} (Cellic CTec2, Novozymes, cellulase activity 154 FPU/mL).

The effect of solid and enzyme loadings on steam exploded *E. grandis* hydrolysis was evaluated using three different enzyme loadings (5, 10 and 25 FPU/g_{glucan}) and two solid concentrations (10% and 16%). In addition, the effect of solid washing and buffer type used (citrate or acetate) on the enzymatic hydrolysis and further butanol fermentation was evaluated. Enzymatic hydrolysis experiments were performed using unwashed and washed steam exploded *E. grandis* as substrates and citrate and acetate buffers (both 0.05 M and pH 4.85). After enzymatic hydrolysis, the supernatants (enzymatic hydrolysate) were recovered by centrifugation at 6,500 rpm for 30 min to remove the solid wastes and collected for chemical analysis. For fermentation assays, the supernatants were then filtered through a 0.22 μm filter to remove suspended particles. All experiments were performed in duplicate.

2.4. Microorganisms and inoculum preparation for butanol fermentations

The microorganisms used in this study (*Clostridium acetobutylicum* DSM 1731, *Clostridium beijerinckii* DSM 6422, *Clostridium beijerinckii* DSM 6423 and *Clostridium saccaroperbutylacetonicum* DSM 14923) were obtained from the German collection of microorganisms (DSMZ, Leibniz, Germany). The lyophilized strains were activated following the supplier's instructions and maintained in stock cultures prepared in reinforced clostridial medium (RCM), containing peptone 10 g/L, beef extract 10 g/L, glucose 5 g/L, sodium chloride 5 g/L, yeast extract 3 g/L, sodium acetate 3 g/L, soluble starch 0.5 g/L and L-cysteine-HCl 0.5 g/L, at 4 °C under anaerobic conditions.

For fermentation inoculum, a pre-culture was prepared by inoculating 2 mL of the stock culture into a serum bottle containing 40 mL of the RCM medium, which was then incubated in orbital shaker (Infors HT Ecotron, Switzerland) until active growth was observed by increased turbidity and vigorous gassing (20–24 h). Fermentation inoculum was prepared in 250-mL bottles with 100 mL of inoculum medium containing 30 g/L glucose, 1 g/L yeast extract and 1% (v/v) of filter sterilized P2 stock solutions. The P2 stock solutions corresponded to buffer (K_2HPO_4 50 g/L, KH_2PO_4 50 g/L, ammonium acetate 220 g/L), vitamin (*p*-amino benzoic acid 0.1 g/L, thiamine 0.1 g/L, biotin 0.001 g/L), and mineral ($MgSO_4{\cdot}7H_2O$ 20 g/L, $MnSO_4{\cdot}H_2O$ 1 g/L, $FeSO_4{\cdot}7H_2O$ 1 g/L, NaCl 1 g/L) solutions. The pH was adjusted to 6.0 ± 0.1 and the medium was swept with O_2-free N_2 over the headspace of the bottles, followed by sterilization at 121 °C for 15 min. Filter-sterilized P2 stock solutions were then added at 1% (v/v) after bottles were cooled down to room temperature, followed by inoculation with 10% (v/v) highly-active cells grown in the pre-culture. Fermentation inoculum was incubated at the cultivation temperature recommended by the supplier for each microorganism (*C. acetobutylicum* DSM 1731 at 37 °C, *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 at 35 °C and *C. saccaroperbutylacetonicum* DSM 14923 at 30 °C) and 150 rpm until a late exponential phase of growth (16–20 h).

2.5. Screening of Clostridium strains for butanol production

The *Clostridium* strains were evaluated based on their capacity to produce butanol in semi-synthetic glucose-based and xylose-based media as well as *E. grandis* enzymatic hydrolysate. Fermentations were conducted in duplicate in 100-mL bottles with 40 mL of the medium under anaerobic conditions. Fermentation medium contained 50–60 g/L of sugar (glucose, xylose or a mixture of both sugars) and was supplemented with yeast extract (1 g/L) and 1% (v/v) of filter sterilized P2 stock solutions. The pH of the medium was initially adjusted to 6.0 ± 0.1 and it was prepared following the conditions described above for inoculum medium preparation. Inoculum size was 10% (v/v) in all fermentations. After inoculation, the bottles were incubated in an orbital shaker at 150 rpm and 37 °C (*C. acetobutylicum* DSM 1731), 35 °C (*C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423) or 30 °C (*C. saccaroperbutylacetonicum* DSM 14923). Fermentation samples were collected routinely and analyzed for biomass concentration by optical density (OD_{600nm}), sugar concentration and fermentation products. Also, microscopic observations were performed periodically to evaluate the morphology and motility of the cells.

Table 1
Experiment conditions (pH, type of resin, and hydrolysate to resin ratio) used for ion-exchange and adsorption resin treatments for XOS purification in hemicellulosic hydrolysate.

Experiment	pH	Resin 1	Resin 2	Hemicellulosic hydrolysate/resin (mL/g)
1	3.2	Amberlite XAD-4	Purolite A 503	3
2	1.0	Amberlite XAD-4	Purolite A 503	3
3	3.2	Purolite A 503	Amberlite XAD-4	3
4	3.2	Dowex 66	Amberlite XAD-4	3
5	3.2	Dowex 66	Amberlite XAD-4	1
6	3.2	Amberlite XAD-4	Dowex 66	1

2.6. Removal of non-saccharide organic compounds in hemicellulosic hydrolysate

The hemicellulosic hydrolysate was treated with resins to remove non-saccharide organic compounds and produce a xylosaccharide-rich liquor stream. Three different types of ion-exchange resins were studied. An adsorptive resin (Amberlite XAD-4 resin) was evaluated at two pH conditions (original pH of the hydrolysate and pH of 1) to remove furfural and phenolics compounds. A strong base anion exchange resin (Purolite A 503) and a weak base anion exchange resin (Dowex 66), both developed for the sugar industry, were evaluated for the removal of aliphatic acids. Assays were done with the resins in columns. Firstly, the solid particles remaining in the hemicellulosic hydrolysate were removed by centrifugation at 4500 rpm for 20 min. Then, the supernatant was added to the column with the resin and agitated for 1 min. The system was left in contact for 30 min to establish all the chemical equilibriums before eluting from the resin at a speed of 1 mL/min. Afterwards, a two-stage washing with 20 mL of distilled water was performed to remove all the chemicals that could be occluded within the resin. The eluted hydrolysate and the two washing waters were analyzed to determine the retention of the XOS and the contaminants in each case. The eluted hydrolysate was treated in a second column with a different type of resin following the procedure described above for the first column. The experiments were performed under the experimental conditions (pH, type of resin, and hydrolysate to resin ratio) listed in Table 1. The resins were regenerated after use according to the manufacturer's recommendations.

2.7. Analytical methods

The chemical composition of *E. grandis* wood and steam exploded *E. grandis* was determined following NREL protocols [28–31]. The chemical composition and total solids content of pretreatment liquid fractions (hemicellulosic hydrolysates and washing waters) was determined following NREL protocols [30,32]. Glucose, arabinose, organic acids (formic and acetic acids), furfural and HMF were measured by an HPLC system (Shimadzu, Kyoto, Japan) equipped with RI and SP detectors and Aminex HPX-87H column (Bio-Rad Laboratories Ltd., USA) operating at 45 °C. The mobile phase used was 5 mM H_2SO_4 at a flow rate of 0.3 mL/min. The carbohydrates, organic acids, furfural and HMF

Table 2
Chemical composition of untreated and steam pretreated *E. grandis* wood (SE) at 180 °C, 190 °C and 200 °C, component recovery in pretreated solid fractions and enzymatic hydrolysis efficiency of steam pretreated substrates at 72 h.

Conditions	Chemical composition (%)							Recovery in pretreated solid[a] (%)				Enzymatic hydrolysis efficiency (%)
	Glucan	Xylan	Arabinan	Lignin (AI + AS)	Acetyl groups	Ash	Extractives	Glucan	Xylan	Lignin	Solids	
Untreated	48.4 ± 2.0	14.8 ± 0.5	0.6 ± 0.1	27.2 ± 1.0	3.6 ± 0.6	0.46 ± 0.07	3.8 ± 0.3	n.a.	n.a.	n.a.	n.a.	8.2 ± 0.5
SE 180 °C	52.3 ± 1.2	5.3 ± 0.8	n.d.	35.2 ± 0.4	1.9 ± 0.1	0.15 ± 0.02	n.a.	86 ± 6	29 ± 5	100 ± 5	79 ± 4	34.1 ± 3.8
SE 190 °C	52.1 ± 4.1	2.4 ± 0.2	n.d.	36.7 ± 0.3	0.9 ± 0.1	0.13 ± 0.02	n.a.	78 ± 5	12 ± 3	97 ± 1	72 ± 1	63.7 ± 5.5
SE 200 °C	59.0 ± 1.4	1.9 ± 0.1	n.d.	37.8 ± 4.0	0.4 ± 0.1	0.16 ± 0.02	n.a.	89 ± 9	9 ± 2	100 ± 12	73 ± 8	83.5 ± 2.0

n.a. not applicable; *n.d.* not detected; AI: acid insoluble; AS: acid soluble; [a]Calculated as g of component in the solid fraction per 100 g of component in the raw material; Enzymatic hydrolysis performed at 16% solid concentration and 30 FPU/g_{glucan} enzyme dosage.

from the eluted hydrolysates and washing waters after the ion-exchange and adsorption resin treatments were analyzed as described above. Molecular weight distribution of xylosaccharides in the liquid fractions was determined by HPLC-GPC system (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-42A column (Bio-Rad Laboratories Ltd., USA) and RI detector operating at 60 °C with water as eluent at a flow rate of 0.6 mL/min. Xylobiose, xylotriose, xylotetraose, and xylopentaose used as standards were obtained from Megazyme International Ireland (Bray, County Wicklow, Ireland). Also, xylose, xylobiose, xylotriose, xylotetraose and xylopentaose produced in the hemicellulosic hydrolysates were analyzed by size exclusion chromatography (HPLC-SEC) using an Aminex HPX-42A column (Bio-Rad Laboratories Ltd., USA) and RID detector. The system was operated at 60 °C using type I deionized water at a flow rate of 0.6 mL/min as mobile phase. Solvents (acetone, isopropanol, butanol, ethanol) and organic acids (acetic and butyric acids) produced by fermentation were measured by a gas chromatograph (Shimadzu GC-2010) equipped with a flame ionization detector and a fused silica column (RTX®-Wax, 30 m long, 0.5 μm film thickness and 0.32 mm ID, Restek). Total phenolics in liquid fractions were determined using the Folin − Ciocalteu method and expressed as gallic acid equivalent [33]. The cellulase activity of Cellic CTec 2 was measured following the NREL protocol [34]. Cell density was measured by OD using a spectrophotometer (Genesys 10S UV–vis) at 600 nm.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed to determine statistical differences ($p \leq 0.05$) using InfoStat software (student version 2013, Universidad Nacional de Córdoba, Argentina, http://www.infostat.com.ar).

3. Results and discussion

3.1. Steam explosion pretreatment

The chemical composition of *E. grandis* wood is shown in Table 2. Carbohydrate fraction accounted for 63%, mainly composed of glucan (48%) and xylan (15%). Remaining constituents were lignin (27%), acetyl groups (4%) and extractives (4%). After steam explosion pretreatment, the composition of pretreated *E. grandis* was found to be in the range of 52–59% for glucan, 2–5% for xylan and 35–38% for lignin. Other minor components, including acetyl groups (0.5–2%) were also detected in the pretreated solids. As expected, steam explosion pretreatment enriched glucan and lignin content compared to the untreated *E. grandis* wood (Table 2). After steam explosion pretreatment, glucan recovery resulted relatively high (78–89%) whereas xylan and acetyls contents were low in the solid fractions due to the extensive xylan and acetyls solubilization (71–91% and 58–92%, respectively). Almost no lignin removal was achieved during steam explosion pretreatment since around 97–100% of lignin remained in the solid fractions. According to the results obtained, higher glucan content and almost complete xylan solubilization (as xylose and XOS) were achieved working at harsh pretreatment conditions (200 °C).

Since the slurry resulting from steam explosion pretreatment had a high solids content (in the range of 80–90%, on wet basis), it resulted difficult to separate all the already dissolved components from pretreated *E. grandis* wood by one-stage filter pressing. Therefore, the water washing stages were performed not only to remove possible inhibitors from the solid fraction, but also to recover the solubilized components from the biomass matrix. The composition of hemicellulosic hydrolysates and washing waters was mainly comprised of xylose (1.2–3.7 g/100 $g_{eucalyptus}$), XOS (3.0–7.0 g/100 $g_{eucalyptus}$) and acetyl groups (1.6–2.2 g/100 $g_{eucalyptus}$) (Fig. 1a). Lower amounts of glucooligosaccharides (GOS) (0.2–0.8 g/100 $g_{eucalyptus}$) were also observed (Fig. 1a), derived from both cellulosic and hemicellulosic components in the *E. grandis* wood. The xylosacharides content determined in the pretreatment liquid fractions resulted slightly lower compared to those obtained by Romaní et al. [35], who reported 11% in pretreatment liquor after steam explosion at 180 °C for 10 min. Byproducts such as formic acid and furfural were also formed during steam explosion pretreatment from the degradation of pentose and hexose sugars. The concentration of these byproducts did not differ significantly among the different pretreatment temperature conditions evaluated. However, even though eucalyptus wood extractives are known to be rich in polyphenols [36], relatively high concentration of phenolic compounds (5.2 g/100 $g_{eucalyptus}$) was observed when steam explosion temperature was increased to harsher conditions (200 °C) which was probably due to the easily cleavage lignin.

The concentration of the main components (XOS and xylose) was determined in the different pretreatment liquid fractions (hemicellulosic hydrolysate and washing water from the three-stage washing step) (Fig. 1b). Only between 16% and 35% of the dissolved xylosaccharides were effectively recovered in the hemicellulosic hydrolysate by filter-pressing the pretreatment slurries. Performing a water washing step allowed to increase the xylosaccharides recovery up to 80–86%, but almost complete recovery was achieved after the second water washing step (95–97%).

Difference in pH values of the different pretreatment liquid fractions were also observed and may represent a good indicator of byproducts removal from the solid framework. The pH values of the hemicellulosic hydrolysates were in the range of 3.19 and 3.31, whereas the pH values of the washing waters were slightly higher (3.30–4.13) and increasing with the number of water washing stages as expected. These relatively low pH values came from the cleavage of acetyl groups from the hemicellulose chains, as well as the release of uronic acid (not shown) and the possible formation of acids by sugars degradation such as formic acid. These facts highlight the importance of performing at least one washing stage after steam explosion pretreatment since byproducts can

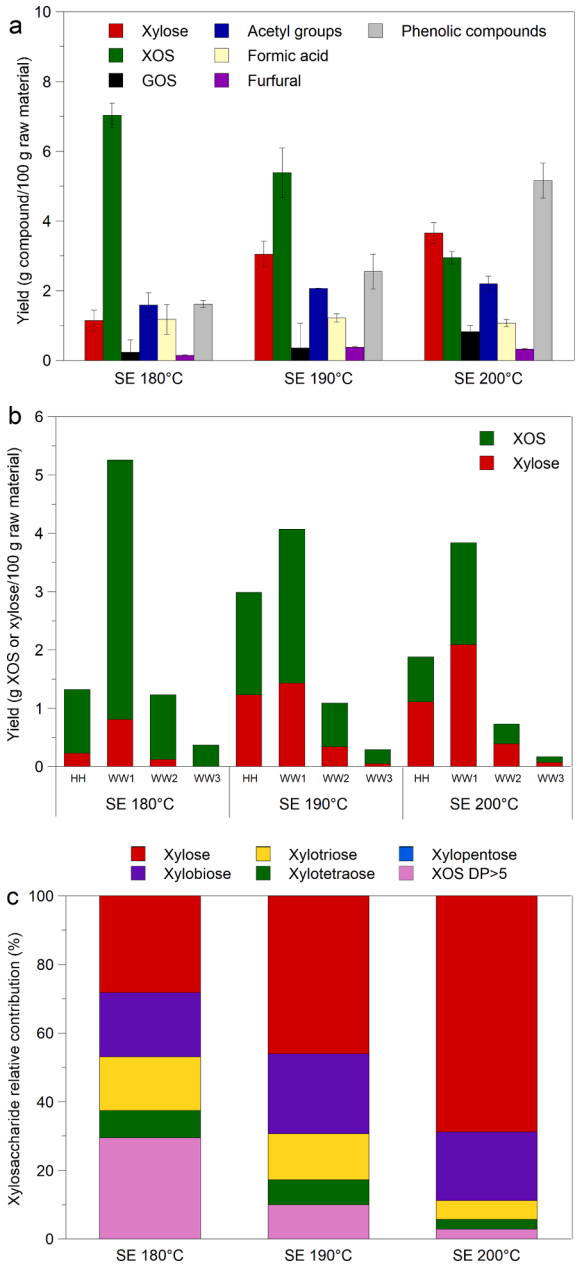

Fig. 1. Composition of hemicellulosic hydrolysates (a), XOS and xylose recovery from xylan hydrolysis in the hemicellulosic hydrolysates and washing waters (b), and relative composition of different xylosaccharide degree of polymerization recovered in the hemicellulosic hydrolysates (c), after steam explosion (SE) of *E. grandis* wood. XOS: xylooligosaccharides (expressed in xylose equivalent); GOS: glucooligosaccharides (expressed in glucose equivalent); HH: hemicellulosic hydrolysate; WW: washing water.

act as impurities and inhibitory compounds to fermenting microorganisms. However, both hemicellulosic hydrolysate and washing waters should be kept for further xylosaccharides valorization.

3.2. Steam explosion pretreatment for xylosaccharides production

The solubilized xylan recovered as xylose and XOS is shown in Fig. 1b. The recovery of xylosaccharides resulted in the range of 6.6–8.4 g/100 $g_{eucalyptus}$ in the hemicellulosic hydrolysate and washing waters, achieving the highest recovery when steam pretreatment was performed at 190 °C. It can be observed that xylan solubilization yield as XOS and xylose varied among the different pretreatment temperature conditions

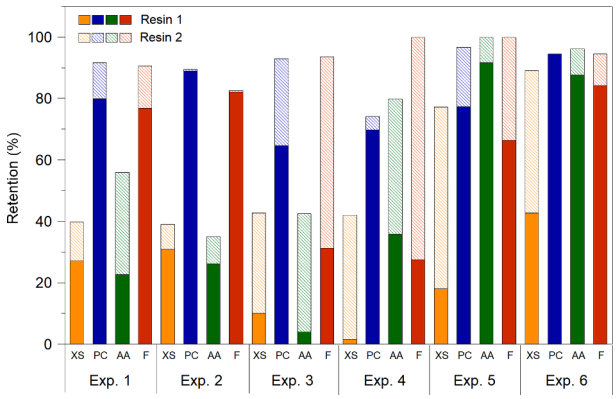

Fig. 2. Retention of xylosaccharides (XS), phenolic compounds (PC), aliphatic acids (AA) and furfural (F) in resin 1 (solid fill) and resin 2 (pattern fill) after the different ion-exchange resin treatments (Exp. 1 to 6, see Table 1).

(180 °C, 190 °C and 200 °C). Pretreatment at 180 °C resulted in solubilized xylan mainly recovered as XOS (86%), even though lower amounts of xylose (14%) were also found as a result of XOS hydrolysis. This demonstrated that steam explosion at 180 °C is useful for hydrolysis of xylan into XOS, resulting in XOS yields up to 7.0 g/100 $g_{eucalyptus}$. By increasing the pretreatment temperature to 190 °C, the xylose content in the pretreatment liquid fractions increased to the detriment of XOS content (3.0 g/100 $g_{eucalyptus}$ and 5.4 g/100 $g_{eucalyptus}$, respectively), as expected. Thus, 64% of solubilized xylan was recovered mainly as XOS under this condition, while 36% was recovered as xylose. Further increasing steam explosion temperature to 200 °C gave a relatively high concentration of xylose (3.7 g/100 $g_{eucalyptus}$) compared to XOS concentration (3.0 g/100 $g_{eucalyptus}$), indicating that solubilized xylan was mostly present as xylose (55%) with a slightly lower proportion of XOS (45%). For the production of value-added XOS compounds, the presence of xylose monomers is usually considered undesirable. However, xylose in hydrolysates could be separated and converted into other value-added chemicals such as ethanol or xylitol.

The utilization of XOS as prebiotic has been reported to be affected by the degree of polymerization (DP) [4]. Due to the nonspecific xylan hydrolysis during steam explosion, xylosaccharides with a variety of DP is expected in the pretreatment liquid fractions (hemicellulosic hydrolysate and washing waters). Fig. 1c shows the different DP of the xylosaccharides found in the pretreatment liquid fractions. Xylosaccharides obtained at 180 °C showed a wide distribution of the degree of polymerization (31 ± 4% of xylose, 42 ± 2% of XOS with DP between 2 and 4 and 30 ± 0% of XOS with DP > 5). As the pretreatment temperature increased, the concentration of xylose and low DP XOS (mainly xylobiose) was higher, while the concentration of XOS with a DP > 5 decreased. Xylosaccharides obtained at 200 °C were mostly composed by xylose (69 ± 2%) followed by xylobiose (20 ± 6%). For XOS production as prebiotic, it is still not clear if its desirably a wide distribution or a higher content of XOS with DP in the range of 2–3 [37–38], so further studies should be performed to evaluate the role of prebiotic of the XOS [4]. For ethanol or xylitol production, the higher amount of xylose or XOS with lower DP is better for the subsequent biological treatment.

3.2.1. Purification of XOS and xylose extracted in hemicellulosic fractions

The hemicellulosic fraction extracted from the *E. grandis* wood after steam explosion pretreatment (200 °C, 10 min) was treated with an adsorptive resin (Amberlite XAD-4 resin), a strong base anion exchange resin (Purolite A 503) and a weak base anion exchange resin (Dowex 66) to remove non-saccharide organic compounds. Fig. 2 shows the retention of the different compounds analyzed (xylosaccharides, aliphatic acids, phenolic compounds and furfural) in the resins employed (Table 1). The retention of phenolic compounds was confirmed with the

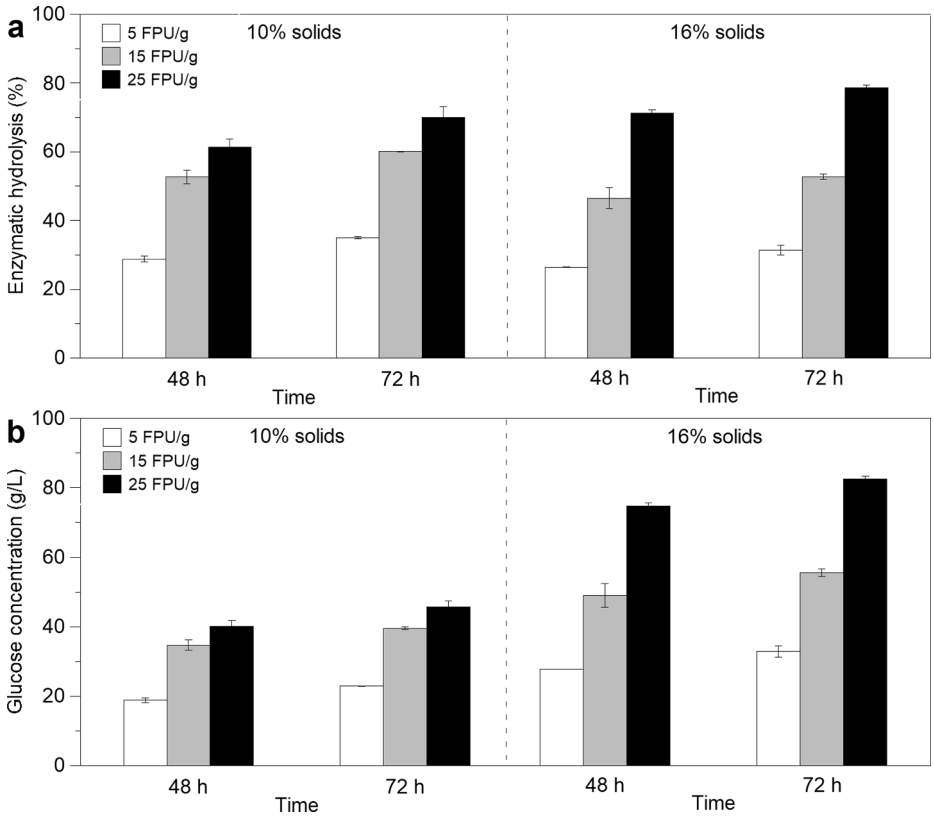

Fig. 3. Effect of enzyme loading (5, 15 and 25 FPU/g_{glucan}) and solid loading (10 and 16%) on enzymatic hydrolysis yield (a) and glucose concentration (b) after 48 h and 72 h of enzymatic hydrolysis of steam exploded *E. grandis* wood. Conditions of steam explosion: 200 °C, 10 min.

Amberlite XAD-4 resin, which allowed to remove at least 80% of them from the hemicellulosic hydrolysates. Also, furfural was removed in a high extension (77–82%). The adsorption capacity of the Amberlite XAD-4 resin for phenolic compounds can be attributed to the hydrophobic interaction between its styrene-DVB matrix and phenolic rings (π-π interaction of the matrix aromatic rings and phenols) [12,39]. However, at least 27% of the xylosaccharides were also retained in this resin, which is detrimental for the aim of the process. According to Schwartz and Lawoko [15], carbohydrates are not strongly retained in this resin, but they are occluded in the void space of the resin bed and can be released by washing the resins after treatment. Performing a washing step allowed the recovery of a higher amount of xylosaccharides, although it was not complete. Moreover, the removal of aliphatic acids (acetic and formic acids), working with an hemicellulosic hydrolysate to resin ratio of 3, was not good (below 30%), which implied that another purification step should be performed. When the hydrolysate to resin ratio was decreased to 1, the removal of aliphatic acids resulted acceptable (88%), but the retention of xylosaccharides increased to 43%. Decreasing the pH to 1 increased the removal of phenolic compounds (89%), mainly by precipitation of acid insoluble phenolics and furfural (82%), but also increased the removal of xylosaccharides (31%). Schwartz and Lawoko [15], who suggested working with the resin at a pH of 1 for more efficient removal, reported a removal of 100% of furfural and 88% of acid soluble lignin, while the retention of xylosaccharides in the resin was 4%. They also reported a 20% retention of acetic acid present in hemicellulosic hydrolysates. The higher non-saccharide compounds removal and low xylosaccharides retention achieved by Schwartz and Lawoko [15] could have been due to the higher lignin and lower saccharides concentration than that used in this work, which may affect the affinity of the resin for the different compounds.

Purolite A 503 resin allowed the removal of only 4% of the aliphatic acids. This resin is a strong anion resin, characterized by removing weak bases from strong acids. In this case, even though the pKa of acetic and formic acid are rather high, removal was not possible. On the other hand, the Dowex 66 resin, a weak anion exchange resin, allowed the removal of>90% of aliphatic acid, especially at the hydrolysate to resin ratio of 1. Also, 77% of the phenolic compounds and 66% of furfural were retained. However, 20% of the xylosaccharides were lost in the treatment. The removal of aliphatic acids in hemicellulosic hydrolysates with weak anion resins has been previously reported by other authors [13–14,40]. Carvalheiro et al. [14] also reported an important removal of phenolic compounds with this type of resin. However, no reported data using the Dowex 66 resin was found in literature.

The addition of a second resin in series (Fig. 2) did not achieve good results. The removal of phenolic compounds (74–97%), furfural (82–100%) and aliphatic acids (35–100%) improved, but unacceptable levels of xylosaccharides retention (39–89%) were observed in the resin. No satisfactory explanation has been found for this phenomenon, so further research should be done on optimizing non-saccharide organic compounds removal while minimizing xylosaccharides retention by resin exchange treatment.

Based on the results obtained, the best condition for resin treatment in terms of maximum removal of non-saccharide organic compounds and minimum loss of XOS xylosaccharides was using the Dowex 66 resin alone at pH 3.2 and hydrolysate to resin ratio of 1. This condition allowed the removal of 77% phenolic compounds, 92% aliphatic acids and 66% furfural, while keeping relatively low xylosaccharide loss (18%) by retention in resin.

3.3. Enzymatic hydrolysis of steam pretreated E. grandis wood

Enzymatic hydrolysis of untreated and steam pretreated *E. grandis wood* was performed at 16% solid loading and 30 FPU/g_{glucan} for 72 h to evaluate the effect of steam explosion pretreatment on the production of fermentable sugars (Table 2). Longer reaction times (up to 96 h) did not

Table 3
Effect of water washing and hydrolysis buffer (citrate or acetate at pH 4.85) on enzymatic hydrolysis of steam exploded *E. grandis* substrate.

Water washing	Conditions	Buffer	Glucose (g/L)	Enzymatic hydrolysis efficiency (%)	Glucan conversion efficiency[a] (%)	Glucose yield[b] (%)
No	–	Citrate	53.1 ± 1.0	50.4 ± 1.0	50.6 ± 2.4	27.2 ± 1.2
		Acetate	46.9 ± 0.1	44.8 ± 0.4	44.7 ± 1.8	24.0 ± 0.9
Yes	3 washing stages with water at a liquid:solid of 5	Citrate	79.8 ± 0.8	78.7 ± 0.8	76.1 ± 2.2	40.9 ± 1.1
		Acetate	78.4 ± 0.1	74.8 ± 0.1	71.3 ± 1.5	38.3 ± 0.8
Yes	Rinsing with water to pH close to neutrality (6–7)	Citrate	79.6 ± 1.4	76.5 ± 1.1	75.9 ± 2.5	40.8 ± 1.4

[a] Calculated as g of glucose per 100 g of glucose in the raw material.
[b] Calculated as g of glucose per 100 g of dry raw material; Enzymatic hydrolysis performed at 16% solid concentration and 25 FPU/g_{glucan} enzyme dosage.

show any contribution to improve enzymatic hydrolysis yields of the pretreated substrates. The enzymatic hydrolysis efficiency of untreated *E. grandis* wood was only 8.2%. Even though increasing pretreatment temperature from 180 °C to 190 °C significantly improved (86% increase) enzymatic hydrolysis efficiency of the pretreated substrate, the glucan to glucose conversion still remained low (64%). Further increase in the pretreatment temperature to 200 °C allowed to achieve the highest enzymatic hydrolysis efficiency (83.5%) and, therefore, the highest release of fermentable glucose (37 g/100 $g_{eucalyptus}$). This may be due to the almost complete hemicellulose (based on xylan and acetyl groups components) removal during pretreatment, since it was previously shown that hemicellulose can hinder the action of enzymes when absorbed onto the cellulose surface [25,41]. Moreover, considering that delignification (3–10%) was limited during steam explosion pretreatment, lignin removal did not necessarily make the pretreated substrates more susceptible to enzymatic hydrolysis. Indeed, improvements in enzymatic hydrolysis efficiency could be related to biomass compositional and structural changes during steam explosion pretreatment, which includes glucan structure rearrangements or lignin fragmentation and recondensation on solid surfaces [42,43].

Concerning fermentable sugars concentration in enzymatic hydrolysates, low xylose concentrations (2–4 g/L) were reached due to the low xylan content in the pretreated substrates, compared to the higher glucose concentrations achieved (almost 90 g/L). Pretreatment at 200 °C allowed to obtain a substrate which can be easily converted into fermentable sugar by enzymatic hydrolysis, in addition to the xylosaccharides effectively extracted in the hydrolysate. Considering that the aim of this study was to simultaneously obtain maximum xylosaccharides yields and fermentable sugars from the hemicellulosic and cellulosic fraction, respectively, pretreatment temperature of 200 °C was selected as the steam explosion condition for further studies.

Effect of solid concentration, enzyme dosage and water washing on enzymatic hydrolysis

The effect of solid concentration (10% and 16%) and enzyme dosage (5, 15, and 25 FPU/g_{glucan}) on glucan enzymatic hydrolysis of washed steam exploded *E. grandis* wood was studied. The experiments were performed using citrate buffer. The enzymatic hydrolysis efficiency and glucose concentration increased with increasing enzyme loadings both for 10% and 16% solid loadings (Fig. 3). The highest enzymatic hydrolysis yield obtained was 70% and 79% at 10% and 16% solid loading, respectively, after 72 h using enzyme loading of 25 FPU/g_{glucan}. Decreasing enzyme loading resulted in lower hydrolysis yields even with a low solid loading of 10% (31–35% for 5 FPU/g_{glucan}).

Along with enzyme loading, solid loading plays an important role for the enzymatic hydrolysis. Increasing the solid loading not only may improve the glucose concentration but also may reduce the equipment size and facilitate downstream processing. However, high solid loadings hydrolysis may lead to problems related to mass transfer efficiency. Results show that higher enzymatic hydrolysis yields (35% and 60%) were obtained at 10% solid loading for 5 FPU/g_{glucan} and 15 FPU/g_{glucan}, respectively. This result may suggest that high concentration of suspended solids decreases enzyme diffusivity into the pretreated biomass, thus decreasing enzymatic hydrolysis yields. However, increasing enzyme loading up to 25 FPU/g_{glucan} allowed to improve enzymatic hydrolysis yield at 16% solid loading. Furthermore, higher glucose concentrations were obtained when solid loading was increased to 16% (33–80 g/L) compared to 10% (23–46 g/L). By comparing the enzymatic hydrolysis results previously described (16%, 30 FPU/g_{glucan}, Table 2) using the same substrate to these results, a small benefit in terms of hydrolysis yield was reached by increasing the amount of enzyme from 25 FPU/g_{glucan} (79%) to 30 FPU/g_{glucan} (83%) at 16% solid

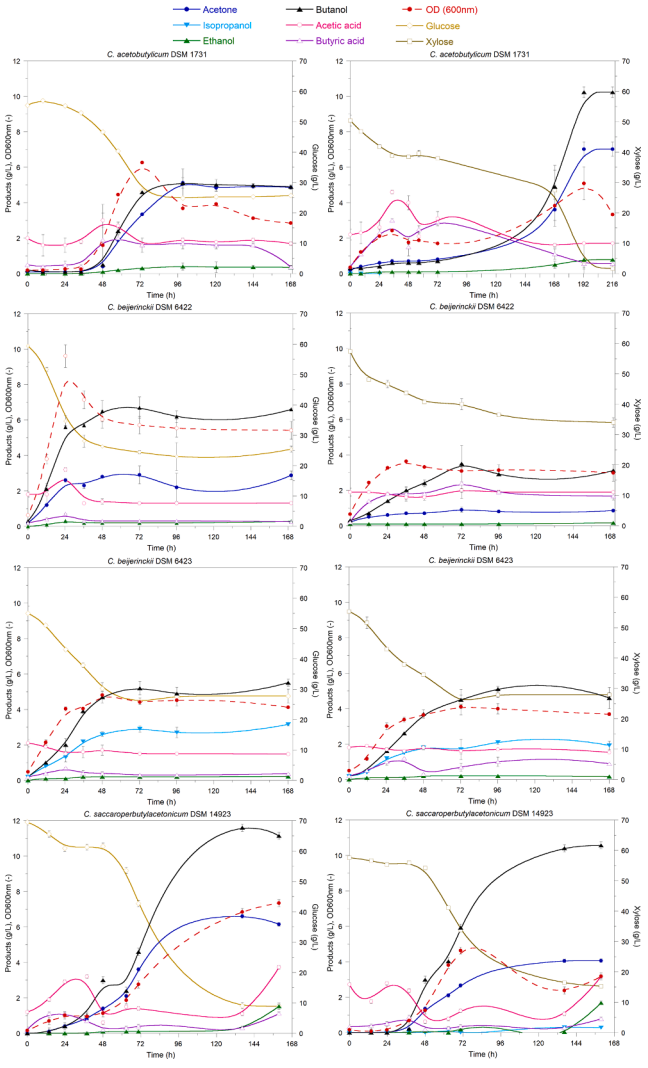

Fig. 4. Production of solvents (butanol, acetone, isopropanol, ethanol), acids (acetic and butyric acid) and biomass, and residual sugars in ABE/IBE fermentations of the *Clostridium* strains assessed using glucose and xylose-based media. (Glucose fermentation on the left side and xylose fermentation on the right side).

concentration. Thus, an enzyme loading of 25 FPU/g_{glucan} may be the optimal enzyme charge for an efficient glucose production (35 g/100 $g_{eucalyptus}$) using a solid content of 16%.

It was previously demonstrated that cellulase activity of enzyme complex can be inhibited by soluble sugars (xylose, XOS and GOS) and byproducts (acetic acid, formic acid, furfural and phenolic compounds) which are being produced during pretreatment [42,43]. Several methods have been proposed for the removal of these inhibitors. However, rinsing the pretreated biomass with enough water to remove these compounds prior to enzymatic hydrolysis is considered the simplest method by many authors [43,44]. To evaluate the effect of solid washing on the removal of possible inhibitors from the solid fraction, the pretreated substrate was subjected to water washing prior to enzymatic hydrolysis under the following conditions: (i) three-stage water washing at a liquid to solid ratio of 5; (ii) rinsing with water until pH value of washing water was close to neutrality. Table 3 shows the results obtained in terms of enzymatic hydrolysis yield and glucose concentration. Only 51% of glucan was hydrolyzed when using unwashed steam pretreated substrate, leading to lower concentrations of released glucose (46.9–53.1 g/L). These results indicated that soluble saccharides and/or byproducts released during pretreatment had significant negative effect on enzymatic hydrolysis of steam exploded *E. grandis* substrates, resulting in a decrease in the hydrolysis efficiency (45–51%) and glucose yield (24–27%). However, the enzymatic hydrolysis increased to almost 80% when using water washed pretreated substrates. By performing water washing stages, inhibitory compounds were effectively removed and, thus, enzymatic hydrolysis efficiency and glucose yield were greatly improved (71–76% and 38–41%, respectively). This suggests that steam exploded *E. grandis* wood should be washed adequately before enzymatic hydrolysis to remove sugars, acids, furans and lignin-derived products for efficient glucan hydrolysis. Furthermore, the enzymatic hydrolysis (76.5%) obtained after rinsing with water to pH close to neutrality was similar to that (74.8%) obtained after three-stage water washing (pH value of 4). Therefore, a three-stage water washing was adopted to minimize the use of water.

3.4. Screening of native Clostridium strains for butanol fermentation

Among the different native *Clostridium* strains evaluated in this study, *C. saccharoperbutylacetonicum* DSM 14,923 and *C. acetobutylicum* DSM 1731 showed better performance in terms of butanol and ABE concentrations and yields achieved in semi-synthetic media (Fig. 4, Table 4). In glucose fermentations, *C. saccharoperbutylacetonicum* DSM 14,923 achieved the highest butanol (11.6 g/L) and ABE (18.6 g/L) concentrations with high glucose consumption (87%). Although relatively high butanol (0.19 g/g) and ABE (0.30 g/g) yields were also achieved, fermentation ceased after 136 h, which resulted in relatively low butanol (0.09 g/L.h) and ABE (0.14 g/L.h) productivities. In xylose fermentations, *C. acetobutylicum* DSM 1731 achieved the highest butanol (10.2 g/L) and ABE (18.0 g/L) concentrations with almost complete xylose consumption (94%). However, as it was also observed for *C. saccharoperbutylacetonicum* DSM 14923, fermentation cease after 192 h, which resulted in relatively low butanol (0.05 g/L.h) and ABE (0.09 g/L.h) productivities. It should be noted that a relatively long lag phase (24–48 h) was observed for both strains, during which low biomass growth and sugar consumption was observed. This lag phase resulted longer in xylose-based semi-synthetic medium rather than in glucose-based semi-synthetic medium (Fig. 4). Similar results were also found by Zetty-Arenas et al. [22] using different *Clostridium* strains.

On the other hand, fermentation was faster when ABE strain

Table 4
Comparison of native *Clostridium* strains for butanol fermentation in semi-synthetic glucose-based and xylose-based medium, and *E. grandis* enzymatic hydrolysate.

Strain	*C. acetobutylicum* DSM 1731			*C. beijerinckii* DSM 6422			*C. beijerinckii* DSM 6423			*C. saccaroperbutylacetonicum* DSM 14923		
Carbon source	Glucose	Xylose	Hydrolysate	Glucose	Xylose	Hydrolysate	Glucose	Xylose	Hydrolysate	Glucose	Xylose	Hydrolysate
Fermentation time (h)	96	192	48	48	72	48	48	96	96	136	136	72
Butanol (g/L)	5.1 ± 0.2	10.2 ± 0.3	0.20 ± 0.03	6.5 ± 0.6	3.5 ± 1.0	5.0 ± 0.1	4.7 ± 0.3	5.1 ± 0.2	2.5 ± 0.5	11.6 ± 0.2	9.5 ± 1.3	0.12 ± 0.08
Isopropanol (g/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	2.6 ± 0.1	2.2 ± 0.1	2.6 ± 0.5	n.a.	n.a.	n.a.
Acetone (g/L)	5.1 ± 0.3	7.0 ± 0.4	0.11 ± 0.01	2.8 ± 0.1	0.91 ± 0.15	3.3 ± 0.1	n.a.	n.a.	n.a.	6.6 ± 0.1	3.7 ± 0.5	0.23 ± 0.10
Ethanol (g/L)	0.37 ± 0.10	0.78 ± 0.08	n.d.	0.22 ± 0.00	0.15 ± 0.00	0.23 ± 0.00	0.20 ± 0.00	0.17 ± 0.00	0.17 ± 0.00	0.28 ± 0.05	1.0 ± 0.1	n.d.
ABE (g/L)	10.7 ± 0.6	18.0 ± 0.8	0.36 ± 0.04	9.5 ± 0.7	4.6 ± 1.2	8.6 ± 0.2	n.a.	n.a.	n.a.	18.6 ± 0.3	13.5 ± 1.8	0.40 ± 0.18
IBE (g/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7.7 ± 0.4	7.5 ± 0.3	5.4 ± 1.0	n.a.	n.a.	n.a.
OD_{600nm}	3.7 ± 0.2	5.1 ± 0.9	2.4 ± 0.1	6.0 ± 0.5	3.1 ± 0.1	3.5 ± 0.6	4.8 ± 0.2	4.0 ± 0.3	3.1 ± 0.1	6.9 ± 0.2	2.2 ± 0.3	1.9 ± 0.1
Glucose conversion (%)	55 ± 4	n.a.	21 ± 1	56 ± 4	n.a.	46 ± 3	45 ± 6	n.a.	25 ± 1	87 ± 5	n.a.	18 ± 2
Xylose conversion (%)	n.a.	94 ± 4	17 ± 1	n.a.	30 ± 6	0 ± 0	n.a.	50 ± 1	0 ± 0	n.a.	63 ± 10	0 ± 0
Butanol yield (g/g)	0.16 ± 0.01	0.21 ± 0.01	0.01 ± 0.00	0.19 ± 0.05	0.18 ± 0.01	0.22 ± 0.01	0.18 ± 0.02	0.18 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	0.26 ± 0.01	0.02 ± 0.00
ABE yield (g/g)	0.35 ± 0.01	0.37 ± 0.01	0.01 ± 0.00	0.28 ± 0.06	0.23 ± 0.01	0.38 ± 0.02	n.a.	n.a.	n.a.	0.30 ± 0.01	0.37 ± 0.01	0.05 ± 0.01
IBE yield (g/g)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.29 ± 0.03	0.26 ± 0.01	0.41 ± 0.05	n.a.	n.a.	n.a.
Butanol productivity (g/Lh)	0.05 ± 0.00	0.05 ± 0.00	0.00 ± 0.00	0.13 ± 0.01	0.05 ± 0.01	0.10 ± 0.00	0.10 ± 0.01	0.05 ± 0.00	0.03 ± 0.01	0.09 ± 0.01	0.07 ± 0.01	0.00 ± 0.00
ABE productivity (g/Lh)	0.11 ± 0.01	0.09 ± 0.01	0.01 ± 0.00	0.20 ± 0.01	0.06 ± 0.02	0.18 ± 0.00	n.a.	n.a.	n.a.	0.14 ± 0.01	0.10 ± 0.01	0.01 ± 0.00
IBE productivity (g/Lh)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.16 ± 0.01	0.08 ± 0.00	0.06 ± 0.01	n.a.	n.a.	n.a.

n.a. not applicable; *n.d.* not detected; Enzymatic hydrolysate obtained at 16% solid concentration and 25 FPU/g_{glucan} enzyme dosage using citrate buffer and washed steam exploded *E. grandis* wood.

C. beijerinckii DSM 6422 and IBE strain *C. beijerinckii* DSM 6423 were used. In glucose and xylose fermentations, *C. beijerinckii* DSM 6423 effectively fermented both carbon sources to produce butanol and isopropanol in the range of 4.7–5.2 g/L and 2.2–2.6 g/L, respectively. Even though complete sugar consumption was not achieved (45% for glucose and 50% for xylose), relatively high butanol (0.18 g/g) and IBE (0.26–0.29 g/g) yields were reached by this IBE strain. Better performance was achieved by ABE strain *C. beijerinckii* DSM 6422 in glucose fermentation, achieving higher butanol concentration (6.5 g/L) and yield (0.19 g/g) at 48 h, even though complete glucose consumption (56%) was also not achieved. However, a poor performance was observed in xylose fermentation, in which only 3.5 g/L of butanol was produced after 72 h fermentation, which corresponded to a butanol yield of 0.18 g/g. Considering that fermentation using glucose-based semi-synthetic medium ceased after 48 h for both strains, using these *Clostridium* strains allowed to increase both butanol and solvent mixture productivities up to 0.10–0.13 g/L.h and 0.16–0.20 g/L.h, respectively. For xylose-based semi-synthetic medium, given that fermentation ceased after 72–96 h, butanol and solvent mixture productivities decreased up to 0.05 g/L.h and 0.06–0.08 g/L.h, respectively, for both strains.

Considering that both product yield and productivity are important parameters to the economic feasibility of the fermentation process, assays using enzymatic hydrolysate as carbon source were performed for a reliable *Clostridium* strain selection (Fig. 4, Table 4). Both *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 effectively fermented the enzymatic hydrolysate from steam exploded *E. grandis* wood at 200 °C, which contained both glucose (42–48 g/L) and xylose (2–4 g/L). *C. beijerinckii* DSM 6422 was able to produce 5.0 g/L of butanol and 8.6 g/L of ABE mixture, consuming 46% of the original glucose at 48 h of fermentation. Butanol (0.22 g/g) and ABE (0.38 g/g) yields resulted higher compared to glucose fermentation, whereas productivities values resulted comparable (Table 4). These results demonstrated that *C. beijerinckii* DSM 6422 represents a promising ABE strain for butanol fermentation using lignocellulosic biomass as feedstock. On the other hand, *C. beijerinckii* DSM 6423 produced 2.5 g/L of butanol and 5.4 g/L of IBE mixture, consuming only 25% of the original glucose after 96 h of fermentation. This may be expected considering that native IBE producers are reported as less effective compared to ABE producers [45–47]. However, butanol (0.19 g/g) and ABE (0.41 g/g) yields resulted higher compared to glucose fermentation, whereas productivities resulted slightly lower (Table 4). The other two strains (*C. acetobutylicum* DSM 1731 and *C. saccharoperbutylacetonicum* DSM 14923) were not able to produce butanol from enzymatic hydrolysate, as they showed poor solvents production (<1 g/L ABE) (Fig. 5). Consequently, low ABE yields (0.01–0.05 g/g) and productivities (0.01 g/L.h) were achieved by these strains. One possible explanation for the poor fermentation performance is that both strains produced relatively high amounts of acids, both acetic acid (4.0–5.5 g/L) and butyric acid (3.0–3.5 g/L), during the growth phase until 48–72 h. Even though this behavior is expected given that acids are produced during acidogenesis phase along with exponential growth for solvent production, the strains were not able to re-assimilate the acids to produce the solvents. Consequently, accumulation of acid end products occurred with a sudden drop in the fermentation broth pH, which may have inactivated biomass growth due to the phenomenon known as “acid crash”. The

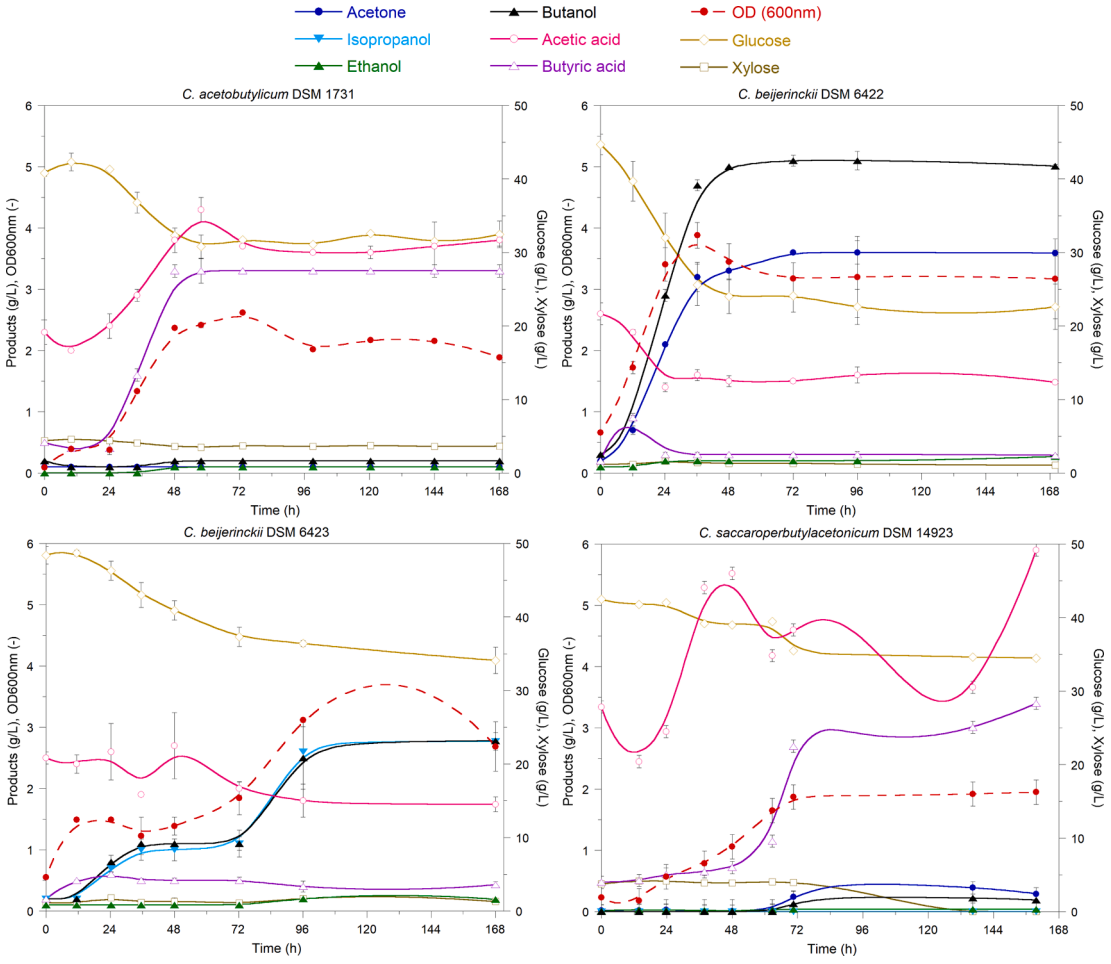

Fig. 5. Production of solvents (butanol, acetone, isopropanol, ethanol), acids (acetic and butyric acid) and biomass, and sugar consumption in ABE/IBE fermentations using *E. grandis* enzymatic hydrolysate as carbon source.

lower performance of *C. beijerinckii* DSM 6423 and poor performance of ABE fermentation by *C. acetobutylicum* DSM 1731 and *C. saccharoperbutylacetonicum* DSM 14923 using the *E. grandis* enzymatic hydrolysate could have resulted from synergistic effects of inhibitory compounds (e.g. acetic acid, formic acid, furfural, HMF, phenolic compounds). For example, acetic acid concentration in the hydrolysate medium (2.4–3.4 g/L) was almost similar to the initial acetic acid concentration in the experiments using semi-synthetic media (1.5–2.5 g/L). Even though furfural and HMF were not detected in the hydrolysate, phenolic compounds were detected in relatively low concentration (1.1 g/L). These compounds, which were liberated from the biomass matrix during enzymatic hydrolysis of steam exploded *E. grandis*, may have a negative effect on *Clostridium* butanol fermentation [19–20].

Considering the highest yields and productivity results achieved both in semi-synthetic medium and enzymatic hydrolysate, *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 were selected for further ABE and IBE fermentations, respectively. Even though better performance was achieved by ABE fermentation rather than IBE fermentation, IBE strain was further studied due to the better properties of IBE mixture over ABE mixture.

3.5. Effects of citrate and acetate buffers on butanol fermentation

The use of acetate buffer (50 mM) instead of citrate buffer (50 mM)

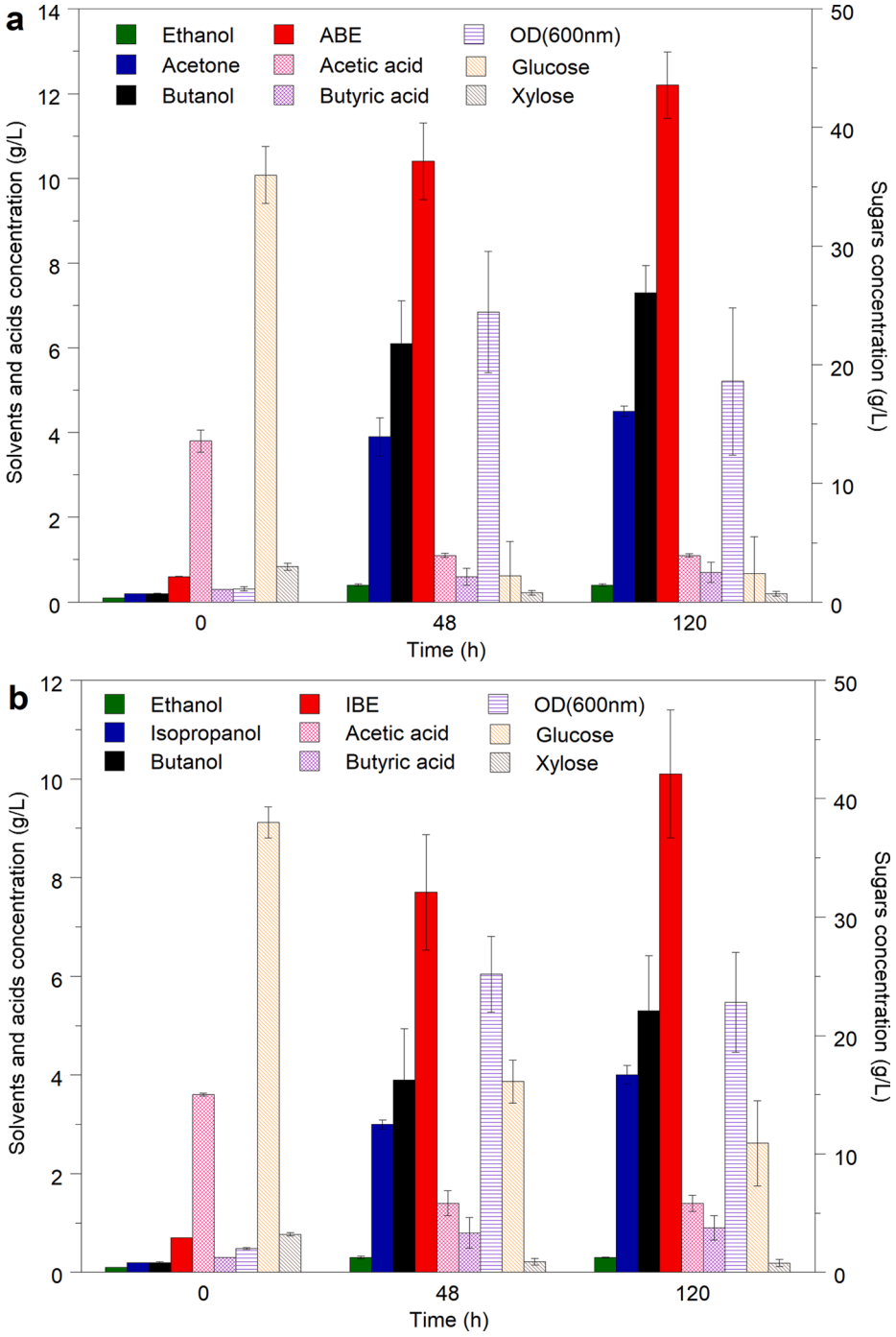

Fig. 6. Solvents and acids produced and sugar concentration during butanol fermentation of steam pretreated *E. grandis* enzymatically hydrolyzed using acetate buffer by *C. beijerinckii* DSM 6422 (a) and *C. beijerinckii* DSM 6423 (b).

during the preparation of enzymatic hydrolysate on the performance of butanol fermentation was evaluated, since it was previously reported that some *Clostridium* strains were not able to grow in glucose-based medium with citrate buffer addition [48]. Results using *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 are shown in Fig. 6 and Table 5. Enzymatic hydrolysis using acetate buffer resulted in the production of additional 2.3–2.8 g/L of butanol. This represents an improvement in butanol and total solvents production of 46% and 42% for *C. beijerinckii* DSM 6422 and 112% and 87% for *C. beijerinckii* DSM 6423, respectively. Moreover, 104% and 184% more glucose were consumed by *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423, respectively, with acetate buffer compared to citrate buffer (Table 5). However, *C. beijerinckii* DSM 6423 achieved lower glucose conversion (71%) compared to almost complete glucose conversion by *C. beijerinckii* DSM 6422. This could be due to the fact that IBE producers are less tolerant to butanol and, thus, low butanol concentrations (6 g/L) are normally produced in batch fermentation [45]. In this case, butanol concentration increased up to 5.3 g/L, which could be close to the butanol tolerance limit for this strain.

On the other hand, relatively high xylose conversions (74–76%) were achieved by both *Clostridium* strains. Even though butanol and total solvents (ABE or IBE) concentrations and sugars (glucose and xylose) conversion were greatly increased, butanol and ABE/IBE yields were not further improved. Considering that the initial acetic acid concentration resulted higher when using acetate buffer (3.6–3.8 g/L) than with citrate buffer (2.5–2.6 g/L), both *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 were able to tolerate and re-assimilate these relatively high acetic acid concentrations for solvent production.

It was previously reported by other authors that the addition of acetate to the fermentation broth enhanced butanol fermentation by *Clostridium* strains [48–50]. Chen and Blaschek [49] reported an acetate tolerance of 100 mM for *C. beijerinckii* NCIMB 8052, achieving the best butanol fermentation performance (18 g/L of ABE) with an acetate concentration of 80 mM. Liu et al. [48] demonstrated that the addition of 50 mM of acetate buffer (pH 5.0) to glucose-based medium with P2 solution enhanced the production of butanol by 40% using both *C. beijerinckii* NCIMB 8052 and *C. acetobutylicum* ATCC 824. Also, Zhou et al. [50] reported that the addition of acetic acid up to 10 g/L increased butanol production by 40% using *C. saccharoperbutylacetonicum* N1-4, reaching a maximum butanol concentration of 4.6 g/L from glucose-based medium (22.5 g/L) after 96 h. However, further increasing acetic acid concentration resulted in substrate inhibition, causing complete inhibition of butanol production by *C. saccharoperbutylacetonicum* N1-4. The improvement in butanol production with acetate addition could be attributed to the fact that acetate can be used as a buffering agent but also as a carbon source [50]. It was also reported that the enzyme activities involved in acetate uptake, acetone production and butanol production can be dramatically increased with acetate addition, which may result in a significant improve in butanol and total solvents production [51].

3.6. ABE fermentation with enzymatic hydrolysate from unwashed steam exploded E. grandis

C. beijerinckii DSM 6422 was selected to study the use of unwashed steam exploded *E. grandi* for butanol production, since it allowed to obtain the highest butanol concentration, sugar conversion and butanol yield. Enzymatic hydrolysates were prepared using citrate and acetate buffers. Fig. 7 and Table 5 show the results obtained. The initial acetic acid and xylose concentrations (6.4–9.1 g/L and 13.4–14.8 g/L, respectively) resulted much higher for enzymatic hydrolysates obtained from washed steam pretreated substrates. Even though significant amounts of pretreatment dissolved products (xylosaccharides, acetic acid, formic acid, furfural and phenolic compounds) were separated by filter pressing from the solid fraction, it was already demonstrated that a considerable amount of these compounds were occluded in the solid framework, requiring multiple water washing steps for adequate separation.

C. beijerinckii DSM 6422 fermented enzymatic hydrolysate from unwashed pretreated substrates using citrate buffer in 48 h, producing 5.5 g/L and 10.2 g/L of butanol and ABE, respectively (Fig. 7). Although the initial acetic acid concentration was of 6.4 g/L in this case, almost no significant difference in butanol production was found compared to results obtained using enzymatic hydrolysate from washed pretreated substrates with acetate buffer (3.8 g/L initial acetic acid) (Table 5). This suggests that the presence of soluble compounds formed during steam explosion pretreatment (e.g. acetic acid, formic acid, furfural and phenolic compounds), which could have acted as inhibitory compounds, did not negatively affect *C. beijerinckii* DSM 6422 fermentation performance. However, poor fermentation parameters were observed in enzymatic hydrolysate with acetate buffer with only 1.6 g/L butanol and 3.9 g/L ABE production after 48 h of fermentation. Even though this longer lag phase certainly resulted from the inhibitory effect of acetic acid (initial concentration 9.1 g/L) in enzymatic hydrolysate formed during steam explosion pretreatment, acetic acid concentration decreased to 4.6 g/L throughout the fermentation, indicating its consumption and re-assimilation for solvent production. After 120 h of fermentation, maximum butanol (6.9 g/L) and ABE (12.5 g/L) production were reached, which demonstrated that inhibitory compounds were not detrimental to biomass growth and butanol fermentation.

Even though similar butanol fermentation performance in terms of

Table 5
Butanol fermentations by *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 from enzymatic hydrolysate prepared using citrate and acetate buffers and washed and unwashed steam exploded *E. grandis* wood after 120 h of fermentation.

Strain	*C. beijerinckii* DSM 6422			*C. beijerinckii* DSM 6423
Enzymatic hydrolysis conditions	Washed substrate acetate buffer	Unwashed substrate citrate buffer	Unwashed substrate acetate buffer	Washed substrate acetate buffer
Butanol (g/L)	7.3 ± 0.6	6.9 ± 0.2	6.9 ± 0.5	5.3 ± 1.1
Isopropanol (g/L)	n.a.	n.a.	n.a.	4.0 ± 0.2
Acetone (g/L)	4.5 ± 0.1	4.8 ± 0.3	5.1 ± 0.5	n.a.
ABE (g/L)	12.2 ± 0.8	12.2 ± 0.4	12.5 ± 0.5	n.a.
IBE (g/L)	n.a.	n.a.	n.a.	10.1 ± 1.3
Maximum OD_{600nm}	6.1 ± 1.4	6.5 ± 0.6	6.5 ± 0.2	5.8 ± 1.0
Glucose conversion (%)	94 ± 2	95 ± 2	99 ± 1	71 ± 4
Xylose conversion (%)	76 ± 3	63 ± 4	59 ± 1	74 ± 2
Butanol yield (g/g)	0.18 ± 0.01	0.17 ± 0.01	0.19 ± 0.01	0.17 ± 0.01
ABE yield (g/g)	0.30 ± 0.02	0.30 ± 0.02	0.34 ± 0.02	n.a.
IBE yield (g/g)	n.a.	n.a.	n.a.	0.32 ± 0.02
Final acetic acid (g/L)	1.1 ± 0.1	1.5 ± 0.2	1.5 ± 0.3	1.4 ± 0.2
Final butyric acid (g/L)	0.7 ± 0.2	0.7 ± 0.1	0.9 ± 0.2	0.9 ± 0.2
Overall butanol yield ($g/kg_{eucalyptus}$)	67.0 ± 4	45.6 ± 3	46.9 ± 2	48.2 ± 3
Overall ABE yield ($g/kg_{eucalyptus}$)	111.6 ± 10	80.5 ± 5	84.0 ± 4	n.a.
Overall IBE yield ($g/kg_{eucalyptus}$)	n.a.	n.a.	n.a.	90.8 ± 5

n.a. not applicable.

product concentration, yield and productivity were achieved in hydrolysates obtained using either unwashed or washed pretreated *E. grandis* substrates, it was already demonstrated that enzymatic hydrolysis performance was negatively affected when unwashed substrates were used (Table 3). This fact decreased glucose yield after enzymatic hydrolysis (from 38 to 40% to 24–27%) and, thus, overall butanol yield (from 112 $g/kg_{eucalyptus}$ to 80–84 $g/kg_{eucalyptus}$) (Table 5).

3.7. Mass balance of selected process conditions

To determine the recovery of the main *E. grandis* components for co–production of butanol and xylosaccharides, the overall mass balance of the proposed process (Fig. 8) was analyzed. The material balance included glucan, lignin and the major component of the hemicellulosic fraction (xylan). As presented in Table 2, 1 kg of oven dried *E. grandis* wood contained 484 g of glucan, 272 g of lignin and 148 g of xylan. After steam explosion process (200 °C, 1.5 MPa, 10 min), 730 g of pretreated *E. grandis* (73% of the raw material) were obtained as solid fraction which contained 431 g of glucan, 276 g of lignin and 14 g of xylan (Table 2). The remaining 270 g (27% of the raw material) were dissolved into the hemicellulosic hydrolysate and washing waters which were comprised of solubilized XOS (30 g) and xylose (37 g) (Fig. 1a). However, considerable amounts of byproducts were formed during steam explosion pretreatment under the selected conditions, such as acetic acid (22 g), formic acid (11 g), furfural (3 g) and phenolic compounds (52 g) (Fig. 1a). An ion-exchange and adsorption resin treatment (Dowex 66

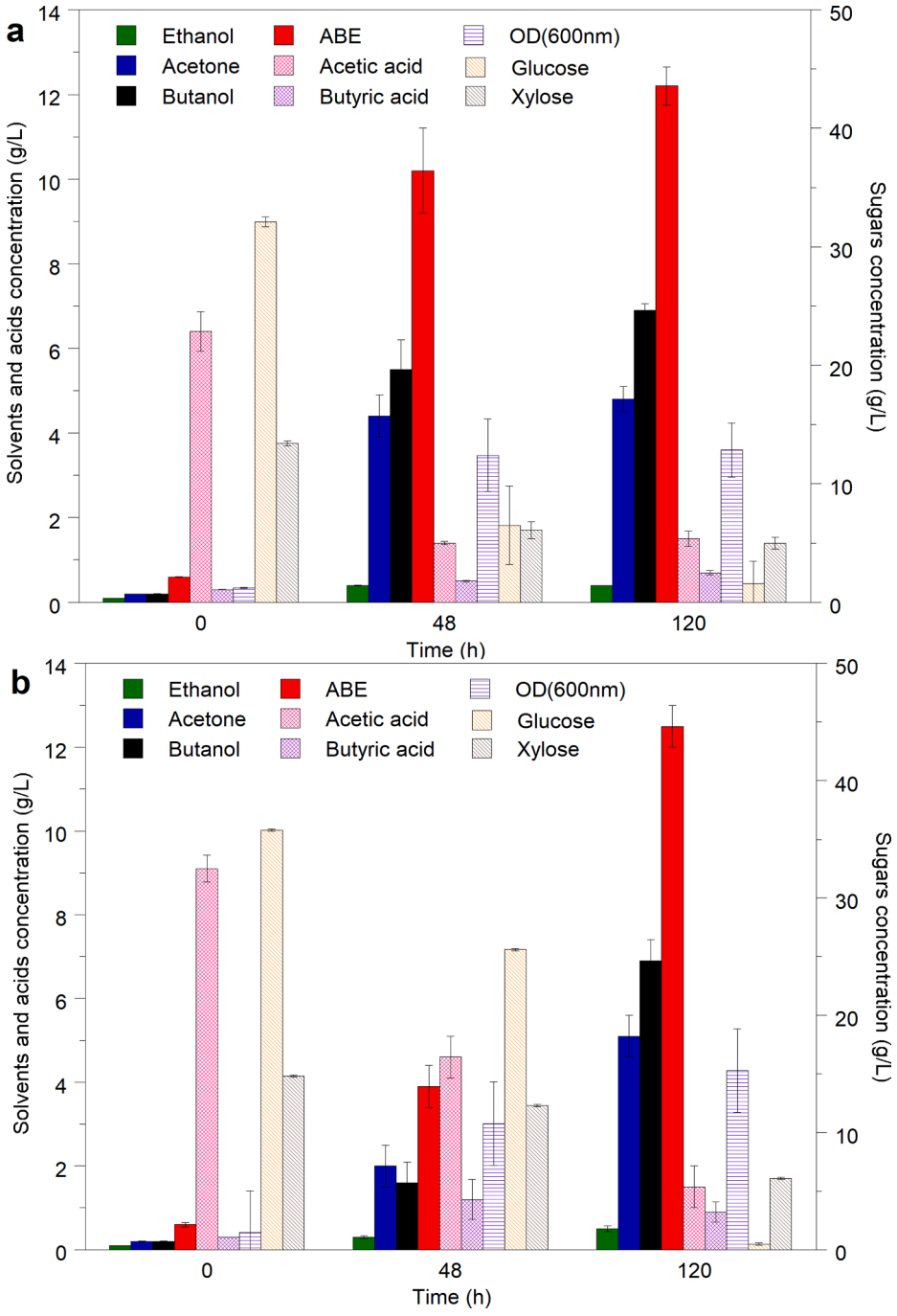

Fig 7. Solvents and acids produced and sugar concentration during butanol fermentation of unwashed steam pretreated *E. grandis* enzymatically hydrolyzed using citrate (a) and acetate (b) buffer by *C. beijerinckii* DSM 6422.

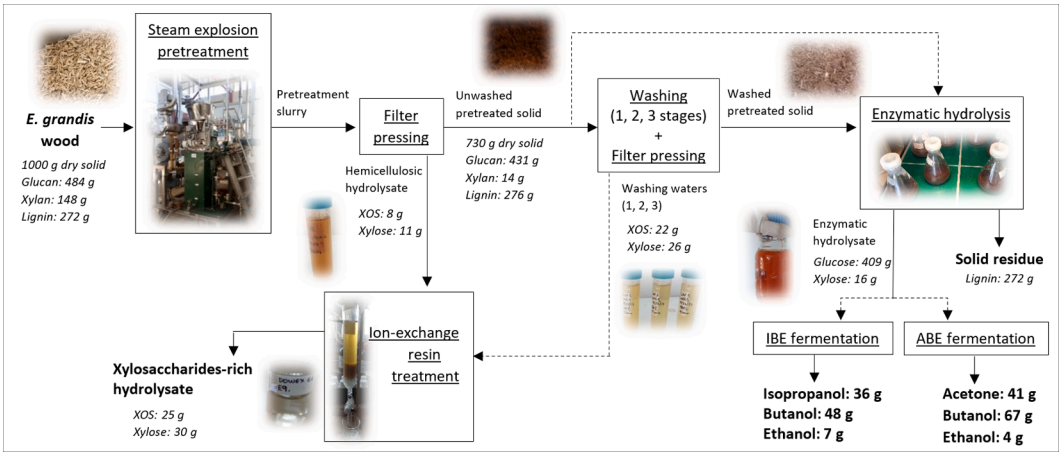

Fig. 8. Overall mass balance of the proposed process for the integrated production of butanol and xylosaccharides from steam exploded *E. grandis* wood.

resin at pH 3.2 and hydrolysate/resin of 1) was then performed, which allowed to obtain a xylosaccharide-rich hydrolysate (25 g of XOS and 30 g of xylose) with lower amounts of impurities (2 g of acetic acid, 1 g of formic acid, 1 g of furfural and 12 g of phenolic compounds) (Fig. 2). The obtained xylosaccharide-rich hydrolysate was mostly composed of xylose (69%) and xylobiose (20%) (Fig. 1c). However, to recover the maximum amount of xylosaccharides after steam explosion pretreatment in a separate xylosaccharides-rich liquid stream by ion-exchange resin treatment for an optimized biorefinery, further investigation is required. Furthermore, the steam pretreated *E. grandis* was subjected to enzymatic hydrolysis (16% solid loading, 25 FPU/g_{glucan} enzyme loading, 72 h) which allowed to obtain an enzymatic hydrolysate containing 409 g of glucose and 16 g of xylose as fermentable sugars (Table 3). The solid residue obtained after enzymatic hydrolysis in this study was not analyzed but this solid fraction should mostly contain lignin (272 g) which could be used for lignin-based materials production or as solid fuel for energy production. Both glucose and xylose sugars (425 g) in enzymatic hydrolysate were fermented by either *C. beijerinckii* DSM 6422 or *C. beijerinckii* DSM 6423 to produce ABE (41 g of acetone, 67 g of butanol and 4 g of ethanol) or IBE (36 g of isopropanol, 48 g of butanol and 7 g of ethanol) solvent mixtures, respectively (Table 5). These results indicate that the proposed process represents a feasible strategy for an integral valorization of *E. grandis* wood by co–production of butanol and xylosaccharides in a biorefinery platform.

4. Conclusions

Steam explosion pretreatment of *E. grandis* wood using a pre-pilot reactor allowed to obtain a good xylosaccharide recovery (50%) and high enzymatic hydrolysis yield (80%) of pretreated solids for glucose release. The xylosaccharides dissolved during pretreatment were separated in a xylosaccharides-rich liquid stream by ion-exchange and resin treatment containing mostly xylose and xylobiose. Steam pretreated *E. grandis* was effectively enzymatically hydrolyzed even at high solid loading, but water washing was required prior to enzymatic hydrolysis to enhance sugar release. The native strains *C. beijerinckii* DSM 6422 and *C. beijerinckii* DSM 6423 presented a remarkable ability to ferment the enzymatic hydrolysate for ABE and IBE production, respectively. The proposed process represents a promising strategy towards an integrated biorefinery by valorization of *E. grandis* wood to co-produce butanol and xylosaccharides.

CRediT authorship contribution statement

Florencia Cebreiros: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Florencia Risso:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Matias Cagno:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Maria Noel Cabrera:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft, Visualization. **Eloísa Rochón:** Conceptualization, Methodology, Validation, Formal analysis, Investigation. **Guillermo Jauregui:** Investigation, Validation. **Elzeario Boix:** Investigation, Validation. **Silvia Böthig:** Conceptualization, Methodology, Validation, Formal analysis, Writing - review & editing. **Mario Daniel Ferrari:** Conceptualization, Methodology, Validation, Formal analysis. **Claudia Lareo:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing - review & editing, Visualization, Supervision, Project administration.

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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