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Second generation bioethanol from *Eucalyptus globulus Labill* and *Nothofagus pumilio*: Ionic liquid pretreatment boosts the yields

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ABSTRACT

The depletion of petroleum reserves and the high level of pollution caused by fossil fuels have led to enhancing renewable energy and fuel production from biomass. *Eucalyptus globulus* and *Nothofagus pumilio* residues could constitute an interesting source of biomass for second generation biofuel production. Lenga residues were pretreated with the ionic liquid (IL) 1-*N*-ethyl-3-methylimidazolium chloride (C_2mimCl), followed by subsequent fermentation using both the strategy of Simultaneous Saccharification and Fermentation (SSF) as well as Separate Hydrolysis and Fermentation (SHF). The SHF process yielded 0.134 g ethanol/g glucose (26.3 wt-% of the theoretical yield) compared to the SSF process which yielded 0.173 g ethanol/g glucose (33.9 wt-% of the theoretical yield) within the first 24 h of fermentation. In case of *Eucalyptus* residues, another IL, 1-*N*-ethyl-3-methylimidazolium acetate (C_2minOAc) was applied. The SSF process was applied for a period of three days. As a result, 3.7 g ethanol/L (corresponding to a yield of 0.19 g of ethanol/g of glucose or 38.0 wt-% of the theoretical maximum) was obtained at 72 h. When fresh Lenga and *Eucalyptus* residues were fermented without any pretreatment, the SSF process yielded 0.017 and 0.002 g of ethanol/g of glucose, respectively (3.33 wt-% and 0.48 wt-% of the theoretical maximum, respectively). Thus, the pretreatment procedures resulted in a significant increase in ethanol production, therefore justifying the need of pretreatment prior to the co-enzyme hydrolysis and fermentation for this type of biomass. Further, the combination of IL pretreatment and use of SSF process demonstrated the high potential for bioethanol production from Lenga and *Eucalyptus* residues. Nevertheless, further improvement by optimization of operational conditions is required to maximize the ethanol yield.

1. Introduction

Bioethanol is among the most produced biofuels worldwide and can be obtained from various types of bioresource. As an alternative to the use of edible crops as raw material, the use of lignocellulosic materials or production of the so- called second generation biofuels, has seen ever increasing research efforts during the last decade (Pinkert et al., 2009; Mussatto et al., 2010). The production of bioethanol from lignocellulosic material usually requires incorporation of an efficient pretreatment, followed by saccharification of the carbohydrates to achieve satisfactory

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efficiency (Mussatto et al., 2010; Gírio et al., 2010; Nigam and Singh, 2011). Different pretreatment strategies have been developed throughout the years for lignocelluloses, including physical, biological, chemical and physicochemical processes (Rayne and Mazza, 2007; Pezoa et al., 2010; Mora-Pale et al., 2011; Romaní et al., 2012; Marzialetti et al., 2014). Ionic liquids (ILs) can be alternatives to the conventional pretreatments (Gírio et al., 2010). ILs are organic salts able to melt under 100 °C, but some even at significantly lower temperatures (Pinkert et al., 2009; Mora-Pale et al., 2011). In terms of chemical processes, they show excellent physical characteristics such as the ability to dissolve polar and non-polar organic or inorganic substances, as well as polymers. Many ILs have been developed for specific processes, thus justifying the name "designer solvents" (Hough and Rogers, 2007; Vancov et al., 2012). A multitude of studies have demonstrated that cellulose is soluble in several ionic liquids such as: 1,8-diazabicyclo-[5.4.0]-undec-7-ene-sulfurdioxide-monoethanolamine (Rogne et al., 2015), *N*-allyl-*N*-methylmorpholinium acetate (Raut et al., 2015), 1-butyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium hydrogen sulfate, 1-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazolium acetate (Gräsvik et al., 2014), 1-ethyl-3-methylimidazolium acetate (Lee et al., 2009; Sun et al., 2009; Arora et al., 2010; Li et al., 2011), 1-allyl-3-methylimidazolium chloride (Mäki-Arvela et al., 2010; Vancov et al., 2012), 1-ethyl-3-methylimidazolium-chloride (Varanasi et al., 2008; Zhao et al., 2009; Varanasi et al., 2009; Pezoa et al., 2010) and other examples are described in Silveira et al. (2015). After cellulose dissolution, it can be regenerated through the addition of anti-solvents like water and organic solvents. It has been demonstrated that cellulose regenerated from ILs solutions is more rapidly saccharified into glucose compared with untreated substrates (Mora-Pale et al., 2011; Silveira et al., 2015). The use of ILs for extraction of cellulose from wood avoids the use of toxic and hazardous chemicals, and can be carried out under mild conditions (Alvira et al., 2010; Wang et al., 2011). Initially, the price of ILs was high, however, in recent years, there are more companies that produce ILs and this has lowered the price.

Hence, an effective pretreatment of lignocellulose materials is needed in order to obtain faster enzymatic hydrolysis of cellulose in the saccharification step (Zhang and Lynd, 2004; Gírio et al., 2010; Wang et al., 2011). After the pretreatment in the bioethanol production process, the saccharification and fermentation steps can be carried out via different process configurations: (a) Separate Hydrolysis and Fermentation (SHF), a process in which hydrolysis of polysaccharides and the fermentation of monosaccharide are performed separately (Pereira, 2008; Olofsson et al., 2008; Santibáñez, 2010). In this case, the hydrolysis is rate-limited by the concentration of generated sugar which inhibits the cellulase activity; (b) Simultaneous Saccharification and Fermentation (SSF), a process in which polysaccharides hydrolysis and monosaccharides fermentation are carried out in one pot; (c) Consolidated BioProcessing (CBP), a process in which the enzymes are produced by the fermenting organisms; (d) Simultaneous Saccharification and Co-Fermentation of hexoses and pentoses (SSCF), a process in which polysaccharides hydrolysis and C5 and C6 fermentation are performed in one pot; and (e) Solid State Fermentation (SoSF). The advantage of the coupling of processes is the immediate consumption of sugar to produce ethanol, which leads to a lower enzyme requirement, less sterile conditions, shorter process time, and smaller reactor volumes (Olofsson et al., 2008; Pejo et al., 2008; Zinoviev et al., 2010).

Eucalyptus is the second most abundant lignocellulosic material in Chile, corresponding to 23% of the total forest plantations in the country, equivalent to 483 Mha. Lenga is a Chilean native tree that grows preferably in the extreme south of the country, covering around 3600,000 ha, representing 26.5% of native forests (CONAF; National Forestry Corporation, 2011). Lenga formations are regarded, in general terms, as over-mature forests due to the high proportion of existing trees under decay and aging (Schmidt et al., 1992). This situation implies low yields of wood for industrial purposes. On the other hand, in the case of Eucalyptus, this type of tree is widely used in the forest industry, therefore there are tons of *Eucalyptus* residues that are usually considered as waste or just burned (García et al., 2011) For these reasons, Lenga and *Eucalyptus* residues could constitute an interesting source of biomass for second generation biofuel production, especially in a country that is characterized by its extreme dependence on foreign sources of energy (Zhao et al., 2009; Fritz, 2009).

The aim of the present work was to study different process configurations and pretreatment strategies (ILs) upon saccharification and fermentation (Separate or Simultaneous) of *Eucalyptus* and Lenga residues aiming for second generation ethanol in Chile.

2. Material and methods

2.1. Materials

Residual Lenga (*Nothofagus pumilio* (POEPP. et. ENDL. KRASSER) of about 40–60 years of age was cut into about 1–2 mm high and 1–3 mm wide as well as 5–7 mm long chips (pin-chip size) and dried at 80 °C overnight prior to pretreatments. The chips had a bulk density of approximately 156 kg/m^3 and an initial moisture content of around 10-15%. The composition of untreated residual Lenga was determined to be as follows. 45.0% of cellulose, 33.2% of hemicellulose, 17.0% lignin, 4.0% of extractives and 0.8% of ash elements (Fritz, 2009).

Residual *Eucalyptus* (*Eucalyptus globulus Labill*) of 15 years of age was cut to pin-chip size (0.5–1 mm wide, 0.5–1 mm high and 10–20 mm long) and dried at 65 °C for 18 h. The composition of untreated residual *Eucalyptus* was determined to be as follows: 47.3% of cellulose, 39.3% of hemicellulose, 17.3% lignin, 4.2% of extractives and 0.9% of ash elements (Santibáñez, 2010).

Ionic liquids: 1-ethyl-3-methylimidazolium-chloride, C_2mimCl, supplied by Merck KGaA, (Germany) was used as received (≥95% purity, the water content was not quantified, but the IL was used immediately to avoid hydration) and 1-ethyl-3-methylimidazolium-acetate, C_2mimOAc, was provided by Sigma–Aldrich® (90% purity, water content below 0.5 wt.%)

Enzymes: A commercial cellulase enzyme complex (Novo Celluclast: Sigma C2730 comprised of endo-β-glucanase, exo-β-glucanase and β-glucosidase) supplied with additional β-glucosidase (Novozyme: Sigma C6105) was used for saccharification.

2.2. Methods

2.2.1. Lignocellulosic materials dissolution with IL and regeneration

Two different pretreatments in addition to blank experiment with Lenga residues were performed with biomass loadings of 1:20 (wt RL/wt IL): a) reference case when no pretreatment was carried out, b) C_2mimCl at 150 °C for 60 min, c) C_2mimCl at 150 °C for 30 min. Subsequently, the remaining solid material was filtered (nylon filters) and washed six times, with 50 mL of distilled water each time, to remove residual ionic liquids from the pretreated material since presence of IL would produce an adverse effect on the subsequent enzymatic hydrolysis (Zhang and Lynd, 2004; Turner et al., 2003). After washing, fibers were freeze-dried overnight.

Additionally, Lenga and the ionic liquid were loaded at different ratios (1:3, 1:5 and 1:10 (wt wood:wt IL)) into test tubes of 10 mL. The tubes were placed in a thermostated oil bath and maintained at 150 °C and reaction times of 15 and 30 min. After incubation the

tubes were removed from the oil bath and left to cool at room temperature and washed. All experiments were carried out in triplicate for better accuracy of the experimental results.

In the case of *Eucalyptus* residues, biomass loadings of 1:3 (wt wood:wt IL) were utilized and three different pretreatments were compared: (a) reference case when no pretreatment was applied (b) C_2mimOAc at 150 °C for 60 min and (c) C_2mimOAc at 150 °C for 30 min. Subsequently, the remaining solid material was filtered and washed six times, with 50 mL of distilled water each time, to remove residual ionic liquid. After washing, the fibers were freeze dried overnight.

2.2.2. Separate hydrolysis and fermentation (SHF)

2.2.2.1. Enzymatic saccharification for Lenga and Eucalyptus. When Lenga residues were used, a suspension of 1 g of IL- pretreated raw material or fresh Lenga (without pretreatment) in 20 mL of sodium acetate buffer (50 mM, pH 4.8 Tween 20, 2.5 g/L) was incubated at 50 °C with stirring (250 rpm). Addition of surfactants (Tween 20) to enzymatic hydrolysis reduced enzyme adsorption to the lignocellulose substrate (Eriksson et al., 2002). The enzymatic hydrolysis reaction was initiated by adding the following enzyme loadings: 37 [FPU/g of cellulose] of cellulase (Celluclast) and 4.9 [CBU/g of cellulose] of β-glucosidase (Novozyme).

When *Eucalyptus* residues were used, a suspension of 1 g of IL pretreated raw material or fresh *Eucalyptus* (without pretreatment) in 20 mL of solution in sodium acetate buffer (50 mM, pH 4.8, with Tween 20, 2.5 g/L) was incubated at 50 °C with stirring (250 rpm). The enzymatic hydrolysis reaction was initiated by adding the following enzyme loadings: 37 [FPU/g of cellulose] of cellulase (Celluclast) and 4.9 [CBU/g of cellulose] of β-glucosidase (Novozyme).

In both cases, saccharification was performed during 72 h and samples were withdrawn at 0, 4, 12, 24, 48 and 72 h for determination of the monomer sugars.

2.2.2.2. Fermentation for Lenga. SHF Fermentations were carried out in anaerobic sterilized Erlenmeyer flasks by adding 20 mL of hydrolyzate from the saccharification with 5 g/L yeast extract; 0.5 g/L of $(NH_4)_2HPO_4$ and 0.025 g/L of $MgSO_4{\cdot}7H_2O$ (fermentation media). Thereafter, the Erlenmeyer flasks were incubated with 0.2 g of *Saccharomyces cerevisiae* Ethanol Red® (kindly provided by S.I. LESAFFRE FERMENTIS, Marq en Baroeul Cedex, France) at 40 °C for 72 h with stirring (250 rpm).

2.2.3. Simultaneous Saccharification and Fermentation (SSF)

When Lenga residues were utilized, a suspension containing 1 g of IL pretreated biomass or fresh Lenga (without pretreatment) plus fermentation media and cellulase mixture was used. SSF was carried out in 250 mL Erlenmeyer flask with 20 mL of media. The Erlenmeyer flasks with the fermentation media were sterilized before adding the IL pretreated biomass. The enzyme loadings were 37 [FPU/g cellulose] of cellulase (Celluclast) and 4.9 [CBU/g of cellulose] of β-glucosidase (Novozyme), respectively. The Erlenmeyer flasks were incubated with 0.2 g of *S. cerevisiae* Ethanol Red® at 40 °C for 72 h with stirring (250 rpm).

When *Eucalyptus* residues were utilized, a suspension containing 1 g of IL pretreated biomass or fresh *Eucalyptus* (without pretreatment) was used. After pretreatment, two different washing methods were compared; the residues were washed with distilled water 2 times or 6 times. SSF was carried out in 250 mL Erlenmeyer flask with 20 mL of media, using the same fermentation media described for Lenga residues. The Erlenmeyer flasks with the fermentation media were sterilized before adding the IL pretreated biomass. The enzyme loadings were 37 [FPU/g cellulose] of cellulase (Celluclast) and 4.9 [CBU/g of cellulose] of β-glucosidase (Novozyme), respectively. The Erlenmeyer flasks were incubated with 0.2 g of *S. cerevisiae* Ethanol Red® at 40 °C for 72 h with stirring (250 rpm).

2.2.4. Carbohydrate analysis in the solid phase

Acid methanolysis method: the content of hemicelluloses and pectins in fresh and pretreated *Eucalyptus* and Lenga biomass were determined by acid methanolysis method. 2 mL of methanolysis reagent containing 2 M of hydrochloric acid – HCl – in methanol was added to 10 mg of freeze dried wood sample and to a calibration solution containing known amounts of carbohydrates. As the next step, the tubes were inserted into an oven operating at 100 °C for 5 h. Once the reaction was completed, 200 μL of pyridine was added to neutralize any excess of HCl, and 2 mL of each internal standard solution containing 0.1 mg/mL of sorbitol and resorcinol in methanol, respectively, were added to each sample. After mixing, methanol was evaporated at 50 °C under nitrogen stream and the sample was further dried under a vacuum (Heraeus VTR 5022) at 42 °C below 50 mbar for 20 min prior to derivatization of the samples (Sundberg et al., 2003).

Acid hydrolysis method: the cellulose content in fresh and pretreated *Eucalyptus* and Lenga biomass were determined by acid hydrolysis method. 200 μL 72 vol-% sulfuric acid was added to each 10 mg wood sample. Also, 10 mg cellulose powder was used as a standard. The standard and the wood samples were placed in vacuum oven and degassed until below 50 mbar. This step was repeated three times. As the next step, the samples were stored under fume hood for 2 h, whereupon 0.5 mL of distilled water was added to each sample. Again, 4 h later 6 mL of distilled water was added to each sample and the samples were stored under fume hood overnight, at room temperature. Next day the samples were placed in an autoclave at 125 °C for 90 min and then cooled down to room temperature. Two droplets of bromocresol green were added to each sample as an indicator and barium carbonate was added to neutralize the samples until the liquid phase turned blue. 1 mL of internal standard containing 5 mg/mL of sorbitol in distilled water was added to each sample, and then centrifuged at 1.200 U/min for 10 min. 1 mL of liquid phase was taken from each sample and transferred to another test tube, where 1 mL of acetone was added. Finally, the samples were evaporated under nitrogen gas stream at 60 °C and further dried under vacuum (Heraeus VTR 5022) at 42 °C below 50 mbar for 15 min prior to derivatization of samples (Sundberg et al., 1996).

2.2.5. Analysis of monosaccharide, sugars and ethanol in liquid phase

Monosaccharide analysis from Lenga and Eucalyptus: The monosaccharides profiles were determined by Gas Chromatography (GC), using a PerkinElmer AutoSystem XL instrument. The analysis was performed in a 20 m × 0.199 mm i.d. column (J&W HP-1) with a film thickness of 0.11 μm. The column oven parameters were as follows: 100 °C, 8 min, ramped by 2 °C/min to 170 °C and thereafter by 12 °C/min to 300 °C (10 min); split injector (25:1), 250 °C; FID detector, 310 °C; injection volume, 1 μl. Hydrogen was used as carrier gas (pressure: 14 psi put bar). In the FI detector hydrogen and air were used with the flow rates of 45 mL/min and 450 mL/min, respectively. GC analysis was carried out after direct silylation of the samples (analysis method without acid methanolysis or acid hydrolysis). Xylitol was used as an internal standard.

The reducing sugars released during the reaction were analyzed by DNS method in an adapted methodology using a 96-microwell format and glucose was measured by the glucose oxidase method (Randox Laboratories), following the suppliers directions (Miller, 1959; Salazar et al., 2006). Ethanol was quantified by high performance liquid chromatography (HPLC) of samples filtered through a 0.22 μm pore size filter. The analyses were conducted with an

Table 1
Carbohydrate content (mg/g of dry mass) in Lenga. Monosaccharides content analyzed by acid methanolysis and acid hydrolysis methods.

Monosaccharides	Fresh Lenga [mg/g dry mass]
Arabinose	6.58
Fructose	0.45
Galactose	9.37
Galacturonic acid	16.89
Glucose[b]	434.02
Glucose (Acid methanolysis[a])	17.54
Glucuronic acid	3.64
Mannose	4.06
Rhamnose	4.43
Xylose	146.78
Total[c]	643.76

[a] Glucose analyzed by acid hydrolysis method.
[b] Total Glucose (calculated with value obtained by acid hydrolysis and acid methanolysis methods.
[c] Total calculated as the total sum of identified monosaccharide and carbohydrate.

Agilent 1100 Series HPLC System. Biorad Aminex HPX-87H column was used with 0.005 M H_2SO_4, flow rate of 0.5 mL/min and column temperature of 45 °C. Standard of ethanol was prepared (1–10 g/L).

3. Results and discussion

3.1. Lenga as the lignocellulosic material

3.1.1. Composition of Lenga biomass and influence of pretreatment in the enzymatic saccharification

The main carbohydrate components in Lenga are glucose 434.02 mg/g dry mass (67.4 wt-%) and xylose 146.78 mg/g dry mass (22.8 wt-%) of total carbohydrates. Table 1 shows the total amount of arabinose, galactose, mannose, rhamnose and uronic acid content in Lenga.

Table 2 shows the effect of different conditions of operation of the pretreatment with IL (temperature, time and type of ILs) on the amount of sugars released from Lenga residues after enzymatic saccharification, using a 1:20 biomass-IL ratio (wt RL/wt IL). These results show that the main monosaccharides released were glucose and xylose under all experimental conditions, because of hydrolysis of cellulose and xylan.

The effect of different enzymatic loading was analyzed (7, 13, 20 and 37 [FPU/g of cellulose) and the maximum production of ethanol was observed with 37 [FPU/g of cellulose] (data not shown).

Additionally, the results show that the amounts of released sugars after enzymatic hydrolysis were higher when Lenga residue was treated with C_2minCl at different temperatures when compared to the fresh Lenga residue, therefore this pretreatment improves the enzymatic digestibility of the biomass. The maximum yields of glucose and xylose obtained from saccharification of pretreated Lenga residues were 56.0 wt-% and 57.9 wt-%, respectively (yield of total reducing sugars: 51.9 wt-%), upon a pretreatment comprising the use of C_2minCl at 150 °C in 30 min. However, when the pretreatment time was increased from 30 to 60 min., a decrease in the amount of released sugars obtained after saccharification of Lenga residues was observed (from 334 mg/g DS to 235 mg/g DS, respectively). Presumably, further carbohydrate hydrolysis occurred during prolonged exposure times.

The influence of different loading ratios of Lenga residues in IL and pretreatment times after enzymatic hydrolysis is shown in Table 3. The maximum amount of glucose released after enzymatic hydrolysis was obtained after C_2mimCl, pretreatment in 15 min at 150 °C; 1:5 biomass-to-IL wt-ratio. Also, increased biomass-to-IL loading (1:10) and longer exposure time (30 min) did not lead to increased yields and, in both cases, $324 \pm 22/24$ mg/g DS was obtained. When accounting for total reducing sugars released in the saccharification step, no significant difference could be observed when using biomass-IL loading ratios between 1:3 and 1:5 and exposure times between 15 and 30 min (reducing sugars average of 457 mg/g of DS). However, when productivity factors are introduced, it can be observed that upon C_2mimCl pretreatment, 1:3 biomass-to-IL loading at 150 °C and exposure time of 15 min gives 0.595 g reducing sugars/h and g of IL. This value implies that less IL can be employed but still obtain the maximum amount of sugars released. Thus, we can conclude that C_2mimCl pretreatment carried out at 150 °C in 15 min and with a 1:3 biomass-to-IL ratio gives best result in case of Lenga.

3.1.2. Comparison between SSF and SHF processes

The differences of SSF and SHF protocols upon ethanol production from Lenga residues are shown in Fig. 1. We can see that when applying the SSF strategy, more ethanol was produced. The reason for the better performance of the SSF strategy might be related to the lower amounts of inhibiting compunds (Olofsson et al., 2008; Manzanares et al., 2011). The yield of ethanol is shown in Table 4. The SHF process yielded 0.134 g ethanol/g glucose whereas only 0.173 g ethanol/g glucose was obtained in case of SSF strategy. However, the maximum obtainable ethanol concentrations were not reached in case of the SSF process (see Fig. 1) and, consequently, longer processing times should be used. On the contrary, SHF gave the maximum within the first 4 h of fermentation. SSF seems to be limited by the inadequate enzymatic hydrolysis of the pretreated Lenga residues. Nevertheless, the fermentation yields can likely be improved by further optimization of the fermentation parameters.

3.2. Eucalyptus as the lignocellulosic material

3.2.1. Different pretreatments with ionic liquids and results of the SSF

Enzymatic saccharification of pretreated *Eucalyptus* residues with C_2mimOAc can be seen in Table 5. The maximum glucose release after enzymatic saccharification was reached after a pretreatment with C_2minOAc at 150 °C, in 30 min and at biomass-to-IL loading of 1:3 (wt:wt). Consequently, 352 mg of glucose/g DS was released (glucose yield of 82.2 wt-% based on the glucan availability in the raw material). Prolonged pretreatment times did not help and less sugars were obtained. Presumably, longer pretreatment times resulted in degradation of carbohydrates thus hampering the subsequent fermentation process. When the mixing time of the washing step was reduced to 5 min, the amount of glucose released after enzymatic saccharification was reduced to 334 mg of glucose/g DS with a glucose yield of 78.2 wt-%. When washing steps were reduced from 3 to 2, amount of glucose released after enzymatic saccharification was significantly reduced in a 61.3 wt-% to 136 mg of glucose/g DS with a glucose yield of 31.8 wt-%. Hence, no proper washing reduced the performance of the enzymatic saccharification, as it has been proposed also by other authors (Wang et al., 2011; Haykir et al., 2013) most probably because of the presence of remaining IL that might have caused enzyme inhibition (Turner et al., 2003).

In case of *Eucalyptus* residues, the SSF process was selected. The pretreatment of choice was selected on the basis of best glucose release upon the saccharification step (C_2mimOAc treatment for 30 min at 150 °C and at a biomass-to-IL loading of 1:3 (wt:wt)). The results after fermentation of non-pretreated and pretreated *Eucalyptus* residues for a period of three days are shown in Table 6. The overall concentration of ethanol achieved after pretreatment was 3.7 g/L, at 72 h (a yield of 0.194 g of ethanol/g of glucose, corresponding to 38.0 wt-% of the theoretical maximum). When the non-pretreated *Eucalyptus* residue was fermented, only 0.002 g of ethanol/g of glucose was obtained (corresponding to 0.48 wt-% of the theoretical maximum). Thus, a 79 fold increase in the amount of

Table 2
Influence of temperature and time of C_2mimCl pretreatment on sugars released from regenerated Lenga (RL) upon enzymatic hydrolysis. 1:20 biomass-to-IL ratio (wt wood/wt IL).

Pretreatment			Released sugars from regenerated Lenga		
Ionic liquid	Temperature[°C]	Time[min]	Monosaccharides[a]	[mg/g DS][b]	Sugar yield[wt-%][c]
No pretreatment			Mannose	2.0 ± 0.6	49.3
			Galactose	6.0 ± 1.3	64.0
			Xylose	7.0 ± 1.0	
			Glucose	40.0 ± 2.6	9.2
			Total sugars	55.0 ± 2.2	8.5
C_2mimCl	150	60	Mannose	1.0 ± <0.1	24.6
			Galactose	2.0 ± 0.1	21.3
			Xylose	50.0 ± 4.6	34.1
			Glucose	182.0 ± 4.3	41.9
			Total sugars	235.0 ± 4.3	36.5
C_2mimCl	150	30	Mannose	3.0 ± <0.0	73.9
			Galactose	2.0 ± 0.1	21.3
			Xylose	85.0 ± 4.6	57.9
			Glucose	243.0 ± 4.3	56.0
			Total sugars	334.0 ± 4.3	51.9

[a] Monosaccharide profile determined by gas chromatography.
[b] DS: dry solid of biomass.
[c] Sugar yield determined as percentage of the theoretical value of carbohydrate content in Lenga biomass.

Table 3
Influence of different biomass-IL ratios and pretreatment times in terms of sugars released from Lenga.

Pretreatment[a]		Released sugars from enzymatic saccharification of pretreated Lenga			Sugar productivity[g/g IL/h]
Biomass/IL ratio[wt:wt]	Time[min]	Sugar	Released sugar[mg/g DS] [b]	Sugar yield[wt-%]	
1:3	15	Glucose	295 ± 2.0	67.9	0.393
		Total reducing sugars	446 ± 8.0	69.3	0.595
1:3	30	Glucose	297 ± 32.0	68.4	0.198
		Total reducing sugars	457 ± 29.0	71.0	0.305
1:5	15	Glucose	324 ± 22.0	74.7	0.259
		Total reducing sugars	469 ± 2.0	72.9	0.375
1:10	30	Glucose	324 ± 24.0	74.7	0.065
		Total reducing sugars	433 ± 30.0	67.3	0.087

[a] Pretreatment: IL was C_2mimCl at 150 °C.
[b] All samples were hydrolyzed with cellulases for 72 h at 50 °C. Glucose was measured by the glucose oxidase method (Randox Laboratories) and reducing sugars were analyzed by DNS method.

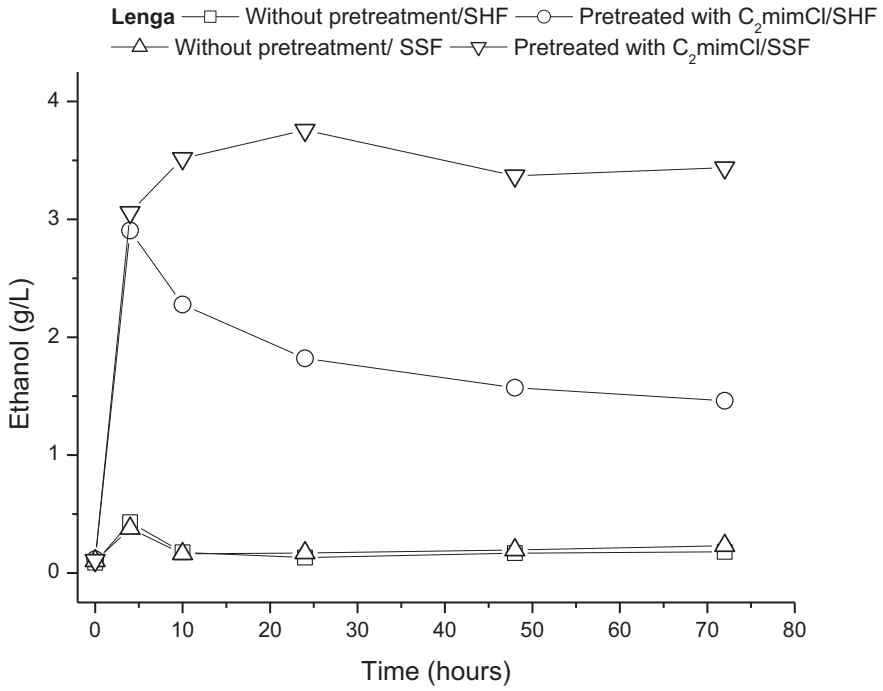

Fig. 1. The influence of the different fermentation and saccharification processes (SHF and SSF) on ethanol production from Lenga. Ethanol production in SHF from Lenga is shown in square and pretreated Lenga is shown in circle; Ethanol production in SSF from Lenga is shown by triangle symbols and pretreated Lenga by inverted triangle symbols. The pretreatment was with C_2mimCl at 150 °C, for 15 min, and a ratio of 1:3 (wt RL/wt IL).

Table 4
The yield of ethanol production for different fermentation and saccharification processes (SHF and SSF) from Lenga.

Pretreatment	Ethanol yield [gr ethanol/gr glucose]	Percentage relative to theoretical yield (wt-%)
Theoretical yield	0.510	100.0
Without pretreatment/SHF[a]	0.020	3.92
Pretreatment with C_2mimCl/SHF[a]	0.134	26.3
Without pretreatment/SFF[a]	0.017	3.33
Pretreatment with C_2mimCl/SSF^	0.173	33.9

[a] Fermentation in 4 h and (^) fermentation in 24 h.

Table 5
Influence of different biomass-IL ratios and pretreatment time over released sugars for *Eucalyptus*.

Pretreatment[a]		Released sugars from enzymatic saccharification of pretreated *Eucalyptus*		
Biomass/IL ratio [wt:wt]	Time [min]	Sugar	Released sugar [mg/g DS][b]	Sugar yield [wt-%]
1:3	30	Glucose	352	82.2
1:3	60	Glucose	281	65.8

[a] Pretreatment: IL was C_2minOAc at 150 °C.
[b] All samples were hydrolyzed with cellulases at 50 °C. Glucose was measured by the glucose oxidase method (Randox Laboratories) and reducing sugars were analyzed by DNS method.

Table 6
Influence of washing cycles on the yield of ethanol production for simultaneous saccharification and fermentation (SSF) of *Eucalyptus* after 72 h.

Pretreatment	Ethanol yield [gr ethanol/gr glucose]	Percentage relative to theoretical yield (wt-%)
Theoretical yield	0.510	100.0
Without pretreatment	0.002	0.48
Pretreatment with C_2minOAc/fibers washed two times	0.083	16.5
Pretreatment with C_2minOAc/fibers washed six times	0.194	38.0

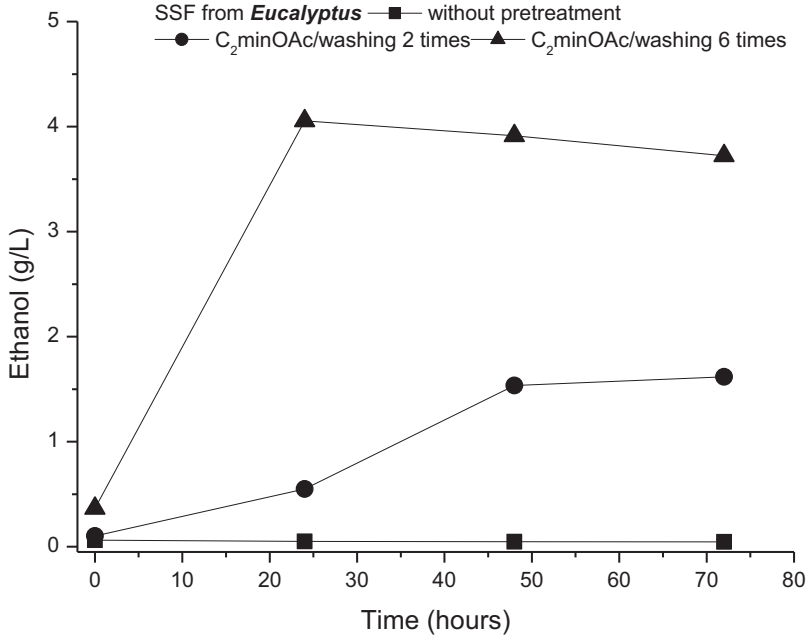

Fig. 2. The influence of simultaneous fermentation and saccharification processes (SSF) for 72 h. Eucalyptus residues without pretreatment is shown in square, *Eucalyptus* pretreated with C_2mimOAc/fibers washed 2 times is shown in circle and *Eucalyptus* pretreated with C_2mimOAc/fibers washed 6 times is shown in triangle; *Eucalyptus* residues were pretreated with C_2mimOAc at 150 °C, for 30 min, and a ratio of 1:3 (wt RL/wt IL).
The fermentation of 9 g/L de carbohydrate was fermented for 48 h at 40 °C.

ethanol produced was reached, justifying the need of pretreatment to co-enzyme hydrolyze and ferment this type of biomass. In fact, it has been postulated that C_2mimOAc can disrupt the inter- and intra-molecular hydrogen bonding (Lynam et al., 2012; Hyvärinen et al., 2014). Fig. 2 shows the evolution of ethanol upon the SSF process. As can be seen, the maximum is reached within the first 24 h (interruption at 72 h). It was also observed that poor washing of the biomass after IL pretreatment severely retarded the subsequent ethanol production. Therefore, care should be taken at this step since cellulose saccharification appears to be severely inhibited by the presence of any residual IL.

For further characterization, the residues were analyzed to quantify the amount of remaining carbohydrates. It can be noticed (Table 7) that the amount of cellulosic glucose was greatly

Table 7
Composition of monomeric units in fresh and processed *Eucalyptus* globulus samples after fermentation. Monosaccharides content analyzed by acid methanolysis method.

Monosaccharide	Fresh sample [mg/g of fresh sample]	Processed sample [mg/g of processed sample][a]
Arabinose	4.14	4.55
Fructose	0.80	2.86
Galactose	13.30	23.23
Galacturonic acid	13.67	12.00
Glucuronic acid	2.54	3.89
4-O-Me-Glucuronic acid	13.15	12.15
Mannose	7.29	27.02
Rhamnose	5.34	2.55
Xylose	146.56	107.24
Glucose[d]	427.55	183.89
Non-cellulosic[b]	21.84	52.51
Cellulosic[c]	405.71	131.38

[a] Calculated by gram of remaining mass after fermentation.
[b] Glucose analyzed by acid hydrolysis method.
[c] Glucose corresponding to cellulose, calculated as difference between value obtained by acid hydrolysis and acid methanolysis methods.
[d] Total calculated as the total sum of identified monosaccharide and carbohydrate.

decreased during the process. It can be also observed that the processed biomass was enriched with arabinose, galactose, glucose and mannose (2.3 fold increase). Plausibly, the occurrence of arabinose, galactose, non-cellulosic glucose and mannose in trees originates from arabinogalactans and galactoglucomannans (Willför et al., 2002, 2008). Consequently, our ionic liquid pretreatment followed by enzymatic saccharification with cellulases could not solubilize, less depolymerize the hemicelluloses. However, a decrease was observed in the amount of xylose. Xylose in wood originates from arabinoglucuronoxylan being the principal carbohydrate of the main chain as well as from xyloglucan being the principal carbohydrate of the branch chain. Therefore, some degradation of them must have occurred. In our previous work reporting C_2mimCl pretreatment coupled to subsequent enzymatic saccharification, xylose was the second most common monosaccharide released (around 50 mg of xylose/g of fresh DS released from *Eucalyptus* biomass). Consequently, this confirms the existence of some hydrolytic activity of the xylanolytic enzymes towards xylans (Pezoa et al., 2010). Further, it might be that partial xylose fermenting occurred since xylose content was decreased. *S. cerevisiae* Ethanol Red® was able to ferment glucose (C6 sugars) with 70.0 wt-% of theoretical yield whereupon also xylose fermenting was observed (6.8 wt-% of the theoretical yield) in 4 h (Supplementary Fig. 1).

Pretreated *E. globulus* wood chips using autohydrolysis at 194 °C for 51 min using optimized conditions for enzymatic hydrolysis, 71.9 wt-% glucose yields was obtained after 72 h (Marzialetti et al., 2014). Also, used autohydrolysis at the temperature range of 210–230 °C as pretreatment for *E. globulus*. Subsequent SSF process delivered ethanol yields up to 91.0 wt-% of the theoretical value (Romaní et al., 2012).

Herein, for the first time bioethanol was produced from Lenga and *Eucalyptus* residues pretreated with ionic liquids. However, the IL C_2mimOAc has previously been successfully used to pretreat lignocellulosic biomass subsequently fermented to ethanol. Goshadrou et al. (2013) treated aspen wood with C_2mimOAc at 120 °C (1–5 h) and subsequently used the SHF process using commercial cellulases and *S. cerevisiae*. They claimed an improvement in the fermentation of aspen wood up to 82 wt-% of the theoretical yield in 24 h (Goshadrou et al., 2013). The differences in the yield obtained by us (38.0 wt-% theoretical yield) could be at least partially explained by the fact that in the above study intensively milled (particle size 177–840 μm) raw material was used whereas in our case relatively large (1–7 mm) chips were used. Naturally, also the wood species was different. Shafiei et al. pretreated Native spruce wood (*Picea abies*) in C_2mimOAc (both chips and powder) at 120 °C in 1 to 15 h. The subsequent SHF process using commercial cellulases and *S. cerevisiae* gave 23.5 wt-% at 120 °C in 1 h and 66.8 wt-% of the theoretical ethanol yield when the pretreatment time was prolonged to 15 h. As expected, when wood powder was used, 72.1 and 81.5 wt-% of the theoretical ethanol yield was achieved for pretreatments times of 1 and 15 h, respectively (Shafiei et al., 2013). The results obtained were analogous to the ones obtained in this study for SSF of *Eucalyptus* pretreated with C_2minOAc at 150 °C in 15 min only.

4. Conclusions

The use of C_2mimCl and C_2mimOAc as "structure-disruptive" solvents upon pretreatment of Lenga and *Eucalyptus* residues was demonstrated. The saccharification of Lenga residues pretreated with C_2minCl at 150 °C for 15 min and at a biomass-to-IL ratio of 1:3 produced 446 mg of reducing sugars/g DS and 295 mg of glucose/g DS under the optimal conditions. It was evident that significant amounts of reducing sugars and glucose (corresponding to almost 69.3 wt-% and 67.9 wt-%, respectively) were released in the two-step process. In case of IL-pretreated Lenga, when the SSF strategy was applied and 0.173 g of ethanol/g of glucose was obtained (33.9 wt-% of the theoretical maximum). This value was 1.3 fold higher than in case of the SHF strategy. In case of C_2mimOAc pretreated *Eucalyptus* residues, at 150 °C for 30 min and using a biomass-to-IL loading of 1:3 (wt:wt), 352 mg of glucose/g DS was obtained (glucose yield of 82.2 wt-%). Interestingly, prolonging the pretreatment time to 60 min resulted in lower carbohydrate yields. After SSF, 0.194 g of ethanol/g of glucose was produced (38.0 wt-% of the theoretical maximum). The analysis of the processed biomass after fermentation demonstrated cellulose hydrolysis during the process as well as partial xylan hydrolysis. Hemicelluloses such as arabinogalactans and galactoglucomannans were enriched in the remaining solids, thus confirming that they were not substantially hydrolyzed either during the pretreatment or the saccharification step. Consequently, the combination of pretreatment with an appropriate ionic liquid followed by the SSF process is a potential process for bioethanol production from Lenga and *Eucalyptus* residues. Still, improvements are possible by further optimization of the operational conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2015.11.039.

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