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USE OF IONIC LIQUIDS IN THE PRETREATMENT OF FOREST AND AGRICULTURAL RESIDUES FOR THE PRODUCTION OF BIOETHANOL

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Pretreatment of lignocellulosic materials is an important step to achieve higher amounts of simple sugars, mono- and disaccharides, for obtaining ethanol as a biofuel, *via* enzymatic hydrolysis. The study introduces a concept that utilizes ionic liquids (ILs) as solvents in the pretreatment step, before enzymatic saccharification, for both forest residues (Eucalyptus *(Eucalyptus globulus* Labill.) and Lenga (*Nothofagus pumilio* (POEPP. EX. ENDL.) KRASSER) and for agricultural residues (wheat and corn). The procedure was evaluated at four different temperatures (80, 121, 150 and 170 °C) for 30 and 60 min, respectively, with 1-ethyl-3-methylimidazolium chloride ([EMIM$^+$][Cl$^-$]). Subsequent enzymatic hydrolysis of these materials was carried out at 47 °C, for 72 h, with commercial cellulases. The results demonstrated that the best experimental conditions found for wheat, corn and Eucalyptus residues were the following: 150 °C, for 60 min, yielding a total of 46, 48 and 30% sugars, respectively; in the case of Lenga residues, the optimum conditions were: 150 °C for 30 min, yielding a total of 40% sugars after saccharification. Finally, an analysis of the solid material after ionic liquid pretreatment is required, to determine the changes related to lignin, cellulose and hemicellulose composition.

Keywords: bioethanol, pretreatment, ionic liquids, forest and agricultural residues

INTRODUCTION

Efficient conversion of lignocellulosic materials into fuel ethanol or other energy fractions has become a priority for producing reasonably priced and renewable energy.[1] The pretreatment of lignocelluloses is known as a key technology, enabling a fast enzymatic hydrolysis of cellulose. The underlying reason is the increased surface area accessible to water and cellulases – a transformation expected to improve hydrolysis kinetics and conversion of cellulose into glucose.[2] Different pretreatment methods – biological, physical, chemical and physico-chemical – are available. Unfortunately, each of these methods has some disadvantages, such as:

(a) the biological processing methods require a long residence time; (b) the physical treatments are energy-demanding, expensive and do not remove the lignin; (c) the chemical methods are costly and mainly suitable for high-value paper products; (d) the physico-chemical pretreatment procedures are considered as very promising, but require high pressures/temperatures and the use of catalysts.

Some time ago, it was discovered that novel, non-volatile solvents called ionic liquids (ILs) are able to dissolve significant amounts of cellulose. Preliminary investigations suggest that celluloses regenerated from IL solutions are subjected to faster saccharification than the untreated substrates.[3,4] These encouraging results indicate the possible utilization of ILs as alternative and non-volatile solvents in cellulose pretreatment processes.[1,5]

Ionic Liquids (ILs)

Ionic liquids (ILs) are organic salts that usually melt below 100 ºC, some of them even at significantly lower temperatures. Thus, frequently, they are also called Room Temperature Ionic Liquids (RTILs). ILs have potential applications as "green solvents", due to their high thermal stability and nearly complete non-volatility under normal conditions. In chemical processes, ILs exhibit excellent physical characteristics, including the ability to dissolve polar and non-polar organic, inorganic, as well as polymeric compounds.[6] Moreover, there exists an almost limitless number of combinations containing anions and cations that can be used to synthesize various ILs.[7] Many ionic liquids have even been developed for specific synthetic problems. For this reason, they have been termed "designer solvents".

Ionic liquids as pretreatment of lignocellulosic materials

ILs have many attractive properties, such as chemical and thermal stability, non-flammability and very low vapor pressure. In contrast to the traditional volatile organic compounds, they have been called "green" solvents, being used in numerous interesting applications. Several studies[1,3,5] have shown that cellulose can be dissolved in a number of hydrophilic ionic liquids, for example, 1-butyl-3-methylimidazolium chloride $[BMIM^+][Cl^-]$ and 1-allyl-3-methylimidazolium chloride $[AIM^+][Cl^-]$. After dissolution, cellulose can be regenerated, *e.g. via* addition of anti-solvents (like acetone or water). However, complete dissolution of the lignocellulosic material (particularly wood) in IL is more difficult and even partial dissolution requires a very long incubation time of the biomass in IL and elevated temperatures. Even then, a high cellulose yield is generally not achieved after regeneration.

An interesting alternative approach comprises a pretreatment permitting the development of the subsequent enzymatic hydrolysis in a relatively short period of time, simultaneously allowing near quantitative yields of glucose and high yields of pentose sugars. Any ionic liquid capable of disrupting the hydrogen bonding structure to reduce cellulose crystallinity in the biomass can be used in the pretreatment strategy. For example, the cation structures including large organic structures, such as imidazolium, pyrroldinium, pyridinium or phosphonium moieties, are obvious choices.[8]

Objective

The present work studied the use of ionic liquids as a pretreatment step for forest (Eucalyptus and Lenga) and agricultural (wheat and corn straws) residues for enhancing enzymatic hydrolysis, the yield of monomeric sugars, pentoses and hexoses. Consequently, especially hexoses can be readily fermented to ethanol.

EXPERIMENTAL

Materials

Lignocellulosic materials

Agricultural residues: wheat and corn residues were obtained from short-rotation assays conducted at the Faculty of Agricultural Sciences – University of Santiago, Chile, from crops harvested in December 2007 and March 2008, respectively. The particle size of the sieved material ranged between 2.3-8.0 mm, and the initial water content was of 10-15%.

Forest residues: residual Lenga (*Nothofagus pumilio* (POEPP. EX. ENDL.) KRASSER) with ages of about 40-60 years, and Eucalyptus

(*Eucalyptus globulus*) with ages of about 8-15 years were selected as representative woody lignocellulosic materials. Residual Lenga was cut into about 1-2 mm high, 1-3 mm wide and 5-7 mm long chips (pin-chip size). The chips had a bulk density of approximately 156 kg/m^3 and a moisture content of around 10-15%. Residual Eucalyptus was cut into about 2 mm high, 2-3 cm wide and 2-4 cm long chips (chip size).

Ionic liquid

A commercial ionic liquid, 1-ethyl-3-methylimidazolium-chloride [EMIM$^+$][Cl$^-$], supplied by Merck KGaA, Germany, was used as received.

Enzymes

A commercial cellulase enzyme complex (Novo Celluclast 1.5 L Sigma C2730 included endo-β-glucanase, exo-β-glucanase and β-glucosidase), supplied with additional β-glucosidase (Novozyme 188, Sigma C6105), was used for saccharification.

Methods

Dissolution with IL and regeneration of lignocellulosic materials

The ionic liquid and various lignocellulosic materials, in a ratio of 5:100 (wt), were introduced into 10 mL test tubes, placed in a thermostated oil bath subjected to different temperatures (80, 121, 150 and 170 °C) and reaction times (30 and 60 min). After incubation, the tubes were removed from the oil bath, followed by an addition of 5 mL deionized water. After mixing by means of an agitator, the tubes were left to cool to room temperature.

Subsequently, the remaining solid material was filtered (on nylon filters), regenerated and washed with 50 mL distilled water, in a Büchner funnel, then freeze-dried. All experiments were carried out three times, for a higher accuracy of the experimental results.

Enzymatic saccharification

A commercial cellulase enzyme complex – Cellulase (Novo Celluclast Sigma C2730) and β-glucosidase (Novozyme, Sigma C6105) – including endo-β-glucanase, exo-β-glucanase and β-glucosidase, was used for saccharification.

A suspension of 100 mg raw material was prepared as a 5% w/v solution in a sodium acetate buffer (50 mM, pH 4.8) and incubated at 47 °C, under stirring (230 rpm). The enzymatic hydrolysis reaction was initiated when the enzyme was added to the solution: for each gram of dry material, 60 [FPU/g of cellulose] of cellulase (Celluclast 1.5 L) and 90 [CBU/g of cellulose] of β-glucosidase (Novozyme 188) were added in the case of agricultural residues, whereas 180 [FPU/g of cellulose] of cellulase and 270 [CBU/g of cellulose] of β-glucosidase were used in the case of forest residues. The reaction was allowed to proceed for 72 h and the samples were taken every 6, 24, 48 and 72 h, for determining the monomeric sugar contents by monosaccharide analysis without acid methanolysis, on a gas chromatograph (GC).

Monosaccharide analysis

The gas chromatography (GC) device used to analyze the monosaccharides was a Perkin-Elmer 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 employed as a carrier gas. 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.

Analysis of reducing sugars

The reducing sugars were quantified by dinitrosalicylic acid (DNSA) reduction.[9] An adapted methodology was carried out,[10] on using a 96 microwell format. 60 μL of the centrifuged, diluted sample was mixed with 120 μL of DNSA and heated at 100 °C for 10 min. Absorbance at 550 nm was registered with an Anthos model 2010 spectrophotometer. To convert the absorbance values to glucose concentrations, the measured values were interpolated using a glucose calibration curve, following a linear correlation between the concentration regimes 0.5-8.5 mM.

RESULTS AND DISCUSSION

Characterization of agricultural and forest residue samples

The materials used in this investigation – wheat residues – contained 35.7% cellulose and 40.2% hemicellulose, yielding a total carbohydrate content of 75.9% on dry lignocellulosic material basis and free extractives. The corn residues contained 41.2% cellulose and 41.5% hemicellulose, yielding a total carbohydrate content of 82.7% on dry lignocellulosic material basis, and the *Eucalyptus* residues contained 42.0% cellulose and 39.0% hemicellulose, yielding

a total carbohydrate content of 81% on dry lignocellulosic material basis. Finally, the Lenga residues contained 38.0% cellulose and 45.0% hemicellulose, corresponding to a total carbohydrate content of 83% on dry lignocellulosic material basis, respectively. A more comprehensive analysis of the samples' chemical composition is shown in Table 1.

Effect of temperature

Agricultural residues

First, the amount of sugars released in the absence of any pretreatment was evaluated, since the aim was to determine the efficiency of saccharification by means of an IL pretreatment, relative to an enzymatic treatment only. Without any IL pretreatment, it was observed that 142 and 156 mg sugars per g of dry solid (DS) were released from wheat and corn residues, respectively, corresponding to 18.5 and 19.0% of the total carbohydrates present in these lignocellulosic materials.

Figures 1 and 2 display the sugars obtained through enzymatic hydrolysis of wheat and corn residues after pretreatment with $[EMIM^+][Cl^-]$, at different temperatures, within 60 min. In general terms, Figures 1 and 2 show that both agricultural residues subjected to a short $[EMIM^+][Cl^-]$ pretreatment are more accessible to commercial cellulase than the untreated samples (s/p).

The best results obtained when applying an $[EMIM^+][Cl^-]$ ionic liquid pretreatment were obtained at a constant temperature of 150 °C for 60 min, 46-48% of the total carbohydrates present in wheat and corn residues being thus released. After the pretreatment at 150 °C, visible changes could be observed in the lignocellulosic material, both for wheat and corn residues. For example, it was evident that the fibers had been shortened and became thinner (Fig. 3). Moreover, a specific odor was detected in the liquid after the pretreatment.

Additionally, the amount of dissolved sugars released into the $[EMIM^+][Cl^-]$ solvent during the pretreatment at 150 °C for 60 min was analyzed for both materials, by reducing sugar assays. In both cases, the amount of dissolved sugars at $[EMIM^+][Cl^-]$ accounted for less than 10% of the original sugars available in agricultural residues (Table 2). Therefore, we consider that the short-term exposure of the samples to the IL, under the experimental conditions applied, was beneficial, mainly resulting in lignin sheathing and disruption, and not in major sugar dissolution.

Forest residues

In the case of forest residues, the amount of sugars released in the absence of any pretreatment amounted to 55 and 29 mg sugars per g of dry solid (DS), from Lenga and Eucalyptus residues, respectively. This value represents 6.8 and 3.5% of the total carbohydrates originally present in these lignocellulosic materials.

Figures 4 and 5 illustrate the sugars released after the enzymatic hydrolysis of Lenga and *Eucalyptus* residues, respectively. The samples were subjected to a pretreatment in an $[EMIM^+][Cl^-]$ solvent, at different temperatures, for 60 min. It is evident that both forest residues subjected to an $[EMIM^+][Cl^-]$ treatment are more accessible to commercial cellulases than the untreated samples (s/p).

Table 1
Composition of wheat and corn straws, Eucalyptus and Lenga residues on dry solids (DS)

Component	Dry solids (% w/w)			
	Wheat straw	Corn straw	Eucalyptus residues	Lenga residues
Cellulose	35.7	41.2	43.6	45.0
Hemicellulose	40.2	41.5	39.6	38.0
Total carbohydrate content	75.9	82.7	81.0	83.0
Lignin	14.7	13.5	19.0	17.0
Extractives	15.7	15.8	5.3	4.0
Ash	7.2	5.5	0.2	0.8

Table 2
Amount and yield of dissolved sugars in [EMIM$^+$][Cl$^-$] after pretreatment of wheat and corn straws

Pretreatment conditions			Amount of dissolved sugars, mg/g DS	Yield of dissolved sugars, %
Type of residue	Temperature, °C	Time, min		
Wheat	150 °C	60	76	10.0
Corn	150 °C	60	18	2.2

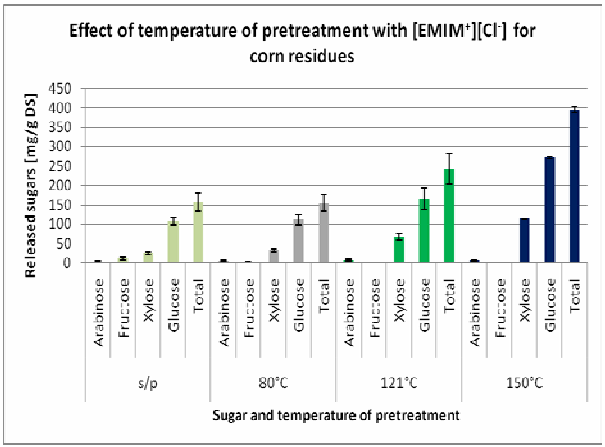

Figure 1: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of wheat straw. Pretreatment with [EMIM$^+$][Cl$^-$], at different temperatures for 60 min; s/p: without IL pretreatment

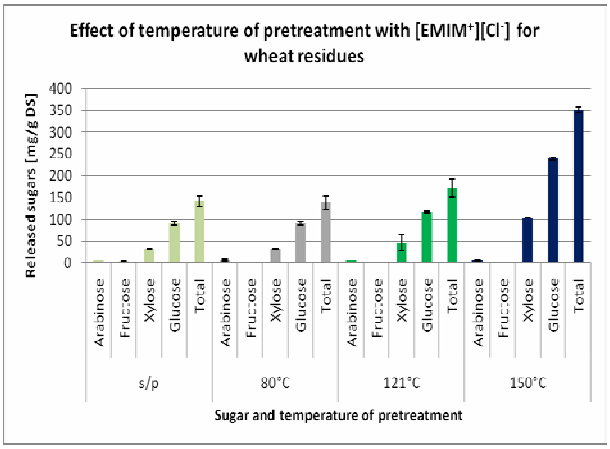

Figure 2: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of corn straw. Pretreatment with [EMIM$^+$][Cl$^-$], at different temperatures for 60 min; s/p: without pretreatment

Figure 3: Wheat residues a) without pretreatment, b) with pretreatment (150 °C for 60 min)

The results obtained show that the best experimental conditions found for Eucalyptus assume a temperature of 150 °C and a treatment time of 60 min, resulting in a 33% yield of total sugars. In the case of Lenga, a temperature of 121 or 150 °C and an incubation time of 60 min yield about 30% sugars in total, after saccharification.

In addition, Figure 6 illustrates that, in the case of pretreated Lenga, when the residues were subjected to a treatment at 170 °C for 30 min, the total amount of sugars obtained was lower than that obtained after the residues were treated at 150 °C for 30 min. A plausible reason could be that cellulose is rather efficiently dissolved in [EMIM$^+$][Cl$^-$] at 170 °C. The amount of dissolved sugars in [EMIM$^+$][Cl$^-$] after pretreatment correlated well with this hypothesis (Table 3). Indeed, the total dissolved sugars present in [EMIM$^+$][Cl$^-$] were analyzed after removal of the materials subject to pretreatments, by means of reducing sugar assays. In all cases, the dissolved sugars were found in less than

6% of the total sugars available in the forest residues (Table 3). Thus, mostly, only lignin sheathing and disruption took place, and no significant sugar dissolution was observed.

In contrast, in the case of *Eucalyptus* residues, the amount of dissolved sugars found in [EMIM$^+$][Cl$^-$] after the pretreatment at 170 °C for 30 and 60 min increased significantly, corresponding to about 15.2 and 22.7% total sugars available, respectively. These results confirm that cellulose dissolution in [EMIM$^+$][Cl$^-$] is rather efficient at 170 °C and, consequently, temperatures approaching 170 °C, as well as prolonged reaction times, are not beneficial if the goal is to preserve the sugars for subsequent enzymatic steps.

Table 3
Amount and yield of dissolved sugars present in [EMIM$^+$][Cl$^-$] after pretreatment of Lenga and Eucalyptus residues

Pretreatment conditions			Amount of dissolved sugars, mg/g DS	Yield of dissolved sugars, %
Type of residue	Temperature, °C	Time, min		
Lenga	150	30	16	1.9
Lenga	150	60	49	5.9
Eucalyptus	150	60	18	2.2
Eucalyptus	170	30	126	15.2
Eucalyptus	170	60	184	22.7

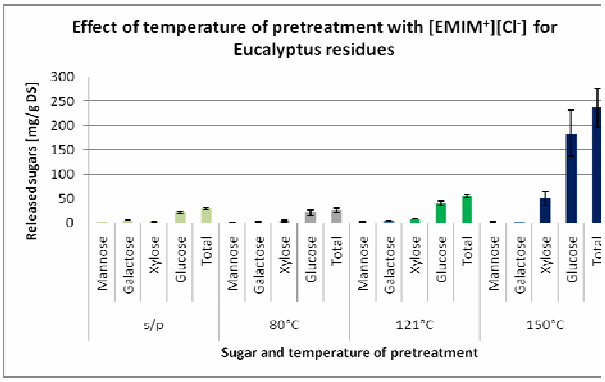

Figure 4: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of *Eucalyptus* residues. Pretreatment with [EMIM$^+$][Cl$^-$], at different temperatures for 60 min; s/p: without pretreatment

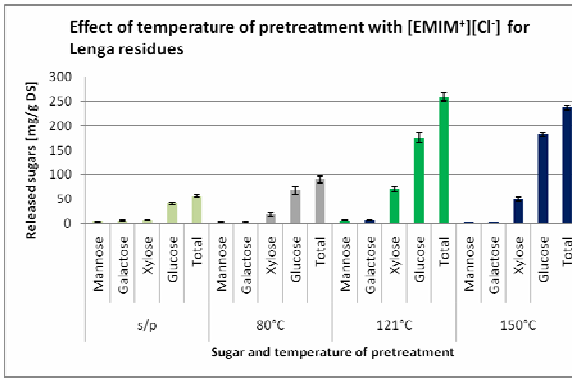

Figure 5: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of Lenga residues. Pretreatment with [EMIM$^+$][Cl$^-$], at different temperatures for 60 min; s/p: without pretreatment

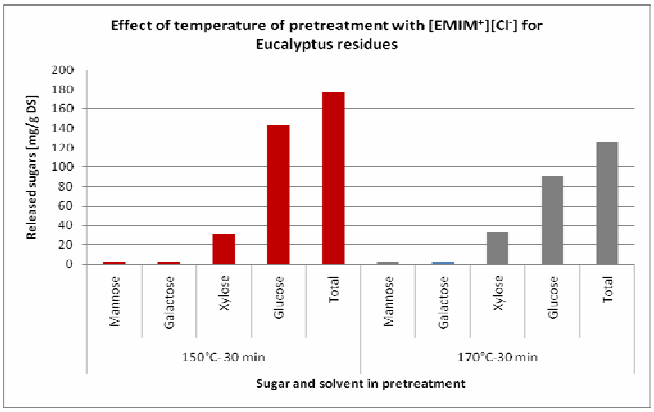

Figure 6: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of *Eucalyptus* residues. Pretreatment with [EMIM$^+$][Cl$^-$], at different temperatures for 30 min

Moreover, it was also observed that, during all pretreatment experiments, the main compounds released were glucose and xylose, together accounting for more than 90% of the total amount of monomers, as illustrated by Figures 1-6. In particular, glucose is the main monomer released, corresponding to almost 70% of the total amount of sugars released in the saccharification step.

Finally, in GC-MS analysis, no furfural was detected for any lignocellulosic materials, when the samples were treated under optimum conditions. Instead, traces of acetone and acetic acid could be detected (data not shown).

Effect of pretreatment time

The effect of IL pretreatment time was evaluated. Two different IL pretreatment time periods (30 and 60 min) were applied to all lignocellulosic materials (Figs. 7 and 8). It is evident, as expected, that prolonged exposure time and temperature lead to a higher sugar release.

Agricultural residues

In the case of agricultural residues, at all temperatures, an increase in the amount of released sugars could be observed at prolonged incubation time (Fig. 7). In particular, during the experiment at 150 °C with [EMIM$^+$][Cl$^-$], an increase in the sugars released in the saccharification step was observed. This corresponded to 100 mg of sugars/g DS for wheat residues, which represents an increase of 40%. Similar results were obtained with corn residues, for which the increase of sugars during enzymatic hydrolysis amounted to 84 mg sugars/g DS, corresponding to an increase of 27%, compared to the case when no pretreatment was applied. For both materials, the increase in glucose (30 and 60 min) is higher than the increase in xylose concentration.

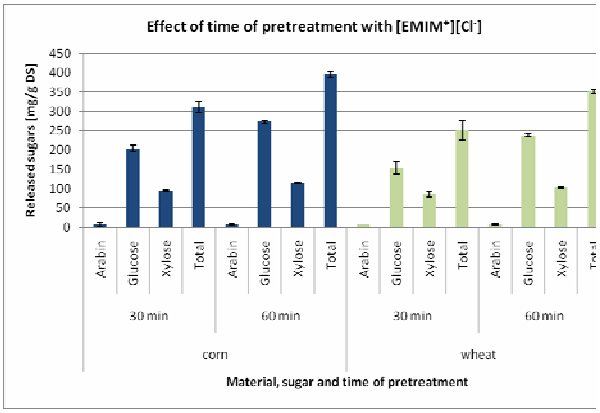

Figure 7: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of corn and wheat straws, pretreated with [EMIM$^+$][Cl$^-$], at 150 °C, for different periods of time

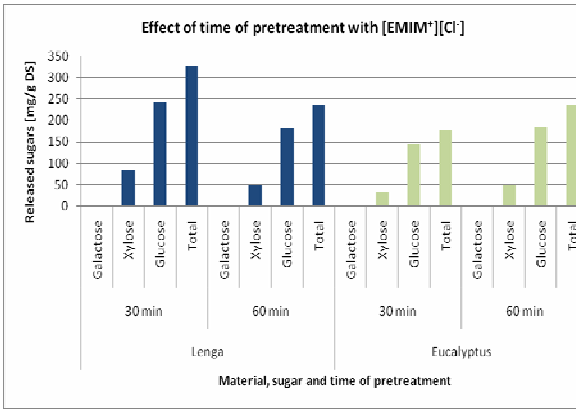

Figure 8: Monomeric sugars obtained after enzymatic hydrolysis (72 h at 47 ºC) of Lenga and *Eucalyptus* residues, pretreated with [EMIM$^+$][Cl$^-$], at 150 °C, for different periods of time

Forest residues

In the case of Eucalyptus, at all temperatures, an increase in the amount of released sugars could be observed at prolonged incubation time (Fig. 8). For instance, in the [EMIM$^+$][Cl$^-$] pretreatment procedure at 150 °C, an increase in the sugars released during the saccharification step corresponded to 59 mg of sugars/g DS for wheat residues, *i.e.*, to an increase of 33%. In contrast, in the case of Lenga, a decrease in the amount of released sugars obtained during saccharification could be observed, as the pretreatment was applied at 150 °C for prolonged incubation time periods. This corresponded to 94 mg of sugars/g DS for Lenga residues, demonstrating a 29% decrease in magnitude. A reason could be that the chip size of Lenga residues (pin-chip) is smaller than that of Eucalyptus residues (chip). Therefore, the cell structure in Lenga residues was more accessible to the IL solvent than in Eucalyptus residues. Therefore, during the next 30 min (after the initial 30 min), an accelerated sugar dissolution in [EMIM$^+$][Cl$^-$]

takes place, as shown in Table 3 (16 mg dissolved sugars/g DS after 30 min and 49 mg of dissolved sugars/g DS after 60 min).

CONCLUSIONS

The use of 1-ethyl-3-methylimidazolium chloride as a structure-disruptive solvent in the pretreatment of some agricultural and forest residues was demonstrated. It was evident that significant amounts of sugars, almost 41%, could be released in the two-step process (IL pretreatment and enzymatic hydrolysis).

The results demonstrate that the best experimental conditions established for wheat, corn and Eucalyptus residues assume a temperature of 150 °C for 60 min, a total sugar yield of 46, 48 and 30%, respectively, being thus attained. For Lenga residues, a temperature of 150 °C, applied for 30 min, resulting in a total sugar yield of 40% after saccharification, was the optimum.

Finally, as a next step, a complete analysis of the solid material after the ionic liquid pretreatment is required, to determine the changes related to lignin, cellulose and hemicellulose composition in detail.

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