ETHANOL PRODUCTION FROM SUGAR LIBERATED FROM PINUS SP. AND EUCALYPTUS SP. BIOMASS PRETREATED BY IONIC LIQUIDS

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Abstract - Pretreatment of lignocellulosic biomass using ionic liquids (ILs) has been widely studied and is considered one of the most promising methods to obtain fermentable sugars. However, few data exist on the fermentation of reducing sugars (RS) obtained by enzymatic hydrolysis of biomass pretreated with ionic liquids for the production of ethanol. Therefore, this study evaluated the production of ethanol from sugars liberated from sawdust of Pinus sp. and Eucalyptus sp. pretreated with ionic liquid [C₂ mim][OAc] and [C₄ mim][OAc], hydrolyzed with enzymes of Penicillium echinulatum employing Saccharomyces cerevisiae and Schizosaccharomyces pombe Y698 yeasts. The data indicate that when the biomass is pre-treated by [C₂ mim][OAc] there is higher production of ethanol that, when treated by [C₄ mim][OAc] for both evaluated yeasts, even though the pretreatment with [C₂ mim][OAc] caused the highest losses of cellulose and hemicellulose. Under the conditions analyzed, it is possible to produce approximately 40 and 47 L of ethanol per ton of Pinus sp. and Eucalyptus sp, respectively. In addition to contributing to knowledge about the physiology of the yeasts in the sugars liberated from biomass pretreated in presence of ionic liquid, this data is also relevant to the development of processes for the production of lignocellulosic ethanol.

Keywords: Ethanol; sawdust; fermentation; ionic liquid; enzymes.

INTRODUCTION

To develop alternative methods of energy generation, enzymatic hydrolysis of sawdust has become increasingly attractive due to the large quantities generated annually (Camassola & Dillon, 2009; Idrees et al., 2014). Moreover, it constitutes a possible source of energy and chemical supplies, and it contributes significantly to the mitigation of serious trinomial pollution problems of soil, water, and air (McKendry, 2002; Reis et al., 2015). In Brazil, it is estimated that 6.4×10⁶ hectares correspond to the area of planted forests, with 4.8×10⁶ ha of Eucalyptus sp. and Pinus sp. The annual productivity of Pinus sp. is about 35 m³ of wood per hectare and Eucalyptus sp. is 41 m³ of wood per hectare (BRACELPA, 2014; Camassola & Dillon, 2007). However, sawdust, originated from the operation of saws, can reach 12% of the total volume of raw material (Cassilha et al., 2004). In Brazil, 620,000 tons of sawdust are generated annually and most of this is burnt in the open, discarded in the environment, or removed to inappropriate landfills, causing damage to the environment, especially to streams, rivers, and springs (Cardoso, 2004).
The process of converting lignocellulosic biomass involves five main stages: selection of suitable biomass, effective pretreatment, production of enzymes such as cellulase and hemicellulase, efficient enzymatic hydrolysis, and fermentation of hexoses and pentoses (Menon & Rao, 2012; Padilha et al., 2015).

Various pretreatment methods have been explored to increase the accessibility of lignocellulosic substrates, such as physical pretreatment (milling and grinding, microwave, and extrusion), chemicals (alkali, acid, organic solvents, ozonolysis, and ionic liquid) physicochemical (steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation, and CO₂ explosion) and biological (Camassola & Dillon, 2016; Haghighi Mood et al., 2013). The fundamental step of pretreatment can reduce the time required for enzyme hydrolysis, increase income (Guilherme et al., 2015; Sant'Ana da Silva et al., 2011), and reduce production costs (Guilherme et al., 2015; Kuhad et al., 2011).

Pretreatment with ionic 1-ethyl-3-methylimidazolium acetate and 1-butyl-3-methylimidazolium is very effective in dissolving lignocellulosic materials, such as grasses (Barr et al., 2012), rice husk (Poornejad et al., 2013), cane sugar bagasse (Sant'Ana da Silva et al., 2011), maple (Lee et al., 2009), pine (Brandt et al., 2011) and eucalyptus (Uju et al., 2012). However, the removal of excess ionic liquid in the lignocellulosic biomass prior to the enzymatic hydrolysis is necessary in order to avoid a negative effect on the cellulase activity and the consequent reduction in final concentrations of total reducing sugars (Alvira et al., 2010; Aver et al., 2014). However, the main reason is the price of ionic liquids (ILs), even the commercial grades. Because of the high price of ILs, the processes are not economically feasible without recovery and recycling of more than 99% of the ILs. Currently, numerous studies are available that employ ILs to perform pretreatment of lignocellulosic biomass, but there is little information about the fermentation processes of the reducing sugars (RS) obtained by enzymatic hydrolysis of biomass pretreated with ionic liquids to obtain ethanol.

The most widely used microorganism for alcoholic fermentation is *Saccharomyces cerevisiae* (Alves Jr et al., 2014), due to its ability to hydrolyze sucrose into glucose and fructose, two easily assimilated hexoses. Other yeasts, such as *Schizosaccharomyces pombe*, have the additional advantage of tolerating high osmotic pressure (large amounts of salts) and high percentage of solids (Sánchez & Cardona, 2008). Therefore, the aim of this study was to evaluate the production of ethanol from sugars liberated from the sawdust of *Pinus* sp. and *Eucalyptus* sp. pre-treated with the ionic liquids [C₄mim][OAc] and [C₅mim][OAc], hydrolyzed with enzymes of *Penicillium echinulatum* (Schneider et al., 2016) using *S. cerevisiae* and *S. pombe* Y-698 yeasts for alcoholic fermentation.

### MATERIAL AND METHODS

#### Reagents

[C₄mim][OAc] and [C₅mim][OAc] were purchased from Sigma-Aldrich. All other chemicals were reagent grade or better.

#### Raw material

The *Eucalyptus* sp. and *Pinus* sp. sawdust were obtained from the Miotto sawmill in the city of Caxias do Sul, RS, Brazil. They had an average particle size of about 5 mm × 3 mm.

#### Enzymes and microorganisms

Cellulases and xylanases produced from *Penicillium echinulatum* S1M29 (Dillon et al., 2011) were used for enzymatic hydrolysis in solid-state cultivation, using 50% wheat bran and 50% sugarcane bagasse (Camassola & Dillon, 2010) The strain used belongs to the microorganism collection of the Enzyme and Biomass Laboratory at the Institute of Biotechnology, University of Caxias do Sul, Brazil. A commercial *S. cerevisiae* culture and *S. pombe* Y 698, kindly donated by the United States Department of Agriculture, were used in the fermentation process.

#### Pretreatment of biomass

[C₄mim][OAc] and [C₅mim][OAc] were separately added to glass tubes containing *Eucalyptus* sp. or *Pinus* sp. sawdust (1:4 w/w) and maintained at 120 °C for 24 h. After pretreatment, the lignocellulosic biomass was washed with distilled water (1:10) and then centrifuged at 800 g and 10 °C for 15 min. The supernatant was removed, and this washing step was repeated five times (Brandt et al., 2010). Weight loss was determined gravimetrically.

#### Enzymatic Hydrolysis and Fermentation

The enzymatic hydrolysis of pretreated *Eucalyptus* sp. or *Pinus* sp. sawdust was performed with an enzyme loading of 15 U Filter Paper Activity (cellulase) and...
108.5 U of b-glucosidases per gram of biomass. The amount of b-glucosidases used was the amount present in the volume of enzyme solution to obtain 15 FPU/g. P. echinulatum enzymes were used and these enzymes were not concentrated. Biomass (2%) and sodium citrate buffer 50 mmol.L⁻¹ (pH 4.8) to complete 50 mL were used. The hydrolysis was carried out at 50 °C for 24 h (Camassola et al., 2004) and 100 rpm. After hydrolysis, the broth not sterilized containing the sugars was subjected to fermentation at a concentration of 10⁶ cells/mL of S. cerevisiae or S. pombe, for 48 h at 28 °C. All experiments were performed in triplicate. Samples were collected at 0, 12, 24, and 48 h.

**Determination of Reducing Sugars (RS)**

RS present in the solutions from enzymatic hydrolysis were measured by the DNS method of Miller (1959). A standard curve of glucose to convert absorbance values to concentration of sugars was produced.

**High Performance Liquid Chromatography (HPLC)**

The dosage of sugars and ethanol by HPLC was performed on a Bio-Rad Aminex HPX- 87H column (Shimadzu) at 60 °C using H₂SO₄ (5 mmol.L⁻¹) as eluent at a flow rate of 0.6 mL min⁻¹ and a refractive index detector. Samples were pre-filtered in polyethersulfone, 0.20 micrometers.

**Characterisation of Biomass**

The characterisation of the chemical composition of Pinus sp. and Eucalyptus sp. was performed according to the methodology proposed by the National Renewable Energy Laboratory (NREL-TP-510-42618, NREL-TP-510-42619, NREL-TP-510-42621), with adaptations described by Menegol et al. (2014).

**RESULTS AND DISCUSSION**

**Pretreatment**

The weight loss after pretreatment with the ILs and subsequent washing, was 33±1.52% with [C₄mim][OAc] and 27±1.03% with [C₂mim][OAc] in the samples of Pinus sp. and 32 ±1.09% with [C₄mim][OAc] and 25±1.91% with [C₂mim][OAc] for samples of Eucalyptus sp. In a study by Li et al. (2013) with [C₄mim][OAc], a loss of mass of 37.2% for Pine and 40.1% for Eucalyptus sp. were verified. These higher losses verified by these authors in relation to those obtained in this work may be related to the higher concentrations of ILs and temperature used. According to Shafiei et al. (2013), about 85-97% of softwood powder with a size between 295 and 833 µm and 89-100% of the wood chips with size less than 2 cm can be recovered after pretreatment with the same ILs used in this work. Other pretreatments of wood chips, such as steam explosion and dilute acid, may have a weight loss ranging from 20-50% (Shafiei et al., 2013).

**Characterisation of Biomass**

In relation to the chemical composition (Figure 1) changes were verified in the composition, especially in the percentage of cellulose, hemicellulose and soluble lignin. For cellulose and hemicellulose, pretreatment with [C₄mim][OAc] had a more pronounced impact on Eucalyptus sp. than for Pinus sp. sawdust. As for soluble lignin, there was an increase for the two ILs and for the two evaluated biomasses, although the values of the increments were reduced. For insoluble lignin there was no difference between pretreatments and controls. Li et al. (2013) obtained greater removals of insoluble lignin than those obtained in this work, which is probably associated with pre-treatment using higher concentrations of ILs and higher temperatures.

**Enzymatic hydrolysis and fermentation**

Figures 1 and 2 show that liberation of RS after enzymatic hydrolysis caused a significant increase in biomass pretreated with ILs compared to non-pretreated biomass. This increased 59.61% for Eucalyptus sp. sawdust (Figure 3) and 60.67 % for Pinus sp. sawdust (Figure 2). At time zero the maximum values of RS obtained with Pinus sp. sawdust pretreated with [C₄mim][OAc] was 319.87 mg/g and 266.59 mg/g for [C₂mim][OAc]. For Eucalyptus sp. sawdust, 280.03 mg/g for [C₄mim][OAc] and 250.72 mg/g for [C₂mim][OAc] was obtained. Comparing with the chemical composition data (Figure 1), it was verified that the sugar release was not proportional to the availability of cellulose and hemicellulose present in the biomasses, since there was a greater availability of polysaccharides in the Eucalyptus sp. but higher amount of RA was observed in the hydrolysed samples of Pinus sp. Yamashita et al. (2010) obtained 462 mg/g of RS from wood chips pretreated by steam explosion (25 atm) and IL [C₄mim][OAc], and a lower concentration of AR when the pretreatment used only the IL 1-butyl-3 methylimidazolium chloride, 69.7 mg/g, with both results obtained for enzymatic hydrolysis of 48 h.
Figure 1. Chemical composition of *Eucalyptus* sp. and *Pinus* sp. sawdust pretreated with [C$_2$mim][OAc] and [C$_4$mim][OAc] and the untreated sawdust (control).

Figure 2. Consumption of reducing sugars by *S. cerevisiae* (A) and *S. pombe* (B) during fermentation of monosaccharides released from *Pinus* sp. sawdust pretreated with ILs and without pretreatment (control).

An interesting result for *S. pombe* was an increase in the amount of reducing sugars 12 h after the start of the fermentation for both *Pinus* sp. and *Eucalyptus* sp. sawdust. *S. cerevisiae* was found to increase the amount of reducing sugars in the presence of [C$_2$mim][OAc] in *Eucalyptus* sp. These increases in the concentration of sugars in the beginning of the fermentation process are due to the remaining non-hydrolyzed biomass, and the presence of enzymes that possibly are derepressed after the initial consumption of sugars by yeasts.

Greater consumption of RS was found during fermentation within the first 24 h (Figures 2 and 3). After this period, the levels of RS were maintained. After 48 h of fermentation, the residual amount of RS for the biomass fermented with both *S. cerevisiae* and *S. pombe* remained steady. The data suggest that both tested yeasts consumed glucose and little metabolized xylose (Figure 4).

*S. cerevisiae* was more efficient in consuming reducing sugar for both sawdusts (*Pinus* sp. and *Eucalyptus* sp.) pretreated with ILs compared to *S. pombe*, showing the highest consumption of RS of 80% obtained with *S. cerevisiae* and the IL [C$_2$mim][OAc].

The *Pinus* sp. sawdust pretreated with [C$_2$mim][OAc] and [C$_4$mim][OAc], and fermented with *S. cerevisiae* yeast (Figure 4A) had similar glucose consumption, 98%. The consumption of xylose was 54% for the biomass pretreated with [C$_2$mim][OAc], and 51% in the pretreatment with [C$_4$mim][OAc]. The ethanol yield from glucose, however, was 37% for the pretreatment with [C$_2$mim][OAc], and about 31% for IL [C$_4$mim][OAc], demonstrating that, despite the use of glucose, the ethanol yield was similar, but IL [C$_2$mim][OAc] contributed to a better yield. Figure 4B shows that *S. pombe* had a glucose consumption of 92% for both the *Pinus* sp. sawdust pretreated with [C$_2$mim][OAc] and [C$_4$mim][OAc]; however, the xylose consumption was greater in the pretreatment with [C$_2$mim][OAc], about 51%, with respect to [C$_3$mim][OAc], about 48%. The ethanol yield considering only glucose was 33% for [C$_2$mim][OAc], and 31% for [C$_3$mim][OAc].
Ethanol production from sugar liberated from *Pinus* sp. and *Eucalyptus* sp. biomass pretreated by ionic liquids

Although yields were reduced, it would be possible to obtain about 47 L of ethanol per ton of *Pinus* sp. sawdust. However, it is still necessary to increase these yields to make the process economically viable to use *Pinus* sp. sawdust.

Figure 5 illustrates that *Eucalyptus* sp. sawdust pretreated with ILs [C₄mim][OAc] and [C₅mim][OAc] enabled the yeast to consume higher glucose content. The increased glucose consumption, about 98%, was observed for the yeast *S. cerevisiae*. The xylose consumption was greater when sawdust was pretreated with [C₄mim][OAc], consumption reaching 62% for *S. cerevisiae* and 59% for *S. pombe*.

The best ethanol yield, considering only the glucose consumption, was obtained with *S. cerevisiae*, with 35% for both ILs. The highest yield of ethanol, about 37%, considering only the glucose consumption, was obtained with the *Pinus* sp. sawdust pretreated with [C₅mim][OAc] and fermented with *S. cerevisiae* and 35% for the *Eucalyptus* sp. sawdust pretreated with both ILs.

The work of Shafiei et al. (2013) with woodchips and sawdust pretreated with [C₅mim][OAc] obtained yields of 66.8% and 81.5% ethanol, respectively. As for their pretreatment with [C₅mim][OAc], the yield was 51.8% for the woodchips and 81% for the sawdust. Although the pretreatment was done with the same ionic liquids and the fermentation was performed with yeast of the same species, the pretreatment time, hydrolysis, and fermentation are different, which may have contributed to the different yields. Yamashita et al. (2010) obtained 30.1 g/L of ethanol using pretreatment with steam explosion (45 atm) and 73.3 g/L for the pretreatment with organic solvents, using an initial substrate concentration of 200 g/L and fermentation time of 48 h with *S. cerevisiae*. Haykir et al. (2013), using cotton rods pretreated with [C₅mim][OAc], and alkali obtained ethanol yields of 22.9 mg/g and 19.8 mg/g, respectively, demonstrating a greater efficiency in the pretreatment with ILs when compared to alkali, although the ionic liquids used are also alkaline. With ethanol yields obtained for the *Eucalyptus* sp. sawdust, it would be possible to produce approximately 40 L of ethanol per ton of waste.

For sawdust of *Pinus* sp. and *Eucalyptus* sp., shown in Figure 6, the concentrations of arabinose and xylitol remained constant during fermentation, with the exception of *Eucalyptus* sp. sawdust pretreated with [C₅mim][OAc] (Figure 5D), where there was a reduction in the concentration of arabinose by *S. cerevisiae*. This suggests that, when the biomass is pretreated with [C₅mim][OAc], this interferes in the metabolism of this yeast, enabling the consumption of pentose arabinose.
Figure 5. Concentration of glucose, xylose, and ethanol determined by HPLC for Eucalyptus sp. sawdust biomass pretreated with \([\text{C}_2\text{mim}][\text{OAc}]\) (A) and \([\text{C}_4\text{mim}][\text{OAc}]\) (B) before (Start - 0 h) and after (48 h) fermentation with \(S.\ cerevisiae\) and \(S.\ pombe\). The concentration of monosaccharides and ethanol is represented in mg released per g of Eucalyptus sp. sawdust, considering the pretreatment losses.

Figure 6. Concentration of cellobiose, acetic acid, glycerol, xylitol, and arabinose for Pinus sp. (A and B) and Eucalyptus sp. (C and D) sawdust biomass pretreated with \([\text{C}_2\text{mim}][\text{OAc}]\) and \([\text{C}_4\text{mim}][\text{OAc}]\) before (Start - 0 h) and after (48 h) fermentation by \(S.\ cerevisiae\) and \(S.\ pombe\). The concentration of substances is represented in mg per g of Pinus sp. and Eucalyptus sp. sawdust considering the pretreatment losses.

In the samples where cellobiose was present, it was consumed by both \(S.\ cerevisiae\) and \(S.\ pombe\), but this consumption was possibly related to the presence of β-glucosidase in the medium. This caused the hydrolysis of the disaccharide to glucose. Cellobiose was not detected in the Eucalyptus samples pretreated with \([\text{C}_4\text{mim}][\text{OAc}]\), indicating that this disaccharide was converted to glucose during the hydrolysis step.

Comparing the yields of ethanol produced by the \(S.\ cerevisiae\) and \(S.\ pombe\) yeasts, although the differences in yields were small, \(S.\ cerevisiae\) had the highest yield.

Regarding the measured assessed biomass, the highest yields of ethanol were from Pinus sp., although this biomass did not contain the highest amount of carbohydrates. Pinus sp. contained 38% cellulose and 16.14% hemicellulose, while Eucalyptus sp. had 40.05% cellulose and 16.55% hemicellulose. In the quantity of lignin, the quantity of the two evaluated biomasses were very similar, 28.7% and 28% for Pinus sp. and Eucalyptus sp., respectively. These data indicate that the concentrations of lignin and carbohydrates are not the most relevant characteristics for the release of sugars from biomass pretreated by ionic liquids.

Figure 6 also shows that all the hydrolyzed samples have acetic acid emanating from the pretreatment process, but the yeasts evaluated consumed all of this acid content. Another aspect seen in Figure 6B and D is the production of glycerol, which was higher for \(S.\ pombe\) in both biomasses pretreated with \([\text{C}_4\text{mim}][\text{OAc}]\). This may have caused a lower ethanol yield, because the formation of glycerol reduces the
efficiency of fermentation as shown by Oura (1977) and Brumm & Hebeda (1988).

The fermentation inhibitors that are commonly formed in pretreatment processes that employ elevated temperatures, 5-hydroxymethyl-2-furfural (HMF) and furfural, were not detected in the samples of this study, but the samples are washed five times and probably the inhibitors were removed. These data are reinforced by data obtained by Shafiei et al. (2013) with woodchips and sawdust pretreated with the same ionic liquids. These authors also did not detect the formation of HMF and furfural.

Comparing the volumetric productivity (QP) data for ethanol, the highest values (0.24 g/L/h) were obtained for Pinus sp. pretreated with [C₃mim][OAc] and fermented by S. cerevisiae. For yield (Yp/s), the highest values were obtained for Pinus sp. pretreated with [C₃mim][OAc] and fermented by S. cerevisiae, when considering the conversion of glucose to ethanol, but considering the total biomass (sawdust), again the sample of Pinus sp. pretreated with [C₂mim][OAc] and fermented by S. cerevisiae was the most promising (Table 1).

**CONCLUSION**

The data obtained in this study indicate the possibility of producing ethanol from Eucalyptus sp. and Pinus sp. pretreated with ionic liquids and fermented by S. cerevisiae and S. pombe yeasts. However, there are different responses to ethanol production according to the ionic liquid used to pretreat the biomass and this production can be related to the modification in biomass, especially in chemical composition. Another possibility, the presence of residual ILs, would have presented greater interference with the metabolism of S. pombe than S. cerevisiae, and the biomass pretreated with [C₂mim][OAc] showed less interference in the production of ethanol by both yeasts evaluated.

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