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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 [C_4mim][OAc] and [C_2mim] [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 [C_2mim] [OAc] there is higher production of ethanol that, when treated by [C_4mim][OAc] for both evaluated yeasts, even though the pretreatment with [C_2mim] [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 6.4×10^6 hectares correspond to the area of planted forests, with 4.8×10^6 ha of *Eucalyptus* sp. and *Pinus* sp. The annual productivity of *Pinus* sp. is about 35 m^3 of wood per hectare and *Eucalyptus* sp. is 41 m^3 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).

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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 CO_2 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 [C_4mim][OAc] and [C_2mim][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

[C_2mim][OAc] and [C_4mim][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

[C_2mim][OAc] and [C_4mim][OAc] were separately added to glass tubes containing *Eucalyptus* sp. or *Pinus* sp. sawdust (1:4 w/w) and maintained at 120 ºC for 24 h. After pretreatment, the lignocellulosic biomass was washed with distilled water (1:10) and then centrifuged at 800 g and 10 ºC 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 ºC 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 10^6 cells/mL of *S. cerevisiae* or *S. pombe*, for 48 h at 28 ºC. 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 ºC using H_2SO_4 (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 [C_2mim] [OAc] and 27±1.03% with [C_4mim][OAc] in the samples of *Pinus* sp. and 32 ±1.09% with [C_2mim] [OAc] and 25±1.91% with [C_4mim][OAc] for samples of *Eucalyptus* sp. In a study by Li et al. (2013) with [C_2mim] [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 [C_2mim] [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 [C_2mim][OAc] was 319.87 mg/g and 266.59 mg/g for [C_4mim][OAc]. For *Eucalyptus* sp. sawdust, 280.03 mg/g for [C_2mim][OAc] and 250.72 mg/g for [C_4mim] [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 [C_4mim][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.

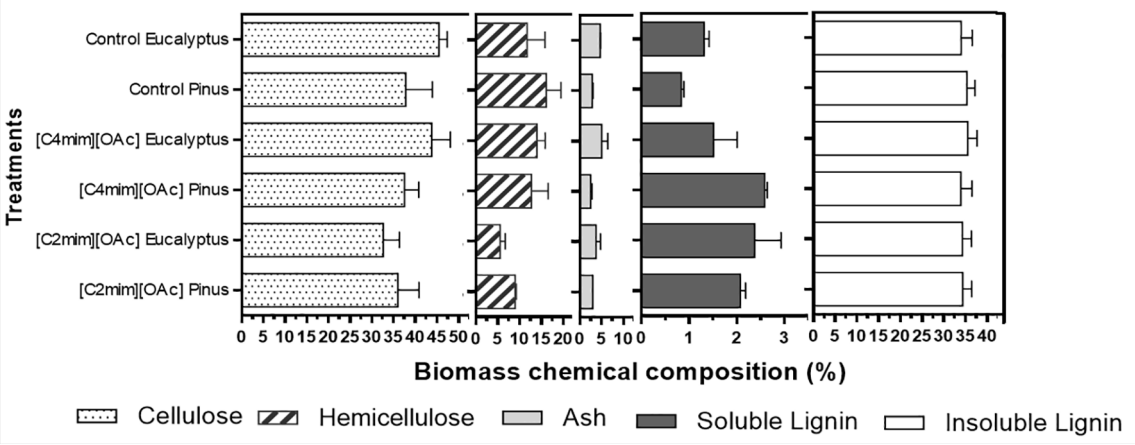

Figure 1. Chemical composition of *Eucalyptus* sp. and *Pinus* sp. sawdust pretreated with [C_2mim][OAc] and [C_4mim][OAc] and the untreated sawdust (control).

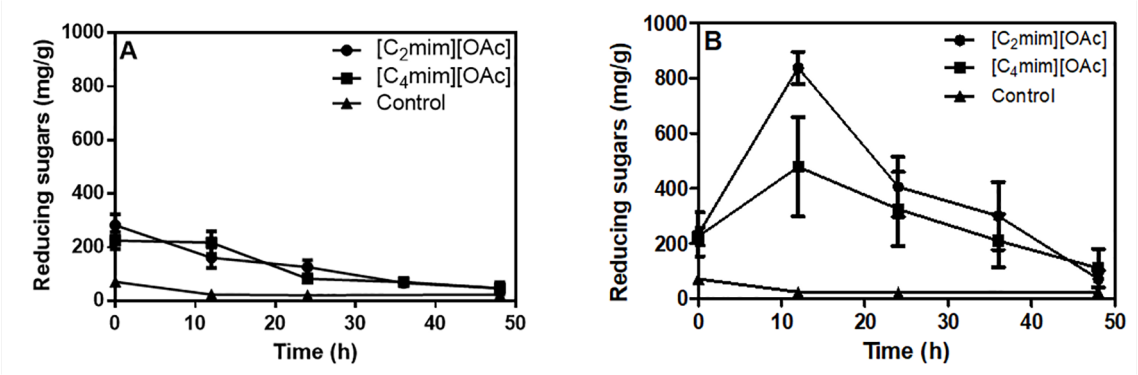

Figure 2. Consumption of reducing sugars by *S. cerevisiae* (A) and *S. pombe* (B) during fermentation of monosaccharides released from *Pinus* sp. sawdust pretreated with ILs and without pretreatment (control).

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_2mim][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_2mim][OAc].

The *Pinus* sp. sawdust pretreated with [C_2mim][OAc] and [C4mim][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_2mim][OAc], and 51% in the pretreatment with [C_4mim][OAc]. The ethanol yield from glucose, however, was 37% for the pretreatment with [C_2mim][OAc], and about 31% for IL [C_4mim][OAc], demonstrating that, despite the use of glucose, the ethanol yield was similar, but IL [C_2mim][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_2mim][OAc] and [C_4mim][OAc]; however, the xylose consumption was greater in the pretreatment with [C_4mim][OAc], about 51%, with respect to [C_2mim][OAc], about 48%. The ethanol yield considering only glucose was 33% for [C_2mim][OAc], and 31% for [C_4mim][OAc].

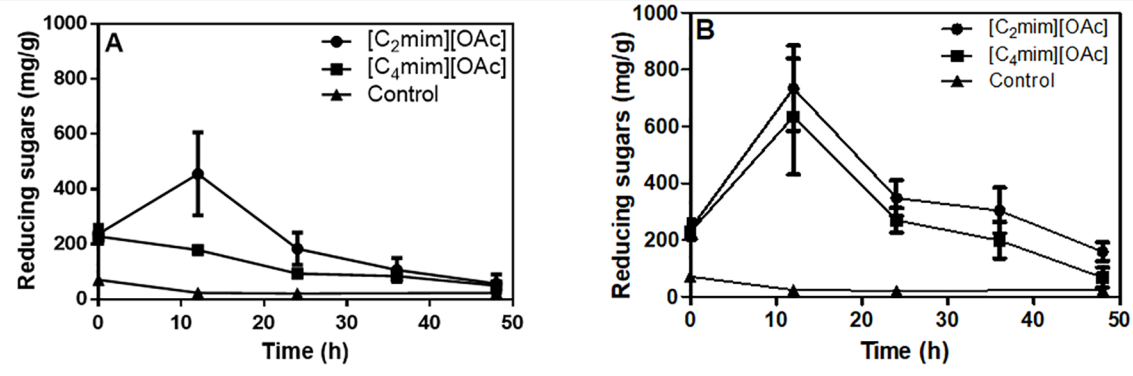

Figure 3. Consumption of reducing sugars by *S. cerevisiae* (A) and *S. pombe* (B) during fermentation of monosaccharides released from *Eucalyptus* sp. sawdust pretreated with ILs and without pretreatment (control).

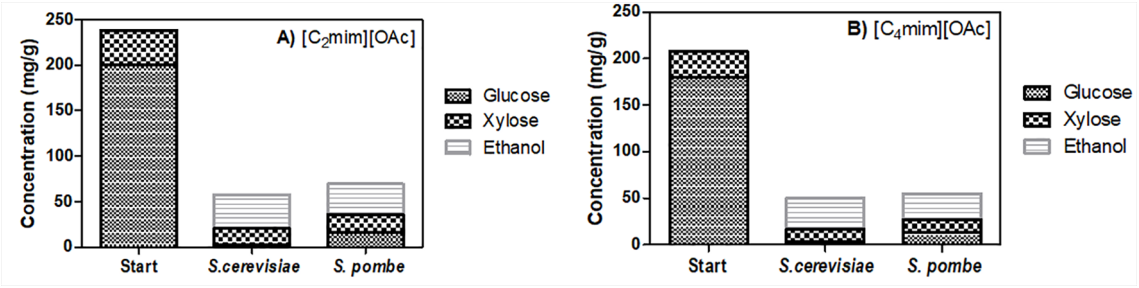

Figure 4. Concentration of glucose, xylose, and ethanol determined by HPLC for *Pinus* sp. sawdust pretreated with [C_2mim][OAc] (A) and [C_4mim][OAc] (B) before fermentation (Start - 0 h) and after (48 h) for *S. cerevisiae* and *S. pombe*. The concentration of monosaccharides and ethanol is represented in mg released per g of *Pinus* sp. sawdust, considering the pretreatment losses.

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 [C_2mim][OAc] and [C_4mim][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 [C_4mim][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 [C_2mim][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 [C_2mim][OAc] obtained yields of 66.8% and 81.5% ethanol, respectively. As for their pretreatment with [C_4mim][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 [C_2mim][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 [C_4mim][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 [C_4mim][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 [C_2mim][OAc] (A) and [C_4mim][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.

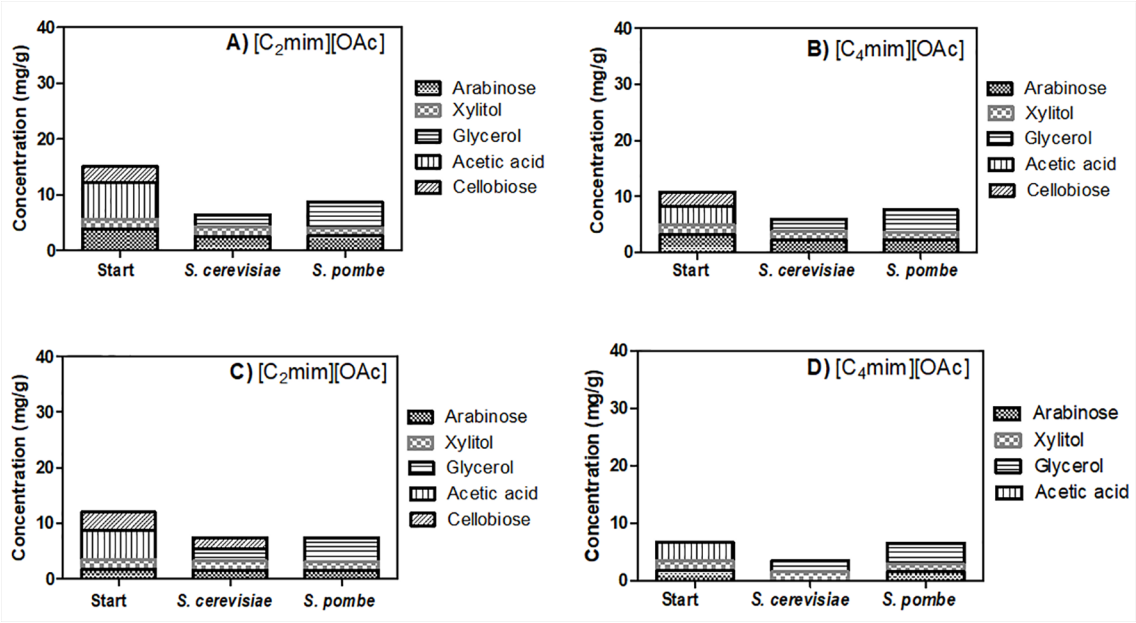

Figure 6. Concentration of cellobiose, acetic acid, glycerol, xylitol, and arabinose for *Pinus* sp. (A and B) and *Eucalyptus* sp. (C and D) sawdust biomass pretreated with [C_2mim][OAc] and [C_4mim][OAc] before (Start - 0 h) and after (48 h) fermentation by *S. cerevisiae* and *S. pombe*. The concentration 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 [C_4mim][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 [C_4mim][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 [C_2mim][OAc] and fermented by *S. cerevisiae*. For yield (Yp/s), the highest values were obtained for *Pinus* sp. pretreated with [C_4mim][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 [C_2min][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 [C_2mim][OAc] showed less interference in the production of ethanol by both yeasts evaluated.

ACKNOWLEDGEMENTS

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REFERENCES

Alves Jr, S.L., Thevelein, J.M., Stambuk, B.U., Expression of *Saccharomyces cerevisiae* B-glucoside transporters under different growth conditions. Brazilian Journal of Chemical Engineering, 31 1-8 (2014).

Alvira, P., Tomás-Pejó, E., Ballesteros, M., Negro, M.J., Pretreatment technologies for an efficient bioethanol production process based on enzymatic hydrolysis: A review. Bioresource Technology, 101 4851-4861 (2010).

Aver, K.R., Scortegagna, A.Z., Fontana, R.C., Camassola, M., Saccharification of ionic-liquid-pretreated sugar cane bagasse using *Penicillium echinulatum* enzymes. Journal of the Taiwan Institute of Chemical Engineers, 45 2060-2067 (2014).

Barr, C.J., Mertens, J.A., Schall, C.A., Critical cellulase and hemicellulase activities for hydrolysis of ionic liquid pretreated biomass. Bioresource Technology, 104 480-485 (2012).

BRACELPA. Associação Brasileira de Celulose e Papel - Panorama do setor. 62 1-5 (2014).

Brandt, A., Hallett, J.P., Leak, D.J., Murphy, R.J., Welton, T. The effect of the ionic liquid anion in the pretreatment of pine wood chips. Green Chemistry, 12 672-679 (2010).

Brandt, A., Ray, M.J., To, T.Q., Leak, D.J., Murphy, R.J., Welton, T. Ionic liquid pretreatment of lignocellulosic biomass with ionic liquid-water mixtures. Green Chemistry , 13 2489-2499 (2011).

Table 1. Ethanol yield from the amount of glucose released from the hydrolysis of *Pinus* sp. and *Eucalyptus* sp. sawdust pretreated with the ILs [C_2mim][OAc] and [C_4mim][OAc] in fermentations employing different yeasts.

		Ethanol yield (Yp/s)	
		Glucose g/g	Biomass (sawdust) g/g
[C_2mim][OAc]	*Pinus* sp. *S. cerevisiae*	0.18	0.04
	Pinus sp. *S. pombe*	0.16	0.03
[C_4mim][OAc]	*Pinus* sp. *S. cerevisiae*	0.19	0.03
	Pinus sp. *S. pombe*	0.16	0.03
[C_2mim][OAc]	*Eucalyptus* sp. *S. cerevisiae*	0.18	0.03
	Eucalyptus sp. *S. pombe*	0.16	0.03
[C_4mim][OAc]	*Eucalyptus* sp. *S. cerevisiae*	0.18	0.03
	Eucalyptus sp. *S. pombe*	0.17	0.03

Brumm, P.J., Hebeda, R.E. Glycerol production in industrial alcohol fermentations. Biotechnology Letters, 10 677-682 (1988).

Camassola, M., De Bittencourt, L.R., Shenem, N.T., Andreaus, J., Dillon, A.J.P. Characterization of the Cellulase Complex of *Penicillium echinulatum*. Biocatalysis and Biotransformation, 22 391-396 (2004).

Camassola, M., Dillon, A.J. Cellulases and xylanases production by *Penicillium echinulatum* grown on sugar cane bagasse in solid-state fermentation. Applied Biochemistry and Biotechnology, 162 1889-1900 (2010).

Camassola, M., Dillon, A.J. Production of cellulases and hemicellulases by *Penicillium echinulatum* grown on pretreated sugar cane bagasse and wheat bran in solid-state fermentation. Journal of Applied Microbiology, 103 2196-2204 (2007).

Camassola, M., Dillon, A.J.P. Biological pretreatment of sugar cane bagasse for the production of cellulases and xylanases by *Penicillium echinulatum*. Industrial Crops and Products, 29 642-647 (2009).

Camassola, M., Dillon, A.J.P. Steam-exploded sugar cane bagasse and wheat bran in solid-state cultivation to produce cellulases and xylanases from Penicillium echinulatum, Vol. accepted manuscript. Brazilian Journal of Chemical Engineering (2016).

Cardoso, A.L., Pirólise lenta de serragem de eucalipto para obtenção de bioóleo e carvão, Dissertação de mestrado em Química - Universidade Federal de Santa Maria, pp. 1-50 (2004)

Cassilha, A.C., Podlasek, C.L., Casagrande Junior, E.F., Silva, M.C., Mengatto, S.N.F. Indústria moveleira e resíduos sólidos: considerações para o equilíbrio ambiental, Revista Educação & Tecnologia, 8 1-21 (2004).

Dillon, A.J., Bettio, M., Pozzan, F.G., Andrighetti, T., Camassola, M. A new *Penicillium echinulatum* strain with faster cellulase secretion obtained using hydrogen peroxide mutagenesis and screening with 2-deoxyglucose. Journal of Applied Microbiology , 111 48-53 (2011).

Guilherme, A.A., Dantas, P.V.F., Santos, E.S., Fernandes, F.A.N., Macedo, G.R. Evaluation of composition, characterization and enzymatic hydrolysis of pretreated sugar cane bagasse. Brazilian Journal of Chemical Engineering, 32 23-33 (2015).

Haghighi Mood, S., Hossein Golfeshan, A., Tabatabaei, M., Salehi Jouzani, G., Najafi, G.H., Gholami, M., Ardjmand, M. Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renewable and Sustainable Energy Reviews, 27 77-93 (2013).

Haykir, N.I., Bahcegul, E., Bicak, N., Bakir, U. Pretreatment of cotton stalk with ionic liquids including 2-hydroxy ethyl ammonium formate to enhance biomass digestibility. Industrial Crops and Products , 41 430-436 (2013).

Idrees, M., Adnan, A., Bokhari, S.A., Qureshi, F.A. Production of fermentable sugars by combined chemo-enzymatic hydrolysis of cellulosic material for bioethanol production. Brazilian Journal of Chemical Engineering , 31 355-363 (2014).

Kuhad, R.C., Gupta, R., Khasa, Y.P., Singh, A., Zhang, Y.H.P. Bioethanol production from pentose sugars: Current status and future prospects. Renewable and Sustainable Energy Reviews , 15 4950-4962 (2011).

Lee, S.H., Doherty, T.V., Linhardt, R.J., Dordick, J.S. Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulose hydrolysis. Biotechnology and Bioengineering , 102 1368-1376 (2009).

Li, C., Sun, L., Simmons, B.A., Singh, S. Comparing the Recalcitrance of *Eucalyptus*, Pine, and Switchgrass Using Ionic Liquid and Dilute Acid Pretreatments. Bioenergy Research, 6 14-23 (2013).

McKendry, P. Energy production from biomass (part 1): overview of biomass. Bioresource Technology , 83 37-46 (2002).

Menegol, D., Scholl, A. L., Fontana, R. C., Dillon, A.J.P., Camassola, M. Potential of a *Penicillium echinulatum* enzymatic complex produced in either submerged or solid-state cultures for enzymatic hydrolysis of elephant grass. Fuel, 133 232-240 (2014).

Menon, V., Rao, M. Trends in bioconversion of lignocellulose: Biofuels, platform chemicals & biorefinery concept. Progress in Energy and Combustion Science, 38 522-550 (2012).

Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31 426-428 (1959).

Oura, E. Reaction products of yeast fermentations, Process Biochem, 12 19-21 (1977).

Padilha, I.Q.M., Carvalho, L.C.T., Dias, P.V.S., Grisi, T.C.S.L., Silva, F.L.H., Santos, S.F.M., Araújo, D.A.M. Production and characterization of thermophilic carboxymethyl cellulase synthesized by *Bacillus* sp. growing on sugarcane bagasse in

submerged fermentation. Brazilian Journal of Chemical Engineering , 32 35-42 (2015).

Poornejad, N., Karimi, K., Behzad, T. Improvement of saccharification and ethanol production from rice straw by NMMO and [BMIM][OAc] pretreatments. Industrial Crops and Products , 41 408-413 (2013).

Reis, L.d., Ritter, C.E.T., Fontana, R.C., Camassola, M., Dillon, A.J.P. Statistical optimization of mineral salt and urea concentration for cellulase and xylanase production by *Penicillium echinulatum* in submerged fermentation. Brazilian Journal of Chemical Engineering , 32 13-22 (2015).

Sant'Ana da Silva, A., Lee, S.H., Endo, T., Bon, E.P. Major improvement in the rate and yield of enzymatic saccharification of sugarcane bagasse via pretreatment with the ionic liquid 1-ethyl-3-methylimidazolium acetate ([Emim] [Ac]). Bioresource Technology , 102 10505-10509 (2011).

Schneider, W.D.H., Gonçalves, T.A., Uchima, C.A., Couger, M.B., Prade, R., Squina, F.M., Dillon, A.J.P., Camassola, M. Penicillium echinulatum secretome analysis reveals the fungi potential for degradation of lignocellulosic biomass. Biotechnology for Biofuels, 9 1-26 (2016)

Shafiei, M., Zilouei, H., Zamani, A., Taherzadeh, M.J., Karimi, K. Enhancement of ethanol production from spruce wood chips by ionic liquid pretreatment. Applied Energy, 102 163-169 (2013).

Sánchez, Ó.J., Cardona, C.A. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresource Technology , 99 5270-5295 (2008).

Uju, Shoda, Y., Nakamoto, A., Goto, M., Tokuhara, W., Noritake, Y., Katahira, S., Ishida, N., Nakashima, K., Ogino, C., Kamiya, N. Short time ionic liquids pretreatment on lignocellulosic biomass to enhance enzymatic saccharification. Bioresource Technology , 103 446-452 (2012).

Yamashita, Y., Sasaki, C., Nakamura, Y. Effective enzyme saccharification and ethanol production from Japanese cedar using various pretreatment methods. Journal of Bioscience and Bioengineering, 110 79-86 (2010).