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Pretreatment of cashew apple bagasse using protic ionic liquids: Enhanced enzymatic hydrolysis

Carla Luzia Borges Reis [a], Lorena Mara Alexandre e Silva [b], Tigressa Helena Soares Rodrigues [b], Anne Kamilly Nogueira Félix [a], Rílvia Saraiva de Santiago-Aguiar [a], Kirley Marques Canuto [b], Maria Valderez Ponte Rocha [a,*]

[a] Departamento de Engenharia Química, Universidade Federal do Ceará, Campus do Pici, Bloco 709, Fortaleza, CE, Brazil
[b] Embrapa Agroindústria Tropical, Rua Doutora Sara Mesquita, 2270 – Pici, CEP 60511-110 Fortaleza, CE, Brazil

HIGHLIGHTS

- Synthesis and characterization of the protic ionic liquid 2-HEAA.
- Different pretreatments using 2-HEAA was studied over cashew apple bagasse.
- 2-HEAA removed lignin from untreated cashew apple bagasse.
- IL-pretreated cashew apple bagasse exhibited higher cellulose crystallinity.
- Enzymatic hydrolysis from CAB pretreated with IL produce a high sugar yield.

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ABSTRACT

To enhance the enzymatic digestibility of cashew apple bagasse (CAB) feedstock in order to produce sugar fermentation-derived bioproducts, the CAB was subjected to three different pretreatments with the ionic liquid 2-hydroxyl-ethylammonium acetate (2-HEAA) and characterized by FTIR, NMR and chemical methods. All conditions were able to delignify CAB, however the best lignin removal (95.8%) was achieved through the method performed with 8.7% w/w of CAB/2-HEAA ratio at 130 °C for 24 h. Although the cellulose crystallinity has been increased in CAB treated with the ionic liquid, but this fact did not influence its digestibility. Nevertheless, the pretreatment with 2-HEAA enhanced significantly the cellulose digestibility, increasing the glucose yield from 48 to 747.72 $mg_{glucose}/g_{CAB}$. Furthermore, 2-HEAA pretreatment was efficient even with reused ionic liquid, obtaining high glucose concentration.

1. Introduction

Lignocellulosic materials represent a key feedstock for production of biofuels and other commodity chemicals. These materials are composed of cellulose, hemicellulose and lignin that form a complex and intricate structure (da Correia et al., 2013). In the valorization process of lignocellulosic biomass, cellulose and hemicellulose can be hydrolyzed to sugars monomers and subsequently converted into alcohols (i.e. ethanol, butanol) as well as xylitol, hydrogen or methane by fermentation processes in biochemical platforms (da Correia et al., 2013; Lopes et al., 2013; de Albuquerque et al., 2015), while lignin provides aromatic compounds for production of resins, for instance (Tan et al., 2009).

Accordingly, many agricultural wastes have been reported as potential sources of lignocellulosic material such as sugarcane bagasse (Jiang et al., 2013; Kimon et al., 2011), cotton stalks (Haykir and Bakir, 2013) and wheat straw (Lopes et al., 2013). Nevertheless, the cashew apple bagasse (CAB), a residue from the cashew apple processing, seems to be a promising alternative. The cashew apple is a pseudofruit with an outstanding economic role in the Northeastern region of Brazil due to the cashew nut exportation. The cashew apple industrial process for juice production yields 15% (w/w) of bagasse, representing one of the major waste from the Brazilian agribusiness (da Correia et al., 2013; de Wanderley et al., 2013; Rocha et al., 2014). Unfortunately, the cashew apple bagasse is highly recalcitrant to both microbial and enzymatic biotransformation, limiting its use and making its con-

* Corresponding author at: Chemical Engineering Department, Universidade Federal do Ceará, Campus do Pici, Bloco 709, Fortaleza, CE 60455-760, Brazil.
E-mail addresses: valderez.rocha@ufc.br, valponterocha@yahoo.com.br (M.V.P. Rocha).

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version into value-added products not economically feasible. Among many methods proposed, the pretreatments of the CAB have been performed by use of diluted sulfuric acid (Rocha et al., 2014, 2011), sodium hydroxide (Rodrigues et al., 2011), alkaline hydrogen peroxide (da Correia et al., 2013; Correia et al., 2015). However, these methods present low yield and high cost (Rocha et al., 2009). Therefore, more efficient pretreatment technologies have been sought, among them the application of ionic liquids.

The development of more efficient pretreatment technologies is a promising way to overcome current challenges on biomass supply. Therefore, ionic liquids (ILs) have evolved as a new type of non-aqueous solvents for biocatalysis, mainly due to their unique and tunable physical properties (Sáez et al., 2013). Ionic liquids are asymmetrical organic salts composed by large organics cations and relatively small inorganic anions that are liquid at room temperature (mp <100 °C) (Santiago et al., 2010). ILs are considered environmentally friendly solvents, since they are non-inflammable, thermally and chemically stable, have extremely low vapor pressures and many of them can be recycled into the process (Santiago et al., 2010). The ILs can be divided into two broad categories, protic ionic liquids (PILs) and aprotic ionic liquids (AILs). The PILs are produced through proton transfer from a Bronsted acid to a Bronsted base, feature low cost and simplicity of synthesis, favoring different applications including industrial usage (Greaves et al., 2006; Brigouleix et al., 2010) An example of a PIL is 2-hydroxyl-ethylammonium acetate (2-HEAA), a heat stable salt so far understudied (Penttilä et al., 2014).

The focus of the pretreatment with ionic liquid has been the reduction of the biomass crystallinity and the extraction of lignin in order to increase its susceptibility to enzymatic hydrolysis (Li et al., 2010a; Lopes et al., 2013). Pretreatment with ILs demonstrated advantages over conventional methods, since it allows to apply different fractionation approaches after the biomass dissolution (Lan et al., 2011; Yang et al., 2013) which leads to the maximization on the biomass recovery and by simultaneously adding value to the fractionated components within the biorefinery concept (Lopes et al., 2013).

In this context, the aim of this study was to evaluate three pretreatment procedures of cashew apple bagasse using the protic ionic liquid, 2-hydroxyl-ethylammonium acetate (2-HEAA), in order to enhance its enzymatic digestibility. The pretreatment methods were also evaluated with respect to the changes on the composition and the crystalline structure of the CAB upon pretreatment.

2. Materials and methods

2.1. Lignocellulosic material

The cashew apple bagasse from the species *Anacardium occidentale* L. was kindly donated by Jandaia Juice Industry (Ceará, Brazil). The cashew apple bagasse was washed three times with water and dried at 60 °C for 24 h. Afterwards, it was milled in a hammer mill, yielding a yellowish solid with average particle size of 20–80 mesh (0.25–0.84 mm), which was named CAB and stored at 26 °C.

2.2. Chemicals and enzyme

Glucose, xylose, arabinose, acetic acid, formic acid, furfural, monoethanolamine and hydroxymethyl-furfural (HMF), dimethyl sulphoxide and KBr were purchased from Sigma–Aldrich (São Paulo, Brazil). Sulfuric acid and ethanol were purchased from Vetec (São Paulo, Brazil). The Cellulase enzyme (NS 22074, 154.77 FPU/mL) from *Trichoderma reesei* was a courtesy of Novozymes.

2.3. Synthesis and characterization of ionic liquid

The protic ionic liquid 2-hydroxyl-ethylammonium acetate was synthesized by an acid-base reaction using acetic acid and monoethanolamine (Alvarez et al., 2011). Briefly, the monoethanolamine was placed in a triple necked flask equipped with a reflux condenser. An equimolar amount of acetic acid was added dropwise into the flask and the reaction mixture was stirred vigorously and kept at 35 °C for 24 h. Then, the reaction mixture was cooled in an ice bath and the ionic liquid was stored at room temperature (26 °C). The product was characterized by nuclear magnetic resonance (NMR) and physical methods.

The ^{1}H and ^{13}C NMR spectra were recorded on an Agilent DD2 600 MHz spectrometer, equipped with an inverse detection One Probe. To assess the formation of the ionic liquid, two samples were analyzed: (I) a 1:1 physical mixture of acetic acid and monoethanolamine and (II) the ionic liquid 2-hydroxyl-ethylammonium acetate. Samples were dissolved in 600 μL deuterated dimethyl sulphoxide (DMSO-d_6, Cambridge Isotope Laboratories) and analyzed in 5 mm tubes (Wilmad). The density and viscosity of 2-HEAA were measured at 25 °C using an electronic densimeter (Anton Paar/DAS-5000) and a digital viscometer (SVM 3000 – Anton Paar), respectively. The pH of the IL was obtained by a pH meter (Tecnal TEC-5, São Paulo, Brazil).

2.4. Pretreatment of cashew apple bagasse

Three methodologies of pretreatment of the CAB were evaluated using the protic ionic liquid 2-hydroxyl-ethylammonium acetate. All experiments were conducted in triplicate.

Method A: this method was based on the methodology described by Silva et al. (2011) with modifications. Five grams of CAB (5% w/w) were placed in 250 mL Erlenmeyer to react with 2-HEAA at 120 °C in an air circulation oven for 3 h. After the reaction, the mixture was cooled and 50 ml of distilled water were added under stirring to enable the precipitation. The solid fraction was separated by centrifugation at 6500*g* for 20 min and designated as CAB-ILa.

Method B: this method was carried out in an air circulation oven at 130 °C for 24 h using solid load of 8.7% w/w (CAB/2-HEAA). After this period, 10% v/w of ethanol was added at 25 °C under constant agitation. The solid fraction was separated by centrifugation at 6500*g* for 20 min and the solids obtained were designated as CAB-ILb.

Method C: this method was based on the methodology described by Perez-Pimienta et al. (2013). The experimental procedure was conducted at 121 °C for 3 h in autoclave using a solid load of 15.23% w/w (CAB/2-HEAA). Then, it was added 12% v/w of distilled water, and the solid fraction was separated by centrifugation at 6500*g* for 20 min. The obtained solid was designated as CAB-ILc.

For all methods, the pretreated biomass was washed until pH 7, dried at 50 °C for 24 h, milled and particles with diameter of 0.25 mm to 0.84 mm were selected.

2.5. Characterization of untreated and pretreated cashew apple bagasse

2.5.1. FTIR spectroscopy characterization

The effect of the pretreatment on the structure of cellulose, hemicellulose and lignin from untreated (CAB) and pretreated materials with ionic liquid (CAB-ILa; CAB-ILb and CAB-ILc) was evaluated by FTIR. Aliquots of 2.7 mg of these solids were mixed with 80 mg of KBr and grounded, until achieving a homogeneous powder. The samples were placed in a press with 8.5 tons for 15 min.

The spectra were recorded in a FTIR Varian 660 spectrometer. The FTIR spectra were acquired in the 4000–400 cm^{-1} region, with a total of 32 scans and a resolution of 4 cm^{-1}.

2.6. Solid nuclear magnetic resonance (NMR) analysis

Cross-polarization/magic angle spinning ^{13}C NMR technique (CP-MAS) was used to obtain structural information and crystallinity from the untreated and treated CAB. Analyses were accomplished on an Agilent DD2 600 MHz spectrometer (^{1}H channel), equipped with a 4 mm solid-state NMR probe and rotating at the magic angle (54.74°). Experiments were conducted in duplicate, using a 4 mm zirconia rotor filled with approximately 50 mg of sample.

The ^{13}C CP-MAS was performed with a contact time of 1 ms, which consisted of high-power pulse (64 spinal) with 6500 scans at a spectral window of 0.37 ppm, 4 s relaxation time between each acquisition and 1216 points. All experiments were performed with 14 kHz magic angle spinning and VNMRJ ™ 4.0 software was used for the acquisition and processing of data. Calibration of ^{13}C NMR spectra was carried out using adamantane as external reference standard.

The C4 carbon of cellulose was used as a probe for the calculation of the crystallinity index (CrI) of cellulose (Newman, 2004). Hence, crystallinity index (CrI) was calculated by dividing the area of the C4 crystalline peaks (92–96 ppm) by a total area of the C4 signals, that is the sum of the crystalline peaks (92–96 ppm) and the amorphous peaks (84–91 ppm), as shown in Eq. (1) (Liitiä et al., 2001).

$$CrI\ (\%) = \frac{A_{92-96\ ppm}}{A_{92-96\ ppm} + A_{84-91\ ppm}} \times 100 \quad (1)$$

2.7. Chemical characterization

The composition analysis and the determination of extractives in untreated and treated CAB (CAB-ILa, CAB-ILb and CAB-ILc) were determined following NREL analytical procedures (NREL/TP, 510-42619 Series) (Sluiter et al., 2008c). The determination of the total solids content was carried out in accordance with NREL procedure TP-510-42621 (Sluiter et al., 2008a), whereas the structural analyses of carbohydrates and lignin were performed in agreement with NREL procedure TP510-42618 (Sluiter et al., 2008b).

The concentrations of carbohydrates (glucose, xylose and arabinose), organics acids, furfural and hydroxymethyl-furfural (HMF) were obtained using a HPLC system (Water, Milford, MA, USA) equipped with a refractive index Waters 2414 detector. The column used was an Aminex HPX-87H (Bio-Rad, Hercules, CA, USA), with column oven at 65 °C and eluted with aqueous 5 mM H_2SO_4 solution at a flow rate of 0.5 mL/min, according to method reported by Rocha et al. (2014). The chromatogram peaks were identified and quantified on the basis of the retention times from authentic analytical standards and calibration curves respectively.

2.8. Enzymatic hydrolysis

Cellulose digestibility of the untreated (CAB) and pretreated bagasses (CAB-ILa, CAB-ILb and CAB-ILc) was evaluated by enzymatic hydrolysis, based on the TP-510-42629 standard NREL procedure (Selig et al., 2008) with some modifications. Briefly, the sample was placed in 50 mL flasks containing 40 μL of 10 mg/mL tetracycline (70% v/v ethanol), and with cellulase enzyme from *Trichoderma reesei* (ATCC) (Novozymes NS 22074, 154.77 FPU/mL) with activity of 30 $FPU/g_{cellulose}$. Tetracycline was added to prevent the growth of organisms during the saccharification. The samples were weighed in amount corresponding to a cellulose loading of 0.1 g/10 mL buffer, previously determined by NREL procedure TP510-42618. Sodium citrate buffer 0.1 mol/L (pH 4.8) was added to fill the total reaction volume of 10 mL. The hydrolysis was conducted in an orbital shaker (Tecnal – TE 422, São Paulo, Brazil) at 45 °C, for 96 h and 150 rpm. Samples were withdrawn and centrifuged at 10,000*g* for 15 min, and the supernatants were used for carbohydrates analysis by HPLC.

The cellulose digested was determined using Eqs. (2) and (3), where 0.9 is a correction factor due to water addition upon hydrolysis of the cellulose polymer, υ is the total volume of the assay, and C is concentration of glucose.

$$g_{cellulose\ \ digested} = C_{glucose} \times v_{(10\ mL)} \times 0.9 \quad (2)$$

$$\%digestion = \frac{g_{cellulose\ \ digested}}{g_{cellulose\ \ added}} \quad (3)$$

2.9. Ionic liquid recovery and reuse

The pretreatment that stood out was selected to evaluate the ionic liquid recovery and its reuse. Initially, the pH of the ionic liquid used in the first pretreatment was adjusted to pH 2.0 using 1 mol/L of sulfuric acid, and then cooled at 17 °C for 24 h to ensure the lignin precipitation (Rabelo et al., 2014). Later on, the lignin was separated by centrifugation at 6500*g* for 20 min and then washed three times with distilled water, dried in an oven at 60 °C for 24 h, and finally stored for future research.

For 2-HEAA recovery, the remaining filtrate was neutralized by the addition of NaOH pellets followed by the removal of volatile fractions through high vacuum. The resulting recovered ionic liquid recovered (ILr) was stored at 17 °C for further use. The ILr was reused in a new pretreatment process maintaining the same solid (CAB)/liquid (IL) ratio.

Three independent replicates of all experiments were performed. To determine the significance of any differences, the results were statistically evaluated at the 95% confidence level ($p < 0.05$) using Analysis of Variance (ANOVA) and Tukey's multiple comparison tests performed in the Microcal Origin 8.1 software (Microcal Software Inc., Northampton, MA, USA).

3. Results and discussion

3.1. Characterization of protic ionic liquid

The synthesized ionic liquid 2-hydroxyl-ethylammonium acetate (2-HEAA) was characterized. The pH of 2-HEAA was 9.71 with a density and viscosity of 1152.4 kg/m^3 and 2.696.9 mPa.s at 25 °C, respectively. The density of synthetized 2-HEAA was in agreement to the density values obtained by Alvarez et al. (2011) and Penttilä et al. (2014).

Table 1
Chemical shifts of the physical mixture of the components of protic ionic liquid 2-HEAA (monoethanolamine and acetic acid) and 2-hydroxyl-ethylammonium acetate protic ionic liquid (2-HEAA).

Number of $^{1}H/^{13}C$	δ $^{1}H/^{13}C$ (ppm) of physical mixture*	δ $^{1}H/^{13}C$ (ppm) of 2-HEAA *
1	*nc*/175.6	176.0
2	1.70/24.4	1.70/24.5
1′	3.53/58.6	3.53/58.5
2′	2.77/41.7	2.77/41.7
NH_2	5.12	6.28

* Values referenced to DMSO-d_6 in $^{1}H/^{13}C$ 2.50/39.51 ppm.

The liquid state NMR was also used to characterize the ionic liquid. The Table 1 shows the chemical shifts of ^{1}H and ^{13}C signals from the physical mixture of acetic acid and monoethanolamine (Figs. A.1 and A.2). There were no significant changes in chemical shifts of the methyl hydrogens (H-2) from acetic acid and the carbons 1′ and 2′ of monoethanolamine, when comparing the physical mixture with the ionic liquid. However, there was a considerable change in the chemical shift of the amine hydrogen from monoethanolamine (5.12 ppm to 6.28 ppm) besides a slight variation in the signal of carboxylic acid carbon (175.6 ppm to 176.0 ppm). This result indicates that the ionic liquid is indeed a product of acid-base neutralization reaction of acetic acid with monoethanolamine, which generated a salt, and not merely a physical equimolar mixture of the two reactants. In addition, the chemical shifts of the remaining hydrogen did not change due to the nature of the interaction (intermolecular) that is not strong enough to cause major changes in the chemical shift (Penttilä et al., 2014).

3.2. Compositional analysis of cashew apple bagasse

This work focused on the chemical characterization of pretreated CAB with the ionic liquid 2-HEAA by three different methods. The compositional analyses of CAB before and after pretreatments are shown in Table 2. The untreated CAB presented 18.16% ± 0.8% of cellulose, 12.83% ± 2.0% of hemicellulose, and 43.28% ± 4.2% lignin. Similar composition was reported earlier (Rodrigues et al., 2011; da Correia et al., 2013).

The percentage of recovered solids after CAB pretreatments was determined through mass balance. The results showed in Table 2 revealed that the highest biomass recovery was achieved using method C, corresponding to 61.28% w/w of the CAB initial mass. For methods A (CAB-ILa) and B (CAB-ILb), the recovery yields were lower (53.4% w/w and 41.11% w/w, respectively), due to the hydrolysis of hemicellulose chains and a more efficient removal of lignin. Therefore, the solids recovery was higher or equal to other pretreatments reported for CAB (da Correia et al., 2013; Rocha et al., 2014).

In addition, the percentage of extractable materials (Table 2) decreased when CAB was pretreated, because proteins and waxes were removed during the pretreatment using 2-HEAA. The cellulose content increased from 18.16% w/w (untreated CAB) to 40.29% w/w, 41.43% w/w, and 47.47% w/w, for the methods A, B, and C, respectively. Similar cellulose contents were found in cashew apple bagasse pretreated with sulfuric acid followed by alkali (Rodrigues et al., 2011) and by alkaline hydrogen peroxide (da Correia et al., 2013).

Lignin content from cashew apple bagasse decreased for all methods owing to the delignification promoted by IL. The CAB-ILa solid and CAB-ILc solids showed the highest lignin percentages of 23.02 ± 3.6% and 25.95 ± 0.7%, and delignification degree of 46.81% (CAB-ILa) and 40.04% (CAB-ILc), respectively. This possibly occurs due to the lower reaction time in unstirred system that did not provide effective action and interaction of the 2-HEAA with the CAB. Lignin extraction of up to 82% was achieved upon ionic liquid pretreatment conducted by method B (CAB-ILb). This finding indicates the effectiveness of this pretreatment for removal of lignin.

Perez-Pimienta et al. (2013) evaluated the pretreatment of agave bagasse (*Agave tequilana* Weber) and switchgrass (*Panicum virgatum*), in oven at 160 °C for 3 h using the ionic liquid 1-ethyl-3-methylimidazolium. Through this method it was achieved 16.6% and 8.2% of delignification. Similar delignification results were obtained by the methods A (CAB-ILa) and C (CAB-ILc) at lower temperature.

Furthermore, it was observed that the mass load did not influence the efficiency of the pretreatment since the method B (CAB-ILb), which had a higher ratio biomass/liquid (8.7% w/w load solid), showed less lignin content in the pretreated solid (7.57% ± 1.1). The lignin acts as a physical barrier by surrounds the cellulose fibers which restricts the access of enzyme to cellulose (Selig et al., 2008; Karagöz et al., 2012). Therefore, the removal of this component enables the activation of the bagasse for subsequent enzymatic hydrolysis.

3.3. FTIR analysis

The FTIR was used to characterize untreated and treated CAB. As the main vibrations from lignocellulosic materials are detected in the region of 1800–800 cm^{-1} (Lopes et al., 2013), this region was chosen for the analysis of all samples considered in this work.

The FTIR spectrum of untreated CAB exhibited vibrations at 1376, 1161, 1107, 1049 and 898 cm^{-1}, which were attributed to carbohydrates. The band at 1376 cm^{-1} was associated to the bending of C—H group in cellulose, a C—O asymmetric band was observed at 1161 cm^{-1} and the band at 898 cm^{-1} corresponded to the vibration of β-glycosidic C—H deformation from glycosidic bonds in carbohydrates (Fig. A.3).

The characteristic vibrations of crude CAB lignin were observed at 1508, 1458 and 1420 cm^{-1} associated to aromatic skeletal vibrations (Fig. A.3a). Bands at 1508 and 1458 cm^{-1} were assigned to C=C stretching vibration and C—H deformations (CH and CH_2) in phenol rings, respectively. The symmetric bending vibrations of C—H bonds in methoxyl groups of syringyl and guaiacyl units corresponded to the band at 1420 cm^{-1}. The strong absorption at 1251 cm^{-1} was originated from the C—O stretching of acetyl groups present in hemicellulose molecular chains.

Cellulose characteristic bands in the region of 1250–850 cm^{-1} were the major vibrations observed in the FTIR spectrum of CAB-ILa (Fig. B.3b). A peak at 1046 cm^{-1} was attributed to hemicellulose absorptions to C—O stretching in C—O—C linkages. Additionally, the absorbance of the lignin bands at 1508, 1458 and 1420 cm^{-1} showed a slight decrease, demonstrating lower lignin content in CAB-ILa than in the raw cashew apple bagasse.

CAB-ILb was essentially composed of carbohydrates due to the extensive lignin extraction in the process. The bands at 1437 cm^{-1} (CH_2 scissoring motion) were evidenced in the spectrum (Figs. A.3b–A.3d). The absorbance peak at 1066 cm^{-1} is associated to the C—O—C ether linkage of pentose and hexose units from hemicellulose and cellulose, while arabinosyl side chains

Table 2
Chemical composition of the untreated cashew apple bagasse and the solid obtained after pretreatment conducted for different methods using 2-HEAA ionic liquid. All values are represented as mean ± standard deviation of three replications.

Method	Conditions of pretreatment			Recovery solids (%)	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractable (%)	Ash (%)
	System	% Load solid (w/w)	Temperature and time (°C/h)						
Untreated	–	–	–	100.0 ± 0.0	18.16 ± 0.8	12.83 ± 2.0	43.28 ± 4.2	5.89 ± 0.3	3.32 ± 0.7
A	Oven	5	120 °C/3 h	53.40 ± 0.2	40.29 ± 0.2	19.59 ± 0.4	23.02 ± 3.6	6.85 ± 0.4	1.38 ± 0.4
B	Oven	8.7	130 °C/24 h	41.11 ± 1.5	41.43 ± 0.8	17.87 ± 0.2	7.57 ± 1.1	15.72 ± 0.0	0.37 ± 0.1
C	Autoclave	15.23	120 °C/3 h	61.28 ± 0.2	47.47 ± 0.2	18.69 ± 0.4	25.95 ± 0.7	5.67 ± 0.1	2.19 ± 0.4

were represented by the absorption peak at 996 cm^{-1} (Lopes et al., 2013). In the spectrum of CAB-ILb, the absorption bands of lignin at 1235 and 1262 cm^{-1} were negligible. Furthermore, the vibration band at 1734 cm^{-1}, which is commonly assigned to acetyl, feruloyl and p-coumaroyl groups esterified with hemicellulose, was absent in the cellulose-rich sample spectrum. In the three IL-treated samples, the bands characteristic of lignin at 1718 cm^{-1} (C=O stretching in unconjugated ketone, carbonyl and ester groups) (Yang et al., 2013) and 1598 cm^{-1} (contribution of the aromatic skeletal) disappeared after the pretreatment (Lopes et al., 2013).

3.4. NMR analysis and crystallinity of cashew apple bagasse

^{13}C NMR technique CP/MAS was used to investigate the structural changes of the main components (cellulose, hemicellulose and lignin) from cashew apple bagasse after pretreatment with 2-HEAA (Fig. A.4).

Initially, the resonance signals observed in the solid-state ^{13}C NMR spectrum from cashew apple bagasse were assigned with basis on the literature data. The region between 62.6 ppm and 68.9 ppm was associated with the C6 carbon of cellulose. This range is particularly relevant, since it indicates the degree of order in the cellulose structure (crystallinity). Furthermore, the C2, C3 and C5 carbon resonated in range from 70.9 ppm to 83.90 ppm, as expected. The C4 carbon, which is also an indicator of ordering of the cellulosic chains, was observed between 83.9 ppm to 91.6 ppm. Signals between 97.2 ppm and 117.8 ppm were attributed to the anomeric carbon (C1) of the cellulose (Atalla and Vanderhart, 1999; Komatsu and Kikuchi, 2013; Johnson and Schmidt-Rohr, 2014; Bernardinelli et al., 2015). Additionally, signals inherent to the lignin residues were located from 128.4 ppm to 166.8 ppm (Bernardinelli et al., 2015; Fu et al., 2015).

The CAB spectrum displayed clearly chemical shifts referring to lignin aromatic rings the region from 128.4 to 166.8 ppm (Fig. A.4a). However, after treatment with the ionic liquid, there was a reduction of these signals in all treated solid (Figs. A. 4b–d).

The ratio of lignin and cellulose was determined by the Eq. (4). This parameter was used to indicate the removal rate of lignin by different pretreatments. Therefore, the ratio was obtained by dividing the regions between 145.4 ppm and 164.9 ppm (A1) that arises from lignin, by the sum of lignin region and cellulose region (97.0–124.0 ppm) (A2) (Fu et al., 2015).

$$Ratio_{lignin/cellulose} = \frac{A_1}{A_1 + A_2} \quad (4)$$

The Table 3 shows the ratio of lignin/cellulose found in the untreated and pretreated cashew apple bagasses. The solid CAB-ILb showed low relative percentage of lignin/cellulose, possibly due to extraction or structure change of lignin caused by the interaction of 2-HEAA with CAB under the conditions provided in the pretreatment B (higher interaction time – 24 h). The CAB-ILa solid showed the highest lignin/cellulose relative percentage (7.22%), possibly due to the lower reaction time and to lack of stirring. The CP/MAS ^{13}C NMR analysis corroborated the results obtained by FTIR (Fig. 3 in ESI) and chemical analysis (Table 2), demonstrating that the CAB after pre-treatment with ionic liquid protic 2-HEAA had significant lignin removal in all analyzed methods.

The CP/MAS ^{13}C NMR was also used to verify the effects of pretreatments on the CAB crystallinity. The values of the crystallinity index (CrI) calculated by Eq. (1) are shown in Table 3. In general, the pretreatment of the CAB with the IL induced an increase in the crystallinity, which was caused by reduction of amorphous components. The highest crystallinity indices (see, Table 3) were found in CAB-ILb and CAB-ILc, 22.39% and 22.79%, respectively.

Nevertheless, in spite of the increase in the crystallinity, the digestibility of the biomass was not enhanced, as will be seen in the enzymatic hydrolysis assays (Section 3.5). Husson et al. (2011) reported a reduction in the crystallinity of a solid pretreated with 1-ethyl-3-methylimidazolium methylphosphonate and verified that the effect of the pretreatment on the cellulose crystallinity depends on the type of ionic liquid. In addition, results obtained with other lignocellulosic biomass demonstrated a progressive increase in the CrI as a function of the pretreatment (Bernardinelli et al., 2015). Although the influence of the CrI on the recalcitrance of biomass has been controversial for a long time, the most recent results point out that the efficiency of pretreatments is not directly correlated with the decrease of crystallinity (Husson et al., 2011).

Table 3
Percentage relative lignin-cellulose and crystallinity indexes (CrIs) obtained from untreated and treated cashew apple bagasse determined by deconvolution of ^{13}C CP-MAS NMR spectra.

Material	% Relative lignin-cellulose	CrI%
BC	9.03 ± 0.3	9.49 ± 1.6
BC-Lia	7.22 ± 1.3	17.46 ± 1.8
BC-LIb	3.87 ± 0.1	22.39 ± 0.6
BC-Llc	3.48 ± 1.3	22.79 ± 0.1

3.5. Enzymatic hydrolysis

The efficiency of the different procedures of pretreatment using 2-HEAA were evaluated by enzymatic hydrolysis. The digestibilities from untreated and pretreated CAB are shown in Fig. 1. It can be observed that glucose was released within 24 h for all investigated solids (CAB, CAB-ILa, CAB-ILb and CAB-ILc). In the beginning of the process, the enzymatic activity was slow down by lignocellulosic barrier. However, the glucose yield increased over hydrolysis due to the breaking of the β-1,4-glycosidic bonds from cellulose molecules which favored the access of the hydrolytic enzymes to substrate, enhancing the digestion of the bagasse (Zhang et al., 2012).

The enzymatic hydrolysis of the pretreated solids revealed that all studied methods were effective in improving the enzymatic convertibility of cellulose. The digestibilities of the CAB-ILa, CAB-

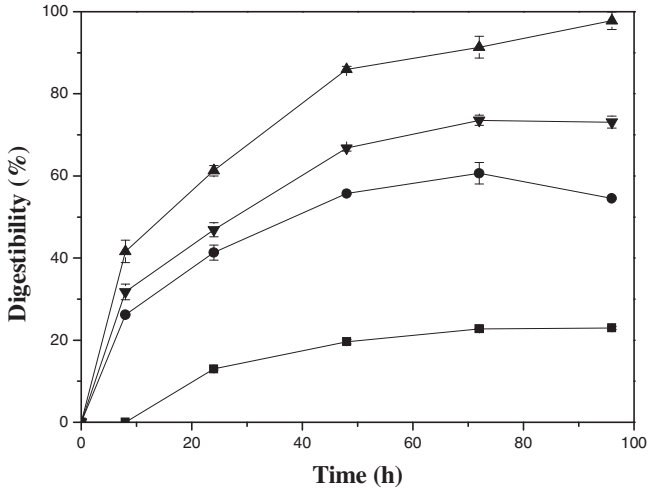

Fig. 1. Digestibility from untreated cashew apple bagasse and treated cashew apple bagasse by different pretreatments released by enzymatic saccharification at 45 °C, 150 rpm and pH 4.8 using the Celluclast 1.5L® enzyme. (■) CAB; (●) CAB-ILa; (▲) CAB-ILb and (▼) CAB-ILc. All values are represented as mean ± standard deviation of three replications.

ILb and CAB-ILc were 48.75%, 95.80% and 71.55% over 96 h of hydrolysis, respectively. As expected, the worst result was observed for the hydrolysis of untreated CAB and was attributed to the low accessibility of the cellulose within the crude lignocellulosic matrix. The native intricate structure of biomass as well as the presence of lignin, hemicellulose and other compounds are known to hinder the access of cellulase to the cellulosic substrate, leading to a poor hydrolysis performance (da Correia et al., 2013; Rocha et al., 2009; Rodrigues et al., 2015).

The glucose yield that took into account the recovered solids after pretreatment was expressed as $mg_{GLUCOSE}/g_{CAB\text{-}IL}$ and $mg_{GLUCOSE}/g_{CAB}$ and is shown in the Fig. 2a and b.

Therefore, it was obtained 48 $mg_{GLUCOSE}/g_{CAB}$ for untreated CAB; 270 $mg_{GLUCOSE}/g_{CAB\text{-}IL}$ (CAB-ILa); 747.72 $mg_{GLUCOSE}/g_{CAB\text{-}IL}$ (CAB-ILb) and 541.84 $mg_{GLUCOSE}/g_{CAB\text{-}IL}$ (CAB-ILc). The corresponding glucose yields (in mg per g of untreated CAB) were 143 $mg_{GLUCOSE}/g_{CAB}$ (CAB-ILa), 314 $mg_{GLUCOSE}/g_{CAB}$ (CAB-ILb) and 335 $mg_{GLUCOSE}/g_{CAB\text{-}IL}$ (CAB-ILc). The method B (CAB-ILb) demonstrated to be the most promising procedure for biomass pretreatment, as higher glucose yield and recovered solids were achieved.

In addition, the hydrolysis from CAB-ILb presented the highest hydrolysis rate with a 95.8% cellulose conversion over 96 h, whereas only 22.9% of the initial cellulose was hydrolyzed (Fig. 1). Indeed, CAB-ILb demonstrated the lowest lignin percentage (see Table 2) and higher crystallinity index. This finding endorses the hypothesis that the removal of lignin is a key factor for enhancing the efficiency of enzymatic hydrolysis by favoring the access of cellulase enzyme to cellulose (Selig et al., 2009; Karagöz et al., 2012). Furthermore, latest studies support the idea that the efficiency of pretreatment to reduce the recalcitrance is not clearly correlated with decreased crystallinity. Other parameters such as the relationship between lignin/cellulose, morphology, porosity, degree of polymerization, distribution of lignin and hemicellulose are more important (Hubbell and Ragauskas, 2010; Foston et al., 2011; Bali et al., 2015). Zhang et al. (2012) reached similar conclusions when treated corn straw in a twin screw extruder, and did not occur a significant reduction in crystallinity index to justify the increase in the yield obtained in the enzymatic hydrolysis (Zhang et al., 2012). The increase in surface area and the deconstruction of cell wall (observed by SEM) were considered responsible for increasing the enzymatic saccharification.

3.6. Recovery and reuse of IL

Considering the feasibility aspect, the possibility of reuse of the IL was investigated in this work through the efficiency of the enzymatic hydrolysis. For this, the recovery and reuse of the IL were examined using the method B (with 85% of IL, w/w), which presented the best results on the lignin removal and the greatest enzymatic digestibility.

The digestibility of the cellulose from CAB-ILb-R1 (first reuse of IL) and CAB-ILb-R2 (second reuse of IL) were 60.51 ± 7% and 66.11 ± 4.5%, respectively, at 72 h. The cashew apple bagasse pretreated with pure IL presented cellulose digestibility of 91.74 ± 3.7% at the same time.

The Fig. 3 shows the yield of glucose in terms of $mg_{glicose}/g_{CAB\text{-}IL}$ of the untreated CAB, CAB-ILb, CAB-ILb-R1 and CAB-ILb-R2. The CAB-ILb-R1 and CAB-ILb-R2 had glucose yields of 463.09 $mg_{glucose}/g_{CAB\text{-}IL}$ and 505.09 $mg_{glucose}/g_{CAB\text{-}IL}$, respectively, which were much higher than the value found in untreated CAB (24.09 $mg_{glucose}/g_{CAB}$) (see Fig. 3). According to statistical analysis by ANOVA, with 95% significance level, the glucose yields exhibited significant difference among the solid from the first cycle of IL (747.72 $mg_{GLUCOSE}/$

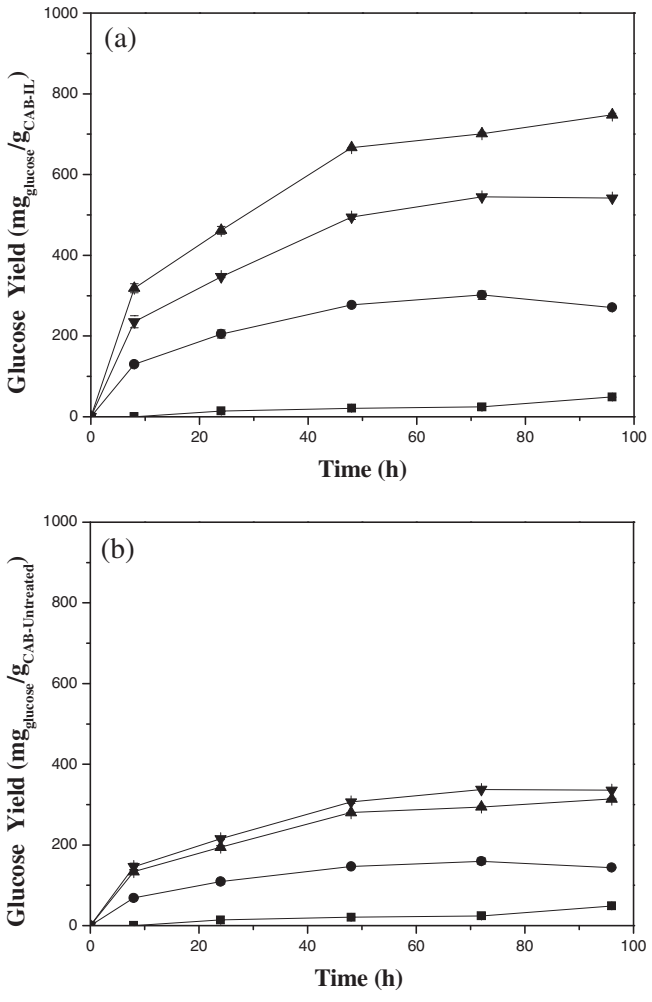

Fig. 2. Glucose yields from cashew apple bagasse with different pretreatments released by enzymatic saccharification at 45 °C, 150 rpm and pH 4.8 from pretreated CAB using the Celluclast 1.5L® enzyme. (■) CAB; (●) CAB-ILa; (▲) CAB-ILb and (▼) CAB-ILc. (a) Glucose yields $mg_{Glucose}/g_{CAB\text{-}IL}$; (b) Glucose yields $mg_{Glucose}/g_{CAB}$. All values are represented as mean ± standard deviation of three replications.

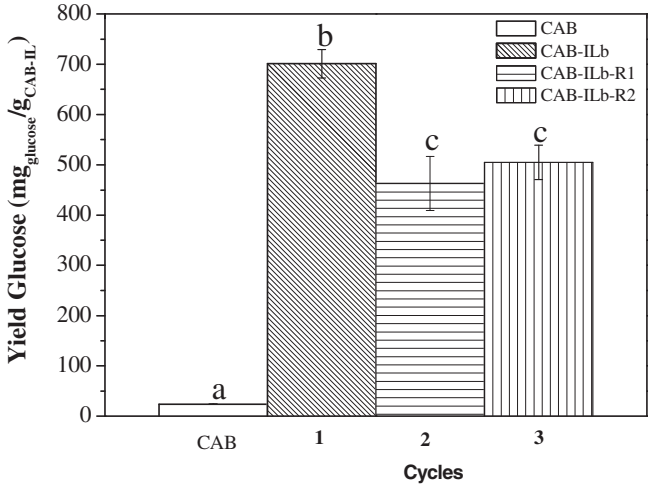

Fig. 3. Results of reuse of ionic liquid: Glucose yield of the enzymatic hydrolysis of untreated CAB, CAB-ILb, CAB-Ilb-R1 and CAB-ILb-R2 conducted at 45 °C, 150 rpm, for 72 h using the cellulase enzyme complex of *Trichoderma reesei* (30 FPU/$g_{cellulose}$). Values with different letters represent statistically significant differences ($p < 0.05$). All values are represented as mean ± standard deviation of three replications.

$g_{CAB\text{-}IL}$) and the solids that arises from CAB pretreated with reused IL. However, the glucose yields of CAB-ILb-R1 and CAB-ILb-R2 were not significantly different indicating that the pretreatment efficiency stabilized after the second IL reuse cycle.

Haykir et al. (2013) and Li et al. (2010b) revealed that the accumulation of components in the recovered and reused ionic liquid 1-ethyl-3-methylimidazolium acetate reduced the efficiency of the lignin removal and yield of glucose obtained in the enzymatic hydrolysis. This occurs because the lignin was not fully removed with the acidification process, which makes it a recalcitrant component. Hence, this residual lignin accumulates over the pretreatment cycles and possibly should have influenced in the enzymatic hydrolysis. However, it was observed that the IL can be reused without substantial losses in the efficiency of pretreatment of CAB for at least two times.

4. Conclusions

The ability of the ionic liquid 2-hydroxyl-ethylammonium acetate to simultaneously remove lignin and hemicellulose indicated a potential solvent for pretreatment of the cashew apple bagasse, which may serve as substrates for the production of higher value added products. The three employed methods demonstrated efficiency as pretreatment agents for CAB, but the method B (conducted in oven at 130 °C for 24 h) affords greater enzymatic digestibility of the cellulose and higher carbohydrates content. Studies on the IL recovery and reuse confirm that the ionic liquid can be reused without losses in the efficiency of the pretreatment from cashew apple bagasse.

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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.biortech.2016.11.019.

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