Research:

Comparison of autohydrolysis and ionic liquid 1-butyl-3-methylimidazolium acetate pretreatment to enhance enzymatic hydrolysis of sugarcane bagasse

Muzna Hashmi[1]; Qining Sun[2]; Jingming Tao[3]; Tyrone Wells Jr[2]; Aamer Ali Shah[1]; Nicole Labbé[3]; Arthur J. Ragauskas[2,3,4*]

Address: [1]Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan; [2]Department of Chemical and Biomolecular Engineering, Department of Forestry, Wildlife and Fisheries, The University of Tennessee, Knoxville, TN 37996–2200, USA; [3]Center of Renewable Carbon, The University of Tennessee, Knoxville, TN, USA; [4]Oak Ridge National Laboratory (ORNL), Oak Ridge, TN, 37831, USA.

Emails: Muzna Hashmi - muznahashmi@gmail.com; *Qining Sun* - qsun6@utk.edu; Jingming Tao - jtao2@utk.edu; Tyrone Wells Jr. - tyrone.wells.phd@gmail.com; Aamer Ali Shah - alishah@qau.edu.pk; Nicole Labbé - nlabbe@utk.edu; Arthur J. Ragauskas* - aragausk@utk.edu

* Corresponding author

Abstract

Background: One of the main challenges for bioethanol production from lignocellulosic material such as sugarcane bagasse is the recalcitrance of the biomass to physical, chemical and biological attacks. Different pretreatment strategies have been employed to enhance sugar yield from sugarcane bagasse, which in turn enhanced ethanol production. This study evaluated the efficiency of an ionic liquid (IL) 1-butyl-3-methyl imidazolium acetate ([C_4mim][OAc]) pretreatment at 110°C for 30 min, and compared it with high temperature autohydrolysis pretreatment in terms of delignification, cellulose crystallinity and digestibility.

Results: It was found that sugarcane bagasse exhibited a substantial decrease in lignin content, reduced cellulose crystallinity, and enhanced glucan and xylan digestibility, when subjected to [C_4mim][OAc] pretreatment. Glucan and xylan digestibility of [C_4mim][OAc] pretreated bagasse was determined as 97.4% and 98.6%, respectively after 72 h of enzymatic hydrolysis. In case of autohydrolysis, the maximum of glucan and xylan digestibility was determined after 72 h as 62.1 and 5.7%, respectively from the bagasse pretreated at 205°C for 6 min. X-ray diffraction analysis also showed a significant reduction in crystallinity of [C_4mim][OAc] pretreated bagasse samples. [C_4mim][OAc] pretreated and autohydrolyzed bagasse (205°C for 6 min) exhibited maximum ethanol production of 78.8 and 70.9 mg/g substrate, respectively after 24 h of fermentation by a newly isolated *S. cerevisiae* strain (MZ-4). However, the fermentation of bagasse autohydrolyzed at 190°C for 10 min and 110°C for 30 min yielded maximum ethanol of 66.0 and 28.4 mg/g substrate, respectively by using *S. cerevisiae* Lalvin EC-118.

Conclusion: [C_4mim][OAc] pretreatment was shown to be comparatively a more promising alternative to high severity autohydrolysis. [C_4mim][OAc] pretreatment weakened the interactions with in lignin structure and also reduced the cellulose crystallinity, which in turn enhanced its digestibility. In case of autohydrolysis, the increase in severity enhanced the

crystallinity of biomass; however, the conditions with high severity exhibited positive effect on glucan digestibility and subsequent ethanol yield, which might be attributed to removal of major hemicelluloses from biomass during autohydrolysis. In comparison, [C_4mim][OAc] pretreatment was more efficient than autohydrolysis even in milder conditions with less processing time.

Key Words: ionic liquid, sugarcane bagasse, hydrolyzability, autohydrolysis, bioethanol

Background

Lignocellulosic biomass is a suitable resource for renewable energy in terms of sustainability and ease of fermentation of enzymatically released sugars that can be converted into bioethanol to substitute for gasoline [1]. This resource is mainly composed of cellulose (30–45%), hemicelluloses (20–30%), and lignin (5–20%) [2]. Cellulose chains are held together by van der Waals interactions and hydrogen bonding which makes it a highly crystalline material [3]. The xylan layer is the most prominent hemicelluloses in grasses and hardwoods and forms covalent linkages to lignin in the cell wall. Xylan also has non-covalent interactions with cellulose, which are believed to play a role in preventing enzymatic degradation [3, 4]. Lignin is a complex and branched aromatic polymer that is associated with hemicellulose and contributes to the recalcitrance of biomass [3, 5]. There are several stages involved in conversion of recalcitrant lignocellulosics into ethanol including physicochemical pretreatment, enzymatic hydrolysis, fermentation, ethanol separation and effluent treatment. Pretreatment is an important step in the overall process and is believed to break down some of the carbohydrate-lignin complexes [3, 6].

Enzymatic hydrolysis of pretreated biomass is one of the most promising means of releasing simple sugars from biomass [7]. Typically, enzymatic hydrolysis of unpretreated biomass is reported to produce less than 20% sugar yield of theoretical value [8]. Different pretreatment

methods have been developed to reduce recalcitrance of lignocellulosic biomass but there are many drawbacks associated with these procedures. For example, biological pretreatments (i.e. lignin degrading fungi) often require long residence times [9, 10], mechanical methods such as grinding and milling techniques are not appropriate due to their high capital costs and intensive energy requirements [11]. Furthermore, various physicochemical techniques (e.g. autohydrolysis, supercritical fluids, steam explosion, dilute acid, and alkali) require high temperature and pressure along with specialized equipment [3, 7, 12]. Another drawback associated with these pretreatments is the release of inhibitors, which negatively affect enzymatic hydrolysis and subsequent fermentation process [7, 13]. These problems highlight the need for a more rapid, environment friendly, cost effective and efficient method for lignocellulosic biomass pretreatment and conversion.

Despite being energy intensive, autohydrolysis is recommended as environmentally benign and clean process [14] which doesn't require any catalyst or corrosive compounds [13]. Biomass and water are heated from 130 to 230ºC for different time periods (from few sec to several h) to carry out this pretreatment [7]. At high temperature (~200˚C) water has an acidic pH and acquires catalytic properties, which eliminates the requirement of catalyst to disrupt biomass [15]. Auto-ionization of water and ionization of acidic species (uronic acid and formic acid) at high temperature generate hydronium ions that catalyze the series of reactions and cause reduction in degree of polymerization (DP) of hemicelluloses and cellulose by hydrolysis of selective glycosidic bonds [7, 16]. During autohydrolysis, acetyl groups are released from substituted xylan chains (along with other organic acids) which act as catalysts to assist in acid-catalyzed hydrolysis of hemicellulose fraction of lignocellulosic biomass [7, 17-19]. The main compositional changes observed after autohydrolysis are lignin transformations and

depolymerization of hemicelluloses and cellulose into oligomers and monomers [7]. These compositional changes induce other structural changes including increasing the reducing ends of plant polysaccharides for efficient exoglucanase activity and thereby increased cellulose digestibility [7, 13, 18].

Ionic liquid pretreatment (IL) is another method of reducing the recalcitrance of biomass that has recently drawn a great deal of attention because of the unique physical and chemical properties of certain ILs that are a very stable class of organic salts with potential application as "green solvents" [8]. The main advantages of using ILs are related to their non-explosive, non-toxic, environment-friendly, low volatility, good recyclability and general stability under severe reaction conditions [1, 8, 20]. IL pretreatment is considered as an effective pretreatment method as it weakens van der Waals interactions between cell wall components [1, 21]. In grasses, the ester linkages that are formed between lignin and arabinoxylan are disrupted during IL pretreatment [1]. It is expected that IL pretreatment imparts compositional changes and interacts with the original biomass by hydrogen, ionic and Π-Π interaction in order to dissolve its components [22]. Anionic moieties of ILs act as hydrogen ion acceptor and interact with hydroxyl groups present within hydrogen bonding network of cellulose; however, cations interact with lignin through Π-Π interaction [3, 23]. IL pretreatment causes dissolution of biomass that can be rapidly precipitated with an anti-solvent and this prevents the reconstruction of the crystalline phase of cellulose resulting in the formation of porous and amorphous structure thus greatly enhanced its digestion [8, 21, 24].

For biomass pretreatment, three of the most cited ILs are imidazolium i.e. [C_4mim][Cl] (1-butyl-3-methylimidazolium chloride), [C_2mim][Cl] (1- ethyl-3-methylimidazolium chloride) and [C_2mim][OAc] (1-ethyl-3-methylimidazolium acetate). All these alkylimidazolium salts have

been reported as most effective agents for lignocellulosics dissolution [22]. It has been reported that the acetate ion in ILs are less viscous and can function as a weak base to remove lignin and de-acetylate biomass [22, 25]. Studies on these three alkylimidazolium salts reveal that shorter alkyl chain of $[C_2mim]^+$ imparts greater extent of saccharification with faster dissolution. However, higher dissolution extent of $[C_2mim]^+$ does not benefit the overall process of pretreatment since losses in $[C_4mim]^+$-treated biomass were much less as compared to $[C_2mim]^+$ pretreatment process. In terms of hemicellulose saccharification yield, $[C_4mim]^+$ ILs perform better as hemicellulose is preserved in its polymeric form and recovered form after pretreatment [22]. Due to these reasons, $[C_4mim][OAc]$ (1- butyl-3-methyl imidazolium acetate) pretreatment was selected for this study. Previously, $[C_4mim][OAc]$ pretreatment of sugarcane bagasse was reported by Silveria et al. (2015), who studied the effect of $[C_4mim][OAc]$ pretreatment in combination with ethanol and supercritical CO_2 (at 110, 145 and 180ºC for 2 h) [26]; while, Aver et al. (2013) used only $[C_4mim][OAc]$ for the pretreatment of sugarcane bagasse at 120ºC for 24 h [27]. However, none of those studies discussed the effect of $[C_4mim][OAc]$ pretreatment on crystallinity of sugarcane bagasse and the efficiency of fermenting microbes for the production of bioethanol from $[C_4mim][OAc]$ pretreated bagasse. In comparison to previous studies, this study evaluated $[C_4mim][OAc]$ pretreatment of sugarcane bagasse at less severe conditions (i.e. 110ºC for 30 min); and compared with high temperature autohydrolysis to investigate the changes it imparts to structure and composition of sugarcane bagasse and its potential to produce bioethanol. High temperature conditions for autohydrolysis were adopted from literature which investigated optimal pretreatment conditions for maximum cellulose conversions [7, 28]. Moreover, the efficiency of various commercially available yeasts was compared with a newly isolated strain to determine a better fermenting strain for enhanced bioethanol production.

Results and discussion

This study investigated structural and chemical characteristics of autohydrolyzed and [C_4mim][OAc] pretreated sugarcane bagasse and the impact of these pretreatment strategies on enzymatic digestibility and production of bioethanol.

Effect of pretreatment on biomass composition

The effect of different pretreatment strategies on composition of sugarcane bagasse is shown in Table 1. The chemical composition of untreated sugarcane bagasse was determined as 39.8% glucan, 16.8% xylan and 31.9% lignin. From Table 1, it can be inferred that the xylan content was reduced from 16.8% (in untreated bagasse) to 6.8% and 3.4% in samples autohydrolyzed at 190ºC and 205ºC, respectively. The maximum xylan dissolution was observed when autohydrolysis was carried out under high severity conditions i.e. 205ºC for 6 min; however, a very slight variation in xylan content was observed in samples autohydrolyzed at 110ºC for 30 min. The xylan content was reduced from 16.8% to 11.1% after [C_4mim][OAc] pretreatment (110°C for 30 min), depicting that the IL exhibited less effect on hydrolysis of hemicellulose, as compared to high severity autohydrolysis pretreatment. The lignin content was determined as 31.9% in untreated bagasse which was increased to 41.8% and 39.4% in samples autohydrolyzed at 190ºC and 205ºC, respectively. The significant increase in lignin content after high severity autohydrolysis could be attributed to the removal of significant amount of hemicellulose while retaining most of the lignin. Li et al. (2007) suggested that the increased lignin content might be due to the repolymerization of polysaccharides degradation products (such as furfural) and/or polymerization with lignin, which forms a lignin like material termed as pseudo-lignin [29]. The

pseudo-lignin can also be generated from carbohydrate without significant contribution from lignin, especially under high severity pretreatment conditions [30]. During our current studies, the content of lignin was increased from 31.9% to 35.3% after [C_4mim][OAc] pretreatment. This increase in lignin content was lesser as compared to the high severity autohydrolysis pretreatment, which might be attributed to lesser dissolution of hemicelluloses by [C_4mim][OAc] as compared to autohydrolysis. However, the bagasse autohydrolyzed under same severity conditions didn't exhibit promising difference in lignin contents as compared to untreated bagasse. Another interesting effect is the increase of acid soluble lignin content that showed that [C_4mim][OAc] weakened the interactions within lignin structure. It has also been previously reported that the IL cation interacts with lignin through Π-Π interaction to help in lignin dissolution; however, complete dissolution of lignin was difficult due to complex lignin-carbohydrate structure and hydrophobicity [8]. [C_4mim][OAc] exhibited better effect on lignin dissolution as compared to autohydrolysis but both pretreatment methods had limited effect on cellulose removal. The increase in cellulose content after high severity autohydrolysis was attributed to the significant removal of hemicelluloses. The effect of both pretreatments on lignocellulosics conversion was further investigated by FTIR and XRD analysis.

Effect of pretreatment on biomass structure

Fourier transform infrared spectroscopy (FTIR) analysis

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR–FTIR) was conducted and different absorption bands were used to monitor the chemical changes of lignin and carbohydrates (Figure 1). Table 2 showed the assignment of various bands of interest to their chemical groups. The bands assigned to acetyl group at 1730 showed reduction of absorbance in

the bagasse pretreated with [C_4mim][OAc] and autohydrolyzed at 205°C for 6 min, confirming the removal of hemicelluloses components; similarly, the reduction in peak at 1161 cm^{-1} was also observed after [C_4mim][OAc] pretreatment. The band position at 1424 cm^{-1} is mainly due to CH_2 scissor motion in cellulose. The increase in band intensity at 1424 cm^{-1} and other cellulose associated band can be attributed to increase in cellulose content due to removal of lignin and hemicelluloses which confirmed the compositional analysis data (Table 1). Significant peaks at 1319 and 1370 cm^{-1} were observed in all pretreated samples that might be attributed to increased cellulose content after pretreatments. Reduction in absorption band at 3336 cm^{-1} after high severity autohydrolysis (i.e. 205°C of 6 min and 190°C for 10 min) represented lower cellulose hydrogen bonding in pretreated samples. The spectra obtained from [C_4mim][OAc] pretreated bagasse showed diminished peaks at 1103 cm^{-1} indicating the reduction of cellulose crystallinity after [C_4mim][OAc] pretreatment.

Figure 1 FTIR spectra of untreated, autohydrolysis and [C_4mim][OAc] pretreated bagasse: (a) from 1800 to 600 cm^{-1}; (b) from 4000 to 1800 cm^{-1} region

Measurement of cellulose crystallinity

The X-Ray diffraction patterns analysis of pretreated and untreated bagasse was performed to examine the cellulose crystallinity index. Two diffraction peaks were noticed at 22º (2θ) and 19º (2θ) which corresponds to I_{002} (crystalline region) and I_{am} (amorphous region), respectively (Figure 2). Crystallinity index (CrI) of untreated bagasse was determined as 0.61, whereas CrI of the samples autohydrolyzed at 110°C, 190°C and 205°C was 0.62, 0.65 and 0.68, respectively (Table 3). The slight increase in CrI can be attributed to removal of amorphous region i.e.

hemicelluloses, lignin or amorphous cellulose and rearrangement of remaining components [38, 39]. Diffraction pattern of [C_4mim][OAc] pretreated sample showed the disappearance of secondary peak (I_{101}) at 2θ value of 15º, and the crystallinity peak (I_{002}) was also found to be weaken that represented the profile of nearly amorphous cellulose [40]. The CrI of [C_4mim][OAc] treated sample was determined as 0.25 (Table 3) demonstrating a reduction in cellulose crystallinity. Previous studies have shown that the crystallinity index of bagasse was reduced from 0.56 to 0.24 after [C_2mim][OAc] pretreatment [8]. It was suggested that anion and cation in the IL were responsible for the disruption of cellulose structure. The cation interacted with lignin though Π-Π interaction and hydrogen bonding whereas anionic acetate acted as hydrogen bond acceptor that attacked the free hydroxyl group of cellulose and deprotonated it, thus reducing cellulose crystallinity [3, 23]. Hydrogen bonding in cellulose structure was presumably disrupted and replaced by hydrogen bonding between cellulose hydroxyl and anions of ionic liquid, thus causing disruption and dissolution of cellulose structure and reduction of its crystallinity [3].

Figure 2 XRD spectra of untreated, autohydrolyzed and [C_4mim][OAc] pretreated bagasse

Enzymatic hydrolysis

The carbohydrate digestibility is considered as an important factor to select the most efficient method of pretreatment. Autohydrolysis was compared with [C_4mim][OAc] pretreatment to obtain kinetics of enzymatic hydrolysis and cellulose digestibility (Figure 3). In case of [C_4mim][OAc] pretreated bagasse 79.8% cellulose was converted after 3 h of enzymatic hydrolysis, whereas in samples autohydrolyzed at 110ºC, 190ºC and 205ºC, the digestibility

231 percentages were noted as only15.6%, 20.8% and 27.3%, respectively. The reaction was
232 completed at 72 h with 97.4%, 19.3%, 46.9% and 62.1% cellulose converted, respectively. Qiu et
233 al. (2012) and Li et al. (2010) reported that enzymatic hydrolysis of [C_2mim][OAc] pretreated
234 bagasse exhibited cellulose digestibility up to 87% and 96%, respectively [1, 8]. The previous
235 studies on [C_4mim][OAc] pretreated bagasse (at 120ºC for 24 h) showed 78% of cellulose
236 digestibility [27]; however, the current study showed the enhanced cellulose digestibility when
237 [C_4mim][OAc] pretreated bagasse (at 110ºC for 30 min) was subjected to enzymatic hydrolysis.
238 In case of autohydrolysis, the limited enzymatic hydrolysis can be attributed to unmodified
239 crystalline cellulosic structure. The increase in amorphous cellulose content as a result of
240 [C_4mim][OAc] pretreatment provided an enhanced surface area leading to better enzyme
241 accessibility and increased digestibility as compared to autohydrolyzed samples.
242 Hemicellulose digestibility of [C_4mim][OAc] pretreated sample was reported as 65.1% after 3 h
243 while for samples autohydrolyzed at 110ºC, 190ºC and 205ºC, hemicellulose conversion
244 percentages were noted as only 4.2%, 2.7% and 3.0%, respectively (Figure 4). The hydrolytic
245 process completed at 72 h and hemicellulose digestion was determined as 98.6% for the
246 [C_4mim][OAc] pretreated bagasse while 8.3%, 4.6% and 5.7% hemicelluloses were converted
247 for samples autohydrolyzed at 110ºC, 190ºC and 205ºC, respectively. Higher hemicelluloses
248 digestibility from [C_4mim][OAc] pretreated bagasse might be attributed to minimal loss of initial
249 xylan and delignification. Silva et al. (2011) reported only 75% xylan digestion when treated
250 with [C_2mim][OAc] [20], which was quite lower as compared to [C_4mim][OAc] treated bagasse.
251 Previous studies on [C_4mim][OAc] pretreated sugarcane bagasse showed 99.5% xylan
252 digestibility [27]. In autohydrolyzed bagasse, hemicelluloses were not accessible to enzymes

because most of the hemicelluloses were already removed and the remaining fraction was covalently linked to lignin [22].

Enzymatic hydrolysis results clearly showed that sugars were released faster to a greater extend when [C_4mim][OAc] pretreatment of sugarcane bagasse was used rather than the autohydrolysis pretreatment. It was also observed that total processing time to reach 60% cellulose digestibility was about 48 h with autohydrolysis but it was reached up to 79.8% within 3 h with [C_4mim][OAc] pretreatment. In comparison to autohydrolysis, [C_4mim][OAc] required lower energy consumption, less processing time and lead to higher glucose yield. The advantages associated with [C_4mim][OAc] offer motivation to explore and develop this pretreatment technique because it prevents dissolution of hemicellulose during pretreatment and also enhances the enzymatic digestibility of cellulose and hemicelluloses.

Figure 3 Comparison of cellulose digestibility percentage between untreated, autohydrolyzed and [C4mim][OAc] pretreated bagasse

Figure 4 Comparison of xylan digestibility percentage between untreated, autohydrolyzed and [C4mim][OAc] pretreated bagasse

Fermentation

Ethanol production from fermentation of pretreated/enzymatically deconstructed samples of sugarcane bagasse showed that different microbial strains have different abilities to produce ethanol from the available sugars (Figure 5). Ethanol production obtained from bagasse autohydrolyzed at 110ºC for 30 min was 28.42 mg/g-substrate when it was fermented with *S.*

Cerevisiae (Lalvin EC-118). The same strain showed maximum ethanol production (66.02 mg/g-substrate) from fermentation of enzymatically hydrolyzed bagasse pretreated at 190ºC for 10 min whereas bagasse pretreated at 205ºC showed maximum ethanol production (70.9 mg/g-substrate) when *S. cerevisiae* (MZ-4) was used as fermenting organism. The differences in ethanol yield by different strains can be attributed to their difference in tolerance against side products released during different pretreatment conditions [41]. *S. cerevisiae* strains usually don't have the ability to ferment xylose but are more efficient in glucose fermentation [42]. *Pichia stipites* is considered as more efficient to ferment xylose into ethanol but its lower production with all pretreated samples can be attributed to its less efficient glucose utilization as compared to *S. cerevisiae* [43]. Moreover, it was also suggested in previous studies that all symporters (i.e. the proteins which are involved in transport of molecules across plasma membrane) in *P. stipites* are competitively inhibited by glucose molecules which makes it difficult to utilize both sugars simultaneously and hinders the conversion of xylose into ethanol [44].

The highest ethanol yield (78.8 mg/g-substrate) was obtained when sugarcane bagasse was pretreated with [C_4mim][OAc] at 110ºC for 30 min and fermented with a newly isolated *S. cerevisiae* (MZ-4) strain. This strain can be considered more promising for [C_4mim][OAc] pretreated bagasse and played important role in production of maximum ethanol. However, it is important to optimize [C_4mim][OAc] pretreatment and fermentation conditions to further enhance ethanol yield.

Figure 5 Production of bioethanol from untreated, autohydrolyzed and [C4mim][OAC] pretreated bagasse by using different yeast strains

Conclusion

A parallel comparison of [C_4mim][OAc] pretreatment and autohydrolysis of sugarcane bagasse showed that this IL is a promising alternative pretreatment technique based on low temperature requirement, high sugar yield, high enzymatic digestibility and less total processing time. In contrast to autohydrolysis, [C_4mim][OAc] pretreated bagasse has more amorphous cellulosic structure, thus enhanced digestibility during enzymatic hydrolysis. During fermentation, glucose derived from [C_4mim][OAc] pretreated bagasse yielded more ethanol when fermented with a newly isolated *S. cerevisiae* (MZ-4) strain, as compared to bagasse pretreated with autohydrolysis. Thusly it can be concluded that [C_4mim][OAc] is a promising pretreatment method for enhanced ethanol production from sugarcane bagasse.

Methodology

Biomass

Sugarcane bagasse was supplied by Green Energy Inc. Vonore, TN USA. Bagasse (5-8 cm size) was air-dried for two-three days. The dried bagasse was milled with Thomas Model 4 Wiley® Mill fitted to get final particle size of less than 0.45 mm. Extractives in sugarcane bagasse were removed using dichloromethane in soxhlet extraction apparatus for 24 h (4-5 cycles per hour) according to TAPPI method T204 cm-07, then biomass was removed from extraction thimble, and solvent was evaporated near to dryness while in the chemical fume hood.

Pretreatment

Ionic liquid (1-butyl, 3-methylimidazolium acetate) was added in sugarcane bagasse to set liquid to solid ratio of 20:1 (5% w/w bagasse solution) and heated (at ~4-5ºC/min). up to 110ºC ±5 for

30 min in 4560 mini-Parr pressure reactor (Parr Instrument Company, Moline, IL, United States) with an agitation set at 100 rpm. After the reaction, 1000 ml deionized water was added to it at room temperature and stirred vigorously to enable regeneration then the biomass was recovered by filtration through VWR Grade 417 filter paper. Recovered biomass was water washed repeatedly, filtered then air-dried before further experiments [38]. To compare the effectiveness of ionic liquid pretreatment to autohydrolysis, sugarcane bagasse was also treated with hot water at the same conditions (110ºC for 30 min) and also to higher temperature i.e. 190ºC ±5 for 10 min and 205ºC ±5 for 6 min. The liquid to solid ratio and equipment used for autohydrolysis were same as previously used for IL pretreatment. The impeller was adjusted at speed of 100 rpm and the reactor was heated to specific temperature (at ~4-5ºC/min). After completion of pretreatment time the reactor was dipped into an ice bath (~5 min) for rapid cooling to terminate the reaction. The wet substrate was recovered from the reactor and washed with approximately 40 ml distilled water during filtration [38]. A part of the biomass was separated for structural and compositional analysis while rest was used for enzymatic hydrolysis. Untreated biomass was used as control sample. All experiments were carried out in duplicates.

Severity factor

In order to compare the efficacy of various pretreatment techniques, the severity factor of all the pretreatments were determined by using the equation:

$$SF = \log (t \times \exp((T - T_{ref})/14.75))$$

In the above equation, t is the treatment time in min, T is the treatment temperature, T_{ref} is the reference temperature (i.e., 100°C) and 14.75 is an empirically determined constant [45, 46].

Chemical composition of Sugarcane Bagasse

All treated and untreated samples were analyzed for lignin, glucan, xylan, arabinan, and mannan content following Laboratory Analytical Procedure (LAP) TP-510-42618 as documented by National Renewable Energy Laboratory (NREL) [47].

FTIR Analysis

To investigate and estimate chemical changes in the sugarcane bagasse samples after each pretreatment, a Perkin Elmer Spectrum 100 FTIR spectrometer equipped with an Attenuated Total Reflectance (ATR) sampling accessory (Perkin-Elmer Inc., Wellesley, MA, United States) was used. Samples (20mg) were pressed uniformly against the crystal surface via a spring-anvil, and spectra were obtained after 32 scans accumulation from 4,000 to 600 cm^{-1} at 4 cm^{-1} resolution [33].

X-ray diffraction (XRD) analysis

Crystallinity index (CrI) of autohydrolyzed and IL treated sugarcane bagasse material was analyzed by X-ray diffractometer (PANalytical 3040/60 X'pert PRO, Netherlands) with CuKα radiation source (k = 0.1505nm). Patterns were collected from 5º to 40º (2θ) with scan step of 0.01º, while the operating voltage and current were 40kV and 30mA, respectively. The crystallinity index (CrI) was defined using the equation:

$$CrI = (I_{002} - I_{am})/I_{002}$$

Where I_{002} is the maximum intensity of crystalline peak at 2θ (22º), whereas I_{am} is the scattered intensity due to the amorphous portion evaluated as the minimum intensity between the main (I_{002}) and secondary peaks (I_{101}) [8].

368

369 **Enzymatic hydrolysis**

370 Enzymatic saccharification was done by adding mixture of cellulases (Celluclast®1.5L; Sigma
371 Aldrich; 23 FPU/ml) and *β*-glucosidase from almond (CAS No: 9001223; Sigma Aldrich). The
372 activity of cellulases was determined as filter paper assay unit (FPU) by LAP TP-510-42628
373 documented by NREL method [48]. All pretreated and untreated bagasse samples were subjected
374 to enzymatic hydrolysis by adding 1g substrate in 100 ml (1% w/v) of 50 mM sodium citrated
375 buffer (pH 4.8) [8]. The dosage of 20 FPU of cellulases and 40 IU of *β*-glucosidase was chosen
376 because further increase in loading did not increase sugar release. Saccharification was carried in
377 rotary incubator with a shaking speed of 150 rpm at 50˚C for 72 h [8]. Enzymatic hydrolysis was
378 carried out in duplicates. Aliquots (500 µl) were collected at 3, 6, 12, 24, 48, and 72 h. Each
379 aliquot was sealed and incubated for 5 min at boiling water to denature the cellulases [38]. The
380 aliquots were filtered through 0.20 µm Nylon syringe filter (Millipore, Billerica, MA) before
381 high performance liquid chromatography (HPLC) analysis. The enzymatic digestibility of
382 cellulose and xylan was calculated as the ratio of glucose in the enzymatic hydrolysis per 100 g
383 of potential glucose in substrate and the ratio of xylose in the enzymatic hydrolysis per 100 g of
384 potential xylose in substrate, respectively [3].

385

386 **HPLC analysis**

387 An HPLC system (Perkin Elmer Flexar HPLC, Perkin Elmer, Shelton, CT) with Bio-Rad
388 Aminex HPX-87P column and refractive index detector was used to determine and quantify the
389 sugars obtained from the compositional analysis of bagasse, and from enzyme hydrolysis of
390 these materials. The temperature for the column was set at 85ºC and H_2O was used as the mobile

phase with a flow rate of 0.25 ml/min. The Chromera® Chromatography Data Systems (CDS) software was used for the analysis and interpretation.

Fermentation

Three strains *S. cerevisiae* and one strain of *P. stipites* (ATCC 58785) were used to determine an efficient strain for enhanced ethanol production in case of each pretreatment study. A self-isolated strain of *S. cerevisiae* (Gene bank accession number: KP970869) labeled as MZ-4 and two commercial strains of *S. cerevisiae* i.e. Lalvin EC-118 and Uvaferm-43 on basis of previously reported studies on wine production from fresh grape juice were selected [49-51]. All strains were cultured on YPD (Yeast extract, Peptone, Dextrose) media [52]. When the culture reached the late stage of exponential growth (~24 h), it was centrifuged at 2500 rpm for 5 min in centrifuge (Beckman CS-6R). The supernatant was removed and cells were washed twice with sterile 0.9% (w/v) NaCl solution in same manner, then the cells were kept in the same sterile solution [6]. After enzymatic saccharification, all samples were subsequently autoclaved and allowed to cool before inoculation [53, 54], and all the four strains were inoculated separately with inoculum size of 5% v/v (containing 300 x 10^6 living cells/mL) [53]. Yeast cultures were stained with methylene blue and live cells were counted with help of haemocytometer [55]. The pH of media was adjusted to 5.0 by drop wise addition of 5N NaOH, and the process of fermentation was carried out at 30°C [18]. Ethanol content was measured from fermentation media after every 24 h with help of Megazyme ethanol Kit.

Abbreviations

IL: Ionic Liquid

414 [C_4mim][OAc]: 1-butyl-3-methylimidazolium acetate

415 [C_2mim][OAc]: 1-ethyl-3-methylimidazolium acetate

416 [C_4mim][Cl]: 1-butyl-3-methylimidazolium chloride

417 [C_2mim][Cl]: 1- ethyl-3-methylimidazolium chloride

418

419 **Competing interests**

420 The authors declare that they have no competing interests.

421

422 **Author's contributions**

423 MH carried out all experimental work under supervision of AJR. AJR and NL contributed in
424 critical review and proofread of the manuscript. QS, JT, TWJ, AAS and NL contributed in
425 analysis and interpretation of various data. The authors have read BioMed Central's guidance on
426 competing interests and have concluded that this manuscript is consistent with these guidelines.

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Tables

Table 1 Chemical composition of untreated, autohydrolyzed and [C_4mim][OAc] pretreated sugarcane bagasse

	Untreated bagasse	Autohydrolyzed at 190°C (for 10 min)	Autohydrolyzed at 205°C (for 6 min)	Autohydrolyzed at 110°C (for 30 min)	[C_4mim][OAc] pretreated at 110°C (for 30 min)
Severity Factor	-	3.64	3.86	1.77	1.77

(SF)					
Acid Insoluble lignin (%)	27.5±0.2	37.7±0.3	36.0±0.4	26.8±0.2	27.3±0.1
Acid Soluble lignin (%)	4.4±0.1	3.1±0.2	3.4±0.1	4.1±0.1	8.0±0.2
Total Lignin (%)	31.9±0.4	41.8±0.7	39.4±0.7	30.9±0.4	35.3±0.4
Glucan (%)	39.8±0.7	48.1±0.6	54.0±0.3	40.5±0.0	38.4±1.0
Xylan (%)	16.8±0.3	6.8±0.4	3.4±0.1	17.2±0.4	11.1±1.0
Arabinan (%)	0.1±0.0	0.05±0.1	N.Q.	5.5±0.1	6.4±0.0
Mannan (%)	N.Q.	N.Q.	N.Q.	0.1±0.0	0.1±0.00
Extractives (%)	2.8±0.2	N.D.	N.D.	N.D.	N.D.

N.Q. = Not quantified
N.D. = Not determined

Table 2 Assignments of FTIR-ATR absorption bands for sugarcane bagasse

Band position (cm^{-1})	Assignment	Source	References
3175-3490	O-H stretching vibration	Cellulose	[21, 31-33]
2850-2970	C-H stretching related to biomass methyl/methylene group	Polysaccharide	[21, 31-33]
1730	C = O stretching vibration in acetyl groups of	Hemicellulose	[22, 31]

	hemicelluloses		
1630-1650	O–H bending due to adsorbed water	Water	[31, 34]
1600-1610	Aromatic skeletal vibration	Lignin	[34, 35]
1514	Aromatic skeletal vibration	Lignin	[31, 34, 35]
1430-1420	CH_2 scissoring motion	Cellulose	[31, 33]
1382-1370	C–H bending vibration	Polysaccharide	[22, 31]
1319	CH_2 wagging	Cellulose	[31, 35, 36]
1243	C-O stretching	Polysaccharide	[34, 37]
1161	C-O-C ring vibrational stretching	Hemicellulose	[34, 36]
1103	O-H association band in cellulose and hemicelluloses (associated with crystalline cellulose)	Cellulose	[21, 22, 35]
898	C–H deformation vibration	Cellulose	[21, 22]

Table 3 Crystallinity of untreated, autohydrolyzed and [C_4mim][OAc] pretreated bagasse

Samples	**Crystallinity Index (CrI)**
Untreated bagasse	0.61
Autohydrolysis (110^{o}C for 30 min)	0.62
Autohydrolysis (190^{o}C for 10 min)	0.65
Autohydrolysis (205^{o}C for 6 min)	0.68

[C_4mim][OAc] pretreated (110°C for 30 min)	0.25

Figure 1

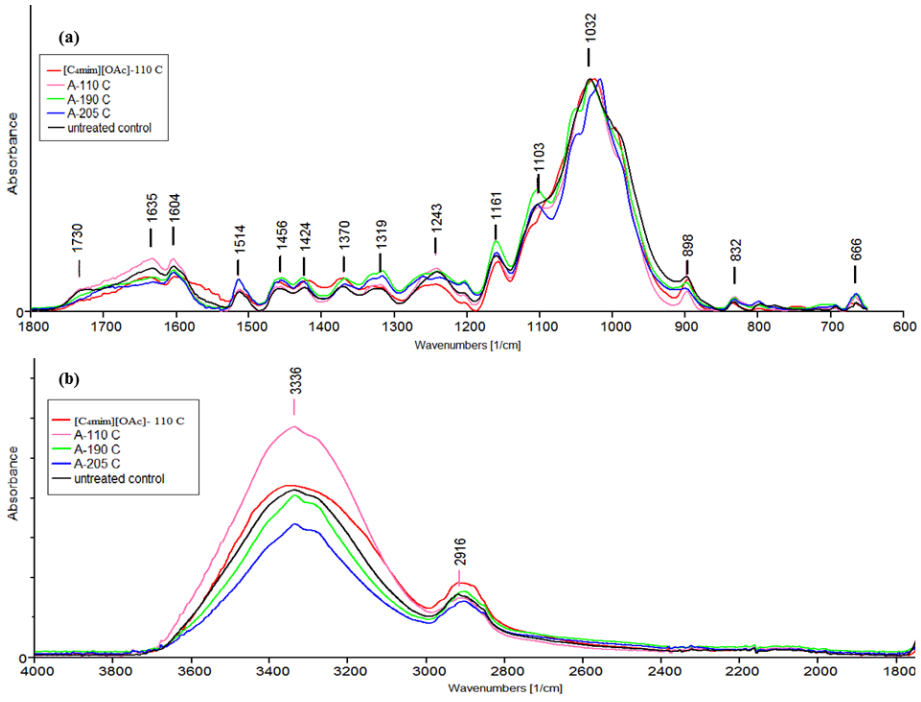


Figure 2

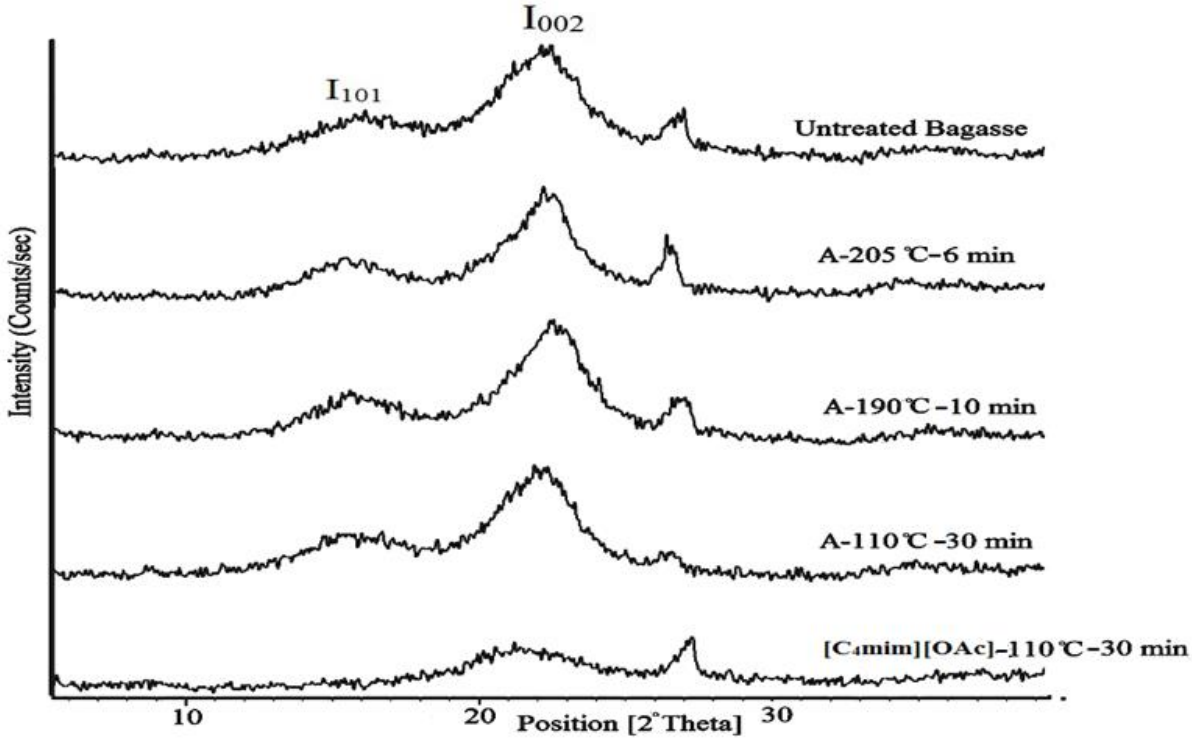

I002
I101
Untreated Bagasse
A-205 ℃-6 min
A-190℃-10 min
A-110℃-30 min
[C4mim][OAc]-110℃-30 min
Intensity (Counts/sec)
10
20
30
Position [2°Theta]

Figure 3

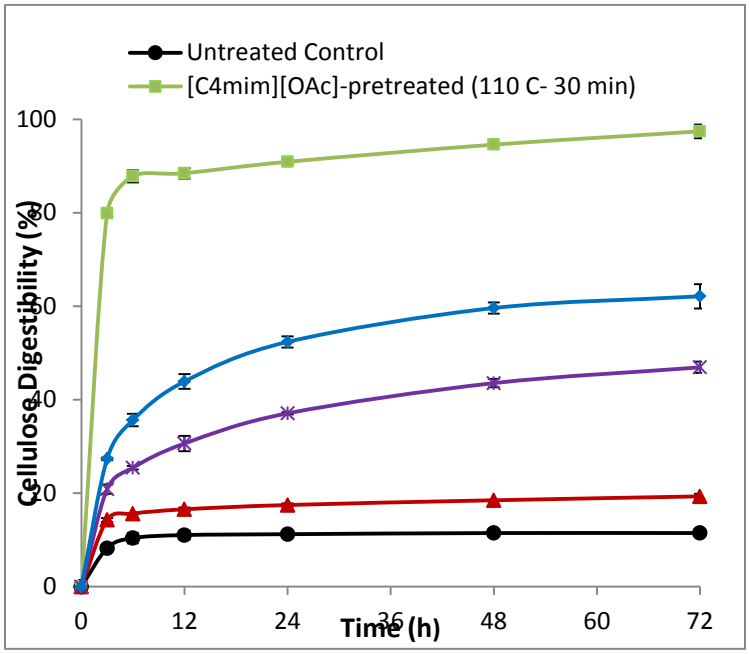
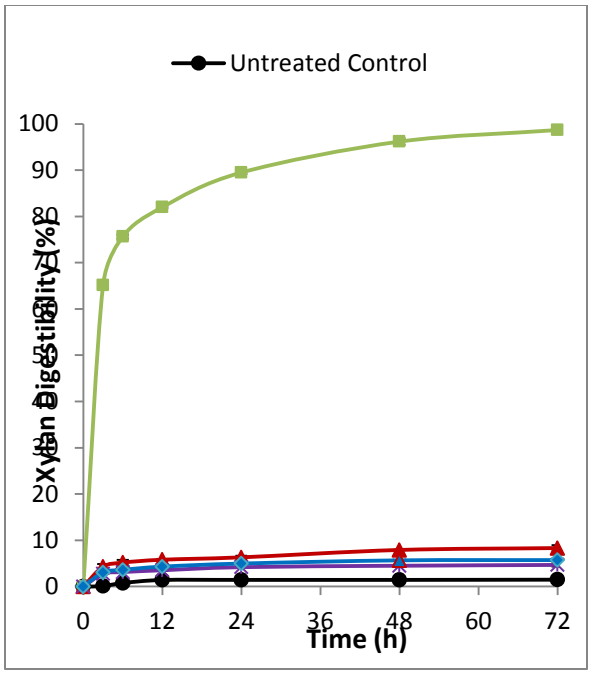

Untreated Control
[C4mim][OAc]-pretreated (110 C- 30 min)
Cellulose Digestibility (%)
Time (h)
0
20
40
60
80
100
0
12
24
36
48
60
72

Figure 4



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669 **Figure 5**

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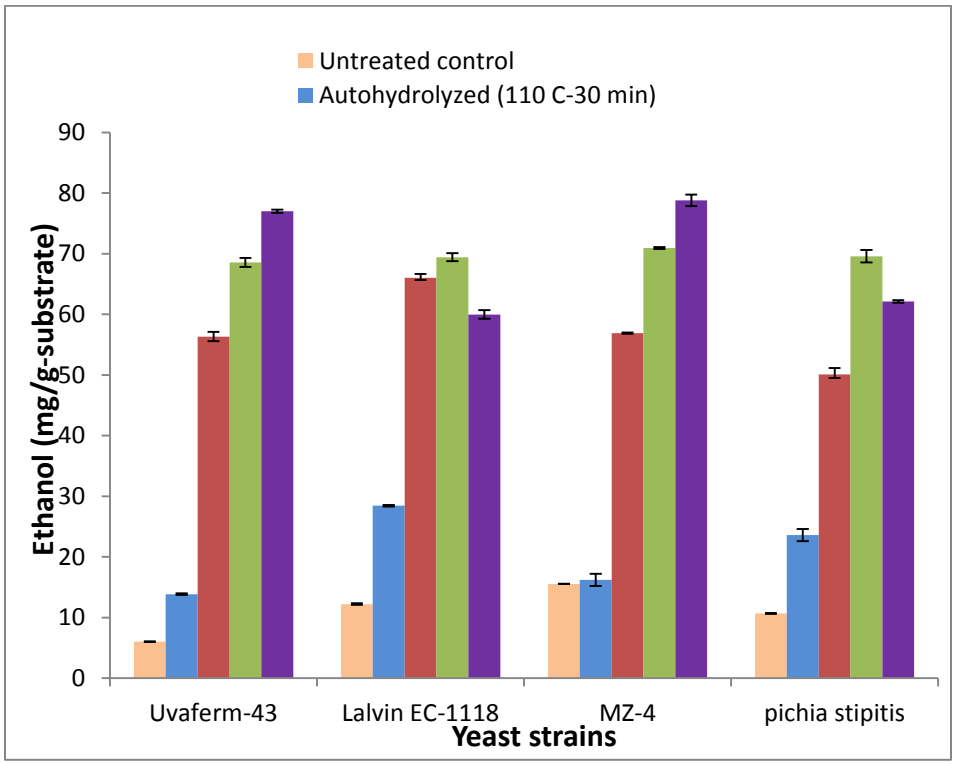

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