Enhanced Production of Bioethanol by Fermentation of Autohydrolyzed and C$_4$mimOAc-Treated Sugarcane Bagasse Employing Various Yeast Strains

Muzna Hashmi $^{1,2}$, Aamer Ali Shah $^1$, Abdul Hameed $^{1,3}$ and Arthur J. Ragauskas $^{2,4,5,*}$

$^1$ Department of Microbiology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan; muznahashmi@gmail.com (M.H.); alishah@qau.edu.pk (A.A.S.); hameedqau@gmail.com (A.H)

$^2$ Department of Chemical and Biomolecular Engineering, University of Tennessee, Knoxville, TN 37996-2200, USA

$^3$ SA-CIRBS, International Islamic University, Islamabad 45320, Pakistan

$^4$ Joint Institute for Biological Sciences, Biosciences Division, Oak Ridge National Laboratory (ORNL), Oak Ridge, TN 37831, USA

$^5$ Center of Renewable Carbon, Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996-4542, USA

* Correspondence: aragausk@utk.edu; Tel.: +1-865-974-2042; Fax: +1-865-974-7076

Abstract: This study examines the fermentation of autohydrolyzed and 1-n-butyl-3-methylimidazolium acetate (C$_4$mimOAc) pretreated sugarcane bagasse, using four different yeast strains to determine the efficiency of bioethanol production. Three strains of Saccharomyces cerevisiae (S. cerevisiae) and one of Scheffersomyces stipitis (S. stipitis) were employed in this study. It was observed that the sugarcane bagasse autohydrolyzed at 205 °C for 6 min with subsequent enzymatic hydrolysis exhibited the maximum ethanol yield of 70.92 ± 0.09 mg/g-substrate when S. cerevisiae MZ-4 was used. However, a slightly higher ethanol yield of 78.78 ± 0.94 mg/g-substrate was obtained from C$_4$mimOAc pretreated bagasse employing S. cerevisiae MZ-4. The study showed that the newly isolated MZ-4 strain exhibited better ethanol yield as compared to commercially available yeast strains S. cerevisiae Uvaferm-43, S. cerevisiae Lalvin EC-1118, and S. stipitis.

Keywords: fermentation; bioethanol; Saccharomyces cerevisiae; ionic liquid; autohydrolysis; pretreatment

1. Introduction

From a global perspective, the immense increase in the production of bioethanol up to 97.1 billion liters in 2016 [1] has proved the significance of bioethanol in the transport sector. In order to broaden the availability of raw materials for cellulosic ethanol production, ongoing research examines new feedstocks, while also looking into reducing the recalcitrance of biomass through pretreatment [2–5]. Despite the advantages of lignocellulosic biomass originating from its sustainability and availability, it is still considered as a challenging material due its recalcitrance. Plant cell wall is a complex structure that is comprised of hemicellulose (heteropolymer of pentoses, e.g., xylose and arabinose; and hexoses e.g., glucose, galactose and mannose), cellulose (β-1, 4-linked glucose), and lignin, and gives tensile strength to plant materials [6,7]. The fermentation of bioethanol from lignocellulose requires prior or concurrent conversion of the plant polysaccharides to monomeric sugars [7].

Bioethanol production from lignocellulosic wastes requires several steps, including the pretreatment of biomass with subsequent enzymatic hydrolysis followed by fermentation [8–10]. During the fermentation process, sugars are converted into ethanol and carbon dioxide with the help of fermenting microbes. A variety of microorganisms are capable of fermenting sugars into bioethanol.
Yeast are eukaryotic organisms that are able to grow on different types of sugars while exhibiting high sugar and ethanol tolerance. *Saccharomyces cerevisiae* (*S. cerevisiae*) is the most promising yeast strain involved in the conversion of hexoses into bioethanol through the Embden-Meyerhof-Parnas pathway [11]. However, xylose, one of the main components of hemicellulose, cannot be converted into ethanol by most of the microbial strains used in industry. *S. cerevisiae* has the ability to utilize both monomeric sugars and sucrose, which make it an efficient microbe for use with a variety of substrates. Other advantages related to its use are resistance against high ethanol concentration, inhibitor resistance, and its ability to consume significant amounts of substrate under industrially preferred conditions. On the other hand, *S. cerevisiae* lacks important genes that are required for the assimilation of xylose, although conversion of this hemicellulose fraction is essential to obtain the desired ethanol yields [12]. The fermentation of all available xylose present in lignocellulosic materials could enhance the overall ethanol yield by up to 25% and could contribute to making this process more economically favorable [13]. There are only a few species of yeast which are able to produce ethanol from xylose—e.g., *Scheffersomyces stipitis* (*S. stipitis*), *Pachysolen tannophilus* (*P. tannophilus*), *Candida shehatae* (*C. shehatae*), and *Candida guillermondii* (*C. guillermondii*); however, only 1% of all known xylose utilizing yeasts are known to display the potential of fermenting xylose to ethanol [12,14].

It was previously reported that the *C. shehatae* exhibited 75% of the maximum theoretical ethanol yield; whereas more than 85% was reported with *S. stipites* [15]. Rouhullah et al. [16] reported that the strain *S. stipitis* has equal ability to ferment glucose to ethanol as observed for *S. cerevisiae*. However, during xylose fermentation *S. stipitis* exhibited far better ethanol production, while a negligible amount of ethanol was reported from *S. cerevisiae*. Silva et al. [17] also demonstrated the fermentation of xylose into ethanol by using *S. stipites*. The strain *S. stipitis* ATCC 58785 was previously reported for the fermentation of a combined mixture of xylose and glucose, exhibiting that this strain has the ability to produce a comparatively greater content of ethanol when xylose was also present in the media along with glucose [18]. Therefore, it was suspected that *S. stipitis* ATCC 58785 might produce better yield as compared to *S. cerevisiae*, which is not usually very efficient in xylose conversion into bioethanol.

This study is the continuation of our previous study that investigated the effects of 1-n-butyl-3-methylimidazolium acetate (C₄mimOAc) pretreatment versus autohydrolysis on sugarcane bagasse [19]. The aim was to study differently pretreated and enzymatically hydrolyzed bagasse to determine the effects of C₄mimOAc pretreatment on sugar release [19]. To clarify this further, different yeast strains were compared to find out which of the strains was working efficiently with a newly-designed C₄mimOAc pretreatment strategy. Thus, this study deals with the use of four different types of yeast on the enhanced production of bioethanol from hydrolyzed sugarcane bagasse.

## 2. Results

### 2.1. Enzymatic Hydrolysis

Our previous study examined sugar release upon cellulase treatment from pretreated bagasse [19], while the current study elaborates on the release of glucose and xylose in terms of concentration (mg/mL), considering that the greater content of glucose in the hydrolysate ultimately leads to improved ethanol production. Variations in glucose and xylose concentration from the enzymatic hydrolysis of sugarcane bagasse pretreated with different strategies are shown in Figures 1 and 2. Glucose concentrations were noted as 0.80 ± 0.02 mg/mL, 2.37 ± 0.06 mg/mL, and 3.53 ± 0.14 mg/mL from samples autohydrolyzed at 110 °C for 30 min, 190 °C for 10 min, and 205 °C for 6 min, respectively. Under these conditions, the xylose concentration was 0.17 ± 0.01 mg/mL, 0.42 ± 0.03 mg/mL, and 0.24 ± 0.002 mg/mL, respectively. During C₄mimOAc pretreatment at 110 °C for 30 min, 4.08 ± 0.06 mg/mL glucose and 1.40 ± 0.006 mg/mL xylose was determined after enzymatic hydrolysis.
Figure 1. Glucose concentration (mg/mL) obtained after enzymatic hydrolysis of autohydrolysis and 1-n-butyl-3-methylimidazolium acetate (C₄mimOAc)-pretreated bagasse.

Figure 2. Xylose concentration (mg/mL) obtained after enzymatic hydrolysis of autohydrolysis and C₄mimOAc-pretreated bagasse.

2.2. Fermentation

Fermentation of the pretreated samples of bagasse for bioethanol production is shown in Figure 3. The results revealed that different yeast strains have different abilities to produce ethanol from the available sugar. Ethanol production on bagasse pretreated by autohydrolysis at 110 °C for 30 min was 28.42 ± 0.12 mg/g-substrate when it was fermented with *S. cerevisiae* (Lalvin EC-118). The same strain exhibited a maximum ethanol production of 66.02 ± 0.65 mg/g-substrate on the fermentation of bagasse pretreated at 190 °C for 10 min. *S. cerevisiae* strains are not able to ferment xylose, but are more efficient in glucose fermentation [12]. The autohydrolysis of sugarcane bagasse at 205 °C for 6 min could support the maximum ethanol production of 70.92 ± 0.09 mg/g-substrate when *S. cerevisiae* MZ-4 was used as the fermenting organism. A comparatively better ethanol production of
78.78 ± 0.94 mg/g-substrate was obtained from C₄mimOAc pretreated bagasse at 110 °C for 30 min, and fermented with S. cerevisiae MZ-4 strain.

Figure 3. Ethanol content (mg/g-substrate) obtained after fermentation of autohydrolyzed and C₄mimOAc-pretreated bagasse by using four different yeast strains.

3. Discussion

When pretreatment temperature was increased, a higher concentration of glucose was obtained. The major effect found by autohydrolysis on lignocellulosic material was removing the hemicellulose and thus reducing its hindrance, and improving the access of enzymes to the cellulose [20,21]. This effect can be clearly observed in Figure 1, where increased pretreatment temperature during autohydrolysis enhanced glucose release during enzymatic hydrolysis but xylose concentration was reduced because some amount of xylan had already been removed during autohydrolysis [19]. Reduction in xylose concentration at 205 °C can be attributed to the removal of some hemicellulose content during high temperature autohydrolysis, while low xylose concentration at 110 °C might be due to more lignin hindrance [19,22]. Many studies have confirmed the same effect of high-temperature autohydrolysis on the reduction of hemicellulose content [21,23]. It was mentioned in our previous study that C₄mimOAc also had a significant effect on hemicellulose; however, compositional analysis and Fourier transform infrared spectroscopy (FTIR) data of pretreated bagasse revealed that the effect of ionic liquid on hemicellulose was still milder as compared to autohydrolysis [19].

As proved by the production of the maximum ethanol titer, S. cerevisiae MZ-4 strain can be considered a more tolerant yeast regarding ionic liquid pretreatment conditions as compared to other yeast strains used in this study. ATCC strain S. stipitis is considered as comparatively more efficient in fermenting xylose to ethanol, but its lower production with all pretreated samples can be attributed to its less efficient glucose utilization as compared to S. cerevisiae [24]. Moreover, it was also suggested in previous studies that all symporters (i.e., the proteins that assist in passage of molecules through the plasma membrane) in S. stipitis are competitively inhibited by glucose molecules, which makes it difficult to utilize both sugars simultaneously, hindering the production of ethanol from xylose [25]. The difference in amount of ethanol produced by these strains can be partially attributed to their difference in tolerance against side-products released during different pretreatment conditions and enzymatic hydrolysis. However, it is very important to optimize both ionic liquid pretreatment and fermentation conditions for this ethanol-producing MZ-4 strain in order to further enhance yields and make the process less expensive. Moreover, previous studies have reported that the formation of inhibitors is limited in ionic liquid (IL) pretreatments, but the remnants of ILs in pretreated materials...
can adversely affect the enzymes and microorganisms [22,26]. Therefore, further studies are required to examine the impact of C₄mimOAc remaining on fermentation, especially under low-process water consumption. Although S. cerevisiae strains were found to be comparatively better for the production of bioethanol from C₄mimOAc-pretreated bagasse, they usually do not have the ability to ferment xylose. Therefore, a large amount of xylose must remain in the fermentation broth. Further research is required to overcome this unutilized xylose. Different molecular strategies such as protoplast fusion or genetic recombination can be tried in the future to induce xylose utilizing abilities in strain MZ-4.

4. Materials and Methods

4.1. Yeast Strains

Three strains of S. cerevisiae and one strain of S. stipitis (ATCC 58785) were used to determine an efficient yeast strain for enhanced ethanol production from autohydrolyzed and C₄mimOAc-pretreated sugarcane bagasse. A new strain of S. cerevisiae (Gene bank accession number: KP970869) labeled MZ-4 isolated from fresh grapes, and two commercial strains of S. cerevisiae (i.e., Lalvin EC-1118 and Uvaferm-43 previously reported for wine production from fresh grape juice [27–29]) were selected. All strains were cultured on YPD (yeast extract, peptone, dextrose) medium [27]. After 24 h, the culture was reached at late exponential phase of growth, which was centrifuged (CS-6R Centrifuge, Beckman, Fullerton, California, United States) for 5 min at 2500 rpm. Cells obtained as pellet were washed twice using sterile saline (0.9% NaCl) [30], and kept in the same solution to use for further experiments. Yeast cultures were stained with methylene blue, and live cells were counted with the help of a haemocytometer [31].

4.2. Fermentation of Pretreated Sugarcane Bagasse

Sugarcane bagasse was pretreated using C₄mimOAc (110 °C for 30 min) and autohydrolyzed (110 °C for 30 min; 190 °C for 10 min, and 205 °C for 6 min). All bagasse samples were washed with water, and then 1 g of substrate was added in 100 mL 50 mM sodium citrate buffer (pH 4.8) for enzymatic hydrolysis. Enzymatic hydrolysis (with 20 FPU cellulase and 40 IU β-glucosidases) was carried out at 50 °C, 150 rpm for 72 h [19]. The hydrolysate was fermented by the aforementioned four yeast strains in separate, and 5% (v/v) (containing 3 × 10⁸ living cells/mL) of each inoculum was added into 50 mL of hydrolyzed broth in each fermentation vessel. Fermentation was carried out after adjusting pH of the medium to 5.0 by drop-wise addition of 5 N NaOH at 30 °C with 30 rpm so that the yeast remained suspended and to avoid shear stress due to high rpm [32]. Fermentation was carried out for 72 h for all sets of experiments; however, no change in ethanol content was observed after 12 h. The ethanol content was measured with the help of Megazyme ethanol kit. All the experiments were carried out in duplicate.

5. Conclusions

It was found that C₄mimOAc pretreatment [33] was more effective than autohydrolysis for sugarcane bagasse, as indicated from maximum ethanol yield. Moreover, S. cerevisiae MZ-4 showed better fermentability of glucose derived from C₄mimOAc pretreated bagasse, hence yielded higher amount of ethanol as compared to the rest of strains.

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