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Effect of post-pretreatment washing on saccharification and co-fermentation from bagasse pretreated with biocompatible cholinium ionic liquid

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ABSTRACT

Choline acetate (ChOAc), a cholinium ionic liquid (IL), was used for biomass pretreatment; thereafter, saccharification of the pretreated biomass and glucose/xylose co-fermentation were conducted with different number of post-pretreatment washes (1–5 and 10 washes). Besides saccharification and co-fermentation data, the inhibitory effect of ChOAc on the biocatalyst was compared with that of 1-ethyl-3-methylimidazolium acetate (EmimOAc). ChOAc showed less inhibitory effect on cellulase and yeast activity compared with EmimOAc. The cellulose and hemicellulose saccharification percentages of the IL-pretreated bagasse were approximately 90% and 60%, respectively, irrespective of ChOAc and EmimOAc with sufficient post-pretreatment washes. The cellulose and hemicellulose saccharification percentages after 5 washes were 82% and 59%, respectively, in the case of ChOAc and 51% and 13%, respectively, in the case of EmimOAc. The overall ethanol yields on the original bagasse basis after saccharification and co-fermentation of the IL-pretreated bagasse when washed 5 times were 54% and 22% in the case of ChOAc and EmimOAc, respectively. ChOAc-pretreated bagasse could be saccharified and co-fermented with fewer washes than EmimOAc-pretreated bagasse.

1. Introduction

Some types of ionic liquids (ILs; generally defined as organic salts that melt below 100 °C) have been demonstrated to dissolve cellulose [1]. Moreover, the reprecipitated cellulose after dissolution in ILs has been shown to have a higher efficiency for enzymatic saccharification, because its crystallinity is significantly decreased [2]. This IL-assisted pretreatment method has been applied to several types of lignocellulosic materials [3]. Moreover, the IL-assisted pretreatment method has been demonstrated to be more effective than the conventional pretreatment method using diluted acid or ammonia [4–7]. In the case of diluted acid pretreatment, most hemicellulose is hydrolyzed and washed out during the pretreatment procedure. On the other hand, hemicellulose can remain in the IL-pretreated biomass, indicating that the IL-pretreated biomass can be suitable for glucose/xylose co-fermentation after enzymatic saccharification [7].

However, the IL-pretreated biomass needs to be washed extensively to remove the residual IL, because even a small amount of residual IL in the pretreated biomass inhibits cellulolytic enzymes and fermentative microbes in the subsequent saccharification and fermentation steps [8,9]. These inhibitory effects are much more marked, particularly in the case of high solid loading of IL-pretreated biomass to the saccharification and fermentation processes. Extensive washing of the pretreated biomass results in large amounts of diluted IL aqueous solution, which leads to high costs for both concentrating the IL from its diluted aqueous solution by evaporation and treating the resultant wastewater.

Completely bio-derived cholinium ILs have been reported, which contain either cholinium cations combined with amino acid-based anions [10] or carboxylic acid-based anions [11]. Moreover, it has been demonstrated that these cholinium ILs could be used for biomass pretreatment to enhance enzymatic saccharification of lignocellulosic biomass [12–18], similar to conventional imidazolium

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ILs. Furthermore, our previous study demonstrated that cholinium ILs were less inhibitory to cellulase enzymes and fermentative microorganisms than imidazolium ILs [13,18]. For imidazolium ILs, there are a few studies reporting the effect of the number of post-pretreatment washes on the resulting inhibition of enzymatic saccharification of the IL-pretreated biomass [19,20]. However, to the best of our knowledge, there are no studies reporting the effect of the number of post-pretreatment washes on both saccharification and co-fermentation of the cholinium IL-pretreated biomass.

Therefore, the present study investigated the effect of the number of post-pretreatment washes on the saccharification and subsequent co-fermentation of the cholinium IL-pretreated biomass. Choline acetate (ChOAc) was used as the biocompatible cholinium IL. The data for enzymatic saccharification and subsequent microbial ethanol co-fermentation were compared with data obtained using 1-ethyl-3-methylimidazolium acetate (EmimOAc), the conventional imidazolium IL, frequently employed for biomass pretreatment.

2. Materials and methods

2.1. Biomass, IL, cellulase, and yeast cells

Bagasse powder with a particle size of >200 μm was purchased from Toyota Motor Corporation (Miyoshi, Japan). ChOAc was prepared using a one-pot neutralization method with minor modifications [21]. EmimOAc was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Commercial cellulase (Cellic® CTec2), a complex blend of cellulase, hemicellulose, and β-glucosidase, was obtained from Novozymes Japan Ltd. (Chiba, Japan). Filter paper unit (FPU) of the cellulase was determined by the NREL method [22]. All other chemicals were from commercial sources and of reagent grade. *Saccharomyces cerevisiae* YPH499XU, which co-expresses xylose reductase from *Pichia stipites*, xylitol dehydrogenase from *P. stipites* and xylulokinase from *S. cerevisiae* [23], was used throughout the study as the test yeast strain assimilating both glucose and xylose.

2.2. Assay of yeast growth inhibition by ILs

For evaluating the inhibitory effects of IL to yeast, the culture was conducted in a 5-mL test tube containing autoclaved YPD medium (10 g/L of yeast extract, 20 g/L of Bacto-peptone, and 20 g/L of glucose; Nacalai Tesque, Kyoto, Japan) supplemented with the IL (EmimOAc or ChOAc) at final concentrations of 0.3–5% (w/w) The yeast cells were precultured aerobically at 30 °C in the test tube containing 5 mL of YPD medium free from IL. The precultured broth was transferred into a test tube at an initial optical density of 0.1 at a wavelength of 660 nm (OD_{660}). The test tube was incubated at 30 °C for 48 h at 48 rpm using a shaking incubator (Compact Rocking Incubator TVS062CA, Advantec Toyo Kaisha, Ltd., Tokyo, Japan) with the OD_{660} value being monitored at 10-min interval. The response to the IL was evaluated based on the relative growth, which was defined as the percentage of the OD_{660} at 48 h in the presence of the IL relative to the OD_{660} at 48 h of a control culture free from the IL. The median effective concentration based on yeast growth (yEC_{50}) was determined as the IL concentration at which the relative growth was reduced to 50%.

2.3. IL-assisted pretreatment and washing of biomass

For biomass pretreatment, 0.5 g of bagasse powder was added to a 15-mL polypropylene tube (Corning Inc., New York, United States) containing 5 g of IL (EmimOAc or ChOAc). After vortexing, the biomass/IL mixture in the tube was sonicated for 30 min at 28 kHz and an emission power of 25 W using an ultrasonic processor (UD-211 with sonotrode TP-040, Tomy Seiko Co., Ltd., Tokyo, Japan) in a water bath maintained at 25 °C, so as to enhance the IL assisted pretreatment [15].

For washing out the IL from the pretreated biomass, 45 mL of deionized water was added to the bagasse/IL mixture in a 50-mL tube, which resulted in the precipitation of the biomass. After stirring, the 50-mL tube was centrifuged (8000 × *g*) for 10 min at 25 °C, and the supernatant was removed. The washing procedure was repeated 1–5 and 10 times. The precipitated wet bagasse was used for subsequent enzymatic saccharification.

2.4. Saccharification and co-fermentation at high loading

Saccharification was performed in a 50-mL polypropylene tube (Corning Inc.) containing the wet pretreated biomass (water content was adjusted to 90%, which resulted in 10% solid loading) and CTec2 at a concentration of 20 FPU/g pretreated biomass. The tube was set in a heat block (Thermo Block Rotator SN-06BN; Nissinrika Co., Tokyo, Japan) and axially rotated at 35 rpm under a controlled temperature of 50 °C. During the enzymatic reaction, liquefied samples were collected after 3, 24, 48, and 72 h and then heated at 90 °C for 5 min to inactivate the enzyme. After centrifugation of the heated samples at 21,500 × *g* for 1 min, the supernatant was subjected to glucose and xylose measurement. Cellulose saccharification was evaluated as the percentage of cellulose hydrolyzed into glucose compared with cellulose in the original bagasse. Similarly, hemicellulose saccharification was evaluated as the percentage of hemicellulose hydrolyzed into xylose compared with hemicellulose in the original bagasse.

After enzymatic saccharification for 72 h, the 50-mL tube was centrifuged (8000 × *g*) for 10 min at 25 °C, and the supernatant was filtered using a 0.45-μm filter to obtain a sterile sugar solution for subsequent fermentation. As an inoculum for fermentation, the yeast was aerobically cultured for 72 h at 30 °C and 150 rpm in 5 mL of YPD medium. The yeast cells were harvested by centrifugation at 3000 rpm for 5 min and washed twice with distilled water. Fermentation was then performed in a 50-mL polypropylene tube closed with a silicon plug (AS ONE, Osaka, Japan), into which a hole was bored using a disposable needle (Terumo Corp., Tokyo, Japan). The obtained sugar solution, water, and yeast suspension were added to a 50-mL test tube to obtain the working volume of 10 mL and a final OD_{600} of 20 (approximately equivalent to 100 g-wet cells/L). Nutrients such as yeast extract and peptone were not added to the fermentation mixture. The tube was set in a reciprocating shaker maintained at 30 °C. During fermentation, samples were collected after 3, 24, and 48 h. After centrifugation of the samples at 21,500 × *g* for 1 min, the supernatant was subjected to glucose, xylose, and ethanol measurement. The overall ethanol yield on a bagasse basis was evaluated as the percentage of ethanol compared with the ethanol theoretically converted from carbohydrate in the original bagasse. Similarly, the ethanol yield on a sugar basis was evaluated as the percentage of ethanol compared with the ethanol theoretically converted from glucose and xylose obtained by saccharification.

2.5. Analysis of biomass composition

The cellulose, hemicellulose, and lignin contents of the original bagasse were determined according to the NREL method [24] with minor modifications. In brief, 0.1 g of the sample was mixed with 2 mL of a 72% (*v*/*v*) H_2SO_4 aqueous solution for 2 h at room temperature. The mixture was transferred to a 200-mL Erlenmeyer flask, diluted with 75 mL of water, and autoclaved at 121 °C for 15 min. The acid-diluted hydrolysate was filtered, after which