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Direct Ethanol Production from Ionic Liquid-Pretreated Lignocellulosic Biomass by Cellulase-Displaying Yeasts

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Abstract Among the many types of lignocellulosic biomass pretreatment methods, the use of ionic liquids (ILs) is regarded as one of the most promising strategies. In this study, the effects of four kinds of ILs for pretreatment of lignocellulosic biomass such as bagasse, eucalyptus, and cedar were evaluated. In direct ethanol fermentation from biomass incorporated with ILs by cellulase-displaying yeast, 1-butyl-3-methylimidazolium acetate ([Bmim][OAc]) was the most effective IL. The ethanol production and yield from [Bmim][OAc]-pretreated bagasse reached 0.81 g/L and 73.4% of the theoretical yield after fermentation for 96 h. The results prove the initial concept, in which the direct fermentation from lignocellulosic biomass effectively promoted by the pretreatment with IL.

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Introduction

Given the inevitable exhausting of fossil fuels and environmental issues such as global warming and acid rain, the utilization of biomass as a source of fuels and fine chemicals has recently become an attractive option. In particular, bioethanol produced from biomass represents a promising alternative fuel. Currently, the main feedstock for bioethanol production is starch-rich biomass, as it is easily and rapidly hydrolyzed [1]. However, lignocellulosic biomass such as bagasse is regarded as a more promising feedstock for bioethanol production, because it is abundant, inexpensive, renewable, and has favorable environmental properties [2]. Despite these advantages, lignocellulosic biomass is much more difficult to process than grains because of the need for extensive pretreatment and relatively large amounts of cellulases for efficient hydrolysis. Therefore, efficient and cost-effective methods for the degradation and fermentation of lignocellulosic biomass to ethanol are required.

Among the many kinds of lignocellulosic biomass pretreatment methods, pretreatment with ionic liquids (ILs) is regarded as a promising strategy [3, 4]. ILs are novel solvents that possess unique properties such as negligible volatility and thermal stability [5]. ILs are reportedly able to dissolve various kinds of biopolymers, including cellulose, under moderate conditions (e.g., mostly under 100 °C, or in some cases at room temperature), and can be regenerated by the addition of poor solvents of cellulose such as water and alcohol [3, 5–7]. The highly crystalline structure of cellulose can be changed to amorphous during dissolution and regeneration. Recent studies have revealed that cellulose pretreated with IL (i.e., regenerated cellulose from an IL solution) can be hydrolyzed by cellulases more rapidly than crystalline cellulose [8, 9]. Furthermore, several reports have mentioned the saccharification of lignocellulosic biomass pretreated with ILs [10–14]. In a previous study, direct ethanol production from pure cellulose, Avicel, which had been pretreated with IL by using cellulase-displaying yeast, was succeeded [15].

In the present study, the effect of four kinds of ILs when used in the pretreatment of lignocellulosic biomass such as bagasse, eucalyptus, and cedar was evaluated. In addition, direct ethanol fermentation from lignocellulosic biomass incorporated with IL by cellulase-displaying yeast was performed.

Experimental Section

ILs and Biomass

1-Ethyl-3methylimidazolium chloride ([Emim][Cl]) and 1-ethyl-3methylimidazolium acetate ([Emim][OAc]) were purchased from Sigma-Aldrich, and 1-ethyl-3methylimidazolium diethyl phosphate ([Emim][DEP]) and 1-butyl-3-methylimidazolium acetate ([Bmim][OAc]) were acquired from Solvionic (Toulouse, France). Commercial cellulase Cellic® CTec1 and HTec1 was supplied by Novozymes Japan. All other chemicals used in this study were of reagent grade.

Bagasse, eucalyptus, and cedar with particle sizes of 200 μm were supplied by Toyota Motor Corporation (Japan). The sugar compositions were determined by Toray Techno Co.,

Ltd. (Japan) using the procedure published by the National Renewable Energy Laboratory [16] and are shown in Table 1.

Pretreatment of Lignocellulosic Biomass with ILs

Bagasse, eucalyptus, and cedar were pretreated with ILs before saccharification and direct fermentation using cellulase-displaying yeast. For pretreatment, biomass (100 mg) was dispersed in IL with a final IL concentration of 200 mM (for example, 0.918 mL for [Emim][DEP]) with magnetic stirring at 120 °C for 30 min using an organic synthesizer, ChemStation PPS-CTRL (EYELA, Japan). After the pretreatment, a highly viscous biomass solution was obtained. Regenerated biomass was formed by adding 5 mL of sodium acetate buffer (200 mM, pH 5.0) to each of the cellulose solutions, which were then used as substrates for saccharification and direct fermentation experiments as described in each of the following sections.

Saccharification of Biomass Pretreated with IL

Concentrated nutrients (40 g/L yeast extract [Difco Laboratories, Detroit, USA] and 80 g/L bacto-peptone [Difco Laboratories], 5 mL), Celic CTec1 (20 μL), and Celic HTec1 (5 μL) were added to a sterilized glass tube containing biomass (100 mg) that had been pretreated with IL, along with acetate buffer (5 mL), as described above. Then, the final volume was adjusted to 20 mL by the addition of sterilized water, and thus, the final biomass concentration was 5 g/L. Saccharification was conducted at 30 °C with stirring at 150 rpm. The samples were periodically collected from the culture medium and centrifuged at 12,000×*g* to remove insoluble residues, and then, glucose and xylose concentrations were analyzed. The production of glucose and xylose was calculated with the exception of the initial sugar that was derived from Celic CTec1 and HTec1. The glucose and xylose yields were calculated as g-(produced glucose or xylose) / g-(glucose or xylose in biomass).

Direct Fermentation of Biomass Pretreated with IL by Cellulase-Displaying Yeast

Previously developed cellulases displaying *Saccharomyces cerevisiae* MT8-1/cocδBEC1 was used for the direct fermentation of biomass that had been pretreated with IL [17]. In brief, MT8-1/cocδBEC1 was constructed as follows. *S. cerevisiae* MT8-1 was used as the host strain for cellulase display. Cellulases displayed on the yeast cell surface were endoglucanase II and cellobiohydrolase II from *Trichoderma reesei*, and β-glucosidase I from *Aspergillus aculeatus*. The three kinds of cellulase genes were simultaneously integrated into the *S. cerevisiae* chromosome. The displaying yeast with the highest cellulase activity at the optimized ratio of the cellulase genes was named MT8-1/cocδBEC1. The cellulase-displaying yeast cells were

Table 1 Sugar composition of lignocellulosic biomass

	Glucose (%)	Xylose (%)	Galactose (%)	Arabinose (%)	Mannose (%)
Bagasse	39.8	25.9	0.7	2.2	0.3
Eucalyptus	43.5	17.8	1.5	0.4	1.2
Cedar	40.8	4.8	1.9	1.0	10.4

grown in an SD medium (6.7 g/L yeast nitrogen base without amino acids [Difco Laboratories] and 20 g/L glucose [Nacalai Tesque, Kyoto, Japan]) supplemented with appropriate amino acids and nucleic acids at 30 °C with shaking at 220 rpm. Cells were collected by centrifugation at 1000×*g* for 5 min and were washed three times with sterilized water. Direct batch fermentation was carried out as with the saccharification experiment, with the exception of the addition of cultivated yeast cells (OD_{600} = 80, 5 mL) to the glass tube.

Direct repeated batch fermentation from bagasse pretreated with [Bmim][OAc] was carried out as with the direct batch fermentation experiment, with the exception of the addition of 2 μL of Celic CTec2 instead of 20 μL of Celic CTec1 and 5 μL of Celic HTec1. After 24 h of fermentation, yeast cells were collected by centrifugation at 50×*g* for 1 min, washed three times with sterilized water, and inoculated into fresh medium and the next batch fermentation was carried out.

Analysis

The supernatant of the saccharification and direct fermentation medium was diluted at the appropriate concentrations before glucose, xylose, and ethanol concentration analysis. The glucose and xylose concentrations were measured using a Glucose CII Test Wako (Wako Pure Chemical, Osaka, Japan) and a D-Xylose Assay Kit (Megazyme, Wicklow, Ireland), respectively. The ethanol concentration was measured by gas chromatography (GC) as described previously [15]. The theoretical ethanol yield was calculated from the glucose included in each biomass and Cellic CTec1 and HTec1, for a theoretical fermentation yield of 0.51 g-ethanol/g-glucose.

Results and Discussion

Enzyme Saccharification of Bagasse Pretreated with IL

To evaluate the degradability of bagasse, IL-pretreated bagasse was digested by a commercial cellulase cocktail of Cellic CTec1 and HTec1. As shown in Fig. 1, the bagasse pretreated with [Emim][OAc] showed the highest degradability. The produced glucose and xylose

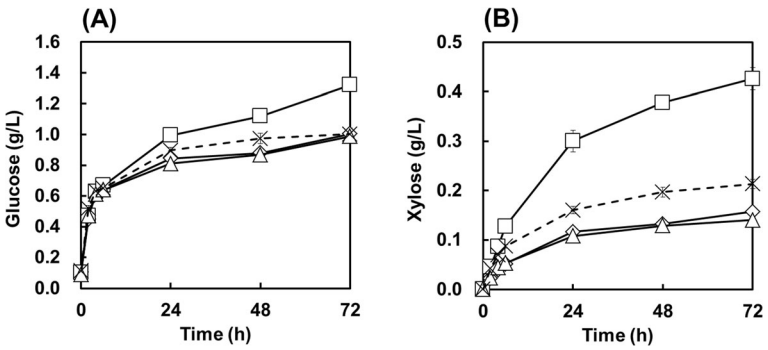

Fig. 1 Time course of glucose (**a**) and xylose (**b**) production in saccharification of IL pretreatment of bagasse: *diamonds*, [Emim][DEP]; *squares*, [Emim][OAc]; *triangles*, [Emim][Cl]; *crosses*, [Bmim][OAc]. Data are averages from three independent experiments (*error bars* represent SD)

concentrations reached 1.3 and 0.43 g/L, respectively, and the yield from the glucan and xylan fraction of the bagasse reached 61.2 and 33.2%, respectively.

The results above show that [Emim][OAc] demonstrated a high ability to convert the highly crystalline structure of cellulose to amorphous cellulose. This could have been because [Emim][OAc] has a low deactivation effect for cellulase compared with other ILs. Similarly, Xiao et al. reported that 200 mM of [Emim][OAc] had no effect on commercial cellulase activity in previous studies [18].

Direct Fermentation of Bagasse After Pretreatment with IL

To examine the ethanol productivity from the bagasse pretreated with IL, direct batch fermentation was carried out. As shown in Fig. 2a, the bagasse pretreated with [Bmim][OAc] showed the highest ethanol productivity after 96 h of fermentation. The ethanol production reached 0.81 g/L and the ethanol yield from glucan reached 73.4% of the theoretical yield. The bagasse pretreated with [Emim][DEP] showed the lowest ethanol productivity—2.2-fold lower than the bagasse pretreated with [Bmim][OAc]. As shown in Fig. 2b, after 24 h of fermentation, glucose concentration was not detectable in any of the bagasse that had been pretreated with IL. By contrast, as shown in Fig. 2c, following fermentation, xylose accumulation was detected in all samples of bagasse that had been pretreated with IL.

To examine the stability of fermentation, direct repeated batch fermentation from the bagasse pretreated with [Bmim][OAc] was carried out. As shown in Fig. 3, ethanol production in 24 h was gradually increased with increasing repetition. In the fourth batch, ethanol production reached 0.69 g/L in 24 h of fermentation.

In the saccharification experiment, the bagasse pretreated with [Emim][OAc] showed the highest degradability (Fig. 1). However, in direct fermentation, the bagasse pretreated with [Bmim][OAc] showed the highest ethanol productivity (Fig. 2). Furthermore, efficient four times repeated batch fermentation from bagasse pretreated with [Bmim][OAc] was achieved (Fig. 3). These were because although [Emim][OAc] has high crystallinity reduction activity, it also exerts a deactivation effect on yeast cells [19], and [Bmim][OAc] may have low inhibitory effect on yeast cells. In a previous study, 200 mM [Emim][OAc] showed a high inhibition effect on yeast fermentation ability [15]. However, eukaryote *S. cerevisiae* seemed to have relatively high tolerance to ILs compared with bacteria such as *Escherichia coli*, *Bacillus subtilis* [20], and *Clostridium sporogenes* [21]. Besides, some other research groups demonstrated the high ILs tolerance of *S. cerevisiae* [22–24]. In a previous study, we also demonstrated the high ethanol productivity of *S. cerevisiae* MT8-1, which is the same host strain used

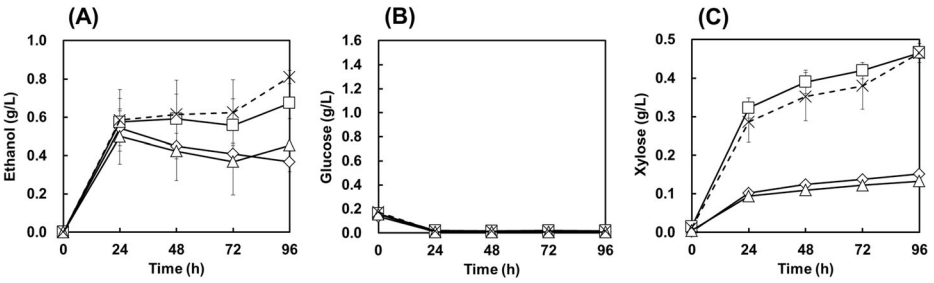

Fig. 2 Time course of ethanol (**a**), glucose (**b**), and xylose (**c**) concentration in direct batch fermentation from IL pretreatment of bagasse: *diamonds*, [Emim][DEP]; *squares*, [Emim][OAc]; *triangles*, [Emim][Cl]; *crosses*, [Bmim][OAc]. Data are averages from three independent experiments (*error bars* represent SD)

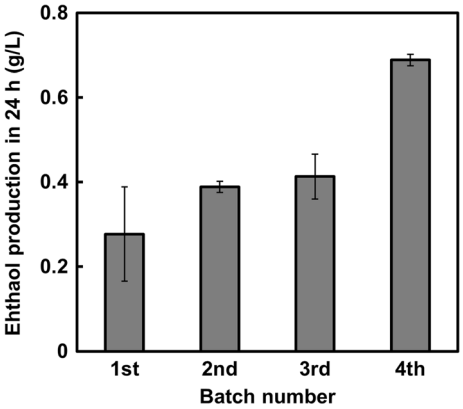

Fig. 3 Direct repeated batch fermentation from [Bmim][OAc] pretreatment of bagasse. Data are averages from two independent experiments (*error bars* represent SD)

in this study, from glucose in the presence of less than 200 mM of [Emim][DEP], [Emim][Cl], or [Emim][OAc] [15]. Thus, yeast *S. cerevisiae* could be a promising microorganism for bioprocess incorporated with ILs. To realize effective fermentation from the IL pretreatment of bagasse, it is important to consider not only the crystallinity reduction activity but also the inhibitory effect on the yeast cells and enzymes of ILs.

The ethanol production from bagasse that had been pretreated with [Bmim][OAc] for 72 h (0.62 g/L) was higher than the theoretical value calculated from the saccharification experiment (0.51 g/L) (Figs. 1 and 2). This could have been because both the amount of glucose and the effect of cellooligosaccharide inhibition for cellulases were reduced and cellulase-displaying yeast had degraded the bagasse. For these reasons, a direct fermentation process using bagasse that has been pretreated with IL could be effective for bioethanol production.

The issues about IL recovery and low concentration of ethanol in direct fermentation are still unsolved in study. Most of the ILs are still expensive compounds, and usage of ILs increases the cost for biomass pretreatment [25]. Thus, recovery and reuse of ILs are important for cost-effective biomass pretreatment. In a previous study, we demonstrated the efficient recovery and reuse of ILs from mixture of culture media and IL by using organic solvents [15]. Furthermore, other research groups demonstrated the recovery of ILs from hydrolysate of lignocellulosic biomass by electrodialysis [26, 27]. The issue about recovery and reuse of ILs from mixture of culture media, yeast cells, lignocellulosic biomass, and IL should be solved for efficient and cost-effective biomass pretreatment with IL in future works. Besides, the produced ethanol concentration in direct fermentation remained low for practical fermentation because of low concentration of initial lignocellulose substrate. The low tolerance of yeast *S. cerevisiae* for high concentration of ILs is one of the bottleneck problems for improving produced ethanol concentration (data not shown). Thus, to improve IL tolerance of yeast *S. cerevisiae* and explore efficient and low toxic ILs for yeast should be important for more practical direct ethanol fermentation from IL-pretreated biomass.

Xylose was accumulated through fermentation (Fig. 2c). The cellulase-displaying yeast used in the present study had no xylose-assimilating ability. Thus, ethanol productivity could be improved by providing xylose assimilation to the cellulase-displaying yeast [28].

Table 2 Profiles of IL pretreatment of eucalyptus and cedar after 72 h of saccharification

Biomass	Ionic liquid	Produced glucose (g/L)	Glucose yield (%)	Produced xylose (g/L)	Xylose yield (%)
Eucalyptus	[Emim][DEP]	0.590 ± 0.022	27.1	0.044 ± 0.002	4.9
	[Emim][OAc]	0.926 ± 0.016	42.6	0.180 ± 0.007	20.2
	[Emim][Cl]	0.628 ± 0.022	28.9	0.043 ± 0.004	4.8
	[Bmim][OAc]	0.796 ± 0.006	36.6	0.096 ± 0.003	10.8
Cedar	[Emim][DEP]	0.680 ± 0.013	33.4	0.018 ± 0.002	7.5
	[Emim][OAc]	0.859 ± 0.025	42.1	0.025 ± 0.004	10.6
	[Emim][Cl]	0.599 ± 0.025	29.4	0.016 ± 0.004	6.5
	[Bmim][OAc]	0.715 ± 0.045	35.0	0.022 ± 0.005	9.0

Data are averages from three independent experiments (± represent SD)

Saccharification and Direct Fermentation from IL-Pretreated Hard Biomass

To evaluate the effectiveness of IL treatment for hard biomass, saccharification and direct fermentation experimentation was carried out using eucalyptus and cedar. As shown in Table 2, the highest glucose production was achieved from eucalyptus and cedar that had been pretreated with [Emim][OAc]. In the case of direct fermentation, however, the utilization of [Bmim][OAc] for pretreatment was better than [Emim][OAc]. Thus, subsequent ethanol production from eucalyptus and cedar pretreated with [Bmim][OAc] was attained (0.22 and 0.24 g/L)—the yield was 18.3 and 21.2% of the theoretical yield after 72 h of fermentation, respectively.

For both eucalyptus and cedar, [Emim][OAc] pretreatment was the most effective for saccharification, as with the IL pretreatment of bagasse (Fig. 1). From these results, [Emim][OAc] could be an effective IL for the saccharification of various forms of lignocellulosic biomass.

The ethanol yield from the IL pretreatment of eucalyptus and cedar remained low by comparison with that from bagasse. This was because hard biomass such as eucalyptus and cedar is less degradable than a soft biomass such as bagasse. It is important to improve pretreatment efficiency and cellulase activity for efficient ethanol production from hard biomass in future work.

Conclusion

The concept was proved by the results of the present study, and direct fermentation from the IL pretreatment of bagasse, eucalyptus, and cedar by using cellulase-displaying yeast was succeeded. [Bmim][OAc] was the most effective IL for direct fermentation. To realize effective direct fermentation, it is important to consider various factors such as the crystallinity reduction effect and the inhibition effect for enzymes and yeasts of ILs. Future work must develop ILs that can fulfill every requirement and cellulase-displaying yeast with the highest cellulase activity, and the direct fermentation process using IL pretreatment of biomass is a promising strategy.

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