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Research Report

Ionic Liquid-based Consolidated Bioprocessing (i-CBP) for Cellulosic Ethanol Production

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ABSTRACT Biomass has received attention for use as a main feedstock in biorefinery processes such as cellulosic ethanol production. The enzymatic hydrolysis of lignocellulose to fermentable sugars is considered to be an effective and environmentally friendly approach to ethanol production. However, the spontaneous crystallization of cellulose and the high degree of hydrogen bonding between cellulose chains make cellulose inaccessible to saccharification enzymes. Ionic liquids have attracted significant attention as potent and effective solvents for the pretreatment of biomass to achieve efficient processing under low energy conditions. We have designed here a novel concept referred to as ionic liquid-based consolidated bioprocessing (i-CBP) for the efficient production of ethanol from biomass. Three fundamental requirements for i-CBP were developed: 1) selection of an optimal ionic liquid for soft or hard biomass pretreatment, 2) recycling and reuse of the ionic liquid, and 3) genetically-engineered yeast that produce optimal cellulolytic enzymes for saccharification of biomass pretreated with ionic liquid. Through a combination of these technologies, we have demonstrated that biomass pretreated with appropriate ionic liquids can be easily hydrolyzed to glucose and directly converted to ethanol using functional transgenic yeasts.

KEYWORDS Bioethanol, Ionic Liquid, Consolidated Bio Processing, Enzymatic Hydrolysis, Transgenic Yeast

1. Introduction

Lignocellulosic biomass has attracted attention as an abundantly available and renewable resource for the production of biofuels and bio-based polymers in sustainable biorefinery processes.[1] There are many challenges related to the use of cellulosic materials for fuels and chemicals. However, one of the most serious problems is the difficulty in hydrolyzing lignocellulose because cellulose in biomass is intermingled with lignin and hemicelluloses. In addition, cellulose in biomass is a highly crystalline polymer because of multivalent inter- and intramolecular hydrogen bonding. The rigid structure of this material hampers the function of saccharification enzymes and makes it difficult to hydrolyze.[2] Therefore, to solve this problem, an effective technology for the pretreatment of lignocellulosic biomass under low energy conditions is required before the cellulose saccharification process. The concept of this lignocellulose pretreatment is to reduce the cellulose crystallinity, and to increase the accessibility of saccharification enzymes to cellulose by the removal of hemicellulose and lignin. For this purpose, various pretreatment technologies including physical, chemical, physicochemical and biological approaches have been reported.[3] However, high energy requirements, slow reaction rates, and the significant cost associated with disposal and recovery of reagents are pointed out as problems in the biomass pretreatment process.

Ionic liquids are novel solvents that have significant scope for biomass pretreatment under low energy conditions. Ionic liquids are molten salts at room temperature, and they have unique properties such as thermal stability, non-volatility, and designable solvent.[4] Ionic liquids have been reported to be able to readily dissolve various types of biopolymers, including cellulose, amylose, and silk fibroin at temperatures under 100°C, and can then be regenerated by the addition of poor solvents such as water and alcohol.[5-7] Recent studies have demonstrated that cellulose pretreated with an ionic liquid can be more rapidly hydrolyzed by cellulase than crystalline cellulose.[8,9] In this reaction, the crystalline cellulose

is changed to amorphous, and the amorphous cellulose can be more accessible to saccharification enzymes and thus more easily degraded (**Fig. 1**). 1-Ethyl-3-methylimidazolium acetate ([Emim][OAc]) is an effective solvent for biomass pretreatment, more than diluted sulfuric acid pretreatment, for the removal of lignin and to increase the hydrolysis performance, and this solvent can also be recycled and reused.[10,11] Moreover, 1-ethyl-3-methylimidazolium diethyl phosphate ([Emim][DEP]) exhibited direct enzymatic saccharification of cellulose in an ionic liquid/water mixture, which indicated that saccharification enzymes can be substantially active in the presence of some ionic liquids.[12]

The use of ionic liquid as a potent reagent for the efficient pretreatment of lignocellulosic biomass has attracted significant attention. However, there has been little research on the use of a continuous process from biomass to ethanol using an ionic liquid pretreatment. The production of cellulosic ethanol from biomass generally consists of separate three steps: 1) pretreatment of biomass by chemical or physical processes, 2) saccharification of cellulose by enzymes, and 3) fermentation to produce ethanol by microorganisms. We have proposed a novel process that combines these three steps using an ionic liquid, and the overall process is referred to as ionic liquid-based consolidated bioprocessing (i-CBP) (**Fig. 2**). In this study, we have developed three fundamental requirements for i-CBP: 1) selection of an optimal ionic liquid for soft or hard biomass, 2) recycling and reuse of the ionic liquid, and 3) the use of genetically-engineered yeast to produce optimal cellulolytic enzymes for biomass pretreated with ionic liquid. Cellulosic ethanol fermentation from biomass pretreated with ionic liquid was performed using transgenic yeast to produce saccharification enzymes.

2. Screening of Effective Ionic Liquid for Biomass Pretreatment.

A recent study showed [Emim][OAc] exhibited better performance for biomass pretreatment than diluted sulfuric acid and increased hydrolysis rates.[13] We expected there could be more efficient ionic liquids for biomass saccharification because there have not been many reports on this subject. Therefore, we have focused on pyridinium-type ionic liquids (1-butyl-3-methylpyridinium chloride; [Bmpy][Cl]). [Bmpy][Cl] has been reported to be capable of dissolving microcrystalline cellulose and up to 12–39% of spruce sulfite pulp with a melting point temperature at 85°C for 12 h treatment.[14] However, there has not been any extensive research on [Bmpy][Cl] as a pretreatment solvent for the enzymatic saccharification of biomass. We first evaluated the potential of [Bmpy][Cl] pretreatment for enzymatic saccharification based on the structural characteristics

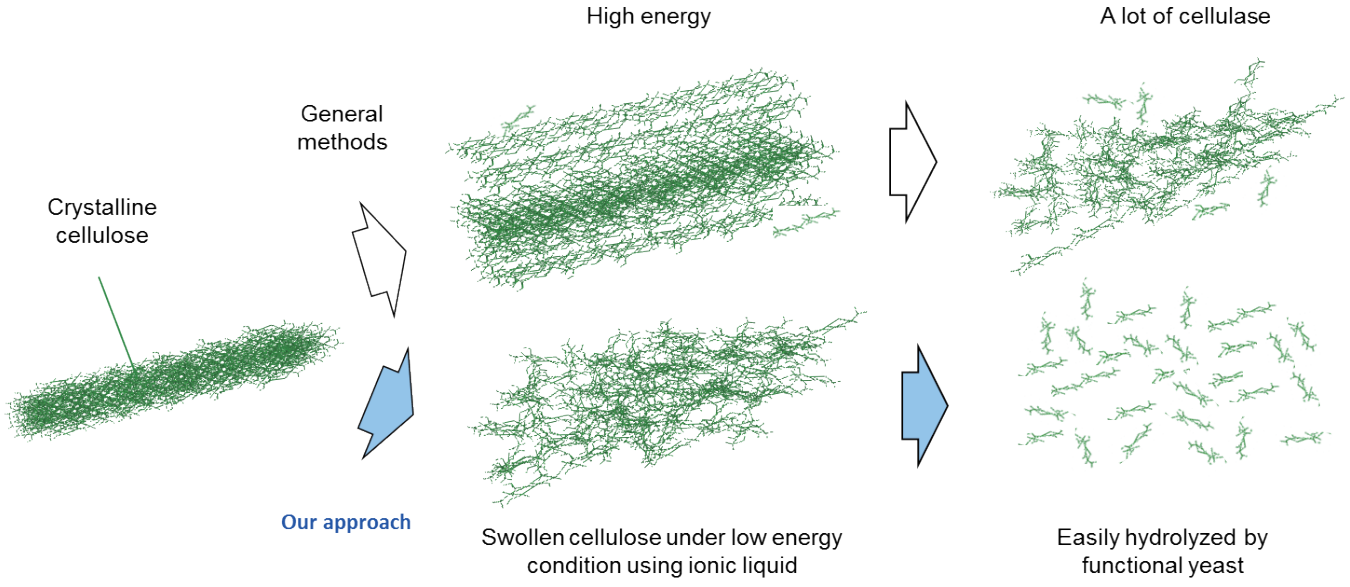

Fig. 1 The concept of the i-CBP (ionic liquid-based consolidated bioprocessing) approach. An ionic liquid is used as a solvent for the pretreatment of cellulose in biomass, whereby it can be hydrolyzed rapidly under low energy conditions.

of pretreated biomass.[15]

A solution of 5% (w/v) biomass was prepared by mixing Bagasse or *Eucalyptus* with [Bmpy][Cl]. The mixture was heated at 120°C for 60 min and then cooled down to room temperature. The material of regenerated biomass was prepared with adding deionized water and freeze dried. For comparison, [Emim][OAc] was used as a control ionic liquid. Enzymatic saccharification was conducted with 5% (w/v) of biomass at 40°C in 10 mM citrate buffer (pH 5.0) with a stirrer rotation of 200 rpm. The dried and regenerated biomass or untreated biomass samples were hydrolyzed with the cellulase mixture at 6 FPU/g of biomass. The commercial cellulase mixture consisted of Celluclast 1.5L and Novozyme 188 (Novozyme) at a volume ratio of 5:1, and the activity of this cellulase mixture was determined by measurement of the soluble reducing sugar. Samples to measure saccharide content, such as cellobiose and glucose, were taken between 0 and 48 h and analyzed using high performance liquid chromatography (HPLC). Comparison of the glucose concentration through biomass saccharification is shown in **Fig. 3**. Differences in the conversion rates between [Emim][OAc] and [Bmpy][Cl] pretreatments were especially noticeable during the initial phase (0 to1 h). For both biomass (Bagasse and *Eucalyptus*), an approximate 6- to 9-fold increase in conversion for 1 h was attained with the [Bmpy][Cl] pretreatment. This may result from a difference in the potential lowering of the cellulose crystallinity, which leads to higher initial rates of enzymatic saccharification. To better understand the kinetic results, the structural characteristics of the regenerated biomass were examined by X-ray diffraction (XRD) analysis.

The XRD analysis of the untreated biomasses exhibited three major peaks, as shown in **Fig. 4**. The first peak at 2θ = 16° was broad, and the next peaks appeared at 2θ = 21.9° and 22.1°, which are known as the crystalline peaks that indicate the presence of cellulose I.[16] The *Eucalyptus* showed higher and sharper diffraction pattern than Bagasse, which implies that *Eucalyptus* is qualitatively more crystalline than Bagasse. The patterns of both untreated biomasses showed type I cellulose or native cellulose with characteristics of highly crystalline cellulose. After pretreatment with ionic liquids, the intensity of the crystalline peaks decreased and shifted slightly to lower 2θ, and the peak at 34.4° disappeared. For the [Emim][OAc] pretreatment, all of the peaks disappeared; however, the crystalline peaks were

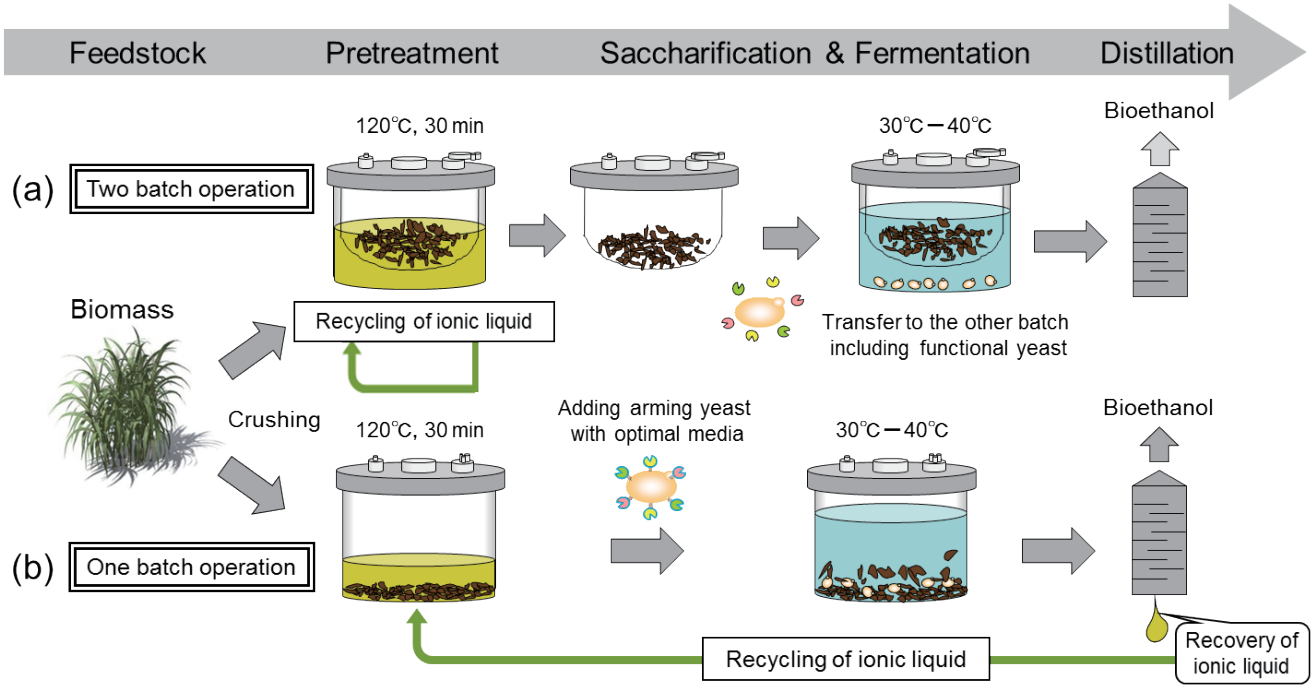

Fig. 2 Overall process of i-CBP for the production of ethanol from biomass. We demonstrated two process. (a) Two batch operation, (b) One batch operation. i-CBP provides effective cellulosic ethanol fermentation using transgenic yeast from biomass pre-treated with an ionic liquid.

reduced to lower intensity after 60 min pretreatment. On the other hand, a significant change in the diffraction patterns was obtained from the pretreatment with [Bmpy][Cl]. The pretreatment of both biomasses with this ionic liquid resulted in the clear splitting of the crystalline peaks (20° and 22° for both biomasses) and a new peak appeared at 12°, which indicates the characteristics of cellulose II.[17] These diffraction patterns could be attributed to mainly amorphous cellulose with a small fraction of cellulose II. For the 10 min pretreatment, although the peak around 12° was not clear, but a clear difference was observed at 2θ = 20°. Based on these results, both biomasses were converted to the cellulose II form by [Bmpy][Cl] pretreatment.

The biomass pretreated with [Bmpy][Cl] exhibited higher formation of glucose than those pretreated with [Emim][OAc] during the initial phase of enzymatic saccharification (Fig. 3). [Bmpy][Cl] pretreatment may promote the structural change from cellulose I to cellulose II, in which short cellulose chains are easier to be recrystallized to thermodynamically favored cellulose II during the cellulose regeneration process. Selection of the optimal operation temperature and reaction time for biomass pretreatment are essential factors for efficient saccharification because they

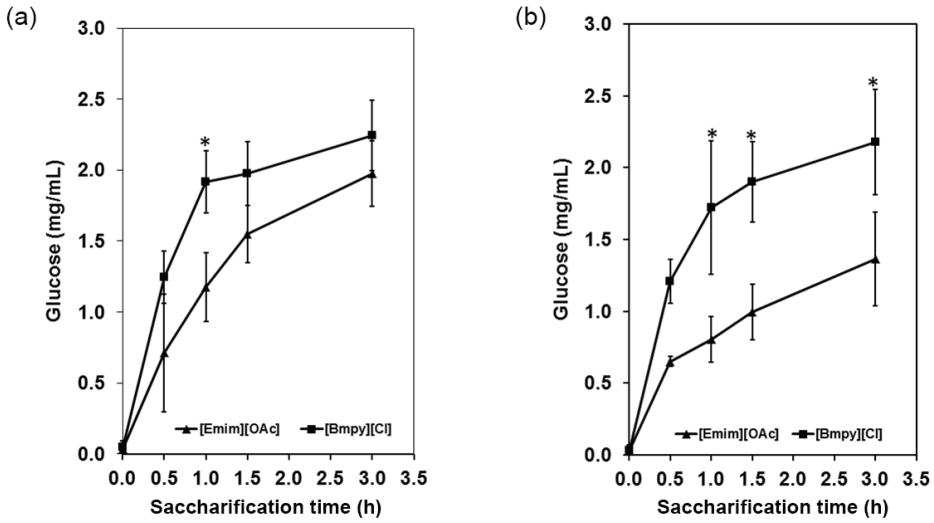

Fig. 3 Initial rates for the saccharification of biomass pretreated with [Emim][OAc] and [Bmpy][Cl] for 10 min (a) = Bagasse, (b) = *Eucalyptus*). Data indicate the mean ± standard deviation (SD) of four independent experiments. *p < 0.05 vs. [Emim][OAc] at the same saccharification time.

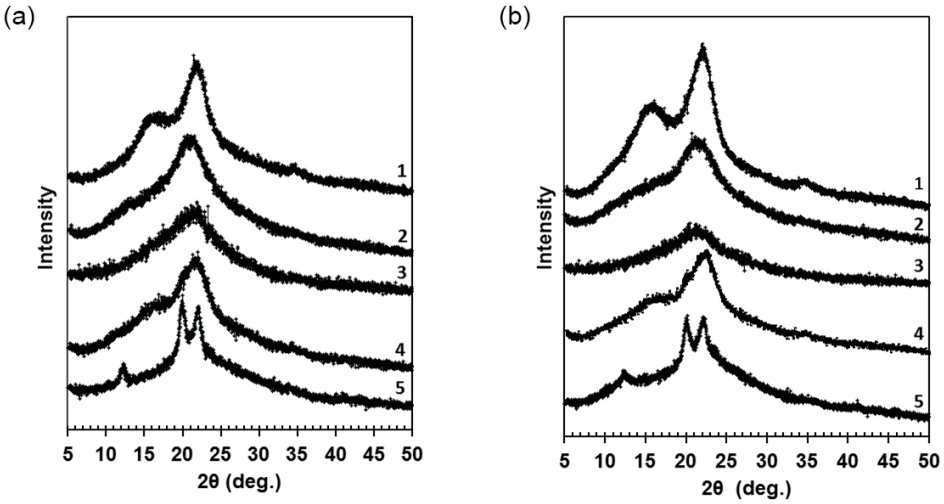

Fig. 4 XRD patterns for untreated and treated biomass (1 = untreated biomass, 2 = [Emim][OAc] for 10 min, 3 = [Emim][OAc] for 60 min, 4 = [Bmpy][Cl] for 10 min, 5 = [Bmpy][Cl] for 60 min) (a) = Bagasse, (b) = *Eucalyptus*).

influence the solubility of the cellulose and the required energy supply. For short time pretreatment, [Bmpy][Cl] exhibited higher potential to increase the initial rates of enzymatic saccharification than [Emim][OAc]. Enhancement of saccharification was clearly demonstrated with *Eucalyptus*, a hardwood. A pyridinium type of ionic liquid such as [Bmpy][Cl] thus has potential to enhance the pretreatment efficiency of biomass.

3. Recycling of Ionic Liquid and Identification of Optimal Cellulase Cocktail for Two Batch Operation

Most ionic liquids are still expensive, so the use of such organic solvents affects the cost for biomass pretreatment. To establish methods for the recovery and reuse of ionic liquid is thus important for the development of i-CBP. Therefore, a two batch operation was designed, which is characterized by separation of the pretreatment process from saccharification and fermentation for the recovery and reuse of ionic liquids (Fig. 2(a)).

We examined the effect of hard biomass saccharification using recycled ionic liquids. In this examination, 1-butyl-3-methylimidazolium acetate ([Bmim][OAc]) was selected as a pretreatment solvent because this ionic liquid was effective for the saccharification of various forms of lignocellulosic biomass (see to following section). A solution of 5 mg Bagasse was prepared with 1.0 g of [Bmim][OAc], and the mixture was heated at 120°C for 30 min. The used [Bmim][OAc] was expelled from the biomass and then reused for the next pretreatment step. This reaction was repeated 15 times at most. Enzymatic saccharification of each pretreated Bagasse was performed at 40°C for 48 h with a rotation speed of 200 rpm in 10 mM citrate buffer (pH 5.0) and the addition of the commercial cellulase mixture consisting of Celluclast 1.5L and Novozyme 188 at a volume ratio of 5:1, at 6 FPU/g of biomass. Each hydrolyzed sample was analyzed using HPLC and the saccharification efficiency was calculated based on the cellulose ratio in the Bagasse.

Figure 5 shows that there was no influence on the efficiency of enzymatic saccharification, even for the [Bmim][OAc] ionic liquid reused after 15 times of pretreatment. This was attributed to [Bmim][OAc] being able to maintain the ability to reduce the cellulose crystallinity, even after reuse. However, a decrease of the saccharification efficiency of approximately 5.0% was confirmed as the number of reuses increased. In this examination, the amount of the ionic liquid was decreased during reuse because the [Bmim][OAc] has soaked into the Bagasse by recycling two or more times. Ionic liquids are generally dissolved in polar organic solvents, such as acetone and ethanol, so that collection of the ionic liquids by solvent extraction from the pretreated biomass can be readily performed. This attempt at reuse is the first finding that confirmed the successful recycling of the ionic liquid for hard-biomass pretreatment over 15 times.

For the two batch operation, genetically engineered yeast that improves the effect of saccharification is also necessary. As for the *S. cerevisiae*, which is the microorganism mainly used for ethanol fermentation, has low ability for the production of secreted proteins. Therefore, the effective combination of the enzyme

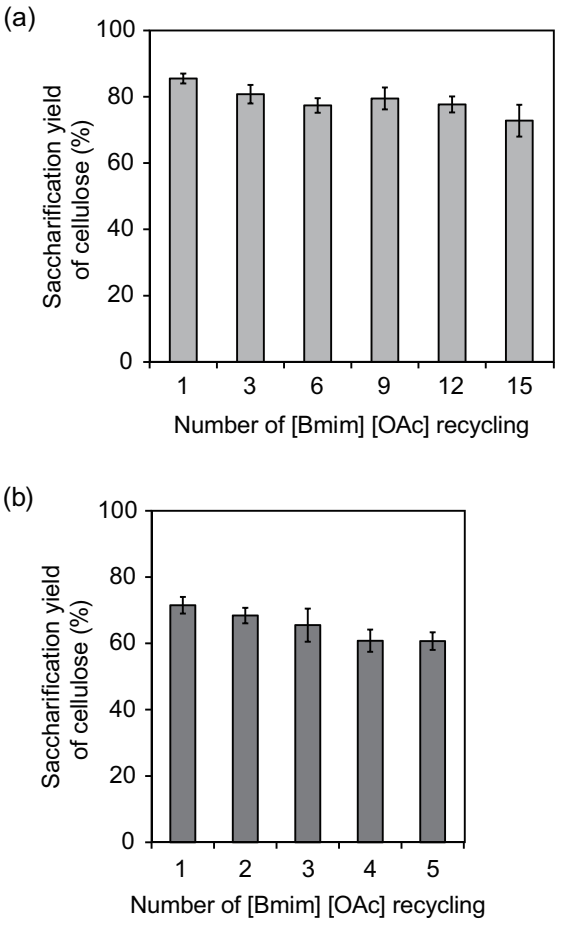

Fig. 5 Woody biomass saccharification using recycled [Bmim][OAc] in two batch operation ((a) = Bagasse, (b) = *Eucalyptus*). Data indicate the mean±SD of three independent experiments.

mixture is required for effective saccharification of biomass. To identify the optimal combination of the cellulase cocktail for saccharification of pre-treated biomass with an ionic liquid, 10 types of transgenic *S. cerevisiae* were constructed that was secretary produced saccharification enzyme, respectively (**Table 1**). These 10 different genes to code the saccharification enzyme were isolated by gene synthesis or polymerase chain reaction (PCR). Each DNA fragment was cloned into the yeast integration vector. This vector, which was based on the commercial pAUR123 vector, consists of a glyceraldehyde-3-phospate dehydrogenase 3 (*TDH3*) promoter, an α-factor fragment (*MFα-1*) for use as a yeast secretory signal, and a cytochrome C (*CYC1*) terminator. Each DNA fragment was isolated by PCR using genomic DNA of the *S. cerevisiae* YPH 499 strain as a template, and then ligated to the pAUR123 vector. The detailed method of molecular cloning was performed according to a standard protocol.[18] Each integration vector was transformed into the *S. cerevisiae* BJ5465 strain by general methods. Selected transgenic yeasts were cultured at 30°C for 72 h in YPD medium, nutritive rich medium, and the culture supernatants were added to the [Bmim][OAc] pretreated Bagasse in various combinations. Enzymatic saccharification was conducted at 40°C for 48 h, and the glucose concentration was then analyzed using HPLC. The efficiency of enzymatic saccharification was calculated based on the cellulose content of the Bagasse. **Figure 6** shows one example to determine the optimal enzyme cocktail. A saccharification efficiency of 66.2% was achieved by various combinations of five different enzymes (Aa. BGL, Ct. Cbh A, Pc. CBH II, Tr. Xyn II, and Tr. EG II). The addition of xylanase that resolves xylan chains was especially effective. It is considered that the xylan chains that surround amorphous cellulose are resolved by xylanase and the accessibility of cellulase to the cellulose chain is thus improved. This result indicates that high saccharification efficiency is achieved only in the culture supernatant of *S. cerevisiae* as same as commercial enzyme mixture.

4. Direct Bioethanol Production with One Batch Operation by the Combination of Ionic Liquid Pretreatment and Arming Yeast

In the ethanol production process, pretreatment, saccharification and fermentation are typically conducted separate to each other with different vessels. To simplify this complicated process, the Kondo group at Kobe University has developed a functional microorganism 'arming yeast' that displays four kinds of cellulase on the yeast cell surface and can produce ethanol from the oligosaccharides degraded with these enzymes.[19,20] Using this arming yeast, all of the production processes (pretreatment, saccharification, and fermentation) can be expected in only one batch (Fig. 2(b)). In our previous study, we demonstrated direct ethanol production from Avicel, a highly crystalline cellulose, with one batch operation by the combination of ionic liquid pretreatment and arming yeast.[21] Therefore, here we verified whether this process could be adopted for use with soft biomass.

Bagasse samples with particle sizes of 200 μm were

Table 1 Breeding of transgenic *S. cerevisiae* expressing the saccharification enzymes.

Type	Name	Source
CBH I	Pc. Cel 7C	*Phanerochaete chrysosporium*
	Ct. Cel 9R	*Clostridium thermocellum*
	Ct. Cbh A	*Clostridium thermocellum*
CBH II	Tr. CBH II	*Trichoderma reesei*
	Pc. CBH II	*Phanerochaete chrysosporium*
EG	Cc. Cel 8C	*Clostridium celllolyticum*
	Tr. EG II	*Trichoderma reesei*
	Tr. EG II-opt	*Trichoderma reesei*
BGL	Aa. BGL	*Aspergilus aculeatus*
Xyn	Tr. Xyn II-opt	*Trichoderma reesei*

CBH: Cellobiohydrolase, , EG: Endoglucanase, BGL: β-glucosidasel, Xyn: Xylanase
opt: Codon usage optimization for *S. cerevisiae* gene expression

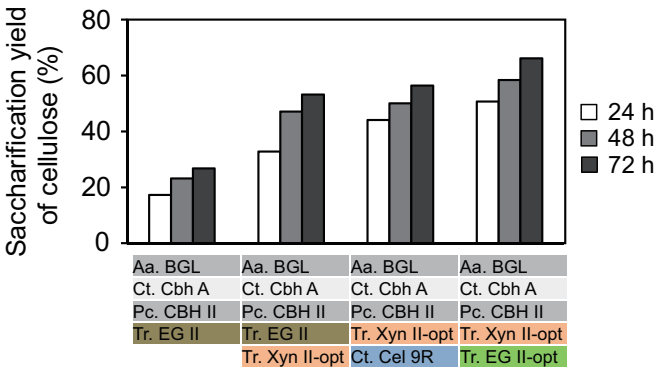

Fig. 6 Identification of the optimal cocktail of cellulolytic enzymes for the saccharification of Bagasse. Detailed enzyme names are summarized in Table 1.

pretreated with four different ionic liquids ([Emim][Cl]; 1-ethyl-3-methylimidazolium chloride, [Emim][OAc]; 1-ethyl-3-methylimidazolium acetate, [Emim][DEP]; 1-ethyl-3-methylimidazolium diethylphosphate, and [Bmim][OAc]; 1-butyl-3-methylimidazolium acetate), at 120°C for 30 min, respectively. Regenerated biomass was formed by adding 200 mM sodium acetate buffer (pH 5.0) to each of the cellulose solutions, which were then used as substrates for fermentation. The arming yeast cells for the fermentation experiment were grown in a synthetic defined (SD) medium with 20 g/L glucose at 30°C and stirring at 220 rpm. The arming yeast exhibits three types of cellulase, endoglucanase II (EG II) and cellobiohydrolase II (CBH II) from *Trichoderma reesei*, and *β*-glucosidase I (BGL I) from *Aspergillus aculeatus*, on the yeast cell surface. Cells were collected by centrifugation and washed with deionized water. This cultivated cells (OD_{600nm} = 80) and YP medium (YPD medium without glucose) were added to the pretreated Bagasse, and one batch fermentation was performed at 30°C for 96 h with stirring at 150 rpm. The glucose, xylose, and ethanol concentrations in the fermentation supernatant were measured using HPLC as described previously.

As shown in **Fig. 7**(a), the Bagasse pretreated with [Bmim][OAc] had the highest ethanol productivity among the four kinds of ionic liquids and the ethanol production reached 0.81 g/L (ethanol yield from glucan was 73.4%).[22] After 24 h of fermentation, the glucose concentration was not detectable in any of the Bagasse that had been pretreated with ionic liquid (Fig. 7(b)). *S. cerevisiae* seems to have relatively high tolerance to ionic liquids compared with other bacteria such as *Escherichia coli*, *Bacillus subtilis*, and [Bmim][OAc] may have a low inhibitory effect on yeast cells. In our previous study, 200 mM [Emim][DEP] also had a high inhibition effect on yeast fermentation.[21] To realize effective fermentation for the Bagasse pretreated with ionic liquid, it is important to consider not only the activity for a reduction in crystallinity but also the inhibitory effect of the ionic liquid on yeast cells and enzymes. In this one batch operation, the issues regarding ionic liquid recovery from the culture medium was still unsolved. In our previous study, we demonstrated the recovery and reuse of [Emim][DEP] from a mixture of culture media and ionic liquid using organic solvents, although many processes were required.[21] Because most ionic liquids are still expensive, more effective methods of the recovery and reuse are necessary. In addition, the accumulation of xylose was detected in all samples (Fig. 7(c)), because it is generally known that *S. cerevisiae* does not have the ability for C5 sugar consumption. A novel enzyme for C5 sugar consumption was recently identified, and research was conducted on a metabolically engineered yeast that produces ethanol from xylose by expression of

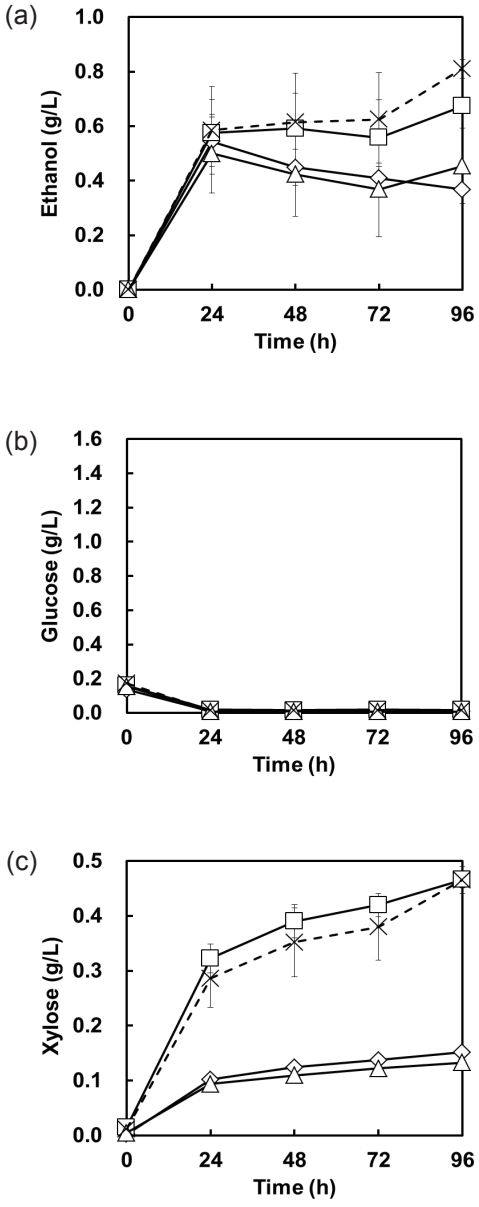

Fig. 7 Time course of the (a) ethanol, (b) glucose, and (c) xylose concentrations in direct batch fermentation from Bagasse pretreated with ionic liquid: diamonds, [Emim][DEP]; squares, [Emim][OAc]; triangles, [Emim][Cl]; crosses, [Bmim][OAc]. Data are averages from three independent experiments (error bars represent SD).

xylose-utilizing gene.[23] Further improvement of the ethanol productivity can be expected in future work by combining with C5 sugar consumption technology with transgenic yeast.

5. Conclusions

Ionic liquid has proven to be effective in reducing cellulose crystallinity and in the removal of hemicellulose and lignin. Ionic liquid can be applied to dissolve a range of lignocellulosic biomass, such as soft or hard biomass. In this study, we have designed a novel process, i-CBP, and developed fundamental technologies necessary for this process.

The pyridinium-type of ionic liquids showed better performance with respect to the initial reaction rates of enzymatic saccharification. Pretreated biomass with [Bmpy][Cl] had either the crystalline polymorphic alteration of cellulose or partial degradation of the crystalline cellulosic fraction. We subsequently demonstrated an ethanol production from soft biomass pretreated with [Bmim][OAc] by one or two batch operation. In this process, recycling of [Bmim][OAc] was examined and the optimal enzyme combination of transgenic yeast was determined. i-CBP shows potential, especially for simplification of the ethanol production process because all of the steps (pretreatment, saccharification, and fermentation) are consolidated into a one or two batch operation. To establish an efficient process for bioethanol production, future work will be necessary to develop low cost ionic liquids that can be reused with ease and cellulase-displaying yeast with higher cellulase activity.

Acknowledgments

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- Young Research Award for Encouragement, The Society for Biotechnology, Japan, 2010

Toru Onishi***

Research Fields:
- Metabolic Engineering
- Fermentation Technology

Academic Society:
- The Society for Biotechnology, Japan

Award:
- Excellent Paper Award, The Society for Biotechnology, Japan, 2007

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