Efficient Hydrolysis of Lignocellulose by Acidic Ionic Liquids under Low-Toxic Condition to Microorganisms

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Abstract: Lignocellulose is known as a renewable resource, and acidic ionic liquids have been highlighted as efficient catalysts for hydrolysis of cellulose. To achieve successive hydrolysis and fermentation, efficient hydrolysis with sufficiently diluted acidic ionic liquids is necessary because acidic ionic liquids are toxic to fermentative microorganisms. *Escherichia coli* was confirmed to grow in 0.05 M dilute acidic ionic liquid—1-(1-butylsulfonic)-3-methylimidazolium hydrogen sulfate ([Sbmim][HSO$_4$])—although the growth was suppressed in more concentrated solutions. Therefore, we applied the 0.05 M [Sbmim][HSO$_4$] solution to hydrolysis of bagasse, leading to a glucose yield of 48% at 190 °C. This value is greater than that obtained with a concentrated [Sbmim][HSO$_4$] solution, which is not suitable for the growth of *Escherichia coli* (yield: 40% in a 1.0 M solution). Efficient hydrolysis with acidic ionic liquids under low-toxic condition was achieved.

Keywords: acidic ionic liquid; hydrolysis; biomass

1. Introduction

Carbohydrates account for 75% of annual renewable biomass. Among the various types of carbohydrates, cellulose is the most attractive because it is inedible and inexpensive; furthermore, it can be obtained on a large scale from biomass (ca. 45% of biomass) [1]. Recently, the efficient conversion of cellulose to glucose for the production of ethanol and critical building blocks such as succinic acid and gluconic acid via fermentation has been extensively studied [1,2]. Acidic or enzymatic hydrolysis has been used to obtain glucose from cellulose. Hydrolysis using dilute acid is known to be a simple, cost-effective method; however, it gives low sugar yields and requires a long reaction time [3]. To improve hydrolysis yield, efficient catalysts are strongly requested.

Recently, certain ionic liquids (ILs) have been applied in the processing of cellulose [4–9]. Because of their remarkable capacity to delignify and solubilize cellulose, the ILs are often used for pretreating lignocellulosic biomass before hydrolysis [10–15]. On the other hand, Davis et al. reported acidic ILs having an acidic group such as a sulfo group in their cationic and/or anionic structures [16–20], and the acidic ILs exhibit higher catalytic activity in cellulose hydrolysis compared to sulfuric acid [21].

Furthermore, it has been reported that microwave heating accelerates hydrolysis of cellulose in 1.0 M acidic IL, 1-(1-butylsulfonic)-3-methylimidazolium hydrogen sulfate ([Sbmim][HSO$_4$]; Figure 1) [22], because the combination of ILs and microwave is known to exert synergistic effects on several reactions [23–25]. In the literature, the glucose yield has increased from a few percent after 30 min to 40% after 12 min at 160 °C when using microwave irradiation.
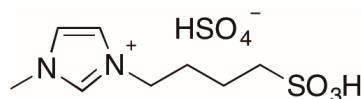

Figure 1. The structure of [Sbmim][HSO$_4$].

However, the concentrated [Sbmim][HSO$_4$] solution (1.0 M in the previous literature) is not suitable for the successive microbial utilization, e.g., the fermentation of glucose to ethanol or other valuable building blocks, because the concentrated [Sbmim][HSO$_4$] solution is lethal to microorganisms due to its acidity and the intrinsic toxicity of ILs [26–29]. Especially, the intrinsic toxicity of IL cannot be reduced unlike the toxicity by acidity (reduced by neutralization): sufficiently dilute solution has to be used. Since there is no report satisfying both conditions—microbial viability and a high yield of sugars—with acidic ILs, in this study, we attempted the efficient hydrolysis of cellulose with a [Sbmim][HSO$_4$] solution that is sufficiently dilute to be undisruptive to the growth of *Escherichia coli* (*E. coli*).

2. Results and Discussion

2.1. Effect of [Sbmim][HSO$_4$] on E. coli Growth

First of all, the toxicity of [Sbmim][HSO$_4$] was investigated because the toxicity of acidic ILs has not been studied. Figure 2 shows the time course of the optical density at 600 nm (OD$_{600}$) of the [Sbmim][HSO$_4$]/medium mixed solutions during culturing *E. coli*. OD$_{600}$ is a conventional indicator of the *E. coli* concentration. At [Sbmim][HSO$_4$] concentrations of 1.00 and 0.50 M, the OD$_{600}$ after 24 h was 0.1 and 0.3, respectively, indicating severe growth inhibition. In contrast, *E. coli* grew and the OD$_{600}$ increased to 2.0 within 24 h in a 0.05 M [Sbmim][HSO$_4$] solution. Although *E. coli* thrived more in a pure medium without [Sbmim][HSO$_4$], resulting in an OD$_{600}$ of 5.0 within 24 h (Figure S1, see Supplementary Materials), it is confirmed that *E. coli* surely grew in the 0.05 M solution with almost half the growth ratio of that in the pure medium. Therefore, we decided to apply the 0.05 M solution to the hydrolysis of cellulose.
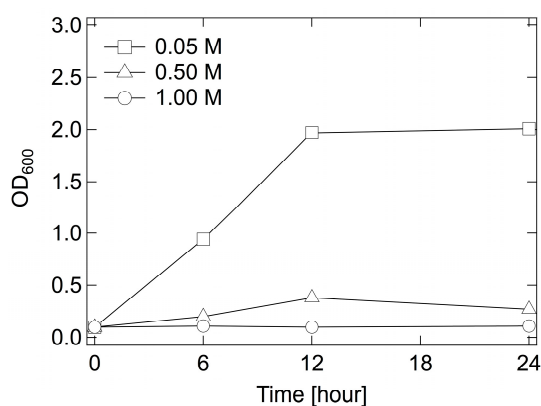

Figure 2. Time course of OD$_{600}$ of [Sbmim][HSO$_4$]/medium mixed solutions during *E. coli* culturing.

As mentioned above, [Sbmim][HSO$_4$] inhibited the growth of *E. coli* because of the IL structure itself and/or the acidity. To investigate the mechanism of toxicity, the same experiments using H$_2$SO$_4$

and 1-ethyl-3-methylimidazolium acetate, an IL that does not contain any acidic part, were also performed. All three compounds, [Sbmim][HSO$_4$], 1-ethyl-3-methylimidazolium acetate, and H$_2$SO$_4$, exhibited a similar inhibitory effect on *E. coli* growth (Figures S2 and S3, see Supplementary Materials). Hence, the toxicity of [Sbmim][HSO$_4$] may be attributed to both the IL structure and the acidity.

2.2. Hydrolysis of Bagasse in a 0.05 M [Sbmim][HSO$_4$] Solution

Figure 3 shows the glucose yield during hydrolysis using 0.05 M [Sbmim][HSO$_4$] solutions at 160 °C. The glucose yield gradually increased in the 0.05 M solution, and reached a maximum of 37%. In a concentrated solution (1.00 M), the glucose yield reached a maximum of 40%, and then decreased. It is confirmed that the 0.05 M solution can hydrolyze bagasse with a comparable yield to that of the 1.00 M solution.
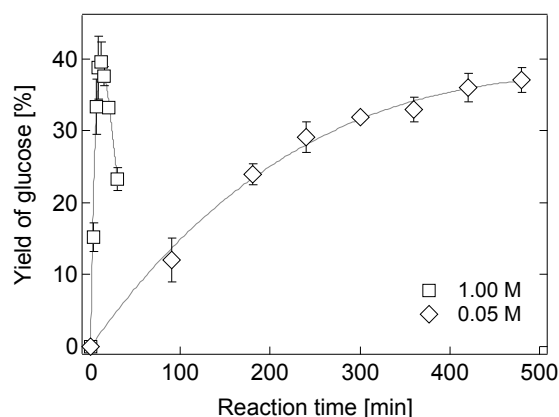

Figure 3. Time courses of glucose yields during bagasse hydrolysis in 1.00 and 0.05 M [Sbmim][HSO$_4$] solutions at 160 °C.

The reactions in the samples proceeded with different rates. Peak yields in 1.00 and 0.05 M solutions were attained after approximately 12 and 500 min, respectively. The [Sbmim][HSO$_4$] concentration was confirmed to affect the reaction rate, and the low reaction rate at 0.05 M is expected to be improved by elevating the reaction temperature.

Figure 4 shows the time courses of the glucose yields during bagasse hydrolysis in the 0.05 M [Sbmim][HSO$_4$] solutions at 190 and 200 °C. The reaction rate improved in both cases, compared to that at 160 °C (see Figure 3): peak yields at 190 and 200 °C were obtained after 35 and 12 min, respectively. The glucose yield was also improved by increasing temperature: the peak yields at 190 and 200 °C were 48 and 47%, respectively. We were able to reduce the [Sbmim][HSO$_4$] concentration to decrease the toxicity and improve the glucose yield, compared to the hydrolysis using the 1.00 M solution. In addition, the glucose yield at 180 °C was 43% within 40 min (Figure S4; see Supplementary Materials). Reaction temperatures of greater than 190 °C are favorable for attaining a high glucose yield.

To investigate the reason behind the improvement of the glucose yield, we estimated k_1 and k_2 (based on Equations (1) and (2), see experimental section; results are shown in Table 1). The k_1 and k_2 depended strongly on both the [Sbmim][HSO$_4$] concentration and the temperature. For example, k_1 and k_2 obtained with the 0.05 M [Sbmim][HSO$_4$] solution at 190 °C were 3.4×10^{-2} and 2.1×10^{-2} min^{-1} (Entry 3), whereas those obtained with a 1.00 M solution at 160 °C were 8.4×10^{-2} and 7.5×10^{-2} min^{-1} (Entry 5, the optimum condition in 1.00 M [Sbmim][HSO$_4$] solution).
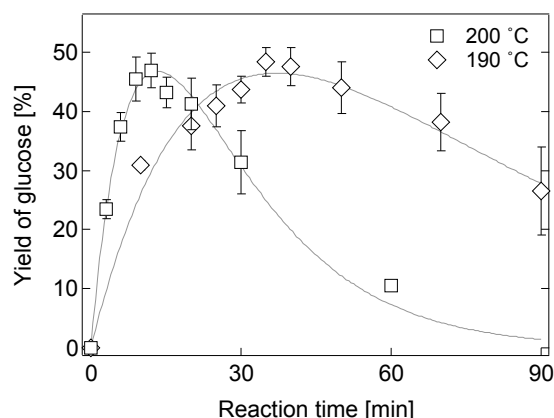
Figure 4. Time courses of glucose yields during the hydrolysis of bagasse in the 0.05 M [Sbmim][HSO$_4$] solution at 190 and 200 °C.

Table 1. k_1, k_2, k_1/k_2, and glucose yield of the samples hydrolyzed in [Sbmim][HSO$_4$] solutions.

Entry	Temperature (°C)	Concentration (M)	k_1 (min^{-1})	k_2 (min^{-1})	k_1/k_2	Yield (%)
1	160	0.05	1.8×10^{-3}	0.17×10^{-2}	1.0	37
2	180	0.05	2.3×10^{-2}	1.7×10^{-2}	1.4	43
3	190	0.05	3.4×10^{-2}	2.1×10^{-2}	1.7	48
4	200	0.05	9.6×10^{-2}	5.7×10^{-2}	1.7	47
5	160	1.00	8.4×10^{-2}	7.5×10^{-2}	1.1	40
6	190	0.10	9.8×10^{-2}	5.9×10^{-2}	1.7	50

A large k_1 and small k_2 are necessary for attaining a high glucose yield; hence, we investigated the ratio of k_1 to k_2 (k_1/k_2). Using 0.05 M [Sbmim][HSO$_4$] solutions at 160, 180, 190, and 200 °C, we obtained k_1/k_2 ratios of 1.0, 1.4, 1.7, and 1.7, which correspond to glucose yields of 37%, 43%, 48%, and 47%, respectively (Entries 1–4; Table 1). These results indicate that a high temperature results in a high k_1/k_2 ratio and thus a high glucose yield. As compared with temperature, the [Sbmim][HSO$_4$] concentration exerts a smaller effect on k_1/k_2. As shown in Entries 1 and 5, k_1/k_2 ratios obtained with 0.05 and 1.00 M solutions at 160 °C were 1.0 and 1.1, respectively; the k_1/k_2 ratios obtained with 0.05 and 0.10 M solutions at 190 °C were both 1.7 (Entries 3 and 6). From these results, the improvement in glucose yield in the 0.05 M solution may be attributed to the high k_1/k_2 ratio due to high temperature.

The activation energy for hydrolysis of bagasse cellulose in the 0.05 M [Sbmim][HSO$_4$] solution was 167 kJ/mol; this value is less than that for cellulose hydrolysis using a diluted H$_2$SO$_4$ solution (180 kJ/mol for Douglas fir [30] and 190 kJ/mol for corn stover [31]). For glucose decomposition in the 0.05 M [Sbmim][HSO$_4$] solution, the activation energy was 144 kJ/mol; this value is greater than that obtained with a dilute H$_2$SO$_4$ solution (138 kJ/mol for Douglas fir [30] and 137 kJ/mol for corn stover [31]). The differences of the activation energies support that the present method is favorable to an increase in the yield.

2.3. Hydrolysis of Various Biomass

To confirm the utility of the present method, we hydrolyzed hardwood and softwood samples, eucalyptus and Japanese cedar, respectively, in addition to bagasse. Because of their abundance and their suitability as biomass for glucose production, these materials have attracted significant interest. Figure 5 shows the time courses of the glucose yields during the hydrolysis of bagasse, eucalyptus, and Japanese cedar in the 0.05 M [Sbmim][HSO$_4$] solutions at 190 °C. As mentioned above, bagasse hydrolysis had a peak yield of 48% at 35 min. With eucalyptus and Japanese cedar, glucose yields were 41% after 25 min and 45% after 30 min, respectively. Because lower glucose yields in 1.00 M

[Sbmim][HSO$_4$] solutions at 160 °C were obtained (37% for eucalyptus and 30% for Japanese cedar), the diluted [Sbmim][HSO$_4$] solution was effective for the hydrolysis of various biomass sources.
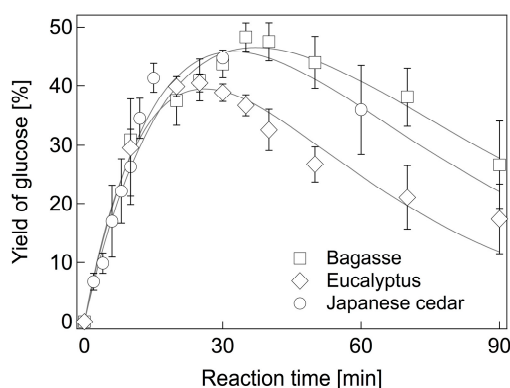

Figure 5. Time courses of glucose yields during the hydrolysis of bagasse, eucalyptus, and Japanese cedar in the 0.05 M [Sbmim][HSO$_4$] solutions at 190 °C.

Figure 5 also shows difference in the duration for which the maximum yield was achieved (t_{max}) and the maximum yields (Y_{max}), depending on the biomass sources. To investigate the reason behind these different values, we measured the crystallinity of biomass. The crystallinity was determined by Fourier transform infrared (FTIR) spectroscopy by calculating the ratio of absorbance at 1437 and 899 cm^{-1}, as reported by O'Connor et al. [32]. The ratio of absorbance was 0.60 (bagasse), 0.87 (Eucalyptus), and 0.67 (Japanese cedar). Figure 6 shows the relation between the crystallinity of the cellulose present in biomass and t_{max} and Y_{max}. As the crystallinity decreased, t_{max} and Y_{max} increased, indicating that the crystallinity of cellulose in biomass is confirmed to be a factor in resisting hydrolysis. It also indicates that a higher glucose yield is expected if pretreatments such as ball milling are applied.
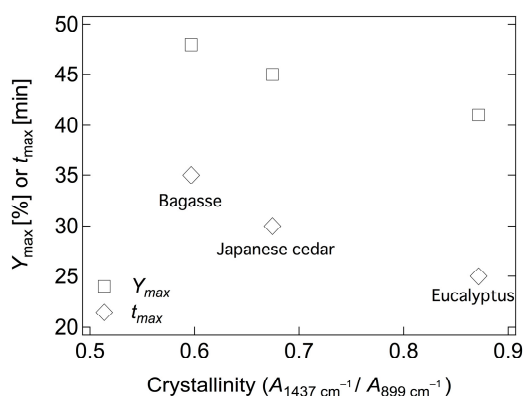

Figure 6. Relationship between the crystallinity of biomass and Y_{max} (square) and t_{max} (diamond).

FTIR measurements may also enable the prediction of t_{max} and Y_{max}. The prediction of t_{max} is particularly useful because excessively short and long reaction times result in a low yield.

2.4. Effect of Biomass Loading on Hydrolysis

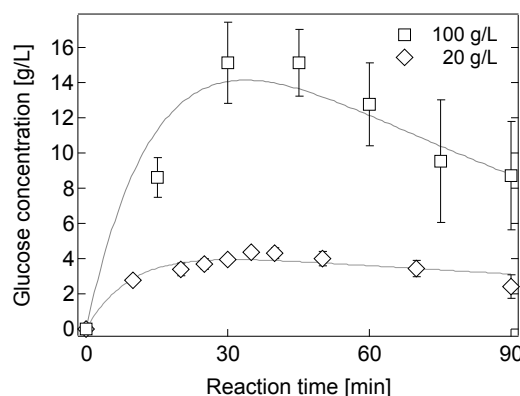To efficiently utilize biomass, high loading is important. Hence, the loading of bagasse was increased from 20 to 100 g/L. Figure 7 shows the time courses of glucose concentrations during hydrolysis with 20 or 100 g/L bagasse using 0.05 M [Sbmim][HSO$_4$] solutions at 190 °C. Even at 100 g/L loading, the glucose concentration increased within approximately 30 min, suggesting that

hydrolysis proceeded despite the absorption of a major part of the [Sbmim][HSO$_4$] solution by the bagasse particles (Table S1 shows photographs of solutions). With the 20 and 100 g/L solutions, the peak glucose concentrations were 4.4 and 15.2 g/L, respectively, corresponding to a 3.5-fold increase in concentration. It is noted that the 100 g/L solution produced a yield of 33%, and the value was less than that obtained with the 20 g/L solution (48%) (Figure S5). In addition, the t_{max} for both the 20 and 100 g/L solutions was around 30 min, indicating that the present method can process a high loading of biomass within this period.

Figure 7. Time courses of glucose concentrations during hydrolysis of 20 and 100 g/L bagasse solutions in the 0.05 M [Sbmim][HSO$_4$] solutions at 190 °C.

3. Materials and Methods

3.1. Materials

Bagasse, eucalyptus, and Japanese cedar powder (particle diameter of approximately 3 mm) were purchased from Sanwa Ceruciron (Yokkaichi, Japan). First, the biomass powder was ground in a mill and then sieved to form a powder with a particle diameter of 250–500 μm. The cellulose content of the original lignocellulosic biomass was determined according to a reported method [33]. [Sbmim][HSO$_4$] (Solvionic, Toulouse, France) was used as received. *Escherichia coli* (*E. coli* KO11, ATCC 55124) was purchased from Summit Pharmaceuticals International Corporation (Tokyo, Japan).

3.2. The Effect of [Sbmim][HSO$_4$] on E. coli Growth

E. coli was aerobically precultured at 37 °C in a test tube containing 5 mL of medium (5 g/L yeast extract, 5 g/L NaCl, and 10 g/L tryptone; Nacalai Tesque, Kyoto, Japan). The precultured broth was transferred to 2 mL of the medium containing [Sbmim][HSO$_4$]. The initial OD$_{600}$ was set at 0.1. Each was incubated at 37 °C for 24 h on a reciprocal shaker at 160 rpm. The culture broth was then sampled at 6, 12, and 24 h, and the OD$_{600}$ was measured.

3.3. Microwave-Assisted Hydrolysis of Biomass in an Aqueous [Sbmim][HSO$_4$] Solution

Biomass (0.30 g) was suspended in [Sbmim][HSO$_4$] solutions (15 mL), and the resulting mixture was then transferred to a 100 mL vessel (HPR-1000/10; Milestone s.r.l., Sorisole, Italy). The vessel was then heated in a microwave synthesizer (StartSYNTH; Milestone s.r.l.).
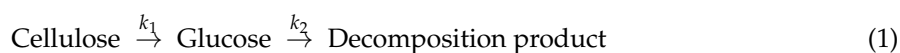
For sampling, the vessel was removed from the microwave system and immediately cooled in an ice bath to quench the reaction. An aliquot of the sample solution (500 μL) was centrifuged at 15,000 rpm for 2 min to precipitate the solids. The supernatant was filtered and then subjected to glucose analysis as described below.

3.4. Analysis of Glucose Yield

We evaluated the efficacy of hydrolysis by the glucose yield because cellulose is considerably difficult to hydrolyze due to its high crystallinity, as compared with hemicellulose. The glucose concentration in the hydrolyzate was determined using a high performance liquid chromatography equipped with a refractive index detector (Shimadzu Co., Kyoto, Japan). A sugar KS-801 column (Showa Denko K.K., Tokyo, Japan) was used in tandem with a sugar KS-G guard column (Showa Denko K.K.). The volume of the injected sample was 10 μL. The column was operated at 80 °C, and ultrapure water was used as the mobile phase (a flow rate of 1.0 mL/min).

3.5. Determination of Reaction Rate Constants

During acid hydrolysis, cellulose is hydrolyzed to glucose, which is then decomposed. Hydrolysis and decomposition of cellulose may be described by the pseudo-homogeneous consecutive first-order reactions [30]:

$$\text{Cellulose} \overset{k_1}{\to} \text{Glucose} \overset{k_2}{\to} \text{Decomposition product} \tag{1}$$

where k_1 and k_2 represent the rate constants for the hydrolysis of cellulose to glucose and for the decomposition of glucose, respectively. The glucose concentration, C_{glucose}, is expressed as follows:

$$C_{\text{glucose}} = a \, \frac{k_1}{k_2 - k_1} \left[e^{-k_1 t} - e^{-k_2 t} \right] \tag{2}$$

where the initial cellulose concentration is expressed as glucose equivalents. The reaction rate constants, k_1 and k_2, were determined by fitting Equation (2) to the experimental data according to the nonlinear least-squares method by using Igor Pro software (WaveMetrics, Inc., Portland, OR, USA).

4. Conclusions

In this study, cellulose present in lignocellulosic biomass was hydrolyzed by using a 0.05 M [Sbmim][HSO$_4$] solution under microwave heating. The 0.05 M [Sbmim][HSO$_4$] solution was sufficiently low to allow for the growth of *E. coli*. The glucose yield during hydrolysis in the 0.05 M solution (48%) was higher than that obtained in the 1.00 M [Sbmim][HSO$_4$] solution (40%), under the optimized conditions. The higher yield was attributed to a high k_1/k_2 ratio due to the maximum temperatures applicable to the 1.00 M and 0.05 M solutions, namely 160 °C and 190 °C, respectively. The dilute [Sbmim][HSO$_4$] solution was also applicable to softwood and hardwood, which is more recalcitrant than herbaceous biomass. The yield at 190 °C was 41% for eucalyptus and 45% for Japanese cedar.

Author Contributions: K.K., K. Takada, K.N., and K. Takahashi conceived and designed the experiments and thoroughly discussed the results and hypotheses; K.I., K.M. and H.S. performed the experiments; K.K. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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