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Effective Concentration of Ionic Liquids for Enhanced Saccharification of Cellulose

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Abstract: In an aqueous enzymatic saccharification using cellulase, the dissolution of crystalline cellulose is one of the rate-limiting steps. Insoluble cellulose powder was preliminarily heat-treated with ionic liquids (ILs), such as [Bmim][Cl] (1-butyl-3-methylimidazolium chloride) and [Amim][Cl] (1-allyl-3-methylimidazolium chloride), which enable the production of soluble cellulose. On the other hand, the presence of ILs leads to a denaturation of enzymes. Using cellulase from *Trichoderma viride*, the effects of [Bmim][Cl] and [Amim][Cl] in the enzymatic saccharification were compared. The production of glucose was optimized with 5 wt%-ILs, both for [Bmim][Cl] and for [Amim][Cl]. The significant inhibiting effects of ILs (IL concentration >10 wt%) could be due to the denaturation of cellulase, because the peak shifts of intrinsic tryptophan fluorescence were observed in the presence of 7.5 wt%-ILs. To analyze kinetic parameters, the Langmuir adsorption model and the Michaelis-Menten model were employed. The investigation suggests that [Amim][Cl] can provide soluble cellulose more efficiently, and can promote enzymatic saccharification in the IL concentration below 5 wt%.

Keywords: ionic liquid; cellulase; insoluble cellulose; hydrolysis; kinetic analysis

1. Introduction

Cellulose is a woody biomass which is attracting attention as renewable energy. Cellulose has high crystallinity and is insoluble both in water and in organic solvents. The enzymatic saccharification is an environmentally-friendly energy generating process, while the irreversible adsorption of the enzyme on the crystalline cellulose surface causes deactivation and reduction of contact efficiency. This prevents continuous glucose production in the aqueous phase [1,2]. To overcome such insolubility of crystalline cellulose, dissolution methods have been developed by utilizing acids (sulfuric acid or hydrochloric acid), alkalis (NaOH, KOH, $Ca(OH)_2$, or ammonia), organic solvents (ethylene glycol, glycerol, tetrahydrofurfuryl alcohol), amphiphilic polymers, and ionic liquids (ILs) [3–5].

ILs are organic salts in the liquid state under standard condition, which are comprised of anions and organic cations. In order to develop a cellulose saccharification process with high efficiency, it is necessary to achieve dissolution of crystalline cellulose and hydrolysis of regenerated cellulose in the aqueous phase [6–11] at the same time. On the other hand, enzymes tend to interact with ILs, and their activities can be decreased due to denaturation. It has been reported that the ternary structure of cellulase, which is monitored by intrinsic tryptophan (Trp) fluorescence (ex: 295 nm, em: 330–350 nm), can be denatured by the presence of tris-(2-hydroxyethyl)-methylammonium methylsulfate and 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) [12,13]. Thus, the competitive effects are derived

by the use of ILs in enzymatic saccharification processes: i.e., a promoting effect due to enhanced dissolution of cellulose, and an inhibiting effect due to the denaturation of enzymes.

The enzymatic saccharification processes of biomass sources have been studied, especially focusing on pretreatment methods: physical, chemical, physico-chemical, biological, and combined pretreatments [14]. Alkaline treatment is popular and relatively easy in large scale reactions, while most cellulase enzymes are optimized in acidic conditions [15]. Ultrasonic irradiation can be applied to various kinds of cellulose sources [16]. A huge number of studies have been carried out on the treatment of cellulose with ILs [17]. In overviewing reported works, it is obvious that the positive effect of ILs, i.e., dissolution of crystalline cellulose, and the negative effect of ILs, i.e., inactivation due to denaturation, are competitive (have a trade-off relationship). However, systematic studies to investigate optimal conditions for IL-enzyme coexisting systems are not well established.

The aim of this study is to investigate the effect of ILs on the enzymatic saccharification of cellulose using cellulase from *Trichoderma viride*, based on kinetic analysis. The efficiency of cellulose saccharification was estimated by monitoring the production of glucose, in the presence of ILs in various concentrations. It is also necessary to discuss the relevance between the conformational stability of cellulase and its enzymatic activity. Based on the kinetic parameters obtained, the differences of [Bmim][Cl] and 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) were discussed.

2. Materials and Methods

2.1. Materials

1-Butyl-3-methylimidazolium chloride ([Bmim][Cl]) and 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) were purchased from Kanto Kagaku (Tokyo, Japan) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Crystalline cellulose powder was purchased from Nacalai Tesque (Kyoto, Japan). Cellulase from *Trichoderma viride* ($\geq$5000 units/g solid) was purchased from Yakult Pharmaceutical Industry (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemical Corporation (Osaka, Japan), and used without further purification.

2.2. Evaluation of IL Concentration Dependence of Cellulose Saccharification Activity

Based on the reference, the reaction mixtures were prepared [18]. Briefly, aliquot amount of IL ([Amim][Cl] or [Bmim][Cl]) and cellulose were heated at 100 °C for 5 min to dissolve cellulose, with stirring at 750 rpm. After cooling down to room temperature, an aliquot amount of acetate buffer (25 mM, pH 4.8) including cellulase was added to adjust the reaction volume to 20 mL. The final cellulose concentration was 2 g/L, and the final cellulase concentration was 9.1 mg/L. The final concentration of IL varied from 0 to 15 wt%. The sample was then incubated at 45 °C in shaking bath, with a stroke speed of 60 rpm. Glucose concentration was measured using Glucose CII Test Wako kit® (Osaka, Japan) [19].

2.3. Measurement of Intrinsic Trp Fluorescence

An IL solution was prepared by adding 25 mM of acetate buffer (pH 4.8) while heating and stirring with [Amim][Cl] or [Bmim][Cl] for 5 min at 100 °C. After cooling down to room temperature, then cellulase solution was added to the prepared IL solution. The total cellulase concentration was 9.1 mg/L. The final concentration of IL varied from 0 to 7.5 wt%. The prepared sample was incubated at 45 °C for 5 min. After that, the fluorescence spectrum of intrinsic tryptophan (Trp) was measured at 45 °C, using a Spectrofluorometer (FP-8500, JASCO, Tokyo, Japan). An excitation wavelength was 295 nm, and the emission spectrum was recorded with a sampling pitch of 0.1 nm.

3. Results and Discussion

3.1. Effect of ILs on Ternary Structure of Cellulase

An intrinsic Trp fluorescence measurement derived from a protein or enzyme can be a good indicator of its ternary structure, because the emission peak of Trp can shift depending on the surrounding dielectric environment: when Trp is exposed to a hydrophobic environment, the emission peak responds by shifting to a lower wavelength [20]. Conformational changes of cellulase in the presence of ILs were determined by monitoring the emission peaks of intrinsic Trp (Figure 1). In both cases of [Amim][Cl] and [Bmim][Cl], the emission peaks were slightly shifted to a higher frequency (red-shift) at 45 °C, just after mixing cellulase with the IL. No significant peak shifts were observed in [Amim][Cl] when the concentration of IL was lower than 5 wt%, while [Bmim][Cl] gradually induced a red-shift of Trp peak, in proportion to the IL concentration. In addition, the fluorescent intensities of Trp drastically decreased with the addition of ILs (data not shown). Turner et al. reported that the denaturation of cellulase from *Trichoderma reesei* can be induced by the presence of sodiumdodecylsalfate, urea, and [Bmim][Cl] [12]. It has been also reported that the intrinsic Trp fluorescence of the hexokinase from *Saccharomyces cerevisiae* can be dependent on the type of the additives, where the wavelength was shifted by 2–4 nm [21]. In the presence of 7.5 wt%-IL, the red-shifts were significant (~10 nm) in the case of both [Amim][Cl] and [Bmim][Cl], suggesting that a high concentration of IL (≥7.5 wt%) could lead to cellulase denaturation at 45 °C. The IL itself could be hydrophobic compared to water. Thus, the red-shift of Trp peak in the presence of 7.5 wt% ILs could be caused by the exposure of intrinsic Trp in cellulase to bulk water, by the denaturation effect of ILs. Although the Trp fluorescence could be influenced by the viscosity of the solution, the solution viscosity was not changed so much even in the case of IL concentration at 15 wt% (Figure S1). Therefore, the peak shift of intrinsic Trp could be evidence of the denaturation of cellulase by ILs.

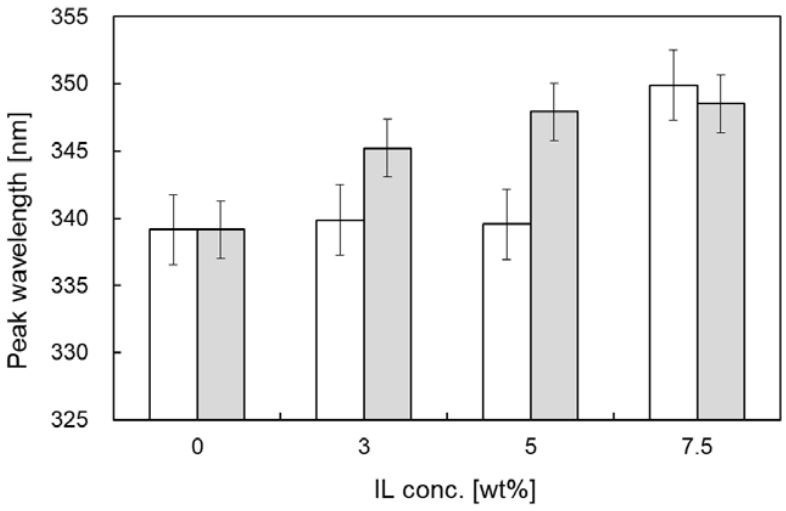

Figure 1. Intrinsic Trp fluorescence emission peaks of cellulase in the presence of ionic liquids (ILs) [Amim][Cl] (open bar) and [Bmim][Cl] (closed bar). The measurements were conducted at 45 °C.

3.2. Effect of ILs on the Saccharification of Cellulose

The dissolution of cellulose is a bottleneck step in the enzymatic saccharification process, in which ILs can promote the dissolution of crystalline cellulose [22]. In the absence of ILs, the saccharification could be considered as the solid-liquid reaction that happens at the crystalline cellulose surface, which usually results in less productivity of glucose. In this case, the reaction of cellobiohydrolase could be the rate-limiting step because it binds to the terminal of crystalline cellulose, however, the hydrolysis reaction at the amorphous part could have a weakening effect. In total, the glucose production could not be promoted without ILs [6]. By pretreatment of cellulose with ILs, most parts of crystalline

cellulose turned to an amorphous state, thus the hydrolysis of soluble cellulose can be drastically improved [13]. Herein, the cellulose hydrolysis reaction was carried out with different concentrations of IL: 0 to 15 wt% in 25 mM acetate buffer (pH 4.8) (Figure 2). The glucose production was most increased with 5 wt%-ILs, in the case of both [Amim][Cl] and [Bmim][Cl]. Since the regenerated cellulose accumulated in the reaction mixture in an amorphous state, the activity of endoglucanase could be enhanced. Although the presence of ILs can promote the dissolution of cellulose, the glucose production was inhibited with higher concentrations of ILs (>10 wt%) (Figure 2c). The inactivation effect was more significant with [Bmim][Cl]. This could be related to the denaturation effect of ILs: the peak shift of intrinsic Trp by [Bmim][Cl] was greater than that by [Amim][Cl] (Figure 1). It is concluded that the enzymatic saccharification of cellulose was optimized with 5 wt%-ILs.

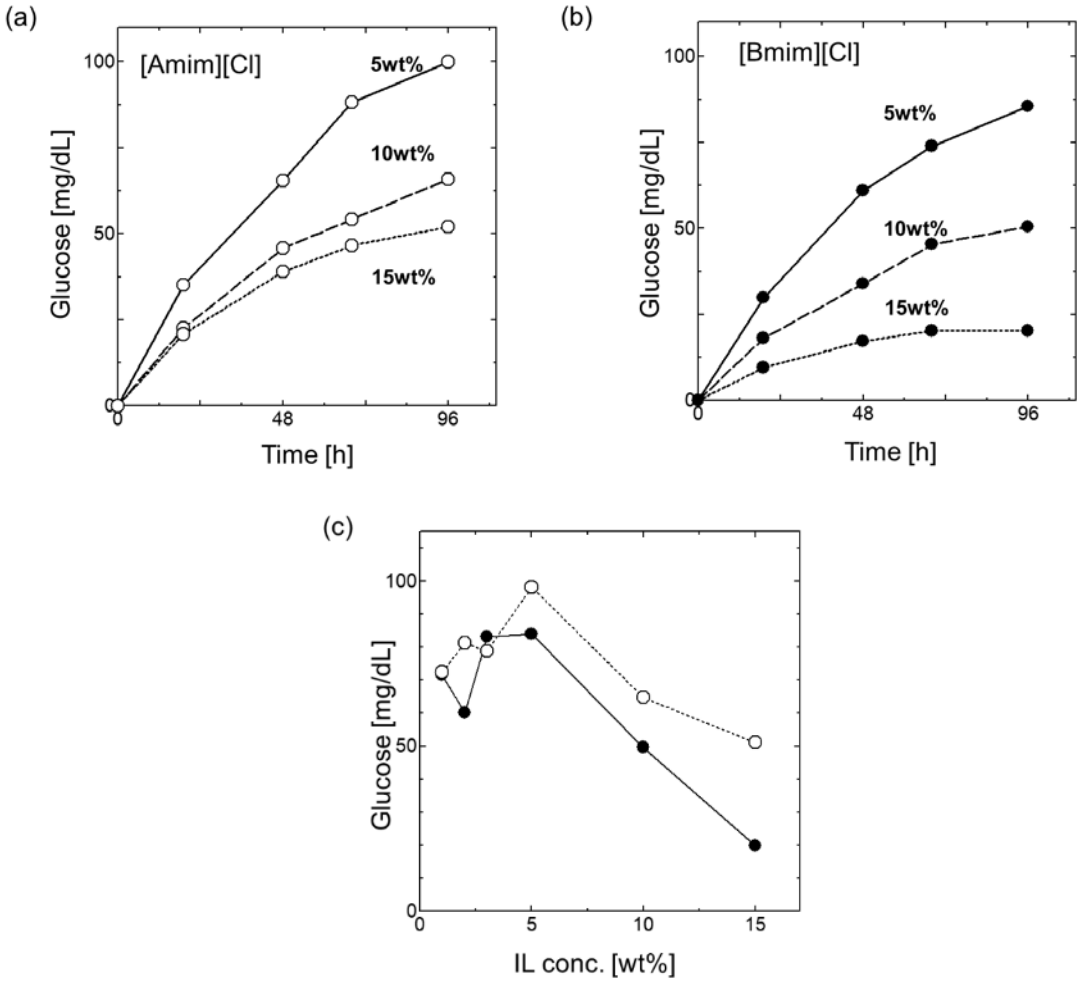

Figure 2. Glucose production in the presence of (**a**) [Amim][Cl] and of (**b**) [Bmim][Cl]. (**c**) Dependence of ionic liquid (IL) concentration on the glucose production, with [Amim][Cl] (open circle) or with [Bmim][Cl] (closed circle). All experiments were conducted at 45 °C with 96 h incubation, 2 g/L of cellulose, 9.1 mg/L of cellulase. The sample volume was 20 mL.

3.3. Kinetic Parameter Analysis for Enzymatic Saccharification of Cellulose

In this reaction system, the initial concentration of the glucose precursor was 1.23×10^{-2} M (2 g/L of ($C_6H_{10}O_5$)), while the obtained glucose concentration was ~6.17×10^{-3} M (~100 mg/dL of ($C_6H_{12}O_6$)). Such saturations in conversion values were due to the adsorption of cellulase onto amorphous cellulose. Herein, the experimentally obtained data were fitted using a Langmuir-type adsorption model, and first-order reaction kinetics. Due to the accessibility of the aqueous enzyme to the substrate, it is assumed that the amount of substrate available for aqueous cellulase could be less

than the initial substrate concentration (C_{ini}). Herein, the effective substrate concentration (C) can be described as followings:

$$C = C_{ini} \times Q_{max}, \quad (1)$$

wherein Q_{max} represents the maximal adsorption value. C_{ini} was 2 g/L, thus 1.23×10^{-2} M. The Q_{max} value was investigated based on the Langmuir adsorption model, as followings:

$$C'/C_{enz} = Q_{max}\, K\, [C_{ini} \times (1 - \text{Conv}\%)]/(1 + K\, [C_{ini} \times (1 - \text{Conv}\%)]), \quad (2)$$

wherein C', C_{enz}, K and Conv% represent the adsorbed substrate concentration, the concentration of enzyme, affinity between enzyme and substrate (i.e., glucose precursor), and obtained conversion value, respectively. $[C_{ini} \times (1 - \text{Conv}\%)]$ indicates the equilibrium concentration of substrate (free cellulose). The constants Q_{max} and K were decided by trial-and-error, which satisfied the R^2 value >0.99. Fitting results are shown in Figure 3 and Table 1. In the case of [Bmim][Cl], the Q_{max} values were decreased in proportion to the IL concentration, suggesting that the amount of substrate (i.e., dissolved cellulose) decreased. This indicate that a high concentration of [Bmim][Cl] can inhibit the binding of enzyme such as endoglucanase. The reaction rate constants, k_c were analyzed using a first-order reaction equation, wherein C (from Equation (1)) was employed as substrate concentration:

$$-dC/dt = k_c\, C, \quad (3)$$

wherein t and k_c represent reaction time and first-order reaction rate constant, respectively. By employing C (Equation (1)) as substrate concentration, the reaction kinetics could be well-fitted by a first-order reaction. The analyzed reaction rate constants, k_c, are summarized in Figure 4. The k_c values increased in proportion to IL concentration ($\leq$5 wt%: region α), while they decreased with higher IL concentration ($\geq$5 wt%: region β). It is suggested that the decrease of saccharification efficiency could be due to the denaturation of enzymes, and as a result, the enzyme could lose the binding affinity toward the substrate.

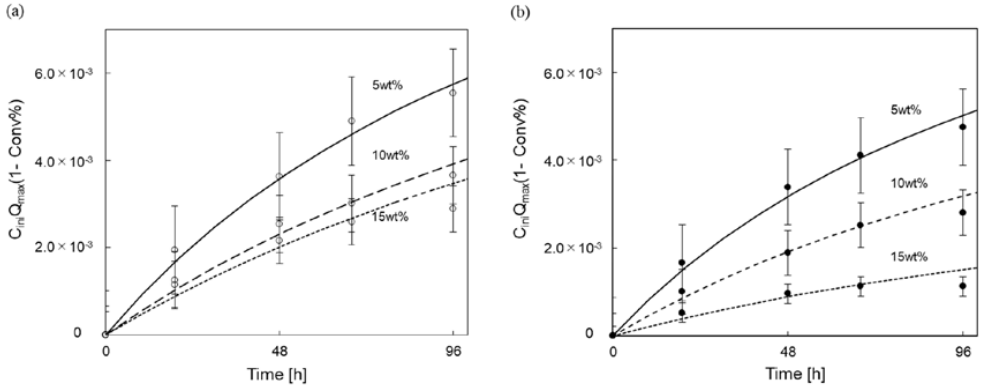

Figure 3. Langmuir adsorption isotherms for [Amim][Cl] (**a**) and for [Bmim][Cl] (**b**). Fitting parameters maximal adsorption value (Q_{max}) and affinity between enzyme and substrate (K) are shown in Table 1, which were analyzed using Equations (1) and (2).

Table 1. Fitting parameters, Q_{max} and K in Langmuir adsorption isotherm.

IL	IL Conc. (wt%)	Q_{max}	K
[Amim][Cl]	5	1.45×10^{-2}	6.85×10^{-2}
[Amim][Cl]	10	1.30×10^{-2}	4.50×10^{-2}
[Amim][Cl]	15	1.25×10^{-2}	4.00×10^{-2}
[Bmim][Cl]	5	1.20×10^{-2}	7.50×10^{-2}
[Bmim][Cl]	10	9.50×10^{-3}	5.25×10^{-3}
[Bmim][Cl]	15	5.00×10^{-3}	4.50×10^{-3}

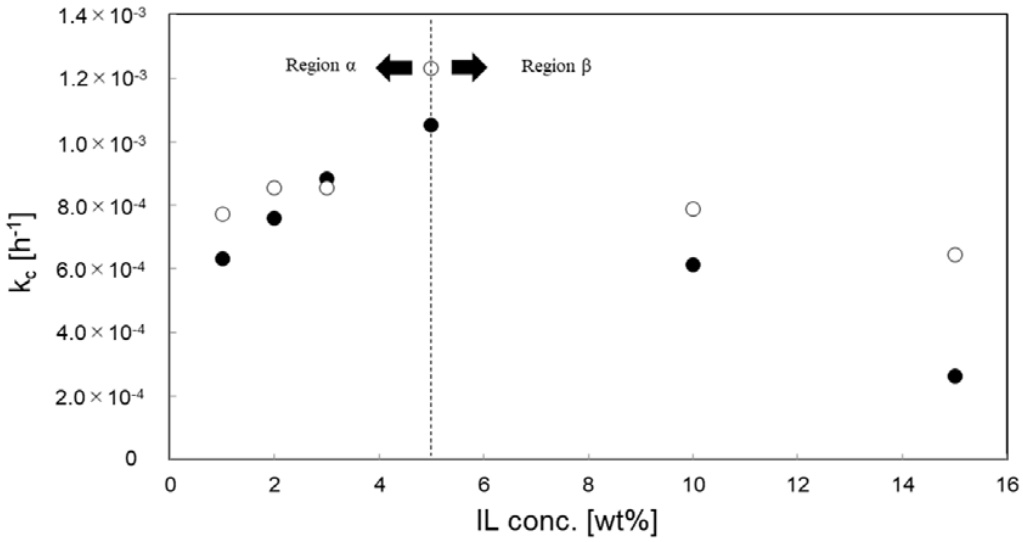

Figure 4. Correlation between reaction rate constant (k_c) and ionic liquid (IL) concentration. [Amim][Cl] (open circle), [Bmim][Cl] (closed circle).

3.4. Effect of IL Species and Concentration for Kinetic Analysis

In region α in Figure 4, the substrate concentration available for saccharification reaction could be proportional to the IL concentration. The hydrolysis by cellulase might be relevant to the dissolution of cellulose. Herein, the kinetic parameters for the enzymatic reaction were also analyzed based on the Michaelis-Menten model. The maximum reaction rate (V_{max}) and substrate affinity (K_m) can be obtained as follows:

$$V = \frac{V_{max}[S]}{K_m + [S]}, \tag{4}$$

wherein V and $[S]$ represent the reaction rate and substrate concentration, respectively. Assuming that the increased glucose production at the α region (Figure 4) is due to the dissolution effect of ILs, the substrate concentration $[S]$ could be proportional to IL concentration ([IL]):

$$[S] \propto [\mathrm{IL}]. \tag{5}$$

The kinetic parameters (V_{max} and K_m) can be analyzed based on the Lineweaver-Burk plot (Figures S2 and S3):

$$\frac{1}{V} = \frac{K_m + [S]}{V_{max}[S]} = \frac{K_m}{V_{max}} \times \frac{1}{[S]} + \frac{1}{V_{max}}. \tag{6}$$

The V_{max} and K_m values obtained from [Amim][Cl] and [Bmim][Cl] in the α region are compared in Table 2. The Michaelis-Menten constant K_m corresponds to the substrate concentration at the time of giving a rate of 1/2 of the maximum reaction rate. The V_{max} values of [Amim][Cl] and [Bmim][Cl] were almost same, indicating that no inhibitory effect of IL occurred at region α. The K_m values were 5.27×10^{-1} for [Amim][Cl] and 8.70×10^{-1} for [Bmim][Cl]. This suggests that [Amim][Cl] can be superior to produce the substrate for enzymatic saccharification. It is reported that [Amim][Cl] exhibits an excellent ability to destroy the crystal structure in cellulose, as compared to [Bmim][Cl] [23]. It is thus suggested that the rate-limiting step in region α is the adsorption of the enzyme to the dissolved substrate.

In this study, the apparent kinetic rate constants (k_c) were obtained with the values of 10^{-3}–10^{-4} (Figure S2). These values are smaller as compared to alkaline-treated cellulose saccharification (k–10^{-2}) [15]. It is also notable that the β-glucosidase activity of cellulase, determined by hydrolysis using cellobiose as the substrate, could not be inhibited by 15 wt% [Bmim][Cl] [18].

Therefore, the rate-limiting step in the enzymatic hydrolysis could be the reaction of endoglucanase or cellobiohydrolase.

Table 2. V_{max} and K_m values obtained from Lineweaver-Burk plot.

IL	V_{max}	K_m	V_{max}/K_m
[Amim][Cl]	5.70×10^{-3}	5.27×10^{-1}	1.08×10^{-2}
[Bmim][Cl]	5.80×10^{-3}	8.70×10^{-1}	0.67×10^{-2}

4. Conclusions

Cellulose hydrolysis using cellulase was promoted or inhibited by ILs, depending on the IL concentration. In the presence of an IL with higher concentration (≥7.5 wt%), the denaturation of cellulase was significant both in the presence of [Amim][Cl] and [Bmim][Cl], which could reduce the enzymatic activity. The Langmuir adsorption model can be applied to understand the actual concentration of substrate (dissolved cellulose) available for hydrolysis reaction. Based on the kinetic parameter analysis, the promoting effect of [Amim][Cl] on cellulose dissolution was greater as compared to that of [Bmim][Cl]. It is important to maintain a lower IL concentration for enzymatic activity, while a higher IL concentration is considered to be superior to generate dissolved substrate. Thus, IL concentration could be altered to optimize the efficiency in enzymatic saccharification in batch reactor processes.

Supplementary Materials: The following are available online at http://www.mdpi.com/2305-7084/2/4/47/s1, Figure S1: Viscosities of solutions including (a) [Amim][Cl] and (b) [Bmim][Cl], in the presence of cellulose. Figure S2: Correlation between IL concentration and reaction rate constant. Figure S3: Lineweaver-Burke plot in region α which IL concentration from 1 to 5 wt%.

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Conflicts of Interest: There are no conflicts of interest to declare.

Abbreviations

IL	ionic liquid
[Bmim][Cl]	1-butyl-3-methylimidazolium chloride
[Amim][Cl]	1-allyl-3-methylimidazolium chloride
Trp	tryptophan

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