

## ***In Situ* Saccharification of Cellulose using a Cellulase Mixture and Supplemental $\beta$ -glucosidase in Aqueous-Ionic Liquid Media**

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*In situ* enzymatic saccharification is one promising approach to efficiently convert non-food biomass to glucose as a platform chemical. The goal of the present research was to illustrate the catalytic behaviors and deactivation mechanisms of cellulase and cellobiase in an aqueous-ionic liquid (IL) media. The hydrolysis reaction rate was considerably increased because of the increased porosity and reduced crystallinity of the substrate. To lower the inhibitory effect of accumulated cellobiose on cellulase, a multi-enzyme synergetic system was explored. Compared with the single cellulase saccharification, addition of cellobiase resulted in a substantial increase in total reducing sugars (TRS) yield (88.0% vs. 59.1%) and glucose yield (40.6% vs. 28.9%) when the activity unit ratio of cellobiase/cellulase was 0.95. Cellulase showed a higher stability in aqueous 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac) media. However, the activity of cellobiase quickly decreased, by 66.5%, after 6 h of incubation.

**Keywords:** *In situ* saccharification; Aqueous-ionic liquid media; Cellulase; Cellobiase; Synergism

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### **INTRODUCTION**

In recent years, with the world population growing rapidly, energy and food shortages are rippling across the world. Development of biomass energy, especially cellulosic resources, has become a hot study program for science and technology workers (Himmel *et al.* 2007). Through photosynthesis, plants convert carbon dioxide into sugar, and approximately  $1.8 \times 10^{12}$  tons of biomass are produced annually. Approximately 40% of the dry weight of biomass is cellulose, but only a small fraction of available cellulosic resources is currently utilized (Imai *et al.* 2004). Cellulosic sources have recently received increasing attention, as they represent inexpensive, non-edible, renewable, and abundant raw materials. Dozens of green “platform chemicals,” such as glucose, 5-hydroxymethyl furfural (5-HMF), and levulinic acid, have been developed from cellulosic sources. However, the strong 3-D hydrogen-bonded frameworks in cellulose have been a big stumbling block to chemical activation and biological/chemical degradation. To resolve this issue, pretreatment of cellulose is inevitable. Pretreatment is one of the common methods used to destroy the crystal structure and to reduce the

polymerization degree of cellulose. In the meantime, the hemicellulose and lignin distribution are also affected (Hendriks and Zeeman 2009), and the pretreatment process may in addition open up the substrate to a less compact form. It is then easier to make the enzyme-catalyst contact with the cellulosic chains favorably (Zhang and Jérôme 2013).

The heterogeneous and recalcitrant nature of cellulosic materials represents an obstacle for their efficient saccharification. To date, processes including mechanical smashing, microwave treatment, hydrothermal treatment, acid and alkali treatment, and solvent treatment have been investigated for pretreatment of cellulose, with ionic liquid (IL) treatment being the most recent method (Mosier *et al.* 2005). The ILs have special physico-chemical properties, such as negligible vapor pressure, high heat capacity, high conductivity, liquid temperature range, good stability, environmental friendliness, and non-toxicity, which provide a feasible method to solve the insolubility of cellulose (Wang *et al.* 2012). ILs can disrupt and break the intramolecular hydrogen-bonding network (Swatloski *et al.* 2002), which provide a feasible way to solve the insolubility of cellulose.

Pretreatment techniques are required to make the polysaccharides more accessible to the catalytic action. The ILs can dissolve cellulose and reduce its crystallinity; both of these factors help improve the surface area for contact between catalyst and substrate (Li *et al.* 2013).

Ionic liquids are potential solvents for the enzymatic saccharification of cellulose. However, the anions of common ILs are nucleophilic and coordinate more strongly with the positively charged sites in the enzyme structure, causing conformational changes (Kaar *et al.* 2003). Moreover, the H-bond basicity of the IL anions affects the enzyme performance. To reduce the effect of ILs on enzyme activity and stability, it is required to dilute the ILs by adding some amount of water. Recently, researchers have paid more attention to studies involving the pretreatment of cellulose using ILs. Soudham *et al.* (2013) proposed an effective technique for hydrolyzing cellulose into glucose using an ionic liquid (1-allyl-3-methylimidazolium formate, [AMIM]COOH) at 120 °C, followed by hydrolysis of regenerated cellulose by cellulases and cellobiase. For purposes of the present discussion, the term “cellulases” will refer to a multi-enzyme complex consisting of exoglucanases and endoglucanases. Many cellulase mixtures also contain  $\beta$ -glucosidases, which have the role of breaking down cellobiose to glucose units. A glucose yield nine times as high as that of untreated cellulose was obtained in a reaction time of 12 h. Xie *et al.* (2012) studied the pretreatment of cellulose in a novel *N*-methylpyrrolidone (NMP)/1-ethyl-3-methyl imidazolium acetate ([EMIM]Ac) mixed solvent that can dissolve up to 10 wt% corn stover at 140 °C in 60 min. Enzymatic saccharification of the regenerated corn stover achieved an 82.9% total reducing sugars yield and a 60.8% glucose yield within 24 h. Even though the pretreatment process afforded a favorable glucose yield, the necessary waste disposal associated with the regeneration process poses a challenge for scaling-up this technology (Xu *et al.* 2015b).

Recently, enzymatic *in situ* saccharification process was proposed by Kamiya *et al.* (2008) to simplify the entire process by eliminating the need to recover the regenerated cellulose. In this strategy, the chosen ILs should be compatible with both cellulose solubility and cellulases activity. Thereafter, several interesting reports have been published concerning enzymatic polysaccharide hydrolysis in IL solutions (Xu *et al.* 2015a; Ninomiya *et al.* 2015). The ILs are believed to be excellent solvents for dissolving cellulose and are likely to induce rapid enzyme deactivation because of the H-bonding between anions ([Cl<sup>-</sup>], [HCOO<sup>-</sup>], and [CH<sub>3</sub>COO<sup>-</sup>]) and enzyme proteins (Xu *et al.* 2015b).

Moreover, the IL used for *in situ* saccharification is limited to imidazolium because enzyme-dissolving ILs usually inactivate enzymes. However, there are few actual studies available that describe the deactivation mechanism. Low concentrations of chloride impurities may drastically decrease enzyme activity, so 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac) was chosen as the reaction solution in the present study. *In situ* saccharification of cellulose by a multi-enzyme system was performed in the presence of high concentrations of imidazolium [EMIM]Ac. The enzymes retained relatively high activities in such aqueous-IL media. The activity and stability of cellulase (a multi-enzyme complexes consist of exoglucanases, endoglucanases, and  $\beta$ -glucosidases) and cellobiase were investigated in 20% (w/v) aqueous-[EMIM]Ac media. Enzyme inhibition kinetics was further analyzed to understand the mechanism of deactivation.

## EXPERIMENTAL

### Materials

Microcrystalline cellulose (Avicel<sup>®</sup> PH-101), cellulase (*T. reesei* ATCC 26921), and cellobiase (*A. niger*) were purchased from Sigma-Aldrich (99% purity, USA). 1-ethyl-3-methylimidazolium acetate ([EMIM]Ac) was acquired from Chengjie (Shanghai, China, 99% purity). Sodium azide (NaN<sub>3</sub>, 99% purity) was purchased from Beijing Dingguo Biotechnology Co., Ltd. Carboxymethyl cellulose (CMC) sodium (99% purity), cellobiose (99% purity), bovine serum albumin (BSA, 98% purity), 3,5-dinitrosalic acid (DNS, 98% purity), and bicinchoninic acid (BCA) hydrate disodium salt (98% purity) were purchased from J&K<sup>®</sup> Scientific Ltd. (Beijing, China).

### Cellulase and Cellobiase Activity Assay

Cellulase (CMCase) and cellobiase activities were determined according to the IUPAC method (Ghose 1987). The difference was that the amount of reducing sugar obtained from the CMCase activity determination was quantified by DNS method using glucose as standard. Furthermore, the released glucose concentration was assayed using a glucose analyzer (Biosensor SBA-50, Shandong). All experiments were done in triplicate. The CMCase and cellobiase activities were calculated using the following equations, respectively. The activity units (U) of these two enzymes are defined as the amount of enzymes that yield 1  $\mu$ mol of glucose per min under the conditions of the specific assay:

$$\text{CMCase activity (U/mL)} = (1000 \times \text{mass of glucose released}) / (\text{molecular weight of glucose} \times \text{sample volume} \times \text{reaction time}) \quad (1)$$

$$\text{Cellobiase activity (U/mL)} = (1000 \times \text{glucose mass released}) / (2 \times \text{molecular weight of glucose} \times \text{sample volume} \times \text{reaction time}) \quad (2)$$

Under similar operating conditions, the assays of CMCase and cellobiase activities were performed in 20% (w/v) aqueous-[EMIM]Ac solution (pH 5.0).

### *In Situ* Enzymatic Hydrolysis of Cellulose in Aqueous-[EMIM]Ac Media

Microcrystalline cellulose (0.1 g) was added to sterilized glass vials containing [EMIM]Ac (2.0 g), and the resulting mixture was stirred for 15 min at 80 °C in a shaking

bath (SHA-C, Baitajinchang Laboratory Instrument Works Co., Jintan, China). Then, 100  $\mu\text{L}$  of 2% (w/v)  $\text{NaN}_3$  solution was introduced to prevent microbial infection and to increase the accuracy of the subsequent determination of reducing sugar content. The supernatant solution was diluted to 10 mL, and 20% (w/v) aqueous-[EMIM]Ac solution was thus obtained. The crystallinity changes in cellulose induced by solubilization and regeneration were determined using an X-ray diffractometer (XRD; Rigaku Rotaflex RAD-C, Tokyo, Japan).

When using cellulase as the only enzyme biocatalyst, 25  $\mu\text{L}$  of cellulase (24.53 U) was added to an aqueous-[EMIM]Ac solution containing 0.1 g of cellulose to initiate the enzymatic reaction at pH 5.0, 50  $^\circ\text{C}$ , and 130 rpm. The hydrolyzate solutions were removed at time intervals of 6, 12, 24, 48, 60, and 72 h. During the cellulase hydrolysis of cellulose, the decelerated enzymatic reaction rate was partially attributed to the inhibition caused by the intermediate product cellobiose. To weaken the inhibition induced by cellobiose accumulation, cellobiase was supplemented to the hydrolysis system to enhance the conversion of cellobiose to glucose. Enzymatic hydrolysis in aqueous-[EMIM]Ac solution, simultaneously adding a certain amount of cellulase (245.28  $\mu\text{L}/\text{g}$  fed substrate) and a gradient concentration of cellobiase (138.9 U/g and 231.5 U/g fed substrates), was carried out at the same reaction condition as that of the single enzyme-catalyzed process. The total hydrolysis time was set at 72 h.

The removed sample was immediately heated at 94  $^\circ\text{C}$  for 5 min to terminate the enzyme reaction. Before the sample analysis, the hydrolysate solution was filtered by using microporous membrane (0.22  $\mu\text{m}$ , pore size), and then constant-volumed to 250 mL with buffer solution.

The total reducing sugars was measured by the DNS method with an ultraviolet (UV) visible spectrophotometer (Shimadzu, UV1800, Japan). Concentrations of glucose and cellobiose were measured using high-performance liquid chromatography (HPLC; LC-20A, Shimadzu, Japan) fitted with a refractive index detector and Aminex HPX-87H column (Bio-Rad, USA) at 60  $^\circ\text{C}$  with 0.005 M  $\text{H}_2\text{SO}_4$  as the mobile phase at a flow rate of 0.6 mL/min. Product yields (wt.%) of glucose, cellobiose, and TR were calculated as follows:

$$Y (\text{wt. } \%) = (\text{mass of product}) / (\text{mass of cellulose substrate}) \times 100\% \quad (3)$$

### Enzyme Catalytic Kinetics in Aqueous-[EMIM]Ac Media

Enzyme activity was determined according to the IUPAC method (Ghose 1987). Enzymatic hydrolysis experiments were carried out at various CMC concentrations (0.5 mL, 2 to 10 mg/mL) with 0.5 mL of cellulase and various cellobiase concentrations (1.0 mL, 0.5 to 6 mg/mL). Specific enzyme activity ( $v$ , U/mg protein) was obtained from enzymatic hydrolyses carried out at 50  $^\circ\text{C}$  and pH 5.0 in aqueous-[EMIM]Ac media with shaking at 130 rpm in a rotary shaker. Protein mass in the enzyme solution was measured using a published bicinchoninic acid (BCA) method (Smith *et al.* 1985). Kinetic parameters including  $V_{\text{max}}$  and the half-saturation constant ( $K_m$ ) were calculated from the Michaelis-Menten equation as follows,

$$\frac{1}{v} = \frac{1}{V_{\text{max}}} + \frac{1}{V_{\text{max}}} \times \frac{K_m}{[S]} \quad (4)$$

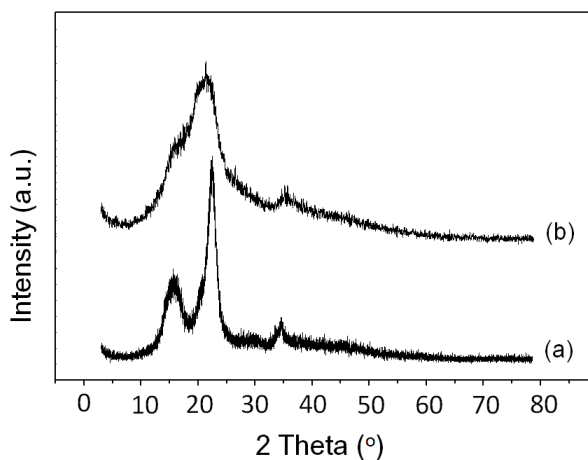
where  $v$  is the reaction rate (U/mg protein);  $V_{\max}$  and  $K_m$  are the maximum specific enzyme activity (U/mg protein) and the half-saturation constant (mg/mL), respectively; and  $S$  is the substrate concentration (mg/mL).

All cellulase and cellobiase activity measurements in this study were performed with pH values adjusted to 5.0.

## RESULTS AND DISCUSSION

The dissolution of cellulose is of great interest. Certain ILs are capable of dissolving a high concentration of cellulose. It is generally known that ILs can effectively break the H-bonding network of cellulose by interaction between the anions of ILs (as H acceptors) and cellulose hydroxyl groups (as H donors), even though the dissolution mechanisms are still not well understood (Remsing *et al.* 2006).

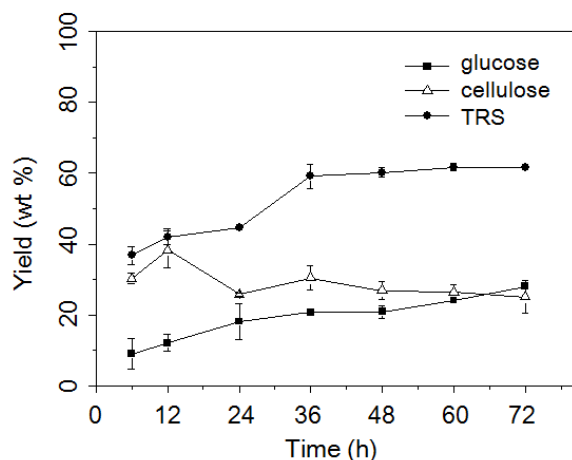
The XRD patterns of cellulose before and after the IL-dissolution are shown in Fig. 1. The XRD pattern of cellulose showed diffraction peaks at  $16^\circ$ ,  $22^\circ$ , and  $35^\circ$ . After the IL treatment, the crystalline peaks near  $16^\circ$  and  $35^\circ$  almost disappeared, and the crystalline peak at  $22^\circ$  turned to an amorphous halo peak (around  $20^\circ$ ). These results demonstrate that the crystallinity of cellulose was noticeably decreased. However, strong hydrogen bonds were being formed between ILs and cellulase as well, which also induced the inactivation of cellulase. The hydrolysis reaction rate would be noticeably affected by the increased porosity and reduced crystallinity of the substrate.



**Fig. 1.** XRD patterns of (a) cellulose and (b) IL-treated cellulose

Because of the low activity of cellulase at higher concentrations of IL, the hydrolysis of cellulose by a single catalyst (cellulase) was performed in aqueous-[EMIM]Ac media with an [EMIM]Ac concentration of 20% (w/v). As illustrated in Fig. 2, the TRS yield reached 59.1 g/L after 36 h of reaction. During this time, the glucose yield reached 20.8 g/L. However, the cellobiose yield increased from 6 to 12 h, but a further increase in the reaction time caused a decrease in the cellobiose yield, from 33.84 g/L to 2.51 g/L. Enzymatic hydrolysis of crystalline and amorphous cellulose requires the synergetic action of endo-1,4- $\beta$ -glucanase (EG), exo-cellobiohydrolases (CBH), and  $\beta$ -glucosidase (BG, cellobiase) (Hamid *et al.* 2015). The EG primarily acts on non-crystalline regions of cellulose, and its activity will be greatly improved with the decrease in cellulose crystallinity caused by IL dissolution. This is one reason why a high

cellobiose yield was obtained at 12 h. The enzyme CBH has the ability to bind to the substrate, and it is beneficial for the degradation of crystalline substrates (Hildebrand *et al.* 2015). This feature was influenced by the transformation of crystalline cellulose to amorphous cellulose.



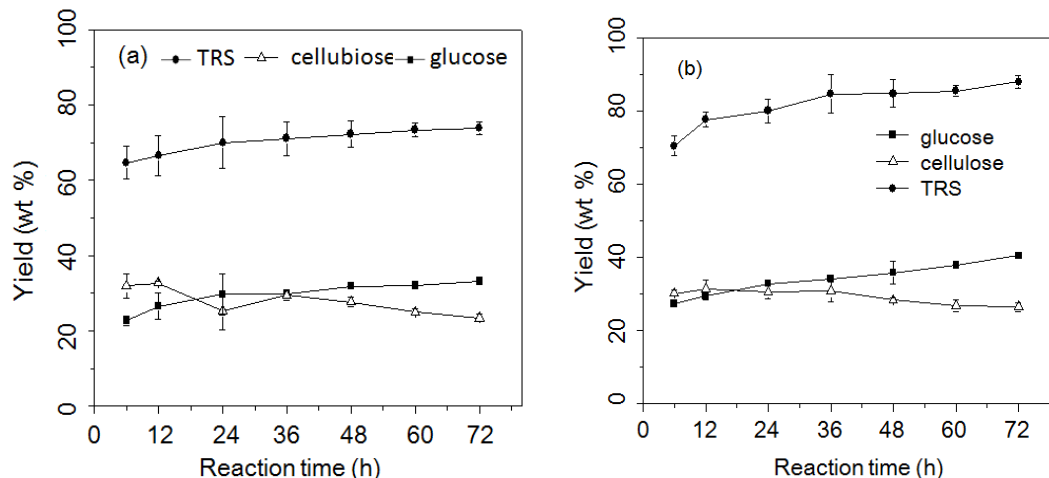
**Fig. 2.** Cellulose hydrolysis in aqueous-[EMIM]Ac medium with single cellulase at pH 5.0, 50 °C, and 130 rpm (EMIM]Ac, 2.0 g; cellulose, 0.1 g; cellulase, 25  $\mu$ L (981.1 U/mL); final reaction volume 10 mL)

Once it was thought that cellulose can be degraded into glucose by the synergic actions of these three types of enzymes (Sinitsyn *et al.* 1991; Dutta and Wu 2014). This view will change in aqueous-[EMIM]Ac medium. An increase in the reaction time from 36 to 72 h led to a nonsignificant increase in the TRS yield. Meanwhile, the glucose yield continued to increase to 2.81 g/L. On the contrary, the cellobiose yield decreased to 2.51 g/L at 72 h. The relatively high yield of cellobiose and low yield of glucose indicated that the currently used cellulase is greatly inhibited by the resultant cellobiose formed. Moreover, the inhibition retards the overall degradation of cellulose. Addition of extra cellobiase is needed to overcome the inhibition issue caused by the accumulation of cellobiose.

Cellobiase (CB), also known as  $\beta$ -glucosidase, has high specificity for hydrolysis of cellobiose into glucose, thus preventing cellobiose accumulation. The CB is responsible for the control of the entire speed of cellulose degradation by removing the product responsible for inhibition of the cellulase. It is necessary to improve the hydrolysis efficiency of cellulase *via* cellobiase.

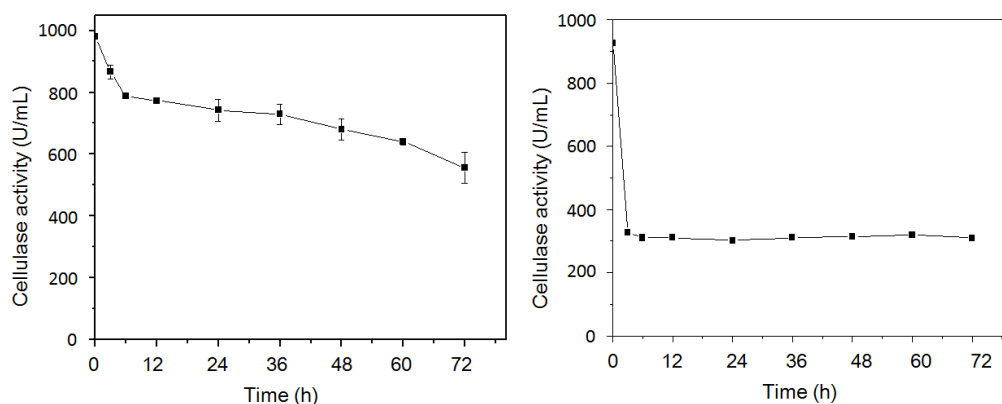
At the beginning of the reaction, the activities of CMCase and CB in the reaction were found at levels of 981.1 U/mL and 926.0 U/mL, respectively. When cellulase was supplemented with cellobiase, 3:5 and 1:1 volume ratios of cellobiase to cellulase were used to have excess cellobiase in the mixture, respectively. The corresponding activity units of cellulase in both fed substrate was 245.28 U/g, while the activity units of cellobiases were 138.9 U/g and 231.5 U/g, respectively. As shown in Fig. 3(a), the supplement of cellobiase decreased the yield of cellobiose (2.35 g/L vs. 2.51 g/L) and highlighted the saccharification results in terms of higher yield of glucose (3.33 g/L vs. 2.81 g/L), and TRS (7.38 g/L vs. 6.18 g/L) compared with single enzymatic saccharification method. Therefore, CB is an essential synergetic enzyme for the saccharification of cellulose.

When cellobiase loading increased from 138.9 U/g fed substrates to 231.5 U/g fed substrates (Fig. 3(b)), the yield of glucose and TRS increased by 4.45 g/L and 4.24 g/L, respectively. This indicates that the yield of glucose and TRS is related to CB level present in cellulase complex. The combined catalytic activity of the cellulase and CB is larger than the individual activities.



**Fig. 3.** Cellulose hydrolysis in aqueous-[EMIM]Ac medium by cellulase and cellobiase with activity ratios of (a) 0.57 and (b) 0.95 (EMIM]Ac, 2.0 g; cellulose, 0.1 g; final reaction volume 10 mL; at pH 5.0, 50 °C and 130 rpm)

Although cellulose can be efficiently converted to glucose, the activities of cellulase and cellobiase become compromised in the presence of aqueous-[EMIM]Ac solvents. Therefore, the activities and stabilities of cellulase and cellobiase were investigated in 20% (w/v) of aqueous-[EMIM]Ac medium. To understand the effect of [EMIM]Ac on enzyme activity, all parameters were kept constant in the former hydrolysis reaction using multi-enzyme synergetic saccharification. The activities of enzymes with incubation time are represented in Fig. 4. The CMCase activity unit gradually dropped to 556 U/mL from 981 U/mL after reaction time of 72 h, for which the average activity decreases by 43.3%. The solvent [EMIM]Ac has a much higher effect on cellobiase than cellulase. It was demonstrated that the activity of cellobiase quickly decreased by 66.5% in aqueous-[EMIM]Ac medium after 6 h incubation.



**Fig. 4.** Stability of (a) cellulase and (b) cellobiase in the presence of 20% (w/v) aqueous-[EMIM]Ac medium (EMIM]Ac, 2.0 g; cellulose, 0.1 g; final reaction volume 10 mL; at pH 5.0, 50 °C, and 130 rpm)

It is presumed that the decrease of enzyme stability in the ionic liquid was attributable to combined effects of high viscosity, salinity, and ionic strength of aqueous-ILs solvents (Engel *et al.* 2010). Moreover, Kudou *et al.* (2014) reported that the structural change of water clusters induced by [EMIM]<sup>+</sup> and Ac<sup>-</sup> resulted in the inhibition of glucosidase. It can be speculated that [EMIM]<sup>+</sup> and Ac<sup>-</sup> have similar inhibitory effect on cellulase and cellobiase.

Because of the inhibition effect of ILs on enzyme activity, a dynamic analysis is required to demonstrate the deactivation mechanism of both tested enzymes. The enzyme kinetics and enzyme inhibition of cellulase and cellobiase were studied, respectively. The Michaelis parameters  $K_m$  and  $V_{max}$  for the isolated enzyme were determined towards the substrates CMC and cellobiose from corresponding Lineweaver-Burk plots (Berghem and Pettersson, 1974). The enzyme reaction subject to [EMIM]Ac inhibition is illustrated in Fig. 5 by transforming the original data to a Lineweaver–Burke plot. To make an appropriate assessment of the pattern of inhibition, making an estimate of  $V_{max}$  and  $K_m$  from the data available is required. From this plot (Fig. 5(a)),  $V_{max}$  and  $K_m$  of cellulase in buffer were calculated to be 1.72 min·mg/mol and 1.00 min·mg/mol, respectively. Correspondingly, cellulase in 20% (w/v) of aqueous-[EMIM]Ac medium have much higher  $V_{max}$  (14.03 min·mg/mol) and  $K_m$  (29.21 min·mg/mol). The increase of  $V_{max}$  originated from a decrease of cellulose crystallinity in aqueous-[EMIM]Ac media. Decrease of  $K_m$  indicated that the affinity of the enzyme to its substrate was decreased. This may be partially due to the fact that [EMIM]<sup>+</sup> could act on inner hydrophobic parts of cellulase molecules leading to the destruction of the natural conformation of the enzyme (Combariza *et al.* 1994; Grossfield *et al.* 2003). Therefore, enzymatic hydrolysis of cellulose in aqueous-[EMIM]Ac media shows many advantages, such as decrease of cellulose crystallinity, high rate of reactions, and high product yields (TRS yield of 88%), except it attracted a certain degree of inhibition.

From the primary Lineweaver–Burk plots of cellobiase (Fig. 5(b)), the straight lines suggested a marked inhibitory effect of [EMIM]Ac on cellobiase. Compared with cellobiase in buffer, cellobiase in aqueous-[EMIM]Ac medium has a lower  $V_{max}$  (0.64 vs. 4.76 min·mg/mol) and  $K_m$  (0.13 vs. 1.32 min·mg/mol). These results suggest that [EMIM]Ac inhibited the enzyme uncompetitively—that is, [EMIM]Ac can only bind with the enzyme-substrate complex, not with the free enzyme.

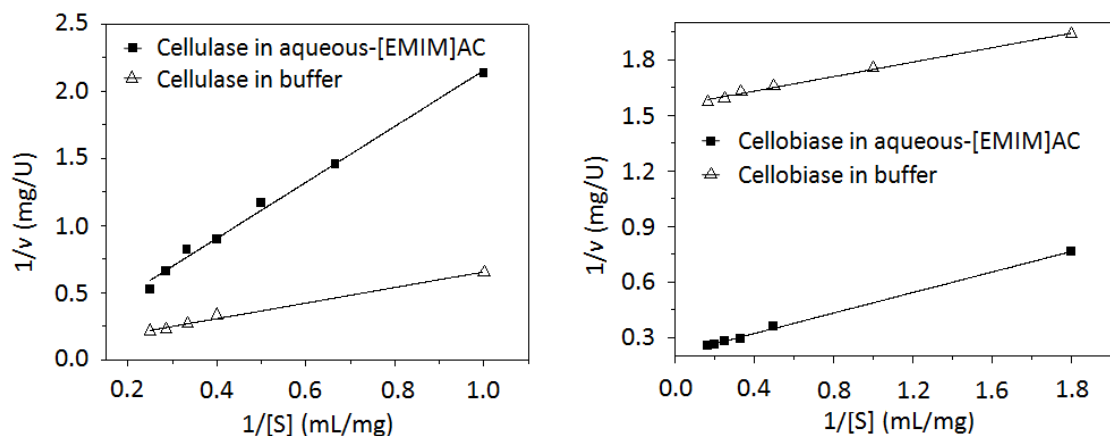


Fig. 5. Lineweaver–Burk plot of inhibition of cellulase (a) and cellobiase (b) by [EMIM]Ac



## CONCLUSIONS

1. A multi-enzyme synergetic system was exploited for cellulose hydrolysis in aqueous-ILs media. Although partly inhibited, cellulase showed acceptable catalytic activity in the presence of concentrations as high as 20% (w/v) [EMIM]Ac.
2. Addition of extra cellobiase is helpful for eliminating the inhibition caused by the accumulation of cellobiose.
3. Compared with cellobiase, cellulase showed a higher stability in aqueous-[EMIM]Ac media.
4. From kinetics analysis of cellulase and cellobiase, [EMIM]Ac inhibited the enzyme uncompetitively.

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## REFERENCES CITED

- Berghem, L. E., and Pettersson, L. G. (1974). "The mechanism of enzymatic cellulose degradation," *Eur J Biochem* 46(2), 295-305. DOI:10.1111/j.1432-1033.1974.tb03621.x
- Combariza, J. E., Kestner, N. R., and Jortner, J. (1994). "Energy-structure relationships for microscopic solvation of anions in water clusters," *J. Chem. Phys.* 100(4), 2851-2864. DOI: 10.1063/1.467231
- Dutta, S., and Wu, K. C. W. (2014). "Enzymatic breakdown of biomass: Enzyme active sites, immobilization, and biofuel production," *Green Chem.* 16(11), 4615-4626. DOI: 10.1039/C4GC01405G
- Engel, P., Mladenov, R., Wulfhorst, H., Jäger, G., and Spiess, A. C. (2010). "Point by point analysis: How ionic liquid affects the enzymatic hydrolysis of native and modified cellulose," *Green Chem.* 12(11), 1959-1966. DOI: 10.1039/C0GC00135J
- Ghose, T. (1987). "Measurement of cellulase activities," *Pure Appl. Chem.* 59(2), 257-268. DOI: 10.1351/pac198759020257
- Grossfield, A., Ren, P., and Ponder, J. W. (2003). "Ion solvation thermodynamics from simulation with a polarizable force field," *J. Am. Chem. Soc.* 125(50), 15671-15682. DOI: 10.1021/ja037005r
- Hamid, S. B. A., Islam, M. M., and Das, R. (2015). "Cellulase biocatalysis: Key influencing factors and mode of action," *Cellulose* 22(4), 2157-2182. DOI: 10.1007/s10570-015-0672-5
- Hendriks, A. T. W. M., and Zeeman, G. (2009). "Pretreatments to enhance the digestibility of lignocellulosic biomass," *Bioresour. Technol.* 100(1), 10-18. DOI:10.1016/j.biortech.2008.05.027

- Hildebrand, A., Szewczyk, E., Lin, H., Kasuga, T., and Fan, Z. (2015). "Engineering *Neurospora crassa* for improved cellobiose and cellobionate production," *Appl. Environ. Microb.* 81(2), 597-603. DOI: 10.1128/AEM.02885-14
- Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., and Foust, T. D. (2007). "Biomass recalcitrance: Engineering plants and enzymes for biofuels production," *Science* 316(5827), 982-982. DOI: 10.1126/science.1137016
- Imai, M., Ikari, K., and Suzuki, I. (2004). "High-performance hydrolysis of cellulose using mixed cellulase species and ultrasonication pretreatment," *Biochem. Eng.* 17(2), 79-83. DOI: 10.1016/S1369-703X(03)00141-4
- Kaar, J. L., Jesionowski, A. M., Berberich, J. A., Moulton, R., and Russell, A. J. (2003). "Impact of ionic liquid physical properties on lipase activity and stability," *J. Am. Chem. Soc.* 125(14), 4125-4131. DOI: 10.1021/ja028557x
- Kamiya, N., Matsushita, Y., Hanaki, M., Nakashima, K., Narita, M., Goto, M., and Takahashi, H. (2008). "Enzymatic *in situ* saccharification of cellulose in aqueous-ionic liquid media," *Biotechnol. Lett.* 30(6), 1037-1040. DOI: 10.1007/s10529-008-9638-0
- Kudou, M., Kubota, Y., Nakashima, N., Okazaki, F., Nakashima, K., Ogino, C., and Kondo, A. (2014). "Improvement of enzymatic activity of  $\beta$ -glucosidase from *Thermotoga maritima* by 1-butyl-3-methylimidazolium acetate," *J. Mol. Catal. B-Enzym.* 104, 17-22. DOI: 10.1016/j.molcatb.2014.02.013
- Li, C., Wang, A., and Zhang, T. (2013). "Progress of conversion of cellulose resource in ionic liquids," *CIESC J.* 1(64), 183-193. DOI: 10.3969/j.issn.0438-1157.2013.01.021
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y, Holtzapple, M., and Ladisch, M. (2005). "Features of promising technologies for pretreatment of lignocellulosic biomass," *Bioresour. Technol.* 96(6), 673-686. DOI: 10.1016/j.biortech.2004.06.025
- Ninomiya, K., Kohori, A., Tatsumi, M., Osawa, K., Endo, T., Kakuchi, R., Ogino, C., Shimizu, N., and Takahashi, K. (2015). "Ionic liquid/ultrasound pretreatment and *in situ* enzymatic saccharification of bagasse using biocompatible cholinium ionic liquid," *Bioresour. Technol.* 176, 169-174. DOI: 10.1016/j.biortech.2014.11.038
- Remsing, R. C., Swatloski, R. P., Rogers, R. D., and Moyna, G. (2006). "Mechanism of cellulose dissolution in the ionic liquid 1-n-butyl-3-methylimidazolium chloride: A  $^{13}\text{C}$  and  $^{35/37}\text{Cl}$  NMR relaxation study on model systems," *Chem. Commun.* 2006(12), 1271-1273. DOI: 10.1039/B600586C
- Sinitsyn, A., Gusakov, A., and Vlasenko, E. Y. (1991). "Effect of structural and physico-chemical features of cellulosic substrates on the efficiency of enzymatic hydrolysis," *Appl. Biochem. Biotechnol.* 30(1), 43-59. DOI: 10.1007/BF02922023
- Smith, P. K., Krohn, R. I., Hermanson, G., Mallia, A., Gartner, F., Provenzano, M., Fujimoto, E., Goeke, N., Olson, B., and Klenk, D. (1985). "Measurement of protein using bicinchoninic acid," *Anal. Biochem.* 150(1), 76-85. DOI: 10.1016/0003-2697(85)90442-7
- Soudham, V. P., Gräsvik, J., Alriksson, B., Mikkola, J. P., and Jönsson, L. J. (2013). "Enzymatic hydrolysis of Norway spruce and sugarcane bagasse after treatment with 1-allyl-3-methylimidazolium formate," *J. Chem. Technol. Biotechnol.* 88(12), 2209-2215. DOI: 10.1002/jctb.4089
- Swatloski, R. P., Spear, S. K., Holbrey, J. D., and Rogers, R. D. (2002). "Dissolution of cellulose with ionic liquids," *J. Am. Chem. Soc.* 124(18), 4974-4975. DOI:10.1021/ja025790m

- Wang, H., Gurau, G., and Rogers, R. D. (2012). "Ionic liquid processing of cellulose," *Chem. Soc. Rev.* 41(4), 1519-1537. DOI: 10.1039/C2CS15311D
- Xie, H., Shen, H., Gong, Z., Wang, Q., Zhao, Z. K., and Bai, F. (2012). "Enzymatic hydrolysates of corn stover pretreated by a N-methylpyrrolidone–ionic liquid solution for microbial lipid production," *Green Chem.* 14(4), 1202-1210. DOI: 10.1039/C2GC00033D
- Xu, J., Liu, X., He, J., Hu, L., Dai, B., and Wu, B. (2015a). "Enzymatic *in situ* saccharification of rice straw in aqueous-ionic liquid media using encapsulated *Trichoderma aureoviride* cellulase," *J. Chem. Technol. Biotechnol.* 90(1), 57-63. DOI: 10.1002/jctb.4458
- Xu, J., Wu, B., Hu, L., Wu, Z., Xu, N., Dai, B., and He, J. (2015b). "Enzymatic *in situ* saccharification of lignocellulose in a compatible ionic liquid-cellulase system," *Chem. Eng. J.* 267, 163-169. DOI: 10.1016/j.cej.2014.12.112
- Zhang, Q., and Jérôme, F. (2013). "Mechanocatalytic deconstruction of cellulose: An emerging entry into biorefinery," *ChemSusChem* 6(11), 2042-2044. DOI: 10.1002/cssc.201300765

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