Research Article

Differential Influence of Imidazolium Ionic Liquid on Cellulase Kinetics in Saccharification of Cellulose and Lignocellulosic Biomass Substrate

Prapakorn Tantayotai
Department of Microbiology, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand

Marttin Paulraj Gundupalli, Elizabeth Jayex Panakkal and Malinee Sriariyanun*
Department of Chemical and Process Engineering, The Sirindhorn International Thai-German Graduate School of Engineering (TGGS), King Mongkut's University of Technology North Bangkok, Bangkok, Thailand
Biorefinery and Process Automation Engineering Center (BPAEC), King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

Kittipong Rattanaporn
Department of Biotechnology, Faculty of Agro-Industry, Kasetsart University, Bangkok, Thailand

Debraj Bhattacharyya
Civil Engineering, Indian Institute of Technology Hyderabad, Telangana, India

* Corresponding author. E-mail: macintous@gmail.com DOI: 10.14416/j.asep.2021.11.003
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Abstract

The effect of [Emim][OAc] on Celluclast 1.5 L, Accellerase 1500, and IL-tolerant (MSL2) cellulase during the saccharification of carboxymethylcellulose (CMC), Avicel (AV), rice straw (RS) was studied in one pot process (pretreatment and saccharification). The inhibition caused by [Emim][OAc] (0.5–2.0 M) with substrate loading (20–50 mg/mL) were also evaluated. In most cases, the inhibition mode of saccharification for CMC and AV was identified to be uncompetitive inhibition when the concentration of [Emim][OAc] was higher than 0.5 M. Under the influence of 0.5 M of [Emim][OAc], the Critical Concentration of Substrate (CCS) values of the Celluclast 1.5 L and Accellerase 1500 on CMC hydrolysis were determined to be at 26.59 and 33.65 mg/mL, respectively. Also, increasing in [Emim][OAc] concentration could increase in CCS values, suggesting the positive effect of [Emim][OAc] on the improvement of enzymatic saccharification. This study provides insight into the process optimization for integration of [Emim][OAc] in one pot process of biorefinery.

Keywords: Cellulase, IL-tolerant, Kinetics, Mechanism, Residual IL, Saccharification

1 Introduction

In recent years, lignocellulosic biomass has been proposed as a potential resource for biofuel and value-added biochemical production in biorefining process [1]. However, one of the major drawbacks of using lignocellulosic biomass is its recalcitrant property and inhibitors [2], [3]. Pretreatment methods enhance hydrolysis of biomass through different mechanisms such as increased swelling of cellulose fibrils [4], removal of lignin [5], modify of cellulose crystallinity [6], degrade of hemicellulose [7], and increase porosity and contact area in biomass [8]. In the past few years, ionic liquid (IL) pretreatment has been extensively studied because ILs have emerged as a new class of chemicals for pretreatment with unique functions and properties, such as incombustibility, thermal stability, non-volatility, high chemical reactivity, high

ionic conductivity, electrochemical (wide range) and recyclability [9]. IL treatment has also been identified as a new, efficient, and environmentally friendly approach for fractionating lignocellulosic biomass [10]. An IL is a combination of organic or inorganic anions and cations. ILs include cations (phosphonium, azolium, pyrrolidinium, pyridinium, and alkylammonium) and anions (nitrate, halide, nitrite, sulfate, perchlorate, tetrafluoroborate, hexafluorophosphate, and azide) having different roles during the pretreatment [11].

Among these ILs, 1-Ethyl-3-methyl-imidazolium acetate, [Emim][OAc], is one of the most widely used IL has imidazolium and acetate as the cation and anion, respectively [12]. The efficiency of hydrogen bond rearrangement by [Emim][OAc] for dissolving cellulose has received much attention in recent years. The solubilized cellulose can be recovered as a precipitate using various anti-solvents, such as alcohols, acetone, or water [13]. The solubilized cellulose is then converted to monosaccharides upon saccharification by enzymatic or acid hydrolysis. [Emim][OAc] was demonstrated to decrease cellulose crystallinity of cotton stalks and increase enzymatic conversion to glucose up to 82–85%. Pretreatment with [Emim][OAc] could handle a high loading biomass ratio up from 3% to 15% w/v with small reduction in glucose yield from 67% to 55% [14]. [Emim][OAc] was shown to work well in ambient temperatures with fast retention time, 30 min, to solubilize pulp fibers with the highest yield of 78% at optimal pretreatment conditions. It is an excellent solvent in the fractionation of hemicellulose from lignocellulose [15].

IL has demonstrated to be an excellent solvent in pretreatment to improve enzymatic saccharification and fractionate cellulose, hemicellulose, and lignin. The use of IL has been reluctant due to evidence of cellulase inactivation [16], [17]. ILs with a lower concentrations of 25–50% was demonstrated to be effective in pretreatment [18]. However, when the residual IL in the solution exceeded 10%, cellulase activity was inhibited [19]. Additions of several types of excellent cellulose-solubilizing ILs, such as [Bmim][Cl], [Amim][Cl], [Emim][Cl], [Emim][OAc], and [Dmim][DMP] showed an inhibitory effect in cellulase saccharification up to 60–80% [16]. Therefore, extensive washing of ILs after pretreatment is necessary to remove remaining residue before processing to enzyme hydrolysis, which leads to extra operational cost, process complexity, and burdens in wastewater treatment. The step toward improvement of IL-mediated biorefining process of lignocellulose has been continuously developed by several strategies, such as using IL-tolerant cellulase [12], [20], low cellulase-inhibition ILs [21], and genetically engineered IL-tolerant cellulase [22].

With all these solutions, a one-pot process combining pretreatment and enzymatic saccharification is expected to have applications in industrial process. Therefore, it is essential to understand the mechanisms of ILs in activation and inactivation of enzymatic saccharification in one-pot reactions. In this study, low concentration of IL, [Emim][OAc] was added to cellulose and lignocellulose to investigate IL's influences on enzymatic saccharification. The mechanism was studied based on monitoring of kinetic parameters (V_m and K_m) of enzymatic saccharification by fitting the experimental data with various linearized Michaelis-Menten models. Furthermore, two types of commercial cellulases, Celluclast 1.5 L and Accellerase 1500, and IL-tolerant cellulase, isolated from *Bacillus sp.* MSL2 [23] were selected in this work to differentially hydrolyze cellulose and lignocellulose under the same conditions of [Emim][OAc] influences. The Critical Concentration of Substrate (CCS) values in activation and inactivation of enzymatic saccharification were determined based on kinetic modeling and calculations, which could further be applied in the process design and optimization of the one-pot biorefining process.

2 Materials and Methods

2.1 *Cellulose and lignocellulosic biomass substrate*

The rice straw (RS) was obtained by collecting the biomass from the paddy fields in Northern Thailand. Carboxymethylcellulose (CMC) and Avicel (AV) was purchased from Sigma-Aldrich (USA). The lignocellulose (RS) was dried at 60 °C overnight for 12 h in a hot air oven to remove moisture until the constant dried weight was obtained. Size reduction of the RS samples was carried out by using the household food processor. The powdered samples were passed through a 20-mesh aluminium sieve to achieve consistent particle size in a homogeneous mixture. The dried sample was stored in an airtight bag until further use.

2.2 *Preparation of crude MSL2 cellulase*

The crude IL-tolerant cellulase enzyme was produced by *Bacillus sp.* MSL2, isolated from a local rice paddy field [23]. The culture of *Bacillus sp.* MSL2 was grown in CMC broth (containing 0.5% CMC, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% $MgSO_4$, 0.05% yeast extract). The culture was incubated at 50 °C for 48 h in an orbital shaker at 200 rpm. The supernatant, which contained extracellular cellulose, was then harvested by centrifugation at 8,000 RPM for 20 min at 4 °C. To concentrate the cellulase, ammonium sulfate was added to 80% concentration and left overnight at 4 °C to allow protein precipitation. The enzyme pellet was harvested by centrifugation at 10,000 RPM for 20 min in at 4 °C, and the pellet was re-dissolved in 10 mL of 0.05 M sodium phosphate buffer (pH 5.0) for dialysis.

Desalting of concentrated cellulase was conducted by using a Float-A-Lyzer dialysis membrane (Spectrum Lab) with 10 kDa MWCO placing in 0.05M phosphate buffer (pH of 5.0). The dialysis buffer was changed three times every two hours. The desalted crude enzyme was concentrated again using a Vivaspin-500 column (GE Healthcare Life Science) and this cellulase fraction was used for testing in this work. The cellulase activity of the crude enzyme was determined by following the NREL protocol [24], and the reducing sugar released by the enzymatic saccharification was determined by the modified Miller's method [25], [26].

2.3 *Enzymatic saccharification of substrates*

The enzymatic saccharification of the CMC, AV and RS were carried out using two types of commercial cellulase enzymes, Celluclast 1.5 L (Sigma-Aldrich, USA) and Accellerase 1500 (Genecor, USA), and IL-tolerant MSL2 cellulase. The saccharification was carried out to understand the effect of [Emim][OAc] in different concentrations (0.5, 1.0, and 2.0 M) on reducing sugar yield from different substrates. The substrate (CMC, AV, and RS) concentrations were varied from 20 to 50 mg/mL. The selected substrate and enzyme (20 FPU/g) was added to a 2 mL centrifuge tube with 1 mL of 50 mM citrate buffer (pH 4.8) [27]. The tubes were mixed thoroughly using a vortex shaker and incubated at 50 °C for 30 min in an inorbital shaker (200 RPM). After the saccharification process, the reducing sugar was quantified using the modified Dinitrosalicylic acid method for the supernatant fractions collected after centrifugation (5,000 RPM, 15 min) [25], [26].

2.4 *Kinetics studies of enzymatic saccharification*

The kinetic parameters (K_m and V_m) of enzymatic saccharification was determined for the saccharification of different substrates concentration (20–50 mg/mL) using different enzymes, with and without the addition of [Emim][OAc]. These kinetic parameters were further evaluated to determine the effect of [Emim][OAc] on saccharification reactions. The types of inhibition in saccharification (including competitive, uncompetitive, non-competitive, and mixed inhibition) were identified by fitting with experimental data. The kinetic parameters, half-velocity constant, (K_m, mg/mL) and maximal velocity (V_m, mg/mL.min) were determined through different fitting models derived from the Michaelis-Menten (MM) equation shown in Equation (1). Three linearized models were derived from Equation (1) that includes Line weaver Burk, Hanes Woolf, and Eddie Hofstie models as expressed in Equations (2)–(4), respectively [28].

$$V_o = \frac{V_m[S]}{K_m + [S]} \tag{1}$$

$$\frac{1}{V_o} = \frac{K_m}{V_m[S]} + \frac{1}{V_m} \tag{2}$$

$$\frac{[S]}{V_o} = \frac{[S]}{V_m} + \frac{K_m}{V_m} \tag{3}$$

$$V_o = \frac{-K_m V_o}{[S]} + V_m \tag{4}$$

3 Results and Discussion

ILs enhance the saccharification efficiency due to modification in the structural arrangements of cellulose fibrils. However, the efficiency varies from biomass to biomass due to the different compositions of hemicellulose, cellulose, and lignin. Therefore, the present study focuses on using different cellulose substrates, CMC and Avicel (with different crystallinity index and degree of polymerization) and lignocellulosic biomass (Rice straw (RS)). Because CMC, AV, and

RS are polymeric molecules and different average molecular weight of CMC are reported by different commercial vendors, therefore this experiment focused on using %w/v, but not a molar unit, to reduce bias in substrate concentration.

Aforementioned, the cellulose precipitation after IL pretreatment requires extensive washing to remove the traces of the residual ILs. It has been reported that for every wash of the pretreated biomass, the concentration of residual IL decreases, and the saccharification efficiency increases [29]. It has been reported that ILs tend to bind to the cellulose structure due to the strong hydrogen bond between the IL and cellulose structure, assisting in the pretreatment process [30]. The residual IL remaining in the pretreated biomass has been reported to inhibit *Saccharomyces cerevisiae* and cellulase enzymes during the saccharification and fermentation process, respectively [31]. The degrees of inhibitory effects of residual ILs were demonstrated to be varied depending on the types of cellulases [32]. Therefore, in this study, commercial cellulase (Celluclast 1.5 L and Accellerase 1500) and IL-tolerant cellulase (MSL2) was used to evaluate the effect of [Emim][OAc] on the one-pot reaction, which combined pretreatment and saccharification together. The present study was carried out using [Emim][OAc] at different concentration such as 0.5, 1.0 and 2.0 M, which equivalent to 8.5, 17.0 and 34% w/v respectively. These concentrations were in the range of efficient biomass pretreatment [18] and inhibition of cellulase in previous studies [20], [33]. Therefore, the positive effect from IL pretreatment and negative effect from cellulase inhibition on saccharification and enzymatic efficiency could be observed simultaneously in a one-pot reaction in this work.

3.1 *Enzymatic saccharification of different substrates*

The effects of [Emim][OAc] under various concentrations on the enzymatic saccharification of cellulose substrates (CMC and AV) and lignocellulose substrate (RS) were studied in detail. Enzymes hydrolyzed the substrates with and without the addition of [Emim][OAc] for 30 min (50 °C). Studies have reported that the initial activity of the enzyme can be studied within 30 min of the hydrolysis reaction [23], [34]. In different literatures, the effect of IL on cellulose structures under lower temperature has been studied and reported [15]. In the present study, the saccharification was carried out at 50 °C with the addition of [Emim][OAc]. ILs, including 1-alkyl-3-methylimidazolium phosphonate ([Amim(MeO)PHO_2], phosphinate-type ILs and [Emim][OAc] were demonstrated to change the cellulose structure in biomass even under lower temperature in the range of 25–50 °C [35], [36]. In the present study, it is hypothesized that the presence of [Emim][OAc] has effect on the structure of cellulose in CMC, AV, and RS to promote accessibility of cellulase to the substrate and, consequently, increases conversion of substrate to sugars. Also, [Emim][OAc] molecules have inhibitory effect on cellulase by providing unfavorable environment to hydrolysis reaction, or they directly interact with cellulase and impair cellulase function.

The sugar concentration varied as the initial concentration of the substrates increased with and without the addition of [Emim][OAc] (Figure 1). The reducing sugar yield also varied for saccharification using different types of cellulases, commercial and IL-tolerant MSL2. The released reducing sugar concentration was higher for CMC and AV compared to RS, indicating to different accessibilities of cellulase to substrates. Without [Emim][OAc], an increase in the reducing sugar was observed as the substrate concentration increased. Therefore, it can be assumed that, without the addition of [Emim][OAc], the saccharification reaction follows the first-order kinetics as shown in Equation (5).

$$-k[S] = \frac{d[P]}{dt} \quad (5)$$

With the addition of 2.0 M [Emim][OAc], reducing sugar amounts released from hydrolysis of CMC by Celluclast 1.5 L and Accellerase 1500 and MSL2 were reduced compared to the control sample [Figure 1(a)–(c)]. Similar patterns of negative effects of [Emim][OAc] on hydrolysis of AV [Figure 1(d)–(f)] and RS [Figure 1(g)–(i)] were observed, but even more sensitive to lower concentrations of [Emim][OAc]. However, comparing to commercial cellulases, the reductions in reducing sugars under the influence [Emim][OAc] in hydrolysis reactions of IL-tolerant MSL2 were smaller [Figure 1(a)–(c)]. It should be noted that the negative effects of [Emim][OAc] on enzymatic saccharification were amplified when higher substrate concentrations were used in types of substrates when using commercial cellulases [Figure 1(a), (b), (d), (e), (g), (h)], but not

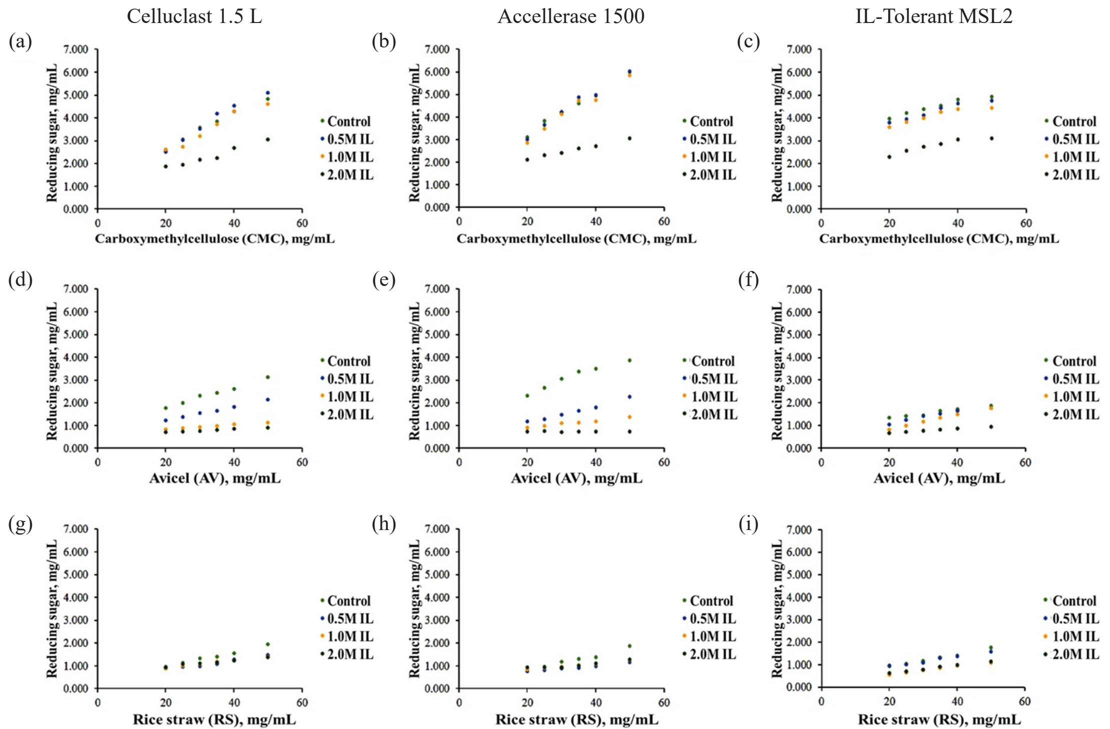

Figure 1: Reducing sugar yield determined for saccharification of CMC, AV, and RS using Celluclast 1.5 L, Accellerase 1500 and MSL2 enzymes with and without addition of [Emim][OAc].

in IL-tolerant MSL2 [Figure 1(c), (f), (i)] . That is the gap between the set with and without [Emim][OAc] at substrate concentration at 50 mg/mL was larger than that of 20 mg/mL.

In our previous studies, it was observed that at lower concentration of CMC substrate, the addition of 1.0 M [Emim][OAc] the enzymatic saccharification by Celluclast 1.5 L, IL-tolerant MSL2, and IL-tolerant CT-1 (obtained from bacterial consortium) was enhanced, but at higher CMC concentration, the reducing sugar yields were reduced by the addition of [Emim][OAc] [12], [20]. This observation could be hypothesized that, among IL-cellulose-water system, at a low concentration of cellulose substrate, there are sufficient amounts of IL to interfere with intramolecular hydrogen bonding of cellulose fibrils and therefore they allow dissociations of cellulose complex to expose with water and cellulase. On the other hand, at high cellulose concentration, less molecules of [Emim][OAc] are available because they are already occupied by cellulose, therefore less water molecules can get access to cellulose, as well as cellulase.

3.2 *Effect of [Emim][OAc] on reaction rate of saccharification*

The reaction rate for the saccharification of CMC, AV, and RS using different cellulase enzymes with and without the addition of [Emim][OAc] were studied. The saccharification rate (V, mg/mL.min) of different cellulases increased as the substrate concentration increased, the addition of [Emim][OAc] resulted in different reaction rates to this trend (Table 1). Similar to reducing sugar yields, saccharification rates were reduced with the addition of [Emim][OAc], except when 0.5 M [Emim][OAc] was added in CMC hydrolysis reaction with Celluclast 1.5 L. This observation suggested that IL pretreatment was less effective than IL inhibition on cellulase. It is observed that the saccharification rates decreased when a higher concentration of [Emim][OAc], (1.0 and 2.0 M) was used. Shi *et al*. evaluated the effect of [Emim][OAc] on enzymatic activity by carrying out the pretreatment and saccharification in a one-pot process. The study was concluded with 81.2% and 87.4% yield of glucose

and xylose when the concentration of [Emim][OAc] was maintained at 10% loading (0.5–0.6 M), respectively [37].

Comparing between commercial cellulases, Celluclast 1.5 L and Accellerase 1500, and IL-tolerant MSL2, the degree of a negative effect of [Emim][OAc] on saccharification yield was different. At condition of 50 mg/mL AV, the saccharification yield for Celluclast 1.5 L and Accellerase 1500 were reduced to 68.27% and 58.91% with the presence of 0.5 M of [Emim][OAc], while the hydrolysis rate of MSL2 was maintained to 95.16% compared to without [Emim][OAc] condition. Likewise, when 2.0 M [Emim][OAc] was added, the hydrolysis rates of Celluclast 1.5 L and Accellerase 1500 were further decreased to 29.81% and 19.38%, while this rate of MSL2 was 50.00%. Similar trend was observed in RS substrate at 50 mg/mL that presence of 0.5 M of [Emim][OAc], the saccharification rates of Celluclast 1.5 L and Accellerase 1500 were dropped to 76.92% and 62.90%, but the rate of MSL2 was still retained at 89.83%.

Therefore, it can be confirmed with our previous study that MSL2 exhibited tolerance towards the lower concentration of [Emim][OAc] [20]. It should be noted that when the [Emim][OAc] concentration was raised to 2.0 M, the hydrolysis rate of MSL2 to CMC at 50 mg/mL was similar to Celluclast 1.5 L and Accellerase 1500 that was 63.35, 51.00, and 62.80%, respectively. This results, added up our previous study that concentration of 2.0 M [Emim][OAc] was over the tolerant limit of MSL2 because only up to 1.0 M [Emim][OAc] was tested. In fact, most of previous research to investigate the inhibitory effect of ILs were conducted with the challenge of IL concentration no more than 20% (2.0 M of [Emim][OAc]) is equivalent to 34% [30], [38]. Saccharification kinetic parameters and inhibition patterns.

The Michaelis-Menten model is generally applied with a homogeneous reaction to monitor enzyme kinetics. The maximal reaction velocity (V_m) of Michaelis-Menten model is identified when saturated

Table 1: Reaction rate (V, mg/mL.min) for saccharification of CMC, AV, and RS using commercial (celluclast 1.5 L and accellerase 1500) and synthesized MSL2 enzyme with and without addition of [Emim][OAc]

Substrate	Concentration (mg/mL)	V, mg/mL/min											
		Celluclast 1.5 L				Accellerase 1500				MSL2			
		Control	0.5M IL	1.0M IL	2.0M IL	Control	0.5M IL	1.0M IL	2.0M IL	Control	0.5M IL	1.0M IL	2.0M IL
CMC	0.161	0.170	0.154	0.102	0.200	0.201	0.195	0.102	0.164	0.159	0.148	0.103	0.161
	0.143	0.152	0.143	0.090	0.166	0.166	0.159	0.091	0.160	0.155	0.146	0.102	0.143
	0.128	0.140	0.124	0.075	0.154	0.163	0.158	0.087	0.151	0.148	0.142	0.096	0.128
	0.119	0.118	0.106	0.073	0.141	0.141	0.138	0.081	0.146	0.137	0.133	0.091	0.119
	0.101	0.101	0.101	0.091	0.065	0.128	0.122	0.117	0.077	0.140	0.131	0.127	0.085
	0.087	0.084	0.084	0.087	0.062	0.104	0.100	0.095	0.071	0.133	0.126	0.120	0.076
AV	0.104	0.071	0.071	0.038	0.031	0.129	0.076	0.046	0.025	0.062	0.059	0.051	0.031
	0.087	0.061	0.061	0.036	0.029	0.117	0.060	0.039	0.025	0.057	0.054	0.049	0.028
	0.082	0.055	0.055	0.033	0.027	0.112	0.055	0.038	0.025	0.055	0.051	0.045	0.027
	0.077	0.052	0.052	0.031	0.025	0.102	0.049	0.037	0.024	0.048	0.044	0.037	0.025
	0.066	0.046	0.046	0.029	0.025	0.089	0.043	0.033	0.025	0.047	0.041	0.033	0.024
	0.059	0.041	0.041	0.028	0.024	0.078	0.040	0.031	0.025	0.045	0.038	0.030	0.022
RS	0.065	0.050	0.050	0.047	0.046	0.062	0.039	0.042	0.042	0.059	0.053	0.046	0.039
	0.052	0.043	0.043	0.041	0.041	0.046	0.033	0.036	0.037	0.047	0.046	0.036	0.033
	0.047	0.037	0.037	0.040	0.039	0.043	0.031	0.034	0.034	0.044	0.043	0.028	0.030
	0.044	0.033	0.033	0.036	0.037	0.040	0.029	0.032	0.032	0.039	0.036	0.025	0.026
	0.038	0.032	0.032	0.033	0.036	0.032	0.027	0.030	0.031	0.035	0.034	0.022	0.023
	0.032	0.030	0.030	0.030	0.031	0.026	0.025	0.028	0.031	0.031	0.032	0.018	0.021

IL – Ionic liquid, [EMIM][OAc]; CMC – Carboxymethylcellulose; AV – Avicel; RS – Rice straw; V – reaction rate, mg/mL.min

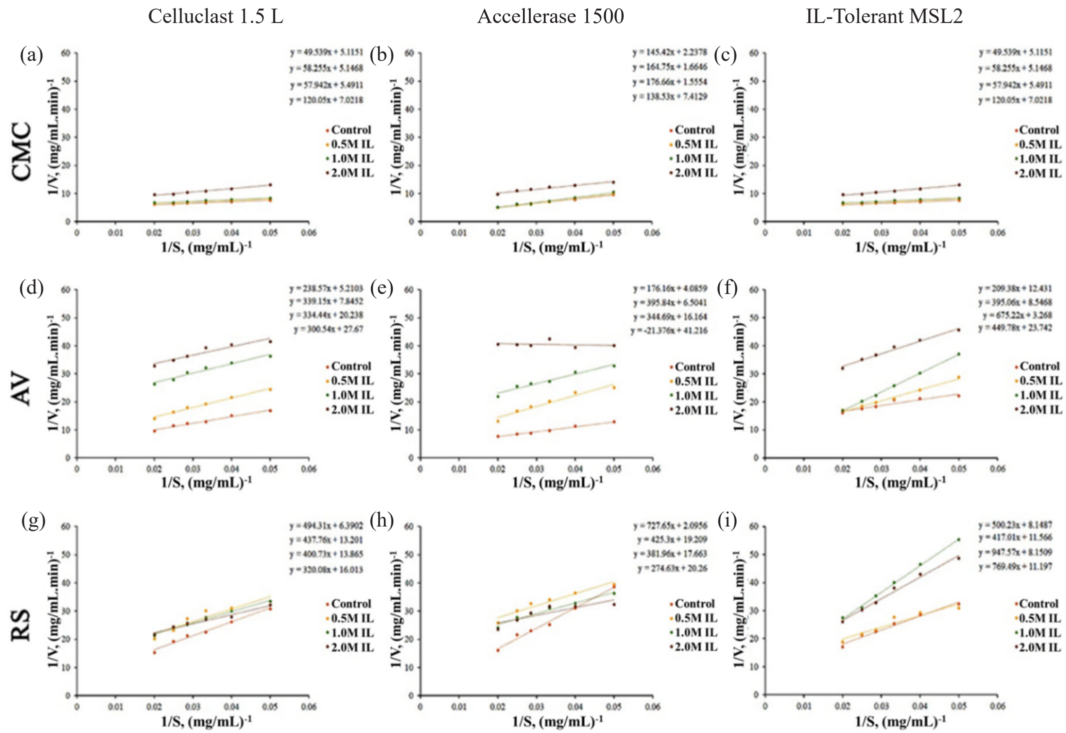

Figure 2: Lineweaver-Burk plot (LWB) to determine the saccharification kinetic parameters for CMC, AV, and RS using Celluclast 1.5 L, Accellerase 1500 and MSL2 enzymes with and without addition of [Emim][OAc].

concentration is used. Michaelis-Menten constant (K_m) is identified at the substrate concentration that reaches half of V_m. With wide application of the Michaelis-Menten model, it is also further applied to explain different biological phenomena, such as cell growth in the fermentation process and enzymatic saccharification of cellulose and lignocellulose [39]–[42]. In this work, the Michaelis-Menten model was applied to explain the saccharification rate of cellulose and lignocellulose substrates by cocktail cellulases.

The kinetic parameters (K_m and V_m) were determined based on model fitting of three linearized models of the Michaelis-Menten model. The suitable model for the current study was selected based on the coefficient of determination (R^2) value. The R^2 values obtained from three different linearized models (LWB, EH, and HW) were determined from the experimental results of enzymatic saccharification with and without the addition of [Emim][OAc]. The R^2 values for the EH model were < 0.9 in all cases of substrates and enzymes. On the other hand, LWB and HW were considered suitable to represent the experimental data of enzymatic saccharification. However, LWB was considered more suitable for all three studies since the R^2 values were nearer to 1.0 than HW. Therefore, the kinetic parameters (K_m and V_m) in this work were determined from the LWB model as shown in Figure 2.

The IL inhibition patterns (Competitive inhibition, Uncompetitive inhibition, Non-competitive inhibition and Mixed inhibition) were analyzed from the modifications of dissociation constants of V_m and K_m determined from the LWB plot [28]. The inhibition caused by inhibitor [I] can be overcome by increasing the substrate or enzyme concentration, which affects the dissociation constant for maximum rate (V^*) and the Michaelis-Menten constant (K^*). The dissociation constant (V^* and K^*) of inhibition in saccharification are determined from the Equations (6) and (7) [43].

$$V^* = \frac{V_m}{V_{m'}} \tag{6}$$

$$K^* = \frac{K_m}{K_{m'}} \tag{7}$$

Where, V^* and K^* are the dissociation constant, V_m is the maximum rate of velocity, K_m is the half velocity constant. $V_{m'}$ and $K_{m'}$ are the maximum rate of velocity and Michaelis-Menten constant for the control samples.

The saccharification kinetic parameters, V_m and K_m, of three types of substrates using three types of cellulases with and without the addition of [Emim][OAc] have been listed in Table 2. It was observed that the V_m values of saccharification of CMC, AV, and RS using Celluclast 1.5 L were reduced for 1.12, 3.91 and 2.16 fold times, respectively. On the other hand, [Emim][OAc] set at 1.0 M resulted in inhibition of cellulase. When [Emim][OAc] concentration was further increased to 2.0 M, the V_m values were also decreased by 2.44, 5.33, and 2.51 fold times, respectively, in comparison to the control. Comparing the V_m reduction of each substrate, it was demonstrated that the IL inhibitory effect depends on the substrate type when hydrolyzed by the same cellulase at the same IL concentration. This finding could be explained by the natural physical, and chemical properties of these tested substrates, such as degree of polymerization, molecular weight, viscosity that was demonstrated to be influenced by substrate solubility in IL [32].

Due to the IL-tolerant property of MSL2, a less inhibitory effect of [Emim][OAc] was observed for substrate hydrolysis. The CMC saccharification by MSL2 enzyme with 0.5M [Emim][OAc] showed no inhibition since the V_m value did not change. Aforementioned, IL tolerance of MSL2 cellulase was clearly impaired at 2.0 M concentration of [Emim] [OAc]. V_m values of saccharification of CMC, AV, and RS by using MSL2 were reduced by 1.37, 1.90, and 1.38 times. It can be inferred that higher IL concentrations represent a greater negative effect on cellulase inhibition than the positive effect of IL pretreatment. The increase in V_m values were observed in some experimental cases in this work. V_m of CMC hydrolysis using Celluclast 1.5 L and Accellerase 1500 with 0.5 M [Emim][OAc] increased 1.73 and 1.34 times, respectively. On the other hand, V_m of AV hydrolysis under the influence of 0.5 M and 1.0 M [Emim][OAc] by MSL2 increased 1.16-fold and 1.37-fold, respectively. Also, 1.0 M [Emim][OAc] in the hydrolysis mixture of RS and MSL2 increased V_m by 6.74 times. It is implied that a lower concentration of [Emim][OAc] activated the enzymes resulting in an increase of V_m compared to the condition without [Emim][OAc]. The increase in V_m is due to the activation of enzymes rather than the inhibitory role of [Emim][OAc] in the mixture. Therefore, it can be concluded that [Emim][OAc] act as enzyme inhibitors and activators at high and low concentrations, respectively. Comparing to inhibitory effect of IL on the dissociation constant in Equations (6) and (7), this constant for the activation of saccharification is determined from Equations (8) and (9) [43].

$$V^* = \frac{V_{m'}}{V_m} \quad (8)$$

$$K^* = \frac{K_{m'}}{K_m} \quad (9)$$

Altogether, the types of inhibition and activation are determined from the dissociation constants, V^* and K^* [44], [45]. The inhibition type was verified from the equation expressed in Equations (10)–(13) [44].

Table 2: The kinetic parameters (V_m and K_m) determined for saccharification of CMC, AV, and RS using commercial (celluclast 1.5 L and accellerase 1500) and MSL2 enzyme with and without addition of [Emim][OAc]

E		Celluclast 1.5 L				Accellerase 1500				MSL2			
S	**P**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**
CMC	V_m	0.386	0.668	0.344	0.158	0.447	0.601	0.643	0.135	0.195	0.194	0.182	0.142
	K_m	69.13	139.0	62.74	33.53	64.98	98.96	113.5	18.68	9.685	11.31	10.55	17.09
AV	V_m	0.192	0.127	0.049	0.036	0.245	0.154	0.062	0.024	0.080	0.093	0.110	0.042
	K_m	45.78	43.23	16.52	10.86	43.113	60.86	21.32	-0.52	16.84	30.00	54.51	18.94
RS	V_m	0.156	0.076	0.072	0.062	0.477	0.052	0.057	0.049	0.123	0.086	0.122	0.089
	K_m	77.3	33.1	28.9	19.9	347.2	22.1	21.6	13.5	61.3	36.0	116.	68.72

IL – Ionic liquid [EMIM][OAc]; S – Substrate; CMC – Carboxymethylcellulose; AV – Avicel; RS – Rice straw; V_m – Maximal velocity, mg/mL.min; K_m – Half velocity constant, mg/mL ; E – Enzyme ; P - Parameter

Competitive inhibition $K^* > 1.0$ $V^* = 1.0$ (10)
Uncompetitive inhibition $K^* < 1.0$ $V^* < 1.0$ (11)
Non-Competitive inhibition $K^* = 1.0$ $V^* < 1.0$ (12)
Mixed inhibition $K^* > 1.0$ $V^* < 1.0$ (13)

Based on Equations (6)–(13), the dissociation constant was calculated, and type of inhibition for saccharification has been summarized in Table 3. Under tested conditions in this study, uncompetitive inhibition in saccharification was fit to most cases of CMC and RS hydrolysis by using three types of cellulases. On the other hand, mixed inhibition was observed when AV was hydrolyzed using Accellerase 1500 with 0.5 M of [Emim][OAc], and also hydrolysis reactions of CMC, AV, and RS by MSL2 enzyme with 2.0 M [Emim][OAc]. While competitive inhibition for CMC and RS hydrolysis by MSL2 was observed at addition of 0.5 M and 1.0 M of [Emim][OAc].

This observation also correlated with previous section that 2.0 M concentration of [Emim][OAc] was the point that impairs MSL2's IL-tolerance. The inhibition of saccharification caused by higher concentration of [Emim][OAc] can be attributed to the high affinity of anions in IL towards to the enzymes with cations causing conformational changes and denaturation of cellulase [46]. Based on molecular dynamics simulation, it was reported that cations ([Bmim]) of IL bind to the active site of the enzyme, causing deactivation and denaturation [47].

3.3 *IL functions as Activator and Inhibitor in saccharification– a particular case*

Under the influence of [Emim][OAc], both K_m and V_m values of enzymatic saccharification of each experimental set was modified, depending on the types of substrates, types of cellulase, and [Emim][OAc] concentration. It was observed that 0.5 M of [Emim][OAc] led to an increase in both V_m and K_m values, suggesting a possibility for both inhibition and activation of saccharification. A similar pattern was observed when CMC and AV were hydrolyzed by using Accellerase 1500 and MSL2 with 0.5 M and 1.0 M of [Emim][OAc]. Again, the dissociation constant was calculated and applied to analyze the inhibition and activation patterns of saccharification, as expressed Equations (14)–(18) [43], [48].

Activated $K^* = 1.0$ $V^* > 1.0$ (14)
Activated $K^* < 1.0$ $V^* = 1.0$ (15)
Inhibited $K^* > 1.0$ $V^* < 1.0$ (16)
Activated and inhibited $K^* < 1.0$ $V^* < 1.0$ (17)
Activated and inhibited $K^* > 1.0$ $V^* > 1.0$ (18)

In the case of hydrolysis with the presence of 0.5 M of [Emim][OAc], both V_m and K_m are increased, so this condition was fit with Equation (18). This implies that the hydrolysis is inhibited below a particular concentration of substrate. However, as the substrate concentration increases, the enzyme is activated. Since

Table 3: The kinetic dissociation constants (V^* and K^*) determined for saccharification of CMC, AV, and RS using commercial (celluclast 1.5 L and accellerase 1500) and MSL2 enzyme with and without addition of [Emim][OAc]

E		Celluclast 1.5 L				Accellerase 1500				MSL2			
S	**P**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**	**Control**	**0.5M IL**	**1.0M IL**	**2.0M IL**
CMC	V^*	1.000	1.730	0.81	0.40	1.000	1.34	1.439	0.32	1.000	0.94	0.932	0.78
	K^*	1.000	2.012	0.98	0.45	1.000	1.53	1.748	0.28	1.000	1.19	1.090	1.75
	IT	-	AI	UI	UI	-	AI	AI	UI	-	CI	NI	MI
AV	V^*	1.000	0.664	0.27	0.18	1.000	0.68	0.253	0.09	1.000	1.15	1.364	0.54
	K^*	1.000	0.944	0.31	0.27	1.000	1.42	0.495	–0.02	1.000	1.72	3.237	1.15
	IT	-	UI	UI	UI	-	MI	UI	E	-	AI	AI	MI
RS	V^*	1.000	0.484	0.41	0.39	1.000	0.19	0.119	0.13	1.000	0.75	0.999	0.78
	K^*	1.000	0.429	0.34	0.28	1.000	0.04	0.062	0.09	1.000	0.57	15.03	1.10
	IT	-	UI	UI	UI	-	UI	UI	UI	-	UI	CI	MI

AI – Activator and Inhibitor; UI – Uncompetitive Inhibition; MI – Mixed Inhibition; NI – Non-competitive Inhibition; E – Experimental error; IT – Inhibition type

increasing the substrate concentration decreases the competitive inhibition effect. However, an increase in the K_m value was observed. It is necessary to determine the concentration of substrate where the enzyme is inhibited and activated in such cases for process optimization purposes. The concentration of substrate for the case as expressed in Equation (18) can be determined from the expression shown in Equation (19) [43].

$$[S] = K_m \times \left[\frac{K^* - V^*}{(V^* - 1)} \right] \quad (19)$$

In such a case, the dissociation constant (V^* and K^*) were determined from Equations (8) and (9) since the K_m and V_m values were increased compared to control. Thus, the dissociation constants (V^* and K^*) for CMC hydrolysis with Celluclast 1.5 L in 0.5 M of [Emim][OAc] were 0.57 and 0.49, respectively. The V^* and K^* for CMC hydrolysis with Accellerase 1500 in 0.5 M of [Emim][OAc] were 0.74 and 0.65, respectively. On the other hand, the V^* and K^* for AV hydrolysis with MSL2 in 0.5 M [Emim][OAc] were 0.68 and 0.36, respectively. Using these dissociation constants, the Critical Concentration of Substrate (CCS) could be calculated based on Equation (19). One example is that the saccharification of CMC with Celluclast 1.5 L in 0.5 M of [Emim][OAc] was inhibited when CMC concentration lower than 26.59 mg/mL, and if CMC concentration was higher than this, the saccharification was activated. Using the same method, CCSs of CMC hydrolysis with Accellerase 1500 in 0.5 M and 1.0 M of [Emim][OAc] were 33.65 and 45.85 mg/mL, respectively. Similarly, CCSs of AV hydrolysis with MSL2 in 0.5 M and 1.0 M of [Emim][OAc] were 46.68 and 50.63 mg/mL, respectively.

A graphical representation of the assumption for exceptional cases as discussed in this section regarding CCS and activation of the saccharification is shown in Figure 3. The V^* and K^* for the experimental sets with IL and without IL addition are the same at the point where the two lines meet. In the present study, when the V^* and K^* were both increased, leading to inhibition before and activation after the CCS point. At this CCS point, the saccharification is either inhibited or activated depending on the substrate concentration used in that process optimization. Therefore, identification of CCS point for each set up of enzymatic saccharification based on the type of substrate, type of cellulase, and

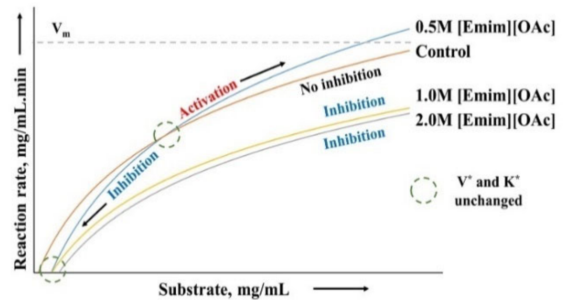

Figure 3: Activation and Inhibition mechanism of saccharification due to the presence of [Emim][OAc] in lower concentration.

IL concentration is suggested to ensure the efficient design of one-pot process combining pretreatment and hydrolysis of cellulose and lignocellulose biomass.

4 Conclusions

In recent years, researchers have tried to reduce the solid washing by implementing one-pot process. In one-pot process, pretreatment, and saccharification are carried without the need to filter and washing of the solids. However, this one-pot process needs well design and calculation to achieve optimized efficiency. In this study, a detailed analysis of the kinetic parameters (K_m and V_m) of enzymatic saccharification of cellulose and lignocellulose with commercial and IL-tolerant cellulases under the influence of [Emim][OAc] were conducted to understand the kinetic mechanism. As expected, the inhibitory effect of [Emim][OAc] on saccharification was clearly observed in most cases of the experimental set up. However, a lower concentration of [Emim][OAc] ranged between 0.5–1.0 M led to both activation and inhibition of enzymatic saccharification depending on the balance of substrate concentration (CCS) and types of substrate. The inhibitory effect caused by [Emim][OAc] was overcome by increasing the substrate concentration, to a point CCS, resulting in a simultaneous increase in K_m and V_m.

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