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RESEARCH ARTICLE

Stability of cellulases in ionic liquids

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Abstract

Lignocellulosic biomass has been used as an alternative source to food crops that serve as feedstock for bioenergy production. The conversion of biomass to bioenergy required pretreatment process. Ionic liquids (ILs) have been recognized as promising solvents that are capable of solubilizing and separating components of lignocellulosic biomass. This research focuses on understanding how ILs affects the activity of cellulases in the enzymatic saccharification process. Sigmacell cellulose was used in the enzymatic saccharification process. Two different ILs were added in the enzymatic saccharification mixtures and the activity of a mixture of commercially available cellulases was measured using high-performance liquid chromatography (HPLC) to measure glucose release. Sulphate based ILs were more harmful for cellulase action than [EMIM][OAc]. [HBIM][HSO_4] inactivated commercial cellulases (Celluclast®) and cellobiase (Novozyme188) in the enzymatic saccharification process. In this research, it was observed that the main factor that affects the activity of cellulase is pH.

Keywords: Lignocellulosic, ionic liquid, pretreatment, enzymatic saccharification, bioenergy

INTRODUCTION

The non-renewable fossil fuels that provide energy for heat, electricity, and transportation are anticipated to run out during the latter half of the 21st century. There is, therefore, a growing interest in the production of transport fuels such as ethanol from renewable resources. Secondary cell walls are generally composed of cellulose (40-50%), hemicellulose (20-30%), and lignin (20-30%), and are therefore, referred to as lignocellulosic feedstocks. Cellulose consists of glucose moieties and is able to produce renewable biomaterial (Furtado *et al*., 2014). In biofuel production, the first process is a pretreatment step. This step is important to separate the components of the biomass into cellulose, hemicellulose and lignin. The second step is enzymatic saccharification which uses enzymes to convert cellulose to sugars. Then, the last step is the fermentation which converts the sugars to biofuel.

Recently, ionic liquids (ILs) have been studied for pretreatment of lignocellulosics. ILs are salts that liquidified at temperatures below 100 °C. The use of ILs in the process of pretreament is to overcome the structural and chemical features that limit the release of carbohydrates. However, ILs used in the current study have caused deactivation of cellulase enzymes from *Trichoderma reesei* and cellobiase from *Aspergillus niger*, resulting in a low amount of glucose release (Engel *et al*., 2010). The objectives of this research were to study the effect of different ILs on the activity of the commercial cellulase (*T. reseei* cellulase mix) and Novozyme 188 (β-glucosidase) on Sigmacell cellulose and to identify the physicochemical causes of the loss of glucan yield of Sigmacell cellulose with the presence of ILs. Wheat, oil palm and corn are food sources for humans and animals. Lignocellulosic biomass, on the other hand, is a structural material for plants. There are four main categories of substances in lignocellulosic biomass: cellulose, hemicellulose, lignin, and extractives. Separation of the lignocellulosic biomass can produce chemicals such as ethanol, butanol, biodiesel and other types of biochemicals (Chen *et al*., 2017).

Lignocellulose is comprised of three polymers interlinked in a dense matrix. Cellulose has a complex and heterogeneous structure and the chemical composition of cellulose consists of D-glucose residues linked by β-1,4-glucosidic bonds. Cellulose is insoluble in water because it has over 10 000 glucose residues. Crystalline microfibrils are formed from the polymeric chains which attach to each other in a parallel fashion. Normally, cellulose contains highly crystalline and amorphous regions along with microfibrils. High crystalline cellulose can only be found in algae or bacteria and these organisms have been used as a model substrate in order to understand how different types of cellulases can attack and degrade crystalline cellulose (Martin, 2000).

Hemicellulose consists of five sugars which are glucose, xylose, mannose, galactose, and arabinose, while lignin consists of phenolic compounds. An example of lignocellulosic biomass normally found in Malaysia is empty fruit bunches of oil palm (EFB) which contains 37 to 46% of cellulose, 25 to 34% hemicellulose and 28 to 32% of lignin (Syafwina *et al*., 2002).

Moreover, the production of bioethanol from biomass relies on the chemistry of the feedstock, for example, herbaceous lignocellulosics are relatively simple to convert when compared to other feedstocks. For instance, biomass for some plants consists of more cellulose and less hemicellulose which indicates that more glucose can be obtained. The hemicellulose in some plants has more xylose, which can be transformed into ethanol (Kamoldeen *et al*., 2017). However,

softwood species are more difficult for bioconversion due to the presence of more lignin which is more difficult to extract from softwoods than from hardwoods. The cell wall of softwoods is thicker and more rigid. Delignification of softwood is very challenging because lignin is very stable (Shimada *et al.*, 1997).

Enzymatic cellulose hydrolysis requires endoglucanase, exoglucanase and β-glucosidase (Acebal *et al*., 1988). Endoglucanases hydrolyze intramolecular β-1,4- glucosidic bonds of cellulose chains randomly to produce new chain ends. Exoglucanases are important in releasing soluble cellobiose or glucose by cleaving cellulose chains at the ends. In order to eliminate cellobiose inhibition, β-glucosidases hydrolyze cellobiose to glucose. Cellulases are produced by a wide variety of organisms which cover a number of ecological niches. This include bacteria and fungi, both living in the presence and absence of oxygen. These can be mesophiles or thermophiles (Payne *et al*., 2015). Within bacteria, industrially relevant organisms such as the Clostridia, are commonly used to manufacture cellulases or used in consolidated bioprocessing to convert cellulose to small molecules by fermentation (Higashide *et al*., 2011). Within fungi, the most widely studied organisms for cellulase production are the *Trichoderma* and *Aspergillus* species.

Bioconversion of lignocellulosic biomass requires a pretreatment step in order to separate carbohydrates and lignin. There are many choices for pretreatment processes namely acid pretreatment, steam explosion and ionic liquids (Brandt *et al*., 2017). Pretreatment is very important in biofuel production as the type of pretreatment has significant impacts on the subsequent enzymatic hydrolysis. Also, an efficient method of pretreatment can help to reduce the cost of biofuel production (Mabee *et al*., 2011; Brandt *et al*., 2011). One of the options is by using ionic liquids (ILs). Ionic liquids are salts in liquid form/phase at ambient temperatures or melt at slightly high temperatures. The separation process of lignocellulose in ILs has been explained in several hydrogen-bonds basic ILs, proposing that ILs can be used for lignocellulose separation (Peleteiro*et al*., 2015). IL pretreatment can be divided into two types of pretreatment. The first one is to dissolve cellulose in ILs. The celluloses recovered from IL pretreatment process are less crystalline and can be accessible by cellulases, which then improved cellulose hydrolysis process *(Zhao et al*., 2009). The second option is a pretreatment that alters or removes lignin from the lignocellulosic materials. A lignin fraction can be transformed into aromatic and beneficial chemicals (Brandt *et al*., 2011). The surface area of the cellulose after the pretreatment is larger and more amorphous. Thus, enzymatic saccharification of the cellulose becomes more effective and helps to increase the amount of sugars compared to the untreated cellulose (Shiga *et al*., 2017). However, enzymes can be deactivated by remaining IL left in the cellulose (Turner *et al*., 2003), probably due to protein deactivation (Bose *et al*., 2010). ILs frequently induce enzyme conformational changes which then cause in inactivation and in some cases, the enzymes will not dissolve in the ILs, leading to a bi- (or tri-) phasic system (Lau *et al*., 2004; Sheldon *et al*., 2002).

Some of the cellulases lose their activity severely in ILs (Johnson *et al*., 2016). A research conducted by Pottkämper *et al*. (2009) exploring on metagenomic cellulases from mesophilic environment demonstrated that many of the enzymes deactivated in 30% (vol/vol) IL. Another study conducted by Liang *et al*. (2011) reported that the thermostable cellulase from *Thermoanaerobacter tengcongensis* MB4 demonstrated 54.4% of its early activity in 1.0 M of 20% (vol/vol) [BMIM]Cl. Moreover, a thermophilic enzyme from *Pyrococcus horikoshii* was very active in 1-ethyl-3-methylimidazolium acetate [EMIM]Ac in which there was 95% relative activity in 20% (vol/vol) IL (Datta *et al*., 2010). Altogether, novel cellulases which have the ability to adapt with high IL concentrations and cellulases that are active over a long time period in the presence of ILs are required by the industries. Therefore, the objective of this study was to investigate the stability of cellulase in ILs.

EXPERIMENTAL

Materials

[C_2C_1im] [Ac] (purity>98%) was purchased from BASF chemical company. The synthesis of [HC_4im][HSO_4] was described in the ESI.

Enzymatic saccharification

The enzymatic saccharification was performed according to LAP"Enzymatic saccharification of lignocellulosic biomass" (NREL/TP-510-42629), which can be accessed from http://www.nrel.gov/biomass/pdfs/42629.pdf. About 100 mg of Sigmacell (20μM) was added to a 50 ml Falcon tube. Then, 5 ml of 0.1M citrate buffer (pH 4.8), antibiotics (30 µl kanamycin and 40 µl tetracycline) and distilled water were mixed to a final volume of 10 ml,asdescribed in the standard protocol. Commercial enzyme preparations were used in which Celluclast (*T. reseei* cellulase mix) and Novozyme 188 (β-glucosidase, both from Sigma) were used. The reactants were incubated at 50°C for 1 hour and the pH of the samples was measured. Subsequently, the samples were placed in the incubator at 50°C for up to 96 hours. Glucose released was analysed on a Jasco HPLC system with refractive index detector equipped with an Aminex HPX-87H column (Biorad). The mobile phase was 10 mM sulphuric acid, the column oven temperature was 35°C, the flowrate was 0.6 ml/min and the acquisition time was 15 min. Glucose yields were then calculated.

Viscosity measurement

The viscosities of ILs were measured using an AR 2000 ex cone-plate viscometer under a nitrogen atmosphere at room temperature. The viscometer was calibrated with Fluka Viscosity & Density Standard Solution N35. The measurements were repeated three times.

Ionic strength

The effect of ionic strength on the activity of the enzyme was studied in order to understand other factors which can influence the activity of the enzyme. Ionic strength was calculated by using a dimensionless ionic strength equation as defined by Atkins (1986):

$$I = \frac{1}{2} \cdot \sum \cdot \frac{m_i}{m^o} \qquad z_i^2$$

I = Ionic strength
m_i = Ionic concentration in units of molality, mol kg^{-1}
m^o = 1 mol kg^{-1}
z_i^2 = Number of charges on the ion

RESULTS AND DISCUSSION

In this research, ILs containing HSO_4^- showed the strongest inhibition with the lowest glucose yield (Table 1 and Figure 1). Research done by other groups has suggested that other parameters such as pH, viscosity and ionic strength, might be responsible for the inactivation of cellulases (Engel *et al*., 2010).

In addition, the pH of ILs can also increase or decrease greatly when the concentration of ILs increases in aqueous solution (Xinjian et al, 2010). Based upon the results (Figure 1), concentrations of 1,2,5 and 10% of [HC_4im] [HSO_4] inhibited cellulase more than [C_2C_1im][Ac]. The results in Table 1 suggested that sulphate based ILs resulted in a very low glucose released and also have a very low pH. Celluclast and Novozyme 188 from Sigma showed a good activity at pH 4.8 (Hu & Catchmark, 2011) and this was the reason why at pH lower than 4.8, the yield of glucose was very low.

Table 1 Average glucose yield after the addition of different types and concentrations of ILs on Sigmacell.

IL	% IL	Glucose yield	pH
[C_2C_1im][Ac]	1%	77	5.12
	2%	70	5.17
	5%	56	5.44
	10%	25	5.71
[HC_4im][HSO_4]	1%	47	3.31
	2%	19	2.41
	5%	16	1.96
	10%	12	1.15
No IL	-	99	4.8

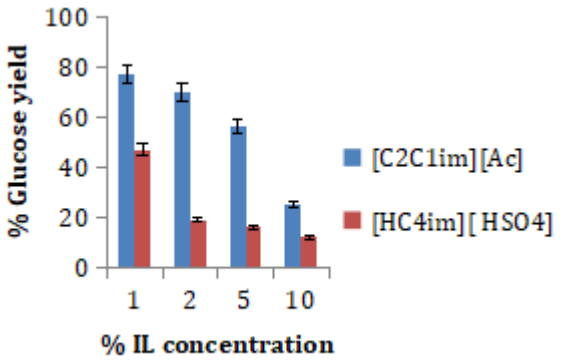

Figure 1 Effect of two types of ILs at 1, 2, 5 and 10% concentration on glucose yield. Glucose released was measured after 72 hours of incubation at 50°C. Error bars are based on standard errors.

The ILs used in this study were all water-miscible and therefore, they are easily dissociated into the respective ions in aqueous solutions. The activity of proteins in aqueous salt solutions can be predicted along the rules of the Hofmeister series (Figure 2), in which ions are classified according to their chaotropicity or kosmotropicity (Hofmeister, 1888; Kunz *et al*., 2004). Chaotropic ions have a low charge density and are normally large in dimension size. Thus, chaotropic ions are known as structure-breakers as they can break the structure of water due to their weak interactions with water molecules. Kosmotropic ions are small and strongly charged. All multivalent ions are highly hydrated and therefore kosmotropic. As their interaction with water is strong, they are called "structure-makers" (Zhao, 2006). Kosmotropic ions induce higher hydrophobic effects in the protein and therefore, stabilising its structure. Nevertheless, at higher concentrations of kosmotropic salts, proteins will precipitate as they are excluded from water (salting out). Many studies have demonstrated that kosmotropic anions and chaotropic cations usually stabilise proteins while kosmotropic cations and chaotropic anions tend to destabilise them (Zhao, 2005). ILs with a hydrogen sulphate anion give the lowest yield of glucose and do not follow the Hofmeister series.

It appears that the main contribution of enzymes activity in this study was pH. The optimum pH for celluclast by *T.reesei* was 4.5-5 (Li *et al*., 2013). Sulphate based ILs had pH lower than 3.5 but [EMIM][OAc] had pH in the range of 5.12-5.7. The activity of cellulase in [EMIM][OAc] was higher than the sulphate based ILs. It can be concluded that the activity of cellulase was significantly affected by the pH.

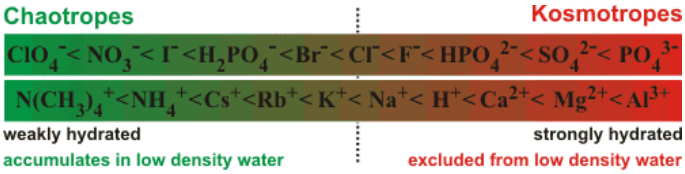

Figure 2 Ionic chaotropes and kosmotropes in approximate order of strength (Chaplin, 2001).

The effect of viscosity on the enzyme was studied as to investigate what are the other physical factors that can influence the activity of the enzyme. The viscosities of 1, 2, 5 and 10% (v/v) of ILs were measured using viscometer at room temperature. Based on the result in Figure 3, as the concentration of ILs was increased, the viscosities of the solutions containing [C_2C_1im][Ac] and [HC_4im][HSO_4] were also increased. It has been reported previously that higher viscosity resulted in low glucose released (Engel *et al*., 2010) and this concept was applied to [C_2C_1im][Ac] and[HC_4im][HSO_4], where higher viscosities resulted in lower percentage of glucose yield.

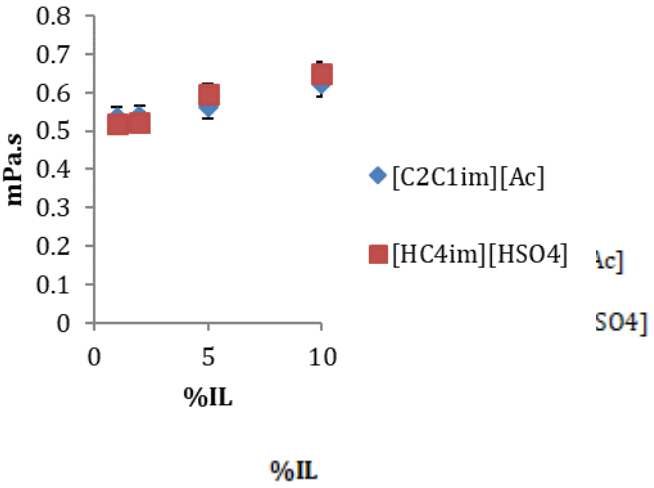

Figure 3 Viscosity of [C_2C_1im][Ac] and [HC_4im][HSO_4]. Error bars are based on standard errors.

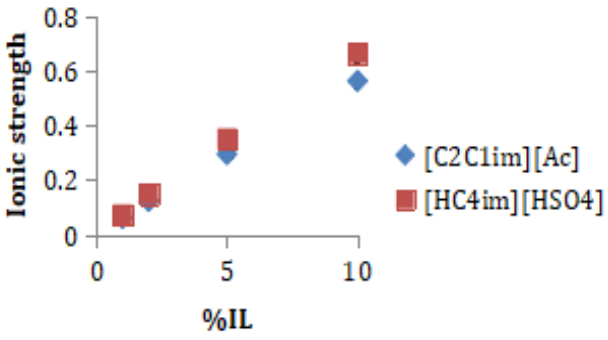

Figure 4 Ionic strength of [C_2C_1im][Ac] and [HC_4im][HSO_4]. Error bars are based on standard errors.

Based from the data obtained in Figure 4, as ionic strength was increased, the enzyme activity was decreased. According to Engel and co-workers (2010), the relative enzyme activity decreased when the ionic strength of [MMIM] [DMP] increased. For each type of IL studied (Figure 4), as the concentration was increased, the ionic strength was increased while the yield of glucosewas decreased, which was similar to the result reported by Engel and co-workers (2010).

CONCLUSION

In conclusion, the findings of this research showed that pH, viscosity and ionic strength of the ILs affected the stability of cellulases. The results demonstrated that reduced pH resulted in the loss of enzyme activity. This suggests that cellulases from acidophilic organisms may be conducted. These findings can provide suitable target to construct genetically modified enzymes that will hopefully improve glucose yield from biomass after IL pretreatment. Perhaps, different ILs with low viscosity and ionic strength can be explored further to investigate the stability of the ILs with cellulases.

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