Chapter 2
Role of Ionic Liquids in the Enzyme Stabilization: A Case Study with *Trichoderma Ressie* Cellulase

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Abstract This chapter discusses the stabilization of *Trichoderma ressie* cellulase (*Tri-Cel*) in ionic liquids (ILs) to enable the *in situ* hydrolysis of cellulosic and lignocellulosic substances. It is well recognized that enzymes tend to lose their activities in ILs, but several methods have been used to increase or minimize the loss of activity in ILs. In this study, cellulase was therefore tested in several ILs. This approach opens an insight for further studies to discover more about the effects of ILs on cellulase and their interactions in the aqueous system. It can also offer successful manufacturing and processing of different biomass biofuels.

Keywords Ionic liquid · Lignocellulose · Cellulase · Hydrolysis · Stability · Activity

2.1 Introduction

ILs may be described as organic liquid salts at room temperature and melt at or below 100 °C. They constitute a carbonic chain that produces a cation that is ionically linked to an anion; thus, a wide variety of ILs may therefore be synthesized. In addition, ILs have customizable features, including thermal stability, miscibility and polarity, which are of significant advantages over traditional organic, non-reusable toxic and volatile solvents. (De Souza Mesquita et al., 2019). ILs have many desirable characteristics, such as enzyme stabilization. Due to their merit, ILs are good media for various reactions (Elgharbawy et al., 2016; Fu et al., 2010). ILs can be

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applied as immobilization and coating agents for enzymes for diverse applications (Moniruzzaman et al., 2015). Enzymes may be activated or stabilized by ILs.

2.2 The Role of ILs in Enzyme-Catalyzed Hydrolysis

Enzyme-catalyzed hydrolysis of IL pretreated substrates involving cellulose transformation from IL solution for enzymatic hydrolysis can be demonstrated in two main pathways (Tan et al., 2011; Zhao et al., 2009). The primary pathway includes a multi-stage process where the biomass is pretreated, washed and then hydrolyzed to the desired product. The secondary pathway is considered a single-step method in which hydrolysis is performed in aqueous IL and cellulase enzymes (Gunny et al., 2014). Multiple ILs have shown impressive outcomes in structural modification of lignocellulose and removal of lignin, including choline acetate [Ch][Ac] (Asakawa et al., 2015). This demonstrates that ILs can be adapted for reliability with certain enzymes (Elgharbawy et al., 2016; Ibrahim et al., 2015).

2.2.1 Principle

Wang and co-workers (Wang et al., 2011a) reported that when analyzed in 1-ethyl-3-methylimidazolium acetate [EMIM][Ac] (15%), certain cellulases were maintained along the process of saccharification. The biomass of yellow poplar and [EMIM][Ac] with the percentage of 10–20%, was used for enzymatic hydrolysis (Shi et al., 2013). In addition, in ionic liquid-enzyme (IL-E) compatible systems, several studies have documented stability of cellulases, for example, [Ch]-based ILs (Ninomiya et al., 2015). Likewise, single-step hydrolysis is preferred as the lignocellulose IL pretreatment is combined with enzymatic hydrolysis to eliminates the stage of cellulose regeneration through washing.

2.2.2 Objective of Experiment

The purpose of this work is to identify the most appropriate cellulase-stabilizing IL to enable lignocellulosic biomass to be hydrolyzed in a single vessel.

2.3 Materials

Tables 2.1, 2.2, and 2.3 list the materials used in this research.

Table 2.1 Consumable items used

No.	Equipment	Aims of usage
1	Pipettes (100 μL, 200 μL, 1 mL)	To add solutions into tubes, microtubes
2	Falcon Tube (15 mL)	To dissolve enzyme and prepare solutions
3	Microcentrifuge tube (2 mL)	To perform enzyme assay and determine total protein
4	Plate/Petri Dish	To contain culture medium (agar)
5	Microplates	Transferring assay solution for absorbance measurement

Table 2.2 Equipment used

No.	Equipment	Usage
1	Weighing balance, Mettler Toledo	To weigh chemicals and reagents
2	Thermomixer, Eppendorf	To incubate and mix the enzyme-IL solution
3	Microplate Spectrophotometer Brand: Multiskan Go™ (Thermo Scientific)	To measure absorbance during determination of enzyme activity and total protein
4	pH meter Brand: Mettler Toledo	To measure pH

Table 2.3 Chemicals and reagents used

No.	Chemicals	Manufacturer
1	1,3-dimethylimidazoliumdimethyl phosphate [DMIM][DMP]	Sigma Aldrich
2	1-ethyl-3-methylimidazolium acetate [EMIM][Ac]	Sigma Aldrich
3	1-ethyl-3-methylimidazolium chloride [EMIM][Cl]	Sigma Aldrich
4	1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP]	Sigma Aldrich
5	3,5-dinitrosalicylic acid	Sigma Aldrich
6	Acetic acid	Friedemann Schmidt
7	Choline hydroxide	Sigma Aldrich
8	Sodium citrate-2-hydrate	Bendosen Laboratory
9	Sodium hydroxide	Bendosen Laboratory
10	Sodium metabisulfite	Fisher Scientific
11	Sodium potassium tartrate	R&M Chemical, UK
12	Sulphuric acid 98%	Fisher scientific
13	Tetrabutyl phosphonium hydroxide	Sigma Aldrich
14	Sodium citrate-2-hydrate	Bendosen Laboratory

2.4 Methodology

2.4.1 Synthesis of Ionic Liquids

[Ch][Ac] was synthesized with slight adjustments using the procedures outlined by Ninomiya et al. (2015). 45.0 wt% of Choline hydroxide [Ch][OH] solution in methanol (Sigma-Aldritch) (100 g) was dispensed dropwise to an equimolar volume of acetic acid (~22.3 g) (Friedemann Schmidt Chemical) in ice-bath. The synthesis was carried out with a round bottom flask with a three-neck that was attached to the condenser and addition funnel. The mixture was left to stir for about 6–12 h before the reaction was stopped. Using the rotary evaporator, methanol was extracted through the vacuum at the time of one hour, at 337 mbar and temperature of 40 °C, while water was evaporated at temperature of 90 °C (2 h, 314 mbar). Using a Freeze dryer (LABCONCO), the resulting residue was vacuum dried to eliminate the residual water. To verify the structure, ^{1}HNMR was used. Choline butanoate [Ch][Bu] was prepared with the same method using butanoic acid in place of acetic acid. Tetrabutyl phosphonium hydroxide was mixed at room temperature with acetic acid to prepare tertabutylphosphonium acetate [TBPH][Ac].

2.4.2 Cellulase Production

Cellulase was prepared at 65% moisture content by fermenting the palm kernel cake (PKC) following the sterilization. The fermentation started with 2% (w/w) of *T. reesei* spore suspension. Solid-state fermentation (SSF) took place for 7 days at a temperature of 30.0 ± 2 °C. Using citrate buffer (pH 4.8 ± 0.2), the crude enzyme proceeded to extraction followed by the centrifugation. In a multi-step procedure, the enzyme was purified using crossflow filtration. A hollow fiber membrane cartridge was used for ultra-filtration and microfiltration of the cell-free supernatant obtained from centrifugation. For the microfiltration process, a 0.45 μm membrane via 0.011 m^2 of active surface area was used. Ultra-filtration was performed through ultra-filtration membranes was used (PALL, MWCO 30, and 10 Kd). To determine endo-β-1,4-D-glucanase activity (cellulase) carboxymethyl sodium salt (CMC) was employed as the reactant substance (Salvador et al., 2010).

2.4.3 Stability of Cellulase ILs

The compatibility of ILs with cellulase was investigated. The enzyme was incubated at: 10, 20, 40, 60, 80 and 100% (v/v) of the ILs. As for the control, *Tri-Cel* was incubated in citrate buffer (50 mM and pH 4.8 ± 0.2). The ILs investigated were 1-ethyl-3-methylimidazolium diethyl phosphate [EMIM][DEP], choline butanoate

[Ch][Bu], choline acetate [Ch][Ac], tetrabutyl phosphonium acetate [TBPH][Ac] and 1,3-dimethyl imidazolium dimethyl phosphate [DMIM][DMP]. CMC hydrolysis was carried out at 45.0 ± 2.0 °C (optimum temperature). For a duration of 6 h, samples were taken every hour, and by using the control (at 100%), which is the enzyme/buffer solution, the activity was described as a residual activity. The activity was evaluated using the dinitrosalicylic acid (DNS) method.

2.4.4 Cellulase Assay

CMC [1.0% (w/v)] was prepared in citrate buffer (pH 4.8 ± 0.2) to assess the activity of endo-β-1,4-D-glucanase. By spectrophotometric quantification of the emitted reducing sugars using DNS, cellulase activity was determined. Substrate solution of 450 μL was prepared with the addition of 35 μL buffer, and the enzyme solution (15 μL) was added. The reaction was terminated after 30 min by adding 1.0 mL of DNS reagent before boiling the solution for 15 min and then cooled before adding water (1.0 mL). The absorbance of the solution was measured at 540 nm. The sugar generated by cellulase was calculated using the glucose standard curve (Fig. 2.1). By measuring different enzyme dilutions, the enzyme concentration that releases approximately 0.5 mg of glucose was recorded. A line was connected for points lower and higher than 0.5 mg, and the enzyme dilution rate (EDR) was defined at 0.5 mg glucose. (Ghose, 1987).

$$\text{CMC} = 6.173/EDR\text{Unit/mL} \tag{2.1}$$

In the CMC reaction, the quantity of glucose is generated by 15 μmL in 30 min, where:

0.5 mg glucose = 0.5 mg/(0.18 mg/μmol) × 0.015 mL × 30 min = 6.173 μmol/min/mL.

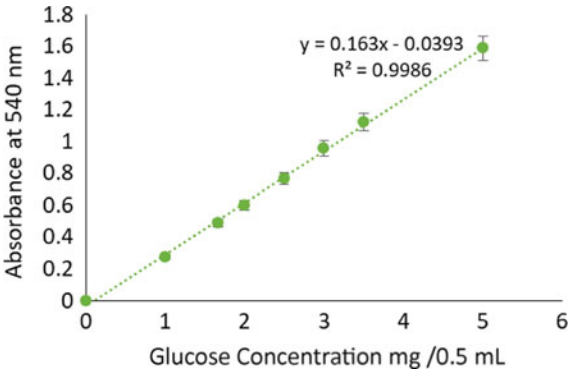

Fig. 2.1 Glucose standard curve for determination of cellulase activity (CMC)

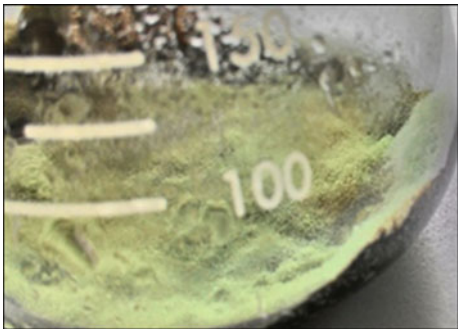

Fig. 2.2 Fermentation of palm kernel cake (PKC) by *Trichodermareesei* (7 days)

Under these assay conditions, only 15 mL of the enzyme solution is being used for the response instead of 0.5 mL, so the formula has been modified accordingly.

2.5 Results and Discussion

2.5.1 Cellulase Activity

The enzyme activity was measured using CMC protocol, resulting in 157.872 ± 1.56 CMC units/mL (789.386 ± 7.8 U/gds) following the fermentation process of 7 days (Fig. 2.2). With maximum stability for 24 h at temperatures between 25 and 50 °C, the optimum pH and temperature were 5.00 and 45 °C, respectively.

2.5.2 Stability of Cellulase in ILs

We analyzed the impacts of different types of ILs for six hours on a few concentrations of locally produced cellulase *Tri-Cel*. Six ILs were analyzed for the effect on *Tri-Cel*; [Ch][Ac], [Ch][Bu] [EMIM][Ac], [EMIM][DEP], [DMIM][DMP] and [TBPH][Ac]. Trends in *Tri-Cel* activity can be seen in Fig. 2.3(a–f)

In [Ch][Ac] (Fig. 2.3a), more than six hours with 20% IL/Buffer, locally generated *Tri-Cel* sustained over than 90% of its activity at 10%. The enzyme retained 80 and 85% at 40, 60, and 80% IL/Buffer. At 100% IL/Buffer, after six hours, enzyme activity was detected at 63.15%. In comparison, in 80 and 100% IL/Buffer, [Ch][Bu] (Fig. 2.3b) attained the initial activity at 50%. *Tri-Cel* sustained its activity (>80%) at low concentrations (10 and 20%), whereas in [EMIM][Ac] (Fig. 2.3c) it preserved 85% of the activity at 10–40% IL/Buffer solution. Although 67% activity was recorded at 60% IL/Buffer, high concentrations resulted in a drastic decrease in the activity. In [TBPH][Ac] (Fig. 2.3d), at low concentrations, *Tri-Cel* retained its activity (90%), while less than 20% was identified at higher concentrations of the

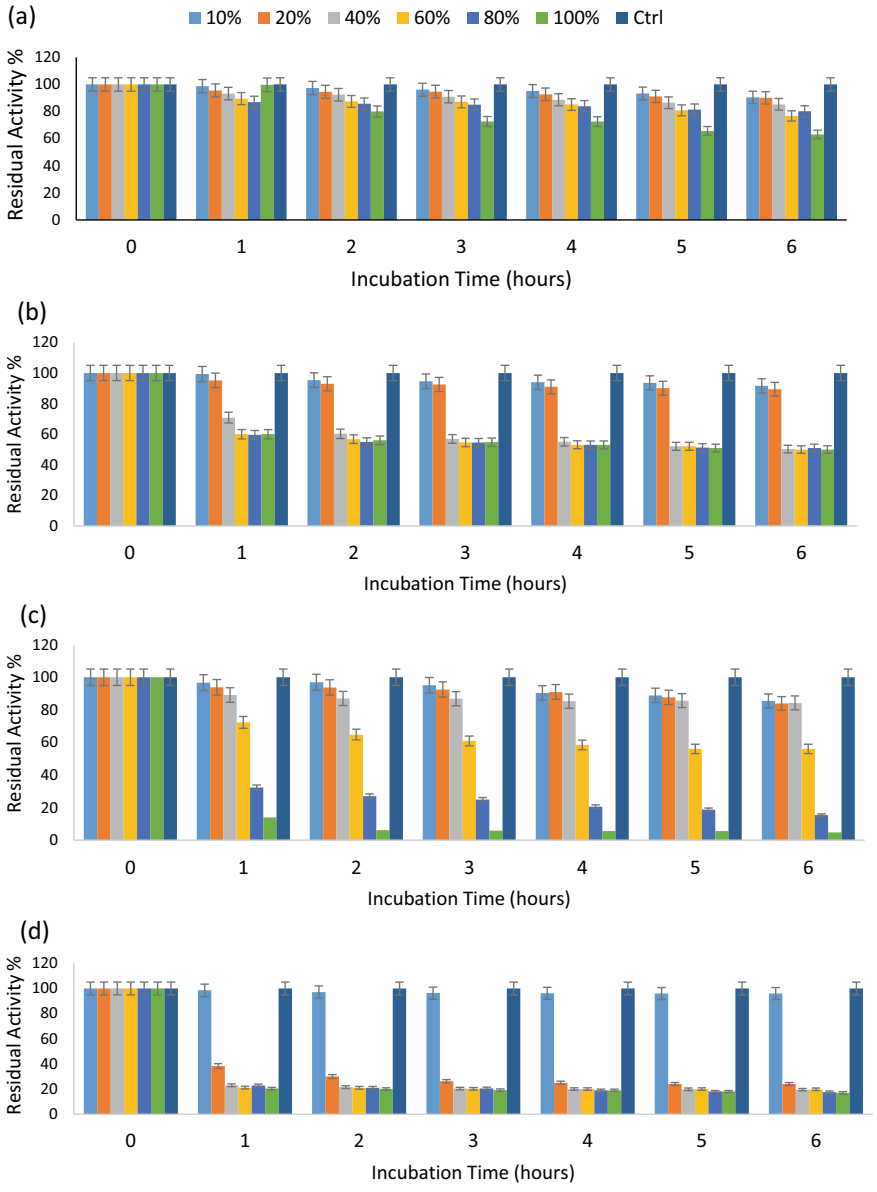

Fig. 2.3 Compatibility of *Tri-Cel* with 6 different ionic liquids (ILs) for a period of 6 h at enzyme optimum conditions (pH 5.0 and 45 °C): **a** [Ch][Ac]. **b** [Ch][Bu]. **c** [EMIM][Ac]. **d** [TBPHA][Ac]. **e** [EMIM][DEP]. **f** [DMIM][DMP]

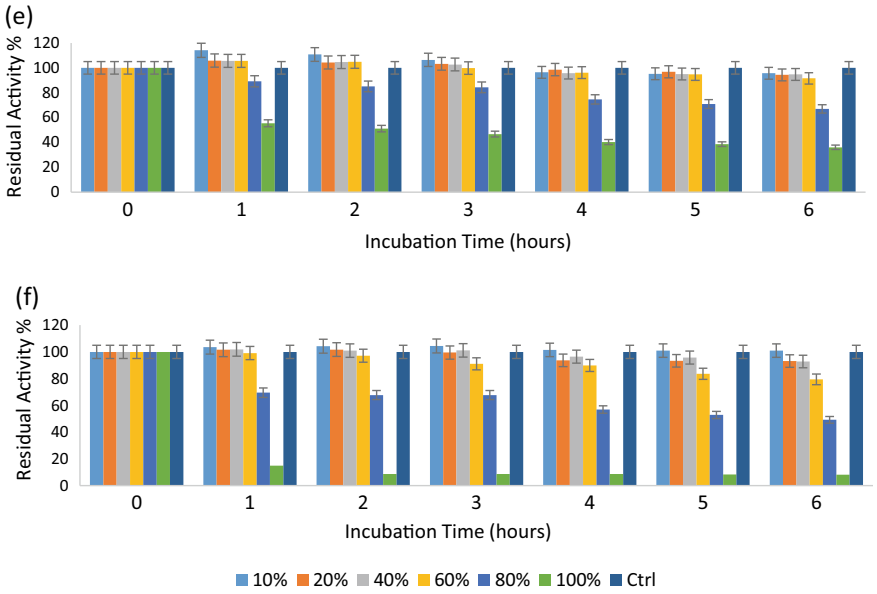

Fig. 2.3 (continued)

IL. Phosphate-based ILs revealed unexpected patterns wherein the [EMIM][DEP] (Fig. 2.3e) stimulated the enzyme in 10–60% IL/Buffer at the first two hours and regulated the activity at 90% in the next six hours. At 80 and 100% IL, the enzyme sustained its activity (70 and 36%), respectively. Similarly, in the initial two hours, a comparable pattern was recorded for [DMIM][DMP] (Fig. 2.3f) at 10–40%, while the activity reduced to 20% in 60% IL/Buffer solution.

In summary, *Tri*-Cel activity was the highest in [Ch][Ac] with an incubation period of six hours, despite being suspended in 100% IL solution. The recorded pattern of low IL concentrations can be in the order: [DMIM][DMP] > [EMIM][DEP] > [Ch][Ac] > [Ch][Bu] > [TBPH][Ac].

2.5.3 Discussion

2.5.3.1 Cellulase Production

Numerous fungal cellulolytic and microbial enzymes have an optimum temperature of 50 °C and optimum activity at pH 4 to pH 6. It was reported (Ni & Tokuda, 2013) that enzyme from *N. koshunensis*; cellobiohydrolase, can function at their best at 45 °C and pH 5.0. Cellulase enzyme from *Trichoderma viride* demonstrated its optimum temperature at 50 °C and at pH 6.0 (Taha et al., 2015). The optimal pH of cellulase agrees with the results of the published studies that the activities

of cellulases exhibits their optimal at pH from 4.0 to 7.0 and temperature range of 30 and 40 °C (Pandey et al., 2015). In the acidic range of pH 3.5–6.5 and with the temperature at 40–60 °C, cellulases of the family of *Bacillus* and *Aspergillus* showed their optimal enzyme activity (Assareh et al., 2012; Lin et al., 2012). At pH 5.0 and 45 °C, extracellular cellulase isolated from the marine bacterium *Pseudoalteromonas* sp. had shown the optimal activity. In the crude enzyme blend, the total cellulase (FPase) was 2.11 U/mL and the activity of cellulase (CMCase) was 6.04 U/mL (Trivedi et al., 2013). The latest findings are following the information documented.

2.5.3.2 Cellulase Stability in ILs

It is a fact that ILs digest the cellulose which act as a biocatalysis reaction medium (Swatloski et al., 2002), but residual ILs in the recovered cellulose have been shown to cause enzymatic hydrolysis by inducing activity loss because of the unfolding of the protein (Bose et al., 2010; Turner et al., 2003).

Trivedi et al. (2013) successfully stabilized the extracellular cellulase from marine bacterium *Pseudoalteromonas* sp. in six different type of ILs; 1-ethyl-3-methylimidazolium methanesulfonate [EMIM][CH_3-SO_3], 1-butyl-3-methylimidazolium chloride [BMIM][Cl], 1-butyl-1-methylpyrrolidinium trifluoromethane sulfonate [BMPL][OTF], 1-ethyl-3-methylimidazolium bromide [EMIM][Br], [EMIM][Ac], and 1-butyl-3-methylimidazolium trifluoromethane sulfonate [BMIM][OTF]. When IL solution was used at 5% (v/v), the enzymatic activity was demonstrating the activity higher than 90% for all ILs. In 20% (v/v) IL solution, it was reported that [EMIM][Ac] carries the highest percentage of the enzyme activity which is 94.37% followed by [BMPL][OTF] with the percentage of 80.2%, [BMIM][OTF] (74.69%), [BMIM][Cl] (73.2%), [EMIM][Br] (67%) and [EMIM][CH_3-SO_3] (59%). In addition, the residual activity of the tested enzyme (*Tri-Cel*) in concentrated IL solution (about 60% v/v) of [EMIM][Ac] is comparable with a previous study in which cellulases sustained 86 and 76% of the activity in 5 and 10% of [EMIM][Ac] (Wang et al., 2011b), which validates that cellulases are gradually losing the activity by rising the IL concentration.

ILs with a hydrophobic origin, cosmoropic anion, chaotropic cation and less viscosity in most cases, tend to boost the enzyme's stability and activity. Even so, because of so many conflicting reports, the theory is not generalized (Naushad et al., 2012). In enzymatic hydrolysis system, [DMIM][DMP] and [EMIM][Ac] were both investigated and revealed that when IL concentration higher than 40% resulted in the cellulase deactivation, endoglucanase sustained its activity (50%) in a solution of 90% (v/v) [DMIM][DMP] (Wahlström et al., 2012). Similarly, after one hour, cellulase sustained about 40% of the activity in [EMIM][Ac] (Ebner et al., 2014). Fukaya et al., (2008) suggested that in enzymatic catalysis, the anionic element of ILs portrayed an important role, whereas a single-step continuous process is used for biomass treatment and saccharification, cellulase in ILs is regarded as a viable alternative. *Tri-Cel* could therefore function as an excellent biocatalysis for biomass

hydrolysis since it is generated locally at minimal cost by optimizing the waste utilization from agro-industrial.

2.6 Conclusion

Tri-Cel has good activity and stability. Of all ILs that were evaluated in this research, [Ch][Bu] and also [Ch][Ac] provided excellent media for the *Tri-Cel*-ILs system. This method is promising on the basis of the analysis and recommended for a one-step process for lignocellulose treatment and hydrolysis. ILs with cholinium cations have shown good compatibility with cellulase enzyme and could be utilized in future studies.

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