Application of Ionic Liquids ss Reaction Media for The Xylanase Reaction

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ABSTRACT

The potential for performing *Thermomyces lanuginosus* xylanase-catalyzed reactions on birchwood xylan dissolved in a range of ionic liquids has been investigated. As apparent from the results, some of the selected hydrophobic ionic liquids performed as better facilitators of the hydrolysis reactions than the hydrophilic ones. Thus, the xylanase reaction profiles in these hydrophobic ionic liquids, including [Py1,4]tfsa, [C4MIm]PF6, [P6,6,6,14]BF4, [P6,6,6,14]dca, [P6,6,6,14]bisphosphinate, and [C1MIm]MeSO4, were investigated in greater detail and the conditions for optimum temperature, optimum pH, and kinetic parameters documented. [P6,6,6,14]bisphosphinate was found to be the best ionic liquid producing the highest xylanase activity (84.98 unit/mg protein) at high temperature and pH, namely at 85°C and pH 9.0, respectively, among all of the ionic liquids tested. In addition, this ionic liquid increases the specificity of the xylanase toward xylan, indicated by lower KM (6.42 mg/mL), though it does not observed well on its enzyme's enhancement rate. Therefore, this study indicates that the application of this ionic liquid for the hydrolysis of real lignocellulosic samples, such as wood pulp, is warranted. Further investigations, outside the scope of this research will be required to validate these observations.

Keywords: Hydrophobic, hydrophilic, ionic liquid, xylanase, xylan

INTRODUCTION

Ions have a dramatic impact on the catalytic efficiency of enzymes because their hydration changes the water structure, and modifies the aqueous environment surrounding the biological macromolecules (Shimizu & McLaren 2006). Ionic liquids, which consist of organic cations and anions, can thus be anticipated to influence the interaction of water and proteins in aqueous environment as well. Nowadays, issues have been addressed for the utilization of chemicals that are environmentally friendly with less wastes and pollution being produced. Hence, the utilization of environmentally acceptable ionic liquids is required.

Enzymes may retain their catalytic activities in ionic liquid solutions presumably as a result of structure conservation. On the other hand, the denaturation of enzymes can also be a result of contact with ionic liquid solutions. The potential range of variability in ionic liquid structures and solution properties makes them attractive materials for macromolecule applications (Pusey *et al*. 2007).

A number of enzymes have been reported to retain catalytic activity in ionic liquid media (Kragl *et al*. 2002, van Rantwijk *et al*. 2003). Several ionic liquids have been found to dissolve and stabilize proteins (Itoh *et al*. 2003, Pusey *et al*. 2007, Fujita *et al*. 2005, Fujita *et al*. 2006). In addition, the preparation of ionic liquid-robed DNAs has been studied by Nishimura *et al.* (2005) while the mechanism of interaction between DNA and ionic liquids has been studied as well by Wang *et al.* (2007).

According to Liu *et al*. (2003) "A co-solvent is an additive that dissolves in the primary solvent." and "…. can increase the solubility of the solute while maintaining the favourable properties of the fluid." This explanation does not suit the description of a co-solvent as used in this paper since the majority of the co-solvents are ionic liquids, and currently have not been shown to assist protein solubility in the main solvent.

However, according to Shimizu (2004a & 2004b), Shimizu & Mclaren (2006), and Shimizu & Smith (2004), the presence of co-solvent in the reaction mixture influences not only the solubility, but also the stability, binding, and crystallization of proteins. As a protein stabilizer, the co-solvent tend to be preferentially excluded from the protein surface, while as denaturants, it is usually preferentially bound to protein surfaces. The protein stability enhancement in co-solvent can be quantified by measuring the preferential hydration parameters and partial molar volume. In this research, the protein stability is maintained in the ionic liquids shown by the maintaining activity of the enzyme towards the reaction.

In recent years, there have been increasing interests in the use of ionic liquids, including their application as solvents in bio-catalytic reactions. One of the areas related to their application is their utilization with lignocellulosic materials. However, research on the hydrolysis of xylan in ionic liquids for biobleaching process has not been reported before. In this study, several ionic liquids have been employed as additives in the reaction mixture to examine their effects on the hydrolysis of birch wood xylan by *Thermomyces lanuginosus* xylanase. In particular, this study thus has as its main aim an examination of the potential of ionic liquids to facilitate the xylanase reaction at high temperature and under high pH conditions. There are several kind of ionic liquid discussed in this paper, including imidazolium-, ammonium-, phosphonium-, and pyrollidinium-based ionic liquids.

METHOD

Materials and reagents

Xylanase from *Thermomyces lanuginosus* (product number X-2753), birchwood xylan (product number X-0502). The ionic liquids were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) including [dmim]$MeSO_4$: 1,3-dimethylimidazolium methylsulfate, [emim]BF_4: 1-ethyl-3-methylimidazolium tetrafluoroborate, [emim]$EtSO_4$: 1-ethyl-3-methyl imidazolium ethylsulfate, [et-Amm]acetate: tetraethylammonium acetate tetrahydrate, [bmim]NO_3: 1-butyl-3-methylimidazo lium nitrate, [bmAmm]$MeSO_4$: tributylmethylamonium methylsufate, [bmim]acetate: 1-butyl-3-methylimidazolium acetate, [bmPyr]tfsa: 1-butyl-1-methylpyrrolidinium bis-(trifluoromethylsulfonyl)amide, [bmim]PF_6: 1-butyl-3-methylimidazolium hexafluorophosphate, [hdP]BF_4: trihexyltetradecylphospho nium tetrafluoroborate, [hdP]dca: trihexyltetradecylphosphonium dicyanamide, [hdP]bisphos phinate: trihexyltetradecylphosphonium bis(2,4,4-trimethylpentyl)phosphinate, [hdP]deca noate: trihexyltetradecylphosphonium decanoate, [hdP]Cl: trihexyltetradecylphosphonium chloride, and [butAmm]benzoate: tetrabutylammonium benzoate, [bmP]$MeSO_4$: tributylmethyl phosphonium methylsulfate. [emim]xs: 1-ethyl-3-methylimidazolium xylenesulfonate, was obtained from the Ionic Liquid Research Group (Professor D. MacFarlane) at Monash University. It has been prepared and characterized for its ability to dissolve lignocellulosic material, particularly lignin (Tan *et al*. 2006). [bmim]lactate: 1-butyl-3-methylimidazolium lactate was synthesized based on Oktavianawati (2009a).

Buffers with pH values 3.5 to 6.5 were prepared from sodium citrate buffer, pH values 7 to 9.5 were from tris-Cl buffer, and pH values 10-10.5 were from glycine-NaOH buffer. The concentrations of all buffers were 0.05 M. A solution of 25 mg/mL of xylan as substrate was prepared by dissolving xylan in sodium citrate buffer 0.05 M, pH 6.5 (Bailey *et al*. 1992). Dinitrosalicylic acid solution (DNS) was prepared for reducing sugar assay (Wang *et al*. 1997).

Xylanase assay in buffer

In brief, the reaction mixture containing 1 mL of xylan (25 mg/mL) and 1 mL of xylanase solution (0.003 mg protein) was incubated in 3 mL of citrate buffer 0.05M at pH and temperature specified for 30 minutes. The amount of reducing sugars produced from the hydrolysis reactions was determined by reducing sugar assay performed based on colorimetric methods (Wang *et al*. 1997) using spectrophotometer UV-Vis (at 540 nm), manufactured by Bio-Rad (Smart Spec^{TM} Plus, catalogue number 170-2525).

The protein content was detected using a microplate reader (Bio-Rad, Model No. 3550, catalogue number 170-6601) at 595 nm and the data were analysed using Microplate Manager®/PC Data Analysis Software, version 3.1 (catalogue number 170-6617). Micro-assay of the protein content of xylanase in the various test samples were conducted based on the Bradford method using Bradford reagent (Bradford 1976).

One unit of enzyme activity is defined as the release of 1.0 mg of reducing sugars from xylan (measured as xylose) per minute under assay condition. Because the same amount of enzyme was used in the experiments, the results (xylanase activities) are comparable. There is a definitions of 100 % activity in this article: the xylanase activity in buffer (ionic liquid-free).

The enzymatic activity values in the various samples were calculated from the following relationship:

$$\text{Units/mL enzyme} = \frac{\text{(mg of reducing sugar liberated) x (vol. assay, mL) x (dilution factor)}}{\text{(time assay) x (vol. enzyme used, mL) x (vol. for red.sugar determination, mL)}}$$

$$\text{Units/mg protein} = \frac{\text{units/mL enzyme}}{\text{mg protein/mL enzyme}}$$

Biochemical characterization and kinetic parameters of xylanase

Optimal conditions including temperature and pH of hydrolysis of xylan and the kinetic parameter to determine K_M, were studied by performing xylanase assay as indicated in Oktavianawati (2009b).

Xylanase reactions in ionic liquids

The enzymatic assay of xylanase was carried out in the mixtures of buffer/ionic liquids at different proportions: 0%, 20%, 40%, 60%, 80% and 100% (v/v). This assay has been discussed in Oktavianawati (2011). The xylanase activity mediated by ionic liquids was then compared with the activity in the aqueous media (100% buffer, ionic liquid-free) in term of percentage activity (in units/mg protein), and the kinetic parameters.

The optimum temperature and pH for the hydrolysis reaction of xylan by xylanase were determined in 20% of ionic liquids based on the method described above. Then, the kinetic parameters of xylanase in the presence of 20% ionic liquids were also determined using enzymatic assay, under the optimum conditions for each selected ionic liquids.

Statistical Analysis

All experiments were conducted in triplicate, and their mean values are presented, along with standard deviations. The statistical analyses were performed by computer using the data analysis tool of Microsoft Excel unless otherwise stated.

RESULTS AND DISCUSSION

Investigation of the xylanase reactions in hydrophilic ionic liquids

In this section protein hydration and water-mediated protein-salt interactions on the xylanase activity in hydrophilic ionic liquids were analysed. The use of ionic liquids as co-solvent in a reaction mixture will influence the solubility, thermal and pH stability, and binding of proteins, which then further influence the enzymatic activity.

This research explores the use of hydrophilic ionic liquids based on cation imidazolium and ammonium. Screening of hydrophilic ionic liquids based on those cations on the xylanase reactions were carried out in the presence of 20% (v/v) ionic liquids at 65°C, pH 6.5.

Figure 1 shows that the xylanase activity was not well maintained in the selected hydrophilic ionic liquids. In the rest of the text, the xylanase activity in buffer (ionic liquid-free), i.e 87.13 unit/mg protein, will be defined as 100% xylanase activity. [C_1MIm]$MeSO_4$ is the best ionic liquid that could maintain the xylanase activity at 65.94 unit/mg protein showing above 75% of xylanase activity in buffer.

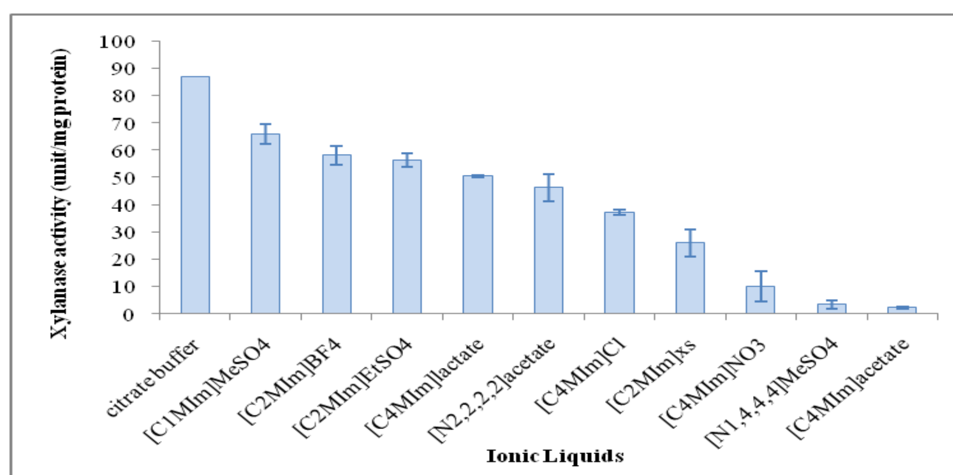

Figure 1. Screening of hydrophilic ionic liquids for xylanase reaction at 65°C and pH 6.5.

Five of the selected ionic liquids, namely, $[C_2MIm]BF_4$, $[C_4MIm]$lactate, $[C_2MIm]EtSO_4$, $[N_{2,2,2,2}]$acetate, and $[C_4MIm]Cl$, kept the enzymatic activity between 50% and 75% of the activity in buffer (37.23 – 58.14 unit/mg protein), while the rest of them resulted in more significant reduction in the xylanase activity.

Dupont mention that aqueous solution of free enzyme might be embedded in the ionic liquid networks, which could protect the essential water of proteins and the solvophobic interactions that is critical for maintaining the native structure (Dupont 2004). The presence of ionic liquids in the aqueous environment will influence the conformation structure or the active sites of the enzyme. In the native folded conformation of proteins, charged groups are on the outside in contact with water, while the non-polar amino acid residues are sequestered into the centre of the structure, out of contact with water producing a cage-like structure. Therefore, the presence of ions, particularly anions in contact with the essential water, in the enzyme solution will influence the structure of the proteins.

When the aqueous solutions of hydrophilic ionic liquids are used as reaction media, the salts will dissociate into individual ions (cations and anions). Thus, the enzyme stability seems to follow the Hofmeister series (Hofmeister 1888). The kosmotropicity and chaotropicity of the ionic liquid anions and cations will explain the effect of these ionic liquids on the hydrolysis reaction of xylan by the xylanase.

$[C_1MIm]MeSO_4$ is a combination of chaotropic cation, $[C_1MIm]^+$, and kosmotropic anion, $MeSO_4^-$. Theoretically, the combination of these ions leads to the stabilization of protein conformation on the enzyme. Indeed, $[C_1MIm]MeSO_4$ was the best of the selected hydrophilic ionic liquids maintaining the xylanase activity up to 75% of its original activity in buffer. Although $MeSO_4^-$ strongly interacts with water and forms high density clusters of water on its surrounding, $[C_1MIm]^+$ does not strongly disrupt the water hydrogen bond network (Zhao 2006). The combination of these ions may result in bulk-water places around the enzyme environment to maintain the protein structure and to help the hydrolysis reaction.

On the other hand, $[C_4MIm]NO_3$, which is a combination of strong kosmotropic cation and chaotropic anion, inhibited the xylanase reaction by more than 85%. As a typical neutral anion, NO_3^- is a very weakly basic anion which exhibits only weak electrostatic interactions with the cation. The inhibition of enzymatic activity may be caused by the strong interaction of both cation and anion with water which resulted in the formation of high density clusters of water around the ionic liquid. Since bulk-water will be concentrated around the ions, there might be less essential water to keep the protein in its native conformation. This fact may lead to destabilization or even denaturation of the protein which then reduced xylanase activity.

Combination of strongly hydrated cation, $[C_4MIm]^+$, with borderline anion, Cl^-, resulted in the inhibition of enzymatic reaction by up to 50% of the activity in buffer. Therefore, the observed effects of the presence of $[C_4MIm]^+$ in the xylanase reaction were similar. This cation is strongly hydrating the protein in the aqueous environment providing low enzymatic activity.

In addition, it is possible that the individual ions of the ionic liquids may affect the enzymatic reaction. For example, apart from the high chaotropicity of BF_4^- ions, deactivation of xylanase in the presence of $[C_2MIm]BF_4$ may also be caused by the low pH of this anion (pH 3-4) which is undesirable for the enzyme (Zhao *et al.* 2006a). At this pH, the active sites of xylanase tend to be protonated which then prevent the enzyme to hydrolyse the substrate (Oktavianawati 2009b).

In summary, the use of large organic cations such as $[C_4MIm]^+$ and $[N_{1,4,4,4}]^+$ should be avoided since they are strong kosmotropes and strongly hydrate the essential water. In this case, smaller cations such as $[C_1MIm]^+$ and $[C_2MIm]^+$ are preferred choices of enzyme stabilizers. However, it must be noted that no matter which ions are present, if the ion concentration is too high, the enzyme could lose its activity due to the high ionic strength.

Hydrophobic ionic liquid

The hydrophobic solvents, such as those ionic liquids used in this research, have a lesser tendency to take away the essential (or critical) water from the enzyme's surface. Enzyme activity is also determined by the water bound to the enzyme rather than the bulk-water content in the system (Zhao *et al.* 2006b, Zhao 2005, Jain *et al.* 2005).

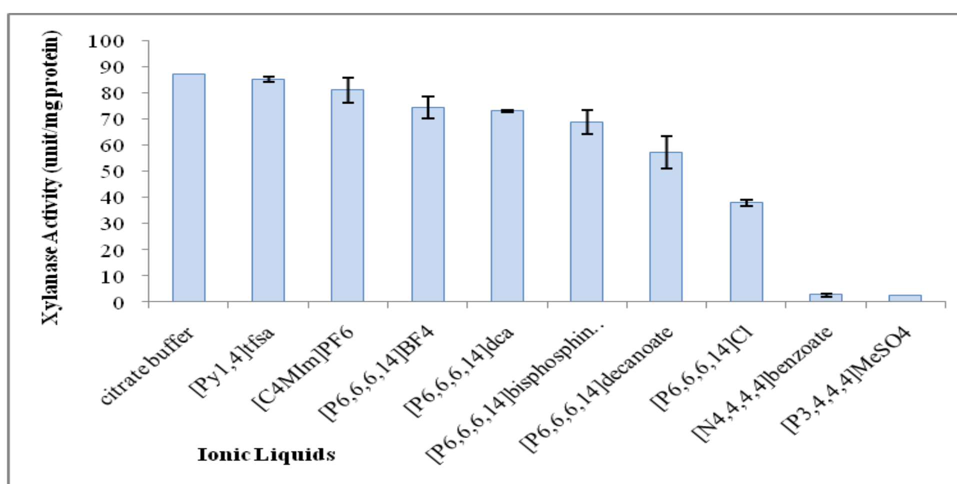

Figure 2. Screening of hydrophobic ionic liquids for xylanase reaction at 65°C and pH 6.5.

According to Fujita *et al.* (2007), in most reports involving hydrophobic ionic liquids, the enzyme is present in a dispersed state rather than dissolved, and is therefore acting as a heterogenous catalyst.

Theoretically, in non-aqueous ionic liquid environments, the xylanase cannot retain its native structure, which then results in the inhibition of enzymatic reaction. The hydrolysis reaction will also not occur since water is not present in the appropriate amount. However, screening of the selected hydrophobic ionic liquids shows the opposite results.

Based on Figure 2, it is evident that some hydrophobic ionic liquids could maintain enzymatic activities above 75% of activity in buffer only. The best two of them are $[Py_{1,4,}]$tfsa and $[C_4MIm]PF_6$. According to some literatures, several enzymes in the presence of hydrophobic ionic liquids based on PF_6 and tfsa salts have shown very high activities and stabilities in a number of applications (Lozano *et al.* 2001, Barahona *et al.* 2006, Eckstein *et al.* 2002, Kamal & Chouhan 2004).

Since tetraalkylphosphonium salts do not have acidic protons and aromatic rings as is the case with imidazolium salts, these salts have less potential for interaction with solutes. The application of those ionic liquids showed that most of them, $[P_{6,6,6,14}]BF_4$, $[P_{6,6,6,14}]$dca, and $[P_{6,6,6,14}]$bisPhosphinate maintained the xylanase reaction above 75% of the original activity in buffer (68.71 unit/mg protein).

There are some possibilities of the interaction between the enzyme and the hydrophobic ionic liquids. The first possibility is that the ionic liquid may interact with the enzyme through electrostatic interaction. The negatively charged protein surface may interact with the cation of the ionic liquid, maintaining the protein conformation. However, it is also possible that the active site of the enzyme could interact with the cation of the ionic liquid leading to inhibition of activity and preventing the binding of the substrate.

The second possibility is that the ionic liquid may interact with the substrate or product in a similar manner to that of an organic solvent. Liquid-liquid extraction method would be the best illustration to describe this process. In this case, the hydrophobic ionic liquid could form a separate layer from the aqueous phase. However, a small percentage of water containing buffer and substrate may be still present in the ionic liquid phase. Moreover, since the enzyme has a number of hydrophobic amino acid residues on the surface, it may interact with or even partly dissolve in this hydrophobic ionic liquid phase. As a result of this, the hydrolysis reaction of xylan by the xylanase may occur in this hydrophobic ionic liquid containing phase as well.

Finally, the product, xylose which is soluble

in water, will go to the aqueous phase. Furthermore, since the substrate and enzyme disperse better over time in the hydrophobic ionic liquid, this leads to a greater number of products compared than within the hydrophilic ionic liquids. In this case, the hydrophobic ionic liquid may assist the hydrolysis reaction of xylan.

$[C_4MIm]PF_6$ is one of the hydrophobic ionic liquid whose physicochemical properties are affected by water saturation and the solute structure. In fact, its viscosity is decreased by an order of magnitude when the water molecules are dissolved in the ionic liquid phase (Carda-Broch 2003). Although $[C_4MIm]PF_6$ is immiscible with water, a small amount of this ionic liquid can dissolve in the aqueous acid. Wong *et al* .(2002) and Baker & Baker (2005), also state that at a temperature below 50°C, less than 2.5 wt% of this ionic liquid is soluble in water.

Therefore, the hydrolysis of xylan in the ionic liquid/water biphasic system may start from the transport of the small amount of substrate and enzyme from the aqueous phase. This transfer involves the interaction of the molecules (substrate and enzyme) with a limited amount of ionic liquid dissolved in water, especially the cation, or with the interface of the ionic liquid phase and aqueous phase.

The third possibility is that the hydrophobic solvents may not as efficiently remove the essential water from the enzyme as the hydrophilic ones do. Practically, the enzyme is then suspended in the hydrophobic media rather than dissolved, resulting in a heterogeneous environment which prevents the enzyme from denaturation by the high ionic strength. However, since the hydrolysis reaction requires a certain number of water molecules to be involved, water is also an important species to be reserved in this occasion.

The optimum condition of the xylanase reaction in the presence of the six best ionic liquids

Based on the results of those screening, six ionic liquids were found to maintain the xylanase activity above 75% of activity in buffer only. Those ionic liquids are listed below and five of them are liquid and one is a solid, i. e $[P_{6,6,6,14}]BF_4$, at room temperature.

One function of ionic liquids in enzymatic reaction is protein solubilizing and stabilization. The secondary structure of the protein may be retained in the ionic liquid. Therefore, the thermal stability of enzyme may increase. In addition, ionic liquid offers both hydrogen donor and acceptor sites to provide a proton activity similar to that in neutral water (MacFarlane *et al.* 2006). Hydrophobic hydration also increases the heat capacity which reflects the strong temperature dependence on the xylanase activity.

The enzymatic reactions vary with pH and often pass through a maximum as the pH is varied. This phenomenon is also occured as the temperature is varied. Then, pH and temperature profiles of the xylanase reaction in the presence of each ionic liquid are required for further studies. The optimum temperature and pH for xylanase reaction in those ionic liquids (table 1) can be determined from their temperature and pH profiles (not shown here). The experiment was carried out in the range of temperature and pH reaction, 40°C - 100°C and pH 3.5 – pH 10.5, respectively. The results was summarized as shown in Table 2.

Table 2 listed that most of the xylanase reaction showed the optimum temperature and pH working range between 65°- 85°C and pH 5 –9. Since this study has its main aim to facilitate the xylanase reaction in ionic liquid at high temperature and alkaline condition, then $[P_{6,6,6,14}]$bisPhosphinate is the best ionic liquid which fulfil this requirement by showing optimum temperature and pH at 85°C and pH 9.0.

Studies of xylanase reaction in the ionic liquid media show that ionic liquids, especially $[P_{6,6,6,14}]$bisPhosphinate potentially has the ability to not only reduce the use of acid for pH adjustment on the xylanase reaction, but also increase the temperature operation.

The use of ionic liquid solvents, especially the hydrophobic ones, also offers the opportunity for recycling the solvents. Since the hydrophobic ionic liquids form separate layer from the aqueous phase, where the standard reaction occurs and product is present, these solvents can be separated at the end of the reaction and used for other reactions.

Table 1. The best ionic liquids for assisting hydrolysis of xylan.

Ionic liquids	xylanase activity (unit/mg protein)
$[Py_{1,4}]$tfsa	85.08
$[C_4MIm]PF_6$	81.09
$[P_{6,6,6,14}]BF_4$	74.49
$[P_{6,6,6,14}]$dca	73.03
$[P_{6,6,6,14}]$bisPhosphinate	68.71
$[C1MIm]MeSO_4$	65.94

Table 2. The optimum temperature and pH for xylanase reaction in 20% of ionic liquids.

Ionic Liquid	Optimum	
	Temperature (°C)	pH
[Py1,4]tfsa	65	7.5
[C4MIm]PF6	65	7.5
[P6,6,6,14]BF4	70	7.0
[P6,6,6,14]dca	65	7.0
[P6,6,6,14]bisphosphinate	85	9.0
[C1MIm]MeSO4	65	5.0

Table 3. The kinetic parameters of the xylanase activity in the presence of ionic liquids.

Medium	V_{max} (unit·mg protein^{-1})	K_M (mg·mL^{-1})	V_{max}/K_M (unit mL·mg^{-1}·mg protein^{-1})	Activation[a]
Buffer (salt free)	14.27	8.97	1.59	1.00
$[Py_{1,4}]$tfsa	7.44	5.93	1.25	0.79
$[C_4MIm]PF_6$	10.58	2.07	5.11	3.22
$[P_{6,6,6,14}]BF_4$	11.11	29.55	0.38	0.24
$[P_{6,6,6,14}]$dca	9.52	19.26	0.49	0.31
$[P_{6,6,6,14}]$bisphosphinate	7.21	6.42	1.12	0.71
$[C_1MIm]MeSO_4$	9.58	21.66	0.44	0.28

One unit is defined as the release of 1 mg of xylose from xylan per minute reaction.
[a] The activation is the ratio of current V_{max}/K_M to that in buffer (ionic liquid free) where V_{max} and K_M are Michaelis-Menten parameters.

Kinetic parameters of the xylanase towards the hydrolysis reaction of xylan in the presence of the six best ionic liquids

One way to measure the effect of ionic liquids on the xylanase reaction is to measure the enzyme's velocity at a variety of substrate concentrations in the presence and absence of ionic liquids. The kinetic of enzymatic reactions in ionic liquid solutions was investigated under the optimum conditions of each ionic liquid (link to Table 2).

K_M and V_{max} can be determined by plotting the enzyme velocity as a function of substrate concentration. These obtained plots appear to satisfy the Michaelis-Menten equation. Table 3 summarizes that the kinetic curves of the enzymatic reaction showed different shapes and values of the kinetic parameters in the presence of different ionic liquids.

If the ionic liquids interact with a site on the enzyme distinct from the site that binds the substrate, the inhibition cannot be overcome by increasing the concentration of the substrate. This inhibition is not competitive, which means that the ionic liquids decrease the V_{max} and may also increase the K_M. These values, V_{max} and K_M of xylanase in the ionic liquids, were compared with V_{max} and K_M of xylanase in buffer. The ionic liquids that follow this trend were $[P_{6,6,6,14}]BF_4$, $[C_1MIm]MeSO_4$, and

[$P_{6,6,6,14}$]dca. The summary of catalytic constants were calculated and illustrated in Table 3.

However, it can be noted that the activation factor is 3.22 in [C_4MIm]PF_6, which is higher than those in buffer and other ionic liquids. Therefore, there was an improved catalytic efficiency in ionic liquids than in buffer only.

The influence of ionic liquids in K_M and V_{max} of xylanase can be seen in each interaction. The data shows that [C_4MIm]PF_6, [$Py_{1,4,4,4}$]tfsa, and [$P_{6,6,6,14}$]bisPhosphinate increases the specificity of the xylanase towards xylan which are indicated by lower K_M values than in buffer alone. However, the enhancement rates of the xylanase were not observed although optimum temperature and pH of each ionic liquid are applied on the system.

Therefore, it is important, for further research, to observe K_M at a variety of concentrations of ionic liquids for more reliable measurements. If the inhibition on the ionic liquid-mediated catalysis is competitive, the plot of the concentrations of ionic liquid and observed K_M will be linear.

CONCLUSION

In this paper, the utility and the effects of different ionic liquids, e.g. hydrophilic and hydrophobic ionic liquids, on the hydrolysis of xylan using xylanase has been examined. It was found that the ionic liquid/water biphasic system (hydrophobic ionic liquids) maintained the xylanase activity to hydrolyse xylan better than the hydrophilic ionic liquids. The data also confirmed that those same hydrophobic ionic liquids maintained the xylanase activity at higher temperature and higher pH than found for the reaction in buffer alone. In this case, [$P_{6,6,6,14}$]bisPhosphinate was found to maintain the xylanase reaction at 85°C and pH 9.0, which then is feasible for application in biobleaching process. However, further investigation using the real sample i.e. pulp, is still needed. The use of some hydrophobic organic salts, [C_4MIm]PF_6, [$Py_{1,4}$]tfsa, [$P_{6,6,6,14}$]bisPhosphinate, increased the specificity of the enzyme, as shown by the lower value of K_M than found if only the buffer as media was used.

Hence, based on the results obtained in this study, it can be concluded that the use of ionic liquids for xylanase reaction could provide an alternative option for providing a reaction media in the biobleaching process.

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