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Full Length Research Paper

Cellulase immobilization properties and their catalytic effect on cellulose hydrolysis in ionic liquid

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Cellulase was immobilized on chitosan by the method of covalent binding. The optimum immobilized conditions were as follow: the pH value was 5.0, the glutaraldehyde concentration was 0.015 (w/v) and the formaldehyde concentration was 0.15 (w/v). Both the free and immobilized cellulase were characterized by determining the pH, temperature, thermal stability and storage stability. The optimum pH of both the free and immobilized cellulase was found as 4. The immobilized cellulase had optimum temperature of 50℃ as compared to 40℃ in case of free enzyme. The immobilized enzyme showed higher thermal stability than the free cellulase, after 120 min, the activity of immobilized cellulose and the free enzyme retained 86.5 and 61% respectively. After 11 cycles, the activity of the immobilize enzyme conserved 80.27%. The immobilized enzyme exhibited slightly better storage stability than the free enzyme. The Km and Vm values for the immobilized and free cellulase were 8.1 and 1.84 mg/L and 0.01 and 0.0036 mg/ml/min respectively. Cellulose hydrolysis by immobilized cellulase in the presence of a 88 ionic liquid (IL), 1,3-dimethylimidazolium dimethylphosphate (MMIM-DMP), was investgated. The result showed that the addition of 20% (v/v) MMIM-DMP gave the highest initial rate, which was 1.3 and 13.9 times higher than the hydrolysis rate in citric acid - sodium hydrogen phosphate buffer and in IL, respectively.

Key words: Renewable energy, cellulose, immobilization, chitosan, cellulose, ionic liquids.

INTRODUCTION

With the development of economy, the renewable energy gets more and more attention. Cellulose is the single largest biomass and greatest renewable resource available on the earth (Lee et al., 2010; Jones and Vasudevan, 2010). Key to the exploitation of this vast resource is the development of methods for the conversion of cellulose polymers into useful sugar products. Compared to chemical methods, enzymatic degradation of cellulose by cellulase has already proven to be the preferred method (Sheldon et al., 1996; Wingren et al., 2003).

However, an effective use of enzyme may be hampered by some peculiar properties of the enzymatic proteins such as their non-reusability, high sensitivity to several denature agents and presence of adverse sensory or toxicological effects. Many of these undesirable constraints may be removed by the use of immobilized enzymes (Iyer and Ananthanarayan, 2008; Brady and Jordaan, 2009; Mateo et al., 2007; Temocin and Yigitoglu, 2009). The originally soluble enzyme is made insoluble by its attachment to the surface of an insoluble carrier, a process called immobilization. This approach has proven to be more advantageous for catalysis than the use of free enzyme (Krajewska, 2004; Cao, 2005; Hanefeld et al., 2009; Sheldon, 2007; Sanjay and Sugunan, 2008). Insoluble carrier, which plays an important role in the utility of an immobilized enzyme, should be readily available and non-toxic and also should

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Abbreviations: IL, Ionic liquid; **MMIM-DMP,** 1,3-dimethylimidazolium dimethylphosphate; **PEG,** polyethylene glycol; **DNS,** 3,5-dinitrosalicylic acid; **HRP,** horseradish peroxidase.

provide a large surface area suitable for enzyme reaction, and substrate and product transport with least-diffusional restriction (Li et al., 2004). Some organic and inorganic supports like montmorillonite (Sanjay and Sugunan, 2008), glass beads (Rojas-Melgarejo et al., 2004), poly (ethylene terephthalate) grafted acrylamide fiber (Temocin and Yigitoglu, 2009), chitosan (Bindhu and Abraham, 2003), eudragit (Gaur et al., 2005) and amberlite (Esawy and Combet-Blanc, 2006) have been used for the immobilization of enzyme.

Ionic liquids are organic salts that are liquid in room temperatures. The anions in ionic liquids bond with cellulose at high temperature, dissolving the cellulose (Youngs et al., 2007). However, the presence of high concentrations of some ionic liquids results in the inactivity of the enzyme (Zhao et al., 2009). It has been found that a mixture of ionic liquids with water is effective in increasing the cellulose hydrolysis yields (Kamiya et al., 2008; Jones and Vasudevan, 2010).

In this study, the cellulase had been covalently immobilized on the chitosan. The activities of the immobilization enzyme and free enzyme were evaluated form the point of pH, temperature, thermal stability and storage stability and the results were compared. The reusable of the immobilized cellulose was also investigated. At last, the cellulose hydrolysis catalyzed by immobilized cellulase in 1,3-dimethylimidazolium dimethylphosphate (MMIM-DMP) was explored.

MATERIALS AND METHODS

Chemicals

The chitosan from shrimp shells (medium molecular weight, degree of deacetylation is range from 75 to 85%) was purchased from Sigma-Aldrich. Polyethylene glycol (PEG 400), glutaraldehyde and formaldehyde were obtained from Shanghai Lanji CO, LTD. Sodium carboxymethyl cellulose was purchased from BASF CO. (Tian Jin). The cellulase (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4, 1.42 mg/ml) was kindly provided by Kangdien CO, LTD. 1,3-dimethylimidazolium dimethylphosphate was prepared by our laboratory.

Immobilization of enzyme

2 g raw chitosan was dissolved in 400 mL 2% (w/v) methylacetic acid. When the solution was adjusted to pH 12 by the addition of sodium hydroxide, the white precipitation was formed. After filtered, the refined chitosan was obtained. 3.2% (w/v) refined chitosan [dissolved in 2% (w/v) methylacetic acid], 2% (w/v) PEG [dissolved in 2% (w/v) methylacetic acid] and different concentrations glutaraldehyde solutions were mixed in the volume proportion of 20:13:6, and the mixtures were placed into the water bath at 40℃ for 2 h. After adjusted to pH 12 using sodium hydroxide, the granular PEG-chitosan carrier was produced. Then the carrier was filtered and washed with double distills water to pH 7.

100 mg PEG-chitosan was immersed in different pH values citric acid - sodium hydrogen phosphate buffer for 4 h, then the support was filtered and the PEG-chitosan carrier was immersed in 1 ml different concentrations formaldehyde for 2 h. After filtered, the carrier was washed with double distill water to pH 7.0. The activated carrier was immersed in 2 ml of cellulase solution and was placed into water bath at 20℃ for 4 h. After filtered, the immobilized cellulase was washed with citric acid - sodium hydrogen phosphate buffer for several times.

Activity measurement of free cellulase and immobilized cellulose

The activities of the free cellualse (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4, the concentration was 1.42 mg/ml) and the immobilized cellualse were determined by using sodium carboxymethylcellulose as substrate. 100 mg immobilized cellulase or 0.073 mL free enzyme was added into 3 ml 1% (w/v) sodium carboxymethylcellulose solution (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4), respectively, and the mixtures were incubated at 40℃ for 20 min. Then the mixtures were filtered. The activities of the immobilized enzyme and free enzyme were determined using the method of 3,5-dinitrosalicylic acid (DNS) as described by Shoemaker and Brown. (1978).

Determination of optimum temperature and pH of immobilized and free enzyme

100 mg of immobilized cellulase and 0.073 ml free cellulase were suspended in 3 ml 1% (w/v) sodium carboxymethylcellulose solution (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4.0), respectively. The mixtures were placed in water bath at 30, 40, 50, 60 70, 80℃ for 20 min. The activities were measured using the aforementioned method.

Immobilized cellulase and free cellulase were added into the 1% (w/v) sodium carboxymethylcellulose solutions (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH values were range from 3 to 7); the solutions were incubated at 50℃ for 20 min. The following procedures were described as previously.

Determination of thermal stability for immobilized and free cellulase

Thermal stability of immobilized cellulase and free cellulase were studied by incubating enzymes at 60℃. Every 20 min, 100 mg immobilized cellulase and 0.073 mL free cellulase were taken out and added into 3 ml 1% (w/v) sodium carboxymethylcellulose solution (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4.0). The solutions were incubated at 50℃ for 20 min. The activities of immobilized cellulase and free cellulase were determined using the method described previously.

Reusability of immobilized cellulase

The immobilized cellulase was added into 1% (w/v) sodium carboxymethylcellulose solution (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH = 4.0). After incubating at 50℃ for 25 min, the reaction solution was centrifuged, and the content of reducing sugar in the supernatant was determined using the method described previously. The precipitate containing immobilized enzyme was washed for three times using 0.1 mol/L citric acid - sodium hydrogen phosphate buffer and this was used for the next cycle of cellulose hydrolysis.

Storage stability of the immobilized and free enzymes

The immobilized and free cellulase was stored at 4°C in 0.1 mol/L

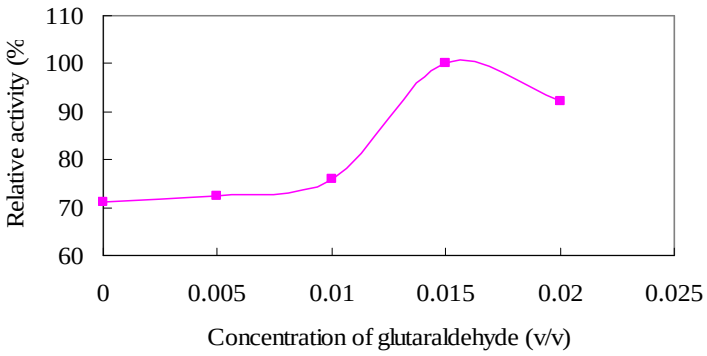

Figure 1. The effect of concentration of glutaraldehyde on cellulase immobilization [Immobilization conditions: pH = 4, formaldehyde concentration = 0.1 (v/v)].

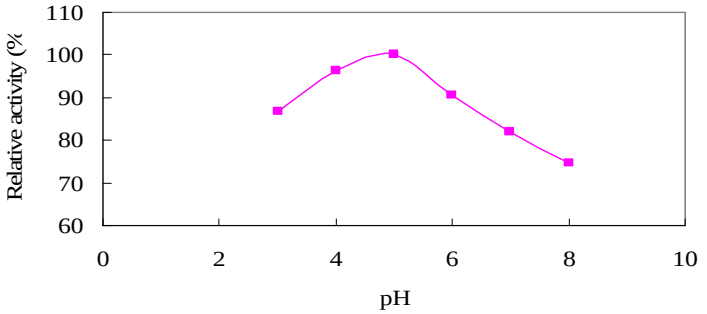

Figure 2. The effect of pH on cellulase immobilization [Immobilization conditions: glutaraldehyde concentration = 0.015 (v/v), formaldehyde concentration concentration = 0.1 (v/v)].

citric acid - sodium hydrogen phosphate buffer with optimum pH. The activities were measured at different time (1-4 weeks) using the method described previously.

Determined of Km and Vm for the free and immobilized enzyme

Km and Vm values of free and immobilized cellulase were determined by measuring initial rate of cellulose hydrolysis at various concentrations. 100 mg immobilized cellulase was added into 9 mL sodium carboxymethylcellulose solution with different concentrations (1, 1/300, 1/500 and 1/700%, dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer, pH was 4.0). The solutions were incubated at 50℃, and the content of reducing sugar in the supernatant was determined using the method as described above every 4 min untile the content of reducing sugar kept constant. A Lineweaver-Burk plot was used to calculate.

Cellulase catalyzed hydrolysis of cellulose in ionic liquids

The mixture with volume ratio of the IL to water (citric acid - sodium hydrogen phosphate buffer) 1:4 was prepared. The sodium carboxymethylcellulose solution was added to the mixture, and the final concentrations were 1% (w/v). In the case of controls, the same amount of cellulose was added to the buffer or the IL. The enzymatic saccharification was initiated by the addition of 100 mg immobilized cellulase to the mixed solutions, the IL or the aqueous. The solutions above were incubated in water bath at 60℃ for 20 min. The quantification of reducing sugar was conducted according to previously described methods (Shoemaker and Brown, 1978).

RESULT AND DISCUSSION

Optimization of immobilization conditions

The enzyme immobilization was carried out at different pH values, glutaraldehyde and formaldehyde concentrations. The results of glutaraldehyde concentrations on the enzyme immobilization were studied and the results were shown in Figure 1. As shown in Figure 2, the maximum activity was obtained when the glutaraldehyde concentration was 0.015 (w/v). Higher concentrations gave lower activity which was expected since glutaraldehyde inactivated cellulase.

The effect of pH values on the enzyme immobilization was investigated in the pH range from 3 to 8 and results are described in Figure 2. The formation point of covalent bonds between the surface of the support and enzyme regions probably depended on the pH value of the coupling media. As could be seen in that figure, the highest immobilization efficient was obtained at the pH 5.0. This was presumably because the amino groups in

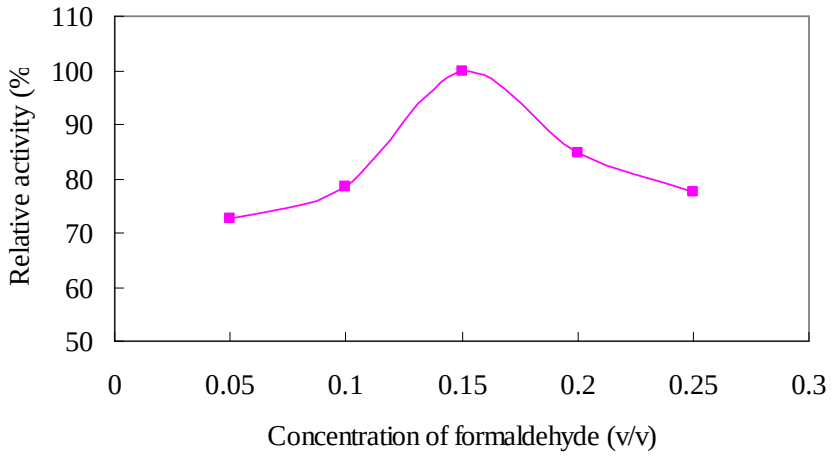

Figure 3. The effect of concentration of formaldehyde on cellulase immobilization (Immobilization conditions: pH = 5, glutaraldehyde concentration = 0.015 (v/v)).

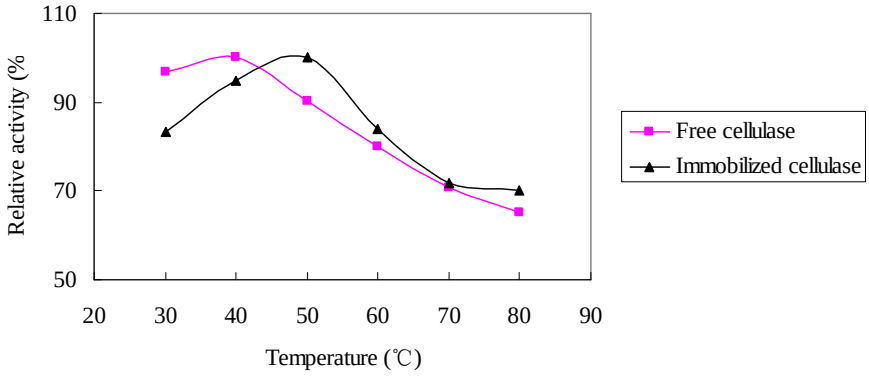

Figure 4. The effect of temperature on enzyme activity.

the enzymatic regions far from the active site might have reacted with glutaraldehyde on the PEG-chitosan during immobilization process in this pH.

The enzyme immobilization was carried out at different formaldehyde concentration values, which is as shown in Figure 3. The formaldehyde was an activator, and it can activate the reactive groups on the surface of the chitosan carrier. As could be seen in that Figure 3, when the formaldehyde concentration was 0.15 (w/v), the maximum activity was obtained.

The characters of free and immobilized cellulase

Effect of temperature on enzyme activity

The effect of temperature on the enzymatic activity of the free cellulose and immobilized cellulase were investigated by varying temperature from 20 to 80℃. Figur e 4 showed the results of maximum activity at 40 and 50℃ for the free cellulase and immobilized cellulase, respectively. For the higher temperature, the free enzyme activity decreased dramatically. In contrast, the decrease in activity of the immobilized enzyme at temperature above 50℃ was much slower than that for the free enzyme. Similar results with the horseradish peroxidase (HRP) enzyme were obtained by Temocin and Yigitoglu (2009).

The effect of pH on the enzyme activity

The activities of the free and immobilized cellulose were assayed at varying pH (3 to 7). The results are shown in Figure 5. It could be seen that for both the free and immobilized enzyme, the optimum pH of was 4. When pH was more or less than 4, the activity of both the immobilized cellulase and free cellulase decreased, however, the downtrend of the immobilized and free cellualse was different (Figure 5). This was explained

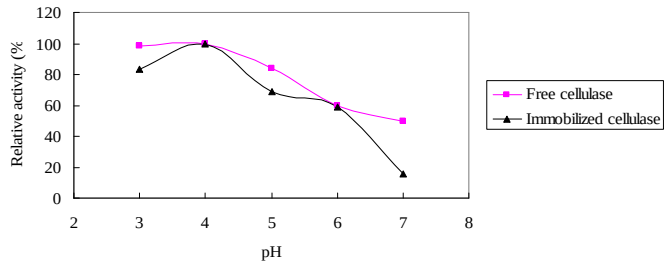

Figure 5. The effect of pH on enzyme activity.

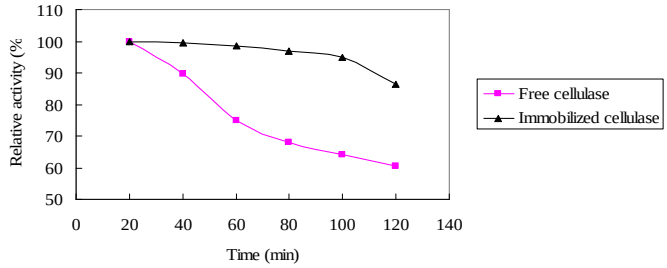

Figure 6. The thermal stability of the free celllulase and immobilized cellulase.

usually by the reason that the residual charges on the solid matrix affected the pH value in the immediate vicinity of the enzyme active site. Similar result was described by previous studies (Esawy et al., 2006; Krajewska and Piwowarska, 2005; González-Sáiz and Pizarro, 2001).

Thermal stability

Cellulase stability at high temperature was more suitable for industrial application. In this study, cellulose immobilized on chitosan showed a significant increase in thermal stability compared to free enzyme (Figure 6). At 60℃, the activity of the immobilized conserved 86. 5% after 120 min whereas the free enzyme conserved 61% of the initial activity only. These data showed that the thermal stability of the immobilized cellualse was much better than that of the free enzyme owing to the covalent bond between the enzyme and support, which prevented the conformation transition of the enzyme at high temperature. An increase in thermal stability of immobilized was also observed (Shukla et al., 2004).

Reusability of immobilized enzyme

For the immobilized enzyme, one of the most important advantages was reuse stability, which can effectively reduce the cost in applications. The result (Figure 7) showed that the relative activity of the immobilized enzyme decreased with the increase of the number of

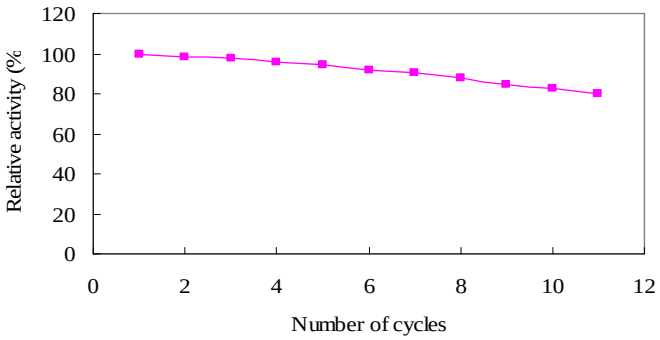

Figure 7. The reusability of immobilized enzyme.

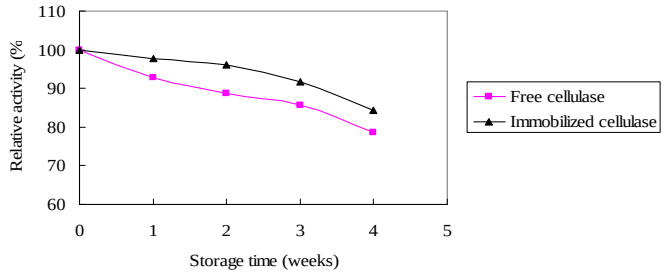

Figure 8. The storage stability of the free and immobilized cellulose.

reuse. After 11 cycles, the immobilized cellualse retained 80.27% of its initial activity. Immobilized of cellulase on chitosan had improved reusability of the enzyme. The results indicated the significance of chitosan as support for immobilization. The number of reuse represented a more satisfying performance when compared to previous research (Temocin and Yigitoglu, 2009).

Storage stability

For enzyme immobilization, another important parameter of measurement when assessing immobilization efficiency was the storage stability. The storage stability of the immobilized cellulose was given in Figure 8. The free enzyme lost about 79% activity in 28 days while the immobilized cellulase showed good storage stability and lost 16% of its initial activity only.

Enzyme kinetics of free and immobilized cellualse

For the two forms of cellulase, linearity of the Lineweaver-Burk plot showed Michaelis-Menten kinetics (Figure 9). The results revealed that the immobilized enzyme decreased its affinity for the substrate (Km=8.1 and 1.84 mg/L for the immobilized enzyme and free enzyme, respectively). Esawy et al. (2006) found that the

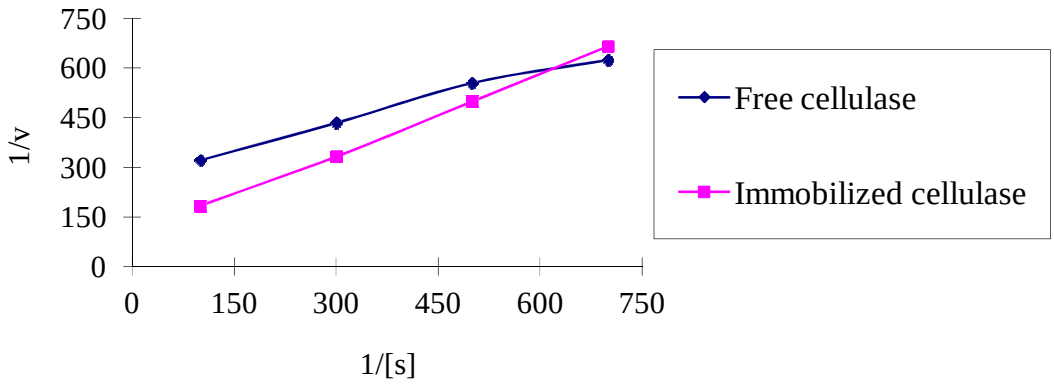

Figure 9. The Lineweaver-Burk plots of free and immobilized cellulose.

Table 1. Cellulose hydrolysis by immobilized cellulase in aqueous-Ionic liquids.

Ratio of IL to water	Initial reaction rate (mg/ml/min)
1:0	0.0069
1:1.5	0.0023
1:2	0.0073
1:2.5	0.0135
1:3	0.0152
1:4	0.0194
1:5	0.0162
0:1	0.0154

Km of immobilized milk-clotting enzyme was lower weakly than the free enzyme. The Vm values for the immobilized and free cellulase were 0.01 and 0.0036 mg/ml/min, respectively. Ruchi et al. (2004) had reported the similar results (Gaur et al., 2005).

The hydrolysis of cellulose in ionic liquid

The initial hydrolysis rate of immobilized cellulase at different ratio of IL is shown in Table 1. 20% (v/v) MMIM-DMP gave the highest initial reaction rate, which was 1.3 times higher than that in water and 13.9 times than that in IL. Kamiya et al. (2008) reported a nearly 2 times increase in cellulose hydrolysis in 20% IL (1-ethyl-3-methylimidazolium diethylphosphate). In our work, we observed only 1.3 –fold increase in cellulose conversion, which may be due to the different IL and the lower activity of the immobilized cellulase. The similar results were reported by Esawy et al. (2006).

Conclusions

In this study, the cellulase was successfully immobilized on chitosan carrier using covalent binding. The immobilization of cellulase showed attractive biocatalytic properties, higher thermal stability, storage stability and better reusability compared to free enzyme. The activity of the immobilized enzyme in 20% IL was increased compared to IL or citric acid - sodium hydrogen phosphate buffer.

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