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Immobilization of cellulase on modified mesoporous silica shows improved thermal stability and reusability

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Cellulase was covalently immobilized using modified mesoporous silica as carrier, and the characters of immobilized cellulase were investigated including the optimum pH, the optimum temperature, thermal stability, reusability, storage stability and enzyme kinetics. As compared to the free enzyme, the optimum pH of the immobilized cellulase showed no changes, the optimum temperature showed a slight increase to 60°C. The immobilized enzymes demonstrated enhanced thermal stability, while the free enzymes lost almost 40% initial activity, the immobilized forms lost only 9% initial activity within a period of 120 min at 60°C. The immobilized cellulase showed excellent reusability. After 11 cycles, the cellulase immobilized on modified mesoporous silica still retained 89% of its initial activity. At optimized conditions, Km and Vmax values for the immobilized enzyme were slightly increased when compared with those of free enzyme. The characters of cellulase immobilized on modified mesoporous silica suggest that the immobilized enzyme has high potential for application in the industrial degradation of cellulose.

Key words: Renewable energy, cellulose, immobilization, mesoporous silica, cellulose.

INTRODUCTION

The growing demand for energy and diminishing fossil fuel reserves have stimulated tremendous interest in finding alternative renewable energy sources (Huang et al., 2011). Over the past few decades, a large number of research efforts have focused on the cellulose, because cellulose is the most abundant renewable biomass in the world, and it can be converted into biofuels, such as ethanol after cellulose is degraded to sugar (Huang et al., 2011). Many methods have been used for the degradation of cellulosic materials to sugars, such as acidolysis

(Li et al., 2010), alkaline hydrolysis (Carrillo et al., 2005) and enzymatic hydrolysis (Park et al., 2002). Among these methods, the enzymatic hydrolysis has received great attention due to the fact that the enzymatic process has the potential to convert the lignocellulosic materials to ethanol with a high yield and low production cost (Wingren et al., 2003; Galbe and Zacchi, 2002; Kannan and Jasra, 2011). However, the cost of ethanol production from lignocellulosic materials is relatively high based on current enzymatic hydrolysis technologies, and the main

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challenges are the low yield and high cost of the hydrolysis process (Sun and Cheng, 2002). The difficulties above can be partly overcome by the immobilization of cellulase due to the fact that cellulase can be reused while retaining its specificity and stability (Kannan and Jasra, 2011). The capacity to retain or recover enzymes also allows biocatalyst separation from product, thereby permitting continuous processes, and prevents carry-through of protein or activity to subsequent process steps (Polizzi et al., 2007; Brady and Jordaan, 2009).

However, an often made observation for immobilization of enzymes is that in comparison to the base preparation, they have reduced specific activity but have acquired extra shelf-lifetime and stability under the operational conditions (Roessl et al., 2010). Many factors can affect the activity of the immobilized enzyme, such as the immobilized procedure, carrier and so on. Different carriers can provide different external backbone for the enzyme molecules, which is expected to improve the stability of enzyme molecules at reaction conditions (Temoçin and Yiğitoğlu, 2009). There are many different materials which have been proposed to immobilize enzymes, including Ca-alginate, agar, K-Carrageenin, polyacrylamide, nylon, cellulose, polysulfone, alumina, silica, porous glass, ceramics, diatomaceous earth, clay and bentonite (Hu et al., 2007). Among these materials, mesoporous silica is the most commonly used supporter for the immobilized enzyme because of its advantages (Hu et al., 2007; Wang et al., 2009). Siso et al. (1990) have reported that after α -amylase was immobilized on porous silica, external diffusional resistances to mass transfer were minimized when the immobilized enzyme was operated in a packed bed reactor.

In this study, the cellulase is immobilized covalently on mesoporous silica, and the optimum temperature, the optimum pH, the reusability, the thermal stability and the storage stability of immobilized cellulase are determined. In addition, the studies have demonstrated that the reusability and thermal stability of immobilized cellulase are significantly improved as compared to the enzyme immobilized onto chitosan. The mesoporous silica may be used as an excellent support for cellulase immobilization.

MATERIALS AND METHODS

Chemicals

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. Mesoporous material SBA-7 was prepared by our laboratory. All other chemicals were of purest grade available commercially.

Immobilization of cellulase

SBA-7 was amino functionalized using the method as described by Gao et al. (2006). The modified SBA-7 was labeled SBA-7-A. The SBA-7-A (0.25 g) was added into 5 ml of solution containing 0.015 mg/mL of glutaraldehyde and vigorously stirred for 1 h at room tem-

perature. It was centrifuged, washed several times with water till all excess of glutaraldehyde was removed. The glutarated SBA-7-A was mixed with 2.5 mL cellulase solution and shaken for 4 h at room temperature. After centrifuging and washed with deionized water, the immobilized cellulase was obtained.

Activity measurement

The activity of immobilized cellulase was estimated according to the method described by Su et al. (2012).

Optimum temperature

The optimum temperature of immobilized cellulase was tested at pH 4.0 using 1% (w/v) CMC (dissolved in 0.1 mol/L citric acid - sodium hydrogen phosphate buffer) as substrate. After 20 min, glucose product in solution was measured at 40, 50, 60, 70 and 80°C. The activity of the immobilized enzyme was determined using the method as described by Shoemaker and Brown (1978).

Optimum pH

The immobilized cellulase was pre-equilibrated by washing with 0.1 mol/L citric acid- sodium hydrogen phosphate buffer of corresponding test pH for several times. Substrate solutions of 1% (w/v) CMC in 0.1 mol/L citric acid- sodium hydrogen phosphate buffer at pH values ranging from 3.0 to 7.0 were prepared. The immobilized cellulases were added into corresponding CMC solutions, and glucose productions were determined after 20 min at 60°C.

Thermal stability

The immobilized cellulase was incubated at pH 4.0 and 60°C, and 100 mg immobilized enzyme was taken out at 20 min intervals. The activity was determined using the method described above.

Reusability

One hundred milligram immobilized cellulase was put into 3 mL of 1% (w/v) CMC solution (dissolved in 0.1 mol/L citric acid- sodium hydrogen phosphate buffer, pH = 4.0) and incubated at 60°C for 25 min all together in a water bath. At the end of the reaction, the immobilized cellulase was removed from the solution, washed with 0.1 mol/L citric acid- sodium hydrogen phosphate buffer and put again into other 3 mL of 1% (w/v) of CMC solution to start a new reaction.

Storage stability

The immobilized cellulase was stored at 4°C in 0.1 mol/L citric acid- sodium hydrogen phosphate buffer (pH = 4.0). The activity was tested at different time using the method as described above.

Enzyme kinetics of immobilized cellulase

These values were determined at the optimum condition, and carboxymethylcellulose with different concentrations (1%, 1/300, 1/500 and 1/700 (w/v), dissolved in 0.1 mol/L citric acid- sodium hydrogen phosphate buffer, pH = 4.0) was used as a substrate. 100 mg immobilized cellulase was added into 9 mL sodium carboxymethylcellulose solutions above, and glucose production in different concentration carboxymethylcellulose solutions after 4, 8, 12, 16

Figure 1. Effect of temperature on immobilized cellulase at pH 4.0 using 1% (w/v) CMC as the substrate. Data are means $\pm$ RSE (relative standard error), $n = 3$, with 1% (w/v) CMC as the substrate.

Figure 2. Effect of pH on immobilized cellulase at 60°C using 1% (w/v) CMC as the substrate. Data are means $\pm$ RSE (relative standard error), $n = 3$.

and 20 min at 60°C was determined. K_m and V_{max} values were calculated from Lineweaver-Burke plots.

RESULT AND DISCUSSION

Effect of temperature and pH on immobilized cellulase

The optimum temperature for cellulose hydrolysis is shown in Figure 1 for free and immobilized cellulases. The immobilized cellulases exhibited maximal activity at 60°C, higher than that of free cellulase (40°C), which indicated that the support material retained the tertiary structure of the enzyme at higher temperature. Other authors have also obtained similar observations (Andriani et al., 2012). The effect of pH on the activity of immobilized and free cellulases are given in Figure 2. The maximum activity for

the immobilized and free cellulases was observed at pH 4. However, when compared with the free cellulase, the relative activity of the immobilized cellulase is higher for the pH value ranging from 4 to 7. The stability of the immobilized enzymes at higher pH values can be due to the residual charges on the solid matrix and the nature of the bound enzyme (Jaiswal and Prakash, 2011). Immobilized cellulase stable at higher pH are more suitable for industrial applications.

Thermal stability

Thermal stability of immobilized enzyme is a very important character for its industrial applications. The thermal stability of immobilized and free cellulases was investigated at 60°C using CMC as the substrate. The results (Figure 3) showed a significant increase in thermal stabi-

Figure 3. Thermostability of free and immobilized cellulase at pH 4.0 and 60°C using 1% (w/v) CMC as the substrate. Data are means $\pm$ RSE (relative standard error), $n = 3$.

Figure 4. Reusability of immobilized cellulase at pH 4.0 and 60°C using 1% (w/v) CMC as the substrate. Data are means $\pm$ RSE (relative standard error), $n = 3$.

lity as compared to the free enzyme. After 120 min, the immobilized cellulase preserved 91.9% initial activity. At the same time, the free cellulase maintained 60.1% initial activity for 120 min. Immobilized cellulase displayed excellent thermal stability. The increase in thermal stability of the immobilized enzyme could be attributed to multi-point attachment of the enzyme to the carrier preventing them against the autolysis (Esawy and Combet-Blanc, 2006). Ruchi and Kharel (2005) have also obtained similar observations.

Reusability

The activity of immobilized cellulase was evaluated using a repeated batch process in order to observe the reuse of the immobilized enzyme (Figure 4). The results showed

that the immobilized cellulase could be used for 7 consecutive cycles without obvious loss of activity. After 11 cycles, it still retained 88% of its initial activity. The immobilized enzyme may have been protected from thermal denaturation by the modified mesoporous silica. As compared to our previous results where the cellulase was immobilized on chitosan (Su et al., 2012), the immobilization of cellulase on modified mesoporous silica has improved reusability of the enzyme. These indicate that the modified mesoporous silica is an excellent material which can be used for cellulase immobilization.

Storage stability

Storage stability of immobilized and free cellulases was studied in 0.1 mol/L citric acid- sodium hydrogen phos-

Figure 5. Storage stability of immobilized and free cellulase. Data are means $\pm$ RSE (relative standard error), $n = 3$.

phate buffer solution of optimum pH and the results are shown in Figure 5. It can be seen that the immobilized cellulase showed a gradual decrease in activity and lost 14% initial activity only after 4 weeks while the free cellulase lost 23% activity in the same time. The immobilized cellulase showed favorable storage stability. The improved storage stability of immobilized enzymes can be attributed to a reduction in the rate of denaturation of the enzyme as a result of fixation on the carrier (Sanjay and Sugunan, 2008; Bayramoglu et al., 2003; Cirpan et al., 2003).

Enzyme kinetics of free and immobilized xylanase

For the two forms of cellulase, linearity of the Lineweaver–Burk plot showed Michaelis-Menten kinetics. The K_m value for the immobilized and free cellulase was 4.03 and 1.84 mg/L, respectively, and the V_m value for the immobilized and free cellulase was 0.0278 and 0.0036 mg/ml/min, respectively. The K_m of immobilized is higher than the free cellulase, suggesting a reduced affinity for the substrate. Tu et al. (2006) had also reported that the K_m of β -glucosidase on Eupergit C was 10 times higher than the free β -glucosidase. The increase in K_m of immobilized enzyme might be due to inaccessibility of the substrate to the enzyme due to limitation in diffusion of substrate into the pores channel of modified mesoporous silica carrier (Andriani et al., 2012). Interestingly, immobilization resulted in about an 8-fold increase in V_m . The increase in the V_m , compared with that of the native enzyme, may be attributed to increased stability of the enzyme after immobilization (Andriani et al., 2012).

Conclusions

Cellulase was successfully immobilized on modified meso-

porous silica carrier using covalent binding. The immobilized cellulase showed higher temperature optima, increased tolerance to higher pH range, and better thermal stability as compared to free enzyme. The modified mesoporous silica carrier employed in this study allows the immobilized enzyme to be effectively reused at least ten times. In conclusion, as compared to other carriers, modified mesoporous silica is a good supporting material for the immobilization of cellulase.

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