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Research Article

Applicability of Coimmobilized Cellulase and Xylanase to Lignocellulose Hydrolysis

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Abstract

Immobilization is a technology for increasing the stability and realizing the reuse of enzymes. In order to clarify the applicability of cellulase and xylanase immobilized simultaneously on the reversibly soluble polymer Eudragit L-100 to lignocellulose hydrolysis, the hydrolysis of pretreated rice straw with the coimmobilized enzymes was analyzed kinetically, and the reusability of the coimmobilized enzymes was investigated. The coimmobilized enzymes were more stable against the changes in pH and temperature than the free enzymes. A holocellulose conversion rate of 61.4 % was obtained in the hydrolysis of pretreated rice straw with both the coimmobilized enzymes and the free enzymes. The maximum reaction rate decreased from 135.1 mmol/(L·min) to 84.7 mmol/(L·min), while the Michaelis–Menten constant increased from 30.6 g/L to 51.7 g/L due to coimmobilization. 54 % of cellulase activity and 46 % of xylanase activity remained after the coimmobilized enzymes were recycled for three times. It is suggested that the coimmobilized enzymes are applicable to lignocellulose hydrolysis, which may lead to an improvement of the efficiency of enzymatic lignocellulose hydrolysis.

Keywords: Cellulase; Xylanase; Immobilization; Biomass; Enzymatic Hydrolysis

Introduction

Enzymatic hydrolysis is an essential operation in the bio-refining of lignocellulosic materials, by which lignocellulosic materials are converted to fuel ethanol and other organic chemicals. The economical efficiency of enzymatic lignocellulose hydrolysis depends on both the effective use of enzymes, and the effective conversion of cellulose and hemicellulose.

In the enzymatic lignocellulose hydrolysis, an enzyme complex basically containing cellulase and xylanase is used. Cellulase and xylanase are usually produced by microorganisms such as *Trichoderma* or *Aspergillus* [1]. However, the production cost of the enzymes is still high, which limits their industrial applications [2]. Immobilization is a technology for increasing the stability and realizing the reuse of enzymes [3]. In our previous work [4], cellulase and xylanase from *Trichoderma reesei* were immobilized simultaneously on Eudragit L-100, a reversibly soluble polymer, depending on the pH of the medium. An activity recovery of 75 % and 59 % was obtained for the cellulase and the xylanase, respectively, under the optimal condition of a polymer concentration at 2 % and a polymer precipitation pH at 4.0. Zymoproteins were connected to the polymer by electrostatic attraction or covalent coupling.

The present research work aimed to clarify the applicability of the coimmobilized enzymes to lignocellulose hydrolysis. First, the pH and thermal stabilities of the coimmobilized enzymes were compared with the free enzymes. Then, the hydrolysis of pretreated rice straw with the coimmobilized enzymes was analyzed kinetically, and the reusability of the coimmobilized enzymes was investigated.

Materials and Methods

Coimmobilization of Cellulase and Xylanase

A commercial enzyme complex (NS50013, Novozymes Investment Co., Ltd.), produced from *Trichoderma reesei,* was used as the test enzymes. One milliliter of the enzyme complex had a cellulase activity of 100 FPU, and a xylanase activity of 800 U under the optimal condition of a temperature at 50°C and a pH at 4.8.

Eudragit L-100 (Evonik Degussa Investment Co., Ltd.) was used as the carrier. A 2 % w/v carrier solution with pH 4.8 was prepared, and then cellulase and xylanase were immobilized simultaneously on the carrier by adding 0.5 mL of the commercial enzyme complex to 50 mL of the carrier solution at room temperature. Under this optimal condition, the activity recovery of cellulase and xylanase was 75 % and 59 %, respectively [4].

Investigation of the pH and Thermal Stabilities of Enzymes

The pH stability of cellulase and xylanase was investigated by incubating the free and coimmobilized enzymes in a solution with different pH (3.0-7.0) at 50°C for 2 h. Upon the completion of incubation period, the remaining activity was measured and expressed as the percentage of original activity before incubation.

The thermal stability of cellulase and xylanase was investigated by incubating the free and coimmobilized enzymes in a solution with pH 4.8 at temperatures ranging from 20 to 70°C for 2 h and measuring the remaining activity.

Enzymatic Hydrolysis of Pretreated Rice Straw

Rice straw was used as the lignocellulosic material. It was obtained from the suburb of Nanjing city, sun-dried to an equilibrium moisture content of 10 %, and then ground to pass through a 40-mesh sieve by a Wiley Laboratory Mill (FZ102, Beijing Yongguangming Medical Instrument Co., China).

The rice straw was treated by wet-milling prior to enzymatic hydrolysis in order to enhance the conversion of cellulose and hemicellulose to reducing-sugars [5]. 40 g of rice straw was wet-milled at room temperature for 1h, using a planet- type ball mill (XQM-4 L, Nanjing Kexi Institute of Experimental Instruments Co., China) with a rotational speed of 500 rpm and a solid concentration of 10 % w/v. Sodium hydroxide (NaOH) solution with a concentration of 1 % was used as the wet-milling medium. After the wet-milling, the rice straw suspension was centrifuged at 7,000 rpm for 30 min. The solid fraction was collected and stored at 4°C until being used for enzymatic hydrolysis. It consisted of 11.8 % water extractives, 46.8 % cellulose, 17.3 % hemicellulose, 10.4 % lignin and 13.8 % ash. The chemical composition of the pretreated rice straw was analyzed with Van Soest's method [6,7], using a fiber analysis system (Fibertec System M6, FOSS, Denmark). By the pretreatment, about half of the lignin in rice straw was removed, while the crystalline structure of rice straw was destroyed.

The pretreated rice straw was hydrolyzed using the free and coimmobilized enzymes. A pretreated rice straw sample containing 4-g dry matter was mixed with 100-mL acetate buffer (0.01 M, pH 4.8) in a 250-mL Erlenmeyer flask. The cellulase loading was controlled at 15 FPU/g-substrate. The enzymatic hydrolysis was performed at 50°C on a thermostated water bath. During the enzymatic hydrolysis, the hydrolysate was sampled and centrifuged at 7,000 rpm for 15 min. Reducing-sugars contained in the supernatant were analyzed with the 3,5-dinitrosalicylic acid (DNS) method [8,9] and expressed by dextrose equivalent.

Kinetic Analysis of Enzymatic Hydrolysis

Reducing-sugar production was determined after 1 h of the enzymatic hydrolysis of pretreated rice straw under the condition of a cellulase loading at 38 FPU, and a substrate loading at 0.20%, 0.25%, 0.33%, 0.50%, 1.00% and 4.00%, respectively. The Michaelis–Menten constant (K_m, g/L) and the maximum reaction rate (r_{max}, mmol/(L·min)) were calculated from the Michaelis–Menten equation:

$$\frac{1}{r} = \frac{1}{r_{\max}} + \frac{K_m}{r_{\max}} \frac{1}{c} \quad (1)$$

where, r is the reaction rate (mmol/(L·min)); c is the substrate concentration (g/L).

Measurement of Enzyme Activity

In the investigation of the stability and reusability of coimmobilized enzymes, cellulase and xylanase activities were measured for the free and coimmobilized enzymes, using the methods reported previously [4]. A unit of FPU for cellulase activity was defined as the amount of cellulase that produces 1 μmol of reducing sugar (as glucose) from 50-mg filter paper in 1 min under the optimal condition of 50°C and pH 4.8. A unit of U for xylanase activity was defined as the amount of xylanase that produces 1 μmol of reducing sugar (as xylose) from 1 %

birchwood xylan in 1 min under the optimal condition of 50°C and pH 4.8.

Results and Discussion

pH and Thermal Stabilities of Coimmobilized Enzymes

Figure 1 shows the remaining activity of free and coimmobilized enzymes after incubation for 2 h at different pH values (temperature: 50°C). In the pH range of 4.0 to 5.0, the activities of free and coimmobilized enzymes did not change. When the pH was lower than 4.0 or higher than 5.0, the activities of both free and coimmobilized enzymes decreased, but the activity decreases in the coimmobilized enzymes were less than those in the free enzymes. At pH 7.0, for example, the free cellulase and xylanase had a remaining activity of 85.4 % and 86.4 %, while the coimmobilized cellulase and xylanase had a remaining activity of 89.1 % and 88.7 %, respectively.

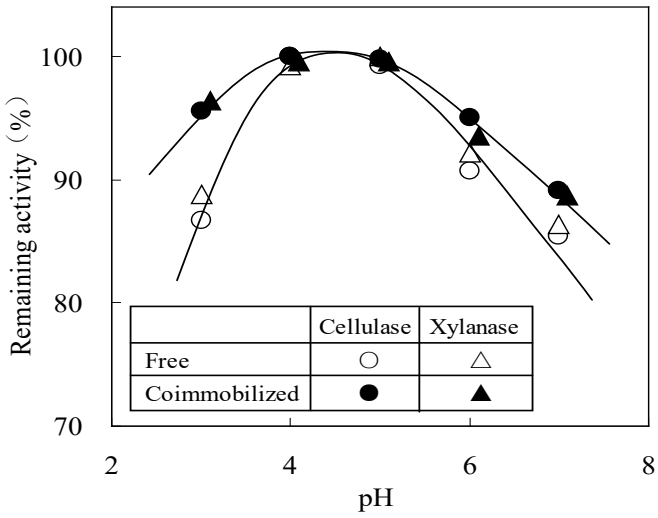

Figure 1. Remaining activity of free and coimmobilized enzymes after incubation for 2 h at different pH values (temperature: 50°C).

Figure 2 shows the remaining activity of free and coimmobilized enzymes after incubation for 2 h at different temperatures (pH: 4.8). At a temperature lower than 50°C, the activities of free and coimmobilized enzymes did not change. When the temperature was higher than 50°C, the activities of both free and coimmobilized enzymes decreased with increasing temperature. The activity decreases in the coimmobilized enzymes were less than those in the free enzymes. At 70°C, for example, the free cellulase and xylanase had a remaining activity of 32.3 % and 62.3 %, while the coimmobilized cellulase and xylanase had a remaining activity of 45.0 % and 69.7 %, respectively.

It was indicated that the coimmobilized enzymes were more stable against the changes in pH and temperature than the free enzymes. The stability improvement of enzymes will be beneficial to the operations of enzymatic hydrolysis and enzyme recovery [10].

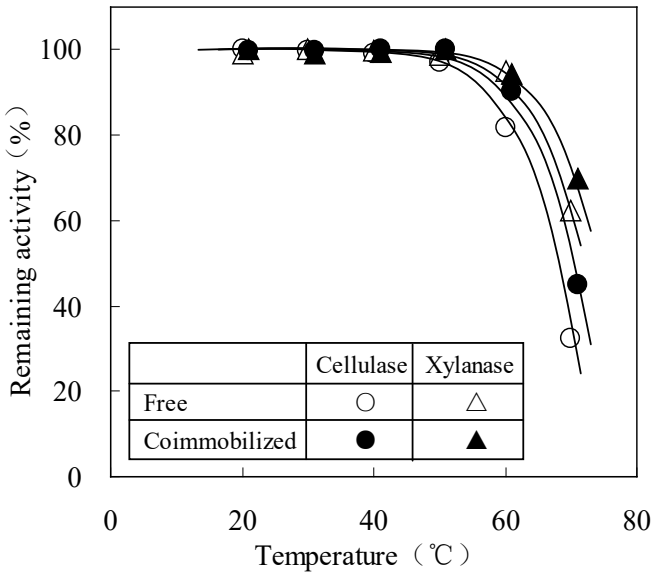

Figure 2. Remaining activity of free and coimmobilized enzymes after incubation for 2 h at different temperatures (pH: 4.8).

Reaction Rate in the Hydrolysis of Pretreated Rice Straw with Coimmobilized Enzymes

Figure 3 shows the changes of reducing-sugar concentration in hydrolysate during the hydrolysis of pretreated rice straw with free and coimmobilized enzymes. The reducing-sugar concentration reached 17.5 g/L in 60 h, and then became constant. The reducing sugars mainly consisted of glucose, xylose and arabinose, which were converted from cellulose and hemicellulose contained in the pretreated rice straw [4]. By defining holocellulose as the sum of cellulose and hemicellulose, it could be found that conversion rate of holocellulose in the pretreated rice straw was as high as 61.4 %.

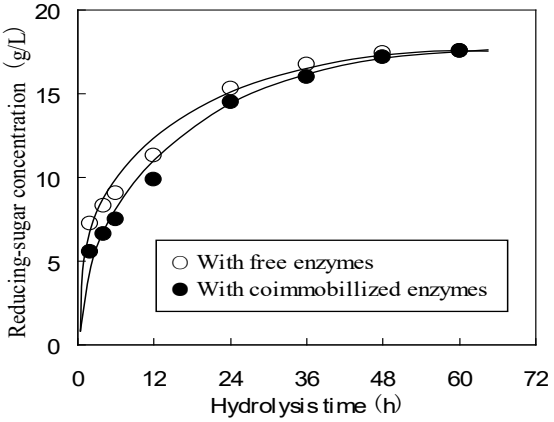

Figure 3. Changes of reducing-sugar concentration during the hydrolysis of pretreated rice straw with free and coimmobilized enzymes.

The Lineweaver-Burk plot for the enzymatic hydrolysis of pretreated rice straw is shown in Figure 4. The relationship of 1/r and 1/c could be expressed by the regression equations as follows:

with the free enzymes,

$$1/r=0.2262/c + 0.0074 \quad (R^2=0.9882) \qquad (2)$$

with the immobilized enzymes,

$$1/r=0.6105/c + 0.0118 \quad (R^2=0.9959) \qquad (3)$$

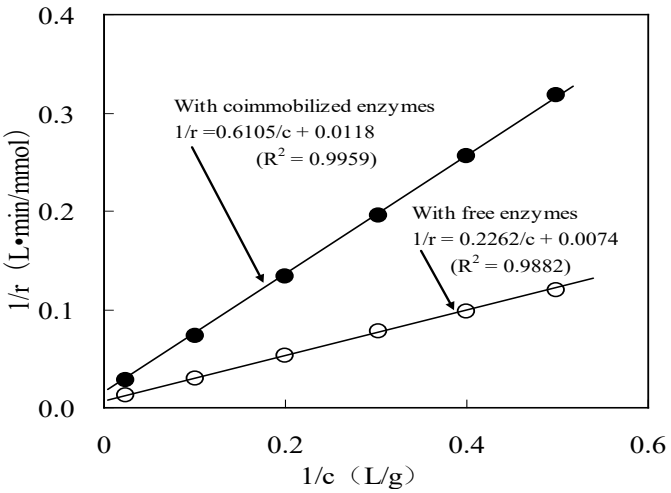

Figure 4. Lineweaver-Burk plot for the enzymatic hydrolysis of pretreated rice straw.

Comparing Equations (2) and (3) with Equation (1), it could be found that the hydrolysis of pretreated rice straw with the free enzymes had a r_{max} of 135.1 mmol/(L·min) and a K_m of 30.6 g/L, while that with coimmobilized enzymes had a r_{max} of 84.7 mmol/(L·min) and a K_m of 51.7 g/L. The reasons for the decrease in r_{max} and the increase in K_m might be that the coimmobilized enzymes had a low xylanase activity, and it was difficult for the coimmobilized enzymes to contact with the substrate, in comparison with the free enzymes.

Reusability of Coimmobilized Enzymes

After each run of the hydrolysis of pretreated rice straw, the coimmobilized enzymes were recycled as shown in Figure 5. The undegraded substrate in the hydrolysate was separated by centrifugation at 7,000 rpm for 15 min. The pH of supernatant was adjusted to 4.0 with 10 % v/v acetic acid for the precipitation of coimmobilized enzymes. Then the precipitate (coimmobilized enzymes) was recovered by centrifugation at 5,000 rpm for 15 min. After being washed three times with acetate buffer (0.01 M, pH 4.8), the precipitate was dissolved by increasing pH with a NaOH solution, and the remaining activities of cellulase and xylanase were measured. The recovered enzymes were used for the hydrolysis of pretreated rice straw under the same condition.

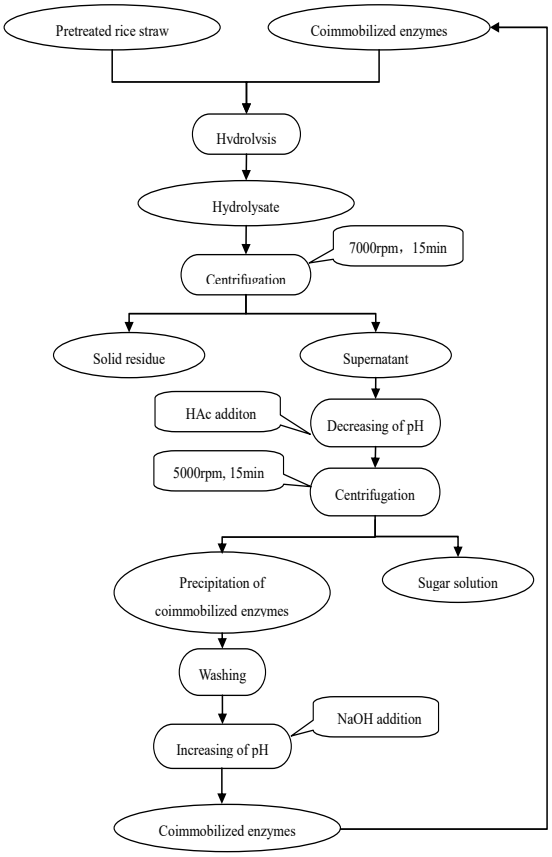

Figure 5. Recycling process of coimmobilized enzymes.

The reusability of coimmobilized enzymes is shown in Figure 6. The activity of fresh coimmobilized enzymes was defined as 100 %, and the reusability of coimmobilized enzymes was expressed by its remaining activity after being recycled. After being recycled for 3 times, the coimmobilized cellulase and xylanase had a remaining activity of 54 % and 46 %, respectively. The coimmobilized enzymes could be recycled, although the remaining activities decreased. It was also reported that the immobilized cellulase on polymer Eudragit L-100 had a remaining productivity of 50% after 5 recycles in the hydrolysis of microcrystalline cellulose (MCC) and rice straw pretreated by boiling in 300 mL of NaOH solution (2% w/v) under normal pressure for 60 min [11]. The decrease in remaining activity possibly resulted from the denature of zymoprotains during the hydrolysis reaction and the loss of zymoprotains during the enzyme recovery. In the coimmobilized enzymes, the zymoproteins was connected to the carrier by electrostatic attraction or covalent coupling [4]. Since a covalent coupling was usually stronger than an electrostatic attraction, the reusability of coimmobilized enzymes could be improved by increasing the covalent coupling connection between zymoprotains and carrier.

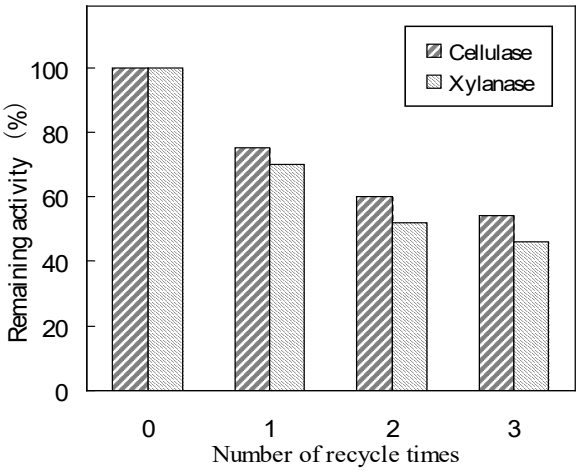

Figure 6. Reusability of coimmobilized enzymes.

In addition to the reversibly soluble polymer used in the present work, water-insoluble compounds such as chitin, chitosan, nylon, polyvinyl alcohol (PVA) membranes and polyacrylonitrile (PAN) membranes have been used as the carriers of immobilized cellulase. In the hydrolysis of microalgal cell walls with immobilized cellulase on a PAN membrane, the immobilized cellulase was recovered on the holder of a reactor, and reducing-sugar yield at the fifth cycle was about 55 % of its original performance [12]. In this case, reaction rate was also low because of great resistance to the contact between immobilized enzymes and substrates. Further studies should be conducted to improve the reducing-sugar yield and hydrolysis rate, as well as the stability and reusability of immobilized enzymes, especially for their industrial applications.

Conclusions

Cellulase and xylanase immobilized simultaneously on the reversibly soluble polymer Eudragit L-100 were applicable to lignocellulose hydrolysis, which might lead to an improvement of the efficiency of enzymatic lignocellulose hydrolysis. The basic information and technical data provided in the present research work were necessary for the development of bioethanol production and biomass refinery industries.

Acknowledgements

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