Biocatalysis and Biotransformation, September–December 2010; 28(5–6): 313–319

ORIGINAL ARTICLE

Preparation and properties of an immobilized cellulase on the reversibly soluble matrix Eudragit L-100

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Abstract

To prepare a smart biocatalyst, cellulase was immobilized on the reversibly soluble matrix Eudragit L-100 by non-covalent and covalent methods. Covalent immobilization using carbodiimide coupling exhibited superior enzyme loading and reusability compared with non-covalent immobilization, and the covalent loading was increased by almost 20% through the addition of *N*-hydroxysuccinimide. The temperature optimum of the cellulase was not improved apparently by immobilization but the pH optimum increased from 4.75 to 5.25. Immobilized cellulase was more active than free cellulase above pH 5.0. Immobilized cellulase was more stable than free cellulase during storage at 4°C, room temperature and 50°C. K_m values of immobilized and free cellulase were 85.55 and 73.84 g L^{-1}, respectively. About 50% productivity was retained after five cycles for hydrolysis of steam-exploded straw.

Keywords: *Immobilized cellulase, Eudragit L-100, N-hydroxysuccinimide, carbodiimide coupling, smart biocatalysis*

Introduction

Cellulosic ethanol is an important future biofuel with the potential to solve the global energy crisis and environmental pollution (Gray 2007; Jørgensen et al. 2007). Excluding the syngas platform, cellulosic ethanol is mainly produced from carbohydrates in three major consecutive steps: pretreatment, saccharification and fermentation (Binod et al. 2010; Talebnia et al. 2010). In order to obtain high-efficiency saccharification, cellulase is always involved in the process (Drissen et al. 2009; Alvira et al. 2010). Cellulase needs to be utilized efficiently in industrial applications because of its high production cost (Zhang et al. 2006; Zhuang et al. 2007). Immobilization could help in this respect, by improving the operational stability of the enzyme and providing the possibility of enzyme reuse (Mateo et al. 2007; Sheldon 2007; Wang et al. 2009). Conventional immobilization of cellulase on insoluble or soluble polymers could not simultaneously provide good contact (during the reaction) and simple recovery (after the reaction). To address this problem matrices of soluble–insoluble (S-IS) polymers have been proposed (Roy & Gupta 2003; Roy et al. 2004).

Eudragit L-100 (a copolymer of methacrylic acid and methyl methacrylate) has been applied extensively as an S-IS polymer to immobilize many enzymes due to its prominent biocompatibility and stability (Taniguchi et al. 1989; Cong et al. 1995; Sardar et al. 2000; Dourado et al. 2002; Roy et al. 2003; Gaur et al. 2005; Rajoka et al. 2007; Smith et al. 2008). The covalent (carbodiimide coupling) immobilization of cellulase on Eudragit L-100 was attempted previously (Taniguchi et al. 1989; Dourado et al. 2002). However, in the presence of acetate, the carboxyl group of acetate was also activated by the carbodiimide agent, inhibiting the coupling between Eudragit and cellulase. The resulting Eudragit-bound cellulase was shown to be non-covalently bound, resulting from simple adsorption (Dourado et al. 2002). However, cellulase was not strongly adsorbed on Eudragit and adsorption was reduced by the presence of xylanase in the crude cellulase (Sardar et al. 2000; Roy & Gupta 2006). Therefore, ideally the immobilization of cellulose should be carried out by a covalent procedure.

In the present work cellulase was immobilized on Eudragit L-100 by carbodiimide coupling in the

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(Received 11 March 2010; revised 19 May 2010; accepted 14 August 2010)

ISSN 1024-2422 print/ISSN 1029-2446 online © 2010 Informa UK, Ltd.
DOI: 10.3109/10242422.2010.516391

absence of acetate and with added *N*-hydroxysuccinimide (NHS) as an enhancer to improve the coupling. The properties of the immobilized cellulase are described herein.

Materials and methods

Materials

Eudragit L-100 was purchased from Degussa Ltd (Essen, Germany), filter paper was provided by Whatman Ltd (Maidstone, UK) and crude cellulase powder was provided by Shanghai Bio Life Science & Technology Co., Ltd (Shanghai, China). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and NHS were obtained from Sigma-Aldrich Co., Ltd (St. Louis, MO, USA).

Enzyme assay and protein estimation

Filter paper activity (FPA) of cellulase was measured according to the Commission on Biotechnology of the International Union of Pure and Applied Chemistry (Ghose 1987). A rolled filter paper strip (1 cm×6 cm, about 50 mg) and 1 mL acetate buffer (0.2 M, pH 5.0) were mixed into each test-tube (13 mm×100 mm). Then 0.5 mL of a diluted enzyme solution series was added into the tube and the mixture was kept at 50°C for 60 min. When 2.0 mg reducing sugars (4% conversion rate) were produced, FPA was calculated by:

$$\text{FPA (U mL}^{-1}) = \frac{0.37}{\text{enzyme dilution rate}} \quad (1)$$

For carboxymethyl cellulase (CMCase) activity, 0.5 mL diluted enzyme solution (protein loading 0.01 g) and 1 mL carboxymethyl cellulose (CMC) solution were mixed at 50°C. Because the initial CMCase V_0 (t=0) is indefinable, the CMCase measured after 10 min reaction was taken as the estimation of V_0. CMCase was calculated by:

$$\text{CMCase} = \frac{\text{reducing sugars amount (μmol)}}{\text{time (min)} \times \text{volume (mL)}} \quad (2)$$

Reducing sugars were measured by the DNS (3,5-dinitrosalicylic acid) method using glucose as standard (Ghose 1987). One unit of FPA and CMCase was defined as the amount of enzyme required for the formation of 1 μmol glucose equivalents per minute.

Protein content was estimated by the Bradford dye-binding method using bovine serum albumin as standard (Bradford 1976).

Preparation of immobilized cellulase on Eudragit L-100

One gram of Eudragit L-100 was dissolved in 40 mL double-distilled water by adding 3 M NaOH dropwise until the pH had increased to 11.0. After the polymer was completely dissolved, cellulase was immobilized on Eudragit L-100 according the following four methods.

- *Method A*: The pH value of the solution was adjusted to 6.5 by adding 3 M acetic acid and the total volume made up to 50 mL with double-distilled water. Then about 100 mg crude cellulase powder was added and the mixture stirred slowly at 20°C for 4 h. The pH of the mixture was then decreased to 3.6 by adding 2 M acetic acid and the mixture centrifuged (6800***g***, 4°C and 10 min). The recovered precipitate was washed three times with 0.02 M acetate buffer (pH 3.6) and then used as immobilized cellulase (Eudragit–cellulase).
- *Method B*: As method A, except that 0.30 g EDC was added to the Eudragit L-100 solution for 10 min at 20°C before adding cellulase.
- *Method C*: As method B, except that the pH value of the polymer solution (2% w/v) was lowered to 6.5 with 3 M HCl instead of acetic acid.
- *Method D*: As method C, except that 0.30 g NHS was also added to the mixture together with EDC.

Supernatant and washing liquids were collected, and their protein content estimated to determine the residual cellulase protein after the immobilization process. Cellulase protein loading was calculated as:

$$\text{Cellulase protein loding (\%)} = \frac{\text{cellulase protein added} - \text{cellulase protein in supernatant and washing liquid}}{\text{cellulase protein added}} \quad (3)$$

Immobilized cellulase FPA was measured as described above, and cellulase activity loading was calculated as:

$$\text{Cellulase activity loading (\%)} = \frac{\text{activity of immobilized cellulase}}{\text{activity of added (free) cellulase}} \times 100 \quad (4)$$

Response of solubility to pH

The solubility of Eudragit and Eudragit–cellulase prepared by method D (similarly hereinafter) was

assessed by adding glacial acetic acid to 2% w/v) suspensions to obtain a final pH range of 5.5 to 3.5. Use of concentrated acid avoided significant volume changes in the two solutions. Solubility was evaluated by measuring the absorbance at 470 nm and expressing this as a turbidity value at each pH.

Determination of optimal pH, temperature and storage stability

Free and immobilized cellulase FPA was assayed over a range of pH values (3.5–8.0) and temperatures (35–80°C). FPA was also assayed every week during storage at 4°C, room temperature (average temperature 29.9°C) and 50°C.

Determination of K_m and V_{max}

K_m and V_{max} values of free and immobilized cellulases were determined using a Lineweaver–Burk plot of measurements of initial CMCase obtained using CMC solutions of different concentrations (from 0.2 to 2%), prepared by 0.2 M acetate buffer.

Hydrolysis of insoluble cellulosic substrates

To check the reusability of immobilized cellulase, one FPU (unit of FPA) of immobilized cellulase was mixed with 0.1 g steam-exploded wheat straw (provided by the National Key Laboratory of Biochemical Engineering, Beijing, China) at 50°C with constant shaking at 120 rpm. The total reaction volume was 5.0 mL. At 60 min intervals, undegraded substrate was separated by centrifugation at 10 625***g*** for 5 min. To recover the enzyme, the pH of the supernatant was reduced to 3.6 with 2 M acetic acid, followed by centrifugation at 6800***g*** for 10 min. The precipitate was washed with 0.02 M acetate buffer (pH 3.6) and used as the recovered cellulase for the next hydrolysis reaction under the same conditions.

Results and discussion

Comparison of immobilization methods

Figure 1 shows the cellulase loading (activity and protein) of immobilized cellulase prepared by methods A to D. EDC, as a cross-linking agent, can activate carboxyl groups for amide bonding with primary amines (Figure 2). However, the cellulase loading on Eudragit L-100 using method B was almost the same as for method A. It has been reported that EDC is ineffective for linking cellulase to Eudragit in an acetate solution (Dourado et al. 2002) as the carboxyl groups of Eudragit L-100 and acetate link with EDC competitively, hindering the coupling of cellulase to Eudragit L-100.

In the absence of acetate, cellulase loading using method C was about 15% higher than that of methods A and B. In method D, the addition of NHS gave nearly 20% higher cellulase loading, presumably due to enhanced carbodiimide coupling arising from stabilization of an intermediate from the activation of Eudragit with EDC by NHS (Figure 2) (Thompson 2004).

Non-covalently bound cellulase protein is easily lost during repeated rounds of precipitation and dissolution, and cellulase protein loading was not more than 10% after five cycles (Figure 3). This is attributed to the weak interaction between Eudragit L-100 and cellulase (Sardar et al. 2000; Roy & Gupta 2006). In contrast, 80% of covalently bound cellulase was retained after eight cycles, reflecting the stability of the carbodiimide coupling.

The difference between the activity and protein loading of immobilized cellulase prepared by non-covalent methods A and B was small, compared with that of cellulase prepared by covalent methods C and D. The reactive conditions involved in covalent binding could chemically modify catalytically or structurally essential residues in the cellulase (Tischer & Wedekind 1999). Thus, the activity loading of the enzyme did not increase in proportion to the protein loading. In contrast, increases in activity loading for non-covalent immobilization were virtually identical to the increases in protein loading, as the cellulase protein structure is not changed by mild adsorption.

Effect of pH value on solubility of Eudragit and Eudragit–cellulase

The solubility profiles of Eudragit and Eudragit–cellulase were similar, with the absorbance values of the two solutions showing a distinct variation at pH 4.2 and 4.6, respectively (Figure 4). Similar effects

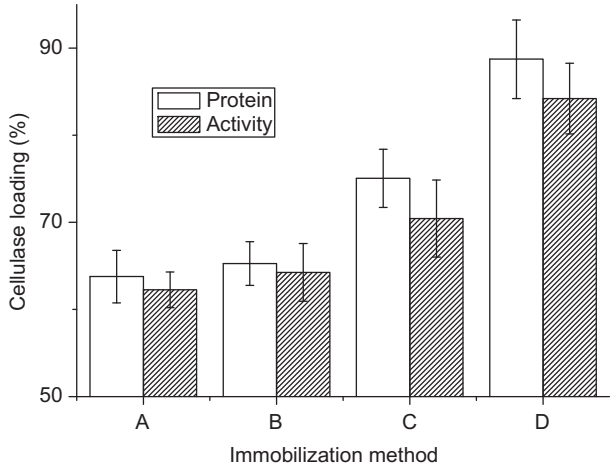

Figure 1. Immobilization of cellulase on Eudragit L-100 by four methods. The initially added cellulase protein and activity was taken as 100%. Data are means ± SD of triplicates.

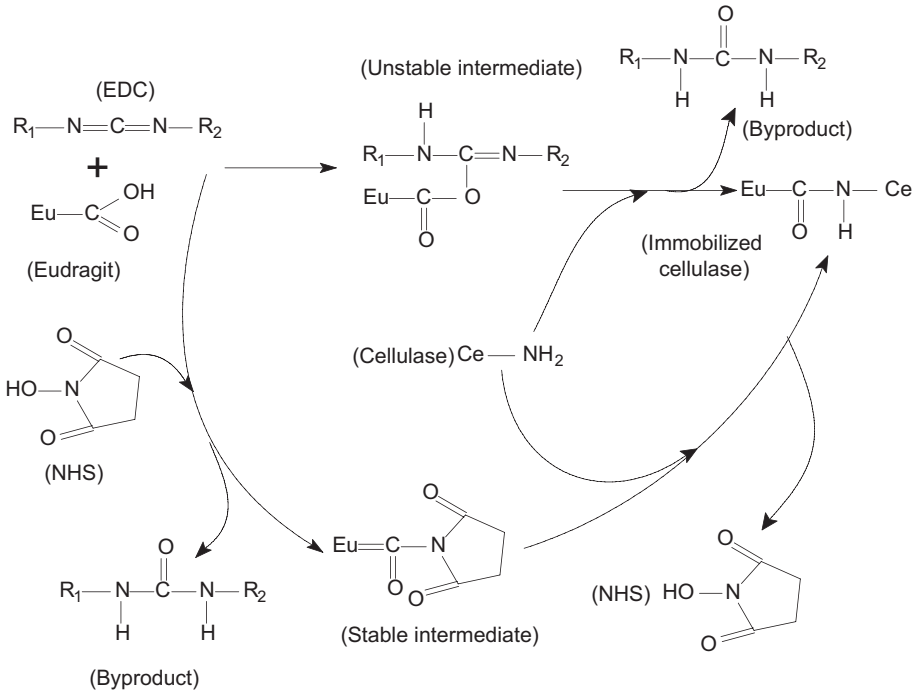

Figure 2. Carbodiimide coupling of cellulase on Eudragit L-100 in the absence (upper) and presence (lower) of NHS.

have been observed previously (Taniguchi et al. 1989; Dourado et al. 2002). Eudragit is reversibly soluble depending on the protonation state of the free carboxyl groups (Roy et al. 2004). Coupling cellulase to Eudragit should decrease the number of free carboxyl groups, causing the solubility profile of Eudragit–cellulase to shift towards more alkaline values compared with Eudragit (Figure 4). Eudragit–cellulase became completely soluble above pH 5.0 and insoluble below pH 4.0, and thus, via immobilization, was converted to a 'smart' biocatalyst that can be used as a homogeneous catalyst during the reaction and recovered after the reaction for reuse.

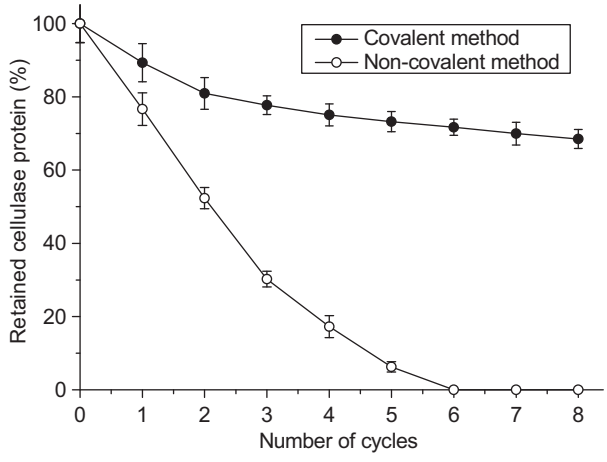

Figure 3. Reusability of immobilized cellulase prepared by non-covalent and covalent methods. The initial cellulase protein was taken as 100%. Data are means ± SD of triplicates.

Effect of pH, temperature and storage time on activity of free and immobilized cellulase

The activity profiles of free and immobilized cellulase at different temperatures were similar (Figure 5). Immobilized cellulase exhibited a slightly higher temperature optimum, in the range 55–60°C, compared with free cellulose with an optimal temperature of 55°C. However, the difference in activity of free and immobilized cellulase at their optimum temperatures was very small.

The optimal pH values for free and immobilized cellulase were 4.75 and 5.25, respectively. The

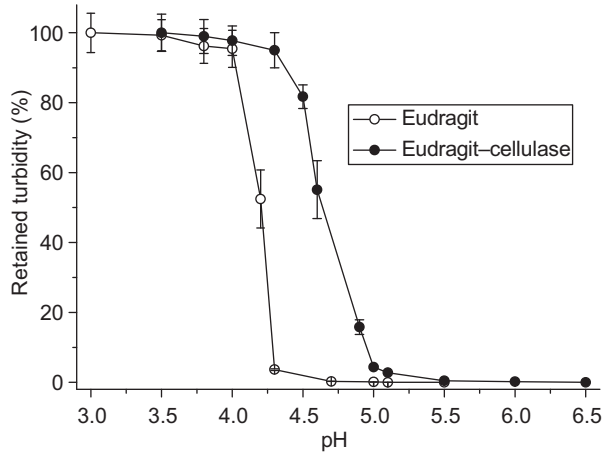

Figure 4. Solubility of Eudragit and Eudragit–cellulase at different pH values. The minimum and maximum absorbance values of the two solutions were 0% and 100%, respectively. Data are means ± SD of triplicates.

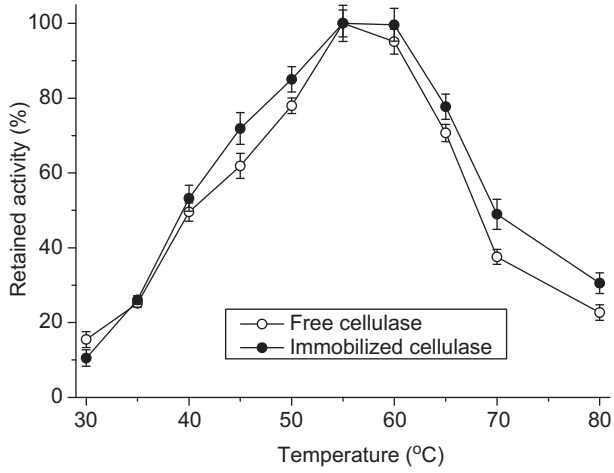

Figure 5. Retained activity of free and immobilized cellulase at different temperatures. The maximum activity of free and immobilized cellulase was taken as 100%. Data are means ± SD of triplicates.

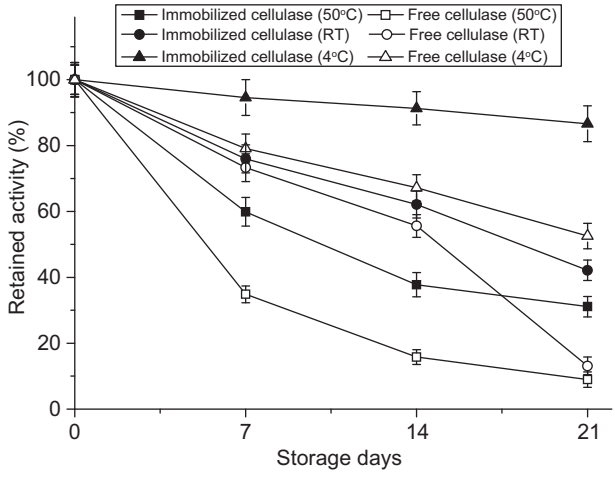

Figure 7. The storage stability of free and immobilized cellulase at 4°C, room temperature (RT) and 50°C. The initial activity of free and immobilized cellulase was taken as 100%. Data are means ± SD of triplicates.

optimum for immobilized cellulase was apparently shifted towards more alkaline values compared with free cellulase (Figure 6), which can be attributed to the anionic nature of the matrix. An anionic matrix can adsorb hydrogen ions from the aqueous solution, giving the diffusion layer around the immobilized enzyme a higher hydrogen ion concentration than the external solution. This micro-environmental effect is compensated for by using a higher pH value in the external solution. The activity of both free and immobilized cellulase decreased quickly at pH values below their optimum. However, immobilized cellulase was much more stable and lost less activity than free cellulase when the pH value was above 5.0.

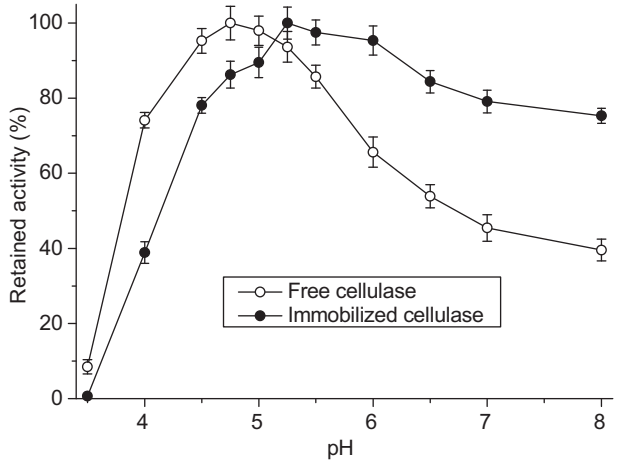

Figure 6. Retained activity of free and immobilized cellulase at different pH values. The maximum activity of free and immobilized cellulase was taken as 100%. Data are means ± SD of triplicates.

The storage stability of free and immobilized cellulase was evaluated over 3 weeks at 4°C, room temperature and 50°C (Figure 7). At 4°C, immobilized cellulase lost its activity more slowly than free cellulase. After 21 days, the remaining activity of free and immobilized cellulase was about 53% and 87% of the initial activity, respectively. At room temperature, the deactivation curves of free and immobilized cellulase were similar over the first 14 days, but then there was a more rapid loss of activity of free cellulase. By the 21st day the activity of free cellulase was 13% of its initial value, compared with 42% for immobilized cellulase. At 50°C, the activity of free cellulase was significantly reduced by the 7th day, but over the next 14 days the loss of activity was similar to that of immobilized cellulase. The retained activity of free and immobilized cellulase after 21 days at 50°C was 9% and 31%, respectively. Similar results have been obtained with other enzymes immobilized on reversibly soluble matrixes (Dourado et al. 2002; Edward et al. 2002; Ai et al. 2005).

In general, the operational stability of the enzyme was notably improved by the immobilization process. Immobilization of cellulase on Eudragit significantly enhanced the pH and storage stability, which increases its suitability for industrial application. Although the optimal temperature of cellulase was not increased through immobilization, the storage stability at elevated temperatures was markedly enhanced, possibly due to the prevention of autolysis (Ferreira et al. 2003; Sharma et al. 2003) and thermal denaturation (He et al. 2000). Immobilization can increase the rigidity of an enzyme, resulting in an increase in thermal stability (Silva et al. 2006).

Kinetics of free and immobilized cellulase

From a Lineweaver–Burk plot analysis of free and immobilized cellulase, the K_m value changed from 73.8 to 85.6 g L^{-1} through immobilization. A marginal increase in K_m value indicates that the covalent coupling of cellulase to Eudragit L-100 causes only a small steric hindrance in the interaction with soluble CMC, effectively retaining the efficiency of the soluble form. This is an advantage of using an S-IS matrix to immobilize cellulase. Similar results were obtained when S-IS matrices including Eudragit L-100 were used to immobilize other enzymes (Sardar et al. 2000; Ai et al. 2005; Gaur et al. 2005; Silva et al. 2006; Chi et al. 2008), whereas a large increase in K_m value is characteristic of an insoluble matrix (Yuan et al. 1999; Mao et al. 2006).

Hydrolysis of insoluble cellulose

Figure 8 shows the practical application in hydrolysis of the insoluble cellulosic substrate. The retained productivity was above 50% after five cycles of use in the hydrolysis of steam-exploded wheat straw. The loss of activity is probably caused by three factors: (1) some cellulase is lost from Eudragit L-100 because of the unavoidable weak linkage; (2) some deactivation of immobilized cellulase occurs during the hydrolysis and sedimentation–dissolution process; and (3) some of immobilized cellulase is lost during the separation of undegraded substrates due to adsorption between them.

Conclusions

Covalent S-IS immobilization is a promising method for cellulase compared with non-covalent adsorption. The immobilized cellulase exhibits a higher pH, temperature and storage stability than free cellulase.

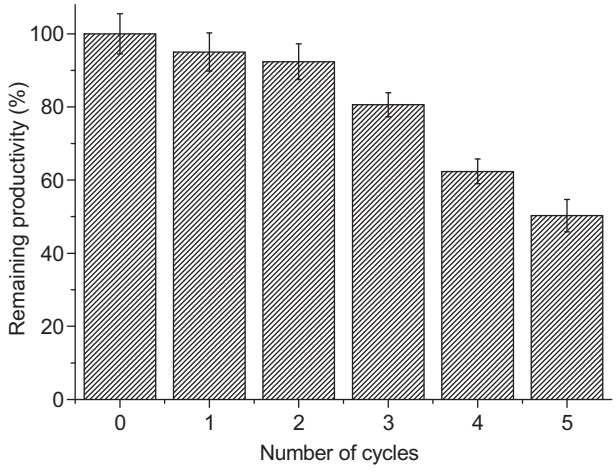

Figure 8. Reusability of immobilized cellulase in the hydrolysis of insoluble celluloses. Data are means ± SD of triplicates.

Covalent coupling of cellulase to the matrix Eudragit L-100 resulted in a slight steric hindrance with soluble CMC. Furthermore, immobilized cellulase performed outstandingly during batch hydrolysis of insoluble cellulosic material.

Declaration of interest: This work was funded by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX-YW-11-A3, KSCX2-YW-G-075-09 and KGCX2-YW-335) and the National High Technology Research and Development Program of China (863 program, 2007AA05Z406). The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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