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**Research Article**

# Immobilization of a 22kDa Xylanase on Eudragit L-100 for Xylo-oligosaccharide Production

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**Abstract:** A commercial xylanase was immobilized on Eudragit L-100 with the recovery activity of 87.3-126.2%. After immobilization, the optimum pH was not changed while its optimum temperature moved from 70 to 75°C. However, the immobilized enzyme did not show a higher thermal stability than the free xylanase. A slightly decrease in the *Km* values of free enzyme was observed upon immobilization. Meanwhile, *Vmax* values of the immobilized enzyme were 38% higher than those of the free xylanase. The result of Fluorescence spectroscopy used to probe the changes in the enzyme structure upon immobilization showed minute change and which may result in increase of immobilized enzyme activity. The application of immobilized enzyme hydrolyzing cottonseed husk and corncob pretreated by alkaline peroxide solution was the basically same as the free enzyme. The reusability of immobilized enzyme in the third cycles produced 163 and 126% reducing sugar hydrolyzing cottonseed husk and corncob.

**Keywords:** Application, eudragit L-100, immobilization, properties, Xylanase

## INTRODUCTION

Xylo-oligosaccharides have been found to have a stimulatory effect on the selective growth of human intestinal *Bifidobacteria* and are frequently defined as prebiotics (Okazaki *et al*., 1990). In particular, xylo-oligosaccharides, with its distinctive characteristics and better function than other oligosaccharides, are right under spotlight.

Most commercial xylases are produced by *Trichoderma, Bacillus, Aspergillus, Penicillium, Aureobasidium,* and *Talaromyces ssp.* (Godfrey and West, 1996). Xylanases (EC3.2.1.8) are glycosyl hydroloses (Davies and Henrissat, 1995) thar break down the backbone of xylan in plant cell wlls. Xylanases generally hydrolyze the β-1, 4-linkages between the xylose units forming the backbone in an endo mode of action, by attacking internal linkages rather than ones at the extremities of the polysaccharide chain, which, in contrast, are referred to as an exo mode of action (Leila and Pickersgill, 1999). Xylanases are not only used commercially in the pulp and paper, food and animal feed industries; but also used to produce low molecular weight xylo-oligosaccharides. In present, the studies on xylanase have attracted considerable research interest because of their potential industrial applications.

Reversibly soluble-insoluble polymers have been successfully used for both protein purification (Fujii and Taniguchi, 1991; Gupta *et al*., 1994; Galaev *et al*., 1996) and immobilization (Taniguchi *et al*., 1989; 1990, 1992; Lina *et al*., 1995; Sardar *et al*., 1997; Gawande and Kamat, 1998). It is shown that a commercial preparation of xylanase could be immobilized on a methyl methacrylate, Eudragit L-100, by simple adsorption (Sardara *et al*., 2000). Immobilization of xylanase to reversibly soluble-insoluble polymer facilitates better hydrolyses of insoluble substrate. Moreover, reversibly soluble-insoluble polymers as carriers for xylanases can be used as a soluble form for mediating the desired reaction. Some researchers have reported that xylanase immobilized on Eudragit S-100 directly hydrolyzed corncob powder pre-treated with dilute alkaline solution (Zhilu *et al*., 2005). In this study, the commercial xylanase is immobilized upon Eudragit L-100 and characteristics of both immobilized and free xylanase are evaluated. Xylanase bound Eudragit L-100 will be utilized to hydrolyze cottonseed husk and corncob pre-treated by alkaline peroxide solution (2.0%). This is the first report of the immobilization of xylanase on Eudragit L-100 for xylo-oligosaccharide production from cottonseed husk and corncob.

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## MATERIALS AND METHODS

Xylan (from Birchwood) was purchased from Sigma, Xylanase was purchased from Novozymes, Eudragit L-100 was a product of Rohm Pharma, Weiterstadt, Germany and was a copolymer of methacrylic acid and methyl methacrylate at a ratio of 1:1. All other chemicals used were of analytical grade. Cottonseed husk and corncob were obtained from local area.

**Xylanase immobilization:** Eudragit L-100 solution was prepared according to the method of Sardar *et al*. (1997). Eudragit L-100 (5 g) was dissolved by constant stirring in 90 mL distilled water and dropwise addition of 3 mol/L NaOH until pH 11.0. After the polymer was fully solubilized, the pH of solution was decreased to pH 7.0 by adding 3 mol/L HCl. Volume of the solution was made up to 100 mL with distilled water. The solution was stored at 4°C for further using.

1 mL of the free enzyme (the free enzyme was added 10 times 0.05 mol/L, sodium citrate buffer pH5.8, mixed and centrifuged at 12000×g for 4 min, the suspension was obtained) was mixed 4 mL of Eudragit L-100 solution at different concentrations (concentration of the polymer varied between 0.5 and 2.0%). The polymer was precipitated after 2 h by adjusting the pH of the solution to pH 4.0 with addition of 0.05 mol/mL acetic acid at 25°C. The suspension was centrifuged for 20 min at 12000×g. The precipitated obtained was washed three times with 0.05 mol/mL acetate buffer, pH 4.5. The final precipitate was dissolved in 0.05 mol/mL acetate buffer, pH5.8; this solution was used to calculate the expressed activity.

**Measurement of enzyme activity:** Xylanase activity was determined by the dinitrosalicylic acid (DNS) method of Bailey *et al*. (1992). The reaction mixture (1 mL) containing 0.1 mL of appropriately diluted enzyme solution and 0.9 mL of 1.0% Birchwood xylan solution in 0.05 mol/mL, pH 5.8, sodium citrate buffer, was incubated at 50°C for 10 min. The reaction was termined by adding 1 mL DNS. The amount of reducing sugar liberated was determined by a DNS method using xylose as the standard (Miller, 1959). One unit of xylanase activity was defined as the amount of enzyme that produces 1 mmol xylose equivalent per minute under the assay conditions.

**SDS-polyacrylamide gel electrophoresis (SDS-PAGE):** SDS-PAGE of the protein samples was performed in a 12% (w/v) polyacrylamide gel by the method of Laemmli (1970). The molecular weight standards used were the low molecular weight calibration kit for SDS electrophoresis (Amersham).

**Fluorescence emission spectra of the immobilized enzyme and free enzyme:** Emission spectra were taken by a Cary Eclipse Spectra-fluoro-photometer at an excitation wavelength of 289 nm, scanning wavelength 220-450 nm.

**Characterization of immobilized and free enzymes:** Effect of pH on free and immobilized was studied by assaying both preparations at different pH values (0.05 mol/L sodium citrate buffer for pH 2.20-4.50; 0.05 mol/L sodium acetate buffer for pH 3.80-6.10; 0.05 mol/L sodium phosphate buffer for pH 6.00-8.00; 0.05 mol/L tris-Hcl buffer for pH 7.00-9.00 and 0.05 mol/L glycine-NaOH buffer for pH 8.80-10.80). To determine pH stability of the free and immobilized xylanases, the enzyme were diluted in appropriate buffers (final concentration 0.05 mol/mL) of different pH values (as mentioned above) and incubated at 35°C for 30 min. The residual activities of these treated enzymes were measured by standard assay procedure.

Effect of temperature on xylanase activity was determined by assaying its activity at different temperature (at 30, 35, 40, 45, 50, 55, 60, 65, 70, 75 and 80°C) in the 0.05 mol/L, pH5.8, sodium citrate buffer. Thermal stability of the enzyme was studied at 75°C by incubating free and immobilized enzymes (in 0.05 mol/L citrate buffer, pH 5.8) at that temperature. Appropriate aliquots of free and immobilized xylanases were with-drown at different time (0, 5, 10, 20, 30, 60 and 120 min) intervals and the activities determined.

**Determination of kinetic parameters:** Km and Vmax values of free and immobilized xylanase were determined by measurement of initial rate of xylan hydrolysis at various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mg/mL) of xylan. The Michaelis constant was calculated using the Lineweaver-Burk equation.

**Pre-treated of cottonseed husk and corncob:** The substrates (10 g) added 10 times alkaline peroxide solution (2.0%, pH11.5) was incubated in RT for 12 h, then were washed to neutral by water and dried within oven.

**Analysis methods:** Thin-Layer Chromatography (TLC) was used to analyze the hydrolysis products according to the method of Jiang *et al*. (2004).

## RESULTS AND DISCUSSION

**Immobilization of commercial xylanase:** Table 1 shows the extent to which the enzyme retained its activity upon immobilization. In order to optimize conditions, different concentration of Eudragit L-100 was added to the same amount of free enzyme. The recovery activity gradually increased as the Eudragit L-100 gradually increased. The recovery activity was increased up 101.4% when the polymer concentration was 1.0%. Although the recovery activity was increased as the concentration of Eudragit L-100 increased, the application of the polymer was limited because of the higher price of it. Therefore, the immobilized xylanase

Table 1: Immobilization of xylanase on Eudragit L-100

| Polymer concentration (%) | Enzyme added (u) X | Enzyme unbound (u) | Enzyme bound (u) (theoretical) | Expressed activity (u)Y | The recovery extent of enzyme immobilization [$(YX^{-1}) \times 100$] (%) |
|---|---|---|---|---|---|
| 0.5 | 3031.3 | 997.9 | 2033.4 | 2644.9 | 87.3±2.6 |
| 1.0 | 3031.3 | 229.9 | 2801.4 | 3074.7 | 101.4±3.0 |
| 1.5 | 3031.3 | 202.7 | 2828.6 | 3555.8 | 117.3±3.5 |
| 2.0 | 3031.3 | 154.1 | 2877.2 | 3826.9 | 126.2±3.8 |

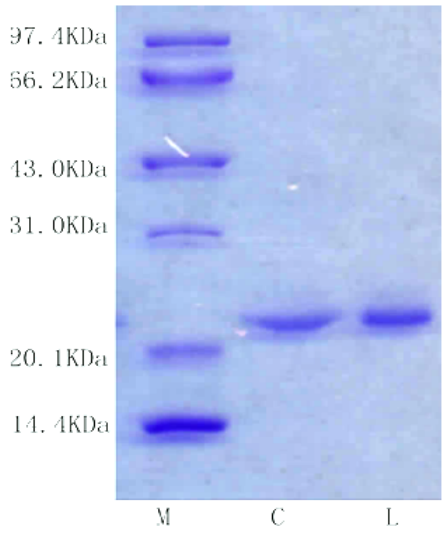


Fig. 1: SDS-PAGE of the free and immobilized xylanase; Lane M: low molecular weight standards; Lane C: free xylanase; Lane L: immobilized xylanase

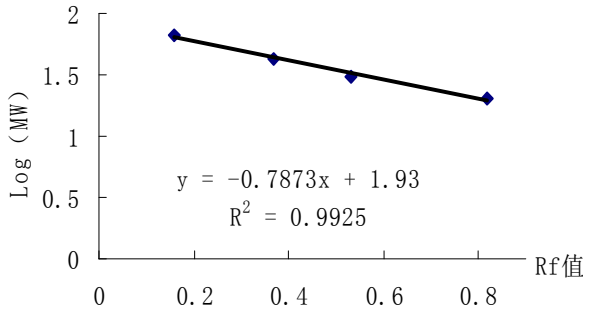


Fig. 2: The relationships between the molecular weight of standard protein and Rf on SDS-PAGE; The standard protein bands: Bovine serum albumin (66,200); Rabbit actin (43,000); Bovine carbonic anhydrase (31,000); Trypsin inhibitor (20,100)

prepared at the polymer concentration of 1% was arbitrarily regarded as the immobilized xylanase and was further characterized and applied in the following study. Dimella *et al*. (1995) immobilized an endo-pectinlyase on Eudragit L-100 whose recovery activity was 80%. Lina *et al*. (1995) added Ca2+ to the α-amylase coupled to Eudragit L-100 which exhibited the higher recovery activity (95.3%). Moreover, Lina *et al*. (1995) also found α-amylase binding to polyethyleneimine resulted in activation of the enzyme (The recovery activity increased up to 132.04%). So it is possible for Eudragit L-100 to activate the xylanase.

SDS-PAGE of the commercial xylanase and immobilized xylanase performed in a 12% (w/v) polyacrylamide gel shows no differences (Fig. 1). Both of them showed a major single band on SDS-PAGE. The molecular weight of them was calculated by the Fig. 2, which were 22.0 kDa.

Fluorescence spectra of the native and immobilized enzymes are shown in Fig. 3. The λmax emission changed from λmax 354 nm of native to λmax 352 nm of immobilized enzyme which suggests that conformation of the protein binded polymer has occurred minute changes in nature. It is also possible that these spectral changes reflect "shielding" by the polymer rather than any significant structural changes (Sardar *et al*., 1997). Wahlgren and Amebrant (1991) suggested that time-dependent structural changes in protein conformation are observed after adsorption on polymeric surfaces.

**Effect of pH on activity and stability of immobilized and free xylanases:** Figure 4 shows the activity of the immobilized and free xylanase at different pH values. The optimum pH values of the immobilized and free xylanase were pH 5.8. The pH stabilities of the immobilized and free xylanase were compared in the pH range between pH2.3 and 11.8 at 35°C during 30 min incubation periods. The pH stabilities of both were agreed with each other (data not shown).

**Effect of temperature on activity and stability of immobilized and free xylanases:** Temperature dependence of the activity of the immobilized and free xylanase was studied at different temperature in 0.05 mol/L sodium citrate buffer (pH5.8). The free enzyme had an optimum temperature of 70°C, whereas that of the immobilized enzyme was shifted to 75°C (Fig. 5). Similarly, Zhilu *et al*. (2005) also reported the increase in optimum temperature.

Figure 6 shows thermal stability of immobilized and free xylanase at 75°C. The rates of heat inactivation of free and immobilized enzyme were investigated in temperature 75°C. The plot of log% remaining activity versus time is linear at the temperature tested, indicating the first-order inactivation kinetics for both the immobilized and free xylanase. Both the immobilized and free xylanase were reasonably stable at between 30°C and 50°C, but the inactivation at 60°C was rapid (data not shown). Thermal stability data at 75°C did not show that free enzyme resulted in significant stabilization compared to the immobilized

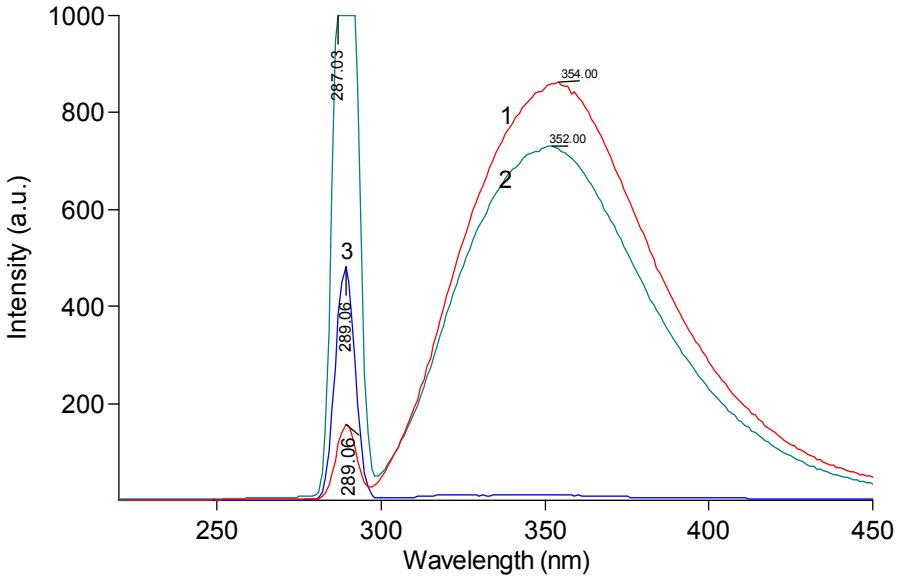


Fig. 3: Fluorescence spectra of immobilized xylanase; 1: Native enzyme; 2: Immobilized xylanase; 3: Eudragit L-100

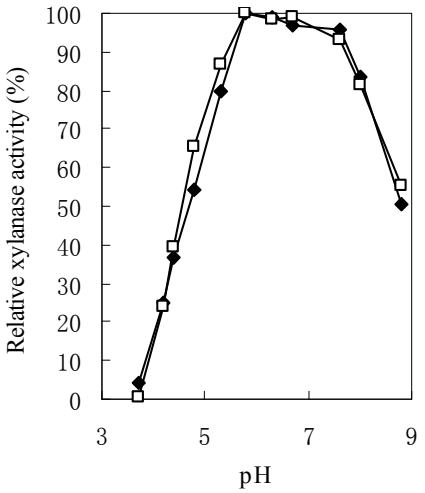


Fig. 4: Effect of on the activities of immobilized and free xylanase; (♦), immobilized enzyme; (□), free enzyme

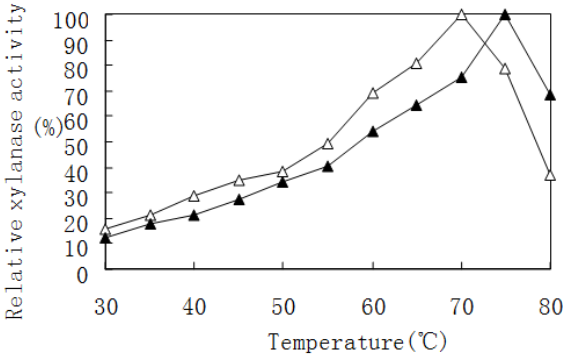


Fig. 5: Effect of temperature on the activities of immobilized and free xylanase; (▲), immobilized enzyme; (△), free enzyme

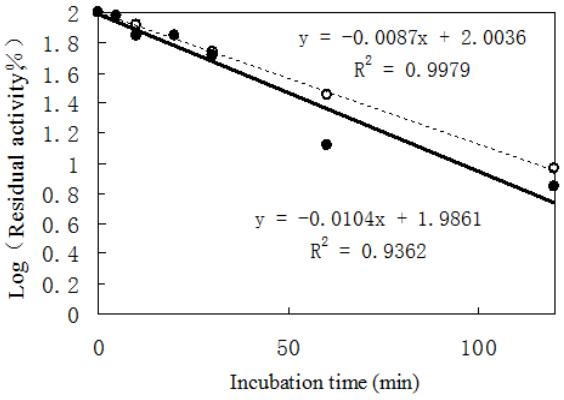


Fig. 6: Thermal stability of immobilized (solid line) and free (dotted line) xylanase at 75°C

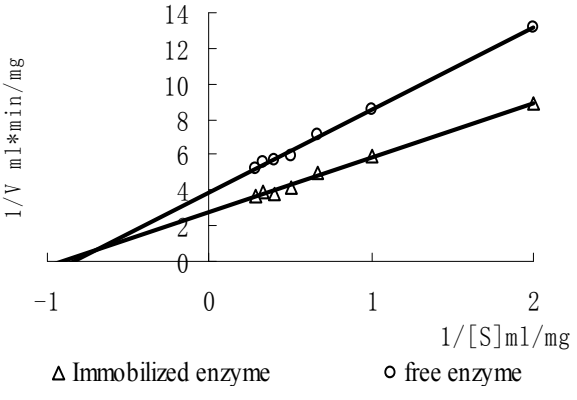


Fig. 7: The determination of Michaelis constant of immobilized and free enzyme

enzyme. Therefore, immobilization decreased the thermal stability of the xylanase.

In general, the immobilization process of the enzyme protects the enzyme against heat inactivation (Gouda and Abdel-Naby, 2002; Edward *et al*., 2002; Roy *et al*., 2003). Immobilization enhanced the optimum temperature of xylanase, while decreased the thermal stability of the xylanase in this study. However, the thermal stabilities of the immobilized and free xylanase have no difference at 50°C. Therefore, the immobilization did not affect the industrial application (Fig. 7).

**Enzyme kinetics of immobilized and xylanases:** Km and Vmax values of immobilized and free enzymes were determined and calculated by Liner wear-Burk equation. The Km values of the immobilized xylanase for Birchwood xylan was slightly decreased to 1.11 mg/mL, compared to 1.20 mg/mL of the free xylanase. The Vmax values of the immobilized enzyme for Birchwood xylan showed significant increases to 2.40 μmol/mL min from 1.73 μmol·mL$^{-1}$min of the free enzyme.

In general, the Km value of the immobilized enzyme should be higher than which of the free enzyme. However, marginal decrease in the value upon immobilization observed here with the soluble matrix confirms that enzyme-substrate binding was more efficient than the free enzyme in this study. This decrease in Km value is likely to be due to the fact that the local concentration of the substrate (with its negative charge) increases because of the positively charged matrix near the immobilized enzyme. Sardar *et al*. (1997) had reported that the Km value decreased in the case of trypsin binding to the Eudragit S-100.

Increases in Vmax upon immobilization are generally not observed. However, Vmax value of the immobilized xylanase was 139% higher than this of free enzyme in this study. Roy *et al*. (2003) reported that there was a marginal increase for immobilized Melanocarpus albomyces II S 68 xylanase on Eudragit L-100 due to the somewhat hydrophobic nature of polymer (Bailey *et al*., 1992). Zhilu *et al*. (2005) also reported that there was a marginal increase for immobilized Streptomyces

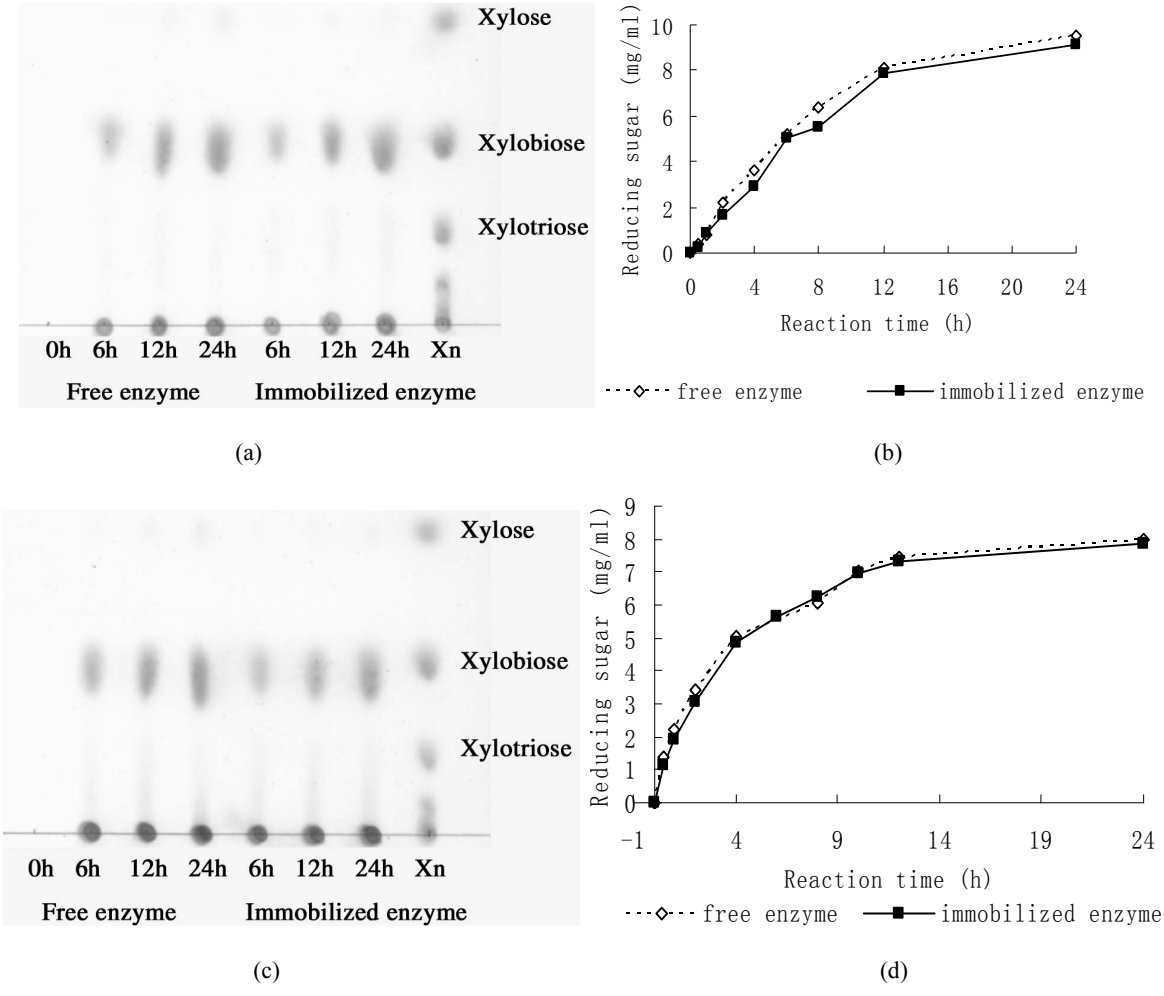


Fig. 8: Time-course of the xylo-oligosaccharide production from pre-treated cottonseed kernel and corncob by immobilized and free xylanase

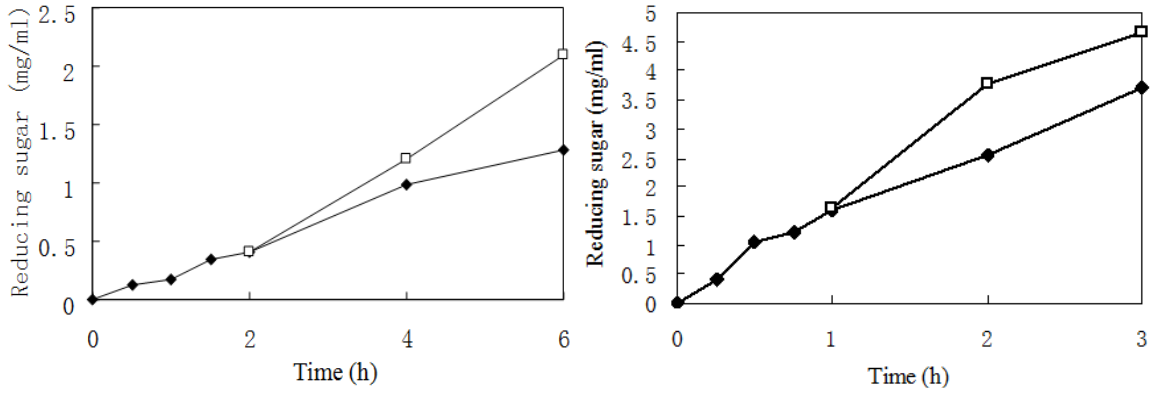


Fig. 9: Reusability of the immobilized xylanase; a) (♦): Two gramme of the pre-treated cottonseed kernel added in 30 mL 0.05 mol/L sodium citrate buffer (pH5.8) was hydrolyzed by immobilized enzyme (40 u/mL); Reducing sugar produced during reaction was determined by DNS method; (□), Before two hours, the process of the hydrolysis is the same as the black dotted; However, the undegraded substrate was filtrated and added the same amount of substrate at 2 h, 4 h, 6 h; b) (♦), Two gramme of the pre-treated corncob added in 40 mL 0.05 mol/L sodium citrate buffer (pH5.8) was hydrolyzed by immobilized enzyme (40 u/mL); Reducing sugar produced during reaction was determined by DNS method; (□), Before one hour, the process of the hydrolysis is the same as the black dotted; However, the undegraded substrate was filtrated and added the same amount of substrate at 1 h, 2 h, 3 h

olivaceoviridis E-86 xylanase on Eudragit S-100 (Zhilu *et al*., 2005).

**Hydrolysis of pre-treated cottonseed kernel for xylo-oligosaccharide production:** Pre-treated cottonseed kernel and corncob (2 g) were incubated with 600 U of the immobilized and free enzyme in 30 mL and 40 mL 0.05 mol/L citrate buffer (pH 5.8) and the reactions were carried out at 50°C for 24 h and then the hydrolyzates were analyzed by TLC (A and C) and DNS (B and D), respectively.

Gould (1984) reported that alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. Therefore, attempts were made to produce xylo-oligosaccharides from cottonseed husk and corncob pre-treated with alkaline peroxide solution.

The products of hydrolysis of both were analyzed by TLC and DNS method. Xylobiose is the major end product found (Fig. 8A and C) from 1 h to 24 h and which also is the major functional factor of the xylo-oligosaccharides. There was few amount of xylose produced with the time prolonged. Figure 8B and D showed the amount of reducing sugar produced within the reaction of hydrolyzing the pre-treated cottonseed kernel and corncob. The amount of reducing sugar of hydrolyzing the cottonseed husk and corncob with immobilized enzyme was slightly lower than that of free enzyme. It was possible that the "shielding" of the Eudragit L-100 leaded to the result.

**Reusability of the immobilized xylanase:** The reusability of the enzyme preparation was further assessed by carrying out hydrolysis of cottonseed husk and corncob using the immobilized xylanase at 50°C. The one sample was hydrolyzed by the immobilized without removing the undegraded sample during process of the reaction. After two (one) hours of hydrolysis, the undegraded substrate of the sample was removed from the reaction mixture by filtrated. The extent of hydrolysis estimated by determining the amount reducing sugars produced in the supernatant. After that, the same sample of immobilized enzyme and the new substrates were mixed again to start the second cycle. Figure 9A and B showed the data corresponding to the cycling of the enzyme preparation thrice. Each cycle consisted of two (one) hours of continuous hydrolysis. The amount of reducing sugar produced after third cycle are 163% (126%) of the first cycle within cottonseed husk (corncob) hydrolyzed by immobilized enzyme.

## CONCLUSION

Commercial xylanase can be no covalently immobilized on a positive copolymer Eudragit L-100 with a high immobilization efficiency of 87.3-126.2%. The performance of immobilized xylanase with respect to catalytic activity is promising compared with that of the free enzyme. Accordingly, it is suggested that xylanase immobilized on Eudragit L-100 is suitable for practical industrial application.

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