STABILITY AND REACTIVITY OF β-GLUCOSIDASE ENZYME IMMOBILIZED ON CHITOSAN-CLAY COMPOSITE

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Abstract

Equal weights of cuttlebone chitosan and activated clay were well mixed and prepared as the wet (without drying) and dried (with freeze-drying) beads. The resulting composite beads were then cross-linked with glutaraldehyde as the supports for β-glucosidase immobilization. It was shown that the storage stability of immobilized enzyme was higher than that of free enzyme. The activity of immobilized enzyme on dried composite was higher those on wet chitosan beads and wet composite after being repeatedly used for 50 times. The thermal deactivation energies of free and immobilized enzymes were also evaluated. For a given enzymatic reaction, the Michaelis constant and maximum rate of reaction using dried-composite immobilized enzyme were larger than those using both free and wet-composite immobilized enzymes.

Introduction

Chitosan is cheap, non-toxic, and not harmful support to the enzymes. It has many advantages such as excellent hydrophilicity, high porosity and large adhesion area. Chitosan possesses hydroxyl and amino ($-NH_2$) groups, which link with enzymes [1] and, can be cross-linked with glutaraldehyde to prevent dissolution in acidic solutions (pH < 2). When chitosan is prepared in the bead form, it can entrap twice as much of enzyme, estimated based on the mass transported and the volume of reactive dyes sorbed onto internal pores [11]. Cetinus and Oztop [2] found that catalase immobilized on chitosan beads exhibits better thermal and pH stabilities than free one, and that there are high activities and long lifetimes when tyrosinase, β-glucosidase, and acid phosphatase are immobilized on chitosan beads [3,4]. Although the cross-linked chitosan beads have greater mechanical strength and are more applicable in biochemical engineering, there are operational defects; for example, the density is similar to that of water (causing it to float easily) and the texture is too soft. This limits industrial applications of chitosan.

The problems of chitosan beads outlined above may be solved when the bead is prepared by mixing with some powders such as clays and activated carbons to increase their densities and mechanical strengths, thereby extending application possibilities [5,6]. Lai and Shin [6] have immobilized acid phosphatase on montmorillonite and chitosan to improve phosphorus content of soil. In this work, activated clay powders were added to chitosan/acetic acid slurry to prepare the composite beads, in either wet (without freeze-drying) or dried (with freeze-drying) manner, to improve the properties of chitosan beads. These beads were then cross-linked with glutaraldehyde for β-glucosidase immobilization. The optimal immobilization conditions (enzyme loading, immobilization time, cross-linking time, amount of glutaraldehye, etc.) and operation stability when used repeatedly of immobilized enzymes were studied and compared with corresponding properties of free enzyme. Thermal deactivation and catalytic kinetics of such immobilized enzymes were finally examined. This is of practical importance for further applications.

Materials and Methods

Preparation of cross-linked composite beads

Chitosan and activated clay were prepared following the methods described earlier [5]. Chitosan flakes (1 g) and activated clay (1 g) were dissolved in 1 M of acetic acid (100 mL) and were agitated with a disperser (IKA, Ultra-Turrax T25 basic) at 24,000 rpm for 10 min. The yielded viscous solution was placed in a vacuum dryer for 3 h to remove bubbles, and then was sprayed drop-wise through a syringe at a constant rate into a neutralizing solution containing 15% NaOH and 95% ethanol in a volume ratio of 4:1. The beads were left in solution for 1 day. The prepared beads were washed with deionized water until the solution was neutral. They are referred to wet composite beads. While they were further dried in a freeze dryer (Eyela FD-550) for 6 h, they are referred to dried composite beads. The diameters of wet and dried composite beads, and wet pure chitosan beads were 3.2, 2.4, and 2.6 mm, respectively.

An aliquot of the wet or dried composite (0.05 g) was placed in a 100-mL vessel containing 50 mL of 5-g/L glutaraldehyde (Acros Co.). The solution was agitated at 150 rpm and 30^{o}C. After cross-linking for 2 h, the beads were washed thoroughly and stored in deionized water.

Immobilization of enzyme and activity determination

An amount of cross-linked wet or dried composite (0.05 g) was in contact with 2 mL of 1 g/L of β-glucosidase (Sigma, E.C.3.2.1.21, from almonds, 3.4 unit/mg) in a shaker for 18 h at 4^{o}C. After washing with deionized water 3 times, the immobilized beads were stored in a vessel containing 0.01 M of acetate buffer (pH 3.5) at 4^{o}C. The activity of β-glucosidase in the solution was determined by adding 0.1 mL sample to 0.9 mL of 0.1-M acetate buffer (pH 3.5), which contains 5 mM of *p*-nitrophenyl β-D-glucopyranoside (Sigma Co., N7006) substrate [4]. The reaction mixture was incubated with stirring at 25^{o}C for 1 min and stopped by adding 2 mL of 1-M Na_2CO_3. The absorbance of the product *p*-nitrophenol was measured at 400 nm using an UV/visible spectrophotometer (Jasco V-500), and the activity was calculated based on a molar extinction coefficient of 18,300 dm^3/(mol cm). One activity unit (*U*) of β-glucosidase is defined to be the amount of this enzyme required hydrolyzing 1 μmol of substrate per minute. The activity of β-glucosidase immobilized on the composite was similarly measured, except that 0.1 mL of the solution was replaced by 0.1 mL deionized water and a given amount of wet or dried composite.

Stability and catalytic reaction experiments

The thermal and pH stability of free and immobilized enzymes were examined by measuring the activity of enzyme after the enzyme had been in the solutions for 1 and 6 h, respectively, at different temperatures (15-75^{o}C) and different pH (2.5-6.0). For an amount of free and immobilized enzymes, the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside substrate at 25^{o}C was monitored at preset time intervals in terms of enzyme activity. In immobilized enzyme systems, the composite (0.05 g) was added to 100 mL of the solution (pH 3.5) containing different amounts of *p*-nitrophenyl β-D-glucopyranoside (0.25-2.5 mM).

Results and Discussions

Effect of enzyme loading and immobilization time on enzyme activity

The BET surface areas of cross-linked and non-crosslinked dried composite beads, and the cross-linked dried chitosan beads are measured to be 114.0, 36.8, and 23.3 m^2/g, respectively, in this work. This indicates that cross-linking step increases the surface area. Table 1 shows that the activity of immobilized enzyme increases when β-glucosidase loading increases up to 30 mg/g chitosan. That is, the immobilized enzymes have almost no activity variation in the enzyme loading range 30-50 mg/g. As shown in Table 1, the activity of dried-composite immobilization enzyme is 1.9 times higher than that of the wet one.

The effect of immobilization time on enzyme activity is listed in Table 2. Dried composite beads have more interior space to absorb enzymes. According to our results, the β-glucosidase quantity of 40 mg/g chitosan and an immobilization time of 18 h are chosen [5].

Table 1. Effect of enzyme loading on the activity of immobilized β-glucosidase

Support	Property	Enzyme loading (mg/g chitosan)				
		10	20	30	40	50
Wet composite	Activity unit (U)	216.4	274.0	286.8	286.3	289.0
	Residual activity (%)	1.5	0.5	0.9	0.6	1.1
Dried composite	Activity unit (U)	394.6	480.8	507.2	512.4	511.9
	Residual activity (%)	6.1	15.4	29.8	48.2	62.1

Table 2. Effect of immobilization time on the activity of immobilized β-glucosidase

Support	Property	Immobilization time (min)							
		3	6	9	12	15	18	21	24
Wet composite	Activity unit (U)	411.2	311.1	355.0	343.8	385.0	339.3	336.9	351.4
	Residual activity (%)	27.8	14.0	13.2	7.1	7.3	6.9	6.9	7.0
Dried composite	Activity unit (U)	582.5	540.3	673.1	763.9	706.0	632.6	766.8	649.7
	Residual activity (%)	99.5	75.5	68.4	60.8	59.6	60.7	64.4	53.1

Effect of added amount of glutaraldehyde on enzyme activity

Fig. 1 shows the activity (U) of immobilized enzyme at different amounts of glutaradehyde and different cross-linking durations during the immobilization process. Under the ranges studied, the activities of dried- and wet-composite immobilized enzymes are maximized at a glutaraldehyde concentration of 1 and 4 g/L, respectively. The activity of the former enzyme is 1.1-2.4 times higher than that of the latter one. A larger amount of glutaradehyde may cause some enzyme denaturation. Jiang et al. [7] have stated that the activity of immobilized laccase reaches its maximum when 8% cross-linked magnetic chitosan microspheres are used as carrier. As glutaraldehyde concentration exceeds 8%, extensive interaction of enzymes with aldehyde groups on the surface of microspheres changes enzyme conformation and cause the drop of enzyme activity.

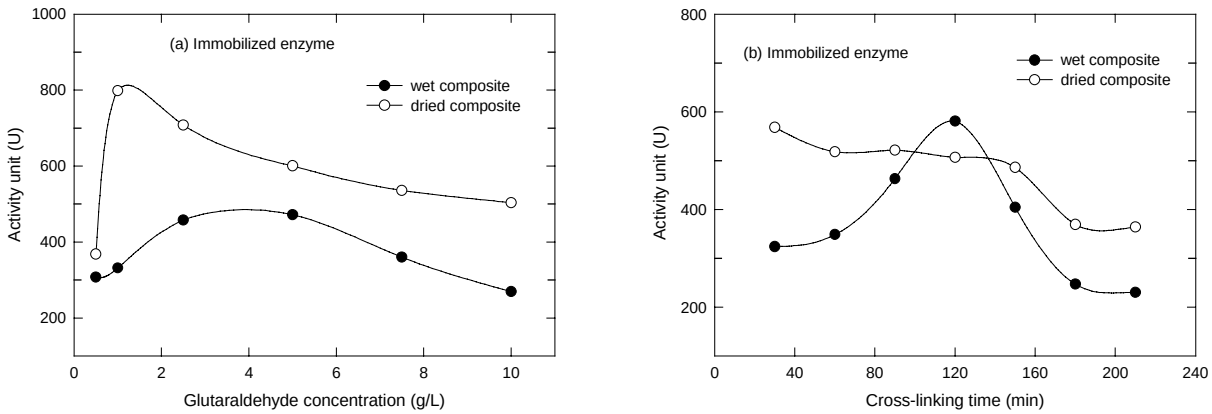

Fig. 1. Effect of amount of glutaraldehyde and cross-linking time on enzyme immobilization

The effect of cross-linking duration on enzyme activity is shown in Fig. 1b. During the period of 30-210 min, the activity of dried-composite immobilized enzyme deteriorates as cross-linking time increases but the activity of wet-composite immobilized enzyme reaches a maximum at 120 min. Thus, the glutaradehyde concentration and cross-linking time are selected to be 5 g/L and 120 min, respectively.

Effect of pH and temperature on enzyme activity

It is observed from Fig. 2 that the relative activity of free enzyme increases when pH is increased up to 6.0. As expected, the immobilized enzymes have either the same or a broader pH range with high activity than free enzymes**.** The optimal pH for wet- and dried composite immobilized enzymes is 4.5, and 5.5, respectively. The activity of wet- and dried-composite immobilized enzymes is comparable at pH 2.5-4.5, but the latter enzyme exhibits higher activity

than the former one at pH 5.0-6.0. For instance, the relative activity of dried-composite immobilized enzyme is over 91.5% in the pH range 4.5-6.0. It has been actually reported that the immobilized α-amylase on wet composite has almost no activity variation (relative activity > 93%) in the pH range 5.8-8.0, but free α-amylase deteriorates when pH is increased [5].

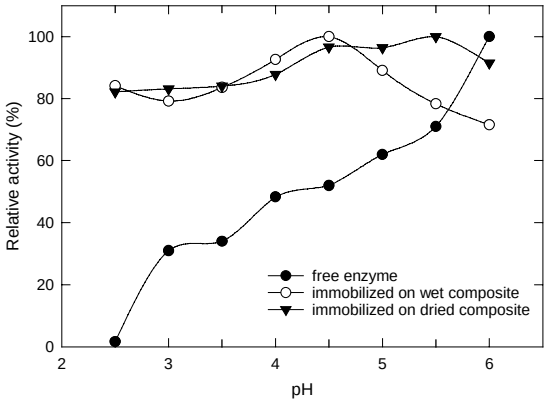

Fig. 2. Effect of solution pH on relative activity of free and immobilized enzyme

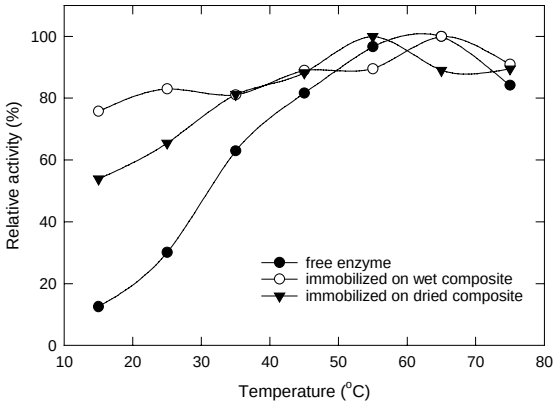

Fig. 3. Effect of temperature on relative activity of free and immobilized enzyme

Fig. 3 show that the immobilized enzymes have a broader tolerance range to heat than free enzymes. The relative activities of free, wet- and dried-composite immobilized enzymes remain over 30.1%, 81.1%, and 65.5%, respectively, in the temperature range 25-75°C. Evidently, the optimal temperature of free and immobilized enzymes is located between 55 and 65°C. In a series of studies, the relative activities of acid phosphatase at 27-87°C, and α-amylase, β-amylase, and glucoamylase at 15-85°C immobilized on wet composite were found to remain over 45%, 71%, 63%, and 74%, respectively [5]. The optimal temperature was shifted from 60°C for free glucoamylase to 50°C for immobilized enzyme; however, the temperature effect was rather small with immobilized glucoamylase but very high with free glucoamylase. Yan and Lin [8] have also indicated that the activity of free β-glucosidase reaches maximum at 55°C but only 70% of the 55°C-activity is obtained in the temperature range 50-60°C.

The loss of the activity of immobilized enzyme is smaller than that of free enzyme at low temperatures. The immobilization support has a protecting effect at high temperatures when enzyme deactivation occurs. The conformational flexibility of the enzyme is affected by immobilization. Immobilization of the enzyme on chitosan-clay composite preserves its tertiary structure, and protects the enzyme from conformational changes that could affect the environment. On the other hand, the high activity of immobilized enzymes at low temperatures is probably a result of favored adsorption of enzymes on activated clay [5].

Operational stability of immobilized enzymes

The reusability of immobilized enzymes is important for economical use of an enzyme. Due to the importance of repeated applications they should be easily separated from product solutions in a batch on a continuous reactor. Fig. 4 shows the activity of β-glucosidase immobilized on wet pure chitosan, wet composite, and dried composite after repeated use. The trends are quite similar in wet chitosan and wet composite. This is also the case for the enzyme acid phosphatase [9]. The activity is lower at the beginning of recycling use, and increases when the beads are repeatedly reused. It reaches a plateau at the eighth reuse. Further reuse of chitosan beads makes them swell and break apart, hence losing activity. But, the activity of immobilized enzyme on dried composite rises rapidly before the eighth reuse.

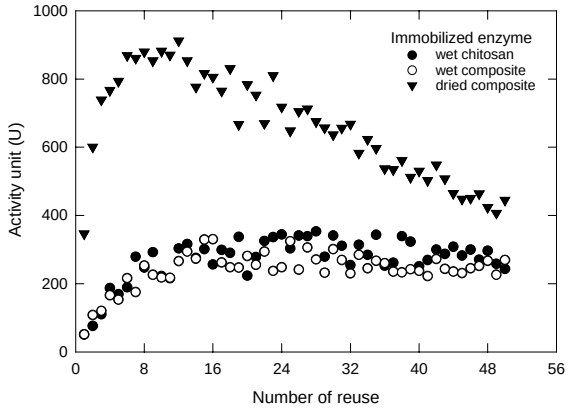

Fig. 4. Activities of immobilized enzymes after reuse

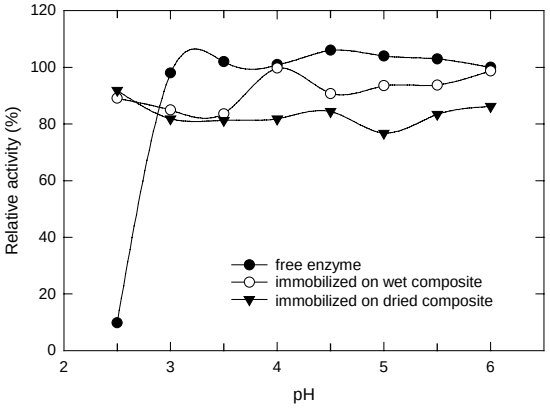

Fig. 5. pH stability of free and immobilized enzymes

In a word, the activity of the enzyme immobilized on dried composite is 1.8 and 1.6 times higher than those on wet chitosan and wet composite after 50-times reuse, and maintains 74% of the activity at the second operation. The dried composite exhibits good mechanical properties and stable enzyme activity. The addition of activated clay in the chitosan matrix greatly increases the density of the composite beads, making them more rigid and indissoluble.

pH stability of free and immobilized enzymes

The pH stabilities of free and immobilized β-glucosidase are compared by immersing them in a 0.1 M of sodium acetate buffer in the pH range 2.5-6 for 1 h at 25°C. The results are shown in Fig. 5. Free enzyme has almost no activity variation (relative activity > 98.6%) at pH 3-6. The pH stability of free enzyme is higher than those of immobilized enzymes; in addition, the dried composite immobilized enzyme exhibits slightly lower pH stability than the wet one, but it still maintains over 76.7% of the original activity at pH 2.5-6. In examining the residual activities of free and immobilized laccase on magnetic chitosan microspheres, Jiang et al. [7] found that immobilized laccase is stable in the pH 5-6 while free laccase is stable in the pH range 7-9. This indicates that immobilization appreciably improves the stability of laccase in acidic region.

Thermal deactivation of immobilized enzymes

Fig. 6 shows time changes of the activities of free and immobilized enzymes at different temperatures. Thermal deactivation of free and immobilized enzymes is described by the pseudo-first-order equation [10]:

$$\ln\left(\frac{A}{A_0}\right) = -k_d t + C_1 \quad (1)$$

where A_0 and A are the activities of enzyme at $t = 0$ and time t, respectively, k_d is the first-order rate constant, and C_1 is a constant. The linear fitting is reasonably acceptable (correlation coefficient, $R^2 > 0.9151$). According to these plots, the half-lives for free and immobilized enzymes are evaluated and also compiled in Table 3. The half-lives for the wet- and dried-composites immobilized enzymes are much longer than that of free enzyme in the range of 50-70°C as also shown in Fig. 7.

Table 3. Deactivation rate constant (k_d) and half -life ($t_{1/2}$) of free and immobilized β-glucosidase

T	Free enzyme			Enzyme on wet composite			Enzyme on dried composite		
(°C)	k_d	R^2	$t_{1/2}$	k_d	R^2	$t_{1/2}$	k_d	R^2	$t_{1/2}$
50	1.07 x 10^{-2}	0.9755	39.1	4.25 x 10^{-3}	0.9708	132.3	2.62 x 10^{-3}	0.9393	131.0
60	6.24 x 10^{-2}	0.9807	8.7	8.80 x 10^{-3}	0.9788	58.6	5.59 x 10^{-3}	0.9748	80.6
70	6.55 x 10^{-1}	0.9947	1.0	5.69 x 10^{-2}	0.9892	6.5	5.24 x 10^{-2}	0.9151	9.4

Units: k_d (min^{-1}) and $t_{1/2}$ (min)

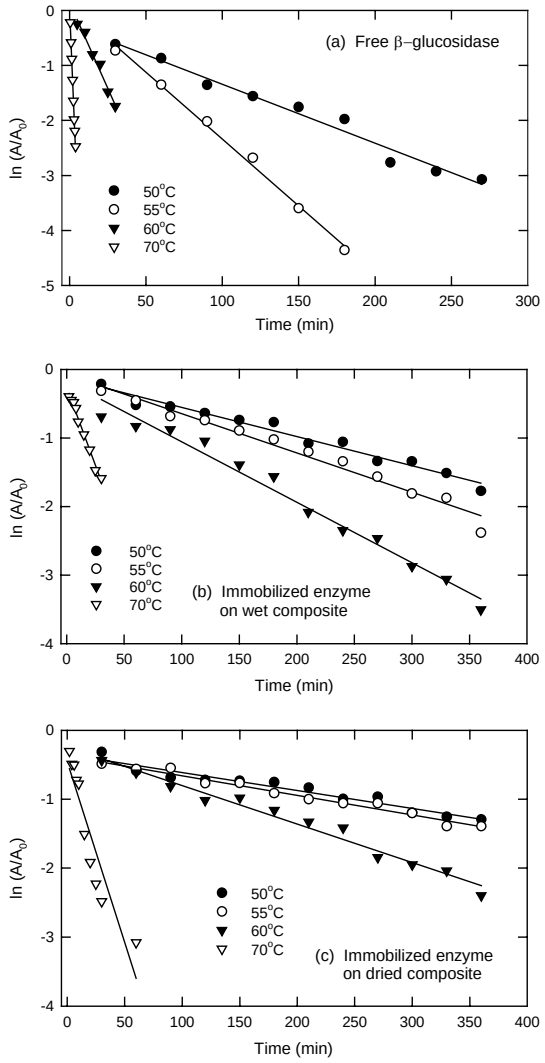

Fig. 6. First-order kinetics of thermal inactivation of free and immobilized enzymes

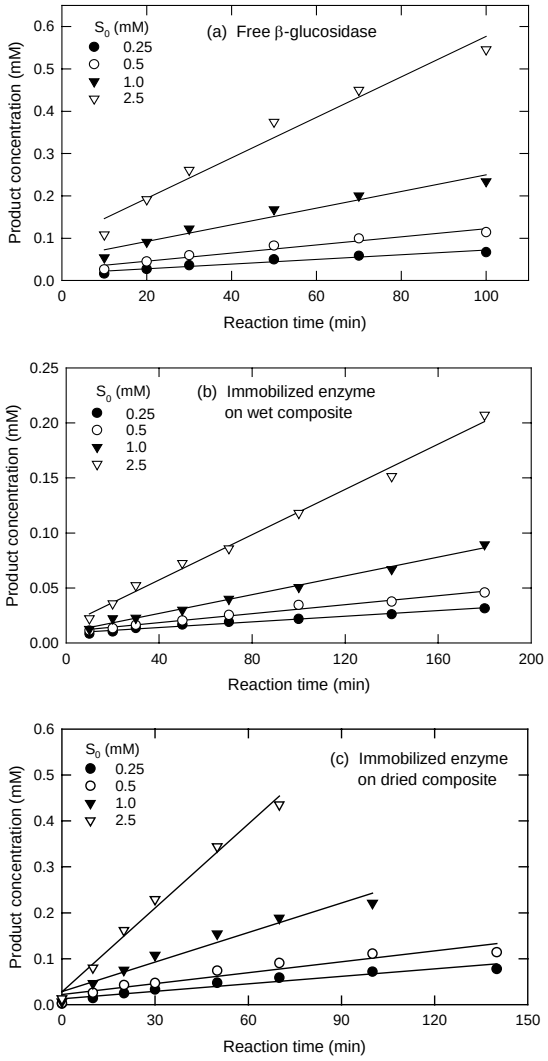

Fig. 9. Time profiles of catalytic reaction of free and immobilized enzymes

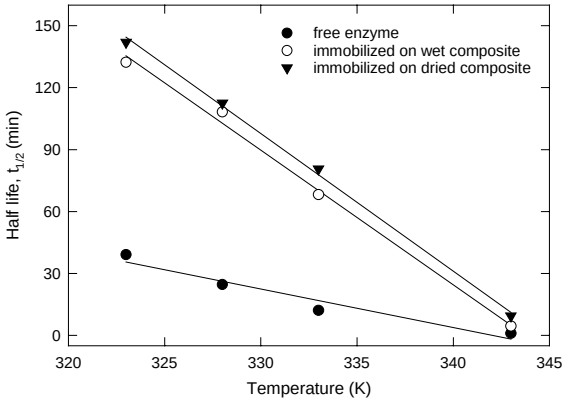

Fig. 7. Half life of free and immobilized β-glucosidase

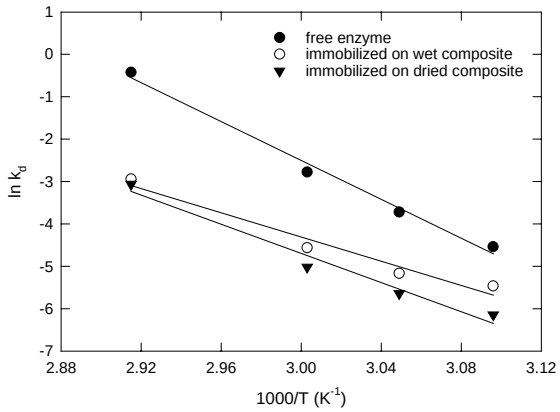

Fig. 8. The Arrhenius plot of deactivation rate constant

Temperature dependence of rate constants is used to evaluate deactivation energy, as shown in Fig. 8:

$$\frac{d(\ln k_d)}{d(1/T)} = -\frac{E_d}{R} \qquad (2)$$

where E_d is the deactivation energy (kJ/mol). The values of E_d for immobilized enzymes (wet, 111.4 kJ/mol; dried, 143.7 kJ/mol) are smaller than that with free enzyme (190.7 kJ/mol), implying that immobilized enzymes are more temperature insensitive. Previous studies have stated that the E_d value for free α-amylase (460 kJ/mol) and glucoamylase (221 kJ/mol) are larger than those for immobilized ones (113 and 36.5 kJ/mol) on wet chitosan-clay composite beads [5].

Kinetics of enzymatic reactions

Time changes of product concentrations (*p*-nitrophenol) at different initial substrate concentrations, S_0 (*p*-nitrophenyl β-D-glucopyranoside), are shown in Fig. 9. The relation between reaction rate and substrate concentration can be described by the Michaelis-Menten equation.

$$V = \frac{V_{max} S}{K_M + S} \tag{3}$$

where V_{max} is the maximum rate of reaction and K_M is the Michaelis constant (mM). These two parameters can be determined from the Lineweaver-Burk plot (Fig. 10).

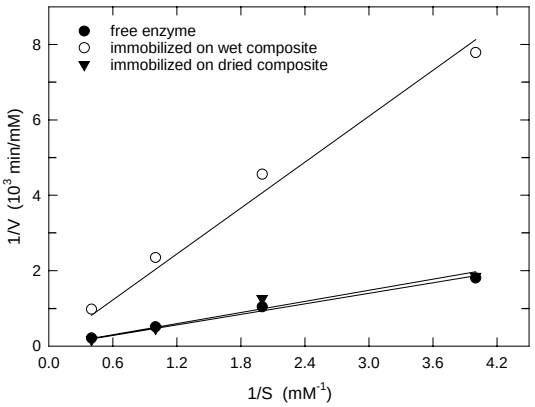

Fig. 10. The Lineweaver-Burk plot of catalytic reaction

As shown in Table 4, the values of K_M and V_{max} decrease in the order dried composite > free > wet composite. A change in freeze-drying affects bead porosity and thus the diffusion of substrate and products to and from the beads. It was reported that the K_M value of immobilized laccase is 4.6 times larger than that of free laccase, but immobilization decreases the V_{max} value from 6.6 x 10^{-3} to 5.9 x 10^{-3} mM/min [7]. Comparison of the K_M value for given free and immobilized enzymes provides information about interaction between an enzyme and its support. An increase in K_M once an enzyme has been immobilized, indicates that the immobilized enzyme has an apparently lower affinity for its substrate than a free enzyme does, which may be caused by steric hindrance of the active site by the support, the loss of enzyme flexibility necessary for substrate binding or diffusion resistance to solute transport near the beads of the support.

Table 4. The maximum rate and Michaelis constant using free and immobilized β-glucosidase

Type of enzyme	V_{max} (mM/min)	K_M (mM)	R^2
Free	1.37 x 10^{-2}	6.0	0.9932
Immobilized on wet composite beads	1.95 x 10^{-3}	3.7	0.9658
Immobilized on dried composite beads	1.60 x 10^{-2}	7.6	0.9724

Cetinus and Oztop [2] have found that the K_M value of immobilized catalase is larger than that of free enzyme but the immobilization decreases the V_{max} value. This is because in their study the chitosan beads are further treated by glutaraldehyde, which reduces enzyme activity and may prohibit substrate diffusion to the enzyme. The change in affinity of the enzyme to its substrate is probably caused by structural changes in the enzyme due to immobilization procedure or by the lower accessibility of the substrate to the active site of immobilized enzymes. The V_{max} value of immobilized enzyme is therefore smaller than that of free enzyme.

Conclusions

The composite beads prepared by mixing equal weights of activated clay and chitosan, in wet or dried manner, and cross-linking with glutaraldehyde were used as the immobilization support for β-glucosidase. The activity of β-glucosidase immobilized on dried composite was significantly higher than that on wet composite at pH 5-6, and the activities for both immobilized enzymes were the same at 45-75^{o}C. The activity of β-glucosidase immobilized on dried composite was 1.8 times higher than that on pure chitosan after 50-times reuse. Thermal deactivation analysis showed that the deactivation energies for immobilized enzyme on wet (111.4 kJ/mol) and dried composites (143.7 kJ/mol) were smaller than that of free enzyme (190.7 kJ/mol), indicating that immobilized β-glucosidase was more temperature insensitive. The half-life of immobilized β-glucosidase was 3~10 times longer than that of free enzyme at 50-70^{o}C. The maximum rate of the given catalytic reaction (V_{max}) using dried-composite immobilized enzyme were 8.2 and 1.2 times larger than those for wet immobilized and free enzymes, respectively. The present work demonstrated a promising application potential of such chitosan-clay composite beads for enzyme immobilization.

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