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Novel and efficient method for immobilization and stabilization of β-D-galactosidase by covalent attachment onto magnetic Fe_3O_4–chitosan nanoparticles

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ABSTRACT

A novel and efficient immobilization of β-D-galactosidase from *Aspergillus oryzae* has been developed by using magnetic Fe_3O_4–chitosan (Fe_3O_4–CS) nanoparticles as support. The magnetic Fe_3O_4–CS nanoparticles were prepared by electrostatic adsorption of chitosan onto the surface of Fe_3O_4 nanoparticles made through co-precipitation of Fe^{2+} and Fe^{3+}. The resultant material was characterized by transmission electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, vibrating sample magnetometry and thermogravimetric analysis. β-D-Galactosidase was covalently immobilized onto the nanocomposites using glutaraldehyde as activating agent. The immobilization process was optimized by examining immobilized time, cross-linking time, enzyme concentration, glutaraldehyde concentration, the initial pH values of glutaraldehyde and the enzyme solution. As a result, the immobilized enzyme presented a higher storage, pH and thermal stability than the soluble enzyme. Galactooligosaccharide was formed with lactose as substrate by using the immobilized enzyme as biocatalyst, and a maximum yield of 15.5% (w/v) was achieved when about 50% lactose was hydrolyzed. Hence, the magnetic Fe_3O_4–chitosan nanoparticles are proved to be an effective support for the immobilization of β-D-galactosidase.

1. Introduction

β-D-Galactosidase (EC3.2.1.23), commonly known as lactase, has been received particular interest because it can mediate the transgalactosylation reaction for the preparation of galactooligosaccharides (GOS) as well as the hydrolytic reaction of lactose for the preparation of low-lactose milk [1,2]. With the enhancement of health awareness, it has become a pressing task for the manufacturers to produce low-lactose milk and GOS in a convenient and inexpensive way. However, the use of both native and soluble enzyme is limited by economic consideration due to their reuse impossibility [3,4]. Such drawback may be overcome by using immobilized enzyme as biocatalysts that can be easily removed from the reaction medium [5,6]. Therefore, various methods for the immobilization of β-D-galactosidase such as adsorption, covalent coupling and cross-linked aggregation have been developed [7–14]. However, most of the works in the literature are related to the hydrolytic reaction of lactose, as requirements for β-galactosidase-mediated transglycosylation are altogether different. Generally, the reaction conditions for the synthesis of GOS should be those favoring transgalactosylation or reverse reaction, namely high lactose concentration, elevated temperature and low water activity in the reaction medium [15,16].

With the development of nanotechnology, the magnetic nanoparticles for the immobilization of enzymes have been paid increasing attention due to their large specific surface area and easy separation from reaction mixture by magnetic field [17,18]. Although the protective effect of porous support, such as preventing inactivation by gas bubbles, is lost in non-porous supports [19–22], these non-porous supports may present some unique advantages, such as no external diffusion [23].

To date, magnetic nanoparticles have been used for the immobilization of many enzymes, such as lipase, protease, glucoamylase, α-amylase, penicillin G acylase and glucose oxidase [23–30]. Nevertheless, a crucial physical property of magnetic nanoparticles is their tendency to aggregate. Magnetic Fe_3O_4 nanoparticles tend to aggregate in liquid media due to the strong magnetic dipole–dipole attractions between particles. Thus, some biocompatible and biodegradable polymers with specific functional groups have been used as stabilizer to modify and increase their stability [31]. Chitosan (CS), known as 2-amino-2-deoxy-(1 → 4)-β-D-glucopyranan, is considered to be one of the most widely distributed biopolymers. Moreover, it is a cationic, non-toxic, biodegradable and biocompatible polyelectrolite. Therefore, it has been extensively investigated for potential applications in the food, cosmetics, pharmaceutical and biotechnological fields [32,33]. The combination of magnetic

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material and CS has been reported and applied in many fields, ranging from affinity isolation of enzyme [34], protein separation [35], cells separation [36,37] to enzyme immobilization [38,39]. Meanwhile, the presence of amino groups turns CS into a good option for the enzyme immobilization through glutaraldehyde technique. Glutaraldehyde activation of supports is one of the most popular techniques to immobilize enzymes [40,41]. The methodology is quite simple and efficient. Furthermore, in some instances, it allows to improve enzyme stability by multipoint immobilization. Multipoint covalent attachment promotes a rigidification of the molecular structure of the immobilized enzyme, which can reduce the conformational change induced by any distorting agent (heat, organic solvents, extreme pH values), thus greatly increase the enzyme stability [20,42,43].

Herein, the preparation and characterization of magnetic Fe_3O_4–CS nanoparticles, immobilization of β-D-galactosidase from *Aspergillus oryzae* and its preliminary application for GOS synthesis are reported in detail. To the best of our knowledge, the immobilization of β-D-galactosidase onto Fe_3O_4–CS nanoparticles is rarely reported.

2. Materials and methods

2.1. Materials

β-D-Galactosidase from *A. oryzae*, lactose, *o*-nitrophenyl-β-D-galactopyranoside (ONPG) and sodium tripolyphosphate (TPP) were purchased from Sigma–Aldrich. CS (molecular mass 100 kDa, degree of deactylation 90%) was obtained from Golden-shell Biochemical Co. Ltd. (Hangzhou, China). Glutaraldehyde (GA, 25% in v/v, aqueous solution), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$, >99%), ferrous sulfate ($FeSO_4{\cdot}7H_2O$, >99%) and ammonium hydroxide (29.4%) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). All the other chemicals used were of analytical grade.

2.2. Preparation of magnetic Fe_3O_4 nanoparticles

Fe_3O_4 nanoparticles were prepared by co-precipitating of Fe^{2+} ($FeSO_4{\cdot}7H_2O$) and Fe^{3+} ($FeCl_3{\cdot}6H_2O$) ions in ammonia solution with N_2 as the protective gas. Briefly, the ferric and ferrous salts (molar ratio 1:1) were dissolved in deionized water at a concentration of 0.3 M iron ions. Chemical precipitation was achieved by adding NH_4OH solution (29.4%) with vigorous stirring at 25 °C, and the final pH was maintained at about 10. After incubation for 30 min at 80 °C, the mixture was cooled to room temperature with stirring, and the resulting magnetic Fe_3O_4 nanoparticles were separated magnetically and washed by deionized water for three times to remove the excess ammonia.

2.3. Preparation of Fe_3O_4–CS nanoparticles

For preparation of Fe_3O_4–CS nanoparticles, the Fe_3O_4 nanoparticles were dispersed in 100 mL of 4 mg/mL CS solution (1% acetate solution, pH was adjusted to 4), and a solution of TPP (50 mL, 0.5 mg/mL) was added simultaneously. The suspension was treated by ultrasonic for 30 min to ensure the nanoparticles coated evenly. The resulted Fe_3O_4–CS nanoparticles were subsequently recovered from the mixture by placing on a permanent magnet, washed with deionized water for three times, freeze-dried and stored at 4 °C for future use.

2.4. Characterization of Fe_3O_4–CS nanoparticles

The average particle size, size distribution and morphology of the nanoparticles were investigated using a high performance digital imaging TEM machine (JEOL H-7650, Hitachi High-Technologies Co., Japan). A drop of well-dispersed nanoparticle dispersion was placed onto amorphous carbon-coated 200 mesh copper grid, followed by drying the sample at ambient temperature. Once evaporated, the sample was loaded into the microscope for imaging. Each sample was prepared at the same concentration. The accelerating voltage used was 100 kV and the images were taken on a Gatan electron energy loss spectrometry system using 6 eV energy slit.

The crystal structure of the nanoparticles was obtained with an X-ray diffractometer (XRD, Thermo ARL X'TRA) using a monochromatized X-ray beam with nickel-filtered Cu Kα radiation. A continuous scan mode was used to collect 2θ data from 5° to 80°. The magnetic properties of nanoparticles were characterized with a vibrating sample magnetometer (VSM, ADE EV5) by measuring the applied field dependence of magnetization between −14,000 and 14,000 Oe at 300 K.

Fourier transform infrared (FT-IR) spectra of CS, Fe_3O_4 nanoparticles and Fe_3O_4–CS nanoparticles were recorded by the potassium bromide pellet method on Tensor-27 (Bruker, Germany) in the range of 400–4000 cm^{-1}. The CS content in Fe_3O_4–CS nanoparticles was determined by a thermogravimetric analyzer (TGA, NETZSCH STA 449C, Germany) with a heating rate of 10 °C/min from 30 to 700 °C in air.

2.5. Immobilization of β-D-galactosidase on Fe_3O_4–CS nanoparticles

The immobilization was carried out by a typical GA activation procedure. In order to investigate the influences of immobilization conditions on the immobilization efficiency and catalytic activity, variables and their levels selected were GA concentration (0%, 2%, 4%, 6%, 8%, v/v), enzyme concentration (0.3, 0.4, 0.5, 0.6, 0.7 mg/mL), cross-link time (1, 2, 3, 4, 5, 8 h), immobilization time (1, 2, 3, 4, 5 h), enzyme solution pH (3, 4, 5, 6, 7, 8), and GA solution pH for activation (3, 4, 5, 6, 7) [40,44]. Briefly, magnetic Fe_3O_4–CS nanoparticles (50 mg) were redispersed in 30 mL GA solution with a shaking for a period of time at 25 °C. The nanoparticles were then separated by magnetic decantation and washed several times with deionized water. An enzyme solution (5 mL) was added to the nanoparticles. At the end of binding, the magnetic nanoparticles bound with β-D-galactosidase were collected and washed several times with phosphate buffer (pH 7, 50 mM). The supernatant was used for protein determination according to the reported method [45]. The immobilized amount of protein was calculated by subtracting the protein recovered in the supernatant from the protein subjected to immobilization.

2.6. Assay of β-D-galactosidase activity

The activity of β-D-galactosidase was determined using ONPG as substrate according to our previous reported method [46]. The reaction was carried out in 5.0 mL HAc–NaAc buffer (50 mM, pH 4.5) containing 2.0 mM ONPG and an appropriate amount of enzyme at 37 °C for 10 min. The reaction was stopped by adding 2.0 mL of 1.0 M Na_2CO_3 solution, and absorbance was recorded at 405 nm. One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol of ONPG/min. The activity of immobilized β-D-galactosidase was measured in a similar manner, except that magnetic nanoparticles were removed by magnetization before measurement. All activity and protein determinations were done at least in triplicate.

2.7. The stability of free and immobilized enzyme

Thermal stability was evaluated by incubating the free and immobilized enzyme in a water bath for 6 h at a temperature rang-

ing from 30 to 80 °C. The pH stability of free and immobilized enzyme was investigated by incubating enzyme at pH ranging from 3 to 8 for 6 h. The storage stability was evaluated by storing the immobilized enzyme at room temperature for 2 months, and possible enzyme leaching during the storage was also examined by measuring the activity and protein content of the supernatant solution at certain interval.

2.8. GOS synthesis

The synthesis of GOS with lactose as substrate was investigated with free and immobilized enzyme. To a solution of lactose (60%, w/v) dissolved in HAc–NaAc buffer (50 mM, pH 4.6) was added immobilized or free β-D-galactosidase from *A. oryzae* (4 U/mL). During the incubation at 40 °C, samples were withdrawn from the reactions at appropriate time intervals and terminated by heating at 100 °C for 5 min to inactivate the enzyme. The resulting samples were analyzed by high performance liquid chromatography (HPLC) with refraction index detector (RID) on an Agilent 1100 series HPLC system, which consisted of a G1311A quatpump, a G1362A RID, a G1379A degasser and a G1316A column oven. The separation was completed on a Sugar-D column (4.6 mm × 250 mm, Nacalai Tesque Inc., Japan) using acetonitrile–water (75:25, v/v) as the mobile phase at a flow rate of 1.0 mL/min [47]. Injection volume was 20 μL. Sugar compounds in the reaction mixture were identified by comparing the retention times with those of standard sugars.

2.9. Reusability assay

The reusability of immobilized β-D-galactosidase was performed as follows: the immobilized enzyme was incubated with lactose (60%, w/v) at 40 °C for 2 h, and then its activity was assayed as mentioned above after magnetic separation and washing with HAc–NaAc buffer. After the activity assay, the enzyme was separated and washed three times with HAc–NaAc buffer, and the fresh lactose solution was added to start the next round. The activity obtained each round was compared with that of the first run (activity defined as 100%).

3. Results and discussion

3.1. Preparation and characterization of Fe_3O_4–CS nanoparticles

3.1.1. Preparation of Fe_3O_4–CS nanoparticles

In the present study, the Fe_3O_4–CS nanoparticles were prepared by two steps summarized as the following. The first step was the synthesis of magnetic Fe_3O_4 nanoparticles by a co-precipitation method, and a schematic representation of the formation of Fe_3O_4 nanoparticles is as follows:

$$Fe^{2+} + 2Fe^{3+} + 8OH^- \rightarrow Fe_3O_4 + 4H_2O$$

The effect of molar ratio of Fe^{2+}/Fe^{3+} on saturation magnetization of nanoparticles was investigated. The saturation magnetization values were 73.7, 32.2 and 12.8 emu/g for Fe_3O_4 nanoparticles obtained with the molar ratio of 1:1, 1:1.5 and 1:2, respectively. Apparently, the suitable molar ratio of Fe^{2+}/Fe^{3+} for preparation of Fe_3O_4 nanoparticles was 1:1. As the reason, it might be due to that Fe^{2+} was easy to oxidize during the preparation, resulting in the alteration of molar ratio of Fe^{2+}/Fe^{3+}.

The second step was the coating of magnetic Fe_3O_4 nanoparticles with CS. The amino groups of CS would protonate into cationic charges (NH_4^+) in condition of pH 4. Therefore, the CS could be adsorbed onto magnetic Fe_3O_4 nanoparticles easily by electrostatic attraction, affording the magnetic Fe_3O_4–CS nanoparticles [48]. Meanwhile, the adding of TPP might cross-link the adsorbed CS through the ionic interactions between the positively charged amino groups and negatively charged counterion, tripolyphosphate. Such cross-link can reduce the solubility of chitosan in aqueous solvents over a broad pH range, and increase resistance to chemical degradation [49].

3.1.2. Characterization of Fe_3O_4–CS nanoparticles

The size, structure and magnetic property of nanoparticles were characterized by TEM, XRD and VSM. In addition, the binding of CS to magnetic Fe_3O_4 nanoparticles was confirmed by FT-IR and TGA.

As shown in Fig. 1, the magnetic Fe_3O_4 nanoparticles had a tendency to aggregate, which might be caused by the coercive forces of magnetite particles. As CS was employed in the preparation of composites, the obtained magnetic Fe_3O_4–CS nanoparticles were almost spherical or ellipsoidal with a mean particle size of 30 nm. They were small in size and exhibited excellent dispersibility, which might be related to the coated CS enhancing the repulsion of magnetite particles. These results are similar to those obtained by Zhu et al. [48]. With this improved dispersibility, the nanocomposites can disperse better in the substrate solution, possibly improving the immobilization efficiency of the enzyme.

For XRD analysis, six characteristic peaks for Fe_3O_4 (2θ = 30.08, 35.42, 43.08, 53.56, 56.98 and 62.62) marked by their indices ((2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1) and (4 4 0)) were observed for both samples. These peaks were consistent with the database in JCPDS file (PDF No. 65-3107), which indicated the resultant nanoparticles were pure Fe_3O_4 with a spinel structure [50]. In addition, the results suggested that the coating with CS process did not cause the phase change of Fe_3O_4.

For the magnetic properties of Fe_3O_4 and Fe_3O_4–CS nanoparticles, the value of saturation magnetization for Fe_3O_4–CS nanoparticles (64.5 emu/g) was slightly lower than that (73.7 emu/g) for the naked ones. The decrease of saturation magnetization might be resulted from the binding of CS to the surface of the naked particles. However, both were able to be absorbed on the wall of a test tube by applying a magnetic field within 10 s in our current experiment. The results indicated that the modification with CS did not influence the magnetic separation of nanoparticles.

The binding of CS to the surface of magnetic Fe_3O_4 nanoparticles was confirmed by FT-IR analysis. Fig. 2 shows the FT-IR spectra of CS (a), the naked Fe_3O_4 (b), and Fe_3O_4–CS nanoparticles (c). For the naked Fe_3O_4, the characteristic absorption peak for Fe_3O_4 was observed at 576.83 cm^{-1} (Fe–O). For the FT-IR spectrum of CS, the characteristic absorption band appeared at 1630.71 cm^{-1} corresponding to the bending vibration of N–H. In the spectrum of Fe_3O_4–CS nanoparticles, peak of N–H bending vibration shifted to 1630.8 cm^{-1} compared with the spectra of CS, and a new sharp peak at 581.31 cm^{-1} related to Fe–O group appeared. It could be noted that the peak at 576.83 cm^{-1} in naked Fe_3O_4 shifted to a higher wavenumber (581.31 cm^{-1}) in Fe_3O_4–CS nanoparticles, revealing the interaction between Fe_3O_4 and CS. Similar result was also reported by Zhu et al. [48]. The results indicated that CS was bound successfully to the magnetic Fe_3O_4 nanoparticles.

The TGA results of the Fe_3O_4 and Fe_3O_4–CS nanoparticles are shown in Fig. 3A and B. For the naked Fe_3O_4, one peak appeared in the differential scanning calorimetric (DSC) curve (Fig. 3B (a)) from 30 to 300 °C, which was caused by the removal of absorbed physical and chemical water. Also, the TGA curve (Fig. 3A (a)) showed that the weight loss from 30 to 700 °C was about 1.3%. For Fe_3O_4–CS nanoparticles (Fig. 3B (a)), besides the peak below 200 °C caused by the loss of residual water, another sharp one appeared at 600 °C and the weight loss was significant, which was related to the degradation of CS. After that, there was no significant weight change,

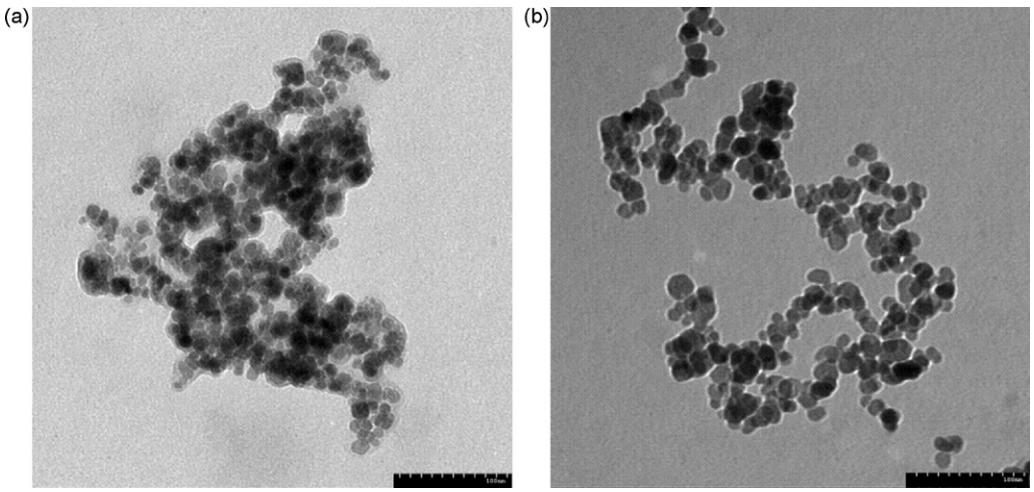

Fig. 1. TEM micrographs for the naked (A) and chitosan coated Fe_3O_4 nanoparticles (B).

implying the presence of only iron oxide within the temperature range. From the percentage of weight loss in the TGA curve (Fig. 3A (b)), the weight loss of Fe_3O_4–CS nanoparticles was 6.5%. Accordingly, the amount of CS bound to the surface of Fe_3O_4 nanoparticles was 5.2%.

3.2. Immobilization of β-D-galactosidase

Although the preparation of carrier is quite important for the immobilization process as well as the performance of immobilized enzyme, the selection of immobilization conditions is also critical for immobilized enzyme to exhibit its maximal activity. Therefore, six factors affecting the immobilization of β-D-galactosidase were investigated in the present study.

3.2.1. Effect of the concentration of GA

In the present study, GA was used as the cross-linking agent since the aldehyde group of GA can interact with both the amino group of CS and enzyme, thus making the enzyme immobilized onto the magnetic composites. To determine the influence of GA concentration, experiment was performed at the following condition: pH of enzyme 5, pH of GA 7, enzyme concentration 0.5 mg/mL, cross-linking time 4 h, immobilize time 3 h, and different GA concentration of 0, 2, 4, 6, 8%. As shown in Fig. 4A, the amount of immobilized enzyme increased with the increase of GA concentration up to 6%. Without GA, the enzyme could be immobilized on the carrier through physical adsorption. However, the immobilized enzyme, in this way, is less stable than that cross-linked by GA, which has been confirmed in previous studies of Batancor et al. [40] and L'opez-Gallego et al. [51]. Moreover, more GA dimer could be formed with the increase of GA concentration [40,52]. The increasing GA dimers made the carrier more reactive [40], so the immobilization yield got improved. However, immobilization efficiency changed slightly with the further increase of GA concentration. It could be explained that the enzyme immobilization has already reached its maximal and the surface of the composites was saturated with enzyme. The relative activity did not change greatly as GA concentration increased from 0 to 4%, but it decreased sharply after that. This decreasing activity might be

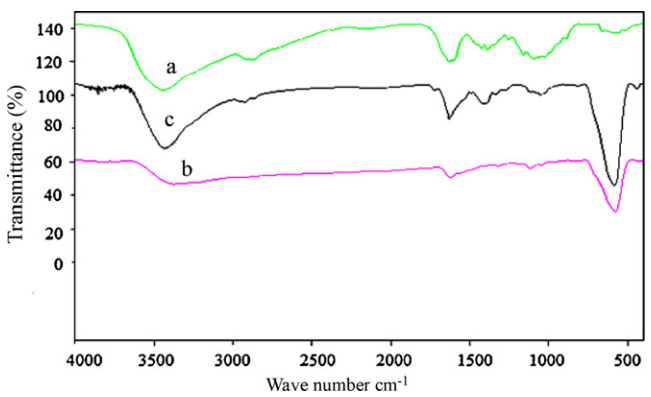

Fig. 2. FT-IR spectra of chitosan (a), Fe_3O_4 (b), and Fe_3O_4–chitosan nanoparticles (c).

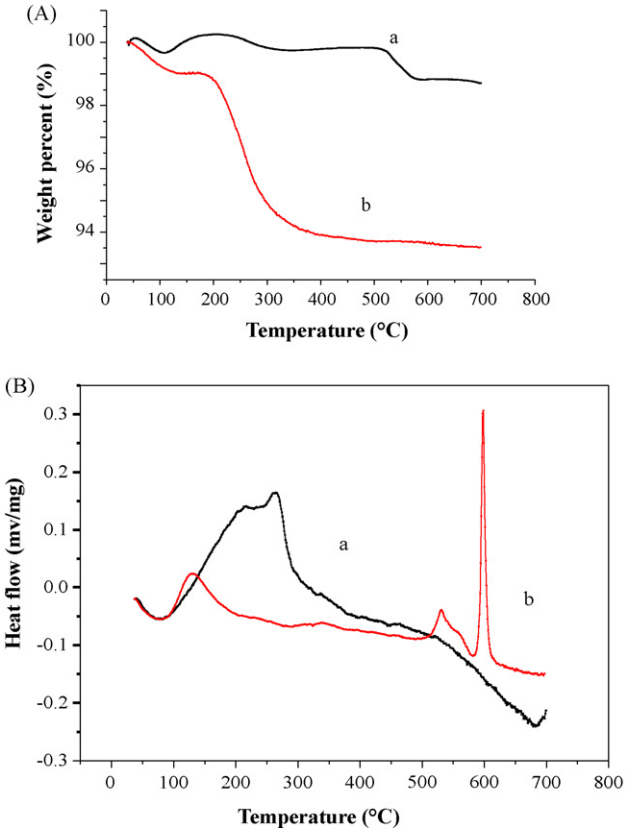

Fig. 3. (A) Thermogravimetric analysis of the naked (a) and chitosan coated Fe_3O_4 nanoparticles (b); (B) differential scanning calorimetry analysis of the naked (a) and chitosan coated Fe_3O_4 nanoparticles (b).

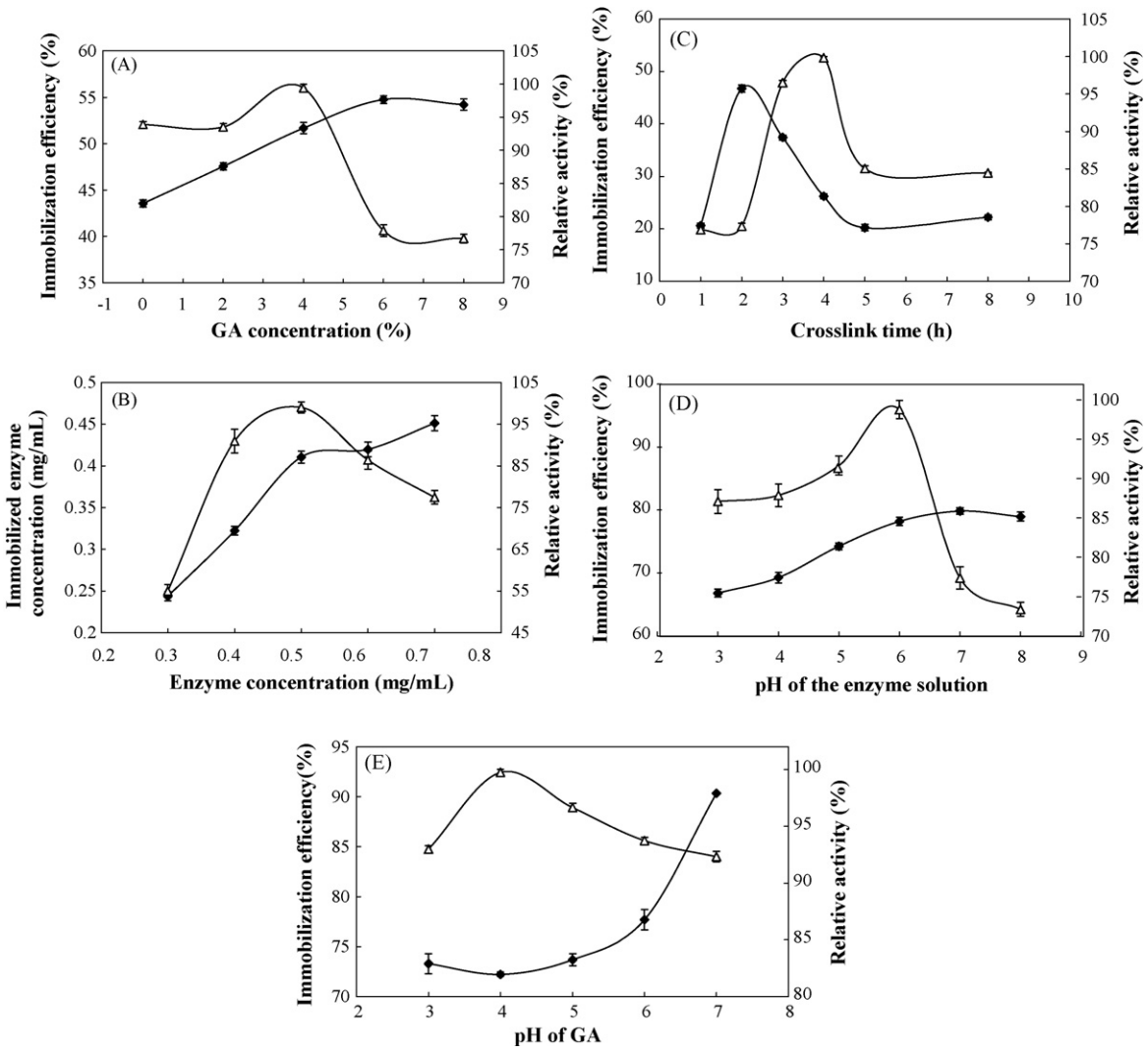

Fig. 4. Effects of immobilization conditions on the immobilization efficiency (♦) and relative activity (△) of β-D-galactosidase. Glutaraldehyde concentration (A), enzyme concentration (B), cross-linking time (C), the initial pH value of enzyme solution (D), and the initial pH value of glutaraldehyde solution (E).

caused by the conformational changes of enzyme resulted from the extensive interaction with GA [53]. Similar results have also been reported by Jiang et al. [54] and Chang and Juang [55]. Meanwhile, in order to demonstrate the influence of GA form on the activity of immobilized β-D-galactosidase, supports with GA monomer and GA dimer were prepared according to the method of Bentancor et al. [40]. After immobilization in GA dimer supports, 20% activity was lost compared to GA monomer supports. Therefore, the decrease of enzyme activity might also be attributed to the increase of GA dimers [42].

3.2.2. *Effect of the concentration of enzyme*

In order to investigate the effect of enzyme concentration, enzyme solution with different concentrations ranging from 0.3 to 0.7 mg/mL was used with other fixed parameters (pH of enzyme 5, pH of GA 7, 4% GA, cross-linking time 4 h, immobilize time 3 h). As shown in Fig. 4B, the immobilization efficiency increased with the increase of enzyme concentration. The phenomenon might simply be explained by the fact that in the solution with higher enzyme concentrations, the chances of their attachment with reactive GA increased, resulting in enhancement of enzyme immobilization. For the relative activity, it reached its maximal at an enzyme concentration of 0.5 mg/mL. After that, it decreased sharply. The decreasing activity might be related to the hidden of some active sites caused by the aggregation of enzyme at high concentration [54].

3.2.3. *Effect of cross-linking time*

The cross-linking time was varied from 1 to 8 h while the other parameters were fixed (pH of enzyme 5, pH of GA 7, 4% GA, 0.5 mg/mL enzyme, immobilize time 3 h). According to Fig. 4C, the maximal amount of enzyme immobilization was achieved when incubation of the carrier with GA was conducted for 2 h. This phenomenon was related to the amount of reactive GA cross-linked on the carrier, which reached its maximal after 2 h incubation. With prolonged incubation, the immobilization efficiency declined rapidly, this might be caused by the instability of GA. The stability of GA would be influenced by higher temperature, oxygen, light or metal [56]. What's more, the GA-NH_2 reaction might conduct between supports, and the reactive GA would be reduced in this case. The release of linked GA from the overcrowded environment caused by external shaking force might be another cause. Besides, the relative activity got to the maximal when the cross-linking time was 4 h. It suggested again that the immobilized enzyme could exhibit its best catalytic activity only with proper immobilized amount. Furthermore, the decrease of activity might also be related to the increase of GA dimers which would appear with prolonged incubation [52].

3.2.4. Effect of immobilized time

To determine the optimal immobilized time, the immobilization was conducted at varied time with other parameters fixed (pH of enzyme 5, pH of GA 7, 4% GA, 0.5 mg/mL enzyme, cross-link time 4 h). The immobilized time had no significant influence on the immobilization efficiency and enzyme activity. The enzyme loading reached 87% within 1 h, and then remained nearly constant with prolonged incubation. This result was in accordance with that reported by Monsan [52], which also proved the immobilization process was swift. The enzyme activity showed a similar tendency, reaching its maximal and remaining unchanged at an immobilization time of 3 h.

3.2.5. Effect of the initial pH value of enzyme solution

The pH of enzyme solution can influence the amount of enzyme immobilized and its activity, so the effect of initial pH value of enzyme solution ranging from 3 to 8 was examined while other factors were fixed (pH of GA 7, 4% GA, 0.5 mg/mL enzyme, cross-linking time 4 h, and immobilize time 3 h). According to Fig. 4D, the optimal pH for immobilization was 6. Immobilization on Fe_3O_4–CS nanoparticles usually proceeds via a two-step immobilization mechanism: a rapid preliminary physical adsorption and then the covalent attachment [40,51]. Mildly acidic pH values, are considered to be unfavorable for the above-mentioned crosslinking reactions as the amino groups of the protein are likely to be protonated [57,58]. Meanwhile, the kinetics of GA cross-linking affected under acidic conditions is known to be slower than the kinetics of cross-linking under neutral or basic conditions [59], which also proved the negative effect of acidic pH values. Moreover, the reaction of the monomeric form of GA with ε-amine groups is considered to be unstable [57,59,60] at low pH. Thus the enhanced stability at higher pH also contributed to the increased immobilization efficiency. However, the activity decreased sharply when the initial pH value was above 6, which might be caused by the denaturation of enzyme at such pH value.

3.2.6. Effect of the initial pH value of GA solution

The immobilization efficiency and relative activity with the varying initial pH value of GA solution was investigated at pH 3, 4, 5, 6 and 7 while other factors were fixed (pH of enzyme 6, 4% GA, 0.5 mg/mL enzyme, cross-linking time 4 h, immobilize time 3 h). As shown in Fig. 4E, the immobilization efficiency increased with the increasing of initial pH value of GA solution. This phenomenon might be attributed to the different protonation degree of amino group under different pH environment. When the pH value was low, the amino group of CS existed as $–NH_3^+$ or $–NH_2^+$, which would inhibit its cross-link interaction with the aldehyde group of GA. Hence, the cross-linked amount of GA reduced with the decrease of pH value, which would directly influence the amount of enzyme immobilized. This was in consistent with the previous reports by Monsan [52] and Agarwal and Gupta [61]. They claimed that a lower pH of reactive medium interfered negatively in the course of the GA–NH_2 reaction and concluded that the best condition of reactivity was connected to neutral or basic media. Furthermore, the amount of GA dimer caused by the polymerization of GA monomer would increase at higher pH [57,59,60], which also improve the immobilization efficiency [40,52]. The optimal pH for the enzyme to perform its activity was 4 in the present study. The reason might be that the immobilized enzyme was not so crowded or scarce at such pH, so that the enzyme could be in its optimal conformation and protect itself from denaturing. The relative small amount of GA dimer might also contribute to the result.

3.3. Properties of immobilized β-D-galactosidase

3.3.1. pH stability

The pH dependence of the free and the immobilized enzyme activity at pH ranging from 3 to 8 at 25 °C was investigated (Fig. 5A). When the pH was between 3 and 4, they exhibited almost the same activity. With the increase of pH, the free enzyme maintained almost the same activity until pH 7. In contrast, the relative activity of immobilized enzyme increased and reached its maximal at pH 6. At pH of 8, the relative activity of free enzyme was 26%, while was 55% for the immobilized one. The enhanced stability in alkaline condition might be caused by multipoint covalent immobilization which seems to be one of the most promising possibilities to improve enzyme stability. By multipoint covalent attachment, the configuration of β-D-galactosidase was fixed on the surface of carriers so the tolerability of enzyme to pH variance in surroundings increased [20,42,43]. What's more, nanoenvironment of the immobilized enzyme on the carrier could have been buffered [62], which also improved the enzyme stability. Below pH 5.4, the isoelectric point (p*I*) value of chitosan, protonation of the $–NH_2$ group on the C-2 position of the D-glucosamine repeat unit occurred. Then hydroxyl in the surrounding would cluster around the carriers with positive charged chitosan. Therefore, the pH in the remainder of the solution decreased. Above pH 5.4, opposite behavior would occur [63,64].

3.3.2. Thermal stability

The effect of temperature on the native and the immobilized β-D-galactosidase activity was investigated. As shown in Fig. 5B, the immobilized β-D-galactosidase was less sensitive to the change of temperature than the free enzyme as the reaction temperature ranged from 50 to 60 °C. The immobilized enzyme maintained almost 65% of its relative activity, while the free enzyme just kept 35%. As was evident from the data, the immobilized enzyme possessed a better heat-resistance than free one. These results would also be related to the improved stabilization by the multipoint cova-

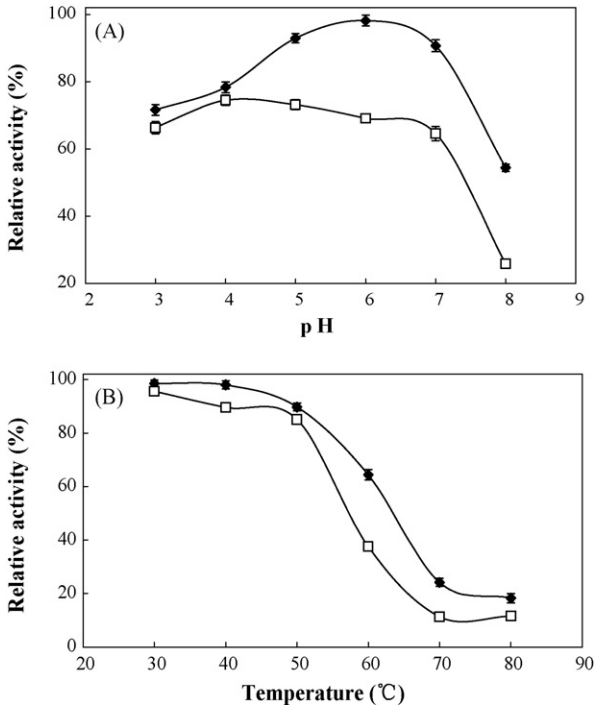

Fig. 5. Characterizations of the immobilized β-D-galactosidase. Effect of pH on the activity of free (□) and immobilized enzyme (♦) (A), thermal stability of free (□) and immobilized enzyme (♦) (B).

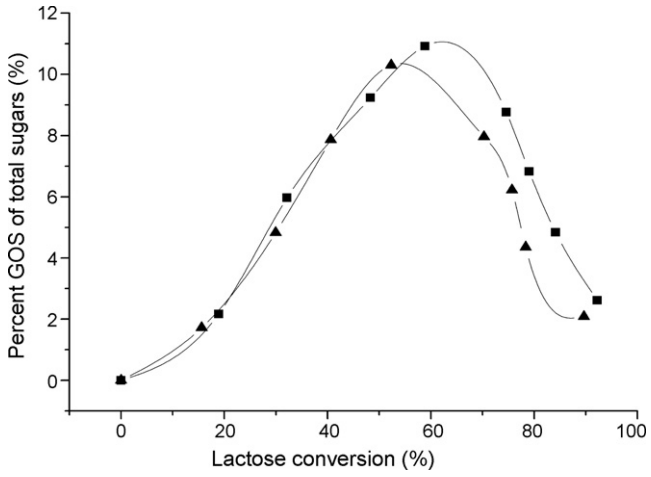

Fig. 6. GOS formation by free (■) and immobilized (▲) β-D-galactosidase.

lent binding between β-D-galactosidase molecule and the support. The immobilized enzyme maintained a greater rigidity and was more resistant to unfolding at higher temperatures than its free form [62–64]. Therefore, the immobilized enzyme could work in harsh environmental conditions with less activity loss compared to its free counterpart [65–67].

3.3.3. Storage stability

The immobilized β-D-galactosidase was stored in HAc–NaAc buffer (0.05 M, pH 4.6) at room temperature and the activity measurements were carried out every 5 days for total 60 days. The immobilized enzyme lost 12% of its initial activity during storage. β-D-Galactosidase immobilized on Fe_3O_4–CS nanoparticles exhibited higher stability than those immobilized on magnetic poly (GMA–MMA) beads [11] and concanavalin, a layered calcium alginate–starch beads [1]. Therefore, the results indicated that the multipoint covalent immobilization on Fe_3O_4–CS nanoparticles definitely kept the enzyme in a stable position.

3.4. Preliminary application of the immobilized enzyme

For preliminary application of the immobilized β-D-galactosidase, the synthesis of GOS by free and immobilized enzyme at 40 °C with lactose (60%, w/v) as substrate was studied. For the free enzyme, the maximum GOS production (16%, w/v) was achieved when about 60% lactose was converted, while for the immobilized enzyme, the maximum yield of 15.5% (w/v) was achieved when about 50% lactose was hydrolyzed (Fig. 6). Both reactions presented the bell-shaped curve due to the balance between transgalactosylation and hydrolytic reaction. The results suggested that the enzyme immobilization on the magnetic Fe_3O_4–CS nanoparticles did not impose any diffusion limitation or alteration on GOS formation from lactose. Similar results were obtained by Neri et al. [12].

3.5. Reusability

The operational stability of the immobilized β-D-galactosidase was evaluated through the repeated process. The immobilized enzyme retained 92% of its initial activity after successively utilization for 15 cycles. Apparently, the immobilized enzyme was quite stable on the surface of the nanocomposites. Therefore, it should be noted that this high operational stability could significantly reduce the operation cost in practical application.

4. Conclusions

Simple and efficient procedures were developed for the preparation of magnetic Fe_3O_4–CS nanoparticles by the electrostatic attraction of CS onto the surface of Fe_3O_4 particles and the immobilization of β-D-galactosidase via GA coupling reaction. The immobilized β-D-galactosidase showed the same or even higher activity in wider ranges of temperature and pH than that of its free form. In addition, the immobilized enzyme could be stored for a long time with little activity loss. Furthermore, the immobilized enzyme retained 92% of its initial activity after successively utilization for 15 cycles. Taking the above into consideration, magnetic Fe_3O_4–CS nanoparticles proved to be an efficient support for β-D-galactosidase immobilization and wide application including GOS production from lactose. Further works are in progress.

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