Journal of Molecular Catalysis B: Enzymatic 57 (2009) 62–66

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Immobilization of lipase on hydrophobic nano-sized magnetite particles

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ARTICLE INFO

Article history:
Received 9 January 2008
Received in revised form 20 June 2008
Accepted 23 June 2008
Available online 1 July 2008

Keywords:
Nano-sized magnetite
Porcine pancreas lipase
Immobilization
Olive oil
Hydrolysis

ABSTRACT

As a tool for the stable enzyme reuse, enzyme immobilization has been studied for several decades. Surface-modified nano-sized magnetite (S-NSM) particles have been suggested as a support for the immobilization of enzyme in this study. Based on the finding that a lipase is strongly adsorbed onto a hydrophobic surface, NSM particles (8–12 nm) were made hydrophobic by binding of sodium dodecyl sulfate via a sulfate ester bond. Various types of measurements, such as transmission electron microscopy, X-ray diffraction, infrared spectroscopy, vibration sample magnetometer, and thermo gravimetric analysis, were conducted in characterizing S-NSM nanoparticles. S-NSM particles were used for the adsorption of porcine pancreas lipase (PPL). A dodecyl carbon chain is expected to form a spacer between the surface of the NSM and the lipase adsorbed. The immobilized PPL showed the higher specific activity of oil hydrolysis than that of free one. Immobilized PPL could be recovered by magnetic separation, and showed the constant activity during the recycles.

1. Introduction

The use of nanophase materials offers many advantages due to their unique size and physical properties. Hybrid nanoscale materials have been used in various bioprocesses, such as nucleic acid detachment [1], protein separation [2], and enzyme immobilization [3]. For more than two decades, nano-sized magnetite (NSM) particles have received great attention [4,5] for such biotechnological applications as sorting or separating cells, proteins, and DNA [6–8], medical diagnosis [9], and controlled drug delivery [10,11]. These applications exploit the paramagnetic properties of NSMs; however, the efficient binding or loading of biomolecules requires the surface functionalization by polymerization [12–15] or sol–gel entrapment [16]. However, such processes may reduce the magnetic response of NSMs. Alternative methods for high NSM content, such as fine synthesis engineering [17–20], and wrapping NSM particles with polymers, such as PEG (polyethylene glycol), copolymers of styrene-acrylic, or oligomers of polysaccharides, have also been suggested. However, the intentional increase of NSM content results in increased particle size, thus limiting their use to some biochemical processes. In contrast, NSM particles coordinated with a low molecular weight ligand are believed to be free of the aforementioned defects.

Lipases (E.C. 3.1.1.3) from different sources are currently used in enzymatic organic synthesis [21,22]. The expanding interest in lipase mainly lies on its wide industrial applications, including detergent formulation, oils/fats degradation, pharmaceuticals synthesis, and cosmetics production [23]. In order to use lipase more economically and efficiently in aqueous as well as in non-aqueous solvents, their activity and operational stability needs to be improved by immobilization.

Immobilization of enzymes, the restriction of enzyme mobility in a fixed space, provides important advantages, such as enzyme reutilization and elimination of enzyme recovery, and purification processes [24]. And it may provide a better environment for enzyme activity. Since enzymes are expensive, catalyst reuse is critical for many processes. Product purity is usually improved, and effluent handling problems are minimized by immobilization. In addition, the enzyme immobilization on to magnetic supports such as NSM particles allows an additional merit compared to other conventional support materials, which is the selective and easy enzyme recovery from the medium under the magnetic force. Hence there is no need for expensive liquid chromatography systems, centrifuges, filters or other equipment.

A few reports have proposed using magnetic nanoparticles directly anchored with a ligand as supports for enzyme immobilization. Acetylated or amino-functionalized γ-Fe_2O_3 magnetic nanoparticles with superparamagnetic properties have been reported [25]. Huang et al. [26] immobilized lipase onto NSM particles through carbodiimide activation. Covalent binding of an enzyme to a support material may be advantageous because it minimizes the amount of enzyme released from the support; however, this type of immobilization often results in structural changes that can greatly reduce the activity of the enzyme. Hence the other

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doi:10.1016/j.molcatb.2008.06.017

technique such as adsorption is needed to avoid this kind of problem [27].

In this study, NSM particles coordinated with low molecular weight ligands were employed as supports for enzyme immobilization via physical adsorption, rather than by covalent bonding. Direct contact of an enzyme to the surface of the magnetites may hinder the flexible enzyme structure. The ligand on the surface of the NSM is presumed to act as a spacer between NSM and the immobilized enzyme, thus mitigating this problem. Porcine pancreas lipase (PPL) was used as the model enzyme in this study. It has been reported that lipases show hyper activation when immobilized on the hydrophobic surface [28,29]. Hence sodium dodecyl sulfate (SDS) was used as the ligand for the preparation of hydrophobic NSM particles, as well as for the spacer between NSM and the immobilized lipase.

To our knowledge, SDS–NSM particles have never been used as a support material for enzyme immobilization through adsorption. Consequently, this work is intended to investigate the preparation of this novel material for enzyme immobilization and to evaluate the stability of these preparations with repeated use.

2. Materials and experimental methods

2.1. Materials

Crude porcine pancreas lipase (E.C. 3.1.1.3) and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich Co. The specific activity of the enzyme was 6.37 U/mg protein. All other materials were of analytical grade and used without further purification. These materials included ferric sulfate ($Fe_2(SO_4)_3{\cdot}nH_2O$), ferrous sulfate ($FeSO_4{\cdot}7H_2O$), ammonium oxalate ($(NH_4)_2C_2O_4$), aqueous ammonia (25%, w/w), olive oil, gum arabic, and ethanol.

2.2. Preparation of hydrophobic nano-sized magnetite

Scheme 1 presents the preparation of SDS-bound NSM particles. The first step is a simple chemical co-precipitation of Fe^{2+} and Fe^{3+} in a concentrated ammonium hydroxide solution (28 wt%) at pH 8–9. A combination of $FeSO_4{\cdot}7H_2O$ (Fe(II) sulfate), dehydrated $Fe_2(SO_4)_3$ (Fe(III) sulfate) and ammonium hydroxide was prepared as reagents with a stoichiometric molar ratio, Fe^{2+}:Fe^{3+}:$C_2O_4{}^{2-}$ = 1:2:0.1. This procedure was typically performed at 75 °C for 1.5 h, and the pH values were set at 9.0 to begin with and changed to 8.6 at the end [30]. Then, oxalate-modified NSM particles were prepared by adding NH_4OH to a solution containing ferric sulfate, ferrous sulfate, and ammonium oxalate at the molar ratio of 2:1:0.1 (pH 9) [29–31]. Oxalate on the particle surface was then replaced with SDS by mixing an aqueous suspension of oxalate-NSM with SDS at the molar ratio of 10:1 in an acidic environment (pH 5). All the reactions were conducted under a nitrogen atmosphere at 75 °C. NSM particles were washed at each step and separated by magnetic decantation.

2.3. Immobilization of lipase on S-NSM particles

For the immobilization of lipase, 200 mg of S-NSM particles were added to 0.85% (w/v) sodium chloride solution containing lipase. The mixture was incubated for 4 h using an over-head stirrer. After washing twice with 0.85% (w/v) sodium chloride solution, the immobilized lipase was separated by magnetic decantation of the supernatants and stored at 4 °C prior to being used. The amount of proteins adsorbed on the supports was determined by measuring the protein concentration of the lipase solution and the supernatant by the Lowry method.

2.4. Assay of enzyme activity

The lipase activity was determined by measuring the degree of oil hydrolysis. An oil emulsion was prepared by mixing 1% (w/v) olive oil (Sigma Chemical Co.) with an aqueous solution of NaCl (20 mM), $CaCl_2$ (1 mM), and gum arabic (1%, w/v) as an emulsifier. The oil hydrolysis reaction was initiated by adding the lipase-immobilized S-NSM particles to the oil emulsion. The reaction pH was maintained at 7.7. Degrees of oil hydrolysis were assayed by measuring fatty acids released from the oil hydrolysis by titration with 10 mM NaOH using a pH-stat titrator (718 Stat Titorino, Metrohm, Switzerland) [32]. One unit (U) of enzyme activity is defined as the amount of enzyme required to liberate a titratable amount of fatty acid equivalent to 1 μmol of NaOH in 1 min. After the reaction, S-NSM particles were separated using magnetic force and used for recycles.

Specific activity of the lipase was defined in this study as the enzyme activity (unit) divided by the protein content and expressed as U/mg protein. Relative specific activity was calculated by dividing the specific activity of the immobilized lipase by that of the free lipase.

2.5. Analytical methods

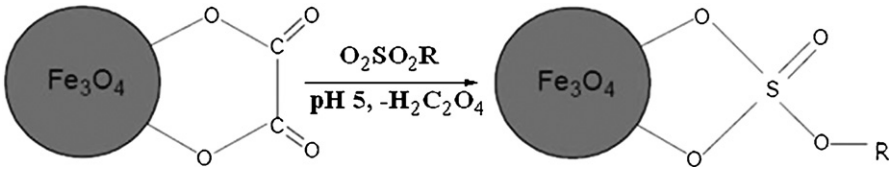
To check the crystallinity of magnetic nanoparticles, X-ray diffractometry (XRD) analysis was performed using a Rigaku X-Ray Diffractometer (model D/max-3A). The size and morphology of magnetic nanoparticles were assessed by high resolution TEM (HR-TEM) using a JEOL model JEM-3010 at 300 kV. The sample for HR-TEM analysis was obtained by placing a drop of the magnetic nanoparticle solution onto a copper grid and evaporating it at room temperature. The magnetic properties were evaluated using a vibration sample magnetometer (VSM; Lake Shore Model 7300). FT-IR analyses were performed on a Varian Excalibur Series. The TGA curves were taken using a thermo gravimetric analyzer from TA Instrument (model Q500) under nitrogen flow. The temperature studied ranged from 40 to 900 °C at a rate of 5 °C/min.

3. Results and discussion

3.1. Characterization of S-NSM particles

The XRD-patterns of our NSM particles were reported in the previous study [30]. Using Debye–Scherrer equation [33], the particle size of the S-NSM particles was calculated to be 12 nm, which was confirmed in the HR-TEM micrograph for S-NSM without the immobilized lipase shown in Fig. 1. Prior to lipase adsorption, S-NSM particles showed spherical or ellipsoidal shapes with a mean diameter of 10 ± 2 nm (Fig. 1a). After lipase adsorption, the degree of particle aggregation increased (Fig. 1b), which was same as the result of covalently immobilized NSM particles [25]; however, a change in particle size was not observed (Fig. 1b).

To verify the binding of SDS to the surface of nanoparticles, infrared spectroscopic analyses of as-synthesized S-NSM particles were performed and the result is shown in Fig. 2. Two peaks at ~595 and 653 cm^{-1} are the characteristic peaks for the Fe–O stretching bands. The peak at 1420 cm^{-1} resulted from the stretching vibration of the C–O bonds in CO_2, which might come from air. The vibration and asymmetric stretching of O–H bonds in water caused the peaks at 1612 cm^{-1} and around 3200–3600 cm^{-1}, respectively [30]. The peak at 2900 cm^{-1} could be assigned to the stretching vibration of C–H bonds in SDS, which indicates its binding to the surface of NSM particles. Fig. 2b shows the IR spectrum of S-NSM with the lipase immobilized. It is noted that the characteristic bands of a protein



Scheme 1. Schematic diagram of SDS-NSM preparation, where R is dodecyl group.

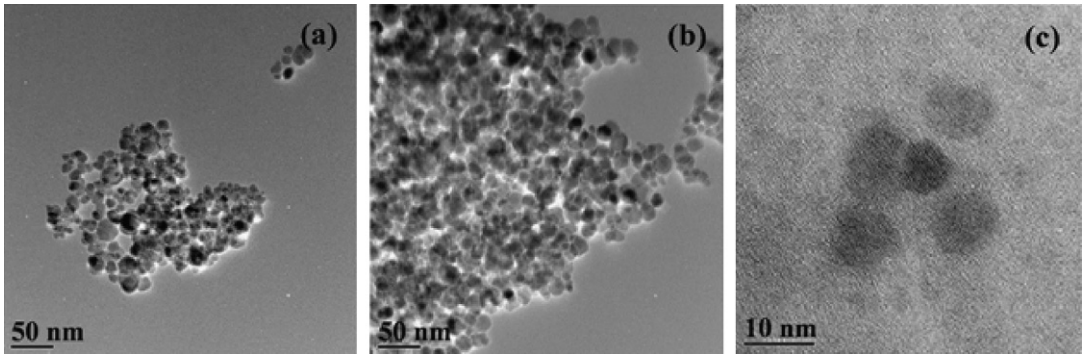


Fig. 1. High-resolution TEM images of S-NSM particles without (a and c) and with (b) immobilized lipase.

(i.e., lipase) at 1652 and 1535 cm^{-1} [26] reveals that the lipase is immobilized on the S-NSM particles.

The immobilization of lipase is further confirmed by TGA. The TGA curve of S-NSM without the lipase (Fig. 3a) shows the maximum weight loss of 3.5% at 200 °C, which is the loss of water molecules. The second weight loss occurred from 200 to 900 °C, which represents the loss of SDS attached on the surface of the NSM particles. In case of lipase immobilized S-NSM particles (Fig. 3b), the weight loss of 5.94% at 200 °C indicates the loss of lipase as well as water molecules. The weight difference mode above 200 °C was same regardless of the lipase immobilization. Therefore it can be concluded that the lipase was immobilized on S-NSM particles via physisorption.

The as-prepared S-NSM particles have a specific saturation magnetization (M_s) of 77 emu/g (Fig. 4), whereas that of commercial magnetic liquid is 123 emu/g [34]. The decrease in the saturation magnetization is most likely featured to the existence of surfactants on the surface of Fe_3O_4 nanoparticles [35].

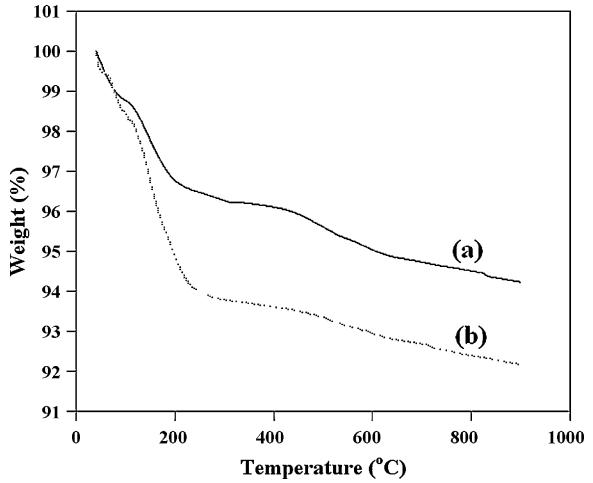


Fig. 3. TGA curves of S-NSM without (a) and with (b) immobilized lipase.

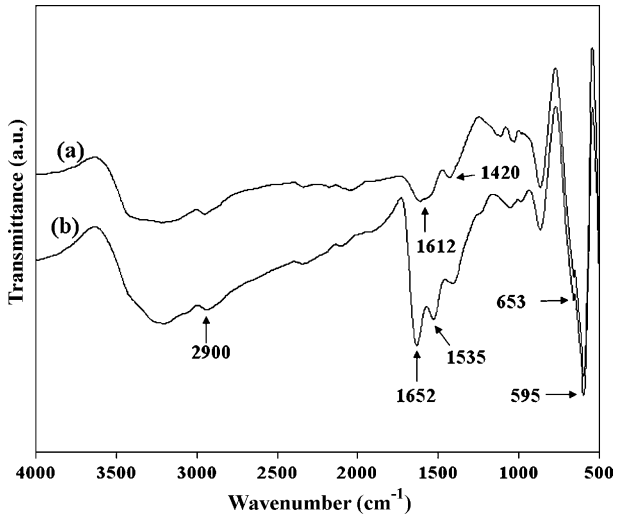


Fig. 2. FT-IR spectra of S-NSM without (a) and with (b) immobilized lipase.

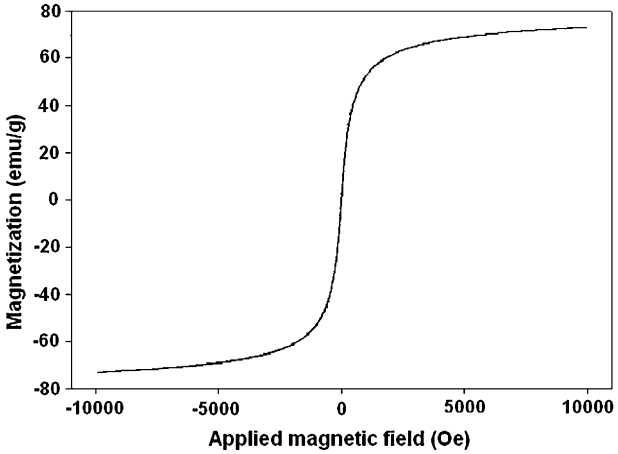


Fig. 4. Magnetization curve of S-NSM particles.

Table 1
Immobilization of PPL on S-NSM particles and its activity

Initial lipase concentration[a] (mg/ml)	Activity of immobilized PPL (μmol/min)	Protein immobilized (mg/g support)	Specific activity[b] (U/mg protein)
0.2	3.69	10.4	5.90
0.4	10.7	23.8	7.52
0.6	18.4	35.3	8.67
0.8	28.4	48.0	9.87
1.0	26.5	57.2	7.72
1.5	26.9	51.2	8.76
2.0	26.7	52.1	8.54

[a] Specific activity of the free lipase was 6.37 U/mg protein.
[b] Error limit ±5%.

3.2. Activity of the lipase immobilized on S-NSM particles

It is expected that the adsorption has been taking place between the hydrophobic carbon chain of the surfactant and hydrophobic area of the lipase via interfacial activation. To determine the optimum enzyme loading, various initial lipase concentrations (0.2–2 mg/ml) were tested for immobilization experiments. As shown in Table 1, the amounts of lipase immobilized increased significantly with the initial lipase concentration and the activities attained a maximum value at an initial lipase concentration of 0.8 mg/ml. The lipase activity is however leisurely decreased when the initial lipase concentration was above 0.8 mg/ml. The excessive enzyme loading is known to hinder the substrate conversion due to the increased protein–protein interaction [15,36]. In the rest of the experiments, the initial lipase concentration was maintained at 0.8 mg/ml.

The pH dependence of the native and the immobilized PPL activities was compared and shown in Fig. 5. The optimum pH of the immobilized lipase was 7.7, whereas that of the free one was 6.7. Also the immobilized lipase showed the higher activity than the free one especially at pH's higher than 7, which implies the lipase became more stable in alkaline conditions after immobilization onto S-NSM particles. The optimum pH value of free lipase shifted 1 unit to the alkaline region after adsorption on the support. This is because, upon immobilization the active site becomes more exposed to solvent than that in the folded-dissolved lipase form. Hence the proton transfer to the amino acid residues at the active site becomes less hindered [37]. The activity of immobilized enzyme was observed to be comparatively more stable than that of free enzyme. For example, at pH 8.7 free lipase shows 4.75 U/mg protein, whereas immobilized lipase shows 8.12 U/mg protein of its activity.

Activities of free and immobilized lipase were measured at various temperatures and the results are shown in Fig. 6. Free and immobilized PPL demonstrated the maximum activity at temperatures between 37 and 40 °C. Comparing the enzyme activities at the higher temperatures with the maximum activity, it can be concluded that the immobilization on S-NSM enhanced the thermal stability of PPL, which might be caused by multipoint attachment of the lipase to the support and/or by the hydrophobic interaction [38,39].

The immobilized lipase was recovered by magnetic separation and reused at least five times. Fig. 7a and b shows the change in the enzymatic activity with increasing usage at 37 and 50 °C, respectively. At 37 °C, PPL immobilized on S-NSM particles showed a significant loss in activity after the first use (Fig. 7a). However, its activity remained constant with the subsequent reuse. Enzymes immobilized to conventional micro-sized support materials through physical adsorption are known to be subject to desorption and exhibit the continuous decrease in the activity with the repeated use [32]. Oil hydrolysis was detected with the supernatant withdrawn after the first use. Hence it is concluded that some enzymes were desorbed from S-NSM during the first reaction. However, the constant PPL activity thereafter implies that a surface-modified NSM particle is a support material for enzyme immobilization superior to conventional micro-sized support materials. At 50 °C, immobilized PPL showed the continuous decrease in the enzyme activity with the repeated use possibly due to the enzyme denaturation at high temperature (Fig. 7b).

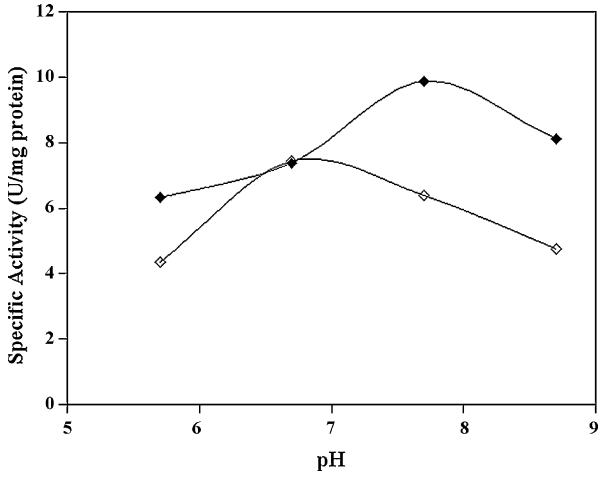


Fig. 5. Effect of pH on the activity of free (◊) and immobilized (♦) lipases.

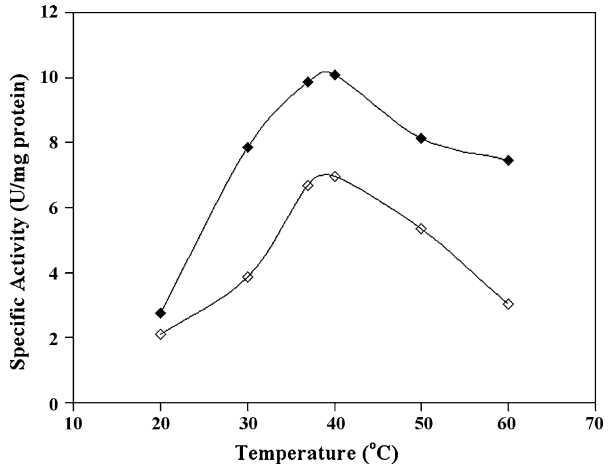


Fig. 6. Effect of temperature on the activity of free (◊) and immobilized (♦) lipases at pH 7.7.

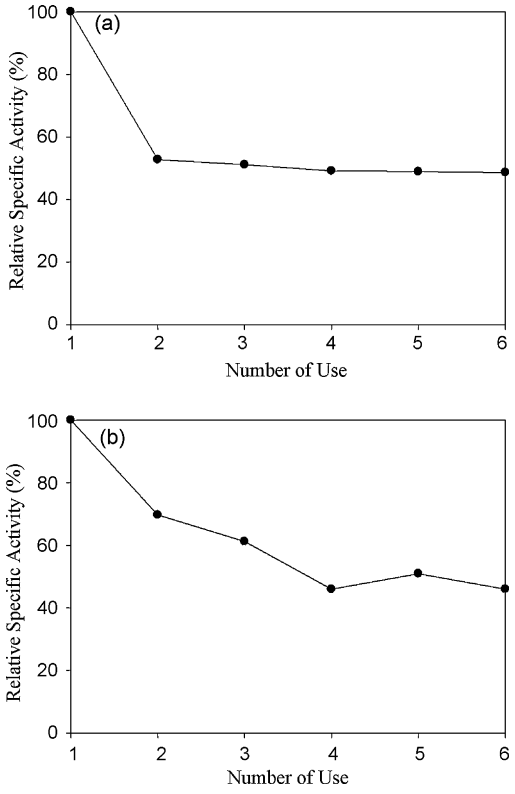


Fig. 7. Changes in the enzyme activities along the repeated use at 37 °C (a) and 50 °C (b).

4. Conclusion

This report describes the development of a novel hydrophobic magnetic nanoparticle for the lipase immobilization. Sodium dodecyl sulfate was used to generate a hydrophobic surface around the NSM particles and to create a spacer between the lipase and the NSM particles. The immobilized PPL showed the higher specific activity and thermal stability than the free one. After an initial drop following the first use at 37 °C, the activity of the immobilized PPL remained constant over the subsequent five uses and recoveries. The stable reuse as well as the convenience in the recovery offered by magnetic separation ensures that a surface-modified NSM particle is a good support material for enzyme immobilization, especially for the physical adsorption of enzymes.

Acknowledgment

The financial supports of the KOSEF (R01-2007-000-11570-0 and 2007-8-1158) are gratefully acknowledged.

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