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Immobilized *Aspergillus niger* Lipase with SiO_2 Nanoparticles in Sol-Gel Materials

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Abstract: Lipase from *Aspergillus niger* was "doubly immobilized" with SiO_2 nanoparticles in sol-gel powders prepared via the base-catalyzed polymerization of tetramethoxysilane (TMOS) and methyltreimethoxysilane (MTMS). The hydrolytic activity of the immobilized lipase was measured using the *p*-nitrophenyl palmitate hydrolysis method. The results showed that the optimum preparation conditions for the gels were made using a MTMS/TMOS molar ratio of 5, 60 mg of SiO_2 nanoparticles, a water/silane molar ratio of 12, 120 mg of enzyme supply, and 120 μL of PEG400. Under the optimal conditions, the immobilized lipase retained 92% of the loading protein and 94% of the total enzyme activity. Characteristic tests indicated that the immobilized lipase exhibited much higher thermal and pH stability than its free form, which shows great potential for industrial applications.

Keywords: sol-gel; SiO_2 nanoparticles; lipase; immobilization; stability

1. Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a group of widely used enzymes distributed in animals, plants, and microorganisms. They can catalyze a variety of reactions, such as esterification, interesterification, and hydrolysis. *Aspergillus niger* lipase is one of the most well-known lipases [1,2]. It displays positional selectivity for the 1- and 3-positions of the glycerol moiety, substrate preference for medium chain length fatty acids, an optimal temperature between 40 °C and 50 °C, an optimal pH from 4 to 7, and is considered GRAS (Generally Regarded As Safe) by the Food and Drug Administration (FDA) of the United States of America [3]. *A. niger* lipase has tremendous potential as a biocatalyst for industrial use with its diverse properties, especially its stability and selectivity. Thus, high-value products (pharmaceuticals, agrochemicals, aroma compounds, etc.) can be obtained [4]. Nowadays, *A. niger* lipase has been successfully used in many industrial applications, including the food industry, dairy industry, structured lipids synthesis, fat hydrolysis, detergent additive, regioselective deacylation, and the production of biodiesel [5–7].

However, in catalytic processes, lipases are highly unstable, and are easily inactivated by many physical and chemical factors. Many methods have been employed to overcome this defect, among which immobilization is one of the promising, as immobilization can promote engineering stability of the lipase and realize its reuse, thus greatly reducing its production costs [8,9]. So far, a few chemical and physical methods have been reported for lipase immobilization. Chemical methods include attaching lipase to a matrix by covalent binding, and physical methods include entrapment of the

lipase in an insoluble gel [10], porous fiber, or microcapsules, and adsorption of the enzyme on a support [11]. However, for *A. niger* lipase, most previous research studies describe its production, purification, crystallization, and applications, and there are few reports on its immobilization [12]. Osuna et al. immobilized *A. niger* lipase with chitosan-coated magnetic nanoparticles; however, the lipase activity was 1.7 times lower than for the free lipase [13].

It has been proven that sol-gel encapsulation is an effective and relatively easy way to immobilize enzymes [14]. The process involves the hydrolysis of the silane-precursor and cross-linking condensation with formation of a SiO_2 matrix, among which the enzyme is encapsulated [15]. Since 1996, the sol-gel production method has been gradually improved and has formed second-generation sol-gel matrixes with the use of a variety of additives [16]. The patent PI0306829-3 introduced a simple and general method to synthesize sol-gel matrices and was used extensively in research [17]. Ranyere et al. [18] have reported the affection of polyethylene glycol (PEG, MW 1500) in various concentrations as additives during immobilization. Ionic liquids as additives were also reported by Cleide's group [19], indicating that ionic liquids with more hydrophobic alkyl groups in the cationic moiety were beneficial for immobilized lipase recovery activity. This sol-gel type of encapsulation works well for many lipases [20]; however, the variability in protein properties and encapsulation conditions highly affects the activities of the encapsulated enzymes. Therefore, it is impossible to form an all-purpose encapsulation protocol. In this work, SiO_2 (or Celite) was used as a porous support, which keeps the lipase dispersion during the cross-linking condensation. Meanwhile, two kind of basic sol-gel materials (methyltrimethoxysilane and tetramethoxysilane) and several additives were employed in the immobilization. Herein, we have tried to develop a "doubly immobilized" and newly immobilized *A. niger* lipase for industrial applications.

2. Results and Discussion

2.1. The Molar Ratio of the Gel Precursors

The ratio of MTMS/TMOS greatly influences enzyme activity, and when the ratio was <75% it resulted in very low enzyme activity (Table 1). The protein immobilization efficiency varied very little from 91.1% to 92.1%. With an increasing ratio of alkyl-modified silane to TMOS, no gel shrinkage occurred during drying; when the ratio was >83.3% it resulted in very high enzyme activity. Lipases are interface-active enzymes with lipophilic domains; they can adopt both open and closed conformations [21]. Lipophilic interactions with the substrate molecules help to induce conformational changes, especially the movement of an α-helical loop. This so-called lid is thereby opened to uncover the active site, and results in the active form lipase [22,23]. A similar effect may occur with the presence of hydrophobic alkyl groups in the gel. Lipophilic interactions between the hydrophobic parts of the gel and lipase during the sol-gel process can fix the encapsulated enzyme in a more active conformation, and enhance the observed activity [24,25]. However, the gelatin behavior was poor with a large excess of MTMS due to the lack of the network-forming ability of TMOS. Only a white plastic-like solid could be formed with pure TMOS. Under these conditions, the substrate could not go into the enzyme active site, and therefore the immobilized lipase exhibited low enzyme activity.

Table 1. The activity of the immobilized lipase derived from different molar ratio of silanes.

Gel Precursors Molar Ratio	Efficiency of Protein Immobilization (%)	Activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$)
TMOS (tetramethoxysilane)	90.1	6.3
MTMS/TMOS (1:1)	91.3	232.2
MTMS/TMOS (3:1)	92.1	281.2
MTMS/TMOS (5:1)	90.9	315.0
MTMS (methyltreimethoxysilane)	90.8	294.0

In the present study, the silane precursor that was sonicated for 30 min before encapsulation showed an apparently shortened gelatin time when compared to the non-sonicated sample, since long gelatin times for the precursors can inactivate the enzyme [26]. Such an ameliorative method could accelerate the gelatin rate, which would protect the protein conformation and make a more suitable microenvironment for the lipase, resulting in the ideal immobilization of lipase.

2.2. Choice of Porous Materials

To form "double immobilization" conditions, an extension of our method was to add some porous solid materials as additives during the sol-gel process [20]. This immobilization condition can bind the lipase-containing gels to the pores of the solid support (celite) or with the lipase-containing support (SiO_2 nanoparticles) in the network of the gels. The effects of the SiO_2 nanoparticles and celite on activity and protein immobilization were investigated. The results indicated that the SiO_2 nanoparticles had a greater effect than the celite on the enzyme activity and the degree of protein immobilization (Table 2). The sample prepared with SiO_2 nanoparticles gave the best results, with a specific enzyme activity of 301.8 μmol·min^{-1}·g^{-1} and a protein immobilization degree of 92.1%, while in the samples with celite, the best data reported were 214.9 μmol·min^{-1}·g^{-1} and 89.1%, respectively. This result was likely attributed to the excellent characteristics of the nanomaterials as well as its interactions with lipase. The activity improved upon increasing the amount of SiO_2 nanoparticles to 60 mg, and then decreased at higher amounts. Under a low SiO_2 loading, not all the enzyme molecular structures could be suitably under the "double immobilization" conditions, and thus, a low enzyme immobilization efficiency was achieved. However, a large excess of SiO_2 nanoparticles would fill up the pores of the gel, leading to an intraparticle diffusion limitation as well as a diffusion limitation of the substrate to the enzyme active site.

Table 2. The effect of additives on the activity and efficiency of protein immobilization.

Additive (mg)	Efficiency of Protein Immobilization (%)	Activity (μmol·min^{-1}·g^{-1})
SiO_2 20	76.2	198.6
SiO_2 40	83.1	227.8
SiO_2 50	89.8	247.0
SiO_2 60	92.1	301.8
SiO_2 80	91.4	224.0
SiO_2 100	90.3	189.1
Celite 20	63.7	169.0
Celite 40	73.4	162.9
Celite 50	78.9	199.2
Celite 60	83.2	213.7
Celite 80	89.1	214.9
Celite 100	89.4	214.3
Controls	77.5	206.7

2.3. Molar Ratio of Water to Silane

The molar ratio of water to silane during the immobilization process had a great effect on the specific activity of the immobilized lipase, but little effect on the degree of protein immobilization (Figure 1). The activity increased with a ratio of up to 12, then decreased at higher ratios. Polymerization at a low water content will result in aggregation of the lipase, where the enzyme will grow around "floc" rather than individual enzyme molecules and lead to low enzyme activity. Additionally, at a low water concentration, alcohol condensation instead of water condensation will probably occur in the period of SiO_2 network growth, and the liberated alcohol may damage the enzyme. For the gelatin process, increasing the water content could promote coarsening, resulting in larger pores and a higher degree of cross-linking within the silicate material. However, at a high water

concentration, more of the lipase was observed to remain in the aqueous supernatant and resulted in lower enzyme loading.

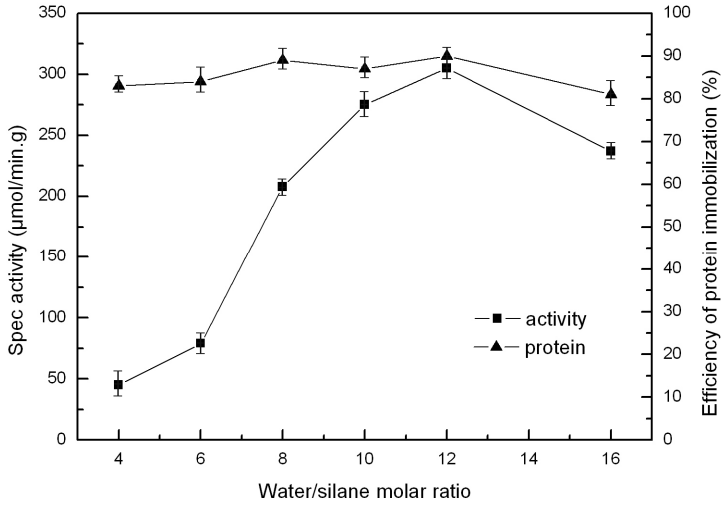

Figure 1. The effect of the water/silane molar ratio. (methyltreimethoxysilane) MTMS/TMOS (tetramethoxysilane) molar ratio of 5, 60 mg of SiO_2, 120 mg of enzyme supply, and 120 μL of PEG400 (polyethylene glycol).

2.4. Choice of Enzyme Supply

The effect of enzyme supply on the immobilization process is shown in Figure 2. The activity increased upon increasing the enzyme supply up to 120 mg, and then decreased at higher supplies. Contrary to this, the amount of immobilized protein decreased. Enzyme denaturation by the support material at low loading may explain the initial increase in enzyme activity; intraparticle diffusion limitation and/or enzyme aggregation may lead to a decrease in activity at high enzyme loading.

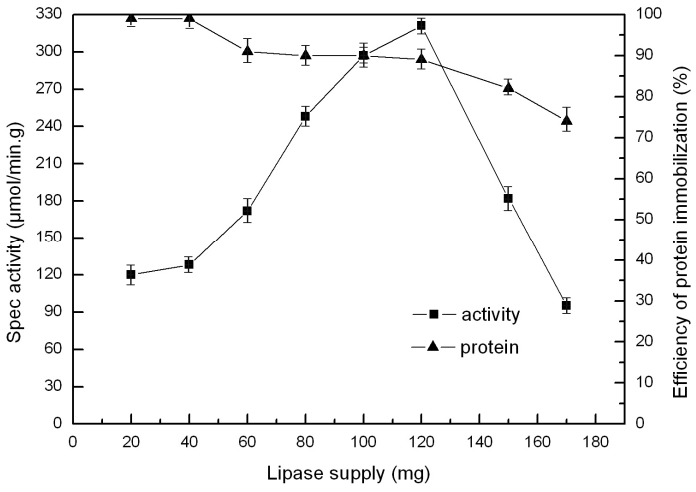

Figure 2. The effect of lipase supply. MTMS/TMOS molar ratio of 5:1, 60 mg of SiO_2, water/silane molar ratio of 12, 120 μL of PEG400.

2.5. The Effect of PEG and PVA

The addition of PVA in the samples of the SiO_2 nanoparticles produced no observable variation of the immobilization efficiency, but even lower data have been obtained in the samples without SiO_2 nanoparticles (Table 3). A possible explanation for this may be that the nanoparticles added during

the formation of the silica matrix protected the enzyme from denaturation. It is also likely due to the presence of hydroxyl groups on the PVA molecules, which interact with the protein and cause the lipase to adopt a "closed" conformation inaccessible to the solvent [27]. This phenomenon is quite interesting because an increase in specific activity has been reported by Reetz et al. When compared with their results, our findings may be explained in three aspects: (i) The silane precursors used in this study were different from those used by Reetz; (ii) the precursors here were sonicated for 30 min before being mixed with the enzyme; (iii) their assay involved the hydrolysis of olive oil, while ours utilizes the hydrolysis of pNPP. The activity of the encapsulated lipase may be affected by many factors, such as the immobilization conditions and the activity assay methods used.

Table 3. The effect of PVA (polyvinyl alcohol) and PEG (polyethylene glycol) on the activity and efficiency of protein immobilization.

Additive (mg)	Sample	Efficiency of Protein Immobilization (%)	Activity ($\mu mol \cdot min^{-1} \cdot g^{-1}$)
SiO_2	IM1	90.8	276.7
	IM2	91.1	343.9
	IM3	90.6	274.1
None	IM1	81.5	71.3
	IM2	84.3	198.6
	IM3	82.2	159.2

IM1: Entrapment of lipase in the presence of PVA. IM2: Entrapment of lipase in the presence of PEG. IM3: Entrapment of lipase in the absence of additives.

However, the sample with PEG showed a higher activity. The reason may be attributed to a conformational change, because less polar environments promote the open conformation of lipase [27]. In addition, PEG may bind water molecules, which can increase the ability of forming a more regular network conformation of the gels.

The immobilization efficiency increased with the molecular weight of PEG up to 400; and then decreased at higher molecular weights (Figure 3). The ability to bind water increased with the molecular weight of PEG < 400. This may have a positive effect on the hydrolysis of the gel precursors to form a regular network conformation, resulting in a high degree of immobilized protein and thus high enzyme activity. However, if the chain of PEG is too long, it will lead to severe contortion at a molecular weight >400. This would decrease its ability to bind water and thus, have a trivial effect on the relationship between the hydrolysis of the precursors and the generation of the sol-gel network conformation.

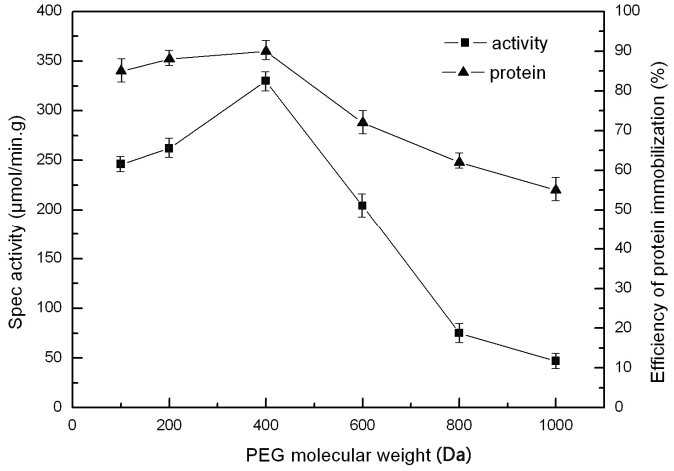

Figure 3. The effect of the molecular weight of PEG. MTMS/TMOS molar ratio of 5:1, water/silane molar ratio of 12, 120 mg of enzyme supply, 60 mg of SiO_2.

The same result has been reported at a lower PEG400 supply (<120 μL) and a higher supply (>120 μL) (Figure 4). This may also involve the mechanism of binding the water. PEG could not bind enough water at a lower level, and would have less of an effect on the hydrolysis of the gel precursors. However, a larger amount of PEG would bind too much water, limiting the hydrolysis of the precursors.

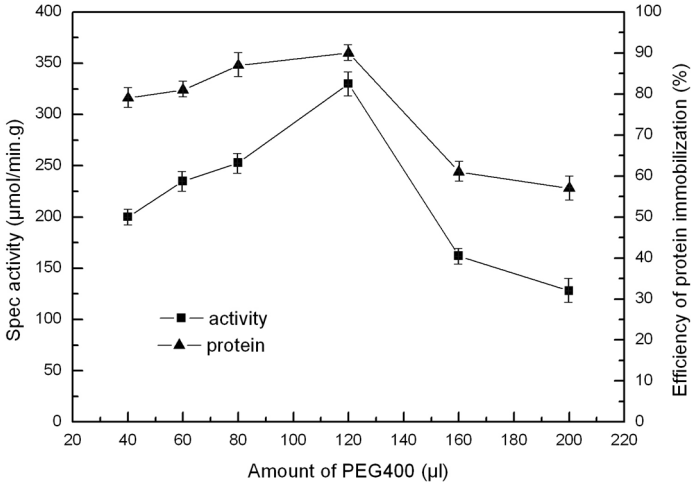

Figure 4. The effect of PEG 400. MTMS/TMOS molar ratio of 5:1, water/silane molar ratio of 12, 120 mg of enzyme supply, 60 mg of SiO_2.

2.6. Stability of the Immobilized Lipase

2.6.1. Optimal Temperature and Thermal Stability of the Free and Immobilized Lipase

The effect of temperature on the enzyme activity and stability was examined. The optimal temperature of the immobilized enzyme was improved to 55 °C, 20 °C higher than that (35 °C) of the free form (Figure 5). After incubation at different temperatures for 2 h, the enzyme activity was measured and the activity of the untreated lipase was taken as 100%. The thermal stability of the immobilized lipase was also improved by 15 °C, much better than that of the free enzyme (Figure 6). The free enzyme lost all of its original activity at 60 °C, while the immobilized enzyme still retained nearly 90% of its original activity at 65 °C.

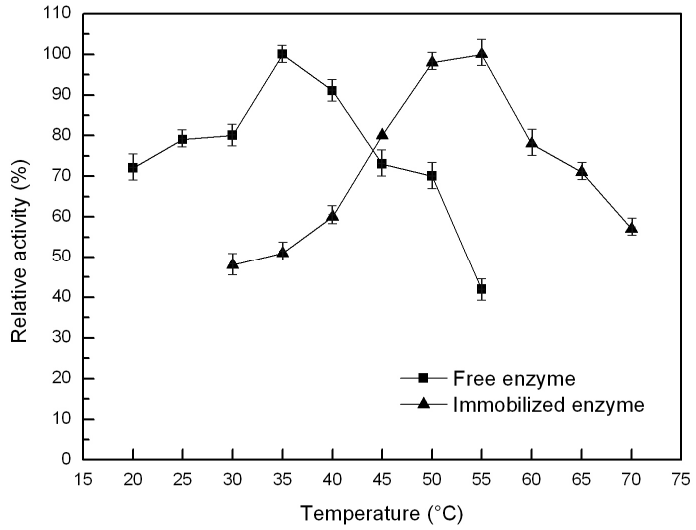

Figure 5. The effect of temperature on the free and immobilized enzyme.

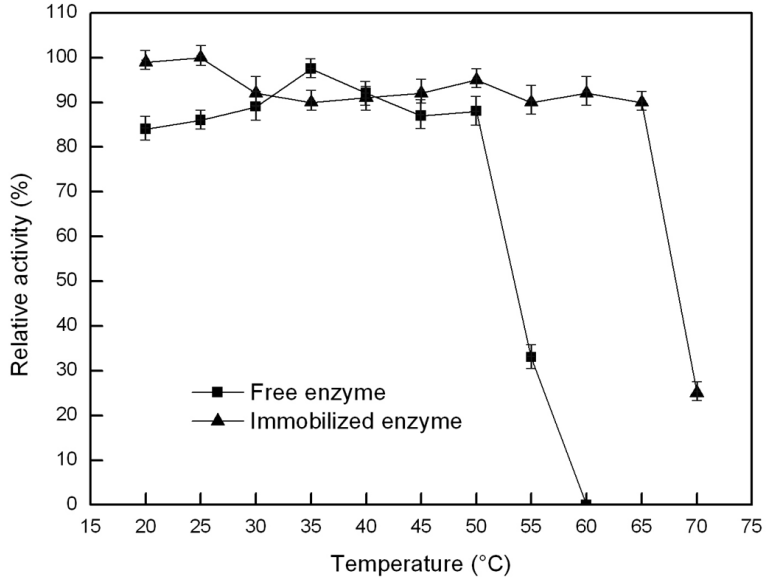

Figure 6. The temperature stability; enzyme activities without treatment were taken as 100%.

2.6.2. Optimal pH and pH Stability of the Free and Immobilized Lipase

The optimal pH of the free enzyme was 8.0, while that of the immobilized form was 8.5 (Figure 7). This may be due to the exposure of the imidazole groups in the active site and the diffusion limitations of the products. The immobilized enzyme exhibited more stability than that found for the free form at a pH range from 3 to 11 (Figure 8). The increased stability of the immobilized lipase might improve the retention ability of the lipase in regards to the free form.

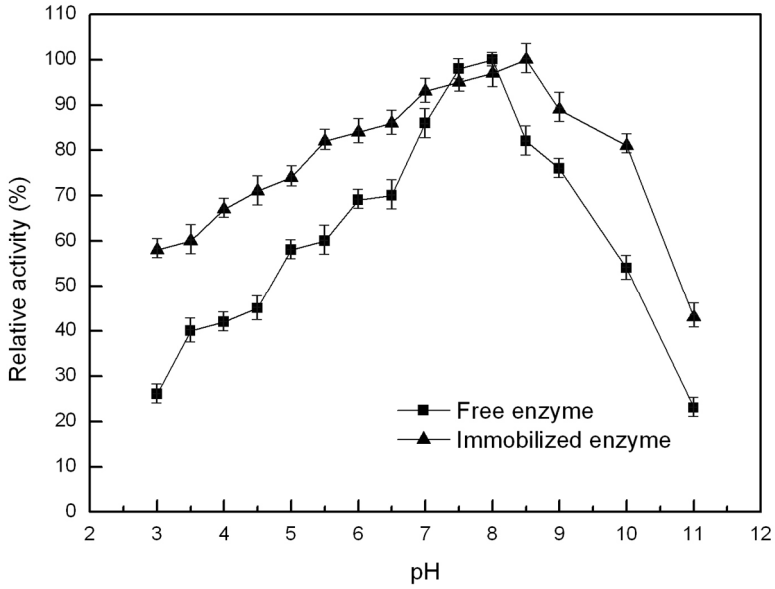

Figure 7. The effect of pH on the free and immobilized enzyme.

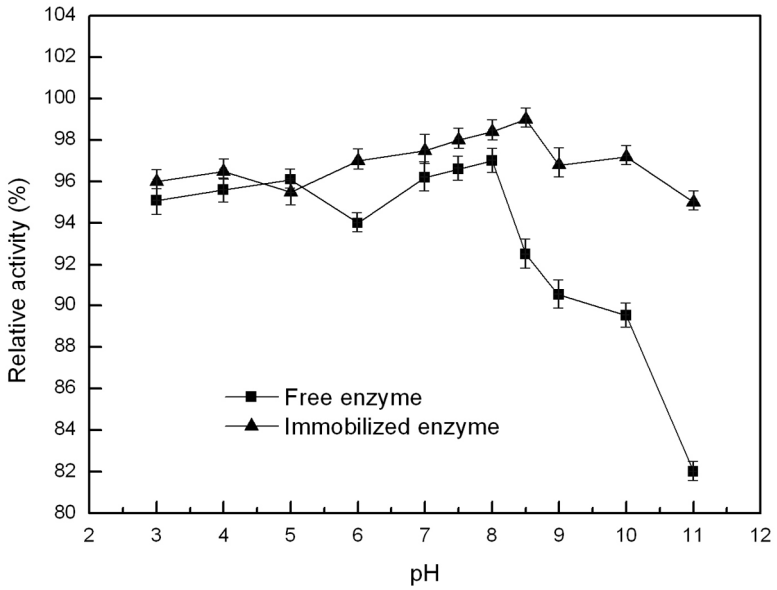

Figure 8. The pH stability; the activities of the untreated enzyme were taken as 100%.

2.7. Comparison of Immobilization Results with Other Related Reports

Sol-gel encapsulation as a traditional method in enzyme immobilization has been used extensively in research studies, and usually results in a positive effect on the enzyme activity recovery. However, reports on immobilizing *A. niger* lipase are rare and most show the immobilized lipase activity being significantly decreased. When compared with other immobilized *A. niger* lipases, the activity recovery in this work was 94.0%, which was basically the same as the original activity of free lipase. Ahmed et al. immobilized *A. niger* lipase with magnetic barium ferrite nanoparticles and increased the enzyme activity [28]. However, the activity was greatly decreased when encapsulating the lipase in a sol-gel matrix [29]. In this work, we designed a different protocol for the sol-gel synthesis which may have been the key factor that contributed to the higher activity recovery (as shown in Table 4). In addition, preliminary tests showed that the immobilized lipase retained a good part of its original activity after five cycles, indicating that it possesses fairly good manipulation stability.

Table 4. A comparison of immobilization results with other related reports.

Reports	Enzyme	Immobilization Method	Activity Recovery (%)	Published Year
1	*Bacillus sp.* ITP-001 lipase	Sol-gel silica matrices encapsulation	177.5	2012 [30]
2	*Rhizomucor miehei* lipase	Adsorbed on Silicalite-1 Sol-gel materials	118.4	2008 [31]
3	*Candida rugosa* lipase	Sol-gel Encapsulation	230.1	2012 [32]
4	*Aspergillus niger* enzyme extraction	Sol-gel encapsulation	71.4	2014 [29]
5	*Aspergillus niger* lipase A	Adsorbed on modified magnetic particles	118.9	2014 [33]
6	*Aspergillus niger* lipase	Adsorbed *Luffa cylindrica* sponges	84.0	2016 [34]
7	*Aspergillus niger* lipase	Sol-gel encapsulation	94.0	This work

3. Materials and Methods

3.1. Materials

Lipase Amano 6 from *A. niger* is an industrial product of Amano Enzyme China Ltd. (Shanghai, China). Tetramethoxysilane (TMOS) and methyltrimethoxysilane (MTMS) were purchased from Wuhan University Silicone New Material Co., Ltd. (Wuhan, China). SiO_2 nanoparticles (model DP1, mean particle diameter is 20 ± 5 nm, specific surface area is 700 ± 30 m^2/g) were purchased from Mingmu Nanomaterials Co., Ltd. (Shaoxin, China). Polyethylene (PEG, MW 400) and polyvinyl alcohol (PVA, MW 20000) were obtained from Wuhan Jiangbei Chemical Reagent Co., Ltd. (Wuhan, China). Bovine serum albumin (Fraction V, Sigma, Shanghai, China), p-nitrophenyl phosphate disodium hexahydrate (pNPP, Amresco, Solon, OH, USA), and the other chemical reagents were of analytical grade.

3.2. Analytical Methods

The enzyme activity and protein content were determined using the following methods. All data are the mean values obtained from at least three measurements and with a standard error of less than 4.32%.

3.3. Hydrolytic Activity on pNPP

The hydrolytic activity was measured using emulsified p-nitrophenyl palmitate (pNPP) [35]. One volume of a 16.5 mM solution of pNPP in 2-propanol was mixed with nine volumes of 50 mM Tris-HCl buffer (pH 7.5) containing 0.4% (*w*/*v*) Triton X-100 and 0.1% (*w*/*v*) arabic gum. Then, 4.5 mL of this mixture was pre-incubated at 35 °C for about 3 min. The reaction was initiated by the addition of 0.5 mL of free enzyme solution at an appropriate dilution in the 50 mM Tris-HCl buffer (pH 7.5) or 0.5 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 100 mg of immobilized enzyme powder. The absorbance was monitored against a blank control at 410 nm after 20 min at 35 °C, using a blank with enzyme without activity. To allow conversion of the absorbance data into concentration, a calibration curve was constructed using standard solutions of p-nitrophenol (pNP). One enzyme activity unit was defined as the releasing amount of pNP catalyzed by per gram of protein per minute under the conditions described above.

3.4. Protein Determination

The protein content of the crude lipase powders was determined using the Bradford method [36] with bovine serum albumin (BSA) as a standard. The amount of entrapped lipase in the sol-gel materials was determined by measuring the amount of protein in the solution before and after entrapment had occurred. The protein remnant was determined as follows: The total protein amount of the immobilization was compared with the total amount of the protein used in the immobilization process.

3.5. The Effect of Temperature on the Enzyme Activity and Stability

The enzyme activity was examined at different temperatures (from 20 °C to 55 °C for the free lipase and from 30 °C to 70 °C for the immobilized lipase). The effect of temperature on the enzyme stability was checked at different temperatures from 20 °C to 70 °C. Both enzyme preparations were incubated in Tris-HCl buffer for 2 h at the desired temperature. After cooling, the remaining activity was analyzed under standard assay conditions.

3.6. The Effect of pH on the Enzyme Activity and Stability

The enzyme activity was first assayed at different pH (from 3 to 11 for both the free and the immobilized form of lipase) and the residual activity was determined using the following standard

assay method after incubating the enzyme at different pH for 2 h. To study the effect of pH, three kinds of buffers were used for pH optimization. They were Na_2HPO_4–citrate acid buffer (0.05 M, pH 3.0–6.0), Tris-HCl buffer (0.05 M, pH 7.0–9.0), and glycine–NaOH buffer (0.05 M, pH 10.0–11.0).

3.7. Lipase Immobilization

A certain amount of *A. niger* lipase powder was firstly dissolved in 390 μL Tris-HCl buffer (0.1 M, pH 7.5) using a 50 mL Falcon tube (Corning, Wuhan, China), and the mixture was vigorously shaken using a Vortex-Mixer (Naze, Shanghai, China). Secondly, a certain amount of SiO_2 (or celite), PEG (or PVA), 50 μL aqueous sodium fluoride (1 M solution), and 100 μL isopropyl alcohol were added, respectively. Then, the mixture was shaken for 2 h. After that, 2.5 mmol of alkylsilane and 0.5 mmol TMOS were sonicated for 30 min respectively, and added to the mixture. Finally, the reaction vessel was shaken for 10 to 15 s and the gelatin could be observed. In addition, if not otherwise indicated above, all parameters used in this procedure were as follows: water/silane molar ratio of 12, MTMS/TMOS molar ratio of 5, 60 mg of SiO_2, 120 mg of enzyme supply, and 120 μL of PEG400. After the formation of the gelatin, the reaction vessel was left to stand closed for 8 to 24 h, and then it was opened and the gel was air-dried at room temperature for 3 days. Isopropyl alcohol (10 to 15 mL) was added to the cube in order to remove the white solid material. The gel was then shaken with 10 mL of Tris-HCl buffer (pH 7.5, 0.1 M) for 2 h, and then filtered out. The obtained gel was washed with 10 mL of distilled water, isopropyl alcohol, and n-pentane, respectively. After drying for 6 to 24 h at room temperature, the gel was ground using a ball mill. The immobilized lipase was obtained and could be used for further experiments.

4. Conclusions

Two cheap sol-gel materials (MTMS and TMOS) and SiO_2 nanoparticles were employed in the immobilization of lipase from *Aspergillus niger* along with some PEG additive. The results proved that this is a fairly good immobilization method for the lipase. The optimum preparation conditions were gels made from a MTMS/TMOS molar ratio of 5, 60 mg of SiO_2 nanoparticles, a water/silane molar ratio of 12, 120 mg of enzyme supply, and 120 μL of PEG400. Under the optimal conditions, the immobilized lipase retained 92% of the loaded protein and 94% of the total enzyme activity. Thermal and pH characteristics tests indicated that the immobilized lipase exhibited higher stability than that of its free form, which shows great potential for industrial applications.

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Author Contributions: Li Xu and Yunjun Yan conceived and designed the experiments; Ying Huang performed the experiments; Caixia Ke analyzed the data and wrote the paper; Li Xu and Yunjun Yan contributed reagents/materials/analysis tools.

Conflicts of Interest: The authors declare no conflict of interest.

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