Covalent Immobilization of Lipase onto Citric Acid-Esterified Loofah Sponge

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Loofah sponge was activated by thermochemical esterification between the carboxyl groups of citric acid and hydroxyl groups of cellulose to introduce free carboxyl groups, which were further reacted with lipase amino groups with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) as the condensing reagent. This resulted in loofah sponge–immobilized lipase. Under the optimized immobilization conditions, the highest activity of immobilized lipase per gram of dry carrier was found to be 45.8 U/g. The immobilized lipase exhibited maximum activity at 40 °C, pH 8.0, while the optimal temperature and pH for the free lipase were 37 °C and 7.5, respectively. The immobilized lipase showed better thermal stability, storage stability, and reusability than free lipase.

Keywords: Lipase; Immobilization; Esterified loofah sponge; Carbodiimide; Amide linkage

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INTRODUCTION

Enzymes are versatile macromolecular biocatalysts that offer high catalytic efficiency and stereospecificity to biochemical reactions under mild reaction conditions. However, the main problems of using soluble enzymes in industry are the instability of enzymes and the difficulty of their recovery and reutilization (Sheldon 2007). To overcome these problems, enzymes are often immobilized onto insoluble solid supports. Immobilization of enzymes not only can increase the stability of enzymes and facilitate the reuse of enzymes, but also can reduce the cost of biocatalytic production.

There are various methods for enzyme immobilization. They include adsorption onto an insoluble carrier (Zheng *et al.* 2012; 2013), entrapment or encapsulation in a polymeric matrix (González-Sáiz and Pizarro 2001; Koszelewski *et al.* 2010), cross-linking with a bifunctional reagent (Šulek *et al.* 2011), and covalent linking to insoluble solid material (Elnashar *et al.* 2009). The immobilization of enzyme by covalent binding offers the following potential advantages: because of the strong interaction between the enzyme and carrier, the enzyme does not leak or detach from the carrier in the process of various applications; the stability of the enzyme towards pH, temperature, and organic solvents are increased markedly; at the same time, the immobilized enzyme can easily make contact with substrate because it is localized on the surface of carrier (Varavinit *et al.* 2001; Kumar *et al.* 2013).

Cellulose, which is composed of a linear chain of β-1,4-linked D-glucose units, is the most abundant natural polysaccharide in the world. In the search for suitable materials for enzyme immobilization, cellulosic materials have been found to be among the top candidates due to their hydrophility, reproducibility, biocompatibility, biodegradability,

and processability (Bryjak *et al.* 2007; Petronijević *et al.* 2007; Wu and Lia 2008; Villalonga *et al.* 2008; Huang *et al.* 2011; Labus *et al.* 2011). Loofah sponge is an inexpensive, biocompatible, hydrophilic cellulosic material and has been used as a carrier for the immobilization of various biological cells (Ahmadi *et al.* 2006; Iqbal and Saeed 2007; Saudagar *et al.* 2008; Meleigy and Khalaf 2009; Pazzetto *et al.* 2011). But so far there has been no report on the immobilization of enzyme on loofah sponge.

In this paper, loofah sponge was activated by thermochemical esterification between the carboxyl groups of citric acid and hydroxyl groups of cellulose in loofah sponge. Lipase (EC 3.1.1.3), an important biocatalyst in food processing, was covalently immobilized to the activated loofah sponge using carbodiimide as the coupling agent to attach carboxyl groups of the esterified surface of loofah sponge to the amino groups of lipase. The enzymatic characteristics, stabilities, and reusability of immobilized lipase were investigated and compared with free lipase.

EXPERIMENTAL

Materials

Loofah sponge, supplied by a local farm, was cut into discs of about 1 cm height and 7 cm diameter. The loofah sponge discs were soaked in boiling water for 30 min, washed thoroughly with tap water to remove impurities, left in distilled water for 24 h, dried overnight at 50 °C, and then stored in a desiccator. Porcine pancreatic lipase, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were purchased from Sigma (St. Louis, MO, USA). *p*-Nitrophenyl palmitate (*p*-NPP) was obtained from Fluka Chemical Corp. (Ronkonkoma, NY, USA). All other chemicals used were of analytical grade from several trademarks.

Activation of Loofah Sponge

The activation of loofah sponge was carried out according to the following method: Loofah sponge discs were mixed with 0.2 M citric acid (CA) at the ratio of 1:50 (disc:acid, w/v) and stirred for 30 min. The mixture was dried at 50 °C for 24 h, followed by performing the optimal thermochemical esterification between citric acid and cellulose at 100 °C for 60 min (Gong *et al.* 2008). The activated loofah sponge was extensively washed with distilled water to remove unreacted citric acid and recovered as the carrier for lipase immobilization. The esterified loofah sponge with different carboxyl group density on it was prepared by changing the conditions of the thermochemical esterification (temperature and time).

Immobilization of Lipase on Esterified Loofah Sponge

Lipase was immobilized onto esterified loofah sponge by EDC/NHS-mediated amide formation between carboxyl groups of esterified loofah sponge and amino groups of lipase. Briefly, 0.1 g of esterified loofah sponge was immersed in 6 mL of phosphate buffer solution (PBS: 50 mM, pH 5.5) containing 10 mg of EDC and 6 mg of NHS. After stirring gently at room temperature for 1 h, the activated loofah sponge was collected, washed several times with the above PBS, and submerged in 6 mL of enzyme solution (enzyme activity of 5.2 U/mg; 1.0 mg/mL in the above PBS). The immobilization process was carried out at room temperature for 4 h with gentle shaking. Finally, the immobilized lipase was recovered and the unbound lipase was removed by washing with the above

PBS and then rinsed with distilled water until no enzymatic activity was detected in the supernatant. The supernatant and washing solutions were pooled for assaying the residual enzyme activity and protein concentration. A reaction scheme for the preparation of the esterified loofah sponge and immobilized lipase is shown in Fig. 1.

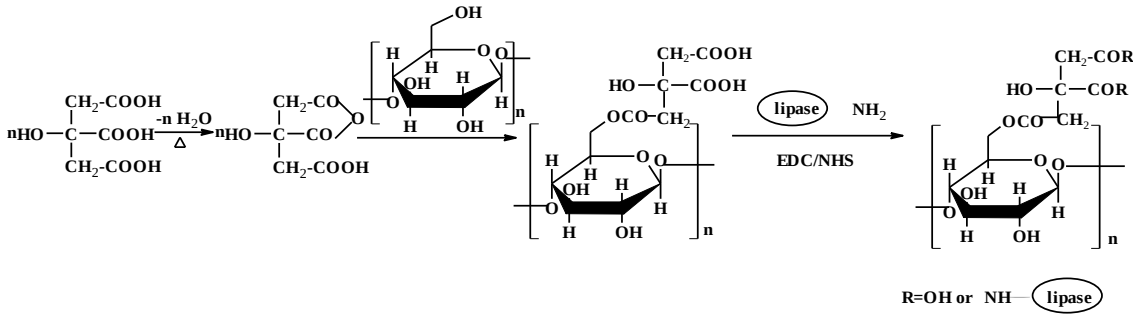

Fig. 1. Schematic illustration of the preparation of the esterified loofah sponge and immobilized lipase

Determination of the Immobilized Protein Amount

Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard protein. The amount of bound protein was indirectly calculated from the difference between the amount of protein used for immobilization and the net amount of protein present in the supernatant and washing solutions after immobilization. The immobilization efficiency was defined as the ratio of the total amount of the immobilized protein to the total amount of the used protein.

Measurement of Lipase Activity

The activities of free and immobilized lipases were analyzed spectrophotometrically by measuring the increment in the light absorbance at 410 nm caused by the release of *p*-nitrophenol because of the hydrolysis of *p*-NPP (Yi *et al.* 2009). The reaction mixture, which was incubated at 37 °C, consisted of 7.7 mL of PBS (50 mM, pH 7.5) containing 0.4% (w/v) Triton X-100, 0.1% (w/v) gum arabic, and 0.1 mL of free lipase solution (1.0 mg/mL), or 0.1 mL of the above PBS as well as 10 mg of immobilized lipase. The reaction was initiated by adding 0.2 mL of *p*-NPP substrate solution (40 mM) prepared with 2-propanol followed by incubation for 5 min at 37 °C, and then the reaction was terminated by adding 4 mL of Na_2CO_3 (0.1 M). The resultant mixture was centrifuged to obtain supernatant. The amount of released *p*-nitrophenol in the supernatant was measured by a UV spectrophotometer (Shimadzu UV-1201, Japan) at 410 nm. The lipase activity was calculated from the standard calibration curve of *p*-nitrophenol. One unit (U) of lipase activity was defined as the amount of enzyme necessary to liberate 1 μM *p*-nitrophenol per minute under described experimental conditions. The retention activity was defined as the ratio of the activity of the immobilized enzyme to the activity of the same amount of free enzyme.

Effect of pH and Temperature on Lipase Activity

The effect of buffer pH on the free and immobilized lipase activities was estimated for reaction mixtures containing 50 mM of the PBS or Gly-NaOH buffer at different pH values (PBS: 6.0 to 8.0; Gly-NaOH: 8.5 to 9.5) at 37 °C. The effect of temperature on both lipase activities was determined at different temperatures in the range of 20 to 50 °C in 50 mM of PBS (pH 7.5) under the same assay conditions.

Stability Detection of Lipases

For determination of thermal stability, both free and immobilized lipases were incubated in substrate-free PBS (50 mM, pH 7.5) at 60 °C for 3 h. The remaining activities of the enzymes were periodically measured according to the above-described method. For the investigation of storage stability, both free and immobilized lipases were stored in a refrigerator at 4 °C for 30 days. The remaining activities of samples were also measured periodically.

Reusability Determination of Immobilized Lipase

The reusability of the immobilized lipase was evaluated in a repeated catalysis process. After each reaction, the immobilized lipase was removed and thoroughly washed with 1% Triton X-100 and 10% 2-propanol in PBS (50 mM, pH 7.5) for the next use. It was then reintroduced into a fresh reaction medium. The residual activity was determined as described above and expressed as a percentage of its residual activity in comparison with the initial activity.

RESULTS AND DISCUSSION

FTIR Spectra of Loofah Sponge and Esterified Loofah Sponge

The FTIR spectra of loofah sponge and esterified loofah sponge are shown in Fig. 2. Compared with loofah sponge, the characteristic stretching vibration absorption band of carbonyl groups at 1739 cm^{-1} increased greatly in the FTIR spectrum of esterified loofah sponge. This result demonstrated that the citric acid had been introduced into loofah sponge.

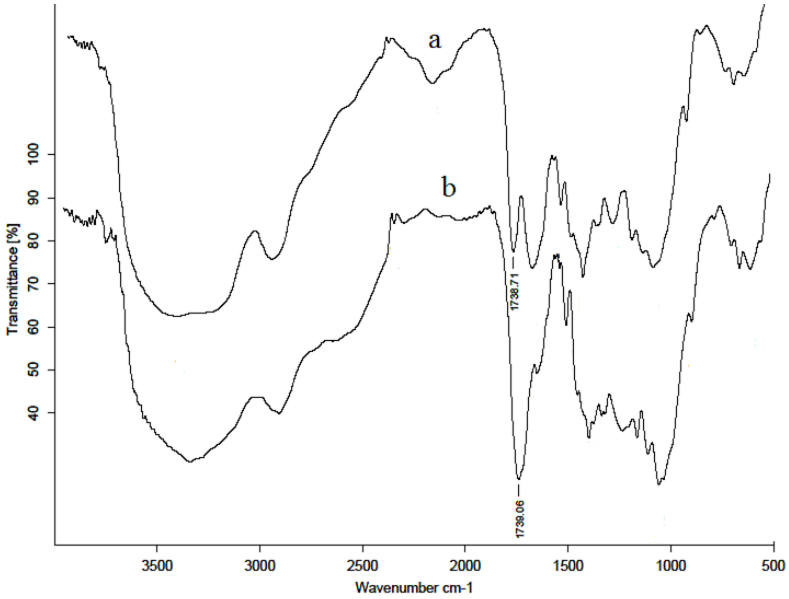

Fig. 2. FTIR spectra of (a) loofah sponge and (b) esterified loofah sponge

Optimal Condition for Lipase Immobilization

To optimize the immobilization conditions of lipase, 0.1 g of esterified loofah sponge with a carboxyl group density of 0.88 mM/g on it was immersed in 6 mL of PBS (50 mM). Then the effect of the lipase amount (6 mL × 0.2 to 2.6 mg/mL), pH value (3.0 to 8.0), EDC amount (2 to 40 mg), and immobilization time (0.5 to 12 h) on the activity

of the immobilized lipase was investigated. The optimal conditions for lipase immobilize-ation were established as follows: 6 mg of lipase (6 mL × 1.0 mg/mL), pH 5.5, 10 mg of EDC, and 4 h of immobilization time. Under these conditions, 0.1 g of esterified loofah sponge with different carboxyl group density (0.24 to 1.42 mM/g) on it was used for lipase immobilization. The highest activity of immobilized lipase per gram of dry carrier with carboxyl group density (1.15 mM/g) was found to be 45.8 U/g (Table 1).

Table 1. Influence of Carboxyl Group Density on Lipase Immobilization

	Carboxyl group density (mM/g)								
	0.21	0.35	0.51	0.69	0.88	1.01	1.15	1.28	1.42
Amount of immobilized protein (mg/g)	7.1	8.4	10.8	12.7	14.9	16.7	18.2	19.3	19.6
Activity of immobilized lipase (U/g)	19.9	24.4	31.5	36.5	40.3	43.1	45.8	44.9	43.8
Retention activity of immobilized lipase (%)	53.9	55.9	56.1	55.3	52.0	49.6	48.4	44.7	43.0.

Properties of Immobilized Lipase

The influence of pH on the activities of free and immobilized lipases is illustrated in Fig. 3. It was observed that the optimal pH for free lipase was close to 7.5 but was approximately 8.0 for immobilized lipase, shifting 0.5 units towards the alkaline region. This shift in the optimal pH often depends on the immobilization method as well as the structure and charge of the carrier (Costa *et al.* 2001). Generally, when the enzyme is coupled with a polyanionic support, the optimal pH usually shifts in the alkaline direction (Yi *et al.* 2009), whereas if the support is polycationic the shift is in the acidic direction. The loofah sponge–immobilized lipase, possibly due to the carboxyl groups introduced by esterification, significantly altered the microenvironment of the enzyme. Furthermore, the immobilized lipase retained a relatively higher activity in the alkaline pH range than the free lipase did, which suggested that the stability of the immobilized lipase was higher than that of the free lipase at the alkaline pH range.

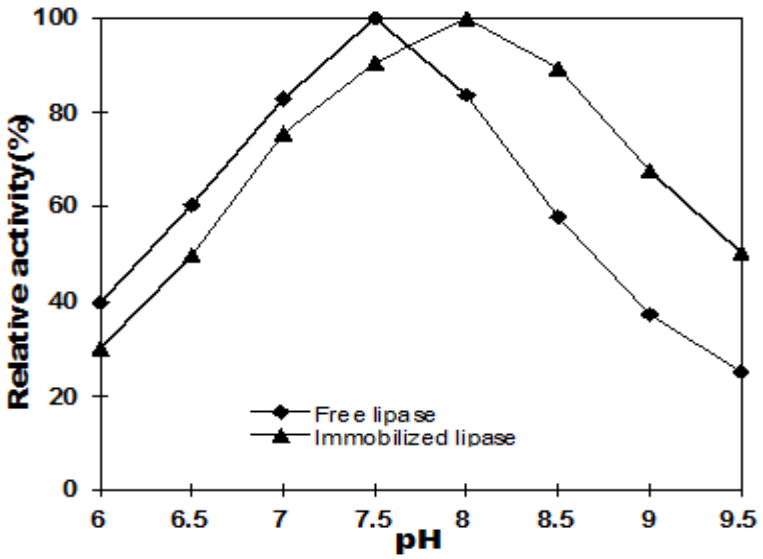

Fig. 3. Influence of pH on the activity of free and immobilized lipases

The effect of temperature on the activities of free and immobilized lipases is shown in Fig. 4. It was found that the optimal temperature of the immobilized lipase was 40 °C, which was 3 °C higher than that of the free lipase. This result was consistent with the literature (Singh *et al.* 2011); the immobilized lipase often exhibits a higher optimal temperature than its free counterpart. The multipoint covalent attachment of the lipase on the esterified loofah sponge by amide linkage would tend to maintain a more rigid conformation of the enzyme molecule. Thus, the immobilized lipase was much more stable than its free counterpart at higher temperature. In addition, the immobilized lipase retained a relatively higher activity in the high temperature range than the free lipase did.

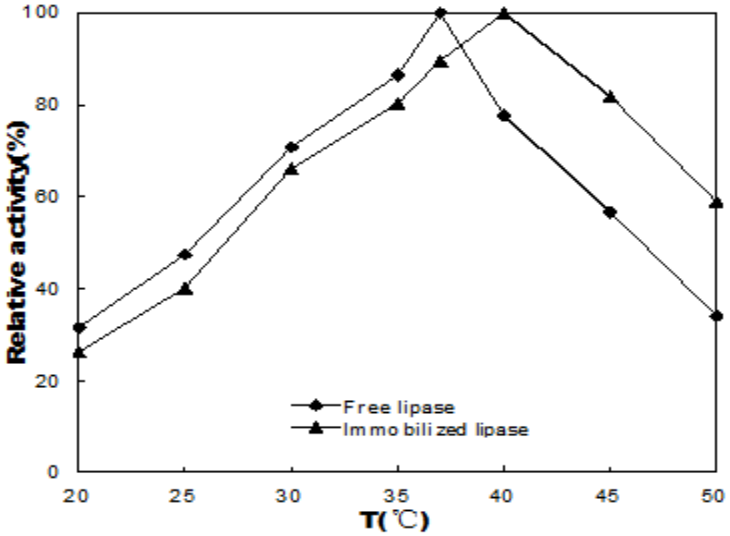

Fig. 4. Effect of temperature on the activity of free and immobilized lipases

Stabilities of the Free and Immobilized Lipase

The results for the thermal stabilities of the free and immobilized lipases are presented in Fig. 5.

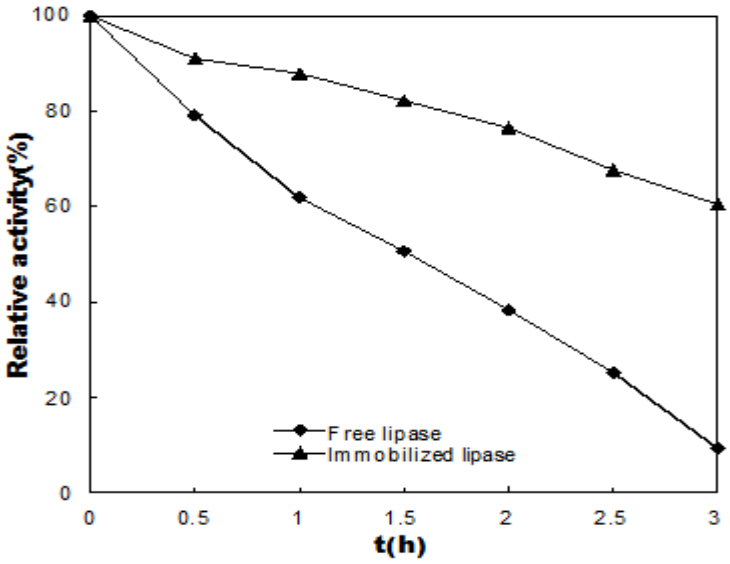

Fig. 5. Thermal stabilities of free and immobilized lipases

The immobilized lipase exhibited smaller thermal inactivation than the free lipase at all time periods. The free lipase lost more than 90% of its initial activity after heat treatment for 3 h at 60 °C, while the immobilized lipase showed significant resistance to thermal inactivation and kept more than 60% of its initial activity under the same conditions. The immobilization of the enzyme to a carrier could prevent the conformation transition of the enzyme at high temperature, thus resulting in increasing the thermal stability of the enzyme towards heat inactivation.

Results for storage stabilities of free and immobilized lipases are shown in Fig. 6. The free lipase lost almost all of its initial activity within 10 days, whereas the immobilized lipase retained more than 73% of its activity, and it still preserved more than 50% of its initial activity after the 30 days of the storage period. This result indicated that the immobilized lipase exhibited better storage stability than free lipase. In general, the enzyme is unstable during storage in solution and its activity decreases gradually over time. This decrease in enzyme activity can be explained as a time-dependent natural loss of enzyme activity. It can be prevented to a significant degree by immobilization.

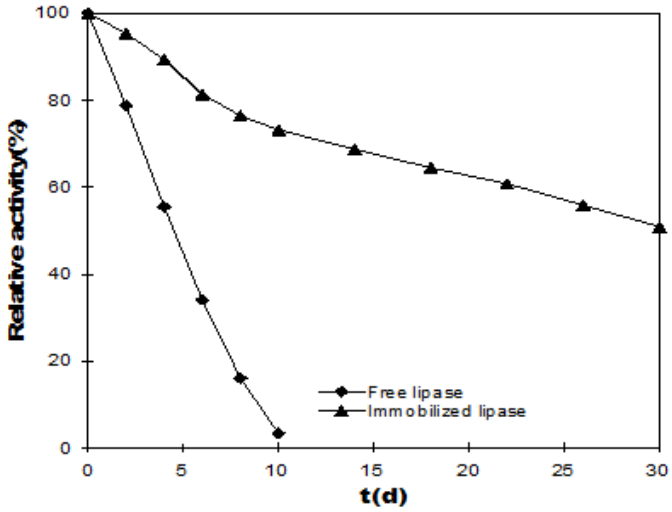

Fig. 6. Storage stabilities of free and immobilized lipases

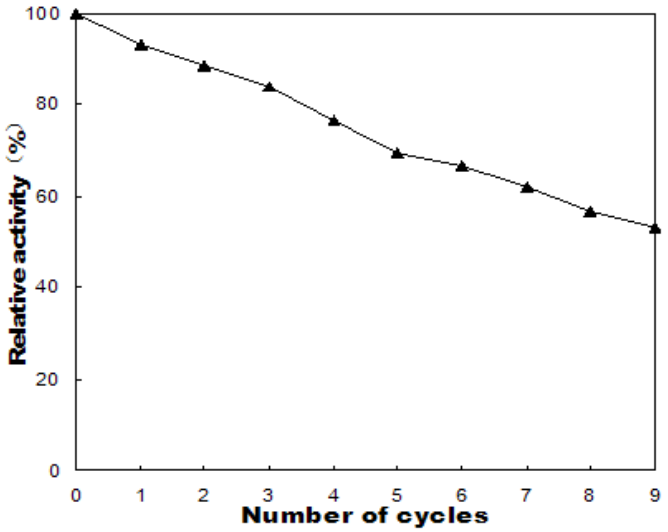

Fig. 7. Reusability of the immobilized lipase

Reusability of the Immobilized Lipase

The reusability of immobilized enzyme is important for industrial use of enzyme. The main advantage of immobilized enzyme is the easy separation and reusability. The effect of repeated use on activity of immobilized lipase is shown in Fig. 7. The activity of immobilized lipase dropped smoothly during the continuous operation process, and the immobilized lipase retained more than 53% of its initial activity after nine cycles of reuse. The loss of activity for immobilized enzyme with repeated use is a common phenomenon (Arasaratnam *et al.* 2000), and it can be attributed to the inactivation of the enzyme caused by denaturation.

CONCLUSIONS

1. Cellulose in loofah sponge was thermochemically esterified by citric acid to introduce free carboxyl groups. The esterified loofah sponge then was employed as a carrier for the covalent immobilization of lipase. Loofah sponge–immobilized lipase was successfully obtained by EDC/NHS-mediated amide formation between carboxyl groups of esterified loofah sponge and amino groups of lipase. Loofah sponge–immobilized lipase exhibited excellent thermal stability, storage stability, and reusability.
2. The lipase immobilized on loofah sponge by amide linkage offers a cheap immobilized biocatalyst suitable for future industrial applications.

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REFERENCES CITED

Ahmadi, M., Vahabzadeh, F., Bonakdarpour, B., Mehranian, M., and Mofarrah, E. (2006). "Phenolic removal in olive oil mill wastewater using loofah-immobilized *Phanerochaete chrysosporium*," *World J. Microbiol. Biotechnol.* 22(2), 119-127.

Arasaratnam, V., Galaev, I. Y., and Mattiasson, B. (2000). "Reversibly soluble biocatalyst: Optimization of trypsin coupling to Eudargit S-100 and biocatalyst activity in soluble and precipitated forms," *Enzyme Microb. Tech.* 27(3-5), 254-263.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," *Analyt. Biochem.* 72(7), 248-254.

Bryjak, J., Aniulyte, J., and Liesiene, J. (2007). "Evaluation of man-tailored cellulose-based carriers in glucoamylase immobilization," *Carbohyd. Res.* 342(8), 1105-1109.

Costa, S. A., Tzanov, T., Paar, A., Gudelj, M., Gübitz, G. M., and Cavaco-Paulo, A. (2001). "Immobilization of catalases from Bacillus SF on alumina for the treatment of textile bleaching effluents," *Enzyme Microb. Tech.* 28(9-10), 815-819.

Elnashar, M. M. M., Danial, E. N., and Awad, G. E. A. (2009). "Novel carrier of grafted alginate for covalent immobilization of inulinase," *Ind. Eng. Chem. Res.* 48(22), 9781-9785.

Gong, R., Zhu, S., Zhang, D., Chen, J., Ni, S., and Guan, R. (2008) "Adsorption behavior of cationic dyes on citric acid esterifying wheat straw: kinetic and thermodynamic profile," *Desalination* 230(1-3), 220-228.

González-Sáiz, J. M., and Pizarro, C. (2001). "Polyacrylamide gels as support for enzyme immobilization by entrapment. Effect of polyelectrolyte carrier, pH and temperature on enzyme action and kinetics parameters," *Eur. Polym. J.* 37(3), 435-444.

Huang, X.-J., Chen, P.-C., Huang, F., Ou, Y., Chen, M.-R., and Xu, Z.-K. (2011). "Immobilization of *Candida rugosa* lipase on electrospun cellulose nanofiber membrane," *J. Mol. Catal. B-Enzym.* 70(3-4), 95-100.

Iqbal, M., and Saeed, A. (2007). "Biosorption of reactive dye by loofa sponge-immobilized fungal biomass of *Phanerochaete chrysosporium*," *Process Biochem.* 42(7), 1160-1164.

Koszelewski, D., Müller, N., Schrittwieser, J. H., Faber, K., and Kroutil, W. (2010). "Immobilization of ω-transaminases by encapsulation in a sol–gel/celite matrix," *J. Mol. Catal. B-Enzym.* 63(1-2), 39-44.

Kumar, D., Nagar, S., Bhushan, I., Kumar, L., Parshad, R., and Gupta, V. K. (2013). "Covalent immobilization of organic solvent tolerant lipase on aluminum oxide pellets and its potential application in esterification reaction," *J. Mol. Catal. B-Enzym.* 87, 51-61.

Labus, K., Turek, A., Liesiene, J., and Bryjak, J. (2011). "Efficient *Agaricus bisporus* tyrosinase immobilization on cellulose-based carriers," *Biochem. Eng. J.* 56(3), 232-240.

Meleigy, S. A., and Khalaf, M. A. (2009). "Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant *Fusarium moniliforme* cells immobilized on loofa sponge," *Bioresource Technol.* 100(1), 374-379.

Pazzetto, R., Delani, T. C. O., Fenelon, V. C., and Matioli, G. (2011). "Cyclodextrin production by *Bacillus firmus* strain 37 cells immobilized on loofa sponge," *Process Biochem.* 46(1), 46-51.

Petronijević, Ž., Ristić, S., Pešić, D., and Šmelcerović, A. (2007). "Immobilization of dextransucrase on regenerated benzoyl cellulose carriers," *Enzyme Microb. Tech.* 40(4), 763-768.

Saudagar, P. S., Shaligram, N. S., and Singhal, R. S. (2008). "Immobilization of *Streptomyces clavuligerus* on loofah sponge for the production of clavulanic acid," *Bioresource Technol.* 99(7), 2250-2253.

Sheldon, R. A. (2007). "Enzyme immobilization: The quest for optimum performance," *Adv. Synth. Catal.* 349(8-9), 1289-1307.

Singh, R. K., Zhang, Y.-W., Nguyen, N.-P.-T., Jeya, M., and Lee J.-K. (2011). "Covalent immobilization of β-1,4-glucosidase from *Agaricus arvensis* onto functionalized silicon oxide nanoparticles," *Appl. Microbiol. Biotechnol.* 89(2), 337-344.

Šulek, F., Fernández, D. P., Knez, Ž., Habulin, M., and Sheldon, R. A. (2011). "Immobilization of horseradish peroxidase as crosslinked enzyme aggregates (CLEAs)," *Process Biochem.* 46(3), 765-769.

Varavinit, S., Chaokasem, N., and Shobsngob, S. (2001). "Covalent immobilization of a glucoamylase to bagasse dialdehyde cellulose," *World J. Microb. Biot.* 17(7), 721-725.

Villalonga, R., Fujii, A., Shinohara, H., Tachibana, S., and Asano, Y. (2008). "Covalent immobilization of phenylalanine dehydrogenase on cellulose membrane for biosensor construction," *Sensor. Actuat. B-Chem.* 129(1), 195-199.

Wu, S.-C., and Lia, Y.-K. (2008). "Application of bacterial cellulose pellets in enzyme immobilization," *J. Mol. Catal. B-Enzym.* 54(3-4), 103-108.

Yi, S.-S., Noh, J.-M., and Lee, Y.-S. (2009). "Amino acid modified chitosan beads: Improved polymer supports for immobilization of lipase from *Candida rugosa*," *J. Mol. Catal. B-Enzym.* 57(1-4), 123-129.

Zheng, M.-M., Lu, Y., Dong, L., Guo, P.-M., Deng, Q.-C., Li, W.-L., Feng, Y.-Q., and Huang, F.-H., (2012). "Immobilization of *Candida rugosa* lipase on hydrophobic/ strong cation-exchange functional silica particles for biocatalytic synthesis of phytosterol esters," *Bioresour. Technol.* 115, 141-146

Zheng, M.-M., Lu, Y., Huang, F.-H., Wang, L., Guo, P.-M., Feng, Y.-Q., and Deng, Q.-C. (2013). "Lipase immobilization on hyper-cross-linked polymer-coated silica for biocatalytic synthesis of phytosterol esters with controllable fatty acid composition," *J. Agric. Food Chem.* 61(1), 231-237.

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