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Immobilization of Lipase using Alginate Hydrogel Beads and Enzymatic Evaluation in Hydrolysis of *p*-Nitrophenol Butyrate

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The immobilization of enzyme is one of the key issues both in the field of enzymatic research and industrialization. In this work, we reported a facile method to immobilize Candida Antarctica lipase B (CALB) in alginate carrier. In the presence of calcium cation, the enzyme-alginate suspension could be cross-linked to form beads with porous structure at room temperature, and the enzyme CALB was dispersed in the beads. Activity of the enzyme-alginate composite was verified by enzymatic hydrolysis reaction of *p*-nitrophenol butyrate in aqueous phase. The effects of reaction parameters such as temperature, pH, embedding and lyophilized time on the reactive behavior were discussed. Reuse cycle experiments for the hydrolysis of *p*-nitrophenol butyrate demonstrated that activity of the enzyme-alginate composite was maintained without marked deactivation up to 6 repeated cycles.

Key Words : Alginate, Enzymatic, Hydrolysis of *p*-nitrophenol, Immobilization, Lipase CALB

Introduction

Enzymes have wide varieties of biotechnological applications. An important application of enzymes is in industrial processes and products due to their high specificity, good reactive behavior, non-toxicity, and water solubility over inorganic catalysts.[1-4] The use of enzymes is limited by their considerably unstable nature and the resulting requirement of stringent conditions, such as particular pH and temperature.[5-7] In addition, purified enzymes are rather costly and discarding them after each use is not economical. Enzyme immobilization has been studied and utilized to overcome these problems.[8-11] The enzyme immobilization is to entrap the enzymes in a semi-permeable supports, which prevents the enzyme from leaving while allowing substrates and products to pass through.[12] Immobilized enzymes typically exhibit greater stability and enzymatic activity over a broad range of pH and temperature.[13,14] In addition, Immobilization of enzymes also contributes to the development of continuous processes, and immobilized enzyme is adaptable to a variety of configurations and specific processes carried out in reactors.[15] In immobilization of enzyme, the enzyme is attached to a carrier; the properties of the carrier (chemical and mechanical) are influencing the entrapment efficiency and catalytic activity of the enzyme as well, making it even more important to find a proper immobilization method. The encapsulation of enzyme into solid support materials generally does not induce severe conformational changes in the enzyme or hinder the diffusion of the substrate.[12] The process for immobilization should be mild enough so as not to denature the enzyme during preparation. Different immobilization methods can lead to different activities and stabilities under identical circumstances. The attempts are being made to optimize the enzyme loading and minimize the loss of enzyme in the reaction medium, at the same time still maintaining the enzyme activity. Immobilization by entrapment involved the enzyme being retained in a membrane device such as a hollow fiber,[16] polymeric network[17] or microcapsule.[1] In this case, the enzyme was covalently attached to the polymer to prevent diffusion out of the carrier.

Alginate is by far the most widely used polymer for immobilization and microencapsulation technologies.[18-20] The main advantage in alginate gels is their thermostability, and thus they can be stored in room temperature. Alginate is the common name given to a family of linear polysaccharides containing 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) acid residues arranged randomly along the chain. Carboxylate (-coo-), ether (-C-O-C-) and hydroxyl (-OH) are generally the most abundant acidic groups in the alginate polymer.[21] Alginate supports are usually made by crosslinking the carboxyl group of the α-L-guluronic acid with a solution of a cationic crosslinker such as calcium chloride, barium chloride.[22] In our previous works, we developed a general and green approach to synthesize and stabilize uniform noble metal nanoparticles in alginate.[23] In the presence of calcium cation, the metal-alginate suspension could be cross-linked to form beads with porous structure at room temperature, and the metal nanoparticles were evenly dispersed in the beads. As-synthesized metal-alginate could be expediently designed in the form of homogeneous or heterogeneous carrier according to different real applications.

An adequate geometrical congruency between enzyme and support is also important: the greater the enzyme-support congruence, the higher the possibility to achieve an intense multipoint attachment. Calcium alginate is one of the most commonly used materials for enzyme encapsulation due to

its natural biocompatibility, ease of formation, and mild physiological gelation conditions. General preparation methods referred to in the literature were more complex, and prone to leakage of the enzyme.[12] In this work, simple and modified encapsulation procedures were applied, the Candida Antarctica lipase B (CALB) was successfully immobilized in alginate carrier. In the presence of calcium cation, the enzyme-alginate suspension could be cross-linked to form beads with porous structure at room temperature. Activity of the enzyme-alginate was verified by enzymatic hydrolysis reaction of *p*-nitrophenol butyrate in aqueous phase. The effects of reaction parameters on the resolution behavior such as temperature, pH, embedding and lyophilized time were discussed. To explore the advantage of the enzyme-alginate beads and their application, the storage and recycling stability were examined for the hydrolysis of *p*-nitrophenol butyrate. The activity of the enzyme-alginate beads was maintained without marked deactivation up to 6 repeated cycles.

Materials and Methods

Materials. Sodium alginate was purchased from Alfa Aesar Co. Anhydrous calcium chloride ($CaCl_2$, analytical reagent grade), *p*-Nitrophenyl butyrate (98%) was purchased from sigma (China). Potassium Phosphate Monobasic (KH_2PO_4), Potassium hydrogen phosphate anhydrous (K_2HPO_4) were supplied by Sinopharm chemical reagent Beijing Co. Ltd. CALB was purchased from novozymes (China).

Fabrication of Enzymatic Catalyst. In a typical experiment, the desired amount of sodium alginate dissolved in pure water, and stirred until the alginate being fully dissolved at room temperature, and then kept standing for about half hours. 50-100 μL of CALB was added into 2 mL of sodium alginate aqueous solution (0.02 g/mL), and the mixture was shaken to evenly state in vibration bed. The enzyme-alginate suspension was dropwise added into 30 mL $CaCl_2$ (10 wt %) aqueous solution without stirring and formed colorless transparent gel beads with diameter about 2-5 mm. After embedding for 60-100 min, these beads were repeated washing with deionized water in the Buchner funnel filtration process. Then, these beads were transferred to a clean glass dish which was sealed with sealing membrane. The beads were stored in a refrigerator (temperature: −11 °C) and used within a week. The top of the sealing cap of the dish was punctured with syringe needle before freeze-drying. The samples environment and samples themself were hold to be clean, and avoided any entry of moisture during the preparation processes.

Hydrolysis Reaction of *p*-Nitrophenol Butyrate. For hydrolysis of *p*-nitrophenol butyrate, a typical experiment was performed as following: 0.06 g of freeze-dried enzyme carefully was placed in 12 mL glass vial, and 3 mL of pure water was added in the vial also. In this case, pure water was both for the reactant and solvent. Taking 10 μL *p*-nitrophenol butyrate (0.06 mmol) instilled into the reaction system. Then, the vial was placed in water bath, and stirred by a magnetic stirrer at the rate of ~800 rpm. After 30 min of reaction, 5 mL of ethanol was added to stop the enzymatic reaction. The resulting solutions were conducted UV-vis spectroscopy analysis. Because sodium alginate carrier could beget autocatalytic hydrolysis reaction, in order to calculate the yield of hydrolysis, the actual quantity of the product was the difference between the sample absorbance minus the blank test (sodium alginate carries without enzyme). The λ_{max} of the hydrolysis product for aqueous medium was found to be at 310 nm.[24] The actual amount of the hydrolysis was deducted the self-reactive amount of alginate.

Results and Discussion

In this study, we took the hydrolysis of *p*-nitrophenol butyrate as a model reaction to evaluate the catalytic activity of the enzymes in different forms (free enzyme, alginate beads with immobilized enzyme, and lyophilized alginate beads with immobilized enzyme). The active order of the enzyme CALB in different forms was shown in Figure 1. In aqueous solutions, the water molecules in form of bound water could maintain the enzyme conformations in active state with the aid of hydrogen bonding, hydrophobic bond, van der Waals forces.[25] Figure 1 demonstrated that lyophilized alginate beads with immobilized enzyme had almost the same activity compared to the free enzyme. Generally, the use of microporous supports might improve the catalytic properties of immobilized lipase compared to those of free lipase because the substrate concentration of the liquid-solid interface is higher than that in solution. In addition, the immobilization could lead to partial coverage of the enzyme active sites, and lost the part of active center. resulting in gel enzyme activity decreased to a certain degree. Alginate itself also had some catalytic activity to hydrolysis of *p*-nitrophenol butyrate as presented in Figure 1.

Optimization of Reaction Time. The enzymatic hydro-

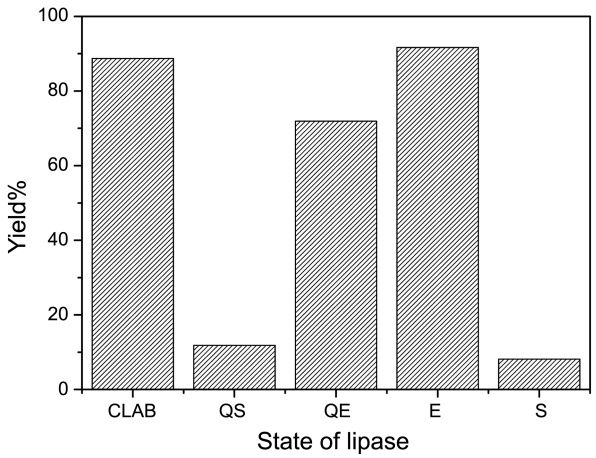

Figure 1. Effect of various enzyme forms on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 2 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 30 min, 40 °C, 800 rpm. CALB: untreated and non immobilised (soluble) CALB; QS: sodium alginate gel; QE: calcium alginate gel enzyme, E: freeze dried calcium alginate gel enzyme, S: freeze dried calcium alginate gel.

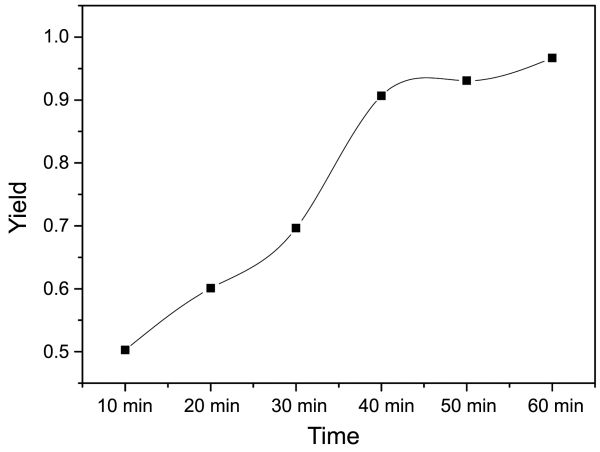

Figure 2. Effect of reaction time on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 2 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 40 °C, 800 rpm. freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

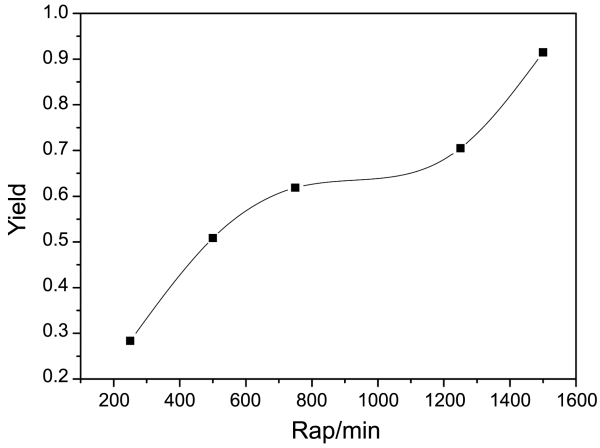

Figure 3. Effect of stirring speed on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 2 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 40 °C, 40 min, freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

lysis is a rapid and sensitive reaction, so this kind of the reaction can be used as a judgment for the activity of enzyme. Reaction time was set from 10 min to 60 min. It could be seen from Figure 2 that the hydrolysis yield increased with an increase of reaction time. As expected, the yield leveled out after 40 min, and thus the reactivity became less sensitive to reaction time. The reaction time was controlled in 40 min in following experiments unless otherwise stated.

Effect of Stirring Speed. Generally, mass transfer effects are relevant for immobilized enzymes in different forms. External mass transfer refers to molecular transport between the bulk reaction mixture and the surface of the enzyme particle through a boundary layer. The external mass transfer rate is inversely proportional to the thickness of the boundary layer around the particle. Increasing the agitation can decrease the thickness of this boundary layer, which leads to an increase in the observed reaction rate. It is clear that external mass transfer limits the enzymatic reaction. Internal mass transfer occurs within the pores of the enzyme and the carrier. The immobilization of an enzyme can affect the flexibility of the enzyme, which can also affect the accessibility of the substrates to the enzymes. Important parameters influencing internal mass transfer are the particle size, the pore size and the effective diffusion coefficient of the substrate inside the pores of the enzyme. In this study, the activity of immobilized enzyme increased with an increase of stirring rate, and leveled out as the stirring rate was greater than 800 rpm (Figure 3). The yield increased again with the increase of the stirring rate as the stirring rate was greater than 1400 rpm. In that case, large number of enzyme beads broke into small pieces because of drastic stirring, and eliminated the internal transfer to a certain degree. The yield subsequently increased. The breaking of the enzyme beads might influence the reuse of the enzymatic catalysts, so in this work, we selected the stirring rate at about 800 rpm.

Effect of Reaction Temperature. It is well known that the enzymes as biocatalysts relying on the external environments is more demanding, higher or lower temperature is not conducive to play a good catalytic activity.[26] The conformation of the enzyme protein, the ionization state of the functional groups, the affinity of the enzyme and substrate are all influenced by the temperature.[27] After immobilization, the protein molecules are fixed in the gels; the molecular overall movement is blocked, thereby increasing the thermal stability of the enzyme. So, immobilization result in a broadening of the temperature range of enzyme activity compared to the free enzyme.[28] The spatial structure of the immobilized enzyme is affected by the carrier. The dependence of temperature on the enzyme spatial structure become weak,making the immobilized enzyme is not very sensitive to temperature change. For the lyophilized alginate beads fabricated by this work, the activity temperature profile of the immobilized enzyme showed the maximum yield at 35 °C (Figure 4). In the temperature range of 30 °C-40 °C, the immobilized enzyme maintained high activity. With increasing the temperature, for example, greater than 40 °C, the activity of the immobilized enzymes decreased

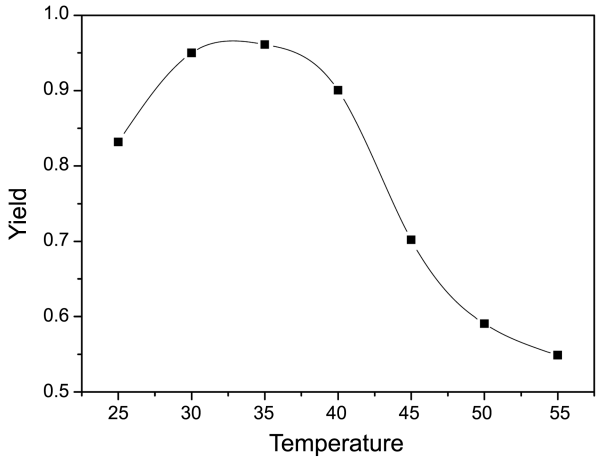

Figure 4. Effect of reaction temperature on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 2 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 40 °C, 40 min, 800 rpm. freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

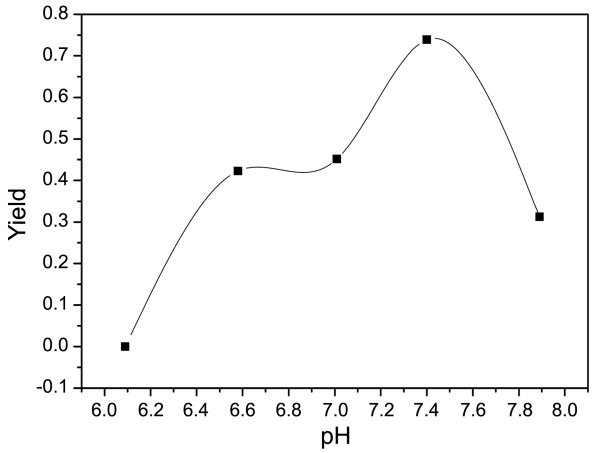

Figure 5. Effect of pH on the hydrolysis of p-nitrophenol butyrate. Reaction conditions: 3 mL of 100 mM potassium phosphate buffer, 0.06 mmol *p*-Nitrophenyl butyrate, 35 °C, 40 min, 800 rpm, freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

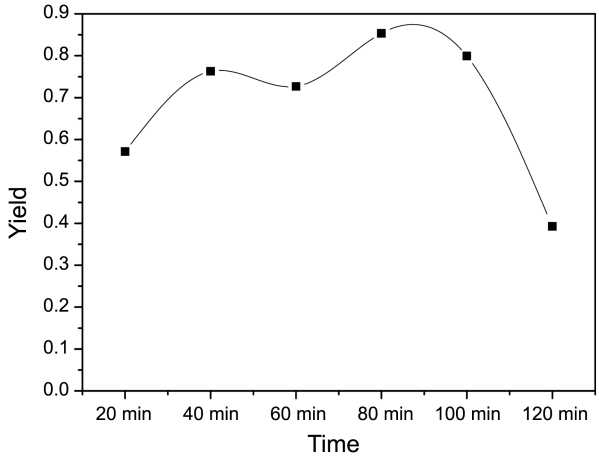

Figure 6. Effect of embedding time on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 3 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 35 °C, 40 min, 800 rpm, freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

significantly. This might be a reason that irreversible degeneration occurred in the enzymes by an increase of temperature.

Effect of the pH. Variation of pH has a significant effect on enzyme stability because the value of pH affects protein dissociation and net charge.[29] For the immobilized enzymatic catalysts, the property of carrier has apparent effect on the optimal pH of enzymatic activity. Although often the reactive activity follows a bell shaped dependence on pH, but more information is required to propound a general theory. To characterize the properties of immobilized enzyme, the effects of pH on the enzymatic activity were assessed. Enzyme activity as a function of pH was determined at 35 °C in 0.1 M phosphate buffer solution over the range of 6.0 to 8.0. Figure 5 demonstrated the activity of the immobilized enzyme beads was preferable at pH 7.4. In the pH range 6.5-8.0, the alginate beads encapsulated with enzyme exhibited acomparable stability. Within a certain pH range, most proteins were stable. As the proteins contacted with a very high pH media, the ionic groups within the protein molecule would produce a strong electrostatic repulsion, which made the protein molecule stretching, degeneration and destruction of the enzyme active center possibly occurred, resulting in the decrease in enzymatic activity. Generally, after immobilization, the lipase enzyme molecules in the alginate pore was restricted, and the stretching of the enzyme molecules was blocked. Thus, the pH stability of the immobilized enzyme expected to be higher than that of the free enzyme.

Effect of Embedding Time. In order to enhance the stability of the alginate beads with enzyme, full cross-linking was needed. Generally, the amount of the enzyme immobilized into the gel increased with the extension of embedding time. Therefore, the activity of immobilized enzyme increased with the increase in embedding time. However, the Ca^{2+} in the solution could interact with the enzymes as the embedding time was too long, and leading to a significant decrease of enzymatic activity. For the hydrolysis reaction in this wok, appropriate embedding time was usually 30-40 min (Figure 6).

Effect of Lyophilized Time. Water content has a great influence on the activity of the immobilized enzyme. Naturally, drying conditions have a significant effect on the final gel properties. From the Figure 7 we could see that the moisture in the enzyme beads failed to fully sublimation when drying time was too short, and excessive free water in enzyme-alginate beads decreased the enzymatic activity to a certain degree. Too long lyophilized time might cause the loss of part of the bonding water in enzyme molecules, resulting in lowered enzymatic activity. For the hydrolysis in this work, about 10 h of drying time was sufficient to maintain the catalytic activity of the enzyme beads.

Lyophilized Enzyme Stability and Reusability. The storage and recycling stability of the enzyme-alginate beads were also examined. The same batches of prepared lyophilized enzyme beads were put in refrigerate. Under the

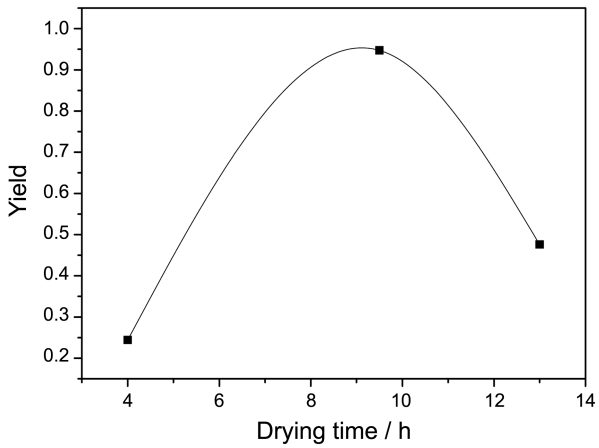

Figure 7. Effect of the lyophilized time on the hydrolysis of *p*-nitrophenol butyrate. Reaction conditions: 3 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 35 °C, 40 min, 800 rpm, freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

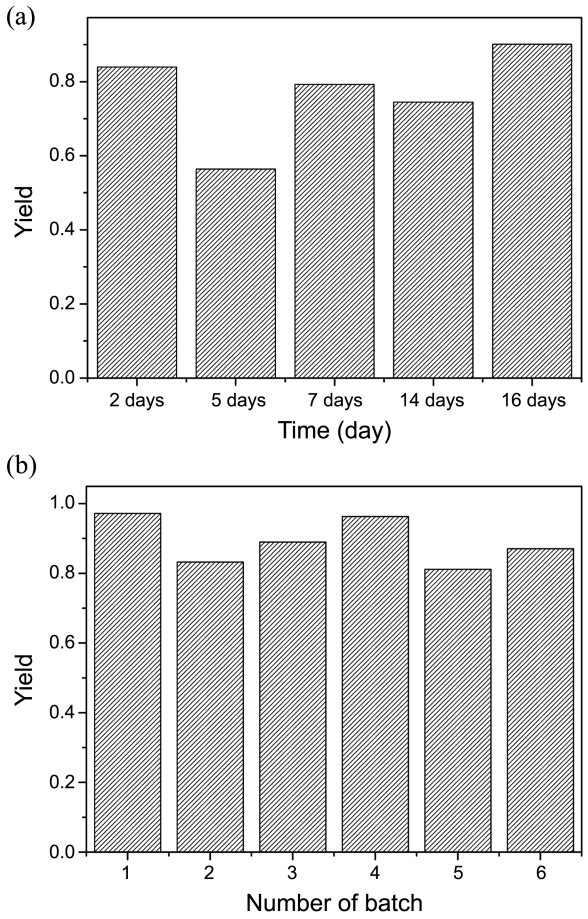

Figure 8. The storage stability (a) and recycling stability (b) of the encapsulated enzyme. Reaction conditions: 3 mL H_2O, 0.06 mmol *p*-Nitrophenyl butyrate, 35 °C, 40 min, 800 rpm, freeze dried calcium alginate gel enzyme contained 50 μL free CALB.

same experimental conditions, the activity of the lyophilized enzyme beads in different storage time was measured. As could be seen from Figure 8(a), the activity of the lyophilized enzyme beads could be well maintained after 16 days storage.

The superiority of the immobilized enzyme is its easy separation with the reaction system, and can be recycled many times. Enzyme beads reuse experiments carried out in following method: the enzyme beads were carefully taken out from the reactor after a batch reaction, and re-placed in a Buchner funnel filtration and repeated washing with distilled water. Under the same conditions, the hydrolysis catalyzed by the same enzyme beads was carried out again. The enzyme activities of each batch reaction were compared in Figure 8(b). The enzyme beads could still maintain a high activity after the six cycles. Carboxyl is the abundant acidic functional group in the alginate, the pK_a of guluronic acid is 3.65, and the pK_a of mannuronic acid is 3.38. Therefore, the alginate gel is usually negatively charged. When alginate and enzyme solution was mixed, if controlling the pH of the solution below the isoelectric point of the enzyme (the isoelectric point for CALB is 6.0[30]) and the enzyme was positively charged, these enzymes could be adsorbed on the alginate network, thereby reducing the leakage of enzymes. In this work, we fabricated the immobilized enzyme catalyst by using buffer solution instead of water to dissolve alginate. In this way, the dense gel was formed; the leakage of the enzyme was effectively blocked. The enzyme immobilization method used in this study could be recycled many times under the premise of maintaining the enzyme activity, suggesting the method could provide a good foundation for further industrial applications.

Conclusion

A simple and green pathway towards the immobilization of lipase CALB in alginate carrier was explored. In the presence of calcium cation, the enzyme-alginate suspension could be cross-linked to form beads with porous structure at room temperature. Activities of the enzyme-alginate beads were verified by enzymatic hydrolysis reaction of *p*-nitrophenol butyrate in aqueous phase. The effects of reaction parameters such as temperature, pH, embedding and lyophilized time on the resolution behavior were discussed. To explore the advantage of the enzyme-alginate beads and their application, reuse cycles were tested for the hydrolysis of *p*-nitrophenol butyrate. The activity of the enzyme-alginate beads was maintained without marked deactivation up to 6 repeated cycles. This work provided a simple way for the design and immobilization of enzyme in catalysis and other applications. The immobilization method provided by this work expected to be expediently used in various organic reaction systems. Studies along these are now in progress.

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References

1. Seema, S. B.; Steven, H. *Biomaterials* **2002**, *23*, 3627.
2. Brian, O. H.; Al-Bahrani, J. L.; Maria, C.; Armando, C. F. B.; Roland, W.; Helen, C. H.; Nicolas, S. *J . Mol. Catal. B-enzym.* **2012**, *77*, 1.
3. Mélanie, B.; Jérôme, B.; Annie, M.; Nicholas, P.; Pierre, M.; Mohammed, D.; Robert, C. *Tetrahedron: Asymmetry* **2012**, *23*, 428.
4. Krienke, H.; Kunz, W.; Xenakis, A.; Schmeer, G. *Biocatalysis Using Lipase Immobilized in Organogels in Supercritical Carbon Dioxide*; 2005.
5. Tang, Y. J.; Li, Y. *China Bio.* **2007**, *27*, 110.
6. Guzmán, F.; Barberis, S.; Illanes, A. *Electron. J. Biotechnol*. **2007**, *10*, 279.
7. Ken, D.; Johnstone, M.; Dieckelmann; Michael, P.; Jennings, J. T. Blanchfield, I. T. *Understanding Biology Using Peptides American Peptide Symposia* **2006**, *9*, 511.
8. Taqieddin, E.; Amiji, M. *Biomaterials* **2004**, *25*, 1937.
9. Carolina, P.; María, C.; Branesb, A. M.; Gloria, F. L.; José, M. G.; Rolando, C. L.; Wilson. *J. Mol. Catal. B-enzym.* **2012**, *78*, 111.
10. Sogani, M.; Mathur, N.; Bhatnagar, P. *Int. J. Environ. Sci. Tec.* **2012**, *9*, 119.

11. Liu, N.; Wang, Y.; Zhao, Q. Z.; Cui, C.; Fu, M.; Zhao, M. *Food Chemistry* **2012**, *134*, 301.
12. Gemeiner, P. *Enzyme Engineering*: Immobilized Biosystems. Chichester, UK, Ellis Horwood, Ltd: 1992.
13. Pencreac'h, G.; Leullier, M.; Baratti, J. C. *Biotechnol Bioeng*. **1997**, *56*, 181.
14. Gawlitza, K.; Wu, C.; Georgieva, R.; Wang, D.; Ansorge-Schumacher, M. B.; Klitzing, R. V. *Phys. Chem. Chem. Phys.* **2012**, *14*, 9594.
15. Hertzberg, S.; Kvittingen, L.; Anthonsen, T.; Skjak-Braek, G. *Enzyme. Microb. Technol*. **1992**, *14*, 42.
16. David, T.; Arazawa, H.; Oh, S. H.; Carl, A.; Johnson, J. R.; Woolley, W. R.; Wagner, W. J.; Federspiel. *J. Membrane. Sci.* **2012**, *25*, 403.
17. Dashevsky, A. *Int. J. Pharm*. **1998**, *1*, 161.
18. Nenad, B.; Milosavic, R. M.; Prodanovic, M. *Methods in Molecular Biology* 2011; p 155.
19. Orive, G.; De Castro, M.; Kong, H. J.; Hernandez, R. M.; Ponce, S.; Mooney, D. J. *J. Control. Release*. **2009**, *135*, 203.
20. Wang, N.; Adams, G.; Buttery, L.; Falcone, F. H.; Stolnik, S. *J. Biotechnol*. **2009**, *144*, 304.
21. Andrea, B.; Barigelli, E.; Dentin, M. *Biomacromolecules* **2009**, *10*, 2328.
22. Hao, X. L.; Xia, Y. Z.; Ji, Q.; Kong, Q. S.; Sui, K. Y. *Sci. Technol. Eng.* **2010**, *10*, 2800.
23. Chen, P.; Zhang, X. G.; Liu, Z. Z. *Nanosci. Nanotech.* **2008**, *1*, 8.
24. Quinn, D. M.; Shirai, K.; Jackson, R. L.; Harmony, J. A. K. *Biochemistry* **1982**, *21*, 6872.
25. Sivalingam, G.; Chattopadhyay, S.; Madras, G. *Chem. Eng. Sci.* **2003**, *58*, 2911.
26. Song, B. D.; Song, L.; Pang, C. X.; Chen, E. X.; Jiang, Y. Q. *China, Chem. Eng.* **2009**, *37*, 8.
27. Abdulkareem, J. H.; Adhami, A.; Jolanta, B.; Beata, G. M. *Process Biochem.* **2002**, *37*, 1387.
28. Cai, J. M.; Wu, K.; Zhang, J. *China, J. Biochem. Mol. Bio*. **2002**, *18*, 548.
29. Xie, H. L.; Zhu, S.; Wang, J. *China. J. Environ. Sci*. **2005**, *26*, 164.
30. Rodrigues, D. S.; Cavalcante, G. P.; Ferreira, A. L. O.; Goncalves, L. R. B. *Chem. Biochem. Eng*. **2008**, *22*, 125.