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Immobilization of lipase on chemically modified bimodal ceramic foams for olive oil hydrolysis

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ABSTRACT

The work reported in this paper is aimed at exploring the feasibility of immobilizing alkali lipase from *Penicillium expansum* on a bimodal ceramic foam, which has both macro- and micro-pore structures. After being chemically modified with a silane coupling agent, the ceramic foam was used as a support for lipase immobilization. To determine the preferable immobilization conditions, the effects of the amount of enzyme for loading, immobilization time, temperature, and pH on enzyme activity were investigated. The results showed that the chemically modified ceramic foam has a high loading capacity and a strong binding ability for the lipase. Thanks to the low internal mass transfer resistance, the ceramic foam has greatly enhanced the rate of immobilization. As a comparison, the immobilized-lipase activity was much higher than that on many frequently used porous materials like diatomite, alumina and activated carbon.

1. Introduction

Lipases (glycerol ester hydrolases E.C.3.1.1.3) are a family of enzymes that catalyze the hydrolysis of fats in their natural environment and constitute an important class of high selectivity and stereo-specificity industrial enzymes [1]. Lipases find useful applications in hydrolysis of fats and oils [2,3], synthesis of many organic intermediates for polymer synthesis [4–6], and preparation of esters for food, cosmetic and pharmaceutical industry [1,7] especially for diesel engine fuels (biodiesel ester) [8]. In many cases, the low catalytic efficiency and stability of native enzymes are considered as main barriers for the development of their large-scale applications [9]. Whereas immobilized lipase generally have good thermal stability and reusability. Moreover, the immobilization of enzyme minimizes the cost of product isolation and provides operational flexibility.

Many supports have been studied including polymers and resins [1,9,10], silica and silica–alumina composites [11–13], and carbonaceous materials [14–16]. These systems generally have a low mechanical strength and often exhibit severe diffusion limitations, leading to a relatively low enzymatic activity [17,18]. To minimize the internal diffusion limitation, porous supports are mostly used in particulate form [1,19]. The size of the carriers is even on the micron scale and cannot be used in fixed-bed reactors [20]. In viewpoint of industrial applications, novel monolithic supports in the form of foams, fibers, membranes and honeycombs have been developed to take the place of traditional granular ones [21,22]. Due to having high mechanical strength and good durability, porous ceramics are often used as supports for enzyme immobilization [22–26]. Macrostructured foam-like ceramics were more favorable primarily because of its low diffusion limitations [16]. In this work, a unique macrostructured ceramic foam with a bimodal pore size distribution was prepared using our patented methodology [27]. In industrial field, it can be potentially applied in monolithic fixed-bed reactors, which greatly facilitate continuous operation and product isolation [28].

For effective enzyme immobilization, the surface of porous ceramics often coated with a carbon layer [16,17,29]. It should be noted that the carbon layer may increase the preparation cost because of the complex procedures to produce the carbon layer [10]. On account of the relatively high surface hydrophobicity of lipases, simple adsorption of lipases on suitably hydrophobic supports has been the more popular strategy over covalent conjugation methods [30,31]. This method [32] has proven very useful to achieve hyperactivation of most lipases: the hydrophobic surface of the matrix induces the conformational change on lipases necessary to enable free access of substrates to their active centers. To obtain suitable hydrophobicity, the surface of inorganic supports may be modified with silane coupling agent [33]. Since the ceramic pore surface with a coating layer possessing methacryloyloxy group was able to immobilize lipase on successfully [34], our ceramic foams for lipase immobilization were first modified with a silane coupling agent also possessing methacryloyloxy group.

In oleochemical industry, the most important application of lipase is to produce fatty acids from oils by hydrolysis [2]. Partial

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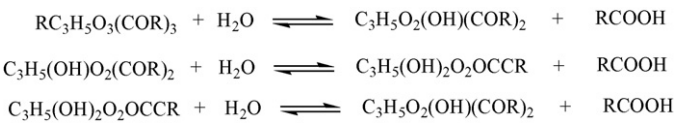

Fig. 1. Reactions of triglyceride hydrolysis catalyzed by lipases.

hydrolysis of triglycerides will yield mono- and diglycerides and fatty acids (Fig. 1). When the hydrolysis is carried to completion, the mono-, di- and triglycerides will hydrolyze to yield fatty acids and glycerol. The traditional method of hydrolysis involves the use of high temperature, pressure and chemical catalysts [35]. Thereupon, enzyme-catalyzed hydrolysis is recently attempted as an energy-saving method, especially for producing high value-added products or heat sensitive fatty acids [36,37]. The focus of this work is to investigate the optimal conditions for the preparation of immobilized lipase and its catalytic properties both in view of hydrolysis activity. An emulsifier was used to overcome the problem of intimate contact between substrate and enzyme [38,39]. On the base of this study, further work can be done in designing and developing a new monolithic fixed-bed reactor for the production of fatty acids.

2. Materials and methods

2.1. Materials

Alkaline lipase from *Penicillium expansum* (2950 U/g lipase) was purchased from Shenzhen Leveking Bio-engineering Co. Ltd., China. Coomassie brilliant blue G250, γ-(methacryloxy)propyhrimethoxy silane (A-174), oxalic acid, ethyl alcohol, 95% ethyl alcohol, sodium hydrogen phosphate, monopotassium phosphate, sodium hydroxide, crystal bovine serum albumin, and sodium chloride were all of analytical grade. Polyethylene glycol (PEG) (WM 1750 ± 50) and olive oil were of chemical grade. The concentration of phosphoric acid was ≥85%. The ceramic foam is prepared by the following method: First, the ceramic raw materials (20 g alumina, 8 g kaolin and 22 g feldspar all in the form of powder) were put into 100 g water, and then 5 g ethanol (95%) was added. After stirring, the suspension of the raw materials was obtained. The flocculating agent, 100 g polyacrylamide (0.3% mass concentration) was dropped into the suspension under agitation. The raw particles in suspension gradually became floccules with water in between them. The floccules were then added into a matrix in the shape of the desired ceramic foam to prepare the earthen ceramic base. By the process of drying and sintering, the water in the ceramic base was removed and cellular structure of the ceramic foam was formed in situ. More detailed procedures are from related literatures [27,40]. In this work, a ceramic cylinder of 100 mm in diameter and 10 mm in thickness was fabricated as shown in Fig. 2. The ceramic foam was treated subsequently by hot concentrated hydrochloric acid. After this treatment, the nanopores can be enlarged to some degree. The pore size distribution of the ceramic foam was measured with the mercury porosimeter, Autopore IV 9500 (Micromeritics Intrement Corp., USA).

2.2. Chemical modification of ceramic foam

The ceramic foam cylinder was cut into cubic forms of 5–10 mm in each edge length as the enzyme supports. The weight of each support was controlled at 200–250 mg. An aqueous alcohol solution was prepared at 1:1 (v/v), and oxalic acid was added into the solution to adjust its pH to 3.5–4.0. A-174 was then put into it to obtain a modification solution at 0.2% (wt%). The supports (total mass ≤5 g) was put into the modification solution (40 ml). In each run, the total amount of A-174 was not less than 1% (wt%) of supports. The mixture was shaken in a 30 °C water bath at 100 rpm for 5 h. After that, the support was washed for several times with alcohol and deionized water separately, and then dried at 110 °C for at least 8 h in an oven. Finally, the modified ceramic foam was cooled to room temperature in a silica gel drier for experimental use.

2.3. Immobilization of lipase

First the amount of crude lipase powder was determined by the total mass of the supports to be used and the enzyme amount per gram of support, generally 12.5 g lipase/g support. Then so much lipase powder was put into a 0.025 M and pH 8.0 phosphate buffer (PBS), to make the enzyme suspension of 0.333 g lipase powder/ml PBS. The enzyme suspension was stirred every 10 min for 30 min, and then centrifuged at 1000 rpm. The resultant supernatant was filtrated with filter paper for further purification. Subsequently, the modified ceramic foams were impregnated in the lipase filtrate at 20 °C and 150 rpm for 4 h. Finally, the ceramic foams with adsorbed lipase were thoroughly rinsed with PBS for three to five times and dried at room temperature for at least 24 h.

2.4. Determination of lipase activity

The enzyme activity of free and immobilized lipase was measured by the classical olive oil emulsion method [41]. Emulsion of olive oil was produced by emulsifying 50 ml olive oil and 100 ml 4% PEG solution (40 g PEG/1000 ml water) at 17,000 rpm.

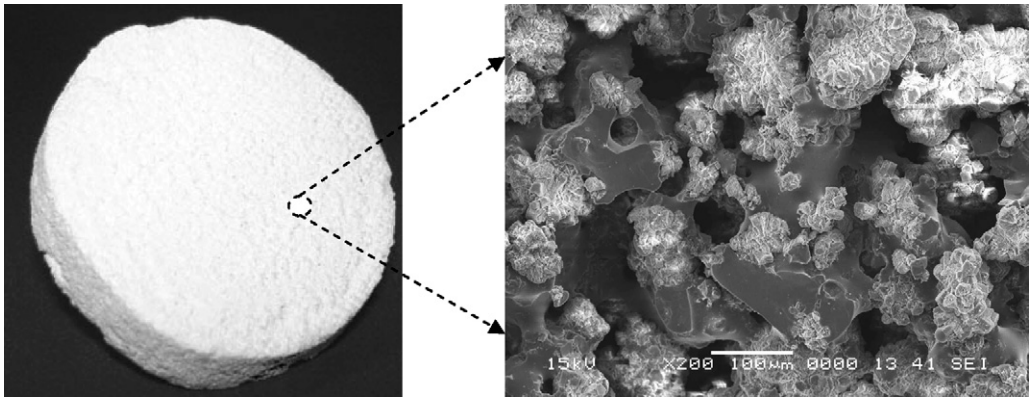

Fig. 2. Photograph of ceramic foam in the shape of cylinder with 100 mm diameter and 10 mm thickness (left) and scanning electronics microphotograph (SEM) of ceramic foam treated by HCl (right).

The emulsion (4 ml) and 5 ml PBS (pH 8.0, 0.025 M) was mixed together in a conical flask of 100 ml and preheated in water bath at 30 °C for 5 min. Then, a piece of lipase-carrying ceramic foam after being weighted was put into the mixture to catalyze the hydrolysis reaction at 150 rpm. Exactly 10 min later, the reaction was refrained by adding 15 ml alcohol (95%). Blank samples were prepared in parallel. The difference was that the alcohol was added just after the pre-heating, but before the addition of the lipase. For the measurement of free lipase, all the steps were the same except that the immobilized lipase was replaced by 1 ml lipase solution that was moderately diluted. The fatty acid produced in the reaction was quantified by the volume of the consumed alkali solution. One unit of lipase activity (U) was defined as the amount of enzyme required to produce 1 μmol fatty acid per minute under the experimental conditions. The activity was expressed in U/g, the value of which can be calculated by the following formula [42]:

$$\frac{50(V_2 - V_1)}{10M}n \quad (1)$$

where 50 is the concentration of the sodium hydroxide solution (μmol/ml), V_1 and V_2 the solution volume of sodium hydroxide which was consumed by the reaction sample and blank sample respectively (ml), n the dilution multiple (for immobilized lipase, $n = 1$), 10 the reaction time (min), and M is the mass of raw lipase powder, support or protein adsorbed (g). The result was expressed as the mean value of at least three independent measurements.

2.5. Protein determination

Protein concentration was determined by the Bradford method [43]. The amount of protein adsorbed on the supports was calculated from that of the protein in the enzyme solution and in the washing solution, as following equation shows:

$$p = \frac{(c_2 - c_1)V - c_w V_w}{M}, \quad (2)$$

where p is the amount of enzyme bound onto supports (mg/g), C_1, C_2 and c_w the concentration of protein in the initial enzyme solution, final enzyme solution and final washing solution, respectively (mg/ml), V and V_w the volume of the enzyme solution and the total final washing solution, respectively (ml), and M is the mass of support (g). The data of protein concentration are an average of at least three experiments.

2.6. Optimization of lipase immobilization

The amount of lipase powder per gram of ceramic supports, time, temperature and pH for lipase immobilization were investigated with hydrolysis activity as objective function. The initial conditions were selected as 15 h of adsorption time, pH 7.5 of PBS, 0.333 g (crude lipase powder)/ml (PBS) of enzyme concentration and 30 °C of water bath. The latter immobilization conditions were based on the former optimized condition(s), and the resultant favorable conditions were obtained after the last optimization experiment was finished. The values of the independent variables were selected as (2, 4, ..., 12 g lipase/g ceramic), (0.33, 1.0, 2.3, 4.0, 5.0 h), (10, 20, 30, 40, 50 °C) and (pH 6, 7, 8, 9, 10). Among them, the immobilization rate was demonstrated by the accumulate lipase activities at different immobilization time. More details are described in the corresponding figure captions.

2.7. Thermal stability and operational stability

The thermal stability was tested according to the residual activity of the lipase, which had been incubated in PBS (pH 8.0, 0.025 M) for 1 h at different bath temperatures. To investigate the operational stability of immobilized lipase, it was repeatedly used in the determination of lipase activity. When one activity measurement was over, the lipase-carrying ceramic foam was thoroughly rinsed with PBS (pH 7.5, 0.025 M). Then, it was added into a fresh reaction mixture to catalyze the hydrolysis reaction as before.

2.8. Optimum working pH and temperature of immobilized lipase

The reaction system was what was used in the measurement of lipase activity. The pH of PBS in hydrolysis was investigated at 36 °C. For the optimum temperature, experiments were carried out at pH 8.0 of PBS. Detailed descriptions are reported in the figure captions.

3. Results and discussion

3.1. Characterization of ceramic foam

The ceramic foam sample shown in Fig. 3 exhibits a bimodal pore size distribution at 45 μm and 77 nm in diameters. The widths of micropores range from 0.3 to 2.0 nm, mesoporous substances have pore sizes from 2 up to 50 nm and macropores range from widths of 50 up to 10^5 nm [44]. So the ceramic foam has a macropore structure. The micrometer pores are the main contribution to the porosity (0.56) and total pore volume (0.4748 ml/g), which minimize the diffusion resistance of the molecules [45]. The nanopores greatly increase the specific surface area for enzyme immobilization, which may eliminate the necessity of coating carbon layer. The total pore area is 7.038 m^2/g. Although it seems to be small, most of the pores are large enough to immobilize lipases. According to some authors, a convenient pore size is around 100 nm for lipase immobilization [46,47]. More suitable pores may be in the diameter range of 50–100 nm because of their excellent comprehensive behavior in enzyme loading and internal diffusion coefficient [48]. The reproducibility of the preparation of the ceramic foam in terms of porosity control is good. For example, when the speed of the stirrer in the flocculation procedure was controlled at 200, 300, 400 and 600 rpm, the porosity of the ceramic foam product is 0.57, 0.60, 0.56 and 0.54, respectively [27]. In a word, the ceramic foams produced with our new method are very suitable for the immobilization of lipases.

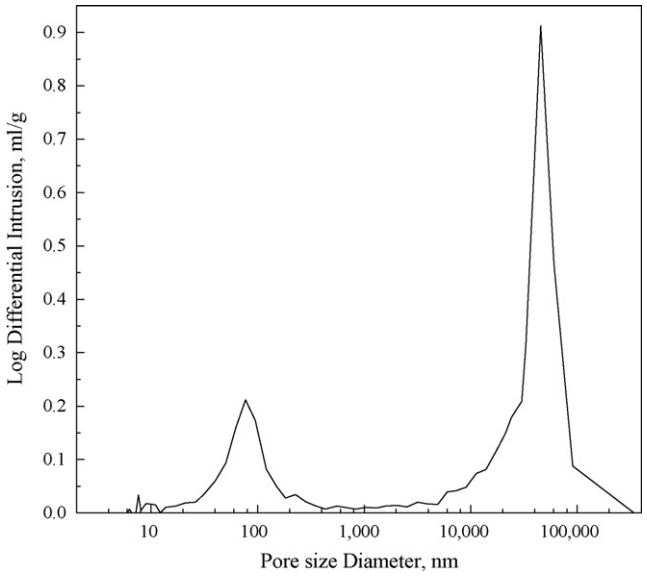

Fig. 3. Pore size distribution of ceramic foam analyzed by Autopore IV 9500.

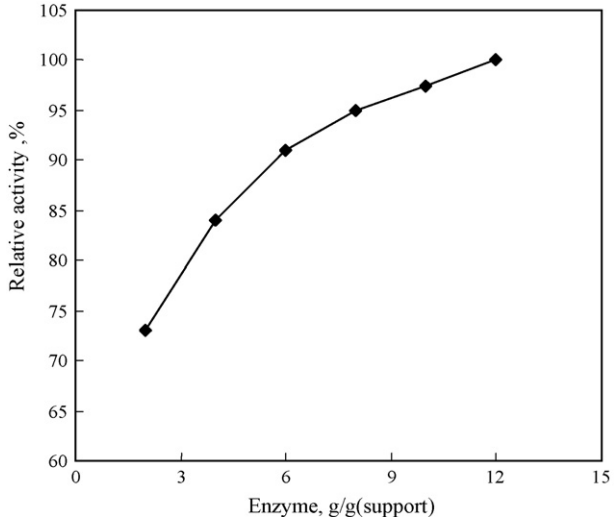

Fig. 4. Effect of enzyme amount on activity of immobilized enzyme: immobilizations were carried out at 0.333 g lipase/ml (PBS of pH 7.5) and 30 °C of water bath for 15 h.

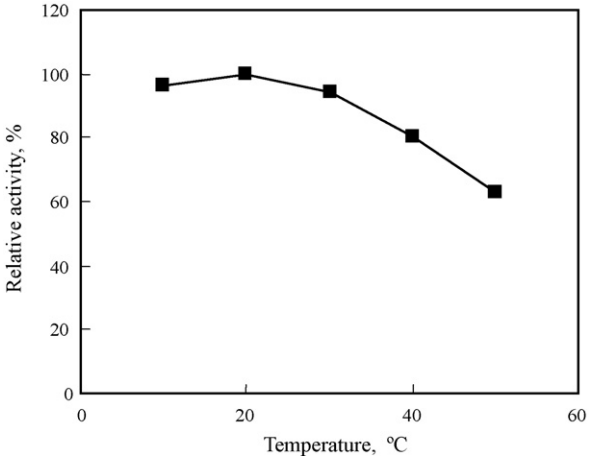

Fig. 6. Effect of temperature on activity on immobilized enzyme: immobilization conditions were that PBS was pH 7.5, enzyme amount was 12.5 g lipase/g ceramic foam, enzyme concentration is 0.333 g lipase/ml PBS and immobilization period was 4 h.

3.2. Enzyme immobilization

3.2.1. Effect of amount of enzyme

When the enzyme concentration and the mass of ceramic foam are selected, the total amount of enzyme for lipase immobilization is dependent on how much lipase powder is assigned to 1 g ceramic foams. In this experiment, the lipase concentration is selected as 0.333 g (native lipase)/ml (PBS). If the concentration is too high, the use rate of the crude lipase may be low because a lot of lipase remains in the enzyme residue after separation. Fig. 4 shows that the activity of immobilized lipase increased with the given amount of the native lipase. At a loading of 12 g lipase/g ceramic, the activity reached to the maximum. The fact that the curve tends to increase implies that the ceramic foam has a good enzyme capacity. However, if enzyme amount per gram of support was too much, the total volume of enzyme solution may be too large, which does not benefit the lipase adsorption.

3.2.2. Effect of immobilizing time

The activities of the lipase immobilized on ceramic foams for different periods of time were measured (Fig. 5). The immobilizations were performed at pH 7.5 of PBS, 30 °C of water bath, 0.333 g lipase/ml (PBS) and 12.5 g lipase/g ceramic foam. Because of the high porosity and the low diffusion resistance of the macropores, the enzyme solution could rapidly diffuse into the internal pore structure of the ceramic foam. Besides, the strong hydrophobic property of the modified ceramic also enhanced the immobilization process [31]. It was found only in 20 min, the activity reached about 61.5% of the maximum, and the immobilization of enzyme completed in 4 h, indicating the equilibrium of adsorption and desorption.

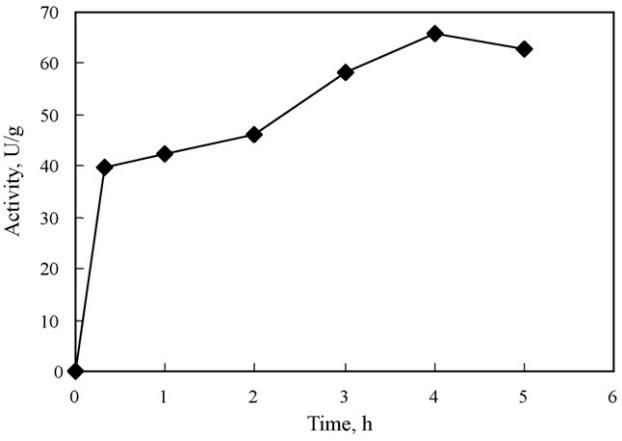

Fig. 5. Effect of immobilization time on enzyme activity: immobilizations were performed at pH 7.5 of PBS, 30 °C of water bath, 0.333 g lipase/ml (PBS) and 12.5 g lipase/g ceramic foam.

3.2.3. Effect of immobilizing temperature

Lipase was immobilized on modified ceramic foams at different water bath temperatures. The results in Fig. 6 indicate that the optimum temperature of enzyme immobilization was 20 °C, and the enzymatic activity was near its maximum value in the range of 10–30 °C. In practice, immobilization temperature can be controlled at 15–25 °C. Temperatures that are too high may cause the thermal deactivation of the enzyme.

3.2.4. Effect of solution pH

Fig. 7 shows that the activity of the immobilized enzyme was highest at pH 8.0. The pH has an effect on the ionic state of the

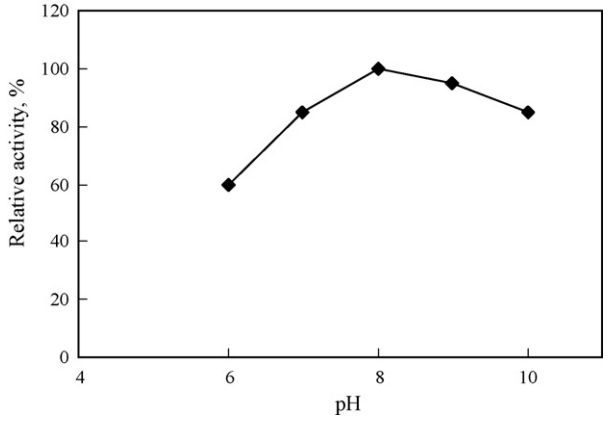

Fig. 7. Effect of pH on activity of immobilized enzyme: immobilization was performed at 30 °C, 0.333 g lipase/ml PBS and 12.5 g lipase/g ceramic foam for 4 h.

Table 1
Preferable conditions of lipase immobilization

Enzyme amount (g/g ceramic)	12
Time (h)	4
Temperature (°C)	20
pH	8

lipase molecules as well as the polarity of the organic groups on the surface of modified ceramic foams. At pH 8.0, the enzymatic polarity might be weakened, which could enhance the lipase binding onto the hydrophobic surface. On the other hand, the pH 8.0 was not too far away from the optimum pH of the free lipase, pH 9.0.

Up to now, the preferable conditions of lipase immobilization were obtained, as what was summarized in Table 1.

3.3. Characteristics of immobilized lipase

3.3.1. Thermal stability

As shown in Fig. 8, the activities of the immobilized lipase remained in a wider range of bath temperatures than that of the free lipase, which means the immobilization procedures have largely improved the thermal stability. For example, at 50 °C, the relative activity of the free lipase was only 17%, whereas that of the immobilized lipase was 46%. The strong hydrophobic interaction between the lipase and the modified ceramic surface made the molecular conformation of the immobilized enzyme more stable than that of the free enzyme, which increased the thermal stability of the immobilized lipase.

3.3.2. Optimal pH of working solution

The activities of the immobilized lipase and free lipase are compared under different working solution pH values (Fig. 9). The optimum pH for immobilized lipase was 8.0, and that of free lipase was about 9.2. That is to say, after being immobilized, the optimum pH was reduced by about 1. This might be because the pore surface was positively charged due to the skeleton of the ceramic foam contains much alumina [49]. The positive charge attracts the hydroxyl ions from the bulk solution to the interface, which causes the pH in the microenvironment of the interface to be higher than that of the bulk phase of the solution. In the catalysis of immobilized enzyme, the pH of the bulk solution must be lowered to counteract the action of the microenvironment.

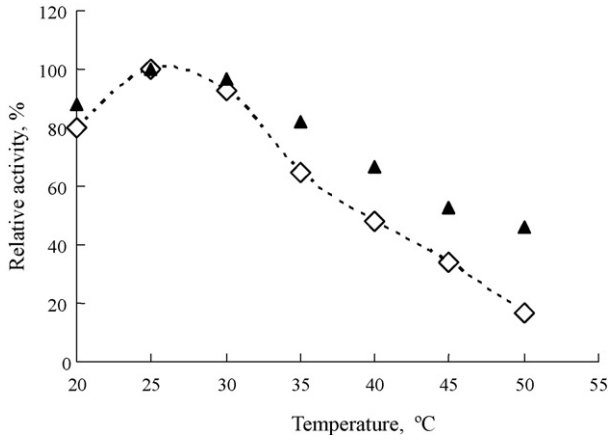

Fig. 8. Thermal stability of immobilized lipase and free lipase: hydrolysis reaction at pH 8.0 PBS, 30 °C water bath and 10 min reaction time. (◊) Free lipase; (▲) immobilized lipase.

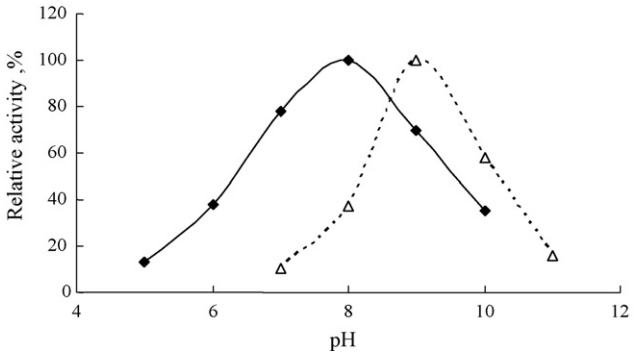

Fig. 9. Effect of reaction pH on hydrolysis activity of lipase-carried ceramic foams: at 0.025 M PBS, 30 °C water bath and 10 min reaction time. (△) Free lipase; (♦) immobilized lipase.

3.3.3. Optimal working temperature

In the measurement of the activity, the hydrolysis of olive oil was carried out at different water bath temperatures (Fig. 10). Generally speaking, the rate of reaction increases as the reaction temperature rises. Temperatures that are too high may result in the deactivation of the enzyme. In this experiment, the optimal working temperature of the immobilized lipase was about 30 °C, which is largely determined by the thermal stability of the immobilized lipase illustrated in Fig. 8.

3.3.4. Operational stability

Operational stability of the immobilized lipase in batch hydrolysis of olive oil was tested by repetitive experiments. As shown in Fig. 11, the activity of the immobilized lipase only decreased in the first repetition by a reduction of 40% from its initial activity, and the activity still kept constant even in the fourth repetition. The trend of the curve indicated that some smaller pores of the ceramic foam were clogged by the particles of the emulsified olive oil in the first cycle, but the rest larger pores were not subject to be blocked again in the other cycles [40]. Fig. 11 also indicated that there is a strong binding force between the enzyme molecules and modified pore surface. Otherwise it was impossible to keep a stable lipase activity since the second batch.

3.3.5. Specific activity and enzymatic recovery

After 1-h immobilization, the protein loading on ceramic foam was 45.36 mg protein/g ceramic and the specific activity was 1215.39 U/g protein. As a comparison, Gao et al. [50] adsorbed lipase from *P. expansum* onto the pore surface of diatomite, the protein

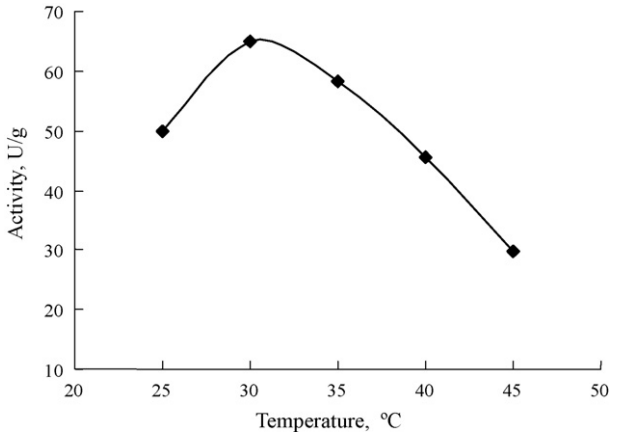

Fig. 10. Effect of reaction temperature on hydrolysis activity of lipase-carrying ceramic foams: 0.025 M and pH 8.0 PBS, 10 min reaction time.

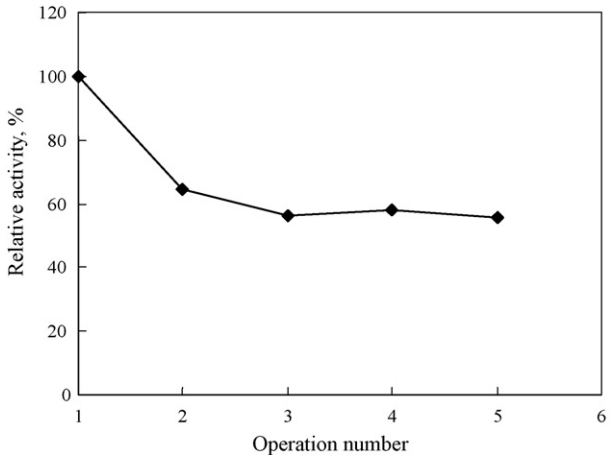

Fig. 11. Stability of immobilized lipase in batch hydrolysis of olive oil: at 0.025 M and pH 8.0 PBS, 30 °C water bath and 10 min reaction time.

loading could be up to 52 mg/g diatomite under the optimal immobilization condition and the specific activity of immobilized lipase was 1301 U/g protein. Compared with Gao's experimental results, the protein loading and the specific activity on the ceramic foam are slightly lower. It seems that the diatomite is superior to the ceramic foam. However, this remains yet to be analyzed further.

In Gao's experiments, the native lipase was purified by 15.75 times from 2465 to 38823 U/g (protein). However, in our experiments, the purchased crude lipase (2950 U/g lipase powder) was not pre-purified. The activity recovery of the lipase on ceramic foam was 41% (1215.39/2950 = 0.412), only a little lower than that on the diatomite (1301/2465 = 0.528). It was mainly owing to the hydrophobic surface property and the favorable pore size distribution of the ceramic foam. The study of Bastida et al. [30] indicated that hydrophobic supports has a purifying function in lipase immobilization through selective adsorption [34,31], and the immobilized lipase could be hyper-activated by the hydrophobic interaction between the lipase molecules and the modified surface [30]. The more suitable pore structure also contributes to the enhancement of the immobilized-lipase activity, as discussed in Section 3.1.

3.3.6. *Compared with other supports*

To assess the performance of the ceramic foam, some typical commercial inorganic supports were compared. The lipase was adsorbed on each of these supports under the same condition and its activity was measured with the same method as described in preceding paragraphs. The results in Fig. 12 shows that the activity of the lipase on modified ceramic foam was comparable to that of silica gel, yet obviously better than diatomite, activated carbon, alumina, and quartz sand.

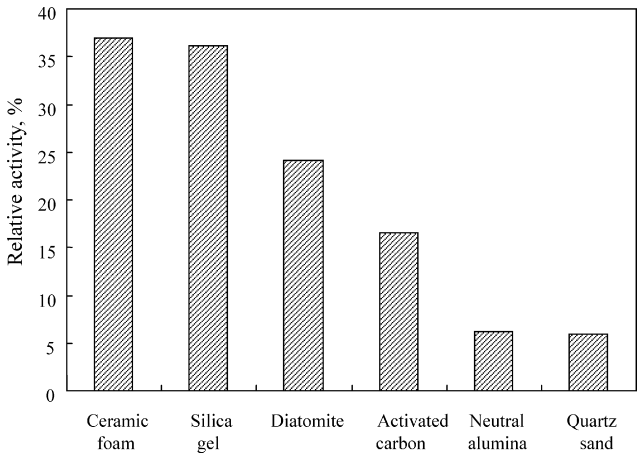

Fig. 12. Activity comparison of lipase immobilized on different inorganic materials: 0.025 M and pH 8.0 PBS, 30 °C water bath and 10 min reaction time.

4. Conclusion

Although enzyme immobilization has been studied for more than half a century, this biotechnology has not been extensively used in modern industrial process due to lack of cheap and efficient materials for enzyme immobilization in view of a feasible reactor operation. To this end, a novel ceramic foam was prepared with bimodal porous structure distributed at 45 μm and 77 nm in diameter. After chemical modification on the foam surface, the conditions for enzyme immobilization were determined under 15–25 °C and pH of PBS at 7.5–8.0. It was showed that the thermal stability of the immobilized lipase was higher than that in its free form. The optimal reaction conditions for hydrolysis of olive oil were found at pH 8.0 and 30 °C. The activity of the lipase immobilized on the ceramic foam was higher than that on some other porous supports. Moreover, the operational stability of the immobilized lipase is also remarkable. The results may be explained from the support structure and surface property: the unique bimodal macrostructure both lowered the diffusion limitation and increased the specific surface area for enzyme immobilization and hydrolysis reaction; the strong hydrophobic interaction between the lipase and the modified ceramic surface makes the molecular conformation of the immobilized lipase more stable and enables free access of substrates to their active centers.

Acknowledgements

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