Efficient Improving the Activity and Enantioselectivity of *Candida rugosa* Lipase for the Resolution of Naproxen by Enzyme Immobilization on MCM-41 Mesoporous Molecular Sieve

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Abstract: *Lipase from Candida rogusa was immobilized on MCM-41 mesoporous molecular sieves in a trapped aqueous-organic biphase system for the resolution of racemic naproxen methyl ester. It was interesting that the activity and enantioselectivity of the immobilized lipase were improved significantly relative to the free enzyme. The proportion of water (ml)/support (g) has a dramatic influence on the activity and enantioselectivity of lipase immobilized onto MCM-41 molecular sieves. It was also found that the activity of immobilized lipase was more sensitive to pH value and temperature than the free one. Higher pH value will increase the activity but decrease the enantioselectivity of the immobilized lipase. The enantioselectivity of the immobilized lipase was not altered significantly within the range of tested temperature. The immobilized lipase can be reused for at least 8 batches without significant lose of activity with the aid of methanotrophic bacteria to eliminate the methanol produced during the resolution process.*

Keywords: *MCM-41, Immobilization, Lipase, Enantioselectivity, Naproxen, Stability.*

Introduction

Increasing the activity and enantioselectivity of enzyme is very important in the enzymatic resolution of chiral compound. The use of crude lipase, often leads to lower activity and/or lower enantioselectivity [17]. Several methods were offered to improve the activity and/or enantioselectivity of lipase [11, 14, 18]. The immobilization of lipase is one of efficient method [15]. Adsorption of a biocatalyst onto a water-insoluble macroscopic support is a frequently used method for enzyme immobilization. While adsorbing forces are relatively weak in some cases. As a result of the weak binding, losses in enzyme activity are usually encountered, and desorption from the support may be caused especially in aqueous system. However, it is not the truth in a trapped aqueous-organic biphase system. In such system, the water content is so low that water is essentially restricted to the pores or interior of solid particles. Since lipase will not dissolve in the organic phase, it will remain in the trapped aqueous phase [20]. So the immobilized efficiency was theoretically 100% in the trapped aqueous-organic biphase system. Moreover, the interfacial area is increased because of the addition of a solid carrier, especially those with high specific area. This will lead to a better distribution and a higher activity of lipase. The contact between enzyme and support will affect the conformation of enzyme and this may alter the selectivity of lipase. Kamori et al. [8] found an increased activity and enantioselectivity of lipase PS in the resolution of chiral

alcohol in organic phase after the immobilization of lipase on TN-M, an inorganic porous ceramics particles.

MCM-41 mesoporous molecular sieve offers properties such as high surface area, tailorable pore size and chemical inertness which should make it an attractive material for enzyme immobilization. The uniform pore size and regular pore channel of molecular sieves [9, 19, 22] offer a distinct advantage over more conventional sol-gel derived oxide supports. The wide range of mesopores that can be engineered allows for the adsorption of quite a variety of biomolecules. All of the above-mentioned unique properties of MCM-41 molecular sieve provide promising candidate for the support of enzymes [2, 6].

In this study, we tried the immobilization of lipase from *Candida rogusa* in silica MCM-41 mesoporous molecular sieves and compared its catalytic activity and/or enantioselectivity with the free enzyme for the resolution of racemic naproxen methyl ester in a trapped aqueous-organic biphase system. The influence of carrier, enzyme loading, water content, temperature and pH on the activity and/or enantioselectivity of lipase were investigated. We also tested the reusability of the immobilized lipase.

Materials and methods

Materials

Candida rugosa lipase (EC 3.1.1.3) from Sigma Chemical Co. with a nominal specific activity of 860 U/mg was used without further purification. MCM-41 mesoporous molecular sieve was kindly provided by Dr. Xin Jing in our laboratory. The textural properties and its application in organic synthesis were published elsewhere [19, 22]. YWG-C_6H_5 was HPLC supports purchase from commercial suppliers. TiO_2 (P25) was purchased from Degussa Co. All other reagents used were obtained from commercial suppliers and were of analytical grade.

Cultivation of Methylomonas IMV 3011

The *Methylomonas* IMV 3011 was grown in the following medium (g/L): NH_4Cl, 0.5; K_2HPO_4, 0.49; $KH_2PO_4 \cdot 7H_2O$, 0.40; $MgSO_4 \cdot 7H_2O$, 0.3; $CaCl_2 \cdot 2H_2O$, 0.02; KNO_3, 1.6; NaCl, 0.3; $FeSO_4 \cdot 7H_2O$, 0.004; $CuSO_4 \cdot 5H_2O$, 0.004; $MnSO_4 \cdot H_2O$, 0.0004; $ZnSO_4 \cdot 7H_2O$, 0.00034; $Na_2MoO_4 \cdot 2H_2O$, 0.00024; pH 7.0. It was incubated for 96 h at 32 °C on 50% methane – 50% air. *Methylomonas* IMV 3011 cells were harvested by centrifugation at 9000 g and washed three times with 0.05 mol/L phosphate buffer, pH 7.0. The washed cells were resuspended in the above phosphate buffer and stored at 4 °C.

Immobilization of lipase from Candida rugosa

0.5 g MCM-41 molecular sieve (or 0.5 g other inorganic support) was suspended in 20 ml isooctane, and then phosphate buffer (from 0.5~1.25 mL, 0.2 M, pH 7.0) dissolving a certain amount (25 mg ~ 125 mg) of lipase was added with mild magnetic stirring. After adsorption (30 min at room temperature), the isooctane was decanted.

Enzymatic hydrolysis of (R, S)-naproxen methyl ester in a batch reactor

Experiment was performed in a flask containing 20 mL isooctane (the concentration of Naproxen methyl ester was 0.036 M). The reaction had been started by adding the immobilized enzyme containing different amounts of phosphate buffer (0.2 M, pH 7.5). The mixture was stirred at 150 r/min and 30 °C for different times.

Operating stability test of immobilized lipase in a repeated batch reactor

In a 250 mL conical flask, 0.6 g immobilized lipase (0.5 g support and 0.1 g *Candida rogusa* lipase) containing 0.75 mL sodium phosphate buffer (0.1 M, pH 7.5) was added to 20 ml isooctane that contained 0.68 mmol naproxen methyl ester. Silicone tubing (50 cm) containing 10 mL suspension of *Methylosinus trichosporium* IMV 3011 (4 mg dry wt /mL) was coiled on the bottom of the conical flask and the two ends of the silicone tubing were kept upward and near the top of the conical flask. The resolution reaction was started by shaking the flask at 30 °C. The substrate solution and suspension of bacteria were replaced with fresh substrate solution and suspension of bacteria every 72 h.

Analytical procedure

The enantiomeric excess of (*S*)-naproxen was determined by HPLC (HP1090) using a chiral column (Chirex R-NGLY & DNB, Phenomenex) capable of separating the (*R*)- and (*S*)-enantiomer of naproxen, the mobile phase was methanol solution (0.03 M ammonium acetate), at a flow rate of 1.0 mL/min. The retention time of (*R*)-naproxen is 11.5 min, for (*S*)-naproxen, it is 15.1 min.

The enantiomeric excess of (*R*)-naproxen methyl ester was determined by HPLC (HP1090) using the same chiral column mentioned above. However, the composition of the mobile phase was changed. And the mobile phase was a V/V mixture of 93.5% n-hexane, 6% 1,2-dichloroethane, 0.5% solution of trifluoroacetic acid (TFA) in ethanol (TFA:ethanol (v/v) = 1:20), at a flow rate of 1.0 mL/min. UV detection at 254 nm was used for quantification at the 25 °C. The retention time of (*R*)-naproxen methyl ester is 12.6 min, for (*S*)-naproxen methyl ester, it is 14.2 min.

The extent of conversion of substrate was monitored by spectrophotometer (UV-120-02, SHIMADZU Co.) at 254 nm or calculated by the ee_s (naproxen methyl ester) and ee_p (naproxen) [5]. *E*-value was calculated by the extent of conversion and ee_p according to Chen et al. [5].

Synthesis of ester

The methyl ester of racemic Naproxen was synthesized by the classical methodology [4] using trimethylchlorosilane and methanol. Trimethylchlorosilane, (0.26 mol) was added to a cooled, stirred suspension of Naproxen (0.12 mol) in methanol (250 mL) under an inert atmosphere. The reaction mixture was stirred at room temperature for 24 h, then the solvent was evaporated under reduced pressure and the residue was purified by recrystallization using petroleum ether as solvent.

Results and discussion

Effect of support on the activity of immobilized lipase

In our previously report, the activity of lipase was increased with increasing the log*P* value of organic solvent [20]. Isooctane was selected as suitable organic solvent. From Table 1 it can be seen that carriers from different sources behave differently. Among them, MCM-41 showed the highest specific surface area. Among the inorganic supports used for the immobilization of the lipase, the MCM-41 immobilized lipase showed the highest activity for the hydrolysis of naproxen methyl ester (Fig. 1). It was even higher than that of the YWG-C_6H_5 immobilized lipase, which was once selected as the best support in our previous experiment [20]. As is known to all, a freely dissolved lipase in the absence of an aqueous/lipid interface resides in its inactive state, and a part of the enzyme molecule covers

the active site. When the enzyme contacts the interface of a biphase water-oil system, a short α-helix – the 'lid'– is folded back and the lipase is rearranged into its active state [7]. It can be speculated that increasing the area of interface will facilitate the increase of reaction rate.

Table 1. Some properties of the support used for the immobilization of lipase

Support	Al_2O_3	MCM-41	Celite	Silica	YWG-C_6H_5	TiO_2
Specific surface area (m^2/g)	158.6	1003	0.8	187	100	52
Pore diameter (Å)	93.5	28.6	–*	105	–*	–*

* Not determined

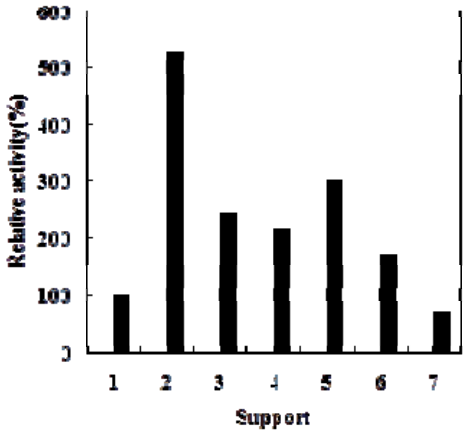

Fig. 1 The relative activity of lipase from *Candida rugosa* immobilized on different support:
1 – Free enzyme; 2 – MCM-41 molecular sieve; 3 – Silica;
4 – Silica-C_6H_5; 5 – Celite; 6 – Alumina; 7 – Titania.
Experiments were performed in a 250 mL flask, 20 mL of isooctane solution
(the concentration of naproxen methyl ester was 10 mg/mL) was added,
and the reaction was started by adding 0.55 g of immobilized enzyme
(50 mg *Candida rogusa* lipase was immobilized on 0.5 g different supports)
containing 0.5 mL sodium phosphate buffer (0.2 mol/L, pH 7.5).

In a trapped aqueous-organic biphase system, a support with a larger surface area offers a larger interfacial area. MCM-41 molecular sieves used for the immobilization of lipase has very large specific surface area (>1000 m^2/g) [19, 22], while other inorganic supports have specific surface area of no more than 200 m^2/g. It seems that the large area of MCM-41 mesoporous is an important factor affecting the lipase activity. From Fig. 1 it can also be seen that the nature of the carrier also plays an important influence on the activity of lipase.

Effect of lipase loading on the immobilization of lipase

Enzyme loading is an important parameter that controls the activity of the immobilized enzymes, particularly with lipases, which have strong affinity for surfaces. To investigate the efficiency of the loading for *Candida rugosa* lipase onto MCM-41 mesoporous molecular sieve, we studied the influence of the amount of lipase in the range of 50-250 mg/g of support (Fig. 2). An almost linear relationship between the activity and the enzyme loading was found within this range of loading. While the efficiency (activity/loading) increased with the increase of enzyme loading from 50-100 mg/g of support. A further increase of loading (100-250 mg/g of support) caused a decrease in efficiency. According to Bosley and Peilow

[3], at low enzyme loadings, the enzyme attempts to maximize its contact with the surface, which results in a change of conformation. As the loading is increased, less area is available for the lipase to spread itself, more of its active conformation is retained, and the loss in activity is reduced.

Similar results were also reported by Soares et al. [16], using the same source of lipase and controlled pore silica as support. But for loadings over 100 mg/g of support, multilayer adsorption may occur which should block the access of substrate to enzyme active site [12].

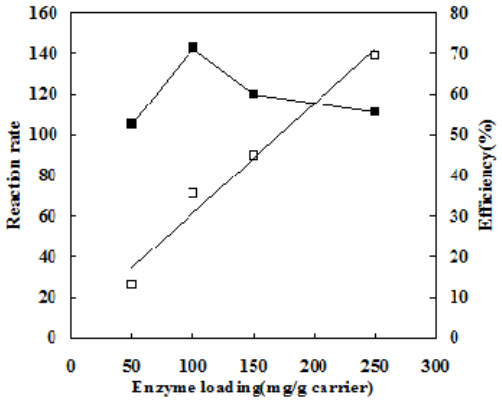

Fig. 2 Effect of enzyme loading on the activity of immobilized lipase:
■ – efficiency and □ – reaction rate (μmol/L/h).
Naproxen methyl ester (0.9 mmol) was dissolved in 20 mL isooctane at 35 °C,
and 25-125 mg lipase dissolved in 0.5 mL sodium phosphate buffer (0.2 M, pH = 7.5)
was immobilized on 0.5 g MCM-41 molecular sieves.

Effects of water content on the activity and selectivity of immobilized lipase

In order to investigate the influence of water content on the activity of MCM-41 immobilized lipase during the resolution of racemic naproxen methyl ester in this trapped aqueous-organic biphase system, different content of phosphate buffer were used for the immobilized lipase and free lipase. It can be seen from Fig. 3, a sharp increase of activity for immobilized lipase was observed when the proportion of aqueous/support increased from 0.5 (mL/g) to 1.5 (mL/g). A further increase of water content caused a decrease in activity of the lipase. For the free lipase, the activity increased steadily and slowly. Although an additional increase of water content for free lipase will enhance its activity, the emulsification in an aqueous/organic two-phase becomes prominent which will complicate work-up. In contrary to the free lipase, the immobilized lipase showed much higher activity and sensitivity to water content. Increased enantioselectivity was also observed when lipase was immobilized in the trapped aqueous-organic biphase system. As it can be seen from Fig. 4, an average *E*-value of about 200 was observed for the immobilized lipase in comparison to an *E*-value of not more than 70 for the free lipase. For example, the enantiomaric excess of naproxen was 99.1% when the conversion reached 18.5% after 72 h reaction for the immobilized lipase with the proportion of aqueous/support 1.5 mL/g. While the enantiomaric excess of naproxen was 96.7% when the conversion reached 5.5% under the same condition for the free lipase.

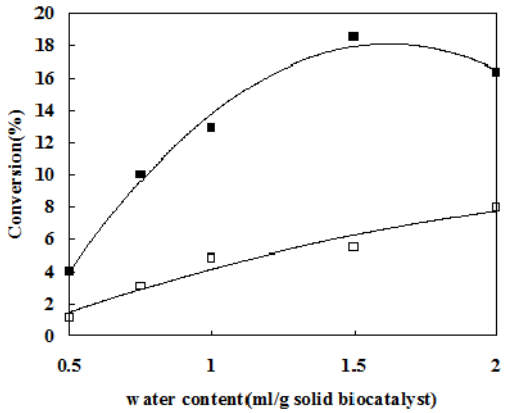

Fig. 3 Effect of water content on the activity
of lipase immobilized on MCM-41 mesoporous molecular sieve:
■ – immobilized lipase and □ – free lipase.
Naproxen methyl ester (0.9 mmol) was dissolved in 20 mL isooctane at 35 °C,
and 50 mg lipase was immobilized on 0.5 g MCM-41 molecular sieves
with different amount of sodium phosphate buffer (pH = 7.5).

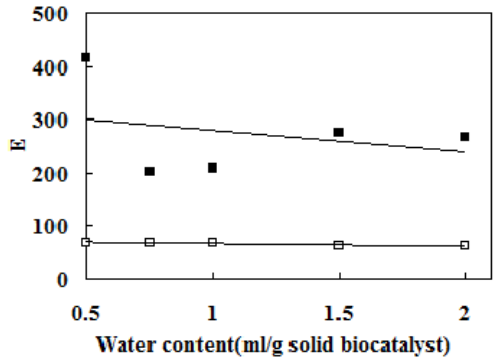

Fig. 4 Effect of water content on the enantioselectivity
of lipase immobilized on MCM-41 mesoporous molecular sieve:
■ – immobilized lipase and □ – free lipase.
Naproxen methyl ester (0.9 mmol) was dissolved in 20 mL isooctane at 35 °C,
and 50 mg lipase was immobilized on 0.5 g MCM-41 molecular sieves
with different amount of sodium phosphate buffer (pH = 7.5).

The higher enantioselectivity may be due to the interaction of lipase and MCM-41 mesoporous molecular sieve, which make the "pocket" of lipase more rigid. Similar results were observed when using other inorganic support such as silica and celite. In the trapped aqueous-organic biphase system, water is not only a reaction media that affects the interfacial area, but also a substrate in the enzymatic hydrolysis of racemic naproxen methyl ester. As a result, water content has complex effect on the activity of lipase. As mentioned above, an increase of the interfacial area will facilitate the reaction. When the water content is very low a monolayer distribution of water to the surface of support appeared, which results to a maxmium interfacial area. However, the thermodynamic reaction equilibrium is unfavorable for the hydrolysis reaction. Increasing water content will facilitate the thermodynamic reaction equilibrium while a decrease in interfacial area can be anticipated since the pores in the interior of MCM-41 are gradually filled with water, so an optimal water content should exist.

Effect of pH and temperature on the activity and selectivity of the soluble and immobilized lipase

It can be seen from Table 2 that both the activity and selectivity of the immobilized lipase was higher than that of the free lipase. For the free lipase, the influence of pH on the activity and selectivity was not significant. A little increase in enantioselectivity was found when the pH value altered from 6.5 to 8.5. For the immobilized lipase, however, the activity increased faster than that of the free enzyme with a change of pH. On the other hand, the enantioselectivity dropped sharply with the increase of pH from 6.5 to 8.5. At pH = 8.5 the enantioselectivity was close to the free enzyme. Lorente et al also found that the enantioselectivity of the enzyme adsorbed on polyethyleneimine (PEI) was greatly increased (from 21 to more than 200) when lowing the pH from 7.0 to 5.0 [10]. These results imply that the interaction of the lipase and MCM-41 mesoporous sieves at different pH values affect the microenvironment of the lipase, which often plays an important role on the activity and enantioselectivity of the immobilized lipase.

Table 2. Effect of pH on the activity and selectivity of free and immobilized lipase in the resolution of racemic naproxen methyl ester

pH[a]	Free lipase			Immobilized lipase		
	Conv., (%)	ee_p, (%)	*E*	Conv., (%)	ee_p, (%)	*E*
6.5	5.54	95.9	51	6.62	99.6	548
7	5.90	96.5	59	8.51	99.1	241
7.5	5.20	96.5	58	9.17	99.0	214
8	4.90	96.8	65	9.18	98.9	195
8.5	4.93	97.8	94	12.5	97.5	92

[a] 0.9 mmol Naproxen methyl ester was dissolved in 20 mL isooctane at 30 °C, and 50 mg lipase was immobilized on 0.5 g MCM-41 molecular sieves with 0.75 mL sodium phosphate buffer at different pH.

From Table 3 it can be seen that the optimum temperature of immobilized lipase was increased from 30 °C (free lipase) to 35 °C and the immobilized lipase was more sensitive to temperature than the free lipase. The result may be due to the interaction of the enzyme with the MCM-41 mesoporous molecular sieve.

Table 3. Effect of temperature on the activity and selectivity of free and immobilized lipase in the resolution of racemic naproxen methyl ester

Temp.	Free lipase			Immobilized lipase		
	Conv., (%)	ee_p, (%)	*E*	Conv., (%)	ee_p, (%)	*E*
25	3.89	97.8	94	4.06	99.0	207
30	5.20	96.5	58	9.17	99.0	214
35	4.91	97.2	75	9.63	98.9	200
40	4.48	96.8	64	6.30	99.1	229
45	4.04	97.0	69	5.15	98.7	160

[a] 0.9 mmol Naproxen methyl ester was dissolved in 20 mL isooctane, and 50 mg lipase was immobilized on 0.5 g MCM-41 molecular sieves with 0.75 mL sodium phosphate buffer (pH = 7.5) at different temperature.

The activity of the immobilized lipase was much higher than that of the free enzyme at the optimum temperature. However, it was close to the free lipase at 25 °C or 45 °C. The enantioselectivity for both forms (immobilized and free) were not changed much except

for a slightly decrease at the higher temperature (45 °C). Lorente et al. [10] also found that the enantioselectivity of the lipase (immobilized on Deca-octyl-Sepabeads) from *Candida Antarctica* (fraction B) increased from 10 to 25 when lowing the reaction temperature from 25 °C to 4 °C in the enantioselective hydrolysis of ethyl mandelate in a macro-aqueous system.

Operating stability of immobilized lipase

We also tested the operating stability of the immobilized lipase. It was found that the residual activity was 26% after 3 batches. The accumulation of methanol – one of the products in the resolution of racemic naproxen methyl ester-is a major reason that causes the deactivation of lipase. But the stability can be enhanced when one of the products – methanol is continuous removed from the reaction system in a CSTR reaction system [20]. Recently [21], an efficient method for the elimination of methanol in situ was established by us using the *methanotrophic* bacteria as catalyst, which can convert methanol into water and carbon dioxide. It was found the stability of lipase was improved and it can be used for 3 times without significant lose of activity. In this study, we tested this method thoroughly and the immobilized lipase showed good operating stability even after 8 batches (Fig. 5) with a half life over 2000 hrs.

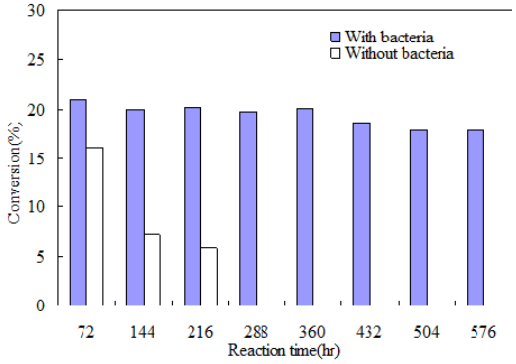

Fig. 5 Conversion of naproxen methyl ester as a function of time
in the trapped aqueous-organic biphase system without or with bacteria
for elimination of methanol.
(reaction condition: see experimental section)

Conclusion

Lipase from *Candida rogusa* was efficiently immobilized on MCM-41 molecular sieve in a trapped aqueous-organic biphase system. Both of the activity and enantioselectivity were improved significantly relative to the free enzyme. Higher pH value will increase the activity but decrease the enantioselectivity of lipase within the range of reaction. For the immobilized lipase, it was more sensitive to temperature than the free enzyme. The immobilized lipase showed good operating stability (the half-life of immobilized lipase reached over 2000 hrs) with the aid of *methanotrophic* bacteria to degrade methanol produced during the resolution process.

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