Appl Biochem Biotechnol (2010) 162:2015–2026
DOI 10.1007/s12010-010-8977-1

Lipase Immobilized on the Hydrophobic Polytetrafluoroethene Membrane with Nonwoven Fabric and Its Application in Intensifying Synthesis of Butyl Oleate

Shu-Guang Wang · Wei-Dong Zhang · Zheng Li · Zhong-Qi Ren · Hong-Xia Liu

Received: 22 September 2009 / Accepted: 19 April 2010 /
Published online: 6 May 2010

Abstract The synthesis of butyl oleate was studied in this paper with immobilized lipase. Five types of membrane were used as support to immobilize *Rhizopus arrhizus* lipase by following a procedure combining filtration and protein cross-linking. Results showed that hydrophobic polytetrafluoroethene membrane with nonwoven fabric (HO-PTFE-NF) was the favorite choice in terms of higher protein loading, activity, and specific activity of immobilized lipase. The factors including solvent polarity, lipase dosage, concentration, and molar ratio of substrate and temperature were found to have significant influence on conversion. Results showed that hexane (log$P$=3.53) was a favorable solvent for the biosynthesis of butyl oleate in our studies. The optimal conditions were experimentally determined of 50 U immobilized lipase, molar ratio of oleic acid to butanol of 1.0, substrate concentration of 0.12 mol/L, temperature of 37 °C, and reaction time of 2 h. The conversion was beyond 91% and decreased slightly after 18 cycles. Lipase immobilization can improve the conversion and the repeated use of immobilized lipase relative to free lipase.

Keywords Lipase immobilization · Butyl oleate · Conversion

Introduction

Butyl oleate is widely used as diesel additive, polyvinyl chloride plastisizer, and water-resisting agent in hydraulic fluids [1–3]. Generally, butyl oleate is chemically synthesized by oleic acid and butanol with the presence of concentrated sulfuric acid. However, chemical synthesis of butyl oleate has many disadvantages, such as discharge of acid wastewater, equipment erosion by concentrated sulfuric acid, byproducts, and etc.

Enzyme-catalysis has attracted much attention because of its mild reaction conditions, high selectivity, few byproducts, and etc. [4, 5]. It has been widely used for the synthesis of

S.-G. Wang · W.-D. Zhang (✉) · Z. Li · Z.-Q. Ren · H.-X. Liu
State Key Laboratory of Chemical Resource Engineering, Beijing University of Chemical Technology, Beijing, China
e-mail: zhangwd@mail.buct.edu.cn

bioproduct, such as L-ascorbyl laurate [6], glucose ester [7], butyl hydroxycinnamate [8], chlorogenate fatty ester [9], xylitol fatty acid ester [10], and butyl oleate [11, 12]. Unfortunately, there are still problems in the biosynthesis of butyl oleate with enzyme, such as low catalysis efficiency and less repeated use of lipase. In Habulin and Knez's [11] studies, after more than 60-h reaction, the highest conversion rate of butyl oleate reached 88.11% with the catalysis of immobilized *Mucor miehei* lipase. Also, the conversion of 75% after 30-h reaction was obtained by Ghamgui et al. [12] with the catalysis of immobilized *Rhizopus oryzae* lipase. However, the conversion rate in chemical synthesis can reach more than 90% in less 24 h. Moreover, due to relatively high cost, higher stability, and use-repeatability of enzyme, forms must be further improved, so as to make the application of enzyme in organic synthesis economically feasible. Therefore, numerous efforts have been focused on the lipase immobilization including support materials and a variety of immobilization methods.

For enzyme immobilization, support has significant impact on enzyme loading and activity. Membrane has attracted more attention as support material with its high surface area, good biocompatibility, and biostability. Membrane with immobilized enzyme can combine its excellent separation capacity and the high catalytic activity of enzyme [13–16]. Enzymes immobilized on membrane have been widely investigated, for instance, pig liver esterase/polysulphone ultrafiltration membrane [17], laccase/poly (vinyldene fluoride) (PVDF) membrane [18], catalase/polyamide membrane [19], and *Arthrobacter* sp. lipase (ABL)/polypropylene membrane [20], all of which show excellent characteristics in application.

The methods of enzyme immobilization have been reviewed in many articles [21]. Adsorption and filtration are used more frequently because of their operational simplicity, low cost, and effectiveness for preserving enzyme activity, while cross-linking is effective and durable in enzyme immobilization, though it sacrifices the enzyme performance to some extent. Therefore, the combination of adsorption or filtration with cross-linking is getting more research interests in enzyme immobilization.

In this work, *Rhizopus arrhizus* lipase is immobilized on five types of membrane by following a procedure combining filtration and protein cross-linking and used for the synthesis of butyl oleate. The effects of solvent, lipase dosage, reaction time, concentration and molar ratio of substrate, and temperature on the conversion are investigated accordingly, and the optimal parameters for butyl oleate synthesis are obtained. The mechanism is also discussed.

Experimental

Materials

R. arrhizus lipase is purchased from Beijing Tianfang Biological Corporation, China. Bovine serum albumin (BSA) is obtained from Beijing Jingke Chemical Reagent Corporation, China. Coomassie Brilliant Blue is purchased from Fluka Corporation, Buchs, Switzerland. Olive oil, oleic acid, hexane, and butanol are purchased from Beijing Chemical Reagents Company, China. All solvents and chemicals are of analytical grade except olive oil, which is of chemical grade, and they are used without further purification.

Five types of flat membrane are used. Their characteristics are shown in Table 1. The membranes are kindly provided by Zhejiang Sci-Tech University, Hangzhou, China.

Table 1 Characteristics of membrane used in experiment

Symbols	Membranes	Contact angle (°)	Thickness (mm)	Pore size (μm)[a]
HO-PTFE-NF	Hydrophobic PTFE membrane with nonwoven fabric[b]	66[c]	1.423	0.20–0.45
HI-PTFE-NF	Hydrophilic PTFE membrane with nonwoven fabric[b]	–[d]	1.590	0.20–0.45
PVDF	Hydrophilic PVDF	78	0.171	0.22
HI-PTFE-CS	Hydrophilic PTFE membrane with chitosan	91	0.054	–
HO-PTFE	Hydrophobic PTFE membrane	109	0.012	0.20–0.45

[a] The data were provided by the manufacturer

[b] Nonwoven fabric is made of polypropylene fiber

[c] Only the side of PTFE membrane was determined

[d] Not determined

Methods

Lipase Immobilization

Lipase solution is prepared by adding a specific amount of crude lipase powder to phosphate buffer (pH=7.0). After 1-h vibration at room temperature, the lipase solution is filtrated through five types of membrane, respectively. Before filtrating, the membranes are immersed into phosphate buffer for 24 h and then air-dried. The pretreated membrane (35 cm^2) is placed on the Busher funnel. A 25-mL lipase solution is completely poured into Busher funnel once and pumped by vacuum pump. During the filtration, the fabric layer of hydrophobic polytetrafluoroethene membrane with nonwoven fabric (HO-PTFE-NF) and HI-PTFE-NF is kept upward in order to increase protein loading on the membrane through fabric adsorption. Filtrated membranes are rinsed with phosphate buffer until no protein is detected in rinsing buffer. All rinsing buffer is collected so as to calculate the protein loading. Filtrated membranes are divided into small pieces of 1×1 cm and then crossing-linked with glutaraldehyde solution (25 mL, 1 wt.%) in order to increase protein fastness on membrane. After 2-h vibration (150 rpm) at room temperature, excess glutaraldehyde is rinsed with phosphate buffer.

Determination of Protein Loading

Protein loading is calculated based on the difference of protein content between initial and filtrated lipase solution. Protein concentration in solution is determined by Bradford assay using a commercialized Coomassie Brilliant Blue G-250 [22]. Bovine serum albumin is used as protein standard.

Determination of Activity of Immobilized Lipase

Activity of immobilized lipase is determined by modified copper soap colorimetry method [23]. The lipase activity is defined as 1 μmol free fatty acid released per minute per

centimeter squared immobilized lipase. The substrates consist of 1 mL olive oil, and 3 mL phosphate buffer (pH7.0) is preincubated for 5 min at 37 °C. Then, immobilized lipase is added to start the reaction, and the reaction was terminated with 8 mL methylbenzene after 15 min. One milliliter of copper reagent [$(CH_3COO)_2Cu$] is added to the methylbenzene phase and analyzed by spectrophotometer at 710 nm.

Determination of Butyl Oleate Yield

Yield of butyl oleate is expressed by the conversion of oleic acid.

$$\text{Conversion } (\%) = (C_0 - C_t)/C_0 \times 100\%$$

where C_0 is the initial oleic acid concentration before reaction, mg/mL, and C_t is the oleic acid concentration after reaction with certain time, mg/mL. Oleic acid concentration is determined by titration with 0.02 mol/L NaOH [24].

Factors Affecting Enzymatic Synthesis of Butyl Oleate

In all experiments, reactions are started by immobilized lipase and 25 mL organic phase composed of oleic acid, butanol and reaction solvent in conical flask (150 mL) at different conditions.

Reaction Solvents

Solvents with different polarity, including acetone, tetrahydrofuran, *tert*-amylalcohol, benzene, hexane, and heptane, are tested to explore their effects on the conversion with free lipase and lipase immobilized on HO-PTFE-NF, respectively. The log*P* values range from −0.24 to 4.0 (Table 2). Reaction conditions are as follows: temperature, 37 °C; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; dosage of immobilized lipase, 34 U (free lipase 5000 U); shaking speed, 200 rpm; and reaction time, 4 h.

Supports

Five types of membrane (Table 1) are used to investigate their effects on protein loading, activity and specific activity of immobilized lipase. Specific activity of the immobilized lipase is used to characterize biofriendliness of support. According to the three parameters

Table 2 Effect of solvent polarity on conversion with free lipase and lipase immobilized on HO-PTFE-NF

Solvents	log*P*	Conversion with free lipase (%)	Conversion with immobilized lipase (%)
Acetone	−0.23	2.7	60.9
Tetrahydrofuran	0.49	0.6	35.9
Tert-amylalcohol	1.15	7.9	61.7
Benzene	2.00	56.8	82.0
Hexane	3.53	66.9	92.7
Heptane	4.00	66.7	91.6

above, the most suitable membrane can be determined. The appropriate reaction conditions are as follows: temperature, 37 °C; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; dosage of immobilized lipase, 50 U; shaking speed, 200 rpm; and reaction time, 2 h.

Lipase Dosage

Dosage of immobilized lipase can be controlled by weight or area of support. However, the unequal distribution of protein on membrane is still a major issue. In order to improve the equal distribution of protein immobilized on membrane, lipase solutions with different concentrations (0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 10.0 g/L) are filtrated through the same area of membrane (35 cm^2), respectively, which would produce immobilized lipase with different activity. Other reaction conditions are as follows: temperature, 37 °C; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; shaking speed, 200 rpm; and reaction time, 2 h.

Reaction Time

The effects of reaction time on conversion are studied through batch experiments. Conversion is determined at 0, 0.5, 1.0, 2.0, 3.0, and 4.0 h, respectively. Other reaction conditions are as follows: temperature, 37 °C; dosage of immobilized lipase, 50 U; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; and shaking speed, 200 rpm.

Reaction Temperature

The conversion is determined at 20, 30, 37, 50, and 60 °C, respectively. Other reaction conditions are as follows: dosage of immobilized lipase, 50 U; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; shaking speed, 200 rpm; and reaction time, 2 h.

Molar Ratio and Concentration of Substrate

In order to observe the effects of different molar ratios and concentrations of substrate on the synthesis of butyl oleate, batch reactions are carried out. Molar ratio of oleic acid to butanol is 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2, respectively. Concentrations of oleic acid and butanol are 0.06, 0.08, 0.10, 0.12, 0.14, 0.16, 0.18, and 0.20 mol/L, respectively. Other reaction conditions are as follows: temperature, 37 °C; dosage of immobilized lipase, 50 U; shaking speed, 200 rpm; and reaction time, 2 h.

Repeated Use of Immobilized Lipase

In this experiment, when one cycle finishes, immobilized lipase is taken out and then put into a new reaction system. Each cycle lasts 2 h. In total, 18 cycles have been conducted. Conversion is determined after each cycle. The reaction conditions of each cycle are as follows: temperature, 37 °C; dosage of immobilized lipase, 50 U; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; and shaking speed, 200 rpm.

Results and Discussion

Selection of Reaction Solvent

As a special kind of protein with catalysis ability, lipase activity is apt to be affected by many factors. Reaction solvent is one of the factors, since polarity of solvent has great impact on lipase activity. In this study, the effects of acetone, tetrahydrofuran, *tert*-amylalcohol, benzene, hexane, and heptane are investigated. Table 2 shows the results of conversion with different solvents. In the reactions with free and immobilized lipases, compared with solvents with log$P$<2, the conversion is low in the solvents with log$P$<2, such as acetone, tetrahydrofuran, and *ter*-amylalcohol. This is consistent with the results in previous work where solvents with log$P$<2 are less suitable for biocatalysis [25, 26]. Contrarily, in the solvents with log$P$>2, such as benzene, hexane, and heptane, the conversion reaches 56.8%, 66.9%, and 66.7% in reactions with free lipase, and 82.0%, 92.7%, and 91.6% in reactions with immobilized lipase, respectively.

However, there are several results that solvents with low log$P$ are also suitable for enzymatic reaction. Zaks and Klibanov [27] found that porcine pancreatic lipase could retain its bound water and be active even in water-miscible solvents. Lv et al. [28] found that cylcohexanone (log$P$=0.96) was the most suitable media to synthesize ascorbyl benzoate. The reason is that lipase activity is affected not only by solvent polarity but also by dielectric constant of solvent, polarity of substrates, and hydrophobicity of activity center of lipase [29–31]. For example, solvents with low log$P$ can facilitate the combination of hydrophobic substrate and lipase with hydrophobic activity center, because hydrophobic substrate can easily escape from the hydrophilic solvent and reach the hydrophobic activity center of lipase. In addition, different lipases might have different tolerance to organic solvent. These factors lead to different results in studies.

Among three solvents with high conversion, benzene is toxic to human health, while the high viscosity makes heptane not suitable for industrialization of enzyme-catalysis. Therefore, hexane is selected as the reaction solvent in the subsequent studies.

Selection of Support

Protein loading on the HO-PTFE-NF, HI-PTFE-NF, and PVDF membrane is about 0.02 mg/cm^2, while on the HI-PTFE-CS and HO-PTFE, protein loading is much lower (Table 3). This indicates that the first three types of membrane are suitable to lipase immobilization. Although the structural difference between HO-PTFE-NF and HO-PTFE is only nonwoven fabric layer, their protein loadings are dramatically different. This indicates that nonwoven fabric layer of HO-PTFE-NF has significant impact on lipase immobilization. In addition, low protein loading of HO-PTFE membrane may result from (1) its strong hydrophobicity, which negatively affects filtration of lipase solution, and (2) fewer groups (such as –OH) on HO-PTFE membrane surface, which reduce protein adsorption. As for HI-PTFE-CS, low protein loading can be attributed by CS dense membrane that impedes filtration process of lipase solution. The limited protein loading on HI-PTFE-CS can be caused by membrane adsorption.

In spite of similar protein loading on HO-PTFE-NF, HI-PTFE-NF, and PVDF membrane, the activities of immobilized lipase are significantly different (Table 3). Lipase immobilized on HO-PTFE-NF shows the highest activity (0.590 U/cm^2) compared with that of HI-PTFE-NF (0.380 U/cm^2) and PVDF membrane (0.336 U/cm^2). It is noted that the correlation between immobilized-lipase activity and protein loading is not linear.

Table 3 Effect of different membranes on lipase immobilization

Membranes	Protein loading (mg/cm^2)	Immobilized-lipase activity (U/cm^2)	Specific activity of immobilized lipase (U/mg)
HO-PTFE-NF	0.021	0.590	28.1
HI-PTFE-NF	0.020	0.380	19.0
PVDF	0.021	0.336	16.0
HI-PTFE-CS	0.005	0.133	26.6
HO-PTFE	–[a]	0.104	–[a]

[a] Under detection limit

This is because the thicker the filtration cake of lipase is, the larger the diffusion resistance of substrates to the reaction zone is. It is also noticeable that even the protein loading is under the detection limit on HO-PTFE membrane, it still has the activity as 0.104 U/cm^2.

Biofriendliness of support is important to reserve enzyme activity. However, neither activity of immobilized enzyme nor protein loading could effectively characterize biofriendliness of support, though they are used widely in studies on enzyme immobilization. In order to evaluate biofriendliness of support, specific activity of immobilized lipase is introduced. As shown in Table 3, HO-PTFE-NF and HI-PTFE-CS have higher specific activity of immobilized lipase than HI-PTFE-NF and PVDF membrane, which is different from the order of activity of immobilized lipase. This indicates that HO-PTFE-NF and HI-PTFE-CS can facilitate the reservation of protein activity. Although the effect of enzyme immobilization depends on protein loading and specific activity of immobilized lipase, the latter one should be considered first, since it is highly correlated with the efficiency of enzyme utilization. Considering protein loading and specific activity of immobilized lipase, HO-PTFE-NF is selected as support in later experiments.

Effect of Lipase Dosage on the Conversion

As shown in Table 4, the protein loading and the activity of immobilized lipase increase with the increase in concentration of lipase solution from 1.0 to 5.0 g/L with high linear correlation (correlation coefficient, R^2 between lipase concentration and protein loading is 0.99; R^2 between lipase concentration and lipase activity is 0.97). The specific activity of immobilized lipase shows downward trend with the increase in concentration of lipase solution. The difference is slight when the concentration of lipase solution is above 4.0 g/L. This indicates that the specific activity of immobilized lipase decreases with increasing lipase concentrations and protein loading. The reasons are that lipase immobilization reduced activity groups of lipase due to its cross-linking with membrane, filtration cake obstructs substrate accessing to the lipase activity center, and glutaraldehyde is toxic to lipase to some extent.

The immobilized lipases with different activity are used for the synthesis of butyl oleate. The results show that the conversion increased when the lipase dosage ranges from 22 to 50 U. The increase is not significant when the lipase dosage is above 50 U, indicating that at present amount of substrates, lipase dosage of 50 U is sufficient for the synthesis of butyl oleate.

Table 4 Effect of free lipase concentration on lipase immobilization on HO-PTFE-NF and the conversion

Lipase (g/L)	Protein loading (mg/cm^2)	Immobilized lipase activity (U/cm^2)	Specific activity of immobilized lipase (U/mg)	Dosage of immobilized lipase (U)[a]	Conversion (%)
0.5	–[b]	–	–	22	68
1.0	0.004	0.798	199.5	28	81
2.0	0.015	0.894	59.6	31	88
3.0	0.030	1.425	47.5	50	91
4.0	0.044	1.774	40.3	62	91
5.0	0.055	2.192	39.9	77	92
10.0	0.073	2.660	36.4	93	92

[a] Dosage used in experiment

[b] Under the detection limit

Effect of Reaction Time on the Conversion

As shown in Fig. 1, the conversion increases slowly within 4-h reaction in the synthetic reaction with free lipase, while in the reaction with immobilized-lipase, the conversion rapidly increases from 0% to 77% in the first hour and almost reaches the highest at the end of second hour. This indicates that lipase immobilization increased the synthesis rate of butyl oleate.

Effect of Molar Ratio of Substrate on the Conversion

The conversion reaches the highest value of 87.1% at the molar ratio of 1.0 (Fig. 2). When the molar ratio range from 0.2 to 1.0, the conversion presented upward trend with the increase of molar ratio. Lower molar ratio means that butanol is excessive in the reaction system, while butanol is toxic to lipase [12, 24], so the lower conversion is obtained. When the molar ratio is above 1.0, the conversion decreases with the increasing of molar ratio. This is because that higher molar ratio means the higher content of oleic acid and the lower

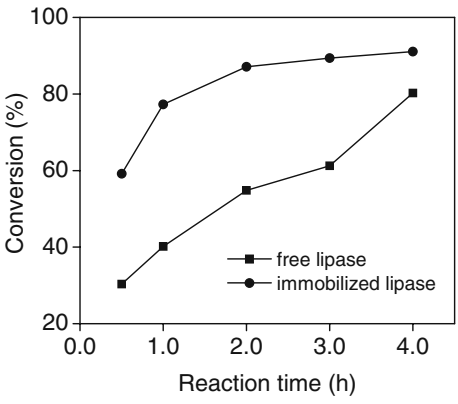

Fig. 1 Effect of reaction time on the conversion catalyzed by lipase immobilized on HO-PTFE-NF

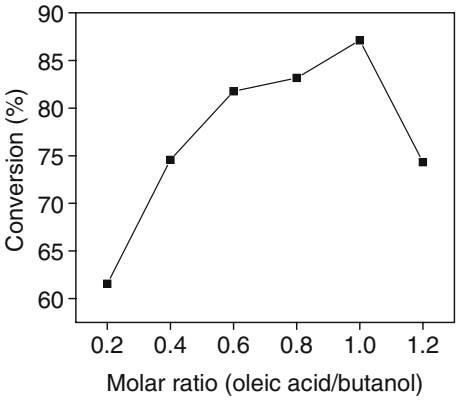

Fig. 2 Effect of molar ratio of oleic acid to butanol on conversion catalyzed by lipase immobilized on HO-PTFE-NF

content of butanol, and less butanol results in lower conversion of oleic acid. This is different from the result of Habulin and Knez [11], who immobilized *M. miehei* lipase on hollow fiber membrane and found that the molar ratio of 0.55 is the optimum molar ratio. This difference may relate to the way of substrates supply and lipase species. In their experiment, butanol is continually added to the reaction mixture, while in the present work, a batch experiment is carried out without any additional of butanol.

Effect of Substrate Concentration on the Conversion

Figure 3 shows that the conversion increases with increasing concentrations of both substrates range from 0.06 to 0.12 mol/L and reaches the highest value of 87.1% at 0.12 mol/L. When both substrate concentrations are above 0.12 mol/L, the conversion decreases. The reason is that lipase is surrounded by high-concentration butanol in the aqueous microenvironment, which can cause lipase deactivation.

Effect of Temperature on the Conversion

It can be observed from Fig. 4 that in the reaction with free lipase, the conversion slightly changes from 20 to 30 °C, while presents significant upwards trend with temperature from 30 to 37 °C, and reaches the highest value of 59.1% at 37 °C. However, when the

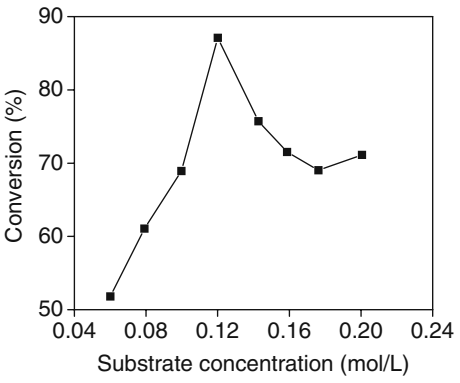

Fig. 3 Effect of substrate concentration on conversion catalyzed by lipase immobilized on HO-PTFE-NF

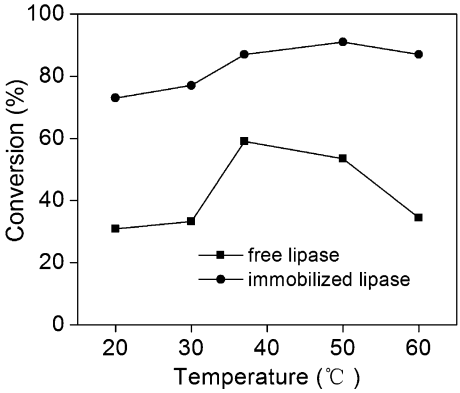

Fig. 4 Effect of temperature on conversion catalyzed by lipase immobilized on HO-PTFE-NF

temperature is above 37 °C, the conversion decreases sharply, due to the fact that lipase configuration is destroyed by high temperature and lipase deactivation occurs. Therefore, the optimum temperature of free lipase is 37 °C.

In the reaction with immobilized lipase, the conversion is significantly higher than that of free lipase at same temperature, which indicates that immobilization improves lipase catalysis ability (Fig. 4). When temperature is lower than 37 °C, the conversion presents similar trend as free lipase. However, when the temperature is above 37 °C, the conversion increases slowly, which is different from that in the reaction with free lipase. This confirms that the linking between lipase groups (such as —OH, —COOH, $–NH_2$) and membrane groups could improve the thermal stability of immobilized lipase. Higher temperatures (above 60 °C) are not tested with the concern of hexane volatilization.

Considering the slight difference in conversion from 37 to 50 °C, 37 °C is chosen as the reaction temperature.

Repeated Use of Immobilized Lipase in the Synthesis of Butyl Oleate

Generally, commercial enzyme is expensive and cannot be used repeatedly, so the application of free enzyme in industrialization is limited. One of the advantages of enzyme immobilization is that it could make the repeated use of enzyme possible in industry. In the present study, the conversion is higher than 90% after 2-h reaction at optimal conditions:

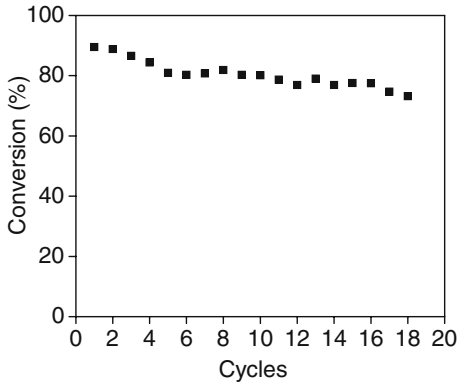

Fig. 5 Repeated use of lipase immobilized on HO-PTFE-NF in the synthesis of butyl oleate

immobilized lipase, 50 U; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; temperature, 37 °C; and reaction time, 2 h (Fig. 5). Compared with the previous work [11, 12], the improvement in both conversion and catalysis efficiency are significant. Conversion slightly decreases after 18 cycles, which indicates that the repeated use of immobilized lipase has been improved significantly compared with the free lipase. This can be benefited from the immobilization of lipase groups (such as –OH, –COOH, –NH_2) on the membrane through adsorption or cross-linking by glutaraldehyde, which increases lipase tolerance to organic solvents.

Conclusions

In this study, *R. arrhizus* lipase immobilized on the HO-PTFE-NF is highly effective in the synthesis of butyl oleate. The highest conversion is beyond 91% after 2-h reaction and slightly decreases after 18 cycles, which is significantly higher than the conversion rate reported in previous work. The optimal reaction conditions in present experiment are as follows: temperature, 37 °C; molar ratio of oleic acid to butanol, 1.0; substrate concentration, 0.12 mol/L; lipase dosage, 50 U; shaking speed, 200 rpm; and reaction time, 2 h. The immobilization significantly improves the conversion and the repeated use of lipase relative to free lipase.

Acknowledgements The authors are grateful to the National High Technology Research and Development Program of China (863) (NO.2007AA06Z310) and the Foundation of Beijing University of Chemical Technology for financial support.

References

1. Kohashi, H. (1990). in: Proceedings of the world conference on oleochemicals: into the 21st Century. Amer Oil Chemists Society, pp 243–250.
2. Linko, Y. Y., Rantanen, O., Yu, H. C., Linko, P., Tramper, J., & Vermüe, M. H. (1992). *in: Biocatalysis in non-conventional media, progress in biotechnology* (pp. 601–608). New York: Elsevier.
3. Linko, Y. Y., Lämsä, M., Huhtala, A., & Rantanen, O. (1995). *Journal of the American Oil Chemists' Society, 72*, 1293–1299.
4. Hills, G. (2003). *European Journal of Lipid Science and Technology, 105*, 601–607.
5. Villeneuve, P., Muderhwa, J. M., Graille, J., & Haas, M. J. (2000). *Journal of Molecular Catalysis. B, Enzymatic, 4*, 113–148.
6. Chang, S. W., Yang, C. J., Chen, F. Y., Akoh, C. C., & Shieh, C. J. (2009). *Journal of Molecular Catalysis. B, Enzymatic, 56*, 7–12.
7. Yu, A., Liang, Z., & Caruso, F. (2005). *Chemistry of Materials, 17*, 171–175.
8. Vafiadi, C., Topakas, E., Alissandratos, A., Faulds, C. B., & Christakopoulos, P. (2008). *Journal of Biotechnology, 133*, 497–504.
9. López Giraldo, L. J., Laguerre, M., Lecomte, J., Espinoza, M. C., Barouh, N., Baréa, B., et al. (2007). *Enzyme and Microbial Technology, 41*, 721–726.
10. Cramer, J. F., Dueholm, M. S., Nielsen, S. B., Pedersen, D. S., Wimmer, R., & Pedersen, L. H. (2007). *Enzyme and Microbial Technology, 41*, 346–352.
11. Habulin, M., & Knez, Z. (1991). *Journal of Membrane Science, 61*, 315–324.
12. Ghamgui, H., Karra-Chaâbouni, M., & Gargouri, Y. (2004). *Enzyme and Microbial Technology, 35*, 355–363.
13. Hughes, J. A., Zhou, S., Bhattacharyya, D., & Jay, M. (1991). *Journal of Membrane Science, 60*, 75–86.
14. Ulbricht, M., & Papra, A. (1997). *Enzyme and Microbial Technology, 20*, 61–68.
15. Magnan, E., Catarino, I., Paolucci-Jeanjean, D., Preziosi-Belloy, L., & Belleville, M. P. (2004). *Journal of Membrane Science, 241*, 161–166.

16. Yu, J. G., Zhang, J. S., Zhao, A., & Ma, X. F. (2008). *Catalysis Communications, 9*, 1369–1374.
17. Sousa, H. A., Crespo, J. G., & Afonso, C. A. M. (2000). *Tetrahedron Asymmetry, 11*, 929–934.
18. Jolivalt, C., Brenon, S., Caminade, E., Mougin, C., & Pontié, M. (2000). *Journal of Membrane Science, 180*, 103–113.
19. Dayal, R., & Godjevargova, T. (2006). *Enzyme and Microbial Technology, 39*, 1313–1318.
20. Abrol, K., Qazi, G. N., & Ghosh, A. K. (2007). *Journal of Biotechnology, 128*, 838–848.
21. Krajewska, B. (2004). *Enzyme and Microbial Technology, 35*, 126–139.
22. Bradford, M. M. (1976). *Analytical Biochemistry, 72*, 248–254.
23. Saisuburamaniyan, N., Krithika, L., Dileena, K. P., Sivasubramanian, S., & Puvanakrishnan, P. (2004). *Analytical Biochemistry, 330*, 70–73.
24. Leitgeb, M., & Knez, Ž. (1990). *Journal of the American Oil Chemists' Society, 67*, 775–778.
25. Laane, C., Boeren, S., Vos, K., & Veeger, C. (1987). *Biotechnology and Bioengineering, 30*, 81–87.
26. Chen, J. P. (1996). *Journal of Fermentation and Bioengineering, 82*, 404–407.
27. Zaks, A., & Klibanov, A. M. (1985). *Proceedings of the National Academy of Sciences of the United States of America, 82*, 3192–3196.
28. Lv, X. X., Pan, Y., & Li, Y. G. (2007). *Food Chemistry, 101*, 1626–1632.
29. Zaks, A., & Klibanov, A. M. (1984). *Sciences, 224*, 1249–1251.
30. Janssen, A. E. M., Padt, A. V., & Sonsbeek, H. M. V. (1993). *Biotechnology and Bioengineering, 41*, 95–103.
31. Chaudhary, A. K., Kamat, S. V., Beckman, E. J., Nurok, D., Kleyle, R. M., Hajdu, P., et al. (1996). *Journal of the American Chemical Society, 118*, 12891–12901.