Journal of Molecular Catalysis B: Enzymatic 72 (2011) 139–144

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Biocatalytic production of ethyl butyrate from butyric acid with immobilized *Candida rugosa* lipase on cotton cloth

Chengliang Shu[a], Jin Cai[a], Lei Huang[a,*], Xiangcheng Zhu[a,b], Zhinan Xu[a,**]

[a] Institute of Biological Engineering, Department of Chemical and Biological Engineering, Zhejiang University, Hangzhou 310027, PR China
[b] Hunan Engineering Research Center of Combinatorial Biosynthesis and Natural Product Drug Discovery, Changsha, PR China

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 27 May 2011
Accepted 27 May 2011
Available online 6 June 2011

Keywords:
Candida rugosa lipase
Cotton cloth
Polyethylenimine
Substrate inhibition
Ping Pong Bi-Bi
Esterification

ABSTRACT

A novel method involving polyethylenimine (PEI) coating and glutaraldehyde cross-linking processes was developed to immobilize *Candida rugosa* lipase onto cotton cloth. After the systematic investigation, the optimal lipase immobilization was achieved when 0.1 g lipase was loaded on 1.5 g support, which was pretreated with 10 ml of 1.0 mg/ml PEI solution at pH 8.0. Subsequent catalytic analysis of immobilized lipase for ethyl butyrate synthesis was also carried out in the Erlenmeyer flasks. The results indicated that when optimal 0.25 M ethanol and 0.6 M butyric acid were catalyzed by the immobilized lipase at 25 °C, the highest conversion yield of 91.2% and 1.27 mmol h^{-1} g^{-1} productivity of ethyl butyrate were obtained. Furthermore, a kinetic model of Ping Pong Bi-Bi mode with inhibition of both substrates was proposed and validated by experimental data. To explore the practical potential of immobilized lipase, its operational stability was evaluated and the residual activity was remained about 50% after 12 repeated recycles, with a half-life time of about 300 h for the immobilized lipase. Finally, a recycle batch reactor using immobilized lipase was developed for ethyl butyrate production. The achieved result of 0.85 M final ethyl butyrate concentration, with the conversion of 70.6% and the productivity of 1.45 mmol h^{-1} g^{-1}, had revealed the promising potential of this immobilized lipase in practical applications.

1. Introduction

Esters derived from short-chain carboxylic acids such as butyric acid are a large group of flavor and fragrance compounds, which are widely used in food, beverage, cosmetic and pharmaceutical industries [1]. Currently, these esters are mainly synthesized via environmentally cost chemical routes. However, enzymatic production of short-chain esters is gaining an increased attention with advantages of its mild reaction conditions, high catalytic efficiency and inherent selectivity [2]. Thus, various lipases have been studied and applied to catalyze desired ester formation [3–6].

Comparing to free enzymes in the solution, the immobilized enzyme has some attractive characteristics including the enzyme reusability and easy accessibility, so this technique has been intensively investigated and utilized in several biocatalysis processes [7–9]. The success of enzyme immobilization relies on several important factors such as the amount of enzyme, type of support as well as the conditions and chemical reagents for the immobilization. Among them, support is generally considered as the point to influence the performance of immobilized enzyme. Considering about the hydrophobic nature of lipases, it is much more simple, economic and selective to immobilize lipases onto hydrophobic supports. However, with increased concentrations of substrates and higher reaction rates in the support microenvironment, hydrophilic supports such as polyurethane foams were also competent for lipase immobilization and catalysis of esterification reaction [2,9]. As an inexpensive and easily available fibrous material, cotton cloth had been successfully used in cell and enzyme immobilization for various fermentations and enzymatic reactions [10–14]. The usage of cotton cloth in enzyme immobilization could provide several profits including large immobilization capacity, simplified configuration, negligible mass transfer resistance, good operational stability and easy post-processing, which are also valuable traits for further industrial application [12]. So far, using cotton cloth as a viable hydrophilic support for lipase immobilization has not been reported yet.

Negatively charged cotton cloth contains various chemical groups including carboxyl, sulfonic, phenolic and hydroxyl. The direct absorption of enzyme on cotton cloth may cause large loss of enzyme activity due to the long reaction period for catalysis and frequent wash process for recycle. Because of the electrostatic interaction between negatively charged supports, polyethylenimine (PEI) has been used as a complementary component for

* Corresponding author.
** Corresponding author. Tel.: +86 571 87951220; fax: +86 571 87951220.
E-mail addresses: lhuangblue@zju.edu.cn (L. Huang), znxu@zju.edu.cn (Z. Xu).

1381-1177/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2011.05.011

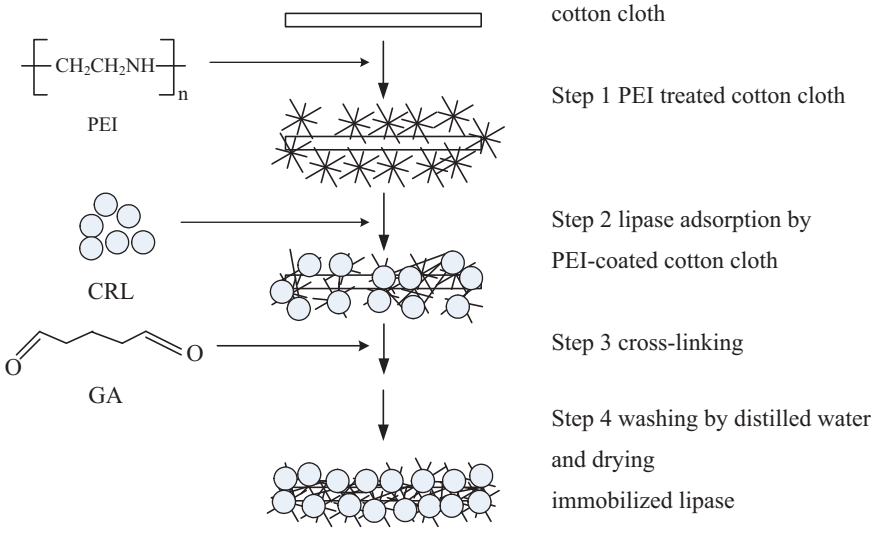

Fig. 1. Procedure of lipase immobilization on cotton cloth.

the cotton cloth treatment to enhance the efficiency of enzyme immobilization [15–17]. When PEI-coated cotton cloth was used as the support, glutaraldehyde (GA) cross-linking was carried out to finally immobilized enzyme. However, the low density of immobilized enzyme and concomitant substrate inhibition, as well as the possible deactivation of immobilized enzyme, are the main challenges need to be solved for the application of this technique in the biocatalysis of short-chain esters.

In this study, the immobilization of *Candida rugosa* lipase (CRL) was carried out, and the immobilization processes were optimized. Moreover, the performance of immobilized lipase on cotton cloth was improved via a comprehensive investigation. Several reaction parameters involved in the synthesis of ethyl butyrate were studied and a kinetic model was constructed. Finally, the operational stability of immobilized lipase was examined, and the usage of immobilized lipase in recycle batch reactor for potential industrial application was attempted.

2. Materials and methods

2.1. Enzyme and reagents

C. rugosa lipase type VII was obtained from Sigma, with a quoted activity of 1449 U/mg of solid. Polyethylenimine (PEI, 80%, ethoxylated, 37 wt% aqueous solution, MW = 50,000) was purchased from Acros Organics. Glutaraldehyde (GA) as 25% (w/v) aqueous solution was purchased from Simopharm Chemical Reagent Co. Ltd., China. Cotton cloth was obtained locally with specific surface area of 28–40 m^2/g and pore diameter of 2–5 nm. All solutions of PEI, GA, and enzyme were prepared in distilled water, and the pH was adjusted using HCl or NaOH solution when necessary.

2.2. Immobilization of lipase on cotton cloth

Briefly, the immobilization of lipase was carried out through three main steps (Fig. 1). 1.5 g cotton cloth was soaked in 10 ml of 1 mg/ml PEI solution (pH 8.0) for 2 h, and then washed with distilled water to remove excess PEI. The treated cotton cloth was wiped with filter paper and soaked into 10 ml of 10 mg/ml enzyme solution for 1 h. Then the PEI-enzyme-coated cotton cloth was cross-linked in 10 ml pre-cooled GA solution (1% (w/v), pH 7.0) for 1 h. The resulted cotton cloth with immobilized enzyme was washed intensively with distilled water, air-dried and conserved at 4 °C until use. When 25 ml Erlenmeyer flask was used for enzyme immobilization, the process was performed in a shaker with 220 rpm rotation speed at 25 °C. In case of recycled batch reactor, the process was performed in a glass column at 25 °C, and the temperature was controlled by water circulation using a peristaltic pump and a thermostatic water bath. The effects of different factors on the immobilization of enzyme, including the concentration and pH of PEI solution, and the amount of cotton cloth, were systematically investigated. The enzymatic activity and the enzyme recovery were also determined.

2.3. Esterification reaction

The enzymatic synthesis of ethyl butyrate catalyzed by immobilized lipase (0.6 g) was initially carried out in a 25 ml flask with 10 ml working volume, using cyclohexane as the reaction medium. The reaction mixture was incubated in a shaker at 220 rpm. The parameters including the reaction temperature, concentrations of substrates and molar ratio of substrates (ethanol to butyric acid) were investigated. Initial reaction rate and conversion yield were also determined.

2.4. Operational stability

A batch-wise fashion was adopted to investigate the operational stability of the immobilized lipase. Each batch was executed for 24 h, and the synthetic activity as well as the conversion yield was determined. The immobilized lipases were recovered by vacuum filtration, and washed intensively with cyclohexane. The resulted enzymes were then mixed with the same volume of fresh reaction mixture to start a new batch. The experiment was not terminated until the residual synthetic activity was lower than 50%.

2.5. Analytical methods

Concentrations of ethyl butyrate, ethanol and butyric acid were determined by the Agilent 6820 gas chromatography, equipped with a flame ionization detector. Propionic acid was taken as the inner standard. The conversion yield was calculated according to the molar ration between the ethyl ester and respective limiting substrate, either alcohol or acid.

Synthetic activity was measured by ester-synthesis method in organic media. It was defined as follows: one unit (U) of activity equals the amount of enzyme catalyzed 1 μmol substrate (ethanol and butyric acid) to form the product (ethyl butyrate) in 1 min under the assay conditions.

Hydrolytic activity of lipase was determined by olive oil emulsion method [18]. 1 U of lipase hydrolytic activity was defined as the amount of enzyme that liberated 1 μmol of free fatty acid per minute under the assay conditions. The immobilization yield (%) was calculated as $1 - A_r/A_i$, A_r is the hydrolytic activity of residual free lipase after immobilization, and A_i is the hydrolytic activity of initial free lipase.

3. Results and discussion

3.1. Immobilization of C. rugosa lipase on cotton cloth

3.1.1. Effects of the concentration and pH of PEI solution on lipase immobilization

C. rugosa lipase is an acidic protein with an isoelectric point of about 4.4 [19]. The electrostatic attraction and concomitant adsorption could occur between negatively charged aqueous enzyme and positively charged PEI-coated cotton cloth. Thus, the intensity of this interaction was directly influenced by the amount of PEI used and the pH of PEI solution. 10 ml of different concentrations (0 mg/ml, 0.1 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml, 4.0 mg/ml and 8.0 mg/ml, all at pH 8) and different pH (6–12, all with 1.0 mg/ml PEI) of PEI solutions were investigated, respectively, to treat with a constant 0.6 g cotton cloth. Then 0.1 g lipase powder was immobilized onto PEI-coated cotton cloth. The immobilization efficiency was evaluated with the relative synthetic activity of loaded enzyme (the maximum value of synthetic activity was 100%) and immobilization yield.

As shown in Fig. 2A, the treatment of PEI could significantly improve the enzymatic activity and the immobilization yield. When 10 mg PEI was used, the synthetic activity could reach maximum 17.8 U/g immobilized lipase, which was 2.3-fold comparing to that of the control. Simultaneously, immobilization yield was also improved with the process of PEI. The maximum immobilization yield could reach 53.8% when 20 mg PEI was applied. However, excess addition of PEI over 20 mg could result in the decrease of both synthetic activity and immobilization yield, which may be due to the occupation of enzyme binding sites by PEI and the repulsion between PEI. Similarly, with the presence of 10 mg PEI, the synthetic activity and the immobilization yield both reached their maximum value at pH 8.0 (Fig. 2B). Considering about the utilization of immobilized lipase, the optimal condition for immobilization was determined with 10 mg PEI treatment at pH 8.0.

3.1.2. Effect of support amount on lipase immobilization

As the main component, support also contributed to the immobilization procedure. The effects of different amounts of support (0.5 g, 0.75 g, 1 g, 1.5 g and 2.0 g) were respectively evaluated, with a constant 0.1 g loading enzyme in 10 ml working volume. The synthetic activity was enhanced from 9.08 U to 16.7 U when the amount of support was increased from 0.5 g to 1.5 g (Fig. 3). But further increase of support amount could lead to the decrease of synthetic activity, which may be caused by the scattered distribution of enzyme on the support and the resulted less contact between enzyme and substrate. The immobilization yield gradually improved with the increase of support amount, and the maximum yield reached 65% when support amount was 2.0 g (Fig. 3). As a result, the optimal load ratio between enzyme and support was 1:15.

3.2. Enzymatic synthesis of ethyl butyrate by immobilized lipase

3.2.1. Time-course of esterification reaction catalyzed by immobilized lipase

With 3.0 g immobilized lipase, 0.5 M ethanol and 0.5 M butyric acid in a 40 ml reaction system, a comprehensive time course of

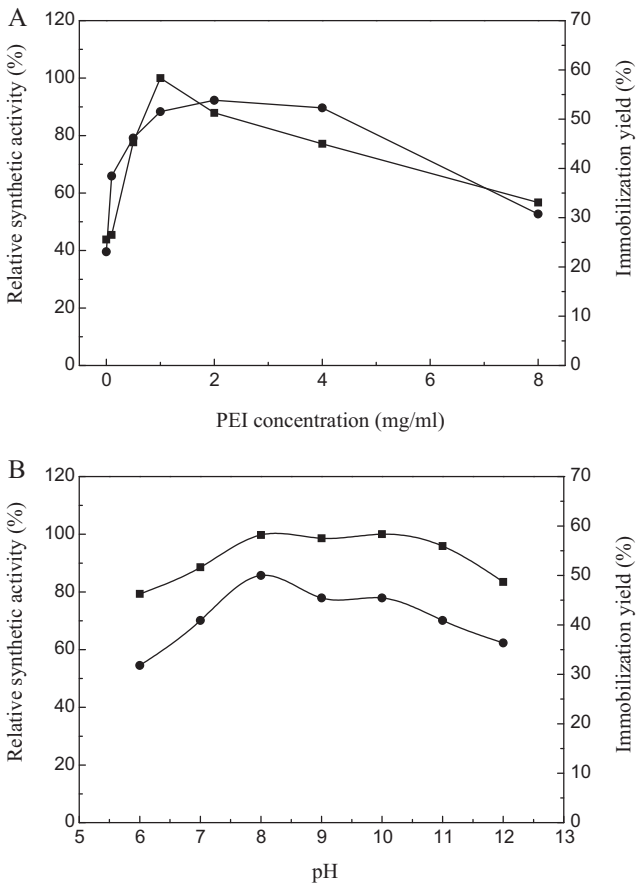

Fig. 2. Effect of PEI concentration (A) and pH (B) on lipase immobilization. –(■)– Relative synthetic activity; –(●)– immobilization yield. The immobilization conditions were: 0.1 g lipase powder, 0.6 g cotton cloth and 10 ml PEI solution. The same pH value (8.0) was set to test the effect of PEI concentration (0–8.0 mg/ml), and the same PEI concentration (10 mg/10 ml) was set to test the effect of different pH value. Esterification conditions: 0.6 g immobilized lipase with 0.3 M ethanol and 0.3 M butyric acid in 10 ml cyclohexane at 25 °C.

24 h esterification reaction was monitored (Fig. 4). The concentrations of both ethanol and butyric acid were decreased steeply during the early stage of reaction, because of the adsorption by cotton cloth. The profile of substrate consumption was similar to the result obtained by using polyurethane foams as the support for CRL

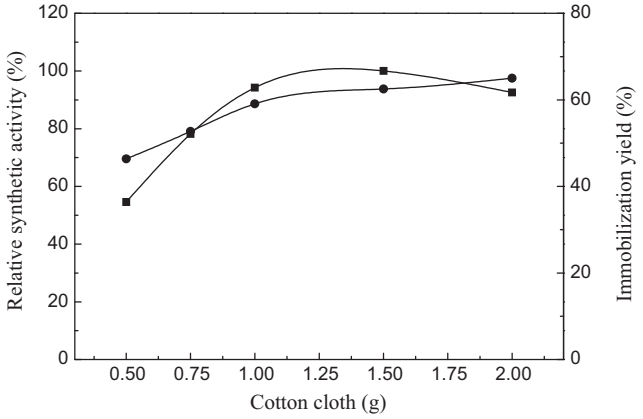

Fig. 3. Effect of support amount on lipase immobilization. –(■)– Relative synthetic activity; –(●)– immobilization yield. The immobilization conditions: 0.1 g lipase powder, 10 ml 1.0 mg/ml PEI solution, pH 8.0 and cotton cloth varied from 0.5 g to 2.0 g. Esterification conditions: 0.6 g immobilized lipase with 0.3 M ethanol and 0.3 M butyric acid in 10 ml cyclohexane at 25 °C.

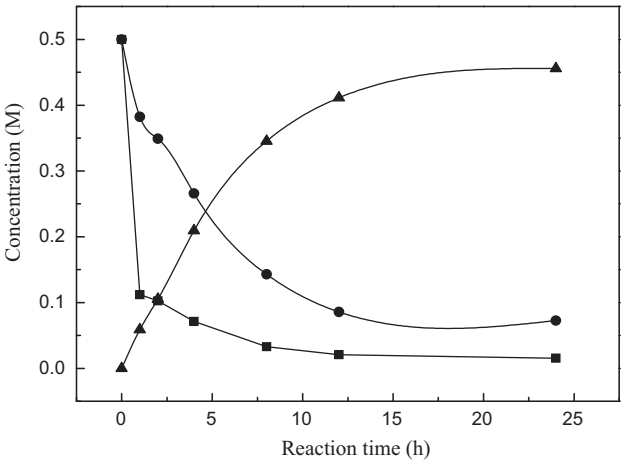

Fig. 4. Time course of esterification reaction catalyzed by immobilized lipase at 25 °C. –(■)– Ethanol; –(●)– butyric acid; –(▲)– ethyl butyrate. Reaction conditions: 3.0 g immobilized lipase, 0.5 M ethanol and 0.5 M butyric acid in 40 ml cyclohexane.

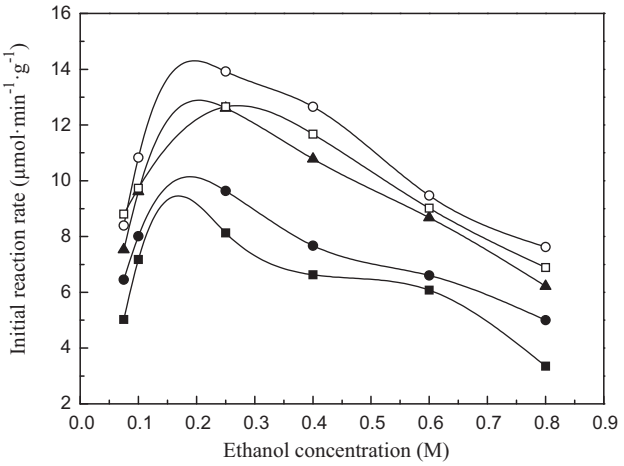

Fig. 5. Effect of molar ratio of ethanol to butyric acid on initial reaction rate at 25 °C. Butyric acid concentration: –(■)– 0.1 M; –(●)– 0.25 M; –(▲)– 0.4 M; –(○)– 0.6 M; –(□)– 0.8 M. The reaction conditions were set in the following: 0.6 g immobilized lipase with appropriate amount of substrates in 30 ml cyclohexane.

immobilization [2]. This so-called positive partition effect behavior could promote the contact between substrates and enzyme, and accelerate the reaction. However, excess substrate may result in the substrate inhibition. The concentration of ethyl butyrate was increased gradually at a relatively constant rate in the early 8 h, and then the reaction was slowed down and reached the equilibrium around 12 h (Fig. 4).

3.2.2. Effect of reaction temperature on ethyl butyrate synthesis

Different reaction temperatures in the range of 15–42 °C were tested to evaluate its effect on the initial reaction rate and conversion yield, with 0.6 g immobilized lipase, equimolar of alcohol and acid (0.1 M) in 30 ml cyclohexane. The initial reaction rate was improved with the increased temperature up to 37 °C, and the highest reaction rate was 16.7 μmol min^{-1} g^{-1} (Table 1). Temperatures beyond 37 °C resulted in the decrease of initial reaction rate. Uncoordinatedly, the maximal 87.9% conversion yield was obtained at 20 °C, higher temperature led to the impairment of conversion yield. Though the higher temperature (less than 37 °C) could enhance the initial reaction rate, the conversion yield was reduced due to the heat deactivation of enzyme. Considering about the cost and efficacy, 20–25 °C was adopted as the optimal reaction temperature. Contrast to the reported highest conversion yield of ethyl butyrate (36% at 37 °C) using other support for lipase immobilization [6], our result offered a higher conversion yield at lower temperatures.

3.2.3. Effect of substrate molar ratio on ethyl butyrate synthesis

The equilibrium of esterification could be pushed forward by the addition of excess alcohol or acid, or by removing products from the reaction mixture [20], but the effect of substrate inhibition must also be considered. Normally, a reaction should have a suitable molar ratio of substrates to ensure the best conversion yield. Therefore, a series of experiments were performed in a binary matrix mode with five different butyric acid concentrations (0.1 M, 0.25 M, 0.4 M, 0.6 M and 0.8 M) and six different ethanol concentrations (0.075 M, 0.1 M, 0.25 M, 0.4 M, 0.6 M and 0.8 M). As shown in Fig. 5, in spite of varied concentration of butyric acid, the highest reaction rates were always achieved when 0.25 M ethanol was initially presented. The initial reaction rate was steadily enhanced with the increase of butyric acid concentration up to 0.6 M. However, further increase of butyric acid could lead to the decrease of initial reaction rate, which was possibly caused by the substrate inhibition. This observation was accordant with the one in the previous literatures [2,21]. So the optimal concentrations of ethanol and butyric acid were 0.25 M and 0.6 M, respectively, and the educed molar ratio was 1:2.4.

3.3. Kinetics of esterification reaction

The investigation of substrates molar ratio had revealed that both ethanol and butyric acid could act as the inhibitor at the high concentration. According to previous literatures [21,22], a kinetic mechanism of Ping Pong Bi-Bi model was proposed with the inhibition of two substrates. A theoretical equation of Ping Pong Bi-Bi mode with the inhibition of alcohol and acid was presented [23].

$$\frac{v_0}{V_{max}} = \frac{[acid][alcohol]}{[acid][alcohol] + K_{macid}[alcohol](1 + [alcohol]/K_{ialcohol}) + K_{malcohol}[acid](1 + [acid]/K_{iacid})} \quad (1)$$

In Eq. (1), V_0 is the initial reaction rate (μmol min^{-1} g^{-1}), V_{max} is the maximal rate, K_m is the Michaelis constant of alcohol or acid (mol/l), K_i is the inhibition constant of alcohol or acid (mol/l). The data collected from two groups of experiments with 0.4 M or 0.6 M ethanol, respectively reacted with six different concentrations of butyric acid (0.075 M, 0.1 M, 0.25 M, 0.4 M, 0.6 M and 0.8 M), were fitted into the Eq. (1) to determine V_0. The kinetic parameters were calculated using Origin 7.5 software through a nonlinear regression

Table 1
The effects of reaction temperature on initial reaction rate and conversion yield.

	Temperature (°C)					
	15	20	25	30	37	42
Initial reaction rate (μmol min^{-1} g^{-1})	8.8 ± 0.4	10.3 ± 0.5	14.8 ± 0.7	15.5 ± 0.7	16.7 ± 0.8	15.4 ± 0.7
Conversion yield (%)	82.3 ± 4.1	87.9 ± 4.4	86.7 ± 4.1	85.9 ± 4.3	84.5 ± 4.2	84.1 ± 4.4

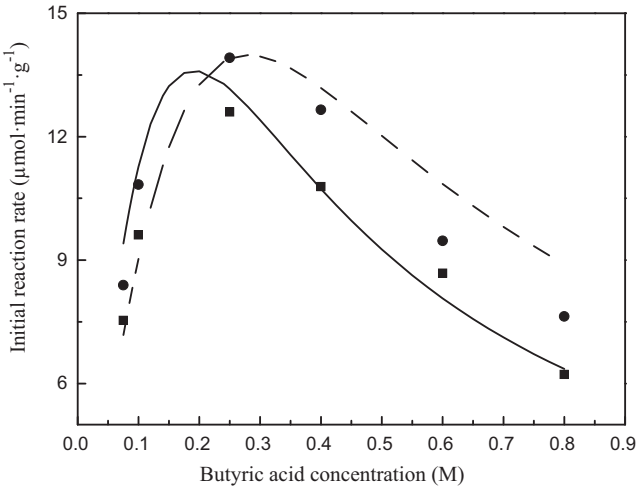

Fig. 6. Initial reaction rate V_0 vs. various butyric acid concentrations at constant ethanol concentrations of 0.4 M and 0.6 M, respectively. Experimental data points: (■) 0.4 M ethanol; (●) 0.6 M ethanol. Theoretical curve: —— 0.4 M ethanol; —- 0.6 M ethanol.

analysis. The obtained results were: V_{max} = 302.7 μmol min^{-1} g^{-1}, $K_{malcohol}$ = 0.3 M, K_{macid} = 0.09 M, $K_{ialcohol}$ = 0.0041 M, K_{iacid} = 0.07 M. The high value of $K_{iacid}/K_{ialcohol}$ suggested that ethanol is a stronger inhibitor than butyric acid. As shown in Fig. 4, a dramatic drop of ethanol concentration steeper than that of butyric acid was observed during the esterification reaction. The possible reason may due to the higher hydrophilicity of ethanol comparing to butyric acid, which makes ethanol easier to be concentrated around the immobilized lipase on cotton cloth and more competent than another substrate butyric acid. The comparison of $K_{malcohol}/K_{ialcohol}$ and K_{macid}/K_{iacid} had further confirmed this deduction, which has also been reported before [24]. As shown in Fig. 6, the experimental V_0 could be approximately fitted with the theoretical model, which suggested that the Ping Pong Bi-Bi mode is a proper kinetic model for the synthesis of ethyl butyrate.

3.4. Operational stability of immobilized lipase

The operational stability of immobilized lipase was examined via 12 recycles. As shown in Fig. 7, approximately half of the initial enzymes maintained their activity even after 12 recycles. Therefore, the half-life time of the immobilized lipase could reach about 300 h. Comparing to the reported data, the half-life time of immobilized CRL on polyurethane foams was 170.3 h [8], and immobilized CRL on poly (N-methylolacrylamide) for butyl butyrate synthesis only had 145 h half-life time [25]. Moreover, the conversion yield of immobilized lipase was well maintained in the range of 83–92% among all cycles. In conclusion, the immobilized lipase on cotton cloth was obviously more endurable to the recycle operation, without significant loss of its conversion efficiency.

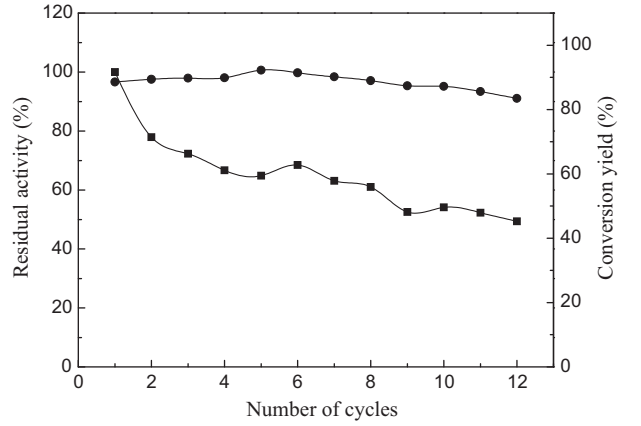

Fig. 7. Operational stability of immobilized lipase on cotton cloth at 25 °C. –(■)– Residual activity; –(●)– conversion yield. Reaction conditions: 0.6 g immobilized lipase with 0.3 M ethanol and 0.3 M butyric acid in 10 ml cyclohexane.

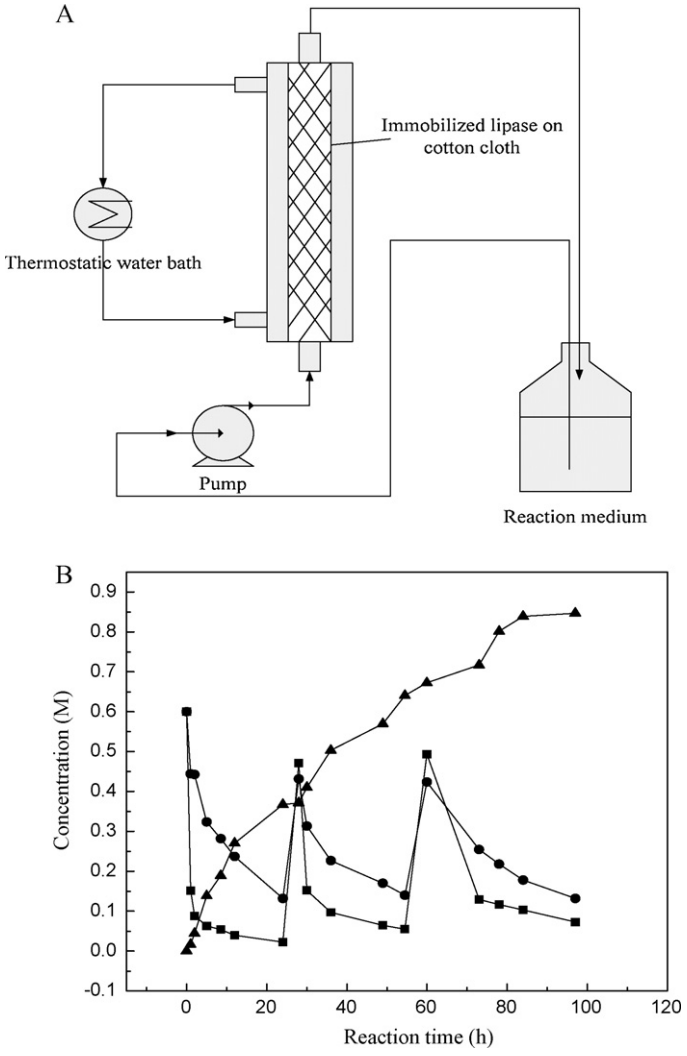

Fig. 8. (A) Schematic diagram of the recycle batch reactor for ethyl butyrate synthesis; (B) time-course of esterification reaction using recycle batch reactor. –(■)– Ethanol; –(●)– butyric acid; –(▲)– ethyl butyrate. Reaction conditions: 36 g immobilized lipase with 1.8 M ethanol and 1.2 M butyric acid in 750 ml cyclohexane, the reaction temperature was 25 °C. A Fed-Batch mode of substrates addition was adopted.

3.5. Ethyl butyrate synthesis in a recycle batch reactor system

To explore the industrial potential and feasible application of immobilize lipase for ethyl butyrate synthesis, a recycle batch reactor was designed (Fig. 8A). Approximately 36 g immobilized lipase was packed into the column to catalyze the synthesis of ethyl butyrate, with the presence of 1.2 M butyric acid and 1.8 M ethanol in cyclohexane. The reaction mixture was supplied following a Fed-Batch mode to eliminate the substrates inhibition. As shown in Fig. 8B, 0.85 M ethyl butyrate was produced after 97 h reaction, with the conversion yield of 70.6% and the productivity of 1.45 mmol h^{-1} g^{-1}. Comparing to other system, the much higher

concentration of final product had indicated a promising industrial application of this recycle batch reactor.

4. Conclusions

A practical immobilization method was developed to load lipase onto PEI-coated cotton cloth. Several factors including the amount of PEI, the pH of PEI solution and the amount of support were investigated to improve the immobilization efficiency. The obtained immobilized lipase was then applied to the synthesis of ethyl butyrate, for the evaluation of its enzymatic performance. The molar ratio of substrates and the reaction temperature were optimized to maximize the conversion yield of immobilized lipase. A kinetic mechanism of Ping Pong Bi-Bi model with inhibition of both substrates was proposed and validated by experimental data. Moreover, with the validated good operational stability of immobilized lipase, a recycle batch reactor was established to make an attempt on the possible industrial application of immobilized lipase for ethyl butyrate production. The achieved result of 0.85 M final ethyl butyrate concentration, with the conversion of 70.6% and the productivity of 1.45 mmol h^{-1} g^{-1}, had revealed the great potential of this immobilized lipase in the practical applications of ester production. Moreover, the bio-production of butyric acid via the fermentation of *Clostridium tyrobutyricum* was greatly hampered by the subsequent separation of product from fermentation broth, due to the high boiling point (163.5 °C) of butyric acid. Considering about the lower boiling point of ethyl butyrate (121.3 °C), the post-processing of *C. tyrobutyricum* fermentation broth via immobilized lipase could be proposed to not only facilitate the isolation of butyric acid, but also directly generate the useful ethyl butyrate for industrial applications. Thus, the system established in this paper has promising capability to develop the bio-production of butyric acid and its ester derivatives.

Acknowledgements

This work was financially supported by the Ministry of Science and Technology of China (Grant Nos. 2009AA02Z206 and 2007CB707805), the National Natural Science Foundation of China (Grant No. 20736008) and the Natural Science Foundation of Zhejiang Province (Grant No. R4090041), the People's Republic of China.

References

[1] Y. Xu, D. Wang, X.Q. Mu, G.A. Zhao, K.C. Zhang, J. Mol. Catal. B: Enzyme 18 (2002) 29–37.
[2] P. Pires-Cabral, M.M.R. da Fonseca, S. Ferreira-Dias, Biochem. Eng. J. 43 (2009) 327–332.
[3] J.A. Rodriguez-Nogales, E. Roura, E. Contreras, Process Biochem. 40 (2005) 63–68.
[4] D.P.C. de Barros, L.P. Fonseca, P. Fernandes, J.M.S. Cabral, L. Mojovic, J. Mol. Catal. B: Enzyme 60 (2009) 178–185.
[5] D. Bezbradica, D. Mijin, S. Siler-Marinkovic, Z. Knezevic, J. Mol. Catal. B: Enzyme 38 (2006) 11–16.
[6] R. Dave, D. Madamwar, Process Biochem. 43 (2008) 70–75.
[7] A. Kumari, P. Mahapatra, G.V. Kumar, R. Banerjee, Bioprocess. Biosyst. Eng. 31 (2008) 291–298.
[8] P. Pires-Cabral, M.M.R. da Fonseca, S. Ferreira-Dias, Biochem. Eng. J. 48 (2010) 246–252.
[9] P. Pires-Cabral, M.M.R. da Fonseca, S. Ferreira-Dias, Biochem. Eng. J. 33 (2007) 148–158.
[10] H. Yamazaki, R.K.H. Cheok, A.D.E. Fraser, Biotechnol. Lett. 6 (1984) 165–170.
[11] S.T. Yang, C.H. Shu, Biotechnol. Prog. 12 (1996) 449–456.
[12] N. Albayrak, S.T. Yang, Biotechnol. Prog. 18 (2002) 240–251.
[13] N. Albayrak, S.T. Yang, Enzyme Microb. Technol. 31 (2002) 371–383.
[14] Y.L. Huang, Z.T. Wu, L.K. Zhang, C.M. Cheung, S.T. Yang, Bioresour. Technol. 82 (2002) 51–59.
[15] J.M. Guisan, P. Sabuquillo, R. Fernandez-Lafuente, G. Fernandez-Lorente, C. Mateo, P.J. Halling, D. Kennedy, E. Miyata, D. Re, J. Mol. Catal. B: Enzyme 11 (2001) 817–824.
[16] S.F. D'Souza, S.S. Godbole, J. Biochem. Biophys. Methods 52 (2002) 59–62.
[17] K.M. de Lathouder, D.T.J. van Benthem, S.A. Wallin, C. Mateo, R.F. Lafuente, J.M. Guisan, F. Kapteijn, J.A. Moulijn, J. Mol. Catal. B: Enzyme 50 (2008) 20–27.
[18] N. Watanabe, Y. Ota, Y. Minoda, K. Yamada, Agric. Biol. Chem. 41 (1977) 1353–1358.
[19] P. Grochulski, Y. Li, J.D. Schrag, M. Cygler, Protein Sci. 3 (1994) 82–91.
[20] F. Ergan, M. Trani, G. Andre, J. Am. Oil Chem. Soc. 68 (1991) 412–417.
[21] S. Maury, P. Buisson, A. Perrard, A.C. Pierre, J. Mol. Catal. B: Enzyme 32 (2005) 193–203.
[22] S.H. Krishna, S.G. Prapulla, N.G. Karanth, J. Ind. Microbiol. Biotechnol. 25 (2000) 147–154.
[23] I.H. Segel, Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems, Wiley Classics Library Ed., Wiley, New York, 1993.
[24] M.N. Varma, G. Madras, J. Chem. Technol. Biotechnol. 83 (2008) 1135–1144.
[25] J.C. Santos, G.F.M. Nunes, A.B.R. Moreira, V.H. Perez, H.F. de Castro, Chem. Eng. Technol. 30 (2007) 1255–1261.