Journal of Molecular Catalysis B: Enzymatic 62 (2010) 15–18

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Pretreatment of immobilized *Candida* sp. 99-125 lipase to improve its methanol tolerance for biodiesel production

Jike Lu, Li Deng, Rui Zhao, Ruisi Zhang, Fang Wang, Tianwei Tan*

Beijing Bioprocess Key Laboratory, College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, 100029, PR China

ARTICLE INFO

Article history:
Received 6 February 2009
Received in revised form 11 August 2009
Accepted 11 August 2009
Available online 18 August 2009

Keywords:
Pretreatment
Immobilized lipase
Biodiesel
Methanol tolerance

ABSTRACT

Candida sp. 99-125 lipase immobilized on textile membrane was pretreated with several methods to improve its activity and methanol tolerance for biodiesel production. Lipase pretreatments with short chain alcohols from n-propyl alcohol to isobutyl alcohol did not have any positive effect on the lipase activity and methanol tolerance. While lipase treated with methanol solutions from 10 to 20% volume concentrations did enhance the enzyme activity and methanol tolerance, and this lipase activation effect did not exist when methanol volume concentration was 40%. 1 mM salt solutions of $(NH_4)_2SO_4$, $CaCl_2$, KCl, K_2SO_4 and $MgCl_2$ pretreatments were the useful tools to improve the lipase activity and methanol tolerance. The reason might be that salts could incorporate with the protein molecular to form a more stable molecular to resist conformation change induced by high methanol concentration. The operational stability of pretreated lipase was improved dramatically for biodiesel production during batch reactions.

1. Introduction

Lipases (EC 3.1.1.3) catalyzed reactions in organic media have drawn great attention in recent years because of its mild reaction condition [1–4]. The process is environmental friendly and green compared with chemical methods. One of the great bottlenecks for industrial application of lipase is its high cost. So immobilization methods have been introduced to improve the lipase stability for repeated utilization.

In addition, several pretreatment methods of both free and immobilized lipase have been investigated to improve their activity, stereoselectivity and stability. Generally, these pretreatment reagents can be classified but not limited to the following four kinds: (1) substrates or their analogues, which can enhance enzyme activity in organic solvents involve tuning the enzyme active site by molecular imprinting [5]; (2) organic solvents, which can increase the total activity in two ways, firstly by removing proteins other than lipases, then secondly by causing a conformational change from the active site "closed form" to the "open form" [6–8]; (3) salts, whose incorporation with the protein molecular could keep the conformation of the lipase and prevent the large change of optimum pH condition happening [9]; (4) enzyme lyoprotectants such as crown ethers, whose binding to the active site of lipase during the dehydration step could keep the enzyme active site in a catalytically active conformation [10]. Moreover, many pretreatment processes are combined together for higher lipase activity and stability.

Biodiesel (fatty acid alkyl esters, FAAEs) can be produced via lipase-catalyzed methanolysis of renewable sources such as vegetable oils, animal fats or even waste oils [11,12]. Methanol is mostly used because of its low cost compared with other alcohols, so FAAEs mainly refer to fatty acid methyl esters (FAMEs). As is known to all, lipase activity inhibition caused by excessive methanol is a big obstacle for process optimization. Therefore in many previous studies, methanol step-addition strategy had to be taken to avoid lipase inactivation [13]. Self-established *Candida* sp. *99-125* lipase immobilized on textile membrane was proved to be quite effective for biodiesel production, and previous studies in our lab were focused on this subject [14–16]. However, lipase inactivation caused by methanol in the system is still a big problem, and this immobilized lipase is not stable especially when it is stored for more than months. So lipase activity and stability still need to be improved through pretreatment process.

In this study, several pretreatment methods such as substrates, organic solvents, and salt solutions were employed to improve methanol tolerance of immobilized lipase *Candida* sp. 99-125 for biodiesel production. The results can contribute to the design of useful strategies for the industrial biodiesel production through enzymatic methods.

2. Experimental methods

2.1. Materials

Myristic acid methyl ester, palmitic acid methyl ester, palmitoleic acid methyl ester, stearic acid methyl ester, oleic acid methyl

* Corresponding author. Tel.: +86 10 6441 6691; fax: +86 10 6441 6691.
E-mail address: twtan@mail.buct.edu.cn (T. Tan).

1381-1177/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.molcatb.2009.08.002

Table 1
Effect of organic solvents on methanol tolerance of immobilized lipase *Candida* sp. 99-125.

Organic solvents		n-Propyl alcohol	n-Butanol	Isopropyl alcohol	t-Butanol	Isobutyl alcohol	Control
Three step methanol addition	Initial rate (%)	5.09	8.99	6.77	7.82	8.06	8.41
	Yield (%)	77.1	80.7	78.4	79.0	77.7	74.4
One step methanol addition	Initial rate (%)	0.84	2.90	0.86	1.56	2.38	2.65
	Yield (%)	0.98	4.34	1.18	2.02	2.33	2.44

Pretreatment conditions: 0.2 g immobilized lipase membrane was immersed in 30 ml organic solvent at 4 °C for 24 h, then dried at room temperature. Determination conditions of methanol tolerance: soybean oil 2 g, 200 μl water, 2 ml n-hexane, temperature 40 °C, 180 rev/min, total reaction time 12 h. For three step methanol addition, every 1/3 molar equivalent of methanol (93 μl) were added to the system at 0, 4 and 8 h, respectively. While for one step methanol addition, total 279 μl methanol was added to the system initially. Initial rate and yield was FAMEs content at 10 min and 12 h, respectively.

ester, linoleic acid methyl ester, and heptadecanoic acid methyl ester were from Sigma (St. Louis, USA) and of chromatographically pure. *Candida* sp. 99-125 (CGMCC 1470) was obtained from our laboratory and registered at the China General Microbiological Culture Collection Center (CGMCC). *Candida* sp. 99-125 immobilized on textile membrane was prepared in our laboratory, and the procedure has been described in detail in our previous paper [14]. This lipase was stored in our lab for more than a month, so it was not as stable as the newly prepared lipase. Soybean oil was purchased from local market (acid value: 0.02, saponification value: 193, water content: less than 0.01). All the other organic solvents and salts including methanol, n-propyl alcohol, n-butanol, isopropyl alcohol, tert-butanol, isobutyl alcohol, $(NH_4)_2SO_4$, $CaCl_2$, KCl, K_2SO_4, and $MgCl_2$ were obtained from Beijing Chemical Factory and were of analytical grade.

2.2. Pretreatment of lipase

Certain amount of the immobilized lipase membrane was immersed in 30 ml different solutions at 4 °C for 24 h. These solutions covered pure organic solvents such as n-propyl alcohol, n-butanol, isopropyl alcohol, tert-butanol, isobutyl alcohol, 1 mM salt solutions of $(NH_4)_2SO_4$, $CaCl_2$, KCl, K_2SO_4, $MgCl_2$, methanol solutions with concentrations from 10 to 40% (v/v). Then the lipase was dried at room temperature for the following experiments. Preliminary experiments showed that 24 h is needed for complete drying, and this lipase has the same weight loss and catalytic properties as lyophilized lipase. So it is unnecessary to employ lyophilization method for lipase drying.

2.3. Determination of activity and methanol tolerance of lipase

Our previous studies showed that methanol stepwise addition was beneficial for lipase activity, so methanol stepwise addition was adopted to determine lipase activity. One step methanol addition strategy was also adopted in this study because higher methanol concentration in the reaction system was introduced to determine the methanol tolerance of varied pretreatment lipase. Typical methanolysis was carried out in a 50 ml stoppered flask, incubated in a reciprocal shaker at 40 °C and 180 rpm. The reaction system contained 2 g soybean oil, 2 ml n-hexane, 0.2 g immobilized lipase, 200 μl distilled water and 279 μl methanol, with a total reaction time of 12 h. These reaction parameters were the optimized methanolysis conditions of this lipase [14,15]. For three step methanol addition, every 1/3 molar equivalent of methanol (93 μl) were added to the system at 0, 4 and 8 h, respectively. While for one step methanol addition, total 279 μl methanol was added to the system initially. At pre-determined time, 20 μl samples were taken and centrifuged to obtain the upper layer. Then 5 μl of the upper layer was dissolved in n-hexane for gas chromatography analysis.

2.4. Operational stability of the pretreated Candida sp. 99-125 lipase

To investigate the operational stability of the pretreated lipase, the methanolysis by single addition and three step addition of methanol were repeated every 12 h. The basic reaction conditions were the same as described before in Section 2.3. After completion of the reaction in 12 h of each cycle, the lipase was transferred directly into the same system for a new cycle. The same operational stability of the lipase without pretreatment was also examined as control.

2.5. GC analysis

The methyl esters content in the reaction mixture were quantified using a GC-2010 gas chromatography (Shimadzu Japan) equipped with a DB-1ht capillary column (30 m × 0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID). The column temperature was held at 100 °C, heated to 180 °C at 15 °C/min, to 230 °C at 10 °C/min and finally to 330 °C at 20 °C/min and then maintained for 5 min. The temperatures of the injector and detector were set at 350 and 360 °C, respectively. Heptadecanoic acid methyl ester purchased from Sigma was used as internal standard.

3. Results and discussion

Various organic solvents were tested to determine whether their pretreatments have positive effects on the methanol tolerance of lipase in this study, and the results were shown in Table 1. In three step methanol addition strategy, there was no obvious enhancement for both the initial rates and the equilibrium yields with pretreatment lipase. On the contrary, pretreated lipase activity was slightly lower than the untreated lipase in most cases. While for one step methanol strategy, similar results were obtained and there was almost no reactions occurred. It could be concluded that lipase

Table 2
Effect of methanol solutions on methanol tolerance of immobilized lipase *Candida* sp. 99-125.

Methanol volume percentage (%)		0	10	20	40	Control
Three step methanol addition	Initial rate (%)	17.5	16.8	16.5	11.7	8.13
	Yield (%)	74.5	79.9	76.9	71.5	74.5
One step methanol addition	Initial rate (%)	10.6	9.52	10.9	3.07	2.77
	Yield (%)	29.0	30.2	39.6	5.88	1.20

Pretreatment conditions: 0.2 g immobilized lipase membrane was immersed in 30 ml methanol solutions at 4 °C for 24 h, then dried at room temperature. Determination conditions of methanol tolerance and lipase activity were the same as that shown in Table 1.

Table 3
Effect of salt solutions on methanol tolerance of immobilized lipase *Candida* sp. 99-125.

Salt solutions		$(NH_4)_2SO_4$	$CaCl_2$	KCl	K_2SO_4	$MgCl_2$	Control
Three step methanol addition	Initial rate (%)	18.7	15.7	17.3	17.2	19.1	7.28
	Yield (%)	82.1	84.3	84.4	83.6	81.9	75.2
One step methanol addition	Initial rate (%)	20.2	18.1	14.3	6.60	22.6	2.63
	Yield (%)	57.2	71.2	56.1	47.2	74.5	1.54

Pretreatment conditions: 0.2 g immobilized lipase membrane was immersed in 30 ml salt solutions at 4 °C for 24 h, then dried at room temperature. Determination conditions of methanol tolerance and lipase activity were the same as that shown in Table 1.

pretreatment with short chain alcohols from n-propyl alcohol to isobutyl alcohol did not have any positive effect neither on the lipase activity, nor on the methanol tolerance. Although previous studies showed that pretreatment with organic solvents such as 2-propanol might increase the activity of *Candida rugosa* lipase [8,17], dissimilar results were obtained for immobilized *Candida* sp. 99-125 lipase. These results demonstrated that lipases from different origins might have distinct properties and one activating methods might not be versatile for other lipases.

Substrates and their analogues can enhance the lipase activity through tuning the enzyme active site by molecular imprinting [18]. However, methanol as a substrate was a strong inhibitor for lipase activity, especially at higher concentration. Therefore in this study, immobilized lipase was pretreated with methanol solutions to avoid lipase inactivation, and the results were shown in Table 2. Lipase treated with methanol solutions from 10 to 20% volume concentrations did enhance the enzyme activity and methanol tolerance, which could be concluded from the higher initial rates and equilibrium yields for both the three step and one step methanol addition strategies. While this lipase activation effect did not exist when methanol volume concentration was 40%, this indicated the complicated effect of methanol to lipase for biodiesel production. Methanol solution pretreatment could slightly improve the lipase activity and methanol tolerance at lower concentration, while inhibiting effect of methanol was dominant at higher concentrations above 40%. Another interesting result was that lipase treated with water without methanol had similar effect as methanol solution pretreatment. Water in the low concentration methanol solutions might play important roles during the pretreatment process. The lipase flexibility might be improved by tuning the water distribution in the immobilized lipase. Thus a more active lipase was formed with improved lipase activity and methanol tolerance.

Salts can incorporate with lipase molecules and keep the conformation of lipase from condition change. Preliminary experiments showed that saturated salt solutions of $(NH_4)_2SO_4$, $CaCl_2$, KCl, K_2SO_4 and $MgCl_2$ did not have any positive effect on the lipase performance. So immobilized lipase was pretreated with 1 mM solutions of $(NH_4)_2SO_4$, $CaCl_2$, KCl, K_2SO_4 and $MgCl_2$, and the reaction results were shown in Table 3. The time course curves of the 1 mM $MgCl_2$ pretreated lipase and untreated lipase were shown in Fig. 1. In three step methanol addition strategy, initial rates increased from 7.28 to 15.7% with 1 mM $CaCl_2$ solutions treatment. All the equilibrium yields were more than 80% while the control yield was 75.2%. These results indicated that 1 mM salts solutions pretreatment was a useful tool to activate the lipase. For one step methanol addition strategy, control experiment without pretreatment showed almost no lipase activity, which was consistent with previous studies that lipase activity was strongly inhibited at higher methanol concentration [13]. While after pretreatments with salt solutions, both the initial rates and equilibrium yields were increased considerably. All the yields were more than 45% with treated lipase, and the most promising results, more than 70% yields, were obtained for $CaCl_2$ and $MgCl_2$ pretreatments. These results showed that methanol tolerance of this lipase had been improved remarkably by salt solution pretreatments. The reason might be that pretreatment could change the lipase aggregation state to an apparent more 'open' form by tuning the water distribution in the lipase. Thus the activity was improved correspondingly. As for the improved methanol tolerance, the explanation might be that salts could incorporate with the protein molecular to form a more stable molecular, which could resist conformation change induced by high methanol concentration. For similar reasons, the activity of *Candida rugosa* lipase lyophilized with LiCl, NaCl or KCl was improved in previous studies [9].

It has been demonstrated that the cost of lipase accounts for a large part of biodiesel production cost, and one of the main advantages of immobilized lipase is that it can be used repeatedly over an extended period of time. Since salt solution pretreatments could improve the lipase activity and methanol tolerance, higher operational stability of pretreated lipase was expected for biodiesel production. To investigate the stability of the pretreated lipase, the methanolysis by single addition and three step addition of methanol were repeated every 12 h, respectively. After completion of the reaction in 12 h of each cycle, the lipase was transferred into the same system for a new batch. The results were shown in Figs. 2 and 3. For three step methanol addition strategy, it was evident that the operational stability of immobilized lipase was improved by 1 mM $MgCl_2$ pretreatment for biodiesel production. The untreated lipase has been stored for more than a month in our lab, its activity decreased sharply during the first three batches. While the lipase treated with 1 mM $MgCl_2$ lost its activity slowly during the first nine batches and biodiesel yield after nine batches was still higher than 50%. For single methanol addition strategy, it could be concluded from Fig. 3 that the lipase could not be reused quite well at higher methanol concentrations even after pretreatment, although the yield of the first batch was much higher than

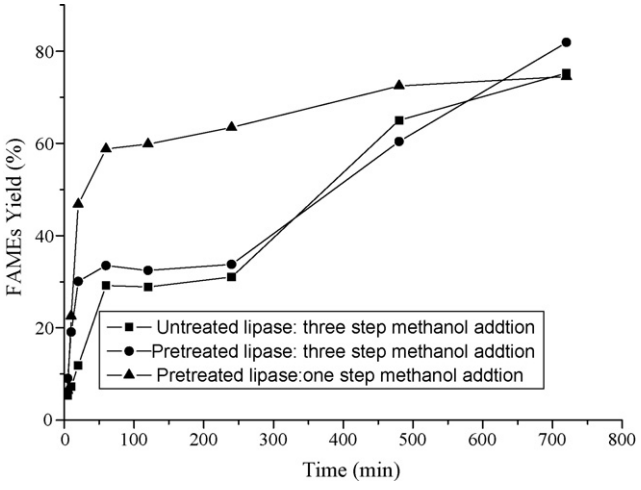

Fig. 1. Time course curves of the methanolysis catalyzed by pretreated and untreated lipases. Pretreatment conditions: 0.2 g immobilized lipase membrane was immersed in 30 ml 1 mM $MgCl_2$ at 4 °C for 24 h, then dried at room temperature. Methanol addition strategies were the same as that shown in Table 1.

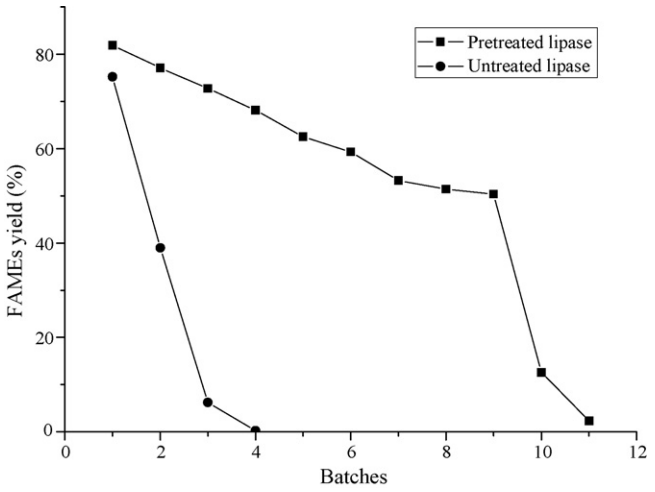

Fig. 2. Operational stability of immobilized *Candida* sp. 99-125 lipase for biodiesel production by three step addition of methanol in batch reactions. Pretreatment conditions were the same as in Fig. 1. Reaction conditions: soybean oil 2 g, 200 μl water, 2 ml n-hexane, temperature 40 °C, 180 rev/min, every 1/3 molar equivalent of methanol (93 μl) were added to the system at 0, 4 and 8 h, respectively, total reaction time 12 h. The lipase was transferred into the same system for a new batch after completion of the former reaction in 12 h.

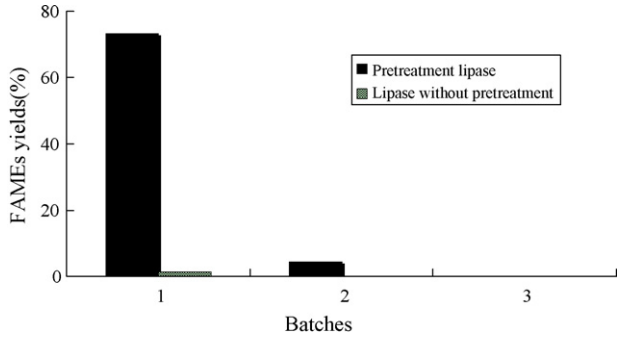

Fig. 3. Operational stability of immobilized *Candida* sp. 99-125 lipase for biodiesel production by single addition of methanol in batch reactions. Pretreatment conditions were the same as in Fig. 1. Reaction conditions: soybean oil 2 g, 200 μl water, 2 ml n-hexane, temperature 40 °C, 180 rev/min, total 279 μl methanol were added to the system initially, total reaction time 12 h. The lipase was transferred into the same system for a new batch after completion of the former reaction in 12 h.

that of the untreated lipase. One step methanol addition strategy was not suitable for complete methanolysis for its high methanol concentration, and the pretreated lipase was rather more stable in batch reactions when methanol was added stepwisely. If the FAMEs yield was lower than 50%, unreacted methanol was accumulated in the system. Higher methanol concentration was a strong inhibitor for lipase activity, so the lipase lost its activity sharply when the FAMEs yield was lower than 50%. These results showed that pretreated method of immobilized *Candida* sp. 99-125 with 1 mM salts solutions could be used for the production of biodiesel fuel.

4. Conclusions

An effective method to improve lipase activity and methanol tolerance was introduced for self-established lipase *Candida* sp. 99-125 immobilized on textile membrane. 1 mM salts solutions of $CaCl_2$ and $MgCl_2$ pretreatments could improve the lipase activity, methanol tolerance and operational stability for biodiesel production. Since lipase inactivation caused by methanol was a major technical obstacle and the high cost of biocatalyst lipase was main bottleneck of biodiesel industrialization, the results obtained in this study might solve the above problems and make biodiesel industrialization in green technology rather feasible.

Acknowledgements

This research was financially supported by the National High Technology Research and Development Program of China (2006AA020203, 2009AA03Z232), the National Nature Science Foundation of China (20576013), the State Key Development Program for Basic Research of China (2007CB714304), the Natural Science Foundation of Beijing, China (2071002).

References

[1] W.J. Ting, K.Y. Tung, R. Giridhar, W.T. Wu, J. Mol. Catal. B: Enzym. 42 (2006) 32–38.
[2] M.A. Zinni, L.E. Iglesias, A.M. Iribarren, J. Mol. Catal. B: Enzym. 47 (2007) 86–90.
[3] T. Kobayashi, W. Furutani, S. Adachi, R. Matsuno, J. Mol. Catal. B: Enzym. 24–25 (2003) 61–66.
[4] Y.B. Tewari, D.J. Vanderah, J.D. Rozzell, J. Mol. Catal. B: Enzym. 21 (2003) 123–131.
[5] J.O. Rich, V.V. Mozhaev, J.S. Dordick, D.S. Clark, Y.L. Khmelnitsky, J. Am. Chem. Soc. 124 (2002) 5254–5255.
[6] Md.M.R. Talukder, S. Tamalampudy, C.J. Li, L. Yanglin, J. Wu, A. Kondo, H. Fukuda, Biochem. Eng. J. 33 (2007) 60–65.
[7] C. Torres, C. Otero, Enzyme Microb. Technol. 19 (1996) 594–600.
[8] M. Goto, H. Ogawa, T. Isobe, M. Kawasaki, T. Kometani, J. Mol. Catal. B: Enzym. 24–25 (2003) 67–73.
[9] H.W. Yu, H. Chen, Y.Y. Yang, C.B. Ching, J. Mol. Catal. B: Enzym. 35 (2005) 28–32.
[10] M. Persson, I. Mladenoska, E. Wehtje, P. Adlercreutz, Enzyme Microb. Technol. 31 (2001) 833–841.
[11] D. Royon, M. Daz, G. Ellenrieder, S. Locatelli, Bioresour. Technol. 98 (2007) 648–653.
[12] P.V. Lara, E.Y. Park, Enzyme Microb. Technol. 34 (2004) 270–277.
[13] Y. Shimada, Y. Watanabe, A. Sugihara, Y. Tominaga, J. Mol. Catal. B: Enzym. 17 (2002) 133–142.
[14] J. Lu, K. Nie, F. Xie, F. Wang, T. Tan, Process Biochem. 42 (2007) 1367–1370.
[15] J. Lu, Y. Chen, F. Wang, T. Tan, J. Mol. Catal. B: Enzym. 56 (2009) 122–125.
[16] J. Lu, K. Nie, F. Wang, T. Tan, Bioresour. Technol. 99 (2008) 6070–6074.
[17] A. Cipiciani, F. Bellezza, F. Fringuelli, M. Stillitano, Tetrahedron: Asymmetry 10 (1999) 4599–4605.
[18] J. Chen, W. Wu, J. Biosci. Bioeng. 95 (2003) 466–469.