Journal of Molecular Catalysis B: Enzymatic 71 (2011) 45–50

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

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

Improving catalytic performance of *Burkholderia cepacia* lipase immobilized on macroporous resin NKA

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ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form 23 March 2011
Accepted 23 March 2011
Available online 8 April 2011

Keywords:
Burkholderia cepacia lipase
Immobilization
Bioimprinting
Interfacial activation
Circular dichroism
Conformation

ABSTRACT

The lipase from *Burkholderia cepacia* adsorbed on macroporous resin NKA was investigated by combined strategies of bioimprinting and interfacial activation to enhance its catalytic performance. The specific activity of the derivative lipase was 211,733.3 U/g-protein, which was 21.7-fold, 19.4% and 47% enhancement over the free lipase powder, non-bioimprinted and non-interfacial activation lipase, respectively. The derivative lipase exhibited a satisfactory thermal stability over a wide range of temperature (from 30 °C to 70 °C) and a strong tolerance to organic solvents such as methanol, ethanol and acetone with 50% concentration. After being used of 50 successive batches (400 h), the derivative lipase still retained over 92% of its original activity (methyl esters yield decreased from 98% to 90%). Circular dichroism analysis indicated that the activity enhancement of the derivative lipase was ascribed to the secondary structure changes. The derivative lipase preparation in this work was probably a promising alternative to produce a biocatalyst of satisfactory thermo-stability, strong solvents tolerance and high operational reusability.

1. Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) constitute a special group of enzymes whose biological function is to catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, free fatty acids (FFA) and glycerol [1]. Numerous studies showed that significant differences were observed in lipase catalytic activity in terms of source, degree of purity, state (free or immobilized), substrate, and reaction medium (solvent-free or biphasic) [2–4]. *Burkholderia cepacia* lipase (BCL) is an extracellular lipase and was widely used for biodegradation of environmental pollutants, biological control of plant diseases and a wide variety of reactions in aqueous and non-aqueous phases [5]. BCL has been recently proven to be among the most versatile lipases and could be used for biofuel synthesis [6]. However, the free form of BCL usually exhibits low stability, bad resistant recovery and no reusability, which will severely lower its feasibility in practical applications [7]. Therefore, immobilized BCL is often used not only for enhancing enzyme stability and activity, but also for easy enzyme recovery and repeated uses for better economical benefits [8]. The methods of BCL immobilization include adsorption, covalent attachment, entrapment, cross-linked enzyme aggregates (CLEAs) and cross-linked enzyme crystals (CLECs) [9–11]. Physicochemical properties of matrix supports are crucial for enzyme immobilization and will influence enzyme loading as well as catalytic behavior. It is very important to choose matrix materials with good properties that will improve enzyme activity and ease suitable immobilization conditions, such as pH, temperature, duration and initial enzyme loading.

Molecular bioimprinting and interfacial activation are the effective approaches to enhance enzymatic activity and stability in non-aqueous media [12]. Bioimprinting consists of loading the enzyme's active site with a substrate analogue in an aqueous solution. A complex similar to an enzyme–substrate complex is formed and small conformational changes are supposed to occur. Then, the ligand is washed away, while the enzyme is unable to recover its former conformation owing to its rigid structure, which results from strong electrostatic interactions in the media with low dielectric constants [13]. Recently determined 3D structures of some lipases show a 'lid' controlling access to the active site. Therefore, the catalytic reactions always occur at lipid–water interfaces. Up to now, interfacial activation has always been employed to improve the performance of lipase in catalytic reactions. Many literatures have proven that non-polar organic solvents, surfactants and divalent cations are usually employed to treat lipases to improve their activities and stabilities [11,12].

In this study, in order to achieve satisfactory catalytic performance of BCL, integrated strategies based on immobilization–bioimprinting–interfacial activation were

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doi:10.1016/j.molcatb.2011.03.007

Table 1
Properties of macroporous resin NKA.

Items	Chemical make-up	Particle size (μm)	Specific surface area (m^2/g)	Pore diameter (Å)
NKA resin	Polystyrene	300	570	200

adopted to modify BCL. The main purposes of this work are: (1) to investigate the preparation of the derivative BCL immobilized on macroporous resin followed with the combined strategies of bioimprinting and interfacial activation; (2) to evaluate the characterization of the derivative BCL, including activation energy, initial esterification activity, thermal stability, organic solvents tolerance and reusability; and (3) to elucidate the conformation variance of the derivative BCL via circular dichroism (CD) analysis.

2. Materials and methods

2.1. Materials

B. cepacia lipase powder was purchased from Amano, Japan. Macroporous resins NKA was purchased from Tianjin Nankai Sci. & Tech. Co. Ltd., Tianjin, China. Bovine serum albumin (BSA) was from Sigma–Aldrich (St. Louis, Missouri, USA). Other reagents, such as lauric acid, dodecanol, methanol, ethanol, isooctane, *tert*-butanol, iso-butanol, *n*-butanol, acetone, propanol, isopropanol, *n*-heptane, hexane, cyclohexane, Na_2HPO_4, NaH_2PO_4 and NaOH were of analytical grade and from Sinopharm Chemical Reagent Co. Ltd. Shanghai, China.

2.2. Immobilization procedures

NKA resin is used as a support matrix to immobilize BCL. The properties of NKA resin such as particle size, specific surface area and pore diameter were shown in Table 1. The procedures of BCL immobilization on macroporous resin matrix were modified according to the method in our previous work [14]. During the BCL immobilization procedure, the effects of pH (pH 3.0–10.0), free lipase loading (2–15%), duration (0.5–7 h) and temperature (25–70 °C) on the immobilization efficiency, specific activity and lipase activity recovery were addressed. To study the effect of pH, three kinds of buffers were used for pH optimization, which were Na_2HPO_4–citrate acid buffer (pH 3.0–5.0), phosphate buffer (pH 6.0–8.0) and glycin–NaOH buffer (pH 9.0–10.0). Immobilization efficiency (%), specific activity (U/g-protein) and lipase activity recovery (%) were estimated as Eqs. (1)–(3).

$$\text{Immobilization efficiency}/\% = \frac{\text{Protein content of immobilized lipase}}{\text{Total protein content of adding free lipase powder}} \times 100\% \quad (1)$$

$$\text{Specific activity (U/g-protein)} = \frac{\text{Initial esterification activity}}{\text{Protein content of immobilized lipase}} \quad (2)$$

$$\text{Lipase activity recovery}/\% = \frac{\text{Total initial esterification activity of immobilized lipase}}{\text{Total initial esterification activity of adding free lipase powder}} \times 100\% \quad (3)$$

2.3. Bioimprinting procedures

0.8 g free BCL powder sample was incubated for 60 min in 50 μL mixture solvent of isopropanol and bioimprinting molecules (decanoic acid, lauric acid, myristic acid, palmitic acid, stearic acid) under the conditions of rotating speed 200 rpm and room temperature. The volume of bioimprinting molecules (*ca.* 30 μmol) ensured the molar ratio of bioimprinting molecules to isopropanol equaling to 0.6:1. After incubation, the bioimprinted lipase was immobilized according to the procedures described in Section 2.2. Afterward, the bioimprinting molecules were removed with 5 mL octane from the surface of lipase, and then the immobilized–bioimprinted BCL was obtained and dried in a FD-1D-50 vacuum desiccator at room temperature.

2.4. Interfacial activation procedures

50 mg of immobilized–bioimprinted BCL was typically incubated at room temperature in nonpolar organic solvents as interfacial activation reagents, such as *n*-hexane, cyclohexane, *n*-heptane and isooctane. The volume of interfacial activation agents kept 5 mL throughout the activation experiments. After activation for 60 min, the interfacial activation reagents were removed and then the immobilized–bioimprinted–interfacial activated BCL (also called 'the derivative BCL') was obtained and dried in a FD-1D-50 vacuum desiccator at room temperature.

2.5. Assay of lipase activity and protein content

Lipase activity of the free and derivative BCL was assayed according to the method described by Foresti et al. [12]. The relative lipase activity was estimated by Eq. (4).

$$\text{Relative activity (\%)} = \frac{\text{Residue lipase activity after treatment}}{\text{Origin lipase activity before treatment}} \times 100\% \quad (4)$$

Protein content of the immobilized BCL was determined by the Bradford protein assay method using Bradford reagent from biorad at a wavelength of 595 nm with a standard calibration curve of BSA [15].

2.6. Measurement of thermal stability, organic solvents tolerance and reusability

Thermal stability assay: The thermal stability tests were carried out when the derivate BCL was kept at predetermined temperatures. At designated time intervals, the residual lipase activity of the derivate BCL was measured at 37 °C as per the methods described in Section 2.5. The residual lipase activity of free BCL treated at the above-mentioned temperature was tested as control. The thermal stability was explained by relative lipase activity (%).

Organic solvents tolerance measurement: The derivate BCL was employed to immerse in organic solvents with 50% volume concentration for 4 h at room temperature. The ratio organic solvent volume and immobilized BCL was 10 mL of organic solvent to 50 mg of the derivative BCL. Then, the organic solvent was removed in vacuum desiccator and residual lipase activity was measured at 37 °C according to the methods described in Section 2.5. The lipase activity of the derivative BCL without treated with organic solvents was tested as control. The thermal stability was explained by relative lipase activity (%).

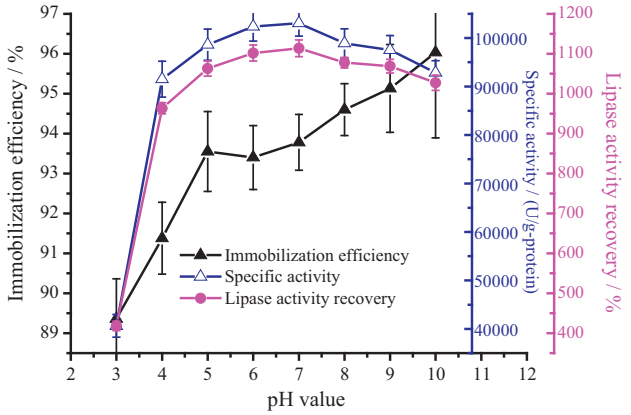

Fig. 1. Effect of pH on the BCL immobilization (conditions: lipase loading 10%, stirring speed 200 rpm, temperature 37 °C, reaction duration 2 h).

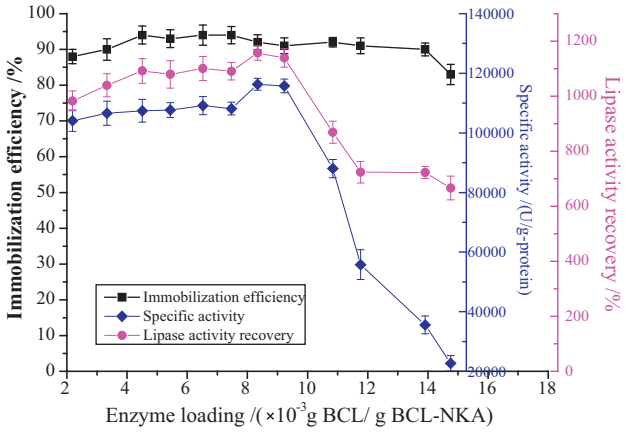

Fig. 2. Effect of lipase loading on the BCL immobilization (conditions: lipase loading 2–15%, stirring speed 200 rpm, temperature 37 °C, reaction duration 2 h).

Operational stability determination: To investigate the reusability of the derivative BCL, batch transesterification of soybean oil and methanol was conducted in solvent free medium. After each reaction period, the derivative BCL was withdrawn by filtering, and reused directly in the next batch. Then, fresh reaction mixture was reintroduced to test the operational stability of the derivative BCL. Each experiment was performed at least three times and the reaction conditions were: the derivative BCL loading 2.5 wt% (based on oil weight, g), soybean oil to methanol molar ratio 1:4, moisture content 7%, reaction temperature 40 °C, reaction time 8 h. The methyl esters yield was calculated to monitor the reusability of the derivative BCL according to the method described in our previous work [1].

2.7. Circular dichroism (CD) measurement

The far-UV (190–250 nm) CD spectra of BCL at 25 °C were recorded in N_2 atmosphere with Jasco J-810 spectrophotometer equipped with a temperature control system (JULABO F-12). For the aqueous sample a quartz cell with path-length 0.1 cm was used. All spectra were obtained at 25 °C with a 1 nm bandwidth and a scan speed of 200 nm/min. For every medium a base line was recorded from the protein spectrum. The free and modified BCL concentration was 0.08 mg/mL. The secondary structure element content was estimated using the DICHROWEB application package [16] based on the SELCON3 algorithm described by Sreerama et al. [17].

2.8. Statistics analysis

All trials were conducted in triplicate and the data were analyzed by the software Origin 7.5 (OriginLab Co., Northampton, MA, USA).

3. Results and discussion

3.1. BCL immobilization on resin matrix

3.1.1. Effect of reaction pH on the BCL immobilization

The pH value is usually a critical factor influencing enzyme immobilization efficiency. The effect of pH on the performance of NKA-BCL (Fig. 1) shows that the maximum NKA-BCL specific activity and lipase activity recovery occur at pH 7.0, which is 95,000 U/g-protein and 96.5%, respectively. It is suggested that a neutral pH condition seems to benefit lipase immobilization onto macroporous resin NKA. In this study, the BCL is mainly immobilized onto resin NKA by physical absorption, and the immobilization efficiency is estimated to be over 93% at pH 7.0. Since lipase immobilization on hydrophobic matrices was carried out by physical adsorption, no ionic interaction is supposed to occur between enzyme and support. The variation of pH of the reaction mixture will affect the reactivity of the functional group (*i.e.*, –COOH (carboxylic groups)) of the enzyme, thereby influencing the binding of lipase on NKA surface [18].

3.1.2. Effect of lipase loading on the BCL immobilization

The lipase immobilization efficiency was examined in terms of lipase loading (from 2% to 15%) in this work. The effect of lipase loading on the BCL immobilization efficiency was studied and the results are shown in Fig. 2.

As shown in Fig. 2, a decrease in NKA-BCL immobilization efficiency, specific activity and lipase activity recovery is observed when the lipase loading is over 10%. The reasonable explanation is probably attributed to the diffusional limitation due to the porous diameter reduction and multilayer immobilized lipases on hydrophobic support [19,20]. Although a higher lipase loading is supposed to result in a higher reaction rate from the aspect of reaction kinetics, the corresponding increase in viscosity for higher lipase loading can lower the mixing efficiency of the reaction mixture containing lipase and resin NKA support. These two effects were competing to each other and it seems that when the lipase loading is too high, the kinetic benefit from higher lipase concentration is downplayed by the poor mass transfer efficiency, leading to a lower lipase activity. According to the foregoing results, a suitable lipase loading of 10% is chosen during BCL immobilization in this work.

3.1.3. Effect of temperature on the BCL immobilization

The effect of temperature on the activity of immobilized NKA-BCL was tested in the range of 30–70 °C. It is observed that the optimum temperature for NKA-BCL is 37 °C, at which temperature the immobilization efficiency, specific activity and lipase activity recovery are 87%, 90% and 92%, respectively. It is also observed that the specific activity, immobilization efficiency and lipase activity recovery of NKA-BCL are relatively high and stable below 60 °C. However, the specific activity, immobilization efficiency and lipase activity recovery of NKA-BC decrease rapidly when the reaction temperature is beyond 60 °C. These observations are attributed to the increase enzymatic reaction rate with the increase of temperature to a certain level in general, and thereafter high temperature causes protein denaturation and thus decreases the reaction rate.

3.1.4. Effect of reaction duration on the BCL immobilization

To examine the effect of reaction duration on the BCL immobilization, a fixed amount of sample was withdrawn from the

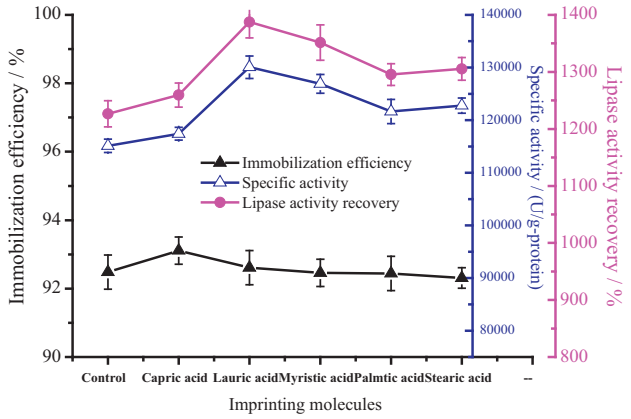

Fig. 3. Effect of bioimprinting on the performance of NKA-BCL (conditions: lipase loading 10%, stirring speed 200 rpm, room temperature, incubated time 60 min, bioimprinting molecular amount 30 μmol).

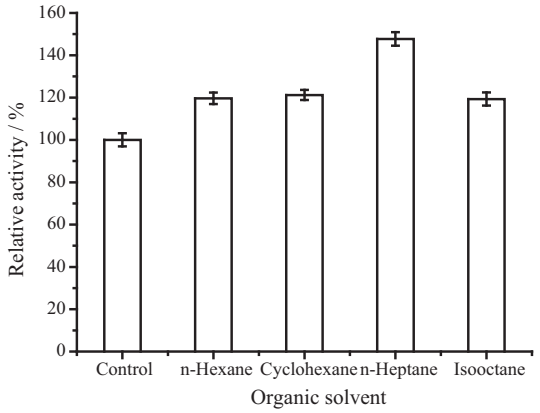

Fig. 4. Effect of interfacial activation on the performance of NKA-BCL (conditions: NKA-BCL loading 50 mg, room temperature, incubated time 60 min, interfacial activation agents amount 5 mL).

reaction mixture at intervals during the immobilization reaction to detect temporal variation of the immobilized lipase activity. The results show that the maximum NKA-BCL specific activity and lipase activity recovery are obtained at the reaction time of 1 h. After 1 h the NKA-lipase specific activity and lipase activity recovery decrease gradually. Simultaneously, the maximum immobilization efficiency is up to 84.3% at 1 h, which indicates that the amount of NKA-lipase combined compound increased initially, reached maximum (84.3%) at 1 h, and then leveled off after 1 h. The slight decrease in lipase specific activity after 1 h is probable a result of enzyme instability over a longer reaction time, whereas is more likely due to the experimental errors (<5%).

3.2. Bioimprinting

The templates used for the bioimprinting NKA-BCL were fatty acid substrate analogues in the range of C_8–C_{18}. The effect of bioimprinting molecules on the performance of NKA-BCL is shown in Fig. 3.

Since lipase activity is measured FFA concentration before and after the esterification of lauric acid and dodecanol, the first fatty acid chosen as template was pure lauric acid. When adding lauric acid concentration is equal to 30 μmol, the maximal specific activity and lipase activity recovery are achieved, which are 159,693 U/g-protein and 1202.96%, respectively. In comparison with non-bioimprinted NKA-BCL, the specific activity and lipase activity recovery of bioimprinted BCL enhance 19.4% and 14.3%, respectively, and the immobilization efficiency is estimated to be 85.67% after imprinting with lauric acid. The reasonable explanations of enhancement of activity imprinted with lauric acid are probably: (1) the dramatic increase of activity is related to the suitable solubility of imprinted molecular in the aqueous phase and its resemblance to the natural substrate of the enzyme [21]. (2) The dramatic increase of activity seems to be related mainly to the formation of an enzyme–support complex with a very suitable open conformation with some restrictions to the access of substrates [22]. Apart from lauric acid, capric acid ($C_{8:0}$), palmitic acid ($C_{16:0}$), and stearic acid ($C_{18:0}$) are used as bioimprinting molecules to enhance the performance of NKA-BCL. However, compared to the control (without fatty acid-bioimprinting), the increase of performance, including immobilization efficiency, specific activity and lipase activity recovery, is slightly higher but not significant (Fig. 3). It is concluded that using lauric acid as bioimprinting molecular can obviously increase specific activity and lipase activity recovery of BCL.

3.3. Interfacial activation

As a guide for the selection of interfacial activation agents to improve the performance of the biocatalysts, the hydrophobicity (log *P*) of agent is mainly considered. Log *P* is a parameter widely used to account for the characteristics of the organic phase. It has been reported that organic solvents with log *P* > 3.0 are suitable for catalytic reaction media, if log *P* is below 2, the organic solvents will strip of the water surrounding the enzyme resulting in decrease of enzyme catalytic activity [23]. Therefore, activation of immobilized–bioimprinted BCL was performed using hydrocarbons organic solvents with log *P* > 3.0, such as *n*-hexane (5.7), isooctane (4.51), *n*-octane (4.5), cyclohexane (3.2), *n*-heptane (5.5) and nonane (5.1). The results (Fig. 4) show that all those tested interfacial activation agents enhance the specific activity of immobilized–bioimprinted BCL to some extent, and the maximal activity of immobilized–bioimprinted BCL is obtained using *n*-heptane as interfacial activation agent, which is 47% enhancement compared with the control (non-interfacial activation). The specific activity of the derivative lipase was 211,733.3 U/g-protein, which was 21.7-fold, 19.4% and 47% enhancement over the free lipase powder, non-bioimprinted and non-interfacial activation lipase, respectively. The effect of organic solvents on the lipase catalytic activity is attributed to the variation of water retained in the microenvironment of the catalytic active site, which is necessary to maintain the conformation of the lipase.

3.4. Activation energy, thermal stability, organic solvent tolerance and operational stability of the derivative BCL

Activation energy is usually a critical factor influencing enzyme activity. The activation energies of the free and derivate BCL according to Arrhenius equation are estimated to be 25.18 kJ/mol and 17.66 kJ/mol, respectively, which indicates that the catalytic rate of the derivate BCL is greatly improved. This phenomenon is accordance with those above-mentioned observations on catalytic performances described before.

The thermal stability of the derivative BCL at the temperature range from 30 to 70 °C was investigated. The results are shown in Fig. 5.

As shown in Fig. 5, the derivative BCL retains almost 100% original activity below 60 °C, and keeps 80% of original activity at 65 °C for 60 min. In contrast, the catalytic activity of free BCL quickly dropped to nearly 40% of its original activity after incubation at 60 °C for 60 min. This observation indicates clearly that the derivative BCL obtains better thermal stability than free lipase. The BCL

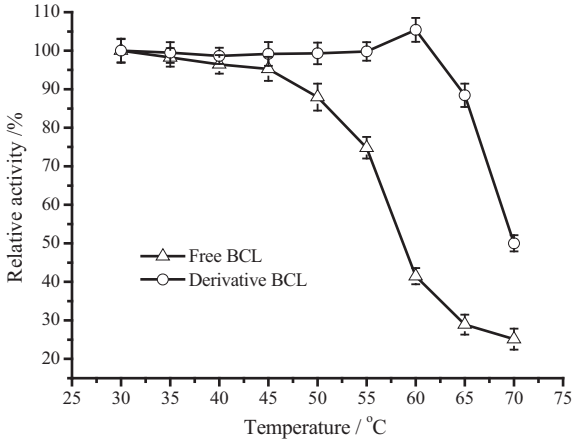

Fig. 5. Comparison of thermostability of derivative BCL and free BCL.

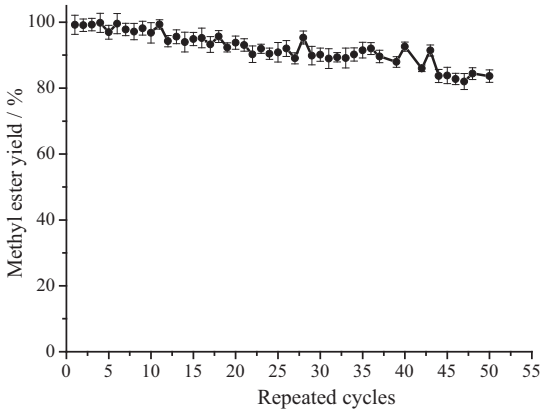

Fig. 7. The operational stability of the derivative BCL (conditions: lipase loading 2.5 wt% (based on oil weight, g), soybean oil to methanol molar ratio 4:1, moisture content 7%, reaction temperature 40 °C, reaction time 8 h).

immobilization on the tested hydrophobic matrices was carried out by physical adsorption, the reason of the high thermal stability is probable that immobilization provides a more rigid external backbone for lipase molecules. Therefore, the effect of higher temperatures in breaking the interactions that are responsible for the proper globular, catalytically active structure becomes less notorious resulting in enhancing the thermal stability. This observation is in good agreement with those reported in literatures [24,25]. High temperature of the resistance of the derivative BCL is an important advantage for potential practical applications.

The derivative BCL was immersed into 50% concentration of organic solvents, including methanol, ethanol, propanol, isopropanol, *n*-butanol, isobutanol, acetone, for 4 h to investigate its organic solvent tolerance. The results (Fig. 6) show that the derivative BCL displays a strong organic solvent tolerance to 50% concentration of methanol, ethanol and acetone, and the relative lipase activity is from 80% to 120% comparing with its original activity. In contrast, the derivative BCL shows weak organic solvent in 50% concentration of propanol, isopropanol, butanol and isobutanol after immersing for 4 h. The reason may be ascribed to the fact that the concentration of methanol, ethanol and acetone surrounding the enzyme is reduced, resulting in lower deactivation of the enzyme when enzyme is immobilized on resin NKA matrix. However, solvent molecule is easy to go through the matrix when the carbon chain of alcohols increases. Thus, the concentration of alcohols surrounding the enzyme increases, which leads to decrease of the enzyme activity. So the relative lipase activity of the derivative BCL is lower in propanol, isopropanol, butanol and isobutanol solution. From Fig. 6, it can be revealed that the derivative BCL shows higher activities in alcohols with side-chain molecular structures compared with the linear ones, e.g. lipase shows higher relative lipase activity in isopropanol and isobutanol than in propanol and butanol. This may be attributed to the stereo-selectivity of lipases. These results agree well with those in our previous work [14].

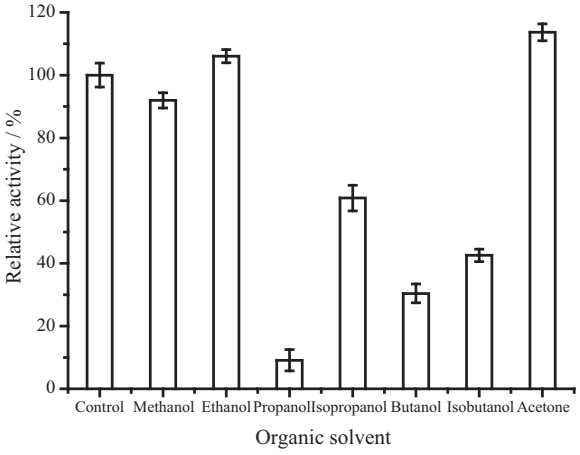

Fig. 6. Organic solvents tolerance of the derivative BCL.

The reusability of the derivative BCL is vital for cost-effective usage in the large-scale applications. As it can be seen in Fig. 7, over 90% methyl esters yield catalyzed by the derivative BCL is obtained after successively repeated use for 50 cycles (400 h). It is suggested that the derivative BCL can be continuously used at least 50 cycles without obvious loss of activity. Therefore, the transesterification reaction catalyzed by the derivative BCL is applicable not only to the batch reaction, but also to the continuous reaction and different reactor configurations.

3.5. Conformation studies

It is logical to consider conformational variation of the derivative BCL as a possible cause for the catalytic performance change. To verify this hypothesis, far UV-CD spectroscopy was conducted to analyze the secondary structure variation of the derivative BCL. From the CD data presented in Table 2, it is observed that the secondary structure of derivative BCL is altered from their native forms when modified with bioimprinting and interfacial activation.

Specifically, in all cases, a substantial decrease in α-helix (up to 8%) and an increase in β-sheet content (up to 4%) are observed. A decrease in α-helix content of the derivative BCL was observed, which probably makes the active site of BCL "open" resulting in enhancing activity. This observation is in accordance with the catalytic activity described in Sections 3.2 and 3.3. An increase in β-sheet content can be attributed to a loss of hydrogen-bonding interactions between the water molecules and the surface of the protein, resulting in a more rigid structure of the derivative BCL, which leads to high thermostability, organic solvent tolerance and

Table 2
Quantitative estimation (%) of the secondary structure elements of the derivative BCL by circular dichroism.

Modified methods	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random coli (%)
Free BCL	30.6	20.34	19.05	29.91
Imprinted-BCL	23.38	24.54	22.99	29.1
Activation-BCL	22.77	23.92	23.15	30.16

Note: BCL—*Burkholderia cepacia* lipase.

operational stability [14]. This observation coincides well with the high reusability observed in Section 3.4.

4. Conclusions

Combined strategies of immobilization, bioimprinting and interfacial activation were employed to explore a high catalytic performance of BCL. It is indicated that the integrated strategy proposed in this work would be a promising modified approach for modification and enhancement of performance of lipases. Compared to the free BCL, the derivative BCL exhibited the satisfactory catalytic performances in activation energy, thermal stability, organic solvent tolerance and operational stability. CD spectroscopy studies show that secondary structure change of the derivative BCL is responsible for the improvement of catalytic performance. It indicates that there should be a close correlation between secondary structure and catalytic performance of BCL after modification, which is significant for its further application.

Acknowledgements

The authors are deeply indebted to the Analytical and Testing Center of Huazhong University of Science and Technology for CD spectra analysis. The financial support is from National Natural Science Foundation of China (NSFC) (31070709), the National High Technology Research and Development Program of China (863 Program) (2009AA03Z232, 2010AA101501), Program for New Century Excellent Talents in University (NCET-07-0336), Doctor Education Fund for New Teachers (20090142120090), Hubei Province Natural Science Fund (2008CDB359, 2009CDA046) and Key Laboratory for Green Chemical Process of Ministry of Education (GCP201001).

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