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Highly enantioselective hydrolysis of DL-menthyl acetate to L-menthol by whole-cell lipase from *Burkholderia cepacia* ATCC 25416

Lijuan Yu, Yan Xu*, Xiaoqing Wang, Xiaowei Yu

Laboratory of Brewing Microbiology and Applied Enzymology, School of Biotechnology, Key Laboratory of Industrial Biotechnology of Ministry of Education, Southern Yangtze University, 1800 Lihu Road, Wuxi 214122, Jiangsu, PR China

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Abstract

Enzymatic hydrolysis of DL-menthyl acetate to L-menthol by whole-cell lipase had been carried out in this work. *Burkholderia cepacia* ATCC 25416 was selected as the efficient experiment strain. Effects of various reaction parameters such as pH, temperature, substrate concentration and co-solvents on the conversion as well as enantioselectivity were studied. A high enantiomeric ratio ($E = 170$) was achieved when 15% (v/v) dimethyl sulfoxide (DMSO) was added, the enantioselectivity was about 3.0-fold higher than that without co-solvent. L-Menthol was obtained with 96% optical purity and 50% conversion under the optimum conditions. *Burkholderia cepacia* whole-cell lipase also exhibited high reaction capacity and good operational stability. The tolerable substrate concentration was 0.23 mol/L; the half-life of activity at 30 °C was about 400 h.

Keywords: *Burkholderia cepacia*; Menthol; Menthyl acetate; Enantioselective hydrolysis; Whole-cell lipase

1. Introduction

L-Menthol is widely used in medicine as constituents of different medical products, in food industry as flavouring additives, in cosmetics as fragrances and in material industry as chiral intermediate [1–3]. The yield and quality of natural menthol are affected seriously by weather and region, etc., synthetic menthol can overcome all these drawbacks. Many techniques of menthol preparation have been successfully utilized in industry [4–8]. The products of racemic mixtures are markedly inferior in taste and odor to the naturally occurring L-menthol [9]. Therefore, there is a great interest in separation of DL-menthol.

The use of biocatalysts for the industrial synthesis of chemicals has been attracting much attention as an environment-friendly synthetic method [10]. Enzymes as biocatalysts have a promising future in chiral resolution for their high stereospecificity and regioselectivity under appropriate conditions. Enantioselective esterification or trans-esterification methods and enantiospecific hydrolysis approaches have been reported for the kinetic resolution of racemic menthol with commercial enzymes [11–14]. However, the commercial enzymes have significant limitations that restrict their industrial applicability including high catalyst cost, low enantiospecificity and limited catalytic efficiency. So it is important to find a new enzyme with a good activity and a high enantioselectivity for L-menthol to satisfy the demands of large-scale production.

Although enzyme has great potential in catalyzing a variety of bio/chemical reactions including lipid hydrolysis, ester synthesis and optical resolution, the applications of isolated enzymes are usually limited for their availability (screening, overexpression and purification), substrate scope and operational stability [15]. These issues can be overcome by using whole cells. Compared with isolated enzymes, whole-cell biocatalysts can be much more readily and inexpensively prepared on an industrial scale. Owing to their diversity and ease of handling, microbial cells have been utilized most commonly for biocatalysis [16].

Herein, we attempted to isolate a stereospecific lipase-production strain with high enantioselectivity and hydrolysis activity for the production of optically active L-menthol. Various reaction parameters affecting the enantioselective hydrolysis of DL-menthyl acetate were discussed, including pH, temperature, substrate concentration and co-solvents; and the stability of whole cells in batch hydrolysis reaction was also observed.

* Corresponding author. Tel.: +86 510 85918197; fax: +86 510 85864112.
E-mail address: yxu@sytu.edu.cn (Y. Xu).

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2. Experimental parts

2.1. Materials

L-Menthol, DL-menthol, DL-menthyl acetate and 1-decanol were purchased from Fluka (Switzerland) and Sigma–Aldrich (Germany) at 99% purity or higher. The other chemicals used in this work were of analytical grade from local sources. Novozym 435 (immobilized lipase from *Candida antarctica*) were donated by Novo Nordisk A/S (Bagsvaerd, Denmark); *Penicillium* lipase and PPL (Porcine pancreas lipase) were purchased from Sigma–Aldrich (Germany); Lipase PS (lipase from *Burkholderia cepacia*), Lipase AK (lipase from *Pseudomonas fluorescens*), Lipase AY (lipase from *Candida rugosa*), Lipase G (lipase from *Penicillium camembertii*) and Lipase PS-C (immobilized lipase from *B. cepacia*) were gifts from Amano Enzyme Inc. (Nagoya, Japan); RCL (whole-cell lipase from *Rhizopus chinesis*) and BCL (whole-cell lipase from *B. cepacia* ATCC 25416) were prepared in our laboratory (shown in Section 2.2).

2.2. Cultivation and preparation of whole-cell lipase

Microorganisms screened were obtained from Spanish Type Culture Collection (CECT), China General Microbiological Culture Collection Center (CGMCC), American Type Culture Collection (ATCC) or isolated from soil. Yeasts were grown in a medium containing 2.0% (w/v) glucose, 1.0% (w/v) yeast extract, 0.5% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$, 0.001% $FeSO_4{\cdot}7H_2O$ and 0.5% NaCl (pH 7); filamentous moulds were grown in a medium containing 2% (w/v) glucose, 0.3% (w/v) yeast extract, 0.5% (w/v) peptone, 0.5% K_2HPO_4 and 0.02% $MgSO_4{\cdot}7H_2O$ (pH 5); bacteria were grown in a medium containing 1.0% (w/v) glucose, 1.0% (w/v) beef extract, 1.0% (w/v) peptone, 0.5% K_2HPO_4, 0.02% $MgSO_4{\cdot}7H_2O$ and 0.5% NaCl (pH 7). All microorganisms were grown in 250 mL flasks with 50 mL medium at 30 °C for 48 h.

After incubation, the microorganism cells were collected by centrifugation at 10,000 × *g* for 15 min and washed twice with physiological saline, then dried with Labconco freeze dry system (USA).

2.3. Screening of microorganisms

In the first screening, the hydrolysis ability and enantioselectivity were estimated by using L-menthyl acetate hydrolysis agar medium and D-menthyl acetate hydrolysis agar medium as the contrast screening plate medium. The transparent zone diameters of L-menthyl acetate hydrolysis agar plate medium produced by some strains were bigger than those of D-menthyl acetate hydrolysis agar plate medium. These strains were collected and used for the secondary screening. Hydrolysis reactions of DL-menthyl acetate were performed in 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0) with 0.05 mol/L DL-menthyl acetate and 15 g/L enzyme at 30 °C for 20 h, the conversion and enantiopurity were determined by gas chromatography as shown in Section 2.4.

2.4. Analysis methods

Samples were extracted with ethyl acetate, enantiomeric purity and conversion were determined by CP3900 gas chromatography (Varian Inc., USA) equipped with a flame-ionization detector. A CP-Chirasil-Dex CB column (25 m length, 0.25 mm i.d.) was used to analyze DL-enantiomers of menthol and their respective esters. The injector and detector were set at 270 °C, respectively. The carrier gas was H_2. The initial column temperature of 100 °C was held for 8 min, then raised to 140 °C at a rate of 4 °C/min and finally held at 140 °C for 2 min. Enantiomeric excess of remaining substrate (ee_s), enantiomeric excess of product (ee_p), enantiomeric ratio (*E*) and conversion based on the GC analyses were calculated as described by Chen et al. [17].

2.5. Enantioselective hydrolysis ability of various lipases

For the production of enantiomerically pure L-menthol, 10 lipases from different sources as described in Section 2.1 were examined. The hydrolysis reactions were performed in 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0) with 0.05 mol/L DL-menthyl acetate and 15 g/L enzyme at 30 °C for 20 h. The conversion and enantiopurity were determined as shown in Section 2.4.

2.6. Enantioselective hydrolysis of DL-menthyl acetate by *B. cepacia* whole-cell lipase

DL-Menthyl acetate (20 mg, 0.101 mmol) and enzyme (30 mg) were homogenized in 2 mL NaH_2PO_4/K_2HPO_4 buffer, and then this reaction mixture was shaken at 200 rpm at a pH range of 4.9–9.2 and a temperature range of 25–50 °C.

The substrate tolerance of whole-cell lipase was investigated with the substrate concentrations ranging from 0.02 to 0.46 mol/L at pH 7.0 and 30 °C.

2.7. Determination of initial reaction rate

Initial reaction rate (mmol/(L min)) was determined using DL-menthyl acetate as substrate at pH 7.0. Reaction was initiated by adding an appropriate amount of DL-menthyl acetate into 2 mL pre-incubated NaH_2PO_4/K_2HPO_4 buffer containing enzyme. The reaction mixture was shaken at 200 rpm in a thermostatted bath at 30 °C for 15 min. L-Menthol was analyzed by GC, and 1-decanol was used as internal standard. The hydrolysis activity of whole-cell lipase was defined using the initial reaction rate. In this study, the amount of the *B. cepacia* ATCC 25416 whole-cell lipase was 30 mg unless otherwise specified.

2.8. Operational stability of whole-cell lipase in batch hydrolysis reaction

DL-Menthyl acetate (20 mg, 0.101 mmol) was added in 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), after addition of 15% (v/v) dimethyl sulfoxide (DMSO) and 15 g/L *B. cepacia* ATCC 25416 whole-cell lipase, the reaction mixture was

homogenized on vortex mixer (USA) for 1 min and shaken at 200 rpm in a thermostatted bath at 30 °C for 20 h. Then the cells were collected by centrifugation at 10,000 × *g* for 15 min and used to catalyze the next batch reaction after washed twice with fresh NaH_2PO_4/K_2HPO_4 buffer. The next batch reaction was initiated by adding 20 mg DL-menthyl acetate in 2 mL fresh NaH_2PO_4/K_2HPO_4 buffer with 15% (v/v) DMSO. After each cycle, a further measurement was taken as described in Section 2.4.

3. Results and discussion

3.1. Screening of microorganisms

The strains capable of hydrolyzing DL-menthyl acetate to L-menthol were screened through combined screening strategy of hydrolysis ability with stereoselectivity. *B. cepacia* ATCC 25416 was the most efficient strain as giving high conversion (>40%) and excellent enantiopurity (>95%). The strains with high catalytic activities and stereospecificity for L-menthol were listed in Table 1. Moreover, the hydrolysis activity and esterification activity of *B. cepacia* ATCC 25416 whole cells were compared. There was hardly any esterification activity for this whole-cell lipase. Wu et al. [18] reported that the esterification activity and hydrolysis activity of the same lipase are not related in many cases, and in extreme cases, a lipase may exhibit no esterification activity while possessing a high hydrolysis activity.

3.2. Enantioselective hydrolysis ability of various lipases

Among the lipases investigated, Lipase PS, Lipase AK, Lipase AY and *B. cepacia* ATCC 25416 whole-cell lipase (BCL) showed higher stereospecificity for L-menthyl acetate in which only Lipase AY and BCL gave higher hydrolysis activity (Table 2). The enantioselectivity of Lipase AY was lower than BCL although Lipase AY gave higher hydrolysis activity. Vorlova et al. [13] ever reported the enantioselectivity of Lipase AY was very low for hydrolysis of DL-menthyl benzoate, which was in accordance with our results. In general, the substrate specificity of lipases varied considerably, and mainly depended on the sources of lipases. BCL gave the maximum ee_p of 96.5% and was the best for splitting DL-menthyl acetate.

Table 1
The strains exhibiting high catalytic activities and enantioselectivity in hydrolysis of DL-menthyl acetate to L-menthol

Microorganism	Enantiomeric excess of L-menthol (%)	Total conversion (%)
Bacteria L12 from soil	95	32
Bacteria L59 from soil	97	33
Burkholderia cepacia ATCC 25416	97	43
Pseudomonas fluorescens CGMCC 1.823	96	20
Candida parapsilosis CECT 1627	89	20
Candida parapsilosis CGMCC 2.491	87	24
Candida boidinii CECT1449	81	18
Candida boidinii CECT10139	87	22
Candida boidinii CECT10437	84	13

Reactions were carried out in 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0) with 0.05 mol/L DL-menthyl acetate and 15 g/L microorganisms dry cells at 30 °C for 20 h. Data are means of at least three determinations.

Table 2
Hydrolysis of DL-menthyl acetate to L-menthol by lipases from various sources

Lipase source[a]	Enantiomeric excess of L-menthol (%)	Total conversion (%)
BCL (whole cell)	96.5	46.4
Novozym 435 (immobilized)	23.3	4.1
PPL	32.1	2.3
RCL (whole cell)	11.0	4.0
Penicillium lipase	0.5	13.0
Lipase PS	90.1	8.2
Lipase PS-C (immobilized)	6.6	95.3
Lipase AK	96.0	5.9
Lipase AY	82.2	41.5
Lipase G	0.5	15.8

Reactions were carried out in 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0) with 0.05 mol/L DL-menthyl acetate and 15 g/L enzyme at 30 °C for 20 h. Data are means of at least three determinations.

[a] BCL, *B. cepacia* ATCC 25416; Novozym 435, *C. antarctica*; PPL, porcine pancreas; RCL, *R. chinesis*; Lipase PS, *B. cepacia*; Lipase PS-C, *B. cepacia*; Lipase AK, *P. fluorescens*; Lipase AY, *C. rugosa*; Lipase G, *P. camembertii*.

3.3. Effects of pH on hydrolysis reaction catalyzed by B. cepacia whole-cell lipase

It is well known that the enantioselectivity and activity of enzyme are highly influenced by pH [19]. In order to determine the optimum pH, the bio-catalytic reaction was performed under different initial pH values at 30 °C. Spontaneous non-enantioselective hydrolysis of DL-menthyl acetate was observed in low levels under harsh pH conditions (pH > 8.0 or <6.5) which resulted in a remarkable decrease of ee_p (Fig. 1). The chemical hydrolysis reaction was enhanced at extremely harsh pH while the total conversion was decreased which presumably due to the activity drop of the key enzyme in whole cells when pH exceeded its optimum range. A high ee_p (>95%) and a good conversion (>40%) were achieved at a pH range of 6.5–8.0. No

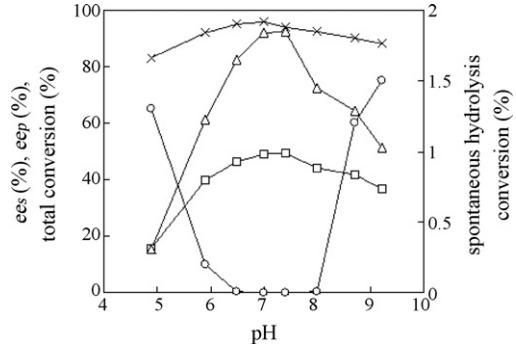

Fig. 1. Effects of pH on total conversion of DL-menthyl acetate (□), ee_p (×), ee_s (△) and spontaneous hydrolysis conversion (◯). Reactions conditions: 0.101 mmol DL-menthyl acetate, 30 mg *B. cepacia* ATCC 25416 whole-cell lipase, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L), 30 °C, 20 h. Data are means of at least three determinations.

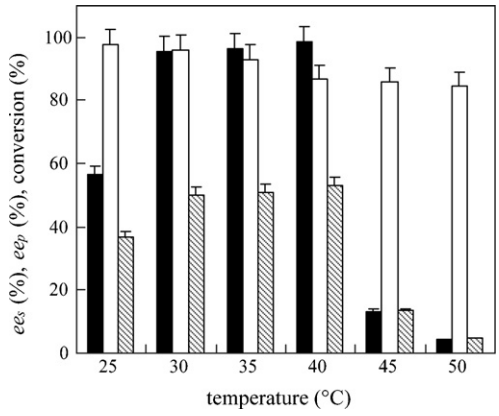

Fig. 2. Effects of temperature on DL-menthyl acetate conversion (▨), ee_p (□) and ee_s (■). Reactions conditions: 0.101 mmol DL-menthyl acetate, 30 mg *B. cepacia* ATCC 25416 whole-cell lipase, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), 20 h. Data are means of at least three determinations.

chemical hydrolysis reaction was detected at pH 7.0, so pH 7.0 was chosen as the favorable pH.

3.4. Effects of temperature on hydrolysis reaction catalyzed by B. cepacia whole-cell lipase

Since the enantioselective hydrolysis of DL-menthyl acetate is a kinetic resolution process, reaction temperature has a serious effect on conversion and enantiomeric excess. The ee_s, ee_p and conversion at different temperature were shown in Fig. 2. There was a 1.4-fold rise of conversion with the increase of temperature from 25 to 40 °C. However, further increase of temperature to 45 °C showed 75% drop of conversion compared with 40 °C. The same effect was found on ee_s which attributed to the deactivation of the enzyme at 45 °C. The ee_p decreased slightly with the rise of temperature indicating that there was a certain temperature range for lipase at which the conformation of the enzyme was optimal. Therefore, 30 °C was established to be the optimum temperature for this reaction system.

3.5. Effects of substrate concentration on hydrolysis reaction catalyzed by B. cepacia whole-cell lipase

Substrate tolerance of enzyme is a crucial factor for its potential industrial applications. A high reaction capacity of enzyme can increase its application efficiency. The reaction capacity of *B. cepacia* whole-cell lipase was examined (Fig. 3). The conversion and ee_s continuously decreased with the rise of substrate concentration because of the increasing rate of substrate exceeding its consuming rate within the test time. The initial reaction rate almost increased linearly till reached the highest point at the substrate concentration of 0.28 mol/L. The ee_p remained higher (>90%) before 0.23 mol/L substrate concentration reached, and the decrease of conversion could be compensated by prolonging the reaction time. As a result, the tolerable substrate concentration was 0.23 mol/L which indicated that the *B. cepacia* whole-cell lipase had a potential to catalyze the hydrolysis of substrate at a higher concentration.

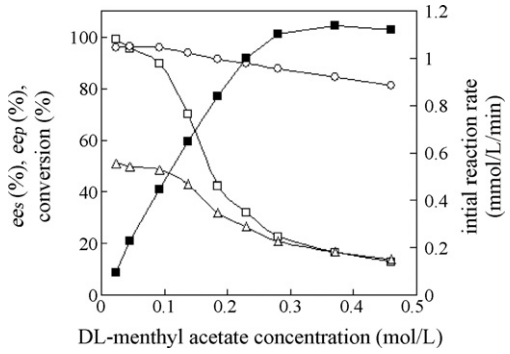

Fig. 3. Effects of substrate concentration on DL-menthyl acetate conversion (△), ee_p (◯), ee_s (□) and initial reaction rate (■). Reactions conditions: 30 mg *B. cepacia* ATCC 25416 whole-cell lipase, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), 30 °C, 20 h. Data are means of at least three determinations.

3.6. Effects of organic co-solvent on hydrolysis reaction catalyzed by B. cepacia whole-cell lipase

In whole-cell biocatalysis, process productivity has been frequently limited because substrates or products of interest are sparingly soluble in water [20]. Aqueous system contained co-solvents provides an alternative methodology for performing efficient bioconversion because they increase the solubility of hydrophobic substrates or products. It has been reported that adding some co-solvents to aqueous buffer can enhance the activity and enantioselectivity of lipase-catalyzed hydrolysis of racemic esters [21–23].

In this work, 12 water-soluble organic solvents with low molecular weight were chosen as the co-solvents in the system, and their effects on enzymatic hydrolysis of DL-menthyl acetate were investigated using *B. cepacia* ATCC 25416 whole-cell lipase (Fig. 4). Compared to the control, the enantioselectivity of the enzyme was improved by several solvents such as *N*,*N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), glycerol and acetone. However, the selectivity was not closely correlated to the solvent hydrophobicity. Among the 12 co-solvents chosen, most co-solvents could improve the activity of enzyme. The relative activities decreased with the increase

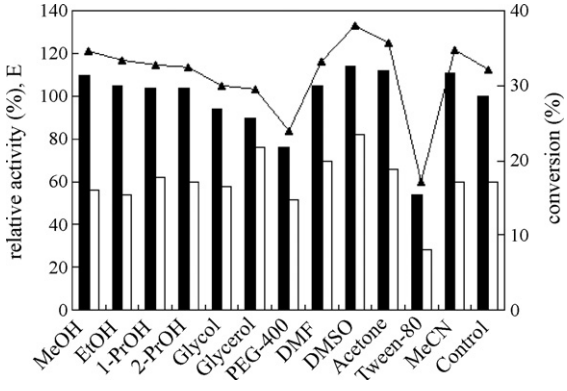

Fig. 4. Effects of organic co-solvents on enantiomeric ratio (*E*, □), relative activity (■) and conversion (▲). Reactions conditions: 0.101 mmol DL-menthyl acetate, 30 mg *B. cepacia* ATCC 25416 whole-cell lipase, 0.02 mL co-solvent, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), 30 °C, 12 h. Data are means of at least three determinations.

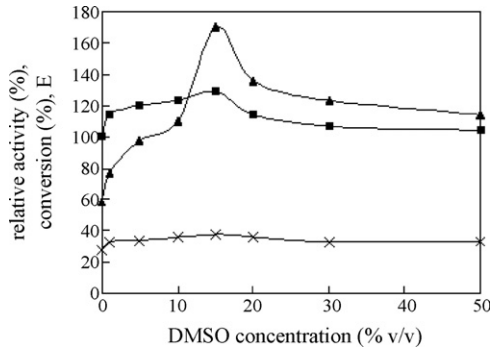

Fig. 5. Effects of dimethyl sulfoxide (DMSO) concentration on enantiomeric ratio (E, ▲), relative activity (■) and conversion (×). Reactions conditions: 0.101 mmol DL-menthyl acetate, 30 mg *B. cepacia* ATCC 25416 whole-cell lipase, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), 30 °C, 12 h. Data are means of at least three determinations.

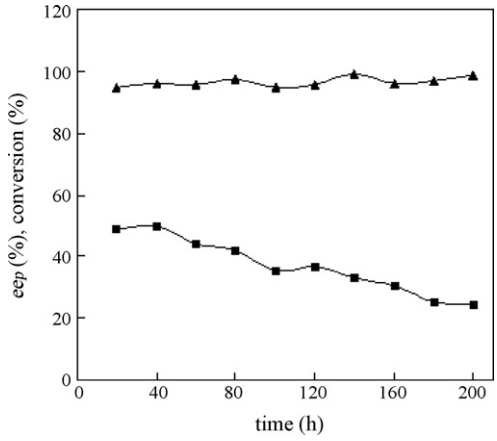

Fig. 6. Changes of DL-menthyl acetate conversion (■) and ee_p (▲) in recycled use of *B. cepacia* ATCC 25416 whole-cell lipase. Reactions conditions: 0.101 mmol DL-menthyl acetate, 30 mg enzyme, 0.3 mL DMSO, 2 mL NaH_2PO_4/K_2HPO_4 buffer (67 mmol/L, pH 7.0), 30 °C, 20 h. Data are means of at least three determinations.

of carbon number of solvents, such as 1-alkanols (methanol, ethanol, and propanol) and polyalcohols (glycol and glycerol). This phenomenon could not be simply explained as the increase of substrate solubility with the help of co-solvents, because not all co-solvents could have the positive effects on the activity. Although methanol is known to have some detrimental effect on lipase, it was found to be an efficient co-solvent for the activity of *B. cepacia* ATCC 25416 whole-cell lipase, which was also reported by other researchers [23]. To explain the results mentioned above, two factors should be considered, the log P values and the dielectric constant of co-solvent mixture. The alcoholysis between primary alkanols and DL-menthyl acetate was not detected after these alkanols were added. DMSO was the favorable co-solvent, similar results were reported with respect to some commercial lipases [24–26].

The concentration of co-solvents also has profound influence on the activity and enantioselectivity. Usually, the enzyme activity increases with the addition of a co-solvent at low concentrations, while co-solvents may also show inhibitory effects at high concentrations [23]. The effects of DMSO concentration on the relative activity, enantiomeric ratio and conversion at 12 h were investigated (Fig. 5). The relative activity of whole-cell lipase rose with the increase of DMSO concentration, up to a maximum of 130% at 15% (v/v) DMSO, then decreased when more (>15%, v/v) DMSO was added. However, even at the DMSO concentration of 50% (v/v), the relative activity was still slightly higher than that of control. Similar effect was observed on the final conversion which increased from 27.9% to 37.7% when DMSO concentration ranging from 0 to 15% (v/v), and did not show any decrease at DMSO concentration of 50% (v/v). The enantiomeric ratio also increased notably with the rise of DMSO concentration, and gave the maximum ($E = 170$) at the DMSO concentration of 15% (v/v), about 3.0-fold higher than that without co-solvent. After addition of DMSO, the E value was always higher than control despite a little decrease when DMSO concentration more than 15% (v/v). The results clearly indicated that 15% (v/v) DMSO showed the best influence on the activity and enantiospecificity of whole-cell lipase. The mechanism responsible for the significant improvement of activity and enantioselectivity by addition of water-miscible organic co-solvents had caused the interest of many research groups. Wehbi et al. [27] proved that the co-solvent can activate the phospholipase by causing some changes in active site polarity along with small conformational changes. Watanabe and Ueji [24] reported that DMSO can cause some conformational change and/or an increase of the flexibility of the lipase.

3.7. Operational stability of whole-cell lipase in batch hydrolysis reaction

The stability of a biocatalyst is an important factor for its potential application in industry. To test the stability of the *B. cepacia* whole-cell lipase in repeated use, batch hydrolysis of DL-menthyl acetate to L-menthol was carried out under the optimum conditions described in Section 2.8. The changes of ee_p and conversion during recycling were shown in Fig. 6. The conversion decreased gradually during recycling due to the mass loss of the enzyme and the activity decrease in the recycling operation. The residual amount of enzyme determined was about 68% of the original after 10 cycles. This issue could be overcome by compensating fresh enzyme after each cycle. The conversion was about 52% after 10 cycles compared to the original, which meant that about 77% of initial enzyme activity retained except the mass loss of the enzyme. The half-life of the whole cells at 30 °C was about 400 h, which was much longer than that reported for *Rhodotorula minuta* var. *texensis* free Cells (50 h at 30 °C) [28]. In addition, the ee_p was not influenced after ten cycles, and retained above 95%, indicating that the conformation of the key enzyme was quite stable under the reaction conditions. As a whole, the whole-cell lipase from *B. cepacia* ATCC 25416 as a biocatalyst had good operational stability and durability.

4. Conclusions

An irreversible resolution of DL-menthol by hydrolysis of the corresponding acetate in phosphate buffer was performed in this work, the whole-cell lipase from *B. cepacia* ATCC 25416

had better hydrolytic activity and enantioselectivity compared to other lipases. The activity and stereospecificity of whole-cell lipase were obviously affected by pH and temperature. Addition of co-solvents to the reaction mixture significantly improved the enantioselectivity and the activity of the whole-cell lipase in most cases. Among the 12 co-solvents chosen, maximum activity and enantioselectivity were achieved by addition of 15% (v/v) DMSO. The tolerable substrate concentration of *B. cepacia* whole-cell lipase was 0.23 mol/L. Furthermore, after ten cycles of reactions, the residual enzyme activity was still about 77%, and the ee_p in all cases was higher than 95%. In conclusion, the whole-cell lipase from *B. cepacia* ATCC 25416 was a useful and promising whole-cell biocatalyst for preparing L-menthol.

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