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An efficient bioprocess for enzymatic production of L-menthol with high ratio of substrate to catalyst using whole cells of recombinant *E. coli*

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ABSTRACT

A gene encoding an esterase of *Bacillus subtilis* ECU0554 previously isolated from soil was cloned and overexpressed in *Escherichia coli* BL21. The recombinant esterase (recBsE) showed the best enantioselectivity ($E > 100$) towards DL-menthyl acetate, in contrast to DL-menthyl esters propionate and butyrate. A high ratio of substrate to catalyst (S/C-ratio, ≥50) was achieved in the kinetic resolution of DL-menthyl acetate by using whole cells of recombinant *E. coli* BL21. Some key parameters of the biocatalytic process, including amount of cosolvent, catalyst loading and substrate loading, were optimized. Compared with the process catalyzed by wild-type whole cells of *B. subtilis* ECU0554, the second-generation bioprocess using whole cells of recombinant *E. coli* BL21 afforded a 40-fold improvement in S/C-ratio and a 75-fold improvement in the volumetric productivity per biocatalyst loading. Moreover, the substrate loading was increased up to 200 g L^{-1} (~1 M), the biocatalyst loading was reduced to 2.5 g L^{-1} and the space-time yield was improved from 54 g L^{-1} d^{-1} to 202 g L^{-1} d^{-1}.

1. Introduction

L-Menthol is one of the most important flavoring chemicals and used extensively in oral products, pharmaceutical, tobacco products, confectionaries, shaving products. Due to the rapidly increasing demand for L-menthol among the global market in recent years, the production of L-menthol also increased. The average annual increase rate of L-menthol production reached 6.1% between 1998 and 2007 and the world total annual production was in excess of 19,000 tons by 2007 (Clark, 1998, 2007). The menthol from natural sources has no longer satisfied the market demand. So considerable efforts have been devoted to the production of L-menthol by synthetic or semi-synthetic means from other more readily available raw materials. For example, Takasago has successfully developed an elegant synthesis of L-menthol from myrcene with catalytic asymmetric isomerization technology as the key step. The key step was the asymmetric isomerization of geranyldiethylamine, catalyzed by an (*S*)-BINAP-Rh complex in THF and forming (*R*)-citronellal enamine. L-Menthol has been successfully industrialized by means of asymmetric synthesis and 1000 tons per year of L-menthol were synthesized by Takasago (Akutagawa, 1997; Noyori, 2003; Trasarti et al., 2004). Another example of industrial synthesis of L-menthol was developed by Haarmann and Reimer. This route utilized the unexpected ability of the racemic menthyl benzoates to be separated by selective crystallization induced by seeding the mixture with one of the optically active menthyl benzoate enantiomers. However, these processes used heavy metal catalyst and extensive solvent, which was hazardous to human health and the environment and violated the principles of green chemistry.

The new alternative biocatalytic processes for production of chiral compounds have increased dramatically in recent years (Carey et al., 2006). This is mainly because biocatalysis has the advantages of environmental friendliness, mild reaction conditions, high catalytic efficiency and often high stereo-, regio- or enantioselectivity (Reetz, 2002). Biocatalysis is becoming more and more promising as an alternative to conventional catalysis and will be more commonly used in the future as a powerful and well recognized tool to complement some significant challenging catalytic processes (Sheldon, 2007; Wong, 2007). Due to above advantages, enantioselective esterification or transesterification methods (Wu et al., 1996; Xu et al., 1995) and enantiospecific hydrolysis approaches (Omata et al., 1981; Vorlova et al., 2002; Yu et al., 2007) have been reported for the kinetic resolution of racemic menthol with commercial lipases. However, these approaches have significant limitations that restrict their practical application including high catalyst loading, low substrate loading, low catalytic efficiency and low S/C-ratio. In general, an S/C-ratio of 20 or higher was beneficial in a typical industrial process (Luetz et al., 2008).

We had reported an enzyme from *Bacillus subtilis* ECU0554 isolated from soil samples, which could efficiently hydrolyze DL-menthyl acetate to product L-menthol at high substrate concen-

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tration (Zheng et al., 2009). However, because of the low esterase activity of *B. subtilis* whole cells, the first-generation bioprocess needed a high catalyst loading (50 g L^{-1}). So high catalyst loading caused serious emulsion in the downstream processing of product, and further resulted in a high manufacturing cost of L-menthol which restricts its practical application.

Herein, we cloned the *B. subtilis* ECU0554 enzyme gene and expressed it in *Escherchia coli* BL21. The gene has been deposited in the GenBank database with accession number BSU34390.

2. Materials and methods

2.1. Materials

All kinds of *p*-nitrophenyl esters were prepared from *p*-nitrophenol and various acids in our laboratory (Zhao et al., 2008). Racemic menthol, D-menthyl acetate and L-menthyl acetate were purchased from Alfa Aesar (Tianjin, China). All other regents or chemicals used were of analytical grade and obtained from local suppliers.

Tryptone and yeast extract were obtained from Oxoid (Shanghai, China). All restriction endonucleases and T4 DNA ligase were provided by TaKaRa (Dalian, China). *B. subtilis* ECU0554 strain, isolated by our laboratory and deposited in China General Microbiological Culture Collection Center (Beijing, China) with an accession number of CGMCC 2548, was used for genomic DNA preparation. *E. coli* strain DH5α from Tiangen (Shanghai, China) and BL21 (DE3) from Invitrogen Life Technologies (Shanghai, China) were used as the host strains for gene cloning and expression, respectively. Both strains were routinely grown in Luria–Bertani medium at 37 °C unless stated otherwise. Kanamycin (50 μg mL^{-1}) was used for the selection of recombinant strains of *E. coli*. The pET24a expression vector was purchased from Novagen (Shanghai, China). *Taq* DNA polymerase was obtained from TaKaRa (Dalian, China).

2.2. Amplification of gene by PCR

The primers for the amplification of the esterase gene were designed based on previously described sequence information from *B. subtilis* subsp. *subtilis str*.168. The forward primer 5′-ACGGATCCATGACTCATCAAATAGTAACG-3′ and the reverse primer 5′-CTTGCGGCCGCTTATTCTCCTTTTGAAGG-3′ were used to amplify a 1470 bp fragment containing the esterase gene. To facilitate cloning of the PCR product, *Bam*HI and *Not*I sites were added to the primers (underlined sequences in the primers represent the restriction site for the respective enzyme). The PCR was performed with initial denaturation at 95 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. The PCR products were separated by electrophoresis using 1% (w/v) agarose gel, and then the DNA band corresponding to the expected length was excised and purified using the gel extraction kit from Tiangen (Shanghai, China). The PCR product was digested with *Bam*HI and *Not*I and inserted into *Bam*HI/*Not*I cleaved plasmid vector pET24a, and then transformed into *E. coli* BL21 (DE3) cells, giving the recombinant strains *E. coli* BL21/pBsE.

2.3. Culture of recombinant E. coli

Cells of *E. coli* BL21/pBsE were grown overnight in 2× LB medium containing kanamycin (50 μg mL^{-1}) at 37 °C. The cells were subsequently diluted (1:14, v/v) in fresh medium (2.8 L) and grown in a 5-L fermenter at 30 °C. IPTG (0.5 mM) was added to induce the esterase gene when the optical density at 600 nm ($OD_{600\,nm}$) of bacterial cultures reached about 10. The cells were harvested by centrifugation (13,500 × *g*, 4 °C, 5 min) when the esterase activity did not increase and washed twice with physiological saline. Then the cells were dried using a vacuum freeze drier and stored at 4 °C for further experiments.

2.4. Preparation of recombinant enzyme

The cells harvested were disrupted 3 times using a high-pressure homogenizer (AH110B, ATS Engineering Inc.) and the homogenate was centrifuged (13,500 × *g*, 4 °C, 30 min) to remove the cells debris. Ammonium sulfate was added slowly into the supernatant (soluble protein) with stirring at 0 °C, and the protein that precipitated between 30% and 60% saturation of ammonium sulfate was collected by centrifugation (13,500 × *g*, 4 °C, 30 min) and dialyzed in sodium phosphate buffer (10 mM, pH 7.0). Then the dialysate was dried using a vacuum freeze drier, and the dry crude esterase powder was stored at 4 °C and used for further experiments.

2.5. Activity assays

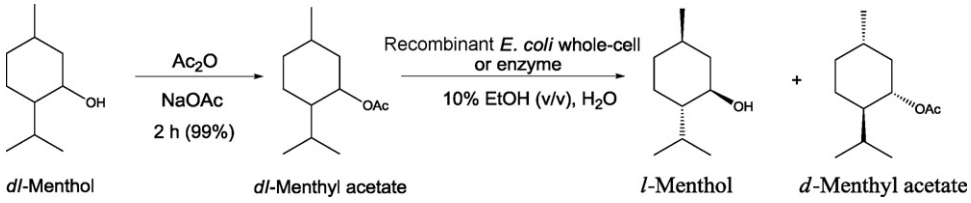
Hydrolytic activities of recombinant enzyme on various *p*-nitrophenyl esters were measured as previously described (Zhao et al., 2008). One unit of activity was defined as the amount of enzyme releasing 1.0 μmol of *p*-nitrophenol per min under the given assay conditions.

2.6. Hydrolysis reaction

Hydrolysis of DL-menthyl esters catalyzed by the recombinant whole cells or enzyme was performed in a 250-mL three-necked, round-bottomed flask with an initial reaction volume of 100 mL containing substrate (100–200 g L^{-1}) in different reactions (Scheme 1). A temperature-controlled water bath was used to control the reaction temperature at 30 °C, and the pH of reaction was adjusted to 8.0 constantly by automatic titration with NaOH (2 M). Samples withdrawn during the reaction were immediately diluted with ethyl acetate, and then centrifuged for 3 min at 12,000 × *g* for GC analysis.

2.7. Scale-up of bioreaction

A 2-L scale reaction was performed in a 3-L stirring-tank reactor at 30 °C under the optimized reaction conditions for the resolution step, containing DL-menthyl acetate (200 g L^{-1}), 10% EtOH of



Scheme 1. Kinetic resolution of DL-menthyl acetate to product L-menthol.

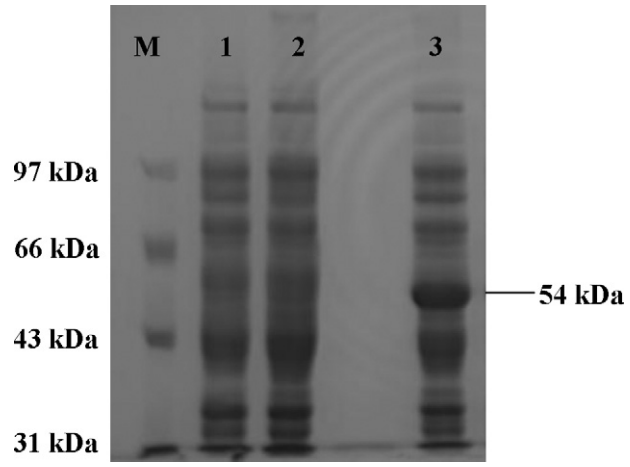


Fig. 1. Expression analysis of recombinant esterase by SDS-PAGE. M represents standard molecular weight markers; line 1, vector control cells of *E. coli* BL21 (pET24a); line 2, uninduced cells of *E. coli* BL21 (pBSE); line 3, cell-free extract of the induced *E. coli* BL21/pBSE cells.

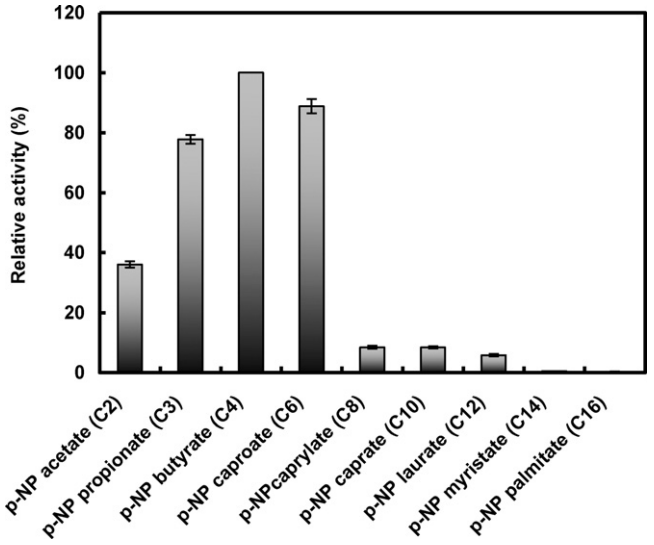


Fig. 2. Relative activity of the esterase from *E. coli* BL21 (pBSE), assayed using *p*-NP esters with different carbon chain-lengths as substrates. The activity towards *p*-NP butyrate was taken as 100%. Results are the means of duplicate experiments.

total initial reaction volume and the lyophilized recombinant *E. coli* whole cell (2.5 g L^{-1}). The pH of reaction was adjusted to 8.0 constantly by automatic titration with NaOH (2 M). The reaction was monitored at regular intervals by GC. The cells were removed from the reaction mixture by centrifugation, and the substrate and product were extracted from the reaction mixture with ethyl acetate. The product was then separated by column chromatography on silica gel using petroleum ether/ethyl acetate (30:1, v/v) as eluent. The solvents were removed through vacuum distillation.

2.8. Gas chromatography analysis

The samples from the hydrolysis reaction were analyzed on a GC-14 gas chromatography (Shimadzu, Tokyo, Japan) equipped with FID detector. The *ee* of remaining substrate was determined with Beta Dex™ 120 Chiral column (30 m × 0.25 mm, 0.25 μm film thickness) from Supelco (Bellefonte, PA, USA) using N_2 as carrier gas. The column, injector and detector were held at 130, 280 and 350 °C, respectively. The *ee* of product was determined with Gamma Dex™ 120 Chiral column (30 m × 0.25 mm, 0.25 μm film thickness) also from Supelco (Bellefonte, PA, USA) using N_2 as carrier gas. The injector and detector were held at 280 and 350 °C, respectively. The oven temperature was programmed from 110 °C, held for 15 min, then raised to 150 °C at a rate of 10 °C min^{-1} and finally held at 150 °C for 1 min.

3. Results and discussion

3.1. Cloning and expression of the esterase from B. subtilis ECU0554

The gene was amplified by polymerase chain reaction (PCR) from the genomic DNA of *B. subtilis* ECU0554 (or CGMCC 2548) by using oligonucleotides derived from the literature (Kunst et al., 1997). Thus, a 1470 bp fragment was obtained that was digested with *Bam*HI/*Not*I and ligated into the expression vector pET24a digested with the same restriction enzymes. The resulting expression plasmid (pBSE) was used to transform *E. coli* BL21 cells and the esterase was induced by adding IPTG. A comparison of SDS-PAGE gels from supernatant of the induced cell-free extract of *E. coli* BL21 (pBSE), uninduced cells of *E. coli* BL21 (pBSE) and the vector of *E. coli* BL21 (pET24a) demonstrated the induction of an additional protein with a molecular mass of about 54 kDa (Fig. 1). Significant esterase activity was found in the induced cells of *E. coli* BL21/pBSE, but no activity was detected in the cells of the blank vector.

3.2. Substrate specificity

Cell extracts from *E. coli* BL21/pBSE were investigated for lipolytic activity using *p*-nitrophenyl esters with chain lengths ranging from C_2 to C_{16} as substrates. As shown in Fig. 2, the enzyme exhibited a marked preference for short-chain fatty acids, yielding the highest activity against *p*-nitrophenyl butyrate (C_4). High hydrolytic activities were also obtained with *p*-nitrophenyl propionate (C_3) and *p*-nitrophenyl caproate (C_6), which exhibited activities of 78% and 89%, respectively. The hydrolytic activity of the protein dropped abruptly towards *p*-nitrophenyl esters with chain lengths of more than 6 carbon atoms, and less than 10% activity could be detected against these long-chain fatty acids. Lipases are, by definition, carboxylesterases that have the ability to hydrolyze long-chain acylglycerols ($\geq C_{10}$), whereas esterases hydrolyze ester substrates with short-chain fatty acids ($\leq C_{10}$) (Verger, 1997). The decrease in activity as the extension of fatty acid chain is a behavior typical of true esterases. These results therefore served to confirm that the enzyme overproduced by *E. coli* BL21/pBSE is indeed an esterase.

3.3. Kinetic resolution of DL-menthyl esters containing short-chain acyl groups

Based on the above results, we knew that the enzyme from *E. coli* BL21 (pBSE) exhibited higher activities towards the esters of short-chain fatty acids. Therefore, three kinds of DL-menthyl esters containing short-chain acyl groups, including DL-menthyl acetate, DL-menthyl propionate and DL-menthyl butyrate, were examined to find the optimal substrate of esterase for production of L-menthol.

Enantiomeric excess of product (ee_p), conversion of substrate (c) and enantiomeric ratio (E) were determined, as listed in Table 1. The reaction time was longer towards DL-menthyl butyrate than DL-menthyl acetate and DL-menthyl propionate at the same conversion (48%), which suggested the recBsE exhibited higher activities towards DL-menthyl acetate and DL-menthyl propionate than DL-menthyl butyrate. This might be attributed to the bulky acyl group which may hinder its movement into the active site of the enzyme.

Table 1
Kinetic resolution of DL-menthyl esters by recBsE.[a]

Substrate	Time (h)	ee_s (%)[b]	ee_p (%)[b]	Conv. (%)[b]	E[c]
DL-Menthyl acetate	4	89	96	48	>100
DL-Menthyl propionate	4	83	89	48	44
DL-Menthyl butyrate	7	80	86	48	32

[a] The recBsE (50 mg) and DL-menthyl acetate (100 g L^{-1}) were added to a 100 mL sodium phosphate buffer (200 mM, pH 8.0) at 30 °C, and pH was kept at 8.0.
[b] Conversion and optical purity were determined by GC.
[c] Enantioselectivity (E) was calculated according to Chen et al. (1982).

What was worse, the enantiomeric excess of product (L-menthol) decreased from 96% to 86% with the increase of acyl group size of esters. Nevertheless, the recombinant esterase of *E. coli* BL21 (pBSE) exhibited the highest hydrolytic activity, the best enantiomeric excess (96%) and the most satisfactory enantioselectivity ($E > 100$) towards DL-menthyl acetate. Therefore, DL-menthyl acetate was selected as the optimal substrate for enzymatic production of L-menthol.

3.4. Biocatalytic hydrolysis of DL-menthyl acetate by whole cells of recombinant E. coli

Compared to isolated enzymes, whole-cell biocatalyst has a number of attributes that are particularly attractive for large-scale applications (Duetz et al., 2001) and has been widely adopted for the commercial synthesis of a variety of compounds, from bulk chemicals to valuable pharmaceuticals (Ishige et al., 2005; Schmid et al., 2001). The use of whole cells eliminates the need for tedious, expensive protein isolation and/or purification (Wichmann and Vasic-Racki, 2005). Additionally, with the protection of cell envelops and walls, enzymes are generally more stable in whole-cell systems.

The stability of the whole-cell biocatalyst and the isolated enzyme was studied under operational temperature (30 °C). These results confirmed that the esterase in whole-cell system was indeed more stable than the isolated enzyme (Fig. 3). The half-life of the esterase in whole-cell system was 11-fold higher than that of the isolated enzyme at 30 °C (Table 2). In this paper, we therefore employed recombinant whole cells of *E. coli* BL21 as biocatalyst for production of L-menthol.

Table 3 shows the comparison between the results of enantioselective hydrolysis of DL-menthyl acetate by whole-cell biocatalysts of recombinant *E. coli* BL21/pBSE and of wild-type *B. subtilis* ECU0554. With 100 g L^{-1} of DL-menthyl acetate, a total of 50 g L^{-1} wild-type dry cells only conversed 38% of substrate in 8 h. Moreover, serious emulsion was formed, which increased significantly the difficulty of product recovery. Although the analytical yield of L-menthol was 38%, the isolated yield, after an hour to allow for some emulsion separation, was only 23%. With the recombinant whole-cell of *E. coli* BL21, as much as 40% of DL-menthyl acetate was converted in 8 h with the same substrate loading, but the biocatalyst loading was reduced to 2.0 g L^{-1}. The S/C-ratio was increased remarkably from 2 to 50 and catalyst yield ($g_{product}/g_{cat}$) was improved by 40-fold. The process catalyzed by recombinant whole-cell with a much higher S/C-ratio had great potential of application in industry, which stimulated us to optimize the biocatalytic process.

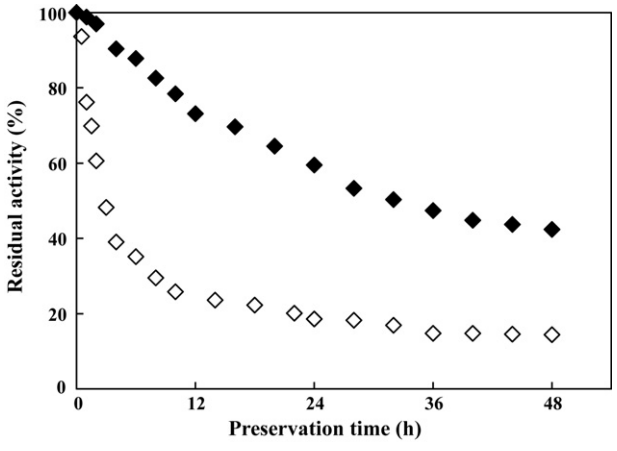


Fig. 3. The stability profiles of the whole cells and the isolated enzyme at 30 °C. Whole-cell of recombinant *E. coli* BL21 (♦), recombinant isolated esterase (◊).

Table 2
First-order inactivation rate constant (k_d) and half life ($t_{1/2}$) of the whole cells and the isolated enzyme at 30 °C.

Biocatalyst form	k_d (h^{-1})	$t_{1/2}$ (h)
Whole cells	0.022	32.4
Isolated enzyme	0.248	2.8

3.5. Optimization of cosolvent concentration

However, compared with the resolution using isolated enzyme, both the enantioselectivity and ee_p decreased (Tables 1 and 3) while using recombinant whole-cell biocatalysts. In many cases, water-miscible organic solvents could improve the enantioselectivity and activity of biocatalyst (Chen et al., 2004; Faber, 2004).

In our previous work, EtOH was used as the cosolvent in the enantioselective hydrolysis of DL-menthyl acetate (Zheng et al., 2009). Therefore, progress curves of the enantioselective hydrolysis of DL-menthyl acetate by recombinant whole-cell were monitored in the presence or absence of 10% (v/v) EtOH (Fig. 4). It was obvious that the L-ester in the EtOH-containing system decreased faster than that in the control system, while the change of D-ester was similar in both systems. In accordance to the non-linear regression of the experimental data, the E-value was more than 100 in EtOH-containing system, which was much higher than that in the absence of EtOH ($E = 31$).

In order to find an optimal cosolvent concentration, the effects of EtOH concentration on both the conversion and the enantioselectivity at 8 h were investigated (Table 4). The *ee* of product rose with the increase of EtOH concentration. The conversion of substrate

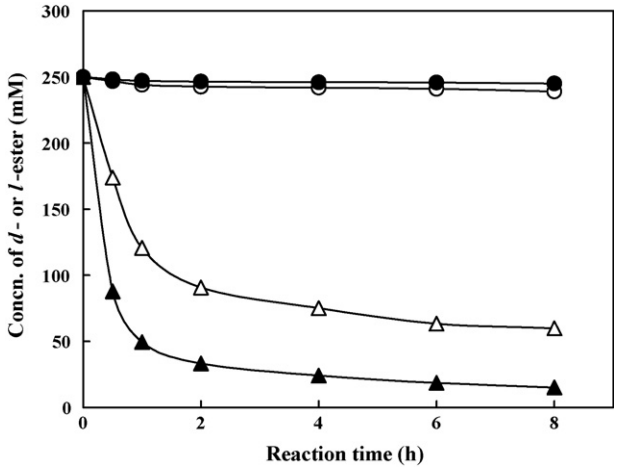


Fig. 4. Biocatalytic hydrolysis of D- or L-menthyl acetate in a medium without or with EtOH (10%, v/v). The dry whole cells of recombinant *E. coli* BL21/pBSE (2.0 g L^{-1}) and DL-menthyl acetate (100 g L^{-1}, 500 mM) were added to a 100 mL sodium phosphate buffer (200 mM, pH 8.0) at 30 °C, and pH was kept at 8.0. (▲) L-ester with EtOH; (△) L-ester without EtOH; (●) D-ester with EtOH; (○) D-ester without EtOH.

Table 3
Comparison of process parameters between those by whole-cell biocatalysts from recombinant *E. coli* BL21 (pBSE) and wild-type *B. subtilis* ECU0554.

Parameters	Process I[a]	Process II[b]	Process III[c]
Substrate loading ($g\,L^{-1}$)	100	100	200
Catalyst loading ($g\,L^{-1}$)	50	2.0	2.5
S/C-ratio	2.0	50	80
Conversion (%)[d]	38.0	40.0	45.0
Reaction time (h)	8.0	8.0	8.0
ee_p (%)[d]	90.0	89.0	95.0 (>99.0)[e]
Time needed for organic phase to separate from aqueous phase containing biocatalyst (min)	>60	<1	<1
Isolated yield (%)	23	38	43
Space–time yield ($g_{product}\,L^{-1}\,d^{-1}$)	54	90	202
Catalyst yield ($g_{product}/g_{cat}$)	0.36	15	27

[a] Catalyzed by whole cells of *B. subtilis* ECU0554 in initial reaction volume of 100 mL without 10% EtOH at 30 °C, and pH was kept at 8.0.
[b] Catalyzed by whole cells of recombinant *E. coli* BL21/pBSE under unoptimized conditions in initial reaction volume of 100 mL without EtOH at 30 °C, and pH was kept at 8.0.
[c] Catalyzed by whole cells of recombinant *E. coli* BL21/pBSE under optimized conditions in initial reaction volume of 100 mL with 10% EtOH at 30 °C, and pH was kept at 8.0.
[d] Conversion and optical purity of the product (ee_p) were determined by GC.
[e] After one recrystalization.

increased from 40% to 48% when EtOH concentration was increased from 0% to 10% (v/v), then decreased significantly when more than 10% (v/v) of EtOH was added. This might be explained as that a low concentration cosolvent reduces mass-transfer resistance from membrane and wall of whole cells, while a high concentration cosolvent inhibits the enzyme activity. The enantiomeric ratio also increased notably with the rise of EtOH concentration, and gave the maximum ($E > 100$) at an EtOH concentration of 10% (v/v). After addition of EtOH, the E value was always higher than the control, despite of a slight decrease observed at EtOH concentrations of more than 10% (v/v). Therefore, 10% EtOH was selected as the optimal concentration of cosolvent in this study.

3.6. Optimization of catalyst loading

The influence of biocatalyst loading on the production of L-menthol was examined. As shown in Fig. 5, the concentration of L-menthol formed was significantly improved with the increase of cells loading in the reaction system. The concentration of product increased from 146 mM to 219 mM when the amount of biocatalyst was increased from 0.67 $g\,L^{-1}$ to 2.50 $g\,L^{-1}$.

To verify the credibility of the optimized conditions, a 2-L scale reaction was implemented in a 3-L stirred-tank reactor. The reaction profile was shown in Fig. 6. The product of 240 mM was formed and the L-isomer of substrate was almost exhausted within 6 h. Moreover, a good optical purity of product ($ee_p > 93\%$) was obtained.

Table 4
Effect of EtOH concentration on the conversion of substrate and enantioselectivity of whole cells.

EtOH added (%, v/v)	ee_s [%][a]	ee_p [%][a]	c [%][a]	E[b]
0	60	89	40	31
5	75	96	44	>100
10	89	96	48	>100
15	40	97	29	97
20	8	99	7	N.A.[c]

[a] Conversion and optical purity were determined by GC.
[b] Enantioselectivity (E) was calculated according to Chen et al. (1982).
[c] Not available.

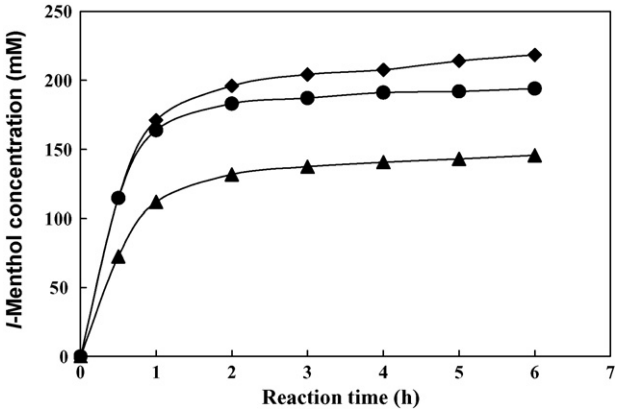


Fig. 5. The concentration of L-menthol formed with different biocatalyst loadings. Reaction conditions: initial reaction volume, 100 mL; substrate, DL-mentyl acetate, 100 $g\,L^{-1}$; pH 8.0, 30 °C. The *E. coli* cells employed: (▲) 0.67 $g\,L^{-1}$; (●) 1.33 $g\,L^{-1}$; (♦) 2.50 $g\,L^{-1}$.

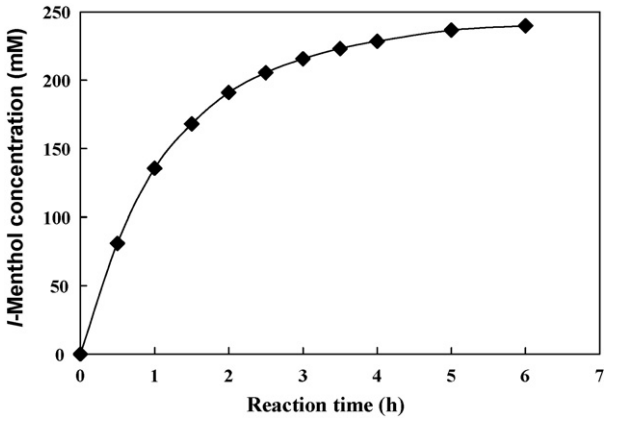


Fig. 6. The time course of 2-L reaction. Reaction conditions: initial reaction volume, 2 L; substrate concentration, 100 $g\,L^{-1}$; whole cells of recombinant *E. coli*, 2.5 $g\,L^{-1}$; pH 8.0, 30 °C.

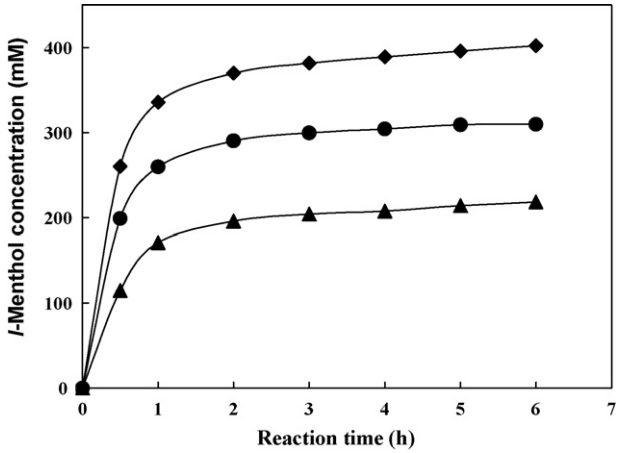


Fig. 7. The concentration of L-menthol formed at different substrate loadings. Reaction conditions: initial reaction volume, 100 mL; S/C-ratio: 40; pH, 8.0, 30 °C. Initial loadings of DL-menthyl acetate: (▲) 100 g L^{-1}; (●) 150 g L^{-1}; (♦) 200 g L^{-1}.

The similar results suggested that the catalyst loading of 2.50 g L^{-1} was the optimal for the kinetic resolution of DL-menthyl acetate of 100 g L^{-1}. Therefore, an S/C-ratio of 40 was adopted for following experiments.

3.7. Optimization of substrate loading

A high substrate concentration in a biocatalytic process is a crucial factor for its potential application, but usually inhibits the activity of biocatalyst as well. The hydrolytic reactions of DL-menthyl acetate with different initial concentrations were investigated.

As shown in Fig. 7, the concentration of product formed increased significantly with the increase of substrate loading. The L-menthol was produced in 402 mM and 96% *ee* with a substrate loading of 200 g L^{-1} and the conversion of substrate was more than 40%. Therefore, a substrate loading of 200 g L^{-1} was adopted for the bioresolution of DL-menthyl acetate.

3.8. Scale-up of optimized bioreaction

To further evaluate the feasibility of the optimized bioprocess for enzymatic production of L-menthol in practical application, a 2-L scale reaction under the above optimal conditions was performed in a 3-L stirred-tank reactor.

The process improvements were summarized in Table 3. A conversion of 45% with an ee_p of 95% was achieved after 8 h, giving a productivity of 211 g L^{-1} d^{-1}. In contrast to the process (I) catalyzed by wild-type whole-cell biocatalyst, the substrate loading was increased from 100 g L^{-1} to 200 g L^{-1} while the biocatalyst loading was decreased from 50.0 g L^{-1} to 2.5 g L^{-1} in the process (III). The space–time yield ($g_{product}$ L^{-1} d^{-1}) and catalyst yield ($g_{product}/g_{cat}$) were improved by *ca.* 4-fold and 75-fold, respectively. The S/C-ratio was significantly increased up to a number of as high as 80, and more importantly, this reduced the formation of emulsions, leading to a faster phase separation during solvent extraction (less than 1 min) and an increase of 2 folds in the total yield of the isolated product (Table 3, entry 7). Finally, 60 g of L-menthol with 99% *ee* were obtained after one recrystallization in acetonitrile.

The new process avoids the use of hazardous heavy metal catalysts and reduces remarkably the toxicity to human health and environment as compared to previous chemically catalytic processes. EtOH and water used in the bioprocess are also safe and environmentally acceptable. The reaction was run under mild conditions, reducing significantly energy consumption. Because of high S/C-ratio, the manufacturing cost of L-menthol was remarkably decreased. The large-scale results further confirmed the optimal process for production of L-menthol was feasibility in practical application.

4. Conclusions

A biocatalytic process for production of L-menthol with high substrate loading (200 g L^{-1}) and S/C-ratio (80) by recombinant *E. coli* cells was developed. The new process prevented the formation of emulsion during the downstream processing and made easier the product recovery. These encouraging results indicate that the biocatalytic process for the production of L-menthol is technologically efficient and environmentally benign with great potential for industrial application.

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