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Enzymatic Production of *l*-Menthol by a High Substrate Concentration Tolerable Esterase from Newly Isolated *Bacillus subtilis* ECU0554

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Abstract: Enzymatic preparation of *l*-menthol has been attracting much attention in the flavor and fragrance industry. A new ideal strain, *Bacillus subtilis* ECU0554, which exhibited high hydrolytic activity and excellent enantioselectivity towards *l*-menthyl ester, has been successfully isolated from soil samples through enrichment culture and identified as *Bacillus subtilis* by 16S rDNA gene sequencing. The esterase extracted from *B. subtilis* ECU0554 (BSE) showed the best catalytic properties ($E > 200$) for *dl*-menthyl acetate among the five menthyl esters examined. Enantioselective hydrolysis of 100 mM *dl*-menthyl acetate at 30°C and pH 7.0, using crude BSE as biocatalyst and 10% ethanol (v/v) as cosolvent, resulted in 49.0% conversion (3 h) and 98.0% *ee* for the *l*-menthol produced, which were much better than those using commercial enzymes tested. Moreover, BSE exhibited strong tolerance against high substrate concentration (up to 500 mM), and the concentration of *l*-menthol produced could reach as high as 182 mM, and more importantly, the optical purity of *l*-menthol produced was kept above 97% *ee*, which were not found in previous reports. These results imply that BSE is a potentially promising biocatalyst for the large-scale enzymatic preparation of *l*-menthol. Using this excellent biocatalyst, the enzymatic production of *l*-menthol will become a mild, efficient, inexpensive and easy-to-use "green chemistry" methodology.

Keywords: *Bacillus subtilis* esterase; enzyme catalysis; high substrate concentration tolerance; hydrolysis; *l*-menthol; *dl*-menthyl acetate

Introduction

l-Menthol is one of the world's largest flavor components[1] and is widely used in many confectionary goods, pharmaceuticals, oral health care products, cosmetics, teas and tobacco products because of its pleasant flavor and aroma, and its cooling-anesthetic effect.[2] However, the yield and quality of natural menthol are affected seriously by weather, region and so on. The menthol produced by chemical synthesis is usually *dl*-menthol, although *d*-menthol has an undesirable taste.[3] Therefore, separation of *l*-menthol from the racemic mixture is necessary.

In the past, chemocatalysis was the main method for the production of *l*-menthol.[4] However, environment pollution and energy pinch arising from chemocatalysis are becoming more and more serious in recent years. In the last few decades, scientists have shown great interest in biocatalysis because of its lower energy consumption, environmental friendliness, lower costs, less waste, shorter synthetic routes and avoidance of functional group protection. Nowadays, biocatalysis, the so-called "white biotechnology", is a fully accepted and validated synthetic method for asymmetric synthesis of chiral molecules using enzymes or microorganisms in pharmaceutical, agrochemical or fine chemical industries[5] and will be more commonly used in the years to come to complement other catalytic processes to attack a number of synthetic problems with significant challenge.[6] Esterases (E.C. 3.1.1.1) and lipases (E.C. 3.1.1.3) are a class of versatile biocatalysts for the preparation of chiral compounds in industry as they are stable, do not require cofactors, have a broad substrate spectrum and frequently exhibit high activities as well as chemo-, regio- or stereoselectivity.[7]

One way of preparing *l*-menthol is enantioselective esterification or transesterification of racemic menthol in organic solvents mediated by lipases or esterases.[8] However, some practical problems still existed in this approach, restricting its industrial application. For example, the esterification rate was low because of a relatively low reactivity of acyl donor and the formation of water as a by-product. Moreover, commercial enzymes exhibited relatively low enantioselectivity. An alternative way of preparing *l*-menthol is enantioselective hydrolysis of racemic menthyl esters in aqueous medium mediated by a specific hydrolase. However, only very few examples of the preparation of *l*-menthol in this manner have been reported so far.[9] Although enzymes and microorganisms used in these reports showed high optical purity, their low activity and low substrate concentration tolerance restricted their industrial application as the costs of both catalyst and downstream separation would be significantly increased.

Although biocatalytic methods for the economical production of optically active menthol under mild conditions are of great interest, the industrial application of many biotransformation processes was limited by lack of ideal biocatalysts. Screening of microorganisms with excellent selectivity, high specific activity and strong tolerance against high substrate/product concentration is of great importance and regarded as the first step for the practical use of enzymes in many related areas. So it is important to discover a new enzyme exhibiting excellent enantioselectivity, high activity and high substrate concentration tolerance to satisfy the demands for *l*-menthol preparation in industry.

In this work, a new stereospecific *l*-menthyl ester hydrolase-producing strain (no. ECU0554) was isolated from soil samples and identified as *Bacillus subtilis*. It showed high hydrolytic activity, excellent enantioselectivity and strong substrate tolerance for the production of optically active *l*-menthol. The effectiveness of the *B. subtilis* esterase (BSE) on different racemic menthyl esters was compared. The reaction parameters affecting the enantioselective hydrolysis of *dl*-menthyl acetate were discussed. And the most tolerable concentration of BSE against *dl*-menthyl acetate was also investigated.

Results and Discussion

Screening of Microbial Strains

Various microbial strains were isolated from soil samples through enrichment culture using *dl*-menthyl acetate as the sole carbon source. The strains capable of hydrolyzing *dl*-menthyl acetate were rapidly screened through thin layer chromatographic (TLC) analysis. Those with an obvious spot of menthol on TLC were marked for further confirmation by gas chromatography (GC). Using this method, 8 strains bearing the higher conversion (>25%) and excellent optical purity (>94% *ee*) for *l*-menthol were selected from 265 active strains in the presence of a lower concentration (10 mM) of the substrate (*dl*-menthyl acetate). These top 8 strains are listed in Table 1. As far as industrial application is concerned, a biocatalyst with stronger tolerance against high substrate or product concentration is more favorable as this will reduce the separation costs of product. So the substrate tolerance of these 8 candidate strains was investigated using elevated concentrations of *dl*-menthyl acetate. As shown in Figure 1, the quantities of *l*-menthol produced by the top 8 strains were all high at a substrate concentration of 100 mM. However, when the substrate concentration was increased from 100 mM to 500 mM, only the *l*-menthol production by the strain ECU0554 increased notably, while those by other strains could hardly increase or even obviously decreased, indicating that ECU0554 was the most tolerable strain against a high substrate concentration among the 8 candidates.

Table 1. Performance of 8 selected microbial strains exhibiting high activity and enantioselectivity in biocatalytic hydrolysis of *dl*-menthyl acetate.[a]

Microorganism	t [h]	ee_p [%][b]	c [%][b]
ECU0554	12	98	41
ECU0532	12	96	41
ECU0515	16	96	35
ECU0561	18	94	34
ECU0526	24	97	32
ECU0530	24	99	25
ECU0544	24	94	25
ECU0555	24	99	25

[a] Reactions were carried out in 10 mL sodium phosphate buffer (100 mM, pH 7.0) with 10 mM *dl*-menthyl acetate and 1.5 g microbial wet cells at 30 °C.

[b] Conversion and optical purity of the produced *l*-menthol were determined by GC.

As shown in Table 1 and Figure 1, strain ECU0554 exhibited the highest hydrolytic activity, the best optical purity (98%) and strong tolerance against a high substrate concentration among the top 8 strains. So it was selected as the best enzyme producer, and was subsequently identified as *Bacillus subtilis* based on its 16S rDNA sequence and hereafter named *B. subtilis* ECU0554. This *l*-menthyl esterase-producing strain is currently deposited in the China General Microbiological Culture Collection Center (Beijing, China), with the accession number CGMCC 2548.

Effect of Acyl Groups on Hydrolysis Activity of BSE

The enzymatic reactivity of a substrate could be extremely influenced by the size, polarity and electrons of the substituent R in the acyl group of menthyl esters (Scheme 1). Racemic menthyl esters **1–5** with different acyl groups were synthesized in order to find

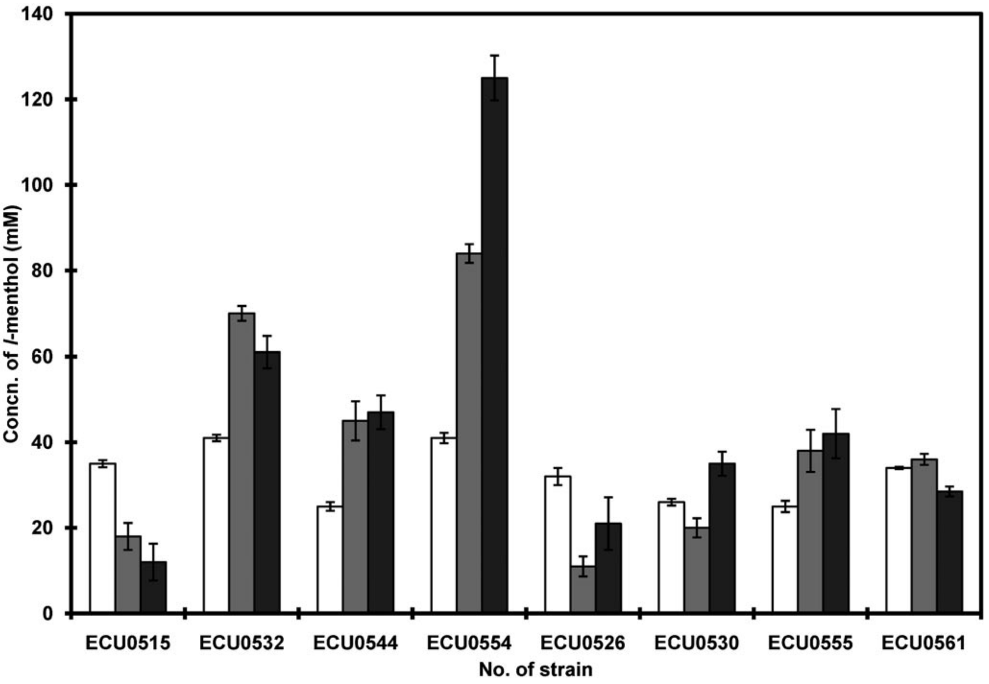
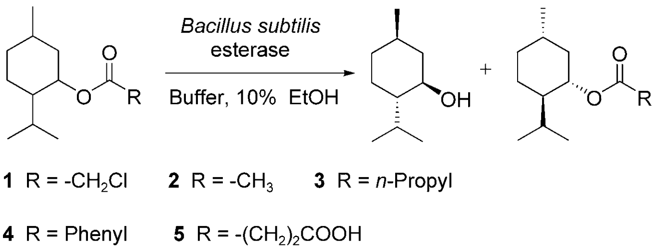


Figure 1. Influence of substrate concentration on *l*-menthol production by 8 selected strains. Reactions were carried out in 10 mL sodium phosphate buffer (100 mM, pH 7.0) with different concentrations of *dl*-menthyl acetate and 1.5 g microbial wet cells at 30 °C for 24 h. Data are means of at least three parallel reactions. Substrate concentrations: (□) 100 mM; (▨) 300 mM; (■) 500 mM.



Scheme 1. Enzymatic kinetic resolution of *dl*-menthyl esters **1–5** using *Bacillus subtilis* esterase (BSE).

Table 2. Enantioselectivity of BSE in kinetic resolution of different *dl*-menthyl esters **1–5**.

Substrate	*t* [h]	ee_p [%][a]	*c* [%][a]	*E*[b]
rac-**1**	3	5.1	97	n.a.[c]
rac-**2**	3	97.7	49	>200
rac-**3**	6	92.0	41	47
rac-**4**	12	78.1	32	11
rac-**5**	24	> 99.0	1	n.a.

[a] Conversion and optical purity of the produced *l*-menthol were determined by GC.

[b] Enantioselectivity (*E*) was calculated according to Chen et al.[11]

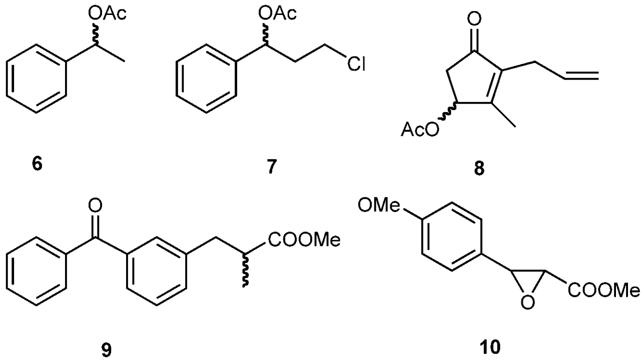
[c] Not available.

an optimal substrate for BSE. Hydrolysis reactions of *dl*-menthyl esters **1–5** were performed in sodium phosphate buffer (pH 7.2, 200 mM) with BSE at 30 °C. In the control experiments in which no enzyme was added, spontaneous non-selective hydrolysis reactions of *dl*-menthyl esters **1–5** were not detected under the same reaction conditions. Enantiomeric excess of product (ee_p), conversion of substrate (*c*) and enantiomeric ratio (*E*) were determined, as listed in Table 2. A remarkable change in hydrolytic activity of BSE was observed when the acyl group of the menthyl ester was changed. The increase in the acyl group size led to a dramatic decrease of BSE activity. The conversions of compounds **3**, **4** and **5** were lower than that of the substrate **2**, even if the reaction time was extended. Moreover, a notably decreased enantioselectivity of BSE for compound **4** ($E=11$) was confirmed. This might be attributed to the bulky acyl group which may hinder its movement into the active site of the enzyme. BSE exhibited an extremely lower conversion towards compound **5** with a polar group (−COOH) since an esterase usually does not accept a highly polar hydrophilic substrate in an aqueous environment.[10]

BSE showed the highest hydrolytic activity on racemic compound **1**, which might be attributed to the electron-withdrawing substituent of the acyl moiety.[12] However, the optical purity of product was very low. In contrast, BSE exhibited not only a high hydrolytic activity, but also the best enantioselectivity ($E>200$) in the resolution of *dl*-menthyl acetate (**2**). So compound **2** (*dl*-menthyl acetate) was selected as substrate in the subsequent work.

BSE-Catalyzed Hydrolytic Kinetic Resolution of Other Racemic Esters

A broad substrate spectrum is also very important for an enzyme. So several other racemic esters (**6–10**, Scheme 2) were also examined using BSE. Kinetic resolution of the racemic esters was performed in sodium phosphate buffer (pH 7.2, 200 mM) with BSE at 30 °C. In the control experiments in which no enzyme was added, spontaneous non-selective hydrolysis reaction of the racemic esters **6–10** was not detected under the same reaction conditions. As shown in Table 3, BSE exhibited excellent hydrolytic activity (up to 46% conversion within 3 h) and moderate enantiomeric excess of (*R*)-product ($ee_p = 85\%$) for racemic compound **8**. Substrates **6** and **7** were also converted at moderate rates (40% and 51% conversions within 5 h and 12 h) but poor enantioselectivity ($E_R =$ 7, 11). However, BSE showed nearly no activity for chiral esters **9** and **10**. Based on the above results, it was found that the size of the substrate has a strong influence on the activity of BSE. The reaction rate remarkably decreased with the increase in the size of the substrate (Table 2 and Table 3). One of the possible reasons is that the bulky substrate may be hindered or prevented in its movement into the active site of the enzyme (entry 5, Table 2; entries 4 and 5, Table 3).



Scheme 2. The structure of chiral esters **6–10** catalyzed by BSE.

Table 3. BSE-catalyzed kinetic resolution of *rac*-esters **6–10**.

Substrate	*t* [h]	ee_S [%][a]	ee_p [%][a]	*c* [%][b]	*E*[c]
(*RS*)-**6**	5	42.1	64.5 (*R*)	40	7
(*RS*)-**7**	12	71.5	69.7 (*R*)	51	11
(*RS*)-**8**	3	71.7	85.9 (*R*)	46	23
(*RS*)-**9**	48	<1.0	–	–[d]	–
(*RS*)-**10**	48	<1.0	–	–[d]	–

[a] Determined by GC or HPLC.

[b] $c = ee_s/(ee_s + ee_p)$.

[c] Enantioselectivity (*E*) was calculated according to Chen et al.[11]

[d] No significant conversion was observed.

Effects of pH and Temperature on BSE Activity

It is well known that pH remarkably influences the activity of an enzyme, which is probably caused by a change in stereochemical configuration in the neighborhood of active sites.[13] In order to determine the optimum pH for BSE-mediated resolution of *dl*-menthyl acetate, the biohydrolytic reaction was performed under different initial pH at 30 °C. As shown in Figure 2, BSE activity was low at pH < 6.5 or pH > 8.0, and the optimum pH of BSE for *dl*-menthyl acetate was 7.0. Moreover, no chemical hydrolysis was detected in the range of pH 6.5–8.0, which was in accordance with the result reported Xu et al.,[14] so pH 7.0 was chosen as the favorable pH for the enzymatic resolution.

The effect of temperature on the kinetic resolution of *dl*-menthyl acetate was also studied. The ee_p and conversion of the reaction at 3 h at different temperatures (20, 30, 40 and 50 °C) are shown in Table 4. The conversion increased from 32% to 47% with the increase of temperature from 20 °C to 30 °C, and it was not significantly changed from 30 °C to 40 °C. However, it rapidly dropped with further increase of the temperature up to 50 °C. Nevertheless, the change of temperature did not remarkably affect ee_p. Based on

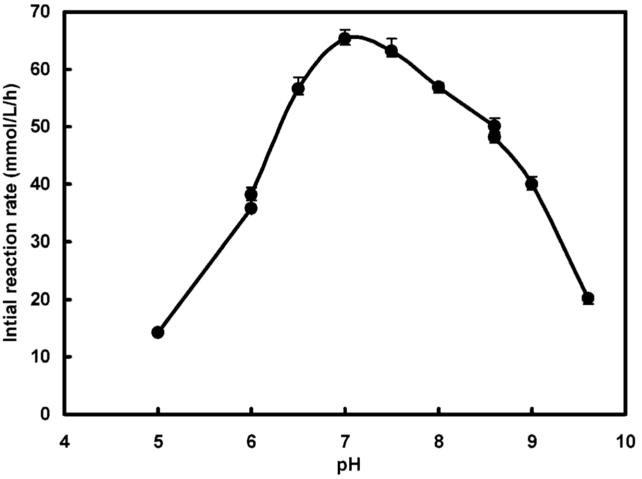


Figure 2. Effect of pH on *dl*-menthyl acetate hydrolysis catalyzed by BSE. Data are means of three independent reactions.

Table 4. Effect of temperature on BSE-catalyzed hydrolysis of *dl*-menthyl acetate.

T [°C]	ee_p [%][a]	c [%][a]	E[b]
20	98.6	32	> 200
30	97.4	47	> 200
40	97.6	43	192
50	99.0	24	> 200

[a] Conversion and optical purity of the produced *l*-menthol were determined by GC.

[b] Enantioselectivity (E) was calculated according to Chen et al.[11]

the above results, pH 7.0 and 30 °C were chosen as the optimal reaction conditions in the following work.

Comparison of Catalytic Performance between BSE and Commercial Enzymes

It was reported that a number of commercially available hydrolases showed high enantioselectivity in the hydrolysis of racemic menthyl esters as well as in the acylation of racemic menthol.[9,15] The kinetic resolution of *dl*-menthyl acetate catalyzed by BSE and various commercial enzymes with equivalent activity was performed in order to compare their catalytic performance. Among the commercial enzymes investigated, only Lipase AS exhibited a high hydrolytic activity but a low enantiomeric excess of product (ee_p = 52.6%), Lipase MY and CRL exhibited moderate hydrolytic activities, while other enzymes showed low or even no hydrolytic activity (< 10% conversion) towards *dl*-menthyl acetate (Table 5). In contrast, BSE exhibited a remarkably higher hydrolytic activity (49% conversion within 3 h) and a much higher enantioselectivity ($E > 200$) in the kinetic resolution of *dl*-menthyl acetate. It is well known that E is the intrinsic property of an enzyme with respect to a specific substrate[11] and a higher enantioselectivity of an enzyme will be much more favorable for its industrial application. Therefore, BSE is considered as a much more promising biocatalyst than the tested commercial enzymes for industrial production of *l*-menthol in the future.

Table 5. Hydrolysis of *dl*-menthyl acetate by enzymes from various sources.

Enzyme	t [h]	ee_p [%][a]	c [%][a]	E[b]
BSE	3	98.0	49	> 200
Lipase AS	48	52.6	50	5
Lipase OF	48	97.0	22	86
Lipase MY	48	93.6	15	36
CRL	48	94.4	11	39
Lipase AYS	48	95.2	8	44
Lipase D	48	97.1	6	54
PPL	48	90.2	7	21
Lipase PS-C	96	98.0	8	108
Lipase PS-D	96	98.5	5	139
Lipase AK	96	> 99.0	< 5	n.a.[c]
Novozym 435	96	–	–[d]	–
Lipase AH	96	–	–[d]	–

[a] Conversion and optical purity of the produced *l*-menthol were determined by GC.

[b] Enantioselectivity (E) was calculated according to Chen et al.[11]

[c] Not available.

[d] No significant conversion was observed.

Strong Tolerance of BSE against High Substrate Concentration

A high substrate/product concentration would be beneficial for practical application of an enzymatic process because it will reduce the cost of product isolation to a large extent.[16] However, enzymatic activity is frequently inhibited by the existence of its own substrate at relatively high concentration.[17] Besides the hydrolytic activity and enantioselectivity, the substrate/product tolerance of an enzyme is also a very important parameter to be evaluated for potential applications in industry. The effect of substrate concentration on the product formation was investigated at a fixed ratio of substrate to enzyme (S/E). The reaction was carried out at pH 7.0 and 30 °C. The pH was kept around 7.0 by manual addition of NaOH (1 M). As shown in Figure 3, the product formation was increased from 49 mM to 182 mM when the substrate concentration was raised from 100 mM to 500 mM, indicating that a high substrate concentration up to

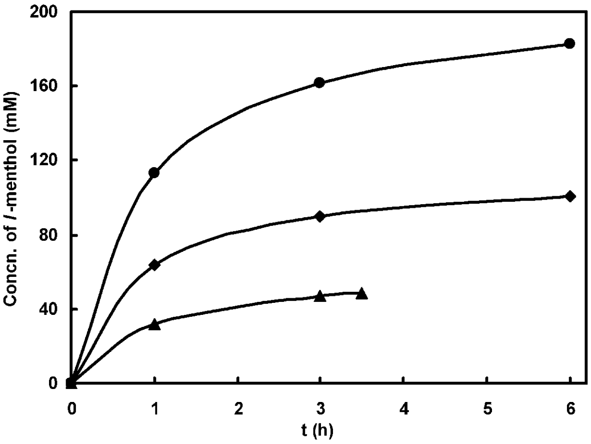


Figure 3. Time courses of *dl*-menthyl acetate hydrolysis catalyzed by BSE at different global volume concentrations of substrate (S) and enzyme (E). The S/E ratio was kept constant. Substrate concentrations: (▲) 100 mM; (◆) 250 mM; (●) 500 mM.

Table 6. Comparison between BSE and Lipase OF for enantioselective hydrolysis *dl*-menthyl acetate at a high concentration (500 mM).

Enzyme	Substrate concn. [mM]	*t* [h]	*l*-Menthol produced [mM]	ee_p [%][a]	Ref.
BSE	500	6	182	97.1	This work
Lipase OF	500	24	3	>99.0	This work
B. cepacia whole cell	480	20	<90	<85.0	[14]

[a] Optical purity of the produced *l*-menthol were determined by GC.

500 mM did not result in any sharp decrease of BSE activity and BSE was tolerant against a pretty high concentration of the substrate and/or the product. Among the commercial enzymes tested, Lipase OF exhibited the best hydrolytic ability towards *dl*-menthyl acetate at a concentration of 100 mM (Table 5), but its hydrolytic ability decreased sharply at a higher concentration (500 mM) (Table 6). On the contrary, the *l*-menthol formed decreased from 22 mM to 3 mM with the increase of substrate concentration from 100 mM to 500 mM. This might be explained in that the activity of Lipase OF was inhibited by a high concentration of the substrate. Besides, it was never reported in previous studies that an enzyme or microorganism could tolerate such a high substrate concentration in the preparation of *l*-menthol *via* enantioselective hydrolysis of racemic menthyl esters. More importantly, the optical purity of *l*-menthol produced was kept above 97% *ee* even at a very high substrate concentration, which was superior to the result that the ee_p decreased with the increase of the substrate concentration, with $<85\%$ *ee* as reported by other researchers[14] at a substrate concentration of 480 mM (Table 6).

Resolution of *dl*-Menthyl Acetate in Preparative Scale

To further evaluate the potential of BSE for practical use, a preparative scale resolution of *dl*-menthyl acetate was conducted on a 3-gram scale. The kinetic profile of this resolution was shown in Scheme 1. A total amount of 500 mg crude enzyme powder was employed. The enzymatic reaction was monitored by chiral GC analysis, and it was stopped at 9 h when the conversion of substrate reached a value of around 50%. After extraction and normal work-up, 1.17 g of the unreacted menthyl acetate was isolated (39% yield), whereas 0.98 g of the enantiopure *l*-menthol was also obtained (98% *ee*, 42% yield).

Conclusions

In this work, a new bacterial strain, *B. subtilis* ECU0554, was successfully isolated from soil samples, which exhibited a high hydrolytic activity on *dl*-menthyl acetate and excellent enantioselectivity towards *l*-menthol. Different kinds of racemic menthyl esters were hydrolyzed by BSE, suggesting that *dl*-menthyl acetate is the best substrate for the preparation of optically pure *l*-menthol. Moreover, BSE has a relatively broad substrate spectrum and might also convert other chiral esters. BSE showed much better performance than the commercially available enzymes tested. Furthermore, BSE also exhibited strong tolerance against high substrate concentrations up to 500 mM. The enzymatic resolution of *dl*-menthyl acetate at a concentration of 99 $\mathrm{g\,L^{-1}}$ (500 mM) in the presence of 10% EtOH over a 6 h period, gave *l*-menthol at a concentration of 28.5 $\mathrm{g\,L^{-1}}$ (182 mM), and more importantly, the optical purity of *l*-menthol produced was above 97% *ee*. In conclusion, BSE was considered as a potentially ideal and promising biocatalyst for practical production of *l*-menthol in the future.

Nonetheless, the industrial application of enzymes is often hampered by the lack of long-term operational stability, and difficult recovery and re-use of the enzyme. These drawbacks can often be overcome by immobilization of the enzyme.[18] So we will try to immobilize BSE in our future work for practical application of this robust biocatalyst.

Experimental Section

Materials and Methods

dl-Menthol and (*RS*)-1-phenylethanol were purchased from Alfa Aesar. (*RS*)-3-Chloro-1-phenylpropan-1-ol was purchased from Shanghai PI Chemicals Co. Ltd., Shanghai, China. (*RS*)-Allethrolon acetate **8** was provided by Yangzhou Agrochemicals Co. Ltd., Jiangsu Province, China. Ketoprofen was provided by Huzhou Konch Pharmaceutical Co. Ltd., Zhejiang Province, China. *trans*-3-(4′-Methoxyphenyl)-glycidic acid methyl ester **10** [(±)-MPGM][19] was synthesized in our laboratory. The other chemicals used in this work were of analytical grade from local suppliers. PPL (porcine pancrease lipase) and CRL (*Candida rugosa* lipase) were purchased from Sigma; Lipase OF (lipase from *Candida rugosa*) was purchased from Meito Sangyo; Lipase PS (lipase from *Burkholderia cepacia*), Lipase AYS (lipase from *Candida rugosa*), Lipase D (lipase from *Rhizopus delemar*), Lipase MY (lipase from *Candida rugosa*), Lipase AS (lipase from *Aspergillus niger*), Lipase AK (lipase from *Pseudomonas fluorescens*), Lipase PS-C, Lipase PS-D and Lipase AH (lipase from *Pseudomonas cepacia*) were obtained from Amano Pharmaceutical Co. Ltd. TLC analysis was carried out on RSG F_{254} silica gel sheets using mobile phase of petroleum ether and ethyl acetate in a ratio of 10:1 (v/v).

Spots were visualized by treatment with 5% (w/v) vanillin/sulphuric acid solution and heating of the dried plates. The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 500 spectrometer (at 500 MHz, Bruker, Karlsruhe, Germany). Mass spectra were recorded on an Agilent 5975 MSD mass spectrograph (electron impact, 70 eV, Agilent, USA). Optical rotations were determined on a Jasco P-1030 polarimeter.

Screening Procedure of Microbial Strains

Different soil samples were collected firstly from the nature, and then enrichment cultured using *dl*-menthyl acetate as the sole carbon source. Those strains capable of hydrolyzing *dl*-menthyl acetate were rapidly screened through TLC analysis. Those with obvious spots of the menthol were marked for further confirmation by GC. The best strains were further compared and used in the subsequent study.

Enzyme Preparation

Cultivation of Bacillus subtilis ECU0554: *B. subtilis* ECU0554 was grown aerobically in a simply optimized medium with the following composition (per liter): glycerol, 30.0 g; peptone, 5.0 g; yeast extract, 5.0 g; NaCl, 1.0 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.2 g; pH 7.0. The microorganisms were cultivated in 500-mL flasks with 100 mL medium at 30 °C for 18 h with shaking at 180 rpm. After cultivation, the microbial cells were harvested by centrifuge under 4 °C at 10,000 × *g* for 10 min and washed twice with physiological saline and stored at 4 °C for further use.

Crude enzyme preparation: The harvested cells of *B. subtilis* ECU0554 were resuspended in sodium phosphate buffer (pH 7.0, 50 mM) and disrupted 3 times by a high-pressure homogenizer (AH110B, ATS Engineering Inc.). Cell debris was removed by centrifugation (12,000 × *g*, 4 °C, 20 min) and the resultant supernatant was used as a crude extract. Ammonium sulphate was added slowly to the crude extract with stirring at 0 °C. The protein that precipitated between 60% and 90% saturation of ammonium sulphate was collected by centrifugation (12,000 × *g*, 4 °C, 20 min) and dialyzed against 10 mM sodium phosphate buffer (pH 7.0). Then the dialysate was dried with a freeze dry system, and the resultant crude esterase was stored at 4 °C.

General Procedure for Chemical Synthesis of Racemic Menthyl Esters

For synthesis of compounds **1**, **2** and **4**, chloroacetyl chloride (1.1 equiv.), acetyl chloride (1.1 equiv.) and benzoyl chloride (1.2 equiv.) were added dropwise to a mixture of *dl*-menthol (7.81 g, 0.05 mol) and pyridine (0.2 equiv.) in dry chloroform (40 mL) on an ice bath, and then the solution was stirred at room temperature. For synthesis of compound **3**, the *dl*-menthol (3.12 g, 0.02 mol) and *n*-butyric anhydride (1.2 equiv.) were added into dry toluene (20 mL), and the mixture was refluxed. The reactions were performed until menthol was completely consumed (as monitored by TLC). The product was extracted into ethyl acetate (100 mL × 3), then washed with an aqueous solution of saturated sodium bicarbonate (100 mL × 3) and dried over anhydrous sodium sulphate. The organic solvent was evaporated under reduced pressure and the product was dried under vacuum. The synthesis of compound **5** was performed as described by Omata.[9]

dl-Menthyl chloroacetate **(1):**[20] This compound was obtained as a colourless liquid; yield: 8.64 g (75%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.77$ (d, 3H, $J = 7.0$ Hz), 0.83–0.92 (m, 7H), 0.99–1.08 (m, 2H), 1.40–1.54 (m, 2H), 1.67–1.71 (m, 2H), 1.85–1.89 (m, 1H), 2.00–2.03 (m, 1H), 4.04 (dd, 2H, $J = 17.2$ Hz, 14.6 Hz), 4.77 (dt, 1H, $J = 10.9$ Hz, 4.4 Hz).

dl-Menthyl acetate **(2):**[21] This compound was obtained as a yellow liquid; yield: 9.02 g (91%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.76$ (d, 3H, $J = 7.0$ Hz), 0.82–1.10 (m, 9H), 1.32–1.39 (m, 1H), 1.43–1.53 (m, 1H), 1.64–1.70 (m, 2H), 1.83–1.90 (m, 1H), 1.96–2.03 (m, 4H), 4.68 (dt, 1H, $J = 10.9$ Hz, 4.4 Hz); MS (EI): $m/z = 198$ (M^+), 183, 165, 138, 123, 109, 95, 81, 67, 55, 43.

dl-Menthyl butyrate **(3):**[22] This compound was obtained as a colourless liquid; yield: 2.77 g (61%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.76$ (d, 3H, $J = 7.0$ Hz), 0.82–1.10 (m, 12H), 1.34–1.39 (m, 1H), 1.43–1.53 (m, 1H), 1.62–1.70 (m, 4H), 1.83–1.90 (m, 1H), 1.96–2.01 (m, 1H), 2.26 (t, 2H, $J = 7.5$ Hz), 4.68 (dt, 1H, $J = 10.9$ Hz, 4.4 Hz); MS (EI): $m/z = 226$ (M^+), 155, 138, 123, 109, 95, 81, 71, 55, 43.

dl-Menthyl benzoate **(4):**[23] This compound was obtained as a colourless solid; yield: 10.81 g (83%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.80$ (d, 3H, $J = 7.0$ Hz), 0.88–0.97 (m, 7H), 1.07–1.18 (m, 2H), 1.52–1.58 (m, 2H), 1.70–1.75 (m, 2H), 1.94–1.99 (m, 1H), 2.11–2.15 (m, 1H), 4.68 (dt, 1H, $J = 10.9$ Hz, 4.4 Hz), 7.42–7.45 (m, 2H), 7.53–7.56 (m, 1H), 8.04–8.06 (m, 2H); MS (EI): $m/z = 260$ (M^+), 162, 147, 138, 123, 105, 95, 81, 67, 55, 41, 29.

dl-Menthyl succinate **(5):**[24] This compound was obtained as a colourless solid; yield: 5.25 g (82%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.74$ (d, 3H, $J = 7.0$ Hz), 0.82–1.08 (m, 9H), 1.35–1.39 (m, 1H), 1.43–1.52 (m, 1H), 1.64–1.70 (m, 2H), 1.83–1.89 (m, 1H), 1.96–2.00 (m, 1H), 2.58–2.62 (m, 2H), 2.67–2.72 (m, 2H), 4.71 (dt, 1H, $J = 10.9$ Hz, 4.4 Hz).

Synthesis of (*RS*)-1-Phenylethyl Acetate (6) and (*RS*)-3-Chloro-1-phenyl-1-propyl Acetate (7)

The (*RS*)-1-phenylethanol (6.11 g, 0.05 mol) or (*RS*)-3-chloro-1-phenyl-1-propanol (8.53 g, 0.05 mol), sodium acetate (0.1 equiv.) and acetic anhydride (1.5 equiv.) were added into chloroform (40 mL), and the mixture was refluxed. The reactions were performed until (*RS*)-1-phenylethanol or (*RS*)-3-chloro-1-phenyl-1-propanol was completely consumed (as monitored by TLC). The product was extracted into ethyl acetate (50 mL × 3), then washed with an aqueous solution of saturated sodium bicarbonate (100 mL × 3) and dried over anhydrous sodium sulphate. The organic solvent was evaporated under reduced pressure and the product was dried under vacuum.

(RS)-1-Phenylethyl acetate **(6):**[25] This compound was obtained as a colourless liquid; yield: 7.34 g (89%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.53$ (d, 3H, $J = 6.6$ Hz), 2.07 (s, 3H), 5.88 (q, 1H, $J = 6.6$ Hz), 7.24–7.40 (m, 5H).

(RS)-3-Chloro-1-phenyl-1-propyl acetate **(7):**[26] This compound was obtained as a colourless liquid; yield: 9.50 g (89%). ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 2.07$ (s, 3H), 2.15–2.21 (m, 1H), 2.35–2.42 (m, 1H), 3.40–3.45 (m, 1H), 3.52–3.57 (m, 1H), 5.92 (dd, 1H, $J = 8.1$ Hz, 5.7 Hz), 7.27–7.36 (m, 5H).

Synthesis of (*RS*)-Ketoprofen Methyl Ester (9)[27]

To a 250-mL round bottom flask was added (*RS*)-ketroprofen (12.74 g, 0.05 mol), methanol (100 mL) and H_2SO_4 (1 mL). The flask was fitted with a reflux condenser and the mixture was refluxed. The reactions were performed until (*RS*)-ketroprofen was completely consumed (as monitored by TLC).The mixture was extracted into ethyl acetate (100 mL × 3), then washed with an aqueous solution of saturated sodium bicarbonate (100 mL × 3) and dried over anhydrous sodium sulphate. The organic solvent was removed under reduced pressure and the product was dried under vacuum. This compound was obtained as a colourless solid; yield: 12.91 g (96%); ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 1.53$ (d, 3H, $J = 7.2$ Hz), 3.67 (s, 3H), 3.81 (q, 1H, $J = 7.2$ Hz), 7.42–7.50 (m, 3H), 7.53–7.61 (m, 2H), 7.67–7.69 (m, 1H), 7.76–7.81 (m, 3H).

General Procedure for Esterase-Catalyzed Resolution of *dl*-Menthyl Esters 1–5

Suspensions of BSE preparation (20 mg) in a solution of *dl*-menthyl ester (0.2 mmol) in ethanol (0.2 mL) and sodium phosphate buffer (1.8 mL, pH 7.2, 200 mM) were shaken at 180 rpm in sealed glass vials at 30 °C for the time indicated in Table 2.

The samples were extracted with ethyl acetate and dried over anhydrous sodium sulphate. Enantiomeric excess of the product and conversion of the substrates were determined by chiral GC analysis.

General Procedure for Esterase-Catalyzed Resolution of Esters 6–10

Suspensions of BSE preparation (20 mg) in a solution of the corresponding (*RS*)-ester (0.1 mmol) in ethanol (0.2 mL) and sodium phosphate buffer (1.8 mL, pH 7.2, 200 mM) were shaken at 180 rpm in sealed glass vials at 30 °C for the time indicated in Table 3.

The samples were extracted with ethyl acetate and dried over anhydrous sodium sulphate. Enantiomeric excess of the product and conversion of the substrates **6–8** were determined by chiral GC analysis and **9**, **10** by HPLC.

Enantioselective Hydrolysis of *dl*-Menthyl Acetate by BSE

dl-Menthyl acetate (40 mg, 0.202 mmol), ethanol (0.2 mL) and BSE preparation (20 mg) were homogenized in 1.8 mL buffer, and this reaction mixture was then shaken at 180 rpm at a pH range of 5.0–9.6 (200 mM sodium citrate, pH 5.0–6.0; 200 mM sodium phosphate, pH 6.0–8.6; and 200 mM glycine-NaOH, pH 8.6–9.6) and in a temperature range of 20–50 °C. The enantiomeric excess of the product and conversion of the substrate were determined by chiral GC analysis.

The substrate tolerance of BSE was investigated with substrate concentrations ranging from 100 mM to 500 mM at pH 7.0 and 30 °C in 2 mL buffer. The ratio of substrate to enzyme ratio (S/E) was fixed in reactions. The conversion was determined by chiral GC analysis.

Enantioselective Hydrolysis of *dl*-Menthyl Acetate by Various Commercial Enzymes

dl-Menthyl acetate (40 mg, 0.202 mmol), ethanol (0.2 mL) and BSE or various commercial enzymes with an equivalent activity (70 U) were homogenized in sodium phosphate buffer (1.8 mL, pH 7.0, 200 mM), and this reaction mixture was then shaken at 180 rpm at 30 °C. The enantiomeric excess of the product and conversion of the substrate were determined by chiral GC analysis.

Enantioselective Hydrolysis of *dl*-Menthyl Acetate by BSE and Lipase OF at a Concentration of 500 mM

dl-Menthyl acetate (198 mg, 1.0 mmol), ethanol (0.2 mL) and the same activity of BSE or Lipase OF preparation (350 U) were homogenized in sodium phosphate buffer (1.6 mL, pH 7.0, 200 mM), and this reaction mixture was then shaken at 180 rpm for the time indicated in Table 6. The enantiomeric excess of the product and conversion of the substrate were determined by chiral GC analysis. The concentration of *l*-menthol formed was calculated according to the internal standard curve.

Resolution of *dl*-Menthyl Acetate in a Preparative Scale

dl-Menthyl acetate (3.0 g, 15.1 mmol) was added into a mixture of ethanol (15 mL) as cosolvent and sodium phosphate buffer (135 mL, pH 7.0, 200 mM). An emulsion was formed by agitation at about 250 rpm before careful addition of BSE (500 mg). The reaction was performed in a 500-mL round-bottom flask at 30 °C. The conversion and optical purity of *l*-menthol were monitored by chiral GC analysis. The reaction was stopped after 9 h at about 50% conversion (relative to the total amount of *dl*-menthyl acetate). After removing the enzyme from the reaction mixture by filtration and extraction of the reaction mixture with ethyl acetate, substrate and product were separated by column chromatography on silica gel using petroleum ether/ethyl acetate (30:1, v/v) as an eluent, and recovered by removal of the solvent under reduced pressure and dried under vacuum. The product was characterized by ^{1}H NMR and the optical purity of product was determined by chiral gas chromatography and polarimetry.

The remaining menthyl acetate was isolated as a yellow liquid; yield: 1.17 g (5.9 mmol, 39%); $[\alpha]_D^{16}$: +66.5 (*c* 1.0, EtOH) {lit.: $[\alpha]_D^{22}$: +79.5 (*c* 1.0, CH_2Cl_2)[28]}. *l*-Menthol was obtained as a colourless solid; yield: 0.98 g (6.3 mmol, 42%; 98% *ee* by GC); $[\alpha]_D^{16}$: −47.5 (*c* 1.0, EtOH) {lit.: $[\alpha]_D^{20}$: −50.0 (EtOH)[29]}; ^{1}H NMR ($CDCl_3$, 500 MHz): $\delta = 0.76$–0.83 (m, 3H), 0.83–1.02 (m, 9H), 1.04–1.14 (m, 1H), 1.38–1.48 (m, 2H), 1.58–1.69 (m, 2H), 1.96–1.99 (m, 1H), 2.14–2.21 (m, 1H), 3.41 (dt, 1H, $J = 10.4$ Hz, 4.3 Hz).

Determination of Lipase/Esterase Activity

The enzyme activity was assayed spectrophotometrically by measuring the conversion of 1 mM *p*-nitrophenyl butyrate to *p*-nitrophenol at 30 °C for 1 min at 405 nm.[19] One unit of the enzyme activity was defined as the amount of enzyme releasing 1 μmol *p*-nitrophenol per minute.

Table 7. Details of chiral GC analyses.

Compound	T_{column} [°C][a]	Retention time [min]	
(*RS*)-1-phenylethyl acetate (**6**)	P1	22.8 (*S*)	23.5(*R*)
(*RS*)-1-phenylethanol	P1	23.8 (*R*)	24.1 (*S*)
(*RS*)-3-chloro-1-phenyl-1-propyl acetate (**7**)	P2	25.7 (*S*)	26.1 (*R*)
(*RS*)-3-chloro-1-phenyl-1-propanol	P2	30.2 (*R*)	30.7 (*S*)
(*RS*)-allethrolon acetate (**8**)	P3	16.6 (*R*)	17.1 (*S*)
(*RS*)-allethrolon	P3	20.0 (*S*)	20.3 (*R*)

[a] P1: temperature program: 22 min 105 °C at 30 °C min^{-1} to 180 °C; P2: temperature program: 25 min 145 °C at 5 °C min^{-1} to 180 °C; P3: temperature program: 16 min 150 °C at 5 °C min^{-1} to 170 °C.

Chiral GC and HPLC Analysis

Samples of compounds **1**, **2**, **3** and **5** (0.5 mL) were extracted with ethyl acetate (0.5 mL) and dried by anhydrous sodium sulphate for 12 h. The samples of compound **4** (0.5 mL) were firstly adjusted to neutral pH with NaOH (2M) as it was acidic and could not be determined by a chiral column, and then extracted with ethyl acetate (0.5 mL) and dried by anhydrous sodium sulphate for 12 h. The optical purity and conversion were determined by GC-14 gas chromatography (Shimadzu, Tokyo, Japan) equipped with FID detector and Gamma Dex^{TM}120 Chiral column (Supelco, 30 m × 0.25 mm, 0.25 μm film of 20% permethylated γ-cyclodextrin) using N_2 as carrier gas. *n*-Octanol was used as an internal standard. The injector and detector temperatures were set at 280 and 350 °C, respectively. For compounds **1**, **2**, **3** and **5**, the initial column temperature of 110 °C was held for 15 min, then raised to 180 °C at a rate of 10 °C/min and finally held at 180 °C for 5 min. For compound **4**, the initial temperature 110 °C was held for 15 min, then raised to 200 °C at a rate of 30 °C/min and finally held at 200 °C for 15 min. Retention times: *d*-menthol, 15.6 min; *l*-menthol, 15.9 min; *rac*-**1**, 24.5 min; *rac*-**2**, 17.2 min; *rac*-**3**, 21.9 min; *rac*-**4**, 31.0 min.

For compounds **6–8**, the ee_s and ee_p were determined similarly by gas chromatography using a Beta Dex^{TM}120 chiral column (Supelco, 30 m × 0.25 mm, 0.25 μm film thickness). Details are given in Table 7.

For compounds **9** and **10**, the enantiomeric excess was determined by HPLC (Agilent, USA) using a chiral column (Chiralcel OJ, 25 cm × ϕ0.46 cm, Daicel Co., Japan).[19,30]

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