Appl Biochem Biotechnol
DOI 10.1007/s12010-015-1632-0

Cloning and Characterization of an Enantioselective *l*-Menthyl Benzoate Hydrolase from *Acinetobacter* sp. ECU2040

Jin-Gang Yin[1] **· Guo-Chao Xu**[1] **· Gao-Wei Zheng**[1] **· Jian-He Xu**[1]

Received: 24 November 2014 / Accepted: 20 April 2015

Abstract A new esterase gene *abmbh*, encoding a benzoate hydrolase which can enantioselectively hydrolyze *l*-menthyl benzoate to *l*-menthol, was recently identified from the genomic library of a soil isolate *Acinetobacter* sp. ECU2040. The *abmbh* gene contains a 1080-bp open reading frame encoding a protein of 360 amino acids with a calculated molecular mass of 40.7 kDa. The corresponding enzyme *Ab*MBH was functionally expressed in *Escherichia coli* BL21 (DE3), purified, and characterized. The *Ab*MBH displayed the maximum activity towards *p*-nitrophenyl butyrate at 50 °C, and an optimum pH of 8.5. A K_M of 2.6 mM and a k_{cat} of 0.26 s^{-1} were observed towards *dl*-menthyl benzoate. The *Ab*MBH exhibited a moderate enantioselectivity (E=27.5) towards *dl*-menthyl benzoate. It can also catalyze the enantioselective hydrolysis of a variety of racemic menthyl esters, including *dl*-menthyl acetate, *dl*-menthyl chloroacetate, and *dl*-menthyl butyrate.

Keywords Esterase · Enzymatic resolution · *l*-Menthol · *dl*-Menthyl benzoate · *Acinetobacter*

Introduction

l-Menthol has unique aroma, refreshing effect, and physiological activity and is therefore widely used in food, medicine, cosmetics, and tobacco industries. From 1998 to 2007, the

Electronic supplementary material The online version of this article (doi:10.1007/s12010-015-1632-0) contains supplementary material, which is available to authorized users.

✉ Gao-Wei Zheng
gaoweizheng@ecust.edu.cn

✉ Jian-He Xu
jianhexu@ecust.edu.cn

[1] Laboratory of Biocatalysis and Synthetic Biotechnology, State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, China

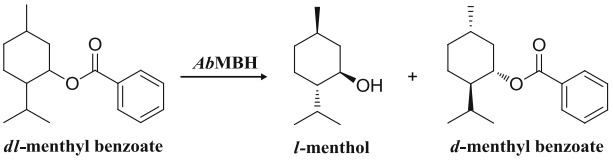
Published online: 01 May 2015

average growth rate of *l*-menthol production was 6.1 %. In 2007, the world total annual production of *l*-menthol reached 19,000 MT, of which 30 % was synthesized by chemical methods [1, 2]. Because the yield and quality of natural menthol depend seriously on weather and region, and its production is no longer able to meet the market demand, more and more researchers and companies focus on chemical synthesis or biotransformation for producing *l*-menthol.

Up to date, chemosynthesis is still the main method for preparing *l*-menthol. For example, Symrise (Germany) developed an industrial route which inoculates racemic supersaturated solution or supercooled melt of *dl*-menthyl benzoate with either *l*- or *d*-menthyl benzoate crystal, making the corresponding enantiomer crystallize preferentially out of the supersaturated solution [3]. Thousands of tons of *l*-menthol per year is being produced via this route. Takasago (Japan) developed an asymmetric synthesis of *l*-menthol by utilizing (*S*)-BINAP-RH as a chiral catalyst for the asymmetric isomerization of geranyldiethylamine, producing 1000 t of *l*-menthol per year [4–6].

However, the use of a large amount of organic solvents and heavy metal catalyst is hazardous to environment and human health. In recent years, as an alternative to chemical catalysis, biocatalysis has attracted an increasing interest because of its high activity; chemo-, regio-, and stereoselectivity; as well as environmental friendliness. Several lipases and esterases have been utilized for either the enantioselective hydrolysis of *dl*-menthyl esters [7–9] or asymmetric acylation of *dl*-menthol to prepare *l*-menthol [10–12]. However, the acyl moiety was mainly an aliphatic carboxylic acid, and seldom enzymes or microbial strains were reported so far that can enzymatically resolve *dl*-menthyl benzoate, an industrially relevant starting compound for production of *l*-menthol in a scale of thousands of tons per year at Symrise. This is probably because there are two bulky groups on both sides of the carbonyl group which are difficult for enzyme to accommodate. Two esterases from *Bacillus* genus, *Bacillus subtilis* BsubE and *Bacillus stearothermophilus* BsteE, were known with poor enantioselectivity (E=10 and 19, respectively) towards *l*-menthyl benzoate [13]. Among 30 commercially available hydrolases, Vorlova and co-workers found that Lipase AY "Amano" could hydrolyze *l*-menthyl benzoate to *l*-menthol (E=15). This is the first lipase reported that is capable of hydrolyzing *l*-menthyl benzoate to *l*-menthol. Taking into account that this commercial lipase is comprised of several isoenzymes that may exhibit opposite stereoselectivities, the most dominant *Candida rugosa* lipase LIP1 was heterogeneously expressed, showing a high enantioselectivity towards *l*-menthyl benzoate (E>100) [7]. An esterase purified from *Burkholderia cepacia* ATCC 25416 could also enantioselectively hydrolyze *l*-menthyl benzoate to *l*-menthol (E=31), although neither the corresponding gene nor the entire protein sequence was reported [14]. An esterase from soil isolate *B. subtilis* ECU0554 showed high hydrolytic activity and excellent enantioselectivity for *dl*-menthyl acetate (E>200); however, the E value for *dl*-menthyl benzoate was merely 11 [9]. Therefore, the discovery of new enzymes hydrolyzing *dl*-menthyl benzoate to produce *l*-menthol is still challenging in view of its importance for menthol industry.

In our previous study, a new bacterial strain, *Acinetobacter* sp. ECU2040, which enantioselectively hydrolyze *dl*-menthyl benzoate to *l*-menthol, was isolated from natural environment [15] and currently deposited in the China General Microbiological Culture Collection Center (deposition number CGMCC8936). In this study, the novel *l*-menthyl benzoate hydrolase from *Acinetobacter* sp. ECU2040 was cloned, expressed, and characterized (Scheme 1). Kinetic constants of the recombinant esterase for *dl*-menthyl benzoate were also determined in this work.



Scheme 1 The enantioselective hydrolysis of *dl*-menthyl benzoate to *l*-menthol by esterase *Ab*MBH

Materials and Methods

Chemicals

dl-Menthol, chloroacetyl chloride, acetyl chloride, *n*-butyric anhydride, and benzoyl chloride were purchased from Alfa Aesar (Tianjin, China). Glycerin tributyrate and Tween-80 were purchased from TCI (Tokyo, Japan). *dl*-Menthyl benzoate, *dl*-menthyl acetate, *dl*-menthyl butyrate, *dl*-menthyl chloroacetate, and *p*-nitrophenyl butyrate (*p*NPB) were synthesized in our laboratory [9, 16]. The other chemicals used in this work were of analytical grade from local sources.

Cloning of *l*-Menthyl Benzoate Hydrolase Gene and Sequence Analysis

The construction of genomic DNA library of *Acinetobacter* sp. ECU2040 was carried out using the shotgun method [17]. The genomic DNA of this strain was prepared using the high-salt method [18] and then partially digested by *Sau*3A I. The DNA fragments of approximately 2–6 kb were pooled, ligated into the pUC118 plasmid (TaKaRa, Dalian) digested by *Bam*H I, and transformed into *Escherichia coli* DH5α competent cells (Tiangen, Beijing). The library was plated onto Luria–Bertani broth (LB) agar plates containing 100 μg mL^{-1} of ampicillin and incubated at 37 °C for 12–16 h. The ampicillin-resistant colonies were picked and inoculated onto LB agar plates containing 1 % (*v*/*v*) glycerin tributyrate as an indicator, and then incubated at 37 °C. Thereafter, the colonies with a colorless and transparent halo, which was the indicator of the enzymatic hydrolysis of glycerin tributyrate, were selected and further verified by measuring their hydrolytic activity and enantioselectivity towards *dl*-menthyl benzoate with gas chromatography (GC). The best colony was used for subsequent study.

The DNA fragments inserted into the positive transformants were sequenced by BGI (Shanghai, China). Nucleotide and deduced amino acid sequence analyses and open reading frame (ORF) determination were performed using the OMIGA (version 2.0) software. BLASTN (http://blast.ncbi.nlm.nih.gov) and UniProt (http://www.uniprot.org) were used to analyze the identities of related nucleotide sequences and deduced amino acid sequences, respectively. Multiple sequence alignment of *Ab*MBH with some related esterase sequences was constructed by the ESPript program (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi).

Gene Expression and Purification of the Recombinant Protein

The *abmbh* gene was amplified from the genomic DNA of *Acinetobacter* sp. ECU2040 using KOD plus DNA polymerase (Toyobo, Tokyo). The forward and reverse primers were 5′-CGGGATCCATGGAGATAGGCATGACAGC-3′ (*Bam*H I) and 5′-CCCAAGCTTACTATTT TTATCCCAGAAC-3′ (*Hind* III), respectively. The resulting PCR products were digested by

*Bam*H I and *Hind* III, ligated into the pET-28a (+) vector (Novagen, Shanghai), and subsequently transformed into *E. coli* BL21 (DE3) competent cells (Tiangen, Beijing). The positive clones were selected and grown in LB media supplemented with kanamycin (50 mg L^{-1}) at 37 °C until an OD_{600} of 0.6–0.8 was reached. The cells were then induced with 0.5 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) and grown for further 12 h at 25 °C.

Thereafter, the cells were harvested, resuspended in buffer A (20 mM sodium phosphate buffer pH 7.4, 1.0 M NaCl, 10 mM imidazole, 10 % glycerol, and 10 mM 2-mercaptoethanol) and disrupted by sonication. The cell lysate was then centrifuged at 12,000×*g* for 20 min. The supernatant was loaded onto a 1-mL NTA-Ni^{2+} agarose column (QIAGEN) pre-equilibrated with buffer A, and then eluted using elution buffer with increasing concentrations of imidazole (10, 30, 50, 100, and 200 mM). Subsequently, the purified enzyme was dialyzed extensively against sodium phosphate buffer containing 0.5 M NaCl to remove imidazole and 2-mercaptoethanol. Finally, the sample was concentrated and stored with 20 % glycerol (*v*/*v*) at −70 °C. The protein concentration was quantified using the Bradford method [19].

Activity Assays

The enzyme activity of *p*NPB was assayed spectrophotometrically using 1.0 mM *p*NPB as substrate. One unit of activity was defined as the amount of enzyme required to liberate 1.0 μmol of *p*-nitrophenol per minute at 30 °C and pH 7.0 [20]. Standard protocol for activity determination of *dl*-menthyl benzoate was carried out in a 0.5-mL reaction mixture, consisting of 10 mM substrate in 100 mM Tris–HCl buffer (pH 8.5) at 30 °C, 1000 rpm for 5 min using a Thermo Shaker Mixer MHR 23 (Digital HLC, Germany). The reaction was stopped and extracted by ethyl acetate. After anhydrated with Na_2SO_4, the conversion rate and enantioselectivity were determined by chiral GC analysis.

The kinetic parameters of the purified *Ab*MBH towards *dl*-menthyl benzoate were determined by assaying the activity (in triplicate) at different concentrations of *dl*-menthyl benzoate (0.5, 1.0, 3.0, 5.0, 7.0, and 10 mM), using standard protocol. The maximal reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_M) of the purified esterase were calculated from the Lineweaver–Burk plot ($1/V=(K_M/V_{max})/[S]+1/V_{max}$, ($V$, mM min^{-1}; S, mM)).

Effects of pH and Temperature on Activity and Stability

The optimum temperature of *Ab*MBH was determined (in triplicate) in 100 mM potassium phosphate buffer (pH 7.0) at different temperatures in the range of 20–60 °C. The thermostability was examined by measuring the residual activity against *p*NPB after incubating the purified esterase (0.1 mg mL^{-1}) at 30, 40, and 50 °C. Aliquots were withdrawn periodically, and the residual activities were assayed as described above.

The optimum pH of *Ab*MBH was determined at 30 °C in triplicate using *dl*-menthyl benzoate as substrate in different buffers (100 mM) within a pH range of 6.0–10.0 (potassium phosphate, 6.0–8.5; Tris–HCl, 8.0–9.0; Na_2CO_3–$NaHCO_3$, 9.0–10.0). A 0.5-mL reaction mixture containing 10 mM *dl*-menthyl benzoate was shaken using a Thermo Shaker Mixer MHR 23 (Digital HLC, Germany). The initial hydrolysis rates of *dl*-menthyl benzoate under various reaction conditions were measured to examine the effect of pH. The spontaneous hydrolysis was determined under the same conditions without the enzyme. The samples were

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

Effect of Metal Ions and Additives on Activity

Influence of various metal ions and additives on enzyme activity was investigated (in triplicate) by adding each compound (final concentration, 1 mM) in the reaction mixture, and subsequently, the enzyme activity was determined. Relative activity was expressed as a percentage of the activity in the absence of any test compound.

Substrate Specificity

The reaction mixture (10 mL) contained the cell-free extract of *Ab*MBH (10 U for *dl*-menthyl acetate, *dl*-menthyl butyrate, and *dl*-menthyl chloroacetate; 100 U for *dl*-menthyl benzoate; the activity was measured using *p*NPB as the substrate), corresponding *dl*-menthyl ester (10 mM) and 0.5 % Tween-80 (*v*/*v*) in potassium phosphate buffer (pH 7.0, 100 mM) in sealed glass vials. The hydrolysis reactions were performed at 30 °C and 180 rpm. The spontaneous hydrolysis and enantiomeric purity were determined by chiral GC analysis.

GC Analysis

The enantiomeric excess of product (ee_p) and conversion of substrate were determined by a gas chromatograph (SHIMADZU GC-2014) equipped with FID detector and CP-Chirasil-Dex CB chiral column (Agilent, 25×0.25×0.25 μm) using N_2 as carrier gas. Both the injector and detector temperatures were set at 280 °C. For *dl*-menthyl acetate, *dl*-menthyl butyrate, and *dl*-menthyl chloroacetate, the column temperature of 130 °C was held for 10, 13, and 25 min, respectively. While for *dl*-menthyl benzoate, the initial column temperature of 130 °C was maintained for 10 min, and then raised to 200 °C at a rate of 10 °C/min, and finally kept at 200 °C for 5 min. Retention times observed were as follows: *d*-menthol, 6.9 min and *l*-menthol, 7.1 min.

Nucleotide Sequence Accession Number

The nucleotide sequences of the *Ab*MBH gene of *Acinetobacter* sp. ECU2040 were deposited in the GenBank database under accession number KJ002562.

Results and Discussion

Cloning and Sequence Analysis of the Esterase Gene

By constructing the genomic library of *Acinetobacter* sp. ECU2040, 50 positive transformants with high activity towards glycerin tributyrate were selected from approximately 14,000 transformants. After rescreening, merely one clone (designated d10) could enantioselectively hydrolyze *dl*-menthyl benzoate to *l*-menthol, which was further investigated.

The inserted fragment in the positive transformant d10 was 2042 bp, which contains three complete open reading frames (ORFs). By BLASTP, only one ORF (ORF_A) shared identities

with many putative or hypothetical esterase genes. The ORF_A was then subcloned into the linear vector pET-28a (+) and transformed into *E. coli* BL21 (DE3). Moreover, the resulting cells could enantioselectively hydrolyze *dl*-menthyl benzoate to *l*-menthol. Therefore, the ORF_A was considered as the target gene and designated as *abmbh* (*Acinetobacter* menthyl benzoate hydrolase gene), and the corresponding encoded protein was designated as *Ab*MBH (*Acinetobacter* menthyl benzoate hydrolase).

Sequence analysis indicated that *abmbh* consisted of 1080 bp with a GC content of 38.2 %, encoding a protein of 360 amino acids with a predicted molecular weight of 40.7 kDa and a predicted isoelectric point of 9.2. Multiple alignment of amino acid sequences showed that *Ab*MBH belongs to the mammalian hormone-sensitive lipase (HSL) family (family IV) [21] (Fig. 1a). The conserved motif GDSAG locates between residues 203–207. A highly conserved catalytic triad of the esterase family is S205, D299, and H329. In addition, a GGG(A)X-motif (GGGF, at sites 132–135) was also found, which can enlarge the space in the oxyanion hole pocket and allow sterically more demanding quaternary alcohol to enter the active site [22]. *Ab*MBH has a higher identity (52–98 %) with the hypothetical hydrolases from multiple *Acinetobacter* strains, however, a lower identity with the enzymes from other genus strains. For instance, only 35 % identity with alpha/beta hydrolase fold family protein from *Mycobacterium yongonense* 05–1390 and 38 % identity with LipN from *Mycobacterium hassiacum* DSM 44199. For some reported esterases, *Ab*MBH has 75 % identity with EstA from *Acinetobacter calcoaceticus* BD413 [23], 82 % identity with EstA from *Acinetobacter lwoffii* I6C-1 [24] and EstA from *Pseudomonas citronellolis* ATCC 13674 [25], and 82 % identity with DPH from *Acinetobacter* sp. M673 [26]. Among the other characterized esterases, *Ab*MBH shared lower identities (34–37 %) with some thermophilic or hyperthermophilic esterases, including Est from *Pyrobaculum calidifontis* VA1 (35 % identity) [27], AFEST from *Archaeoglobus fulgidus* ATCC 49558 (37 %) [28], Sto-Est from *Sulfolobus tokodaii* DSM 16993 (36 %) [29], Est2 from *Alicyclobacillus acidocaldarius* (36 %) [30], and EstE1 from a metagenomic library (34 %) [31]. The phylogenetic analysis of *Ab*MBH with some reported menthyl benzoate hydrolase showed that *Ab*MBH forms a branch with but distinct from LIP1 of *C. rugosa* [7], BsubE of *B. subtilis* [13], BsteE of *B. stearothermophilus* [13], and BSE of *B. subtilis* [32] (Fig. 1b). Alignment of protein sequence showed that *Ab*MBH had no significant sequence homology (<12 %) to the reported *dl*-menthyl benzoate lipase/esterases.

Expression and Purification of the Recombinant Esterase *Ab*MBH

The *l*-menthyl benzoate hydrolase gene was expressed in *E. coli* BL21 (DE3). The His-tagged recombinant protein was then purified from the cell-free extract using NTA-Ni^{2+} agarose affinity chromatography and further analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which corresponded reasonably well to the calculated His-tagged *Ab*MBH molecular mass of 45.7 kDa (Fig. 2).

Effect of pH and Temperature on Activity and Stability

The effect of pH on the crude enzyme activity was examined in the range of pH 6.0–10.0, controlling the hydrolysis degree of *dl*-menthyl benzoate within 10 % conversion. Spontaneous hydrolysis of substrate was not detected. The enzyme exhibited excellent activity in a pH ranging from 8.0 to 9.0 (Fig. S1). The optimal pH of *Ab*MBH was observed at 8.5 in Tris–HCl buffer.

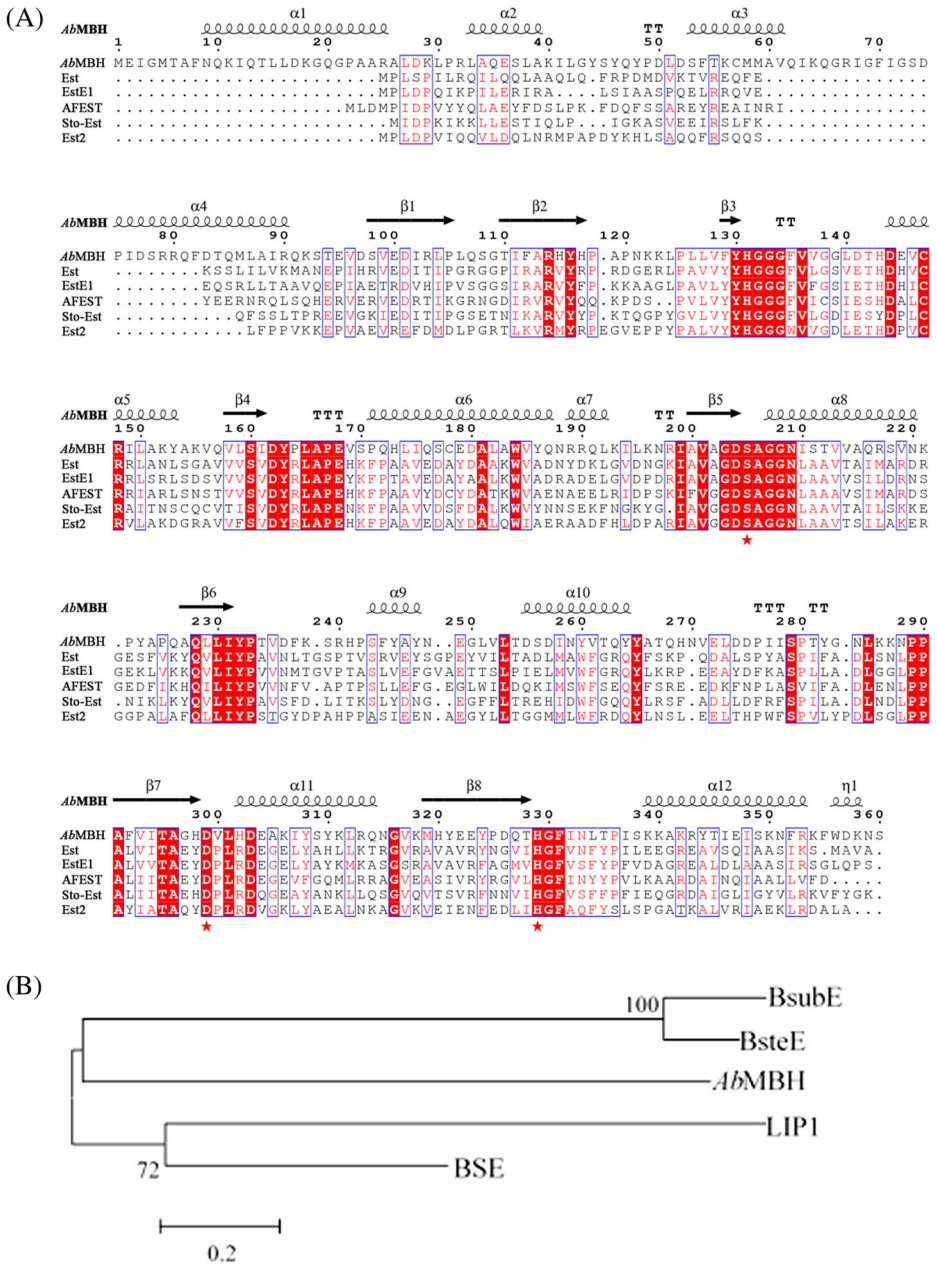


Fig. 1 **a** Multiple sequences alignment of *Ab*MBH with some related esterases: Est of *P. calidifontis* (Q8NKSO), AFEST of *A. fulgidus* (O28558), Sto-Est of *S. tokodaii* (Q976W8), Est2 of *A. acidocaldarius* (Q7SIG1), and EstE1 of uncultured archaea from metagenomic library (Q5G935). The catalytic triad is indicated by the symbol *star*. **b** The phylogeny tree among closely related proteins with menthyl benzoate hydrolysis activity was drawn and tested with 1000 bootstrap. LIP1 of *C. rugosa* [7], BsubE of *B. subtilis* [13], BsteE of *B. stearothermophilus* [13], and BSE of *B. subtilis* [32]

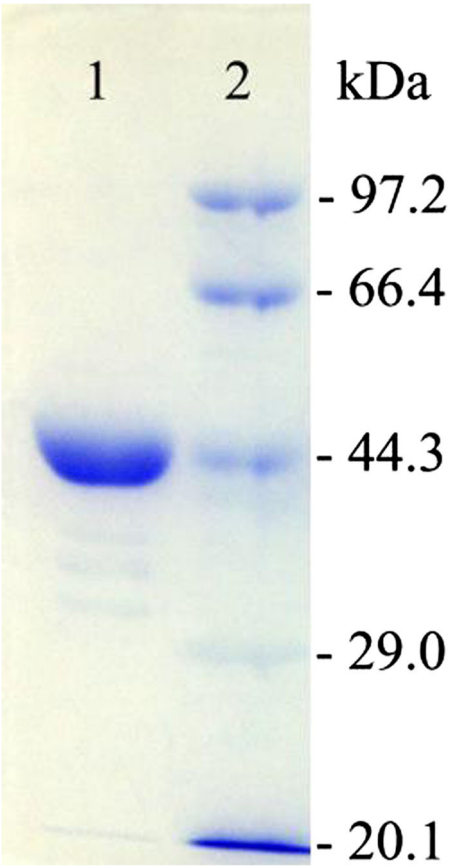


Fig. 2 SDS-PAGE analysis of purified *Ab*MBH. *Lane 1*, purified *Ab*MBH; *lane 2*, protein markers

The effect of temperature on activity and thermostability of *Ab*MBH was determined using *p*NPB as substrate. The maximal activity was observed at 50 °C (Fig. S2). *Ab*MBH was unstable at 40 and 50 °C but quite stable at 30 °C, retaining about 55 % activity after incubation at 30 °C for 15 h (Fig. S3). At 30 °C, the half-life of purified *Ab*MBH was 16.2 h.

Effect of Metal Ions and Additives on Activity

The activity was determined using *p*NPB as the substrate. Relative activity was expressed as a percentage of the activity obtained in absence of any test compound. The enzyme was severely reduced by Ag^{+} and phenylmethylsulfonyl fluoride (PMSF). No ion was found that can activate the esterase. It was surprising that metal-chelating agent EDTA showed 42.7 % inhibition (Table 1).

Activity and Enantioselectivity Towards Various *dl*-Menthyl Esters

Because the size, polarity, and electrophilicity of the substituent (*R*) in the acyl moiety of esters can affect the reactivity of enzymes, we tested the activity and

Table 1 Effects of metal ion and additive on esterase *Ab*MBH activity

Metal ion (1 mM)	Relative activity (%)	Metal ion (1 mM)	Relative activity (%)
None	100±0.4	Al^{3+}	75.9±5.6
Pb^{2+}	97.9±2.2	Fe^{3+}	74.9±1.6
Ca^{2+}	97.5±1.0	Cu^{2+}	72.3±0.7
Mn^{2+}	90.4±4.7	Ag^{+}	0.0
Zn^{2+}	87.1±2.3	EDTA	57.0±3.0
Mg^{2+}	79.1±3.0	PMSF	29.8±7.3
Ni^{2+}	77.6±3.0		

Each compound was added into the reaction solution and subsequently, the enzyme activity was determined. The relative activity was determined under standard conditions. Relative activity was expressed as a percentage of the activity observed in absence of any test compound

enantioselectivity of *Ab*MBH towards various *dl*-menthyl esters with different acyl groups. No spontaneous hydrolysis of these *dl*-menthyl esters was detected under the control experiments in which no enzyme was added. The results revealed that the activity and enantioselectivity of *Ab*MBH differ considerably depending on the acyl group of the menthyl ester (Table 2). *Ab*MBH showed the highest hydrolytic activity on *dl*-menthyl chloroacetate, which might result from the electron-withdrawing substituent of the acyl moiety [34]. However, the enantioselectivity was very poor (E= 4.2). *Ab*MBH exhibited the highest enantioselectivity (E=44.2) towards *dl*-menthyl acetate. The activity of *Ab*MBH decreased dramatically with the increasing of the acyl group size, especially for *dl*-menthyl benzoate. This might be attributed to the two bulky groups, which hinder its movement into the active site of *Ab*MBH.

As shown in Table 3, several esterases or esterase-producing strains were proved with the ability of enantioselective resolution of *l*-menthyl benzoate. Among the recombinant esterases, BsubE, BsteE, and BSE, this newly identified *Ab*MBH displayed the highest enantioselectivity (E=27.5). The specific activity of A*b*MBH was at the same level with BsteE in the hydrolysis of *dl*-menthyl benzoate.

Kinetics of *Ab*MBH-Catalyzed *dl*-Menthyl Benzoate Hydrolysis

The maximal reaction rate (V_{max}) and apparent Michaelis–Menten constant (K_M) of the purified *Ab*MBH were calculated from the Lineweaver–Burk plot. The V_{max} and K_M values are 18.7 μM min^{-1} and 2.6 mM, respectively. The k_{cat} and k_{cat}/K_M values are 0.26 s^{-1} and 0.1 mM^{-1} s^{-1}, respectively.

Table 2 The enantioselectivities of *Ab*MBH towards various *dl*-menthyl esters

Substrate	Time (h)	Conv. (%)	ee_p (%)	E[a]
dl-Menthyl chloroacetate	1	48.2	46.8	4.2
dl-Menthyl acetate	2	40.8	91.7	44.2
dl-Menthyl butyrate	4	39.3	74.3	10.9
dl-Menthyl benzoate	12	43.7	86.3	27.5

[a] Enantioselectivity (E) was calculated according to Chen et al. [33]

Table 3 Comparison on various hydrolases for the preparation of *l*-menthol from *dl*-menthyl benzoate

Enzyme	Spec. activity (U/mg protein)[a]	Conc. (mM)	Time (h)	Conv. (%)	ee_p (%)	*E*	Ref.
LIP1	–	0.5	8	45	>99	>100	[7]
BsubE	0.32	10	4.5	30	–	10	[13]
BsteE	0.34	10	5.5	40	–	19	[13]
Esterase	–	50	20	32.8	90.4	31	[14]
BSE	–	100	12	32	78.1	11	[9]
*Ab*MBH	0.34	10	12	43.7	86.3	27.5	This work

[a] Activity was defined as the amount of enzyme required to liberate 1.0 μmol of menthol per minute

Conclusions

In this work, a novel *l*-menthyl benzoate hydrolase gene *abmbh* was cloned from the genomic library of *Acinetobacter* sp. ECU2040 by shotgun method. *Ab*MBH could efficiently hydrolyze various menthyl esters including *dl*-menthyl acetate, *dl*-menthyl chloroacetate, and *dl*-menthyl butyrate as well as *dl*-menthyl benzoate. *Ab*MBH exhibited the highest activity towards *dl*-menthyl chloroacetate and the highest enantioselectivity towards *dl*-menthyl acetate. After heterogeneous expression in *E. coli* BL21 (DE3) and high-density cultivation in jar fermenter, the *Ab*MBH production increased by more than 100 folds as compared with that of the wild-type strain. Comparing with other reported *dl*-menthyl benzoate lipases/esterases, the enantioselectivity of *Ab*MBH towards *dl*-menthyl benzoate (E=27.5) is moderate. However, since the sequence identity was relatively low (<12 %) with other active hydrolases, this provides a novel *dl*-menthyl benzoate esterase as a starting biocatalyst for directed evolution and rational design, which may provide solutions to remarkably enhance the catalytic activity, enantioselectivity, and/or thermostability [35–39]. All these inspiring results aroused our interest to engineer the enantioselectivity, activity, and operational stability of *Ab*MBH for potential application in menthol industry.

Acknowledgments This work was financially supported by the National Natural Science Foundation of China (Nos. 31200050 and 21276082) and Ministry of Science and Technology, People's Republic of China (Nos. 2011AA02A210 and 2011CB710800).

Ethical Statement This manuscript is original and has not been published and will not be submitted elsewhere for publication. No data has been fabricated or manipulated (including images) to support our conclusions. No data, text, or theories by others are presented as if they were our own.
The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results.

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Clark, G. S. (1998). Menthol. *Perfumer & Flavorist, 23*, 33–46.
2. Clark, G. S. (2007). Aroma chemical profile: menthol. *Perfumer & Flavorist, 32*, 38–47.

3. Fleischer, J., Bauer, K., & Hopp, R. (1976). Separating optically pure *d*- and *l*-isomers of menthol, neomenthol and isomenthol. *US Pat*. 3943181.
4. Akutagawa, S. (1997). Enantioselective isomerization of allylamine to enamine: practical asymmetric synthesis of (−)-menthol by Rh–BINAP catalysts. *Topics in Catalysis, 4*, 271–274.
5. Noyori, R. (2003). Asymmetric catalysis: science and opportunities. *Advanced Synthesis & Catalysis, 345*, 15–32.
6. Trasarti, A. F., Marchi, A. J., & Apesteguia, C. R. (2004). Highly selective synthesis of menthols from citral in a one-step process. *Journal of Catalysis, 224*, 484–488.
7. Vorlova, S., Bornscheuer, U. T., Gatfield, I., Hilmer, J. M., Bertram, H. J., & Schmid, R. D. (2002). Enantioselective hydrolysis of D, L-menthyl benzoate to L-(−)-menthol by recombinant Candida rugosa lipase LIP1. *Advanced Synthesis & Catalysis, 344*, 1152–1155.
8. Yu, L. J., Xu, Y., Wang, X. Q., & Yu, X. W. (2007). Highly enantioselective hydrolysis of *dl*-menthyl acetate to *l*-menthol by whole-cell lipase from *Burkholderia cepacia* ATCC 25416. *Journal of Molecular Catalysis B: Enzymatic, 47*, 149–154.
9. Zheng, G. W., Yu, H. L., Zhang, J. D., & Xu, J. H. (2009). Enzymatic production of *l*-menthol by a high substrate concentration tolerable esterase from newly isolated *Bacillus subtilis* ECU0554. *Advanced Synthesis & Catalysis, 351*, 405–414.
10. Xu, J. H., Kawamoto, T., & Tanaka, A. (1995). High-performance continuous operation for enantioselective esterification of menthol by use of acid anhydride and free lipase in organic solvent. *Applied Microbiology and Biotechnology, 43*, 639–643.
11. Lu, Z. X., Chu, Y., Han, Y. C., Wang, Y. H., & Liu, J. L. (2005). Enzymatic esterification of DL-menthol with propionic acid by lipase from *Candida cylindracea*. *Journal of Chemical Technology and Biotechnology, 80*, 1365–1370.
12. Brady, D., Reddy, S., Mboniswa, B., Steenkamp, L. H., Rousseau, A. L., Parkinson, C. J., Chaplin, J., Mitra, R. K., Moutlana, T., Marais, S. F., & Gardiner, N. S. (2012). Biocatalytic enantiomeric resolution of L-menthol from an eight isomeric menthol mixture through transesterification. *Journal of Molecular Catalysis B: Enzymatic, 75*, 1–10.
13. Henke, E., & Bornscheuer, U. T. (2002). Esterases from *Bacillus subtilis* and *B. stearothermophilus* share high sequence homology but differ substantially in their properties. *Applied Microbiology and Biotechnology, 60*, 320–326.
14. Yu, L. J., Xu, Y., & Yu, X. W. (2009). Purification and properties of a highly enantioselective L-menthyl acetate hydrolase from *Burkholderia cepacia*. *Journal of Molecular Catalysis B: Enzymatic, 57*, 27–33.
15. Ngo-Thi, M. T., Yin, J. G., Pan, J., Zheng, G. W., & Xu, J. H. (2013). Enantioselective hydrolysis of *dl*-menthyl benzoate by cell-free extract of newly isolated *Acinetobacter* sp. ECU2040. *Applied Biochemistry and Biotechnology, 170*, 1974–1981.
16. Qian, L., Liu, J. Y., Liu, J. Y., Yu, H. L., Li, C. X., & Xu, J. H. (2011). Fingerprint lipolytic enzymes with chromogenic *p*-nitrophenyl esters of structurally diverse carboxylic acids. *Journal of Molecular Catalysis B: Enzymatic, 73*, 22–26.
17. Wang, B. Z., Guo, P., Hang, B. J., Li, L., He, J., & Li, S. P. (2009). Cloning of a novel pyrethroid-hydrolyzing carboxylesterase gene from *Sphingobium sp*. strain JZ-1 and characterization of the gene product. *Applied and Environmental Microbiology, 75*, 5496–5500.
18. Miller, S. A., Dykes, D. D., & Polesky, H. F. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research, 16*, 1215.
19. Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.
20. Gao, L., Xu, J. H., Li, X. J., & Liu, Z. Z. (2004). Optimization of *Serratia marcescens* lipase production for enantioselective hydrolysis of 3-phenylglycidic acid ester. *Journal of Industrial Microbiology and Biotechnology, 31*, 525–530.
21. Arpigny, J. L., & Jaeger, K. E. (1999). Bacterial lipolytic enzymes: classification and properties. *Biochemistry Journal, 343*, 177–183.
22. Henke, E., Pleiss, J., & Bornscheuer, U. T. (2002). Activity of lipases and esterases towards tertiary alcohols: insights into structure-function relationships. *Angew Chemical International Edition, 41*, 3211–3213.
23. Kok, R. G., Christoffels, V. M., Vosman, B., & Hellingwerf, K. J. (1993). Growth-phase-dependent expression of the lipolytic system of *Acinetobacter calcoaceticus* BD413: cloning of a gene encoding one of the esterases. *Journal of General Microbiology, 139*, 2329–2342.
24. Kim, H. E., Lee, I. S., Kim, J. H., Hahn, K. W., Park, U. J., Han, H. S., & Park, K. R. (2003). Gene cloning, sequencing, and expression of an esterase from *Acinetobacter lwoffii* I6C-1. *Current Microbiology, 46*, 291–295.
25. Chao, Y. P., Fu, H. Y., Wang, Y. L., Huang, W. B., & Wang, J. Y. (2003). Molecular cloning of the carboxylesterase gene and biochemical characterization of the encoded protein from *Pseudomonas citronellolis* ATCC 13674. *Research in Microbiology, 154*, 521–526.

26. Wu, J., Liao, X. W., Yu, F. B., Wei, Z. B., & Yang, L. Y. (2013). Cloning of a dibutyl phthalate hydrolase gene from *Acinetobacter* sp. strain M673 and functional analysis of its expression product in *Escherichia coli*. *Applied Microbiology and Biotechnology, 97*, 2483–2491.
27. Hotta, Y., Ezaki, S., Atomi, H., & Imanaka, T. (2002). Extremely stable and versatile carboxylesterase from a hyperthermophilic archaeon. *Applied and Environmental Microbiology, 68*, 3925–3931.
28. Manco, G., Giosue, E., D'Auria, S., Herman, P., Carrea, G., & Rossi, M. (2000). Cloning, overexpression, and properties of a new thermophilic and thermostable esterase with sequence similarity to hormone-sensitive lipase subfamily from the archaeon *Archaeoglobus fulgidus*. *Archives of Biochemistry and Biophysics, 373*, 182–192.
29. Suzuki, Y., Miyamoto, K., & Ohta, H. (2004). A novel thermostable esterase from the thermoacidophilic archaeon *Sulfolobus tokodaii* strain 7. *FEMS Microbiology Letters, 236*, 97–102.
30. Manco, G., Adinolfi, E., Pisani, F. M., Ottolina, G., Carrea, G., & Rossi, M. (1998). Overexpression and properties of a new thermophilic and thermostable esterase from *Bacillus acidocaldarius* with sequence similarity to hormone-sensitive lipase subfamily. *Biochemistry Journal, 332*, 203–212.
31. Rhee, J. K., Ahn, D. G., Kim, Y. G., & Oh, J. W. (2005). New thermophilic and thermostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. *Applied and Environmental Microbiology, 71*, 817–825.
32. Zheng, G. W., Pan, J., Yu, H. L., Ngo-Thi, M. T., Li, C. X., & Xu, J. H. (2010). An efficient bioprocess for enzymatic production of L-menthol with high ratio of substrate to catalyst using whole cells of recombinant *E. coli*. *Journal of Biotechnology, 150*, 108–114.
33. Chen, C. S., Fujimoto, Y. F., Girdaukas, G., & Sih, C. J. (1982). Quantitative analyses of biochemical kinetic resolutions of enantiomers. *Journal of the American Chemical Society, 104*, 7294–7299.
34. Barnier, J. P., Blanco, L., Guibejampel, E., & Rousseau, G. (1989). Preparation of (*R*)-veratryl- and (*R*)-(3-methoxybenzyl)succinates. *Tetrahedron, 45*, 5051–5058.
35. Manco, G., Carrea, G., Giosue, E., Ottolina, G., Adamo, G., & Rossi, M. (2002). Modification of the enantioselectivity of two homologous thermophilic carboxylesterases from *Alicyclobacillus acidocaldarius* and *Archaeoglobus fulgidus* by random mutagenesis and screening. *Extremophiles, 6*, 325–331.
36. Valinger, G., Hermann, M., Wagner, U. G., & Schwab, H. (2007). Stability and activity improvement of cephalosporin esterase EstB from *Burkholderia gladioli* by directed evolution and structural interpretation of muteins. *Journal of Biotechnology, 129*, 98–108.
37. Ema, T., Kamata, S., Takeda, M., Nakano, Y., & Sakai, T. (2010). Rational creation of mutant enzyme showing remarkable enhancement of catalytic activity and enantioselectivity toward poor substrates. *Chemical Communication, 46*, 5440–5442.
38. Jochens, H., Aerts, D., & Bornscheuer, U. T. (2010). Thermostabilization of an esterase by alignment-guided focused directed evolution. *Protein Engineering, Design & Selection, 23*, 903–909.
39. Godinho, L. F., Reis, C. R., van Merkerk, R., Poelarends, G. J., & Quax, W. J. (2012). An esterase with superior activity and enantioselectivity towards 1,2-*O*-isopropylideneglycerol esters obtained by protein design. *Advanced Synthesis & Catalysis, 354*, 3009–3015.