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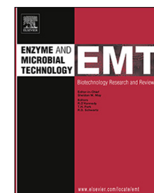
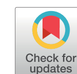


Efficient microbial resolution of racemic methyl 3-cyclohexene-1-carboxylate as chiral precursor of Edoxaban by newly identified *Acinetobacter* sp. JNU9335



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ABSTRACT

Optically active 3-cyclohexene-1-carboxylic acid (CHCA) derivatives are important pharmaceutical intermediates. Due to the special rotatable structure, enantioselective preparation of chiral CHCA is hard to achieve. To identify efficient and enantioselective hydrolases for the biosynthesis of CHCA from methyl 3-cyclohexene-1-carboxylate (CHCM), target-oriented screening from soil samples and gene mining from genome database were explored. All putative hydrolases attempted displayed low enantioselectivity. A hydrolase-producing strain JNU9335 was successfully identified with relatively high enantioselectivity, and was designated as a strain of *Acinetobacter* sp. according to 16S rDNA sequence and phylogenetic analysis. After optimization, strain JNU9335 could produce 233 $U{\cdot}L^{-1}$ hydrolase with *E* value of 21. Isooctane/aqueous biphasic system is favorable for the enzymatic resolution of CHCM, the *E* value of JNU9335 could further be increased to 36. The newly identified JNU9335 could tolerate as high as 1.0 M CHCM, producing (*S*)-CHCM with ee_s of 99.6% and isolation yield of 34.7%. This study provides an efficient biocatalyst for the preparation of chiral 3-cyclohexene-1-carboxylic acid derivatives.

1. Introduction

Optically active 3-cyclohexene-1-carboxylic acid (CHCA) derivatives contain a cyclohexene ring with structural properties distinguished them from cyclohexane, cyclohexandiene, and benzene derivatives, and display special bioactivity [1]. CHCA derivatives have been widely used as building blocks in pharmaceutical and fine chemical industries. For instance, (*S*)-CHCA could be facilely converted into (*S*)-3,4-diaminocyclohexanecarboxylic acid, the key building block for the inhibitor of coagulant factor Xa (fXa), commercially known as Edoxaban for the treatment of cancer-associated venous thromboembolism with superior oral adsorption and lower bleeding risk than other anticoagulants [2–4]. Moreover, it could also be applied in the synthesis of C24-C34 fragment of Prograf and perfume materials such as iris oil [5]. As a result, efficient and enantioselective synthesis of chiral CHCA derivatives is of special interest.

(*S*)-CHCA can be synthesized through chemical resolution of the corresponding racemic methyl 3-cyclohexene-1-carboxylate (*rac*-CHCM) or by asymmetric Diels-Alder reaction [6]. However, these processes depend on stoichiometric amount of expensive chiral resolving agents such as (*S*)-α-methylbenzylamine [7] and (*R*)-α-phenethylamine [8], resulting in low atomic economy. Moreover, these procedures are complicated and discharge toxic acetone etc. Biocatalysts with intrinsically stereoselective structures are superior alternatives to chemical catalysts for the synthesis of chiral building blocks, and have been widely applied in pharmaceutical, agrochemical, materials, flavour and fragrance etc [9–10]. Among various types of stereoselective enzymes, hydrolases have been regarded among the most promising biocatalysts in organic synthesis due to their readily available, mild reaction condition, no requirement of coenzymes and suitability for the synthesis of both enantiomers [11–12]. It is of special interest to identify efficient hydrolases for the preparation of chiral acids, especially (*S*)-CHCA, which are hard to be achieved by other biocatalysts such as oxidoreductases [13–14]. However, although biocatalytic approaches are generally accepted as highly enantioselective and environmentally begnin, few enzymes have been identified with desired activity and enantioselectivity toward CHCM. It might be attributed to its special cyclic and rotatable structures, and highly similar substituents linked to the prochiral carbon. The spatial conformations of (*R*)- and (*S*)-CHCM are quite similar, and there is only tiny difference in steric and electrostatic properties (Fig. S1), which are generally accepted as hard to be discriminated by biocatalysts [15–16]. Commercial

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esterases from pig liver (PLE) and horse liver (HLE) were reported to catalyze the resolution of *rac*-CHCM. About 10 g·L^{-1} *rac*-CHCM (71 mM) was converted into (*S*)-CHCA by 2 mL·L^{-1} PLE with 43% conversion ratio or by 0.2 g·L^{-1} HLE with 41% conversion ratio [17]. The low substrate loading, high price and unstable performance of different batches of commercial enzymes, however, restrict their scale-up application. In fact, commercial PLE and HLE were also evaluated in our previous study. At elevated substrate concentrations, significantly decreased enantioselectivity was observed. Very recently, carboxylesterase BioH involved in biotin synthesis in *Escherichia coli*, was reported to catalyze the hydrolysis of *rac*-CHCM, however, with ee_p of 32.3% and *E* value of 2.1 [18]. Through combinatorial modulation of steric and aromatic interactions, the best mutant Mu3 (L24A/W81A/L209A) was obtained with 70.9% ee_p and *E* value of 7.1 at 40 mM *rac*-CHCM. Moreover, the low aqueous solubility of CHCM and its high saturation vapor are disadvantageous for the biosynthesis at high substrate loading. Therefore, the stereoselective preparation of (*S*)-CHCA or (*S*)-CHCM by biocatalysts remains challenging.

Identification of efficient biocatalysts with desirable enantioselectivity is critical for the biocatalytic synthesis of (*S*)-CHCM, which could be facilely converted into (*S*)-CHCA. Various strategies such as traditional screening from commercial enzymes or enrichment from soil samples, novel screening by genome data mining from genomic or metagenomic libraries, have been developed [19–21]. So far, few hydrolases have been reported with desirable enantioselectivity and activity toward CHCM. In this study, target-oriented systematical screening work including traditional and novel screening methods was attempted to identify novel hydrolases and microbial strains for biocatalytic preparation of (*S*)-CHCM (Fig. 1A). A novel hydrolase-producing strain, *Acinetobacter* sp. JNU9335, was identified, and the fermentation and reaction conditions were optimized to achieve efficient and enantioselective biotransformation. Finally, the potential of the newly identified strain was evaluated in the preparation of (*S*)-CHCM at gram-scale.

2. Materials and methods

2.1. Reagents and strains

Soil samples for enrichment were collected from different provinces of China such as Jiangsu Province, Shaanxi Province, Shandong Province and Jiangxi Province. Racemic methyl 3-cyclohexene-1-carboxylate was purchased from Shanghai Maclean Biochemical Technology Co. Ltd. Peptone and yeast extract were purchased from Oxoid Co. Ltd. Restriction endonucleases and DNA ligase were purchased from Takara Co. Ltd. Bacterial genomic DNA extraction kit and PCR product recovery kit were purchased from Tiangen Biochemical Technology Co., Ltd. Strains for genome data mining were stored in our laboratory. All the metal ions were of analytic grade with purity of > 99.0% and purchased from Sinopharm Reagent Co. Ltd.

2.2. Media

Enrichment medium (per liter): 2 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.5 g $MgSO_4$, 1 g NaCl, 0.1 mmol (the first and second rounds of enrichment) or 0.5 mmol (the third round of enrichment) *rac*-CHCM as the sole carbon source.

Tributyrin agar plate medium (per liter): 10 g of tributyrin, 10 g Tween-80, 16 g peptone, 10 g yeast extract, 5 g NaCl and 15 g agar, pH 7.0, sterilized at 121 °C for 20 min.

Rich medium (per liter): 15 g glycerol, 5 g peptone, 5 g yeast extract, 1 g NaCl and 0.5 g KH_2PO_4, pH 7.0, sterilized at 121 °C for 20 min.

2.3. Genome data mining of hydrolases

Amino acid sequence of *Rh*Est1 was submitted to Uniprot protein database and nine putative hydrolases with 57.4%–77.1% sequence identities were selected. All the nine hydrolases coding genes were amplified from genomic DNA by primers (Supplementary files). The resultant PCR products were ligated by Exnase II (Vazyme Co. Ltd) into pET28a double-digested with *Bam*H I and *Xho* I, and transformed into *E. coli* BL21(DE3). Positive transformants, evaluated by colony PCR and

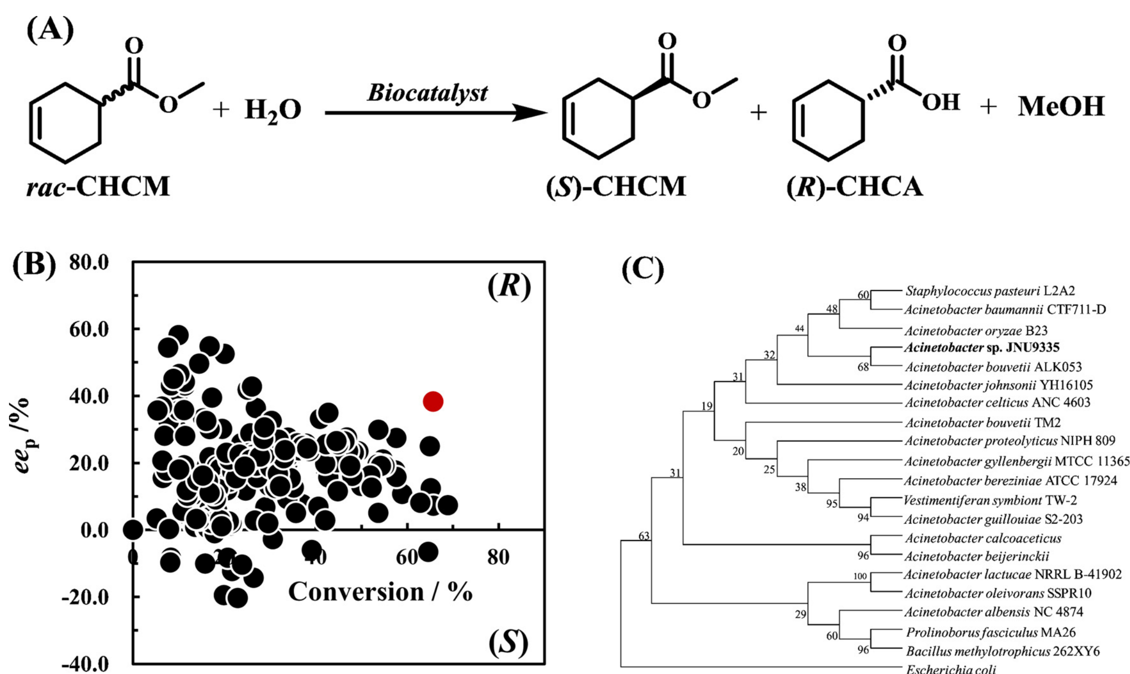

Fig. 1. Screening results of CHCM hydrolase-producing strains.
(**A**) Enantioselective hydrolysis of *rac*-CHCM for the preparation of chiral CHCA derivatives. (**B**) Screening results with *rac*-CHCM as substrate. Strain JNU9335 was shown with red dot. (**C**) Phylogenetic tree of *Acinetobacter* sp. JNU9335. Bootstrap values were based on 1,000 replicates.

sequencing, were inoculated into LB medium supplemented with 50 μg·mL^{-1} kanamycin and cultivated at 37 °C and 180 rpm. When OD_{600} reached 0.6–0.8, 0.2 mM IPTG was added and further cultured at 25 °C and 180 rpm for 12 h. Cells were harvested by centrifuge (8000 × *g*, 4 °C, 10 min), washed with physiological saline solution and disrupted by sonication. Cell debris was removed by centrifuge to obtain the crude extract.

2.4. Determination of enzyme activity

The activity of recombinant hydrolases toward CHCM was determined in reaction mixture containing 50 mM *rac*-CHCM, 5% (v/v) DMSO, PBS (100 mM, pH 7.0) and appropriate amount of hydrolases at 30 °C and 180 rpm. Reactions were carried out at 20 °C and 180 rpm until conversion ratios reached 50%. Samples were withdrawn, treated by addition of 1.0 M HCl to terminate the reaction, and extracted with equal volume of ethyl acetate (1 mM dodecane as the internal standard), dried over anhydrous sodium sulfate and analyzed by chiral gas chromatography. One unit (U) of activity was defined as the amount of enzymes required for producing 1.0 μmol of CHCA per minute at above-mentioned condition. Protein concentration was determined by Bradford method with BSA as standard protein.

2.5. Enrichment and screening of wild strains

Three rounds of enrichment were conducted to screen for hydrolase-producing bacteria with capacity in enantioselective resolution of *rac*-CHCM. In the first round of enrichment, fresh soil samples were suspended in the enrichment medium, supplemented with 10 mM of *rac*-CHCM and 5% (v/v) dimethyl sulfoxide (DMSO) as co-solvent, and cultured for 48 h at 30 °C and 180 rpm. Afterwards, appropriate amount of cultures was transferred into fresh enrichment medium, with supplement same as the first round of enrichment, and further cultured for 48 h. In the third round of enrichment, the concentration of *rac*-CHCM was enhanced to 50 mM due to the strong volatility, and the culture was further cultivated at 30 °C and 180 rpm for 48 h. Then 100 μL of enriched cultures was spread on a tributyrin agar plate and further cultured at 30 °C for 24 h until bright halos appear. All single colonies with bright halos were inoculated into the rich medium and cultivated at 30 °C and 180 rpm for 24 h. Cells were collected by centrifuge (8,000 × *g*, 4 °C, 10 min), lyophilized under vacuum and −40 °C to obtain cruder power of JNU9335 and stored at 4 °C for further use. Appropriate amount of cells was suspended in a mixture of 9.5 mL of PBS (100 mM, pH 7.0). To start the reaction, 0.5 mL of 50 mM (*R*, *S*)-CHCM and 5% (v/v) dimethyl sulfoxide (DMSO) were added and further stirred at 30 °C and 180 rpm. Samples were extracted and analyzed as above described.

Morphology of strain JNU9335 was observed by microscope (Model CME, Leica Co. Ltd, USA). Genome DNA of JNU9335 was extracted, and its 16S rDNA was amplified with primers (27F: 5'-AGAGTTTGATCCT GGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3'). The PCR program consisted of pre-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 1 min, elongation at 72 °C for 2 min, and further elongation at 72 °C for 10 min. The resultant PCR product was extracted and purified from the agarose gel by DNA recovery kit, and sequenced by Genewiz Co. Ltd.

2.6. Optimization of the fermentation medium

Effects of carbon sources, nitrogen sources, metal ions and fermentation time of strain JNU9335 on the production and enantioselectivity of hydrolase were investigated. Different carbon sources including glucose, mannose, glycerol, maltose, xylose, lactose, sucrose or fructose were added in the rich medium at 15 g·L^{-1}. Various nitrogen sources and combinatorial ratios including yeast extract, peptone, beef extract, yeast extract/peptone (1:1), peptone/beef extract (1:1), yeast extract/beef extract (1:1) were supplemented in the rich medium. Different metal salts including $Al_2(SO_4)_3$, $MgSO_4$, $MnSO_4$, $FeSO_4$, $CuSO_4$, $CaCl_2$ or $ZnSO_4$ were also tested and without addition of metal ions was regarded as control. Culture was withdrawn from fermentation broth at 6, 12, 18, 24, 30 and 36 h to test the influence of fermentation time on hydrolytic activity and enantioselectivity of the hydrolase produced by JNU9335.

For each reaction, about 0.025 g·mL^{-1} resting cells were suspended and applied in the activity assay with 50 mM *rac*-CHCM as above mentioned. Reactions were carried out at 30 °C and 180 rpm for 0.5 h. Then, reactions were acidified, extracted and analyzed as above described. All reactions were conducted in triplicate.

2.7. Effect of reaction conditions on the kinetic resolution of rac-CHCM by JNU9335

Influence of reaction conditions including pH, temperature and two-phase were also investigated, including pH range of 4.0 to 10.0 using Na_2HPO_4-Citric acid buffer (pH 4.0–6.0), Na_2HPO_4-NaH_2PO_4 buffer (pH 6.0–8.0) and CHES-NaOH buffer (pH 8.0–10.0), temperatures ranging from 20 to 40 °C, nine co-solvents (5%, v/v) including ethanol, methanol, isopropanol, acetonitrile, THF, acetone, dioxane, METB and DMF, and six different organic solvents (50% v/v) including isopropyl ether, toluene, cyclohexane, n-hexane, n-heptane and isooctane as the organic phase. Different ratios of isooctane and aqueous phase from 5:5, 6:4, 7:3, 8:2 to 9:1 were also evaluated in the resolution of *rac*-CHCM. Control was conducted without addition of organic phase. All reactions were performed in triplicate. The analytical yield and *ee* values were determined by chiral GC analysis.

Effect of substrate concentrations (100 mM, 200 mM, 500 mM and 1.0 M) on the resolution of *rac*-CHCM by JNU9335 was also investigated under optimized reaction conditions. The pH of reaction mixture was maintained at pH 8.0 by titration with 1.0 M NaOH. Reactions were stopped when conversions reached 50%.

2.8. Biocatalytic preparation of (S)-CHCM at gram scale

Preparation of (*S*)-CHCM in an isooctane/aqueous biphasic system was carried out in a 250 mL sealed three-necked flask. One gram of JNU9335 dry cells was suspended in 25 mL PBS (200 mM, pH 8.0), and the reaction was initiated by addition of 7.0 g *rac*-CHCM (1.0 M, dissolved in 25 mL isooctane). Samples were withdrawn periodically and subjected to analysis of conversion ratio and enantioselectivity. After 24 h, reaction was terminated and extracted for three times with equal volume of isooctane. The organic phases were combined and dried over anhydrous Na_2SO_4 and evaporated under vacuum to obtain purified (*S*)-CHCM. Structure of (*S*)-CHCM was characterized by ^{1}H-NMR and ^{13}C-NMR.

2.9. Analysis of the CHCM and CHCA

Both enantiomers of CHCM and CHCA were analyzed by GC equipped with a B-DM column (0.25 mm × 30 m), with N_2 as carrier gas. The temperatures of injector and detector were 280 °C, and the column temperature was kept at 50 °C for 5 min, increased to 180 °C at 5 °C·min^{-1} and kept for 5 min. The retention time of (*S*)-CHCM, (*R*)-CHCM, dodecane, (*R*)-CHCA, (*S*)-CHCA was 18.58, 18.73, 20.49, 27.46 and 27.68 min respectively. The enantiomeric excess (*ee*) and enantioselectivity (*E*) value were calculated according to the following formula.

$$ee_p(\%) = \frac{A_R - A_S}{A_R + A_S} \times 100,$$

(A_R and A_S denote the peak area of (*R*)- and (*S*)-CHCA).

Table 1
Comparison of putative hydrolases identified by genome data mining in the resolution of *rac*-CHCM.

Entry	Source strain	Identity [%]	Specific activity [U mg^{-1}]	Time [h]	ee_p [%]	*E* value
*Rh*Est1	*Rhodococcus* sp. ECU1013	100	17.2 ± 1.0	0.5	5.5 (*S*)	1.1
Hyd1	*Rhodococcus pyridinivorans*	68.8	10.8 ± 0.5	2.0	5.3 (*S*)	1.1
Hyd2	*Rhodococcus opacus*	77.1	42.9 ± 3.4	0.5	5.6 (*R*)	1.1
Hyd3	*Rhodococcus koreensis*	76.0	0.7 ± 0.1	6.0	13.3 (*S*)	1.3
Hyd4	*Rhodococcus fascians*	73.5	7.1 ± 0.4	3.0	0.4 (*S*)	1.0
Hyd5	*Rhodococcus kyotonensis*	73.5	4.2 ± 0.2	3.0	2.0 (*R*)	1.0
Hyd6	*Rhodococcus tukisamuensis*	73.5	2.5 ± 0.1	6.0	13.5 (*S*)	1.4
Hyd7	*Rhodococcus hoagii*	75.3	0.7 ± 0.1	6.0	2.2 (*S*)	1.0
Hyd8	*Rhodococcus ruber*	72.6	21.0 ± 0.8	0.5	2.4 (*R*)	1.1
Hyd9	*Hoyosella subflava*	57.4	0.5 ± 0.1	6.0	25.7 (*S*)	1.7

$$E\ \text{value} = \frac{\text{Ln}[1-\text{Conversion ratio}\times(1+ee_p)]}{\text{Ln}[1-\text{Conversion ratio}\times(1-ee_p)]}$$

3. Results

3.1. Genome data mining for CHCM hydrolases

*Rh*Est1 from *Rhodococcus* sp. ECU1013 belongs to carboxylesterase family, and exhibited hydrolytic activity toward esters of cyclopropanecarboxylate, cyclobutanecarboxylate and cyclopentanecarboxylate for producing corresponding chiral acids [22]. Herein, *Rh*Est1 was evaluated in the hydrolysis of *rac*-CHCM. Activity analysis revealed that it could catalyze the hydrolysis of *rac*-CHCM with specific activity of 17.2 U·mg^{-1}. Thus, nine putative hydrolases with 57.4–77.1% identities toward *Rh*Est1 were cloned and heterogeneously expressed in *E. coli* BL21(DE3) (Fig. S2).

Activities of all nine hydrolases were evaluated in the hydrolysis of CHCM (Table 1). The specific activities of Hyd2 from *Rhodococcus opacus* and Hyd8 from *Rhodococcus ruber* were 42.9 and 21.0 U·mg^{-1}, representing 2.49- and 1.22-fold of *Rh*Est1 respectively. To determine the enantioselectivity accurately, reaction time was strictly controlled to maintain conversion ratio of less than 30%. Hyd2, Hyd5 and Hyd8 exhibited (*R*)-preference, while other hydrolases including *Rh*Est1, Hyd1, Hyd3, Hyd4, Hyd6, Hyd7 and Hyd9 were (*S*)-preference. The highest enantioselectivity of 25.7% ee_p was found with Hyd9, much higher than 15.5% of *Rh*Est1 and 8.2% of HLE. However, the specific activity of Hyd9 was merely 0.5 U·mg^{-1}. It can be concluded that CHCM can be readily hydrolyzed by putative recombinant hydrolases, however, the enantioselectivity is relatively low.

3.2. Traditional screening from soil samples

To obtain microbial strains with high substrate tolerance, three rounds of target-oriented enrichment was adopted with increasing concentrations of CHCM from 10 mM to 50 mM. A total of 248 strains with clear halos on tributyrin plate and different morphology were isolated to evaluate their capacity in the hydrolysis of CHCM. The conversion and ee_p values of all strains were determined and illustrated in Fig. 1B. The ratio of (*R*)-preferred strains (91.1%) is much higher than that of (*S*)-preferred strains (8.9%), which is different from putative hydrolases identified by genome data mining. One strain, designated as JNU9335, exhibited 38.3% ee_p and 60.6% conversion ratio in the primary screening (red dot in Fig. 1B). For JNU9335, it should be noted that only (*S*)-CHCM was residual in the reaction mixture with over 99% ee_s at conversion ratio over 60%. Further evaluation at elevated substrate concentrations revealed that JNU9335 could efficiently catalyze the enantioselective hydrolysis of *rac*-CHCM. Considering the catalytic efficiency and enantioselectivity, JNU9335 was selected for the development of biocatalytic synthesis of (*S*)-CHCM or (*S*)-CHCA.

The 16S rDNA of JNU9335 was sequenced and submitted to NCBI under accession number of MK571155. BLAST of nucleotide sequence in NCBI database revealed that all strains, except for uncultured bacterium, with > 99% sequence identity to the 16S rDNA of JNU9335 belong to *Acinetobacter* clan, such as 99.4% identity of *Acinetobacter johnsonii* strain YH16105. The phylogenetic tree of JNU9335 with *Acinetobacter*, *Escherichia*, and *Bacillus* species was constructed (Fig. 1C) to indicate their close relationship with *Acinetobacter* species. Considering its high evolutional homology with *Acinetobacter* species, strain JNU9335 was named as *Acinetobacter* sp. JNU9335 and deposited at China General Microorganisms Collection and Management Center under accession number of CGMCC No. 17220.

3.3. Effect of fermentation conditions on activity and enantioselectivity of JNU9335

Effect of various fermentation conditions including carbon sources, nitrogen sources, metal ions and fermentation time were investigated on the production of CHCM hydrolase by JNU9335 (Fig. 2). All the carbon sources were supplemented at the same loading. Different carbon sources had significant influence on enzyme production and enantioselectivity of JNU9335. Glucose is favorable for the hydrolase production, with enzyme production of 224 U·L^{-1}, followed by 220 and 210 U·L^{-1} of mannose and glycerol respectively (Fig. 2A). The enzyme production of JNU9335 is 166 U·L^{-1} with xylose, 25.9% lower than that of glucose. With regard to enantioselectivity, sucrose is the best carbon source with *E* value of 20, followed by glycerol, xylose and lactose with *E* values of 17. The *E* value of enzymes produced with glucose as carbon source was 13, much lower than that of sucrose. The lowest *E* value was 12 with maltose as carbon source. In view of high enzyme production level and enantioselectivity, glycerol was selected as the carbon source for CHCM hydrolase production of JNU9335.

Different nitrogen sources including peptone, yeast extract and beef extract were also examined. As shown in Fig. 2B, addition of organic nitrogen sources had no promotive effect on hydrolase production. The highest hydrolase production of 233 U·L^{-1} was achieved with combination of peptone and yeast extract. However, the enantioselectivity of JNU9335 was not enhanced. Addition of beef extract resulted in decreased production and enantioselectivity of hydrolase. As a result, complex nitrogen of peptone and yeast extract was selected as nitrogen source for enzyme production of JNU9335.

Considering metal ions might activate the activity of hydrolase, various metal ions were supplemented in the medium. As shown in Fig. 2C, almost all the tested metal ions led to decreased enzyme production except for Cu^{2+} with enzyme production slightly increased to 228 U·L^{-1}. However, addition of metal ions exhibited distinct influence on enantioselectivity of JNU9335. The *E* value was increased from 17 (control) to 20 in the presence of Ca^{2+}, Al^{3+}, Mn^{2+} and Fe^{2+}. In favor of high enzyme production and enantioselectivity, Al^{3+} was supplemented in the fermentation medium.

Fermentation time might also affect CHCM hydrolase production and enantioselectivity of JNU9335. In Fig. 2D, enzyme production

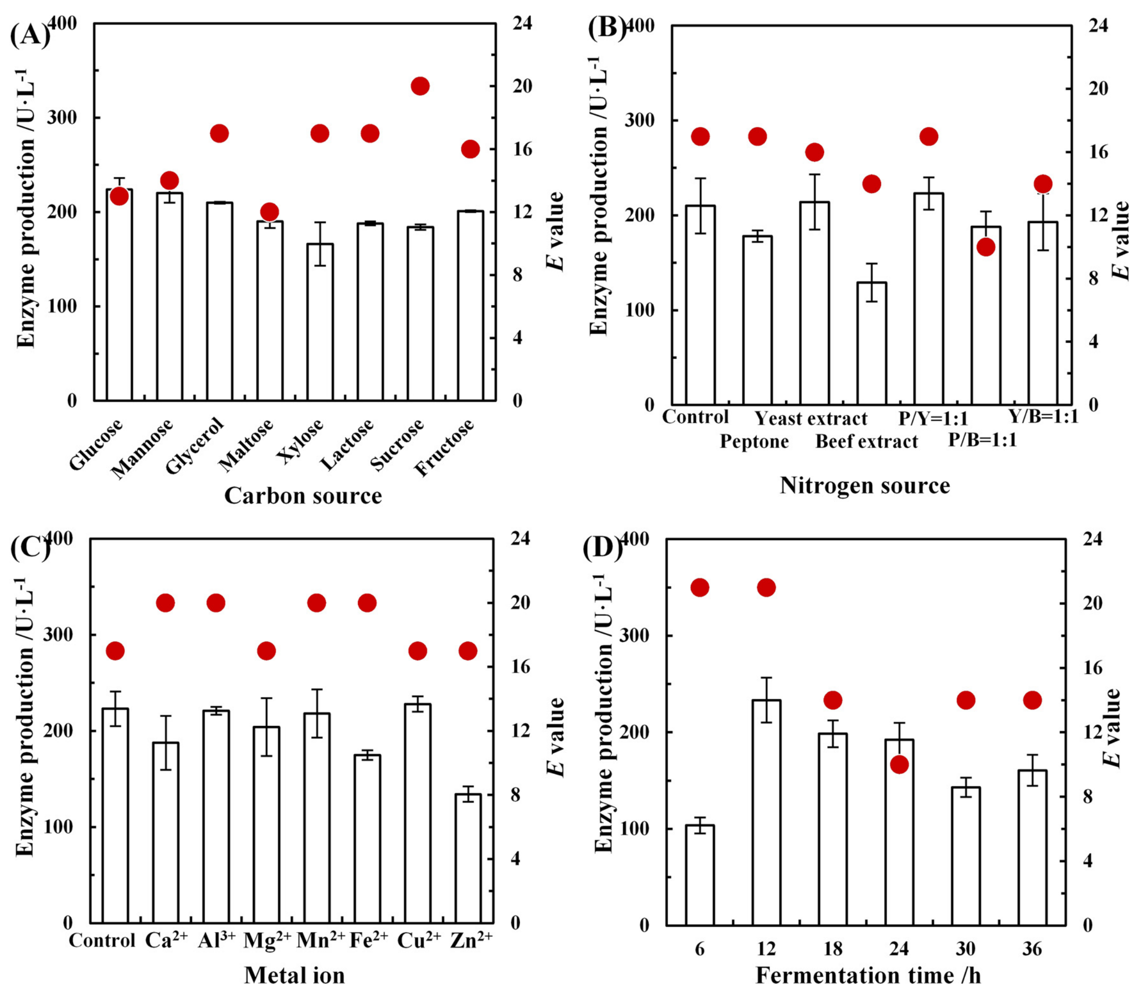

Fig. 2. Effect of fermentation conditions on enzyme production and enantioselectivity of JNU9335.
(**A**) Carbon sources (15 g·L^{-1}); (**B**) Nitrogen sources (10 g·L^{-1}), P/Y: peptone/yeast extract, P/B: peptone/beef extract, Y/B: yeast extract/beef extract; (**C**) Metal ions (0.2 g·L^{-1}), (**D**) Fermentation time. Column: enzyme production, red dot: *E* value.

increased rapidly in the first 12 h to 233 U·L^{-1} which is 2.24-fold of that at 6 h (104 U·L^{-1}), then gradually decreased with prolonged fermentation time. Fermentation time also plays an important role in enantioselectivity, since there might be multiple hydrolases with hydrolytic activity toward CHCM in JNU9335. The *E* value was 21 at 6 and 12 h, then decreased to 14 at 36 h. Thus, 12 h is regarded as the preferable fermentation time for production of CHCM hydrolase by JNU9335.

3.4. Effect of reaction conditions on conversion and enantioselectivity of JNU9335

With the increase of temperature, conversion ratios raised accordingly, and the highest conversion ratio of 30.3% was detected at 35 °C (Fig. 3A). However, the *E* values decreased alongside the increase of reaction temperature. To achieve high conversion ratio and *E* value, 20 °C was regarded as the optimal temperature for resolution of *rac*-CHCM catalyzed by JNU9335. Different buffers with pH ranging from 4.0 to 10.0 were also examined (Fig. 3B). First of all, no spontaneous hydrolysis of *rac*-CHCM was found in all the tested buffers. Low conversion ratios were noted at pH ≤ 6.0 and pH ≥ 10.0. Higher conversion ratios were observed at pH 7.0–9.0, and the optimal pH is pH 8.0 with the highest conversion ratio of 32.7% and *E* value of 19. At pH 8.0, compared with NaH_2PO_4-Na_2HPO_4 buffer, JNU9335 displayed higher conversion ratio and *E* value in CHES-NaOH buffer. In addition, the hydrolysis of CHCM into CHCA results in decreased pH. To develop an efficient biotransformation approach, the reaction pH should be maintained at around pH 8.0 especially at high CHCM loading.

Due to the low solubility and high saturation vapor pressure of CHCM in aqueous system, co-solvents or organic aqueous biphasic system were introduced to improve the mass transfer and decrease the loss of substrate. However, all the co-solvents resulted in decreased conversion (Fig. 4A). Furthermore, the supplemented co-solvents are hard to be separated from the products. Biphasic systems consisted of organic solvents and aqueous phase could be more suitable [23]. Six organic solvents including isopropyl ether, toluene, cyclohexane, hexane, n-heptane and isooctane were investigated. As illustrated in Fig. 4B, activity of JNU9335 was significantly influenced by isopropyl ether and toluene, giving only 5.7% and 4.0% conversion ratios respectively. Although no organic solvent exhibited positive effect on the activity of JNU9335, its enantioselectivity could be enhanced by hexane, n-heptane and isooctane. The *E* value of JNU9335 in the presence of isooctane was 36, much higher than that of the control. Furthermore, different ratios of isooctane and aqueous were also investigated (Fig. 4C). With the increase of isooctane to 90% (v/v), *E* values decreased significantly. Ratio of 5:5 was desirable for the preparation of chiral CHCA derivatives catalyzed by JNU9335.

3.5. Resolution of rac-CHCM into (S)-CHCM catalyzed by JNU9335

Substrate loading and biocatalyst dosage were also optimized to establish an efficient biocatalytic approach for practical synthesis of the chiral precursor of Edoxaban. Initial experiments were conducted with

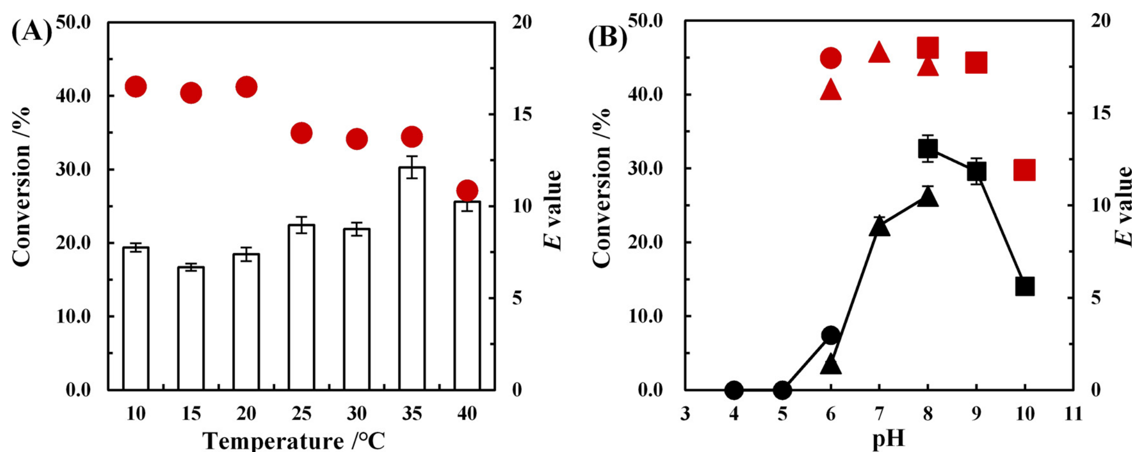

Fig. 3. Effect of temperature and pH on the resolution of CHCM.
(**A**) Temperature, column: conversion, red dot: *E* value. (**B**) pH, black & red dots: conversion and *E* values at pH 4.0–6.0 of Na_2HPO_4-citric acid buffer, black & red triangles: conversion and *E* values at pH 6.0–8.0 Na_2HPO_4-NaH_2PO_4 buffer, black & red squares: conversion and *E* values at pH 8.0–10.0 of CHES-NaOH buffer.

4 g·L^{-1} JNU9335 dry cells and 0.1 M *rac*-CHCM dissolved in 10 mL PBS buffer (pH 8.0, 100 mM) and 10 mL isooctane respectively, and the pH of reaction mixture was maintained at 8.0 by titration with 1.0 M NaOH at 0.1 M *rac*-CHCM (Table 2, **entry 1**). The conversion ratio reached 50.1% rapidly in 6 h. Further experiments were performed at 0.2 M *rac*-CHCM employing 8 g·L^{-1} JNU9335 dry cells (Table 2, **entry 2**). At the end of 6 h, conversion ratio reached 51.5%, and the ee_p and *E* value were 82.8% and 30.6 respectively. Stable performance of the newly identified biocatalyst encouraged us to further increase substrate loading, since the substrate loading of over 100 g·L^{-1} is essential for an efficient biocatalytic process [24]. At 0.5 M *rac*-CHCM, 51.6% of conversion ratio could be achieved in the end of 8 h, with ee_p of 82.2% and *E* value of 29.2 (Table 2, **entry 3**). Longer reaction time was required for higher substrate loading. Further reaction was attempted at 1.0 M *rac*-CHCM (140 g·L^{-1}) with 20 g·L^{-1} biocatalyst dosage. Within 12 h, the conversion ratio reached 50.5% and ee_p was 82.5%, resulting in *E* value of 28.6 (Table 2**, entry 4**). It should be noted that JNU9335 could tolerate as high as 1.0 M *rac*-CHCM without significant sacrifice of enantioselectivity. Chiral (*S*)-CHCM could be obtained by precisely controlling conversion ratios for a complete hydrolysis of (*R*)-CHCM.

Application of JNU9335 for the synthesis of (*S*)-CHCM at gram-scale was further evaluated. In a 50-mL reaction mixture, 7.0 g *rac*-CHCM (1.0 M) dissolved in 25 mL isooctane was thoroughly mixed with 1 g JNU9335 cells in 25 mL PBS buffer (Table 2, **entry 5**). Reaction was mechanically agitated at 200 rpm and 20 °C. During the initial 6 h, the conversion ratio rapidly increased to 45.8%, with ee_s of 68.3% (Fig. 5). Then the hydrolytic reaction slowed down because most of the preferable enantiomer (*R*)-CHCM had been hydrolyzed by JNU9335 after 6 h. The conversion ratio slightly increased to 62.8% at 24 h, with ee_p of 59.0% and *E* value of 21.9 toward product. At this time point, almost all the (*R*)-CHCM was hydrolyzed into (*R*)-CHCA (Fig. 5), while the unreacted (*S*)-CHCM was mainly remained in isooctane phase with ee_s of 99.6% and *E* value of 910 toward CHCM. Then, the reaction was terminated by adjusting the pH to 10.0, and (*S*)-CHCM were isolated and extracted with equal volume of isooctane for three times. About 2.43 g (*S*)-CHCM was obtained with isolated yield of 34.7% and characterized by ^{1}H-NMR and ^{13}C-NMR. **(*S*)-CHCM**: ^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.54–5.80 (m, C*H*=C*H*, 2 H), 3.68 (d, *J* =5.6 Hz, OCH_3, 3 H), 2.46–2.65v (m, C*H*COOCH$_3$, 1 H), 2.17–2.32 (m, =CHCH_2CH, 2 H), 2.04–2.16 (m, =CHCH_2CH2, 2 H), 1.87–2.02 (m, CH$_2$CH_aH$_b$CH, 1 H), 1.54–1.74 (m, CH$_2$CH$_a$$H_b$CH, 1 H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 175.71, 126.35, 124.96, 51.18, 38.95, 27.24, 24.87, 24.22. **(*S*)-CHCA**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 11.66 (s, COO*H*, 1 H), 5.47–5.85 (m, C*H*=C*H*, 2 H), 2.76–2.51 (m, C*H*-CH$_2$, 1 H), 2.25 (d, *J* = 25.6 Hz, CH_2-CH, 2 H), 2.08 (t, *J* = 32.2 Hz, CH$_a$$H_b$-C$H_2$, 3 H), 1.54–1.80 (m, C$H_a$Hb-CH$_2$, 1 H). ^{13}C-NMR (101 MHz, $CDCl_3$) δ 182.78, 126.75, 125.03, 39.22, 27.20, 24.86, 24.35.

4. Discussion

Optically active 3-cyclohexene-1-carboxylic acid (CHCA) and derivatives are important chemical compounds with wide application as building blocks in pharmaceutical and fine chemical industries [1]. Development of synthetic process for the efficient synthesis of CHCA is of special interests. However, the chemical routes dependent on sacrifice of stoichiometric amount of chiral resolution reagents. Moreover, no biocatalytic route has been reported at high substrate loading with high enantioselectivity for the synthesis of chiral CHCA, although biocatalysts are generally favored due to various advantageous [11,12]. Hence, efficient and enantioselective synthesis of chiral CHCA remains challenging.

To establish biocatalytic process for the synthesis of chiral (*S*)-CHCA, various strategies were attempted in this study to explore naturally evolved biocatalysts, including putative enzymes from databases and microorganism from soil samples. Genome data mining is regarded as an efficient method to identify new enzymes. This method, however, requires sequences of known enzymes as probe. Since few recombinant hydrolyases capable of hydrolyzing *rac*-CHCM have been reported, protein sequence of *Rh*Est1, with high activity and enantioselectivity in the preparation of chiral 2,2-dimethylcyclopropanic acid, was adopted as the probe for genome data mining. It should be noted that all the putative enzymes were active in the hydrolysis of *rac*-CHCM, especially Hyd2 from *R. opacus* with specific activity of 42.9 U·mg^{-1}. However, the enantioselectivity was not satisfactory. Only Hyd9 from *Hoyosella subflava* displayed ee_p of 25.7% (*S*) and *E* value of 1.7 in the resolution of *rac*-CHCM. Enantioselective synthesis of chiral CHCA are hard to be achieved by above recombinant biocatalysts.

Traditional screening from soil samples by enrichment with substrates as the sole carbon source is the most straightforward and target-oriented method to identify novel microorganisms with desirable activity, especially in the circumstance of lacking recombinant enzymes. Moreover, to obtain robust biocatalysts with high tolerance to high concentration of substrate and/or product. Three rounds of enrichment with elevated CHCM concentration were adopted. Finally, a strain, designated as *Acinetobacter* sp. JNU9335, was obtained. Fermentation conditions were investigated. Glycerol, complex nitrogen source of peptone and yeast extract, Al^{3+} and fermentation for 12 h were the most suitable conditions for production of CHCM hydrolase in JNU9335. The CHCM hydrolase in JNU9335 might be constitutionally expressed. Furthermore, various reaction factors, such as temperature

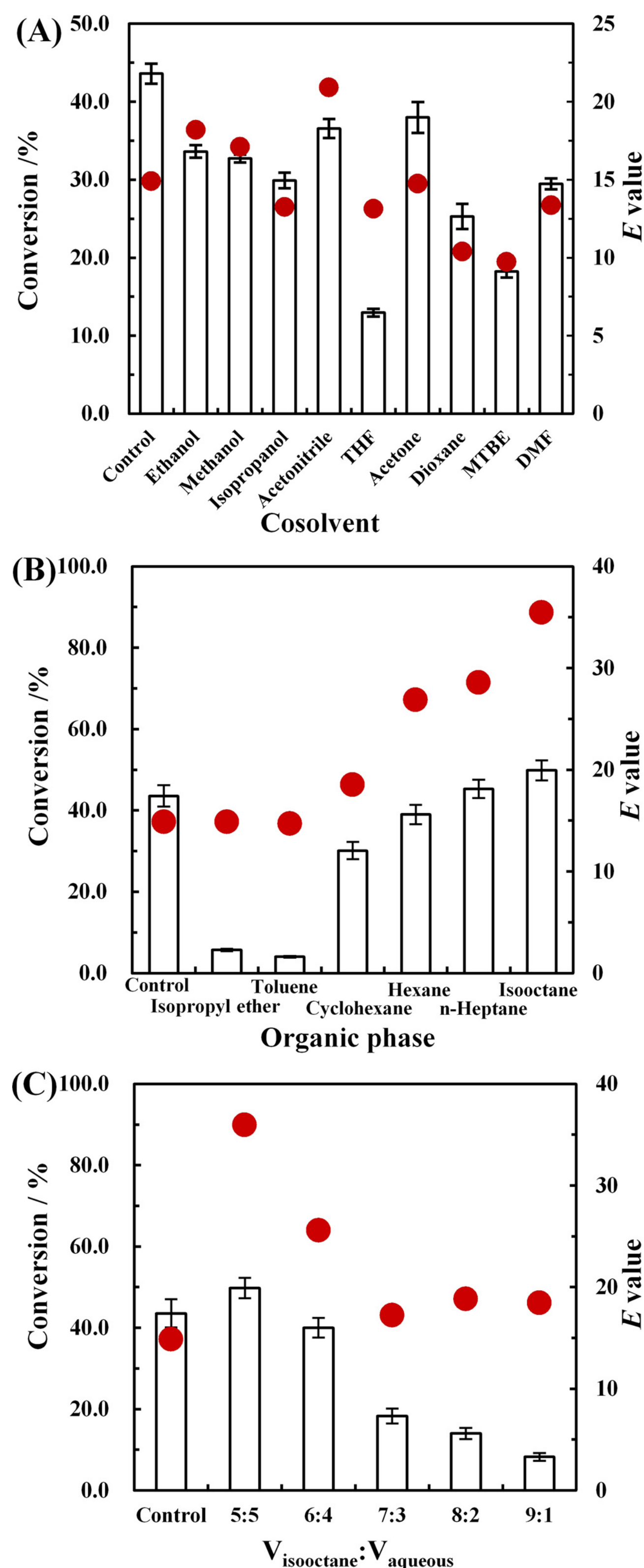

Fig. 4. Effect of co-solvents and organic phases on the conversion and *E* value of JNU9335 catalyzed enantioselective resolution of *rac*-CHCM.
(**A**) co-solvents, 5% (v/v). (**B**) organic phases, 50% (v/v). (**C**) ratios of isooctane and aqueous. Column: conversion, red dot: *E* value.

and pH which might also influence the enantioselectivity through manipulating fast and slow reactions [25], were systematically explored. The optimal temperature and pH were 20 °C and 8.0 for the high enantioselectivity. Isooctane was the most suitable organic phase for the kinetic resolution of *rac*-CHCM. At isooctane and aqueous ratio of 5:5, the highest *E* value of 36 could be achieved. The reaction rate of slow reaction (hydrolysis of (*S*)-CHCM) might be decreased. Under the optimal conditions, the newly identified JNU9335 could tolerate as high as 1.0 M *rac*-CHCM (140 $g{\cdot}L^{-1}$), exhibiting promising potential in practical synthesis of chiral CHCA [24].

To the best of our knowledge, the newly identified *Acinetobacter* sp. JNU9335 was the first reported microorganism capable of enantioselective resolution of *rac*-CHCM. The enantioselectivity of JNU9335 is higher than putative hydrolases obtained by genome data mining. Biotransformation process for the synthesis of (*S*)-CHCM, a key precursor of Edoxaban, was developed at 1.0 M *rac*-CHCM catalyzed by JNU9335, significantly higher than 40 mM of recombinant BioH mutants (Table 3) [18]. The space-time yield of JNU9335 was 48.6 $g{\cdot}L^{-1}{\cdot}d^{-1}$, 2.7-fold of commercial PLE, ranking the highest record for the preparation of chiral (*S*)-CHCA derivatives. Identification of the key hydrolase responsible for the hydrolysis of CHCM in JNU9335 is being conducted to further improve the performance of biocatalytic synthesis of optically active CHCA derivatives.

5. Conclusions

Genome data mining and traditional enrichment from soil samples were explored to identify efficient and enantioselective biocatalysts for the synthesis of chiral 3-clycohexene-1-carboxylic acid. A hydrolase-producing strain *Acinetobacter* sp. JNU9335 was identified by target-oriented three rounds of enrichment. Conditions for enzyme production and microbial resolution were optimized. Isooctane/aqueous biphasic system was proved to be the most effective for the reaction. Enantioselective resolution of as much as 1.0 M *rac*-CHCM to produce (*S*)-CHCM was achieved by JNU9335 with ee_s of 99.6% and isolation yield of 34.7%.

Author Agreement

All authors have seen and approved the final version of this submission. This article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants performed by any of authors.

CRediT authorship contribution statement

Zhe Dou: Investigation, Formal analysis, Writing - original draft. **Guochao Xu:** Methodology, Validation, Writing - review & editing. **Ye Ni:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

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Table 2
Kinetic resolution of *rac*-CHCM employing JNU9335 at elevated substrate loadings.

Entry	Substrate (M)	Cell ($g{\cdot}L^{-1}$)	Time (h)	Conv. (%)	ee_p (%)	*E* value
1	0.1	4	6	50.1	85.8	36.2
2	0.2	8	6	51.5	82.8	30.6
3	0.5	16	8	51.6	82.2	29.2
4	1.0	20	12	50.5	82.5	28.6
5	1.0	20	24	62.8	59.0 (99.6)[a]	21.9 (910)[b]

[a] Number in parentheses denotes ee_s.
[b] Number in parentheses denotes *E* value calculated according to CHCM.

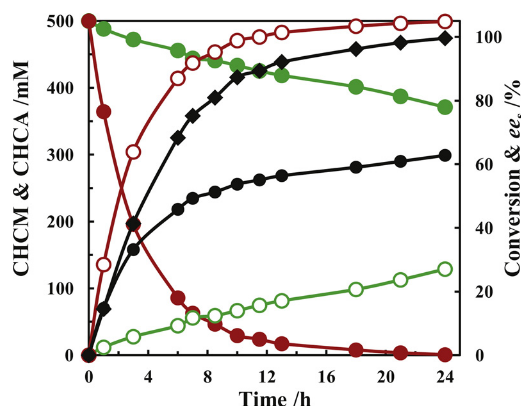

Fig. 5. Gram-scale enantioselective preparation of (*S*)-CHCM at 1.0 M *rac*-CHCM catalyzed by JNU9335 in isooctane/aqueous biphasic system. Black solid dot: conversion, black diamond: ee_s, red solid dot: (*R*)-CHCM, red hollow dot: (*R*)-CHCA, green solid dot: (*S*)-CHCM, green hollow dot: (*S*)-CHCA. Reaction consisted of 7.0 g *rac*-CHCM (50 mmol) dissolved in 25 mL isooctane and 1.0 g JNU9335 dry cells dissolved in PBS buffer (pH 8.0, 100 mM). (*R*)- and (*S*)-CHCA, (*R*)- and (*S*)-CHCM were determined by GC equipped with a chiral B-DM column (0.25 mm × 30 m) and dodecane as the internal standard.

Table 3
Comparison of microbial kinetic resolution of *rac*-CHCM.

Biocatalyst	*rac*-CHCM [mM]	Time [h]	Yield [%]	*E* value	S. T. Y. [$g\,L^{-1}\,d^{-1}$]	Reference
PLE	71	4	43	*n.a.*[a]	18	[17]
HLE	71	6	41	*n.a.*[a]	16.4	[17]
BioH	40	1	24.9[b]	2.2	*n.a.*[c]	[18]
$BioH_{Mu3}$	40	1	21.3[b]	7.1	*n.a.*[c]	[18]
JNU9335	1000	24	34.7	21.9 (910)[d]	48.6	This study

[a] *n.a.*: *E* value was not available because of lacking conversion ratios.
[b] Numbers refer to conversion ratios.
[c] *n.a.*: Space-time yield (S. T. Y.) was not available because of lacking isolation yield.
[d] Number in parentheses denotes *E* value calculated according to CHCM.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.enzmictec.2020.109580.

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