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A novel lipase from *Aspergillus oryzae* catalyzed resolution of (*R*,*S*)-ethyl 2-bromoisovalerate

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Abstract

In this study, a novel lipase M5 derived from *Aspergillus oryzae* WZ007 was prone to exhibit high hydrolytic activity and stereoselectivity towards racemic substrate (*R*,*S*)-ethyl 2-bromoisovalerate. (*R*)-ethyl 2-bromoisovalerate was obtained by enzymatic resolution, which is the key chiral intermediate for highly efficient enantiomerically fluvalinate. The results showed that the enzymatic reaction was carried out in 120mM racemic substrate for 3 hours, the enantiomeric excess reached 98.6%, the conversion was 51.7%, and *E* value above 120. Therefore, the novel lipase M5 has the ability to efficiently produce (*R*)-ethyl 2-bromoisovalerate, which greatly reduces the industrial production cost of the highly efficient counterpart of fluvalinate.

KEYWORDS

Aspergillus oryzae, biocatalysis, enantioselectivity, lipase, (R)-ethyl 2-bromoisovalerate

1 | INTRODUCTION

Chiral pesticides are pesticides with chiral characteristics, including racemates and nonracemates. A large number of drugs currently including pesticides are chiral molecules and are composed of enantiomers having different biological activities. The enantiomers of chiral pesticides often exhibit stereoselectivity in the biological systems, because all biomacromolecules (such as proteins, polysaccharides, and nucleic acids) produced by the organisms are chiral. These can be identified under the selective action of various environmental carriers, thereby inhibiting biological activities such as insecticidal, acaricidal, and environmental toxicity, and enrichment, distribution, degradation, and metabolism show significant enantioselectivity differences.[1,2] Because of the existence of a chiral center, although the physical and chemical properties of the two enantiomers may be similar, their biological activities may vary diversely or completely. For example, one enantiomer has high activity or complete activity, while other enantiomers have low or no activity at all, such as diclofop-methyl, glufosinate, and fluvalinate. There is an antagonistic or counteracting effect between the activities of the enantiomers, resulting in a mixture that is much less active or completely inactive than the individual enantiomers.[3,4] As mentioned above, the biological activities of different enantiomers may vary greatly; therefore, the development of optically pure pesticides based on highly active substances is beneficial to greatly improve their effectiveness and efficiency.

(*R*)-ethyl 2-bromoisovalerate is an important pharmaceutical intermediate for the preparation of the highly efficient counterpart of fluvalinate (its structure as shown in Figure 1), which a highly efficient, broad-spectrum non–cyclopropanecarboxylic acid pyrethroid insecticide, acaricide. There are many methods for synthesizing fluvalinate, but it is known that in the process of synthesizing fluvalinate, the raw material generally adopts the more expensive *D*-valine.[5] The synthesis of 2-(2-chloro-4-(trifluoromethyl)phenylamino)-3-methylbutanoic acid

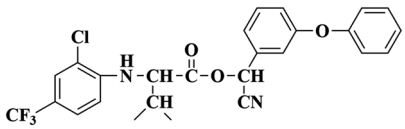

FIGURE 1 The structure of fluvalinate

by *D*-valine can directly obtain the chiral product dextrorotatory acid, which can directly obtain a pair of high efficiency of fluvalinate composed of dextrorotatory acid and racemic cyanohydrin from industrial applications without resolution, but the raw materials are more expensive.[6] Therefore, it is necessary to explore cheap raw materials and simple reaction routes to reduce costs and improve economic efficiency. This study attempts to synthesize the fluvalinate intermediate (*R*)-ethyl 2-bromoisovalerate, obtained by enzymatic catalyzed highly efficient resolution of the cheaper raw material (*R*,*S*)-ethyl 2-bromoisovalerate, which it is rarely reported.

Lipases (triacylglycerol hydrolase, EC 3.1.1.3) are the most popular enzymes in biocatalysis that catalyzes ester hydrolysis.[7-11] So far, lipases have been used in many fields, and its source can be derived from bacteria such as *Aspergillus* sp, *Streptomyces* sp, *Pseudomonas* sp, and many more microbes.[12] It is well known that resolution racemates are one of the main applications of lipases.[13-15] In previous experiments, we have performed studies on the recombinant *Pichia pastoris* that highly efficient express the *Aspergillus oryzae* lipase gene to produce lipase, which was specifically applied to the substrate (*R*,*S*)-methyl 2-(4-hydroxyphenoxy) propanoate.[16] The *Escherichia coli* Rosetta (DE3) competent cell strain has been specially treated that supplemented six rare codons (AUA, AGG, AGA, CUA, CCC, and GGA) lacking in *E coli* and enhances the expression level of foreign genes in the prokaryotic system, especially eukaryotic genes.[17,18] The expression of foreign genes in *E coli* Rosetta makes the construction of engineering bacteria more convenient and effective, which is suitable for constructing a large number of engineering bacteria. In this study, we used the novel recombinant *E coli* Rosetta cells expressing *A oryzae* lipase gene as the biocatalysts and investigated the influences of reaction factors, such as reaction temperature and substrate concentration, on the enzyme catalyzes enantioselective hydrolysis of racemic substrate (*R*,*S*)-ethyl 2-bromoisovalerate. Although we have done the experiments of enzyme purification and enzymatic properties, while limited by the length of the article, this content has not been included in the paper.

2 | MATERIALS AND METHODS

2.1 | Materials

A oryzae WZ007 was isolated from soil samples by our laboratory, which was deposited at China-type culture collection (Wuhan, China) under the accession number of CCTCC No: M 206105. Novozym 435 (CAL-B, derived from lipase B of *Candida antarctica* immobilized on macroporous polyacrylate resin; 10 000 U/g), Lipozyme RM IM (RML, derived from lipase of *Rhizomucor miehei* immobilized on ionic resin; 20 000 U/g), and Lipozyme TL IM (TLL, derived from lipase of *Thermomyces lanuginosus* immobilized on silica; 50 000 U/g) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Lipase PS (PCL, lipase from *Pseudomonas cepacia*; 10 500 U/g) was purchased from Purolite Ltd (Wales, UK). (*R*,*S*)-ethyl 2-bromoisovalerate was purchased from Aladdin Chemistry Co Ltd (Shanghai, China). Other chemical reagents were analytically pure and were commercially available.

2.2 | Gene cloning and overexpression

The cells of *A oryzae* WZ007 preserved in our laboratory were first grown, and mRNA was extracted and was converted to cDNA through reverse transcriptase. The coding sequence (Figure S1; GenBank: MN399852) was cloned on the basis of the base sequence of the *A oryzae* lipase gene (GenBank: NWUI02000090.1), which was obtained at National Center for Biotechnology Information (NCBI) database. The amino acid sequence alignment showed that it was 98.34% similar to sequence ID: AP007159.1 and 99.14% similar to sequence ID: XM_023235897.1 from *A oryzae* and the similarity to sequence (GenBank: RAQ53993.1) from *Aspergillus flavus* was 100%.[19] The lipase gene was amplified from *A oryzae* WZ007 using the forward primer PaEST F (5′-ATGAAGATTCAAAAACTCATTCTGTACA-3′) and reverse primer PaEST R (5′-CTCGAGTTAA-TATGCATACTTTGCAATGTCAGGC-3′). The polymerase chain reaction (PCR) was performed under the following conditions: DNA template was denatured at 98°C for 5 minutes, 30 cycles of 98°C for 10 seconds, 60°C for 10 seconds, and 72°C for 1.5 minutes, and finally extended by 72°C for 10 minutes. Next, the obtained PCR products was purified using a genomic DNA fragment rapid purification kit, and inserted into a pET-28b(+) vector, and then transformed into *E coli* Rosetta competent cells. The positive transformants were incubated in kanamycin-added medium at 37°C, 200 rpm. When the OD_{600} values reached 0.4 to 0.6, the inducer isopropyl-

β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.2mM, and then the gene overexpression was induced at 22°C for 8 to 10 hours. The expression level of the protein was 1.0 ± 0.05 mg/mL with good repeatability in different fermentations. Subsequently, the cells were collected by centrifugation (8000 rpm, 4°C, and 10 min) and were lyophilized using a vacuum freeze dryer (cold trap temperature −80°C, vacuum degree 10 Pa) as the biocatalyst (lipase M5-containing *E coli* whole cell biocatalyst) for further experiments.

2.3 | Substrate specificity of lipase M5

The substrate specificity was determined by using saturated fatty acid ethyl esters with varying chain length (C4, C6, C8, C10, C12, C14, C16, and C18) as substrates at the final concentration of 30mM, and the enzyme activity was measured by gas chromatography (GC) under standard assay conditions.

2.4 | Biocatalytic hydrolysis of (*R*,*S*)-ethyl 2-bromoisovalerate

The biocatalytic reactions were carried out in 50-mL three-necked flask by adding 30mM to 270mM substrate, 0.4 g of lyophilized lipase M5 cells, and 5% (v/v) cosolvent in 10-mL (0.2M, pH 8.0) phosphate buffer (PB) system. Reaction was performed at 30°C and stirred at 600 rpm using a magnetic stirring device. The pH was controlled by autotitration using 0.5M NaOH solutions. Scheme 1 shows that the enzymatic hydrolysis protocol towards substrate (*R*,*S*)-ethyl 2-bromoisovalerate. After a prescribed reaction time period, 1-mL reaction solution was taken out as the sample and immediately inactivated by acidification with 4M HCl solution, then extracted with ethyl acetate, and the product concentration of (*R*)-ethyl 2-bromoisovalerate was further determined by GC. Figure 2A,B shows the gas chromatograms of racemic (*R*,*S*)-ethyl 2-bromoisovalerate and the hydrolytic product (*R*)-ethyl 2-bromoisovalerate, respectively.

To validate the separation and quantification, standard solutions (30mM-270mM substrate concentration) were prepared and analyzed by GC. The peak areas were calculated versus substrate concentration against substrate. Standard curves were prepared using the concentrations of (*R*,*S*)-ethyl 2-bromoisovalerate and the corresponding peak area as the abscissa and the ordinate, respectively. There was a good linear relationship between substrate concentration and peak area, as shown in Figure S2.

2.5 | Analytical method

The conversion and enantiomeric excesses of the substrate (*R*,*S*)-ethyl 2-bromoisovalerate were measured by GC. The GC used was an Agilent 7890A with a chiral capillary column (BGB-175, 30.0 m × 0.25 mm × 0.25 μm), an autoinjector (Agilent 7683B), and a flame ionization detector (FID) and was operated in split mode with the split ratio of 20:1. The injector and FID temperatures were maintained at 250°C. The column temperature was held at 90°C to 120°C at a rate of 1°C/min. The carrier gas was high-purity nitrogen, and the flow rate was 0.5 mL/min. The air flow rate and the hydrogen flow rate were 300 and 30 mL/min, respectively. The amount of each injection was 1 μL. The retention times of (*R*)-ethyl 2-bromoisovalerate and (*S*)-ethyl 2-bromoisovalerate were 17.9 and 18.2 minutes, respectively.

One unit (U) of lipase hydrolysis activity was defined as the amount of enzyme that hydrolyzes 1-μmol substrate per minute under assay conditions (30°C, pH 8.0). The enantiomeric excess ($e.e._s$), conversion (C), and enantiomeric ratio (E) of the substrate (*R*,*S*)-ethyl 2-bromoisovalerate were calculated according to the equations reported by Chen et al[20] as follows:

$$\text{e.e.}_s = \frac{[R]-[S]}{[R]+[S]} \times 100\% \quad (1)$$

$$C\frac{C_0 - C_t}{C_t} \times 100\% \quad (2)$$

$$E = \frac{\ln[(1-C)(1-e.e._s)]}{\ln[(1-C)(1-e.e._s)]} \quad (3)$$

where [*R*] and [*S*] refer to the peak areas of GC corresponding to (*R*)-ethyl 2-bromoisovalerate and (*S*)-

SCHEME 1 The lipase M5-containing *E coli* whole cell biocatalyst catalyzed the resolution of substrate (*R*,*S*)-ethyl 2-bromoisovalerate

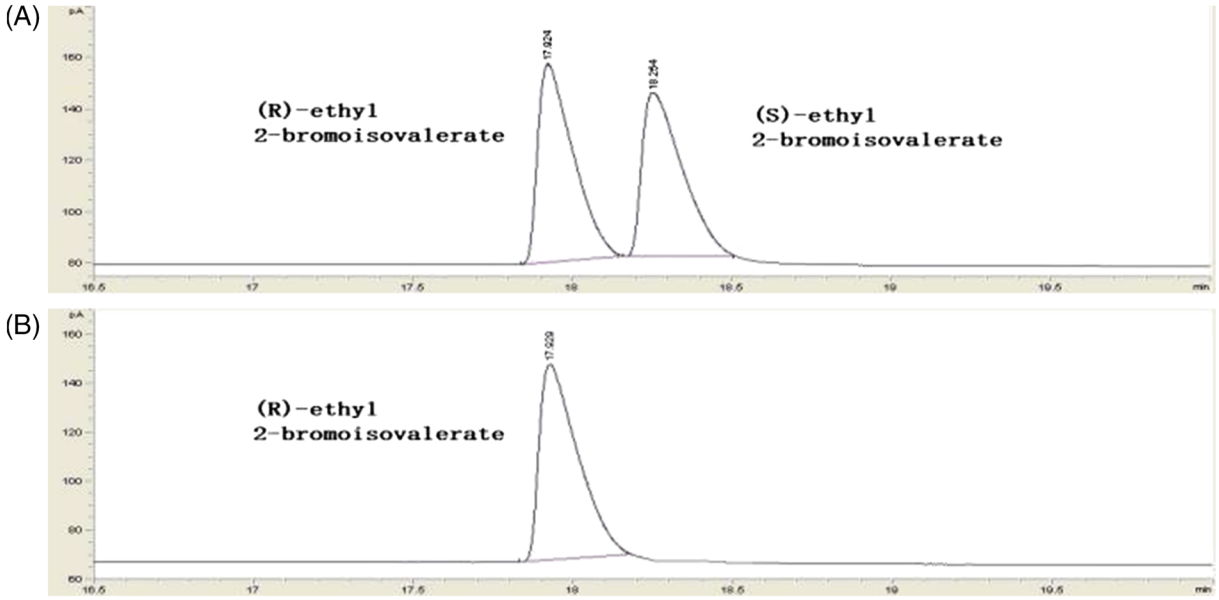

FIGURE 2 Gas chromatogram analysis of (*R*,*S*)-ethyl 2-bromoisovalerate and the hydrolysis products. A, Raceme of (*R*,*S*)-ethyl 2-bromoisovalerate. B, The hydrolytic product (*R*)-ethyl 2-bromoisovalerate

ethyl 2-bromoisovalerate, respectively; C_0 is the initial reaction concentration (mM) of the substrate, and C_t is the remaining substrate concentration (mM) after a certain period of reaction.

2.6 | Experimental reproducibility

All experiments in this study were performed in triplicate, and the standard deviation was calculated by the software package SPSS Statistics 20 (SPSS Inc) to confirm the obtained results. Multivariate analysis of variance (ANOVA) and Tukey test were performed to determine significant differences for each sample, $P < .05$. Data presented in the tables and figures were expressed as an average ± standard deviation of the three values obtained.

3 | RESULTS AND DISCUSSION

3.1 | Screening of lipases

Lipases are acceptable for nonnatural substrates, and they exhibit different enantioselectivity to the substrate.[21] (*R*)-ethyl 2-bromoisovalerate is an important intermediate for the synthesis of fluvalinate; the different commercial lipases (Lipase M5, Novozym 435, Lipozyme RM IM, Lipozyme TL IM, and lipase PS) were screened for enantioselective hydrolysis of (*R*,*S*)-ethyl 2-bromoisovalerate. As shown in Table 1, among the four commercially available lipases, lipase PS has exhibited the highest specific activity on the substrate, but the selectivity was poor. However, the lipase M5-containing *E coli* whole cell biocatalyst (lipase M5) has a higher catalytic activity than lipase PS, and *E* value was the highest.

TABLE 1 Results of hydrolysis of (*R*,*S*)-ethyl 2-bromoisovalerate catalyzed by different lipases[a]

Lipases	Source	Specific Activity,[b] U/mg	Conversion, %	$e.e._s$, %	E
Lipase M5	*A oryzae*	6.5	51.8	93.6	50.4
Novozym 435	*C antarctica*	4.5	36.2	10.2	1.6
Lipozyme TL IM	*T lanuginosus*	2.9	22.9	8.4	1.9
Lipozyme RM IM	*R miehei*	1.3	10.5	5.2	2.7
Lipase PS	*P cepacia*	7.5	60.1	73.2	6.0

[a]Conditions: 0.02-g lipase, 5-μL (30mM) racemic substrate, 1000 rpm, 35°C, pH 7.0 PB, reaction time 2 h.
[b]The specific activity of lipases was calculated as units of enzyme hydrolytic activity possessed by 1 mg of biocatalyst.

Under the catalytic reaction of lipase M5, the substrate conversion and enantiomeric excess were significantly higher than commercial lipase. Thus, lipase M5 has the potential to hydrolyze the substrate (*R*,*S*)-ethyl 2-bromoisovalerate and was selected for further study.

3.2 | Substrate specificity

Substrate specificity is a parameter important for the enzyme in the application process. It is generally measured by catalyzing the activity of esters of different carbon chain lengths.[22] In this study, the substrate specificity of lipase M5-containing *E coli* whole cell biocatalyst was investigated using saturated fatty acid ethyl esters with different acyl chain lengths (C4-C18) as substrates. The data showed that the enzyme showed more specificity towards medium chain fatty acid ethyl esters (maximum specificity for C8, 100%, set as reference) (Figure 3). However, with the extension of the acyl chain (>C8), the hydrolytic activity of the enzyme gradually decreases. Since the ability to catalyze the hydrolysis long acyl chain substrates (≥C10) was a typical behavior of lipases,[23] the enzyme M5 from *A oryzae* WZ007 was noted to be a lipase.

3.3 | Optimization of biocatalytic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate

3.3.1 | Effect of pH on biocatalytic resolution

pH can change the charge density on the cell surface and the molecular structure of the enzyme. This will result in a change in the rate at which the substance enters and exits the cell, followed by a change in the activity and selectivity of the enzyme.[24,25] Therefore, pH plays an important role on enzyme activity. As shown in Figure 4, we can see that the activity of the enzyme gradually increases in the pH range of 6.5 to 8.0, the conversion increases from 30.8% to 43.3%, and $e.e._s$ also increases significantly. When the pH of the reaction medium was 8.0, the enzymatic activity and the optical purity of substrate reached a maximum, the conversion and $e.e._s$ reached 43.3% and 89.9%, respectively. However, when pH >8.0, the substrate began to hydrolyze spontaneously, $e.e._s$ decreased with increasing pH, and the enantioselectivity of the enzyme decreased. Therefore, it was optimal to control the reaction pH to 8.0.

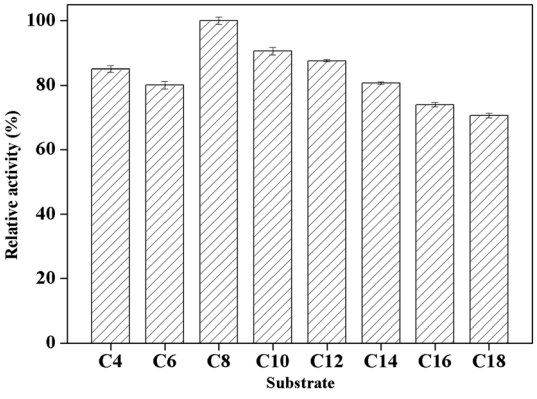

FIGURE 3 Substrate specificity of lipase M5-containing *E coli* whole cell biocatalyst to different fatty acid ethyl esters

3.3.2 | Effect of temperature on biocatalytic resolution

Temperature plays an important role in the thermodynamic equilibrium of the reaction and greatly affects the catalytic activity of the enzyme.[26,27] The effect of temperature on the enzymatic resolution of racemic substrates was found to be in the range of 15°C to 60°C (Figure 5). It was perceived that, as the temperature gradually increases, the enzyme activity first rises and then starts declining. The enzyme activity continuously increased at temperatures ranging from 15°C to 30°C. Maximum activity was noted at 30°C, at which time the conversion was 44.6%, and $e.e._s$ was 92.6%. However, when the temperature exceeded 40°C, the enzyme activity began to decrease rapidly, and the conversion rate and $e.e._s$ show a consistent trend with the enzyme activity. This indicates that high temperatures lead to partial inactivation of the enzyme, which seriously impairs the activity and

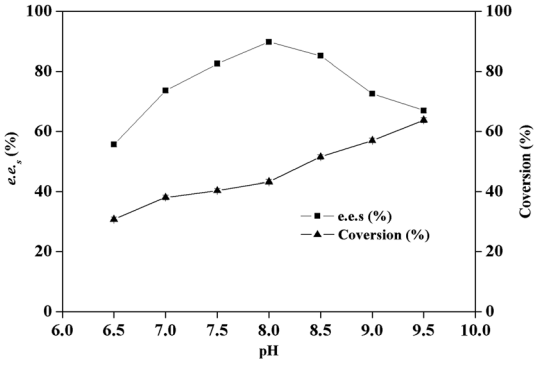

FIGURE 4 Effects of pH on the enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 0.02-g lipase M5-containing *E coli* whole cell biocatalyst, 5-μL (30mM) racemic substrate, 1000 rpm, 35°C, reaction time 2 h, and reaction volume 1 mL. 0.2M phosphate buffer: pH 6.5-8.0; 0.2M Tris-HCl buffer: pH 8.5-9.5

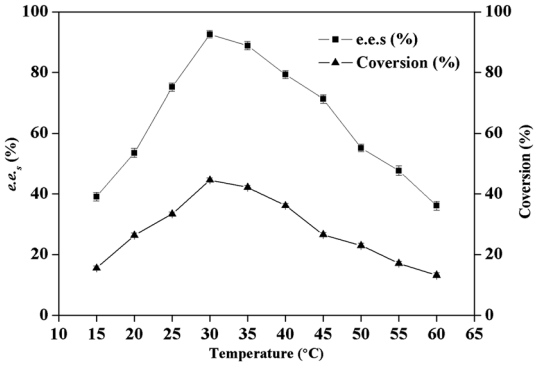

FIGURE 5 Effects of temperature on the enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 0.02-g lipase M5-containing *E coli* whole cell biocatalyst, 5-μL (30mM) racemic substrate and pH 8.0 PB, 1000 rpm, reaction time 2 h

selectivity of the enzyme. Thus, the reaction temperature of 30°C was selected for subsequent experiments.

3.3.3 | Effect of biocatalysts amount on biocatalytic resolution

The effect of the biocatalyst amount on the conversion and enantiomeric excess of the substrate (*R*,*S*)-ethyl 2-bromoisovalerate was tested in the range from 5 to 50 g/L. The amount of lipase M5-containing *E coli* whole cell biocatalyst was significantly affected on the conversion and enantiomeric excess of the reaction (Figure 6). When the amount of biocatalyst reached 40 g/L, the conversion no longer increased. As the amount of biocatalyst was further increased, the enantiomeric excess of the substrate began to decrease. Therefore, 40 g/L of the biocatalyst amount was sufficient for the reaction and was selected.

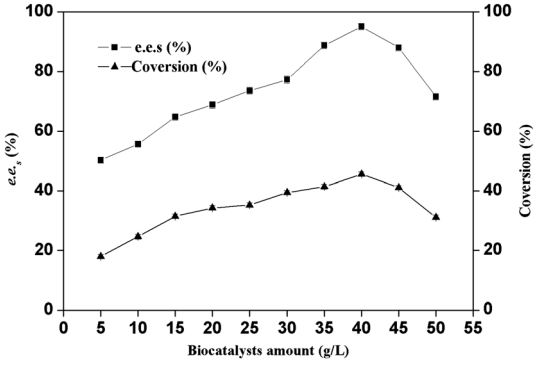

FIGURE 6 Effects of biocatalysts amount on the enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 5-μL (30mM) racemic substrate and pH 8.0 PB, 5-50 g/L biocatalysts amount, 1000 rpm, 30°C, reaction time 1 h

3.3.4 | Effect of cosolvent on biocatalytic resolution

Cosolvents have become an effective way to enhance the activity and selectivity of lipases.[23,28,29] Therefore, in order to find the optimal cosolvent, the effects of different cosolvents on biotransformation were investigated experimentally. The conversion and enantioselectivity of reaction were improved only in the presence of 5% (v/v) dimethyl sulfoxide (DMSO) compared with the cosolvent–free control experimental group, and other cosolvents did not promote the enzyme activity, of which acetonitrile and tetrahydrofuran have a strong inhibitory effect on enzyme activity (Figure 7). Therefore, DMSO was selected as the optimal cosolvent in this study.

3.3.5 | Effect of substrate concentration on biocatalytic resolution

Because of the substrate concentration is too high, it may lead to substrate inhibition, so substrate concentration is also one of the important factors affecting the enzymatic reaction. In addition, the substrate tolerance is a key indicator of the potential of enzymes for industrial applications.[27,30] We explored the effect of a substrate concentration range of 30mM to 270mM on the enzymatic catalytic reaction. The experimental results are shown in Figure 8. When the substrate concentration was 30mM to 120mM, the conversion and $e.e._s$ increased significantly with the increase of substrate concentration, reaching a maximum of 47.1% and 94.3%, respectively.

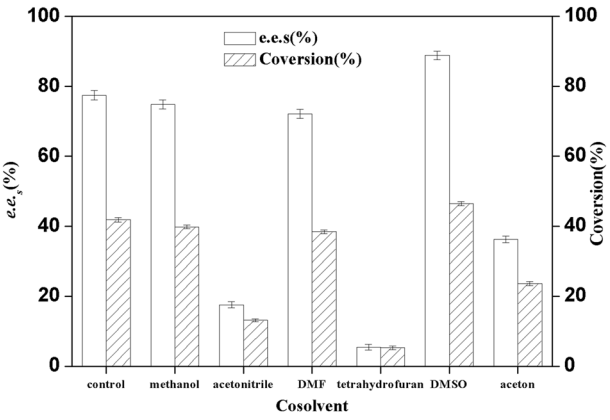

FIGURE 7 Effects of cosolvent on the enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 0.04-g lipase M5-containing *E coli* whole cell biocatalyst, 5-μL (30mM) racemic substrate and pH 8.0 PB. 5% cosolvents (methanol, acetonitrile, acetone, DMF, DMSO, tetrahydrofuran) were added to 30°C, 1000 rpm, reaction time 1 h and reaction volume 1 mL. Take no cosolvent as a control

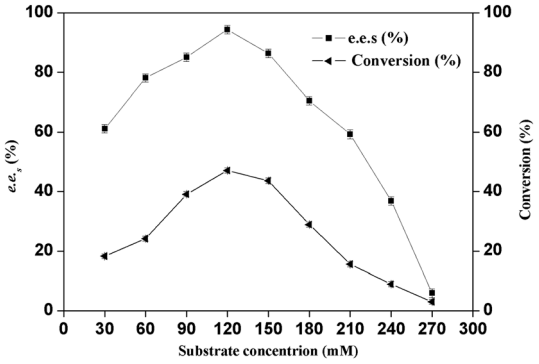

FIGURE 8 Effects of substrate concentrations on the enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 0.4-g lipase M5-containing *E coli* whole cell biocatalyst, 30mM-270mM substrate concentrations in 10-mL PB (0.2M, pH 8.0) and 5% DMSO, 30°C, 600 rpm, reaction time 2 h

When the substrate concentration exceeded 120mM, the conversion and $e.e._s$ of the reaction had a significant drop. Therefore, high concentration of substrate causes the substrate to be inhibited, the catalysis of the enzyme reaches the saturation point, and the reaction rate was lowered. Therefore, a substrate concentration of 120mM was adopted for bioresolution (*R*,*S*)-ethyl 2-bromoisovalerate.

3.3.6 | Time course on biocatalytic resolution

The time course of the enantioselective hydrolysis of (*R*,*S*)-ethyl 2-bromoisovalerate catalyzed by lipase M5-containing *E coli* whole cell biocatalyst was estimated (Figure 9). The reaction was carried out in a 50-mL system at 30°C and 600 rpm, including 120mM racemic substrate, 2-g lyophilized recombinant *E coli* Rosetta cells, and 5% (v/v) cosolvent DMSO. In the course of the reaction, an automatic titration was carried out using a 0.5M NaOH solution to control the pH of the reaction system to be maintained at 8.0. Samples were taken every 30 minutes, inactivated with 4M HCl solution, then extracted with ethyl acetate, and analyzed by GC. It was observed from the graph that the reaction rate was faster in the first hour, and the conversion rate and $e.e._s$ were significantly increased. When the reaction time exceeded 2 hours, the conversion rate and $e.e._s$ were still increasing, but the speed was slowed down. After 3 hours of reaction, the substrate concentration remained almost unchanged, indicating that the reaction was completed, at which time the conversion of the reaction reached 51.7%, $e.e._s$ was 98.6%, and *E* value was 120.1. Therefore, recombinant *A oryzae* lipase M5 has great industrial application prospects for the resolution of the racemic substrate (*R*,*S*)-ethyl 2-bromoisovalerate to produce (*R*)-ethyl 2-bromoisovalerate.

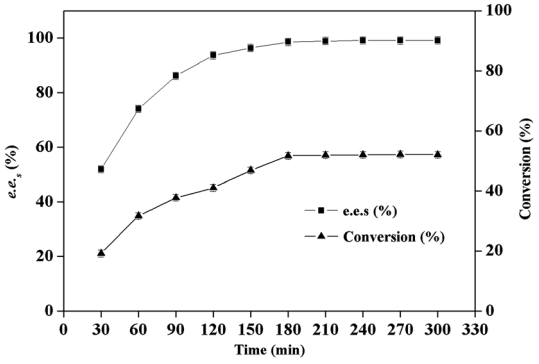

FIGURE 9 Time course of enzymatic resolution of (*R*,*S*)-ethyl 2-bromoisovalerate. Reaction conditions: 2-g lipase M5-containing *E coli* whole cell biocatalyst, 120mM racemic substrate in 50-mL PB (0.2M, pH 8.0) and 5% DMSO, 30°C, 600 rpm

4 | CONCLUSION

In summary, lipase catalyzed resolution of (*R*,*S*)-ethyl 2-bromoisovalerate by recombinant *E coli* Rosetta cells expressing *A oryzae lipase gene* was evaluated. A series of enzymatic resolution experiments showed that lipase M5 has high activity and significant enantioselectivity to (*R*,*S*)-ethyl 2-bromoisovalerate. Under the substrate concentration of 120mM, the conversion reached 51.7% and the $e.e._s$ exceeded 98.6% after 3 h of enzymatic reaction. Therefore, the preparation of (*R*)-ethyl 2-bromoisovalerate (an important intermediate for the high-efficiency enantiomer of fluvalinate) by recombinant lipase in the resolution of (*R*,*S*)-ethyl 2-bromoisovalerate has potential industrial prospects.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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