Effects of inducible and constitutive expression vectors on heterologous expression of LEH mutants BE3 and BG5

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Research

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Abstract

Background

Limonene epoxide hydrolase (LEH) is a class of enzymes in Epoxide hydrolases (EHs) that can form chiral products only by one step catalysis. High purity *S* or *R* chiral intermediates play an important role in the application of biomedical industry, and can be used to synthesize various drugs such as ibuprofen, linezoline, cilastatin, etc. Therefore, it has application value to find the expression system which can realize the stable and efficient conversion of LEH to produce high purity *S* and *R* chiral products.

Results

We explored the constitutive expression system of LEH for the first time, and tried to realize the expression of LEH in *B.subtilis* WB800. Firstly, the LEH mutant on the original inducible vector pBAD-Myc-HisA was obtained and connected to the constitutive vector pHY-p43 containing strong promoter p43. *E. coli* TOP10 and *B.subtilis* WB800 were used as the host bacteria to realize the non induced and secretory expression of LEH.

Conclusions

The results showed that the non induced expression of LEH could be achieved successfully by using the constitutive promoter vector pHY-p43, and the substrate affinity and enzyme catalytic efficiency of the mutants have increased. The catalytic decomposition of the substrate and the formation of chiral products by LEH were determined by GC-MS, which also had stable enzyme activity in the system. This study laid a foundation for large-scale fermentation of LEH and catalytic production of chiral products.

Background

EHs play an important role in microorganisms, plants, insects and mammals. The three main function of EHs are detoxification, catabolism, and regulation of signaling molecules[1].Which is different from other EHs enzyme reactions requiring two steps, and the LEH from *Rhodococcus erythropolis* DCL14[2] only one step complete catalytic reaction. So this reaction doesn't require any other coenzyme to participate, the catalytic mechanism is that the active center is composed of Asp–Arg–Asp triad[3], which involves epoxide protonation by Asp101, nucleophilic attack by water, and abstraction of a proton the water by Asp132. Tyr53, Asn55 and Asp132 position water molecules in a favorable position for epoxide attack and activated water molecules by hydrogen bonds. Asp132 abstracts a proton from the water molecules. Meanwhile, Asp101 supplies of protons to the epoxide ring of the substrate to activate the epoxide ring, promoting nucleophilic attack on epoxide carbon. Arg99 locates the carboxyl groups of Asp101 and Asp132 as a proton shuttle assisted proton balance and charge stabilization (Fig. 1).[3, 4].

Epoxides as important substrates for EHs also play an important role in chiral pharmaceuticals synthesis and rubber product development[5]. Among them, the (*R*)- or (*S*)-enantiomers of the absolute configuration produced by chiral epoxides usually have different functions and effects, such as anti-inflammatory antalgica drugs ibuprofen and β-receptor blocker propranolol ,compared with (*S*)-ibuprofend, (*R*)-ibuprofen can achieve significant effect at lower concentration[6]; (*S*)-propranolol has a β-receptor blocker effect about 100 times stronger than (*R*)-propranolol, and is an enantiomer that plays a major role in the treatment of angina pectoris[7]. The experiment will be based on two of the mutants that have significant enantioselectivity, and explore the influence of different expression systems on LEH expression in the form of organic phase catalysis in accordance with the requirements of environmental protection and production application, in order to obtain high purity chiral enantiomers through stable and efficient enzyme catalysis.

Additionally *Escherichia.coli* (*E.coli*) is a commonly used host strain of prokaryotic expression systems, which has significant advantages[8], but it also has disadvantages that are difficult to overcome, such as the presence of the target protein often in the form of inclusion bodies leads to difficulty in product purification and low product biological activity[9]–[10]. For the absence of *Bacillus subtilis (B. subtilis*) outer membrane, *B. subtilis* can simplify the protein secretion pathway and secrete high-level extracellular protein. As early as 2007, due to the unique protein secretion ability of *B. subtilis*, about 60% of the enzymes on the market were produced by *B. subtilis* [11]. Therefore, *E. coli* and *B. subtilis* as a heterogenic expression host has considerable research and application value.

Materials And Methods

Strains, plasmids and chemicals

The plasmid pHY-P43 was purchased from YouBio (Changsha, China); The limonene epoxide hydrolase (LEH) mutants library were constructed by professor Dick B. Janssen group of University of Groningen[12]; The clone strain competent cells *E.coli* Top10 was purchased from Tiangen (Beijing, China); The expression strain *B. subtilis* WB800 was presented by professor Wei xuetuan of Huazhong Agricultural University; The substrates cyclohexene oxide (CHO) was purchased from TCI (Tokyo, Japan); The media used in the experiment were Luria-Bertani (LB) medium and Terrific Broth (TB) medium.

Construction of recombinant heterologous expression vector

According to the sequencing results of the pBAD-LEH mutant, a pair of primers were designed to amplification the LEH mutants BE3 and BG5, restriction enzyme *BamH* I and *EcoR* I was selected as the restriction site at the multipe cloning site of the pHY-p43, Forward primer(*BamH* I)：5’-CGGGATCCCGTTGGGCTAACAGGAGGAATTAC-3’, Reverse primer (*EcoR* I): 5’-CGGAATT

CCGCAAGCTGGAGACCGTTTAAACT-3’, specific amplification of the target gene from BE3 and BG5, the full length of the segment is 570 bp, PCR system:94℃, 5 min; 94℃, 30 s, 65℃, 30 s, 72℃, 30 s, 30

cycles; 72℃, 10 min, target gene purified and digested by *BamH* I and *EcoR* I at 37℃ for 4 h, the expression vector pHY-p43 also digested by *BamH* I and *EcoR* I , then ligated into the vector pHY-p43.The recombinant plasmids be transformed into *E.coli* Top10 and the successfully sequenced recombinant was extracted extracted and transferred into *B.subtilis* WB800 competent cell by electrical transformation[13] , get the recombinant pHY-p43-BE3 map (Fig. 2A) and pHY-p43-BG5 map (Fig. 2B)

Protein structure homology simulation and molecular docking

The protein crystal structure of the limonene-1,2-epoxide hydrolase （LEH） subunit of *Rhodococcus erythropolis* was obtained from the PDB database (PDB ID: 1NWW, 5CF2). The protein monomer sequence was obtained by bGI gene sequencing. The structure models of the variants BE3 and BG5 were constructed by homology modeling and downloaded from SWISSMODEL Workspace (http://swiss model .expasy.org/)[14]-[15]. Molecular docking of the LEH mutant with the substrate cyclohexene oxide was completed by Autodock4.2.6. All water molecules were removed, nonpolar hydrogen atoms added and protein displayed by Pymol (http://www.pymol.org).

Screening for high activity variants

All the plasmids pBAD-LEH were transformed into *E. coli Top10* and cultured in TB medium. When the culture reached OD_{600} of 0.6, 0.02% L-arabinose was added for LEH production inducer and the culture was incubated for a further 16 h at 30°C. pHY-p43-LEH culture by LB medium, and after transformed into *E. coli* Top10, the culture was incubated for a further over 12 h at 37°C. Cells were harvested by centrifugation (10 min, 6,000 × g, 4°C), and each 100 mg of cells was resuspended in 1 mL buffer (50 mM Hepes, pH 8.0), lysed by sonication (total time 20 min, cycles of 5 s on 10 s off, 60Watt, 4°C). The cell debris was removed by centrifugation (30 min, 6,000 × g, 4°C) and the supernatant was collected into the eppendorf tubes.

For *B. subtilis* WB800, the culture was incubated at 37°C for a further 20 h after being transformed and the supernatant was collected. Proteins were purified by the Protein purification kit (His-tagged Protein Purification Kit, China); The cell-free extract was loaded on the column and washed by the loading buffer (followed by the kit's instruction) to remove unbound contaminants and the eluting solution between the 5 ml to 10 ml was collected. The proteins were desalted and concentrated by the ultrafiltration centrifuge tubes (Milipore). The protein concentration was determined using the Bradford[16] procedure and purity was analyzed using 12% SDS-PAGE.

Enzyme assay and GC analytics

After desalination and concentration, LEH from *E. coli* TOP 10 was obtained to detect enzyme activity and kinetic parameters by gas chromatograph-mass spectrometry (GCMS-QP2020，Japan). Substrate solution was prepared with 1 M substrate in acetonitrile to make the stock solution, then diluted 20-fold in 50 mM potassium phosphate buffer (final pH 7.0 and final concentration 50 mM). In a 4 mL glass vial with cap, 980 μL substrate solution is mixed 20 μL enzyme stock solution (LEH (1~5 mg/mL) in 10 mM

HEPES buffer (pH 7.5)) and incubate at 30°C. After 90 min incubation, in duplo, take a sample of 400 µL and add it to 400 µl ethylacetate containing 1 mM IS (ethylacetate with 1 mM hexadecane as internal standard) in a 2 mL Eppendorf tube. Add 120 µl of 5 M NaCl in water, Mix well, vortex 20 s, centrifuge (8000 rpm, 2 min) and using a Pasteur pipette place the supernatant in a new eppendorf vial. Repeat the extraction with another 400 µl of ethylacetate containing IS. Combine the two ethylacetate extracts and dry with solid $MgSO_4$. 150 µl of the water-free supernatant is analyzed by chiral gas chromatography. Injector (split) and detector (FID) at 200°C, sample temperature 200℃, carrier is nitrogen at a flow rate of 1 mL/min. For cyclohexene oxide :temperature starts from 40°C, hold for 1 minute, increase to 150°C at 10°C/min; hold 6 min at 150°C; Measure peak area's and using the internal standard peak area's and a calibration curve, calculate concentrations. One unit (U) is the amount of enzyme that produces 1 µmol of product per min.

Determination of the Extracellular expression LEH mutants enzyme properties

The optimum temperature of LEH from *E.coli* TOP 10 was determined using 50 mM HEPE buffer (pH 7.5) with temperature ranging from 50 to 85°C. The optimum pH was measured by assaying the enzyme activity at various pH values (0.05 M KP (potassium phosphat) buffer, pH 5.8-8.0) at 30°C. Kinetic parameters of the LEH variants were determined in KP buffer (0.05 M, pH 7) at 30 °C with varied concentration of the substrate cyclohexene oxide (with concentration range from 1 mM to 70 mM). Kinetic parameters V_{max} value , k_{cat} value and K_m value were obtained with the help of software Originpro 2021 by plotting enzymatic activity versus substrate concentrations and fitting them using the Michaelis–Menten equation.

Results And Discussion

LEH characteristics and screeing mutant library

Based on the mutant library constructed with the vector pBAD/Myc-His A[12], all mutants were transformed, cultured and purified to verify the enantioselectivity (Table 1). Since mutant BE3 had significant (S) - enantioselectivity and mutant BG5 had (R) – enantioselectivity in our experiment, they were selected for the subsequent test.

Table 1 Screening and verification of LEH mutant library

Mutants	Mutation site	*ee* (%)	C (%)
AG9	T76K/T85V/N92K/Y96F/E124D/ I5C/E84C/G89C/S91C	10.59±0.29 (*R,R*)	99.15
AH6	T76K/T85V/N92K/Y96F/E124D/ I5C/A17C/E84C/N92C	16.68±0.25 (*R,R*)	99.99
BE3	M32L/L74F/I80F/L103I/I116V/F139L	86.44±0.78 (*S,S*)	22.06
BE5	M78L/I80F/I116V/F139W	14.05±0.67 (*S,S*)	91.25
BE6	M32A/L35W/M78I/I80A/V83A/I116V	34.86±6.49 (*S,S*)	10.99
BE7	M32L/I80W/L103I/I116V/F139W	6.67±5.77 (*R,R*)	17.34
BE9	M32L/L35M/L74I/M78I/I80F/V83I/ L103V/I116V/F139L	83.30±8.53(*S,S*)	8.57
BF2	M32L/M78L/I80F/I116V/F139W	0.61 (*S,S*)	61.17
BF3	M78W/F139W	41.56±1.27 (*R,R*)	32.15
BF4	M32A/L35M/M78L/I80W/L103V/ I116V/F139W	1.79±0.21 (*R,R*)	19.74
BF5	M32L/L35M/L74I/M78V/I80F/V83G/ L103V/F139L	49.66±14.27 (*R,R*)	0.29
BF6	L74I/I80F/I116V/F139W	5.87±2.71(*R,R*)	99.04
BF7	M32L/L35M/M78L/I80F/L103I/I116V/F139L	60.11±2.11 (*S,S*)	79.84
BF8	M32L/L35M/M78L/I80W/V83I/ L103I/I116V/F139W	11.28(*S,S*)	10.62
BF9	M32L/L35W/L103I/I116V/F139W	9.31±0.46 (*S,S*)	64.32
BG1	M32L/L74F/M78A/I80F/L103I/ I116V/F139L	73.63±2.27 (*S,S*)	64.43
BG2	M32L/M78I/I80W/L103I/I116V/F139L	60.58±4.97 (*S,S*)	13.24
BG3	M32L/L35W/M78I/I80A/L103I/I116V/F139L	25.51±5.89 (*R,R*)	37.48
BG5	M32L/L74I/I80V/L103F/F139L	79.41±0.92 (*R,R*)	62.37
BG6	M32L/L74I/I80V/L103F/F139W	70.89±1.58 (*R,R*)	73.90
BG7	M32L/L74I/L103F/F139W	60.40±1.20 (*R,R*)	58.34
BG8	M32L/M78G/L103F/F139M	30.35±0.28 (*R,R*)	84.36
BG9	M32L/M78G/L103F/I116V/F139L	54.63±1.80 (*S,S*)	97.61
BH2	M32A/M78G/L103F/F139L	18.46±0.91 (*R,R*)	97.85

Note: for the substrate CHO; C%: conversion rate

LEH catalyzes the hydrolysis of cyclohexane oxide to vicinal diol cyclohexane-1,2-diol (Fig. 3). The substrate CHO was molecular docked with BE3 and BG5. The docking grids of BE3 was set to 46×54×64

Å, and the docking grids of BG5 was set to 54×52×40 Å. For the docking results, CHO was located on the surface of the protein to the catalyst. BG5 has a larger cavity than BE3, which can reduce steric hindrance and is more conducive for CHO to enter the ligand channel (Fig.4).

Construction of pHY-p43-LEH expression vector

Mutants BE3 and BG5 genes were amplified from plasmid pBAD-LEH mutants library. The double enzyme digestion products of target fragments BE3, BG5 and vector pHY-p43 were successfully recovered (Fig. 5A). After connection and transformation, some single colony was selected by bacteria liquid PCR, then the transformed plasmids were extracted for plasmid PCR and double enzyme digestion (Fig. 5B-C).

LEH heterologous expression in *E. coli* Top10 and *B. subtilis* WB800

Plasmids pHY-p43-BE3 and pHY-p43-BG5 transformed into *E. coli* TOP10 were cultured over 12 h, and proteins were purified by 6×His affinity chromatography. SDS-PAGE detection showed that pHY-p43 /TOP10 system expressed LEH successfully (Fig. 6A), and the sequencing results also showed that the target fragments were correctly connected.

Meanwhile, pHY-p43-BE3 and pHY-p43-BG5 were transformed into *B. subtilis* WB800 by electrotransformation. From the SDS-PAGE results, the expression in *B. subtilis* WB800 was lower than the expression in *E. coli*, therefore the latter strain was considered for the optimized system (Fig. 6B).

Enzymatic characteristics of mutants BE3 and BG5 in different systems

For the protein expression of pBAD/Myc-HisA–LEH system needs to add a certain concentration of inducer and control the corresponding induction temperature and time, also the culture time is more than 20 h. In order to make an efficient and economic procedure, the expression system of LEH was considered to be optimized. The constitutive vector pHY-p43-LEH containing strong promoter p43 was our selection for promoting the expression,pHY-p43 system did not need inducer, and the amount of protein needed in the experiment could be obtained by conventional temperature 37°C and LB medium for cultured more than 12 h.

In our experiment, LEH mutants were designed to catalyze substrate cyclohexene oxide (CHO). During the gas chromatography, retention times of cycloheptene oxide ca. 9.3min; acetonitrile ca. 8.8 min; (S,S)-cycloheptane diol ca. 13.7 min; (R,R)-cycloheptane diol ca. 14.0 min; IS ca. 16.3 min. Measure peak area's and the internal standard peak area's and a calibration curve, calculate concentrations (Table 2).

Table 2 The substrate conversion and enantiomeric excess of mutants BE3 and BG5

Mutant	CHO conversion (%)	CHO ee(%)
pBAD-BE3	46.212	-56.987
pBAD-BG5	33.477	40.886
pHY-P43-BE3	37.71	-71.57
pHY-P43-BG5	39.93	69.55

The mutants under the expression system were purified from *E.coli* TOP10, and the LEH expressed by different expression systems was determined to be affected by pH, temperature and reaction time In the experiment, the 1M substrate stock solution was diluted with 50 mM potassium phosphate buffer to 50 mM, and 990 μL substrate dilution and 10 μL LEH purified enzyme solution were incubated in a 5 mL glass bottle at 30°C for 90 min. First, the optimal reaction temperature was determined, and the temperature was set at 30 to 85℃. It was found that the same mutant had the same optimal temperature in different expression systems The optimal reaction temperatures for pBAD-BE3 and pHY-p43-BE3 are both at 65°C, and the optimal reaction temperatures for pBAD-BG5 and pHY-p43-BG5 are both at 55°C (Fig. 7). Then the optimal pH was determined and configure the substrate diluted potassium phosphate buffer at 5.8, 6.2, 6.6, 7, 7.4, 7.8, 8. For pHY-p43-BE3 and pBAD-BG5, the catalysis was at the highest level at pH 6.2, and while the optimal pH of pBAD-BE3 is 5.8, pHY-P43-BG5 is 7.4 (Fig. 8).

Finally, the change of the different reaction time of each mutant was determined, and it basically belonged to a steady ascending state, and there were no significant changes (Fig. 9). Therefore, the reaction time of the subsequent experiment kept for 90min. The enzyme-catalyzed reaction was carried out under the above-mentioned single-factor optimal conditions, and it was found that the substrate conversion rate was significantly increased, and the conversion rate of pBAD-BE3 on CHO reached 100%, but after the conversion rate increased, the *ee* value decreased (Fig. 10). It was speculated that the optimal temperature and pH of single factor had certain influence on the conformation of LEH, and the formation of chiral products was also affected by the increase of catalytic efficiency.

Kinetic analysis of LEH mutants expressed in *E. coli* Top10

The kinetic parameters of mutant BE3 and BG5 were determined using different concentrations of substrate CHO from 1 mM to 70 mM, and the reaction system was 990 μL substrate dilution plus 10 μL LEH solution in 5 mL incubater at 30°C for 90 min, in which the substrate concentration was unique variable. The K_m value and V_{max} value are calculated by the Michaelis-Menten equation in the software Originpro 2021. From the table 3, that the mutants of the p43 system have stronger substrate affinity and better catalytic efficiency than the pBAD system. Therefore, the p43 system was selected as the optimized plasmid expression system.

Table 3 The kinetic parameters of mutants BE3 and BG5

Mutant	Enzyme concentration(μmol/L)	K_m(mM)	Vmax (umol/min·mg)	K_{cat}(s^{-1})	K_{cat}/K_m ($mM^{-1}s^{-1}$)
pBAD-BE3	3.557	20.425 ± 5.870	19.149	0.822	0.040
pBAD-BG5	2.548	10.930 ± 0.403	5.212	0.313	0.029
P43-BE3	1.962	9.797 ±1.640	5.670	0.546	0.056
P43-BG5	1.188	9.08 ± 4.11	4.735	0.369	0.045

Note: for the subatrate cyclohexane oxide

Conclusions

In summary, mutants BE3 and BG5 can catalyze the production of chiral enantiomeric products with distinct *S* and *R* configurations of the substrate CHO. The change of expression system did not affect the enzymatic properties of LEH, but the mutants expressed in pHY-P43 expression system had higher substrate affinity and enzyme catalytic efficiency than PBAD/Myc-HisA, a the constitutive expression system significantly shortened the culture time and simplified the experimental procedures without affecting the protein expression level. Moreover, in the p43 constitutive expression system, the substrate affinity and enzyme catalytic efficiency of the mutants have increased. Combined with the enzymatic properties of mutants, p43-BE3 will give the highest catalysis at pH 6.2 and 65 °C, and otherwise p43-BG5 will be at pH 7.4 and 55 °C.

Declarations

Acknowledgments

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Authors' contributions

Jie Yong was responsible for the conception of the experimental framework, the construction and detection of protein expression vector and the writing of the paper. Binghuan Liu screened mutants. Kunlun Wang assisted in carrying out the experiment. Hui Yang guided bioinformatics related content such as homology modeling. Professor Yun Tian provides an experimental platform. Professor Haiyan Zhou is responsible for monitoring the progress of the experiment, guiding the article in detail and providing financial support for the experiment.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

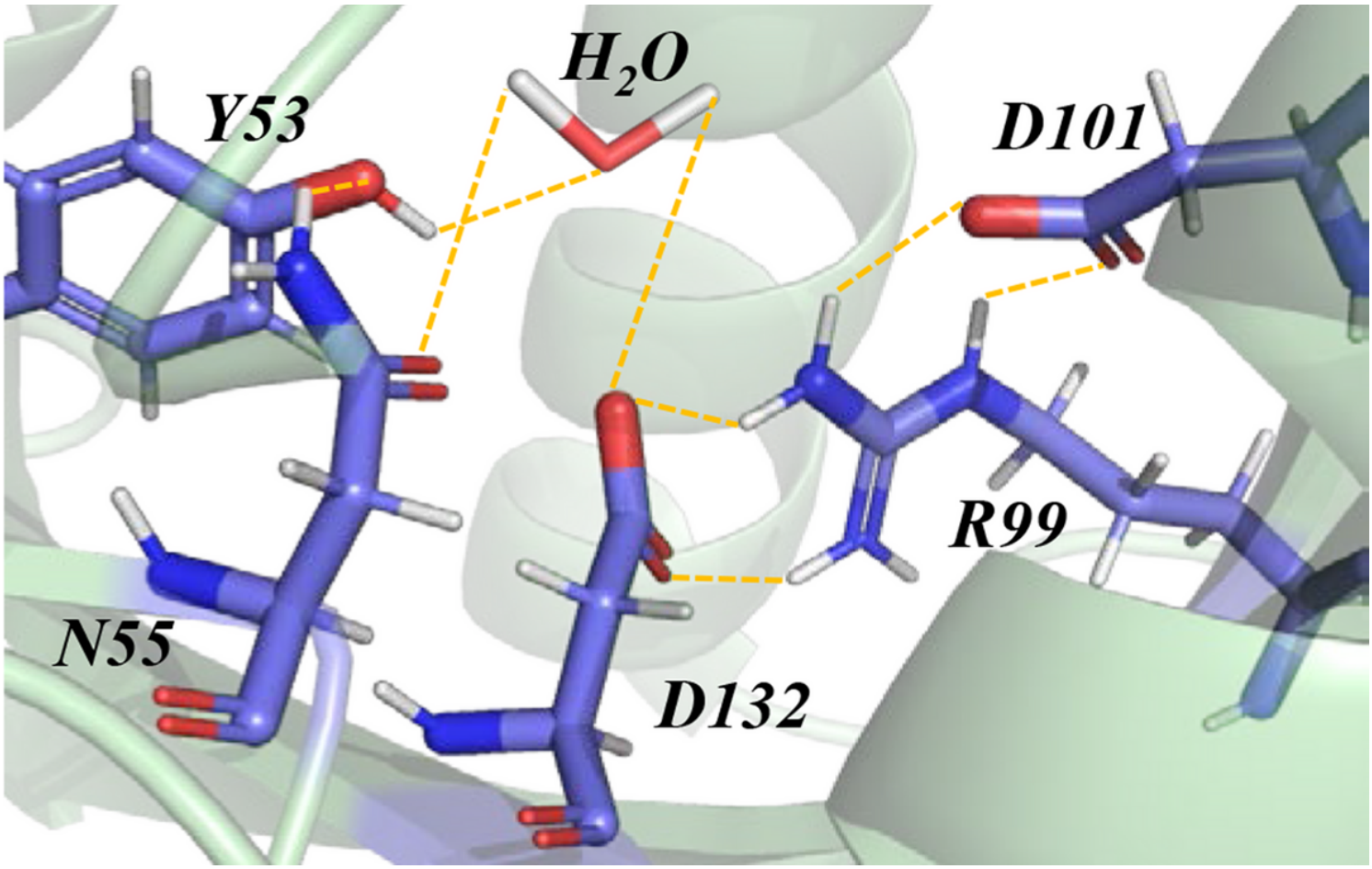

Figure 1

The catalytic mechanism of LEH

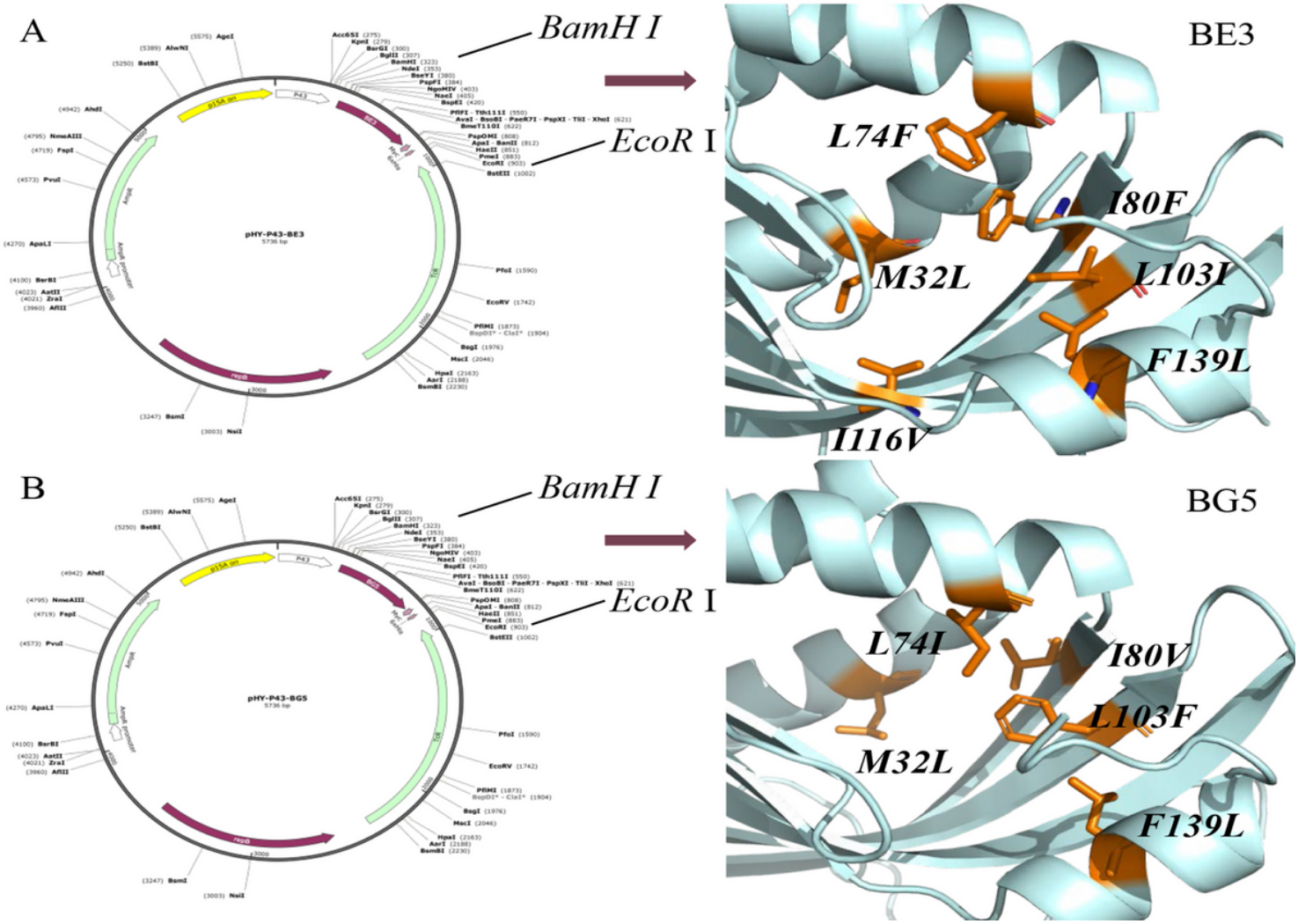

Figure 2

The pHY-p43-LEH map and structure models of LEH mutants BE3 and BG5

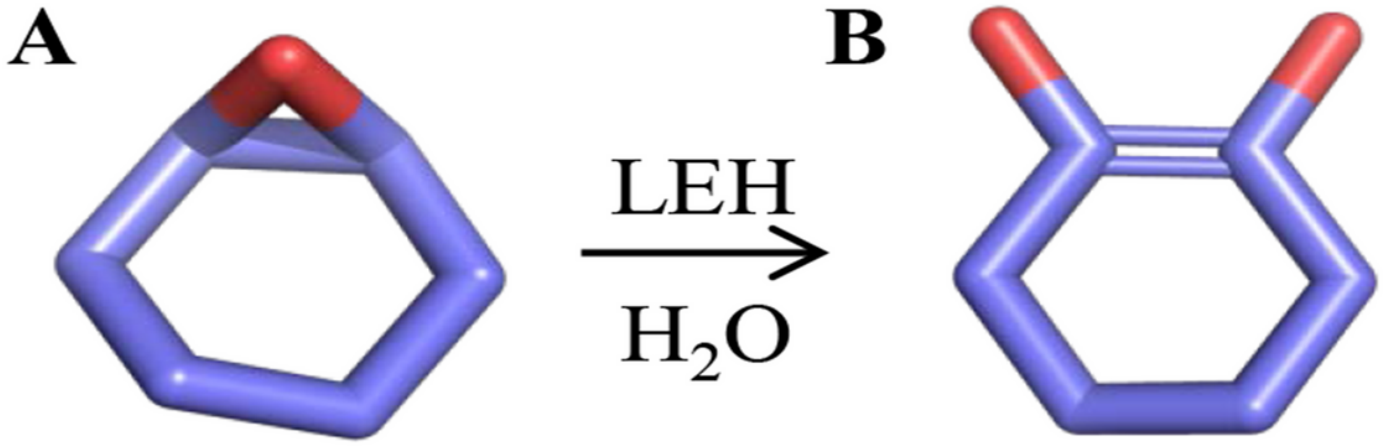

Figure 3

The catalytic of CHO by LEH. A: Cyclohexane oxide; B: Cyclohexane-1,2-diol

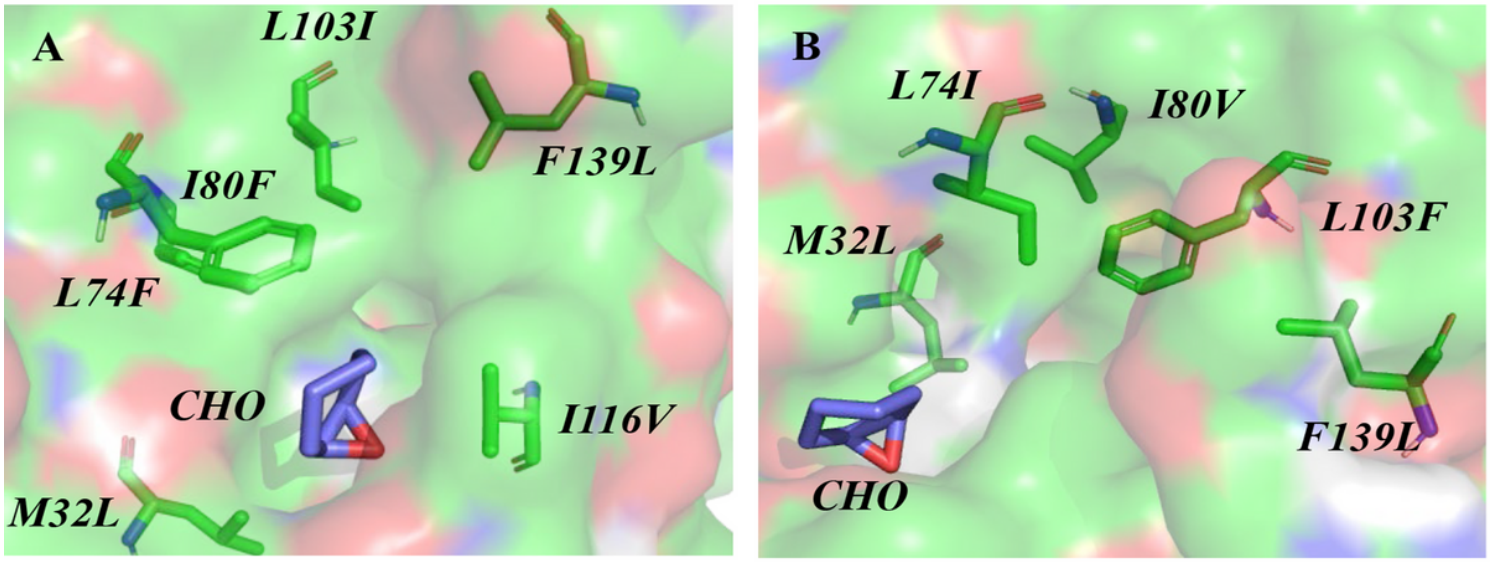

Figure 4

Molecular docking the substrate CHO with LEH mutants. A: Docking of substrate CHO and BE3; B: Docking of substrate CHO and BG5;

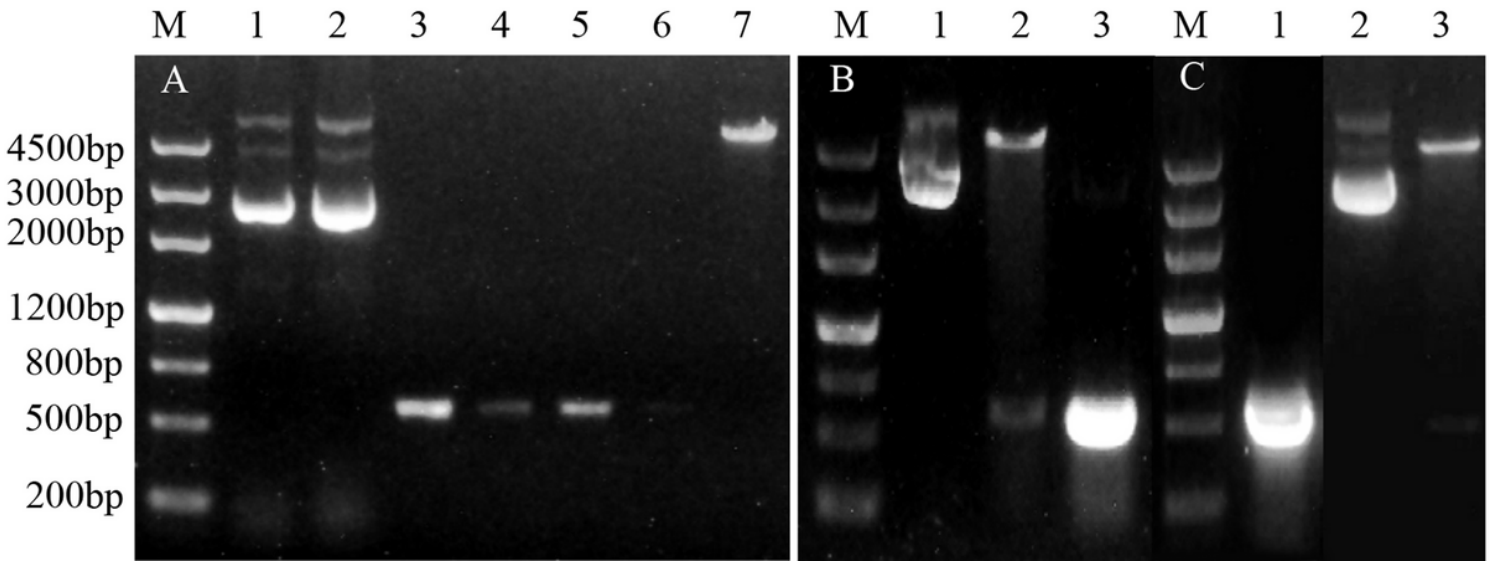

Figure 5

Target gene and transformant electrophoresis of pHY-p43-LEH system. A: M: Marker, 1: pBAD-BE3, 2: pBAD-BG5, 3-4: pBAD-BE3 PCR amplification and restriction enzyme digestion, 5-6: pBAD-BG5 PCR amplification and restriction enzyme digestion, 7: pHY-p43 restriction enzyme digestion; B: 1: recombinant plasmid pHY-p43-BE3, 2: PCR amplification pHY-p43-BE3, 3: restriction enzyme digestion of pHY-p43-BE3; C: 1: recombinant plasmid pHY-p43-BG5, 2: PCR amplification pHY-p43-BG5, 3: restriction enzyme digestion of pHY-p43-BG5.

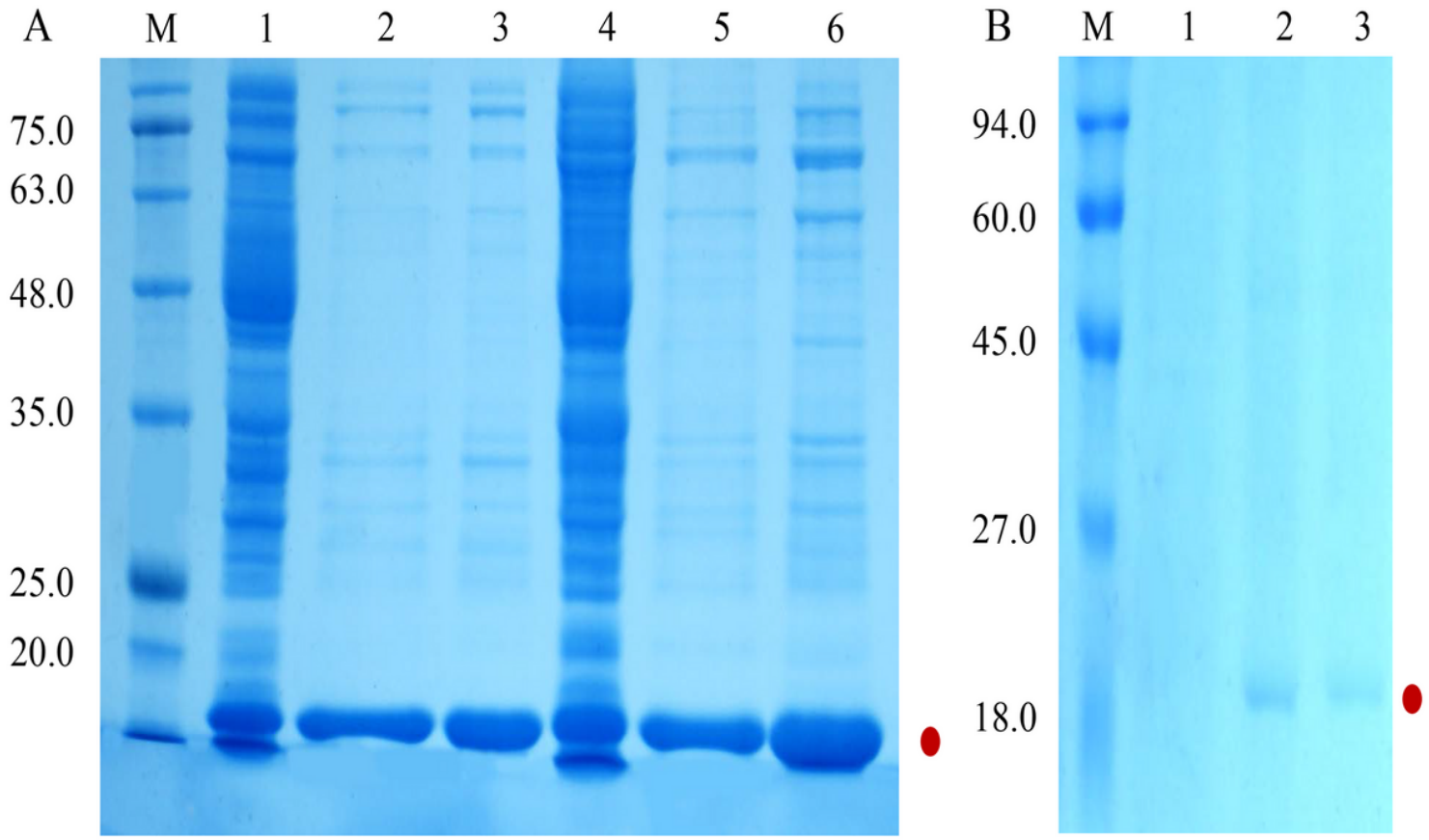

Figure 6

pHY-p43-LEH heterologous expression in E.coli Top10 and B.subtilis WB800. A: M: Marker, 1: crude enzyme of pHY-p43-BE3, 2: purification enzyme of pHY-p43-BE3, 3: desalination enzyme of pHY-p43-BE3, 4: crude enzyme of pHY-p43-BG5, 5: purification enzyme of pHY-p43-BG5, D6: desalination enzyme of pHY-p43-BG5. B: M: Marker, 1-3: purification enzyme of pHY-p43-BE3

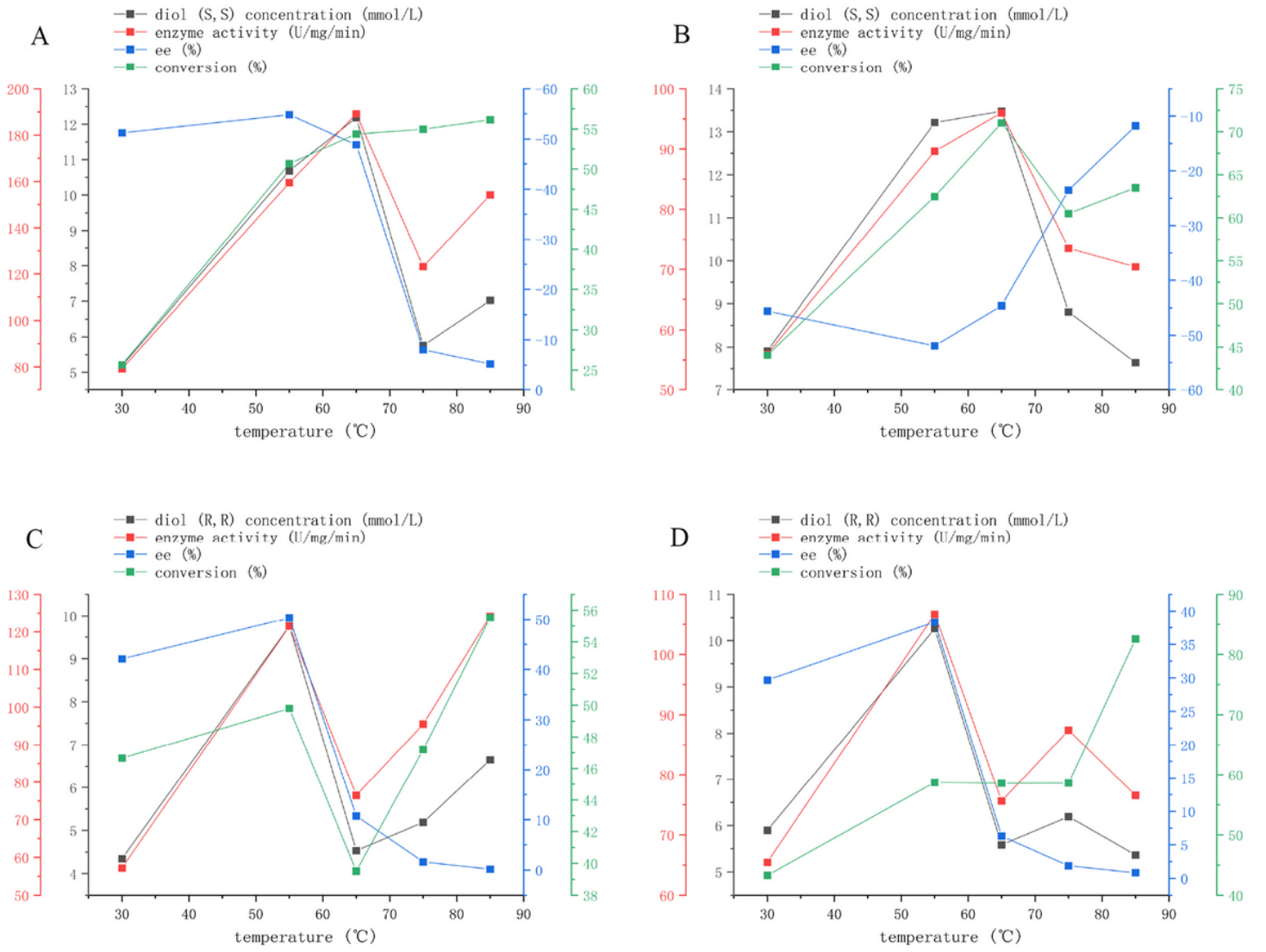

Figure 7

Temperature of enzymatic characteristics of mutants. A: p43-BE3; B: pBAD-BE3; C: p43-BG5; D: pBAD-BG5

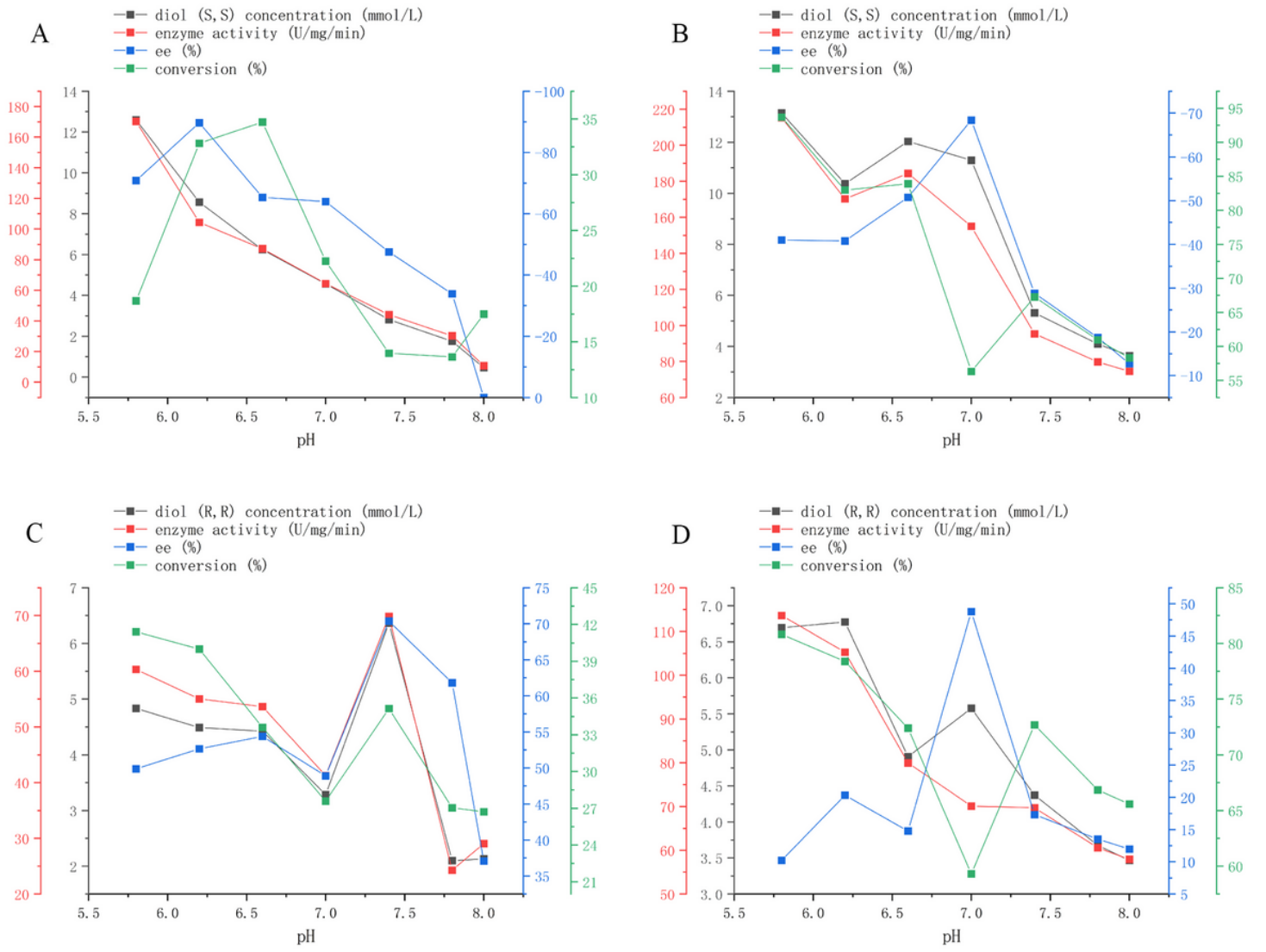

Figure 8

pH of enzymatic characteristics of mutants. A: p43-BE3; B: pBAD-BE3; C: p43-BG5; D: pBAD-BG5

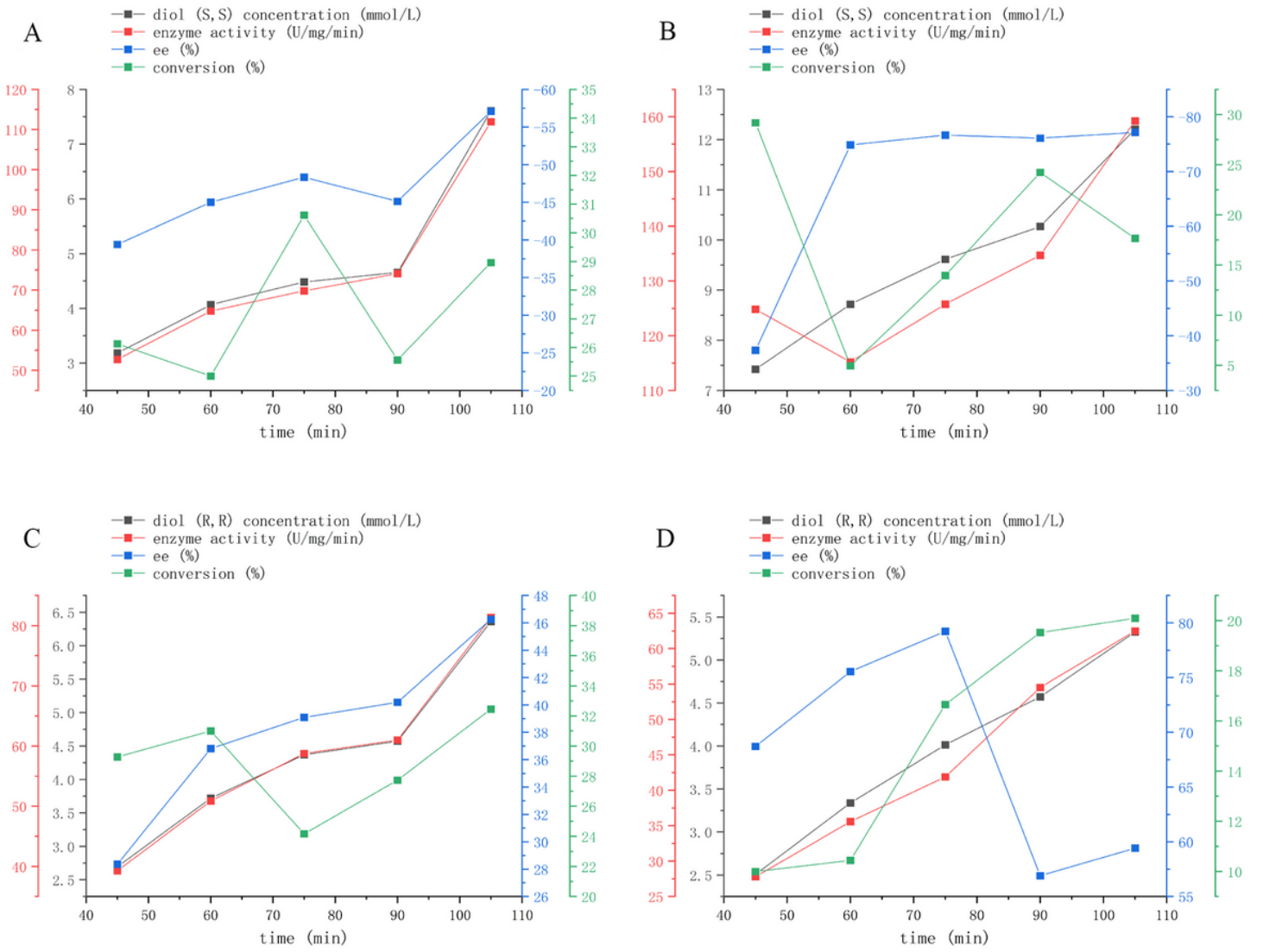

Figure 9

Reaction time of enzymatic characteristics of mutants. A: p43-BE3; B: pBAD-BE3; C: p43-BG5; D: pBAD-BG5

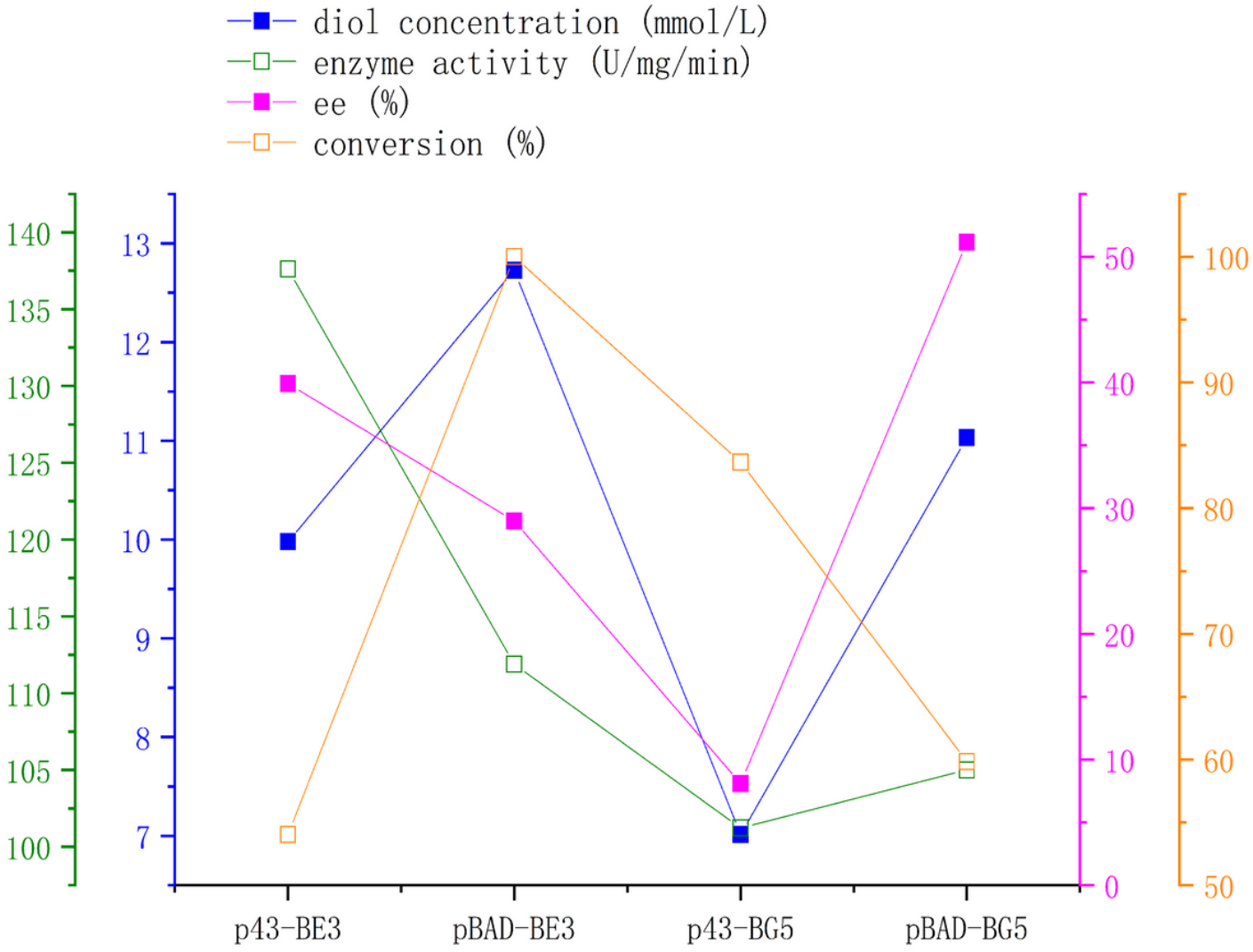

Figure 10

Enzyme-catalyzed reaction in single-factor optimal conditions