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Development and characterization of recombinant whole-cell biocatalysts expressing epoxide hydrolase from *Rhodotorula glutinis* for enantioselective resolution of racemic epoxides

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Abstract

Various recombinant whole-cell biocatalysts expressing the epoxide hydrolase (EH) gene of *Rhodotorula glutinis* were developed and characterized for preparing enantiopure epoxides. Double copies of EH gene were integrated into the chromosome of *Pichia pastoris* to overexpress EH protein under the control of alcohol oxidase (AOX) promoter. The EH gene was also expressed and displayed on the surface of *Saccharomyces cerevisiae*. *Escherichia coli* Rosetta which has tRNA genes for rare codons was employed as a host cell to express the EH gene at a high level. The kinetic characteristics of the recombinant whole-cell biocatalysts were analyzed and relevant parameters were determined. Enantiopure (*S*)-styrene oxide with an enantiomeric excess higher than 99% was readily obtained by enantioselective hydrolysis of racemic styrene oxides at an initial concentration of 20 mM using the recombinant EHs.

Keywords: Enantioselective hydrolysis; Enantiopure epoxides; Epoxide hydrolase; Recombinant yeast; Recombinant *E. coli*

1. Introduction

Enantiopure epoxides are important enantiopure synthons in organic syntheses for preparing optically active compounds [1]. Enantiopure epoxides can be obtained by kinetic resolutions of racemic epoxides via enantioselective hydrolysis reactions by epoxide hydrolase (EH) [2–4]. EH catalyzes the addition of a water molecule to the epoxide ring to form the corresponding diol. The catalytic mechanism of EH consists of two-step hydrolytic cleavages of the epoxide ring, a nucleophilic opening of the epoxide ring to form a covalent glycol-monoester intermediate by the aspartic residue in the catalytic active site, and then a hydrolysis of the monoester intermediate by a water molecule activated by the histidine-aspartic acid pair to generate diol as a product [5,6].

EHs are ubiquitous and found in mammals [7], aquatic fishes [8], plants [9], yeasts [10,11], fungus [12,13], and bacteria [14,15]. High enantioselectivity and wide availability of EHs have made them highly promising biocatalysts for producing enantiopure epoxides with a high optical purity from cheap and readily available racemic epoxides by enantioselective hydrolysis.

Most of EH-catalyzed enantioselective hydrolysis of racemic epoxides were performed using either whole-cells or EH enzymes. Whole-cell applications have been carried out using microbial cells [16]. In general, microbial cells as biocatalysts have intrinsic limitation as they have only small amounts of EHs in cells. Various EH enzymes have been purified and used as biocatalysts for enantioselective hydrolysis reactions [17]. However, purification of enzyme to homogeneity is time-consuming procedure, and requires additional cost. In some cases, purified enzymes loose their activity during isolation procedure. To compromise the advantages and drawbacks of wild-type whole-cell and purified enzyme biocatalysts, we can employ recombinant whole-cell biocatalysts expressing heterologous EH gene at a high level [14,18]. The use of recombinant whole-cell biocatalyst can stabilize activity of EH during reactions at high substrate concentrations constituting a separate phase by themselves [19]. In this study, we constructed and characterized various recombinant whole-cell biocatalysts including recombinant *Pichia pastoris* and *Escherichia coli* expressing

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the EH gene of *Rhodotorula glutinis* to explore potential industrial application of recombinant EHs. The EH gene was also expressed and displayed on the cell surface of *Saccharomyces cerevisiae*. We evaluated enantioselective hydrolysis reactions of racemic styrene oxides by EH activities from wild-type *R. glutinis*, recombinant *P. pastoris*, recombinant *E. coli* and the surface displayed *S. cerevisiae*.

2. Experimental

2.1. Strains, plasmids and DNA manipulations

R. glutinis ATCC 201718 was used as a source for the EH gene. *E. coli* Rosetta (DE3) pLysS strain (Novagen, USA), *S. cerevisiae* EBY100 (Invitrogen, USA) and *P. pastoris* GS115 (*HIS*$^-$) (Invitrogen, USA) were used for expression of the EH gene. The vector pET21b(+) (Novagen), pYD1 (Invitrogen), pPICZ B and pPICZ C (Invitrogen) were used for expression of the EH gene in *E. coli*, *S. cerevisiae* and *P. pastoris*, respectively. Isolation of plasmid DNA from *E. coli* was conducted using Qiagen Miniprep Kit (Qiagen, Hilden, Germany). Isolation of DNA from agarose gel was conducted using Qiaquick Gel Extraction Kit (Qiagen, Hilden).

2.2. Construction of expression plasmid harboring EH gene from R. glutinis

To clone the EH gene from *R. glutinis*, isolation of total RNA was performed as described previously [19]. A cDNA library was synthesized from the total RNA using the first strand cDNA kit (Gibco, USA) as described previously and used to amplify the EH gene by PCR [19]. The primers were designed based on the EH sequences of *R. glutinis* in GenBank (ID: AF172998) as shown in Table 1. After PCR amplification, the DNA was inserted into pGEM-T easy vector (Promega, USA) and transformed into *E. coli* DH5α cells. The *E. coli* cells were cultivated on LB agar plate containing X-gal, IPTG and ampicillin for 16 h at 37 °C and white colonies were selected as transformants containing the recombinant plasmid. Each pGEM-T/RgEH plasmid DNA (here, RgEH denotes the EH gene of *R. glutinis*) was digested with suitable restriction enzymes, and the RgEH DNA fragment was ligated into respective expression vectors, pPICZ, pYD1 or pET21b.

For construction of multimeric expression cassette containing the EH genes into pPICZ C vector, we followed the manufacturer's protocol (Invitrogen). First, PCR-mediated site-directed mutagenesis on *Bgl*II site of *R. glutinis* EH cDNA was carried out by the method of Tomic et al. [20], because *Bgl*II restriction site is needed for the construction of multimeric expression cassette. Primers used for the introduction of mutation are given in Table 1. The PCR product of RgEH without *Bgl*II site was digested with *Eco*RI/*Xho*I and ligated to pPICZ C vector. The resulting pPICZ C/RgEH (no *Bgl*II site, G1077A mutation) was digested with *Bgl*II and *Bam*HI. The DNA fragment containing RgEH gene was self-ligated for the construction of multimeric expression cassette with *Bgl*II–*Bam*HI ligation.

2.3. Transformation of the recombinant plasmids containing R. glutinis epoxide hydrolase gene into various host cells

The resulting plasmid designated as pPICZ C/RgEH (multicopies) was linearized by digesting with *Bst*XI for transformation. Competent cells of *P. pastoris* GS115(*his4*) was made by treatment with *Pichia* EasyComp^{TM} Transformation kit (Invitrogen) and was transformed with the linear plasmid DNA by heat shock. The recombinant *P. pastoris* was cultivated at 30 °C for 4 days on the YPDS/zeocin agar plate (1% (w/v) yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 100 μg/ml zeocin and 2% agar).

Recombinant pYD1/RgEH DNA was transformed to *S. cerevisiae* strain EBY100 (a *GAL1-AGA1::URA3 ura3-52 trp1 leu2Δ1 his3 Δ200 pep4::HIS3 prb1 Δ1.6R can1 GAL*), in which Aga1 protein expression is inducible under the control of *GAL1* promoter with galactose. A pYD1 vector has Aga2 protein gene fused to the various epitopes like hexahistidine tag for yeast surface display. Competent cells of *S. cerevisiae* were made by treatment with *S. cerevisiae* EasyComp^{TM} transformation kit (Invitrogen). Transformants containing pYD1/RgEH were selected on YNB-CAA agar plate (ura$^-$ and trp$^-$) with 2% (w/v) glucose for 48 h at 30 °C.

E. coli BL21(DE3), *E. coli* BLR(DE3) and *E. coli* Rosetta(DE3)pLysS were used for expression of the EH gene

Table 1
Oligonucleotides used for constructions of various recombinant plasmids containing the EH gene of *R. glutinis*[a]

Vector	Primer	Sequences
pPICZ C (double copies)	Forward (*Eco*RI)	5′-TCGAATTCAAAA**ATG**GCGACACACACATTC-3′
	Reverse (*Xho*I)	5′-TCCTCGAGCTTCTCCCACATGACGCCAA-3′
For *Bgl*II site-directed mutation	Forward (G → A)	5′-CTTTGCCCTCTCGCTCTACCCAGGGCGA**A**ATCTACT-3′
	Reverse (C → T)	5′-AGTAGAT**T**TCGCCCTGGGTAGAGCGAGAGGGCAAAG-3′
pYD1	Forward (*Kpn*I)	5′-GAGGTACCA**ATG**GCGACACACACATTC-3′
	Reverse (*Xho*I)	5′-5′-TCCTCGAGCTTCTCCCACATGACGCCAA-3′
pETb(+)	Forward (*Eco*RI)	5′-TCGAATTCG**ATG**GCGACACACACATTCGCT-3′
	Reverse (*Xho*I)	5′-5′-TCCTCGAGCTTCTCCCACATGACGCCAA-3′

[a] Restriction enzyme recognition sites are underlined. Mutated residues are in underlined bold letters. Start codon (ATG) in forward primers is in bold letters. Reverse primers with ending sequence were designed without stop codon for expression of the fused His-tag peptide.

of *R. glutinis*. The recombinant plasmid pET21b(+)/RgEH containing the EH gene from *R. glutinis* was introduced into *E. coli* hosts by electroporation. The recombinant *E. coli* strains were selected on the LB agar plate containing ampicillin for 16 h at 37 °C.

2.4. Expression of R. glutinis epoxide hydrolase in various host cells

For inoculum culture, *P. pastoris* harboring pPICZ C/RgEH (double copies) was grown at 30 °C for 20 h in BMGY medium (1% (w/v) yeast extract, 2% peptone, 2% glucose, 0.1 M sodium phosphate buffer (pH 6.0), 1.0% (v/v) glycerol, and 0.2% of 0.2 mg/ml biotin) to OD_{600} = 5–6. Main culture to express EH proteins was conducted in a two-step mode using BMGY medium and BMMY medium (1% (w/v) yeast extract, 2% peptone, 0.1 M sodium phosphate buffer (pH 6.0), 0.5% (v/v) methanol, and 0.2% of 0.2 mg/ml biotin, pH 6.0). The gene encoding the EH of *R. glutinis* was expressed under the control of AOX1 promoter that is turned on in presence of methanol. For the first step of main culture, the inoculum was transferred into 50 ml of fresh BMGY medium in a 500 ml flask at an OD_{600} of 0.25 and cultivated at 300 rpm for 6–8 h to be an OD_{600} of 6–8. Afterwards, the cells were harvested, washed twice with sterile DDW and transferred into 50 ml of BMMY medium in which EH activity was induced. The cells were further cultured for 84 h with the addition of methanol for every 12 h to a final concentration of 1.0% (v/v).

For seed culture, *S. cerevisiae* EBY100 harboring pYD1/RgEH was grown at 30 °C for 24 h in YNB-CAA medium (ura^- and trp^-) with 2% (w/v) glucose to OD_{600} = 2–5. Centrifuged cell pellet ($5000 \times g$) was resuspended with the same medium containing 2% (w/v) galactose instead of glucose to OD_{600} = 0.5–1.0 and cultured for a given time at 25 °C and 250 rpm.

After seed culture for 16 h, the recombinant *E. coli* strains were cultured in 50 ml Luria–Bertani (LB) medium supplemented with 50 μg ampiciline ml^{-1} at 37 °C and 250 rpm until OD at 600 nm was about 0.5–1. IPTG induction was started to express the EH gene at 0.1 mM for 4 h. The recombinant cells were harvested by centrifugation, washed with distilled water, and then used for enantioselective hydrolysis reactions.

2.5. Polyacrylamide gel electrophoresis and immunoblotting

Recombinant yeast cells with *R. glutinis* EH gene were cultured in the appropriate medium as described in Section 2.4. A 100 μl of breaking buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA, 5% (v/v) glycerol) and equal volume of acid-washed glass beads (size 0.5 mm) were added to cell pellets of 200 μl culture and vortexed vigorously for 30 s for 8 times. The 50 μl of each supernatant was cooked at 95 °C for 10 min with Laemmli buffer, separated on 12% SDS-polyacrylamide gel, and blotted onto nitrocellulose membranes. In case of recombinant *E. coli* cells, 100 μl culture without induction or 200 μl culture with induction at OD_{600} = 2 was used on SDS-PAGE. The membrane was incubated with polyclonal antibody against hexahistidine (H-15, Santa Cruz Biotechnology, Inc., USA) and peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch, USA), and then visualized with CN/DAB (4-chloronaphthol/3,3′-diaminobenzidine) substrate kit (Pierce, USA).

2.6. Epoxide hydrolase-catalyzed enantioselective hydrolysis reaction

The kinetic resolution of racemic styrene oxides was conducted in 5 ml screw-capped vials with the working volume of 1 ml. The cultured cells were suspended in a 100 mM KH_2PO_4 buffer (pH 8), and enantioselective hydrolysis reactions were initiated with the addition of 20 mM racemic styrene oxide in a shaking incubator at 30 °C and 250 rpm. The reaction was then stopped by extraction with an equal volume of cyclohexane. The progression of the enantioselective hydrolysis reaction was analyzed by the analysis of samples withdrawn periodically from the reaction mixtures. The spontaneous chemical degradation of styrene oxide throughout recombinant cell-catalyzed enantioselective hydrolysis was negligible within short reaction time below 30 min.

The kinetics on the styrene oxides hydrolysis was studied by measuring the initial rates with enantiopure (*R*)- or (*S*)-styrene oxide (Aldrich Chemical Inc., USA). The concentration of (*R*)- or (*S*)-styrene oxide was in the range of 0.75 and 6.5 mM, since the solubility was about 7.0 mM at 35 °C.

2.7. Analyses

Cell concentration was measured by a spectrophotometer at 600 nm (UV1240, Shimazhu, Japan). Enantiomeric excess ($ee = 100 \times (S - R)/(S + R)$) and yield (yield = 100 × residual (*S*)-epoxide concentration/initial racemic epoxide concentration) of enantiopure styrene oxides were determined by a chiral GC analysis. The reaction mixture was extracted with an equal volume of cyclohexane, and 1 μl of the organic layer was analyzed using chiral GC with a fused silica capillary beta-DEX 120 column (0.25 mm i.d. × 30 m, 0.25 μm film thickness, Supelco Inc., USA) fitted with a FID detector. The temperatures of the column, injector, and detector were 100, 220 and 220 °C, respectively.

3. Results and discussions

3.1. Development of recombinant P. pastoris expressing epoxide hydrolase of R. glutinis

In general, an enhanced expression of EH gene in a recombinant cell confer a higher activity and specific productivity of a recombinant whole-cell biocatalyst. Compared with a recombinant *E. coli* containing multicopy plasmids with a strong promoter, the expression level of the target gene in a recombinant *Pichia* can be restricted since only single copy of the target gene is integrated into the chromosome of *Pichia* host. One simple way to enhance the expression level in a recombinant *Pichia*

is to build multimer of the target gene by ligating each additional expression cassette. To generate multiple copies of EH expression cassette, head-to-tail multimers were constructed as described in Section 2. We could isolate the recombinant plasmids with two copies of the EH gene from *E. coli* DH5α, and the recombinant plasmid was linearized and transformed into *P. pastoris* GS115 (*HIS*$^-$) to be integrated into the chromosome of the host cells. We compared expression levels to see whether multiple copies increase the amount of the expressed EH protein by SDS-PAGE and immunoblotting, however, only a faint band was observed.

The degradation kinetics on the recombinant *P. pastoris* containing double copies of the EH gene of *R. glutinis* was evaluated (Table 2). We used Michaelis–Menten kinetic model to evaluate the kinetic characteristics of the recombinant cell at initial concentrations of (*S*) and (*R*)-styrene oxide from 0.5 to 6 mM. The maximum hydrolysis rate (V_{max}) and saturation constant (K_m) for both isomers were determined from Lineweaver–Burke plots, and summarized in Table 2. In case of the recombinant *P. pastoris* containing double copies of the EH gene, the maximum hydrolysis rate (V^R_{max}) and saturation constant (K^R_m) for (*R*)-isomer were 387.1 nmol min^{-1} (mg dcw)$^{-1}$ and 3.2 mM, respectively. In comparison, the whole-cell activity of the recombinant *P. pastoris* with double copies of the EH gene was improved by about 11-fold compared to wild-type *R. glutinis*. The higher activity is mainly due to the higher expression of the EH gene in *Pichia* host strain.

3.2. Development of recombinant S. cerevisiae surface-displaying epoxide hydrolase of R. glutinis

When whole-cells are used as biocatalysts for enantioselective hydrolysis reactions, there is one possible drawback that the racemic substrates should diffuse across cell membranes, which cause mass transfer limitation. In general, K_m values are much lower in the purified enzymes, indicating the mass transfer resistance conferred by cell walls of whole-cells is high. In accordance with this assumption, the affinity constants of whole-cell biocatalysts such as wild-type *R. glutinis* and recombinant *P. pastoris* expressing the EH gene of *R. glutinis* were rather high in the range 3.5–7.5 mM. We also observed that K^S_m value of the recombinant *P. pastoris* decreases by more than two orders of magnitude when measured after the cells are broken by a bead beater (unpublished data). The resistance to mass transfer of substrates across cell membranes can be overcome by expressing the enzyme of interest to the surface of a cell. This approach that requires no need for enzyme purification and minimizes mass transport resistance has clear advantages over purified enzymes and wild-type whole-cells.

To develop a surface-displayed EH whole-cell biocatalyst, we used *S. cerevisiae* Aga2 protein as the surface-anchoring motif to display the EH protein of *R. glutinis*. The EH gene was inserted into *Kpn*I and *Xho*I site of pYD1 vector containing the gene of Aga2 protein for yeast surface display. The recombinant pYD1/RgEH DNA was transformed to *S. cerevisiae* strain EBY100 in which the EH protein expression is inducible from *GAL*1 promoter. When expression of the EH protein was induced with 2% (w/v) galactose, the recombinant *S. cerevisiae* exhibited (*R*)-specific enantioselective hydrolysis activity toward racemic styrene oxides. The degradation kinetics on enantiopure styrene oxide was evaluated. In case of the surface displayed *S. cerevisiae*, the maximum hydrolysis rate (V_{max}) and saturation constant (K_m) for (*R*)-enantiomer were 27.2 nmol min^{-1} (mg dcw)$^{-1}$ and 2.6 mM, respectively. In comparison with the recombinant *P. pastoris*, the maximum hydrolysis rate (V_{max}) was very low and saturation constant was similar. The relative ratio of the first-order reaction rate for (*R*)- and (*S*)-enantiomer was also similar with those of the recombinant *P. pastoris* and *E. coli* (Table 2). When the recombinant *S. cerevisiae* was analyzed by SDS-PAGE, the expression level of the EH was very low, indicating that the recombinant *S. cerevisiae* exhibited a low whole-cell EH activity per unit cell mass. Therefore, we concluded that low level expression of the EH at the surface of yeast does not enhance the catalytic efficiency.

3.3. Development of recombinant E. coli expressing epoxide hydrolase of R. glutinis

Recently, Vissor et al. reported kinetic resolutions of racemic epoxides using the recombinant *E. coli* BL21(DE3) containing the EH gene of *R. glutinis* [21]. They used 1,2-epoxyhexane as the substrate and obtained the specific activity of 200 nmol min^{-1} (mg protein)$^{-1}$ of the recombinant *E. coli*, which is only 1.7 times higher than that of wild-type *R. glutinis*. In general, each microorganism has its own bias in usage of codons [22]. Differences in codon usage can hamper efficient and correct expression of heterologous genes. We analyzed

Table 2
Kinetic constants for the enantioselective hydrolysis of styrene oxide by wild-type *R. glutinis*, recombinant *P. pastoris*, recombinant *S. cerevisiae* and three types of recombinant *E. coli*[a]

	Wild-type *R. glutinis*	Recombinant *P. pastoris*	Recombinant *S. cerevisiae*	Recombinant *E. coli* BL21	Recombinant *E. coli* BLR	Recombinant *E. coli* Rosetta
V^S_{max} (nmol min^{-1} mg^{-1} dcw)	10.5	135.3	6.2	390.3	176.1	1439.2
V^R_{max} (nmol min^{-1} mg^{-1} dcw)	34.5	387.1	27.2	840.8	514.7	2287.6
K^S_m (mM)	7.7	4.1	3.9	5.7	1.57	5.7
K^R_m (mM)	3.5	3.2	2.6	5.4	1.67	2.0
Rate ratio[b]	7.2	3.7	6.6	2.3	2.9	4.5

[a] The kinetic data were calculated from experiments repeated three times.
[b] Ratio of the first-order reaction rate, $(V^R_{max}/K^R_m)/(V^S_{max}/K^S_m)$.

the codons present in the EH gene of *R. glutinis* whether low level protein expression was due to the differences in codon usage between *E. coli* and *R. glutinis*. The EH gene of *R. glutinis* contains 1 CGA, 6 GGA and 10 CCC for a total of 19 *E. coli* rare codons (4.7%). In case of proline, the frequency of rare codon, CCC, was about 25.6% of total proline-encoding codons. Correct expression of genes with many rare codons can be enhanced if copy numbers of the corresponding tRNAs for rare codons increase within host cells. We employed *E. coli* Rosetta(DE3)pLysS strain as the host strain to express the EH gene at a high level. *E. coli* Rosetta(DE3)pLysS harbors the pRARE plasmid encoding tRNA genes for rare codons, especially for Arg, Ile, Gly, Leu and Pro together with the gene encoding T7 lysozyme [23].

The pET21b(+)/RgEH plasmid was constructed for expression of *R. glutinis* EH gene in *E. coli*, and then transformed into various *E. coli* host strains, BL21(DE3), BLR(DE3) and Rosetta(DE3)pLysS for an attempt to increase the expression level. Fig. 1 shows SDS-PAGE and immunoblotting analysis of the EH gene expressed in three different *E. coli* strains. A 1 mM of IPTG was added at the mid-log phase (0.6–0.8 OD at 600 nm) of the cultures and the cells were cultured for 4 h at 37 °C. As shown in Fig. 1, the expression levels of the EH gene in *E. coli* BL21 and Rosetta hosts were clearly higher than that in *E. coli* BLR host. The band intensities of the recombinant *E. coli* BL21 and Rosetta were similar to each other.

The degradation kinetics on enantiopure styrene oxides by various recombinant whole-cell biocatalysts were evaluated (Table 2). In all cases, enantioselective hydrolysis reactions by the recombinant *E. coli* yielded (*S*)-styrene oxide as the residual enantiomer. Of the three recombinants, the recombinant *E. coli* Rosetta containing rare-tRNA genes in its plasmid DNA gave the highest initial hydrolysis rate (Table 2). The maximum hydrolysis rate (V_{max}) and saturation constant (K_m) of the recombinant *E. coli* Rosetta for (*R*)-enantiomer were 2287.6 nmol min^{-1} (mg dcw)$^{-1}$ and 2.0 mM, respectively. Whereas the relative ratio of the first-order reaction rates for (*R*)- and (*S*)-enantiomer was rather lower than that of wild-type *R. glutinis*, the maximum hydrolysis rate of the recombinant *E. coli* Rosetta for (*R*)-enantiomer was 66-fold higher than that of wild-type *R. glutinis*, indicating that the expression level was clearly enhanced. Although the expression levels of the EH gene in *E. coli* BL21 and Rosetta were similar to each other, the maximum hydrolysis rate of the recombinant *E. coli* Rosetta for (*R*)-enantiomer was 2.7-fold higher than that of the recombinant *E. coli* BL21. From the above results, it can be inferred that correct expression level of the EH gene in *E. coli* Rosetta was probably higher compared to the recombinant *E. coli* BL21, resulting in the higher whole-cell EH activity of the recombinant *E. coli* Rosetta.

3.4. Kinetic resolution of racemic styrene oxides using various recombinant EHs

Enantioselective resolutions of 20 mM racemic styrene oxide using various recombinants EHs developed in this study were performed under the reaction conditions of 30 °C, pH 8.0, and cell concentration of 10 mg dcw ml^{-1}. Time course studies revealed that enantiopure (*S*)-styrene oxide with 99% ee was obtained for all recombinant EHs within various reaction times (Fig. 2). The yields were in the range from 16.3 to 34.6%. Of the various recombinant whole-cell biocatalysts, the recombinant *E. coli* Rosetta showed much higher hydrolysis activity. We conclude that the recombinant whole-cell EH biocatalysts

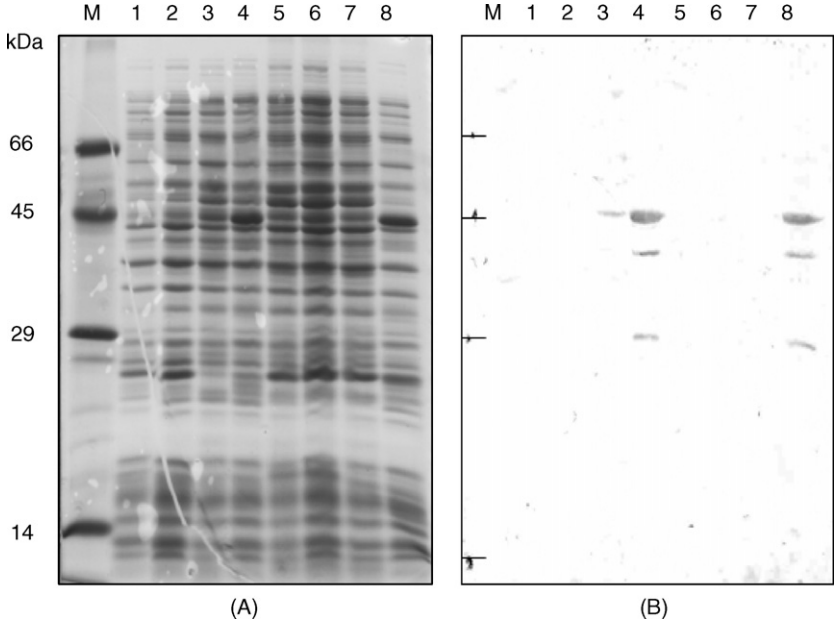

Fig. 1. SDS-PAGE and immunoblotting analysis of recombinant *E. coli* containing the EH gene of *R. glutinis*. EH proteins were expressed as the cells were induced with 1 mM IPTG for 3.5 h. Proteins were separated on 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 (A) and immunoblotted with polyclonal hexahistidine antibody (B). Lanes 1 and 2 indicate BL21 recombinant with pET21b(+) vector only. EH proteins were expressed in *E. coli* BL21 (lanes 3 and 4), BLR (lanes 5 and 6) and Rosetta strain (lanes 7 and 8). Lanes 1, 3, 5 and 7: without induction; lanes 2, 4, 6 and 8: with IPTG induction.

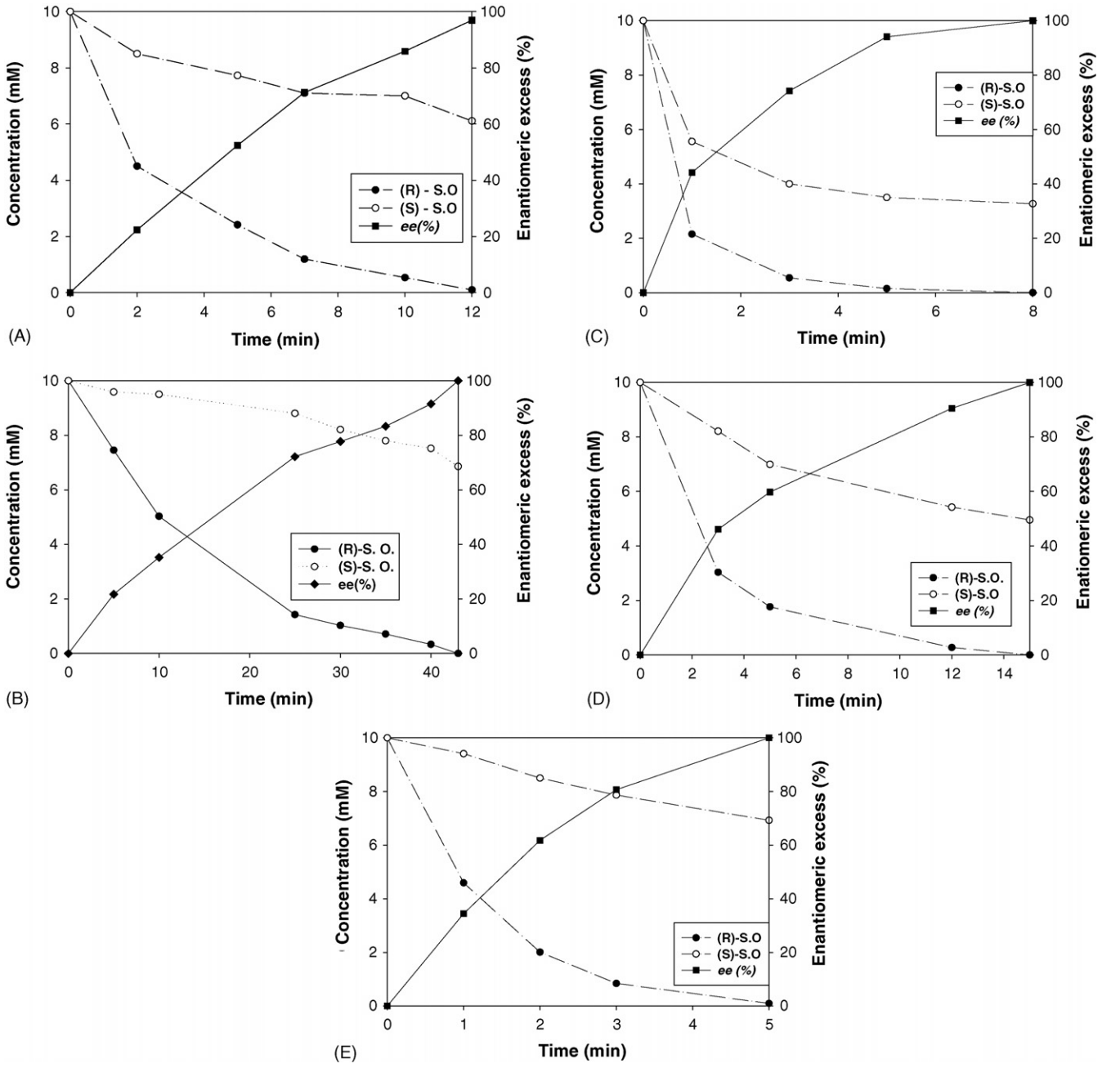

Fig. 2. Enantioselective resolutions of racemic styrene oxide at 20 mM by various recombinant whole-cell biocatalysts. Symbols: (●) (*R*)-styrene oxide, (○) (*S*)-styrene oxide and (■) enantiomeric excess. (A) Recombinant *P. pastoris* (double copies), (B) recombinant *S. cerevisiae*, (C) recombinant *E. coli* BL21, (D) recombinant *E. coli* BLR and (E) recombinant *E. coli* Rosetta.

developed in this study are highly active and have industrial potential under the conditions where the purified EH or wild-type whole-cells cannot be used.

4. Conclusions

In this study, we developed highly active recombinant whole-cell biocatalysts that can be used for enantioselective hydrolysis reactions of racemic epoxides. The recombinant *P. pastoris* harboring double copies of *R. glutinis* EH gene was constructed. The surface-displayed EH biocatalyst was constructed using *S. cerevisiae* EBY100 as the host strain. We employed *E. coli* Rosetta(DE3)pLysS strain harboring pRARE plasmid encoding tRNA genes for rare codons as the host strain to express the EH gene of *R. glutinis* at high level in *E. coli* host. The recombinant strains showed 11–66-fold higher hydrolytic activities on (*R*)- and (*S*)-styrene oxides than wild-type *R. glutinis*. All of the recombinant strains could successfully perform enantioselective resolutions for racemic styrene oxide of 20 mM. The recombinants developed in this study are expected to be useful biocatalysts for enantioselective hydrolysis of racemic epoxides for preparing enantiopure epoxides.

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