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Highly Efficient Deracemization of Racemic 2-Hydroxy Acids in a Three-Enzyme Co-Expression System Using a Novel Ketoacid Reductase

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Abstract Enantiopure 2-hydroxy acids (2-HAs) are important intermediates for the synthesis of pharmaceuticals and fine chemicals. Deracemization of racemic 2-HAs into the corresponding single enantiomers represents an economical and highly efficient approach for synthesizing chiral 2-HAs in industry. In this work, a novel ketoacid reductase from *Leuconostoc lactis* (*Ll*KAR) with higher activity and substrate tolerance towards aromatic α-ketoacids was discovered by genome mining, and then its enzymatic properties were characterized. Accordingly, an engineered *Escherichia coli* (HADH-*Ll*KAR-GDH) co-expressing 2-hydroxyacid dehydrogenase, *Ll*KAR, and glucose dehydrogenase was constructed for efficient deracemization of racemic 2-HAs. Most of the racemic 2-HAs were deracemized to their (*R*)-isomers at high yields and enantiomeric purity. In the case of racemic 2-chloromandelic

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acid, as much as 300 mM of substrate was completely transformed into the optically pure (*R*)-2-chloromandelic acid (> 99% enantiomeric excess) with a high productivity of 83.8 g L^{-1} day^{-1} without addition of exogenous cofactor, which make this novel whole-cell biocatalyst more promising and competitive in practical application.

Keywords Biocatalysis · 2-Hydroxy acid · Deracemization · Ketoacid reductase · Co-expression · (*R*)-2-Chloromandelic acid

Introduction

Enantiopure 2-hydroxy acids (2-HAs), a class of particularly important chiral building blocks, are widely used in the synthesis of pharmaceuticals and fine chemicals [1–5]. For instance, (*R*)-2-chloromandelic acid is a valuable intermediate in the manufacture of anti-thrombotic agent, (*S*)-clopidogrel [6–8]. (*R*)-2-Hydroxy-4-phenylbutyric acid plays an important role in the production of angiotensin-converting enzyme (ACE) inhibitors such as cilazapril and benazapril [9, 10].

Due to their importance, a series of approaches for the preparation of enantiopure 2-HAs have been explored, and a great progress has been achieved in the past decades. Compared with the traditional chemical approaches, several biocatalyst-mediated strategies are more potential and preferable due to their high yields, strict enantioselectivity, mild reaction conditions, and environmental friendliness [1, 11], mainly involving kinetic resolution of racemic 2-HAs and derivatives with oxidases or hydrolases [12–14], asymmetric reduction of prochiral 2-ketoacids with ketoacid reductases [15], and cascade deracemization of racemic 2-HAs by coupling oxidation and reduction reactions with targeted oxidoreductases [16, 17]. Among these enzymatic routes, deracemization by coupling redox reactions in one pot represents an economical and highly efficient fashion, which can efficiently transform racemates of 2-HAs into the corresponding single enantiomers, thus circumventing yield-reducing and time-consuming isolation of intermediates in multiple reactions [18–20]. In recent years, multi-enzyme cascade reaction has become a very important synthetic strategy in the field of biocatalysis and attracted a lot of attentions [21–25]. With the advance of genetic and metabolic engineering tools, redox cascade reactions can be implemented by multi-gene co-expression in a target host cell, such as *Escherichia coli* [26–29]. This method is more efficient, cost-saving, and easy to operate compared with multi-strain cascade. In previous studies, the redox cascade reaction was used for the synthesis of (*R*)-2-HAs including (*R*)-2-chloromandelic acid in one pot by multi-strain cascade or a single recombinant *E. coli* co-expressing (*S*)-2-hydroxy acid dehydrogenase (HADH), ketoacid reductase (KAR), and glucose dehydrogenase (GDH) [20, 30]. However, the substrate concentration was only 20 mM due to the poor activity of KAR used in the reduction reaction.

In this work, we firstly discovered a novel ketoacid reductase from *Leuconostoc lactis*, *Ll*KAR, with higher activity and substrate tolerance by means of genome mining, and then constructed a recombined *E. coli* co-expressing HADH, *Ll*KAR, and GDH for highly efficient deracemization of racemic 2-HAs (Fig. 1). (*S*)-2-HAs in the racemic mixtures were enantioselective oxidized to the corresponding 2-ketoacids by HADH (FMN-dependence) [31]. Then prochiral ketoacids were asymmetric reduced to (*R*)-2-HAs by *Ll*KAR (NADH-dependence), and NADH-regeneration was implemented by GDH. The substrate loading and

productivity were significantly improved due to the high activity of *Ll*KAR, indicating that this synthetic method is more competitive and promising in the industrial application.

Materials and Methods

Materials

The gene of HADH was cloned from *Pseudomonas aeruginosa* (AGM49308.1). The gene of GDH was cloned from *Exiguobacterium sibiricum* (WP_012369122.1). The kits for rapid extraction of plasmid and purification of DNA were purchased from Axygen Biotechnology Co., Ltd. (Hangzhou, China). ClonExpress® II (One Step Cloning Kit) was supplied by Vazyme Biotech Co., Ltd. (Nanjing, China). Plasmids pET28b and pCDFDuet (Novagen, Darmstadt, Germany) were used as expression vectors. Both *E. coli* DH5α and *E. coli* BL21 (DE3) were cultured in Luria-Bertani (LB) medium as cloning host and expression host, respectively. All the chemicals and agents used in experiments were obtained commercially.

Mining and Screening of KARs

The genome mining strategy was adopted for searching novel KARs with high activity and enantioselectivity. Two functionally known KARs (*Le*KAR and *Sc*KAR) was used as templates for the NCBI-pBLAST search. All the potential genes were synthesized in vitro, cloned into pET28b, and then transformed into *E. coli* BL21 (DE3).

The specific activity and enantioselectivity of KARs were tested using the standard enzyme activity assay and enantiomeric excess (*e.e.*) determination protocol, respectively. The further comparison of KARs from different sources was carried out in a reaction system (10 mL) containing potassium phosphate buffer (PPB, 100 mM, pH 7.0), phenylglyoxylic acid (100 and 400 mM), glucose (2.0 equiv. vs. substrate), NAD^+ (0.5 mM), KARs (800 U/mL), and

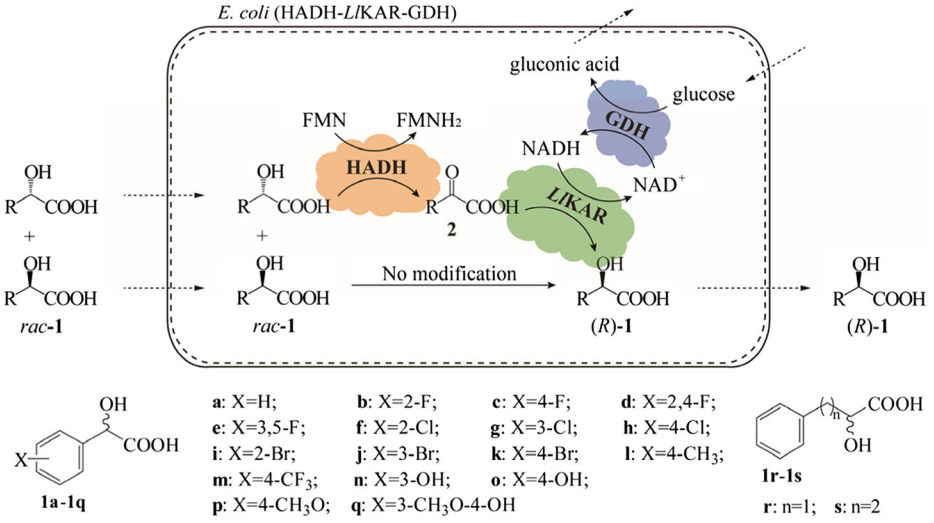


Fig. 1 Whole-cell of recombinant *E. coli* (HADH-*Ll*KAR-GDH) used for highly efficient deracemization of racemic 2-HAs

GDH (800 U/mL, for regeneration of cofactor). Reactions were performed for 3 h at 35 °C, 700 rpm and titrated automatically with 3.0 M NaOH for maintaining pH at 7.0. Samples were centrifuged (12,000 ×*g* for 3 min), diluted, and filtered for chiral HPLC analysis to calculate conversion and *e.e.* values.

Cultivation of Recombinant *E. coli*

All the engineered *E. coli* strains were cultured in LB medium containing a certain amount of antibiotics (50 μg/mL kanamycin for pET28b, 50 μg/mL streptomycin for pCDFDuet, and both antibiotics for both vectors existing simultaneously) at 37 °C. When the OD_{600} of the cultures reached 0.6–0.8, IPTG was added into the medium to a final concentration of 0.1 mM. The cultures were incubated continually at 28 °C, 150 rpm for another 12 h. The cells were collected by centrifugation (9000 ×*g*, 4 °C, 10 min) and then washed twice with physiological saline.

Purification and Kinetic Parameters Analysis of KARs

Resting cells were resuspended in 100 mM PPB (pH 7.5) and disrupted by sonication. Then the crude enzyme solution was obtained via centrifugation and stored at 4 °C for further use. Bio-Scale™ Mini Nuvia™ IMAC Ni-charged cartridges (Bio-Rad Laboratories, Inc.) were used for the purification of KARs. Detailed operating procedures referred to the instruction manual and recommended protocol in the quick start guide. The fractions containing target protein were pooled and dialyzed against 20 mM PPB (pH 7.0) for desalting. The purified protein samples were analyzed by SDS-PAGE.

The kinetic parameters of purified enzymes were investigated by determining the initial velocities of the enzymatic reactions as described previously [32, 33]. The concentration of phenylglyoxylic acid, as a mode substrate, was varied from 0.5 to 20 mM with a fixed NADH concentration (5 mM).

Catalytic Properties Characterization of Purified *Ll*KAR

The optimum pH of *Ll*KAR was examined in different buffers (100 mM, pH 5.0–9.0): CH_3COOH-CH_3COONa (pH 5.0–6.0), KH_2PO_4-K_2HPO_4 (pH 6.0–8.0), and Tris-HCl (pH 8.0–9.0). The pH stability was investigated by determining the residual activity after pre-incubating the purified *Ll*KAR in different buffers (pH 5.0–9.0) at 4 °C for 12 h. The optimum temperature was examined at various temperatures (20–55 °C). Thermal stability was investigated by determining the residual activity of purified *Ll*KAR at different temperatures (25–65 °C), and then the corresponding parameters, such as thermal inactivation rate constant (k_d) and half-lives ($t_{1/2}$), were obtained according to the reported methods [34]. In addition, the thermal stability was further studied by recording the CD spectra at different temperatures (25–65 °C, pre-incubating for 30 min, respectively) and variable wavelengths (190–350 nm), using a spectropolarimeter J-815 (Jasco Co., Tokyo, Japan).

The effects of metal ions and chemical agents on *Ll*KAR activity were evaluated. The residual activity was determined using the standard enzyme activity assay after the interaction of purified *Ll*KAR and different compounds (1.0 mM) for 20 min at 35 °C. Relative activity here represents a percentage of the enzyme activity in the absence of any tested compound.

Several α-ketoacid substrates with aliphatic or aromatic substituents were chosen to investigate the substrate specificity of *Ll*KAR using the standard enzyme activity assay and *e.e.* determination protocol. The activity towards phenylglyoxylic acid was regarded as 100%.

Analytic Methods

The standard enzyme activity assays of KARs were carried out in a reaction system (1.0 mL) including 100 mM PPB (pH 7.0), 10 mM phenylglyoxylic acid, 5 mM NAD(P)H, and enzyme at appropriate concentration. Reaction mixture and enzyme were preheated at 35 °C for 5 min, respectively. Also, reactions were conducted at 35 °C and 700 rpm for 2 min and terminated by 6.0 M HCl. Samples were centrifuged (12,000 ×*g* for 3 min), diluted, and filtered for chiral HPLC analysis. One unit (U) of enzyme activity was defined as the amount of enzyme required to catalyze the reduction of phenylglyoxylic acid for producing 1.0 μmol of mandelic acid in 1.0 min under the standard enzyme activity assay conditions.

The *e.e.* values were determined via a reaction system (1.0 mL) consisted of 100 mM PPB (pH 7.0), 10 mM phenylglyoxylic acid, 15 mM NAD(P)H, and enzyme at suitable concentration. Reactions were performed at 35 °C and 700 rpm for 10 h and terminated with 6.0 M HCl. Samples were centrifuged (12,000 ×*g* for 3 min), diluted, and filtered for chiral HPLC analysis, which was performed according to the method reported previously [20].

Construction of Recombinant *E. coli* (HADH-*Ll*KAR-GDH)

The gene of GDH from *E. sibiricum* and the gene of *Ll*KAR from *L. lactis* were successively cloned and linked to the expression plasmid pCDFDuet using seamless cloning technology with ClonExpress® II (Vazyme Biotech Co., Ltd., Nanjing, China). Then the resulting pCDFDuet-*Ll*KAR-GDH was transformed into *E. coli* BL21 (DE3), and the target recombinant transformants (*E. coli* (*Ll*KAR-GDH)) were screened on the LB plates with 50-μg/mL streptomycin.

The gene of HADH from *P. aeruginosa* was linked to the expression plasmid pET28b. Then recombinant plasmids pET28b-HADH and pCDFDuet-*Ll*KAR-GDH were extracted and co-transformed into the expression host *E. coli* BL21 (DE3). Kanamycin (50 μg/mL) and streptomycin (50 μg/mL) were added to the medium simultaneously for the selection of positive transformants (*E. coli* (HADH-*Ll*KAR-GDH)). The co-expression of three enzymes (HADH, *Ll*KAR, and GDH) was determined using SDS-PAGE.

Deracemization of Racemic 2-HAs with *E. coli* (HADH-*Ll*KAR-GDH)

The cascade deracemization of racemic 2-HAs (**1a**-**1s**) was performed in a 10 mL reaction system involving PPB (100 mM, pH 7.0), substrate (20 mM), glucose (20 mM), and whole-cell catalyst (8 g DCW/L). Reactions were carried out at 30 °C, 700 rpm for 6 h. Samples were taken once an hour, centrifuged (12,000 ×*g* for 3 min) for removing the catalyst, diluted, and filtered to determine yields without isolation and *e.e.* values by chiral HPLC analysis.

Whole-Cell Biocatalytic Synthesis of (*R*)-1f

The synthesis of (*R*)-**1f** with whole-cell biocatalyst *E. coli* (HADH-*Ll*KAR-GDH) was conducted in a 10 mL reaction system including PPB (100 mM, pH 7.0), *rac*-**1f**, glucose (1.0

equiv. vs. substrate), and whole-cell catalyst. A series of reactions were performed under various conditions as listed in Table 5 at 30 °C and 700 rpm. The process of deracemization was monitored by chiral HPLC analysis and stopped until no further conversion. Whole-cell biocatalytic synthesis of (*R*)-**1f** with recombinant *E. coli* (HADH-*Ll*KAR-GDH) by adding *rac*-**1f** in a fed-batch mode was started in a 10 mL potassium phosphate buffer (100 mM, pH 7.0) containing *rac*-**1f** (100 mM), glucose (100 mM), and catalyst (20 g DCW/L) at 30 °C and 700 rpm. *Rac*-**1f** (100 mM) and glucose (100 mM) were added per 4 h. All the reactions were titrated automatically with 3.0 M NaOH for maintaining pH at 7.0.

Results and Discussion

Mining and Screening of KARs

In previous work, a HADH (AGM49308.1) with high activity has been used for the oxidation of 2-HAs. In order to match the activity of HADH in the three-enzyme co-expression system, it is necessary to search for a KAR with high activity towards 2-keto acids to avoid its accumulation. The strategy of genome mining, as an effective method, has been widely used in the discovery of novel biocatalysts and could largely shorten the mining period [35]. In view of this, a mini-library of KARs was constructed by a NCBI-pBLAST search with two amino acid sequences of *Le*KAR from *Leuconostoc mesenteroides* and *Sc*KAR from *Saccharomyces cerevisiae* as templates. Six predicted KARs owning 62–84% identities of amino acid sequence with probe proteins were selected, and its genes were synthesized and overexpressed in *E. coli* BL21 (DE3).

For screening an ideal biocatalyst from the mini-library, the activity and enantioselectivity of all these KARs were tested with phenylglyoxylic acid as a mode substrate (Table 1). *Le*KAR and KARs from entry1–3 exhibited better specific activities and perfect selectivity, and among them *Ll*KAR was the best one, giving a specific activity of 3.7 kU/mg crude enzyme and *e.e.* value of > 99%. The substrate loadings of these KARs were further compared using 100 and 400 mM phenylglyoxylic acid, respectively (Fig. 2). We found only *Ll*KAR could transform phenylglyoxylic acid at two various concentrations to the corresponding (*R*)-**1a** with excellent conversion (> 99%) and enantioselectivity (> 99%) within 3 h.

Four recombinant KARs were purified from crude enzyme solution by IMAC Ni-charged cartridges to determine their kinetic parameters (Table 2). SDS-PAGE analysis displayed that the electrophoretic pure KARs were obtained, and a single band of about 32 kDa was presented in the corresponding lane (Fig. S1). Results of kinetic parameters analysis revealed that *Ll*KAR owned the maximum V_{max} (7.29 mmol min^{-1} mg^{-1}) and the minimum K_m (4.30 mM) towards phenylglyoxylic acid compared with other KARs. The catalytic efficiency (K_{cat}/K_m) of *Ll*KAR reached 981.48 mM^{-1} s^{-1}, up to 3.7 times of the original enzyme (*Le*KAR), which indicated the application of *Ll*KAR in cascade deracemization system was of great potential. Thus, *Ll*KAR from *L. lactis* was used in the following experiments.

Catalytic Properties Characterization of Purified *Ll*KAR

In order to better understand and utilize *Ll*KAR with promising activity and substrate tolerance, we did a series of experiments to characterize its catalytic properties. *Ll*KAR has been proved to be a NADH-dependent reductase in initial experiments, the same as a α-

Table 1 Evaluation of KARs using phenylglyoxylic acid as a mode substrate

Entry	Name	GenBank accession no.	Microbial strain	Identity to probe enzyme (%)	Specific activity (kU/mg)	*e.e.* (%)
Probe enzyme	*Le*KAR	ANN45946.1	*Leuconostoc mesenteroides*	–	1.1138	> 99 (*R*)
	*Sc*KAR	AAS56366.1	*Saccharomyces cerevisiae*	–	0.7849	> 95 (*R*)
1	*Ll*KAR	WP_068852189.1	*Leuconostoc lactis*	84 to *Le*KAR	3.7077	> 99 (*R*)
2	*Lm*KAR	WP_041775202.1	*Leuconostoc mesenteroides*	74 to *Le*KAR	3.3692	> 99 (*R*)
3	*Ko*KAR	WP_047720787.1	*Klebsiella oxytoca*	49 to *Le*KAR	2.8895	> 99 (*R*)
4	*Sk*KAR	EJT41757.1	*Saccharomyces kudriavzevii*	85 to *Sc*KAR	0.1689	> 99 (R)
5	*Ka*KAR	XP_003955441.1	*Kazachstania africana*	69 to *Sc*KAR	0.1317	> 99 (R)
6	*Cg*KAR	KTB10995.1	*Candida glabrata*	62 to *Sc*KAR	Not detectable	Not detectable

ketoacid reductase from *L. mesenteroides* [15], but unlike most other reported reductases for catalytic reduction of ketoacids/esters especially from eukaryotic yeasts [32, 36–38].

The effect of pH on activity of purified *Ll*KAR was determined in different buffers (pH 6.0–9.0). As shown in Fig. S2a, the optimal activity was observed at pH 7.0 and over 80% enzyme activity could be examined at pH 6.0–7.5. The pH stability of *Ll*KAR was excellent in a neutral environment, and over 90% activity was still retained after being incubated at pH 6.0–8.0 and 4 °C for 12 h (Fig. S2b). The effect of temperature on activity was assayed at 20–55 °C

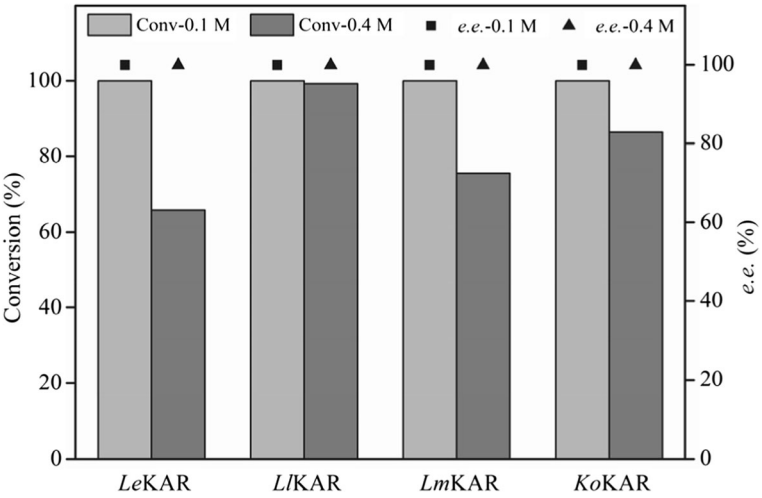


Fig. 2 Conversion and enantioselectivity of *Le*KAR, *Ll*KAR, *Lm*KAR, and *Ko*KAR-catalyzed asymmetric reductions of phenylglyoxylic acid at 0.1 and 0.4 M. Reaction conditions (35 °C, 700 rpm, 3 h): phenylglyoxylic acid (100 and 400 mM), glucose (2.0 equiv. vs. substrate), NAD^+ (0.5 mM), KARs (800 U/mL), and GDH (800 U/mL) without purification, in a 10 mL potassium phosphate buffer (100 mM, pH 7.0). Reactions were titrated automatically with 3.0 M NaOH for maintaining pH at 7.0

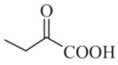
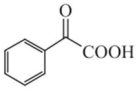
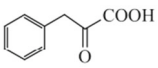
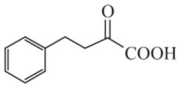
Table 2 Kinetic parameters analyses of KARs with phenylglyoxylic acid as the substrate

Enzyme	V_{max} (mmol min^{-1} mg^{-1})	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m (mM^{-1} s^{-1})
*Le*KAR	3.71	8.22	2168.07	263.78
*Ll*KAR	7.29	4.30	4220.91	981.48
*Lm*KAR	7.25	5.11	4254.29	832.38
*Ko*KAR	4.47	5.90	2525.64	427.93

(Fig. S2c). The maximum activity of *Ll*KAR was detected at 35 °C, and it retained over 90% catalytic activity at a relatively broad temperature (30–40 °C). Thermostability of purified *Ll*KAR was studied at 25–65 °C (Fig. S2d). The enzyme activity decreased relatively slowly at less than 45 °C and still maintained about 30% of initial activity even being incubated at 45 °C for 24 h. The rates of inactivation at 55 and 65 °C markedly accelerated with half-lives of 0.42 and 0.22 h, respectively (Fig. S2e & Table S1). In addition, CD spectra of *Ll*KAR at various temperatures were also used for the thermostability analysis. Obviously, the shapes of characteristic peaks changed greatly at 55 and 65 °C, which was very consistent with the inactivation curve above (Fig. S2f).

The effects of metal ions and chemical agents on *Ll*KAR activity were evaluated as illustrated in Fig. S3. The presence of Mn^{2+}, Fe^{2+}, and Ca^{2+} has relatively slight stimulative effects on the activity of *Ll*KAR. But, it was significantly inhibited by Cu^{+}, Cu^{2+}, and Ni^{+}, which maybe cause by the interaction with key residues in enzyme structure, such as His [33, 39]. All the chemical agents tested have the inhibitory effects on the enzyme activity to varying degrees. Among them, water miscible DMSO, water immiscible *n*-heptane, and EDTA exhibited relatively small impacts, meanwhile indicating that *Ll*KAR is not a metalloenzyme.

Table 3 Substrate specificity of purified *Ll*KAR

Substrate	Relative activity (%) [a]	Yield (%) [b]	*e.e.* (%) [b]
	49.7	65.2	>99 (*R*)
	56.4	71.8	>99 (*R*)
	100.0	>99	>99 (*R*)
	85.6	97.7	>99 (*R*)
	83.5	96.0	>99 (*R*)

[a] Reactions were carried out at the standard enzyme activity assay conditions and the activity towards phenylglyoxylic acid was regarded as 100%

[b] Reaction conditions (35 °C, 700 rpm, 10 h): substrate (10 mM), NADH (15 mM) and purified *Ll*KAR (80 U), in a 1.0 mL potassium phosphate buffer (100 mM, pH 7.0)

The substrate specificity of *Ll*KAR was researched using several various α-ketoacid substrates with aliphatic or aromatic substituents. As described in Table 3, *Ll*KAR exhibited higher activity and conversion towards aromatic α-ketoacids than aliphatic α-ketoacids, the same as a phenylpyruvate reductase from *Lactobacillus* sp. CGMCC 9967 [33], probably because the presence of benzene ring in the substituent group could promote hydrogen transfer process. In addition, the increased distance between the benzene ring and the carbonyl group could decrease enzyme activity and catalytic efficiency to a certain extent. All tested substrates were reduced with perfect *e.e.* values (> 99%) following the anti-Prelog's rule.

Construction of Recombinant *E. coli* (HADH-*Ll*KAR-GDH)

The efficient and economical regeneration of NAD(P)H, due to their high cost and large usage, is always critical for industrial applications of a large number of reductases. So, many related strategies have been developed at present [40, 41]. Herein, we adopted the co-expression of GDH from *E. sibiricum* and *Ll*KAR selected above with promising activity and substrate tolerance in one *E. coli* cell for the regeneration of NADH in the asymmetric reduction reaction. Therefore, the recombinant plasmid pCDFDuet-*Ll*KAR-GDH was constructed and transformed into *E. coli* BL21 (DE3), and then SDS-PAGE analysis indicated the co-expression of *Ll*KAR and GDH in one cell was successfully realized with two clearly visible bands of 32 and 28 kDa (Fig. S4). Subsequently, to establish redox cascade reaction system for highly efficient deracemization of racemic 2-HAs, the resulting pCDFDuet-*Ll*KAR-GDH and recombinant plasmid pET28b-HADH (the gene from *P. aeruginosa*) with different antibiotic resistances were co-transformed into the expression host *E. coli* BL21 (DE3). SDS-PAGE analysis confirmed that the co-expression of HAHD, *Ll*KAR, and GDH in one cell was successfully achieved with three clearly visible bands of

Table 4 Enantioselective cascade biocatalysis for deracemization of racemic 2-HAs with recombinant *E. coli* (HADH-*Ll*KAR-GDH)[a]

Entry	Substrate	Reaction time (h)	Yield of (*R*)-**1** (%)	*e.e.* of (*R*)-**1** (%)
1	*rac*-**1a**	2	98.16	> 99
2	*rac*-**1b**	2	98.19	> 99
3	*rac*-**1c**	2	98.37	> 99
4	*rac*-**1d**	2	98.60	> 99
5	*rac*-**1e**	2	99.52	> 99
6	*rac*-**1f**	2	98.36	> 99
7	*rac*-**1g**	2	98.75	> 99
8	*rac*-**1h**	2	99.40	> 99
9	*rac*-**1i**	2	98.19	> 99
10	*rac*-**1j**	2	98.99	> 99
11	*rac*-**1k**	2	99.27	> 99
12	*rac*-**1l**	2	98.96	> 99
13	*rac*-**1m**	2	98.98	> 99
14	*rac*-**1n**	4	85.27	95.1
15	*rac*-**1o**	4	81.98	94.3
16	*rac*-**1p**	4	90.53	> 99
17	*rac*-**1q**	4	86.64	> 99
18	*rac*-**1r**	4	96.70	93.4
19	*rac*-**1s**	4	95.80	91.6

[a] Reaction conditions (30 °C, 700 rpm, 6 h): each substrate (**1a**-**1s**, 20 mM), glucose (20 mM), and whole-cell catalyst (8 g DCW/L), in a 10 mL potassium phosphate buffer (100 mM, pH 7.0)

42, 32, and 28 kDa (Fig. S4). This strategy of one-cell three-enzyme co-expression could avoid, to a certain extent, cumbersome processes of cell cultivation and mass transfer difficulties in multi-cellular system.

Deracemization of Racemic 2-HAs with *E. coli* (HADH-*Ll*KAR-GDH)

To investigate the applicability of recombinant *E. coli* coexpressing three enzymes, the cascade deracemization of racemic 2-HAs (**1a**-**1s**) was conducted using the resting cells of *E. coli* (HADH-*Ll*KAR-GDH) as catalysts. The yields and *e.e.* values of (*R*)-**1** without isolation at different reaction times were determined by chiral HPLC as listed in Table 4. The nature, position, and number of substituent on the benzene ring and the distance between benzene ring and hydroxy group have different effects on the yields and *e.e.* values of target products. For racemic **1a**-**1m**, the corresponding (*R*)-2-HAs were obtained with excellent yields (> 98%) and *e.e.* values (> 99%) at 2 h. Interestingly, all the yields towards 2-HAs (**1b**-**1m**) with different substituents on benzene ring, either electron-withdrawing or electron-donating groups, were slightly higher than that without substituent (**1a**). The yields for 2-HAs with *o*-, *m*-, and *p*-substituents are higher than 98% although the steric effects (**1f**-**1h**, **1i**-**1k**). For racemic **1n**-**1s**, the yields and *e.e.* values (except for **1p**-**1q** with *e.e.* values of > 99%) of products were relatively lower than that of other substrates even when the reaction time was extended to 4 h, as results of the (*S*)-isomer of 2-HAs residues (**1r**-**1s**), or intermediate ketoacids accumulation (**1p**-**1q**), or both (**1n**-**1o**). In general, the recombinant *E. coli* coexpressing HADH, *Ll*KAR, and GDH could achieve highly efficient deracemization of most racemic 2-HAs with satisfactory yields and *e.e.* values. The results demonstrated that this newly constructed system has a great potential for application.

Whole-Cell Biocatalytic Synthesis of (*R*)-1f

(*R*)-**1f** is a significant chiral synthon for preparation of clopidegrel, one of salable anti-thrombotic agents [6, 7, 14]. In order to further confirm the application potential of this newly built cascade deracemization system, the resting cells of recombinant *E. coli* (HADH-*Ll*KAR-GDH) coexpressing three enzymes were cultured and collected for the preparation of (*R*)-**1f**. As shown in Table 5, when the catalyst dosage was 10 g DCW/L, 150 mM of *rac*-**1f** was completely transformed into the corresponding (*R*)-**1f** with perfect yields (> 98%) and *e.e.*

Table 5 Whole-cell biocatalytic synthesis of (*R*)-**1f** with recombinant *E. coli* (HADH-*Ll*KAR-GDH)[a]

Entry	Catalyst (g DCW/L)	*rac*-**1f** (mM)	Time (h)	Yield[b] (%)	*e.e.* (%)
1	10	50	8	> 99	> 99
2	10	100	14	> 99	> 99
3	10	150	18	> 99	> 99
4	10	200	20	95.7	> 99
5	20	200	16	> 99	> 99
6	20	300	36	94.8	> 99

[a] Reaction conditions (30 °C, 700 rpm): *rac*-**1f** (various concentrations as listed above), glucose (1.0 equiv. vs. substrate), catalyst (various dosages as listed above), in a 10 mL potassium phosphate buffer (100 mM, pH 7.0). Reactions were performed until no further conversion. pH was kept at 7.0 by adding automatically with 3.0 M NaOH in the process of reaction

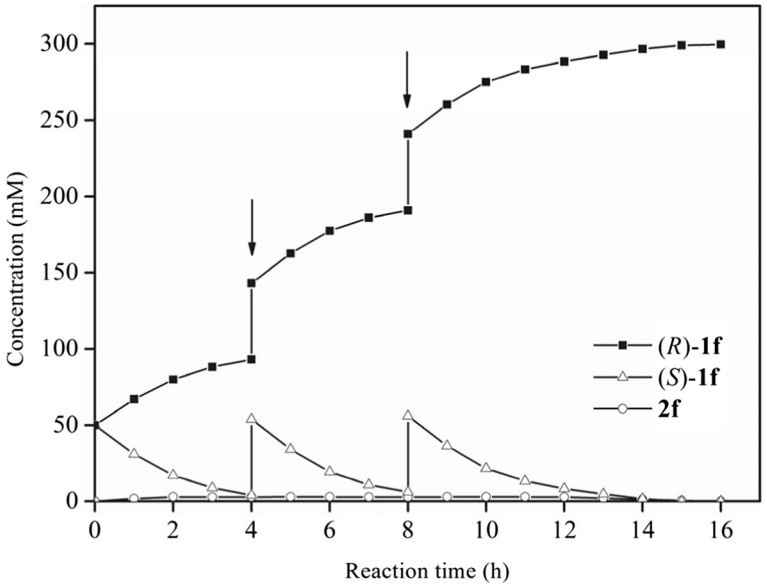


Fig. 3 Whole-cell biocatalytic synthesis of (*R*)-**1f** with recombinant *E. coli* (HADH-*Ll*KAR-GDH) by adding *rac*-**1f** in a fed-batch mode. Reaction was started in a 10 mL potassium phosphate buffer (100 mM, pH 7.0) containing *rac*-**1f** (100 mM), glucose (100 mM), and catalyst (20 g DCW/L) at 30 °C and 700 rpm. Also, *rac*-**1f** (100 mM) and glucose (100 mM) were added per 4 h. pH was kept at 7.0 by adding automatically with 3.0 M NaOH in the process of reaction

values (> 99%). In the case of 200 mM *rac*-**1f**, (*R*)-**1f** could be produced with a yield of > 99% and an *e.e.* value of > 99% at 16 h after increasing the catalyst dosage to 20 g DCW/L, and a productivity of 55.9 g L^{-1} day^{-1} was achieved. Subsequently, the substrate loading was further increased up to 300 mM and only a yield of 94.8% was obtained until 36 h, and further increasing cell dosage was not beneficial to the biotransformation. However, when the strategy of adding substrate in a fed-batch mode was used in this experiment, 300 mM *rac*-**1f** could be completely transformed into (*R*)-**1f** with a yield of > 99% and an *e.e.* value of > 99% within 16 h as shown in Fig. 3, giving a higher productivity of 83.8 g L^{-1} day^{-1}. Compared with the previous work for the deracemization of *rac*-**1f** (substrate concentration:20 mM, productivity of 44.0 g L^{-1} day^{-1}) [20], The substrate concentration and the productivity were increased 14 times and 90.6%, respectively. The result indicated that the newly constructed *E. coli* (HADH-*Ll*KAR-GDH) has a greater potential for practical application.

Conclusion

In summary, a novel ketoacid reductase (*Ll*KAR) exhibiting higher activity and substrate tolerance towards aromatic α-ketoacids has been successfully discovered and characterized. The newly reconstructed recombinant *E. coli* co-expressing HADH, *Ll*KAR, and GDH exhibited higher catalytic efficiency for the deracemization of racemic 2-HAs. As much as 300 mM *rac*-**1f** could be completely transformed into (*R*)-**1f** with high yield and enantiopurity without addition of exogenous cofactor, making this novel whole-cell biocatalyst more promising and competitive in practical application.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that there is no conflict of interest.

Ethical Statement The authors declare that there are no studies conducted with human participants or animals.

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