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Highly stereoselective biosynthesis of (*R*)-α-hydroxy carboxylic acids through rationally re-designed mutation of D-lactate dehydrogenase

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An NAD-dependent D-lactate dehydrogenase (D-nLDH) of *Lactobacillus bulgaricus* ATCC 11842 was rationally re-designed for asymmetric reduction of a homologous series of α-keto carboxylic acids such as phenylpyruvic acid (PPA), α-ketobutyric acid, α-ketovaleric acid, β-hydroxypyruvate. Compared with wild-type D-nLDH, the Y52L mutant D-nLDH showed elevated activities toward unnatural substrates especially with large substitutes at C-3. By the biocatalysis combined with a formate dehydrogenase for *in situ* generation of NADH, the corresponding (*R*)-α-hydroxy carboxylic acids could be produced at high yields and highly optical purities. Taking the production of chiral (*R*)-phenyllactic acid (PLA) from PPA for example, 50 mM PPA was completely reduced to (*R*)-PLA in 90 min with a high yield of 99.0% and a highly optical purity (>99.9% e.e.) by the coupling system. The results presented in this work suggest a promising alternative for the production of chiral α-hydroxy carboxylic acids.

Enantiomerically pure α-hydroxy carboxylic acids are valuable chemicals and versatile building blocks for the synthesis of a variety of significant compounds with retention of chirality at C-2[1,2]. 3-Phenyllactic acid (PLA), for instance, is a new type antiseptic agent and a useful precursor for the synthesis of drugs, including Danshensu (3,4-dihydroxyphenyllactic acid) that can inhibit platelet aggregation and coronary artery disease[3]. Another optically active α-hydroxy carboxylic acid, α-hydroxybutyric acid, is an important material for the production of isoleucine, poly(α-hydroxybutyric acid), and some kinds of medicines, such as azinothricin family of antitumour antibiotics[4]. In addition, α-hydroxy-4-phenylbutyric acid is an important precursor to angiotensin-converting enzyme inhibitors[5]. Thus, production of chiral α-hydroxy carboxylic acids has attracted researchers' attention[2,6,7].

Many routes have been developed for the preparation of chiral α-hydroxy carboxylic acids, such as traditional chemical methods[8,9], chemoenzymatic routes[5,7,10], enzymatic kinetic resolution of racemic α-hydroxy carboxylic acids[4,11–14], and asymmetric bioreduction of α-keto carboxylic acids[7,15–17]. Currently, asymmetric bioreduction of α-keto carboxylic acids by using dehydrogenases is the method of choice because of its excellent stereoselectivity, high theoretical yield up to 100%, and environment-friendly nature. However, the used dehydrogenases exhibited low activities, especially toward substrates with large aliphatic or aromatic groups at C-3[16–18]. Therefore, there is still a requirement for new dehydrogenases that can reduce a broad spectrum of α-keto carboxylic acids with high efficiency. In addition, the used dehydrogenase is NADH-dependent, which reduces α-keto carboxylic acids into α-hydroxy carboxylic acids and oxidizes NADH into NAD^+ in the meanwhile. Due to the high cost of the NADH, an *in situ* regeneration system of NADH should be introduced for its recycle. Formate dehydrogenase (FDH), which catalyzes the oxidation of formate to carbon dioxide while NAD^+ is reduced to NADH, could be used in enzymatic synthesis of chemical compounds as a versatile biocatalyst for NADH regeneration[19–21].

Although candidate dehydrogenases can be isolated from the organisms in the environment[3,22] or identified from genomic databases by genome mining[23], rational re-design of existing dehydrogenases that have been well characterized in crystal structures offers a promising alternative approach. Such a strategy has been successfully applied to xylose reductase and D-fructose-6-phosphate aldolase[24,25].

Based on this knowledge, the NAD-dependent D-lactate dehydrogenase (D-nLDH) of *Lactobacillus bulgaricus* ATCC 11842, an efficient D-lactic acid producing strain[26,27], which has been extensively studied and its crystal structure is known[28], was selected as a target to create new biocatalysts in this study. The resultant D-nLDH mutant exhibited high activity and high enantioselectivity toward α-hydroxy carboxylic acids with larger groups at C-3. Furthermore, D-nLDH mutant was combined with FDH from *Candida boidinii* NCYC 1513 for the production of chiral (*R*)-PLA to evaluate the enzyme's potential for industrial applications.

Results

Rational re-design of D-nLDH. D-nLDH exhibits high activity and stereoselectivity toward pyruvic acid and β-hydroxy pyruvic acid, but not toward bulkier substrate analogs[29]. In order to improve the reduction activity of D-nLDH toward α-hydroxy carboxylic acids with larger groups at C-3, certain residues of D-nLDH, which are around the C-3 group of α-hydroxy carboxylic acids, should be redesigned. Previous studies of D-nLDH stereo structure revealed that Tyr52 and Phe299 are mainly responsible for hindering larger substrates because of their short distance from the side chain of α-keto carboxylic acids and their steric orientation[28,30–32]. Moreover, Tokuda et al. have succeeded in converting the D-nLDH of *Lactobacillus pentosus* into D-α-hydroxyisocaproate dehydrogenase by replacing of Tyr52 with an aliphatic amino residue, Leu[33]. Thus, these two residues, Tyr52 and Phe299, were selected for site-directed mutagenesis in this study. Tyr was substituted with Leu to abate the steric exclusion effect, and Phe was replaced by Tyr, which was considered to facilitate the binding of aromatic substrates[28]. These D-nLDH Y52L and F299Y mutants were constructed and investigated as biocatalysts for the reduction of α-keto carboxylic acids (Fig. 1A) in the following study.

Expression and purification of D-nLDH and D-nLDH mutants. Using the primers ldhD1.f and ldhD1.r, *ldhD* was amplified by PCR from the genome of *L. bulgaricus* ATCC 11842. Using the primers ldhDY52L.f and ldhDY52L.r, TAC of *ldhD* was replaced by CTC to obtain $ldhD^{Y52L}$. Using the primers ldhDF299Y.f and ldhDF299Y.r, TTC of *ldhD* was replaced by TAC to obtain $ldhD^{F299Y}$. The $ldhD^{Y52L/F299Y}$ double mutant was constructed by combining the upper two steps. The DNA fragments were sequenced to confirm that only the expected mutation had occurred. Then, D-nLDH, Y52L mutant, F299Y mutant, and Y52L/F299Y double mutant were purified to homogeneity through the Ni-affinity chromatography (Fig. 1B).

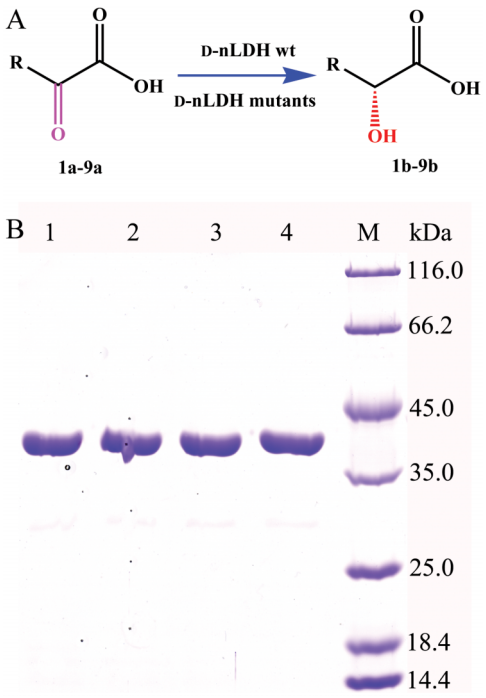

Figure 1 | The reduction reaction of α-keto carboxylic acids and the enzymes used for this reaction in this study. (A) Scheme for reduction of α-keto carboxylic acids by D-nLDH wild-type and D-nLDH mutants. 1a and 1b, R = CH_3; 2a and 2b, R = CH_3OH; 3a and 3b, R = CH_3CH_2; 4a and 4b, R = $CH_3CH_2CH_2$; 5a and 5b, R = $CH(CH_3)_2$; 6a and 6b, R = $C(CH_3)_3$; 7a and 7b, R = C_6H_5; 8a and 8b, R = $C_6H_5CH_2$; 9a and 9b, R = *p*-OH-$C_6H_4CH_2$; (B) SDS-PAGE analysis of the purified enzymes. From lane 1 to 4, D-nLDH wild-type (wt), D-nLDH Y52L, D-nLDH F299Y, and D-nLDH Y52L/F299Y, respectively.

Substrate specificity and enantioselectivity of D-nLDH and D-nLDH mutants. Table 1 summarizes the results of specific activity determination of D-nLDH wild-type and mutants toward various α-keto carboxylic acids. The Y52L mutant exhibits robust activities toward the selected bulky substrates (3a to 9a), including aliphatic (longer than three carbons) and aromatic α-keto carboxylic acids. The specific activities are 16–1476-fold higher than those of D-nLDH wild-type. Furthermore, we found that the optimal substrate for this mutant switches to 4a, compared to the wild-type enzyme that is selective for 1a. Although the F299Y mutant is generally less active than the Y52L mutant, it also provides the improved activities toward

Table 1 | Specific activity of D-nLDH wild-type and D-nLDH mutants for α-keto carboxylic acids

α-Keto carboxylic acids (R)	Specific activity (U mg^{-1} protein)[a]				e.e.[b]
	Wild-type	Y52L	F299Y	Y52L/F299Y	
1a (CH_3)	771.4	669.0	1067	70.5	>99.9% *R*
2a (CH_2OH)	545.5	796.3	153.7	73.5	>99.9% *R*
3a (CH_3CH_2)	52.4	854.1	17.6	207.0	>99.9% *R*
4a ($CH_3CH_2CH_2$)	2.93	1,121	20.0	738.3	>99.9% *R*
5a ($CH(CH_3)_2$)	0.08	10.6	0.08	1.01	>99.9% *R*
6a ($C(CH_3)_3$)	0.06	1.27	0.13	0.06	-[c]
7a (C_6H_5)	0.05	3.40	0.11	0.11	>99.9% *R*
8a ($C_6H_5CH_2$)	18.1	1,016	10.4	1,519	>99.9% *R*
9a (*p*-OH-$C_6H_4CH_2$)	0.04	60.5	0.40	62.8	>99.9% *R*

[a]The enzymes (D-nLDH wild-type, Y52L mutant, F299Y mutant, and Y52L/F299Y double mutant) used for assay were N-terminal His-tagged fusion and purified to apparent homogeneity.
[b]The e.e. values were determined using D-nLDH Y52L mutant. HPLC analysis of the products was performed with a chiral column by using the corresponding racemic α-hydroxy carboxylic acids as authentic standards.
[c]Lack of authentic standard.

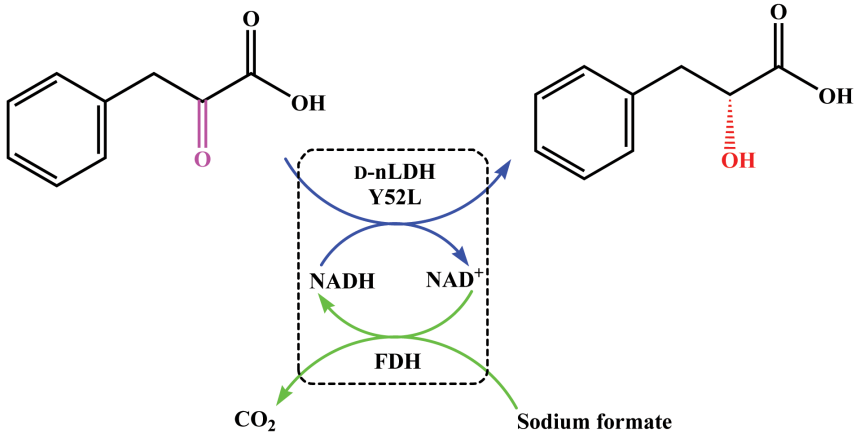

Figure 2 | Asymmetric reduction of PPA (8a) with NADH cofactor recycling by using whole-cell system.

the substrates 4a, 6a, 7a, and 9a. Therefore, a Y52L/F299Y double mutant was constructed to investigate the combined effect of these two mutations.

The Y52L/F299Y double mutant also gives better activities toward the tested large substrates than the wild-type. However, its activities toward the tested substrates are weaker, compared to the Y52L mutant except for 8a and 9a. The optimal substrate of the Y52L/F299Y double mutant is substrate 8a. Table 1 shows that the Y52L/F299Y double mutant has a unique and robust activity toward substrate 8a (1,519 U mg^{-1}). This activity is higher than that of the wild-type enzyme toward its preferred substrate, 1a (771.4 U mg^{-1}). The most obvious distinction between the Y52L mutant and Y52L/F299Y double mutant is their activities toward substrates 1a and 2a. Although both the Y52L and F299Y mutants showed excellent activities toward 1a and 2a, the activity dramatically decreased in the context of the Y52L/F299Y double mutant.

To sum up, the Y52L mutant, which exhibits good activities toward all tested α-keto carboxylic acids, is superior to other mutants. Therefore, it is a good biocatalyst, suitable for the reduction of a homologous series of α-keto carboxylic acids with broad tolerance for the substitute at C-3. Enantiomeric excess (e.e.) values for the α-hydroxy carboxylic acids were also determined. The results indicate that the Y52L mutant retains its high stereoselectivity and produced the chiral (*R*)-α-hydroxy carboxylic acids with e.e. > 99.9% (Table 1).

Asymmetric reduction of phenylpyruvic acid (PPA) to (*R*)-PLA. Asymmetric reduction of PPA (8a) was investigated in order to further explore the potential of D-nLDH mutants in the synthesis of important chiral (*R*)-α-hydroxy carboxylic acids. The resultant product, (*R*)-PLA (8b), can be used as a new type of natural antiseptic agent and as a key precursor for synthesis of hypoglycemic and anthelmintic reagents[34–36]. We previously found that PLA could be efficiently produced by a lactic acid bacterium (LAB), but the optical purity was poor[3]. Here, production of optically pure (*R*)-PLA was studied by using a whole-cell system with NADH regeneration (Fig. 2). In this coupled reaction system, FDH can be utilized to regenerate NADH because of its low cost substrate, formate, and easily separated product, carbon dioxide.

Table 2 | Kinetic parameters of D-nLDH wild-type and D-nLDH mutants for PPA (8a)

D-nLDH	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
Wild-type	11.4	17.1	11.3	1.0×10^3
Y52L	0.27	3,049	2,013	7.5×10^6
F299Y	0.32	2.7	1.8	5.7×10^3
Y52L/F299Y	1.4	2,191	1,447	1.0×10^6

The kinetic parameters of purified D-nLDH wild-type and D-nLDH mutants for PPA (8a) were evaluated. The results indicate that the Y52L mutant exhibits the highest catalytic efficiency (Table 2). Then, $ldhD^{Y52L}$ and *fdh* were coexpressed on pETDuet-1 in *E. coli* BL21(DE3) (Fig. 3). In addition, *E. coli* BL21(DE3) harboring pETDuet-$ldhD^{Y52L}$ was used as control. The biocatalysis system consisted of whole cells of recombinant *E. coli*, PPA, and sodium formate (Fig. 4 and Fig. S1). As shown in Fig. 4A, when $ldhD^{Y52L}$ and *fdh* were coexpressed in *E. coli* BL21(DE3), 50 mM PPA was completely reduced to (*R*)-PLA in 90 min with a high yield of 99.0%. In contrast, the reaction rate was slower and only 23.8 mM (*R*)-PLA was produced with a yield of 63.7% after 360 min in the absence of FDH (Fig. 4B). The optical purity of (*R*)-PLA was monitored and e.e. values were excellent (>99.9% e.e.) throughout in both biocatalysis systems (Fig. S2). Therefore, this coupling system provides the excellent bioreduction efficiency and high enantioselectivity compared with other systems.

Discussion

Enzymes have been widely accepted as useful biocatalysts for synthesis of a series of valuable organic compounds, especially their capacities for asymmetric catalysis. However, despite the widespread uses of enzymes in biosynthesis, the candidate enzymes with desirable characteristics are scarce. Many methods for exploitation and screening of the target enzymes have been reported in previous reports. Here, we demonstrated the utility of rational re-design to modify D-nLDH by using bioinformatics and gene cloning techniques. The resultant biocatalysts, D-nLDH mutants, possess good catalytic activities capable of transforming targeted substrates. The approach adopted in this study is efficient and the preconditioning is based on the structure and related catalytic mechanism of the objective enzyme. D-nLDH has been widely researched, including its property, characteristic, and structure. Based on these preconditions, Tyr52 and Phe299 were considered as pivotal residues for substrate spectrum and chosen for site-directed mutagenesis.

Enantiomerically pure α-hydroxy carboxylic acids are important synthons for fine chemicals. Stereospecific nLDHs responsible for enantiomerically pure lactic acid production are of interest, as optically pure lactic acid is the most common α-hydroxycarboxylic acid. In fact, in addition to catalyzing the reduction of pyruvate, D-nLDH has been reported to reduce other α-keto carboxylic acids. For example, the D-nLDH in *B. coagulans* has proved to be responsible for transformation 8a into 8b[3]. D-nLDH of *L. pentosus* has showed high activities toward hydroxypyruvate (2a), α-ketobutyrate (3a) and 8a[29]. However, the catalytic efficiencies of these D-nLDHs were poor

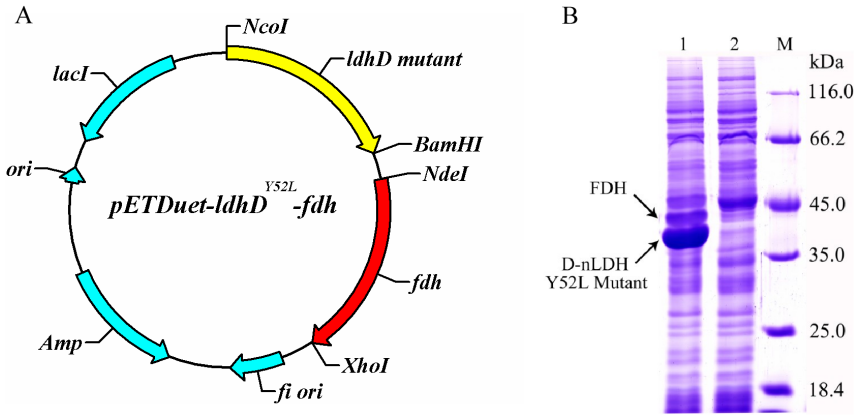

Figure 3 | Construction of *E. coli* BL21(DE3) harboring pETDuet-*ldhD*^{Y52L}-*fdh*. (A) Map of plasmid pETDuet-*ldhD*^{Y52L}-*fdh*; (B) SDS-PAGE analysis of the expressed enzymes. Crude cell extract of *E. coli* BL21(DE3) harboring plasmid pETDuet-*ldhD*^{Y52L}-*fdh* and pETDuet-1 for lane 1 to 2, respectively.

on non-natural substrates. Therefore, the mutants obtained in this study are attractive for production of α-hydroxy carboxylic acids.

Because conversion of α-keto carboxylic acids to α-hydroxy carboxylic acids is accompanied by the oxidation of NADH to NAD, a cosubstrate is necessary to supply NADH. Therefore, the *E. coli* transformant coexpressing both *ldhD* mutant and *fdh* was constructed. In the absence of FDH, the reduction of 8a was slow and then stopped after the exhaustion of intracellular NADH (Fig. 4B). Thus, this process provides excellent bioreduction efficiency and high enantioselectivity for the production of α-hydroxy carboxylic acids.

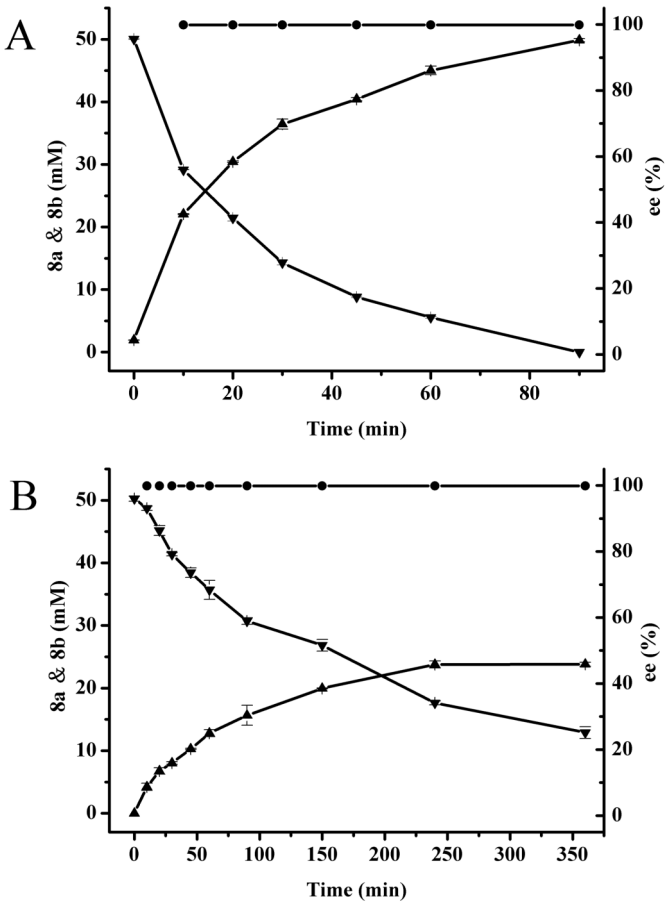

Figure 4 | Time course of the production of (*R*)-PLA. (A) The biocatalyst was *E. coli* BL21(DE3) harboring pETDuet-*ldhD*^{Y52L}-*fdh*; (B) The biocatalyst was *E. coli* BL21(DE3) harboring pETDuet-*ldhD*^{Y52L}. ▼, PPA, 8a; ▲, (*R*)-PLA, 8b; ●, ee.

In summary, the substrate selectivity of D-nLDH was successfully altered, and its activity toward substrates with large aliphatic or aromatic groups at C-3 was drastically improved. This study expands its range of application in the production of (*R*)-α-hydroxy carboxylic acids. More importantly, because of the high yield and high stereoselectivity of D-nLDH Y52L mutant, the whole-cell catalysis system containing D-nLDH Y52L mutant and FDH was successfully applied to the direct synthesis of (*R*)-phenyllactic acid. The method developed in this study could be used as a promising alternative for the production of highly optically pure α-hydroxy carboxylic acids. Our results for D-nLDH mutants might open up a way to reconstruct other enzymes, such as NAD-dependent L-lactate dehydrogenases and NAD-independent lactate dehydrogenases, based on their structures in order to modify the substrate spectra and improve the catalytic efficiency for the synthesis of valuable chiral compounds.

Methods

Chemicals. Pyruvate, β-hydroxypyruvate, 2-oxobutyrate, phenylpyruvate, 4-hydroxyphenylpyruvate, racemic lactate, racemic 2-hydroxybutyrate, racemic 2-hydroxy-3-methylbutyrate, racemic phenyllactate, and racemic 4-hydroxyphenyllactate were purchased from Sigma-Aldrich. 2-Oxovalerate, 3,3-dimethyl-2-oxobutyrate, and racemic glycerate were purchased from TCI. 3-Methyl-2-oxobutyrate, and racemic 2-hydroxyvalerate were purchased from Acros Organics. Benzoylformate and racemic mandelate were purchased from J&K Chemical. All other chemicals were of reagent grade.

Bacterial strains, plasmids and primers. Bacterial strains, plasmids, and oligonucleotide primers used in this study were listed in Table 3. *Escherichia coli* was grown at 37°C in Luria-Bertani (LB) medium and ampicillin was added at a concentration of 100 μg ml^{-1}, if necessary. *L. bulgaricus* ATCC 11842 was cultured in MRS media at 42°C[29] and *C. boidinii* NCYC 1513 was incubated in YPD media at 30°C[15].

Cloning and site directed mutagenesis of *ldhD*. Genomic DNA of *L. bulgaricus* ATCC 11842 was extracted with the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The *ldhD* gene was amplified using primers ldhD1.f and ldhD1.r with genomic DNA of *L. bulgaricus* ATCC 11842 as template and cloned into pMD18-T to construct pMD18-*ldhD*. Three *ldhD* mutants (*ldhD*^{Y52L}, *ldhD*^{F299Y}, *ldhD*$^{Y52L/F299Y}$) were generated by using the TaKaRa MutanBEST Kit (Takara Biotechnology Dalian Co. Ltd., China) according to the manufacturer's protocol. The point mutant at Tyr52 was introduced into primer ldhDY52L.f and the point mutant at Phe299 was introduced into primer ldhDF299Y.f. Plasmid pMD18-*ldhD* was used as template for single mutant construction and the resultant plasmid pMD18-*ldhD*^{Y52L} was used as template for double mutant construction. The mutants were confirmed by DNA sequencing.

Purification of D-nLDH and D-nLDH mutants. The resulting plasmids pMD18-*ldhD*, pMD18-*ldhD*^{Y52L}, pMD18-*ldhD*^{F299Y}, and pMD18-*ldhD*$^{Y52L/F299Y}$ were digested with *Pst*I-*Xho*I and cloned into the *Pst*I-*Xho*I sites of pETDuet-1 separately to construct four different expression plasmids, pETDuet-*ldhD*, pETDuet-*ldhD*^{Y52L}, pETDuet-*ldhD*^{F299Y}, and pETDuet-*ldhD*$^{Y52L/F299Y}$. The recombinant plasmids were separately transformed into *E. coli* BL21(DE3) for protein expression. Cells were

Table 3 | Strains, plasmids, and oligonucleotide primers used in this study

Strain, plasmid, or primer	Relevant characteristics	Source or reference
Strain		
E. coli DH5α	φ80 *lacZ*ΔM15 Δ(*lacZ*YA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17 *sup*E44λ- *thi*-1	Invitrogen Life Technologies
E. coli BL21(DE3)	F^- *ompT gal dcm lon hsdS*$_B$($r_B^-m_B^-$) λ(*DE3*)	Novagen
L. bulgaricus ATCC 11842	Wild-type, source of *ldhD* gene	ATCC[a]
C. boidinii NCYC 1513	Wild-type, source of *fdh* gene	NCYC[b]
Plasmid		
pMD18-T	Cloning vector, Amp^r	TaKaRa
pETDuet-1	Expression vector, Amp^r	Novagen
pMD18-*ldhD*	Wild-type *ldhD* gene in pMD18	This study
pMD18-*ldhD*^{Y52L}	*ldhD*^{Y52L} gene in pMD18	This study
pMD18-*ldhD*^{F299Y}	*ldhD*^{F299Y} gene in pMD18	This study
pMD18-*ldhD*$^{Y52L/F299Y}$	*ldhD*$^{Y52L/F299Y}$ gene in pMD18	This study
pETDuet-*ldhD*	N-terminal His-tagged *ldhD* gene in pETDuet-1	This study
pETDuet-*ldhD*^{Y52L}	N-terminal His-tagged *ldhD*^{Y52L} gene in pETDuet-1	This study
pETDuet-*ldhD*^{F299Y}	N-terminal His-tagged *ldhD*^{F299Y} gene in pETDuet-1	This study
pETDuet-*ldhD*$^{Y52L/F299Y}$	N-terminal His-tagged *ldhD*$^{Y52L/F299Y}$ gene in pETDuet-1	This study
pETDuet-*ldhD*^{Y52L}-*fdh*	Both *ldhD*^{Y52L} and *fdh* without His-tag in pETDuet-1	This study
Oligonucleotide primer	Sequence (5′ → 3′) and properties[c]	
ldhD1.f	CTGCAGATGACTAAAATTTTTGCTTACGCA (*Pst*I)	
ldhD1.r	CTCGAGTTAGCCAACCTTAACTGGAGTTT (*Xho*I)	
ldhDY52L.f	GTTGTT***CTC***CAACAACTTGACTACACCGCT	
ldhDY52L.r	AACACCGTCAGCACCCTTTGCCAAAGCAA	
ldhDF299Y.f	CACACACTGCT***TAC***TACACTACTCACGCTGT	
ldhDF299Y.r	GAGTTACCAGAACGTTTGGACGAGCGATTAAGT	
ldhD2.f	CCATGGTGACTAAAATTTTTGCTTACGCA (*Nco*I)	
ldhD2.r	GGATCCTTAGCCAACCTTAACTGGAGTTT (*Bam*HI)	
fdh1.f	CATATGAAGATCGTTTTAGTCTTATATGATGCTGGTA (*Nde*I)	
fdh1.r	CTCGAGTTATTTCTTATCGTGTTTACCGTAAGCTTTG (*Xho*I)	

[a]ATCC, American Type Culture Collection.
[b]NCYC, National Collection of Yeast Cultures.
[c]For site directed mutagenesis, mutagenised codons were introduced (highlighted in italic and bold); For protein expression, recognition sites were introduced for restriction endonucleases (recognition sites underlined, restriction endonucleases indicated in parentheses).

incubated aerobically in LB medium (100 μg ml^{-1} ampicillin) at 37°C to an optical density of 0.6 at 600 nm. 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression, and cultures were grown at 16°C for a further 10 h. Then, cells were harvested and suspended in a binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 20 mM imidazole [pH 7.4]) and disrupted by sonication. Thereafter, intact cells and cell debris were removed by centrifugation, and the resultant supernatant was filtered and loaded onto a HisTrap HP 5-ml column (GE Healthcare). Purification was performed with gradient elution by using an elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 500 mM imidazole [pH 7.4]). All enzymes were purified to electrophoretic homogeneity for the activity assay.

Biotransformation by whole cells coexpressing *ldhD* mutant and *fdh*. The plasmid pETDuet-*ldhD*^{Y52L}-*fdh* was constructed as follows: *ldhD*^{Y52L} gene was amplified using primers ldhD2.f and ldhD2.r with plasmid pMD18-*ldhD*^{Y52L} as template. The *fdh* gene was amplified using primers fdh1.f and fdh1.r with genomic DNA of *C. boidinii* NCYC 1513 as template. The resulting PCR products *ldhD*^{Y52L} and *fdh* were digested with *Nco*I-*Bam*HI and *Nde*I-*Xho*I, respectively, and cloned into the MCS1 and MCS2 of pETDuet-1 to construct pETDuet-*ldhD*^{Y52L}-*fdh*, which was then transformed into *E. coli* BL21(DE3). Recombinant *E. coli* BL21(DE3) harboring pETDuet-*ldhD*^{Y52L}-*fdh* was cultured for protein expression and harvested for biotransformation. The reaction was carried out at 30°C and 120 rpm in phosphate buffer solution (PBS, 1/15 M [pH 7.4]) containing 50 g DCW l^{-1} cells, 50 mM phenylpyruvate, and 100 mM sodium foramte. The concentrations of phenylpyruvate and phenyllactate in the reaction mixtures were quantitatively analyzed by high-performance liquid chromatography (HPLC).

Analytical methods. The reduction activity of D-nLDH wild-type and mutants was assayed at 37°C in 1 ml of 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADH, 10 mM α-keto carboxylic acids and the purified enzyme. The rate of NADH decrease in initial 1 min was determined by measuring the absorbance change at 340 nm. One unit of D-nLDH activity was defined as the amount that catalyzed the oxidation of 1 μmol NADH per minute. Protein concentration was determined by the Bradford method using bovine serum albumin for calibration.

The enantiomeric excess (ee) value of α-hydroxy carboxylic acids is defined as follows.

$$\frac{(R\text{–}\alpha\text{–hydroxy carboxylic acid}) - (S\text{–}\alpha\text{–hydroxy carboxylic acid})}{(R\text{–}\alpha\text{–hydroxy carboxylic acid}) + (S\text{–}\alpha\text{–hydroxy carboxylic acid})} \times 100\%$$

Phenylpyruvate and phenyllactate were measured by HPLC (Agilent 1100 series, Hewlett-Packard, USA) equipped with an Agilent Zorbax SB-C18 column (150 × 4.6 mm, 5 μm) and a variable-wavelength detector at 210 nm. The mobile phase consisted of 1 mM H_2SO_4 and acetonitrile with a ratio of 85 : 15 (v/v) at a flow rate of 0.7 ml min^{-1} at 30°C. Stereoselective assays of α-hydroxy carboxylic acids were performed by HPLC equipped with a chiral column (MCI GEL CRS10W, Japan) and a tunable UV detector at 254 nm. The mobile phase was 2 mM $CuSO_4$ for lactate and glycerate, 2 mM $CuSO_4$ and methanol with a ratio of 90 : 10 (v/v) for 2-hydroxybutyrate and 2-hydroxyvalerate, 2 mM $CuSO_4$ and methanol with a ratio of 85 : 15 (v/v) for 2-hydroxy-3-methylbutyrate, mandelate, phenyllactate, and 4-hydroxyphenyllactate, respectively, at a flow rate of 0.5 ml min^{-1} and at 25°C.

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Author contributions

P.X., C.M., Z.Z. and C.G. conceived and designed the experiments. Z.Z., B.S., H.Z. and T.Q. performed the experiments. C.M., C.G. and P.X. contributed reagents and materials. C.M., Z.Z., C.G. and P.X. analyzed the data. Z.Z., C.M. and P.X. wrote the paper. All authors reviewed the manuscript.

Additional information

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