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Enzymatic preparation of D-phenyllactic acid at high space-time yield with a novel phenylpyruvate reductase identified from *Lactobacillus* sp. CGMCC 9967

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ABSTRACT

An NADH-dependent phenylpyruvate reductase (*La*PPR) was identified through screening the shotgun library of *Lactobacillus* sp. CGMCC 9967. It belongs to D-3-phosphoglycerate dehydrogenase (PGDH) subfamily of 2-hydroxy acid dehydrogenase superfamily. *La*PPR was stable at pH 6.5 and 30 °C, with a half-life of 152 h. *La*PPR has a substrate preference towards aromatic to aliphatic keto acids, and various keto acids could be reduced into D-hydroxy acids with excellent enantioselectivity (>99%). By construction the coexpression system with glucose dehydrogenase, as much as 100 g L^{-1} phenylpyruvic acid was asymmetrically reduced into D-phenyllactic acid with 91.3% isolation yield and 243 g L^{-1} d^{-1} productivity. The results suggest that *La*PPR is a promising biocatalyst for the efficient synthesis of optically pure D-phenyllactic acid.

1. Introduction

Phenyllactic acid (PhLA, 2-hydroxy-3-phenylpropanic acid) is an important class of organic acids with versatile applications. Since 1998, PhLA was isolated from honey and fermented foods, and emerged as a natural and green alternative to chemical antibiotics. PhLA has a broad inhibitory-spectrum against bacterial and fungal contaminations through destroying the cell membrane (Dieuleveus et al., 1998; Tuberoso et al., 2011). The potentials of PhLA as biological antiseptic agents in the food industry have been well established (Mu et al., 2012b). Besides, PhLA is of substantial interest as a monomer for bio-polymers of poly-PhLA. Compared with poly-lactic acids, poly-PhLA contains a bulky aromatic side chain and displays enhanced thermostability and excellent ultraviolet-absorbing property (Kawaguchi et al., 2014). Especially, chiral PhLA is an α-hydroxyl acid and can be applied as building blocks in the synthesis of pharmaceuticals and fine chemicals, such as Englitazone, Statine, Danshensu, anti-HIV reagents, phenylalanine (Fig. 1) (Coppola and Schuster, 1997; Urban and Moore, 1992; Weckwerth et al., 2000; Sato et al., 2015).

Promising applications of PhLA stimulated great endeavors in the development of synthesis strategies, including traditional chemical methods, chemo-enzymatic routes, enzymatic kinetic resolution of racemic α-hydroxy carboxylic acids and α-hydroxy nitriles, and asymmetric reduction of α-keto carboxylic acids (Gröger, 2001). By contrast, biological methods, including fermentation and biocatalysis, are much greener, due to its high selectivity (enantio- and regioselectivity), environmentally benign and mild reaction conditions (Zheng and Xu, 2011). PhLA has been reported to be produced by fermentation with lactic acid bacteria (LAB) or non-LAB strains, such as *Bacillus coagulans* SDM, *Propionibacterium jensenii* DSMZ 20535, *Geotrichum candidum* etc. PhLA is a by-product of phenylalanine metabolism in LAB, in which phenylalanine is transaminated to phenylpyruvic acid (PhPA) and further reduced to PhLA (Mu et al., 2012a). Through fermentation, 17.4 g L^{-1} PhLA was produced from *Lactobacillus* ssp. SK007 by fed-batch fermentation with feeding of PhPA and pH-control strategies, ranking the highest record (Mu et al., 2009). However, the chemical and enantioselective purities of PhLA produced by fermentation are usually at moderate level and difficult to purify. Biotransformation by whole cells or isolated enzymes provides efficient solutions for the synthesis of fine chemicals. Among the biocatalytic approaches, hydrolyases (hydroxynitrilases and lipases) catalyzed processes are the commonly used industrial routes (Schmid et al., 2002). Various hydrolases have been identified with desired properties, however, with limitation of 50% theoretical yield (Schmidt and Griengl, 1999; Larissegger-Schnell et al., 2006). The stereospecific reduction of 2-oxo carboxylic acid by reductases/dehydrogenases is a highly

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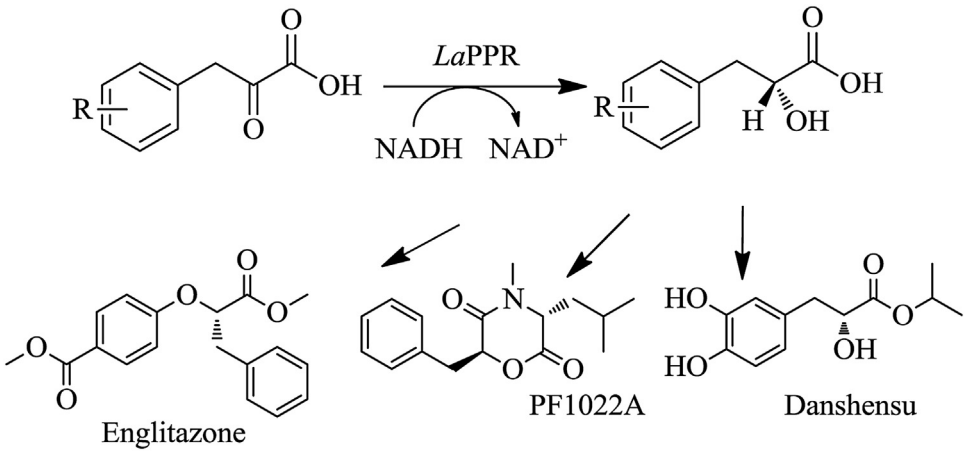


Fig. 1. Asymmetric preparation of D-phenyllactic acid and three target pharmaceuticals.

Table 1
Comparison of various enzymes for the bioreductive preparation of D-PhLA from PhPA.

Entry	Enzyme	Family	Strain	K_m[mM]	k_{cat}[s^{-1}]	k_{cat}/K_m[s^{-1} mM^{-1}]	*ee*[%]	Productivity [$g\,L^{-1}\,d^{-1}$]	Reference
1	D-HicDH	HicDH	Lactobacillus casei	0.15	*n. a.*[a]	*n. a.*	*n. a.*	*n. a.*	Hummel et al. (1985)
2	LDH	D-LDH	*L. plantarum*	20	11.3	0.565	*n. a.*	*n. a.*	Taguchi and Ohta, (1991)
3	LDH	D-LDH	*L. plantarum* SK002	5.4	*n. a.*	*n. a.*	96	2.90	Jiang et al. (2010)
4	LDH	D-LDH	*P. pentosaceus* ATCC 25745	1.73	173	10	*n. a.*	*n. a.*	Yu et al. (2012)
5	LDH	D-LDH	*P. acidilactici* DSM 20284	2.9	3.05	1.05	*n. a.*	*n. a.*	Mu et al. (2012a,b)
6	LDH	D-LDH	*L. confusus* 20196	3.0	143	47.7	*n. a.*	*n. a.*	Hummel et al. (1983)
7	LDH	D-LDH	*L. pentosus* JCM1558	0.8	40	50	*n. a.*	*n. a.*	Tokuda et al. (2003)
8	LDH	D-LDH	*Bacillus coagulans* SDM	4.4	16.5	3.75	*n. a.*	37.3	Zheng et al. (2011)
9	ManDH1	D-MDH	*Enterobacter. faecalis*	11	235	21.4	*n. a.*	*n. a.*	Tamura et al. (2002)
10	ManDH2	D-MDH	*E. faecalis*	3.4	233	68.5	*n. a.*	*n. a.*	Tamura et al. (2002)
11	ManDH	D-MDH	*E. faecalis*	5.7	353	61.9	*n. a.*	*n. a.*	Wada et al. (2008)
12	ManDH	D-MDH	*L. curvatus*	0.15	101	673	*n. a.*	*n. a.*	Hummel et al. (1988)
13	YiaE	GHPR	*E. coli* K-12	7.9	29.5	3.73	98	*n. a.*	Yun et al. (2005)
14	PPR	GHPR	*Wickerhamia fluorescens* TK1	0.40	150	375	99.9	*n. a.*	Fujii et al. (2011)
15	*La*PPR	PGDH	*Lactobacillus* sp. CGMCC 9967	0.82	47.3	57.7	99.9	243	This work

[a] *n. a.*: not available.

efficient and straightforward asymmetric synthetic strategy (Fig. 1). A number of robust oxidoreductases with promising potential in biomanufacture have been identified. Li et al. reported a non-LAB strain *Straphylococcus haemolyticus* T01 that catalyzed the asymmetric reduction of PhPA into D-PhLA, with 1.59 $g\,L^{-1}$ product yield and over 99% *ee* (Mu et al., 2012a). Employing resting cells of *B. coagulans* SDM, 37.3 $g\,L^{-1}$ PhLA could be produced from PhPA, however with no mention of the enantioselectivity (Zheng et al., 2011). Various enzymes belonging to different subfamilies have been discovered with PhPA reducing activity and heterogeneously expressed in *Escherichia coli* (Table 1). Most of the enzymes are originated from LAB, and could be classified into D-lactate dehydrogenase (D-LDH) family. An NAD-dependent D-LDH was cloned from *Lactobacillus bulgaricus* and exhibited excellent enantioselectivity, whereas with low activity towards bulkier substrates such as PhPA (Razeto et al., 2002). After rational re-design, one D-LDH mutant (Y52L) was developed, which could completely reduce 50 mM PhPA (*c.a.* 8.2 $g\,L^{-1}$) to D-PhLA in 90 min with 99.0% yield, 99.9% *ee*, and space-time yield of 131 $g\,L^{-1}\,d^{-1}$ (Zheng et al., 2013).

To achieve high productivity of optically pure D-PhLA, we endeavored to discover novel and efficient phenylpyruvate reductase (PPR). One LAB strain *Lactobacillus* sp. CGMCC 9967 with D-PhLA producing ability was isolated. A phenylpyruvate reductase (*La*PPR) was identified through shotgun library screening. Gene *ppr* was recombinant expressed and co-expressed with glucose dehydrogenase from *Bacillus mageterium* in *E. coli* for efficient production of D-PhLA with internal cofactor regeneration (Xu et al., 2013). The application potential of this newly established bioreductive system was also investigated.

2. Materials and methods

2.1. Materials

Phenylpyruvic acid (PhPA) and racemic 3-phenyllactic acid (PhLA) were purchased from Sigma–Aldrich. Other chemical compounds were from Tokyo Chemical Industry (Shanghai, China). NADH, NAD^+ and bacterial and yeast genomic DNA extraction kits were obtained from Sangon (Shanghai Sangon Biotech Co., China). Polymerase, endonucleotidases, T4 DNA ligase and vectors were purchased from Takara (Dalian, China).

2.2. Construction and screening of shortgun library

A *Lactobacillus* sp. strain capable of producing D-PhLA was isolated from a local farm (Wuxi, China), and was deposited at China General Microbiological Culture Collection Center (CGMCC) as CGMCC 9967. *Lactobacillus* sp. CGMCC 9967 was cultured in rich medium containing the following ingredients (per liter of deionized water): 10 g beef extract, 10 g peptone, 5 g yeast extract, 20 g glucose, 5 g sodium acetate, 2 g diammonium citrate, 0.58 g magnesium sulfate, 0.28 g manganese sulfate, 0.1 g tween 80, 2 g dipotassium hydrogen phosphate (pH 6.2–6.4). After cultivated at 30 °C for 24 h, the genomic DNA of *Lactobacillus* sp. CGMCC 9967 was extracted with bacterial genome extraction kit. Through

strictly controlling the genomic DNA amount, enzyme amount and digestion time, a shotgun genes' library with 2–6 kb fragments was constructed by enzymatic digestion with *Sau*3AI and recovered with nucleotide recovery kit. The genes were ligated into pUC118 digested with *Bam*HI and bacterial alkaline phosphatase. The resultant recombinant plasmids were transformed into *E. coli* JM109 to form the shotgun library expressed under the control of endogenous promoters. 16 S rDNA analysis suggests this strain shares high similarity with *Lactobacillus casei*. The average genome size of *L. casei* was about 3.0 Mb (2.62–3.36 Mb). To reach 99% coverage, the size of library should be about 2300–6905. About 6000 monocolonies were picked up and inoculated into 96-deep well plates supplemented with 0.6 mL LB, then cultivated at 30 °C and 200 rpm for 24 h. After centrifugation, the collected cells were resuspended in Tris–HCl buffer (pH 7.4, 10 mM) and disrupted with lysozymes. High throughput screening were performed with 200 μL reaction mixture including 170 μL KPB buffer (pH 7.0, 100 mM), 10 μL PhPA (20 mM, dissolved in KPB), 10 μL NADH (10 mM in deionized water) and 10 μL crude enzymes extracts by monitoring the absorbance at 340 nm for 5 min. The colonies with obvious activities were further cultured in 30 mL LB and rescreened for the activity and enantioselectivity of reduction of PhPA.

2.3. Cloning, expression and purification of recombinant La*PPR*

Cloning of gene coding for *La*PPR was performed according to general molecular cloning manual. The phenylpyruvate reductase gene (*ppr*) was amplified from *Lactobacillus* sp. CGMCC 9967, with a pair of forward primer 5′-GGAATTCCATATGATGAAGATTCTAAACAG-3′ (*Nde*I) and reverse primer 5′-GGAATTCTCAGTATCCGCGCGTGAGATCTT-3′ (*Eco*RI). The resultant product was further inserted into pET28a to form pET28-*ppr* and further transformed into *E. coli* BL21(DE3). Glucose dehydrogenase was cloned from *Bacillus megaterium* using the following primers (Forward primer: GGAATTCCATATGTATCCGGATTTAAAAGC [*Nde*I], Reverse primer: CCCAAGCTTTTAACCGCGGCCTGC [*Hind*III]) as above described and expressed in *E. coli* BL21(DE3) harboring pET28-*gdh*. Recombinant proteins with *N*-terminal his-tag were heterogeneously overexpressed under 0.5 mM IPTG at 30 °C and purified to homogeneity as previously described (Xu et al., 2012).

2.4. Construction of coexpression plasmid

Since the order in polycistron of coexpressed *La*PPR and GDH may influence the expression level, two different coexpression plasmids were constructed, pET-*ppr*-T7-*gdh* and pET-*gdh*-T7-*ppr*. Oligonucleotides primers (T7-F: AAGGAAAAAAGCGGCCGCTAATACGACTCACTATAG [*Not*I], coGDH-R: CCGCTCGAGTTAACCGCGGCCTGCCTGG [*Xho*I] and coPPR-R: CCGCTCGAGTCAGTATCCGCGCGTGAGATC [*Xho*I]) were used to amplify the fragments of *gdh* and *ppr* with respective T7 promoter for expression, and digested with *Not*I and *Xho*I. The plasmids of pET28-*ppr* and pET28-*gdh* were digested with the same pair of restriction enzymes. Two coexpression plasmids, pET28-*ppr*-T7-*gdh* and pET28-*gdh*-T7-*ppr*, were constructed through ligation of linearized pET28-*ppr*, pET28-*gdh* and the corresponding fragments. The resultant plasmids were transferred in to *E. coli* BL21(DE3) and induced with 0.6 mM IPTG at 30 °C. The cells were harvested, lyophilized to get the dry cells and stored at 4 °C for further use.

2.5. Enzyme activity assay

The activity of *La*PPR and GDH was determined by measuring the decrease or increase of NADH at 340 nm and 30 °C. The reaction mixture was 200 μL, consisted of 10 μL NADH (10 mM), 10 μL PhPA (20 mM), 10 μL *La*PPR in 170 μL PBS buffer (pH 7.0, 100 mM). For GDH, the reaction mixture consisted of 10 μL NAD^+ (10 mM), 10 μL glucose (100 mM) and 10 μL of GDH in 170 μL PBS buffer (pH 7.0, 100 mM). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 μmol of NADH per minute or the reduction of 1 μmol of NAD^+ per minute under the standard activity assay condition.

2.6. HPLC analysis

Conversion was analyzed using reverse HPLC equipped with Agilent Zorbax SB-C18 column (250 mm × *Φ*4.6 mm). Methanol/H_2O (60/40) supplemented with 0.05% trifluoroacetic acid (TFA) was used as elution phase with flow rate of 1 mL min^{-1} at 210 nm and 290 nm for PhLA and PhPA respectively. The retention times for PhLA and PhPA were 9.180 min and 17.467 min. The enantioselectivity of PhLA was measured using HPLC with Chiralcel OD-H column (Daicel Co., Japan; 250 mm × *Φ*4.6 mm). Racemic PhLA was eluted with hexane/isopropanol/TFA (98/2/0.05) at 1 mL min^{-1} and 210 nm. Retention times of D- and L-PhLA were 38.2 min and 41.2 min respectively. The enantiomeric excess was calculated based on peak areas of D-PhLA and L-PhLA according to the following formula.

Enantiomeric excess (ee) = $\frac{A_{D-PhLA}-A_{L-PhLA}}{A_{D-PhLA}+A_{L-PhLA}} \times 100\%$

2.7. Characterization of La*PPR*

Effect of pH on the activity of *La*PPR was carried out using standard assay except at various pHs, including sodium citrate buffer (pH 2.0–6.0, 100 mM), sodium phosphate buffer (pH 6.0–8.0, 100 mM) and Glycine–NaOH buffer (pH 8.0–10.0, 100 mM). pH-stability of *La*PPR was studied by incubating the purified *La*PPR in above mentioned buffers for 24 h at 30 °C. Then the residual activity was determined at standard assay condition, and highest activity was regarded as 100%.

The optimum temperature of *La*PPR was investigated at different temperatures, ranging from 25 °C to 40 °C, using standard activity assay protocol. Thermostability profiles were performed by incubating the purified *La*PPR at 30 °C, 40 °C or 50 °C in KPB buffer (pH 6.5, 100 mM). Appropriate amount of enzyme was removed at interval to determine the residual activity.

Various metal ions (0.2 mM) and additives (1% v/v) were incubated with about 0.1 mg/mL purified *La*PPR at 30 °C for 2 h. Afterwards, the residual activity was determined at the standard activity assay condition. Control experiment was also kept at 30 °C for 2 h without addition of metal ions or additives, and regarded as 100%.

Kinetic parameters of purified *La*PPR were assayed at various concentrations of PhPA and NADH. To determine the apparent K_m values, the concentrations of PhPA were varied from 0.1 to 10 mM with a fixed concentration of NADH at 2.0 mM. While in the case of NADH, the concentrations of NADH were ranged from 0.1 mM to 1.0 mM under 5.0 mM PhPA. Apparent kinetic parameters were calculated based on the double reciprocal Lineweaver–Burk plots. All the data was measured at three replicates.

Several α-ketoacids with aliphatic or aromatic substituents were chosen to characterize the substrate spectrum of *La*PPR. Standard activity assay protocol was adopted with 1.0 mM of each substrate. The conversion and *ee* analysis were performed with 1 mL reaction mixture containing 5 mM substrates, 1 mM NADH, 10 mM glucose, 100 μL purified *La*PPR (1 mg/mL) and 10 U GDH in KPB buffer (pH 6.5, 100 mM) and agitation at 30 °C and 120 rpm for 12 h. Then the reactions were terminated by adding 9% HCl, extracted with equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and analyzed by chiral HPLC analysis or GC analysis.

2.8. General protocol for the asymmetric reduction of PhPA

General method for the asymmetric preparation of D-PhLA was conducted as following: reactions mixture consisted of 0.2 g DCW of *E. coli* BL21(DE3) harboring pET-*ppr*-T7-*gdh*, 0.2–5.0 mmol PhPA, 1.5 equiv. glucose (0.3–7.5 mmol) in 10 mL KPB buffer (pH 6.5, 100 mM) at 30 °C, titrated with 0.5 M Na_2CO_3 to maintain the pH at 6.5. Samples were removed from reaction mixture and acidified with 9% HCl to end the reaction. Then, samples were centrifuged at 12000 rpm for 5 min, filtrated through 0.22 μm filter to remove protein and subjected to HPLC for conversion and enantioselecivity analysis.

2.9. Preparative synthesis of chiral D-PhLA

Preparative reaction was carried out with 10 g DCW of *E. coli* BL21(DE3) harboring pET-*kar*-T7-*gdh*, 1.5 equiv. glucose of PhPA in 1 L KPB (pH 6.5, 100 mM). The reaction was started by addition of 50 g PhPA, and magnetically stirred at 30 °C until complete. Na_2CO_3 (0.5 M) was added to maintain the pH at 6.5. After the reaction was terminated, the reaction mixture was acidified with 9% HCl to pH 3.0 and extracted with equal volume of ethyl acetate for three times. The upper organic layers were collected, combined and dried over anhydrous Na_2SO_4, further evaporated and dried under vacuum to obtain purified D-PhLA (white powder).

2.10. Homology modeling and molecular docking analysis

Homology structure of *La*PPR was constructed with EasyModeller 4.0 using protein 3ETV as template and verified with SAVES (The Structure Analysis and Verification Server version 4). All docking calculations were accomplished with AutoDock Vina 1.1, with the docking algorithm that took account of ligand flexibility while kept the protein rigid. For NADH, the parameters were set as: center_*x* −1.528, center_*y* 20.389, center_*z* 42.583, size_*x* 26, size_*y* 24, size_*z* 30; for PhPA the parameters were set as center_*x*: −0.62, center_*y* 9.84, center_*z* 54.71, size_*x* 20, size_*y* 20, size_*z* 22. Stereo views were constructed using Pymol and LIGPLOT (Wallace et al., 1995).

3. Results

3.1. Identification of phenylpyruvate reductase

Using 10 g L^{-1} resting wet cells of *Lactobacillus* sp. CGMCC 9967, 10 mM PhPA (1.64 g L^{-1}) could be asymmetrically reduced into D-PhLA with 99% *ee* and 90% conversion. This good performance in the preparation of D-PhLA encouraged us to identify the functional phenylpyruvate reductase from *Lactobacillus* sp. CGMCC 9967.

The shotgun genomic library of *Lactobacillus* sp. CGMCC 9967 was developed to screen for phenylpyruvate reductase coding gene. Most of the clones displayed little activity by determination the decrease of NADH in absorbance at 340 nm. Only five clones were isolated with obvious reducing activity. They were sequenced, assembled and predicted over NCBI online tool to search for ORF. About five 700–1000 bp ORFs predicted to be dehydrogenase in NCBI were cloned and expressed in *E. coli* BL21(DE3). However, four of them were expressed either in insoluble forms or with no apparent activity. One ORF coding for dehydrogenase was soluble expressed and displayed reductase activity towards substrate PhPA. The gene and its coding protein were designated as *ppr* and *La*PPR. The nucleotide sequence and protein sequences of *La*PPR have been deposited in the GenBank database under accession no. of KP735960. The length of *ppr* gene is 939 bp with a GC content of 49.9%, encoding a dehydrogenase of 312 amino acids. Gene *ppr* is located on the complementary strand, and has a putative ribosomal binding sequence (GAAAAGGAGTGCGACATG, underlining indicating the Shine–Dalgarno sequence) on the 5 bp upstream of the start codon. BLASTp analysis in the non-redundant protein sequences database indicates *La*PPR shares more than 90% sequence identity to dehydrogenase from *L. casei*, however with little knowledge about its function and applications. Further BLAST against protein database bank (PDB) reveals that *La*PPR displays the highest identity (45%) to phosphoglycerate dehydrogenase (PGDH) from *Lactobacillus plantarum* (PDB: 3EVT), 29% to PGDH from *Pyococcus horikoshi* (1WWK), and PDGH from *Sulfolobus tokodaii* (2EKL). *La*PPR is presumed to be a new member of PGDH subfamily, which belongs to the family of D-isomer 2-hydroxy acid dehydrogenase, including glyoxylate/hydroxypyruvate reductase (GHPR), D-lactate dehydrogenase (D-LDH), erythronate-4-phosphate dehydrogenase (PDXB), D-2-hydroxyisocaproate dehydrogenase (D-HicDH), formate dehydrogenase (FDH), and C-terminal binding protein (CTBP) (Fig. 2). Physiologically, PGDH plays vital role in the metabolism of L-serine by utilizing NADH as cofactor for the conversion of 3-phosphoglycerate into 3-phosphohydroxy pyruvic acid, key precursors for serine. As shown in Table 1 and Fig. 2, most of the enzymes catalyzing the asymmetric reduction of PhPA were classified into D-LDH, D-HicDH and GHPR family, which are distant from PDGH evolutionally.

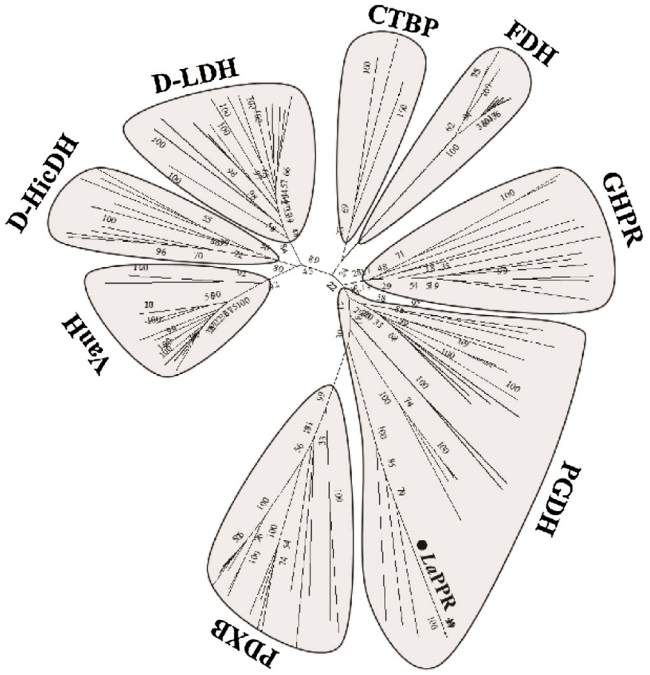


Fig. 2. Phylogenetic analysis of D-isomer-specific 2-hydroxyacid dehydrogenase superfamily.

3.2. Enzymatic properties of purified La*PPR*

This newly discovered *ppr* gene from *Lactobacillus* sp. CGMCC 9967 was soluble expressed in *E. coli* BL21 harboring pET28-*ppr*. The recombinant *La*PPR with *N*-terminal His-tag was purified to homogeneity through nickel affinity chromatography, desalt column and superdex chromatography. The specific activity after affinity purification was 7.3 U mg_{prot}^{-1}, 7.8 folds to that of crude extract (0.93 U mg^{-1}). After desalting and superdex separation, the specific activity was further increased to 19.6 U mg_{prot}^{-1}. A single band was migrated on the SDS-PAGE, corresponding to a molecular weight (MW) of about 37 kDa (Fig. 3). Further separation through gel exclusion chromatography (Superdex^{TM} 200) returned a single

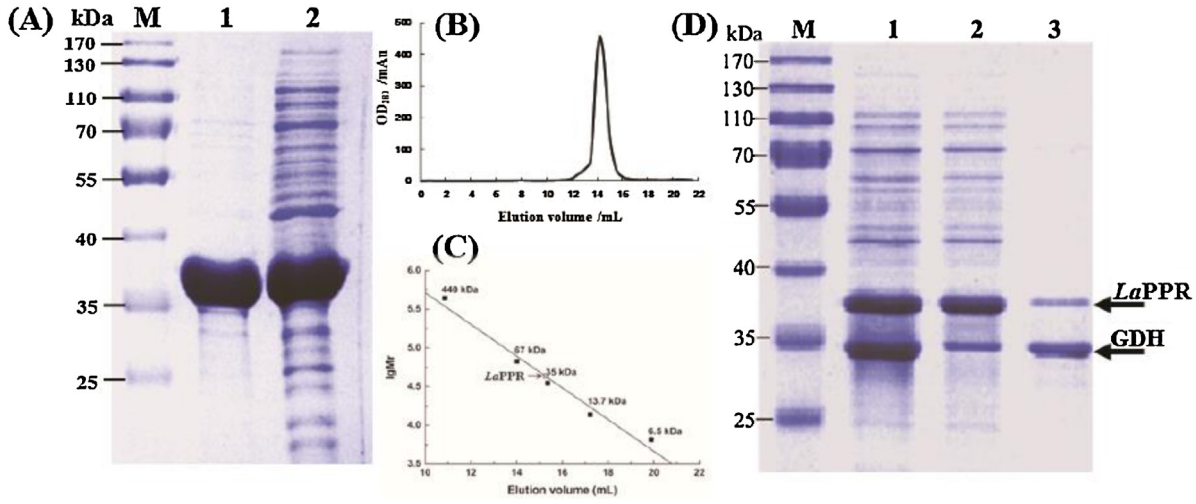


Fig. 3. Purification of the recombinant *La*PPR and coexpression of *La*PPR and glucose dehydrogenase.

Table 2
Effect of metal ions and additives on the activity of purified *La*PPR.

Metal ions [0.2 mM]	Relative activity [%]	Additives [1%, v/v]	Relative activity [%]
Control	100	Control	100
K^+	97.8 ± 2.8	Tween80	69.9 ± 1.9
Ca^{2+}	59.5 ± 1.3	PEG4000	84.7 ± 2.1
Mg^{2+}	113.7 ± 2.3	Methanol	81.4 ± 1.9
Ba^{2+}	68.0 ± 1.1	Ethanol	72.9 ± 1.3
Zn^{2+}	97.7 ± 1.4	Glycerol	91.8 ± 1.8
Mn^{2+}	107.2 ± 1.6	Isopropanol	96.4 ± 2.0
Fe^{2+}	89.2 ± 0.5	Hexane	91.3 ± 1.8
Fe^{3+}	77.3 ± 1.5	DMSO	48.5 ± 1.8
Cu^{2+}	93.4 ± 0.5	DMF	49.3 ± 1.8
Co^{2+}	103.2 ± 2.0	THF	87.3 ± 1.5
Ni^{2+}	60.8 ± 2.6	β-Mercaptoethanol	80.1 ± 1.2
Al^{3+}	39.3 ± 3.4	L-Cystein	83.8 ± 2.2
EDTA	62.5 ± 2.3	L-Glutathione	65.5 ± 1.7

peak with 35.8 kDa *ca.* in MW based on the molecular mass curve of standard proteins, indicating *La*PPR is a monomeric protein.

The characteristics of purified *La*PPR were investigated. No activity was detected using NADPH as reducing cofactor, while full activity was found with NADH, revealing that *La*PPR is a NADH-dependent reductase. According to the pH-profiles (Fig. 4(A)), the highest activity of *La*PPR was measured at around pH 7.0 in KPB buffer (100 mM). However *La*PPR was much more stable under slightly acidic condition. After 24 h incubation, the highest residual activity was determined at pH 6.5 (Fig. 4(B)). Therefore, the pH of the following reaction was maintained at pH 6.5. As shown in Fig. 4(C), *La*PPR was mesophilic. Along with the increase of temperature, the relative activity was decreased. Thermostability analysis indicated that *La*PPR was relatively stable at 30 °C and 40 °C, while liable at 50 °C. The half-lives of *La*PPR at 30, 40 and 50 °C were calculated to be 152 h, 42.6 h and 6.57 h respectively. The activity decreased markedly during the initial 24 h, and then decreased slowly as the elongation of incubation time.

Metal ions dependence was also characterized (Table 2). None metal ion was detected with significant enhancement on the activity of *La*PPR, which was in consistence with other PGDH members (Singh et al., 2014). Under 0.2 mM of Mg^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}, the residual activity was more than 90% compared with the control. Al^{3+} was toxic to *La*PPR, leading to residual activity of 39.3%. For all the additives (1%), including organic solvents, reductants and surfactants, the activity of *La*PRR was decreased to certain extent. *La*PPR was relatively stable in glycerol, isopropanol, and hexane, and could retain more than 90% activity. DMSO and DMF were not suitable cosolvents for the asymmetric reduction of PhPA catalyzed by *La*PPR, resulting in over 50% decrease in activity.

Kinetic constants of purified *La*PPR were determined at various substrate concentrations. K_m values towards PhPA and NADH were 0.82 mM and 0.43 mM respectively. *La*PPR displayed high affinity for PhPA, relatively lower than other phenylpyruvate reductases (Table 1). The V_{max} of purified *La*PPR was 79.3 μmol min^{-1} mg^{-1} towards PhPA, and the k_{cat} and k_{cat}/K_m were 47.3 s^{-1} and 57.7 s^{-1} mM^{-1}.The catalytic efficiency of *La*PPR on PhPA was at moderate level compared with other phenylpyruvate reductases.

Seven α-keto acids with aliphatic or aromatic side chains were chosen to explore the substrate spectrum of *La*PPR (Table 3). Relative activity analysis showed that *La*PPR preferred aromatic α-keto acids to the aliphatic keto acids. Among three aromatic α-keto acids tested, PhPA was reduced more efficiently than benzoylformic acid (BFA). However, the activity on *o*-chlorobenzoyformic acid (CBFA) was much higher than PhPA (3.85-fold) and BFA (19.2-fold). The asymmetric reduction product of CBFA, *o*-chloromandelic acid (CMA), is a vital chiral synthon for preparation of Clopidegrel, one of the best-selling pharmaceuticals (Yun et al., 2005). The electron withdrawing substituent on the *ortho*-position of phenyl ring could promote the asymmetric reduction with *La*PPR. Despite the structural similarity with PhPA, pyruvic acid was merely reduced by *La*PPR (Table 3), and no obvious activity was detected. This phenomenon manifests the importance of aromatic group for the activity of *La*PPR. The enantioselectivities towards seven keto acids were analyzed at 5 mM substrate concentration. Except for pyruvic acid and *tert*-butylpyruvic acid, all the keto acids were asymmetrically reduced into pure D-hydroxyl acid with >99% *ee*. Especially for PhPA, BFA and CBFA, 5 mM substrate could be completely reduced into D-PhLA, D-mandelic acid (MA) and D-CMA in 12 h.

3.3. *Coexpression of* La*PPR and GDH in* E. coli *BL21(DE3)*

Although bioreductive process is a promising approach for biomanufacture, the adoption of high price cofactors ($NAD(P)^+$ or FAD/FMN) is one of the main obstacles for its industrialization. To reduce the cost of cofactors, another enzyme was introduced for internal cofactor regeneration. A tandem system of *gdh* and *ppr* in one plasmid was employed, due to its higher compatibly and stability than two-plasmid systems.

The coexpressed *E. coli* BL21(DE3) strains harboring pET28-*ppr*-T7-*gdh* and pET28-*gdh*-T7-*ppr* were constructed. According to the

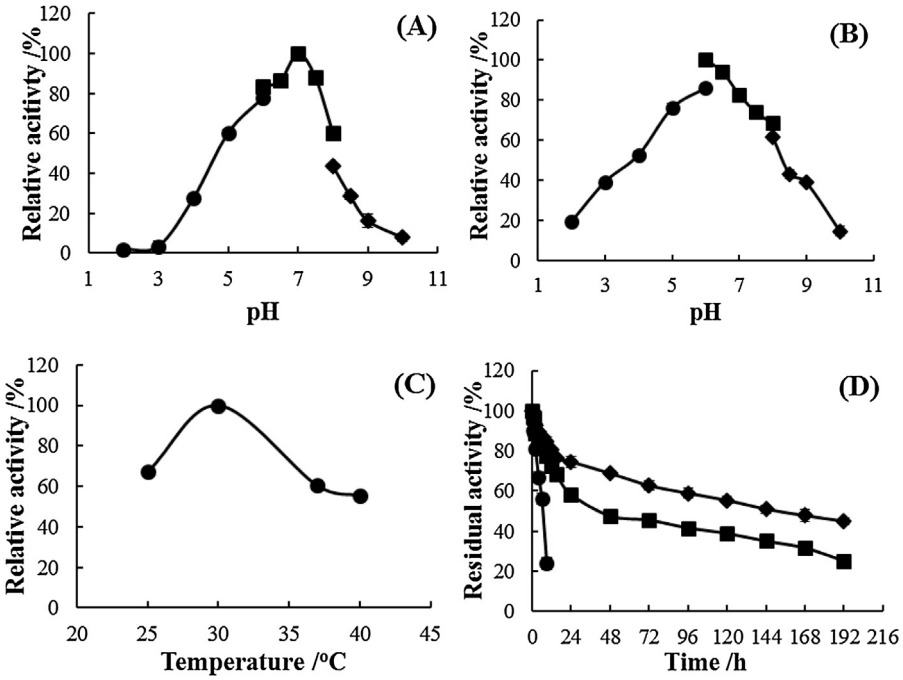
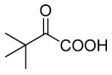
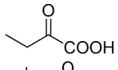
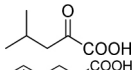
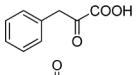
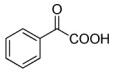
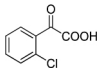


Fig. 4. The pH and temperature profiles of *La*PPR.

Table 3
Substrate specificity of purified *La*PPR towards various α-keto acids.

Substrate	Relative activity [%][a]	Conversion [%]	*ee* [%]
	*n. d.*[b]	*n. d.*	*n. d.*
	*n. d.*	*n. d.*	*n. d.*
	23.6	8.74	>99 (D)
	38.2	14.5	>99 (D)
	100	>99	>99 (D)
	75.0	>99	>99 (D)
	385	>99	>99 (D)

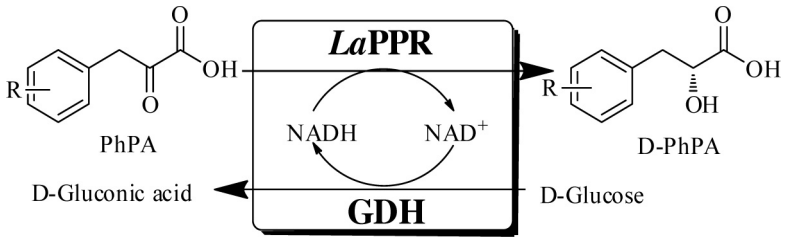
[a] Activity towards PhPA (U mg^{-1}) was regarded as 100%.
[b] *n. d.*: no activity was detected.

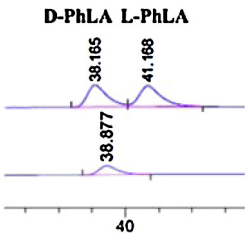
SDS-PAGE analysis, GDH and *La*PPR were successfully co-expressed in *E. coli*, with obvious bands on the SDS-PAGE. However, with regard to the solubility, GDH was mostly insolubly expressed by *E. coli* containing pET28-*ppr*-T7-*gdh*, while partially soluble in *E. coli* carrying pET28-*gdh*-T7-*ppr* (Fig. 3(D)). To balance the enzymatic activity between GDH and *La*PPR and achieve high cofactor regeneration for the reduction of PhPA, the recombinant coexpressed *E. coli* carried pET28-*gdh*-T7-*ppr* was used for the preparation of D-PhLA.

3.4. Optimization on the asymmetric preparation of D-PhLA

The potential of *La*PPR in the asymmetric preparation of D-PhLA was investigated by simply optimization on the substrate loadings using above constructed recombinant *E. coli* whole cells (Table 4), aiming at 100 g L^{-1} PhPA with no addition of external cofactors. Initially, 20 mM PhPA was added into the reaction mixture with 10 g L^{-1} DCW of recombinant *E. coli* cells. Within 0.5 h, all PhPA was converted into D-PhLA (99% *ee*) at 30 °C and 120 rpm. The isolation yield and productivity were 86% and 153 g L^{-1} d^{-1} respectively. Considering its good performance, the reaction was optimized at elevated substrate loading. At 100 mM PhPA (18.6 g/L), full conversion was achieved in 2 h, with 91.9% yield and 205 g L^{-1} d^{-1} productivity (Table 4, entry 3). Then the substrate concentration was further increased to 200 mM PhPA under 20 g L^{-1} biocatalyst. The productivity of as much as 394 g L^{-1} d^{-1} was attained. In order to improve the efficiency of biocatalyst, concentration of PhPA was raised to 500 mM (93.1 g L^{-1}). All the PhPA could be asymmetrically reduced into D-PhLA with 93.9% yield and 300 g L^{-1} d^{-1} productivity in 7 h. When PhPA loading was further increased to 100 g L^{-1}, no residual PhPA was detected in the reaction mixture at 9 h. The isolation yield and productivity were 91.3% and 243 g L^{-1} d^{-1}. Although

Table 4
Asymmetric reduction of PhPA employing *La*PPR.





Entry	Substrate			Catalyst	Time	Conv.	*ee*	Yield	Productivity
	[M]	[g L^{-1}]	[g]	[g DCW L^{-1}]	[h]	[%]	[%]	[%]	[g L^{-1} d^{-1}][a]
1	0.02	3.7	0.037	10	0.5	>99	>99	86.0	153
2	0.05	9.3	0.093	10	1	>99	>99	90.8	203
3	0.1	18.6	0.186	10	2	>99	>99	91.9	205
4	0.2	37.2	0.372	20	2	>99	>99	88.2	394
5	0.5	93.1	0.931	20	7	>99	>99	93.9	300
6	0.5	100	1.00	20	9	>99	>99	91.3	243
7	0.5	50	50.0	10	9	>99	>99	87.0	116

[a] Productivity was calculated according to space-time yield.

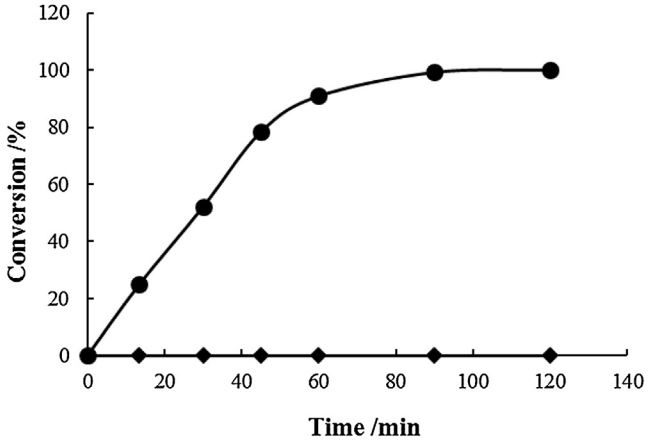


Fig. 5. Time course of the asymmetric reduction of 50 g PhPA at 1 L scale employing recombinant *E. coli* BL21 whole cells harboring pET28-*gdh*-T7-*ppr* (●) and pET28-*gdh* (◊).

the productivity at 100 g·L^{-1} was lower than that at 37.2 g L^{-1} PhPA, the D-PhLA concentration in the reaction mixture was significantly enhanced by 2.7-fold. No PhLA was produced using recombinant *E. coli* harboring pET28-*gdh*, which proved the effect of *La*PPR in the asymmetric reduction of PhPA.

To examine the possibility of the newly developed bioreductive system for the preparation of D-PhLA, the reaction was carried out at 1 L scale (Fig. 5). The PhPA loading and biocatalyst amount was 50 g L^{-1} and 10 g L^{-1}, the same substrate to biocatalyst ratio achieved the best result at 10-mL scale. Within 9 h, 50 g PhPA in 1 L KPB was completely reduced into D-PhLA. After simply extraction and purification, the isolation yield was 87.0%. The product was confirmed as D-PhLA by ^{1}H NMR and specific rotation analysis as following: Compound D-2-hdyroxy-3-phenylpropanoic acid (87% yield). $[\alpha]_D^{25} = +1960^\circ$ (*c* 1.00, H_2O) {$[\alpha]_D^{20} = +38.6^\circ$ (*c* 1.0, *N*,*N*-dimethylformamide) (Xu et al., 2005)}. ^{1}H NMR (500 MHz, DMSO) (Cohen-Arazi et al., 2008): δ/ppm: δ 2.78 (dd, *J* = 6.3, 5.2 Hz, 1H, C*H*2), 2.94 (dd, *J* = 6.3, 5.2 Hz, 1H, CH2), 4.15 (dd, *J* = 8.3, 4.4 Hz, 1H, CHOH), 5.28 (s, 1H, OH), 6.41–8.12 (m, 5H, Ph-H), 12.49 (s, 1H, COOH).

4. Discussion

Phenyllactic acid (PhLA) is an ideal antimicrobial compound with broad inhibition activity against various bacteria, yeasts and fungi, and has less side-effect on the health of human being. One of the important applications of PhLA has been focused in the food industry, especially in the inhibition microbial contaminants. In addition, the aromatic group endues the poly-phenyllactic acid much advantageous characteristics, such as higher thermostability than poly-lactic acid. Especially, chiral PhLA can be used as a key building block for the synthesis various pharmaceuticals and fine chemicals. Considering the significant application, various studies have been committed on the production of PhLA. PhLA was firstly identified in honey with a content of 100–800 mg/kg (Tuberoso et al., 2011). First report on the fermentation production of PhLA was published by Lavermicocca and coworkers by using *L. plantarum* 21B (Lavermicocca et al., 2000). Since then, various LAB strains or non-LAB strains have been discovered in the production of PhLA. However the relative low product concentration (0.1–17.3 g L^{-1}), high product purification cost and low enantioselectivity are the main issues for the fermentative production of PhLA. Biocatalytic preparation by phenylpyruvate reductases is regarded as the one of the most promising solutions for the synthesis of PhLA, especially optically pure PhLA, due to its high efficiency, enantioselectivity and easy operation. Most of the phenylpyruvate reductases belong to D-lactate dehydrogenase, D-2-hydroxyisocaproate dehydrogenase, glyoxylate/hydroxypyruvate reductase and D-mandelate dehydrogenase subfamilies. The highest productivity was reported at 37.3 g L^{-1} using D-LDH mutant from *B. coagulans* (Zheng et al., 2011).

A *ppr* gene coding for a novel phenylpyruvate reductase (*La*PPR) was identified through screening the shotgun genomic library of *Lactobacillus* sp. CGMCC 9967. Compared with other phenylpyruvate reductases, this *La*PPR was distant phylogenetically and fallen into D-PGDH subfamily (EC 1.1.1.95) as shown in Fig. 2, which is much different from D-LDH. There are three different structural forms of PGDH, Type I, Type II and Type III, based on the length of the sequence and the domain organization (Singh et al., 2014). Consensus sequence analysis reveals that *La*PPR is a member of Type III$_H$ PGDH, with His-Glu-Arg as catalytic triad forming a charge relay system for hydride transfer in most PGDH enzymes. In *La*PPR, the

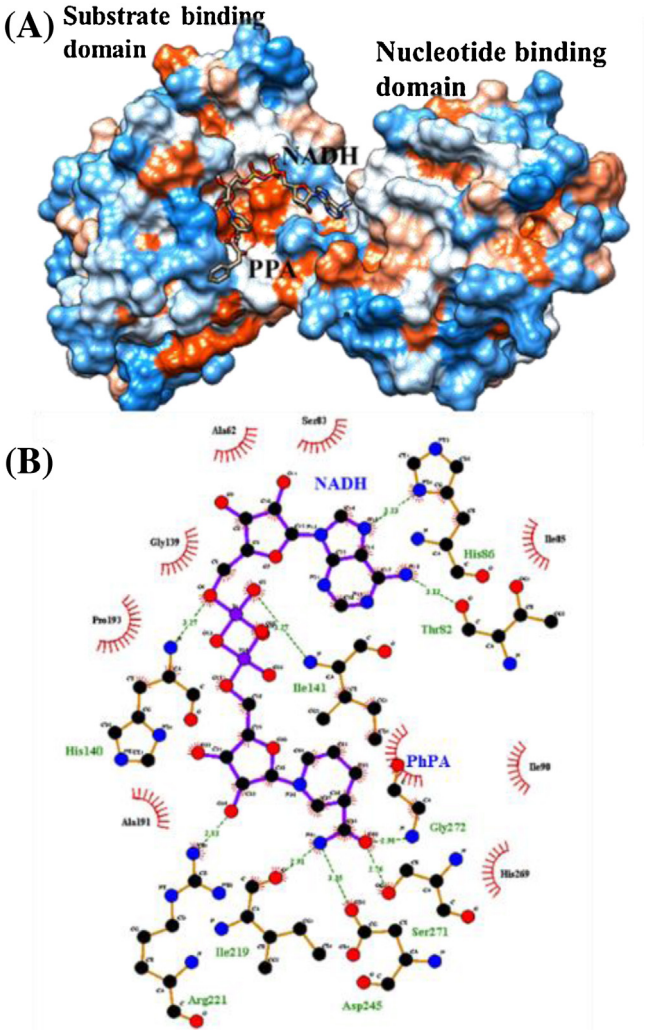


Fig. 6. Homology structure of *La*PPR and the interaction residues around NADH and PhPA.

catalytic triad is His272, Glu253 and Arg224. Among them, Arg224 plays vital role in orienting the substrate for a favorable conformation, while His272 is essential for the catalysis and functions as an acid/base catalyst. After binding to the substrate, His272 could act as proton donor to the prochiral carbonyl oxygen of substrate. However, the detailed catalytic mechanism of PGDH enzymes in the asymmetric reduction of PhPA is not completely understood. To get a deep insight into the relationship between structure and function, the homology structural model of *La*PPR was established using phosphoglycerate dehydrogenase (45% identity) from *L. plantarum* (PDB: 3EVT, Bonanno JB et al. unpublished) as template. More than 95% of the residues of *La*PPR were located in the maximum allowable region according to the Ramachandran chart. As illustrate in Fig. 6(A), *La*PPR constitutes two apparent domains, nucleotide-binding domain (NBD) and substrate-binding domain (SBD) (Singh et al., 2014). The interface between the two domains formed the catalytic cleft, where the cofactor and substrate are close to each other. According to the docking result of *La*PPR (Fig. 6(B)), the glycine-rich sequence (140–145, GTGHIG) is identified in the cleft of two domains. Residues T85 and H89 stabilize the adenine ring through two hydrogen bonds, while I219, D245, S271 and G272 interact with the nicotiamide structure through polar interact with the N and O atoms. The R221 of the catalytic triad acts on the NADH to reduce the pK_a and promote the electron transferring.

Enzymatic characteristics of *La*PPR were systemically investigated. *La*PPR is NADH-dependent, with no promoting metal ions. It is quite stable at pH 6.5 and 30 °C, which is important for the biocatalytic reaction. *La*PPR prefers aromatic keto acids to aliphatic keto acids. Also, the aromatic group is important for the activity. All three tested aromatic keto acids could be reduced with 99% conversion. Most importantly, excellent enantioselectivity was observed with *La*PPR. All five prochiral keto acids with obvious activity are asymmetrically reduced into optically pure hydroxyl acids. The prominent enzymatic performance provides key evidences for the potential application of *La*PPR in the preparation of chiral hydroxyl acids.

Since phenylpyruvate reductase is cofactor dependent, it is of vital importance to introduce a cofactor regeneration system to achieve the efficiently bioreductive preparation of D-PhLA. Despite that a number of reductases have been reported capable of reducing PhPA, less was focused on the preparation of PhLA. Zheng et al adopted formate dehydrogenase (FDH) for the cofactor regeneration. Although the cosubstrate/coproduct was easy to be removed, the efficiency was relatively low (Zheng et al., 2013). Glucose dehydrogenase, with much higher regeneration efficiency, was employed in this work. Through titration of Na_2CO_3 into the reaction mixture, the pH was maintained at 6.5 to guarantee the conversion of PhPA by *La*PPR. Compared between two tandem systems in one plasmid, pET28-*gdh*-T7-*ppr* was proved to be much more suitable, considering the protein solubility and activity of the two enzymes. Using this biocatalyst, as much as 100 g L^{-1} PhPA could be asymmetrically reduced into D-PhLA under no assistance of external cofactors. The isolation yield and productivity could reach 91.3% and 243 g L^{-1} d^{-1} respectively, ranking the highest record in the preparation of D-PhLA from PhPA.

5. Conclusions

In summary, a novel phenylpyruvate reductase, belonging to PGDH family, was identified from *Lactobacillus* sp. CGMCC 9967. It is highly stable and efficient in the asymmetric preparation of D-PhLA from PhPA. As much as 100 g L^{-1} phenylpyruvate could be asymmetrically reduced into D-phenyllactic acid with 91.3% isolation yield, >99% *ee*, and space-time yield of 243 g L^{-1} d^{-1}. This study provides useful guidance for further application of *La*PPR in the synthesis of enantiopure D-PhLA with excellent productivity.

Ethical statement

I certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Journal of Biotechnology. And the study is not split up into several parts to increase the quantity of submissions and submitted to various journals or to one journal over time. No data have been fabricated or manipulated (including images) to support your conclusions. No data, text, or theories by others are presented as if they were our own. The submission has been received explicitly from all co-authors. And authors whose names appear on the submission have contributed sufficiently to the scientific work and therefore share collective responsibility and accountability for the results. The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors. Informed consent was obtained from all individual participants included in the study.

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References

Cohen-Arazi, N., Katzhendler, J., Kolitz, M., Domb, A.J., 2008. Preparation of new α-hydroxy acids derived from amino acids and their corresponding polyesters. Macromolecules 41, 7259–7263.

Coppola, G.M., Schuster, H.F., 1997. α-Hydroxy Acids in Enantioselective Synthesis. Wiley-VCH, Weinheim.

Dieuleveus, V., Lemarinier, S., Guéguen, M., 1998. Antimicrobial spectrum and target site of D-3-phenyllactic acid. Int. J. Food Microbiol. 40, 177–183.

Fujii, T., Shimizu, M., Doi, Y., Ito, T., Miura, D., Wariishi, H., Takaya, N., 2011. Novel fungal phenylpyruvate reductase belongs to D-isomer specific 2-hydroxyacid dehydrogenase family. Biochim. Biophys. Acta 1814, 1669–1676.

Gröger, H., 2001. Enzymatic routes to enantiomerically pure aromatic ö-hydroxy carboxylic acids: a further example for the diversity of biocatalysis. Adv. Synth. Catal. 343, 547–558.

Hummel, W., Schütte, H., Kula, M.R., 1983. Large-scale production of D-lactate dehydrogenase for the stereospecific reduction of pyruvate and phenylpyruvate. Eur. J. Appl. Microbiol. Biotechnol. 18, 75–85.

Hummel, W., Schütte, H., Kula, M.R., 1985. D-2-hydroxyisocaproate dehydrogenase from *Lactobacillus casei*: a new enzyme suitable for stereospecific reduction of 2-ketocarboxylic acids. Appl. Microbiol. Biotechnol. 21, 7–15.

Hummel, W., Schütte, H., Kula, M.R., 1988. D-(−)-mandelic acid dehydrogenase from *Lactobacillus curvatus*. Appl. Microbiol. Biotechnol. 28, 433–439.

Jiang, J.H., Mu, W.M., Zhang Tao, Jiang, B., 2010. Bioconversion of phenylpyruvate to phenyllactate: gene cloning, expression, and enzymatic characterization of D- and L-lactate dehydrogenase from *Lactobacillus plantarum* SK002. Appl. Biochem. Biotechnol. 162, 242–251.

Kawaguchi, H., Uematsu, K., Ogino, C., Teramura, H., Niimi-Nakamura, S., Tsuge, Y., Hasunuma, T., Oinuma, K.I., Takaya, N., Kondo, A., 2014. Simultaneous saccharification and fermentation of kraft pulp by recombinant *E. coli* for phenyllactic acid production. Biochem. Eng. J. 88, 188–194.

Larissegger-Schnell, B., Glueck, S.M., Kroutil, W., Faber, K., 2006. Enantiocomplementary deracemization of (±)-2-hydroxy-4-phenylbutanoic acid and (±)-3-phenyllactic acid using lipase-catalyzed kinetic resolution combined with biocatalytic racemization. Tetrahedron 62, 2912–2916.

Lavermicocca, P., Valerio, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M., 2000. Purification and characterization of novel antifungal compounds from the sourdough *Lactobacillus plantarum* strain 21B. Appl. Environ. Microbiol. 66, 4084–4090.

Mu, W.M., Liu, F.L., Jia, J.H., Chen, C., Zhang, T., Jiang, B., 2009. 3-Phenyllactic acid production by substrate feeding and pH-control in fed-batch fermentation of *Lactobacillus* sp. SK007. Bioresour. Technol. 100, 5226–5229.

Mu, W.M., Yu, S.H., Jiang, B., Li, X.F., 2012a. Characterization of D-lactate dehydrogenase from *Pediococcus acidilactici* that converts phenylpyruvic acid into phenyllactic acid. Biotechnol. Lett. 34, 907–911.

Mu, W.M., Yu, S.H., Zhu, L.J., Zhang, T., Jiang, B., 2012b. Recent research on 3-phenyllactic acid, a broad-spectrum antimicrobial compound. Appl. Microbiol. Biotechnol. 95, 1155–1163.

Razeto, A., Kochhar, S., Hottinger, H., Dauter, M., Wilson, K.S., Lamzin, V.S., 2002. Domain closure, substrate specificity and catalysis of D-lactate dehydrogenase from *Lactobacillus bulgaricus*. J. Mol. Biol. 318, 109–119.

Sato, K., Ito, H., Ei, H., Microbial conversion of phenyllactic acid to L-phenylalanine. 1986, Brevet, Ajinomoto Co, Inc. JP 86212293.

Schmid, A., Hollmann, F., Park, J.B., Bühler, B., 2002. The use of enzymes in the chemical industry in Europe. Curr. Opin. Biotechnol. 13, 359–366.

Schmidt, M., Griengl, H., 1999. Oxynitrilases from cyanogenesis to asymmetric synthesis. Top. Curr. Chem. 200, 193–226.

Singh, R.K., Raj, I., Pujari, R., Gourinath, S., 2014. Crystal structures and kinetics of Type III 3-phosphoglycerate dehydrogenase reveal catalysis by lysine. FEBS J. 281, 5498–5521.

Taguchi, H., Ohta, T., 1991. D-lactate dehydrogenase is a member of the D-iosmer-specific 2-hydroxyacid dehydrogenase family. Cloning, sequencing and expression in *E. coli* of the D-lactate dehydrogenase gene of *Lactobacillus plantarum*. J. Biol. Chem. 266, 12588–12594.

Tamura, Y., Ohkubo, A., Iwai, S., Wada, Y., Shinoda, T., Arai, K., Mineki, S., Iida, M., Taguchi, H., 2002. Two forms of NAD-dependent D-mandelate dehydrogenase in *Enterococcus faecalis* IAM 10071. Appl. Environ. Microbiol. 68, 947–951.

Tokuda, C., Ishikura, Y., Shigematsu, M., Mutoh, H., Tsuzuki, S., Nakahira, Y., Tamura, Y., Shinoda, T., Arai, K., Takahashi, O., Taguchi, H., 2003. Conversion of *Lactobacillus pentosus* D-lactate dehydrogenase to a D-hydroxyisocaproate dehydrogenase through a single amino acid replacement. J. Bacteriol. 185, 5023–5026.

Tuberoso, C.I., Bifulco, E., Caboni, P., Sarais, G., Cottiglia, F., Floris, I., 2011. Lumichrome and phenyllactic acid as chemical markers of thistle (*Galactites tomentosa Moench*) honey. J. Agric. Food Chem. 59, 364–369.

Urban, F.J., Moore, B.S., 1992. Synthesis of optically active 2-benzyldihydrobenzopyrans for the hypoglycemic agent englitazone. J. Heterocycl. Chem. 29, 431–438.

Wada, Y., Iwai, S., Tamura, Y., Ando, T., Shinoda, T., Arai, K., Taguchi, H., 2008. A new family of D-2-hydroxyacid dehydrogenases that comprises D-mandelate dehydrogenases and 2-ketopantoate reductases. Biosci. Biotechnol. Biochem. 72, 1087–1094.

Wallace, A.C., Laskowski, R.A., Thornton, J.M., 1995. LIGPLOT: a program to generate schematic diagrams of protein-ligand interactions. Protein Eng. 8, 127–134.

Weckwerth, W., Miyamoto, K., Iinuma, K., Krause, M., Glinski, M., Storm, T., Bonse, G., Kleinkauf, H., Zocher, R., 2000. Biosynthesis of PF1022A and related cyclooctadepsipeptides. J. Biol. Chem. 275, 17909–17915.

Xu, G.C., Yu, H.L., Zhang, X.Y., Xu, J.H., 2012. Access to optically active aryl halohydrins using a substrate tolerant carbonyl reductase discovered from *Kluyveromyces thermotolerans*. ACS Catal. 2, 2566–2571.

Xu, G.C., Yu, H.L., Zhang, Z.J., Xu, J.H., 2013. Stereocomplementary bioreduction of β-ketonitrile without ethylated byproduct. Org. Lett. 15, 5408–5411.

Xu, Y.J., Chen, L.G., Duan, X.M., Meng, Y., Jiang, L.Q., Li, M.L., Zhao, G.L., Li, Y., 2005. Total synthesis of hirsutellide A. Tetrohedron Lett. 46, 4377–4379.

Yun, H.D., Choi, H.L., Fadnavis, N.W., Kim, B.G., 2005. Stereospecific synthesis of (*R*)-2-hydroxy carboxylic acids using recombinant *E. coli* BL21 overexpressing YiaE from *E. coli* K12 and glucose dehydrogenase from *Bacillus subtilis*. Biotechnol. Prog. 21, 366–371.

Yu, S., Jiang, H., Jiang, B., Mu, W.M., 2012. Characterization of D-lactate dehydrogenase producing D-3-phenyllactic acid from *Pediococcus pentosaceus*. Biosci. Biotechnol. Biochem. 76, 853–855.

Zheng, G.W., Xu, J.H., 2011. New opportunities for biocatalysis: driving the synthesis of chiral chemicals. Curr. Opin. Biotechnol. 22, 784–792.

Zheng, Z.J., Ma, C.Q., Gao, C., Li, F.S., Qin, J.Y., Zhang, H.W., Wang, K., Xu, P., 2011. Efficient conversion of phenylpyruvic acid to phenyllactic acid by using whole cells of *Bacillus coagulans* SDM. PLoS One 6, e19030.

Zheng, Z.J., Sheng, B.B., Gao, C., Zhang, H.W., Qin, T., Ma, C.Q., Xu, P., 2013. Highly stereoselective biosynthesis of (*R*)-α-hydroxy carboxylic acids through rationally re-designed mutation of D-lactate dehydrogenase. Sci. Rep. 3, e3401.