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ORIGINAL ARTICLE

Efficient synthesis of D-phenyllactic acid by a whole-cell biocatalyst co-expressing glucose dehydrogenase and a novel D-lactate dehydrogenase from *Lactobacillus rossiae*

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Abstract

D-Phenyllactic acid is a versatile natural organic acid, which is used as an antiseptic agent, monomer of the biodegradable material poly-phenyllactic acid and in the synthesis chiral intermediate of pharmaceuticals. In this report, the novel NADH-dependent D-lactate dehydrogenase *Lr*LDH was identified by screening a shotgun genome of *Lactobacillus rossiae*. To improve cofactor regeneration, the *Exiguobacterium sibiricum* glucose dehydrogenase *Es*GDH was overexpressed together with *Lr*LDH in *E. coli* BL21(DE3)-pCDFDuet-1-*gdh-ldh*. The total enzyme activity in the fermentation broth of *E. coli* BL 21(DE3)-pCDFDuet-1-*gdh-ldh* peaked at 2359.0 U l^{-1} when induced by 10 g l^{-1} lactose at 28 °C and 150 rpm for 14 h. The biocatalytic reduction of sodium phenylpyruvate to D-phenyllactic acid was successfully carried out using whole cells of the engineered *E. coli*. Under the optimized biocatalysis conditions, 50 g l^{-1} sodium phenylpyruvate was completely converted to D-phenyllactic acid with a space-time yield and enantiomeric excess of 262.8 g l^{-1} day^{-1} and > 99.5%, respectively. To our best knowledge, it is the highest productivity reported to date, with great potential for the mass production of D-phenyllactic acid.

Keywords D-Phenyllactic acid · Whole-cell transformation · Sodium phenylpyruvate · Asymmetric reduction · Lactate dehydrogenase

Introduction

Phenyllactic acid (PLA; 2-hydroxy-3-phenylpropanoic acid), is an organic acid that is widely distributed in honey and some fermented foods such as cheese, pickles, and milk (Mu et al. 2012a). It has an inhibitory effect against a wide range of fungi and bacteria by destroying the cell membrane (Lavermicocca et al. 2000; Lavermicocca and Valerio 2003; Dieuleveux et al. 1998a, b; Wang et al. 2018). Because the molecule contains a chiral carbon atom, PLA has two enantiomers, whereby D-PLA has shown better antimicrobial activity and gained more attention than L-PLA (Mu et al. 2012a; Valerio et al. 2004). Owing to its low molecular mass and high hydrophilicity, PLA can diffuse comparatively easily (Wang et al. 2016). Moreover, the excellent antimicrobial activity, good stability, board effective pH range, and good safety make D-PLA an ideal antiseptic agent, which is widely used in the food industry and as a green alternative to antibiotics added to livestock feed (Schnürer and Magnusson 2005; Wang et al. 2009, 2010). In addition, D-PLA can be used as a monomer for the synthesis of biocompatible and biodegradable poly-phenyllactic acid. Due to the presence of a bulky aromatic side chain, poly-phenyllactic acid displays enhanced toughness, improved thermostability, and outstanding ultraviolet absorption properties compared to poly lactic acid (Kawaguchi et al. 2014; Fujita et al. 2013; Kawaguchi et al. 2017). Furthermore, as chiral building blocks, D-PLA plays an important role in the synthesis of pharmaceuticals and fine chemicals such as englitazone, ragaglitazar, danshensu, and the anthelmintic agent PF1022A, among others (Urban and Moore 1992; Ebdrup et al. 2003; Xu et al. 2016; Weckwerth et al. 2000).

D-PLA is a versatile compound with promising applications, accompanied with great commercial demands, which inspired a growing research interest in D-PLA synthesis. Due to its mild reaction conditions, exquisite

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stereoselectivity, environmental friendliness, and energy-effective operation, biocatalytic asymmetric ketone reduction was identified as a reliable, scalable, and straightforward route to optically active alcohols (Wang et al. 2017a; Choudhury et al. 2014; He et al. 2014; Ni and Xu 2012). Natural microorganisms are low-cost and easily available biocatalysts, which are widely distributed in the environment. In recent years, numerous bacteria with the ability to produce D-PLA from phenylpyruvic acid have been identified such as *Lactobacillus plantarum* (Dallagnol et al. 2011; Zhang et al. 2014), *Lactobacillus pentosus* (Zhu et al. 2015), *Leuconostoc mesenteroides* (Li et al. 2014), *Pediococcus acidilactici* (Mu et al. 2012b), *Pediococcus pentosaceus* (Yu et al. 2015), and *Bacillus coagulans* (Zheng et al. 2011). However, the D-PLA yields obtained using these organisms were relatively low level, because of poor enzyme expression. Moreover, two PLA-producing enzymes with opposite stereoselectivity coexist in the cells, so that the optical purity of D-PLA produced by natural microorganisms was moderate to low. In the catabolism of lactic acid bacteria, PLA is produced from phenylpyruvic acid in a reaction catalyzed by lactate dehydrogenase (LDH) (Wang et al. 2016). *Escherichia coli* is a versatile host for heterologous protein expression and easy to manipulate. Using recombinant *E. coli* expressing a D-LDH encoding gene as biocatalyst can overcome the shortcomings of biotransformations catalyzed by natural microorganisms. The reductive activity of LDH depends on nicotinamide adenine dinucleotide (NADH), which acts as a hydride donor (Jia et al. 2018). Due to the high price of NADH, an efficient and economic internal cofactor recycling system is essential for large-scale synthetic applications (Wang et al. 2017b). Glucose dehydrogenase (GDH) is one of the enzymes most frequently used for cofactor regeneration due to its high activity, wide pH range, prominent stability, and tolerance of many organic solvents (Xu et al. 2007; Wichmann and Vasic-Racki 2005). In addition, GDH uses cheap glucose as hydride source and the byproduct gluconic acid does not interfere with the synthesis reaction (Zhou et al. 2015). As a result, numerous biocatalysis systems were constructed with cofactor regeneration based on GDH, which exhibited excellent catalytic efficiency. For example, Cui et al. (2018) achieved the efficient synthesis of methyl-(*R*)-3-hydroxybutyrate with a productivity of 265 g l^{-1} day^{-1} and enantiomeric excess above 99.9% using whole cells of engineered *E. coli* expressing carbonyl reductase and GDH for the in situ regeneration of the coenzyme. Tang et al. developed a coupled system consisting of carbonyl reductase and GDH and produced optically pure (*S*)-3-chloro-1-phenyl-1-propanol from 100 g l^{-1} 3-chloro-1-phenyl-1-propanone with 100% conversation within 12 h (Tang et al. 2018). Wang et al. coupled the mutational aldo–keto reductase *Km*AKR with GDH to catalyze the asymmetric reduction of *t*-butyl 6-cyano-(5*R*)-hydroxy-3-oxohexanoate and produced up to 237.4 mmol l^{-1} *t*-butyl 6-cyano-(3*R*,5*R*)-dihydroxyhexanoate with a productivity of up to 372.8 g l^{-1} day^{-1} (Wang et al. 2017c; Yu et al. 2019).

Screening new biocatalysts is an important task in biosynthesis. To achieve high productivity of optically pure D-PLA, we endeavored to discover novel and efficient D-LDH enzymes. In this work, a novel strain of *Lactobacillus rossiae* was isolated which exhibited the ability to reduce sodium phenylpyruvate (PPA) to D-PLA. Subsequently, a D-LDH encoding gene (*ldh*) was identified through shotgun library screening and co-expressed in *E. coli* together with glucose dehydrogenase from *Exiguobacterium sibiricum*. A whole cell biotransformation for D-PLA synthesis with in situ cofactor regeneration was implemented and the application potential was investigated.

Materials and methods

Reagents

Sodium phenylpyruvate (PPA), kanamycin, streptomycin, and the Plasmid Extraction Kit were purchased from Sangon Biotech (Shanghai, China). D- and L-phenyllactic acids (PLA) were from Sigma-Aldrich (St. Louis, MO, USA). Tryptone and yeast extract were obtained from Oxoid (United Kingdom). Protein size markers, 2 × Protein SDS-PAGE loading buffer and the Fast DNA SPIN Kit for Soil were purchased from TAKARA (Beijing, China). The DNA Gel Extraction Kit, PCR Cleanup Kit and Plasmid Miniprep Kit were purchased from Axygen (Suzhou, China). Restriction enzymes were from Thermo Fisher Scientific Co., Ltd (Beijing, China). Oligonucleotides synthesis and DNA sequencing were provided by Sangon Biotech (Shanghai, China). Unless otherwise specified, all other reagents and chemicals used in this study were obtained from general commercial suppliers and used without further purification.

Escherichia coli BL21 (DE3) and the plasmids pCDF-Duet-1 and pET28a used for recombinant protein expression were purchased from Novagen (Shanghai, China). The pMD-19-T vector for gene cloning was purchased from TAKARA (Beijing, China). *E. coli* BL21 (DE3)/pET-28b-*gdh* was stored in our laboratory.

LB medium (pH 7.0) contained 10.0 g l^{-1} tryptone, 5.0 g l^{-1} yeast extract and 10.0 g l^{-1} NaCl.

Cloning of *ldh* and the construction of *E. coli*-pET28a-*ldh*

The LDH gene (GenBank: KRL56345.1) with a length of 1014 bp was amplified by PCR from the genomic DNA of *L. rossiae* using the primers P1 and P2 listed in Table 1. PCR conditions were as follows: initial denaturation at 95 °C for

Table 1 Primers used in this work

Serial number	Primers	Restriction site
P1	CCATGGGCATGGAGGTGTCTGCATTGA	Nco I
P2	CTCGAGCTAGTTAAAGGCCACAACAT	Xho I
P3	AGATCTATGGAGGTGTCTGCATTGA	Bgl II
P4	GCGGCCGCCTAGTTAAAGGCCACAACAT	Not I
P5	CCATGGGCATGGGTTATAATTCTCTGAAAGG	Nco I
P6	GCGGCCGCTCAACCACGGCCAGCCT	Not I
P7	CATATGGGTTATAATTCTCTGAAAGG	Nde I
P8	CTCGAGTCAACCACGGCCAGCCT	Xho I

Sequences of restriction sites were underlined

5 min, followed by 30 amplification cycles (95 °C for 30 s, 56 °C for 30 s, and 72 °C for 1.5 min), and a final extension at 72 °C for 10 min. The amplified PCR product was ligated into the pMD-19-T vector, double digested with *Nco* I and *Xho* I, and then inserted into the expression vector pET28a. The resulting expression vector pET28a-*ldh* was used to transform the competent *E. coli* BL21 (DE3) and the positive clone was verified by DNA sequencing.

Construction of the GDH/LDH co-expression strains

For the *E. coli* BL21 (DE3)/pCDFDuet-1-*gdh-ldh* strain, the *ldh* was amplified by PCR from genomic DNA of *L. rossiae* using the primers P2 and P3 listed in Table 1. The *gdh* gene from *Exiguobacterium sibiricum* (GenBank: AIZ68241.1) was amplified by PCR from pET28a-*gdh* using the primers P5 and P6 listed in Table 1. The PCR used the same temperature program as above. The amplified PCR products were inserted into the multiple cloning sites (MCS 1 and MCS 2) of the pCDFDuet-1 vector between BglII and XhoI and NcoI and NotI recognition sites (Fig. 1a). The resulting recombinant co-expression plasmid pCDFDuet-1-*gdh-ldh* was used to transform the competent cells of *E. coli* BL21 (DE3) to produce *E. coli-gdh/ldh*.

For *E. coli* BL21 (DE3)/pCDFDuet-1-*ldh-gdh*, the *ldh* was amplified using the primers P1 and P4 and *gdh* was amplified using the primers P7 and P8 using the same PCR conditions as above. The amplified PCR products were inserted into pCDFDuet-1 vector between the NcoI and NotI, and NdeI and XhoI recognition sites (Fig. 1b). The resulting recombinant co-expression plasmid pCDFDuet-1-*ldh-gdh* was used to transform the competent cells of *E. coli* BL21 (DE3) to produce *E. coli-ldh-gdh*.

Cultivation of recombinant strains

For protein expression, *E. coli*/pET28a-*ldh*, *E. coli*/pET28a-*gdh*, *E. coli-gdh-ldh*, *E. coli-ldh-gdh* and *E. coli*/pCDFDuet-1 were incubated in LB medium containing 50 μg ml^{-1} kanamycin or streptomycin, at 37 °C and 200 rpm for 10 h. Then 4% (v/v) of the pre-culture was transferred to fresh, sterile LB medium containing 50 μg ml^{-1} kanamycin or streptomycin and cultivated under the same conditions. To initiate expression, lactose was added into the broth to a final concentration of 8 g l^{-1} when the culture reached an OD600 of 0.8. After induction at 28 °C and 150 rpm for 8 h, the cells were harvested by centrifugation at 8000×*g* for 10 min at 4 °C and washed twice with potassium phosphate buffer (100 mM, pH 7.0).

To examine the expression of heterologous proteins, cell pellets of the recombinant strains were suspended at a concentration of 20 g DCW l^{-1} in potassium phosphate

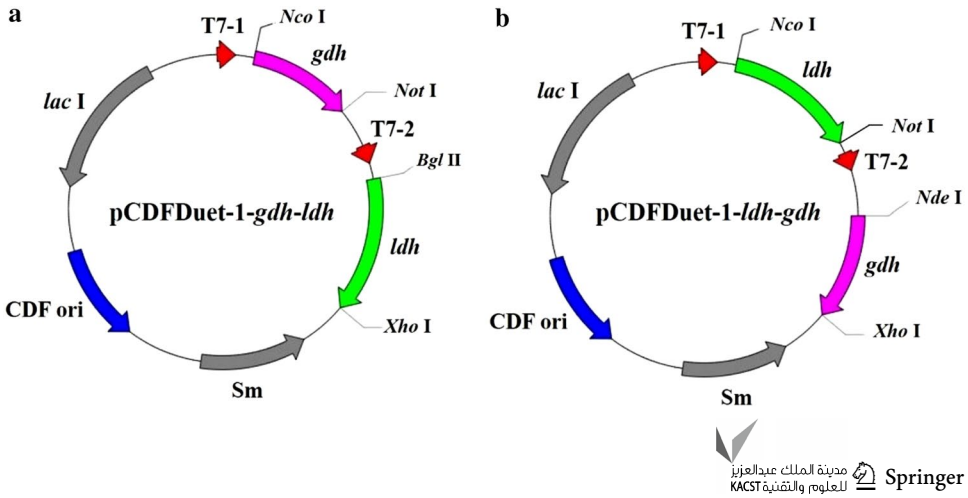


Fig. 1 Schematic presentation of the co-expression plasmids contained *ldh* and *gdh* genes. **a** Plasmids pCDFDuet-1-*gdh-ldh*. **b** Plasmids pCDFDuet-1-*ldh-gdh*

buffer (100 mM, pH 7.0) and disrupted by sonication. The cell debris was removed by centrifugation at 10,000×*g* for 20 min at 4 °C and the resulting supernatant was analyzed by SDS-PAGE with 12% separating gel under denaturing conditions. One unit of biocatalyst activity was defined as the amount of cells (measured by dry cell weight) required for the generation of 1 μmol PLA per minute.

The optimum expression conditions for *E. coli-gdh-ldh*, including inducer concentration and induction time, were determined. The optimum inducer concentration was examined by adding lactose at concentrations from 2 to 14 g l^{-1} and culturing at 28 °C for 10 h. The optimum induction time was examined by harvesting the induced cells after 2–16 h. The cells were harvested and the activity and biomass were measured.

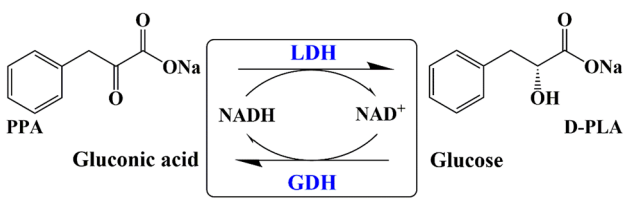
The catalytic efficiency of *E. coli*/pET28a-*ldh*, *E. coli-gdh-ldh,* and *E. coli-ldh-gdh* in the conversion of PPA into D-PLA was assayed by monitoring the generation of PLA using high-performance liquid chromatography (HPLC) as described below. The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.0), 10 g l^{-1} PPA, 10 g l^{-1} glucose, and 10 g DCW l^{-1} cells in a total volume of 10 ml and incubated at 30 °C and 200 rpm for 15 min.

Optimization of reaction conditions for D-PLA production

The basic conditions for bioconversion were as follows: cells were resuspended in 10 ml of 100 mM potassium phosphate buffer containing glucose and 10 g l^{-1} PPA, and then incubated at 200 rpm for 15 min at an appropriate temperature (Scheme 1). To optimize the bioconversion conditions, reaction temperature (25–45 °C), pH (6.0–8.0; 100 mM potassium phosphate buffer), glucose concentration (0–30 g l^{-1}), and whole-cell biocatalyst loading (5–30 g l^{-1}) were investigated. Samples were withdrawn and the concentration and enantiomeric excess (e.e.) of D-PLA were assayed by HPLC as described below.

Biotransformation by whole cells of *E. coli-gdh-ldh*

The biotransformation reactions were performed in 100 ml round bottom flasks with 50 ml of the reaction mixtures, which comprised potassium phosphate buffer (100 mM, pH 7.0), cells of *E. coli-gdh-ldh* at a total cell density of 15 g DCW l^{-1}, PPA loading at concentrations ranging from 30 to 60 g l^{-1} and a twofold molar excess of glucose, without external cofactor addition. The reaction mixtures were incubated at 35 °C under magnetic stirring at 300 rpm and the pH was automatically adjusted to 7.0 by titrating 1.0 M Na_2CO_3 solution. Samples were withdrawn every 20 min and the concentration and e.e. of D-PLA were assayed by HPLC as described below.



Scheme 1 Scheme for the asymmetric preparation of D-PLA from PPA

Analytical methods

The concentrations of PLA and PPA were determined by HPLC on an LC-20A system (Shimadzu, Japan) equipped with an ODS HYPERSIL column (4.6 × 250 mm, 5 μm, Thermo, USA) and an SPD-10A VP Plus ultraviolet detector (Shimadzu, Japan) set at a wavelength of 210 nm. The column temperature was maintained at 40 °C. The mobile phase consisted of 1:4 (v/v) acetonitrile and 0.1% formic acid in water (v/v) at a flow rate of 1.0 ml min^{-1}.

The optical purity of D-PLA was measured by HPLC on a CHIRALCEL OJ-RH column (4.6 × 150 mm, 5 μm; Daicel, Japan) maintained at 40 °C. The mobile phase was composed of acetonitrile, methanol, trifluoroacetic acid, and water at a volumetric ratio of 50:50:1.5:898.5 and run at a flow rate of 0.6 ml min^{-1}.

The extent of reaction, optical purity of D-PLA, and productivity were indicated by Conversion (%), enantiomeric excess (e.e., %) and space-time yield ($g_{D\text{-}PLA}$ l^{-1} day^{-1}), respectively, which were defined as follows:

$$\text{Conversion} = \frac{C_0 - C_s}{C_0} \times 100\% \quad (1)$$

$$\text{e.e.} = \frac{C_D - C_L}{C_D + C_L} \times 100\% \quad (2)$$

$$\text{space-time yield} = \frac{C_p}{t}, \quad (3)$$

where C_0 is the initial molar concentration of substrate (PPA), C_s is the final molar concentration of substrate (PPA), C_D and C_L are the final molar concentrations of D-PLA and L-PLA respectively, C_p is the final molar concentration of product (PLA), and t is the reaction time.

Results and discussion

Co-expression of *ldh* and *gdh* in *E. coli*

In this work, a D-lactate dehydrogenase gene was identified by searching the whole genome sequence of *L. rossiae* in the

NCBI database (https://www.ncbi.nlm.nih.gov/), and named as *ldh*. The coding sequence was amplified and inserted into the expressed vector pET-28a for heterologous expression in *E. coli*. The recombinant strain *E. coli*/pET28a-*ldh* displayed an excellent stereoselectivity, producing D-PLA with an e.e. > 99.5%. However, it had low catalytic efficiency and only 0.85 g l^{-1} D-PLA was generated within 15 min of reaction (Table 2). As LDHs are NADH-dependent oxidoreductases, cofactor recycling is one of the key limitations of biocatalytic D-PLA production. GDH is well known and frequently used for cofactor regeneration. Accordingly, whole cells co-expressing LDH along with GDH would be a preferable system for the cofactor recycling and D-PLA preparation. To establish a corresponding biocatalyst, the *ldh* from *L. rossiae* and *gdh* from *E. sibiricum* were amplified by PCR and cloned in two different relative positions into the pCDFDuet-1 vector with two MCS. The resulting recombinant plasmids pCDFDuet-1-*gdh-ldh* and pCDFDuet-1-*ldh-gdh* were subsequently used to transform to *E. coli* competent cells and the corresponding strains *E. coli-gdh-ldh* and *E. coli-ldh-gdh* were obtained. The strains were then induced with 8.0 g l^{-1} lactose at 28 °C for 8 h and harvested by centrifugation. Biotransformation reactions were performed using whole cells of *E. coli*/pET28a-*ldh*, *E. coli-gdh-ldh*, and *E. coli-ldh-gdh*, respectively. As indicated in Table 2, the D-PLA yields obtained using the two co-expression strains were much higher than that of *E. coli*/pET28a-*ldh*. It was therefore obvious that the coupling with *Es*GDH significantly improved the catalytic efficiency. Furthermore, the D-LDH titers produced by *E. coli-gdh-ldh* and *E. coli-ldh-gdh* within 15 min were 3.33 g l^{-1} and 2.47 g l^{-1}, respectively and the former strain exhibited a better catalytic activity.

The cells of the strains were resuspended and disrupted by ultrasonication and the cleared lysates were examined by SDS-PAGE. As shown in Fig. 2, both *E. coli-gdh-ldh* and *E. coli-ldh-gdh* showed soluble expression of LDH and GDH. By comparing the amounts of both enzymes, it was

Table 2 Catalytic efficiency of recombinant strains

Strain	D-PLA (g l^{-1})	e.e. (%)
E. coli/pET28a-*ldh*	0.85 ± 0.12	> 99.5
E. coli-gdh-ldh	3.33 ± 0.21	> 99.5
E. coli-ldh-gdh	2.47 ± 0.26	> 99.5

The cells were induced with 8 g l^{-1} lactose added when the culture reached an optical density of 0.8 at 600 nm at 28 °C and 150 rpm for 8 h. The reaction mixture was composed of 100 mM potassium phosphate buffer (pH 7.0), 10 g l^{-1} PPA, 10 g l^{-1} glucose, and 10 g DCW l^{-1} cells in a total volume of 10 ml and incubated at 30 °C and 200 rpm for 15 min. Samples were withdrawn and assayed by HPLC. All reactions were performed in triplicate and error bars represent the standard error of mean

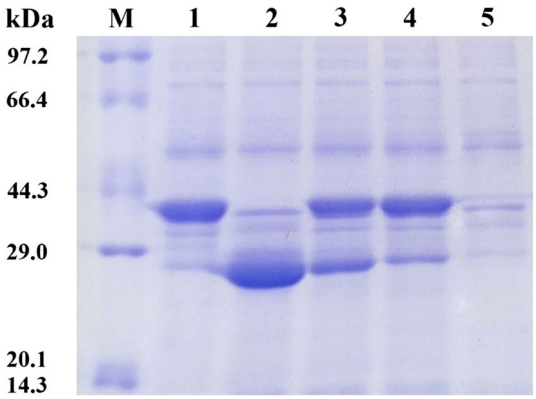


Fig. 2 SDS-PAGE analysis. M: protein molecular weight marker; Lane 1–5: soluble fractions from lactose induced *E. coli*/pET28*a-ldh*, *E. coli*/pET28*a-gdh*, *E. coli* BL21-*gdh-ldh*, *E. coli-ldh-gdh*, and *E. coli*/pCDFDuet-1 respectively, induced by 8.0 g l^{-1} lactose at 28 °C for 8 h

obviously that more GDH, but less LDH was expressed in *E. coli-gdh-ldh* than in *E. coli-ldh-gdh*. This result suggested that the order of the two genes in pCDFDuet-1 can

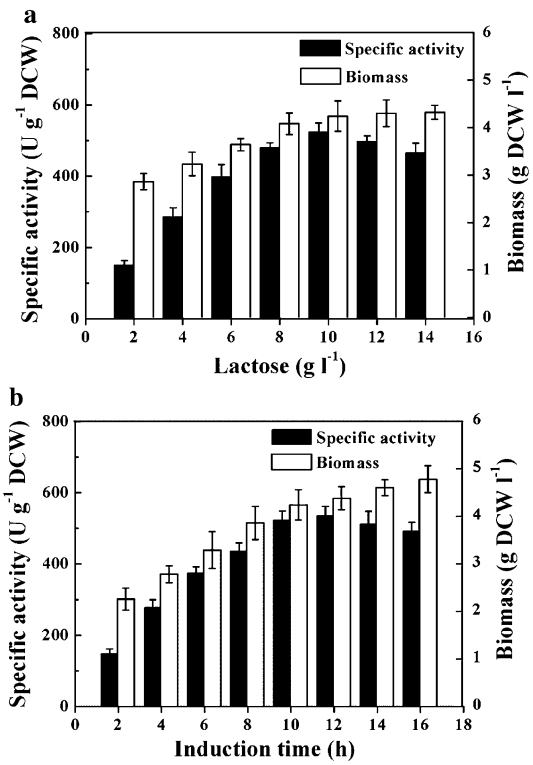


Fig. 3 Effect of induction conditions on specific activity and biomass. **a** Optimization of lactose concentration. Lactose were added as inducer at the range of 2–14 g l^{-1} and cultured at 28 °C for 10 h. **b** Optimization of induction time. The induction time was examined within a range of 2–16 h. All reactions were performed in triplicate, and error bars represent the standard error of mean

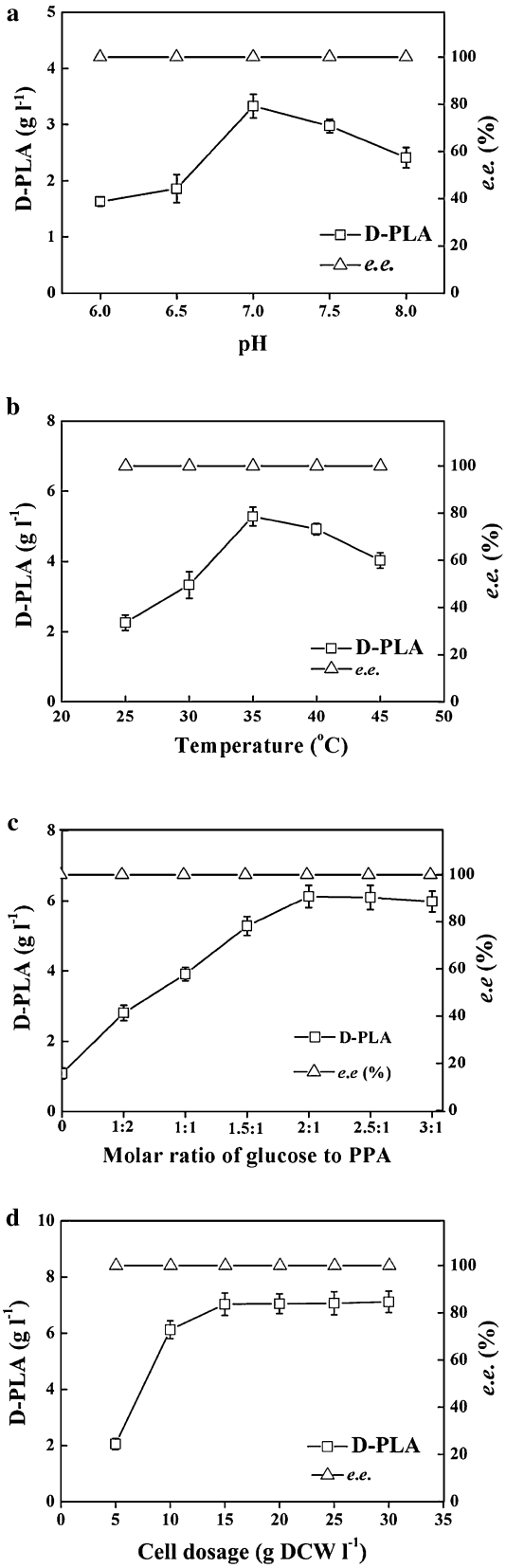


Fig. 4 Optimization of biocatalysis conditions. Effect of initial pH (**a**), conversion temperature (**b**), ratio of glucose to PPA (**c**) and cell dosage of *E. coli-gdh-ldh* (**d**) on D-PLA production. Samples were quantitatively analyzed after biocatalysis for 15 min. All reactions were performed in triplicate, and error bars represent the standard error of mean

significantly affect the expression levels of the enzymes. *E. coli-gdh-ldh* had better relative expression levels of the two enzymes, which led to a relatively high PPA reduction activity (Table 2).

Optimization of expression conditions for *E. coli-gdh-ldh*

To obtain the optimal protein expression conditions, the impact of lactose concentration and induction time on the specific biocatalyst activity and biomass of *E. coli-gdh-ldh* cells were studied. As shown in Fig. 3a, the specific activity increased at relatively low concentration of lactose and peaked at 10 g l^{-1}, reaching 523.4 U g^{-1} DCW. Lactose addition at the tested concentrations was clearly beneficial to cell proliferation. Even though the cells kept proliferating, the maximal total activity 2219.2 U l^{-1} was obtained following induction with 10 g l^{-1} lactose for 10 h. Since the total activity is the decisive factor affecting biocatalytic efficiency, lactose with a concentration of 10 g l^{-1} was subsequently used for the induction. The optimal induction time was evaluated in a time range of 2–16 h. As shown in Fig. 3b, the specific activity and biomass were time dependent. The maximum total activity of 2359.0 U l^{-1} was obtained at 14 h. Therefore, *E. coli-gdh-ldh* was induced with 10 g l^{-1} lactose and allowed to express the recombinant enzymes at 28 °C and 150 rpm for 14 h to produce the optimized whole-cell biocatalyst for the following experiments.

Optimization of biocatalysis conditions

E. coli-gdh-ldh cells were cultured, induced, and harvested as described above. To improve the efficiency of PLA production, the biocatalysis conditions, including reaction pH, temperature, molar ratio of glucose to PPA, and biocatalyst loading were investigated.

The reaction pH is a crucial parameter affecting enzyme activity and stability. Therefore, the effects of pH on PLA production from PPA using resting cells of *E. coli-gdh-ldh* were determined in a pH range from 6.0 to 8.0. As shown in Fig. 4a, the highest PLA production was achieved at pH 7.0, which indicated that neutral pH gives the best catalytic efficiency.

Temperature also plays an important role in biocatalytic reactions, because it affects the activity and stability of enzymes, as well as the solubility of substrates. Thus, the

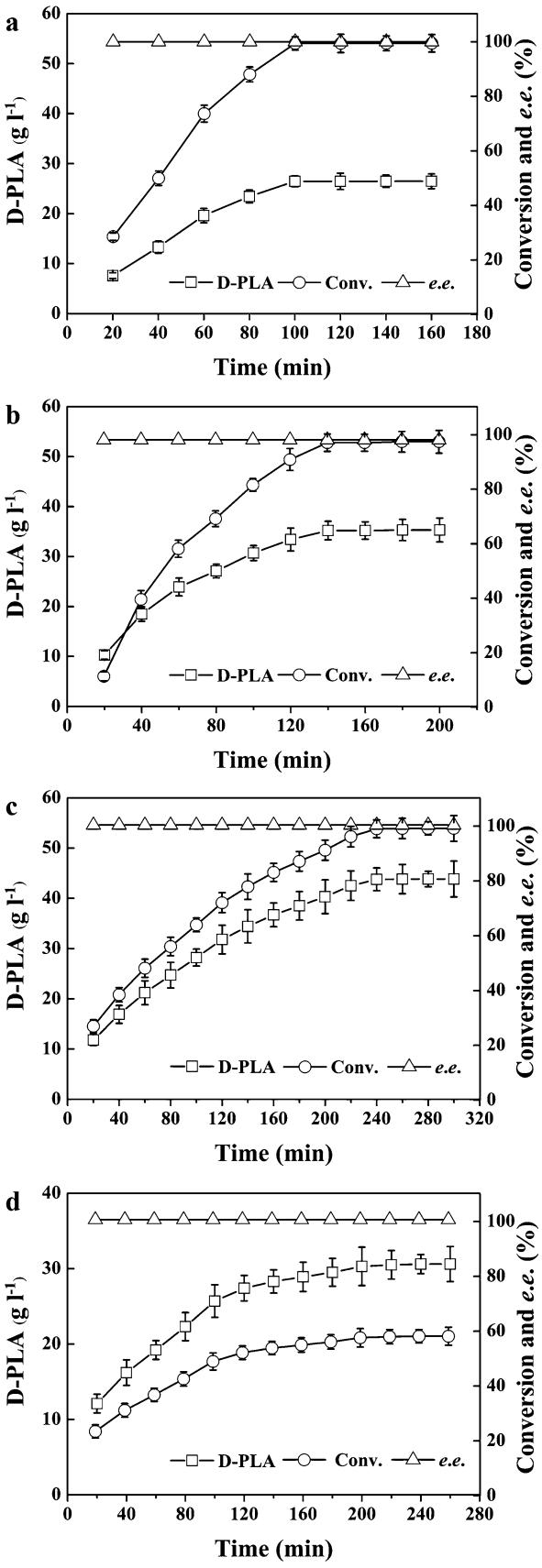


Fig. 5 Time course of D-PLA production from different concentration of PPA. The biotransformation reactions were carried out in 100 ml round bottom flasks with 50 ml of the reaction mixtures, which comprised potassium phosphate buffer (100 mM, pH7.0), cells of *E. coli-gdh-ldh* at a total cell density of 15 g DCW l^{-1}, PPA loading at concentrations of 30 (**a**), 40 (**b**), 50 (**c**) and 60 g l^{-1} (**d**), respectively, and 2-fold glucose. The resulting mixture was maintained at 35 °C, 300 rpm in magnetic stirring apparatuses without external cofactor added, and the pH was automatically adjusted to 7.0. Samples were withdrawn every 20 min and the concentration and e.e. of D-PLA were assayed by HPLC. All reactions were performed in triplicate, and error bars represent the standard error of mean

optimum temperature was examined in a range of 25–45 °C. The concentration of PLA rose, when the temperature was increased from 25 to 35 °C, but dropped sharply above 35 °C (Fig. 4b). The optimal temperature was therefore 35 °C.

Glucose was used as the substrate of GDH, which drives NADH regeneration. The effect of the molar ratio of glucose to PPA on PLA generation was also investigated as shown in Fig. 4c. At molar ratios below 2:1, the PLA production was significantly improved with increasing amounts of glucose added and reached the maximum value of 6.1 g l^{-1}. However, further increasing the glucose concentration led to a dramatic decrease of PLA generation, probably because the addition of glucose increased the viscosity of the reaction solution, reducing the mass transfer efficiency.

The effect of biocatalyst loading on PLA production was also investigated. At biocatalyst concentrations below 15 g DCW l^{-1} in the reaction mixture, the reaction was significantly enhanced with the increase of cell dosage. However, biocatalyst loading exceeded 15 g DCW l^{-1} had no apparent effect on the PLA production (Fig. 4d) indicating that the catalyst was already saturated. As a result, an optimum biocatalyst loading of 15 g DCW l^{-1} was used for the following experiments.

Asymmetric synthesis of PLA

Based on the results of single-factor optimization experiments, asymmetric reduction of PPA at a substrate concentration of 30–60 g l^{-1} was conducted and the time courses of the bioconversions are illustrated in Fig. 5. PPA was completely converted within 100 min at a substrate concentration of 30 g l^{-1}, 140 min at 40 g l^{-1}, and 240 min at 50 g l^{-1} (Fig. 5a–c). However, further increasing the PPA concentration to 60 g l^{-1} led to a sharp decrease of D-PLA yield, suggesting that excess PPA loading brought about a detrimental substrate inhibition effect on enzyme activity (Fig. 5d). Substrate loading of 50 g l^{-1} afforded the highest D-PLA yield of 43.8 g l^{-1} after 240 min of the biocatalytic reduction, with an e.e. and space-time yield of > 99.5% and 262.8 g l^{-1} day^{-1}, respectively. Table 3 summarizes the results and compares D-PLA production using different recombinant strains and processes reported in the literature.

Table 3 Comparison of various recombinant strains for the preparation of D-PLA from phenylpyruvic acid or sodium phenylpyruvate

Strain	D-LDH source	Process	D-PLA (g l^{-1})	Productivity (g l^{-1} day^{-1})	References
E. coli/pET28-*gdh*-T7-*ppr*	*Lactobacillus* sp.	Batch	91.1	243.0	Xu et al. (2016)
E. coli/pET28-*dldh*744^{M307L}	*S. inulinus*[a]	Fed-batch	21.4	37.9	Wang et al. (2016)
E. coli/pET28-*ldh*^{Y52V}	*L. pentosus*	Fed-batch	15.6	124.8	Zhu et al. (2015)
E. coli/pETDuet-*ldhd*^{Y52L}-*fdh*	*L. bulgaricus*	Batch	8.2	131.7	Zheng et al. (2013)
E. coli/pET-28a-*d-ldh*^{Y52V}-*fdh*	*L. pentosus*	Batch	17.5	83.7	Zhu et al. (2017)
E. coli/*pET28-gdh-ldh*	*L. rossiae*	Batch	43.8	262.8	This work

[a]*Sporolactobacillus inulinus*

It is obvious that the batch catalytic process using resting cells co-expressing GDH and LDH in this work afforded the highest space-time yield for the production optically pure D-PLA.

Conclusions

The *E. coli* strain overexpressing only the NADH-dependent LDH catalyzes the asymmetric reduction of PPA to D-PLA with low efficiency. To improve the biotransformation efficiency, the cofactor regeneration enzyme GDH was introduced and co-expressed with LDH. Among the two recombinant strains with the two enzymes in different MCSs of the vector, *E. coli-gdh-ldh* showed a higher PPA reduction activity. After optimizing the expression and reaction conditions, asymmetric biocatalytic reduction of PPA for the production of D-PLA was performed with in situ NADH regeneration using the GDH intrinsic in the whole-cell biocatalyst and glucose as a co-substrate, which completely converted 50 g l^{-1} PPA, yielding D-PLA with a space-time yield of 262.8 g l^{-1} day^{-1} and e.e. of over 99.5%. This work provides a valuable whole-cell biocatalysis method for efficient D-PLA synthesis with great potential for industrial application.

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Compliance with ethical standards

Conflict of interest On behalf of all the authors, the corresponding author states that there is no conflict of interest.

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