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Article

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Biosynthesis of Phenylglyoxylic Acid by LhDMDH, a Novel *D*-Mandelate Dehydrogenase with High Catalytic Activity

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ABSTRACT

D-mandelate dehydrogenase (DMDH) has the potential of converting *D*-mandelic acid to phenylglyoxylic acid (PGA), which is a key building block in the field of chemical synthesis and is widely used to synthesize pharmaceutical intermediates or food additives. A novel NAD^+-dependent *D*-mandelate dehydrogenase was cloned from *Lactobacillus harbinensi* (LhDMDH) by genome mining and expressed in *Escherichia coli* BL21. After being purified to homogeneity, the oxidation activity of LhDMDH toward *D*-mandelic acid was approximately 1200 $U \cdot mg^{-1}$, which was close to four times the activity of the probe. Meanwhile, the k_{cat}/K_m value of LhDMDH was 28.80 $S^{-1} \cdot mM^{-1}$, which was distinctly higher than the probe. By co-culturing two *E. coli* strains respectively expressing *LhDMDH* and *LcLDH*, we developed a system for the efficient synthesis of PGA, achieving a 60% theoretical yield and 99% purity without adding coenzyme or co-substrate. Our data supports the implementation of a promising strategy for the chiral resolution of racemic mandelic acid and the biosynthesis of PGA.

KEYWORDS: mandelate dehydrogenase, biosynthesis, phenylglyoxylic acid, whole cell catalysis, green chemistry

INTRODUCTION

Phenylglyoxylic acid (PGA) plays an important role as a building block in the field of chemical synthesis and is widely used to synthesize pharmaceutical intermediates or food additives.[1, 2] Compared with chemical preparation via dehydrogenation of mandelic acid, hydrolysis of benzoyl cyanide, or oxidation of styrene,[2, 3] the microbial synthesis of PGA from mandelic acid provides an eco-friendly approach to industrial-scale production,[2, 4] which addresses the concerns about the environmental aspects of chemical manufacturing. Enantioselective conversion of D-mandelic acid to PGA has been reported in *Alcaligenes bronchisepticus*, *Pseudomonas polycolor*, *Gibberella fujikuroi*, and *Pseudomonas putida*.[5, 6] *D*-mandelate dehydrogenase (DMDH), a member of the ketopantoate reductase family, has much potential for use in the conversion D-mandelic acid to PGA.[3, 6, 7] It also has tremendous potential in the biosynthesis of enantiopure mandelic acids, which are also important intermediates for synthesis of pharmaceutical and natural products.[8, 9] The heterologous expression of DMDH genes from *Lactobacillus brevis*[7], *Pseudomonas putida*[6] and *Pseudomonas aeruginosa*[3] in *E. coli* was capable of oxidizing mandelic acid into PGA. However, the poor activity of these recombinant mandelate dehydrogenases impedes their industrial application. Genome mining in microbial sequences could be an economical strategy to identify novel mandelate dehydrogenases with higher catalytic activity.[10, 11] In addition, recombinant DNA techniques can be used to overproduce a desired enzyme in microorganisms.

Based on the techno-economic impact analysis, NAD^+ has to be regenerated from NADH for oxidation reactions with dehydrogenases.[12] To address this problem, the systems

alcohol dehydrogenase (ADH)/ acetone or acetaldehyde,[13] lactate dehydrogenase (LDH)/pyruvate,[14] and NADH oxidase (NOX)/O_2[12] are now used universally. Theoretically, such a system could also be applied to the biosynthesis of PGA by coupled enzymatic catalysis, as shown in **Scheme 1**. However, large amounts of these co-substrates are needed in the coupled enzymatic catalysis, which can impede their industrial application and make these methods inefficient and expensive. In addition, complex processes for enzyme separation can be omitted by utilizing microbial whole cells as a biocatalyst, in addition to the supplementary addition of cofactor regeneration enzymes.[15, 16] Therefore, whole-cell catalysis is recognized as a most promising approach for the applied biosynthesis of PGA.

In this work, we identified a novel NAD^+-dependent *D*-mandelate dehydrogenase (LhDMDH) from *L. harbinensis* and successfully expressed it in *E. coli*. The purified recombinant LhDMDH exhibits higher catalytic activity and better kinetic properties than the probe. Furthermore, we developed a co-culture system consisting of two recombinant *E. coli* strains, which can efficiently biosynthesize PGA without adding coenzyme or co-substrate. Taken together, our results provide an alternative strategy for the chiral resolution of racemic mandelic acid and the biosynthesis of PGA.

MATERIALS AND METHODS

Reagents and Kits

PrimeSTAR® HS (Premix), DNA Marker Restriction enzymes and DNA Ligation Kit Ver.2.1 were purchased from TaKaRa (Dalian, China). *Bam*H I and *Xho* I were purchased from New England Biolabs, Inc. (Ipswich, MA, England). D-mandelic acid, L-mandelic acid, D-2-chloromandelic acid, L-2-chloromandelic acid, L-lactic acid and PGA were

purchased from Aladdin (Shanghai, China). NAD^+, $NADP^+$, NADH and NADPH were purchased from Bontac-bio (Shenzhen, China). BCA-200 protein assay kit, IPTG and protein markers were purchased from Sangon (Shanghai, China). Axygen® AxyPrep™ PCR Clean-Up Kit, Axygen® AxyPrep™ DNA Gel Extraction Kit and Axygen® AxyPrep™ Plasmid Miniprep Kit were purchased from Corning (New York, United States). EasyPure Bacteria Genomic DNA Kit was purchased from TransGen Biotech (Beijing, China). All other chemicals were of analytical grade and purchased from Solarbio (Beijing China).

Strains, Plasmids and Culture Media

E. coli BL21 and *E. coli* DH5α/pET28a, preserved by our group, were cultured in LB medium containing 10 $g \cdot L^{-1}$ tryptone, 5 $g \cdot L^{-1}$ yeast extract and 10 $g \cdot L^{-1}$ NaCl, pH 7.2. *Lactococcus lactis* CICC 20209 and *Leuconostoc citreum* CICC 23234, purchased from China Center of Industrial Culture Collection (Beijing, China), were used for genomic DNA extraction. *Lactococcus lactis* CICC 20209 was cultured in skim milk medium containing 120 $g \cdot L^{-1}$ skim milk powder with natural pH. *Leuconostoc citreum* CICC 23234 was cultured in MRS medium containing 10 $g \cdot L^{-1}$ tryptone, 10 $g \cdot L^{-1}$ beef extract, 5 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ sodium acetate, 2 $g \cdot L^{-1}$ ammonium citrate, 1 $g \cdot L^{-1}$ Tween 80, 2 $g \cdot L^{-1}$ K_2HPO_4, 0.2 $g \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 0.05 $g \cdot L^{-1}$ $MnSO_4 \cdot H_2O$, 20 $g \cdot L^{-1}$ $CaCO_3$, pH 6.8. *Klebsiella oxytoca* (gifted by Prof. Jian-Ping Wu) and *Staphylococcus aureus* (gifted by the group of Hong-Fei Shi) were cultured in LB medium and used for genomic DNA extraction.

Genome Mining for Putative *D*-mandelate Dehydrogenases

LbDMDH, a NAD^+-dependent *D*-mandelate dehydrogenase with high activity encoded by *Lactobacillus brevis* (GenBank accession no: WP_011668914.1),[7] was used as probe for the BLAST search in the NCBI database (http://www.ncbi.nlm.nih.gov) for 2-dehydropantoate 2-reductase and potential novel *D*-mandelate dehydrogenases, which was mainly from a bacterial genome and an uncharacterized sequence. The phylogenetic tree of the potential novel *D*-mandelate dehydrogenases was constructed using both the ClustalX2 program and MEGA 6.0 software. Based on the phylogenetic analysis, ten potential novel *D*-mandelate dehydrogenases with 40–70% identical amino acid sequences to LbDMDH were selected.

Gene Cloning and Expression of Selected *D*-mandelate Dehydrogenases

Based on the results of genome mining, a series of paired primers shown in **Table S1** were designed and synthesized by Synbio Technologies Genes for Life (Suzhou, Jiangsu, China), and used to amplify the gene fragments of potential novel *D*-mandelate dehydrogenases. The other potential *D*-mandelate dehydrogenase genes from unobtainable or pathogenic microorganisms were codon optimized and artificially synthesized by Synbio Technologies Genes for Life. The original strains of potential *D*-mandelate dehydrogenases were cultivated in the abovementioned media at 37°C for 14 h on a rotary incubator at 220 rpm and harvested through high speed centrifugation. The collected microbial cells were thoroughly washed with sterile deionized water. The genomic DNA was extracted from the collected microbial cells using the EasyPure Bacteria Genomic DNA Kit according to the manufacturer's instructions. Using these genomic DNA as template, the gene fragments of potential *D*-mandelate dehydrogenases were amplified with the above primers. The amplified and artificially synthesized genes were cloned into pET28a, and transformed into

E. coli BL21. The recombinant transformants were confirmed by DNA sequencing and selected for culturing.

The selected recombinant transformants were cultured for 14 h in tubes containing 4 mL LB medium with 50 μg mL^{-1} kanamycin at 37°C and 200 rpm, and then the cultures were transferred into flasks containing 100 mL fresh LB medium containing 50 μg mL^{-1} kanamycin. After cultivation at 37°C for 2.5 h, IPTG was added to a final concentration of 0.1 mM, and induction was allowed to proceed for 20 h at 16°C. The induced cells were harvested by centrifugation and lysed by ultrasonication, and the cell lysate was centrifuged at 10000 ×g and 4°C for 30 min. The supernatant was then purified to homogeneity by Ni-chelating affinity chromatography. [17]

Enzyme Activity and Protein Assays

The dehydrogenase activity of *D*-mandelate dehydrogenase on D-mandelic acid was assayed by coupling the increase of NADH.[6, 7] The reaction mixture contained 100 mM glycine-NaOH buffer, pH 9.5, 1.0 mM NAD^+ and 6.0 mM substrate. The oxidation activity was assayed spectrophotometrically at 30°C by monitoring the increase of NADH absorbance at 340 nm. The molar absorption coefficient of NADH at 340 nm is 6.22×10^3 M^{-1} cm^{-1}.[6, 18] The reduction activity of *L*-lactate dehydrogenase on sodium pyruvate was assayed by coupling the decrease of NADH.[15] One unit of enzyme activity was defined as the amount of enzyme reducing or oxidizing 1 μmol NADH per minute under the above reaction conditions.[7] The protein concentration was measured with the BCA-200 Protein Assay Kit (Pierce, Rockford, IL), using bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a

12.5% gel using the reported method,[19] and the isolated protein bands were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA). The apparent molecular weights were estimated using Quantity One software based on the standard marker proteins.[10]

Biochemical Characterization of Purified Recombinant Enzymes

The temperature optima of these recombinant *D*-mandelate dehydrogenases were determined under the standard assay conditions described above, except for temperatures ranging from 20 to 60°C. To estimate their thermostability, these recombinant *D*-mandelate dehydrogenases were incubated at pH 9.0 and various temperatures (20–50°C) for 1.0 h, and then, the residual enzyme activity was measured under the optimal reaction temperatures. Here, thermostability was defined as a temperature, at or below which the residual activity retained more than 85% of its original activity. The pH optima of these recombinant *D*-mandelate dehydrogenases were assayed by the standard activity assay method as stated above with 100 mM glycine-NaOH buffer over the pH range of 7.0−11.0. To estimate the pH stability, aliquots of these recombinant DMDHs were preincubated at 0°C for 1.0 h in varied pH values from 7.0 to 11.0, and the residual activities were assayed under the optimal reaction temperatures and pH values. The pH stability, in this work, was defined as the pH range over which the residual activities were more than 85% of the original activity.

The kinetic parameters of the purified *D*-mandelate dehydrogenases were determined by assaying (in triplicate) the activities on various substrate concentrations (1–12 mM) at a fixed NAD^+ concentration of 2 mM. On the other hand, the kinetic parameters of the purified dehydrogenase were determined by assaying (in triplicate) their activities on

various NAD^+ concentrations (0.2–1.2 mM) at a fixed substrate concentration of 10 mM. The correlation curve of reaction rate to substrate or NAD^+ concentration was plotted and analyzed using nonlinear fitting by the Origin 9.0 software.

The substrate specificities of the purified *D*-mandelate dehydrogenases were determined by measuring the enzyme activities, as described above, toward different substrates, including D, L-mandelic acid, L-mandelic acid, D-2-chloromandelic acid, L-2-chloromandelic acid and L-lactic acid.

Analysis of PGA

PGA was dissolved in deionized water at concentrations ranging from 0.5 to 2.5 mM, and determined using an HPLC system equipped with a Thermo Hypersil C18 column (250 mm × 4.6 mm, 5 μm, ThermoFisher Scientific), and a UV detector at 215 nm. The mobile phase contained 20% methanol and 80% ddH_2O, and the flow rate was 1.0 mL min^{-1}. Retention time of PGA was 3.1 min. The correlation curve of peak area to PGA concentration was plotted and analyzed using Origin 9.0, which provided one linear regression equation. Meanwhile, PGA was dissolved in deuterium dimethylsulfoxide at a concentration of 1 mM and fully characterized by ^1H NMR spectroscopy at 400 MHz.[20]

'One□pot' Bioconversion of D-mandelic acid by Recombinant *E. coli* Co-culture

To enhance the capacity of NAD^+ regeneration, another recombinant *E. coli* strain containing the gene for LcLDH, an efficient *L*-lactate dehydrogenase (GenBank accession no. OJF74586) from *Lactobacillus casei*,[21] was constructed and named as *E. coli* BL21/pET28a-*LcLDH*. These recombinant *E. coli* strains were co-cultured and induced as described above. Then, D-mandelic acid was added to a final concentration of 50 mM, and

transformed for 48 h at 30°C. During this period, 100 μL samples were removed for HPLC analysis every 4 h. The *E. coli* BL21/pET28a and *E. coli* BL21/pET28a-*LhDMDH* were used as control respectively.

'One□pot' Bioconversion of D,L-mandelic acid

To evaluate the application potential of LhDMDH in the chiral resolution of racemic mandelic acid, these recombinant *E. coli* strains were co-cultured and induced as described above. Then, D,L-mandelic acid was added to a final concentration of 50 mM, and transformed for 48 h at 30°C. During this period, 100 μL samples were removed for HPLC analysis every 4 h.

Purification and Identification of Biotransformation Products

After the catalytic reaction was terminated, the resulting solution was centrifuged at 8228 ×g for 10 min to remove the cells, and the supernatant was extracted three times by isovolumetric acetate. The extracts were combined and vacuum evaporated to obtain the crude products. The crude products were purified to homogeneity by silica gel filtration chromatography, [9] and the yield was determined by HPLC analysis as described above. The product was fully characterized by ^{1}H NMR spectroscopy at 400 MHz.

RESULTS AND DISCUSSION

Gene Cloning and Expression of *D*-mandelate Dehydrogenases

To search for an efficient *D*-mandelate dehydrogenase for the biosynthesis of PGA, a genome mining strategy was adopted. Four gene fragments of potentially novel *D*-mandelate dehydrogenases were amplified, as shown in **Figure S1**, and four gene fragments were artificially synthesized. Eight potential NAD^{+}-dependent mandelate

dehydrogenases were cloned and heterogeneously over-expressed in *E. coli* BL21 and SDS-PAGE analysis of four representative recombinant dehydrogenases are shown in **Figure 1**. Interestingly, several of them displayed oxidation activity toward D-mandelic acid. Among these, a putative 2-dehydropantoate 2-reductase (GenBank accession no: WP_027828400.1) from *L. harbinensis*, herein designated LhDMDH, was confirmed with the highest oxidation activity (230 $U \cdot mL^{-1}$ fermentation liquor on *D*-mandelic acid), which was close to ten times that of the probe.[7] Sequence alignment revealed a relatively low (<55%) amino acid sequence identity with the probe **(Figure S2)**, indicating that it is a novel *D*-mandelate dehydrogenase. The highest sequence identity (39.9%) found using BLASTp in the protein database of NCBI was with a putative 2-dehydropantoate 2-reductase (PDB accession no. 2EW2) from *Enterococcus faecalis* V583, belonging to the ketopantoate reductase family. [7]

To characterize their enzymatic properties, the recombinant *D*-mandelate dehydrogenases were purified through Ni-NTA affinity chromatography. As shown in **Figure 1**, they were purified to homogeneity, seen as a single band on an SDS-PAGE gel, corresponding to an apparent molecular weight of 38.0 kDa. The specific oxidation activity of purified LhDMDH was 1200 $U \cdot mg^{-1}$, which was close to four times that of the probe[7] and significantly higher than the other reported *D*-mandelate dehydrogenases.[3, 6, 22] The specific oxidation activity of LcDMDH, a *D*-mandelate dehydrogenase cloned from *Leuconostoc citreum*, was 40.6 $U \cdot mg^{-1}$. On the other hand, the specific oxidation activity of LlDMDH-2, a *D*-mandelate dehydrogenase cloned from *Lactococcus lactis*, was 11.7 $U \cdot mg^{-1}$. Among the purified, recombinant *D*-mandelate dehydrogenases, no activity was

234 detected with $NADP^+$ as cofactor, indicating that LhDMDH is an NAD^+-dependent
235 *D*-mandelate dehydrogenase.

236 **Comparative Enzymatic Properties of *D*-mandelate Dehydrogenases**

237 The enzymatic properties of purified recombinant *D*-mandelate dehydrogenases were
238 investigated using D-mandelic acid as the model substrate. The temperature properties of
239 these purified *D*-mandelate dehydrogenases are shown in **Figure 2**, which shows higher
240 catalytic activity at a temperature range of 25-30°C. The temperature optimum of LhDMDH
241 and probe was 30°C, while the other was 25°C **(Figure 2a)**. All of the purified *D*-mandelate
242 dehydrogenases were highly stable at 20°C after being incubated for 1 h, and they retained
243 levels of residual activities at 80% or above **(Figure 2b)**. Unfortunately, the residual activity
244 of LhDMDH was less than 50% after being incubated at 30°C for 1 h, and its low
245 thermostability would be impeded during biosynthesis of PGA. Hence, whole cell catalysis
246 is a preferred option to eliminate the influence of low thermostability, in the biosynthesis of
247 PGA.[16, 23] It should be noted that with the emergence of various bioinformatics software, *in*
248 *silico* design is playing a more significant role in molecular modification of thermostability.
249 In particular, molecular dynamics simulation has become a more mature tool [24]. Our future
250 research will focus on the use of *in silico* design to modify LhDMDH at the molecular level
251 to improve its thermostability.

252 The pH properties of the purified *D*-mandelate dehydrogenases are shown in **Figure 3**.
253 As shown in **Figure 3a**, all of them are alkalophilic dehydrogenases, and the pH optimum of
254 LhDMDH was 9.5. Except for LlDMDH-2, they were highly stable at pH values ranging
255 from 9.5 to 10.5 retaining more than 80% of their original activity (**Figure 3b**). The

LlDMDH-2 was stable only within a narrow pH range, and its residual activity declined markedly at pH values less than 8.5 or more than 9.5.

The kinetic parameters of the purified *D*-mandelate dehydrogenases toward D-mandelic acid and NAD^+ were determined and summarized in **Table 1** and **Table 2**, respectively. The K_m value of LhDMDH toward D-mandelic acid was 1.05 mM, which was distinctly lower than that of the probe, indicating that LhDMDH had a higher a□nity for *D*-mandelic acid.[7] In addition, the k_{cat}/K_m value was 28.80 $S^{-1}\cdot mM^{-1}$, which was distinctly higher than the one of probe, which further indicates the advantage of using LhDMDH in the biosynthesis of PGA.

The substrate specificity of the purified *D*-mandelate dehydrogenases were determined and summarized in **Table 3**. They all clearly displayed the greatest oxidation activity toward D-mandelic acid, and only extremely low activity was detected toward D-2-chloromandelic acid. In addition, nearly no activity was detected toward L-mandelic acid, L-2-chloromandelic acid and L-lactic acid, indicating that these *D*-mandelate dehydrogenases could be applied to chirally resolve racemic mandelic acid during the biosynthesis of PGA. Furthermore, the absence of activity toward L-lactic acid is beneficial to NAD^+ regeneration with the aid of an efficient *L*-lactate dehydrogenase.[14, 15]

'One□pot' Biosynthesis of PGA by *E. coli-E. coli* Co□cultures

Biosynthesis at the industrial scale will benefit from reducing the amount of coenzyme or co-substrate used and from enhancing the regeneration of NAD^+. Therefore, we investigated the feasibility of the biosynthesis of PGA in a 'one-pot' fermentation system by co-culturing the two strains *E. coli* BL21/pET28a-*LhDMDH* and *E. coli*

BL21/pET28a-*LcLDH*. In this co-culture, *E. coli* BL21/pET28a- *LhDMDH* is dedicated to the conversion of D-mandelic acid into PGA and *E. coli* BL21/pET28a- *LcLDH* is dedicated to enhance the regeneration of NAD^+ by utilizing the pyruvic acid generated by itself. To detect the expression level of the co-culture, SDS-PAGE and enzyme activities analysis were carried out. As shown in **Figure 4**, SDS-PAGE analysis of the co-cultured bacteria found two specific bands with apparent molecular weights of 38.0 and 40 kDa, which indicates that LhDMDH and LcLDH were successfully expressed in the process of co-culturing. In addition, the oxidation activity toward D-mandelic acid of the co-cultured bacteria was 190 $U \cdot mL^{-1}$ fermentation liquor, which showed a slight decline compared with single culture. Meanwhile, the co-cultured bacteria's reduction activity toward sodium pyruvate was 210 $U \cdot mL^{-1}$ fermentation liquor, which further indicates that LhDMDH and LcLDH were successfully expressed in activated states.

D-mandelic acid was bioconverted in 48 h by the co-culture, and the product was purified to chromatographically pure homogeneity (**Figure S3a**), which means that purity was over 99%. As shown in **Figure S3,** the retention time of the purified product was 3.1 min, which was in accordance with the retention time of PGA (**Figure S3b**). This result, combined with the ^1H NMR spectra of D-mandelic acid (**Figure S4**) and the product (**Figure S5**), sufficiently demonstrates that the D-mandelic acid had been converted to PGA by co-culturing the two strains *E. coli* BL21/pET28a-*LhDMDH* and *E. coli* BL21/pET28a-*LcLDH*. These results also indicate that the PGA was successfully synthesized without adding coenzyme or co-substrate, implying that the synthesis cost of

299 PGA can be significantly reduced by this strategy, thus fully demonstrating the advantage of
300 whole cell catalysis. [16, 25, 26]

301 To fully assess the productivity of *E. coli*-*E. coli* co-cultures in comparison with *E. coli*
302 BL21/pET28a-*LhDMDH* or *E. coli* BL21/pET28a single culture for PGA synthesis, three
303 different culture modes were performed, and their yields measured. As shown in **Figure 5**,
304 after co□culturing for 48 h, the yield of PGA was over 60%, which was significantly higher
305 by approximately 20% than the yields of the single cultures. This improvement is attributed
306 to that the enhanced the capacity of NAD^+ regeneration by utilizing the pyruvic acid
307 generated within the co□cultured *E. coli*/pET28a-*LcLDH*.[14] Moreover, this result also
308 indicates that the NAD^+ and NADH can be transmitted between cells. Unfortunately, the
309 yield of PGA by co-culture could not be sustained after 48 h under these culture conditions.
310 These conditions may restrict the regeneration or transmission capacity of NAD^+, thus the
311 next research priority will be optimizing the culture conditions or co-expressing the
312 LhDMDH and LcLDH. In addition, the strategy for L-phenylglycine biosynthesis from PGA
313 by co-expressing leucine dehydrogenase could be used to improve the capacity to regenerate
314 NAD^+ and synthesize the derivatives of PGA [7, 27].

315 We also used the co-culture system to transform racemic mandelic acid into PGA. As
316 shown in **Figure 6**, after co□culturing for 48 h, the yield of PGA was approximately 40%
317 toward 50 mM D, L-mandelic acid, which is close to the theoretical yield of 50%. Therefore,
318 the LhDMDH has tremendous application potential in the chiral resolution of racemic
319 mandelic acid and the biosynthesis of PGA, which is an important intermediate for the
320 synthesis of pharmaceutical and natural products.[8, 9]

In conclusion, a novel D-mandelate dehydrogenase (LhDMDH) from *L. harbinensis* was successfully mined and expressed in *E. coli* BL21. The LhDMDH displayed high catalytic activity and excellent kinetic parameters. Interestingly, the LhDMDH had no activity toward L-lactic acid and L-mandelic acid which, with the aid of an efficient *L*-lactate dehydrogenase, is suitable for NAD^+ regeneration and in the chiral resolution of racemic mandelic acid and the biosynthesis of PGA. We successfully biosynthesized PGA by co-culturing two recombinant *E. coli* strains without adding coenzyme or co-substrate. All of these results establish a solid foundation for the chiral resolution of racemic mandelic acid and the biosynthesis of PGA.

ASSOCIATED CONTENT

Supporting Information

Additional experimental results and other data are available free of charge via the Internet at http://pubs.acs.org. These include: sequences of the primers used for cloning and expression of novel *D*-mandelate dehydrogenases (Table S1); the PCR amplification for the four *D*-mandelate dehydrogenase genes (Figure S1); multi-sequence alignment of the representative *D*-mandelate dehydrogenases (Figure S2); the HPLC analysis spectrum of the product (Figure S3); the ^{1}H NMR analysis spectra of the D-mandelic acid (Figure S4) and product (Figure S5).

AUTHOR INFORMATION

Authors' Contributions

Cun-Duo Tang, Hong-Ling Shi and the first author contributed equally to this work.

Notes

The authors declare that they have no competing interests.

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445 **Scheme and Figure Legends**

446 **Scheme 1**. **Coupled enzymatic synthesis of PGA from D-mandelic acid.**

447

448 **Figure 1. The SDS-PAGE analysis for the representative recombinant *D*-mandelate**
449 **dehydrogenases.** Lane M, PageRuler Prestained Protein Ladder; lane 1, expressed products
450 of *E. coli*/pET28a; lane 2, crude LbDMDH; lane 3, purified LbDMDH; lane 4, crude
451 LhDMDH; lane 5, purified LhDMDH; lane 6, crude LcDMDH; lane 7, purified LcDMDH;
452 lane 8, crude LlDMDH-2; lane 9, purified LlDMDH-2.

453

454 **Figure 2. The temperature properties of the representative recombinant *D*-mandelate**
455 **dehydrogenases.** (**a)** temperature optima of the representative recombinant *D*-mandelate
456 dehydrogenases. The temperature optima were assayed spectrophotometrically by
457 monitoring the increase of NADH absorbance at 340 nm at reaction temperatures ranging
458 from 20 to 60°C. (**b)** temperature stabilities of the representative recombinant *D*-mandelate
459 dehydrogenases. The temperature stabilities were determined by incubating them at various
460 temperatures (20-50°C) for 1.0 h, and then the residual enzyme activities were measured as
461 describe in the text.

462

463 **Figure 3. The pH properties of the representative recombinant *D*-mandelate**
464 **dehydrogenases.** (**a)** pH optima of the representative recombinant *D*-mandelate
465 dehydrogenases. The pH optima were assayed by the standard activity assay method as
466 described in the text with 100 mM glycine-NaOH buffer over the pH range of 7.0−11.0. (**b)**

pH stabilities of the representative recombinant *D*-mandelate dehydrogenases. To estimate the pH stability, aliquots of these recombinant *D*-mandelate dehydrogenases solution were preincubated at 0°C for 1.0 h at various pH values from 7.0 to11.0, and the residual activities were assayed under the optimal reaction temperatures and pH values.

Figure 4. SDS-PAGE analysis for the expressed products of single culture and co-culture. Lane M, PageRuler Prestained Protein Ladder; lane 1, expressed products of single cultured *E. coli*/pET28a-*LhDMDH*; lane 2, expressed products of co-cultured *E. coli*/pET28a-*LhDMDH* and *E. coli*/pET28a-*LcLDH*.

Figure 5. The yield of different culture mode. The reactions were carried out at 30°C and 200 rpm for 48 h in a 100-mL fermentation liquor containing 50 mM D-mandelic acid without adding coenzyme or co-substrate.

Figure 6. The conversion curve of co-culture toward D, L-mandelic acid. The reactions were carried out at 30°C and 200 rpm for 48 h in a 100-mL fermentation liquor containing 50 mM D, L-mandelic acid without adding coenzyme or co-substrate.

Tables

Table 1 The kinetic parameter of *D*-mandelate dehydrogenases for D-mandelic acid.

Enzyme	Kinetic parameters for substrate			
	K_m (mM)	k_{cat} (S^{-1})	K_i (mM)	k_{cat}/K_m (S^{-1} mM^{-1})
LbDMDH	1.45	8.82	24.37	6.08
LhDMDH	1.05	30.28	26.40	28.80
LcDMDH	1.75	1.56	24.86	0.89
LlDMDH-2	1.00	2.29	16.25	2.29

Table 2 The kinetic parameter of *D*-mandelate dehydrogenases for NAD^+.

Enzyme	Kinetic parameters for NAD^+			
	K_m (mM)	k_{cat} (S^{-1})	K_i (mM)	k_{cat}/K_m (S^{-1} mM^{-1})
LbDMDH	0.78	9.23	N	11.83
LhDMDH	0.40	29.05	N	72.00
LcDMDH	0.85	1.86	N	2.19
LlDMDH-2	0.42	1.85	N	4.40

Table 3 The substrate spectrum of *D*-mandelate dehydrogenases

Substrate	Relative activity (%)			
	LbDMDH	LhDMDH	LcDMDH	LlDMDH-2
D-mandelic acid	100	100	100	100
D, L-mandelic acid	75	82	85	80
D-2-chloromandelic acid	0.59	0.28	2.51	0.05
L-mandelic acid	0.04	0.02	0	0
L-2-chloromandelic acid	0	0	0	0
L-lactic acid	0	0	0	0

Scheme 1.

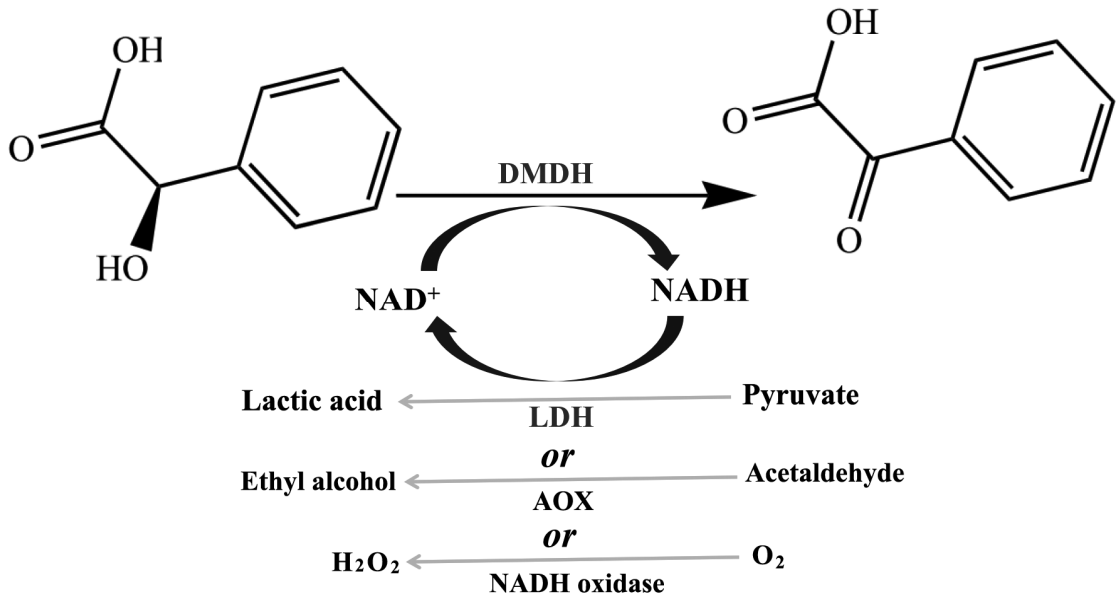

OH
O
HO
DMDH
OH
O
O
NAD+
NADH
Lactic acid
LDH
Pyruvate
or
Ethyl alcohol
AOX
Acetaldehyde
or
H2O2
NADH oxidase
O2

Figure 1.

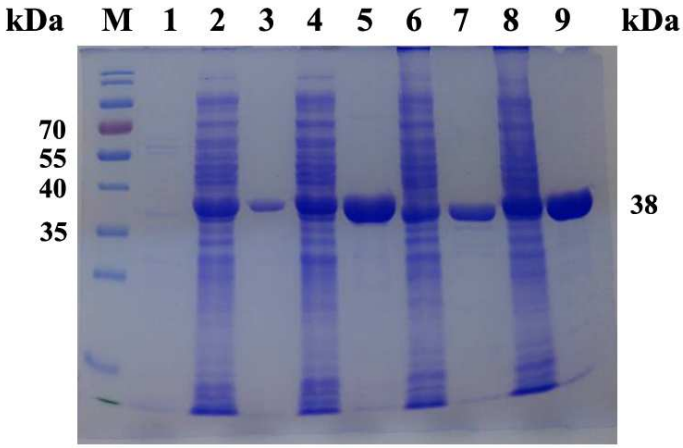


Figure 2a.

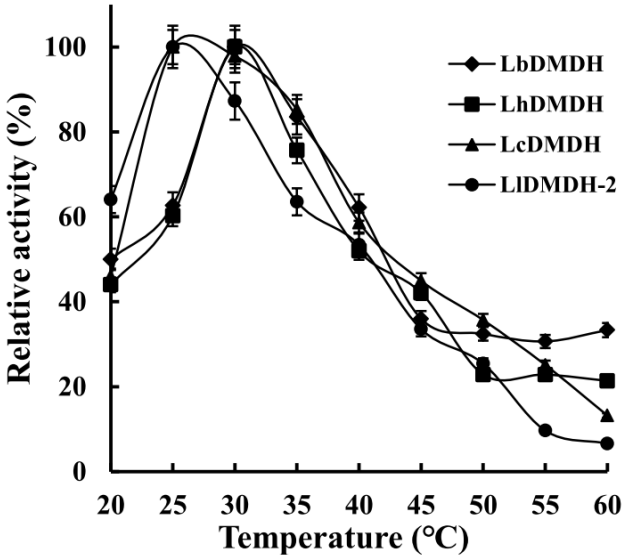

Relative activity (%)
100
80
60
40
20
0
20
25
30
35
40
45
50
55
60
Temperature (°C)
LbDMDH
LhDMDH
LcDMDH
LlDMDH-2

Figure 2b.

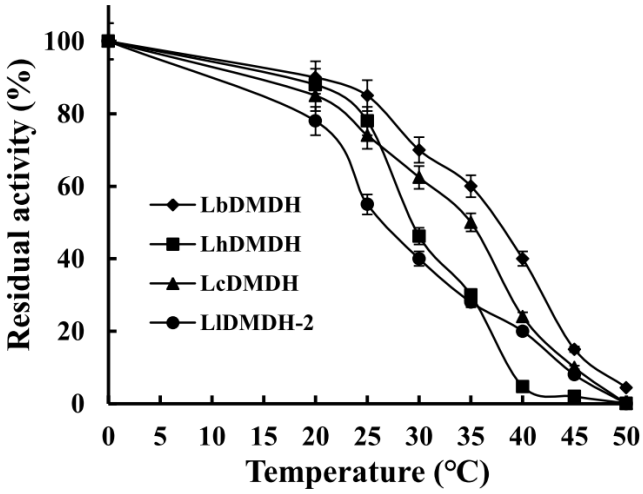

Residual activity (%)
100
80
60
40
20
0
0 5 10 15 20 25 30 35 40 45 50
Temperature (°C)
LbDMDH
LhDMDH
LcDMDH
LlDMDH-2

Figure 3a.

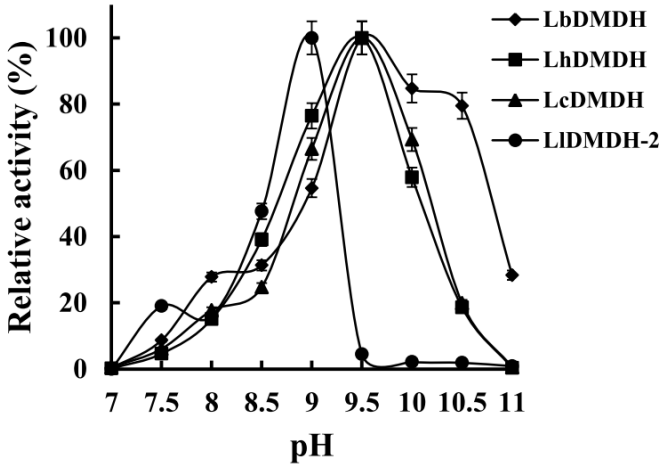

LbDMDH
LhDMDH
LcDMDH
LlDMDH-2
Relative activity (%)
100
80
60
40
20
0
7
7.5
8
8.5
9
9.5
10
10.5
11
pH

Figure 3b.

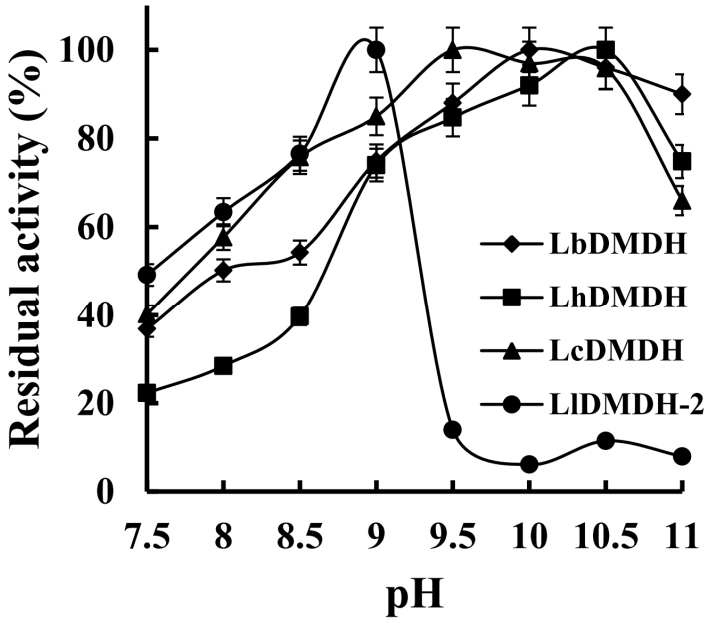

Residual activity (%)
100
80
60
40
20
0
7.5
8
8.5
9
9.5
10
10.5
11
pH
LbDMDH
LhDMDH
LcDMDH
LlDMDH-2

Figure 4.

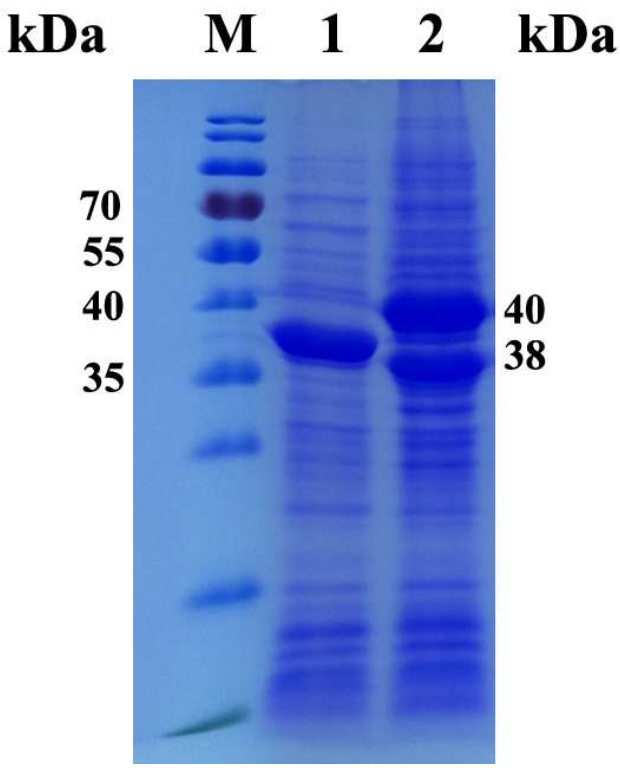


Figure 5.

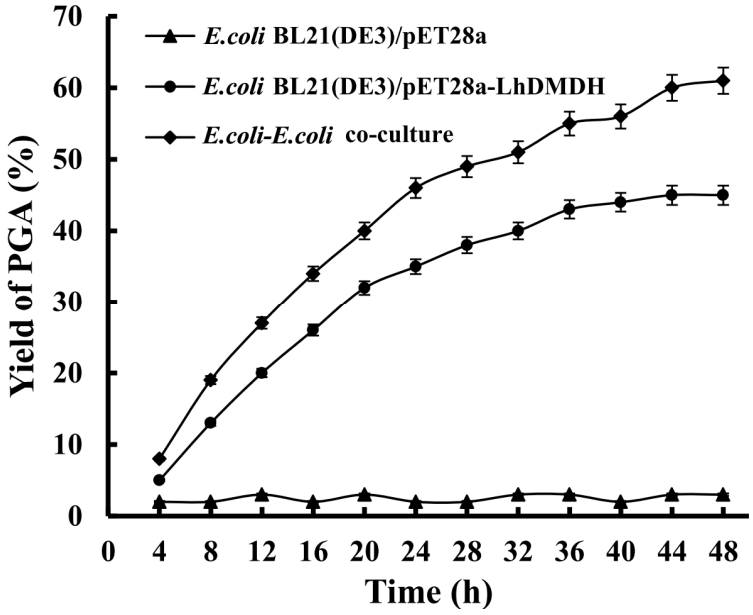
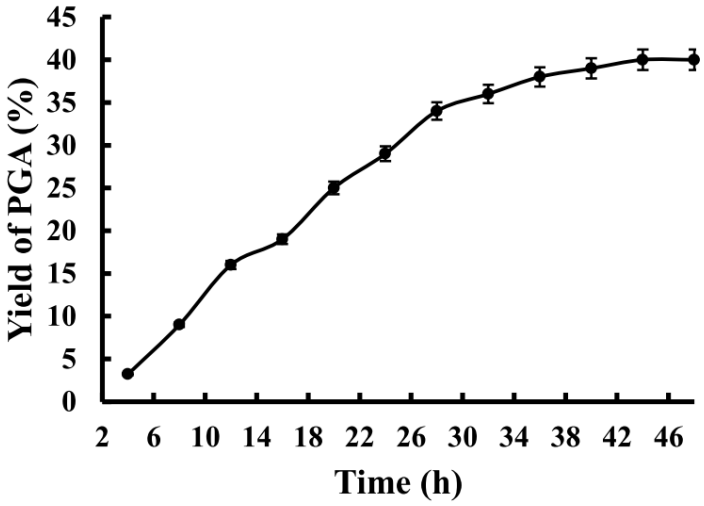
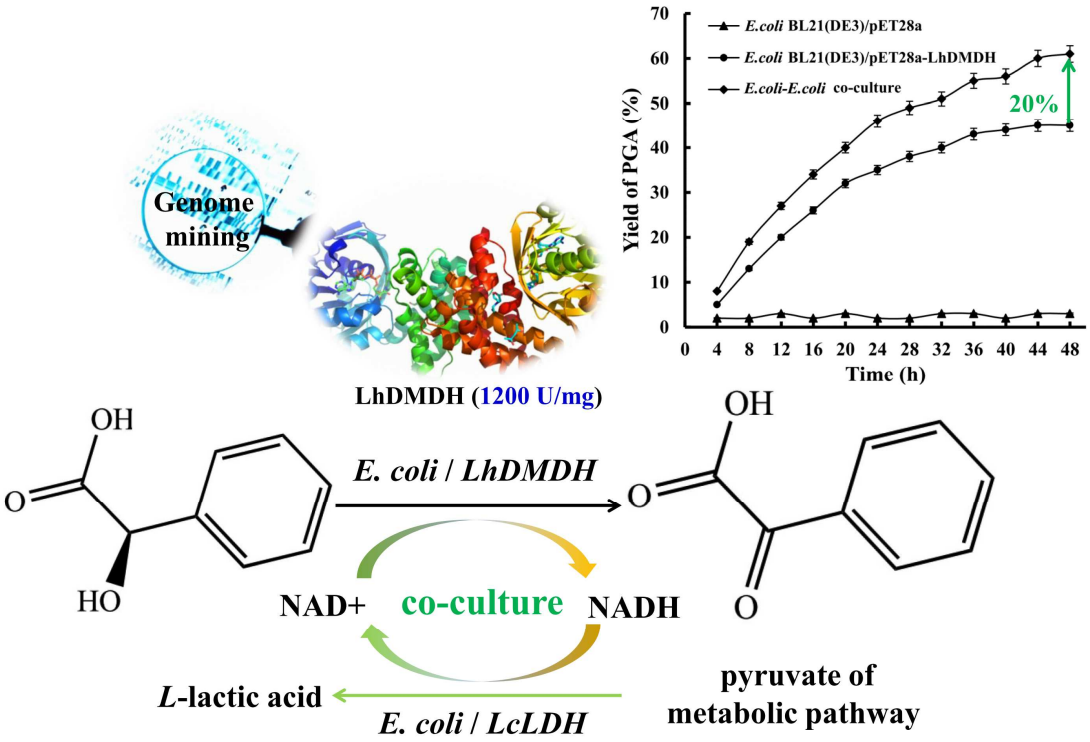

E.coli BL21(DE3)/pET28a
E.coli BL21(DE3)/pET28a-LhDMDH
E.coli-E.coli co-culture
Yield of PGA (%)
Time (h)
0
10
20
30
40
50
60
70
0 4 8 12 16 20 24 28 32 36 40 44 48

Figure 6.


Yield of PGA (%)
0
5
10
15
20
25
30
35
40
45
2
6
10
14
18
22
26
30
34
38
42
46
Time (h)

TOC graphic


Genome mining
LhDMDH (1200 U/mg)
E. coli / LhDMDH
NAD+
co-culture
NADH
L-lactic acid
E. coli / LcLDH
pyruvate of metabolic pathway
E.coli BL21(DE3)/pET28a
E.coli BL21(DE3)/pET28a-LhDMDH
E.coli-E.coli co-culture
20%
Yield of PGA (%)
Time (h)