JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Biotechnology and Biological Transformations

One-Pot Synthesis of Phenylglyoxylic Acid from Racemic Mandelic Acids via Cascade Biocatalysis

Cunduo Tang, Peng-Ju Ding, HongLing Shi, Yuanyuan Jia, Maozhi Zhou, Hui-Lei Yu, Jian-He Xu, Lunguang Yao, and Yunchao Kan

J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b07295 • Publication Date (Web): 26 Feb 2019

One-Pot Synthesis of Phenylglyoxylic Acid from Racemic Mandelic Acids *via* Cascade Biocatalysis

Cun-Duo Tang[†§#*], Peng-Ju Ding[†#], Hong-Ling Shi[†#], Yuan-yuan Jia[†], Mao-zhi Zhou[§], Hui-lei Yu[§], Jian-He Xu[§], Lun-Guang Yao [*†] and Yun-Chao Kan[*†]

[†] Henan Provincial Engineering Laboratory of Insect Bio-reactor and Henan Key Laboratory of Ecological Security for Water Source Region of Mid-line of South-to-North, Nanyang Normal University, 1638 Wolong Road, Nanyang, Henan 473061, People's Republic of China

[§] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 200237, People's Republic of China

[#] These authors contribute equally to this work

***Corresponding Author:** Dr. Cun-Duo Tang, Prof. Lun-Guang Yao and Yun-Chao Kan

Corresponding Address:

Henan Provincial Engineering Laboratory of Insect Bio-reactor, Nanyang Normal University, 1638 Wolong Road, Nanyang, Henan 473061, People's Republic of China

Phone: +86-377-63525087, Fax: 86-377-63525087, E-mail: tcd530@126.com; kanyunchao@163.com; lunguangyao@163.com

ABSTRACT

Phenylglyoxylic acid (PGA) are key building blocks and widely used to synthesize pharmaceutical intermediates or food additives. However, the existing synthetic methods for PGA generally involve toxic cyanide and complex processes. In order to explore an alternative method for PGA biosynthesis, we envisaged cascade biocatalysis for the one-pot synthesis of PGA from racemic mandelic acid. A novel mandelate racemase named *Ar*MR showing higher expression level (216.9 $U \cdot mL^{-1}$ fermentation liquor) was cloned from *Agrobacterium radiobacter* and identified. And six recombinant *E. coli* strains were engineered to co-express three enzymes of mandelate racemase, *D*-mandelate dehydrogenase and *L*-lactate dehydrogenase, and transform racemic mandelic acid to PGA. Among them, the recombinant *E. coli* TCD 04, engineered to co-express three enzymes of *Ar*MR, *Lh*DMDH and *Lh*LDH, can transform racemic mandelic acid (100 mM) to PGA with 98% conversion. Taken together, we provide a green approach for one-pot biosynthesis of PGA from racemic mandelic acid.

KEYWORDS: mandelate dehydrogenase, mandelate racemase, cascade reaction, phenylglyoxylic acid, whole cell catalysis

INTRODUCTION

Phenylglyoxylic acid (PGA) are key building blocks in the chemical synthesis process, and widely used to synthesize pharmaceutical intermediates or food additives.[1-4] Compared with chemical preparation generally involve toxic cyanide and complex processes,[2, 5] microbial or enzymatic biosynthesis of PGA provides an eco-friendly approach to industrial-scale production.[2, 6-7] It is reported that many microorganisms can selectively transform mandelic acid to PGA, including *Gibberella fujikuroi*, *Alcaligenes bronchisepticus*, *Pseudomonas polycolor*, and *Pseudomonas putida*.[8-9] *D*-mandelate dehydrogenase (DMDH) plays a key role in the process of converting *D*-mandelic acid to PGA, and it is a member of ketopantoate reductase family.[5, 9-10] Heterologous expression of DMDH genes from many microorganisms in *E. coli* was capable to oxidize mandelic acid into PGA.[4, 10] In our previous study, the PGA yield was about 40% from 50 mM racemic mandelic acid catalyzed by co-culturing two engineered *E. coli* strains.[4] Meanwhile, we have verified that the NAD^+ regeneration capacity could be enhanced by utilizing the pyruvic acid based on co-culturing two engineered *E. coli* strains. However, the residual mandelic acid significantly increases the cost of product purification. Therefore, enhancing the transformation efficiency of substrates can not only improve the utilization rate of raw materials, but also reduce the cost of purification.

Mandelate racemase (EC 5.1.2.2) is well-characterized racemases[10-11] and an ideal candidate for the racemization of non-natural α-hydroxycarboxylic acids under physiological reaction conditions, which had been applied in deracemization protocols in connection with a kinetic resolution step.[12] So far, the *Pp*MR from *Pseudomonas putida*

ATCC 12633 (Uniprot No. P11444) is well-characterized and widely-applied mandelate racemase, which k_{cat} values towards *D*- mandelic acid and *L*- mandelic acid were 822 and 756 s^{-1}. [12-13] And many cascade reactions had been designed and applied using the *Pp*MR as the core catalyst.[10, 14-15] However, the industrial application of *Pp*MR was impeded due to its poor soluble expression level. It had been reported that genome mining could be an economical strategy in discovering novel enzyme with higher expression level,[4, 16-18] therefore it should also be used to discover novel mandelate racemase with higher expression level. In addition, the mature DNA synthesis and sequencing techniques have enabled the overproduction of a desired enzyme in microorganisms.

In this work, we tried to discover novel mandelate racemases with higher expression level, and achieve a eco-friendly synthesis of PGA from racemic mandelic acid via one-pot cascade biocatalysis (**Scheme 1**) combined with the previous research basis.[3] As known to us, the PGA is more expensive than racemic mandelic acid, thus the process of converting racemic mandelic acid to PGA is highly value-added and attractive. Taken together, our results will build a solid basis for future studies, including chiral resolution of racemic mandelic acid, biosynthesis of PGA derivatives and so on.

MATERIALS AND METHODS

Reagents and Kits

PrimeSTAR® HS (Premix), DNA Marker and DNA Ligation Kit Ver.2.1 were purchased from TaKaRa (Dalian,China). Restriction enzymes *Eco*R I, *Bam*H I, *Sal* I, *Hin*d III, *Nde* I and *Xho* I were purchased from New England Biolabs, Inc. (Ipswich, MA, England). *D*-mandelic acid, *L*-mandelic acid, racemic mandelic 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

Escherichia coli BL21, *E. coli* DH5α/pACYCDuet-1, *E. coli* DH5α/pET28a, *E. coli* BL21/ pET28a-*LhDMDH*[4], *E. coli* BL21/ pET28a-*PpMR*[10] and *E. coli* BL21/ pET28a-*LbDMDH*[10] preserved by our Lab were cultured in LB medium.[4] *E. coli* BL21/ pET28a-*LhLDH* contained an *L*-lactate dehydrogenase encoding gene from *Lactobacillus helveticus,* which was artificially synthesized and constructed by Synbio Technologies Genes for Life after codon optimization. *Agrobacterium radiobacter* DSM 30147*, Bradyrhizobium japonicum* CGMCC 1.2550*, Clostridium ljungdahlii* DSM 13528*, Halomonas sp.* S23321 and *Herbaspirillum rubrisubalbicans* ATCC 19308 preserved by our group were used for genomic DNA extraction after culturing in LB medium.

Genome Mining for Putative Mandelate Racemases

*Pp*MR, a well-characterized mandelate racemase with high specific activity encoded by *Pseudomonas putida* ATCC 12633 (Uniprot No. P11444), was used as probe for the BLAST search for potential novel mandelate racemases, which were mainly from bacterial genome sequences and unidentified. In order to ensure the diversity of sequences, candidate

sequences with the similarity of probe sequences between 20% and 65% were selected. And the functions of these sequences were not commented and verified in detail.

Gene Cloning and Expression of Selected Mandelate Racemases

Based on the results of genome mining, many paired primers shown in **Table S1** were designed and synthesized, which were used to amplify the gene fragments of potential novel mandelate racemases. The cultivation, collection and genomic DNA extraction of original strains were performed as reported before.[4] Nextly, the gene fragments of potential mandelate racemase were respectively amplified based on these genomic DNA. The amplified genes were transformed into *E. coli* BL21 by the pET28a plasmid, and those recombinant transformants were identified by DNA sequencing. The expressions of these transformants were performed as reported before,[4] and then the lysate supernatant was purified to electrophoretic purity by Ni-chelating affinity chromatography.[4]

Enzyme Activity and Protein Assays

The oxidation activity of *D*-mandelate dehydrogenase on *D*-mandelic acid and reduction activity of *L*-lactate dehydrogenase on sodium pyruvate were assayed as reported before.[4] The racemization activity of mandelate racemase on *L*-mandelic acid was assayed by determining the production of *D*-mandelic acid. 1 mL reaction mixture contained 100 mM Tris-HCl buffer, pH 7.5, 3.3 mM Mg^{2+}, proper quantity of enzyme and 2.0 mM *L*-mandelic acid. The conversion reaction was maintained at 30 °C and 1000 r/min for 30 min. Then the reaction was stopped by adding 20 μL 2 M sulfuric acid, and extracted with equivoluminal ethyl acetate, and the production of *D*-mandelic acid or residual quantity of *L*-mandelic acid was determined by HPLC analysis in organic phase using a Shimadzu LC2010A HPLC

system with a UV detector (working wavelength 228 nm) at 30 °C, equipped with a CHIRALCEL® OJ-H (250 mm × 4.6 mm, 5 μm, DAICEL INVESTMENT CO., LTD.). The mobile phase contained 92% hexane, 8% isopropanol and 0.1% TFA, and the flow rate of mobile phase was 1.0 mL min^{-1}. Retention time of *D*-mandelic acid was 19.9 min (**Figure S1**), and the one of *L*-mandelic acid was 23.0 min (**Figure S2**).

The protein concentration and SDS-PAGE were carried out as reported before.[4] Then the apparent molecular weight of subunit was estimated with the Quantity One software based on the standard marker proteins.[16] Meanwhile, the actual molecular weights of these recombinant enzymes were determined by mass spectrometric analysis at Xevo G2-XS Q-TOF. And the subunit numbers of these enzymes were calculated by comparing the apparent molecular weight and actual molecular weight.

Biochemical Characterization of Recombinant Mandelate Racemases

To estimate the temperature optima of the recombinant mandelate racemases, their activities were determined under the standard assay conditions as above, except temperatures ranging from 20 to 60°C with an interval of 5 °C. To estimate the thermostability of these recombinant mandelate racemases, they were incubated with 100 mM, pH 7.5 HEPES buffer at 30, 40 and 50 °C, respectively, and the residual enzyme activity was determined by sampling at different time. Then, the half-lives of these recombinant mandelate racemases were calculated. The pH optimum of the mandelate racemase was assayed by the standard activity assay method with 100 mM glycine-NaOH buffer over the pH range of 8.0−10.5, 20 mM citric acid-sodium citrate buffer over the pH range of 5.0−6.0 and 100 mM Tris-HCl buffer over the pH range of 6.5−7.5. To estimate influence of metal ions and EDTA on

mandelate racemase, the activity of mandelate racemase was assayed by the standard activity assay method with various metal ions and EDTA at a final concentration of 1.0 mM. The kinetic parameters of the mandelate racemases were determined by assaying the activity on various substrate concentrations. The correlation curve was fitted in nonlinear fitting by the Origin 9.0 software.[4, 19]

Genetic Engineering of the Plasmids Containing DMDH and LDH Encoding Gene

Based on the analysis of multiple cloning sites of plasmid and restriction enzyme sites of the target gene, many paired primers shown in **Table S2** were designed and synthesized, which were used to amplify the gene fragments of *D*-mandelate dehydrogenase and *L*-lactate dehydrogenase. *LhLDH* was amplified from the strain of *E. coli* BL21/ pET28a-*LhLDH* using primers LhLDH-F and LhLDH-R with PrimeSTAR® HS (Premix) Kit. The PCR product was double-digested with *Bam*H I and *Sal* I, and then ligated to the *Bam*H I and *Sal* I digested MCS1 of pACYCDuet-1 plasmid with DNA Ligation Kit Ver.2.1. The ligation product was transformed into *E. coli* BL21 expression competent cells to give pACYC-*LhLDH*. Then, the *LbDMDH* and *LhDMDH* was respectively amplified, double-digested and then ligated to the *Nde* I and *Xho* I digested MCS2 of pACYC-*LhLDH* plasmid to give pACYC-*LhLDH-LbDMDH* and pACYC-*LhLDH-LhDMDH* using similar methods.

Engineering of *E. coli* (TCD01–06) Strains

Each of the *LDH-DMDH* plasmids (pACYC-*LhLDH-LbDMDH* and pACYC-*LhLDH-LhDMDH*) and each of the *MR* plasmids (pET28a-*PpMR* and the recombinant plasmid constructed as above) were co-transformed into competent cells of *E.*

coli BL21 (DE3) to give the six *E. coli* strains co-expressing DMDH, MR and LDH. The details are provided in **Table S3** of the Supporting Information.

'One-pot' Biosynthesis of PGA from Racemic Mandelic Acid

To evaluate application potential of the engineered *E. coli* strains in transforming racemic mandelic acid to PGA, these engineered *E. coli* strains were cultured and induced as above. And then, 100 mM racemic mandelic acid was added and transformed for 48 h at 30°C. During this transformation process, 100 μL samples were extracted for HPLC analysis every 4 hours.[4] After the catalytic reaction was terminated, the purification of biotransformation products was performed as reported before.[4] And then, the product yield was determined by HPLC analysis as above, and the product was characterized by ^{1}H NMR spectroscopy at 400 MHz.

Analysis of PGA

PGA was determined by HPLC analysis and ^{1}H NMR spectroscopy at 400 MHz as reported before.[4] Retention time of PGA was 3.1 min. The correlation curve was fitted in linear fitting by the Origin 9.0.

RESULTS AND DISCUSSION

Gene Cloning and Expression of Mandelate Racemases

To search for an efficient mandelate racemase for deracemization of racemic mandelic acid, genome mining strategy was adopted. Ten gene fragments of potentially novel mandelate racemases were amplified and detected by agarose gel electrophoresis as shown in **Figure S3**, and then they have been cloned and over-expressed in *E. coli* BL21. To detect recombinant protein expression patterns, SDS-PAGE was carried out on a 12.5% gel, and its

results were shown in **Figure 1**. As shown in **Figure 1**, eight of them were successfully expressed except *BjMR-B and BjMR-F*. Interestingly, two of them displayed racemization activity toward *L*-mandelic acid. Among them, a putative mandelate racemase (UNIPROT accession no: B9JQ28) from *Agrobacterium radiobacter*, herein designated as *Ar*MR, was confirmed with the highest racemization activity. It was examined and converted that the *Ar*MR had the racemization activity on *L*-mandelic acid of 216.9 $U \cdot mL^{-1}$ fermentation liquor, which was near to four times that of the probe. Meanwhile, another putative mandelate racemase (GenBank accession no: WP_058894565.1) from *Herbaspirillum rubrisubalbicans*, herein designated as *Hr*MR, was confirmed with racemization activity of 33.7 $U \cdot mL^{-1}$ fermentation liquor, which was slightly lower than that of the probe. In addition, as shown in **Figure 1C**, the soluble expression level of *ArMR* using pET28a plasmid was significantly higher than that of *Pp*MR. Sequence alignment revealed a relatively low amino acid sequence identity (about 60%) with the *Pp*MR **(Figure S4)**, indicating that they are novel mandelate racemases.

In order to characterize the enzymatic properties of the recombinant mandelate racemases, they were firstly purified to homogeneity through Ni-NTA affinity chromatography. As shown in **Figure 1D**, they were purified to homogeneity, and their apparent molecular weights were about 43.0 kDa. Based on the results of high resolution mass spectrometer analysis, the actual molecular weight of *Pp*MR, *Ar*MR and *Hr*MR was 308460.8, 154811.2 and 157253.2 Da, respectively. Comparing with the apparent molecular weight and actual molecular weight, the *Ar*MR and *Hr*MR were all comprised of four subunits, while the *Pp*MR was comprised of eight subunits as previously reported. [20]

The specific racemization activity of purified *Ar*MR and *Hr*MR was 107.4 and 21.5 $U \cdot mg^{-1}$ for the *L*-mandelic acid, respectively. They were slightly lower than the one of the *Pp*MR determinated in this study, which specific racemization activity toward *L*-mandelic acid was 324.8 $U \cdot mg^{-1}$. Nevertheless, the *Ar*MR can still reflect a higher level of enzyme activity in fermentation liquor owe to its higher proportion of soluble expression comparing with the probe, which further indicated that the racemases obtained, especially the *Ar*MR, has a great application prospect in deracemization of racemic mandelic acid.

Comparative Enzymatic Properties of Mandelate Racemases

In order to compare the enzymatic properties of these recombinant mandelate racemases, they were investigated using *L*-mandelic acid as the model substrate. The temperature properties of these mandelate racemases were shown in **Figure 2**. These purified mandelate racemases exhibited higher catalytic activity from 45 to 55°C, and the temperature optimum of *Ar*MR was 50°C, while the one of *Hr*MR was 55°C. The half-life of these recombinant mandelate racemases at various temperatures was summarized in **Table 1.** Unfortunately, the half-life of *Ar*MR at 50 °C was 0.17 h, and its thermostability was too bad to be impeded in enzymatic racemization of racemic mandelic acid. Hence, to eliminate the influence of bad thermostability, whole cell catalysis was preferred at 30 °C in enzymatic racemization of racemic mandelic acid. In addition, with the emergence of various bioinformatical software, the computer-aided rational design is playing a more significant role in molecular modification of thermostability. Particularly, molecular dynamics simulation has become more mature and reliable.[3, 21] In next research, the molecular

233 modification of *Ar*MR for improving its thermostability will be carried out by *in silico*
234 design.

235 The pH properties of these mandelate racemases were shown in **Figure 3**. The pH
236 value of buffer can significantly influence the activities of these enzymes as shown in
237 **Figure 3**. These purified mandelate racemases had no racemization activity at pH value of 6
238 or below, which could be attributed to that mandelate racemase catalyzes deracemization of
239 substrate by a two-base mechanism.[22] Proton gains and losses are catalyzed by two typical
240 alkaline amino acid residues, so it is speculated that the more acidic environment is not
241 conducive to proton exchange and significantly reduces the catalytic activity. In the alkaline
242 pH range, these enzymes activities were higher, and even in the glycine - NaOH buffer with
243 a pH value of 10, it still retained more than 10% of the relative activity. And the pH
244 optimum of the *Ar*MR was 8.0, while the one of the *Hr*MR was 9.0.

245 The kinetic parameters of these mandelate racemases toward *L*-mandelic acid were
246 summarized in **Table 2**. The K_m toward *D*-mandelic acid and *L*-mandelic acid of *Ar*MR was
247 1.44 and 0.81 mM, which was slightly higher than the one of the probe, [13] indicating that
248 *Ar*MR displayed lower affinity toward *D*-mandelic acid and *L*-mandelic acid. Moreover, the
249 k_{cat} values toward *D*-mandelic acid and *L*-mandelic acid of *Ar*MR were 409.8 and 218.3 s^{-1},
250 which were also distinctly lower than the ones (822 and 756 s^{-1}) of the probe. [13, 20]
251 Nevertheless, the *Ar*MR can still reflect a higher level of enzyme activity in fermentation
252 liquor attribute to its higher proportion of soluble expression comparing with the probe,
253 which further indicated that the *Ar*MR could display further advantage in the
254 deracemization process of racemic mandelic acid by whole-cell catalysis. Meanwhile, it also

means that the catalytic activity of *Ar*MR still needs to be improved on the premise of ensuring the proportion of soluble expression.

It is reported that the crystal structure of *Pp*MR contained a Mg^{2+} in the catalytic center, which is connected to the side chain of catalytic triad in the active center and played an important role in the catalytic process.[23] In this paper, we investigated the influence of Mg^{2+} and other metal ions on the activity of mandelate racemase (**Figure 4**)**.** As shown in **Figure 4**, there is no effect of the tested metal ions and EDTA on enzyme activity but the activity of racemase was improved by adding Mg^{2+} and Zn^{2+}. These results indicated that Mg^{2+} and Zn^{2+} also played an important role in the catalytic processes of *Ar*MR and *Hr*MR, and they might have the same catalytic mechanism as the *Pp*MR, which would be further verified in our subsequent studies.

Engineering and Expressing of *E. coli* (TCD01–06) Strains

Based on the techno-economic impact of reducing addition of coenzyme and cosubstrate, and enhancing regeneration of NAD^+ and conversion efficiency of substrate, we investigated the feasibility of co-expressing three relevant enzymes in an *E. coli* cell to achieve the purpose of **Scheme 1**. Each of the *LDH-DMDH* plasmids (pACYC-*LhLDH-LbDMDH* and pACYC-*LhLDH-LhDMDH*) and each of the *MR* plasmids (pET28a-*PpMR,* pET28a-*ArMR* and pET28a-*HrMR*) were co-transformed into competent cells of *E. coli* BL21 (DE3) to give the six *E. coli* strains co-expressing DMDH, MR and LDH. In the co-expression system, DMDH was dedicated for converting *D*-mandelic acid to PGA, and LDH was designated to enhancing the regeneration of NAD^+ by utilizing the pyruvic acid generated by *E. coli*. Meanwhile, the MR was dedicated for converting

277 *L*-mandelic acid to *D*-mandelic acid, when the *D*-mandelic acid had been converted to PGA.
278 After DNA sequencing identification, all of these *E. coli* strains were respectively named as
279 *E. coli* TCD 01 to *E. coli* TCD 06, the details of these *E. coli* strains are shown in **Table S3**
280 of the Supporting Information. To detect the expression level of co-expression system,
281 catalytic activities of three enzymes were analyzed and summarized in **Table 3**. As shown
282 in **Table 3**, the three enzymes activities of recombinant *E. coli* TCD 04, engineered to
283 co-express three enzymes of *Ar*MR, *Lh*DMDH and *Lh*LDH, were the highest and best
284 proportion, which may be more conducive to the cascade reaction.

285 **'One-pot' Biosynthesis of PGA from Racemic Mandelic Acid**

286 To assess application potential of the engineered *E. coli* strains in transforming racemic
287 mandelic acid to give PGA, these engineered *E. coli* strains were cultured and induced as
288 above. Then, racemic mandelic acid was added to a final concentration of 100 mM, and
289 transformed 48 h at 30°C. The racemic mandelic acid was bioconverted 48 h by engineered
290 *E. coli* whole-cell, and the product was purified to chromatographically purity, which purity
291 was over 99%. And the retention time of the purified product was 3.1 min, which was in
292 accordance with the retention time of PGA. Meanwhile, combined with ^{1}H NMR spectra of
293 racemic mandelic acid (**Figure S5**) and the product (**Figure S6**), these results sufficiently
294 demonstrated that the racemic mandelic acid had been converted to PGA by one-pot cascade
295 biocatalysis. These results also indicated that the PGA had been successfully synthesized
296 without adding coenzyme or cosubstrate, implying that the synthesis cost of PGA can be
297 significantly reduced by this strategy, which fully demonstrated the advantage of whole cell
298 catalysis. [24-26]

In addition, the conversion curves of racemic mandelic acid catalyzed by these engineered *E. coli* strains were measured and analyzed. As shown in **Figure 5**, the yield of PGA assisted by the mandelate racemase was significantly higher than the one that was only catalyzed by *D*-mandelate dehydrogenase and *L*-lactate dehydrogenase.[4] Among the strains, the *E. coli* TCD04 containing pET28a-*ArMR* and pACYC-*LhLDH-LhDMDH* gave the highest conversion to PGA, thus it was selected as the best catalyst for this cascade biotransformation. Under the optimized conditions, 100 mM of racemic mandelic acid were reacted with *E. coli* TCD04 to give PGA in 98% conversion in 48 h, and its space-time yield was up to 7.36 g L^{-1} d^{-1}. Compared with the results we reported earlier,[4] the significantly increased yields of PGA should mainly be attributed to that the co-expressed mandelate racemase can continuously convert *L*-mandelic acid to *D*-mandelic acid, and maintain the reaction so that the substrate can be transformed as much as possible. Moreover, co-expression can avoid intercellular transmission of NAD^+ and NADH in regeneration NAD^+ comparing with the co-culture mode, which can enhance the efficiency of NAD^+ regeneration. It was hardly surprising that these *E. coli* strains containing *Pp*MR encoding genes failed to show better productivity, although the *Pp*MR can display excellent kinetic parameters. This result is consistent with that the *Pp*MR did not reflect a higher level of enzyme activity in fermentation liquor due to its higher proportion of inclusion bodies in recombinant *E. coli* cells, which further confirmed that the *Ar*MR could display further advantage in the racemization process of racemic mandelic acid by whole-cell catalysis.

Furthermore, these *E. coli* strains containing *Lb*DMDH encoding genes showed lower productivity than those *E. coli* strains containing *Lh*DMDH, which should be due to the

lower catalytic activity and soluble expression proportion of *Lb*DMDH expressed in *E. coli* cells. Meanwhile, this result also indicated that the mandelate dehydrogenase was the rate-limiting enzyme in the whole cascade reaction for the one-pot synthesis of PGA from racemic mandelic acid. All of these results showed that the engineered *E. coli* strain TCD 04 had tremendous application potential in chiral resolution of racemic mandelic acid and biosynthesis of PGA, which are also important intermediates for synthesis of pharmaceutical and natural products.[4, 27-28]

In conclusion, a novel mandelate racemase (*Ar*MR) from *A. radiobacter* was mined and expressed in *E. coli* BL21. The *Ar*MR displayed high catalytic activity and soluble expression proportion. Interestingly, we achieved a green and efficient synthesis of PGA from racemic mandelic acid via one-pot cascade biocatalysis using the engineered *E. coli* strain coexpressing three enzymes of mandelate racemase, *D*-mandelate dehydrogenase and L-lactate dehydrogenase. Among of these, the recombinant *E. coli* TCD 04, engineered to coexpress three enzymes of *Ar*MR, *Lh*DMDH and *Lh*LDH, can transform 100 mM racemic mandelic acid to give PGA with 98% conversion. The high-yielding synthetic methods use cheap and green reagents, and *E. coli* whole-cell catalysts, thus providing green and useful alternative methods for manufacturing PGA. Taken together, we provide a green approach for one-pot biosynthesis of PGA from racemic mandelic acid.

ASSOCIATED CONTENT

Supporting Information

Additional experimental results and other data are available free of charge via the Internet at http://pubs.acs.org. Sequences of the primers used for cloning and expression of novel

mandelate racemases (Table S1); Sequences of the primers used for genetic engineering of the plasmids containing DMDH and LDH encoding gene (Table S2); List of the recombinant strains and the plasmids contained (Table S3); The HPLC analysis spectrum of the *D*- mandelic acid (Figure S1); The HPLC analysis spectrum of the *L*- mandelic acid (Figure S2); The PCR amplification for the ten mandelate racemase genes (Figure S3); Multi-sequence alignment of the representative mandelate racemases (Figure S4); The ^{1}H NMR analysis spectrum of the racemic mandelic acid (Figure S5); The 1H NMR analysis spectrum of the product (Figure S6).

AUTHOR INFORMATION

Authors' Contributions

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

Notes

The authors declare that they have no competing interests.

ACKNOWLEDGMENTS

We are grateful to Prof. Minchen Wu (Wuxi Medical School, Jiangnan University) and Prof. Ting Lei (Nanyang Normal University) for providing technical assistance. This work was supported by the grants from Scientific and Technological Project of China (31870917), Scientific and Technological Project of Henan Province (182102110084 and 162102210116), Scientific research and service platform fund of Henan Province (2016151), fund of scientific and technological innovation team of water ecological security for Water

Source Region of Mid-line of South-to-North Diversion Project of Henan Province (17454), and the Special Funded Projects of Nanyang Normal University (2018QN004).

REFERENCES

1. He, Y. C.; Pan, X. H.; Xu, X. F.; Wang, L. Q., Biosynthesis of benzoylformic acid from benzoyl cyanide with a new bacterial isolate of Brevibacterium sp. CCZU12-1. *Appl Biochem Biotechnol* **2014,** *172* (6), 3223-33.

2. He, Y.-C.; Zhou, Q.; Ma, C.-L.; Cai, Z.-Q.; Wang, L.-Q.; Zhao, X.-Y.; Chen, Q.; Gao, D.-Z.; Zheng, M.; Wang, X.-D.; Sun, Q., Biosynthesis of benzoylformic acid from benzoyl cyanide by a newly isolated Rhodococcus sp. CCZU10-1 in toluene–water biphasic system. *Bioresource Technology* **2012,** *115* (Supplement C), 88-95.

3. Tang, C.; Shi, H.; He, Z.; Ding, P.; Jiao, Z.; Kan, Y.; Yao, L., Green biosynthesis of phenylglyoxylic acid by biotransformation using recombinant Escherichia coli whole cells. *CIESC Journal* **2018,** *69* (6), 2627-2631.

4. Tang, C. D.; Shi, H. L.; Xu, J. H.; Jiao, Z. J.; Liu, F.; Ding, P. J.; Shi, H. F.; Yao, L. G.; Kan, Y. C., Biosynthesis of Phenylglyoxylic Acid by LhDMDH, a Novel d-Mandelate Dehydrogenase with High Catalytic Activity. *J Agric Food Chem* **2018,** *66* (11), 2805-2811.

5. Li, D.; Zeng, Z.; Yang, J.; Wang, P.; Jiang, L.; Feng, J.; Yang, C., Mandelate racemase and mandelate dehydrogenase coexpressed recombinant Escherichia coli in the synthesis of benzoylformate. *Biosci Biotechnol Biochem* **2013,** *77* (6), 1236-9.

6. Bornscheuer, U. T.; Huisman, G. W.; Kazlauskas, R. J.; Lutz, S.; Moore, J. C.; Robins, K., Engineering the third wave of biocatalysis. *Nature* **2012,** *485* (7397), 185-194.

7. Chen, X.; Yang, C.; Wang, P.; Zhang, X.; Bao, B.; Li, D.; Shi, R., Stereoselective biotransformation of racemic mandelic acid using immobilized laccase and (S)-mandelate dehydrogenase. *Bioresources and bioprocessing* **2017,** *4* (1), 2-2.

8. Miyamoto, K.; Ohta, H., Enantioselective oxidation of mandelic acid using a phenylmalonate metabolizing pathway of a soil bacteriumAlcaligenesbronchisepticus KU 1201. *Biotechnology Letters* **1992,** *14* (5), 363-366.

9. Wang, J.; Feng, J.; Li, W.; Yang, C.; Chen, X.; Bao, B.; Yang, J.; Wang, P.; Li, D.; Shi, R., Characterization of a novel (R)-mandelate dehydrogenase from Pseudomonas putida NUST506. *Journal of Molecular Catalysis B: Enzymatic* **2015,** *120*, 23-27.

10. Fan, C. W.; Xu, G. C.; Ma, B. D.; Bai, Y. P.; Zhang, J.; Xu, J. H., A novel D-mandelate dehydrogenase used in three-enzyme cascade reaction for highly efficient synthesis of non-natural chiral amino acids. *J Biotechnol* **2015,** *195*, 67-71.

11. Gu, J.; Liu, M.; Guo, F.; Xie, W.; Lu, W.; Ye, L.; Chen, Z.; Yuan, S.; Yu, H., Virtual screening of mandelate racemase mutants with enhanced activity based on binding energy in the transition state. *Enzyme Microb Technol* **2014,** *55*, 121-7.

12. Felfer, U.; Goriup, M.; Koegl, M. F.; Wagner, U.; Larissegger-Schnell, B.; Faber, K.; Kroutil, W., The Substrate Spectrum of Mandelate Racemase: Minimum Structural Requirements for Substrates and Substrate Model. *Advanced Synthesis & Catalysis* **2005,** *347* (7-8), 951-961.

13. Gu, J.; Yu, H., The role of residue S139 of mandelate racemase: synergistic effect of S139 and E317 on transition state stabilization. *Journal of biomolecular structure & dynamics* **2012,** *30* (5), 585-93.

14. Resch, V.; Fabian, W. M. F.; Kroutil, W., Deracemisation of Mandelic Acid to Optically Pure

Non-Natural L-Phenylglycine via a Redox-Neutral Biocatalytic Cascade. *Advanced Synthesis & Catalysis* **2010,** *352* (6), 993-997.

15. Zhou, Y.; Wu, S.; Li, Z., One-Pot Enantioselective Synthesis of D-Phenylglycines from Racemic Mandelic Acids, Styrenes, or Biobased L-Phenylalanine via Cascade Biocatalysis. *Advanced Synthesis & Catalysis* **2017,** *359* (24), 4305-4316.

16. Tang, C. D.; Shi, H. L.; Tang, Q. H.; Zhou, J. S.; Yao, L. G.; Jiao, Z. J.; Kan, Y. C., Genome mining and motif truncation of glycoside hydrolase family 5 endo-beta-1,4-mannanase encoded by Aspergillus oryzae RIB40 for potential konjac flour hydrolysis or feed additive. *Enzyme Microb Technol* **2016,** *93-94*, 99-104.

17. Ma, B.-D.; Yu, H.-L.; Pan, J.; Liu, J.-Y.; Ju, X.; Xu, J.-H., A thermostable and organic-solvent tolerant esterase from Pseudomonas putida ECU1011: Catalytic properties and performance in kinetic resolution of α-hydroxy acids. *Bioresource Technology* **2013,** *133*, 354-360.

18. Liang, C.; Nie, Y.; Mu, X.; Xu, Y., Gene mining-based identification of aldo–keto reductases for highly stereoselective reduction of bulky ketones. *Bioresources and bioprocessing* **2018,** *5* (1), 33.

19. Tang, C.-D.; Shi, H.-L.; Jiao, Z.-J.; Shi, H.-F.; Yao, L.-G.; Xu, J.-H.; Kan, Y.-C., Exploitation of cold-active cephalosporin C acylase by computer-aided directed evolution and its potential application in low-temperature biosynthesis of 7-aminocephalosporanic acid. *Journal of Chemical Technology & Biotechnology* **2018,** *93* (10), 2925-2930.

20. Landro, J. A.; Gerlt, J. A.; Kozarich, J. W.; Koo, C. W.; Shah, V. J.; Kenyon, G. L.; Neidhart, D. J.; Fujita, S.; Petsko, G. A., The role of lysine 166 in the mechanism of mandelate racemase from Pseudomonas putida: Mechanistic and crystallographic evidence for stereospecific alkylation by (R)-.alpha.-phenylglycidate. *Biochemistry* **1994,** *33* (3), 635-643.

21. Gao, S.-J.; Wang, J.-Q.; Wu, M.-C.; Zhang, H.-M.; Yin, X.; Li, J.-F., Engineering hyperthermostability into a mesophilic family 11 xylanase from Aspergillus oryzae by in silico design of N-terminus substitution. *Biotechnology and bioengineering* **2013,** *110* (4), 1028-1038.

22. Schafer, S. L.; Barrett, W. C.; Kallarakal, A. T.; Mitra, B.; Kozarich, J. W.; Gerlt, J. A.; Clifton, J. G.; Petsko, G. A.; Kenyon, G. L., Mechanism of the Reaction Catalyzed by Mandelate Racemase: Structure and Mechanistic Properties of the D270N Mutant. *Biochemistry* **1996,** *35* (18), 5662-5669.

23. Mitra, B.; Kallarakal, A. T.; Kozarich, J. W.; Gerlt, J. A.; Clifton, J. R.; Petsko, G. A.; Kenyon, G. L., Mechanism of the Reaction Catalyzed by Mandelate Racemase: Importance of Electrophilic Catalysis by Glutamic Acid 317. *Biochemistry* **1995,** *34* (9), 2777-2787.

24. Cardillo, A. B.; Perassolo, M.; Sartuqui, M.; Rodríguez Talou, J.; Giulietti, A. M., Production of tropane alkaloids by biotransformation using recombinant Escherichia coli whole cells. *Biochemical Engineering Journal* **2017,** *125* (Supplement C), 180-189.

25. de Carvalho, C. C. C. R., Enzymatic and whole cell catalysis: Finding new strategies for old processes. *Biotechnology Advances* **2011,** *29* (1), 75-83.

26. Lee, P. G.; Kim, J.; Kim, E. J.; Jung, E.; Pandey, B. P.; Kim, B. G., P212A Mutant of Dihydrodaidzein Reductase Enhances (S)-Equol Production and Enantioselectivity in a Recombinant Escherichia coli Whole-Cell Reaction System. *Appl Environ Microbiol* **2016,** *82* (7), 1992-2002.

27. He, Y.-C.; Ma, C.-L.; Zhang, X.; Li, L.; Xu, J.-H.; Wu, M.-X., Highly enantioselective oxidation of racemic phenyl-1,2-ethanediol to optically pure (R)-(−)-mandelic acid by a newly isolated Brevibacterium lutescens CCZU12-1. *Applied Microbiology and Biotechnology* **2013,** *97* (16), 7185-7194.

28. Zhang, Z.-J.; Pan, J.; Li, C.-X.; Yu, H.-L.; Zheng, G.-W.; Ju, X.; Xu, J.-H., Efficient production of (R)-(−)-mandelic acid using glutaraldehyde cross-linked Escherichia coli cells expressing Alcaligenes sp. nitrilase. *Bioprocess and biosystems engineering* **2014,** *37* (7), 1241-1248.

449

Scheme and Figure Legends

Scheme 1. One-pot synthesis of PGA from racemic mandelic acids via cascade biocatalysis with recombinant *E. coli* cells containing the MR, DMDH and LDH.

Figure 1. The SDS-PAGE analysis for the potential recombinant mandelate racemases. (A) Lane M, PageRuler Prestained Protein Ladder; lane 1, the bacteria lysate supernatant of *E. coli*/pET28a-*ArMR*; lane 2, the bacteria lysate supernatant of *E. coli*/pET28a-*HrMR*; lane 3, the bacteria lysate supernatant of *E. coli*/pET28a-*BjMR-A*; lane 4, the bacteria lysate supernatant of *E. coli*/pET28a-*BjMR-C*; lane 5, the bacteria lysate sediment of *E. coli*/pET28a-*ArMR*; lane 6, the bacteria lysate sediment of *E. coli*/pET28a-*HrMR*; lane 7, the bacteria lysate sediment of *E. coli*/pET28a-*BjMR-A*; lane 8, the bacteria lysate sediment of *E. coli*/pET28a-*BjMR-C*. **(B)** Lane M, PageRuler Prestained Protein Ladder; lane 1, the bacteria lysate supernatant of *E. coli*/pET28a-*BjMR-D*; lane 2, the bacteria lysate supernatant of *E. coli*/pET28a-*BjMR-E*; lane 3, the bacteria lysate supernatant of *E. coli*/pET28a-*HhMR*; lane 4, the bacteria lysate supernatant of *E. coli*/pET28a-*ClMR*; lane 5, the bacteria lysate sediment of *E. coli*/pET28a-*BjMR-D*; lane 6, the bacteria lysate sediment of *E. coli*/pET28a-*BjMR-E*; lane 7, the bacteria lysate sediment of *E. coli*/pET28a-*HhMR*; lane 8, the bacteria lysate sediment of *E. coli*/pET28a-*ClMR*. **(C)** Lane M, PageRuler Prestained Protein Ladder; lane 1, the bacteria lysate supernatant of *E. coli*/pET28a-*ArMR*; lane 2, the bacteria lysate sediment of *E. coli*/pET28a-*ArMR*; lane 3, the bacteria lysate supernatant of *E. coli*/pET28a-*PpMR*; lane 4, the bacteria lysate sediment of *E. coli*/pET28a-*PpMR*. **(D)** Lane M, PageRuler Prestained Protein Ladder; lane 1, the

473 bacteria lysate supernatant of *E. coli*/pET28a-*ArMR*; lane 2, the purified recombinant *Ar*MR;
474 lane 3, the bacteria lysate supernatant of *E. coli*/pET28a-*HrMR*; lane 4, the purified
475 recombinant *Hr*MR.
476
477 **Figure 2. The temperature optima of the recombinant mandelate racemases.** The
478 temperature optima were measured using the above method except the reaction temperatures
479 ranging from 20 to 60°C.
480
481 **Figure 3. The pH optima of the recombinant mandelate racemases.** The pH optima
482 were assayed by the standard activity assay method as stated above with 100 mM
483 glycine-NaOH buffer over the pH range of 8.0−10.5, 20 mM citric acid-sodium citrate
484 buffer over the pH range of 5.0−6.0 and 100 mM Tris-HCl buffer over the pH range of
485 6.5−7.5.
486
487 **Figure 4. The effect of the tested metal ions and EDTA on activity of the recombinant**
488 **mandelate racemases.** The activity of mandelate racemase was assayed by the standard
489 activity assay method with various metal ions and EDTA at a final concentration of 1.0 mM.
490
491 **Figure 5. The conversion curves of racemic mandelic acid catalyzed by these**
492 **engineered *E. coli* strains.** The reactions were carried out at 30°C and 200 rpm for 48 h in
493 a 100-mL fermentation liquor containing 100 mM racemic mandelic acid without adding
494 coenzyme or cosubstrate.

Tables

Table 1 The half-life of mandelate racemases at various temperatures.

Enzyme	Half-life $t_{1/2}$ (h)		
	30 °C	40 °C	50 °C
*Ar*MR	70.7	27.2	0.17
*Hr*MR	85.2	53.1	1.4

Table 2 The kinetic parameters of mandelate racemases towards different substrates.

Enzyme	Substrates	K_M (mM)	k_{cat} (s^{-1})	V_{max} (μmol $min^{-1}mg^{-1}$)	k_{cat}/K_M ($mM^{-1}s^{-1}$)
*Ar*MR	D- mandelic acid	1.44 ± 0.2	409.8	582 ± 26.5	284.6
	L- mandelic acid	0.81 ± 0.1	218.3	310 ± 8.0	269.5
*Hr*MR	D- mandelic acid	1.68 ± 0.3	80.2	102 ± 14	47.7
	L- mandelic acid	1.37 ± 0.2	38.5	62 ± 8	28.1

Table 3 The catalytic activities of three enzymes by co-expression.

Strains	Catalytic activity (U·mL^{-1} fermentation liquor)		
	mandelate racemase	*D*-mandelate dehydrogenase	*L*-lactate dehydrogenase
TCD 01	40.7	25.8	194.2
TCD 02	45.2	192.4	188.1
TCD 03	190.4	23.2	213.8
TCD 04	**185.6**	**208.2**	**198.3**
TCD 05	36.1	26.9	210.4
TCD 06	33.2	212.6	206.5

Scheme 1.

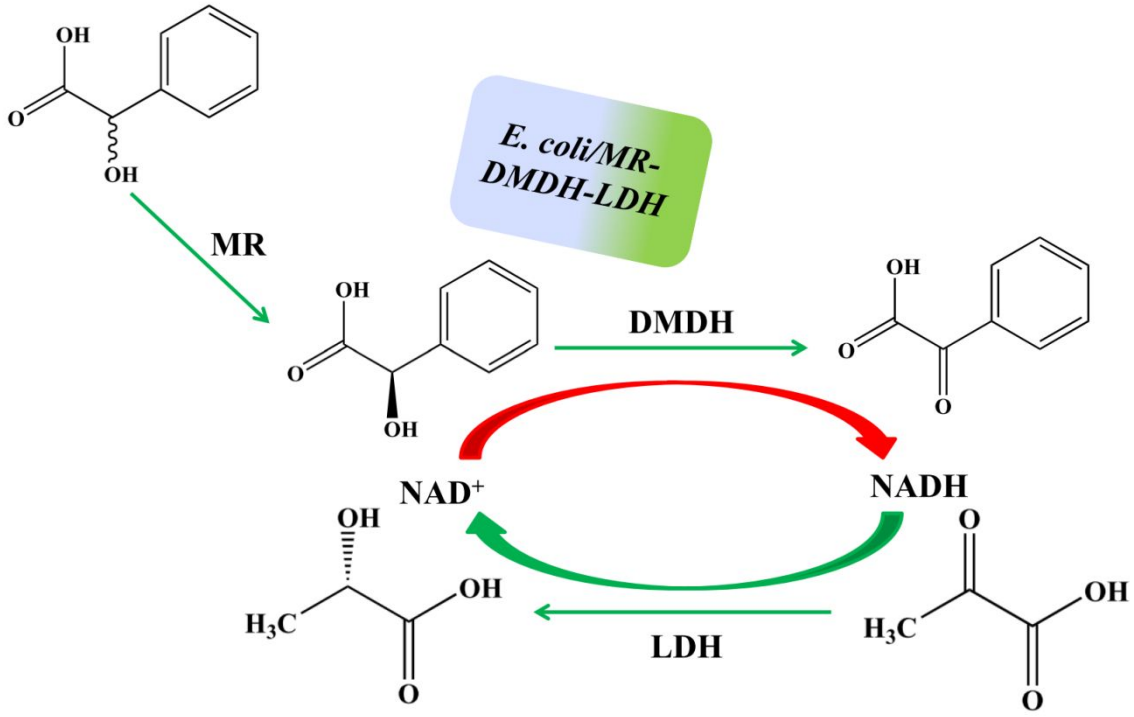

OH
O
OH
MR
E. coli/MR-DMDH-LDH
OH
O
OH
DMDH
OH
O
O
NAD+
NADH
OH
H3C
OH
O
LDH
O
H3C
OH
O

Figure 1A.

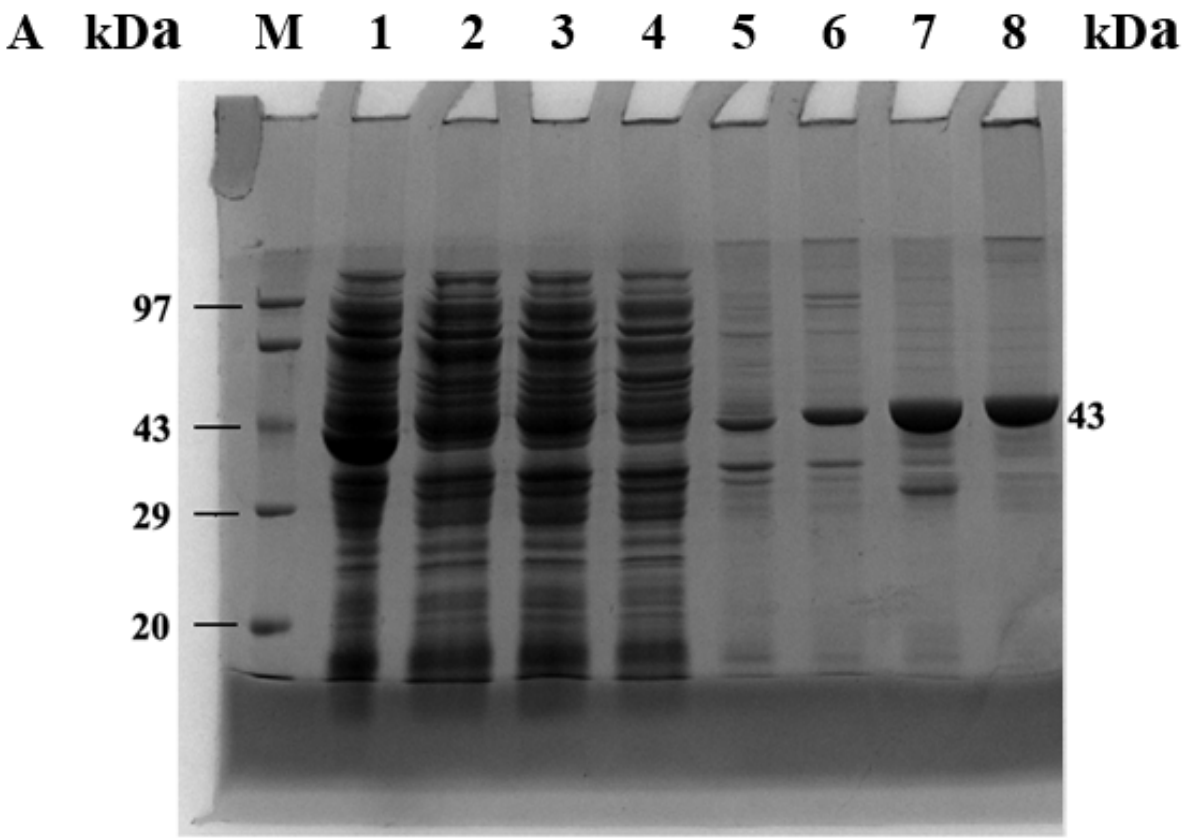


Figure 1B.

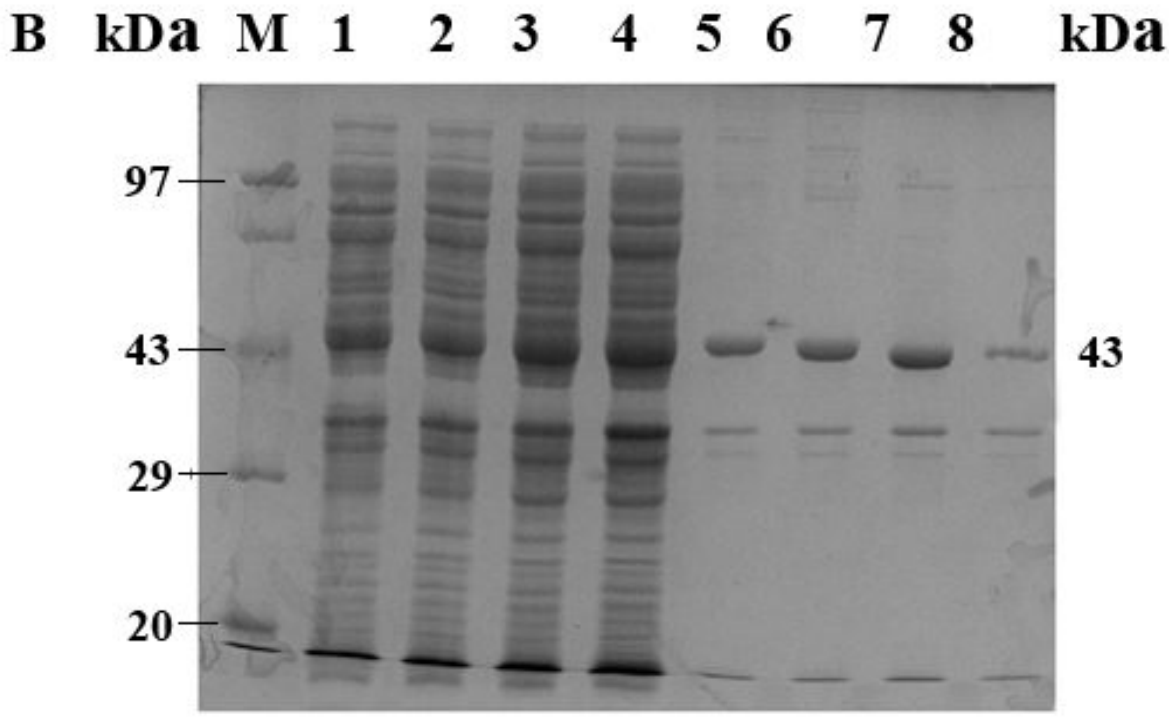


Figure 1C.

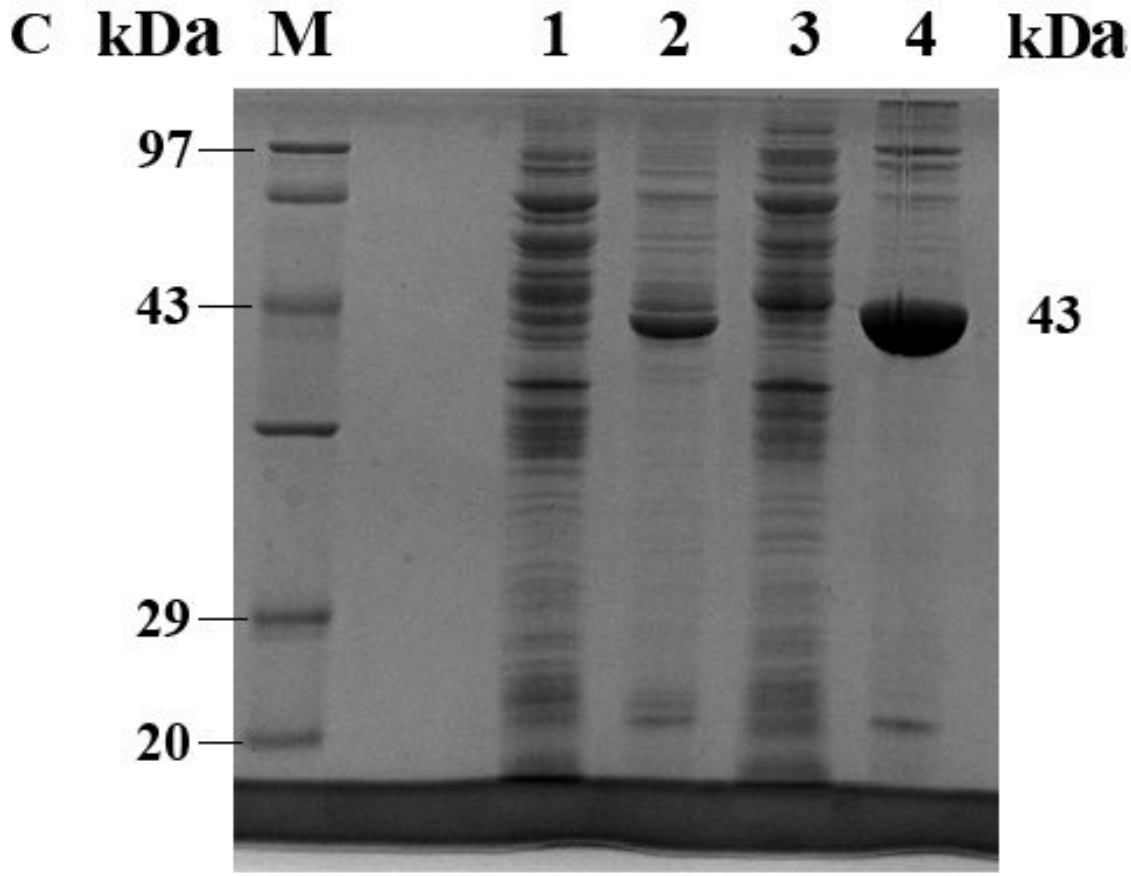


Figure 1D.

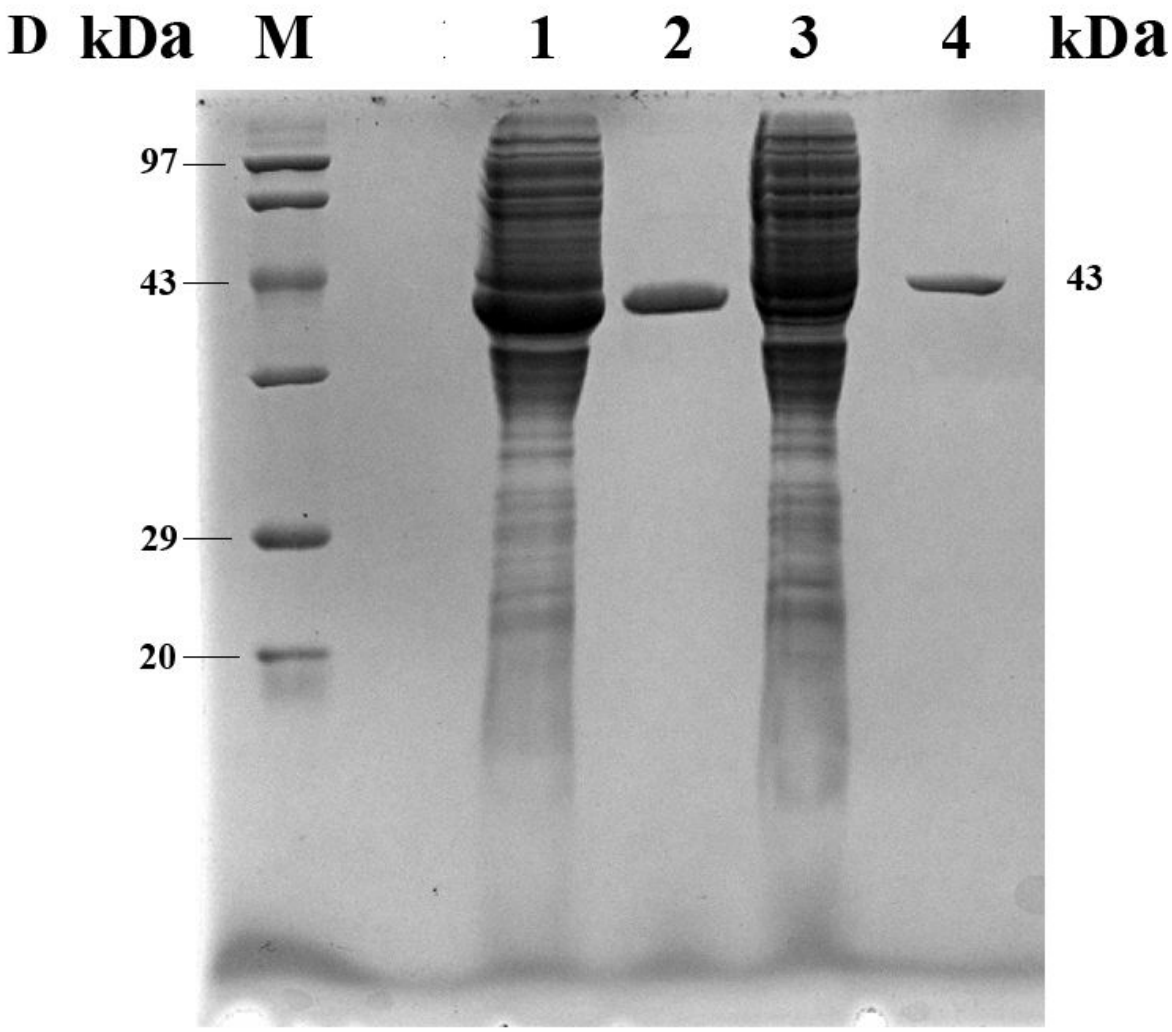


Figure 2.

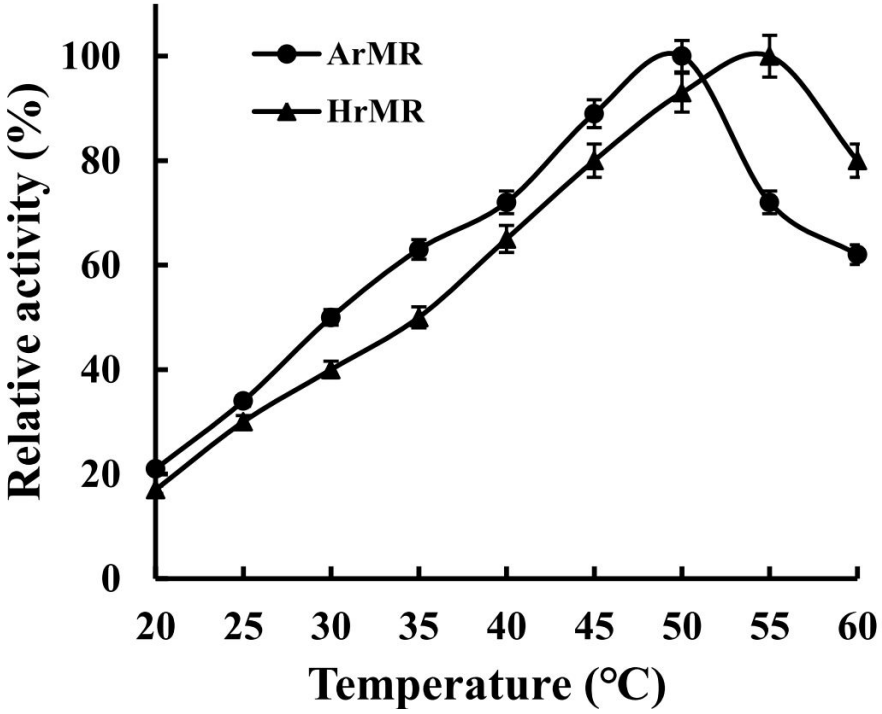

ArMR
HrMR
Relative activity (%)
100
80
60
40
20
0
20
25
30
35
40
45
50
55
60
Temperature (°C)

Figure 3.

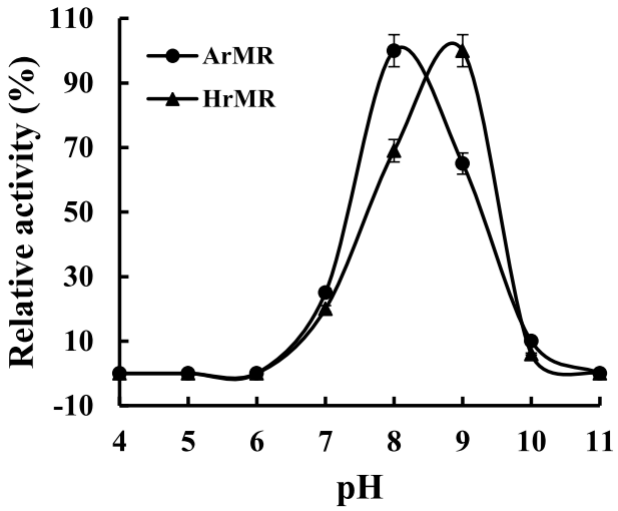

ArMR
HrMR
Relative activity (%)
110
90
70
50
30
10
-10
4
5
6
7
8
9
10
11
pH

Figure 4.

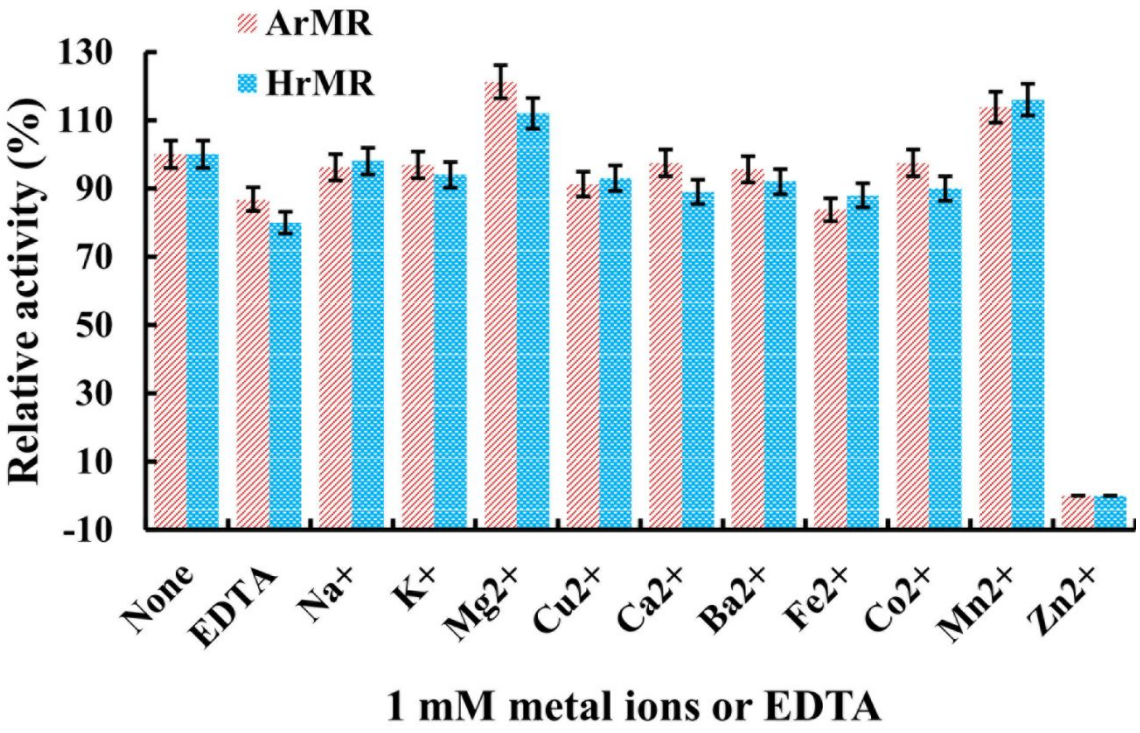
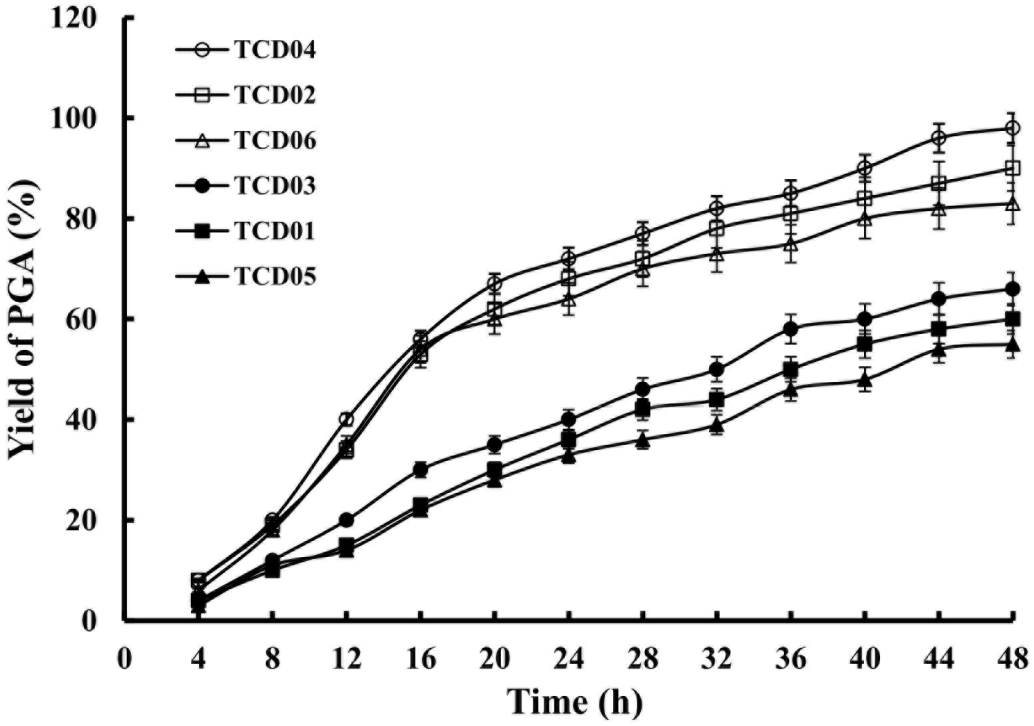
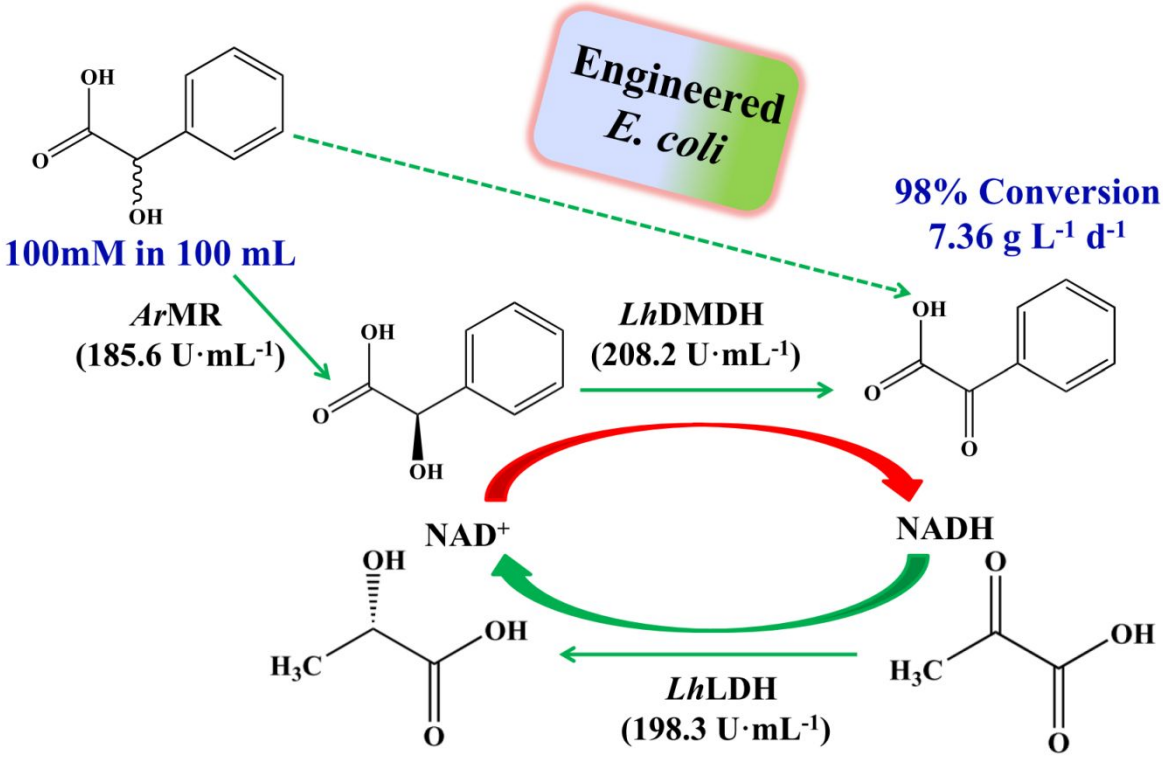

ArMR
HrMR
Relative activity (%)
130
110
90
70
50
30
10
-10
None
EDTA
Na+
K+
Mg2+
Cu2+
Ca2+
Ba2+
Fe2+
Co2+
Mn2+
Zn2+
1 mM metal ions or EDTA

Figure 5.


TCD04
TCD02
TCD06
TCD03
TCD01
TCD05
Yield of PGA (%)
Time (h)
0
20
40
60
80
100
120
0
4
8
12
16
20
24
28
32
36
40
44
48

TOC graphic


Engineered
E. coli
98% Conversion
7.36 g L-1 d-1
100mM in 100 mL
ArMR
(185.6 U·mL-1)
LhDMDH
(208.2 U·mL-1)
NAD+
NADH
LhLDH
(198.3 U·mL-1)