Simultaneous Directed Evolution of Coupled Enzymes for Efficient Asymmetric Synthesis of L-Phosphinothricin

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ABSTRACT The traditional strategy to improve the efficiency of an entire coupled-enzyme system relies on the separate direction of the evolution of enzymes involved in their respective enzymatic reactions. This strategy can lead to enhanced single-enzyme catalytic efficiency but may also lead to a loss of coordination among enzymes. This study aimed to overcome such shortcomings by executing a directed evolution strategy on multiple enzymes in one combined group that catalyzes the asymmetric biosynthesis of L-phosphinothricin. The genes of a glutamate dehydrogenase from Pseudomonas moorei (PmGluDH) and a glucose dehydrogenase from Exiguobacterium sibiricum (EsGDH), along with other gene parts (promoters, ribosomal binding sites [RBSs], and terminators), were simultaneously evolved. The catalytic efficiency of PmGluDH was boosted by introducing the beneficial mutation A164G (from 1.29 s⁻¹ mM⁻¹ to 183.52 s⁻¹ mM⁻¹), and the EsGDH expression level was improved by optimizing the linker length between the RBS and the start codon of gdh. The total turnover numbers of the bioreaction increased from 115 (GluDH_WT with low expression of GDH) to 5,846 (A164GNADPH coupled with low expression of EsGDH) and to 33,950 (A164GNADPH coupled with high expression of EsGDH). The coupling efficiency was increased from ~30% (GluDH_WT with low expression of GDH) to 83.3% (GluDH_A164G with high expression of GDH). In the batch production of L-phosphinothricin utilizing whole-cell catalysis, the strongest biocatalytic reaction exhibited a high space-time yield (6,410 g · liter⁻¹ · day⁻¹) with strict stereoselectivity (>99% enantiomeric excess).

IMPORTANCE The traditional strategy to improve multienzyme-catalyzed reaction efficiencies may lead to enhanced single-enzyme catalytic efficiencies but may also result in the loss of coordination among enzymes. We describe a directed evolution strategy for an entire coupled-enzyme system to simultaneously enhance enzyme coordination and catalytic efficiency. The simultaneous-evolution strategy was applied to a multienzyme-catalyzed reaction for the asymmetric synthesis of L-phosphinothricin, which not only enhanced the catalytic efficiency of GluDH but also improved the coordination between GluDH and GDH. Since this strategy is enzyme independent, it may be applicable to other coupled-enzyme systems for chiral chemical synthesis.

KEYWORDS directed evolution, coupled-enzyme system, L-phosphinothricin, asymmetric biosynthesis

The coupled-enzyme system for chiral chemical synthesis by whole-cell biocatalysis has exhibited several advantages over traditional step-by-step synthesis, including low catalyst cost, convenient downstream processing, and elimination of intermediate isolation steps (1–5). However, natural-enzyme-based biocatalytic reactions usually
exhibit low catalytic efficiencies and poor coordination between enzymes. Therefore, protein engineering, including directed evolution and semirational engineering, has been widely used to obtain the desired biocatalysts and biocatalytic reactions, such as KnowVolution (knowledge-gaining directed evolution) (6), MORPHING (mutagenic organized recombination process by homologous in vivo grouping) (7), CASTing (combinatorial active-site saturation) (8), TCSM (triple-code saturation mutagenesis) (9), and FRESCO (framework for rapid enzyme stabilization by computational libraries) (10).

The traditional sequential strategy of protein engineering to improve the efficiency of multienzyme-involved whole-cell biocatalysis relies on the separate direction of the evolution of enzymes involved in their respective enzymatic reactions; (B) the simultaneous strategy: functional enzyme genes and other gene parts are mutated simultaneously; (C) workflow of the simultaneous strategy for multiple enzymes in one combined group: (i) mutagenesis of all the gene parts simultaneously, (ii) gene expression for the generation of a gene part library, (iii) screening of the gene library, and (iv) isolation of the genes from improved mutants.
The commercialized PPT contains two isomers, but only the L-enantiomer (L-PPT) isomer exhibits herbicidal activity (13). Thus, the production of optically pure L-PPT is of considerable significance for improving atomic utilization, decreasing the cost, and reducing the pressure on the environment. Enzyme kinetic resolution of racemic D,L-PPT or its precursors to produce L-PPT is a convenient route when using acylhydrolase, amidase (15), deacetylase, and nitrilase for L-PPT biosynthesis (16). It often reaches a high enantiomeric excess (ee) value of the product but demonstrates only a theoretical maximum yield of up to 50%. To overcome this restriction, glutamate dehydrogenase (GluDH) (EC 1.4.1.X) with a glucose dehydrogenase (GDH)-catalyzed enzymatic reaction has attracted considerable attention from chemists (17, 18). In this route, L-PPT is synthesized by the asymmetric reductive amination of PPO {2-oxo-4-[(hydroxy)(-methyl)phosphinyl]butyric acid} with strict enantioselectivity (19, 20) and a 100% theoretical maximum yield (21–23). NADP⁺ is also generated in this process. GDH is coupled with β-α-glucose, which is oxidized to D-glucono-δ-lactone, to recycle the coenzyme (24, 25) (Fig. 2). Enzyme-associated asymmetric reductive amination, which exhibits many advantages such as high stereoselectivity, a high theoretical maximum yield (100%), and a high equilibrium constant, is a promising method for the synthesis of chiral amino acids (22, 23). Currently, due to the low activity and narrow substrate scope of GluDH, protein engineering works are primarily focused on GluDH activity improvement and substrate spectrum extension (26). However, for a coupled-enzyme system, the engineering of only one enzyme is insufficient. The coordination between GluDH and GDH is an important factor that affects the efficiency of a biocatalytic reaction. The increased similarities of coenzyme preference and optimal enzymatic properties would boost the reaction efficiency and increase the product yield (27, 28). In contrast, directed enzyme evolution without considering the coordination between enzymes may decrease the efficiency of the biocatalytic reaction.

Here, we developed and executed a directed evolution strategy on a coupled-enzyme system in which functional enzyme genes (Pseudomonas moorei GluDH [PmGluDH] and Exiguobacterium sibiricum GDH [EsGDH]) and other gene parts (promoters, RBSs, and terminators) were mutated simultaneously, followed by library construction and screening (Fig. 1C). The resulting beneficial mutants not only increased the enzyme catalytic efficiency but also improved the cofactor recycling efficiency in this coupled-enzyme system.

RESULTS AND DISCUSSION

Construction of a multienzyme-catalyzed reaction for L-PPT synthesis. Previously, directed enzyme evolution was executed in a sequential manner (Fig. 1A) on PmGluDH and EsGDH, which rendered an improved variant (PmGluDH_WT+V375S, resulting from a V375S substitution in wild-type PmGluDH) but a low coordination efficiency between GluDH and GDH (14). In this study, the directed evolution strategy was first performed on EsGDH; however, no beneficial substitutions were obtained after screening more
than 5,000 clones. Given that the beneficial mutations may not exist in the functional gene region, we intended to mutate the entire coupled-enzyme system simultaneously, including functional enzyme genes (PmGluDH and EsGDH) and other gene parts (promoters, RBSs, and terminators). The PmGluDH and EsGDH genes were cloned into a commercialized pETduet-1 vector to construct a multienzyme-catalyzed reaction for L-PPT synthesis (Fig. 2). The GluDH gene was inserted into the first open reading frame (ORF) between SacI and NotI, and the GDH gene was cloned into the second ORF between BglII and PacI (see Fig. S1 in the supplemental material). After validation by gene sequencing, the recombinant plasmid was introduced into Escherichia coli BL21 (DE3) for gene expression. The two enzymes were produced by isopropyl-β-D-thiogalactopyranoside (IPTG) induction after 16 h. Using lyophilized E. coli cells containing PmGluDH and EsGDH as biocatalysts, the conversion from 300 mM PPO to L-PPT showed a value of 11.5% after 12 h (with no exogenous NADP⁺ added) (Fig. S2). However, the theoretical maximum yield of phosphinothricin is higher than 99.9% when the concentrations of glucose and amine ion are both in excess because the equilibrium constant (Kₑq) of the glucose dehydrogenase-catalyzed reaction is remarkably high (>1 × 10¹⁵) (29), and gluconolactone (the product of the glutamate dehydrogenase reaction) spontaneously hydrolyzes to gluconic acid, thereby providing a favorable equilibrium. Therefore, the efficiency of the entire coupled-enzyme system (PmGluDH_WT and EsGDH) for L-PPT synthesis needs to be improved.

Development of an evolutionary strategy to simultaneously improve enzyme catalytic efficiency and coordination between enzymes. Many factors affect the final yield of phosphinothricin, such as the catalytic efficiencies of enzymes and gene expression levels. A simultaneous strategy was applied to evolve the entire coupled-enzyme system, which contains four phases (Fig. 1C). In the first phase, random mutagenesis was performed at the whole-gene level, including functional gene parts and promoters (30). Three different concentrations of MnCl₂ (0.10, 0.15, and 0.20 mM) were used for the generation of libraries using error-prone PCR (epPCR) for mutagenesis. After the screening of 96 clones in a microtiter plate for each library, active mutant ratios of the three libraries were 78%, 46%, and 24%, respectively. The library (0.15 mM Mn²⁺ used) generated 54% active mutants, of which a 1.2% average mutation rate was used to select for further screening because ~50% active and inactive clones had previously been used successfully in directed evolution to improve enzymatic properties (31). Subsequently, one round of simultaneous evolution was executed. About 8,000 clones were screened by using our previously developed fluorescence-based high-throughput screening assay (32) in which PPT was derivatized and detected in a microtiter plate reader. Forty-eight clones were selected after 1st-round screening because they displayed >1.5-fold-higher apparent conversion (toward 300 mM PPO) than the PmGluDH_WT-catalyzed reaction. After rescreening, three beneficial clones were obtained that showed a >3-fold improvement in PPO conversion. Among them, one clone with the A164G substitution showed ~6-fold-higher apparent conversion than the PmGluDH_WT-catalyzed reaction toward 300 mM PPO. Another clone, which possessed R205K, displayed 3-fold-higher apparent conversion. These two substitutions (A164G and R205K) were located in the functional gene (PmGluDH gene) region. The third clone harbored both R205K and a base deletion. The base deletion was located in the linker between the RBS and the start codon of GDH, which led to the shortening of the linker from 5'-ATATACATCCAGAT-3' to 5'-ATATACATCCAGA-3'.

Identification of the improved mutants and recombination. To identify the beneficial substitution and investigate its role, we generated three mutants with single mutations or deletions using the conversion of PPO amination as an indicator to evaluate the reaction efficiency. The mutant with a single substitution, A164G in GluDH, exhibited improved conversion from 11.5% to 63.6% (with no exogenous NADP⁺ added [a 12-h reaction]) toward 300 mM PPO, indicating that a small and nonpolar residue was preferred at this position. Site saturation mutagenesis was then used to investigate the best amino acid substitution at this position, and it was found that only Gly at this position would boost conversion (Table S1). Although the structural change
introduced by the Ala-to-Gly substitution is small, it causes significant effects on GluDH properties. The beneficial substitution of Ala to Gly has also been reported in other studies; for example, Scrutton et al. reported that a single A179G mutation in a dehydrogenase decreased its $K_m$ value by half toward NADPH (33). Another study reported that the A284G mutation (as one of the beneficial mutations) was obtained in a transaminase variant, ATA-117 Rd11, used for sitagliptin manufacture (34). The mutant with R205K in GluDH showed a slight increase in PPO conversion (from 11.5% to 21.3%, with no exogenous NADP$^+$ added [a 12-h reaction]), and only the positively charged residues were preferred at this position (Table S1). In the third mutant, the short linker between the RBS and the starting codon resulted in improved conversion (from 11.5% to 35.1% toward 300 mM PPO, with no exogenous NADP$^+$ added [a 12-h reaction]) and increased GDH gene expression. Given that no other mutation was present in the composite gene part, we hypothesized that the improvement was caused by the single-base deletion. To verify this hypothesis, we designed different lengths of linkers and inserted them between the RBS and the starting codon of GDH. Table S2 and Fig. S3 show that an 8-bp linker led to the highest PPO conversion in GDH as well as increased gene expression. The expression level of GDH was increased from 0.09 g/liter (with a 14-bp linker) to 2.87 g/liter (with an 8-bp linker). Importantly, the gene expression element was ignored by our previous sequential evolution. Furthermore, Howard Salis’s RBS Calculator (https://salislab.net/software/predict_rbs_calculator) was used to predict the translation initiation rates of the mRNAs containing the linkers with different lengths. Interestingly, the 8-bp linker led to the highest translation rate of mRNA (Table S2), which matched the experimental results (an 8-bp linker resulted in the highest PPO conversion).

Recombination of the two beneficial substitutions (A164G and R205K) in the GluDH gene did not further increase the L-PPT yield (A164G, 63.6%; R205K, 21.3%; A164G + R205K, 54.1% [toward 300 mM PPO with no exogenous NADP$^+$ added [a 12-h reaction]]). In the final gene mutant for the multienzyme-catalyzed reaction, A164G was introduced into GluDH with the optimal linker length (8 bp), which achieved the highest conversion of 300 mM PPO (99.1% with no exogenous NADP$^+$ added [a 4-h reaction]) (Table 1).

**TABLE 1** Comparison of the conversions of different GluDHs with EsGDH$^a$

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Conversion (%)</th>
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<tbody>
<tr>
<td>GluDH_WT + low expression of EsGDH</td>
<td>11.5$^a$</td>
</tr>
<tr>
<td>GluDH_WT + high expression of EsGDH</td>
<td>35.1$^b$</td>
</tr>
<tr>
<td>GluDH_A164G + low expression of EsGDH</td>
<td>63.6$^a$</td>
</tr>
<tr>
<td>GluDH_A164G + high expression of EsGDH</td>
<td>99.1$^c$</td>
</tr>
<tr>
<td>GluDH_R205K + low expression of EsGDH</td>
<td>21.3$^b$</td>
</tr>
<tr>
<td>GluDH_R205K + high expression of EsGDH</td>
<td>67.7$^b$</td>
</tr>
</tbody>
</table>

$^a$Reaction conditions were as follows: 0.5 g dry cell weight (DCW) of lyophilized *E. coli* cells, 300 mM PPO, 750 mM ammonium, and 360 mM glucose in phosphate buffer (pH 7.5) at 40°C.

$^b$Reaction time of 12 h.

$^c$Reaction time of 4 h.

Evaluation of the coordination between GluDH and GDH in the PPO amination reaction. Cofactor recycling and coupling efficiencies are two key factors for evaluating the coordination between GluDH and GDH. The coupling efficiency of GluDH_WT with low expression of GDH was about 30% (the molar ratio of glucose to PPO is about 3:1). After simultaneous evolution, the coupling efficiency was increased to 83.3% (molar ratio of about 1:2:1). Table 2 summarizes the cofactor recycling efficiency of GluDH_WT, and GluDH_A164G$^{NADPH}_{-}$-catalyzed reactions in the absence of GDH or in the presence of low or high expression of GDH. For the generation of the GDH-absent system, genes of *PmGluDH_WT* and GluDH_A164G were cloned into empty pETDuet-1 with the same cloning sites. Without the NADPH regeneration system (GDH/glucose), the total turnover numbers (TTNs) remained at a low level. The TTNs of the *PmGluDH_WT*- and the variant *PmGluDH_A164G*-catalyzed reactions were <200. The TTN was notably increased to 5,846 when the coenzyme regeneration system was
linked to the \( \text{PmGluDH}_{A164G} \)-catalyzed reaction. The highest TTN (33,950) was observed for the engineered GluDH coupled with high expression of GDH, indicating that the optimal cofactor recycling efficiency was achieved. The TTN of \( \text{PmGluDH}_{V375S} \)-catalyzed reactions (obtained from directed enzyme evolution in a sequential manner) was 63% lower than that of the \( \text{PmGluDH}_{A164G} \)-catalyzed reaction with low expression of GDH (3,679 versus 5,846) and only 8.7% of the TTN of the \( \text{PmGluDH}_{A164G} \)-catalyzed reaction with high expression of GDH (2,979 versus 33,950). The high expression of GDH could not be obtained by sequential directed evolution because the region from the RBS to the start codon was not covered in the evolution strategies. This is one of the advantages of simultaneous evolution over sequential evolution.

**Characterizations of \( \text{PmGluDH}_{WT} \) and the variant \( \text{PmGluDH}_{A164G} \).** The effects of temperature and pH on purified GluDH activity were evaluated. The \( \text{PmGluDH}_{WT} \) activity was tested at temperatures ranging from 25°C to 60°C, and the optimal temperature was 40°C (Fig. S4A). Within the pH range of 5.0 to 10.0, the highest activity of \( \text{PmGluDH}_{WT} \) was observed at pH 7.0 in phosphate buffer (Fig. S4B). When the pH was lower than 6.0 or higher than 8.5, the relative activities sharply decreased to less than half. A similar pH spectrum was also observed for \( \text{PmGluDH}_{A164G} \) (Fig. S4D). The optimal temperature of the variant \( \text{PmGluDH}_{A164G} \) was also 40°C, but its relative activity was lower than that of \( \text{PmGluDH}_{WT} \) at 45°C (Fig. S5). The half-life values of GluDH\(_{A164G}\) were lower than those of GluDH\(_{WT}\), but its \( k_p \) (dissociation constant) values were higher (Fig. S5 and Table S3). This suggested that GluDH\(_{A164G}\) had lower thermostability than GluDH\(_{WT}\), which was also observed in the circular dichroism spectroscopy analysis (Fig. S6). The change in the protein folding free energy (\( \Delta \Delta G \)) is an important characteristic directly related to protein thermostability. FoldX software was used for \( \Delta \Delta G \) prediction and calculation (\( \Delta \Delta G = \Delta G_{\text{mutant}} - \Delta G_{\text{WT}} \)). The positive \( \Delta \Delta G \) value (\( \Delta G_{\text{A164G}} - \Delta G_{\text{WT}} = 1.11 \text{ kcal/mol} \)) indicated that GluDH\(_{A164G}\) was less stable than GluDH\(_{WT}\). The kinetic parameters of purified \( \text{PmGluDH}_{WT} \) and its “best” variant, \( \text{PmGluDH}_{A164G} \) (both with His tags), were determined using phosphate buffer (pH 7.0 at 40°C) (for sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE] results, see Fig. S7 in the supplemental material). The variant \( \text{PmGluDH}_{A164G} \) showed considerable improvements in turnover numbers (\( k_{\text{cat}} \)) toward PPO, from 8.33 s\(^{-1}\) (WT) to 958.1 s\(^{-1}\) (A164G) (Table 3 and Fig. S8). In comparison with other reported GluDHs toward the same substrate, PPO, the \( k_{\text{cat}} \) of the obtained \( \text{PmGluDH}_{A164G} \) was 150-fold higher than that of GluDH\(_{WT}\) from *Pseudomonas putida* (\( \text{PpGluDH}_{WT} \)) (\( k_{\text{cat}} = 6.33 \text{ s}^{-1} \)) (26). The substrate affinity of the best variant, \( \text{PmGluDH}_{A164G} \), was slightly greater than that of the WT \( (K_m \text{ values of } 6.17 \text{ mM versus } 5.22 \text{ mM}) \). The high \( k_{\text{cat}} \) and moderate substrate affinity resulted in a high catalytic efficiency (\( k_{\text{cat}}/K_m \) ratio) toward PPO, which was significantly increased from 1.29 s\(^{-1}\) · mM\(^{-1}\) (WT) to 183.52 s\(^{-1}\) · mM\(^{-1}\) (A164G) (Table 3). However, the \( k_{\text{cat}} \) value of \( \text{PmGluDH}_{A164G} \) toward NADPH was slightly higher than

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**TABLE 2** Total turnover numbers of PPO amination with GluDH \( \text{WT}^{\text{NADPH}}, \text{A164G}^{\text{NADPH}}, \) and \( \text{V375S}^{\text{NADPH}} \) in the absence of GDH, in the presence of low expression of GDH, and in the presence of high expression of GDH

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mean TTN (( \mu \text{mol total product} \cdot \mu \text{mol catalyst}^{-1} )) ± SD(^{\text{a}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Only GluDH(^{b})</td>
<td>115 ± 7</td>
</tr>
<tr>
<td>GluDH + low expression of GDH(^{b})</td>
<td>281 ± 13</td>
</tr>
<tr>
<td>GluDH + high expression of GDH(^{b})</td>
<td>547 ± 35</td>
</tr>
</tbody>
</table>

\(^{a}\)TTN, total turnover number. The total product is the sum of all quantified \( \mu \text{-PPT} \) in 5 min: TTN = product/

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\(^{b}\)The reaction mixture contained PPO (100 mM) in phosphate buffer (100 mM; pH 7.5), NADPH (0.5 mM), \( \text{NH}_4^+ \) (400 mM), and dry cells (0.5 g/liter).

\(^{c}\)“Glucose” (120 mM) in addition to GluDH.
that of \( Pp \text{GluDH\textunderscore WT} \), where the \( K_m \) values were similar (Table 3). In the \textit{in silico} analysis, the PPO was docked into the active sites of \( Pm \text{GluDH\textunderscore WT} \) and \( Pm \text{GluDH\textunderscore A164G} \). The results showed that the methyl phosphinyl group of PPO was properly accommodated in the enlarged cave (volume increased from 402 Å\(^3\) to 432 Å\(^3\)) (Fig. 3), thereby contributing to the increased catalytic efficiency.

**Characterizations of the engineered coupled-enzyme system harboring GluDH\textunderscore A164G with high expression of GDH.** The effects of several parameters were investigated for the high conversion of the engineered bioreaction. The molar ratio between glucose and PPO was optimized, and conversion reached a high level when this ratio was higher than 1.2:1 (Fig. S9). The best ammonium donor for PPOamination was found to be \((\text{NH}_4)_2\text{SO}_4\) after analysis of five different donors (Fig. S9). The optimal molar ratio of \( \text{NH}_4^+ \) to PPO was more than 4:1. The optimal temperature and pH were 40°C and 7.5 (phosphate buffer) (Fig. S9), respectively. The engineered bioreaction showed a broad applicable range of temperatures (from 25°C to 55°C) and preferred an alkaline environment (pH 7.0 to 8.0), suggesting the potential practical applications of this bioreaction under industrial conditions.

**Asymmetric amination synthesis of \( \text{L-PPT} \) by the improved bioreaction.** To evaluate the practical applications of the biocatalytic bioreactions, we used 100 to 1,000 mM PPO as the substrate for the desired asymmetric amination synthesis of \( \text{L-PPT} \). For the reaction, 100 mM PPO (17.8 g · liter\(^{-1}\)) was mixed with 120 mM glucose as the cosubstrate, 150 mM \((\text{NH}_4)_2\text{SO}_4\) as the amino donor, and 0.5 mM extra cofactors. The substrate was completely converted to \( \text{L-PPT} \) (\( >99\% \) conversion and \( >99\% \) ee) within 5 min by using 0.5 g · liter\(^{-1}\) lyophilized \( E. \text{coli} \) possessing the improved bioreaction mixture. The conversion rate can reach only \( \sim 26\% \) (25.68 mM after 3 h) by using the initial bioreaction (GluDH\textunderscore WT with low GDH expression) under the same reaction conditions (Fig. 4). When substrate loading was increased to 500 mM (89.04 g · liter\(^{-1}\)) and 1 M (178.08 g · liter\(^{-1}\)), the conversion rates were close to 100% after 20 min and 40 min, respectively, by using the same amount of the biocatalyst (0.5 g · liter\(^{-1}\) dry cells) harboring GluDH\textunderscore A164G with high expression of GDH, yielding \( \text{L-PPT} \) with an ee of \( >99\% \). In comparison with other reported GluDH-involved bioprocesses, the reaction time was shortened from 15 h to <1 h (26). The space-time yield (STY) of the best bioprocess (GluDH\textunderscore WT+ A164G with high expression of GDH) reached up to 6,410 g · liter\(^{-1}\) · day\(^{-1}\), which was 173-fold higher than that of the initial bioreaction (37 g · liter\(^{-1}\) · day\(^{-1}\)) and 1.35-fold higher than that of the GluDH3-GDH-catalyzed reaction (14). In comparison with the amidase- and transaminase-catalyzed reactions, the engineered bioreaction displayed the highest conversion and shortest reaction time even against the highest PPO concentration (178.08 g/liter) (Table 4). Therefore, because of the excellent enantioselectivity of \( \text{L-PPT} \) (\( >99\% \) ee) (Fig. S10 and S11) and the highest STY documented thus far, the improved biocatalytic asymmetric amination is promising for the synthesis of \( \text{L-PPT} \) on an industrial scale.

**Conclusions.** The coupled-enzyme-catalyzed reaction for the asymmetric synthesis of \( \text{L-PPT} \) is an interesting alternative to chemical synthesis routes despite existing challenges such as the poor catalytic efficiency and insufficient cofactor recycling efficiency. Here, a directed evolution strategy was executed on multiple enzymes in one combined group, which yielded a catalytic-efficiency-improved \( Pm \text{GluDH} \) variant (A164G)
with a high expression level of GDH. The TTN of the multienzyme-catalyzed reaction increased from 281 (GluDH_WT with low expression of EsGDH) to 33,950 (GluDH_A164G with high expression of EsGDH), which suggested that the cofactor recycling efficiency of the reaction was substantially increased. In the batch production of L-PPT toward 1 M PPO, the engineered bioreaction exhibited a high STY (6,410 g · liter⁻¹ · day⁻¹) with strict stereoselectivity (>99% ee) of L-PPT. This STY appears to be the highest among all reported values for L-PPT synthesis. As the simultaneous strategy is an enzyme-independent strategy, it may shed light on other multiple-enzyme evolution strategies that aim to enhance enzyme catalytic efficiency and improve coordination.

MATERIALS AND METHODS

Experimental materials. The compound PPO (purity of 95%) was synthesized by Lubachem Co., Ltd. (Shandong, China), and characterized by nuclear magnetic resonance (NMR) (see Fig. S12 in the supplemental material). The d,l-phosphinothricin standard and NADPH (NADP⁺) were purchased from Sigma-Aldrich (Shanghai, China). All other chemicals and reagents were at least chemically pure and obtained commercially. Taq DNA polymerase, Phanta Max Super-Fidelity DNA polymerase, and a one-step cloning kit were purchased from Vazyme (Nanjing, China). The PCR cleanup kit and plasmid miniprep kit were purchased from Axygen (Shanghai, China). T4 DNA ligases were purchased from Thermo Fisher Scientific (Shanghai, China). Restriction endonucleases and DpnI were purchased from New England BioLabs, Inc. (Shanghai, China).

Genes, plasmids, and microorganisms. The gene of an amino acid dehydrogenase (GenBank acces-
The amplified PCR products were doubly digested with the corresponding restriction endonucleases and inserted into the expression vector pETDuet-1. The PCR program was operated as follows: 95°C for 3 min (1 cycle); 95°C for 15 s, 53°C to 58°C for 15 s, and 72°C for 1.5 min (25 cycles); and 1 cycle of 72°C for 10 min. The recombinant plasmid (pETDuet-PmGluDH) was transformed into E. coli BL21(DE3), and its sequence was verified by sequencing. Positive strains verified by sequencing were stored in a −80°C freezer. The amplified PCR products of EsGDH genes were inserted into the second multiple-cloning site of the pETDuet-1 vector to couple PmGDH with EsGDH (GenBank accession number KM817194.1) using the inFusion HD cloning kit (for primers, see Table 5).

TABLE 5 Site-directed mutagenesis primers used in this work

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
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<tbody>
<tr>
<td>PmGluDH-F</td>
<td>GAGGCTAGATGAGGACGCTGAGCTCTTCTT</td>
</tr>
<tr>
<td>PmGluDH-R</td>
<td>GCCGCGCCTGAGACGCCCCCTGTCGCA</td>
</tr>
<tr>
<td>EsGDH-F</td>
<td>GAGATATACATCCAGATATGGGTATAATTCTCTGAAGGCAAGT</td>
</tr>
<tr>
<td>EsGDH-R</td>
<td>GCAGCAGCTAGGTAAATATATCAACCCAGGCCAGCTGAAAGCT</td>
</tr>
<tr>
<td>(EsGDH)pETduet-F</td>
<td>TTAATACCTAGGTCGTGCAACCG</td>
</tr>
<tr>
<td>(EsGDH)pETduet-R</td>
<td>ATCTGGATGATATCTCTCTTTAT</td>
</tr>
<tr>
<td>SimVolution-F</td>
<td>ATGATTGAGAGCGTCGAGCTCTTCTT</td>
</tr>
<tr>
<td>SimVolution-R</td>
<td>TCAACCCACGGCCAGCTGAAAGC</td>
</tr>
<tr>
<td>PmGluDH-205-F</td>
<td>GCCGATTGTTGATNTNCCAGAAAGCTACC</td>
</tr>
<tr>
<td>PmGluDH-205-R</td>
<td>GTGACATCTCGGMINATCAACTG GCC</td>
</tr>
<tr>
<td>PmGluDH-164-F</td>
<td>GTCGATGTGCCANNGKGAGATTTGGGG</td>
</tr>
<tr>
<td>PmGluDH-164-R</td>
<td>ACCGCCATATCTCCMNNTGGGCAATCGA</td>
</tr>
</tbody>
</table>

This work
Construction of mutant libraries and screening. Three error-prone PCR (epPCR) libraries (with 0.10, 0.15, and 0.20 mM Mn

were generated under standard conditions using pETDuet-PmGDH-EsGDH as a template. The purified epPCR products were cloned into the pETduet-1 vector by using the MEGAWHOP (megaprimer PCR of whole plasmid) cloning method (35). The DpnI-digested MEGAWHOP product was transformed into E. coli BL21(DE3) for expression and screening. Single colonies were picked from agar plates and inoculated into 1.0 ml of LB medium (containing 135 μM ampicillin) in each well of a 96-well plate. After culturing at 37°C for 8 h, 200 μl of the seeding culture was transferred to another 96-well plate containing 800 μl of LB medium (with 135 μM ampicillin and 0.1 mM IPTG) and shaken at 22°C for protein production (200 rpm for 16 h). The cultures were centrifuged at 4,000 rpm for 10 min, and the cells were resuspended in 0.1 M phosphate buffer (pH 7.5). The reaction using a mixture (500 μl) containing 300 mM PPO, 750 mM ammonium, and 360 mM glucose was performed at 40°C in a Thermomixer compact instrument (Eppendorf, Germany) for 1 h. α-Phthalaldehyde (OPA) (4 mM) and N-acetyl-l-cysteine (NAC) (4.5 mM) were added to the supernatants for derivatization, and the mixture was incubated at 30°C for 5 min, followed by a measurement of the derivatized PPT at an excitation wavelength (λex) of 340 nm and an emission wavelength (λem) of 450 nm. The improved variants were sequenced and stored in a 30% glycerol solution at −80°C.

Site-directed mutagenesis and site saturation mutagenesis. Site-directed/saturation mutagenesis of GluDH at positions 164 and 205 was performed using a QuikChange mutagenesis kit with Phanta Max Super-Fidelity DNA polymerase (Vazyme, Nanjing, China). PCR was performed as previously reported (6), and the primers are listed in Table 5. The PCR products were digested with FastDigest DpnI at 37°C for 15 min and then transformed into E. coli BL21(DE3) competent cells.

Assays for GluDH activity detection. Standard assays were executed using a mixture of the substrate PPO (100 mM final concentration), an amino donor (200 mM NH4+/SO42~), the coenzyme NADPH (10 mM), and the purified enzyme in phosphate buffer (100 mM at pH 7.0). The reaction mixture was incubated at 40°C for 10 min with shaking at 600 rpm in the Thermomixer, and the reactions were stopped by the addition of a 6 M HCl solution.

The concentration and enantiomeric excess (ee) of products (D,L-PPT) were determined by a precolumn derivatization method. The derivatized sample (10 μl) was eluted at 35°C with 45 mM ammonium acetate buffer (pH 5.7)–methanol (9:1, vol/vol) as the mobile phase at a flow rate of 1 ml/min in a high-performance liquid chromatography (HPLC) system equipped with a C8 column (5 μm by 250 mm by 4.6 mm; Acchrom Technologies Co., Ltd.). The parameters used for the detection of D,L-PPT by a fluorescence detector were set at 340 nm (λem) and 305 nm (λex) of products (D,L-PPT) were determined by a precol

multi-enzyme-catalyzed biosynthesis of L-PPT in a whole-cell catalysis form. E. coli cells harboring PmgluDHs and GDH were used as the catalyst for the synthesis of L-PPT. The reaction mixture consisted of 1 M PPO, 1.2 M glucose, 1 M ammonium sulfate, 0.5 mM NADPH, and a certain amount of recombinant E. coli cells. The reaction mixtures were incubated at 40°C at 500 rpm in a pH-Stat bioreactor (902 Titrandot; Metrohm, Shanghai, China). The reaction pH was automatically adjusted to 7.5 during the whole reaction by adding 0.13 M NH4+/H2O. At fixed time intervals, conversion was determined by measuring the l-PPT concentration using the HPLC. According to previous reports (36, 37), the formed product was purified and characterized using polarimetry ([α]D25° = +16.9 (c = 1, H2O)), and NMR analysis was performed using an NMR spectrometer with D2O as the solvent with carbon and proton determinations at 500 and 126 MHz.

Effects of different temperatures and pHs on the activity of purified enzymes. The effects of temperature on GluDH activity were determined at different temperatures (25°C to 60°C) in phosphate buffer (100 mM at pH 7.0). The reaction mixture was incubated at 40°C for 10 min with shaking at 600 rpm in the Thermomixer, and the reaction was initiated by the addition of a 6 M HCl solution.

Determination of enzyme kinetic parameters and total turnover numbers. Kinetic analyses of wild-type and mutant PmgluDHs toward PPO and NADPH were carried out using 100 mM phosphate buffer (pH 7.0) at 40°C. A pseudo-one-substrate kinetic model was used under a fixed concentration of the cosubstrate to obtain the kinetic parameters (26, 38). To calculate the kinetic data toward PPO, three independent initial rate (the first 10-min reaction period) measurements were performed with the same batch of purified enzyme over 0.01 to 0.5 mM NADPH with 100 mM NH4+/SO42~ and 100 mM PPO. Initial rate data were fitted to the Michaelis-Menten equation, and the Km and Vmax values were calculated by using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). The following equation was used for total turnover number (TTN): calculation of the single-enzyme- and double-enzyme-catalyzed reactions for whole-cell catalysis: TTN = product/(bio)catalyst = L-PPT (mol)/[GluDH (g)/49,060 (g/mol)].

Protein homology modeling and molecular docking. The homology model of PmgluDH_WT was built by using the HHpred server based on the crystal structure of a GluDH protein from Corynebacterium

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glutamicum (PDB accession number 5GUD) (1.7 Å) (39, 40). All the homology models of variants were built using the BuildModel tool implemented in FoldX software (41, 42). The PPO structure was drawn using YASARA software and energy minimized under the AMBER03 force field (43, 44). The stereochemical quality of GluDHs was checked by using PROCHECK (43). Docking of PPO into GluDHs was executed by using Autodock 4.2 to predict the binding energy and fine-tune the ligand placement in the protein binding site (45, 46).

Computational analysis of RBS strength. Howard Salis’s RBS Calculator (https://salislab.net/software/predict_rbs_calculator) was used to predict the translation rate of mRNA (47, 48). In the calculation, the definition of the RBS genetic part begins 35 nucleotides (position -35) before the start codon of GOD, up to the start (position +1) of the mRNA transcript.

Data availability. The cDNA gene and amino acid sequences of GluDH and GDH have been deposited under GenBank accession numbers WP_090325311.1 and KMB17194.1, respectively.

SUPPLEMENTAL MATERIAL

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

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