Improved \( \text{l-phenylglycine} \) synthesis by introducing an engineered cofactor self-sufficient system

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**ARTICLE INFO**

**Keywords:**
- \( \text{l-phenylglycine} \)
- Whole-cell biocatalyst
- Self-sufficient
- Protein scaffold
- Hydroxymandelate synthase

**ABSTRACT**

\( \text{l-phenylglycine} \) (L-phg) is a valuable non-proteinogenic amino acid used as a precursor to \( \beta \)-lactam antibiotics, antitumor agent taxol and many other pharmaceuticals. L-phg synthesis through microbial bioconversion allows for high enantioselectivity and sustainable production, which will be of great commercial and environmental value compared with organic synthesis methods. In this work, an L-phg synthesis pathway was built in *Escherichia coli* resulting in 0.23 mM L-phg production from 10 mM \( \text{l-phenylalanine} \). Then, new hydroxymandelate synthases and hydroxymandelate oxidases were applied in the L-phg synthesis leading to a 5-fold increase in L-phg production. To address \( \text{2-oxoglutarate, } \) \( \text{NH}_4^+ \), and NADH shortage, a cofactor self-sufficient system was introduced, which converted by-product \( \text{l-glutamate} \) and NAD\(^+\) to these three cofactors simultaneously. In this way, L-phg increased 2.5-fold to 2.82 mM. Additionally, in order to reduce the loss of these three cofactors, a protein scaffold between synthesis pathway and cofactor regeneration modular was built, which further improved the L-phg production to 3.72 mM with a yield of 0.34 g/g L-phe. This work illustrated a strategy applying for whole-cell biocatalyst converting amino acid to its value-added chiral amine in a cofactor self-sufficient manner.

1. Background

To date, more than 900 naturally occurring amino acids have been identified [1]. Most of them are non-proteinogenic chiral amino acid, which are becoming increasingly important to modern drug discovery. These amino acids are used as antimitabolites of common amino acids and serve as effective inhibitors for a series of metabolic targets [2]. It is estimated that optically active amine including amino acids make up approximately 40% of all chiral intermediates involved in the production of active pharmaceutical ingredients [3].

\( \text{l-phenylglycine} \) (L-phg) is a non-proteinogenic amino acid, which is used as the building block of virginiamycin S, pristinamycin I, penicillin, and antitumor compound taxol [4–6]. Currently, the synthesis of L-phg is based on petrochemical feedstocks. The process uses environmentally harmful organic solvents and poisonous reagents and the low enantioselectivity which does not meet the demands of environmental safety and green chemistry [7]. A fermentative process to produce chiral amino acids is preferable because of its high enantioselectivity, sustainable raw material, and relatively reduced environmental detriments. Previous studies have reported overproduction of L-phg based on glucose, \( \text{l-phenylalanine} \) (L-phe), and other substrates [2,8–10].

There are two natural biosynthetic pathways of L-phg production from phenylypyruvate (PPA). One was discovered in *Streptomyces pristinaeapsiralis* [11]. In this pathway, PPA is converted to phenylacetyl-CoA by the PglB/C pyruvate dehydrogenase-like complex. In the next step, Phenylacetyl-CoA is oxidized to benzoylformyl-CoA by PglA. CoA residue from benzoylformyl-CoA is released by the thioesterase PglD, yielding phenylglyoxyxlate (benzoylformate, PG). With L-phe as an amino donor, PglE further converts PG to L-phg (Fig. 1a the pathway in blue background). This natural L-phg operon is expressed in different actinomycetal host strains by genetic engineering and fermentation optimization, with L-phg reaching 1.6 \( \mu \)g/L in 96 h\(^2\). The other pathway is characterized in *Streptomyces coelicolor* and *Amycolatopsis orientalis*. PPA is converted to \( \text{l-mandelate} \) (L-MA) by 4-hydroxymandelate...
synthase (HmaS). L-MA is then converted to PG by 4-hydroxymandelate oxidase (Hmo) [9,12,13]. Finally, the PG is further converted to L-phg or D-phg by 4-hydroxyphenylglyoxylate aminotransferase (HpgAT) or D-phenylglycine aminotransferase (DAT) with L-phe and l-glutamate (L-glu) as the amino donor, respectively (Fig. 1a pathway in red background) [8,14]. In some research, this pathway has been employed to produce D/L-phg in E. coli. However, the poor activity of HmaS and Hmo (<11 mU/mg) [9] and the low expression level of these heterologous enzymes in E. coli hinder the L-phg production. The L-phg production was 51.6 mg/g dry cell weight and 91.4 mg/L from glucose in l-phenylalanine as substrate [6,8].

In the meantime, researchers have developed other biological routes for L-phg synthesis. In one, racemic mandelate is used as the substrate. Mandelate racemase (MR) coupled with d-phenylglycine dehydrogenase (MD) catalyzes the substrate to produce PG, which is converted to L- or D-phg by HpgAT or DAT (Fig. 1b) [10,15,16]. A recent example for L-phg production used leucine dehydrogenase (LeuDH) to catalyze the conversion of PG to L-phg using NADH and NH₄⁺ as cofactor. To get sufficient redox supply, formate dehydrogenase was co-expressed, which regenerated NADH by oxidizing formate to CO₂. By co-expressing of FDH with LeuDH, 60.2 g/L L-phg was obtained with 99% yield, which showed a satisfactory productivity and enantioselectivity towards L-phg (Fig. 1c) [7,17]. These examples showed us the application of transferases and amine dehydrogenases in prochiral ketones reduction for chiral amines synthesis. In this process, the sufficient supply of amine donor and redox were important for successful engineering.

The cascade (or “domino”) one-pot reaction is successfully performed by sequential or simultaneous reactions to produce chiral alcohols, amines, as well as amino acids [16]. Cascading reaction often involves the participation of oxidoreductase, together with generation or consumption of NAD(P)H, which can lead to a stoichiometric redox imbalance. In many cases, formate dehydrogenase and glucose dehydrogenase are used to recycle NAD (P) H by oxidation of sacrificial substrates [18–20]. In our previous work, a synthesis pathway from L-phe to 2-phenylethanol (2-PE) was coupled with glutamate dehydrogenase (GDH) as a cofactor regeneration module [21]. Using this module, no external cofactor or redox source was required as GDH utilized the by-product L-glu and NAD(P)⁺ to simultaneously synthesize the co-substrates 2-oxoglutarate (2-OG) and NAD(P)H (Fig. 2a) [21]. Theoretically, this redox self-sufficient system is also feasible for any amino acid to its value-added amine derivatives, if this biocconversion process meets the following two conditions, (a) the substrate amino acid deamination was catalyzed by a transaminase with 2-OG as amino group acceptor, (b) the product amine derivatives use NAD(P)H and NH₄⁺ as cofactor for its amination. We suppose that L-phg synthesis from L-phe is suitable for this system. The TyrB catalyzes L-phe and 2-OG to PPA and L-glu, then PPA is converted to PG following the pathway in S. coelicolor and A. orientalis. At last, LeuDH convert PG to L-phg with the utilization of NADH and NH₄⁺. By introducing the cofactor regeneration module, by-product L-glu could be converted to 2-OG, NADH and NH₄⁺, which are all needed redox and co-substrate in L-phg synthesis. In this manner, no redox needs to be added, even NH₄⁺ in this system stoichiometric balanced (Fig. 2b).

In this study, we exemplified a self-sufficient biotransformation system from amino acid L-phe to its value-added chiral amine derivatives L-phg. Firstly, an L-phg pathway was built in E. coli BW25113. New HmaS and Hmo were expressed to improve the synthesis efficiency. Then, the cofactor regeneration module was introduced to maintain redox balance and amino group recycling. Finally, a protein scaffold was used to enhance the connection between the synthetic pathway and cofactor regeneration module to obtain better L-phg production.

2. Results

2.1. Construction of L-Phg biosynthetic pathway in E. coli

A heterologous L-phg pathway was constructed by co-overexpressing the aromatic transaminase (tyrBₑ) from E.coli, hydroxymandelate synthase (hmaSc) and hydroxymandelate oxidase (hmoSc) from S. coelicolor, and a codon-optimized leucine dehydrogenase (leuDHₑopt) from Bacillus clausii (Fig. 2b). Based on our previous research, the enzyme activity of TyrBₑ and LeuDHₑ were much higher than HmaSₑ and HmoSc [9,17]. Therefore, these genes were divided into two operons, hmaSc-hmoSc were gibson assembled into high copy plasmid pYB1s, and leuDHₑopt-tyrBₑ were gibson assembled into a medium copy plasmid pRB1a. These

![Fig. 1](image_url)  
**Fig. 1.** Current L-phg synthesis pathways based on different substrates. (a) Summary of natural L-phg synthesis pathway from PPA as substrate. The pathway in blue or red background was the L-phg synthesis pathway identified from Streptomyces pristinaespiralis or Streptomyces coelicolor and Amycolatopsis orientalis respectively. (b) L-phg synthesis pathway from racemic MA. (c) L-phg synthesis pathway from PG.
two plasmids have the same arabinose-inducible promoter, but the origin and selective marker of pYB1s were P15A and streptomycin while the pRB1a were R6K and ampicillin. The resulting E. coli BW25113 harboring pYB1s-hmaS\textsubscript{Sc}-hmo\textsubscript{Sc} and pRB1a-leuDH\textsubscript{Bc}-opt-tyrB\textsubscript{Ec} (designated SC-LT, protein expression results showed in Fig. S2c) was used as the whole-cell biocatalyst.

After a 12-h biotransformation, 0.23 mM (35.3 mg/L) L-phg was obtained from 10 mM L-phe. As Fig. 2c shows, only 2 mM L-phe was catabolized in the first 12 h. Over time, the substrate L-phe decreased slowly, while the final product, L-phg did not further accumulate. And the intermediates L-MA and PG kept in a low concentration. These results indicated that L-phg production was hampered by the insufficiency of enzymes in this pathway. TyrB was a protein from E. coli, SDS-PAGE showed that it had a very high soluble expression (Fig. S1). Previous study showed that the specific activity of TyrB was 170 μmol/min/mg towards L-phe \[22\]. In our previous study on 2-PE synthesis, TyrB could catalyze the conversion of 20–40 mM L-phe to PPA in 12 h \[21\]. At the same time, the residual LeuDH enzyme activity after 12 h of bioconversion was measured. As shown in Fig. 2d, the residual LeuDH activity of SC-LT was 247.3 U/mL. Based on SDS-PAGE, there were solubility problems in HmaS and Hmo expression (Fig. S1). Given the fact that the enzymes activity of HmaS and Hmo were low \[6\], we supposed that the insufficiency of HmaS and Hmo were the bottlenecks in this L-phg biosynthesis pathway.

2.2. Optimization L-phg production using different combinations of HmaS and Hmo

In previous studies, HmaS and Hmo from S. coelicolor and A. orientalis were expressed in E. coli for phenylglycine synthesis. The activity of HmaS\textsubscript{Sc} and Hmo\textsubscript{Sc} were 2.2 mU/mg and 4.7 mU/mg to PPA and L-MA respectively \[9\], which may be improved by screening new HmaS and Hmo. Protein sequences annotated as HmaS in Kyoto Encyclopedia of Genes and Genomes were downloaded and used for phylogenetic analysis. As Fig. 3a showed, HmaS could be divided into six main branches. We selected six HmaS from each branch, including previously reported HmaS from S. coelicolor, A. orientalis, Actinosynnema mirum, Micromonospora tulbaghiae, Myxococcus stipitatus and Herpetosiphon aurantiacus. The genes encoding six HmaS were codon optimized based on E. coli preference, namely hmaS\textsubscript{Sc}\textsubscript{opt}, hmaS\textsubscript{Ao}\textsubscript{opt}, hmaS\textsubscript{Am}\textsubscript{opt}, hmaS\textsubscript{Mt}\textsubscript{opt}, hmaS\textsubscript{Ms}\textsubscript{opt} and hmaS\textsubscript{Ha}\textsubscript{opt} (numbered as hmaS1-hmaS6). According to references, S. coelicolor, A. orientalis, A. mirum, M. tulbaghiae, were widely used host for β-lactam antibiotics and glycopeptide antibiotics fermentation indicating that they may have a more active L-phg synthesis pathway \[23–26\]. Therefore, the Hmo encoding genes from these four organisms were synthesized too (hmo\textsubscript{Sc}\textsubscript{opt}, hmo\textsubscript{Ao}\textsubscript{opt}, hmo\textsubscript{Am}\textsubscript{opt}, hmo\textsubscript{Mt}\textsubscript{opt}, hmo\textsubscript{Ms}\textsubscript{opt} numberered as hmo1-hmo4). The expression level of each hmaS1-6 and hmo1-4 were analyzed by SDS-PAGE (Shown in Fig. S2a and b). hmaS1-6 and hmo1-4...
were gibson assembled to each other under a pBAD promoter in expression vector pYB1s resulting plasmids HHxy (the x denotes the hmaS and y denotes the hmo assembled in plasmid, for example HH23 represents the plasmid expressing hmaS2 and hmo3). The HHxy together with pRB1a-leuDHBcopt-tyrBEc formed 24 different combination strains (i.e., HH11-LT to HH64-LT, Fig. 3b) (HH11-LT and HH31-LT protein expression results showed in Fig. S2c).

After 12-h biotransformation, the substrate distribution of 24 strains was measured. As heatmap showed in Fig. 3c–f, significant differences in L-phe utilization were observed according to different hmaS-hmo combinations. In most hmaS-hmo combinations, only 20–50% of L-phe was used (Fig. 3c), except that hmaS3 utilized up to 60%–71% of the L-phe and produced significantly higher intermediate L-MA, PG (Fig. 3d and e). Strain HH33-LT produced 6.1 mM L-MA, accounting for 85.4% of the L-phe utilized. Strain HH31-LT produced 4.9 mM PG, which was 73.4% of L-phe utilized.

Different intermediates distributions were observed when hmaS3 was accompanied with different hmo too. When hmo2, hmo3 or hmo4 was coupled with hmaS3, 3.3–6.1 mM L-MA was accumulated (Fig. 3d) and only a small amount of L-MA (0.5–2.5 mM) was subsequently

Fig. 3. L-phg production from L-phe by whole-cell bioconversion using different recombinant E. coli strains. (a) The phylogenetic tree analysis of HmaS. HmaSs were chosen from each phylogenetic branches for L-phg production. (b) Operons built in this study, LT represents the plasmid harbouring operon leuDHBcopt-tyrBEc. The HHxy represents the plasmid harbouring operon hmaSx-hmoy, x (1–6) represents different hmaS, y (1–6) represents different hmo. HHxy coexpressed with LT form 24 combinations. (c–e) The concentration of metabolites L-phe, MA, PG, L-phg of these 24 combinations. The abscissa axis represents the hmaS used and the ordinate axis represents the hmo used. The concentration of each metabolite was visualized by a heat map.
converted to PG and L-phg (Fig. 3e and f). In contrast, hmo1 converted almost all L-MA to the subsequent steps (Fig. 3d). These differences suggested that hmo1 had the best catalytic performance among hmo1-4. Among 24 hmaS-hmo combinations, hmaS3-hmo1 was the most efficient. This strain produced 1.13 mM (172.9 mg/L) L-phg (Fig. 3f), which was 5-times higher than that of SC-LT.

Another result should be noticed that PG accumulation in strain HH31-LT was markedly higher than other strains (4.86 mM, Fig. 3e). The reason for this result could be the low catalytic activity of LeuDH or inadequate cofactor NADH or NH₄⁺. For further analysis of the main cause, the LeuDH₄ activity in strain 31-LT was tested after a 12-h biotransformation. The activity was 231 U/L (Fig. 2d). This result indicated that under ideal conditions, LeuDH₄ could catalyze 13.9 mM PG to L-phg per hour. Thus, cofactor NADH or NH₄⁺ shortage was considered as the major bottleneck in HH31-LT biotransformation process, rather than LeuDH activity.

2.3. Introduction of a NADH regeneration module for higher L-phg production

The LeuDH₄ activity was proved to be not the main problem in HH31-LT, leading to the shortage of cofactor NADH or NH₄⁺ might block L-phg synthesis from PG. An NAD⁺-dependent formate dehydrogenase (FDH) from Candida boidinii was used to enhance the NADH supply. FDH is widely used as an NADH regeneration module as it catalyzes the oxidation of formate to simultaneously produce CO₂ and NADH [27,28]. fdh₄₈opt was codon optimized and inserted into either plasmid containing the leuDH₄₈opt-tyrB₄ operon or hmaS₃-hmo1 operon and resulted in plasmid LFT and F31 (Fig. 4a). Strains HH31-LFT, F31-LT, and F31-LFT were used for L-phg production. When fdh₄₈opt was overexpressed, 20 mM sodium formate and 50 mM NH₄Cl were added for efficient supply of NADH and NH₄⁺.

Integrating fdh₄₈opt to leuDH₄₈opt-tyrB₄ operon or hmaS₃-hmo1 operon made a big difference in L-phg utilization. When fdh₄₈opt coexpressed with leuDH₄₈opt-tyrB₄ (HH31-LFT) 2.6 mM L-phe left (Fig. 4b). When fdh₄₈opt was coexpressed with hmaS₃-hmo1 in strain F31-LT, a majority of L-phe (7.3 mM) remained unused. Strain F31-LFT which harboring two copies of fdh₄₈opt had similar metabolite distributions with F31-LT. We believed the expression of fdh₄₈opt in hmaS₃-hmo1 operon took up the resources originally used for hmaS₃-hmo1 expression, which turned down the L-phg synthetic pathway. Therefore, strains F31-LT and F31-LFT utilized significantly lower L-phe than HH31-LT and HH31-LFT (Fig. 4b). When operon leuDH₄₈opt-fdh₄₈opt-tyrB₄ was overexpressed (HH31-LFT), L-phg production reached 2.38 mM, which was 2.1-times that of the HH31-LT. At the same time, PG decreased 11% due to the increased NADH supply (Fig. 4b).

2.4. Construction of a self-sufficient module for L-phg production

Co-expression of fdh₄₈opt with the L-phg synthesis pathway increased L-phg production, indicating that insufficient NADH supply was the major reason for PG accumulation. We noticed that, there were two co-substrates that were also essential for L-phg production. One was 2-OG, which acts as the amino acceptor in L-phe deamination. The other was 2-OG, NH₄⁺ dependence for external co-substrate or redox source. (Fig. 2a). We believed that introducing the cofactor regeneration module would further improve the L-phg production.

An NADH-dependent GDH encoded by rocG₄ from B. subtilis was inserted into leuDH₄₈opt-tyrB₄ operon (Fig. 4a, designated as LRT) and coexpressed with HH31 [29] (protein expression results showed in Fig. 5c). The strain HH31-LRT used 7.8 mM substrate, 18% higher compared with strain had no rocG₄ (Fig. 4c). This result indicated that more 2-OG was regenerated by rocG₄. In the meantime, the concentrations of L-MA and PG increased from 0.20 to 4.86 mM to 0.68 and

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Fig. 4. L-phg production using different NADH regeneration strategies. (a) The plasmids carrying fdh₄opt and rocG₄s. (b) Production of L-phg by strains with or without FDH. When FDH was coexpressed, 50 mM NH₄Cl and 20 mM HCOONa were added simultaneously. (c) Production of L-phg by strains with FDH or GDH. L-glu (2 mM) was added to enhance the cofactor regeneration by GDH.

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From the text, we can infer that synthetic and systems biotechnology is a field that involves the design and application of biological systems, often using genetic engineering and metabolic engineering techniques to produce valuable compounds or fuels.
5.33 mM, respectively, and L-phg production increased by 58% from 1.13 to 1.79 mM compared with strain had no rocGb (Fig. 4b). Furthermore, L-glu was proved to have a positive effect on cofactor regeneration in our previous work [21]. Therefore, 2 mM L-glu was used to enhance cofactor regeneration. The addition of L-glu accelerated the utilization of L-phe and PG amination to L-phg. L-phg production increased dramatically from 1.79 mM to 2.82 mM, which was 12.3-times higher than that of the original strain, SC-LT (Fig. 4c).

Although the cofactor regeneration system improved the production of L-phg, accumulation of 4.93 mM PG remained. LeuDH activity was measured to determine whether enzyme activity or NADH and NH₄⁺ caused insufficient conversion of PG to L-phg. When co-expressed with rocGb or fdhₜₐₐ, LeuDH activities were 150.1 and 139.5 U/mL, respectively (Fig. 3b). The enzyme assay showed that the activity of LeuDH was sufficient to convert more PG to L-phg. Thus, NADH and NH₄⁺ supply was further enhanced.

2.5. Spatial organization of cofactor self-sufficient module and LeuDH

Synthetic scaffolds have been widely used to enhance product synthesis by avoiding the intermediate loss that occurs by competing pathways [30,31]. In this study, the cofactor regeneration module and LeuDH were spatially organized by a synthetic protein scaffold to form a substrate channel for NADH and NH₄⁺ which conserved more 2-OG and NH₄⁺ for L-phg production rather than used by cell central metabolism (Fig. 3b). SH₃ and slig protein-protein interaction domains were fused to the C-terminus of rocGb and leuDHopt by a flexible linker (GGGGS) [32]. By altering the number of slig, one to three rocGb conjoined with one leuDHopt to adequately supply NADH and NH₄⁺. The strains with recombinant plasmids were designated slig-1, slig-2, and slig-3 (Fig. 3b).

The scaffold SH₃-slig closely colocalized leuDHopt and rocGb. This prevented the diffusion of NADH and NH₄⁺ and facilitated the conversion of PG to L-phg. This strategy resulted in an increase of L-phg to 3.28, 3.72, and 3.01 mM in slig-1, slig-2, and slig-3, respectively. Compared with 31-LRT, slig-2 produced more than 2-fold of L-phg. The molar yield of L-phg reached 37.2%. The accumulation of PG decreased from 5.3 mM to approximately 4 mM, demonstrating that spatial organization could be an effective method to improve target compound production by reducing the loss of intermediate metabolites.

3. Discussion

L-phg is an important building block of antibiotics [2,33], antitumor drug taxol [34], drugs used to treat Alzheimer’s and many other diseases [35,36]. Many progresses have been made on synthesis of L-phg in a biological way [8,10,17]. In this study, we built a L-phg synthesis pathway in E. coli. However, only a small amount of L-phe was utilized to synthesis L-phg. As study showed that enzyme activities of HmaS and Hmo from S. coelicolor were 0.501 and 1.822 U/mg. At the same time, the solubility of these two enzymes in E. coli were low. Thus, HmaS and Hmo was considered as the major bottleneck for L-phg production.

To address this problem, new HmaS and Hmo from different organisms were introduced into L-phg synthesis. 6 HmaS and 4 Hmo were chosen and each HmaS was co-expressed with every Hmo. After enumerating all the combinations of HmaS and Hmo, HmaS₄₃ and Hmo from A. mirum was found to catalyze the most amount of L-phe to L-MA and following intermediates. A. mirum produces a variety of biologically active compounds including ansamitocins [37], β-lactam antibiotics valdoxylamine A [38] and nocardicin A [39]. The pentapeptide precursor of Nocardicin A has 4-hydroxy-γ-phenylglycine at position 1, 3, and 5 [40]. Which indicating A. mirum may have an active L-phg synthesis pathway. Recently, HmaS from another nocardicin producer, N. uniformis, was utilized in S. cerevisiae for MA and HMA production which led to a 42% and 90% increase in HMA and MA production [41]. These results indicate that there may be more efficient HmaS and Hmo from other nocardicin producers. L-phg biomanufacturing maybe benefit by further screen new HmaS and Hmo in these strains.

Although more than 6 mM L-phe was converted to PG in HH31-LT, only 1.1 mM L-phg was obtained. The reason for this result could be the low LeuDH activity or shortage of NADH and NH₄⁺. The LeuDH activity assay showed it was adequate. Therefore, the limited cofactor and NH₄⁺ were believed to be the major bottlenecks. Two approaches were applied to address these bottlenecks. One was coupling an NADH regeneration enzyme FDH with L-phg pathway. The FDH could oxidize sodium formate and regenerate NADH simultaneously. This strategy has been successfully applied in L-tert leucine, ethanol, phenyllactic acid synthesis [42-45]. As a result, more than 2-fold L-phg was obtained, indicating that NADH and NH₄⁺ were the major problems in L-phg synthesis.

The other strategy was inspired by our previous work (Fig. 2a), an Ehrlich pathway was established in E. coli to produce 2-PE from L-phe. By co-expressing GDH, the L-glu and NAD(P)⁺ byproducts were able to simultaneously regenerate to 2-OG and NADPH, which lead to 3.8-fold 2-PE increased [21]. In this study, we adopted this strategy to an amino acid to its valued-added chiral amine derivatives. Similar to 2-PE biosynthesis, 2-OG and NAD(P)⁺ were essential in L-phg, while NH₄⁺ was also necessary. Therefore, the NH₄⁺ generated by GDH was reused, and no waste accumulated in this conversion (Fig. 2b). Thus, a self-sufficient cofactor system based on a bridging mechanism was developed to simultaneously rebalance the co-substrate and redox equivalents, so that no external cofactor or redox source was required.

Compared with FDH co-expression approach, L-phg production increased by 20% and no additional sodium formate and NH₄Cl were needed, which was a big advantage.

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**Fig. 5.** L-phg production by spatial colocalization of LeuDH and GDH. (a) Metabolite distribution of strains with different ratios of LeuDH to GDH. (b) Schematic diagram of the spatial colocalization of LeuDH and GDH. By altering the number of slig in the C-terminus of LeuDH, different numbers of GDH colocalized with LeuDH, leading to different biocatalytic performance.
Unlike in 2-PE production, when the cofactor regeneration module was applied in L-phg production, we noticed an accumulation of intermediate PG. Even though we designed a stoichiometric balanced reaction, the accumulation of intermediate reminded us that the cofactor regeneration module and the synthetic pathway should function at the same pace. In this way, the generated cofactor and redox regenerated could be used by L-phg synthesis rather than used by competing reactions. In L-phg synthesis, cofactor regeneration was faster than the synthetic pathway, leading to the faster regeneration of 2-OG and NADH than the synthetic pathway. The 2-OG was used to convert L-phe to PPA and L-glu. L-glu recycled and produce 2-OG and NADH, which further accelerate the conversion of L-phe to PPA (Fig. 2b). In this situation, NADH in the engineered bacteria did not wait until PG generated, but catabolized by other competing reactions instead. Therefore, when PG generated, there were not enough NADH. Compared with HH31-LT, HH31-LRT produced 2.5 folds L-phg, and PG decrease 8%. The problem of PG accumulation was not totally solved.

To retain more NADH, an orthogonal interaction pair slig-SH3 was applied in order to colocalize LeuDH and GDH. This protein scaffold strategy was first used for mevalonate biosynthesis. By altering the stoichiometry of the SH3, PDZ, and GBD domains, three mevalonate biosynthetic enzymes were arranged in a designable manner, which lowered the host-expressing burden and increased the production of mevalonate 77-fold [32]. Protein scaffolds have been applied to incorporate sequential enzymes in gamma-aminobutyric acid, polyhydroxybutyrate, and resveratrol production [46–48]. In this work, a scaffold was built between the synthetic pathway and cofactor regeneration module. In this way, cofactor NADH, and co-substrate NH4+ generated by GDH could be directly utilized by LeuDH. Thus, L-phg increased by 2.1-fold compared with no scaffold. The final L-phg level reached 3.72 mM (562.5 mg/L), which was the highest L-phg titer from a single strain.

These results exemplified an application of self-sufficient cofactor regeneration strategy in amino acids to its value-added chiral amine derivatives. Moreover, enzyme colocalization was used between synthesis pathway and cofactor regeneration modular and proved to be an effective way to enhance product formation.

4. Material and methods

4.1. Chemicals and reagents

L-phe, L-glu, sodium formate, NH4Cl, and Tris base were purchased from Shanghai ShengGong Bio-chemical Co. Ltd. (Shanghai, China). For high-performance liquid chromatography (HPLC) analysis, standards for L-phe, PPA, L-MA, PG, and L-phg were purchased from Sigma-Aldrich (Steinheim, Germany). Restriction endonucleases and DNA polymerase were purchased from New England Biolabs (Ipswich, MA, USA). The analysis was performed on a XBridge C-18 3.5 μm column (4.6 × 150 mm; Waters, Milford, MA, USA). The analysis was performed at 30 °C with a mobile phase comprising 10% methanol in 20 mM phosphate buffer (pH = 6.5) at a flow rate of 0.5 mL/min. The analytes were detected at OD210.

4.2. Bacterial strains and culture conditions

All strains and plasmids used in this study are listed in Supplementary Table S1. E. coli strains were grown at 37 °C on a shaker at 200 rpm in Luria–Bertani (LB) medium with ampicillin and streptomycin (50 μg/mL) added as required. For protein expression, overnight cultures were inoculated (1% inoculum) into auto-induction ZYM medium [49] and incubated with constant shaking at 30 °C for 16 h.

4.3. Plasmid and strain construction

The genes hmasS<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, hmas<sup>opt</sup>, leuDH<sup>opt</sup>, fth1<sup>opt</sup>, (translated protein sequence ID: 3ZGJ_A, WP_016331888.1, ACU38423.1, WP_120569635.1, WP_015349166.1, ABX04531.1, WP_011028840.1, WP_016331889.1, ACU37897.1, WP_120569648.1, WP_000171355.1, and CAA94664.2) were codon-optimized based on the E. coli codon preference and synthesized by GenScript (Nanjing, China). The hmas<sub>opt</sub> and hmas<sub>opt</sub> genes were amplified from genome Streptomyces coelicolor A3(2) (GI: 30407153). The other genes for L-phg production, including tyrB and rocG, were amplified from E. coli BW25113 (GI: 749300132) and Bacillus subtilis subsp. subtilis str. 168 (Sequence ID: CP053102.1) genomic DNA. String DNA fragments with a 20-base pair (bp) overhang were inserted into pYB1a and pRBI1s derivatives using the Gibson assembly method. The primers were designed by CE design (https://crm.vazyome.com/etool/simple.html). They are listed in Supplementary Table S2.

4.4. Bioconversion conditions

For L-phg bioconversion, cells were collected after 16 h of culture by centrifugation at 8000 × g for 10 min, washed twice with ice-cold 0.85% NaCl solution, and suspended in 200 μL (optical density at 600 nm = 30) of a reaction mixture containing 10 mM L-phe and 50 mM Tris-HCl buffer (pH = 7.0). L-glu, NH4Cl, and sodium formate were added as required. The bioconversion reactions were performed at 37 °C with shaking at 200 rpm for 12 h.

4.5. Phylogenetic analysis

Gene sequences were downloaded from KEGG (Entry: K16421). Multiple alignments were obtained using Mega5 ClastalW with default parameter (gap open penalty 10, gap extension penalty 0.1). The phylogenetic tree was built using the Neighbor-joining method with the Bootstrap option. Finally, the editing tool iTOL was used.

4.6. Analytical methods

Cell density was estimated by measuring the OD<sub>600</sub> (1 OD/L = 0.23 g (dry cell weight)/L). To determine LeuDH enzyme activity assay, 200 μL of cell lysate was added to 2 mL of enzyme reaction mixture containing 25 mM Tris-HCl buffer, 1 mM NH4Cl, 0.1 mM NADH, and 0.2 mM PG (pH = 7.0). One unit of enzyme activity was defined as the amount of enzyme catalyzing the reduction of 1 μmol NADH/min. The concentrations of L-phe, L-MA, PG, and L-phg were measured by HPLC using the Agilent 1260 series device (Hewlett–Packard, Palo Alto, CA, USA) equipped with an XBridge C-18 3.5 μm column (4.6 × 150 mm; Waters, Milford, MA, USA). The analysis was performed at 30 °C with a mobile phase comprising 10% methanol in 20 mM phosphate buffer (pH = 6.5) at a flow rate of 0.5 mL/min. The analytes were detected at OD210.

CRediT authorship contribution statement

**Pengchao Wang:** Data curation, Formal analysis, Investigation, Conceptualization, Writing – original draft, Writing – review & editing, Funding acquisition.

**Xiwen Zhang:** Data curation, Investigation, Formal analysis, Writing – original draft. **Yucheng Tao:** Data curation, Investigation, Formal analysis. **Xubing Lv:** Data curation, Investigation.

**Shengjie Cheng:** Data curation, Investigation. **Chengwei Liu:** Project administration, Resources, Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled “Improved L-phenylglycine synthesis by introducing an engineered cofactor self-sufficient system”
Acknowledgements

We thank Prof. Tao Yong and Prof. Lin Baixue for valuable advises and vectors used in this research. This work was supported in part by the National Natural Science Foundation of China (Project No. 31900064), as well as the Natural Science Foundation of Heilongjiang Province of China (Project No. LH2019C012). The authors declare that they have no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2021.12.008.

Abbreviations

L-phe t-phenylalanine
PPA p-phenylpyruvate
L-MA l-mandelate
PG phenylpyruvate oxidase
L-phenylglycine
L-glut glutamate
2-OG 2-oxo-glutarate
2-PE 2-phenylethanol
TyR aromatic transaminase
PglB/C pyruvate dehydrogenase
PglA benzyloformyl-CoA synthetase
PglD thioesterase
PglE l-phenylglycine aminotransferase
HmaS 4-hydroxymandelate synthase
Hmo 4-hydroxymandelate oxidase
HpgAT 4-hydroxymandelate synthase
DAT d-phenylglycine aminotransferase
MR mandelate racemase
MD S-mandelate dehydrogenase
LeuDH leucine dehydrogenase
GDH glutamate dehydrogenase

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