Chassis engineering of *Escherichia coli* for trans-4-hydroxy-L-proline production

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Summary

Microbial production of *trans*-4-hydroxy-L-proline (Hyp) offers significant advantages over conventional chemical extraction. However, it is still challenging for industrial production of Hyp due to its low production efficiency. Here, chassis engineering was used for tailoring *Escherichia coli* cellular metabolism to enhance enzymatic production of Hyp. Specifically, four proline 4-hydroxylases (P4H) were selected to convert L-proline to Hyp, and the recombinant strain overexpressing DsP4H produced 32.5 g l\(^{-1}\) Hyp with α-ketoglutarate addition. To produce Hyp without α-ketoglutarate addition, α-ketoglutarate supply was enhanced by rewiring the TCA cycle and L-proline degradation pathway, and oxygen transfer was improved by fine-tuning heterologous haemoglobin expression. In a 5-l fermenter, the engineered strain *E. coli*ΔsuCDΔputA-VHDLΔDsP4H showed a significant increase in Hyp titre, conversion rate and productivity up to 49.8 g l\(^{-1}\), 87.4% and 1.38 g l\(^{-1}\) h\(^{-1}\) respectively. This strategy described here provides an efficient method for production of Hyp, and it has a great potential in industrial application.

Introduction

*trans*-4-Hydroxy-L-proline (Hyp), one of the hydroxyproline isomers, is a useful chiral building block in medicine, biochemistry, food, cosmetic and other aspects of industry (Yi et al., 2014). Hyp is generally produced in industry by acid hydrolysis of animal collagen, which is a complex process with many bottleneck problems such as low efficiency and heavy environmental pollution (Zhao et al., 2017). To overcome these problems, Hyp biosynthesis is regarded as a promising method due to its high catalytic efficiency and environmental compatibility (Shibasaki et al., 2000b; Zhao et al., 2017).

Recently, four metabolic engineering strategies have been developed to reconstruct an efficient cell factory for Hyp production, and they mainly relate to a key enzyme, proline 4-hydroxylase (P4H) that can catalyse the hydroxylation of L-proline to Hyp (Table 1). Strategy I is to screen Hyp-producing strains. Some bacteria or fungi have been found to form Hyp via fermentation directly (Serizawa et al., 1995). In addition, *Escherichia coli* NA45 was isolated from *E. coli* BL21(pUC19-TTP-P4H by chemo-physical combination mutagenesis, which could convert L-proline to Hyp with glyceral as a sole carbon source (Wang et al., 2016). Strategy II is to select and express P4H. When the P4H gene from *Dactylosporangium* sp. RH1 (DsP4H) was expressed in *E. coli* W1485ΔputA, *E. coli* BL21(DE3) and *Corynebacterium glutamicum* (Shibasaki et al., 2000b; Yi et al., 2014), the highest concentration of Hyp was up to 41 g l\(^{-1}\) with L-proline and glucose as substrates (Shibasaki et al., 2000b). In view of this, Hyp production from glucose was firstly achieved in an L-proline-producing *E. coli* by expressing DsP4H (Shibasaki et al., 2000a). Additionally, Hyp production (45.83 g l\(^{-1}\)) was largely enhanced from glucose by expressing P4H from *Altemonas mediterranea* and a γ-glutamyl kinase (proB) mutation in *E. coli* MG1655ΔputA (Wang et al., 2018). Strategy III is to introduce haemoglobin into *E. coli* for oxygen transfer. When the *Vitreoscilla* haemoglobin (VHb) gene was integrated into the chromosome of the DsP4H-expressing strain *E. coli* WD3(pTrc99a-p4H), Hyp production (14.4 g l\(^{-1}\)) was increased by 73.2% compared to that of *E. coli* strain without VHb (Zhao et al., 2017). Strategy IV is to engineer Hyp biosynthetic pathway. Hyp titre (21.72 g l\(^{-1}\)) was improved in an L-proline-producing *C. glutamicum* by expressing and...
optimizing DsP4H, deleting succinyl-CoA synthetase (SucCD) gene, and expressing feedback-resistant proB gene (Falcioni et al., 2015; Zhang et al., 2019). Similarly, the final Hyp production of 31.0 g l \(^{-1}\) was obtained by overexpressing DsP4H, proB and glutamate-semialdehyde dehydrogenase (proA), and knocking out proline dehydrogenase (PutA), \(\alpha\)-ketoglutarate dehydrogenase (SucAB), isocitrate lyase (AceA) and isocitrate dehydrogenase kinase/phosphatase (AceK; Zhang et al., 2015). The above research results have indicated that Hyp production can be successfully improved by enzymatic transformation and microbial fermentation. However, the hydroxylation of proline is strongly interconnected with central carbon metabolism in host (Loenarz and Schofield, 2011; Falcioni et al., 2013; Falcioni et al., 2015), and thus many physiology-related factors potentially can interfere with the catalytic performance.

In this study, we described chassis engineering strategies to optimize the catalytic performance of E. coli for Hyp production. By combining central carbon metabolism with enzymatic transformation, its interconnection was established with \(\alpha\)-ketoglutarate (\(\alpha\)-KG; Fig. 1). Based on this interconnection, the whole-cell biocatalysis was optimized systematically by metabolic engineering to enable an efficient production of Hyp. Under controlled culture conditions, the engineered strain E. coli\(\Delta\)sucCD\(\Delta\)putA\(-\text{VHb}_{\text{L}-}\)DsP4H produced up to 49.8 g l \(^{-1}\) Hyp in a 5-L fermenter.

Results

Constructing the synthetic pathway for Hyp production

Proline 4-hydroxylase (P4H) can catalyse the hydroxylation of \(L\)-proline at the 4-position to produce trans-4-hydroxy-\(L\)-proline (Hyp) in the presence of \(\alpha\)-ketoglutarate (\(\alpha\)-KG), oxygen and ferrous ion (Lawrence et al., 1996; Fig. 2A). P4H from Dactylosporangium sp. RH1 (DsP4H) has been used for enzymatic production of Hyp with \(L\)-proline as substrate (Shibasaki et al., 2000b). To identify the superior enzyme, three P4Hs from Bacillus mega-terium (BmP4H), Aspergillus oryzae (AoP4H) and Aspergillus flavus (AfP4H) were selected from Uniprot database with DsP4H as a probe. Then, we cloned and overexpressed DsP4H, BmP4H, AoP4H and AfP4H respectively. Next, the activities of four P4Hs were assayed, and they showed hydroxylation activities of 68.5, 56.2, 22.3 and 25.4 U mg \(^{-1}\) respectively (Fig. 2B).

Finally, the effect of four P4Hs on Hyp production was investigated with 50 g l \(^{-1}\) \(L\)-proline as substrate, and strain E. coli–DsP4H produced the highest concentration of Hyp up to 32.5 g l \(^{-1}\) with its conversion rate 57.1%, which was 26.5%, 162.1% and 108.3% higher than that of E. coli–BmP4H, E. coli–AoP4H and E. coli–AfP4H respectively (Fig. 2C). Thus, the recombinant E. coli–DsP4H was selected for further research.

Engineering the \(\alpha\)-KG-supplying pathways to enhance Hyp production

E. coli–DsP4H could be used for efficiently converting \(L\)-proline to Hyp with \(\alpha\)-KG addition. Thus, the effect of \(\alpha\)-KG addition on Hyp production was analysed with whole-cell biocatalyst E. coli–DsP4H. Hyp titres were increased with \(\alpha\)-KG addition from 0 to 10 g l \(^{-1}\), and the maximal Hyp production was observed at 10 g l \(^{-1}\) \(\alpha\)-KG (Fig. 2D). When the addition of \(\alpha\)-KG was over 10 g l \(^{-1}\), Hyp production was slightly reduced (Fig. 2D). In this process, the main by-product, succinate, was also measured during the enzymatic production of Hyp. With the increase of Hyp titres, succinate concentrations were increased gradually (Fig. 2D). When 10 g l \(^{-1}\) \(\alpha\)-KG was added, the maximal concentration of succinate was up to 15.5 g l \(^{-1}\), which was 3.8-fold higher than that of no \(\alpha\)-KG addition (Fig. 2D). To sum up, conversion ratio with E. coli–DsP4H was up to 57.1% with \(\alpha\)-KG addition, but this conversion ratio was reduced to 22.5% without \(\alpha\)-KG addition. These results showed that additional supply of \(\alpha\)-KG was necessary for efficient production of Hyp, possibly due to the fact that E. coli host did not produce enough \(\alpha\)-KG for this transformation reaction.

\(\alpha\)-KG can be supplied through two metabolic pathways, the tricarboxylic acid (TCA) cycle from glucose and \(L\)-proline degradation pathway from \(L\)-proline (Shibasaki et al., 2000b; Fig. 3A). The conversion of \(\alpha\)-KG to

| Table 1. Comparison of Hyp production by the engineered microorganisms |
|------------------|----------------|----------------|----------------|----------------|
| Strains | Hyp titre (g l \(^{-1}\)) | Conversion rate (%) | Productivity (g l \(^{-1}\) h \(^{-1}\)) | References |
| E. coli NA45 | 25.4 | 38.1 | 0.59 | Wang et al. (2016) |
| E. coli W1485 putA/pWFH1 | 41.0 | 87.0 | 0.41 | Shibasaki et al. (2000b) |
| E. coli W1485 putA/pWF1 | 25.0 | – | 0.25 | Shibasaki et al. (2000a) |
| E. coli SEch(pTrc-B74A-alp4h) | 45.83 | 62.6 | 1.27 | Wang et al. (2018) |
| E. coli W35-VGB(pTrc99a-p4h) | 14.4 | – | 0.25 | Zhao et al. (2017) |
| C. glutamicum Hyp-7 | 21.72 | – | 0.36 | Zhang et al. (2019) |
| E. coli 3aW3110/pTrc99a-p4h-proba | 31.0 | – | 0.60 | Zhang et al. (2018a) |
| E. coli\(\Delta\)sucCD\(\Delta\)putA\(\Delta\)VHb\(_{\text{L}-}\)DsP4H | 49.8 | 87.4 | 1.38 | This study |

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succinate in the TCA cycle is sequentially catalysed by α-KG dehydrogenase complex and succinyl-CoA synthetase (sucCD) in *E. coli*, and this conversion can also be achieved by one-step reaction with P4H (Lawrence et al., 1996). Thus, when the sucC and sucD genes are simultaneously deleted in *E. coli*ΔDsP4H, flux partitioning at the α-KG node may be mainly redirected towards Hyp synthesis under the driving force of P4H that also functions as another bypass route for succinate formation. To demonstrate this idea, the sucC and sucD genes were simultaneously deleted in *E. coli*ΔDsP4H, and the resulting strain *E. coli*ΔsucCD-DsP4H showed a 139.1% increase in Hyp production up to 30.6 g l\(^{-1}\) without α-KG addition compared to that of *E. coli*ΔDsP4H, and

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succinate accumulation was only increased to 5.8 g L\(^{-1}\) (Fig. 3B). To our surprise, the concentration of glutamate was up to 10.2 g L\(^{-1}\), which was 5.8-fold higher than that of \(E.\ coli\)-DsP4H (Fig. 3B). To identify sources of glutamate, the activities of proline dehydrogenase (PutA) and glutamate dehydrogenase (GDH) were assayed. PutA activity in \(E.\ coli\)-sucCD-DsP4H was increased by 37.9% compared to that of \(E.\ coli\)-DsP4H, but GDH activity was kept consistent with that of \(E.\ coli\)-DsP4H (Fig. 3C). These results indicated that glutamate formation was from L-proline degradation, but not \(\alpha\)-KG.

To reduce L-proline degradation and convert more L-proline to Hyp, putA in L-proline degradation pathway was deleted in \(E.\ coli\)-sucCD-DsP4H, and its activity in the resulting strain \(E.\ coli\)-sucCD-DsputA-DsP4H was decreased significantly (Fig. 3C). In addition, the formation of glutamate was resulted in a 3.6-fold decrease compared to that of \(E.\ coli\)-sucCD-DsP4H (Fig. 3B). Based on this, Hyp production and its conversion rate were up to 41.3 g L\(^{-1}\) and 72.5%, both of which were increased by 35.0% compared to that of \(E.\ coli\)-sucCD-DsP4H (Fig. 3B). In addition, succinate accumulation was only increased to 8.4 g L\(^{-1}\) (Fig. 3B). To further improve conversion rate from L-proline to Hyp without \(\alpha\)-KG addition, glucose addition was optimized to balance flux partitioning at the \(\alpha\)-KG node between cell growth and Hyp production. With the increase of glucose addition from 10 to 30 g L\(^{-1}\), Hyp production was increased, and the maximal Hyp titres and conversion rate reached 47.6 g L\(^{-1}\) and 83.6% at 30 g L\(^{-1}\) glucose (Fig. 3D). When the addition of glucose was over 30 g L\(^{-1}\), Hyp production was reduced slightly (Fig. 3D). These abovementioned results demonstrated that \(E.\ coli\)-sucCD-DsputA-DsP4H could efficiently convert L-proline to Hyp without \(\alpha\)-KG addition. However, Hyp productivity was only 0.66 g L\(^{-1}\) h\(^{-1}\) that did not meet the needs of industrial application, possibly due to the fact that Hyp production with P4H is a high-oxygen-demand process (Zhao et al., 2017).

Fig. 3. Engineering the \(\alpha\)-KG-supplying pathways to enhance Hyp production.
A. The \(\alpha\)-KG-supplying pathways for enhancing Hyp production.
B. Effect of sucCD and putA gene deletion on Hyp, succinate and glutamate production.
C. The specific activities of putA and GDH in different recombinant strains.
D. Effect of glucose addition on Hyp production and conversion rate with strain \(E.\ coli\)-sucCD-DsputA-DsP4H.
AcCoA, acetyl-CoA; CIT, citrate; gdh, glutamate dehydrogenase gene; OAA, oxaloacetate; PEP, phosphoenolpyruvate; proA, glutamate-semialdehyde dehydrogenase gene; proB, \(\gamma\)-glutamyl kinase gene; proC, \(\Delta^1\)-pyrroline-5-carboxylate reductase gene; PYR, pyruvate; SUCC, succinyl-CoA.

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Expressing haemoglobin to improve Hyp production

Vitreoscilla haemoglobin (VHb) is an oxygen-binding protein with an oxygen dissociation rate constant of 5600/s (Orii and Webster, 1986), which has been widely used in recombinant strains to improve growth and production of target compounds (Zhang et al., 2013; Akbas et al., 2014; Li and Zhang, 2015). Thus, VHb was overexpressed in E. coliΔsucCDa·putA·VHb·DsP4H to enhance cell respiration by promoting oxygen transfer to the intracellular terminal oxidases (Fig. 4A). The respiration intensity of E. coliΔsucCDa·putA·VHb·DsP4H was increased by 55.2% compared to that of E. coliΔsucCDa·putA·DsP4H (Fig. 4B). Further, Hyp production (48.8 g l⁻¹) was only 2.5% higher than that of E. coliΔsucCDa·putA·DsP4H (Fig. 4C), and Hyp productivity (1.02 g l⁻¹ h⁻¹) was increased by 54.5% (Fig. 4D). However, E. coliΔsucCDa·putA·VHb·DsP4H still accumulated 10.8 g l⁻¹ succinate (Fig. 4C). These results revealed that VHb overexpression tended to balance cell respiration and Hyp production, but this balance should be further optimized.

To realize the optimal balance between cell respiration and Hyp production, the expression of VHb was further improved by ribosome binding sites (RBSs) with different strengths from our previous study (Zhang et al., 2018b). Each RBS was assembled into operons and cloned into plasmids with the same promoter, and then all these combinations could be expressed respectively. With the increase of RBS strengths, the respiration intensity was decreased gradually (Fig. 4B). Further, Hyp production (> 48 g l⁻¹) and conversion rate (> 85%) were similar to each other, but Hyp productivity with E. coliΔsucCDa·putA·VHb(L)·DsP4H (1.38 g l⁻¹ h⁻¹) was increased by 35.3% compared to that of E. coliΔsucCDa·putA·VHb·DsP4H (Fig. 4C and D). In addition, its succinate accumulation was reduced to 4.6 g l⁻¹ (Fig. 4C). These results displayed that the optimization of RBS strengths could improve the balance of cell respiration and Hyp production, thus enhancing Hyp productivity.

Producing Hyp with E. coliΔsucCDa·putA·VHb(L)·DsP4H in a 5-l bioreactor

Based on the above experiments, we further explored the potential of using whole-cell biocatalyst of the recombinant strain E. coliΔsucCDa·putA·VHb(L)·DsP4H for the transformation of L-proline to Hyp in 5-l bioreactors. In this batch culture, glucose and L-proline were rapidly consumed during cell growth and Hyp synthesis and were depleted completely at 36 h (Fig. 5A). In addition, strain E. coliΔsucCDa·putA·VHb(L)·DsP4H grew continuously from 0 to 36 h, and obtained a maximal OD of 32.3 (Fig. 5A). Hyp accumulated gradually in the broth from 0 to 36 h, and the final Hyp titre, conversion rate and productivity were up to 49.8 g l⁻¹, 87.4% and 1.38 g l⁻¹ h⁻¹ respectively (Fig. 5A). Further, the highest oxygen uptake rate (OUR) of E. coliΔsucCDa·putA·VHb(L)·DsP4H was 38.1% higher than that of E. coliΔsucCDa·putA·DsP4H (Fig. 5B), due to VHb overexpression. These results indicated that E. coliΔsucCDa·putA·VHb(L)·DsP4H was stable for scale-up culture, suggesting that it has great potential for industrial production of Hyp in the future.

Discussion

Hyp production can be catalysed by proline-4-hydroxylase (P4H) to hydroxylate L-proline with α-ketoglutarate (α-KG) and oxygen as co-substrates to generate succinate and carbon dioxide (CO₂) in the presence of ferrous ion. In this study, to improve the catalytic performance of E. coli, chassis engineering was used to enhance α-KG supply by rewiring the TCA cycle and L-proline degradation pathway and promote oxygen transfer by fine-tuning heterologous haemoglobin expression. These strategies resulted in a significant increase in Hyp titre, conversion rate and productivity up to 49.8 g l⁻¹, 87.4% and 1.38 g l⁻¹ h⁻¹ respectively. These results lay a good foundation for industrial production of Hyp in the future and pave the way to the development of whole-cell biocatalysis through combining metabolic engineering with enzymatic transformation for microbial production of other chemicals.

Metabolic engineering plays an important role in improving the catalytic efficiency of whole-cell biocatalyst through modifying cellular metabolic network to overcome potential metabolic bottlenecks. Interconnection between cellular metabolism and enzymatic transformation can be bridged tightly by co-substrates or redox cofactors required for enzyme activity (Blank et al., 2010; Falcioni et al., 2013; Schrewe et al., 2013; Theodosiou et al., 2015). As a typical example, Hyp biosynthesis from L-proline can be catalysed by P4H with α-KG and oxygen as co-substrates to generate succinate and CO₂ in the presence of ferrous ion. α-KG can be supplied to P4H from the TCA cycle and L-proline degradation pathway, and in turn central carbon metabolism can assimilate and recycle the coproduct of this enzymatic reaction succinate (Theodosiou et al., 2017). Generally, the availability of L-proline and α-KG in this hydroxylation will be influenced through five pathways (Zhang et al., 2018a): (i) degradation of L-proline with PutA (Falcioni et al., 2013), (ii) biosynthesis of L-proline from glutamate caused by GDH and proBAC, (iii) oxidation of citrate via the TCA cycle to form α-KG, followed by oxidation of α-KG to succinate with SucAB and SucCD, (iv) glyoxylate pathway from isocitrate to succinate via AceA, and (v) phosphorylation of
isocitrate dehydrogenase under the action of AceK (Smirnov et al., 2010). An effective method to improve the conversion of l-proline is to reinforce l-proline biosynthesis pathway by expressing proBAC genes (Zhang et al., 2018a) and interrupt l-proline degradation pathway by knocking out putA gene (Theodosiou et al., 2015) and the TCA cycle by deleting SucAB and SucCD genes (Zhang et al., 2018a, 2019). In this study, sucCD and putA genes were deleted in E. coli-DS4H, and the final engineered strain E. coliΔsucCΔputA-DS4H could efficiently convert l-proline to Hyp without α-KG addition. The reason is that overexpression of DS4H in E. coli drive the redirection of carbon flux partitioning at the α-KG node towards the Hyp biosynthesis as another bypass route for succinate formation. In addition, SucAB deletion could impair cell growth (Theodosiou et al., 2017; Zhang et al., 2018a), but these strategies in this study had no significant impact on cell growth due to the formation of succinyl-CoA via SucAB and succinate via DS4H.

Oxygen transfer is generally regarded as one of the main limiting factors in oxygen-demand process. P4H catalyses hydroxylation of proline to form Hyp with the co-substrate of α-KG undergoing oxidative decarboxylation to succinate. Due to the catalytic characteristics of P4H, Hyp production is a high-oxygen-demand process. In addition, Hyp fermentation broths also exhibit high viscosity during culture, which can further hinder oxygen transfer (Shibasaki et al., 1999; Falcioni et al., 2015). During microbial fermentation, oxygen transfer can be generally improved by increasing agitation and aeration rates (Falcioni et al., 2015), but this will cause high energy consumption and result in physical damage to cells (Smith et al., 1990). To overcome this issue, Vitreoscilla haemoglobin (VHb), an oxygen-binding protein (Orii and Webster, 1986), provides a good choice, which can enhance respiration and energy metabolism by promoting oxygen transfer to the intracellular terminal oxidases (Dikshit et al., 1992; Chi et al., 2009). VHb has been widely used to improve cell growth and chemical
production such as Hyp (Zhao et al., 2017), poly(L-lysine; Xu et al., 2015), polysaccharide (Li et al., 2016), natamycin (Wang et al., 2014) and fatty acids (Suen et al., 2014). In this study, VHb expression in recombinant E. coli ΔsucCDΔputA-DsP4H improved Hyp production, presumably by enhancing oxygen transfer. On the one hand, VHb expression enhances the level of dissolved oxygen, thus weakening the anaerobic fermentation pathway and enhancing the aerobic fermentation pathway (Clark, 1989). On the other hand, VHb expression improves cell growth and extends the period of exponential growth in both shake flasks and bioreactor fermentation (Zhang et al., 2013). Third, succinate is the major by-product resulting from hydroxylation of proline with P4H, and VHb expression was able to reduce the concentration of succinate in this study, suggesting that the VHb-mediated improvement of oxygen transfer helps the TCA cycle to match up with proline hydroxylation in E. coli ΔsucCDΔputA-VHb(L)-DsP4H. This strategy provides new insights into engineering

Fig. 5. Hyp production with E. coli ΔsucCDΔputA-VHb(L)-DsP4H in a 5-l bioreactor.
A. Hyp, L-proline, glucose and OD.
B. Comparison of OUR between E. coli ΔsucCDΔputA-VHb(L)-DsP4H and E. coli ΔsucCDΔputADsP4H.
E. coli host by flux coupling for the enzymatic production of Hyp and its related high-value-added products.

Experimental procedures

Strains and plasmids

E. coli F0901 was constructed to produce pyruvate, α-ketoglutarate (α-KG) and L-malate (Dong et al., 2017). The engineered E. coli strains used for trans-4-hydroxy-L-proline (Hyp) production in this study were derived from E. coli F0901, in which lactate dehydrogenase (ldhA), pyruvate oxidase (poxB), pyruvate formate lyase (pflB), phosphotransacetylase (pta), acetate kinase A (ackA), fumarate reductase (frdBC) and fumarase (fumB and fumAC) genes were all deleted (Dong et al., 2017). E. coli JM109 and plasmid pETM6R1 (Zhang et al., 2017). The expression of VHB was optimized by ribosome binding sites (RBSs) with different strengths (RBS03: CGACATAACGTAGAAAGAA-TAAGGTAGTTTCC; RBS09: TATTTAAACTACGACATAACGTAGTTTCC); RBS10: AAGAGGCCGGCGAAGAAGGAGGTAGTTTCC) from our previous study (Zhang et al., 2018b).

DNA manipulation

Standard molecular cloning was used for plasmid construction according to the protocol of EasyPath Brick Vectors Assembly (Xu et al., 2012). Proline 4-hydroxylase (P4H) gene from Dactylosporangium sp. RH1 (DsP4H, Gene ID: D78338.1) was artificially synthesized with codon optimization by Shanghai Sunny Biotechnology. P4H gene from Bacillus megaterium WSH-002 (BmP4H, Gene ID: BWSH-2348) was amplified from the corresponding chromosomal DNA by PCR. AoP4H (Gene ID: AOR_1_1350154) and AP4H (Gene ID: AFLA_030540) genes were amplified by PCR using the cDNA of Aspergillus oryzae RIB40 and Aspergillus flavus NRRL3357 as a template respectively. Haemoglobin gene from Vitreoscilla sp. HG1 (VhB, Gene ID: AF292694.1) was artificially synthesized by Shanghai Sunny Biotechnology. All gene deletions were performed according to the classical red homologous recombination method (Datkeno and Wanner, 2000). The expression of VHB was optimized by ribosome binding sites (RBSs) with different strengths (RBS03: CGACATAACGTAGAAAGAA-TAAGGTAGTTTCC; RBS09: TATTTAAACTACGACATAACGTAGTTTCC; RBS10: AAGAGGCCGGCGAAGAAGGAGGTAGTTTCC) from our previous study (Zhang et al., 2018b).

Table 2. Strains and plasmids used in this study

| Strains and plasmids          | Relevant characteristics | References       |
|-------------------------------|--------------------------|------------------|
| Strains                       |                          |                  |
| E. coli W3110                 | F^- λ- rph-1 INV(rmD, mE) | CGSC             |
| E. coli F0901                 | E. coli W3110            |                  |
| E. coli-frdBC                | ΔldhA::poxB::pFl::ptA-ackA::frdBC::fumB::fumAC |                  |
| E. coli-BmP4H                | E. coli F0901(pETM6R1-DsP4H) | This study      |
| E. coli-AoP4H                | E. coli F0901(pETM6R1-BmP4H) | This study      |
| E. coli-AP4H                 | E. coli F0901(pETM6R1-AoP4H) | This study      |
| E. coli-suCD-DsP4H           | E. coli F0901(SuCD::DsP4H) | This study      |
| E. coli-suCD::pA::DsP4H      | E. coli F0901(SuCD::pA::DsP4H) | This study      |
| E. coli-suCD::pA::VhB(L, M)  | E. coli F0901(SuCD::pA::VhB(L, M)) | This study      |
| Plasmids                      |                          |                  |
| pKD3                          | R6K::ori, cm^R, rnb(Ter)  | Invitrogen       |
| pKD4                          | R6K::ori, Km^R, rnb(Ter)  | Invitrogen       |
| pKD46                         | R101::ori, Amp^R, araBp-gam-bet-exo, repA101(ts) | Invitrogen       |
| pCP20                         | Amp^R, cm^R, FLP recombination | Invitrogen       |
| pETM6R1                      | ColE1::ori, Amp^R, Tc^R, DesP4H | This study      |
| pETM6R1-BmP4H                | ColE1::ori, Amp^R, Tc^R, BmP4H | This study      |
| pETM6R1-AoP4H                | ColE1::ori, Amp^R, Tc^R, AoP4H | This study      |
| pETM6R1-AP4H                 | ColE1::ori, Amp^R, Tc^R, AP4H  | This study      |
| pETM6R1-DsP4H-VhB(L)         | ColE1::ori, Amp^R, Tc^R, DesP4H-VhB(L) | This study      |
| pETM6R1-DsP4H-VhB(M)         | ColE1::ori, Amp^R, Tc^R, DesP4H-VhB(M) | This study      |
| pETM6R1-AoP4H-VhB(L)         | ColE1::ori, Amp^R, Tc^R, AoP4H-VhB(L) | This study      |
| pETM6R1-AP4H-VhB(M)          | ColE1::ori, Amp^R, Tc^R, AP4H-VhB(M)  | This study      |

Medium Luria–Bertani (LB) used for seed cultures: 5 g l^-1 yeast extract, 10 g l^-1 peptone, 5 g l^-1 NaCl. Ampicillin (100 mg ml^-1) was added appropriately when needed.

Modified AM1 mineral salts medium A used for fermentation in shake flasks: 50 g l^-1 α-KG, 20 g l^-1 glucose, 20 g l^-1 tryptone, 10 g l^-1 yeast extract, 2.63 g l^-1 (NH_4)_2HPO_4, 0.87 g l^-1 NH_4H_2PO_4, 0.15 g l^-1 KCl, 0.3 g l^-1 FeSO_4, 0.37 g l^-1 MgSO_4, 7H_2O, and 1 ml trace element solution (2.4 g l^-1 FeCl_3-6H_2O, 0.3 g l^-1 CoCl_2-6H_2O, 0.3 g l^-1 CuCl_2, 0.3 g l^-1 ZnCl_2-4H_2O, 0.3 g l^-1 NaMnO_4, 0.075 g l^-1 H_3BO_3, 0.5 g l^-1 MnCl_2-4H_2O, dissolve in 0.12 M HCl).
Ampicillin (100 mg ml⁻¹) and IPTG (0.4 mmol l⁻¹) were added appropriately when needed.

Modified AM1 mineral salts medium B used for fermentation in a 5-l bioreactor: 50 g l⁻¹ L-proline, 30 g l⁻¹ glucose, 20 g l⁻¹ tryptone, 10 g l⁻¹ yeast extract, 2.63 g l⁻¹ (NH₄)₂HPO₄·12H₂O, 0.87 g l⁻¹ NH₄H₂PO₄, 0.15 g l⁻¹ KCl, 0.3 g l⁻¹ FeSO₄, 0.37 g l⁻¹ MgSO₄·7H₂O, and 1 ml trace element solution (2.4 g l⁻¹ FeCl₃·6H₂O, 0.3 g l⁻¹ CoCl₂·6H₂O, 0.3 g l⁻¹ CuCl₂·3H₂O, 0.3 g l⁻¹ ZnCl₂·4H₂O, 0.3 g l⁻¹ Na₂MoO₄·2H₂O, 0.075 g l⁻¹ H₂BO₃, 0.5 g l⁻¹ MnCl₂·4H₂O, dissolve in 0.12 M HCl). Ampicillin (100 mg ml⁻¹) and IPTG (0.4 mmol l⁻¹) were added appropriately when needed.

**Culture conditions**

The seed culture inoculated from a slant was cultivated on a reciprocal shaker (200 rpm) at 37°C in a 250-ml flask containing 25 ml medium LB for 12 h. The broth was centrifuged, and then the supernatant liquid was discarded. The optical density at 600 nm was measured using a spectrophotometer. Glucose concentration was quantified by high-performance liquid chromatography (HPLC; Zhang et al., 2017). The glutamate, succinate and α-KG concentrations were determined by high-performance liquid chromatography (HPLC; Zhang et al., 2009). L-proline and Hyp were assayed by HPLC with a Zorbax Eclipse XDB-C18 column (Agilent, Santa Clara, CA, USA) at 40°C after derivatization with 2,4-dinitrofluorobenzene (Zhang et al., 2019).

**Measurement of conversion rate**

Conversion rate was determined using the following equation:

\[
\text{Conversion rate (\%)} = \frac{M3}{M1 - M2} \times 100
\]

where \(M1\) is the concentration of L-proline before conversion, \(M2\) is the remaining concentration of L-proline after conversion, and \(M3\) is the concentration of L-proline used for Hyp production.

**Measurement of oxygen consumption rate**

Oxygen consumption rate measurements were performed as described by Srikumar et al. (2013) with minor modifications. *E. coli* cells were cultured at 37°C in medium A until they reached the exponential growth phase. Then, cultures were collected, washed with 50 mM potassium phosphate buffer (pH 6.8), and resuspended to OD₆₀₀ = 0.1. Resuspended cells were used to seed XF 96-well microplates (Seahorse Biosciences, Santa Clara, CA, USA). Plates were centrifuged at 2000 rpm for 2 min and then allowed to rest for 30 min at 37°C. Oxygen consumption rate was measured according to the manufacturer’s manual on a Seahorse XF96 Analyzer. XF96 culture plates and the corresponding sensor cartridge were placed in Seahorse instrument, and temperature was maintained at 37°C. To equilibrate culture to instrument conditions, an initial wait time (20 min) was added. To allow for cell settling, a wait time (1 min) was also included after mixing 1 min. Before adding azide to a final concentration of 0.05% in media, three measurements were taken for the basal reading. Then, three additional readings were taken. The mean of the three readings across the span of 2 min was calculated for each well.

**Measurement of oxygen uptake rate**

Oxygen uptake rate (OUR) was determined in a 5-l bioreactor using the dynamic gassing-out method as described by Bhave and Chattoo (2003), Chen et al. (2007), and Wu et al. (2018). The airflow to bioreactor was stopped with simultaneous reduction in agitation rate to 100 rpm, and dissolved oxygen (DO) was measured by DO electrode (Mettler, Columbus, OH, USA). Aeration and agitation were restored before DO values reached critically low values. Time course of DO decrease was recorded, and the slope of DO vs time plots was used to determine OUR. 100% DO saturation indicated an oxygen concentration of 8 mg l⁻¹ (Bhave and Chattoo, 2003).

**Enzymes activity assays**

P4H activity was measured as reported by Yi et al. (2014). The reaction mixture containing 80 mM MES buffer (pH 6.5), 4 mM L-proline, 8 mM α-KG, 2 mM FeSO₄, 4 mM L-ascorbic acid, and cells were incubated at 35°C for 10 min with shaking, and then, cellular activity was inactivated completely by heat treatment at 100°C for 5 min. The concentration of Hyp in this mixture was
determined after centrifugation. One unit of P4H activity was defined as the amount of enzyme that forms 1 nmol of Hyp in one minute.

Proline dehydrogenase (PutA) activity was determined in toluene-treated whole cells as described by Deutch et al. (1985) with minor modifications. The reaction mixture containing toluene-treated cells, L-proline (1.0 M), α-aminobenzaldehyde (50 mM) and ethanol (20%) was incubated at 37°C for 2 h with vigorous shaking, and then, this assay was terminated by adding trichloracetic acid (20%). The concentration of Δ1-pyrroline-5-carboxylate in this mixture was measured to calculate PutA activity.

Glutamate dehydrogenase (GDH) activity was assayed by measuring spectrophotometrically the oxidation of NADPH at 340 nm at 22°C (Veronese et al., 1975). The reaction system containing 0.1 M Tris-HCl buffer (pH 8.0), 0.1 M NH4Cl, 2.5 mM a-KG and 0.1 mM NADPH was initiated by adding cell extracts. One unit of GDH activity was defined as the amount of enzyme that converts 1 pmol of NADPH in one minute.

Protein concentrations in cell extracts were determined by the Lowry method using bovine serum albumin as the standard (Lowry et al., 1951). The specific enzyme activity was defined as the enzyme activity per milligram of protein under the assay conditions.

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Conflict of interest

The authors declare no conflict of interest.

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