RESEARCH ARTICLE

Biosynthesis of (R)-(−)-perillyl alcohol by *Escherichia coli* expressing neryl pyrophosphate synthase

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Abstract
(R)-(−)-perillyl alcohol is widely used in agricultural and anticarcinogenic fields. Microbial production of (R)-(−)-perillyl alcohol was investigated in this study. We optimized biosynthesis of (R)-(−)-perillyl alcohol in *Escherichia coli* by using neryl pyrophosphate synthase and NADPH regeneration. Engineering neryl pyrophosphate (NPP)-supplied pathway resulted in a 4-fold improvement of (R)-(−)-perillyl alcohol titer. Subsequently, combined engineering of p-cymene monooxygenase (*CymA*) expression and module for NADPH regeneration exhibited a 15.4-fold increase of titer over the initial strain S02. Finally, 453 mg/L (R)-(−)-perillyl alcohol was achieved in fed-batch fermentation, which is the highest (R)-(−)-perillyl alcohol titer in *E. coli*.

KEYWORDS
(R)-(−)-perillyl alcohol, *Escherichia coli*, NADPH regeneration, neryl pyrophosphate synthase

INTRODUCTION

Perillyl alcohol, a monoterpene, has enantiomers of R and S. (R)-(−)-perillyl alcohol has been proved to have anticarcinogenic properties [1]. The breakthroughs of anticancer mechanism of (R)-(−)-perillyl alcohol have recently been made in oncology of lung, liver breast, lymphoma, and other diseases [2–4]. As such, the biosynthesis of (R)-(−)-perillyl alcohol has great significance. However, low yield (plant extracts) and environmental pollution (chemical synthesis) limit its development. Hence, more effective microbial production strategies may be an alternative to increase the production of (R)-(−)-perillyl alcohol.
NPP is the cis-isomer of GPP. The isomerization is principally occurring also if GPPS is employed but takes time and thus limits the space-time-yield. A previous study has shown that the engineered strain produced 87 mg/L (R)-(+)-perillyl alcohol in a 5 L bioreactor [5]. However, the (R)-(+)-perillyl alcohol produced by *Escherichia coli* is still below the level of industrialization. In recent decades, several efficient methods have been proposed for terpenoid production, focusing on NPPS instead of GPPS and improving NADPH regeneration.

GPP or NPP from the condensation of IPP and DMAP is the first step in the synthesis of isoprenoids (Figure 1) [6,7]. Overexpression of GPP synthase catalyzes substrate rearrangement to produce a large number of structures, resulting in low conversion efficiency [8]. However, NPP was orthogonal to the growth-associated pathway [9]. Anthony et al. [6] reported that NPP is another substrate for several monoterpenes including 2-carene, limonene, and α-terpinene. The study has shown that the KM value of PHS for NPP was 9.1 μM, with the KM value of PHS for GPP was 2900 μM in phellandrene production [6]. It shows that NPPS has stronger substrate affinity.

In recent years, NPPS has been used to synthesize terpenoids in *Saccharomyces cerevisiae* and *Yarrowia lipolytica* to achieve a higher production [9–12]. Meanwhile, production of monoterpenes from NPP was somehow better than production of monoterpenes from GPP.

**PRACTICAL APPLICATION**

The expression of neryl pyrophosphate (NPP) synthase provides an effective approach for converting renewable glucose to (R)-(+)-perillyl alcohol. After a series of engineering efforts, the optimized strain S11 biosynthesized 453 mg/L (R)-(+)-perillyl alcohol in fed-batch fermentation. In addition, our results indicated that biosynthesis of (R)-(+)-perillyl alcohol by expressing neryl pyrophosphate synthase is a powerful strategy to achieve the efficient yield in *E. coli*, which will promote the production of other biobased monoterpenes.

![Figure 1](image_url)
NADPH is the important cofactor of the mevalonate pathway and increasing cofactor NADPH supply can extend central metabolic targets and regulate metabolic balance [13,14]. A total 4 mol NADPH is required to produce 1 mol (R)-(+-)-perillyl alcohol (Figure 4A). A total 35%–45% of the NADPH required for biosynthesis is produced by pntAB expressed in E. coli [15]. Through the overexpression of pntAB to enhance the NADPH availability, the production of chemicals (i.e., β-carotene, 3-hydroxypropionate and poly-(3-hydroxybutyrate)) were improved [16–18]. In addition, MaeB, UdHA, tPos5 genes amplification, and yiguB gene deletion had also been defined to enhance availability of NADPH and experimentally verified by the improvement of protoilludene production [19].

In this study, we reported the engineering of the mevalonate pathway in E. coli for increased biosynthesis of (R)-(+-)-perillyl alcohol from renewable carbon source. To reach the target, the gene encoding NPPS from Solanum lycopersicum was codon optimized and expressed in E. coli. Secondly, to enhance (R)-(+-)-perillyl alcohol production, the expression of cymA gene was increased. Lastly, enhancing NADPH regeneration pathway to improve (R)-(+-)-perillyl alcohol production was discovered. This study may set a foundation to explore a platform for economical production of (R)-(+-)-perillyl alcohol and other terpenoids in the long-term run.

2 MATERIALS AND METHODS

2.1 Strains and culture conditions

Strains used in this study were listed in Table 1. Cultures were cultivated in LB medium (per liter: 10 g tryptone, 5 g yeast extract, and 10 g NaCl) containing 10 g/L glucose in shake-flask fermentation. The bioreactor fermentation medium contained (per liter) 20 g glucose, 9.8 g K2HPO4, 0.3 g ferric ammonium citrate, 2.1 g citric acid monohydrate, 0.12 g MgSO4, and 1 mL trace element solution, containing (NH4)6Mo7O24⋅4H2O 0.37 g/L, ZnSO4⋅7H2O 0.29 g/L, H3BO3 2.47 g/L, CuSO4⋅5H2O 0.25 g/L, and MnCl2⋅4H2O 1.58 g/L. The media were supplemented with appropriate antibiotics (100 or 50 µg/mL kanamycin) to maintain corresponding plasmids.

2.2 Plasmid construction

The npps gene (nerlyl pyrophosphate synthase, GenBank: NM_001247704.1) from S. lycopersicum was codon optimized by BGI and synthesized by GeneWiz (Suzhou, China), which was cloned into PUC57. Plasmid pS00 was constructed in previous study [5]. The mva and npps genes were assembled into pET-28a(+) using Cl15 (Vazyme Biotech Co., Ltd., Nanjing, China) at the BamHI/SalI sites, generating plasmid pET28a(+-mva-npps). In the same way, clls and cymA genes assembled into pET-28a(+) to generate pET28a(+-clls-cymA. mva and npps genes from pET-28a(+-mva-npps, clls and cymA genes from pET-28a(+-clls-cymA were cloned into pET28a(+) with BamHI/SalI restriction sites, creating pS01 (Table 1). The cymAa and cymAb from Pseudomonas putida were codon optimized for all plasmids. cymAa and cymAb were cloned into pACYCDuet-1-amp with BamHI/SacI restriction sites, constructing pS02 (pACYCDuet-1-cymAa-cymAb). The pntAB gene from E.coli BL21(DE3) was ligated into pACYCDuet-1-amp to creat pS03 (pACYCDuet-1-pntAB). pntAB was cloned from pS03 into pS02 at the BamHI/SacI sites to produce pS04 (Table 1). All primers are shown in Table S1.

2.3 Shake-flask fermentation

First, single colony was picked up from the LB agar plate and inoculated into a tube containing 4 mL LB broth and cultivated to the exponential phase. Then, seed cultures were inoculated into 250 mL baffled triangular flask containing 50 mL LB and 10 g/L glucose at 37°C, 200 pm with OD600 of 0.6–0.8, the temperature was set at 30°C inducted with 0.2 mM IPTG. OD600, glucose levels, perillyl alcohol and perillyl aldehyde production were then assessed for the induction of 24 and 48 h. To investigate the effect of pyruvate on (R)-(+-)-perillyl alcohol production in the shake flask, pyruvate concentrations varying from 0 to 4 g/L were added to the initial medium.

2.4 Fed-batch fermentation in a bioreactor

The engineered strain was cultured for 12 h for 37°C in 250 mL shake flask with 50 mL LB medium. Fed-batch fermentations were applied in a 5 L fermenter (ez-control, Applikon) containing 2 L fermentation broth at 0.5–1.5 vvm aeration and 400–800 rpm, respectively. The fermentation was inoculated with 5.0 % (v/v) seed culture, and performed at 37°C and pH 7.0 with addition of NH4OH (25%, v/v). When the OD600 reached 20 (initial glucose was consumed), 0.2 mM IPTG was added and the temperature was set at 30°C. The feeding speed was variable for the induction time with 70% glucose supplement (0–24 h, 2%, 24–50 h, 3%). The glucose concentration was less than 1 g/L and dissolved oxygen (DO) levels were not less than 20%. The concentration of glucose, cell density (OD600),
TABLE 1  Strains and plasmids used in this study

| Name      | Relevant characteristics | References |
|-----------|--------------------------|------------|
| Strains   |                          |            |
| E. coli DH5α | F− recA endA1Δ80dlacZ/ΔM15hsdR17(τ− mK+)λ− | Invitrogen |
| E. coli BL21(DE3) | F− ompT hsdSB (rB− mB−) gal dcm rne131Δ(DE3) | Invitrogen |
| S00       | E. coli. BL21(DE3)::Trc-Low | [25]       |
| S01       | E. coli. BL21(DE3)::Trc-Low △YjGB | This study |
| S02       | S00 /pS00                 | This study |
| S03       | S00 /pS01                 | This study |
| S04       | S00 /pS00, pS02           | This study |
| S05       | S00 /pS00, pS03           | This study |
| S06       | S00 /pS00, pS04           | This study |
| S07       | S01 /pS00, pS04           | This study |
| S08       | S00 /pS01, pS02           | This study |
| S09       | S00 /pS01, pS03           | This study |
| S10       | S00 /pS01, pS04           | This study |
| S11       | S01 /pS01, pS04           | This study |
| S12       | S00 /pS01, pS04           | This study |
| Plasmids  |                          |            |
| pTrcHis2B | pBR322 origin, Amp        | Invitrogen |
| pET28a(+) | Kan’ ori pBR322lacI1T7p   | Novagen    |
| pACYCDuet-1 | Cm’ p15A lacI T7lac   | Novagen    |
| pACYCDet-1-Amp | Amp’ p15A lacI T7lac | This study |
| Trc-Low   | pTrcHis2B carrying ERG12, ERG8, ERG19 and IDI from S. cerevisiae | [25]       |
| pS00      | pET28a(+) carrying mvaE and mvaS from Enterococcus faecalis, gpps from Abies grandis, cls from Citrus limon, cymAa and cymAb from P. putida | This study |
| pS01      | pET28a(+) carrying mvaE and mvaS from Enterococcus faecalis, npps from S. lycopersicum, cls from Citrus limon, cymAa, and cymAb from P. putida | This study |
| pS02      | pACYCDet-1-Amp carrying cymAa and cymAb from P. putida | This study |
| pS03      | pACYCDet-1-Amp carrying pntAB from E. coli BL21(DE3) | This study |
| pS04      | pACYCDet-1-Amp carrying cymAa and cymAb from P. putida, pntAB from E. coli BL21(DE3) | This study |

(R)-(+)–perillyl alcohol, perillyl aldehyde, and acetate were detected every 4 h.

For the effect of pyruvate on fermentation, 3 g/L pyruvate was added into the fermentation medium in induction time. For extractive fermentation, 10% (w/v) dioctyl phthalate (DINP) was added after 2 h of induction.

2.5 Analytical methods

The biomass of E. coli was determined by OD600 on a spectrophotometer (Cary 50 UV-vis, Varian). When OD600 was 1, the dry cell weight (DCW) was calculated as 0.323 g DCW/L.

Limonene, (R)-(+)–perillyl alcohol and perillyl aldehyde were determined by gas chromatography (GC) analysis. Specific test methods and sample treatment were reported [5].

Glucose, pyruvate, and acetic acid were detected by HPLC (LC-20A, Shimadzu, Japan) equipped with an Aminex HPX-87H ion exchange column with 5 mM sulfuric acid and a refractive index detector (RID) at 0.4 mL/min. NADP+ and NADPH were determined by the assay kits (KTB1010, CheKine NADP/NADPH Assay Kit, Shanghai). For the assay, 1 mL was sampled at 24 h and then centrifuged 10,000 rpm for 10 min at 4°C.

3 RESULTS AND DISCUSSION

3.1 Improvement of production by enhancing NPP-supplied pathway

The (R)-(+)–perillyl alcohol synthetic pathway was constructed in engineered E. coli (Figure 1). GPP has been widely used as a monoterpenesubstrate, including
geraniol, sabinene, pinene, and limonene [20–22], while there were a few reports on NPP. It is reported that the solubility of GPPS is low, which may affect the production of terpenoids [23]. Another study showed that NPP has advantages over GPP in the synthesis of other monoterpenes [24]. gpps, npps, and cls genes were codon-optimized. ERG8, ERG9, and ERG12 genes were successfully integrated into E. coli BL21 (DE3) strain [25]. npps gene codon-optimized was integrated to S00 to obtain S03 strain. As shown in Figure 2B, strain S03 harboring npps gene produced 12 mg/L (R)-(+)-perillyl alcohol, 4-fold higher than strain S02 harboring gpps gene (3.1 mg/L) in 48 h postinduction. These results showed that (R)-(+)-perillyl alcohol production for NPPS was higher than GPPS.

Wu et al. [11] described that limonene production of the strain harboring npps and ls in the downstream module was improved by 110-fold compared with the original strain expressed gpps. Terpenes (1,8-cineole, camphene) synthases have a greater $K_{cat}$ for NPP [9]. GPP may catalyze the rearrangement of the substrate to produce other structures, involve in cell growth, and then reduce monoterpenes production [26]. Therefore, the engineered strain carrying npps may have efficient effect on monoterpene production, while the strain harboring gpps get more flux toward FPP and promote synthesis of sesquiterpenes.

3.2 Increasing (R)-(+) -perillyl alcohol production by increasing cymA expression

It has been reported that higher yields of (R)-(+) -perillyl alcohol and perillyl acid chemicals were obtained by using limonene as substrate, especially in P. putida and Y. lipolytica [27–29]. (R)-(+) -perillyl alcohol gradually reached a high titer with the addition of limonene in the report [5]. Even though the production of limonene was 1.29 g/L in engineered E. coli [11], the yield of (R)-(+) -perillyl alcohol from limonene was not maximized. Therefore, limonene hydroxylase, CymA, was the rate-determining step to the titer of (R)-(+) -perillyl alcohol in this study. We proposed to increase the expression of cymA and construct new strains (i.e., S04 and S08) (Figure 3A). The expression of cymAa and cymAb in S04 and S08 significantly increase compared to the control by RT-PCR (Figure S1). The (R)-(+) -perillyl alcohol production of the optimized strains (S04 and S08) reached a titer of 4.8 mg/L and 18 mg/L (1.5-fold and 6-fold increase over the control strain), and OD600 was respectively 7.0 and 8.2 at 24 h after induction (Figure 3B and C). By increasing expression of cymA gene, the production of (R)-(+) -perillyl alcohol was further improved (Figure 3B and C). Increasing cymA in pACYCDuet-1 may balance the remaining pathway and transform the intermediate product into (R)-(+) -perillyl alcohol to the uttermost.

3.3 Effect of NADPH regeneration on the production of (R)-(+) -perillyl alcohol

A total amount of 2 mol NADPH is required for the synthesis of 1 mol mevalonate from 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) (Figure 4A). In the glycolytic pathway, about 6 mol NADH is synthesized for every 1.5 mol of glucose [30]. Therefore, how to convert the excess NADH into NADPH to increase (R)-(+) -perillyl alcohol production is the main problem. The NADPH regeneration can be promoted by the reduction of NADH to NADPH and decreasing the consumption of NADPH.
Plasmid pS03 was constructed by inserting pntAB (Table 1). Overexpression of pntAB in the strain S05/S09 also harboring plasmids pS03 resulted in an improvement on (R)-(+-)perillyl alcohol production. Strain S05 and S09 produced 7.7 and 22 mg/L of (R)-(+-)perillyl alcohol after 48 h postinduction, respectively more than the initial strain S02 (2.5 and 7-fold) (Figure 4C). Whereas the cell concentration of S09 was 13.5, a little higher than strain S05 (OD600 = 12.0) (Figure 4B). These results confirmed the importance of the overexpression of pntAB for (R)-(+-)perillyl alcohol production. PntAB has been reported to improve NADPH for several NADPH-dependent oxidation-reduction reactions in an engineered E. coli [17,31].

Aldehyde reductase (YjgB) was unnecessary NADPH-dependent for broad substrate specificities [32]. Previous studies have explained the deletion of yjgB, which signifies decreasing the consumption of NADPH, to increase protoilludene and β-carotene production in E. coli [19,33]. Therefore, the gene yjgB of the host strain S00 was knocked out to become S01. Strain S06 and S10 were on the basis of strain S01 (Table 1). (R)-(+-)perillyl alcohol production in the strains S06 and S10 was 8.9 and 27 mg/L. In addition, the ratio of (R)-(+-)perillyl alcohol to the by-product perillyl aldehyde production was 1.8:1 in the strain S10, while the ratio was 1:1 in the strain S09 (Table S2). We found that the deletion of yjgB could reduce the by-product perillyl aldehyde amount to a certain extent. Intracellular concentrations of NADPH were 21.2 and 27.8 µmol/g CDW by overexpression of pntAB and deletion of yjgB, which were 21% and 58% higher than those in strain S08 (Table 2). It was proved that NADPH regeneration was a restrictive factor of (R)-(+-)perillyl alcohol production.

3.4 Combined engineering of cymA expression and module for NADPH regeneration to further increase (R)-(+-)perillyl alcohol production

Combing engineering of cymA expression and module for NADPH regeneration, it is to test whether there is a synergistic effect on (R)-(+-)perillyl alcohol production. The strains S07 and S11 with combined modulation of cymA and pntAB, had a (R)-(+-)perillyl alcohol titer of 16 and 32 mg/L, which were 5- and 10-fold higher than strain S02 (Figure 4C). It was proved that strain S11 for NPP substrate was more suitable for the titer and the cell growth than the strain S07 for GPP substrate (Figure 4B).
3.5 Effects of pyruvate on (R)-(+)-perillyl alcohol production

Pyruvate is a precursor of acetyl-CoA in the mevalonate pathway. In previous reports, the addition of pyruvate as an auxiliary carbon source can increase the yield of terpenoids [24,34,35]. In order to investigate the effect of pyruvate additions on (R)-(+)-perillyl alcohol production, pyruvate concentrations (0, 1, 2, 3, and 4 g/L) were added to the shake flask with LB medium. Although there were no obvious changes in cell growth, the highest titer of (R)-(+)-perillyl alcohol was 48 mg/L with the addition of 3 g/L pyruvate, 15.4- and 1.5-fold increase over the initial strain S02 and the cultivation without pyruvate (Figure 4D). The results may indicate adding pyruvate as an auxiliary carbon source can promote (R)-(+)-perillyl alcohol production by strain S11. Since pyruvate is a precursor of the mevalonate pathway, the addition of pyruvate may greatly improve IPP and DMAPP production, and thus significantly increased the titer of (R)-(+)-perillyl alcohol.
Fed-batch production of \((R)\)-\((+)-\)perillyl alcohol in 5 L bioreactors. Strains S12 (A) and S11 (B) were used for the experiments, respectively. (C) Fed-batch production by the strain S11 with the addition of 3 g/L pyruvate. (D) Fed-batch production by the strain S11 with tyruvic acid addition and DINP extraction after 2 h of induction.

### 3.6 \((R)\)-\((+)-\)perillyl alcohol production from an engineered strain in a 5 L bioreactor

In order to obtain a high titer of \((R)\)-\((+)-\)perillyl alcohol, fed-batch fermentation was carried out in a 5 L bioreactor with pH at 7.0. We constructed strains S12 and S11 to verify whether \(yjgB\) deletion reduces the by-product perillyl aldehyde. When strain S12 was used, it produced 162 mg/L of perillyl alcohol and 142 mg/L of perillyl aldehyde at 27 h of induction in fed-batch fermentation (Figure 5A). Meanwhile, strain S11 produced 242 mg/L of perillyl alcohol (49% higher than S12) and 125 mg/L of perillyl aldehyde at 30 h of induction (Figure 5B). The ratio of \((R)\)-\((+)-\)perillyl alcohol to the by-product perillyl aldehyde was 2:1 in S11, higher than the ratio in S12 (1:1). It may be concluded that strain S11 (\(yjgB\) deletion) can reduce perillyl alcohol to perillyl aldehyde and improve the production, while \(yjgB\) deletion increased geraniol production in the report [21]. S11 achieved the highest titer of 304 mg/L \((R)\)-\((+)-\)perillyl alcohol and OD600 reached 47 at 31 h of induction with the addition of 3 g/L pyruvate in the fermentation medium (Figure 5C). The content of pyruvate was maintained at 1.0–2.5 g/L between 6 and 13 h and rarely after 13 h (Figure 5C). While pyruvate production by glycolysis was a little without the addition of pyruvate (Figure 5B). It concluded that adding pyruvate could promote the supply of acetyl CoA, metabolic engineering of mevalonate pathway and \((R)\)-\((+)-\)perillyl alcohol production. At the later stage of fermentation, the production was decreased. It may be due to transportation of intermediates [1] and cytotoxicity induced by residual acetic acid [36]. We detected the titer of acetic acid was 4–5 g/L (Figure 5C) in the later fermentation period, which may lead to other metabolic pathways and reduce \((R)\)-\((+)-\)perillyl alcohol production. Furthermore, optimization of fermentation process and efflux pumps (intermediates transportation and cellular export systems) may provide a mechanism to improve \((R)\)-\((+)-\)perillyl alcohol production.

In recent study, DINP was selected as an advantageous solvent for limonene and perillyl alcohol extraction, avoiding evaporative loss of limonene [37,38]. When S11 was used by addition pyruvate and DINP overlay, the maximum \((R)\)-\((+)-\)perillyl alcohol concentration was
**TABLE 3** Summary of microbial production of (R)-(+)-perillyl alcohol in *E. coli*

| Strains     | Gene modifications                        | Substrate | Cultivation mode | Titer (mg/L) | Yield (mg/g glucose) | Reference |
|-------------|-------------------------------------------|-----------|------------------|--------------|----------------------|-----------|
| *E. coli*   | Expression of limonene hydroxylase from *Bacillus stearothermophilus* BR388 | (R)-(+)-limonene | Shake flask | 0.51 | NA          | [39]      |
| *E. coli*   | **gpps, cls, and cymA expression**       | Glucose   | Fed-batch       | **87**       | 1.5                  | [5]       |
| *E. coli*   | **npps, pntAB expression and yjgB deletion** | Glucose   | Fed-batch       | **453**      | 4.0                  | This study |

453 mg/L and the yield was 4.0 mg/g glucose after 35 h of induction, 5.2- and 2.6-fold, compared to the previous report, respectively [5] (Table 3, Figure 5D). We found that (R)-(+)-perillyl alcohol production has a decreasing tendency in the time course of fed-batch fermentation (Figure 5D). DINP could be quite cytotoxic to cell survival. It could be better to use in situ stripping methods or particulate adsorbed materials. This should definitely be a task for future experiments to develop a usable productive process.

### 4 CONCLUDING REMARKS

In this study, we reported the engineering of the mevalonate pathway in *E. coli* for (R)-(+)-perillyl alcohol. First, the (R)-(+)-perillyl alcohol production was 12 mg/L by NPP-supplied pathway, second, 32 mg/L of (R)-(+)-perillyl alcohol was achieved by increasing cymA expression and NADPH regeneration. Finally, the production raised to 48 mg/L when 3 g/L pyruvate was added. Through combined engineering of npps, cymA regulation, and NADPH regeneration, S11 was obtained which proved 146-fold titer improvement of the initial strain S02 in fed-batch fermentation. The best strain S11 produced 453 mg/L (R)-(+)-perillyl alcohol with a yield of 4.0 mg/g glucose in a 5 L bioreactor (Table 3). The yield of (R)-(+)-perillyl alcohol is still relatively low for the strain S11. Together, these results indicate that other monoterpenes can be synthesized by current technical strategies using NPP as substrate. However, the method of NPP-supplied way is not available to reach industrial applicability, which is probably due to focus on the optimization of rate-limiting enzymes, metabolic imbalances, and accumulation of toxic intermediates.

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**CONFLICTS OF INTEREST**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**REFERENCES**

1. Das BC, Mahalingam SM, Panda L, Wang B, Evans T. Design and synthesis of potential new apoptosis agents: hybrid compounds containing perillyl alcohol and new constrained retinoids. Tetrahedron Lett. 2010;51:1462–1466.

2. Fischer JDSDG, Carvalho PC, Neves-Ferreira AGDC, Fonseca COD, Domont GB. Anti-thrombin as a prognostic biomarker candidate for patients with recurrent glioblastoma multiform under treatment with perillyl alcohol. J Exp Ther Oncol. 2008;7:285–290.

3. Pyrczak-Felczykowska A, Narlawar R, Pawlik A, Guzow-Krzemińska B, Kassiou M. Synthesis of usnic acid derivatives and evaluation of their antiproliferative activity against cancer cells. J Nat Prod. 2019;82(7):1768–1778.

4. Cho H, Wang W, Jhaveri N, et al. Perillyl alcohol for the treatment of temozolomide-resistant gliomas. Mol Cancer Ther. 2012;11(11):2462–2472.

5. Sun C, Dong X, Zhang R, Xie C. Effectiveness of recombinant *Escherichia coli* on the production of (R)-(−)-perillyl alcohol. BMC Biotechnol. 2021;21:3.

6. Schilmiller AL, Schauvinhold I, Larson M, et al. Monoterpenes in the glandular trichomes of tomato are synthesized from a neryl diphosphate precursor rather than geranyl diphosphate. Proc Natl Acad Sci USA. 2009;106:10865–10870.

7. Akhtar T, Matsuba Y, Schauvinhold I, Yu G, Pichersky E. The tomato cis-prenyltransferase gene family. Plant J. 2013;73:640–652.

8. Croteau R. Biosynthesis and catabolism of monoterpenoids. Chem Rev. 1987;87:929–954.

9. Ignea C, Raadam M, Motawia M, Makris A, Kampranis S. Orthogonal monoterpenoid biosynthesis in yeast constructed on an isomeric substrate. Nat Commun. 2019;10(3799):1–15.

10. Cheng S, Liu X, Jiang JG, Wu J, Zhang J. Orthogonal Engineering of Biosynthetic Pathway for Efficient Production of Limonene in *Saccharomyces cerevisiae*. ACS Synth Biol. 2019;8(5):968–975.
11. Wu J, Cheng S, Cao J, Qiao J, Zhao G. Systematic optimization of limonene production in engineered Escherichia coli. J Agric Food Chem. 2019;67(25):7087–7097.
12. Kang JH, Gonzales-Vigil E, Matsuba Y, Pichersky E, Barry CS. Determination of residues responsible for substrate and product specificity of Solanum habrochaites Short-Chain cis-Prenyltransferases. Plant Physiol. 2014;164:80–91.
13. Alper H, Fischer C, Nevoigt E, Stephanopoulos G. Tuning genetic control through promoter engineering. Proc Natl Acad Sci USA. 2005;102:12678–12683.
14. Choi HS, Lee SY, Kim TY, Woo HM. In silico identification of gene amplification targets for improvement of lycopene production. Appl Environ Microbiol. 2010;76:3097–3105.
15. Sauer U. The soluble and membrane-bound transhydrogenases UdhA and PntAB have divergent functions in NADPH metabolism of Escherichia coli. J Biol Chem. 2004;279:6613.
16. Zheng Y, Yuan Q, Yang X, Ma H. Engineering Escherichia coli for poly-(3-hydroxybutyrate) production guided by genome-scale metabolic network analysis. Enzyme Microb Technol. 2017;106:60–66.
17. Liu B, Xiang S, Zhao G, et al. Efficient production of 3-hydroxypropionate from fatty acids feedstock in Escherichia coli. Metab Eng. 2019;51(2019):121–130.
18. Zhao J, Li Q, Sun T, Zhu X, et al. Engineering central metabolic modules of Escherichia coli for improving β-carotene production. Metab Eng. 2013;17:42–50.
19. Zhou J, Yang L, Wang C, Choi ES, Kim SW. Enhanced performance of the methylethylerythritol phosphate pathway by manipulation of redox reactions relevant to IspC, IspG, and IspH. J Biotechnol. 2017;248:1–8.
20. Zhang H, Liu Q, Cao Y, et al. Microbial production of sabinen—A new terpene-based precursor of advanced biofuel. Microb Cell Fact. 2014;13(20):1–10.
21. Zhou J, Wang C, Yang L, Choi ES, Kim SW. Geranyl diphosphate synthase: an important regulation point in balancing a recombinant monoterpene pathway in Escherichia coli. Enzyme Microb Technol. 2015;68:50–55.
22. Sarria S, Wong B, Martin HG, Keasling JD, Peralta-Yahya P. Microbial synthesis of pinene. ACS Synth Biol. 2014;3:466–475.
23. Willrodt C, David C, Cornelissen S, Bühler B, Julsing MK, Schmid A. Engineering the productivity of recombinant Escherichia coli for limonene formation from glycerol in minimal media. Biotechnol J. 2014;9:1000–1012.
24. Cao X, Lv Y, Chen J, Imanaka T, Wei L, Hua Q. Metabolic engineering of oleaginous yeast Yarrowia lipolytica for limonene overproduction. Biotechnol Biofuels. 2016;9(214):1–11.
25. Cheng T, Zhao G, Xian M, Xie C. Improved cis-Abienol production through increasing precursor supply in Escherichia coli. Sci Rep. 2020;10:1–11.
26. Croteau R. Biosynthesis and catabolism of monoterpenoids. Chem Rev. 2002;87:929–954.
27. Mars AE, Gorissen JPL, Beld IVD, Eggink G. Bioconversion of limonene to increased concentrations of perillyl acid by Pseudomonas putida GSI in a fed-batch reactor. Appl Microbiol Biotechnol. 2001;56:101–107.
28. Mirata MA, Heerd D, Schrader J. Integrated bioprocess for the oxidation of limonene to perillyl acid with Pseudomonas putida DSM 12264. Process Biochem. 2009;44:764–771.
29. Alonso-Gutierrez J, Kim E-M, Bath TS, et al. Principal component analysis of proteomics (PCAP) as a tool to direct metabolic engineering. Metab Eng. 2015;28:123–133.
30. Zhang S, Yang W, Chen H, Liu B, Tao Y. Metabolic engineering for efficient supply of acetyl-CoA from different carbon sources in Escherichia coli. Microb Cell Fact. 2019;18(130):1–11.
31. Hao Y, Ma Q, Liu X, et al. High-yield production of L-valine in engineered Escherichia coli by a novel two-stage fermentation. Metab Eng. 2020;62:198–206.
32. Pick A, Rühmann B, Schmid J, Sieber V. Novel CAD-like enzymes from Escherichia coli K-12 as additional tools in chemical production. Appl Microbiol Biotechnol. 2013;97:5815–5824.
33. Wu Y, Yan P, Li Y, Liu X, Zhao X. Enhancing β-Carotene production in Escherichia coli by perturbing central carbon metabolism and improving the NADPH supply. Front Bioeng Biotechnol. 2020;8(585):1–13.
34. Du FL, Yu HL, Xu JH, Li CX. Enhanced limonene production by optimizing the expression of limonene biosynthesis and MEP pathway genes in E. coli. Bioreour Bioprocess. 2014;1:10.
35. Zhou K, Zou R, Zhang C, Stephanopoulos G, Too HP. Optimization of amorphadiene synthesis in bacillus subtilis via transcriptional, translational, and media modulation. Biotechnol Bioeng. 2013;110:2556–2561.
36. Lee M, Chun J. Effects of glucose and acetic acid on the growth of recombinant E. coli and the production of pyruvate dehydrogenase complex-E2 specific human monoclonal antibody. Korean J Biotechnol Bioeng. 2000;15(2):482–488.
37. Cornelissen S, Julsing M, Volmer J, Riechert O, Schmid A. Whole-cell-based CYP153A6-catalyzed (S)-limonene hydroxylation efficiency depends on host background and profiles from monoterpane uptake via AlkL. Biotechnol Bioeng. 2013;110:1282–1292.
38. Rolf J, Julsing M, Rosenthal K. A gram-scale limonene production process with engineered Escherichia coli. Molecules. 2020;25(1881):1–25.
39. Chang H, Gage D, Oriel P. Cloning and expression of a limonene degradation pathway from Bacillus steatorhermophilus in Escherichia coli. J Food Sci. 1995;60(3):551–553.

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