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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

1 | 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.

Abbreviations: ClLS, limonene synthase from *Citrus limon*; CymA, p-cymene monoxygenase hydroxylase and p-cymene monoxygenase reductase; CymAa, p-cymene monoxygenase hydroxylase; CymAb, p-cymene monoxygenase reductase; DMAPP, dimethylallyl diphosphate; ERG12, mevalonate kinase; ERG19, mevalonate pyrophosphate decarboxylase; ERG8, phosphomevalonate kinase; FPP, famesyl diphosphate; GPP, geranyl diphosphate; GPPS, Abies grandis geranyl diphosphate synthase; IDI, IPP isomerase; IPP, isopentenyl diphosphate; LB, lysogeny broth; LS, limonene synthase; mvaE, acetyl-CoA acetyltransferase/HMG-CoA reductase; mvaS, HMG-CoA synthase; NPP, neryl pyrophosphate; NPPS, neryl pyrophosphate synthase; PHS, phellandrene synthase; pntAB, NAD(P) transhydrogenase; yjgB, aldehyde reductase

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.

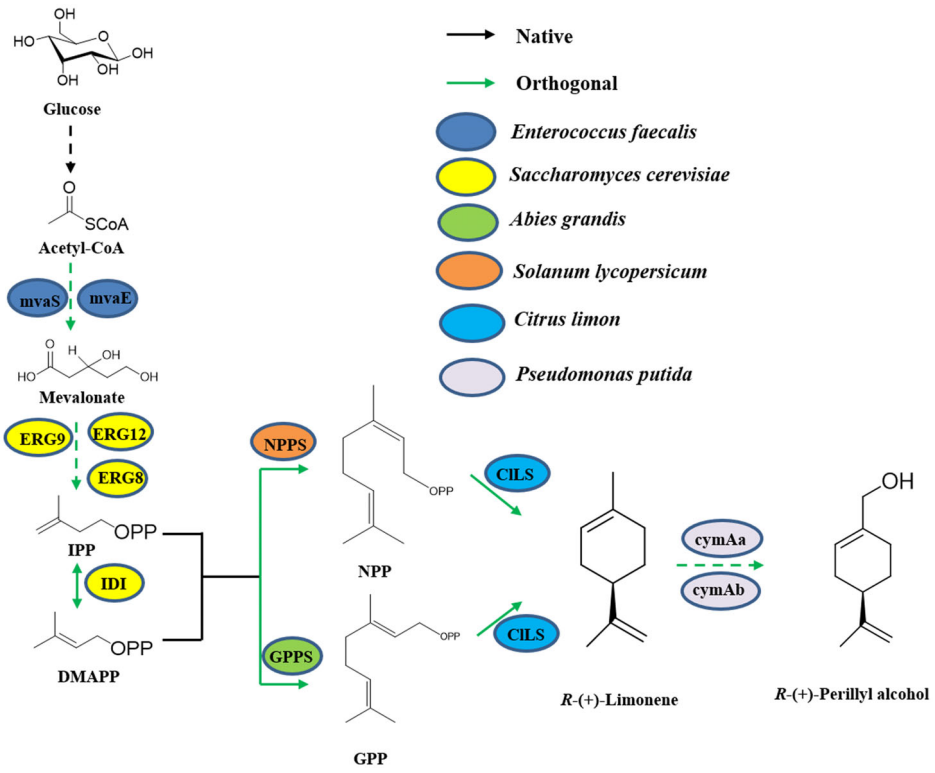

FIGURE 1 (*R*)-(+)-perillyl alcohol production in engineered *E. coli*. Enzymes are described in the legend. The dashed arrows indicate multiple steps. Metabolite abbreviations: ClLS, limonene synthase; cymAa, p-cymene monoxygenase hydroxylase; cymAb, p-cymene monoxygenase reductase; DMAPP, dimethylallyl diphosphate; ERG8, phosphomevalonate kinase; ERG12, mevalonate kinase; ERG19, mevalonate pyrophosphate decarboxylase; GPP, geranyl diphosphate; GPPS, *Abies grandis* geranyl diphosphate synthase; IDI, IPP isomerase; IPP, isopentenyl diphosphate; mvaE, acetyl-CoA acetyltransferase/HMG-CoA reductase; mvaS, HMG-CoA synthase; NPP, neryl pyrophosphate; NPPS, neryl pyrophosphate synthase

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 *yjgB* 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 K_2HPO_4, 0.3 g ferric ammonium citrate, 2.1 g citric acid monohydrate, 0.12 g $MgSO_4$, and 1 mL trace element solution, containing $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ 0.37 g/L, $ZnSO_4{\cdot}7H_2O$ 0.29 g/L, H_3BO_4 2.47 g/L, $CuSO_4{\cdot}5H_2O$ 0.25 g/L, and $MnCl_2{\cdot}4H_2O$ 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 (neryl 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 C115 (Vazyme Biotech Co., Ltd., Nanjing, China) at the *Bam*HI/*Sal*I 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 *BamH*I/*Sal*I 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 *BamH*I/*Sac*I 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 *BamH*I/*Sac*I 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 applicated 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 NH_4OH (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⁻ *rec*A *endA*1Φ80d*lacZ*△*M15hs*dR17($r_k^-m_k^+$)λ^-	Invitrogen
E. coli BL21(DE3)	F- ompT hsdSB (rB – mB -) gal dcm rne131 λ(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^roripBR322lacI^qT7p	Novagen
pACYCDuet-1	Cm^r p15A lacI T7lac	Novagen
pACYCDet-1-Amp	Amp^r 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*, *clls* from *Citrus limon, cymAa* and *cymAb* from *P. putida*	This study
pS01	pET28a(+) carrying *mvaE* and *mvaS* from *Enterococcus faecalis*, *npps* from *S. lycopersicum*, *clls* 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 AminexHPX-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 monoterpene substrate, including

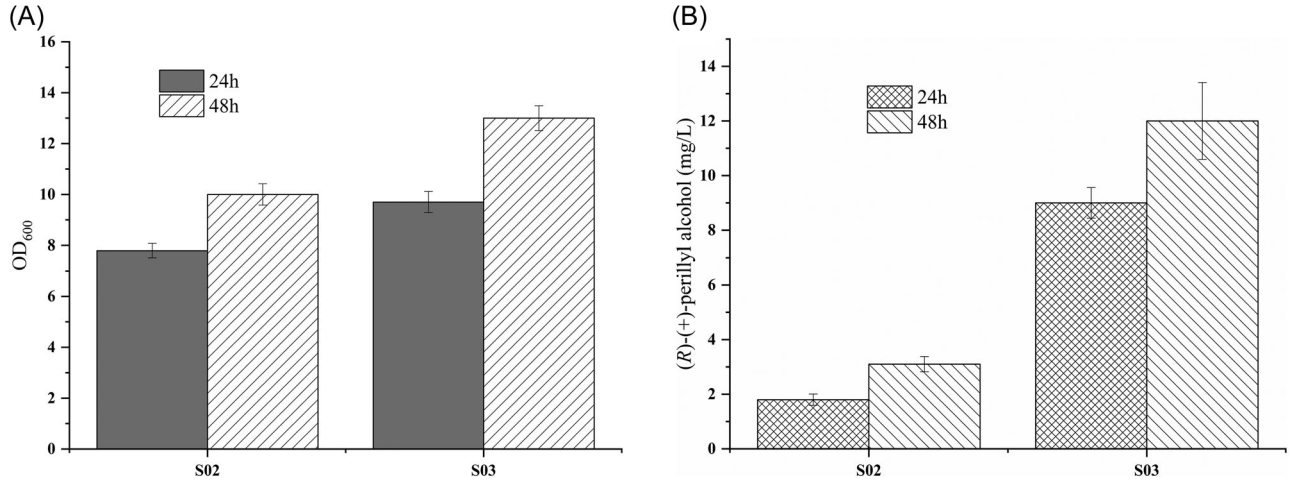

FIGURE 2 (*R*)-(+)-Perillyl alcohol production using strains S02 and S03. The OD600 (A) and production (B) of (*R*)-(+)-Perillyl alcohol were measured after 24 and 48 h of IPTG induction in the shake flask. Data are presented as the mean ± s.d. ($n = 3$)

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 *clls* 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 monoterpenes 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 expreesion 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.

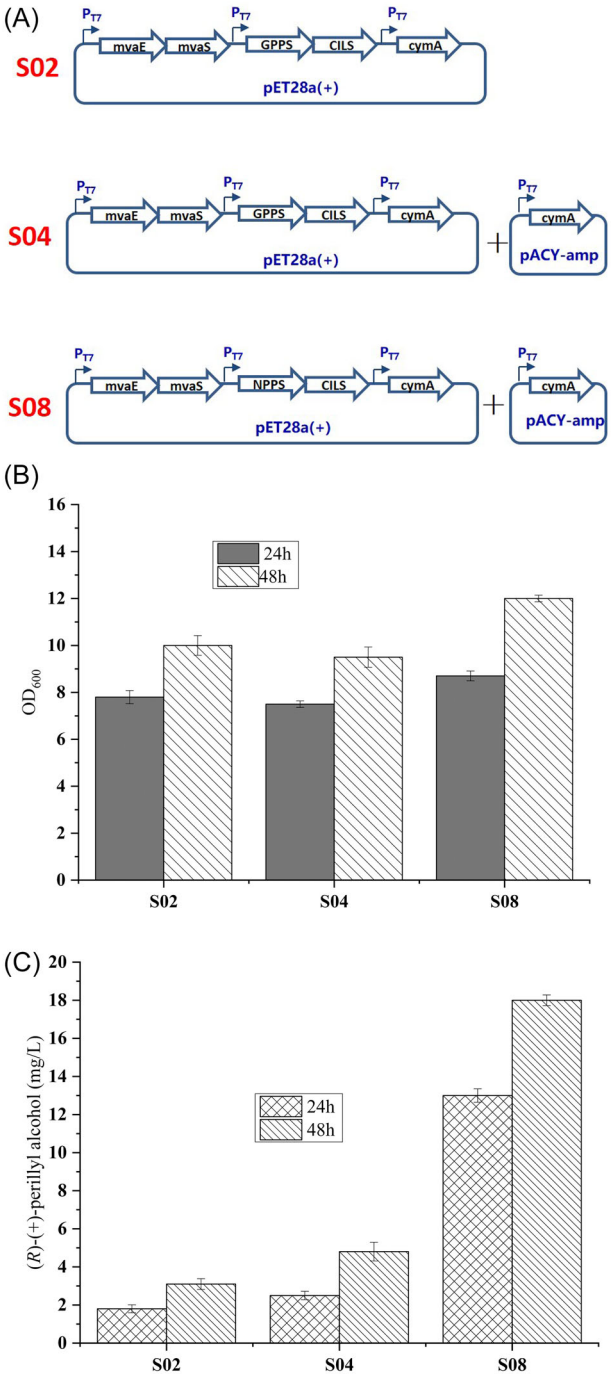

FIGURE 3 (*R*)-(+)-perillyl alcohol production by regulating *cymA* expression. (A), Strains for (*R*)-(+)-perillyl alcohol production. The OD600 (B) and total production (C) of (*R*)-(+)-perillyl alcohol were measured after 24 h and 48 h of induction in the shake flask. Data are presented as the mean ± s.d. ($n = 3$)

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].

TABLE 2 Intracellular concentrations of NADPH and NADPH/NADP$^+$ at 24 h in stains S08, S09, and S10

strains	NADPH (μmol/g CDW)	NADPH/NADP$^+$
S08	17.5 ± 0.2	1.12
S09	21.2 ± 0.4	0.66
S10	27.8 ± 0.6	0.84

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).

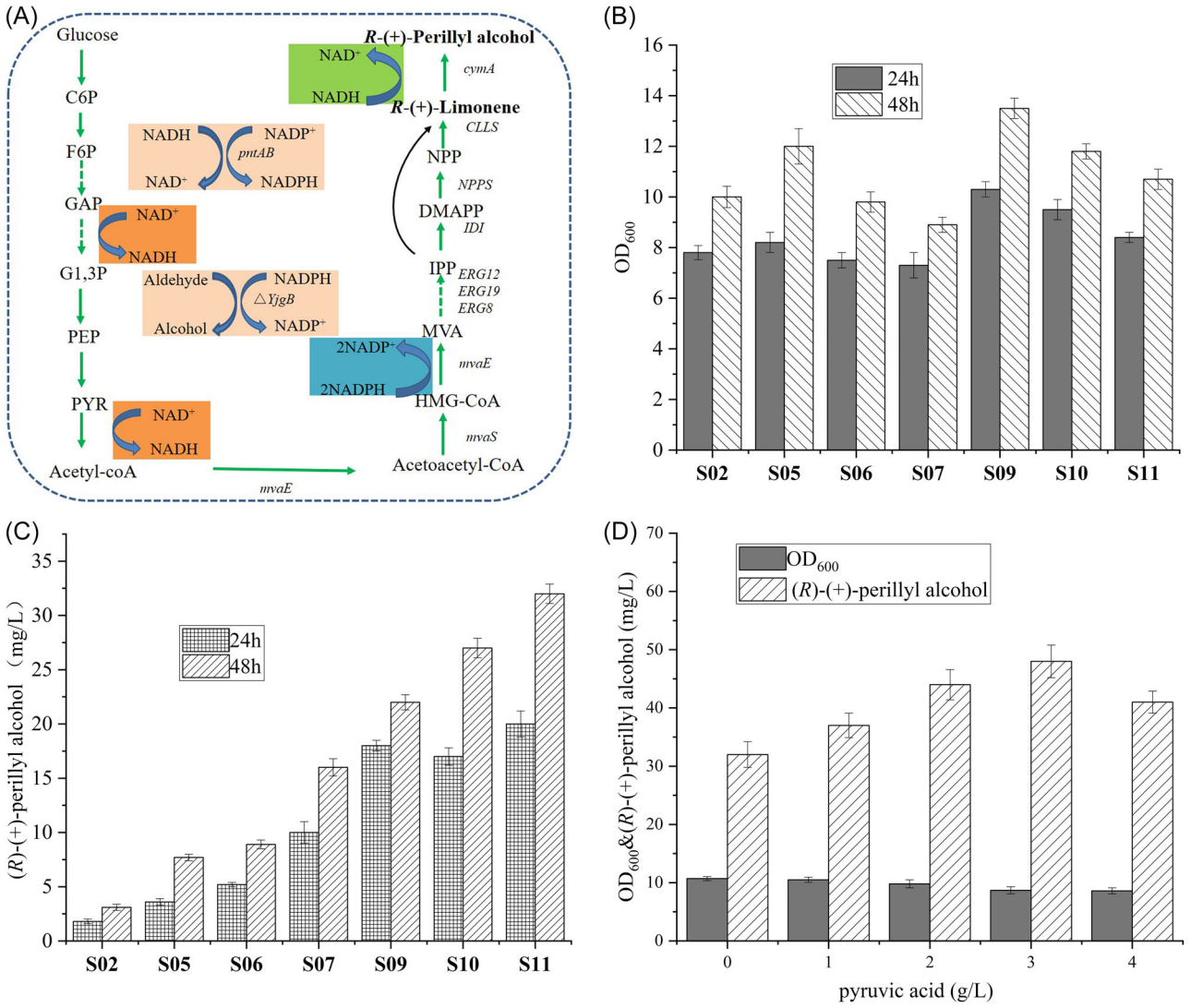

FIGURE 4 (*R*)-(+)-perillyl alcohol production by cofactor regeneration. (A) Cofactor regeneration for enhancing *pntAB* gene and knockout of *yjgB*. The OD600 (B) and total production (C) of (*R*)-(+)-perillyl alcohol were measured after 24 and 48 h of induction in the flask. (D) Pyruvate was added to the LB medium of the strain S11 to a final concentration of 1, 2, 3, or 4 g/L. Data are presented as the mean ± s.d. ($n = 3$). Metabolite abbreviations in (A): ClLS, limonene synthase; cymA, p-cymene monoxygenase hydroxylase and p-cymene monoxygenase reductase, DMAPP, dimethylallyl diphosphate; ERG8, phosphomevalonate kinase; ERG12, mevalonate kinase; ERG19, mevalonate pyrophosphate decarboxylase; GPP, geranyl diphosphate; GPPS, *Abies grandis* geranyl diphosphate synthase; IDI, IPP isomerase; IPP, isopentenyl diphosphate; mvaE, acetyl-CoA acetyltransferase/HMG-CoA reductase; mvaS, HMG-CoA synthase; NPP, neryl pyrophosphate; NPPS, neryl pyrophosphate synthase; pntAB, NAD(P) transhydrogenase; *yjgB*, aldehyde reductase

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.

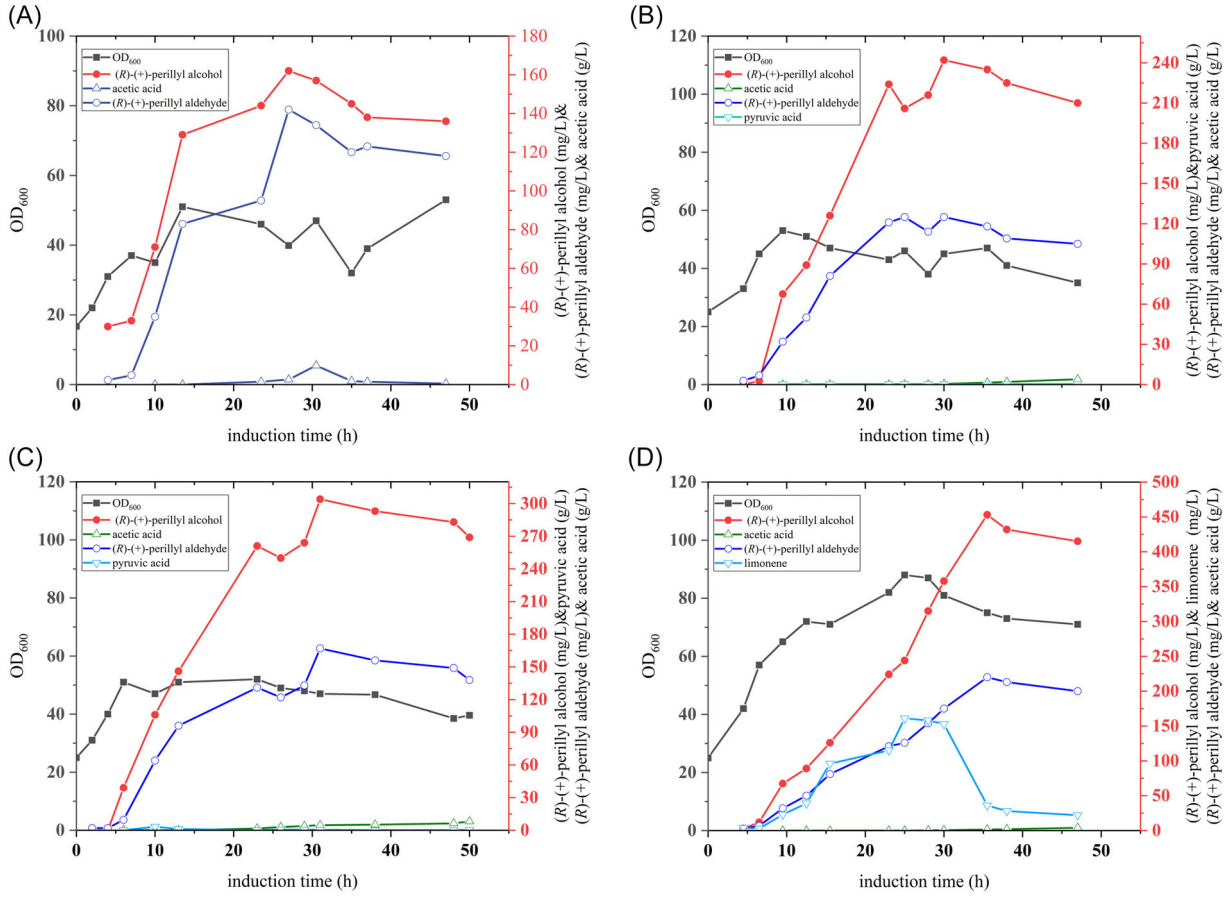

FIGURE 5 Fed-batch production of (*R*)-(+)-perillyl alcohol in 5 L bioreactors. Strains S12 (A) and S11 (B) were used for the experiments, respectively. (C) Fed-batch production by the strain S11 with the addition of 3 g/L pyruvate. (D) Fed-batch production by the strain S11 with tyruvic acid addition 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*, *clls*, 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 useable 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.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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