Effectiveness of recombinant Escherichia coli on the production of (R)-(+)-perillyl alcohol

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Research

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Abstract

Background : (R)-(+)-perillyl alcohol is a naturally oxygenated monoterpene that is produced in perilla leaves, citrus, lemon and lavender. Perillyl alcohols have many uses, including as natural flavor additives, insecticides, jet fuels and anti-cancer therapies. The bioconversion of readily available monoterpene precursors, such as (R)-(+)-limonene, are recognized as valuable oxygenated derivatives. However, as this natural product is present at low concentrations in plant oils, alternative microbial production methods are required for its extraction from natural plant sources. Results : We engineered Escherichia coli to possess a heterologous mevalonate (MVA) pathway, including limonene synthase , P-cymene monoxygenase hydroxylase and P-cymene monoxygenase reductase for the production of (R)-(+)-perillyl alcohol. The concentration of (R)-(+)-limonene (the monoterpene precursor to (R)-(+)-perillyl alcohol) reached 27.3 mg/L from glucose. Enhanced (R)-(+)-perillyl alcohol production was therefore achieved. The strain produced (R)-(+)-perillyl alcohol at a titer of 45.7 mg/L in a 5 L bioreactor fed batch system. Conclusions : These data highlight the efficient production of (R)-(+)-perillyl alcohol through the mevalonate pathway from glucose. This method serves as a platform for the future production of other monoterpenes.

Background

Perillyl alcohol is a natural monoterpene that exists in two optical forms. (*R*)-(+)-perillyl alcohol is produced in perilla leaves, citrus, lemon and lavender [1-3] and has extensive applications. It serves as a natural flavor additive for food, as an insecticide in agricultural fields, as a jet fuel in aviation fields, and as a healing agent for anti-cancer therapeutics [4-7]. The bioconversion of readily available monoterpene precursors (such as (*R*)-(+)-limonene) are recognized as valuable oxygenated derivatives [8]. Up to 70%-97% of (*R*)-(+)-limonene is present in citrus oils [9], produced to levels that exceed 60000t per year [10]. Many terpenes are synthesized from limonene, such as perillyl alcohol, carvone and α-terpineol. The enantiomers of carvone cost US $ 30–60 per kg; whilst (*S*)-(-)- and (*R*)-(+)-perillyl cost US $4500/kg [7,11]. As the chemical synthesis of perillyl alcohol is of high cost and leads to environmental pollution, the biosynthesis of perillyl alcohol from renewable carbon sources is regarded as an economically feasible industrial process. New and more effective synthesis procedures are however required.

Perillyl alcohol can be synthesized in *Escherichia coli* [12,13], *Pseudomonas putida* [5,14-16], *Yarrowia lipolytica* [17,18], *Mortierella minutissima* [19], *Fusarium verticilloides* [20], *Aspergillus* strain [21,22] from the biotransformation of limonene to perillyl alcohol. The maximum yield of (*R*)-(+)-perillyl alcohol was 258.1 mg/L for 3 days using *Mortierella minutissima* cultivation containing 0.5% (*R*)-(+)-limonene at 15 °C [19]. This produced the highest levels of (*R*)-(+)-perillyl alcohol currently reported. During (*S*)-(-)-perillyl alcohol production, titers can increase up to 3.4 g/L for *Mycobacterium* sp. strain HXN-1500 [23]. It has been reported that *Bacillus pallidus* BR425 [24] can be used during the synthesis of perillyl alcohol with α-pinene as the substrate. Metabolic engineering for natural compound production can be enhanced through gene modifications that increase enzyme activity. Many terpenoids have been produced at high

titers by metabolic engineering, including monoterpenes, sesquiterpenes, diterpenes and tetraterpenes [25-34].

Isopentenyldiphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) form the building blocks of monoterpenes, sesquiterpenes, and diterpenes during synthesis, the precursors of which include geranyl pyrophosphate (GPP), farnesylpyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP), respectively. These precursors are derived from mevalonate (MVA) and are catalyzed by geranyl pyrophosphate diphosphate synthase (GPPS) (Fig. 1). However, the microbial mediated production of monoterpenes is limited by toxicity [35] and poor GPPS expression [36-37]. In this study, we initially engineered *E. coli* to produce (*R*)-(+)-perillyl alcohol from glucose by the heterologous expression of P-cymene monoxygenase hydroxylase (CymAa) and a P-cymene monoxygenase reductase (CymAb) (Fig. 1). Genetic modifications to enhance the production of (*R*)-(+)-perillyl alcohol were also performed. These included the codon optimization of *cymAa*, *cymAb* and *ClLS* and exogenous GPPS expression. The highest performing strain SC04 was then cultured under fed-batch conditions for the assessment of its potential for large-scale production.

Results

Characterization of perillyl alcohol by GC-MS

It is well established that during (S)-(-)-perillyl alcohol production, titers can be increased by 100 mg/L using *Escherichia coli* [12]. Many terpenoids have been produced to high titers through metabolic engineering, including monoterpenes, sesquiterpenes, diterpenes and tetraterpenes [25-34]. In this study, P-cymene monoxygenase hydroxylase (CymAa) and P-cymene monoxygenase reductase (CymAb) derived from *Pseudomonas putida* were introduced into BL21 (DE3) [13,16]. Recombinant monoterpene synthase was used with (*R*)-(+)-limonene as the substrate in *in vitro* enzyme assays. (*R*)-(+)-limonene exists in more than 70% of the citrus fruits. Limonene, one of the simplest monoterpenes, has a chiral center, containing (*R*)-(+) and (*S*)-(-), respectively [38]. C*ymAa* and *cymAb* were ligated into pET28a (+) to create pSC01. BL21 (DE3) containing pSC01 was inoculated into 250 ml sealed flasks at 37 °C with shaking. At an OD_{600} of 0.6, IPTG and (*R*)-(+)-limonene were added for 24 h at 30°C. Products were identified by GC-MS after organic extraction by ethyl acetate. As shown in Fig. S1, control assays with extracts of *E. coli* lacking a cDNA clone did not yield (*R*)-(+)-perillyl alcohol, whilst *E. coli* carrying *cymAa* and *cymAb* from *Pseudomonas putida* produced (*R*)-(+)-perillyl alcohol to detectable quantities. The biosynthetic pathway for (*R*)-(+)-perillyl alcohol production was successfully constructed using (*R*)-(+)-limonene as a substrate.

Perillyl alcohol production from limonene

Limonene is toxic to most microorganisms and its concentrations regulate the growth of BL21 (DE3). It was therefore important to assess the optimal concentrations of limonene for fed-batch production. Different (*R*)-(+)-limonene concentrations were assessed (0.2 mM to 3 mM) in growth assays (Fig. 2).

When (*R*)-(+)-limonene was used at 0.2 mM, the maximum conversion yield of perillyl alcohol was 58%. When (*R*)-(+)-limonene was used at 2 mM, the maximum production was 86.9 mg/L. When limonene was used at ≥ 2 mM, the production and yield of (*R*)-(+)-perillyl alcohol decreased. At increased limonene concentrations, cell growth declined. At (*R*)-(+)-limonene concentrations of 1.0 mM, the OD_{600} decreased from 2.5 to 2.0. SC01 could catalyze the synthesis of (*R*)-(+)-perillyl alcohol using (*R*)-(+)-limonene as the substrate. The optimum limonene concentration was therefore 0.2 mM.

Limonene production from glucose

We optimized the yields of limonene to improve the production of perillyl alcohol. pTrcHis2B and pET-28a (+) were assessed. pYJM14 contains *ERG8, ERG19, IDI* from *Saccharomyces cerevisiae* [39]. pSC02 contains geranyl pyrophosphate synthase GPPS from *Abies grandis* that converts IPP/DMAPP to GPP, and (*R*)-(+)-limonene synthase CILS from *citrus limon* that converts GPP to limonene [40] (Table 1). SC02 was cultured in fermentation medium under sealed shake-flask conditions.

The levels of (*R*)-(+) limonene in the culture media were plotted against known concentrations of (*R*)-(+)-limonene. The (*R*)-(+)-limonene concentration of SC02 reached 10.4 mg/L and 27.3 mg/L (mg limonene per liter of culture) with 0.2 mM IPTG and 10% n-dodecane overlay at 48 h and 72 h of induction. When 10% DINP (diisononyl phthalate) was used instead of the n-dodecane overlay, the limonene titers were 0.9 mg/L and 2.0 mg/L respectively at 48 h and 72 h (Fig. 3). The enantiomer forms produced by the strain cultures were analyzed using GC with an Cyclosil-B column, and the production of (*R*)-(+)-limonene by SC02 was confirmed (Fig. S2).

Microbial perillyl alcohol production using the MVA pathway

CymAa and CymAb catalyze the biosynthesis of (*R*)-(+) perillyl alcohol using (*R*)-(+) limonene as a direct substrate through a hydroxylated reaction at the 7 position. We cloned *cymAa* and *cymAb* from *Pseudomonas putida* into pSC04&pSC05. Terpenoid synthase is expressed to low levels in *E. coli* [41], so *cymA and cymAb* were codon optimized (Table 1). According to the analysis of chirality of the intermediate limonene produced by SC02, we concluded that the configuration of perillyl alcohol was R-type (Fig. S2). Expression levels in the perillyl alcohol pathway for SC04 were investigated by qRT-PCR. The results indicated that *ERG8, ERG19 ERG12,* and *IDI* significantly increase compared to the control. Moreover, changes in the expression of *mvaE, mvaS, CILS, GPPS, cyma* and *cymAb* were comparable to the control (Fig. 4). We used n-dodecane or DINP as the extraction solvent and the organic phase was collected and centrifuged to remove cell contaminants. Samples were then subjected to GC-MS analysis and trace levels of perillyl alcohol were detected. pYJM14 and pSC04&pSC05 were co-transformed into *E. coli* BL21 (DE3), resulting in recombinant strain SC04&SC05. SC04 that was cultured in sealed shake flasks to assess perillyl alcohol production. The (*R*)-(+)-perillyl alcohol levels for SC04 reached 5.2 mg/L after induction by 0.2 mM IPTG and 10% DINP overlay for 72 h, which was 2.4-fold higher than SC05 (2.1 mg/L for 72 h) (Supplementary Fig. 5). In the absence of DINP, SC04 produced 0.6 mg/L and 0.4 mg/L of perillyl alcohol. We included n-dodecane overlays in the cultures, and thus no perillyl alcohol was detected

[12]. The production of perillyl alcohol was 0.6 mg/L in SC04 and 0.4 mg/L in SC05, mainly due to the rapid extraction of limonene to the organic phase, and as n-dodecane overlay could not be used for perillyl alcohol fermentation.

Fed-batch culture of engineered E.coli strains

High cell densities for (*R*)-(+)-perillyl alcohol production were achieved through fed-batch fermentation using SC04 in a 5 L bioreactor. According to the residual glucose concentrations, feeding rates were maintained at ≤ 1 g/L. Figure 6 shows the time-course of (*R*)-(+)-perillyl alcohol production and cell density during fermentation. (*R*)-(+)-perillyl alcohol biosynthesis was initiated from 5 h of induction and 10% DINP was added during the fed-batch process. After 60 h of cultivation, the production of (*R*)-(+)-perillyl alcohol peaked at 45.7mg/L with DINP overlay. However, the engineered strain achieved a cell-density OD_{600} of 108 for two-phase fermentation. The accumulation of IPP and DMAPP may have influenced the growth of the engineered strain [29,42,43]. However, the two-phase fermentation of (*R*)-(+)-perillyl alcohol fermentation eliminated toxic intermediates. (*R*)-(+)-perillyl alcohol biosynthesis requires many genes which when overexpressed, influence host cell viability and reduce productivity [44].

Discussion

(*R*)-(+)-perillyl alcohol is produced in perilla leaves, citrus, lemon and lavender and has a range of applications. Many terpenes are synthesized from limonene, such as perillyl alcohol, carvone and α-terpineol. As the chemical synthesis of perillyl alcohol is of high cost and is environmentally unfriendly, the biosynthesis of perillyl alcohol from renewable carbon sources is regarded as an economically feasible industrial process. Indeed, the development of (*R*)-(+)-perillyl alcohol as an anti-cancer drug has been limited by the costs associated with extracting the compound from its natural oil. Terpenes are produced by microorganisms and can be scaled-up to increase drug production [45]. We used *E.coli* as a host and confirmed the feasibility of (*R*)-(+)-perillyl alcohol production. The levels produced were however, too low for industrial requirements.

(*R*)-(+)-perillyl alcohol synthesis requires CymAa and CymAb. Both convert limonene derived from the metabolism of *p*-cymene [13]. Enzyme activity was lower than other terpene synthases, that reduce perillaldehyde or perilla acid [16]. We initially engineered SC0 and showed successful (*R*)-(+)-perillyl alcohol production using GC-MS (Fig. S1). The maximum production of (*R*)-(+)-perillyl alcohol composed with (*R*)-(+)-limonene as substrate was 86.9 mg/L and lethal concentrations of limonene to *E. coli* were 2 mM (Fig. 2). (*R*)-(+)-Limonene accumulates in biological membranes leading to damage. The compound is also insoluble in water [46]. It has been reported that limonene is inhibitory to multiple microorganisms [47]. A recent report [48] revealed that 0.025% (v/v) limonene inhibited the growth of *E. coli* [35]. Thus, the tolerance and solubility of limonene requires improvement to increase both the concentrations and yields of (*R*)-(+)-perillyl alcohol.

In previous studies, terpenoids have been studied using the MVA pathway [25,29,30,49]. Our research group constructed engineered *E.coli* which synthesized isoprene by the mevalonate (MVA) pathway, and produced isoprene to 665.2 mg/L under flask conditions [50,51]. GPPS and ClLS were engineered to improve (*R*)-(+)-limonene production, and SC03 produced no detectable limonene (data not shown), highlighting the need for terpenes. When no extracts were added during limonene fermentation, no detectable SC02 was observed (data not known), indicating that limonene is rapidly lost from the culture medium. As limonene from the fermentation system was highly volatile and anti-microbial [52,53] other collection methods have been reported including culture extraction, solvent overlay, solid-phase micro-extraction, gas shipping to a cold trap, and adsorbent polydimethlsiloxane bars [52,54,55]. Whilst not all of these methods are appropriate, they do prevent product inhibition and toxicity, avoiding evaporative loss of the limonene product using the two-phase system. Davies et al. [56] showed that an overlay of n-dodecane enhanced limonene recovery. N-dodecane was selected as a favorable solvent for terpenoid extraction experiments due to its low volatility [56]. In engineered *E.coli,* DINP fermentation prolongs both the growth and production phases, leading to (*S*)-(-)-limonene concentrations of 1.35 g/L [57]. (*R*)-(+)-limonene obtained by n-dodecane extraction was 27.3 mg/L for 72 h, 3-fold higher than that of DINP extraction (Fig. 3). It may be that the distribution coefficient of (*R*)-(+)-limonene in n-octane is larger than that of the DINP phase. The strain fermented by the DINP overlay also inhibited cell growth, and the OD_{600} decreased from 3.4 to 2.5 (Fig. 3). Jongedijk et al. [52] engineered Saccharomyces cerevisiae to express (*R*)-(+)-limonene synthase from *Citrus limon*. Trapping of the headspace in limonene synthase expressing strains resulted in 0.12 mg/L (*R*)-(+)-limonene. Pang et al. [58] engineered *Yarrowia lipolytica* to achieve (*R*)-(+)-limonene at 11.705 mg/L, through the overexpression of *HMGR* and the optimization of the fermentation conditions. Cao et al. [59] engineered *Yarrowia lipolytica* to increase limonene titers to 23.56 mg/L by encoding neryl diphosphate synthase1 (NDPS1) and limonene synthase (LS). Here, we engineered *E. coli* to produce (*R*)-(+)-limonene to 27.3 mg/L, close to the maximum titers reported.

We confirmed that the chirality of perillyl alcohol was (*R*)-type in SC04, which was performed with (*R*)-(+)-limonene as an intermediate (Fig. S2). We then engineered SC04 & SC05 and investigated the effects of copy numbers on (*R*)-(+)-perillyl alcohol production. We hypothesized that *cymAa* and *cymAb* genes produced by the lower copies of SC05 could not repress all promoters. In contrast, SC04 had 40 copies encoding the repressor from medium and middle-copy plasmids. The genes contributed to higher levels in SC04 than in SC05 (Fig. 4). This hypothesis was supported by the production of perillyl alcohol, which was 2.4-fold in SC04 and SC05 (from 2.1 mg/L to 5.2 mg/L) (Fig. 5). In addition, the levels of *ERG12*, *ERG8*, and *ERG19* in the MVA pathway were higher than those of the control strain, whilst the levels of *GPPS*, *ClLS*, *cymAa*, and *cymAb* showed no obvious changes compared to controls (Fig. 4). Most intermediates can be efficiently converted to IPP, but the production from IPP to perillyl alcohol was weak. This suggests the enhanced production of (*R*)-(+)-perillyl alcohol through regulating metabolic imbalances.

In summary, (*R*)-(+)-perillyl alcohol production was achieved by assembling biosynthetic genes encoding a heterologous MVA pathway. The highest-performing strain (SC04) accumulated up to 45.7 mg/L of (*R*)-

(+)-perillyl alcohol under fed-batch fermentation conditions.

Conclusions

We have engineered *E. coli* to produce (*R*)-(+)-perillyl alcohol from glucose through the MVA pathway. SC04 produced 45.7 mg/L of (*R*)-(+)-perillyl alcohol, higher titers than the previously reported system, which was 0.51 mg/L of (*R*)-(+)-perillyl alcohol during the growth on limonene by the *E.coli* transformant EC409A [13]. This study provides new methods for the synthesis of chiral perillyl alcohol. Efforts should now be directed towards the optimization of the MVA pathway.

Methods

Strains and culture conditions

Metabolic engineering for natural compound production can be enhanced through gene modifications that increase enzyme activity. All experimental materials used in this study are listed in Table 1*. E.coli* were grown in LB (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L). During the production of (*R*)-(+)-limonene and (*R*)-(+)-perillyl alcohol, strains were cultivated in shake-flasks in medium containing glucose 10 g/L, $MgSO_4$ 1 mM and riboflavin 0.05 mM during fed-batch fermentation in glucose 20 g/L, K_2HPO_4 9.8 g/L, ferric ammonium citrate 0.3 g/L, citric acidmonohydrate 2.1 g/L, $MgSO_4$ 1 mM riboflavin 0.05 mM and 1 mL trace element solution, including $(NH_4)_6Mo_7O_{24}.4H_2O$ 0.37 g/L, $ZnSO_4.7H_2O$ 0.29 g/L, H_3BO_4 2.47 g/L, $CuSO_4.5H_2O$ 0.25 g/L, and $MnCl_2.4H_2O$ 1.58 g/L. As required, ampicillin (100 μg/ml) and kanamycin (50 μg/ml) were added for selection.

Plasmid construction

P-cymene monoxygenase hydroxylase (*cymAa*, GenBank Accession No.:AAB62299.1) and P-cymene monoxygenase reductase (*cymAb*, GenBank Accession No.:AAB62300.1) from *Pseudomonas putida* were codon optimized on (www.jcat.de), and cloned into pUC57. C*ymAa* and *cymAb* were PCR amplified and subcloned into pET28a (+) with *Bam*HI/*Sac*I restriction sites, creating pSC01 (pET28a-cymAa-cymAb). *ClLS* (GenBank Accession No.:AF514287.1) of *Citrus limon* and *GPPS* (GenBank Accession No.:AF513112.1) of *Abies grandis* were amplified and ligated into pUC57 using Genewiz Biotech Co., Ltd. (Suzhou, China), producing pUC57-ClLS&pUC57-GPPS. M*vaE-mvaS* was then excised from pYJM20 [50] and ligated into pET-28a (+) to create pET28a-mvaE-mvaS. *GPPS* and *ClLS* were cloned and assembled into pET28a-mvaE-mvaS at the *Sac*I/*Aat*II sites to generate pSC02 (Table 1). C*ymAa-cymAb* fragments were obtained through *Pseudomonas putida* using *Aat*II and *Pac*I and ligated into pSC02 to create pSC03 (Table 1). M*vaE, mvaS, and GPPS* were cloned from pSC02 into pCOLADuet-1 at the *Bam*HI/*Xho*I sites, generating pcolaDuet-mvaE-mvaS-GPPS-ClLS. C*ymAa, cymAb* were cloned from pSC03 into pcolaDuet-mvaE-mvaS-GPPS-ClLS at the *Xho*I/*Pac*I sites to produce pSC05 (Table 1).

pYJM14 was constructed from pTrcHis2B through the introduction of *ERG8, ERG12, ERG19* and *IDI* from *S. cerevisiae* [39,60]. All plasmids and primers are shown in Table S1.

RT-PCR analysis

Total RNA was isolated from 24 h cultures using commercially available SPAKeasy RNA kits. RNA was reverse transcribed using TaKaRa Primer Script RT reagent Kit and RT-PCRs were performed. Each reaction contained 1 μL cDNA, 5μL TB green Premix Ex TaqII, 0.2μL 50×ROX Reference Dye, 0.2 μM for/rev primer, and ddH_2O up to 10 μl. RT-PCR conditions were as follows: 30 s at 95 °C, 40 cycles of 95 °C for 5 s, 60 °C for 30 s. Gene expression was normalized to the absolute transcript levels of *rpoD.* qRT-PCRs were performed on a Primer 5.0 program (Table S2). Relative gene expression was calculated using the $2^{\Delta\Delta}$Ct method for each treatment. Reactions were repeated a minimum of 3 times.

Shake-flask fermentation

Cultures were produced in in 25 ml of LB. *E. coli* strains with each recombinant plasmid inoculated in a gyratory shaker at 37 °C and 180 rpm. IPTG (0.2 mM) was added to induce recombinant protein expression upon an OD_{600} of 0.6. Cultures were incubated at 30°C with 2-phase fermentation [61] used for limonene and perillyl alcohol extraction from the aqueous broth due to the toxicity of terpenes. We added 10% (v/v) n-dodecane or DINP (disononyl phthalate) following IPTG induction and cultures were incubated for 72 h. Cell densities, glucose levels, limonene levels and perillyl alcohol production were then assessed.

Fed-batch fermentation

For (*R*)-(+)-perillyl alcohol production on a larger scale, fed-batch cultivations were performed in a 5 L bioreactor system (Biostat B plus MO 5L) using 2 L of fermentation fluid. Seed cultures (100 ml, 10 g NaCl, 5 g yeast extract, and 10 g of tryptone per 1 L) were added to shake flasks overnight at 37 °C and Sparger aeration was performed to maintain high dissolved oxygen (DO) levels. Post-fermentation, the pH of the broth was maintained at 7.0 through ammonia addition. Fermentation was performed during the growth stages under the following conditions: 37 °C, agitation 400 rpm and airflow at 1 L/min. Antifoam 204 was added as required. DO was maintained at 20% saturation through the control of air flow and stirrer speed (1-2 L/min and 400-900 rpm, respectively). When cells reached an OD OD_{600} of ~20, the temperature was switched to 30 °C and 0.2 mM IPTG and 0.05 mM riboflavin were added. DINP (10%) was added after 4 h when the initial glucose levels were exhausted, as indicated by the increase in DO. The fed batch mode was initiated through the feeding of 60% glucose at appropriate rates. Residual glucose levels were maintained to low levels through the addition of acetic acid. Samples were periodically collected and OD_{600} values were determined prior to centrifugation for the separation of the organic and aqueous phases. Organic layers were removed for all GC–MS analysis.

Analytical methods

E. coli growth was determined through OD_{600} measurements on a spectrophotometer (Cary 50 UV-vis, Varian). The Shimadzu GC-MS system (TQ8050) was used for Limonene and perillyl alcohol identification. GC-MS conditions were as follows: 30 m DB-5MS column (internal diameter 0.32 mm, film thickness 0.25 μm); temperature: 50 °C hold, ramped up 10 °C/min to 250 °C with a final hold at 250 °C for 10 min. Highly pure helium was used as a carrier at a linear velocity of 1 ml/min; an injector temperature of 250 °C; a split ratio of 1:10; an ion source temperature of 230 °C and mass range of *m/z* 40–500. Limonene and perillyl alcohol peaks were identified through the retention times of external standards and MS comparisons via the National Institute of Standards and Technology (NIST) database. Fermentation broths were mixed, centrifuged, and the organic layer was taken for GC-MS analysis.

The enantiomeric distribution of limonene was analyzed using the Agilent Technologies 7890B GC System on a Cyclodex B column (30m×0.25mm internal diameter; film thickness=0.25μm). GC conditions were as follows: 50 °C hold, 2°C/min ramping to 160 °C; carrier: high-purity helium, linear velocity: 1 ml/min; temperature of the injector: 250 °C; split ratio: 1:20. Compounds of interest: (*S*)-(-)-limonene at 21.2 min and (*R*)-(+)-limonene at 22.02 min. The fermentation broth was mixed and centrifuged, and the organic layer was analyzed.

Declarations

Ethics approval and consent to participate

Not applicable

Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CS, RZ and CX conceived and designed research. XD contributed analytical tools. CS performed experiments and analyzed data. CS wrote the manuscript. All authors read and approved the manuscript.

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Table 1

Table 1 Strains and plasmids used in this study

Name	Relevant characteristics	References
Strains		
E.coli DH5α	F^- *rec*A *end*A1 *Φ80dlac*Z△*M15hs*dR17($r_k^-m_k^+$)λ^-	Invitrogen
E.coli BL21(DE3)	F- ompT hsdSB (rB – mB -) gal dcm rne131 λ(DE3)	Invitrogen
SC01	*E.coli* BL21(DE3)/pSC01	This study
SC02	*E.coli* BL21(DE3)/pSC02,pYJM14	This study
SC03	*E.coli* BL21(DE3)/pSC03	This study
SC04	*E.coli* BL21(DE3)/pSC04,pYJM14	This study
SC05	*E.coli* BL21(DE3)/pSC05,pYJM14	This study
Plasmids		
pTrcHis2B	pBR322 origin, Amp	Invitrogen
pET28a(+)	Kan^roripBR322lacI^qT7p	Novagen
pCOLADuet-1	Kan^r ColA lacI T7lac	Novagen
pYJM14	pTrcHis2B carrying ERG12, ERG8, ERG19 and IDI from *Saccharomyces cerevisiae*	[39]
pSC01	pET28a(+)carring cymAa and cymAb from *Pseudomonas putida*	This study
pSC02	pET28a(+)carrying mvaE and mvaS from *Enterococcus faecalis*, ,GPPS from *Abies grandis*,ClLS from *Citrus limon.*	This study
pSC03	pET28a(+) carrying GPPS from *Abies grandis*,ClLS from *Citrus limon.*	This study
pSC04	pET28a(+)carrying mvaE and mvaS from *Enterococcus faecalis*,,GPPS from *Abies grandis*,ClLS from *Citrus limon,* cymAa and cymAb from *Pseudomonas putida*	This study
pSC05	pCOLADuet-1-carrying mvaE and mvaS from *Enterococcus faecalis*, ,GPPS from *Abies grandis*,ClLS from *Citrus limon,* cymAa and cymAb from *Pseudomonas putida*	Tis study

Figures

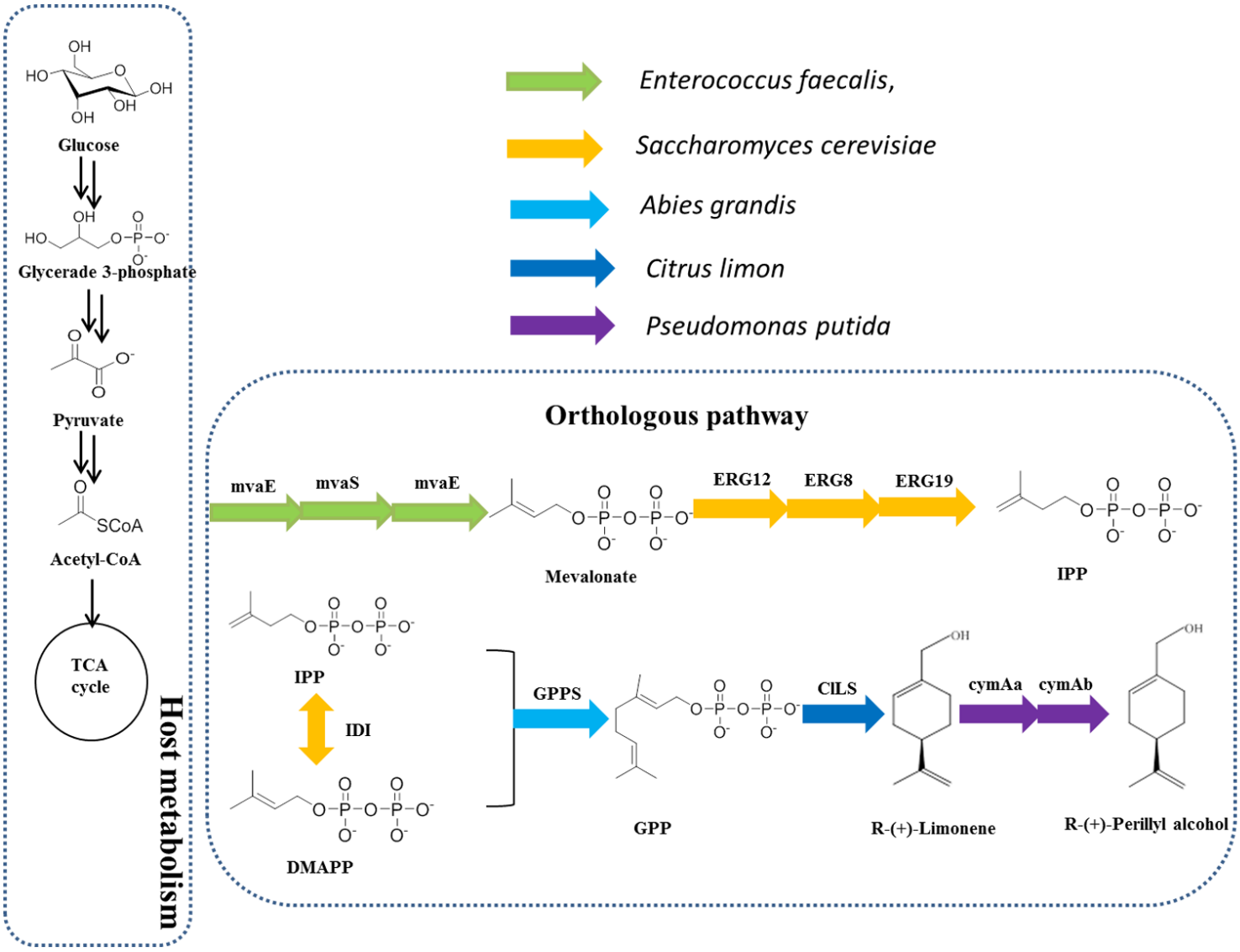

Figure 1

(R)-(+)-perillyl alcohol production via the MVA pathway. Enzymes are depicted in the legend.

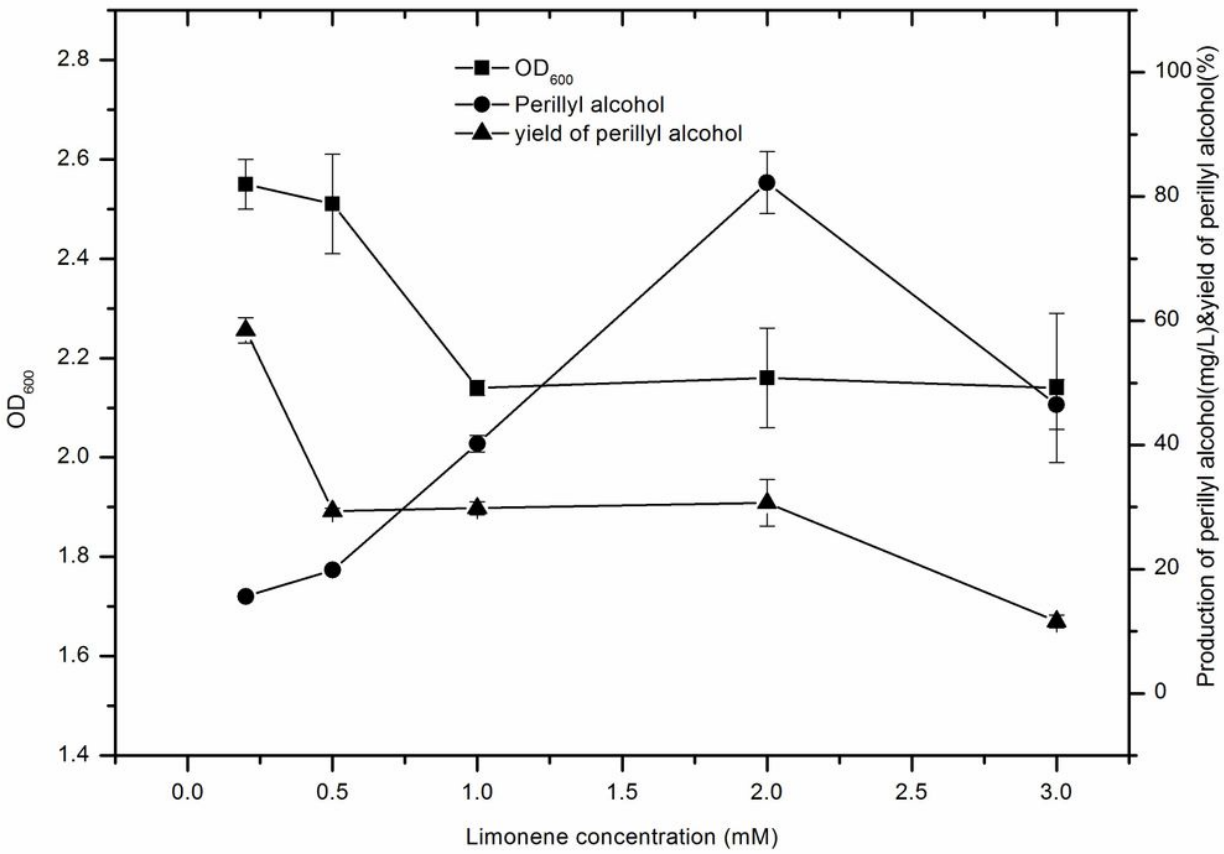

Figure 2

Effect of limonene concentration on the activity of SC01. When the OD600 reached 0.6, samples were induced at 30℃ for 24 h using 0.2 mM IPTG and 0.1 mM (R)-(+)-limonene. Error bars: SD from three independent cultivations.

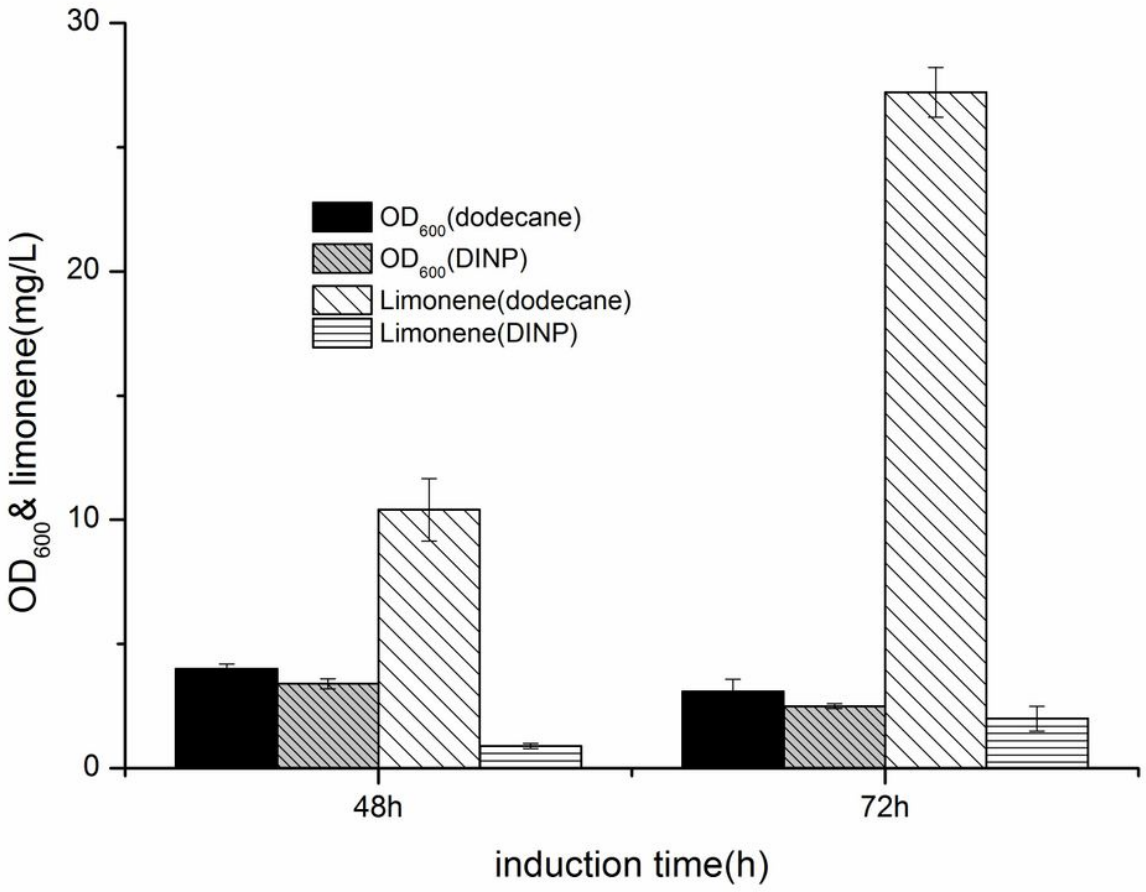

Figure 3

Limonene production using the SC02 strain. The enantiomeric distribution of limonene was analyzed using the Agilent Technologies 7890B GC System on a Cyclodex B column (30m×0.25mm internal diameter; film thickness=0.25μm). GC conditions were as follows: 50 °C hold, 2°C/min ramping to 160 °C; carrier: high-purity helium, linear velocity: 1 ml/min; temperature of the injector: 250 °C; split ratio: 1:20. Compounds of interest: (S)-(-)-limonene at 21.2 min and (R)-(+)-limonene at 22.02 min. The fermentation broth was mixed and centrifuged, and the organic layer was analyzed. The total production of Limonene was measured after 2 and 3 days of IPTG induction and 10% n-dodecane or DINP induction.

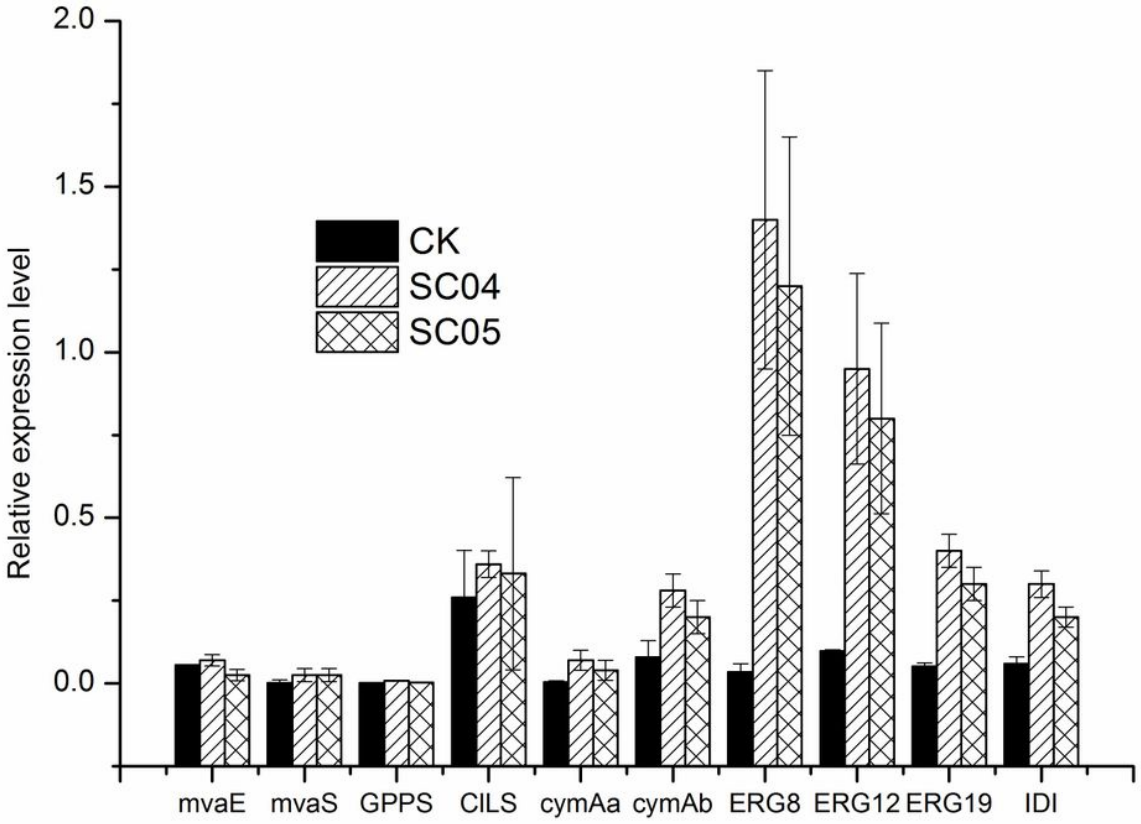

Figure 4

Relative expression levels in SC04 and SC05 engineered for (R)-(+)-perillyl alcohol production. Data was for 24 h in LB medium with 0.2 mM IPTG and 20 g/L glucose. Gene expression was calculated using the 2-△△CT. system and are shown as the mean ± SD (n≥3). Identical letters indicate no significant differences ($p<0.05$).

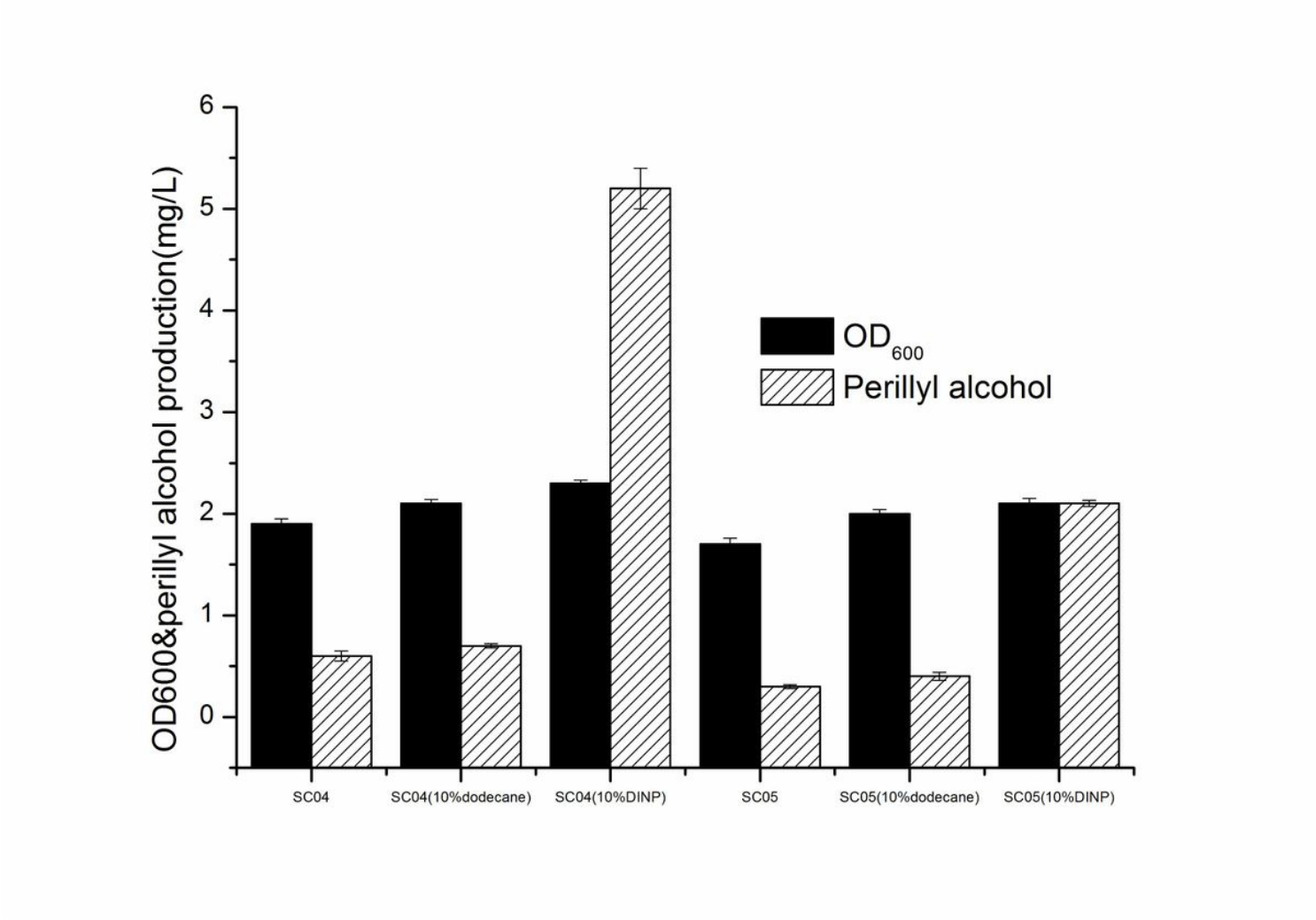

Figure 5

5 Perillyl alcohol production using SC04 and SC05 strains. Total production of (R)-(+)-perillyl alcohol was measured at 72 h after induction with 0.2 mM IPTG and10% n-dodecane or 10% DINP, Data are the means of 3 repetitions ± SD.

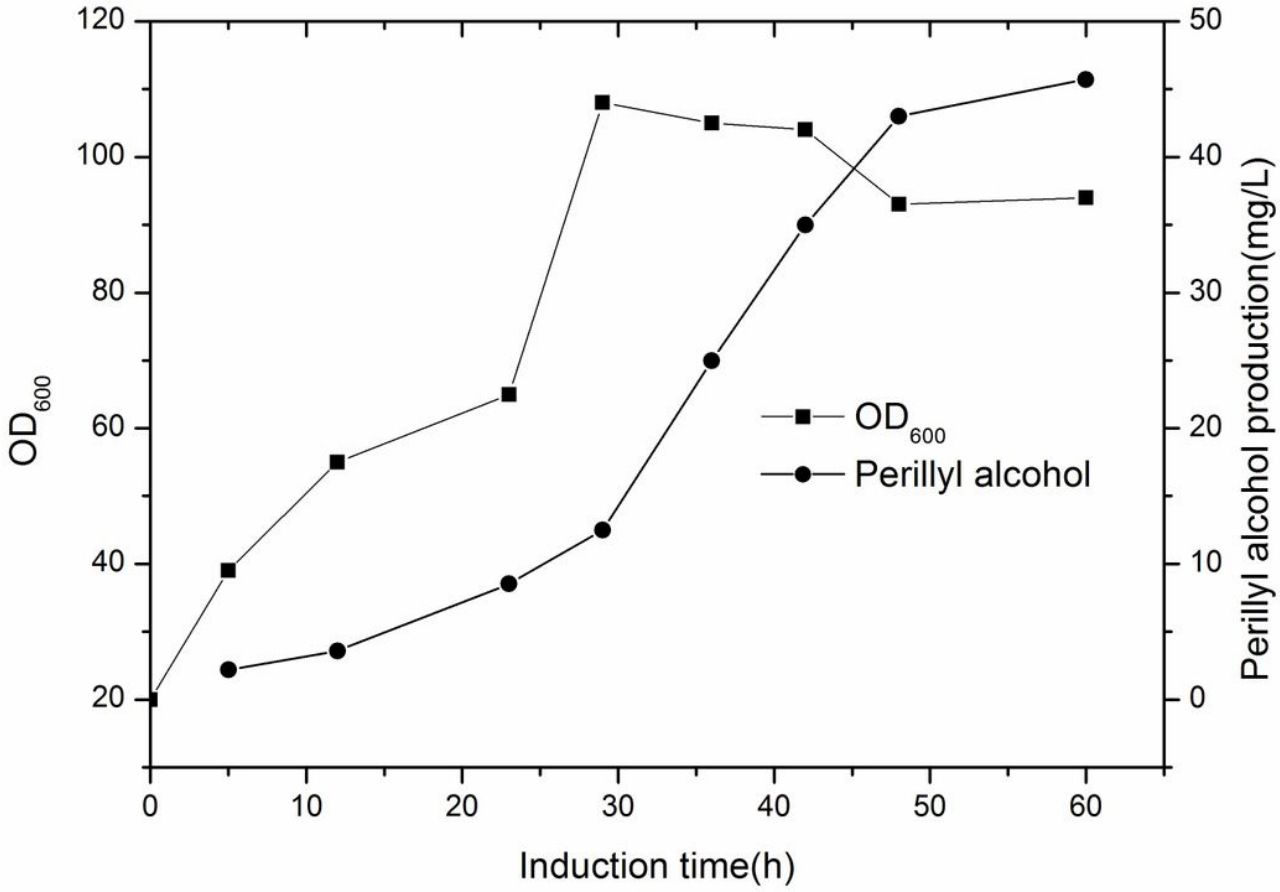

Figure 6

Time-course of cell density (OD600), and perillyl alcohol production during fed-batch fermentation of SC04.

Supplementary Files

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- supplementarymaterials0203.docx