

Biosynthesis of Pinene in Purple Non-Sulfur Photosynthetic Bacteria

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Abstract

Background: Pinene is a monoterpene, that is used in the manufacture of fragrances, insecticide, fine chemicals, and renewable fuels. Production of pinene by metabolic-engineered microorganisms is a sustainable method. Purple non-sulfur photosynthetic bacteria belong to photosynthetic chassis that are widely used to synthesize natural chemicals. To date, researches on the synthesis of pinene by purple non-sulfur photosynthetic bacteria has not been reported, leaving the potential of purple non-sulfur photosynthetic bacteria synthesizing pinene unexplored.

Results: *Rhodobacter sphaeroides* strain was applied as a model and engineered to express the fusion protein of heterologous geranyl diphosphate synthase (GPPS) and pinene synthase (PS), hence achieving pinene production. The reaction condition of pinene production was optimized and 97.51 $\mu\text{g/L}$ of pinene was yielded. Then, genes of 1-deoxy-D-xylulose 5-phosphate synthase, 1-deoxy-D-xylulose 5-phosphate reductoisomerase and isopentenyl diphosphate isomerase were overexpressed, and the ribosome binding site of GPPS-PS mRNA was optimized, improving pinene titer to 539.84 $\mu\text{g/L}$.

Conclusions: In this paper, through heterologous expression of GPPS-PS, pinene was successfully produced in *R. sphaeroides*, and pinene production was greatly improved by optimizing the expression of key enzymes. This is the first report on pinene produce by purple non-sulfur photosynthetic bacteria, which expands the availability of photosynthetic chassis for pinene production.

Background

Terpenes are natural compounds that can be used as fragrances, flavorings agents, medicines and potential biofuels [1]. Terpenes are isoprenoids, synthesized from isopentenyl intermediates by corresponding terpene synthases, and these isopentenyl intermediates are condensed from C5 isoprene units, namely isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [2]. IPP and DMAPP are common building blocks of isoprenoids, which are synthesized from three molecules of acetyl-CoA via the mevalonate pathway or from pyruvate and glyceraldehyde-3-phosphate via the 2C-methyl-D-erythritol 4-phosphate (MEP) pathway shown in Fig. 1a [3]. These two isoprenoid pathways exist in different species. The mevalonate pathway is dominant in eukaryotes, archaea, and cytoplasm of higher plants, and the MEP pathway is found in most bacteria, green algae, and chloroplasts of higher plants [2, 3].

Pinene (C₁₀) is a monoterpene, which is used in the manufacture of fragrances, insecticide and fine chemicals. Besides, it has potential as a feedstock for high-density renewable fuels, for pinene dimers contain high volumetric energy [4]. Pinene is generally synthesized in plants, converted from geranyl diphosphate (GPP) by pinene synthase (PS), while GPP is synthesized by geranyl diphosphate synthase (GPPS) from condensing IPP and DMAPP shown in Fig. 1a [5]. In addition, the pinene could also be produced by microorganisms through metabolic engineering, which is thought to be a sustainable method [6]. Yang et al, constructed the mevalonate pathway, GPPS and PS genes in *E.coli*, which led to

pinene production (5.44 mg/L) under shake-flask conditions [7]. Then, pinene yield in *E.coli* was further improved by introducing GPPS-PS protein fusions [5] and evolved geranyl diphosphate synthase and pinene synthase with high activity [8, 9]. With the expression of pinene synthase, pinene can also be produced in other microorganisms, e.g. Cyanobacterial [8] and *Corynebacterium glutamicum* [10].

The purple non-sulfur photosynthetic bacteria are non-oxygen-producing bacteria, with a variety of metabolic modes that enable them to grow under phototrophic conditions or in darkness conditions by respiration, fermentation, or chemolithotrophy [11]. The diversity of metabolism allows purple non-sulfur photosynthetic bacteria to use a variety of carbon sources [12], and they have been studied as phototrophic platform organisms for valuable chemicals production [13]. For example, purple non-sulfur photosynthetic bacteria have been used to produce poly- β -hydroxyalkanoates [14], membrane proteins [15], hydrogen gas [16], and carotenoids [17]. Moreover, purple non-sulfur photosynthetic bacteria have abundant inner membrane system, which act as a container for hydrophobic metabolites [13]. They have been used to study the synthesis of sesquiterpenes, triterpenes, tetraterpenes, etc., through the endogenous MEP pathway or heterologous mevalonate pathway [13, 18, 19]. However, researches on the synthesis of pinene by purple non-sulfur photosynthetic bacteria have not been reported, leaving the potential of purple non-sulfur photosynthetic bacteria in pinene production undetermined.

Rhodobacter sphaeroides is a kind of widely studied purple non-sulfur photosynthetic bacteria, which is applied as a bio-factory to synthesize farnesol [20], coenzyme Q₁₀ [21], lycopene [19] and valencene [22]. In this paper, *R. sphaeroides* strain was used as a model and a fusion protein of GPPS and PS was introduced to it to allow pinene synthesis. Through genetic engineering and reaction conditions optimization, the potential of pinene production in purple non-sulfur photosynthetic bacteria was tested.

Results

Pinene production using protein fusions

Purple non-sulfur photosynthetic bacteria use the endogenous MEP pathway to produce IPP and DMAPP, which are further catalyzed by CrtE to synthesize GPP. Then, GPP can be catalyzed to pinene by introducing an exogenous PS gene, as shown in Fig. 1a. However, CrtE have both GPP synthase and farnesyl diphosphate (FPP) synthase activity. Thus, CrtE can convert IPP and DMAPP to GPP, then to FPP. As an intermediate, GPP is more readily catalyzed to FPP, instead of generating monoterpenes, e.g. pinene[23].

To elevate the metabolic flux from GPP to pinene, a feasible strategy is to construct a fusion protein of GPPS and PS to make it easier for GPP to enter PS active site from GPPS active site [5, 8]. In this study, a pBBR- α GppsPs plasmid was constructed, in which a fusion gene of *Abies grandis* GPPS and PS was expressed under the strong trc promoter and with the ribosome binding site of RBS α . The pBBR- α GppsPs plasmid was transferred to *R. sphaeroides* by conjugation and the final strain (*R. sphaeroides*:pBBR- α GppsPs) was grown under light anaerobic conditions to produce pinene. Pinene titers were detected over

time at various temperatures to obtain the optimal culturing temperature and duration. The results showed that maintaining the temperature at 30°C was more suitable for pinene production as the rate of pinene production and the final pinene titers were higher at this condition (Fig. 2). It was observed the pinene titers gradually increasing to maximum and then reached plateau after culturing for 132 h at 30°C, so the optimal culturing duration was 132 h.

As the transcription of *gpps-ps* was induced by IPTG, we tested the effect of IPTG concentrations on pinene production. Figure 3 showed pinene production using GPPS-PS fusion protein at various IPTG concentrations, ranging from 0 to 1000 µM. At the IPTG concentration of 3 µM, the highest pinene titer was obtained, which was 97.51 µg/L in total (32.17 µg/L α-pinene and 65.34 µg/L β-pinene). When IPTG concentration was higher than 10 µM, the pinene titers were much lower than the value at the IPTG concentration of 3 µM. This suggests that the increase in IPTG concentration will inhibit the synthesis of pinene in *R. sphaeroides*. Thus, in the subsequent pinene production reaction, the concentration of IPTG was set to 3 µM.

Improving Pinene Production By Overexpression Of Key Genes

As most genes in isoprenoid pathway are expressed at low level under normal growth conditions, the common method is to increase the expression of key genes to improve the metabolic flux [24]. The genes coded for MEP pathway enzymes were studied and four genes (*dxs*, *idi*, *ispD* and *ispF*) were deemed to be the rate-limiting in *E. coli* [24, 25]. Lu et.al overexpressed *dxs*, *dxr*, *ispD* and *idi* simultaneously and increased Q_{10} yield by two folds in *R. sphaeroides* [21]. However, there is limited information about the MEP pathway in purple non-sulfur photosynthetic bacteria and the key genes were not confirmed by experiments.

In this study, the genes in MEP pathway were overexpressed and pinene production was tested, respectively, in order to determine the key genes. Then, the key genes were co-expressed to increase metabolic flux towards IPP and DMAPP.

Eight plasmids with the expression of *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* and *idi* were constructed based on pBBR-αGppsPs (Fig. 1b), and then transferred to *R. sphaeroides*, respectively. Figure 4a showed pinene production with overexpression of these genes. Pinene titers were improved to 105.34 and 109.73 µg/L with overexpression of *dxs* and *idi*, respectively, indicating that Dxs and Idi are rate-limiting enzymes of MEP pathway in *R. sphaeroids*, which is consistent with the reports that Dxs and Idi enhanced carbon flux of isoprenoid biosynthesis in *E.coli* [26, 27]. We therefore co-expressed *idi* and *dxs* together (pBBR-αGppsPs-IdiDxs) and further improved pinene production to 148.83 µg/L (Fig. 4b).

In contrast, overexpression of *dxr*, *ispD*, *ispE*, *ispF*, *ispG* and *ispH*, respectively, resulted in a decrease in pinene production. This suggests overexpression of these genes might cause metabolic burden or toxicity.

Considering that Dxr was reported to be a key enzyme in *E.coli* [28], *dxr* was overexpressed with *idi* and *dxs* together (pBBR- α GppsPs-IdiDxsDxr) to improve pinene production. As shown in Fig. 4b, the strain overexpressing *idi*, *dxs* and *dxr* produced a higher amount of pinene (174.08 $\mu\text{g/L}$) than the one overexpressing *idi dxs* combination. This result indicates that Dxr turns into a rate-limiting enzyme, when the primary rate-limiting steps are released, and overexpression of *dxr* may further improve metabolic flux. In summary, overexpressing *idi*, *dxs* and *dxr* simultaneously causes a great increase of pinene output.

Improving Pinene Production By Altering Rbs Of Gpps-ps Mrna

The expression level of GPPS-PS protein can be altered by changing ribosome binding site, as the ribosome binding site plays an important role in protein translation. Therefore, we considered increasing pinene production by optimizing ribosome binding site. Two standard RBS sequences (RBS β and RBS γ) as well as RBS α , were all taken from iGEM toolbox. The strength of RBS α , RBS β , and RBS γ were tested with GFP as the reporter protein. As shown in Fig. 5a, the fluorescent intensity of GFP is stronger with RBS γ compared to RBS α , but is weaker with RBS β . Then, the ribosome binding site of GPPS-PS mRNA (RBS α) was replaced by a stronger RBS (RBS γ) and a weaker RBS (RBS β), and two plasmids (pBBR- β GppsPs, pBBR- γ GppsPs) were transferred to *R.sphaeroides*, respectively, then the resulting pinene titers were compared. Figure 5b shows that the pinene production can be improved by using a stronger RBS, but decreased with a weaker RBS. Thus, overexpression of GPPS-PS protein could increase pinene production, which suggests the metabolic fluxes of downstream pathway might be inadequate in the strain harboring pBBR- α GppsPs.

Therefore, RBS α was replaced with RBS γ , and pBBR- γ GppsPs-IdiDxs and pBBR- γ GppsPs-IdiDxsDxr were constructed and transferred to *R.sphaeroides*, respectively. As shown in Fig. 6, overexpressing *idi* and *dxs* increased the production of pinene to 401.43 $\mu\text{g/L}$ and overexpressing *idi*, *dxs* and *dxr* together further improved the yield of pinene to 539.84 $\mu\text{g/L}$, which was 5.54 folds compared to *R.sphaeroides*:pBBR- α GppsPs strain. Thus, overexpression of key enzymes of the MEP pathway and the downstream pathways simultaneously can substantially increase pinene production.

Discussion

To date, heterogeneous terpenoids production were mainly achieved in yeast [29] and *E. coli* [30]. Researches on the synthesis of pinene in other microorganisms were limited. Previous study used cyanobacteria to synthesize pinene with an engineered pinene synthase under phototrophic conditions and reached 80 $\mu\text{g/L}$ pinene production[8]. In our work, we showed 539.84 $\mu\text{g/L}$ pinene production in engineered *R. sphaeroides* under phototrophic conditions. Thus, photosynthetic bacteria can be used as an alternative photosynthetic chassis.

Compared with *E.coli*, the production of pinene in phototrophic microorganisms is much lower. In fact, phototrophic microorganisms synthesize a large amount of compounds with IPP and DMAPP as precursors, such as carotenoids [31], coenzyme Q₁₀ [21], lycopene [19], through the MEP pathway and downstream pathways. Researchers improved the yield of target products by weakening some competitive pathway. For example, Zhu YQ increases the production of coenzyme Q₁₀ by reducing the synthesis of carotenoids [32]. For pinene production, we can reduce the synthesis of the above metabolites to guide more metabolism flux to the direction of pinene synthesis. In addition, the growth rates of phototrophic microorganisms are much slower than *E. coli*. Increasing the growth rate is significant for phototrophic microorganisms to be developed into bioproduction platforms. Researchers have constructed fast-growing cyanobacteria by expressing key rate-limiting enzymes, with a doubling time of 2–3 hours [33]. Therefore, understanding the rate-limiting enzymes of purple non-sulfur photosynthetic bacteria growth is of great significance for improving the growth rate.

In addition to optimizing the endogenous MEP pathway, introduction of a heterogeneous mevalonate metabolic pathway is also a common method [34]. We tried to use plasmids to introduce the mevalonate pathway genes with *gpps-ps* gene simultaneously, but the pinene production was severely reduced. A similar phenomenon was also found in the synthesis of patchoulol by *R. capsulatus* [13]. When a plasmid was used to express the mevalonate pathway and multiple other genes simultaneously, patchoulol could not be detected [13]. The possible reason might be that the overexpression of mevalonate pathway genes brought great metabolic burden and growth pressure to the host. However, when the exogenous mevalonate pathway was recombined into the genome, patchoulol production can be increased [13]. Therefore, an effective way to introduce the heterogeneous mevalonate pathway is to recombine it into the genome. In addition, improving the activity of pinene synthase can greatly increase the output of pinene [8]. It is also very important to further screen for pinene synthase with high activity.

Conclusions

In our work, we successfully synthesized pinene in purple non-sulfur photosynthetic bacteria for the first time by heterologous expressing a fusion protein of geranyl diphosphate synthase and pinene synthase. The optimal temperature and IPTG concentration for pinene synthesis were 30 °C and 3 μM, respectively, and the optimal duration for the reaction was 132 h. Using the appropriate RBS and overexpressing *idi*, *dxs* and *dxr* simultaneously, the pinene titer can be increased by 5.54 folds to 539.84 μg/L. However, for the synthesis of pinene in purple non-sulfur photosynthetic bacteria, there is still a lot of work that can be done, such as expressing and optimizing a heterogeneous mevalonate pathway, screening for highly active pinene synthase, weakening the competitive metabolic pathway, and increasing the growth rate of purple non-sulfur photosynthetic bacteria.

Methods

Microorganisms and growth conditions

E.coli DH5α and *E.coli* S17-1 were applied for plasmid construction and di-parental conjugation, respectively. *R. sphaeroids* 2.4.1 (ATCC17023) was used for pinene production. *E. coli* strains were grown at 37 °C in Luria-Bertani medium consisting of yeast extract (5 g/L), tryptone (10 g/L), NaCl (10 g/L). For conventional cultivation and di-parental conjugation, *R. sphaeroides* strains were cultivated in modified Sistrom's medium [35] with or without agar. For pinene production, *R. sphaeroides* was grown photoheterotrophically with light intensity around 4000 lux in modified Sistrom's medium supplied with glucose (30 g/L) as carbon source. In addition, the media were supplemented with 50 mg/ L of kanamycin (Km) for both *R. sphaeroides* and *E.coli* when necessary.

Plasmids And Ribosome Binding Sites

The plasmids and ribosome binding sites (RBSs) used in this study are listed in Table 1. The *gpps* and *ps* gene were both from *Abies grandis* [5], and the *gpps-ps* fusion gene were synthesized by Sangon Biotech (Shanghai) Co. Ltd., after codon optimization. For plasmids construction, all the gene fragments were ligated using the In-Fusion PCR cloning system (Clontech). All the information for plasmids construction, including the method, all the primers and sequences of synthetic genes and original plasmids are available from Mendeley Data [36].

Table 1
Plasmids and RBSs used in this study

Name	Description	References
pBBR- α GppsPs	<i>Ptrc:lacO-RBSα-gpps-ps, Placlq-laclq</i>	This work
pBBR- α GppsPs-Idi	<i>Ptrc:lacO-RBSα-gpps-ps, Ptac:lacO-idi, Placlq-laclq</i>	This work
pBBR- α GppsPs-Dxs	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-dxs, Placlq-laclq</i>	This work
pBBR- α GppsPs-Dxr	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-dxr, Placlq-laclq</i>	This work
pBBR- α GppsPs-lspD	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-lspD, Placlq-laclq</i>	This work
pBBR- α GppsPs-lspE	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-lspE, Placlq-laclq</i>	This work
pBBR- α GppsPs-lspF	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-lspF, Placlq-laclq</i>	This work
pBBR- α GppsPs-lspG	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-lspG, Placlq-laclq</i>	This work
pBBR- α GppsPs-lspH	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-lspH, Placlq-laclq</i>	This work
pBBR- α GppsPs-IdiDxs	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-idi dxs, Placlq-laclq</i>	This work
pBBR- α GppsPs-IdiDxsDxr	<i>Ptrc:lacO-RBSα-gpps-ps,, Ptac:lacO-idi dxs dxr, Placlq-laclq</i>	This work
pBBR- β GppsPs	<i>Ptrc:lacO-RBSβ-gpps-ps, Placlq-laclq</i>	This work
pBBR- γ GppsPs	<i>Ptrc:lacO-RBSγ-gpps-ps, Placlq-laclq</i>	This work
pBBR- γ GppsPs-IdiDxs	<i>Ptrc:lacO-RBSγ-gpps-ps, Ptac:lacO-idi dxs, Placlq-laclq</i>	This work
pBBR- γ GppsPs-IdiDxsDxr	<i>Ptrc:lacO-RBSγ-gpps-ps, Ptac:lacO-idi dxs dxr, Placlq-laclq</i>	This work
pBBR- α GFP	<i>Ptrc:lacO-RBSα-gfp, Placlq-laclq</i>	This work
pBBR- β GFP	<i>Ptrc:lacO-RBSβ-gfp, Placlq-laclq</i>	This work
pBBR- γ GFP	<i>Ptrc:lacO-RBSγ-gfp, Placlq-laclq</i>	This work
RBS α (BBa_J95021)	GAGCAGAGGAGA	Registry of Standard Biological Parts
RBS β (BBa_J95016)	CCTGGGGGAGGG	Registry of Standard Biological Parts

Name	Description	References
RBSy (BBa_J95015)	CATCAACGGAGGT	Registry of Standard Biological Parts

Pinene Production And Analytical Methods

The appropriate plasmid was transferred into *R. sphaeroids* 2.4.1 before pinene production using diparental conjugation. *R. sphaeroids* 2.4.1 transformants carrying the proper plasmids were cultured and harvested at logarithmic growth phase and then inoculated into the pinene producing medium at a ratio of 1:100 (v/v). Sealable transparent bottles with the volume of 155 mL were used as reactors and filled with the culture. Dodecane with the content of 1.2% (v/v) and IPTG with proper concentration were added to the culture when cells reached an OD₆₆₀ of 0.3–0.4. Each test was conducted in triplicate. After 132 hs' cultivation, dodecane were collected and centrifuged, and then mixed at the ratio of 1:1(v/v) with limonene/ethyl acetate mixture in which the concentration of limonene was 6 mg/L. The concentration of pinene was tested with limonene as an internal standard by GC-MS (Agilent 7890A with Agilent 5975 MS detector) using the method proposed by Sarria et al [5].

Abbreviations

GPPS
geranyl diphosphate synthase; PS:pinene synthase; IPP:isopentenyl pyrophosphate; DMAPP:dimethylallyl pyrophosphate; MEP:2C-methyl-D-erythritol 4-phosphate; GPP:geranyl pyrophosphate; RBS:ribosome binding site; CrtE:prenyltransferase; FPP:farnesyl pyrophosphate; Pyr:pyruvate; G3P:glyceraldehyde-3-phosphate; DXP:1-deoxy-D-xylulose 5-phosphate; Dxs:1-deoxy-D-xylulose 5-phosphate synthase; Dxr:1-deoxy-D-xylulose 5-phosphate reductase; IspD:2C-methyl-D-erythritol 4-phosphate cytidyltransferase; IspE:4-diphosphocytidyl-2C-methyl-D-erythritol kinase; IspF:2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG:2C-methyl-D-erythritol 2,4-cyclodiphosphate reductase; IspH:4-hydroxyl-3-methylbut-2-enyl diphosphate reductase; Idi:isopentenyl diphosphate isomerase; AcCoA:acetyl-CoA; AACT:acetoacetyl-CoA thiolase; HMGS:3-hydroxy-3-methyl-glutaryl-CoA synthase; HMGR:3-hydroxy-3-methyl-glutaryl-CoA reductase; MK:mevalonate kinase; PMK:phosphomevalonate kinase; PMD:diphosphomevalonate decarboxylase.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The datasets for plasmids construction are available from Mendeley Data (<http://dx.doi.org/10.17632/m7r93c3tsh.1>). All other data generated or analyzed during this study are included in this published article.

Competing interests

The authors have declared that no competing financial interests exist.

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Authors' contributions

XW and GM conceived the idea and designed experiments. GM constructed the clones used in this study and performed experiments. XW wrote the manuscript. CL drew the graphic abstract. XQ, CL and LM critically revised the manuscript. XW, JK and LZ supervised the research.

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Figures

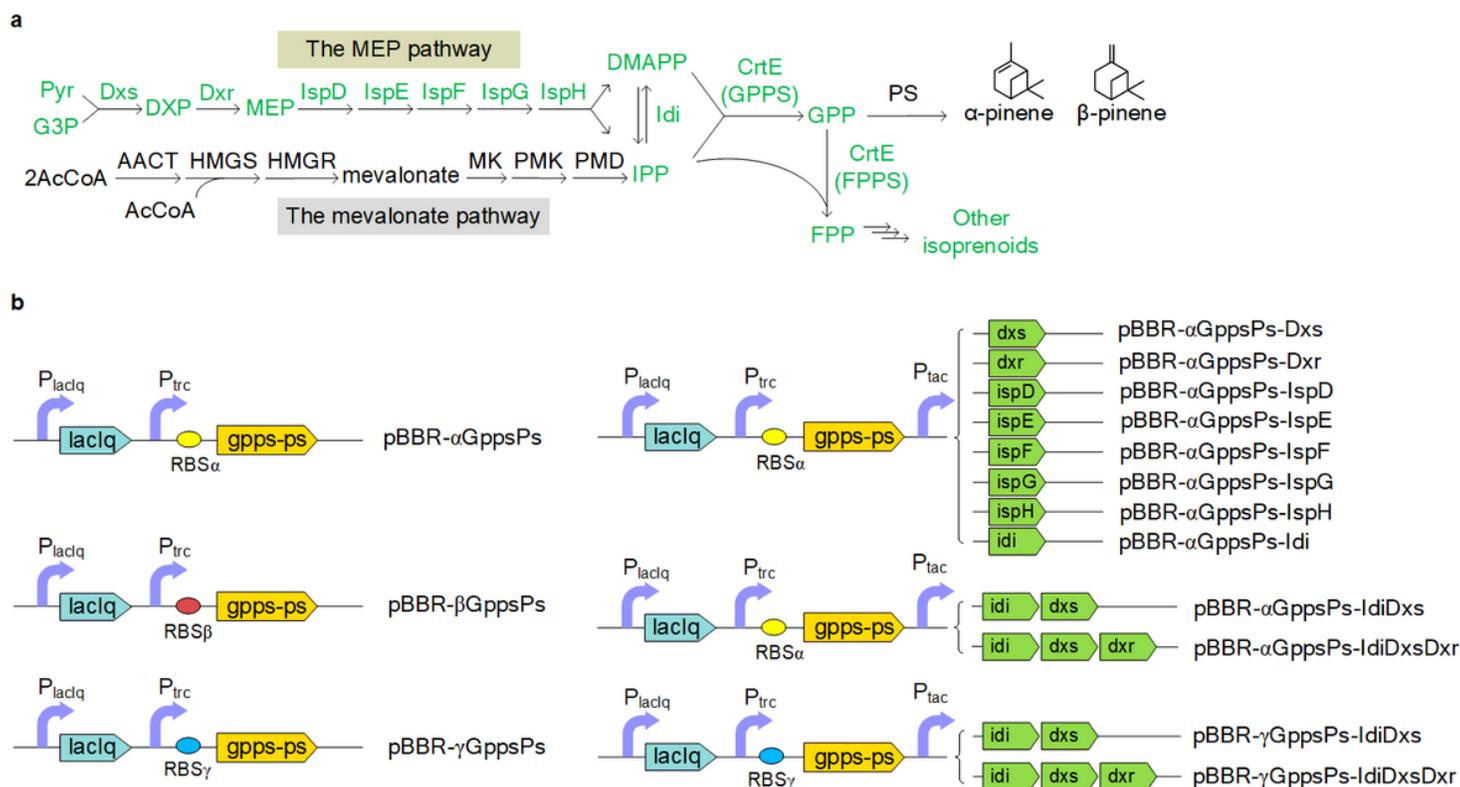


Figure 1

The schematic of the terpene synthetic pathway and the functional operons of plasmids used in this study. (a) Biosynthetic pathway of isoprenoids. The intrinsic terpene biosynthesis of photosynthetic bacteria is shown in green. (b) The functional operons of plasmids used for the biosynthesis of pinene in *R. sphaeroides*. Pyr, pyruvate; G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2C-methyl-D-erythritol 4-phosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; Dxs, 1-deoxy-D-xylulose 5-phosphate synthase; Dxr, 1-deoxy-D-xylulose 5-phosphate reductase; IspD, 2C-methyl-D-erythritol 4-phosphate cytidyltransferase; IspE, 4-diphosphocytidyl-2C-methyl-D-erythritol kinase; IspF, 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, 2C-methyl-D-erythritol 2,4-cyclodiphosphate reductase; IspH, 4-hydroxyl-3-methylbut-2-enyl diphosphate reductase; Idi, isopentenyl diphosphate isomerase; CrtE, prenyltransferase, having both geranyl diphosphate synthase (GPPS) and farnesyl diphosphate synthase

(FPPS) activity; PS, pinene synthase; AcCoA, acetyl-CoA; AACT, acetoacetyl-CoA thiolase; HMGS, 3-hydroxy-3-methyl-glutaryl-CoA synthase; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; MK, mevalonate kinase; PMK, phosphomevalonate kinase; PMD, diphosphomevalonate decarboxylase.

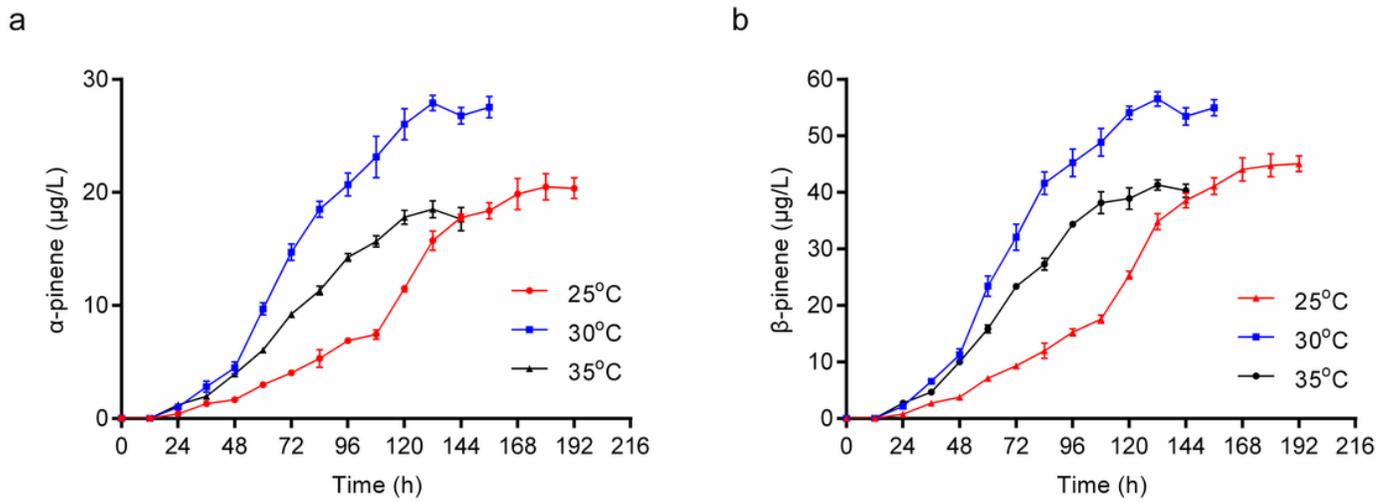


Figure 2

Pinene production over time at various temperatures by *R. sphaeroides* harboring pBBR- α GppsPs plasmid. (a) The titers of α -pinene titers, (b) The titers of β -pinene. Errors indicate s.d. (n = 3).

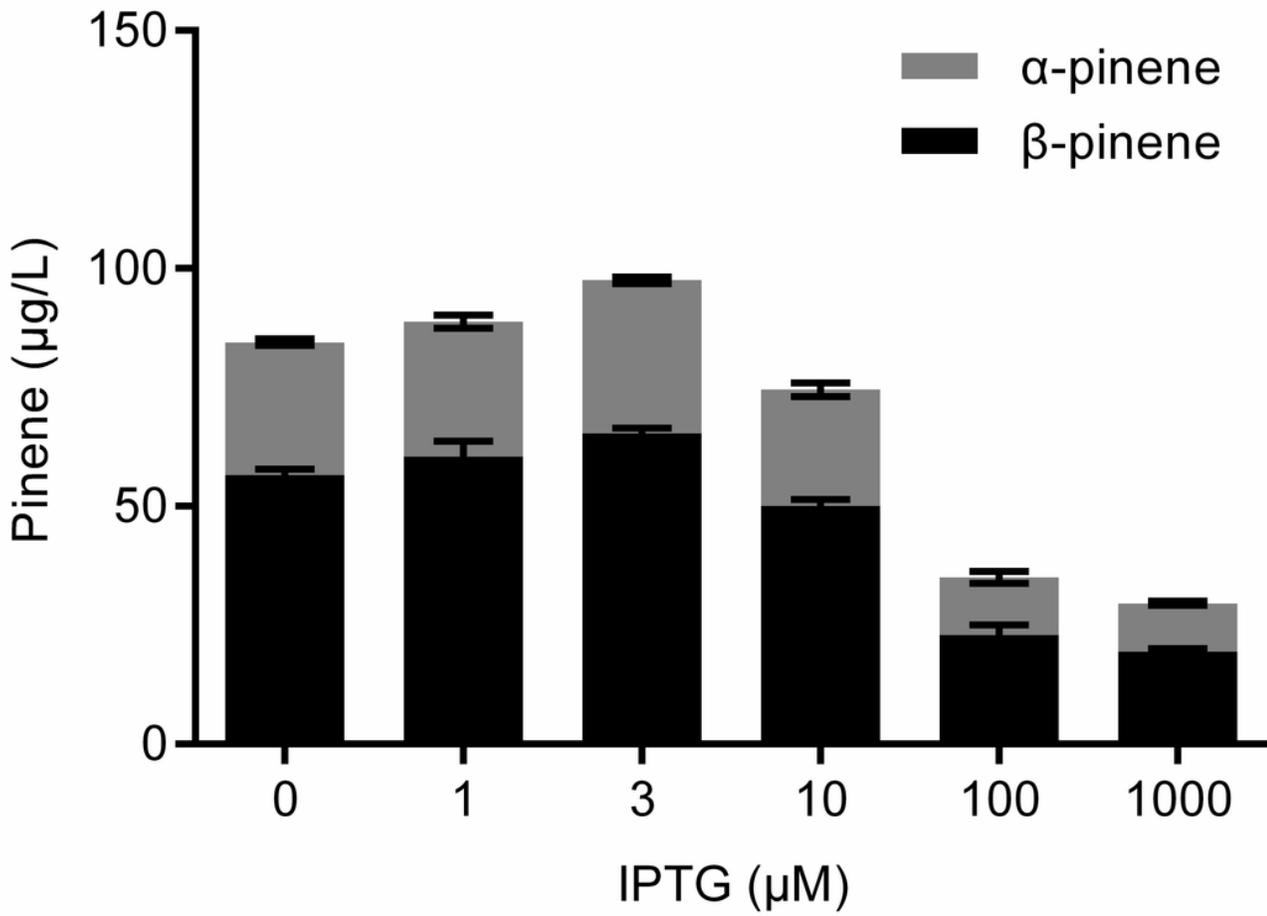
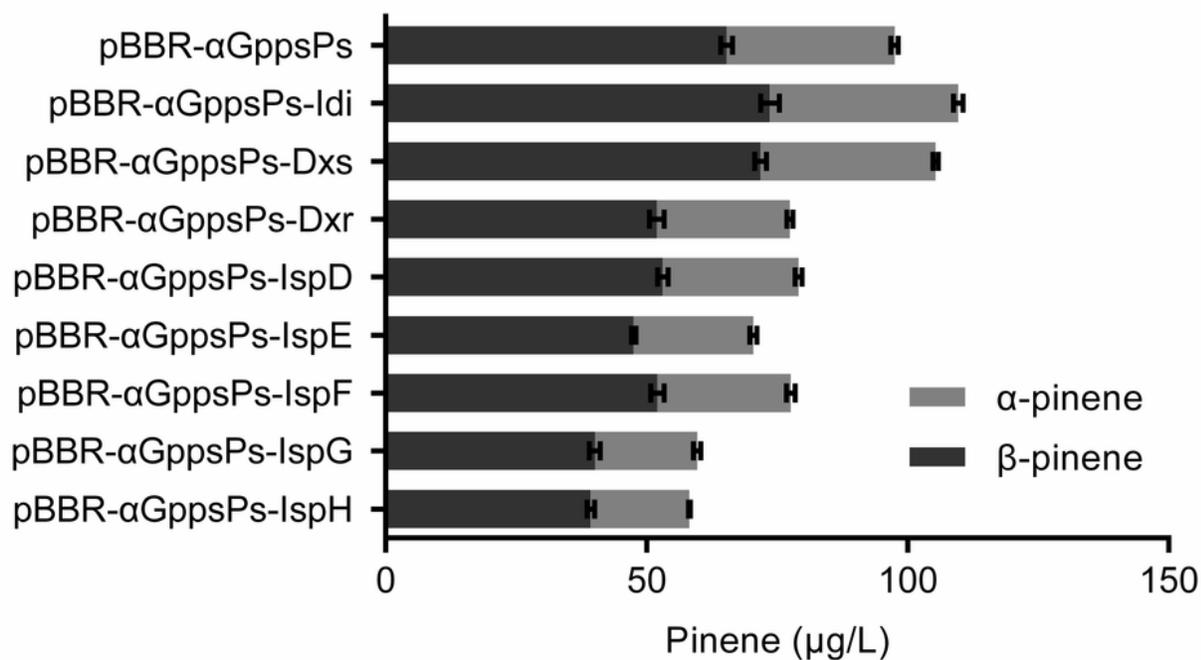


Figure 3

Pinene production at various IPTG concentrations by *R. sphaeroides* harboring pBBR-αGppsPs plasmid. Errors indicate s.d. (n = 3).

a



b

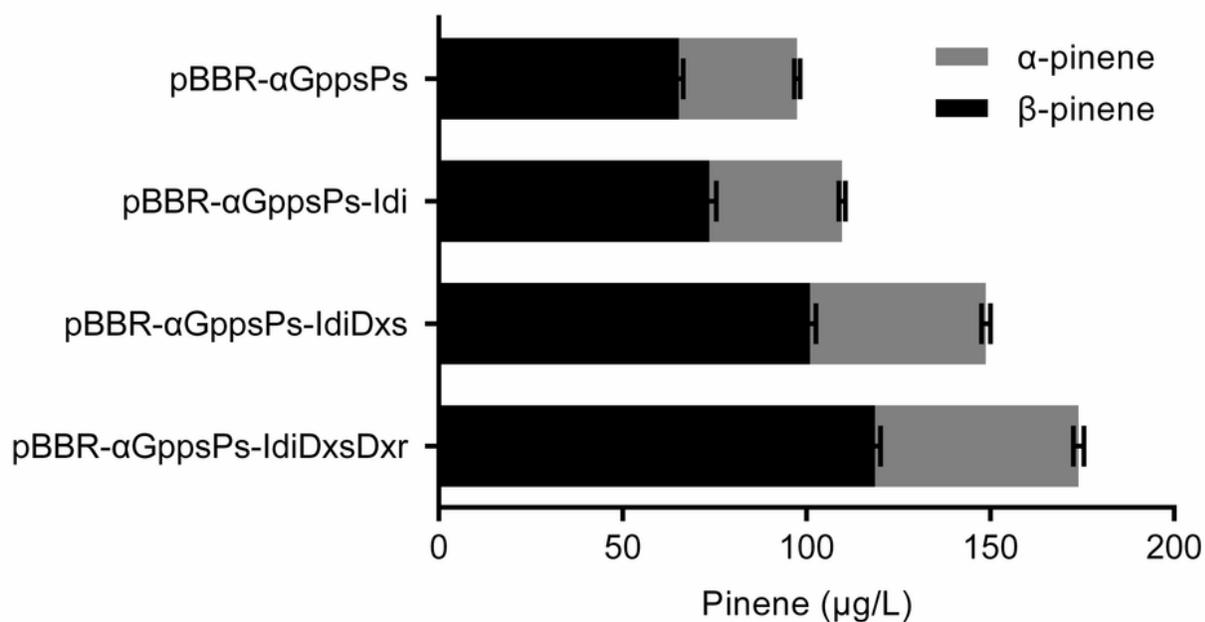


Figure 4

Pinene production by *R. sphaeroides* harboring plasmids derived from pBBR-αGppsPs with overexpression of *dxs*, *dxr*, *ispD*, *ispE*, *ispF*, *ispG*, *ispH* and *idi* genes (a), and genes of rate-limiting enzymes (b), respectively. Errors indicate s.d. (n = 3).

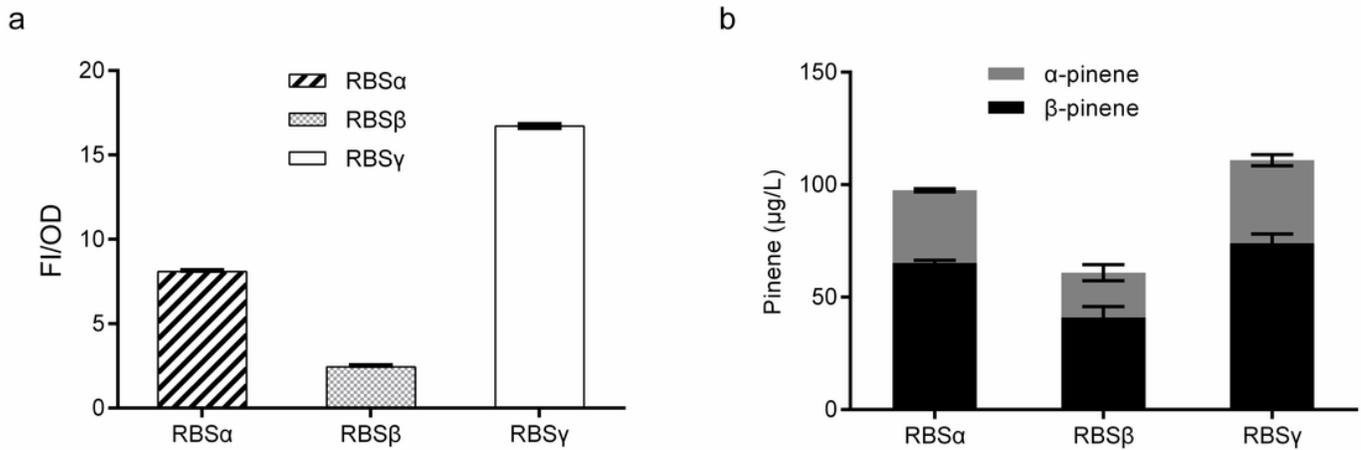


Figure 5

Test of various RBS. (a) The fluorescent intensity of GFP with various RBS. (b) Pinene production via GPPS-PS fusion protein with various RBS. Errors indicate s.d. (n = 3).

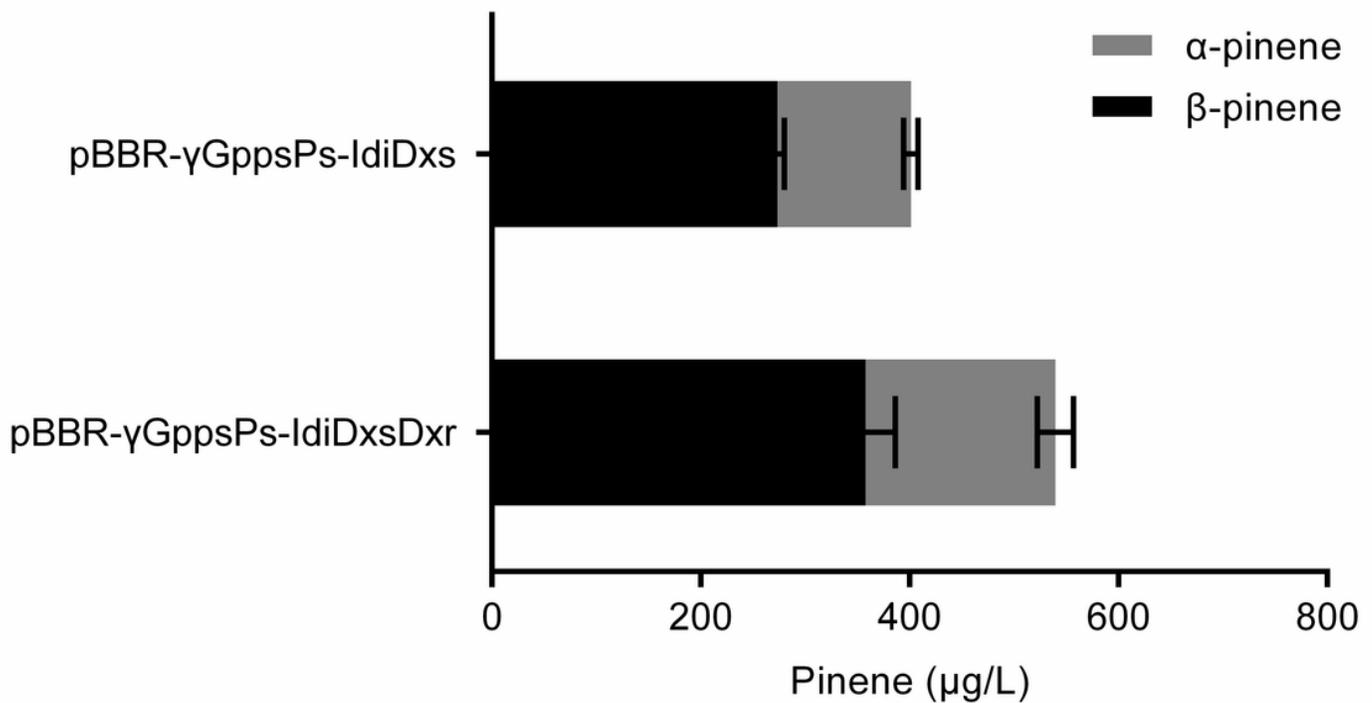


Figure 6

Pinene production by *R. sphaeroides* harboring plasmids derived from pBBR- γ GppsPs with overexpression of genes of rate-limiting enzymes. Errors indicate s.d. (n = 3).