



Engineering *Escherichia coli* for the production of terpene mixture enriched in caryophyllene and caryophyllene alcohol as potential aviation fuel compounds

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ABSTRACT

Recent studies have revealed that caryophyllene and its stereoisomers not only exhibit multiple biological activities but also have desired properties as renewable candidates for ground transportation and jet fuel applications. This study presents the first significant production of caryophyllene and caryolan-1-ol by an engineered *E. coli* with heterologous expression of mevalonate pathway genes with a caryophyllene synthase and a caryolan-1-ol synthase. By optimizing metabolic flux and fermentation parameters, the engineered strains yielded 449 mg/L of total terpene, including 406 mg/L sesquiterpene with 100 mg/L caryophyllene and 10 mg/L caryolan-1-ol. Furthermore, a marine microalgae hydrolysate was used as the sole carbon source for the production of caryophyllene and other terpene compounds. Under the optimal fermentation conditions, 360 mg/L of total terpene, 322 mg/L of sesquiterpene, and 75 mg/L caryophyllene were obtained from the pretreated algae hydrolysates. The highest yields achieved on the biomass basis were 48 mg total terpene/g algae and 10 mg caryophyllene/g algae and the caryophyllene yield is approximately ten times higher than that from plant tissues by solvent extraction. The study provides a sustainable alternative for production of caryophyllene and its alcohol from microalgae biomass as potential candidates for next generation aviation fuels.

1. Introduction

Caryophyllene, a natural bicyclic sesquiterpene (C₁₅) compound, is a common component present in the essential oils of various plants (Kpadonou Kpoviessi et al., 2012; Meccia et al., 2009; Rodrigues et al., 2012). It is widely used for flavoring and personal healthcare applications (Sabulal et al., 2006; Sköld et al., 2006). Several studies have revealed that beta-caryophyllene and caryophyllene essential oils exhibit a wide range of potential therapeutic applications (Rufino et al., 2015; Klauke et al., 2014; Paula-Freire et al., 2014; Alvarez-Gonzalez et al., 2014; Cheng et al., 2014). Caryolan-1-ol (caryophyllene alcohol) is a hydroxylation product of caryophyllene (Nakano et al., 2011). Similar to caryophyllene, caryolan-1-ol is also a fragrance ingredient widely used in personal and house healthcare (Bhatia et al., 2008). In addition to their biological activities, recent studies suggest that the hydrocarbons derived from terpenes are structurally similar to the compounds in petroleum distillate fuels, and often share similar combustion properties (Edwards et al., 2010). Blending of hydrogenated sesquiterpanes, carophyllane and its stereoisomers, in particular, which have a moderate cetane number and only moderately high viscosity,

with synthetic branched paraffins has been found to raise cetane number and reduce viscosity for biosynthetic fuels that meet applicable jet and diesel specifications (Harvey et al., 2015). Additionally, current bio-based fuel molecules such as ethanol have very high oxygen content (up to 2:1 of C:O), which introduces significant cost and material property hurdles for blending into the petroleum-derived fuels infrastructure (http://www1.eere.energy.gov/bioenergy/pdfs/algal_bio-fuels_roadmap.pdf). Compared with fuel alcohol, the terpene mixtures of caryophyllene, caryophyllene alcohol and other caryophyllene stereoisomers are either oxygen free or have very low oxygen hydrocarbon and hydrocarbon-like compounds (C:O > 10), making them particularly attractive candidates as “drop-in” replacements for non-renewable ground transportation and aviation fuels. Based on this observation, caryophyllene and its isomers have been deemed to be among the top three most promising jet fuel compounds with high energy density (<http://www.biofuelsdigest.com/bdigest/06/18/9-advanced-molecules-that-could-revolutionize-jet-and-missile-fuel/>, 2014). It often requires blending of different types of hydrocarbons to achieve satisfactory combustion properties of the fuel. Harvey et al. (Harvey et al., 2015) recently determined that blending hydrogenated heterogeneous

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sesquiterpenes (particularly, caryophyllene and its stereoisomers) with synthetic branched paraffins could yield fuel with improved combustion properties.

The high heterogeneity of the sesquiterpene profile produced by the endophytic caryophyllene synthase makes it a desirable terpene synthase (TPS) for the production of terpene mixture enriched with caryophyllene and its stereoisomers as the next generation renewable aviation and diesel fuels. In our previous studies (Gladden et al., 2013; Wu et al., 2016, 2017), we discovered a suite of novel caryophyllene synthases from endophytes, which produced a wide spectrum of terpenes, of which caryophyllene accounted for 40%, indicating low product specificity and high heterogeneity of the terpene profile. Therefore, in this study, we selected a highly non-specific caryophyllene synthase and a caryolan-1-ol synthase with high product heterogeneity and reported the production of caryophyllene and caryophyllene alcohol enriched terpene mixture as potential compounds for aviation fuel through the heterologous expression of the mevalonate pathway with a geranyl diphosphate (GPP) synthase (Burke and Croteau, 2002), an endophytic caryophyllene synthase (Gladden et al., 2013; Wu et al., 2016), and a caryolan-1-ol synthase (Nakano et al., 2011).

Microalgae, due to its high lipid content, high growth rate, no competition for agriculture land use, and the ability to utilize CO₂, represents a promising renewable resource for the sustainable production of bioproducts and fuels. Under conditions supporting robust growth, microalgae accumulate carbohydrate and protein as major components, up to 80% of total biomass, with lipid contents typically less than 20% (Laurens et al., 2014; Luque, 2010). Recently, a few studies have reported bioconversion of the carbohydrates or proteins from algae biomass into ethanol (de la Cruz et al., 2014; El-Mashad, 2015; Fasahati et al., 2015; Martin and Grossmann, 2014) and isobutanol (Huo et al., 2011). In this study, we demonstrated the viability of bioconversion of the algae hydrolysate into caryophyllene enriched terpene mixture using the newly developed bioconversion strain, which paves the way for the cost-effective and sustainable production of fuels from algae biomass.

2. Material and methods

2.1. Stains and plasmids

The *E. coli* strain DH10B was purchased from Invitrogen and the strain DH1 was obtained from Joint BioEnergy Institute (JBEI). The plasmids JBEI3122, pBbE1a, and pBbE2k were provided by the courtesy of Dr. Jorge Alonso-Gutierrez from JBEI. The plasmid JBEI3122 contains the genes of the mevalonate pathway encoding seven enzymes (Alonso-Gutierrez et al., 2013) (AtoB, HMGS, HMGR, MK, PMK, PMD, and IDI) except the geranyl diphosphate synthase (GPPS) and caryophyllene synthase. The protein sequences of caryophyllene synthase from *Hypoxylon* sp. CI4A (JGI protein ID: 6706, CI4A-CS) and GPPS (GenBank: AF513112.1, GPPS_{Ag}) from *Abies grandis* (Burke and Croteau, 2002) with the chloroplast signal peptide truncated were converted into DNA sequences and codons were optimized based on *E. coli* codon bias. A Ribosome Binding Site for GPPS_{Ag} and caryophyllene synthase (CI4A-CS) were created and optimized by online RBS calculator developed by Dr. Salis Lab (<https://salis.psu.edu/software/>). All the gene sequences containing RBS sites were synthesized by Genscript. Strains used in this study are listed in Table 1.

2.2. Reconstruction of the terpene biosynthetic pathway into *E. coli* DH1

The open reading frames of caryophyllene synthase CI4A-CS and GPPS_{Ag} including the optimized ribosome binding sites were synthesized by Genscript. The ORF of CI4A-CS was sub-cloned into plasmid pBbE1a under *EcoRI* and *BamHI* cutting sites to obtain vector pBbE1a-

Table 1
Strains and plasmids used in this study.

Strains	Plasmids contained	Description
DH1-CS1	pJBEI3122, pBbE1a-CI4A-CS, pBbE2k-GPPS _{Ag}	Construct 1
DH1-CS2	pJBEI3122- GPPS _{Ag} , pBbE1a-CI4A-CS	Construct 2
DH1-CS3	pJBEI3122, pBbE1a-GPPS _{Ag} - CI4A-CS	Construct 3
DH1-CS4	pJBEI3122, pBbE1a -CI4A-CS-GPPS _{Ag}	Construct 4
DH1-gcoA	pJBEI3122- GPPS _{Ag} , pBbE1a-gcoA	Construct 5
DH1-CS-gcoA	pJBEI3122- GPPS _{Ag} , pBbE1a-CI4A-CS-gcoA	Construct 6
Control	pJBEI3122- GPPS _{Ag}	

CI4A-CS. The GPPS_{Ag} ORF was sub-cloned into vector pBbE2k under *EcoRI* and *BamHI* cutting sites to obtain plasmid pBbE2k-GPPS_{Ag} as well. The plasmids pJBEI3122, pBbE1a-CI4A-CS, and pBbE2k-GPPS_{Ag} were co-transformed into expression host DH1 as the construct1 of the caryophyllene biosynthesis pathway. The plasmids pJBEI3122 and pBbE2k-GPPS_{Ag} were co-transformed into strain DH1 as negative control as well.

The gene GPPS_{Ag} was further amplified using Primers GPPS-F/R (GPPS-F: 5-GAG TCT GAA TTCAAAGA TCT GAG ATT TAT CAC ATA GAG CGG-3, GPPS-R: 5-GGA TCCCTC GAG TCA ATT TTG TCT GAA TGC CAC G -3) with the plasmid pBbE2k-GPPS_{Ag} as the template. The amplicons were sub-cloned into plasmid pJBEI3122 at the downstream of gene isoprenyldiphosphate isomerase (*idi*), under the restriction cutting sites *BamHI* and *XhoI*, to obtain plasmid pJBEI3122-GPPS_{Ag}. The plasmids pJBEI3122-GPPS_{Ag} and pBbE1a-CI4A-CS were co-transformed into host strain DH1 as the construct 2 of the caryophyllene biosynthesis pathway. The plasmid pJBEI3122-GPPS_{Ag} was transformed into the strain DH1 as the negative control. Furthermore, the amplicon of gene GPPS_{Ag} was sub-cloned, under *EcoRI* cutting site, into plasmid pBbE1a-CI4A-CS to obtain plasmid pBbE1a-GPPS_{Ag}-CI4A-CS. The right orientation of gene GPPS_{Ag} was confirmed by diagnostic PCR using checking primer 1 (5-CAT CCG GCT CGT ATA ATG TGT GG-3) and primer 2 (5-GCTC CTC GGT TCC TCC AAC AAG-3). The plasmid pJBEI3122 and pBbE1a-GPPS_{Ag}-CI4A-CS were co-transformed into *E. coli* strain DH1 as the construct 3 of the caryophyllene biosynthesis pathway. The amplicon of gene GPPS_{Ag} was sub-cloned into plasmid pBbE1a-CI4A-CS under the cutting sites of *BamHI* and *XhoI* to obtain pBbE1a-CI4A-CS-GPPS_{Ag}. The plasmids pJBEI3122 and pBbE1a-CI4A-CS-GPPS_{Ag} were co-transformed into host strain DH1 as the construct 4 of the caryophyllene biosynthesis pathway.

The protein sequence of caryolan-1-ol synthase (Nakano et al., 2011) (caryophyllene alcohol synthase, GenBank: B1W019) was extracted from GenBank. The protein sequence was converted into DNA sequence and codon was optimized based on the *E. coli* codon bias. A Ribosome Binding Site for caryolan-1-ol synthase (*gcoA*) was created and optimized with the online RBS calculator developed by the Salis Lab. The sequence of gene *gcoA* containing RBS site was synthesized by Genscript and sub-cloned into the plasmid pBbE1a under cutting sites *EcoRI* and *BglII* to achieve the plasmid pBbE1a-*gcoA*. The gene *gcoA* with RBS was amplified using primers (*gcoA*-F: 5-ATGGAATTCGGATC CGCCAGTTCATATACTAACCTAAGGAGGACC-3, *gcoA*-R: 5-ATGGAATTCCTCGAG TCA GGC TGC GAA GTA TTG CGA C-3) and sub-cloned into the plasmid pBbE1a-CI4A-CS under the cutting sites *BamHI* and *XhoI* to achieve the plasmid pBbE1a-CI4A-CS-*gcoA*. The plasmids pJBEI3122-GPPS_{Ag} and pBbE1a-*gcoA* or pBbE1a-CI4A-CS-*gcoA* were co-transformed into strain DH1 for the caryophyllene alcohol production. The codon-optimized synthetic genes of caryophyllene synthase (JGI protein ID: 6706), GPPS (GenBank: AF513112.1), and caryolan-1-ol synthase (caryophyllene alcohol synthase, GenBank: B1W019) were submitted into GenBank with an accession number: KX525265, KX578222, and KX759400, respectively.

2.3. Production of caryophyllene and caryolan-1-ol by the engineered *E. coli* strains

The transformants of the different pathway constructs containing caryophyllene synthase (CI4A-CS) or caryolan-1-ol synthase (*gcoA*), shown in Table 1, were cultured into 15 mL of LB medium with 100 µg/L of ampicillin, 34 µg/L chlorophenicol, or 25 µg/L of kanamycin as necessitated by the pathway constructs. The cultures were incubated at 37 °C at 220 rpm overnight. Then, 1 mL of overnight culture was inoculated into 25 mL of fresh EZ-rich medium (TeKnova, CA). The culture was continued to incubate at 37 °C, 220 rpm until the OD_{600 nm} reached 0.8 and then caryophyllene, caryophyllene alcohol, and terpene production were induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) at the final concentration 0.5 mM for another 72 h at 30 °C, 180 rpm. The flasks were cap-sealed with a hole at the middle covered by multiple layers of paper tape.

To optimize IPTG concentration for enzyme expression, the cultures were induced at serial concentrations of IPTG at 30 °C, 180 rpm for 72 h. In terms of induction temperature optimization, the cultures were induced under optimized IPTG concentration under different induction temperatures, at 180 rpm for 72 h. For production of terpenes from algal biomass, the hydrolysate from the marine microalgae *Nannochloropsis* sp. was prepared according to NREL's protocol (http://www.nrel.gov/biomass/microbial_procedures.html). The pretreated hydrolysate was digested with 2 mg/mL Pronase (Promega, CA) at 55 °C for 48 h and the enzyme was deactivated by incubating at 80 °C for 30 min. The Pronase digested hydrolysate was filtered through 0.2 µm PTFE membrane and used as medium for terpene production. The fermentation was done as described above.

2.4. LC-MS analysis of pathway metabolites

20 mL of one-day culture after induction was collected and spun down at 6000 g for 10 min. The cell pellets were rinsed with icy DI water three times and resuspended with 1 mL of methanol and the cells were disrupted with bead beater (BioSpec, CA) at speed 4 for 30 s, twice. The mixture was centrifuged at 10,000 g for 10 min and the supernatant was transferred to a new 2 mL vial. 750 µL of Ultrapure DI water was added into sediment cell lysate and vortexed vigorously at 4 °C. The mixture was centrifuged and the supernatant was combined with the methanol portion. The methanol in the combined supernatant was blown out by nitrogen gas and the leftover mixture was filtered through a 3KDa molecular weight cutoff (MWCO) spin column (Millipore). The filtrates were analyzed by LC-MS using the method described by Rodrigues et al. (Rodrigues et al., 2014).

2.5. Quantification of terpene products with GC/MS

The volatile terpene compounds in the headspace of each culture were analyzed with GC-MS using a solid-phase micro-extraction (SPME) syringe consisting of 50/30 divinylbenzene/carboxen on polydimethylsiloxane on a Stable Flex fiber. The SPME fiber was explored into the headspace of each culture flask for 10 min to absorb the terpene compounds. Then, the syringe was inserted into the injection port (220 °C) of an Agilent gas chromatograph 7890 A containing a 30 m × 0.25 mm i.d DB wax capillary column with a film thickness 0.25 µm to elute the terpene. The column was temperature programmed as follows: 60 °C for 2 min, increasing to 80 °C at 5 °C/min and holding for 3 min, then increasing to 120 °C at 5 °C/min and holding for 3 min, then increasing to 220 °C at 10 °C/min and holding for 2 min and then increasing to 240 °C at 40 °C/min and holding for 2 min. The carrier gas was ultra-high purity helium at a constant flow rate of 1.8 mL/min, and the initial column head pressure was 16.188 psi. A 4-min injection time was used to desorb the caryophyllene and other terpene compounds from the sampling fiber into a split/splitless injection (splitless mode, 220 °C) of the chromatograph

coupled with quadrupole MS 5975 C. The MSD parameters were set as EI at 70 eV, mass range of 50–650 Da, and the scan speed at 2 scans/s.

Spectral components were searched against the NIST 2015 mass spectral library, and only components with mass spectra match factors > 85% were reported as tentatively identified compounds. Compounds with peak areas > 1% of the total peak area in the chromatogram are reported.

SPME has become more popular in the quantitative analysis of volatile chemicals (Ai, 1997) due to its distinct features, such as relatively consistent adsorption ability of both mono- and sesquiterpene. In this study, the SPME was calibrated for the estimation of the concentrations of terpenes as following. A serial dilution of pinene and caryophyllene (five concentrations) were added into the known quantities of the culture media inoculated with a negative control strain to simulate the liquid-gas phase equilibrium of terpene compounds produced in the culture. The flasks were cap-sealed and incubated under identical conditions used for caryophyllene and caryolan-1-ol formation strains. The terpene compounds in the headspace were collected by SPME as described above. The concentrations of caryophyllene and other terpene compounds produced in the culture were calculated through reference to the standard curves. The titer of caryolan-1-ol was also estimated by referring to the caryophyllene standard curve since no commercial standards are available. Due to the lack of the commercial standards for all other terpene compounds produced in this study, the mono-, sesqui- and total terpene titers or yields presented were calculated based on corresponding standard curves of pinene and caryophyllene, respectively. All the experiments were performed in triplicate. The R square values of the standard curves were all higher than 90%. All error bars were presented as the standard deviation of the replicates.

3. Results

3.1. Biosynthesis of caryophyllene and related terpenes through the mevalonate pathway in *E. coli*

In this study we aimed to increase caryophyllene production titer by reconstruction of a heterologous mevalonate (MEV) pathway and a caryophyllene synthase into *E. coli* (Fig. 1(A)). First, we constructed the full caryophyllene biosynthesis pathway into three plasmids (denoted as “construct 1”, Table 1) to demonstrate the feasibility of caryophyllene production, as shown in Fig. 1. In construct 1, the seven enzymes from the mevalonate pathway were constructed into a medium-copy-number plasmid pJBEI3122 (p15A) with the first three enzymes (AtoB, HMGS, HMGR) expressed under a medium strength promoter LacUV5 and last four enzymes (MK, PMK, PMD, and IDI) under a strong promoter P_{trc} to achieve optimal intermediate metabolite flux (Alonso-Gutierrez et al., 2013; Lee et al., 2011). *E. coli* genome contains *ispA* encoding for GPP synthase/farnesyl diphosphate (FPP) synthase, which can provide GPP and FPP precursors for monoterpene and sesquiterpene biosynthesis (Yang and Nie, 2016). In addition, we observed higher titer of intermediate metabolite farnesyl diphosphate (FPP) was produced with the co-expression of MEV pathway with GPPS than that with GPPS and FPPS in the host strain DH1 in our previous study. Therefore, only a geranyl diphosphate synthase (GPPS) from *Abies grandis* (Burke and Croteau, 2002) without the farnesyl diphosphate synthase (FPPS) was cloned into a medium copy plasmid pBbe2K (ColE1) under the strong T7 promoter. The caryophyllene synthase CI4A-CS (JGI protein ID: 6706) was cloned into a medium-copy-number plasmid pBbe1a (ColE1) under a strong promoter P_{trc}. *E. coli* strain DH1 containing these three plasmids was inoculated into fermentation medium (EZ-rich, TeKnova, CA) and induced with 1 mM IPTG when the OD_{600 nm} reached 0.8. After 72 h' culture, the total terpene produced in the headspace of culture was collected using SPME and analyzed with GC-MS. Strain DH1-CS1 containing construct 1 produced ~205 mg/L of total terpene including

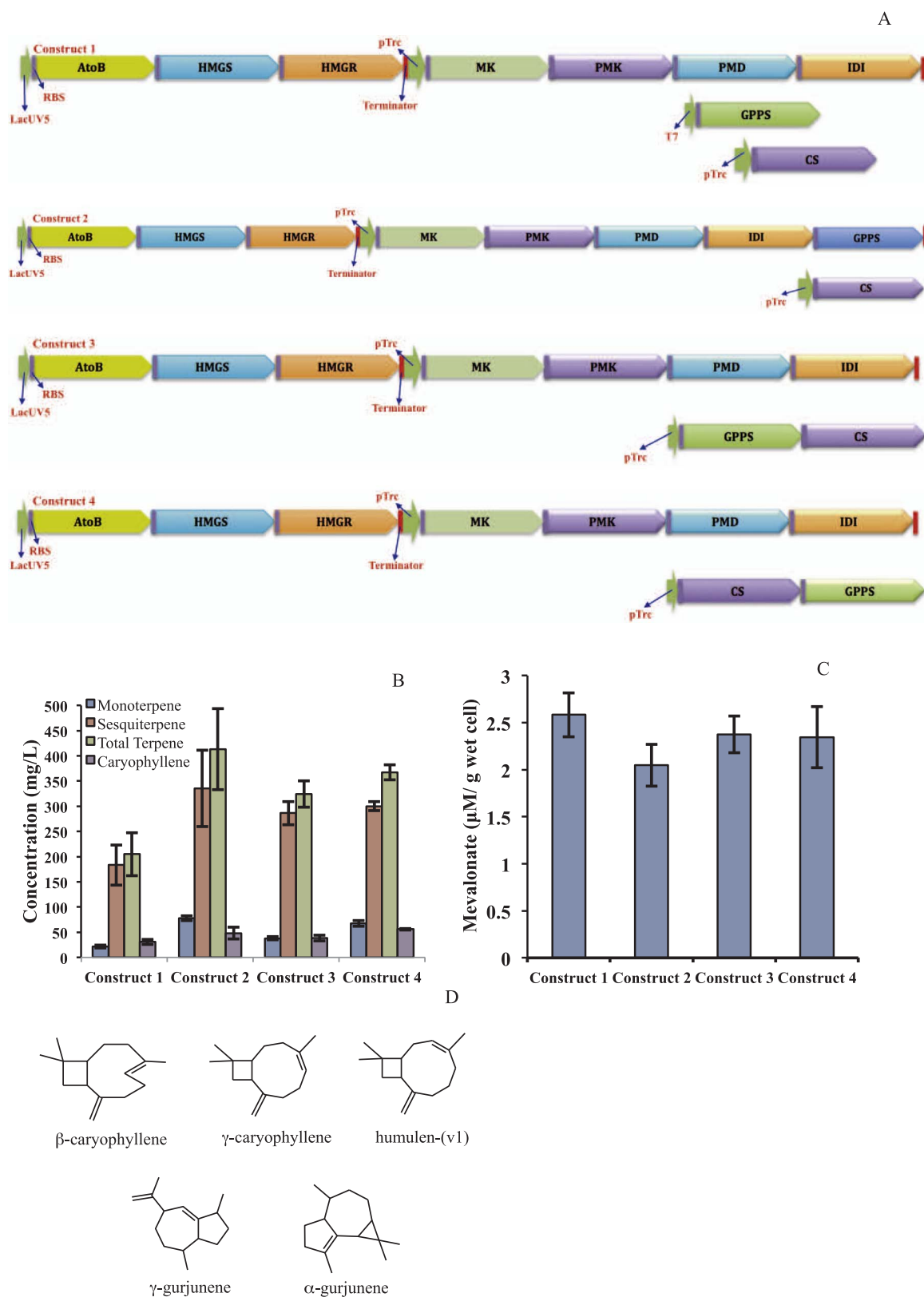


Fig. 1. Enhancement of terpene production through four different pathway constructs. A: constructs of the four different caryophyllene biosynthesis pathway expression strategies; B: terpene titers of four different caryophyllene biosynthesis pathways constructs; C: The accumulated mevalonate in the strain containing four different caryophyllene biosynthesis pathways constructs, respectively D: the most abundant terpene compounds produced by caryophyllene synthase, Cl4A-CS. AtoB: acetoacetyl-CoA acetyltransferase; HMGS: hydroxymethylglutaryl-CoA synthase; HMGR: hydroxymethylglutaryl-CoA reductase; MK: mevalonate kinase; PMK: mevalonate-5-phosphate kinase; PMD: mevalonate diphosphate decarboxylase; IDI: isopentenyl-diphosphate isomerase; GPPS: geranyl diphosphate synthase.

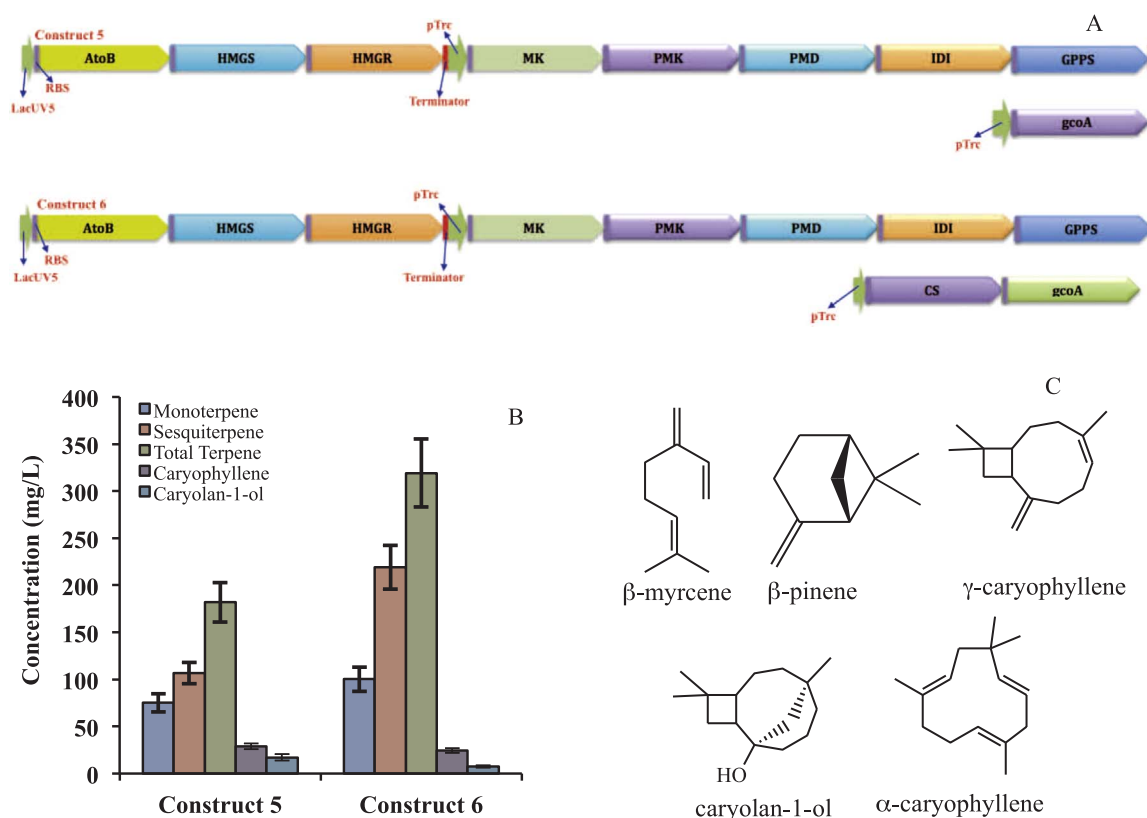


Fig. 2. Comparison of caryophyllene alcohol production from two different pathway constructs. A: constructs of two different caryolan-1-ol biosynthesis pathway expression strategies; B: terpene titers of two different caryolan-1-ol biosynthesis pathway constructs; C: the most abundant terpene compounds produced by caryolan-1-ol biosynthesis pathways.

22 mg/L of monoterpene, 183 mg/L sesquiterpene, and 31 mg/L caryophyllene. The caryophyllene synthase C14A-CS produced more than 25 terpene compounds in the culture (Wu et al., 2016), suggesting the high heterogeneity of the terpene mixture preferably for the aviation fuel applications. Among the over 25 terpenes, caryophyllene accounted for 15% of the total (Fig. 1(B)). In addition to caryophyllene, humulen-(V1) and gurjunene were the other two most abundant sesquiterpene compounds (Fig. 1(D)). No obvious terpene compounds were detected in the culture of the control strain.

3.2. Improvement of caryophyllene and total terpene production titer using different pathway enzyme expression strategies

In construct 1, all pathway enzymes were cloned into three different plasmids. Maintaining multiple plasmids in the engineered strain increases the metabolic burden from DNA, RNA, and protein synthesis as well as multiple antibiotic resistance proteins that the strain must produce (Rozkov et al., 2004) and therefore results in low production titer of the target products (Moon et al., 2010). To address this issue, the plasmids with three other pathway enzyme expression strategies were constructed and introduced into the *E. coli* bioconversion chassis in order to increase the metabolic flux driving force to the final product formation, as shown in Fig. 1 (A). In construct 2, GPPS_{Ag} was cloned downstream of the gene encoding for enzyme IDI in plasmid pJBEI3122 under the strong promoter P_{trc} to achieve homologous expression of all intermediate pathway enzymes. In construct 3 and construct 4, GPPS_{Ag} was cloned upstream and downstream of caryophyllene synthase C14A-CS, respectively, in the plasmid pBbE1a-C14A-CS under the same strong promoter P_{trc} to achieve the high expressions of both GPPS and C14A-CS. The strains (DH1-CS2, 3, and 4, Table 1) containing these three constructs were cultured and induced at the same conditions used for strain DH1-CS1. Among these three constructs, the strain DH1-CS2 containing construct 2 produced the highest concentration of total

terpene, up to 414 mg/L, including 336 mg/L of sesquiterpene, 78 mg/L of monoterpene, and 48 mg/L of caryophyllene (Fig. 1(B)). Strains DH1-CS3 and DH1-CS4 produced similar amount of sesquiterpene (286 and 300 mg/L, respectively). However, DH1-CS4 produced up to 67 mg/L monoterpene which was 76% higher than from DH1-CS3. Additionally, strains DH1-CS3 and DH1-CS4 produced 38 mg/L and 56 mg/L of caryophyllene, respectively. Compared with DH1-CS1, DH1-CS2 strain produced 101% more total terpene as well as 83% more sesquiterpene; DH1-CS3 and 4 also produced 58% and 79% more of total terpene, respectively. To investigate the metabolic flux of the four different constructs, we performed target metabolomics analysis of pathway intermediate metabolites, as shown in Fig. 1 (C). LC-MS analysis of the pathway metabolites indicated the accumulation of mevalonate for all four constructs. Among them DH1-CS1 strain had the highest level of mevalonate (2.6 $\mu\text{mol/g}$ wet cell) accumulated intracellularly (Fig. 1(C)), suggesting the least efficient conversion of mevalonate into downstream metabolites in construct 1. Correspondingly, DH1-CS1 strain also produced the lowest titer of total terpene and caryophyllene. In contrast, DH1-CS2 accumulated the lowest amount of mevalonate (2.0 $\mu\text{mol/g}$ wet cell, Fig. 1(C)) *in vivo*, and produced the highest titer of total terpene and caryophyllene among the four strains. Both DH1-CS3 and DH1-CS4 produced similar amount of total terpene and accumulated similar level of mevalonate as well. Mevalonate was detected as the only intermediate metabolite, suggesting that phosphorylation of mevalonate catalyzed by mevalonate kinase is likely the rate limiting step.

3.3. Biosynthesis of caryophyllene alcohol through expression of caryolan-1-ol synthase

Caryolan-1-ol (caryophyllene alcohol) is a hydroxylated derivative of caryophyllene, which is currently used as an aroma component in shampoos, soaps, and household detergent. To produce caryophyllene

alcohol, a caryolan-1-ol synthase gene (*gcoA*) from *Streptomyces griseus* (Nakano, 2011) was cloned into vectors pBbE1a and pBbE1a-CI4A-CS as constructs 5 and 6 (Fig. 2(A)). Both strain DH1-*gcoA* (construct 5) and DH1-CS-*gcoA* (construct 6) produced more than 10 terpene compounds including monoterpene and sesquiterpene in the culture (Table S1), indicating that both caryophyllene synthase and caryolan-1-ol synthase are bi-functional terpene synthases. However, strain DH1-CS-*gcoA* produced twice as many compounds as by strain DH1-*gcoA* in which only caryolan-1-ol synthase was expressed. Caryophyllene and caryolan-1-ol were detected in the culture of both strains. The titers of caryophyllene (29 and 24 mg/L) were about 1.7–3.3-fold as high as that of caryolan-1-ol (17 and 7 mg/L) (Fig. 2 (B)). Besides caryophyllene and caryolan-1-ol, DH1-*gcoA* strain produced more monoterpene than sesquiterpene, with β -myrcene and β -pinene the most abundant terpene compounds (Fig. 2(B) and Table S1 (A)). DH1-CS-*gcoA* produced a similar terpene profile as DH1-CS2 except for the presence of caryolan-1-ol (Table S1 (B)). However, both DH1-CS-*gcoA* and DH1-*gcoA* produced less amount of total terpene than DH1-CS2, which is probably due to the slow reaction kinetics of caryolan-1-ol synthase (Nakano, 2011). DH1-*gcoA* and DH1-CS-*gcoA* produced 50% and 25% less of total terpene than strain DH1-CS2, respectively.

3.4. Effects of inducer concentration and temperature on caryophyllene, caryolan-1-ol and terpene production

In this study, a one-factor-at-one-time (one way) optimization method was applied to determine the optimal induction temperature and IPTG concentration sequentially. First of all, four culture temperatures (18 °C, 25 °C, 30 °C, 37 °C) were investigated to identify the optimal temperature for terpene production in DH1-CS2 and DH1-*gcoA* under 1 mM IPTG induction. The results indicated that DH1-CS2 produced the highest amounts of total terpene at 37 °C, up to 403 mg/L of total terpene which was 1.7 times higher than that produced at 18 °C. The caryophyllene produced at 37 °C was 50 mg/L which was 2-fold higher than that produced at 18 °C. In contrast, DH1-*gcoA* produced the least amount of terpenes at 37 °C (110 mg/L, Fig. 3(B)). Similar levels of terpene were detected in the cultures at 25 °C and 30 °C where 187 mg/L and 172 mg/L of total terpene were produced respectively. The highest titer of 22 mg/L caryophyllene was achieved at 25 °C while caryolan-1-ol was 3 mg/L which was lower than the highest value (5 mg/L) obtained at 30 °C. Therefore, 37 °C and 25 °C was selected respectively as the optimal induction temperature for DH1-CS2 and DH1-*gcoA* for further optimization.

A range of IPTG concentrations, from 0.5 to 2 mM, were investigated to optimize inducer concentration under previously determined optimal culture temperatures. The results (Fig. 3(C)) showed that DH1-CS2 produced the highest titers of total terpene (482 mg/L) as well as caryophyllene (76 mg/L) when induced by 1.5 mM IPTG at 37 °C in EZ rich medium (TeKnova, CA). The optimal IPTG concentration for strain DH1-*gcoA* was 0.5 mM, where 237 mg/L of total terpene, 36 mg/L caryophyllene, and 7 mg/L caryolan-1-ol (Fig. 3(D)) was produced at 25 °C in the EZ rich medium. Both DH1-CS2 and DH1-*gcoA* have the same pathway enzyme expression strategy, with the entire intermediate pathway enzymes cloned into plasmid pJBEI3122 and CI4A-CS or *gcoA* expressed in another separate plasmid pBbE1a. However, the two constructs achieved the highest terpene titers under different fermentation conditions, suggesting different enzyme properties and kinetics between CI4A-CS and *gcoA* as well as varied metabolic burden and flux to terpene formation.

The highest terpene production titers from DH1-CS2 and DH1-*gcoA* were determined under the previously determined optimal conditions. DH1-CS2 produced 449 mg/L of total terpene, 406 mg/L sesquiterpene, 43 mg/L monoterpene as well as 100 mg/L caryophyllene (Fig. 3(E)). DH1-*gcoA* yielded 243 mg/L of total terpene, including 143 mg/L monoterpene and 100 mg/L sesquiterpene in which caryolan-1-ol was 10 mg/L.

3.5. Caryophyllene production from a microalgae hydrolysate by *E. coli* DH1-CI4A-CS2

We further investigated the production of caryophyllene and other terpene products by the engineered *E. coli* strain DH1-CI4A-CS2 using biomass from marine microalgae *Nannochloropsis* sp. The engineered strain DH1-CI4A-CS2 produced a wide spectrum of terpene compounds with sesquiterpenes as the major components. As shown in Fig. 4(A), the titer of total terpene was up to 360 mg/L, including 323 mg/L of sesquiterpene, 38 mg/L of monoterpene as well as 75 mg/L of caryophyllene. Correspondingly, DH1-CI4A-CS2 consumed 43% of total algal carbohydrates and 14% of total algal proteins in the media, indicating incomplete consumption of the substrates in algal hydrolysate (Fig. 4(B)). Compositional analysis of the *Nannochloropsis* sp. biomass indicated that the biomass contained 20% carbohydrates and 58% proteins (data not shown). Based on this data, the highest terpene yield achieved corresponded to ~48 mg total terpene/g algae with 43 mg sesquiterpene/g algae, 5 mg monoterpene/g algae, and 10 mg caryophyllene/g algae.

4. Discussion

To optimize the metabolic flux driving force for increased total terpene production, three expression strategies were applied and the constructs were co-expressed into *E. coli* strains. Among the four engineered strains, DH1-CS2 yielded the highest titer of terpenes while DH1-CS1 produced the lowest concentration of terpenes. The lower terpene titer of DH1-CS1 is probably due to metabolic flux imbalance, likely resulting from the non-optimized expression levels of GPPS and TPS as well as the higher metabolic burden induced by the three plasmids. In construct 1, both GPPS and caryophyllene synthase were expressed in two plasmids containing the same origin of replication ColE1. The incompatibility of these two plasmids in the strain may cause the low expression levels of either GPPS_{Ag} or caryophyllene synthase, which can further contribute to a low flux (Selzer et al., 1983; Tolia and Joshua-Tor, 2006). In constructs 2, 3, and 4, the pathway enzymes were expressed in two plasmids with different but compatible origin of replications - pJBEI3122 with p15A and of pBbE1a with ColE1 origin. In addition, in construct 2, the GPPS was expressed in the vector pJBEI3122 (p15A) which can yield 10–12 copies of the plasmid in the strain, while the plasmid pBbE1a (ColE1) containing caryophyllene synthase can produce 15–20 copies of the plasmid (Tolia and Joshua-Tor, 2006). pJBEI3122 expressing all intermediate pathway enzymes with relatively lower copy numbers could reduce the potential toxicity of intermediate metabolites on the cell (Jones et al., 2000). Furthermore, the higher-copy-number plasmid pBbE1a expressing caryophyllene synthase could possibly generate a large metabolic flux driving force to the final product formation, which in turn reduced the intermediate accumulation. Most likely, the combination of these two factors resulted in the highest production titer of terpene from the DH1-CS2 strain. In addition, DH1-CS2 accumulated the lowest level of mevalonate (Fig. 1(C)) *in vivo*, indirectly indicating the highest metabolic flux driving force to final products formation. The construct 3 and 4 have similar expression strategies for the pathway enzymes. Consequently, strains DH1-CS3 and 4 accumulated similar levels of mevalonate *in vivo* and produced similar titers of terpene in the culture.

Optimization of fermentation conditions is imperative for improving the product yield of the engineered terpene producing strains. Generally, the chemical reaction rate increases as the reaction temperature rises. However, previous studies showed that lower induction temperatures reduce the formation of inclusion bodies in metabolically engineered strains and can enhance the activities of pathway enzymes and improve the product yield (de Groot and Ventura, 2006; Vera et al., 2007). Therefore, optimization of inducer concentration and induction temperature for pathway enzyme express was identified as important means to achieve the maximal terpene production yield. In this study,

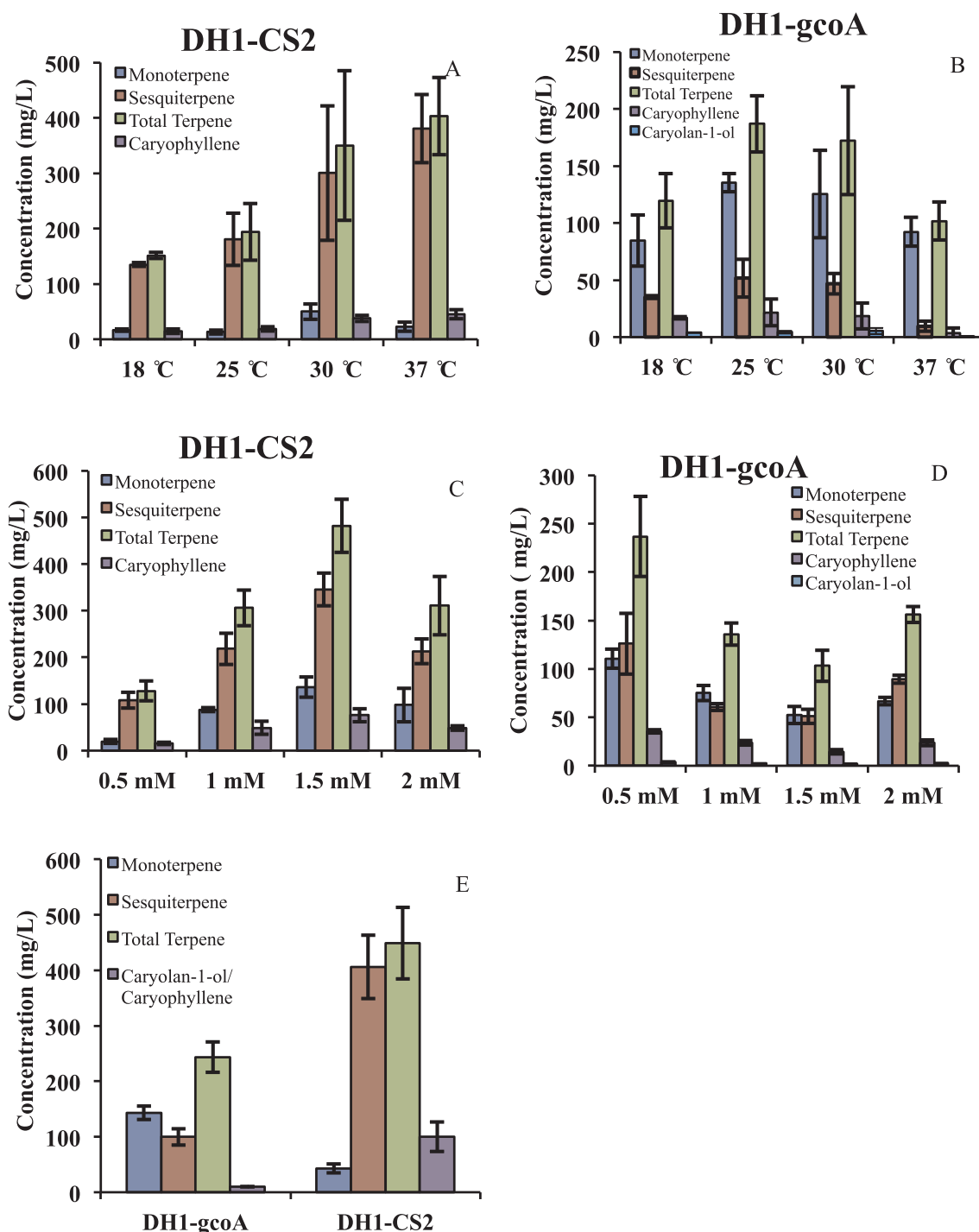


Fig. 3. Effects of inducer concentration and temperature on caryophyllene, caryolan-1-ol and terpene production. A: terpene titers of caryophyllene biosynthesis construct 2 at different temperatures, B: terpene titers of caryolan-1-ol biosynthesis construct 5 at different temperatures, C: terpene titers of caryophyllene biosynthesis construct 2 induced by different concentrations of IPTG, D: terpene titers of caryolan-1-ol biosynthesis construct 5 induced by different concentrations of IPTG, E: terpene titers of construct 2 and 5 under optimal fermentation conditions.

DH1-CS2 produced the highest amount of total terpene at 37 °C while DH1-gcoA produced the highest amounts of terpenes at 25 °C, indicating that the properties of the expressed terpene synthase was dependent on the production temperature. In most cases, exogenously introduced pathways increase the metabolic burden on the cells (Glick, 1995; Neubauer et al., 2003; Sharma et al., 2011). The levels of inducer (in this case, IPTG) can modulate the transcription of the heterologous pathway genes and, therefore, the optimal inducer concentration can minimize the extent of metabolic burden on the engineered strains and

therefore maximize the product yield (Donovan et al., 1996; Huang et al., 2014; Liu et al., 2011; Zhang et al., 2009). The different optimal inducer concentrations for different TPSs also suggested distinct properties among the expressed terpene synthases.

Ohnishi and collaborators (Nakano et al., 2011) previously reported that gcoA catalyzes the biosynthesis of caryolan-1-ol via formation of β -caryophyllene but that the major portion of β -caryophyllene does not interact with the binding site of gcoA during the catalysis process. This indicates that the gcoA has faster kinetics for the conversion of FPP into

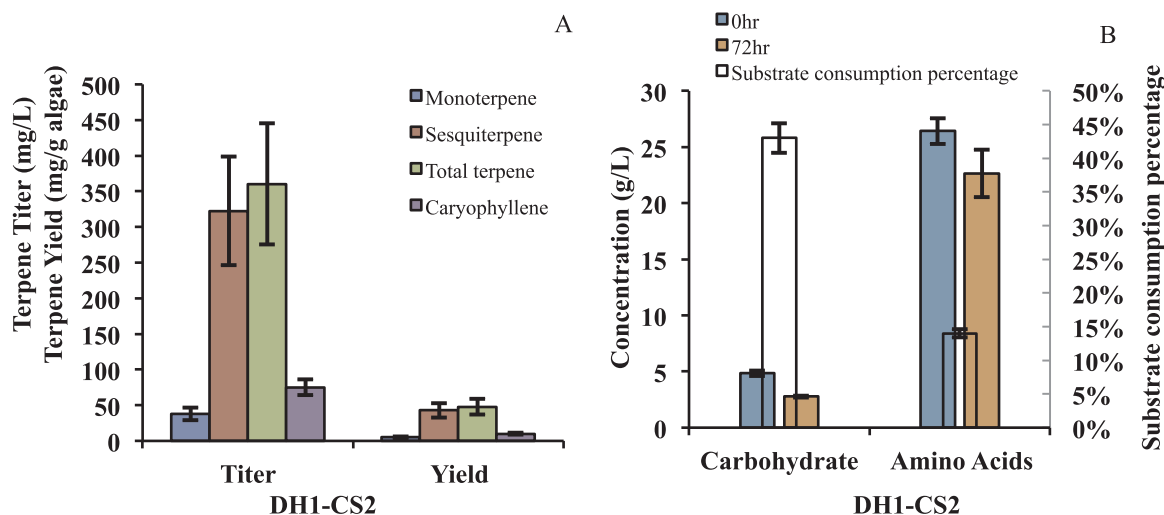


Fig. 4. Terpene and caryophyllene production from the hydrolysate of *Nannochloropsis* sp. A: terpene and caryophyllene titers and yields of the engineered strain DH1-CI4A-CS, B: algal carbohydrate and protein consumption.

caryophyllene than the hydroxylation of caryophyllene into caryophyllene alcohol, which results in the major portion of β -caryophyllene being released into the medium before being hydroxylated into caryophyllene alcohol. This is likely the underlying reason for the higher terpene titer from DH1-gcoA strain expressing caryophyllene compared with the one expressing caryolan-1-ol. In DH1-CS-gcoA, it was expected that the caryophyllene synthesized by CI4A-CS could be hydroxylated by caryolan-1-ol synthase (gcoA) to form caryophyllene alcohol, resulting in the increase of the caryophyllene alcohol titer by supplying gcoA with more caryophyllene as substrates. However, DH1-CS-gcoA produced lower titers of caryolan-1ol and caryophyllene than DH1-gcoA, which is probably due to the competition of CI4A-CS against caryolan-1-ol synthase in construct 6 for FPP as substrate. The competition most likely resulted in the formation of other sesquiterpene compounds by CI4A-CS such as humulen, which was indicated by the higher heterogeneity of the terpene mixture profile, and therefore lower amount of caryophyllene and caryophyllene alcohol could be converted from FPP.

In this study the terpene titer from algal hydrolysate was reduced compared with the EZ-rich medium. This is likely due to the fact that algal hydrolysate without any supplements is nutrient limited compared with rich EZ-rich medium or other commercial media formulation. We observed that the culture required over double the time to reach the induction cell density than in EZ-rich medium. Compared with terpene production from sugars, the current yield of total terpene based on algae biomass from DH1-CI4A-CS2 is approximately one order of magnitude lower. However, compared with the current state-of-the-art technology of essential oil production where the extraction yields of essential oils ranged from 0.1% to 1% of plant tissues (corresponding to 1–10 mg essential oil/ g plant tissue) (Gong et al., 2014; Moncada et al., 2016), the engineered strain in this study increased the terpene yield by 4–40 folds (algae biomass based), which makes it a promising alternative pathway for terpene production.

In summary, caryophyllene, caryolan-1-ol, and their stereoisomers were produced by the engineered *E. coli* strains as potential candidates for aviation fuel. The total terpene and caryophyllene titers were increased after improving the metabolic flux through four different biochemical pathway regulation strategies as well as the optimization of fermentation parameters. Moreover, this is the first demonstration of caryophyllene-enriched terpene mixture production from an algae biomass hydrolysate. This study provides an alternative pathway for caryophyllene caryolan-1-ol enriched terpene production as potential aviation fuel compounds from renewable sources.

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Competing interests

The authors declare no competing interests.

Contributions

W.W.H conceived and designed the study, performed the experiments, collected and analyzed the data, wrote and revised the manuscript. R.W.D conceived, designed, supervised the study, and revised the manuscript. All authors read and approved the manuscript.

Ethical approval

This article does not contain any studies with animals performed by any of the authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.meteno.2018.01.001>.

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