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Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2

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ABSTRACT

Isoprenoids, major secondary metabolites in many organisms, are utilized in various applications. We constructed a model photosynthetic production system for limonene, a volatile isoprenoid, using a unicellular cyanobacterium that expresses the plant limonene synthase. This system produces limonene photosynthetically at a nearly constant rate and that can be efficiently recovered using a gas-stripping method. This production does not affect the growth of the cyanobacteria and is markedly enhanced by overexpression of three enzymes in the intrinsic pathway to provide the precursor of limonene, geranyl pyrophosphate. The photosynthetic production of limonene in our system is more or less sustained from the linear to stationary phase of cyanobacterial growth for up to 1 month.

1. Introduction

Isoprenoids that are composed of isoprene units are major secondary metabolites in many organisms. They are major components of essential oils (Fornari et al., 2012), steroids (Ghayee and Auchus, 2007) and carotenoids (Takaichi, 2011) and are also useful for various commercial applications. These include the production of perfumes, medicines, materials, and biofuels (Bohlmann and Keeling, 2008). These products can be manufactured in part from petroleum or other materials via chemical synthesis, and many of them are extracted and purified from plant materials such as barks, leaves, roots and essential oils of certain plants. However, isoprenoid production in plants is limited to their slow growth. To overcome this, isoprenoid production has been studied using genetically engineered microorganisms such as *Escherichia coli* and yeast. Isoprenoids that have commercial use or other benefits can be produced using these organisms (Curran and Alper, 2012). For example, carvone (Carter et al., 2003), artemisinin (Martin et al., 2003), bisabolane (Peralta-Yahya et al., 2011), astaxanthin (Miura et al., 1998) and sterols (Wriessnegger and Pichler, 2013). Production levels in these heterotrophic organisms reached several grams per liter. However, although metabolic engineering of these organisms achieved such high productivity in synthesizing exogenous isoprenoids, these microbes require an organic carbon source and emit carbon dioxide during their growth and production. As carbon dioxide is one of the major greenhouse gases, it has become imperative to develop methods to photosynthetically produce isoprenoids directly from carbon dioxide using phototrophic microbes.

For the photosynthetic production of isoprenoids, we took advantage of the model cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis*), which is highly tractable to genetic engineering. It is becoming more widely known that metabolic engineering enables cyanobacteria to photosynthetically produce various important biofuels such as isopropyl alcohol (Kusakabe et al., 2013), isobutyl aldehyde (Atsumi et al., 2009), fatty acids (Liu et al., 2011), alkanes (Wang et al., 2013), ethylene (Takahama et al., 2003) and hydrogen (Masukawa et al., 2012). However, there have been few studies of the potential for photosynthetic production of functional isoprenoids as commercially useful compounds and also as

Abbreviations: GPP, geranyl pyrophosphate; MEP, methyl erythritol-4-phosphate; LMS, limonene synthase; GC–MS, gas chromatography–mass spectrometry.

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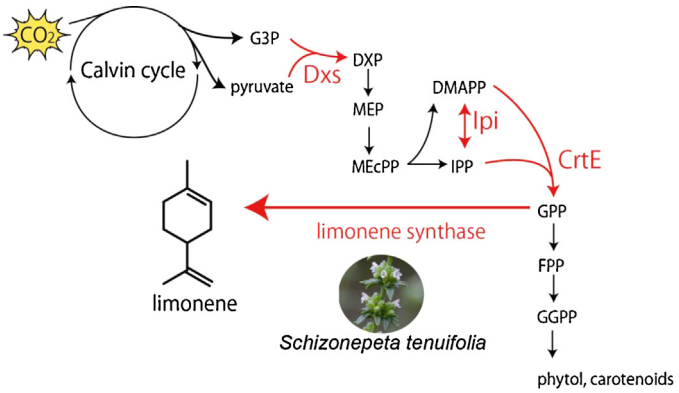

Fig. 1. Biosynthesis of limonene from GPP by limonene synthase derived from *Schizonepeta tenuifolia*. GPP is a metabolic intermediate of the MEP pathway, which is supplied from CO_2 fixation via the Calvin cycle. Three engineered enzymes of the MEP pathway are highlighted in red. G3P, glyceraldehyde-3-phosphate; DXP, 1-deoxyxylulose-5-phosphate; MEP, 2-methyl erythritol-4-phosphate; MEcPP, 2-methyl erythritol-2,4-cyclodiphosphate; DMAPP, dimethylallyl pyrophosphate; IPP, isopentenyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

potential biofuels. Cyanobacteria naturally accumulate functional isoprenoids such as carotenoids, the phytol of chlorophyll and quinones as essential cofactors for photosynthesis (Nowicka and Kruk, 2010). Some cyanobacteria also produce odorous monoterpenes (Li et al., 2012), sesquiterpenes (Agger et al., 2008) and hopanoids (Saenz et al., 2012). Thus, the cyanobacteria may provide a potential platform for the large-scale photosynthetic production of various isoprenoids. There is a pioneer work to produce a hemiterpene isoprene (C_5H_8) in *Synechocystis* by plant enzyme isoprene synthase (Bentley and Melis, 2012; Lindberg et al., 2010).

Here, we chose to test the production of the popular volatile monoterpene limonene ($C_{10}H_{16}$) in *Synechocystis* as this compound can be used in various applications. Limonene is a water-insoluble liquid under atmospheric pressure and may be released from cells without specific treatments. There are two naturally occurring enantiomers, (*R*)-limonene and (*S*)-limonene, which are produced by stereo specific enzymes in plants. (*R*)-limonene has a lemon-like fragrance, whereas (*S*)-limonene has a petroleum-like odor. These limonene enantiomers have been used not only in perfumes but also as a solvent for polystyrene. Limonene can be produced by a single enzyme, limonene synthase (LMS) (EC 4.2.3.20) from geranyl pyrophosphate (GPP), which is a natural intermediate for the biosynthesis of carotenoids and phytol (Fig. 1).

In our current study, we constructed recombinant *Synechocystis* strains that would heterologously produce this monoterpene using a plant-derived LMS. These recombinant strains stably express the enzyme and successfully produce limonene. We evaluated the production and accumulation of limonene in our cyanobacterial system using a gas-stripping method. This is the first study to describe the construction of cyanobacteria that heterologously produce a plant monoterpene and that showed increased production of this compound through genetic engineering.

2. Materials and methods

2.1. Strains and plasmid construction

A glucose tolerant strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 was used as a platform in this study. The LMS gene from the medicinal herb *Schizonepeta tenuifolia* (GenBank accession number AF282875) (Maruyama et al., 2001) was used for expression in *Synechocystis*. The initial experiments were done using the native DNA sequence of this plant LMS gene after truncation of the transit region for targeting to chloroplasts. However, our subsequent experiments were done using a synthetic DNA (Table S1 and Fig. S1) in which the codons had been optimized for *Synechocystis* (GenScript, Piscataway, USA). The optimized *LMS* gene was cloned into an expression vector (pT31CTH-TePixJ) that incorporated a 6xHis-tag epitope, a trc promoter and a chloramphenicol resistant cassette (Ishizuka et al., 2006). The *LMS* insert in the resulting plasmid (pT31CTH-LMS) was confirmed by nucleotide sequencing. The resultant plasmid DNA was introduced by double homologous recombination into the *Synechocystis* chromosome, yielding transformants in which part of a silent *slr2031* gene was replaced (Satoh et al., 2001).

The genes encoding three enzymes of the methyl erythritol-4-phosphate pathway (MEP pathway) in *Synechocystis*, *dxs* (sll1945 for deoxyxylulose-1-phosphate synthase), *crtE* (slr0739 for GPP synthase) and *ipi* (sll1556 for isopentenyl pyrophosphate isomerase) were cloned into pT7blue (Merck, Darmstadt, Germany) and then combined as a single operon (Fig. 2A, Fig. S2) in another expression vector, pPCRScript-slr0846-1/9, by replacing the slr0846 gene insert (Midorikawa et al., 2012). The resulting plasmid, pS46KT-MEP, was used to integrate these three genes with the kanamycin-resistant cassette by double homologous recombination into the chromosome at an intergenic neutral site between sll0821 and slr0846. In the recombinant chromosome, an extra copy of *dxs-crtE-ipi* is expressed from the trc promoter at this intergenic neutral region in addition to the intrinsic copies. Since cyanobacterial cells possess multiple copies of identical chromosomes (Watanabe et al., 2012), complete segregation of the integrated genes was confirmed by PCR.

2.2. Western blotting

Cells were collected by centrifugation. Protein extraction buffer (100 mM NaCl, 10% (w/v) glycerol, 20 mM HEPES–NaOH (pH 7.5)) including 0.5 μM phenylmethylsulfonylfluoride and zirconia beads were then added to the cell pellet, followed by vortexing twice for 30 s with a 1 min interval. The resulting homogenate was centrifuged and the supernatant was subjected to SDS-PAGE and Western blotting as described previously (Yoshihara et al., 2004). We used peroxidase conjugated HisProbe (Pierce, Rockford, USA) to detect the LMS protein.

2.3. Limonene extraction from cells

Limonene was extracted from cells using chloroform and the organic layer was collected and subjected to gas chromatography–mass spectrometry (GC–MS) analysis.

2.4. GC–MS analysis

GC–MS was performed using QP2010 Plus GC-MS (Shimadzu, Kyoto, Japan) equipped with a ZB-AAA column (Phenomenex, Torrance, USA). The analytical conditions were as follows: He (1.55 ml/min) as a carrier gas, ionization voltage 70 kV, split ratio of 15:1, injector temperature of 150 °C, and an oven program of 50 °C for 1 min increasing at 20 °C min^{-1} to 150 °C in 5 min and a held at 150 °C for 2 min. Analyses were carried out in the selected ion monitoring mode (m/z = 68, 93, 121, 136). The limonene peak was identified as the retention time in the selected ion chromatogram. In the quantitative analysis, we used α-pinene as an internal standard.

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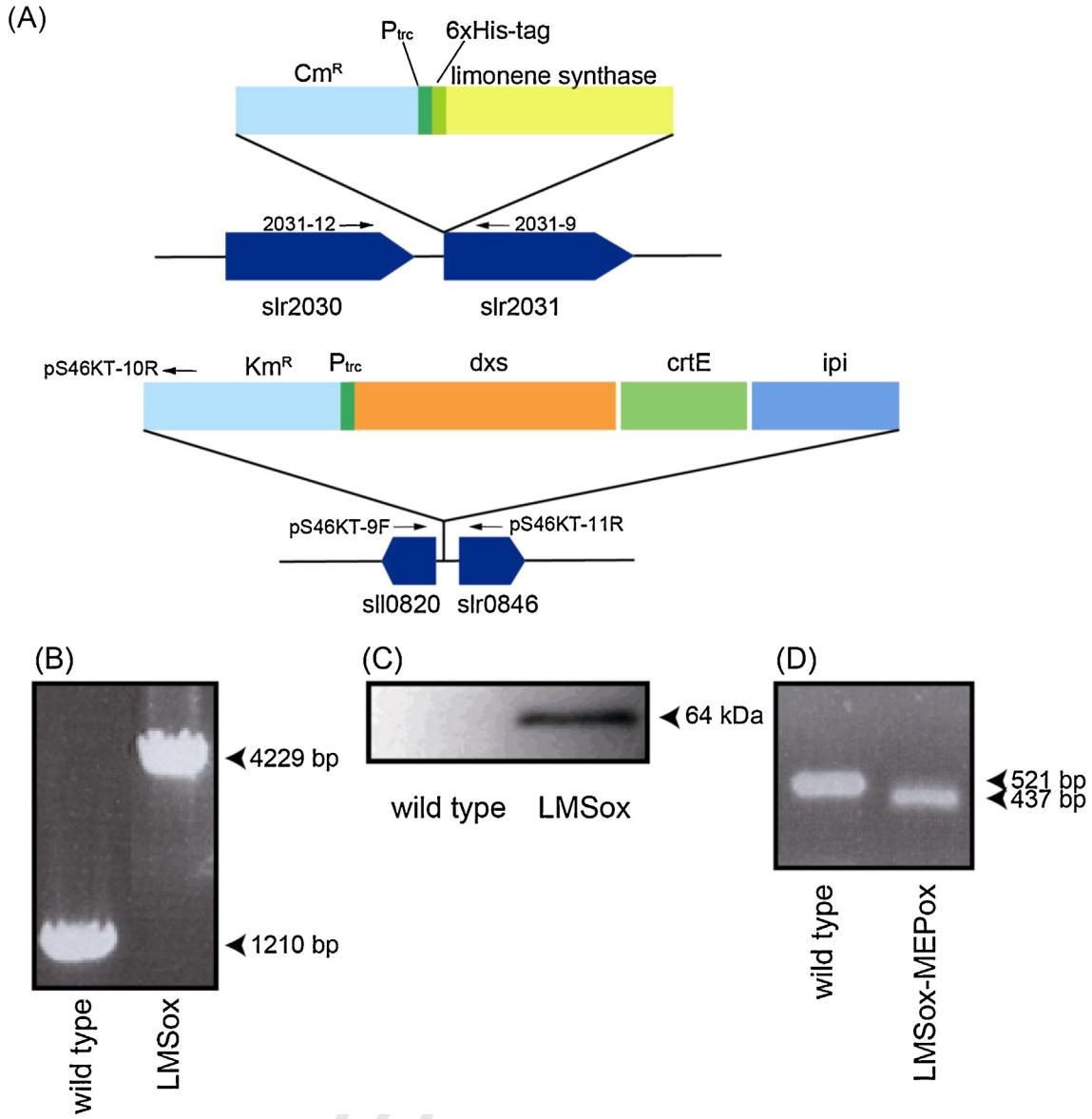

Fig. 2. Integration and expression of engineered genes in the *Synechocystis* genome. (A) Schematic representation of gene integration into the cyanobacterial chromosome by homologous recombination. P_{trc}, trc promoter; Cm^R, chloramphenicol resistance cassette; Km^R, kanamycin resistance cassette. Primers for PCR analysis are highlighted by arrows. (B) PCR analysis of the integration of the limonene synthase using the primers 2031-9 and 2031-12. (C) Western blotting of His-tagged limonene synthase. (D) PCR analysis of the integration of the *dxs-crtE-ipi* genes for the MEP pathway using the primers pS46KT-9F, pS56KT-10R and pS46KT-11R.

2.5. Culture conditions and gas-stripping method

Cyanobacterial cells were grown under normal conditions at 30 °C in BG11 medium supplemented with 20 mM HEPES-KOH (pH 7.8) (Rippka, 1988) with bubbling of 1% (v/v) CO_2 and continuous illumination with white fluorescent lamps at 50 μmol photons s^{-1} m^{-2}. Recombinant cells were maintained in the presence of antibiotics (20 μg/ml chloramphenicol and/or 20 μg/ml kanamycin) but cultivated in the absence of antibiotics for limonene production. To start the experiments, growing cells were collected by centrifugation and resuspended in a fresh BG11 medium at a density of 2×10^8 cells/ml. The cultures were bubbled with 1% (v/v) CO_2 under continuous illumination with white fluorescent lamps at 100 μmol photons s^{-1} m^{-2}. We used a polytetrafluoroethylene tube to connect the culture flask and the cold trap. Limonene was trapped using a Dimroth condenser at −15 °C and recovered into the octane phase (cold trap).

3. Results

3.1. Construction of LMSox cyanobacterial strain

We introduced the plant *LMS* gene into a silent locus on the *Synechocystis* chromosome (Fig. 2A) and confirmed complete segregation by PCR analysis (Fig. 2B). We used the segregated strain as the LMSox strain. The *LMS* gene is expressed from a strong trc promoter and expression of the LMS protein was confirmed by Western blotting to occur in the LMSox strain (Fig. 2C).

3.2. Limonene detection

First, we detected limonene accumulation in LMSox cells. The cells were collected by centrifugation and limonene was extracted using chloroform. Fig. 3 shows GC–MS chromatograms of three mass fragments, which could be derived from limonene (m/z = 68,

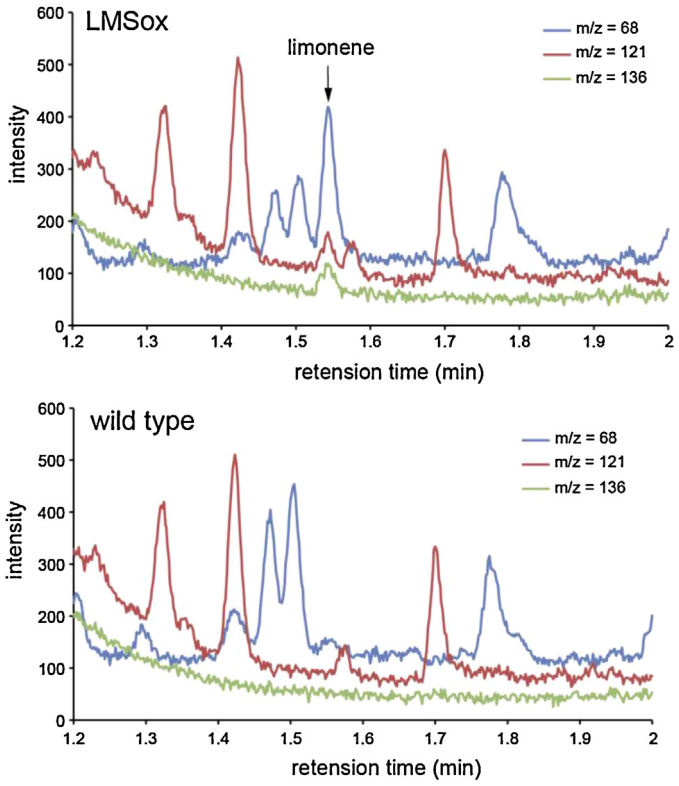

Fig. 3. GC–MS analysis of extracts from LMSox and wild type cyanobacterial cells. Each line indicates a selected ion chromatogram.

121, 136). The limonene peak at 1.58 min was unambiguously detected in each chromatogram in LMSox but not in wild type cells. In contrast, the other peaks were not commonly detected, implying an origin other than limonene.

3.3. Gas-stripping method

For efficient photosynthesis and carbon assimilation of *Synechocystis* cells, CO_2 must be supplied continuously. However, the volatile limonene product must be collected from the exhaust. To achieve both, we chose the gas-stripping system (Fig. 4A) in which the bubbling snatches limonene from the medium and releases it into the octane phase through the cold trap. The octane phase was analyzed by GC–MS. Limonene was detected as a single peak at 1.58 min in the GC chromatogram of LMSox cells, but was not detected in wild type cells (Fig. 4B). The mass spectrum of this peak gave fragments of 136, 121, 93 and 68, which matched the fragments of authentic limonene (Fig. 4C). The complete recovery of limonene in this trap was confirmed by its absence in the second trap. The recovery of limonene was 99.4% in the octane phase, 0.6% in the cell and 0% in the culture medium, indicative of exclusive recovery in the trap (Fig. 4D).

3.4. Limonene production

The photosynthetic growth of LMSox cells was found to be almost identical to wild type under various growth conditions. As shown in Fig. 5, the growth of both cell types measured from inoculation at a low cell density showed no difference in the log phase. It is important to note that the limonene production and/or

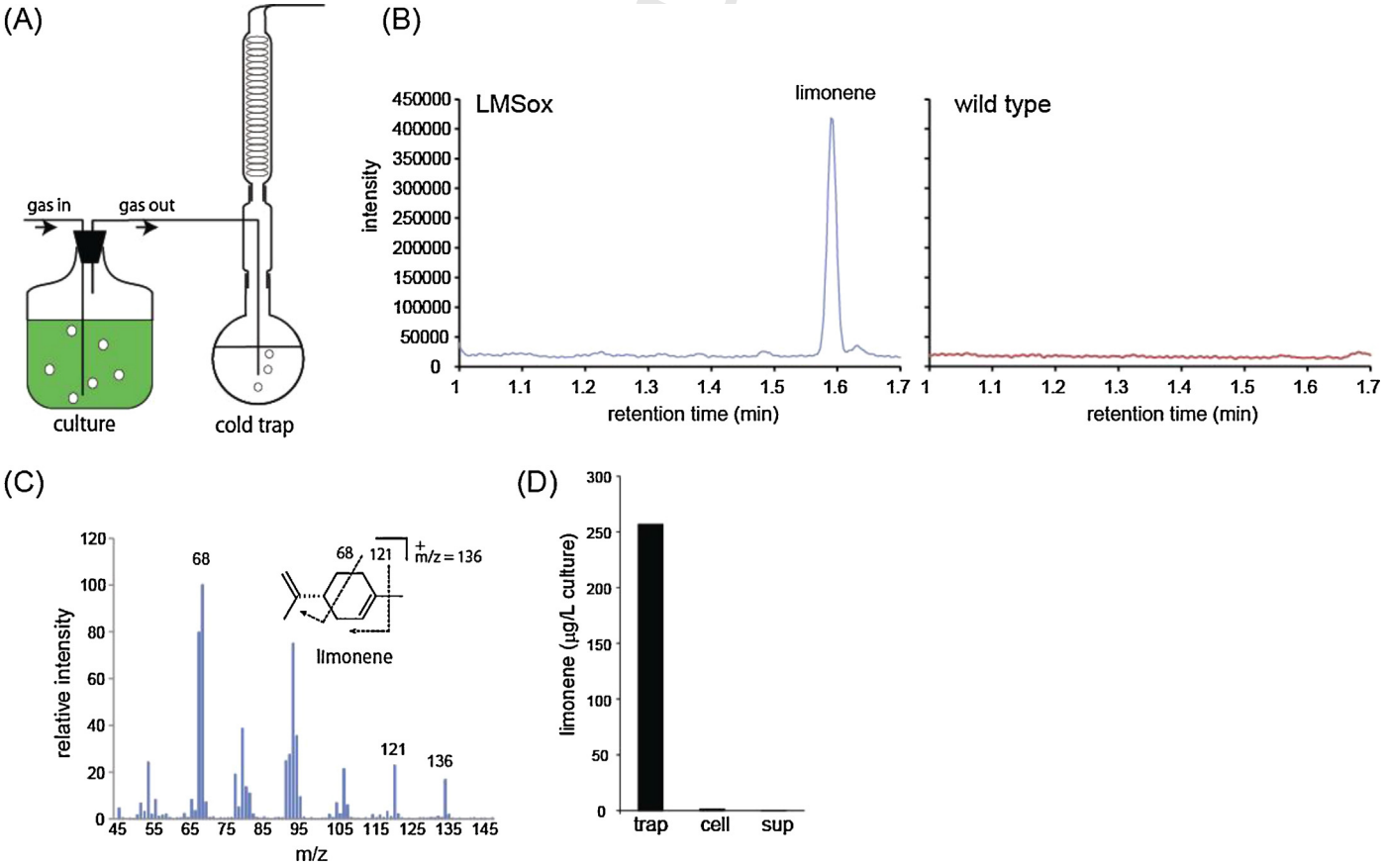

Fig. 4. Extraction of limonene produced from cyanobacterial cultures using the gas-stripping method. (A) Schema for the gas-stripping system. (B) GC–MS total ion chromatogram of the octane phase in the cold trap. (C) Mass spectrum of the limonene peak in panel B. (D) Distribution of the accumulated limonene in the octane phase of the trap (trap), cells collected from the culture medium (cell) and supernatant of the culture medium after centrifugation (sup).

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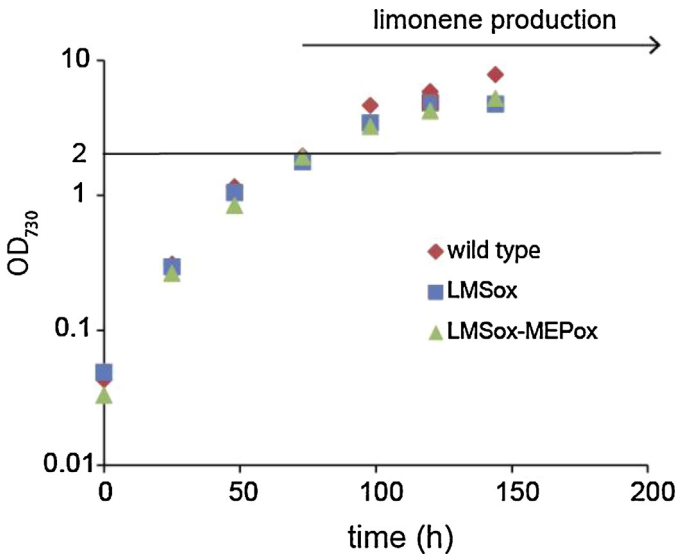

Fig. 5. Growth of LMSox and LMSox-MEPox cyanobacterial cells under conditions of bubbling with light at 50 μmol photons m^{-2} s^{-1}. The growth phase for the limonene production is denoted with an arrow.

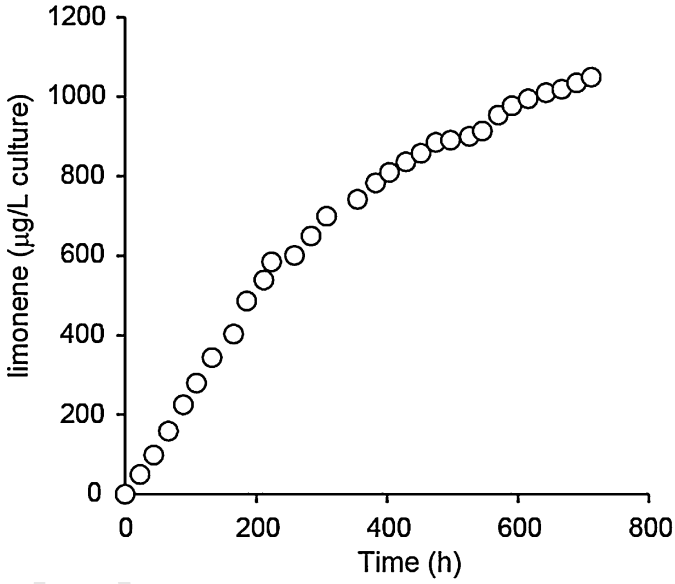

Fig. 7. Augmented production of limonene in LMSox-MEPox cells.

accumulation did not repress the growth of *Synechocystis*. Time course analysis of the limonene accumulation in the trap is shown in Fig. 6. The accumulation of limonene increased continuously for 168 h, at a nearly constant rate of 41 μg L culture^{-1} day^{-1}.

3.5. Overexpression of the MEP pathway

Synechocystis possesses an active MEP pathway to biosynthesize its own isoprenoids, such as carotenoids and phytol. We introduced an additional copy of three genes (*dxs*, *crtE* and *ipi*) under the control of a strong trc promoter at a neutral site of the *Synechocystis* chromosome to increase the supply of GPP, which is the direct substrate of LMS (Fig. 1). Dxs and CrtE catalyze reactions with a large free energy difference and Ipi enhances the interconversion between isopentenyl pyrophosphate and dimethylallyl pyrophosphate, which combine to produce GPP (Ajikumar et al., 2010; Kim and Keasling, 2001). We generated a double recombinant cyanobacterial strain, LMSox-MEPox. We confirmed its complete segregation by PCR (Fig. 2D). In this LMSox-MEPox strain, the rate of limonene production (56 μg L culture^{-1} day^{-1}) was improved by 1.4-fold compared with the parent LMSox strain (Fig. 6). Again, this improvement was achieved without growth inhibition (Fig. 5).

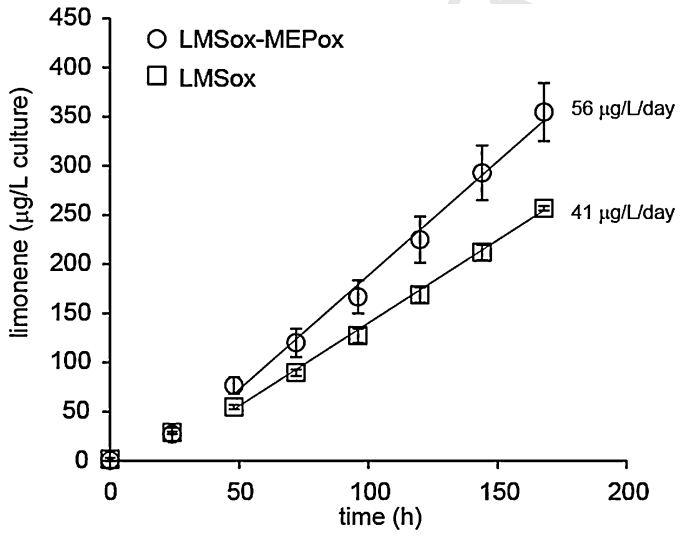

Fig. 6. Limonene production in LMSox and LMSox-MEPox cyanobacterial cells. The standard error for three independent experiments is shown.

3.6. Long-term production

We investigated the sustainability of the limonene production in our system without changes of the culture medium for up to 1 month (Fig. 7). The LMSox-MEPox strain produced limonene linearly for 300 h after which this productivity gradually attenuated and was measured at 19 μg L culture^{-1} day^{-1} at 712 h. The overall production achieved was 1 mg/L. Thus, limonene can be produced sustainably in cyanobacteria during the stationary phase of growth.

4. Discussion

We successfully constructed a system to produce limonene at a rate of 41 μg L culture^{-1} day^{-1} (LMSox) and 56 μg L culture^{-1} day^{-1} (LMSox-MEPox) using genetically engineered cyanobacterial cells of the *Synechocystis* strain that exogenously express a plant limonene synthase and three intrinsic enzymes to promote supply of the substrate for this enzyme. Although limonene is growth inhibitory for microorganisms (Aggarwal et al., 2002; Chambon et al., 1990), we achieved its sustainable production in cyanobacteria without any defect in growth, pigmentation, or other properties, likely because the produced limonene was efficiently removed from the culture system by gas-stripping.

We employed the gas-stripping system (Atsumi et al., 2009) to collect volatile limonene and to sustain its production in cyanobacteria without medium exchange. Our system supported the sustained production of this compound for up to 300 h. Even at 700 h, the *Synechocystis* cells produced limonene at nearly one-third of the initial rate. In this system, the produced limonene does not stay in the cell nor in the culture medium and is exclusively recovered in the trap due to air bubbling to supply CO_2 (Fig. 4D). This likely underlies why a sustained production could be achieved for weeks. Further, our method would also help to avoid possible product-induced feedback inhibition, which is often observed in many metabolic pathways.

Bentley and Melis (2012) have reported the photosynthetic production of a hemiterpene (isoprene) in a recombinant *Synechocystis*

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system. In that report, the culture system was semi-closed, and needed to be exchanged with fresh medium every 48 h. In our current report, we demonstrated the sustained production of limonene using *Synechocystis* cells at high cell density (late log to stationary phase). It is important to separate the biomass production from the cell growth when we use microalgae, because growth is very costly in terms of nutrients. This will be an important consideration in the future when constructing an efficient bioreactor for the photosynthetic production of limonene and other related isoprenoids.

Regulation of the intrinsic MEP pathway or bottlenecks in this pathway has not been studied in cyanobacteria to date, although the genes in this pathway have been identified (Cunningham et al., 2000; Harker and Bramley, 1999; Yin and Proteau, 2003). It is generally known that various protective carotenoids accumulate whilst the chlorophyll content decreases under high light conditions (Kilian et al., 2007). In many cyanobacteria, myxoxanthophylls and zeaxanthin often accumulate under high light (Montero et al., 2012). In terms of gene regulation, only two genes (*crtB* for phytoene synthase and *crtP* for phytoene desaturase) are reported to be induced by high light in cyanobacteria (Fernandez-Gonzalez et al., 1998). Carotenoids and phytol of chlorophylls are derived separately from a common precursor, geranylgeranyl pyrophosphate, which is the end product of the MEP pathway. In *E. coli*, several enzyme steps have been implicated as rate-limiting in the MEP pathway in gene engineering trials, which leads to the overproduction of carotenoids and other isoprenoids (Ajikumar et al., 2010; Kim and Keasling, 2001). We chose these putative rate-limiting enzymes (Dxs, CrtE and Ipi) to enhance the MEP pathway in *Synechocystis* and, indeed, obtained marked improvement of the limonene production (1.4-fold) in our cyanobacterial system. However, we did not see any effects on the accumulation of chlorophylls or carotenoids, likely because their biosynthesis is regulated after geranylgeranyl pyrophosphate as mentioned above. Further fine-tuning of our operon design and screening of other genes are now in progress.

Native isoprenoids in cyanobacteria comprise mostly carotenoids and phytol of chlorophyll *a*, in addition to minor cofactors (plastoquinone and phylloquinone). We estimated the accumulation of these compounds during the early phase of the limonene production as follows: carotenoids ca. 1000 $\mu g\,L\,culture^{-1}\,day^{-1}$ and phytol ca. 600 $\mu g\,L\,culture^{-1}\,day^{-1}$. This indicated that the flow to limonene is approximately 2.5% (LMSox) or 3.4% (LMSox-MEPox) of the total isoprenoid biosynthesis. During this period, the cyanobacterial cells grow rather linearly instead of exponentially with an approximate rate of 94 mg dry cell weight $L\,culture^{-1}\,day^{-1}$. Assuming that the carbon content of the dry cell weight is approximately 50%, the carbon flow to limonene would be 0.1% (LMSox-MEPox) of the total fixed carbon. It should be noted however that the content of carotenoids and chlorophyll in our overexpression mutants were comparable to wild type. This may suggest that the LMS activity is limiting the flow to limonene. We are now studying this possibility by further overexpressing LMS in *Synechocystis*.

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