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Diffusion-Based Process for Carbon Dioxide Uptake and Isoprene Emission in Gaseous/Aqueous Two-Phase Photobioreactors by Photosynthetic Microorganisms

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ABSTRACT: Photosynthesis for the generation of fuels and chemicals from cyanobacteria and microalgae offers the promise of a single host organism acting both as photocatalyst and processor, performing sunlight absorption and utilization, as well as CO_2 assimilation and conversion into product. However, there is a need to develop methods for generating, sequestering, and trapping such bio-products in an efficient and cost-effective manner that is suitable for industrial scale-up and exploitation. A sealed gaseous/aqueous two-phase photobioreactor was designed and applied for the photosynthetic generation of volatile isoprene (C_5H_8) hydrocarbons, which operates on the principle of spontaneous diffusion of CO_2 from the gaseous headspace into the microalgal or cyanobacterial-containing aqueous phase, followed by photosynthetic CO_2 assimilation and isoprene production by the transgenic microorganisms. Volatile isoprene hydrocarbons were emitted from the aqueous phase and were sequestered into the gaseous headspace. Periodic replacement (flushing) of the isoprene (C_5H_8) and oxygen (O_2) content of the gaseous headspace with CO_2 allowed for the simultaneous harvesting of the photoproducts and replenishment of the CO_2 supply in the gaseous headspace. Reduction in practice of the gaseous/aqueous two-phase photobioreactor is offered in this work with a fed-batch and a semi-continuous culturing system using *Synechocystis* sp. PCC 6803 heterologously expressing the *Pueraria montana* (kudzu) isoprene synthase (*IspS*) gene. Constitutive isoprene production was observed over 192 h of experimentation, coupled with cyanobacterial biomass accumulation. The diffusion-based process in gaseous/aqueous two-phase photobioreactors has the potential to be applied to other high-value photosynthetically derived volatile molecules, emanating from a variety of photosynthetic microorganisms.

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Introduction

Isoprene (C_5H_8) is the monomeric five-carbon building block of the largest and most diverse group of naturally occurring organic compounds referred to as terpenoids. It is biologically important as the building block of more than 25,000 different naturally occurring compounds. It has significant commercial potential, as it is feedstock in the synthetic chemistry industry, where it is used to manufacture products ranging from rubber to adhesives and perfumes. There is also potential for isoprene to be developed as a renewable drop-in biofuel, where oligomerization of isoprene units may generate second-order fuel molecules, suitable for use as supplements of gasoline, jet fuel, and diesel. Pure hydrocarbons also store greater relative energy than alcohols (Berg et al., 2002; Schakel et al., 1997). Currently, the industrial supply of isoprene is limited to petrochemical sources. Sustained availability of these resources is being questioned by the increasing global demand for energy and synthetic chemistry feedstock. Accordingly, there is an urgent need to develop methods for creating renewable biofuels and synthetic chemistry feedstock, such as isoprene.

A variety of herbaceous, deciduous, and conifer plants naturally produce isoprene via the process of leaf photosynthesis. As a small hydrophobic and volatile molecule, isoprene easily passes though chloroplast and cellular membranes and is released into the atmosphere though the stomata of leaves (Sharkey et al., 2008; Sharkey and Yeh, 2001). The undesirable effects of isoprene as an atmospheric pollutant are well discussed in the literature (see Sharkey et al., 2008 for a review). The biological significance of isoprene release by plants is unclear; however, it has been suggested that plants utilize isoprene emission as a thermotolerance mechanism (Behnke et al., 2007; Sasaki et al., 2007; Sharkey et al., 2001; Singaas et al., 1997). The

methyl-erythritol-4-phosphate (MEP) biosynthetic pathway, found in bacteria, and plant and algal plastids (Lichtenthaler, 2000) is responsible for the flux of metabolites, leading to the synthesis of isoprene in isoprene-producing plants. Immediate precursors of isoprene are the 5-carbon molecules dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP). In isoprene-producing plants, isoprene synthesis is linked to photosynthetic carbon assimilation. This is evidenced by the light-dependent emission of isoprene (Sanadze, 1969), the incorporation of ^{13}C-labeled CO_2 into isoprene (Mgalobilishvili et al., 1978; Sanadze et al., 1972), and the role of glyceraldelyde-3-phosphate (G3P) and pyruvate (two early products of photosynthesis) in forming the carbon skeleton of the MEP pathway intermediates (Lichtenthaler, 1999; Rohmer et al., 1996). The isoprene synthase IspS enzyme catalyzes the terminal step of isoprene production upon removal of pyrophosphate from DMAPP to produce the hydrophobic isoprene molecule in the chloroplast (Silver and Fall, 1991). Nuclear-encoded isoprene synthase genes *(IspS)* have been cloned and characterized from two plant families; the kudzu vine (*Pueraria montana*) (Sharkey et al., 2005) and poplar (*Populus alba*, *P. tremuloides* and *P. nigra*) (Fortunati et al., 2008; Miller et al., 2001; Sasaki et al., 2005).

Given the volatile nature of isoprene (boiling point = 34.1°C), it is impractical to attempt to harvest this hydrocarbon from plants. However, microorganisms are amenable to cultivation in fully enclosed bioreactors that offer advantages in product containment and sequestration. Although some microorganisms naturally produce isoprene (Kuzma et al., 1995) the quantities are small, and the enzyme/pathway responsible for the formation of isoprene from DMAPP has yet to be elucidated. However, over-expression of a plant *IspS* gene in a photosynthetic or non-photosynthetic microorganism can effectively endow the property of isoprene production from the cell's own metabolism (Lindberg et al., 2010; Miller et al., 2001; Whited et al., 2010).

Work from this lab previously detailed the heterologous expression of kudzu (*Pueraria montana*) *IspS* in the cyanobacterium *Synechocystis* sp. PCC 6803 for photosynthetic isoprene production (Lindberg et al., 2010). There are major advantages for using a photosynthetic microorganism, such as cyanobacteria or microalgae, to synthesize isoprene entirely via photosynthesis from sunlight, carbon dioxide, and water, as it eliminates the need for expensive organic carbon feedstock (e.g., glucose) and multi-step fermentation processes. Isoprene has a major advantage over other targeted bio-products, such as fatty acids, as it is a volatile hydrocarbon that is naturally emitted by the cells, eliminating the need for costly dewatering and product extraction processes, or the establishment of a new culture following harvesting and cell lysis.

We describe here isoprene production from the cyanobacterium *Synechocystis* sp. PCC 6803 in a sealed fed-batch bioreactor that operates spontaneously on the principle of a diffusion-based method for CO_2/isoprene exchange in a gaseous/aqueous two-phase photobioreactor. According to this concept, photosynthesis, isoprene production, and biomass accumulation are supported by the diffusion of concentrated (100%) CO_2 from the gaseous headspace into the aqueous cell-containing medium. The absorbed and assimilated CO_2 from the gaseous headspace is replaced by isoprene (C_5H_8) and O_2, in a process that is driven merely by diffusion, allowing for high rates of cell growth and productivity, while permitting the sequestration of isoprene in the headspace of the bioreactor. Importantly, this method allows for sustained photoautotrophic metabolism and growth despite the sealed bioreactor headspace (required for accumulation of a volatile product), which would otherwise induce anaerobic metabolism. Briefly sparging the reactor with 100% CO_2 once every 24 or 48 h replenishes the CO_2 supply in the gaseous headspace while flushing away the isoprene and O_2 products of photosynthesis. This method was further applied to a semi-continuous culturing system in a fed-batch bioreactor, enabling the continuous accumulation and harvesting of isoprene, proving suitability of this approach in industrial scale-up for the production of renewable photosynthetically generated isoprene hydrocarbons.

Materials and Methods

Strains and Growth Conditions

The glucose-tolerant cyanobacterial strain *Synechocystis* sp. PCC 6803 (Williams, 1988) was used as the recipient strain in this study, and is referred to as the wild type. The codon-optimized isoprene synthase gene from *Pueraria montana* (the kudzu vine *IspS* gene) was employed for the transformation of the wild type (Lindberg et al., 2010). The heterologous transformed cyanobacteria are referred to as *SkIspS* transformants. Wild type and *SkIspS* transformant strains were maintained on solid BG-11 media supplemented with 10 mM TES-NaOH (pH 8.2), 0.3% sodium thiosulfate, and 5 mM glucose. Where appropriate, kanamycin was used at a concentration of 25 μg/mL. Liquid cultures, including those used for aerated photoautotrophic growth experiments, were grown in BG-11 containing 25 mM HEPES–NaOH, pH 7.5. Liquid cultures for inoculum purposes were maintained at 25°C under a slow stream of constant aeration and illumination at 20 μmol photons m^{-2} s^{-1}.

Generation of *Synechocystis* Transformants With Heterologous Expression of the *IspS* Gene

Wild-type *Synechocystis* sp. PCC 6803 was transformed as described by Lindberg et al. (2010), where the *Synechocystis PsbA2* gene was replaced with the *Synechocystis* codon optimized kudzu *IspS* (*SkIspS*) gene via double homologous recombination. The *SkIspS* transformants described in this

work differ from the *SkIspS* transformant by Lindberg et al. (2010) only in terms of the wild-type genetic background. (The *SkIspS* transformants by Lindberg et al. were generated from the glucose-sensitive *Synechocystis* sp. PCC 6803 strain.) Transformations were carried out according to established procedures (Eaton-Rye, 2004; Williams, 1988). Successful transgene incorporation and complete DNA cyanobacterial copy segregation for the *SkIspS* gene was verified by genomic DNA PCR, using primers designed within the upstream and downstream regions of the *PsbA2* gene that were used for homologous recombination: A2us_F, 5′-TGGATATTTGCTGGGGGTTA-3′, and A2ds_R, 5′- GACTCTCTAATGGTAACTGCCC-3′.

Chlorophyll Determination, Photosynthetic Productivity, and Biomass Quantitation

Chlorophyll *a* concentrations in cultures were determined spectrophotometrically in 90% methanol extracts of the cells according to Meeks and Castenholz (1971). Photosynthetic productivity of the cultures was tested polarographically with a Clark-type oxygen electrode (Rank Brothers, Cambridge, England). Cells were harvested at mid-exponential growth phase, and maintained at 25°C in BG11 containing 25 mM HEPES–NaOH, pH 7.5, at a chlorophyll *a* concentration of 10 μg/mL. Oxygen evolution was measured at 25°C in the electrode upon yellow actinic illumination, which was defined by a CS 3–69 long wavelength pass cut-off filter (Corning, Corning, NY). Photosynthetic activity of a 5 mL aliquot of culture was measured at varying actinic light intensities in the presence of 15 mM $NaHCO_3$, pH 7.4, to generate the light saturation curve of photosynthesis. Culture biomass accumulation was measured gravimetrically as dry cell weight, where 5 mL samples of culture were filtered through 0.22 μm Millipore filters and the immobilized cells dried at 90°C for 6 h prior to weighing the dry cell weight.

Diffusion-Based Growth in Fed-Batch or Semi-Continuous Growth Bioreactors

One-liter fed-batch bioreactors were designed by us and custom-made (Adams and Chittenden Scientific Glass, Berkeley, CA) from 1-L Corning Pyrex bottles that were modified to have two-side arm openings (Fig. 1). A long aerator tube was fixed within one side arm to enable delivery of gases (CO_2 or air), which were slowly bubbled though the bottom of the liquid medium. All gases provided to the culture were first passed though a 0.2-μm filter unit (Millex-FG Filter Unit, Millipore) to maintain sterility. The second side arm functioned as a headspace sampling and/or photoproduct removal tube, from which headspace gases (isoprene and O_2) were vacated and replaced with fresh CO_2. Alternatively, the headspace sampling/product removal tube could also be used for liquid culture sampling through a

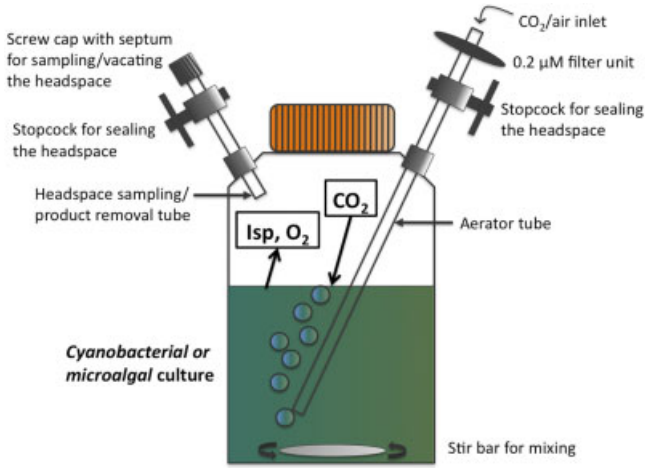

Figure 1. Custom-designed fed-batch bioreactor for diffusion-based CO_2/O_2 gas exchange and volatile hydrocarbons production and trapping. A 100% CO_2 gas stream was slowly fed into the gaseous/aqueous two-phase bioreactor via the aerator tube to fill the reactor headspace. Efficient and spontaneous uptake and assimilation of headspace CO_2 by the cells occurred by diffusion and was concomitantly exchanged for photosynthetically produced O_2 and isoprene during photoautotrophic growth. Accumulation of isoprene over time takes place in the sealed bioreactor headspace. Slow continuous mechanical mixing was employed to keep cells suspended and to promote balanced cell illumination and gaseous CO_2 diffusion in the liquid culture. Gas samples were taken from the headspace though the septum of the sampling/product removal tube for isoprene concentration analysis by gas chromatography (GC). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

septum. Both side arms were fitted with stopcocks so that the headspace of the bioreactor could be sealed.

Bioreactors were seeded with 700 mL culture of *Synechocystis* cells at an $OD_{730\,nm}$ of 0.05 in BG11 medium, containing 25 mM HEPES–NaOH, pH 7.5, and grown under continuous illumination at 75 μmol photons $m^{-2}\,s^{-1}$, and continuous bubbling with air, until an $OD_{730\,nm}$ of approximately 0.5 was reached. Inorganic carbon delivery to the culture was made in the form of 500 mL aliquots of 100% CO_2 gas, slowly aerated through the bottom of the liquid culture and into the bioreactor headspace (Fig. 1). As CO_2 gas is heavier than N_2 and O_2, slow bubbling was necessary and sufficient to purge N_2 and O_2 in the headspace through the sampling/product removal tube, and to replace them with CO_2 in the headspace. Once atmospheric gases were replaced with 100% CO_2 in the headspace, the bioreactor was sealed and the culture was incubated upon illumination at 150 μmol photons $m^{-2}\,s^{-1}$ at 35°C. Periodically, as required by the culture growth and productivity conditions, CO_2 gas bubbling through the culture was repeated to recharge the CO_2 supply in the headspace of the culture and to remove the isoprene and O_2 products of photosynthesis from the reactor.

For continuous growth and productivity experiments, daily CO_2 flushes were administered to the culture as described above and, after every 48 h of CO_2 diffusion-supported growth, cultures were diluted with fresh growth

medium, down to an $OD_{730\,nm}$ of 0.5 (dry cell weight of $\sim 0.2\,g\,L^{-1}$), to ensure cells were always in an actively dividing stage.

Isoprene Production Assays

Gas from the headspace of sealed bioreactors was periodically sampled and analyzed by gas chromatography using a Shimazu 8A GC (Shimazu, Columbia, MD) equipped with a flame ionization detector (FID) and a Porapak N 80/100 column appropriate for detection of short-chain hydrocarbons. Quantitation of isoprene production was performed on the basis of an isoprene vapor calibration curve (Fig. 2) constructed by the GC analysis of a series dilution of a vaporized pure isoprene standard (Acros Organics, Fair Lawn, NJ). Isoprene in the gaseous headspace was further identified by gas chromatography–mass spectrometry (GC–MS) analysis through the comparison of retention time and mass spectrum with the vaporized pure isoprene standard. GC–MS analyses were performed with an Agilent 6890GC/5973 MSD equipped with a DB-XLB column (0.25 mm i.d. × 0.25 μm × 30 m, J &W Scientific). Oven temperature was initially maintained at 40°C for 4 min, followed by a temperature increase of $5^{\circ}C\,min^{-1}$ to 80°C, and a carrier gas (helium) flow rate of 1.2 mL per minute.

Results

Transformation, DNA Copy Homoplasmy, and *IspS* Transgene Expression

Substitution of the *PsbA2* gene with the *Synechocystis* codon-optimized kudzu *IspS* gene construct (*SkIspS*) was implemented by double homologous recombination (Fig. 3A), as previously described (Lindberg et al., 2010). Segregation of the *Synechocystis* DNA copies (homoplasmy) was achieved upon cultivation of the transformants for several generations under antibiotic selective pressure, which promoted deletion of wild type copies of the *Synechocystis* DNA and replacement with the *IspS* transgene-containing copy. DNA copy homoplasmy was tested by genomic DNA PCR analysis with primers designed to the upstream and downstream regions of *PsbA2* gene, which amplified a larger product in the transformant than in the wild type DNA, corresponding to the replacement of the endogenous 1,083 bp *PsbA2* with the larger 1,718 bp *SkIspS* gene fused to a 836 bp NptI kanamycin-resistance cassette (Fig. 3A). Results from this genomic DNA PCR analysis are shown in Fig. 3B, where the untransformed wild type yielded a 1.7 kb product (Fig. 3B, lane 1), whereas four independent *SkIspS* transformant lines yielded a 3.2 kb product (Fig. 3B, lanes 2–5). Absence of wild-type size products from the transformants is evidence of complete segregation of the *Synechocystis* DNA copies, all of which now contain the *SkIspS* transgene.

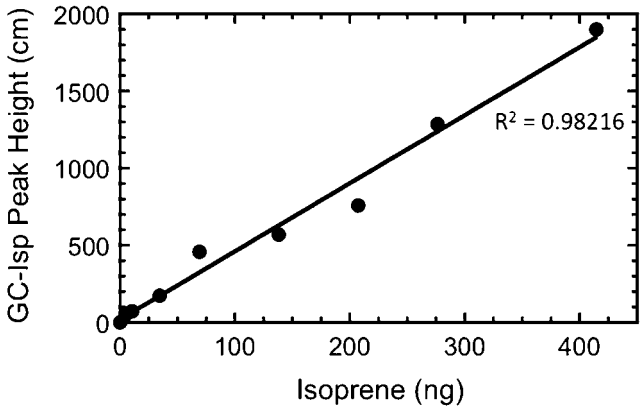

Figure 2. Calibration curve plotting the isoprene peak height as a function of isoprene amount injected in the GC. A serial dilution of vaporized pure isoprene standard of known concentrations was prepared. A 1 mL sample of each standard was injected and analysed by the GC. The calibration curve was based on the amount of isoprene (ng) in the 1 mL sample relative to the isoprene peak height from the chromatogram. At higher isoprene concentrations, the GC sensitivity was attenuated to enable the measurement of the large amplitude peaks, and this attenuation is corrected for in the presentation of the calibration curve.

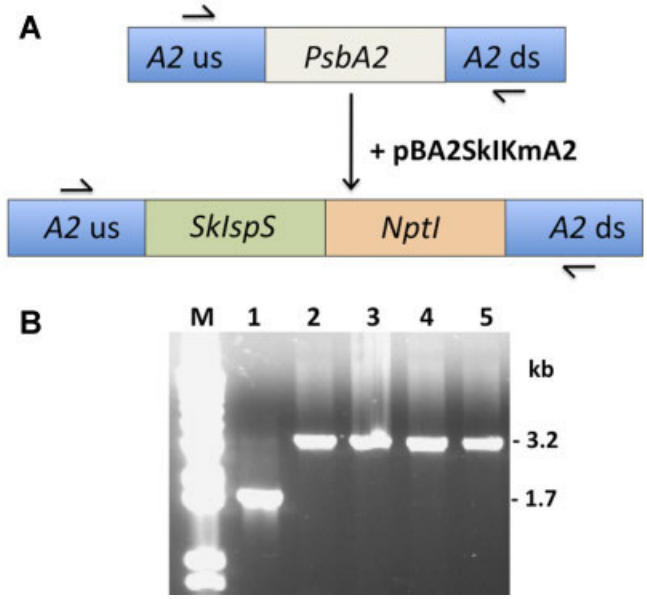

Figure 3. Double homologous recombination and *Synechocystis* DNA copy segregation. **A**: Maps of the *PsbA2* gene locus in wild-type *Synechocystis* and of the *SkIspS-NptI* gene construct for double homologous recombination. Cells were transformed with plasmid pBA2SkIKmA2 (Lindberg et al., 2010) designed to replace the endogenous *PsbA2* with a heterologous *SkIspS* gene and a kanamycin antibiotic selectable *NptI* marker. Genomic PCR primers (arrows) to the upstream and downstream regions of the *PsbA2* gene (*A2*us, *A2*ds) were designed to amplify a 3.188 kb product in the *SkIspS* transformant compared to a 1.717 kb in the wild type. **B**: A PCR product of ~1.7 kb was amplified in the wild type containing the endogenous *PsbA2* (lane 1), whereas larger products of ~3.2 kb were amplified in four different *SkIspS* transformant lines (lanes 2–5). Absence of the 1.7 kb product from the latter show DNA copy segregation in the transformants following the replacement of *PsbA2* with the heterologous *SkIspS* transgene construct. M, 1 kb plus marker. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

Cultivation of wild type and *SkIspS* transformants under the conditions of the gaseous/aqueous two-phase bioreactor described in Figure 1 tested for the accumulation of isoprene hydrocarbons in the culture headspace. Analysis of the accumulated reactor headspace gases in the wild type after 48 h incubation showed no evidence of isoprene hydrocarbons (Fig. 4, left panel). The headspace of the *SkIspS* transformant showed substantial isoprene accumulation, as evidenced by the GC peak with a 3.8 min retention time (Fig. 4, middle panel). Isoprene was the sole short-chain volatile hydrocarbon generated by photosynthesis in the transformant (compare with the isoprene standard shown in Fig. 4, right panel). Isoprene was positively identified by GC–MS analysis as the volatile product accumulating in the gaseous headspace of the *SkIspS* transformants. This was evidenced upon comparison of peaks with ~1.8-min retention time in the GC analysis of headspace gases with an isoprene standard (Fig. 5A and B) and also upon MS analysis of products with 1.8 min retention time showing distinct mass spectral lines [53, 67, and 68] that signify isoprene hydrocarbons (Fig. 5C and D). Isoprene vapor was not detected in the wild type (not shown). These results are evidence that the *SkIspS* transgene and its encoded isoprene synthase enzyme are responsible for the catalysis of isoprene production in the transformant strains.

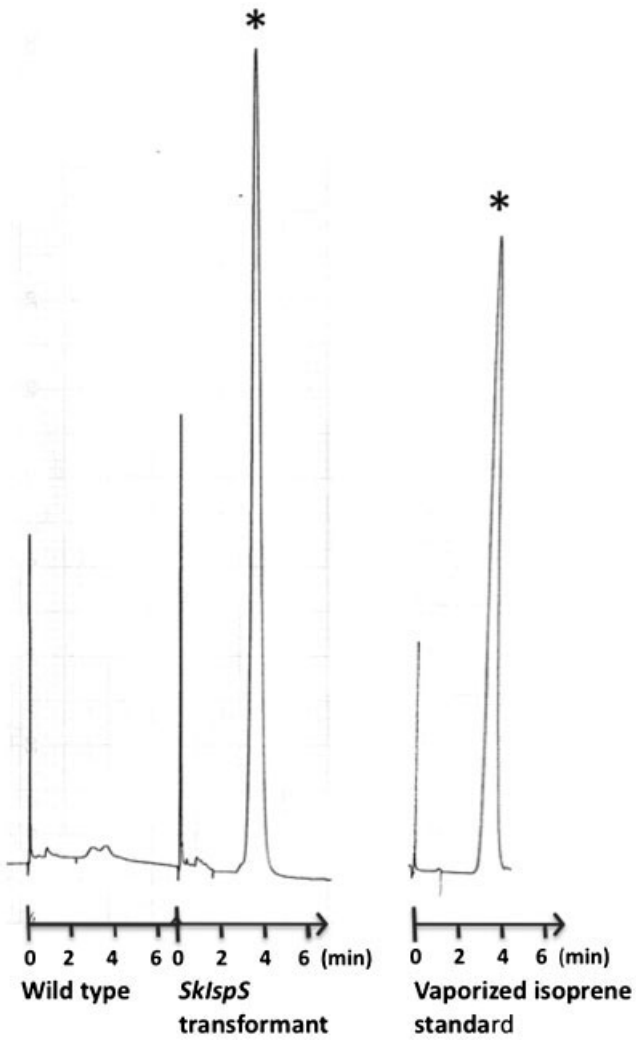

Figure 4. GC analysis of gases in the headspace of wild-type and *SkIspS* transformant cultures. Cultures were sealed for 48 h in the presence of CO_2 in the reactor headspace. GC analysis of headspace gases from wild type (left panel) and the *SkIspS* transformant (middle panel). Isoprene peaks were identified by comparison with an isoprene vapor standard (right panel), and are labeled with asterisks.

Growth and Photosynthesis of *Synechocystis* Wild Type and *IspS* Transformants

The photoautotrophic cell growth kinetics of the *SkIspS* transformants were about the same as that of the wild type, with a doubling time of 23 h under a light intensity of 20 μmol photons $m^{-2} s^{-1}$ and continuous bubbling with air (Fig. 6A). The light saturation curves of photosynthesis of wild type and the *SkIspS* transformants were also similar to one another (Fig. 6B), where oxygen evolution saturated at about 500 μmol photons $m^{-2} s^{-1}$, with an average P_{max} of 263 μmol O_2 (mg Chl)$^{-1}$ h^{-1} in wild type and 287 μmol O_2 (mg Chl)$^{-1}$ h^{-1} in the *SkIspS* transformant (Fig. 6B). Similarly, rates of oxygen consumption during dark respiration were about the same in the wild type and *IspS* transformants and equal to about −17 μmol O_2 (mg Chl)$^{-1}$ h^{-1}. Importantly, at sub-saturating light intensities between 0 and 250 μmol photons $m^{-2} s^{-1}$, rates of oxygen evolution and the initial slopes of photosynthesis as a function of light intensity were comparable in wild-type and *SkIspS*-transformant cells (Fig. 6B), suggesting similar quantum yields of photosynthesis (Melis, 2009). These results clearly showed that deletion of the endogenous *PsbA2* coding region from the *Synechocystis* genome, with the attendant replacement/integration and expression of the *SkIspS* transgene in the cell, as well as the subsequent generation and accumulation of isoprene, had no adverse effects on the growth and photosynthetic productivity parameters of the transformants.

CO_2 Diffusion-Based Photoautotrophic Growth and Isoprene Production in a Gaseous/Aqueous Two-Phase Fed-Batch Bioreactor

A 1-L fed-batch bioreactor was employed, where a single dose of 500 mL of 100% CO_2 was dispersed upon bubbling slowly through the 700 mL aqueous culture volume (Fig. 1). Upon the subsequent sealing of the culture and incubation at 150 μmol photons $m^{-2} s^{-1}$, CO_2 was spontaneously taken-up from the headspace and assimilated in photosynthesis by the cells in the liquid phase, leading to autotrophic biomass accumulation and isoprene production by *Synechocystis*. Exchange of CO_2 in the headspace for molecular oxygen and isoprene maintained equilibrium of gas pressure in the sealed headspace. When a single dose of 100% CO_2 was fed to cells at an $OD_{730\,nm}$ of 0.5 (Fig. 7A),

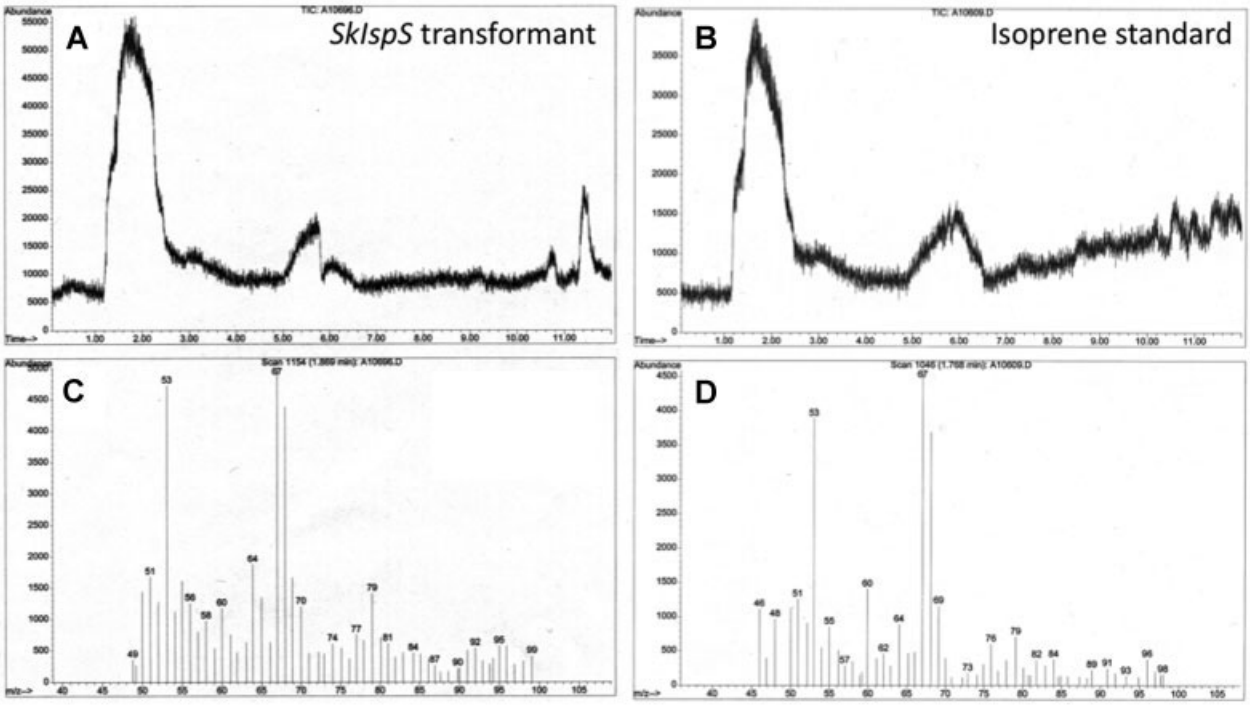

Figure 5. GC–MS identification of isoprene in the gaseous headspace of the *SkIspS* transformants. **A**: GC analysis of headspace gases from a sealed *SkIspS* culture after 48 h autotrophic growth in the presence of CO_2. **B**: GC analysis of vaporized pure isoprene standard. **C**: MS analysis of headspace gases from a sealed *SkIspS* culture showing the signature [53, 67, and 68] MS lines attributed to isoprene. **D**: MS lines of vaporized pure isoprene standard. The GC profiles (upper panels) showed that isoprene had a retention time of around 1.8 min, and the mass spectra of products eluted at 1.8 min from the *SkIspS* transformant culture matched that of the isoprene standard, showing that isoprene is enriched in the headspace of this culture.

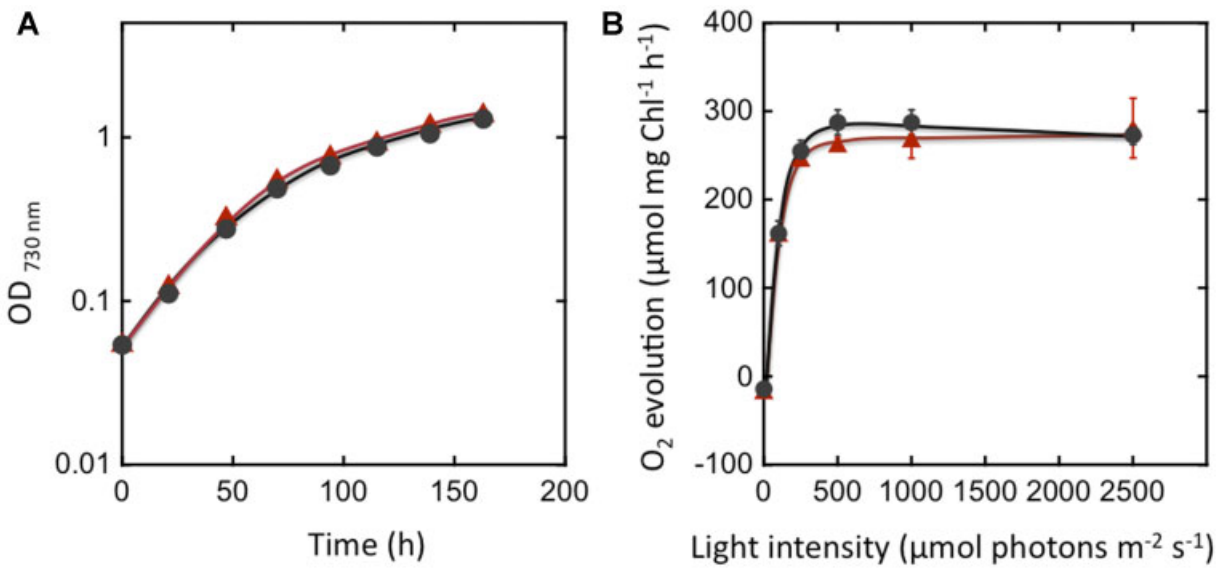

Figure 6. Growth and photosynthesis of wild type and *SkIspS* transformants in liquid culture. **A**: Photoautotrophic growth kinetics of wild-type (triangles) and *SkIspS* transformant cells (circles), as measured by optical density at 730 nm, when grown with continuous aeration and continuous illumination at 20 µmol photons $m^{-2} s^{-1}$. **B**: Light saturation curves of photosynthesis for wild type and *SkIspS* transformant cells, as measured by the oxygen-evolution activity of an aliquot of the cultures in the presence of 15 mM $NaHCO_3$, pH 7.4 under a range of actinic light intensities.

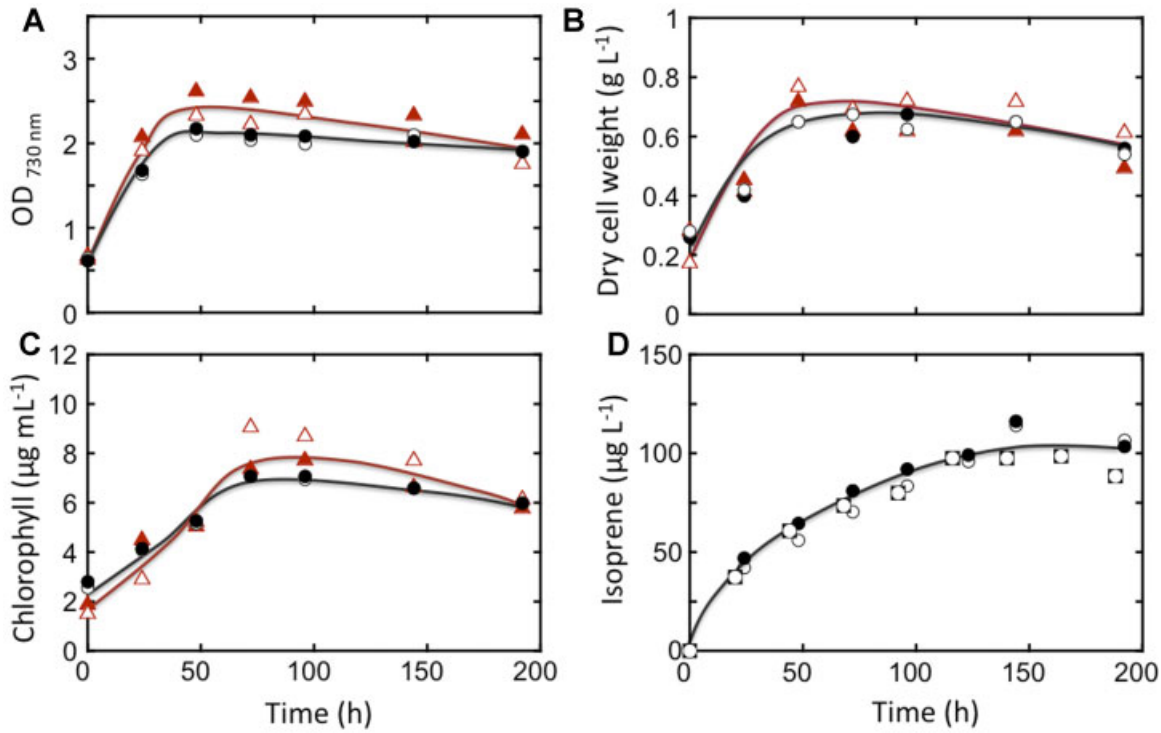

Figure 7. CO_2 diffusion-supported photoautotrophic growth and isoprene production in a fed-batch bioreactor. Photoautotrophically grown cultures of *Synechocystis* were slowly bubbled with 500 mL of 100% CO_2 to fill the headspace of the culture upon displacement of other headspace gases. Measurements started with a culture $OD_{730\,nm} = 0.5$. After administration of the 500 mL CO_2, the headspace of the gaseous/aqueous two-phase system was sealed and a time course of the following parameters were undertaken: **A**: cell density as $OD_{730\,nm}$, (**B**) dry cell weight, (**C**) chlorophyll concentration, and (**D**) amount of isoprene accumulated in the headspace per liter culture. Two independent measurements with separate samples are shown for each strain. Wild type (triangles), *SkIspS* transformant (circles). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

cell growth and biomass accumulation was supported for ~48 h in both wild type and *SkIspS* transformant cultures. This was evidenced by measurements of the $OD_{730\,nm}$ of the culture, which increased from 0.5 to about 2.5 in 48 h (Fig. 7A), increase in dry cell weight from 0.25 to about 0.7 g L^{-1} (Fig. 7B), and increase in the chlorophyll content from 0.25 to about 0.75 mg mL^{-1} culture (Fig. 7C). A doubling time of 23 h was measured over the growth phase, and after about 48 h of growth the cells entered the stationary phase of growth (Fig. 7A,B, and C). Sealing of the fed-batch bioreactors without prior feeding with CO_2 prompted cells to almost immediately enter a photoinhibition state, which progressed into bleaching of the cells after about 48 h incubation under illumination conditions (results not shown).

Isoprene concentration measurements from such fed-batch *SkIspS* cultures (Fig. 7D) showed that a yield of 100 µg isoprene L^{-1} culture could be achieved from a single dose of CO_2. The highest rate of isoprene production (2 µg isoprene L^{-1} culture h^{-1}) occurred during the initial 48 h of cell growth, with the rate of isoprene production slowing gradually as cells entered the stationary growth phase. Interestingly, isoprene accumulation continued well into the stationary phase, suggesting that internal cellular metabolism (possibly the breakdown of cellular glycogen) suffices to sustain isoprene production for extended periods of time, although net biomass increase is no longer observed. Enhanced rates of isoprene production were observed at the higher light intensity of 500 µmol photons $m^{-2}\,s^{-1}$ (data not shown), where photosynthesis is saturated in *Synechocystis* (Fig. 6B). The increase in isoprene production was proportional to the increase in P_{max} and to biomass accumulation observed at the higher light intensities. Incubation under a 12 h light (150 µmol photons $m^{-2}\,s^{-1}$)/12 h dark cycle showed that isoprene production was strictly light dependent (data not shown).

Entry into the stationary phase of growth at 48 h after the administration of a single dose of CO_2 was found to be due to the efficient and spontaneous diffusion-based uptake and assimilation of CO_2 from the culture headspace, leading to CO_2 depletion in the bioreactor. This interpretation was supported by the recovery of growth upon repetitive administration of equivalent amounts of CO_2 as shown in Figure 8., where the $OD_{730\,nm}$ of the culture increased from 0.5 to about 6.5 in 192 h upon repetitive administration of 100% CO_2 every 48 h (Fig. 8A). Also observed were increases in dry cell weight from 0.2 to about 1.8 g dw L^{-1} (Fig. 8B) and in the chlorophyll content of the culture from 0.5 to about 10 µg mL^{-1} (Fig. 8C). Isoprene concentration measurements (Fig. 8D) showed a yield of 150 µg

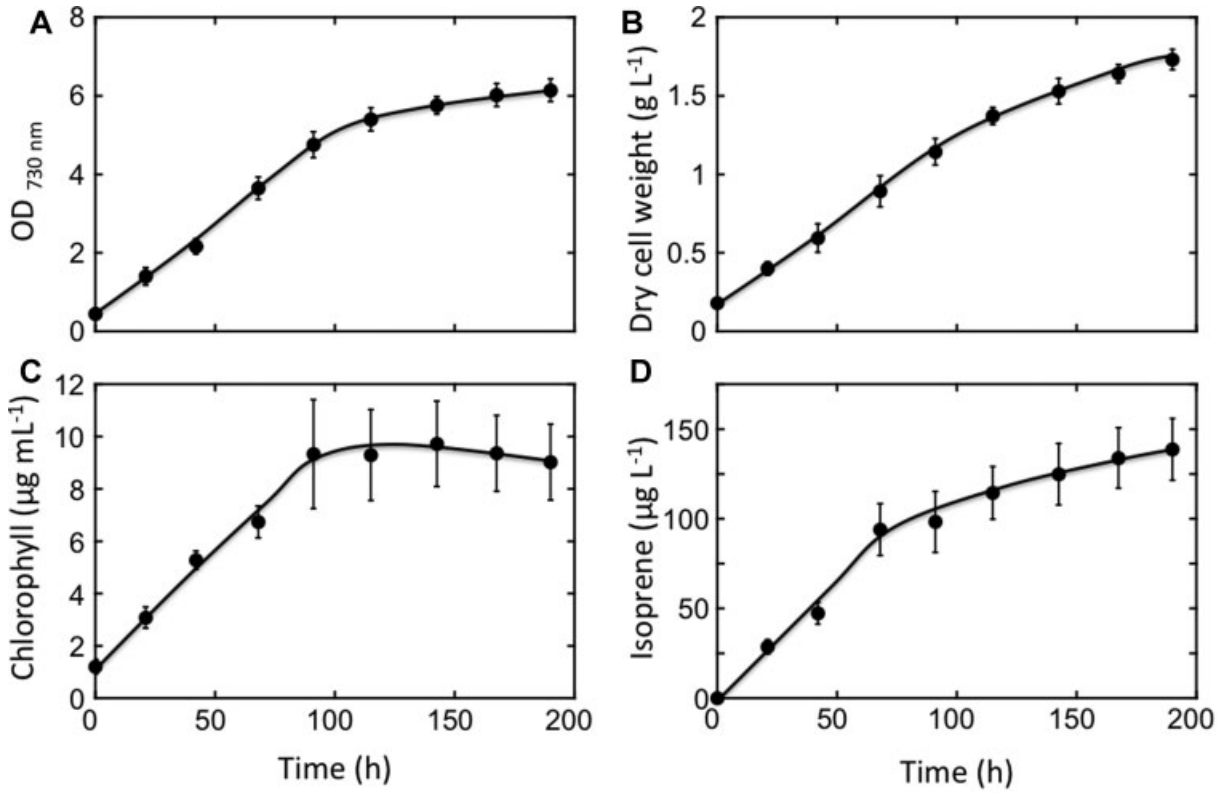

Figure 8. Cell growth and isoprene production upon periodic supplementation of the culture with CO_2 in a fed-batch gaseous/aqueous two-phase bioreactor. Cultures were bubbled slowly with 500 mL of 100% CO_2, sufficient to fill the headspace of the bioreactor upon displacement of other headspace gases. Supplementary doses with 500 mL of 100% CO_2 were administered approximately every 48 h, after which the headspace was sealed to allow CO_2 diffusion-based photoautotrophic growth and emission/accumulation of isoprene hydrocarbons. Each treatment of CO_2 effectively flushed accumulated O_2 and isoprene vapor from the headspace. Accordingly, isoprene content was measured before and after flushing with CO_2 to give an estimation of isoprene accumulation over each 24-h period. Over the time course of the experiment the following culture variables were measured: **A**: cell density as $OD_{730\,nm}$, (**B**) dry cell weight, (**C**) chlorophyll concentration in the cultures, and (**D**) cumulative amount of isoprene produced per liter culture. Results are presented for the *SkIspS* transformant only and show the average of three independent experiments, for which error bars represent the standard deviation of the mean.

isoprene L^{-1} culture, and kinetics of isoprene accumulation that matched the kinetics of cell growth. Coupled with the extended growth phase is a prolonged period of maximal isoprene production, where 100 µg isoprene L^{-1} culture is achieved after only about 70 h of growth. The slower rate of biomass accumulation and isoprene production at times longer than 70 h in this experiment was a result of nutrient depletion in the medium rather than a C-limitation, as growth in 2× concentration of nutrients sustained growth and isoprene production for a longer period of time (results not shown). However rates of growth and isoprene production were not entirely improved under these nutrient-enhanced conditions, due to the high-density of pigments in the cultures, limiting irradiance penetration and utilization (Melis, 2009; Mitra and Melis, 2008, 2010).

Continuous Growth for Biomass and Isoprene Production

The preceding suggested that, under photoautotrophic growth conditions, maximum rates of isoprene accumulation were obtained when cell growth was maintained in a metabolically active state (Figs. 7 and 8). Therefore, a semi-continuous culturing system was devised to maintain cultures in an actively dividing stage. Cultures were fed CO_2 every 24 h to support high photoautotrophic growth rates and, in addition, were diluted with fresh growth medium every 48 h, down to an $OD_{730\,nm}$ of 0.5 (~0.2 g dw L^{-1}). Using this approach, a constant rate of isoprene production of 2 µg isoprene L^{-1} culture h^{-1} was achieved over 192 h of experimentation (Fig. 9A). Over this period of time, three dilutions were administered, bringing the culture biomass density from about 0.7 g dw L^{-1} prior to the dilution, to between 0.1 and 0.2 g dw L^{-1} after the dilution with fresh growth media (Fig. 9B). It is evident from the results of Figure 9 that both isoprene and biomass accumulation increased linearly with time after each dilution. These results suggest constancy in the partition of photosynthate between isoprene and biomass, serving as proof of principle that the isoprene synthase *IspS* transgene can be stably expressed in a photosynthetic microorganism and function for product generation continuously.

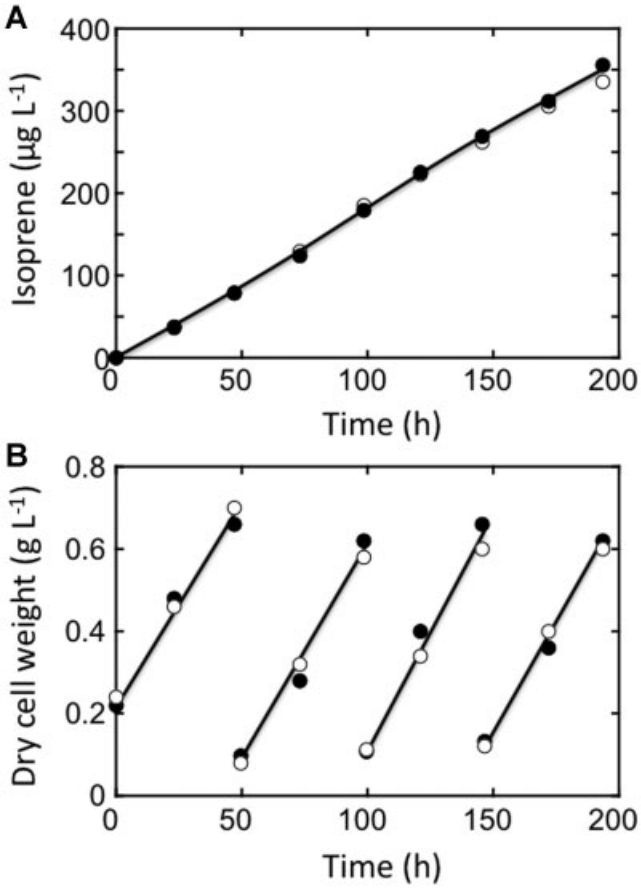

Figure 9. Continuous growth for biomass and isoprene production in a gaseous/aqueous two-phase bioreactor. *SkIspS* transformant cultures were administered 500 mL of 100% CO_2 approximately every 24 h to maintain photoautotrophic growth while the bioreactor was sealed for isoprene accumulation. Cultures were also diluted with fresh growth media every 48 h to an $OD_{730\,nm} = 0.5$ ($\sim$0.2 g dw L^{-1}) in order to maintain cells in the actively dividing stage. **A**: Isoprene hydrocarbons accumulated over the time course of the experiment. **B**: Culture dry cell weight as a function of time during continuous growth in the bioreactor. Dilutions of the culture were implemented at 48, 96, and 144 h. Results shown are for two different *SkIspS* transformant lines.

Yield of Isoprene in Sealed Gaseous/Aqueous Two-Phase Photobioreactors

It is important to note that rates of isoprene production reported here are based on the amounts accumulated in the gaseous headspace of the sealed cultures. Isoprene, being a pure hydrocarbon, has very limited solubility in a water-based growth medium. However, Henry's Law stipulates that the solubility of a gas in a liquid at a particular temperature is proportional to the partial pressure exerted by that gas above the liquid phase. Since the approach described here entails a sealed gaseous/aqueous two-phase photobioreactor, one would predict that, at any given time, a quantity of isoprene is dissolved in the aqueous media that is proportional to the partial pressure of gaseous isoprene in the headspace. Using Henry's constant for isoprene at 35°C (Lindinger et al., 1998), Henry's Law predicts that a 700 mL *Synechocystis* culture that accumulates 35 μg isoprene in 24 h in the sealed headspace of a 1 L gaseous/aqueous two-phase photobioreactor ($\sim$40% total bioreactor volume) would accumulate an additional 38 μg isoprene in the aqueous media ($\sim$60% total bioreactor volume). Predictions of isoprene solubility according to Henry's Law were experimentally validated by measuring the amount of isoprene vapor that dissolved in sealed flasks containing water, BG-11 media, or a suspension of biomass in BG-11 media (data not shown). The total amount of photosynthetically derived isoprene in the gaseous/aqueous two-phase photobioreactor may, therefore, be $\sim$twofold greater than what we measured by our sampling method in the gaseous headspace.

Discussion

Application of photosynthesis for the direct generation of fuel offers the advantage of a shortcut in the solar-to-biofuel generation process. Photosynthetic microorganisms, e.g., cyanobacteria and microalgae, are the organisms of choice for such direct solar-to-product processes, as they grow to high densities in sealed photobioreactors (Angermayr et al., 2009; Beer et al., 2009; Chisti, 2007), and have better solar energy conversion efficiencies in photosynthesis than land plants (Melis, 2009). Isoprene is promising as a high-value bio-product, acting both as a chemical feedstock and a biofuel. However, commercial exploitation requires that a "scale-up" method for isoprene generation and sequestration be developed. In particular, the problems associated with the maintenance of photoautotrophic metabolism, whilst allowing for product accumulation and concentration in a sealed headspace, must be addressed.

Here, we described a diffusion-based method for spontaneous gas exchange in gaseous/aqueous two-phase photobioreactors, using carbon dioxide as a feedstock for the photosynthetic generation of isoprene. The headspace of the bioreactor was filled with 100% CO_2 and sealed, allowing the diffusion-based CO_2 uptake and assimilation by the cells via photosynthesis, and the concomitant replacement of the CO_2 in the headspace with isoprene vapor and O_2. Importantly, this approach allows for sustained photoautotrophic metabolism in parallel with isoprene accumulation in the headspace at much higher concentrations than would be achieved upon continuous bubbling and collection of isoprene in "real time". Physicochemical condensation of isoprene vapor into liquid form, and/or isoprene trapping in solvents such as methanol, ethanol, butanol, hexane, heptane, octane, or dodecane are potential methods for harvesting the accumulated isoprene and allowing retention of the molecule as a stable blendstock.

Repetition of the carbon dioxide replenishing and isoprene removal protocol enabled cells to continuously produce isoprene but also to reach high biomass density in the bioreactor (Fig. 8). Accordingly, a semi-continuous culturing method was devised and employed, which involved the periodic dilution of the culture to ensure it is always in the actively dividing stage. A medium density

biomass mitigates the negative effects of reduced light penetration to cells that would prevail at high biomass density in photobioreactors (Melis, 2009; Mitra and Melis, 2008). This method allows cells to stay in a photosynthetically active state that permitted maintenance of maximum rates of isoprene production over an indefinite period of time (Fig. 9). These results provided evidence that the *SkIspS* transgene is stably incorporated into the *Synechocystis* genome, and retains stability of expression over multiple generations in the absence of antibiotic selection. Taking into consideration the isoprene dissolved in the growth medium (Henry's law), we estimated consistent rates of 4 μg isoprene L^{-1} culture h^{-1} was achieved using this method, which equates to 0.08% of assimilated carbon partitioning as isoprene (assuming that carbon contributes to approximately half the weight of dw biomass). In plant species that naturally produce isoprene under heat stress, this carbon-partitioning ratio is higher; for example, leaves of oak (*Quercus robur*) are reported to partition around 0.6% of assimilated carbon in the form of isoprene (Li et al., 2011). Further work to enhance carbon partitioning toward isoprene production by metabolic engineering and/or physiological manipulation is currently in progress.

The method described in this work for the generation and sequestration of photosynthetically derived isoprene hydrocarbons from a cyanobacterial culture is based on the principle of diffusion-driven spontaneous gas exchange in a fed-batch gaseous/aqueous two-phase photobioreactor. This method, using solar energy to drive the assimilation and conversion of CO_2 into the valuable bio-product isoprene, would have a positive impact on the quest for renewable energy generation. More importantly, this technology has the potential to be extended for use with any other photosynthetic microorganism, and for the generation and harvesting of other high-value volatile bio-products.

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