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Isoprene production in *Synechocystis* under alkaline and saline growth conditions

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Abstract Photosynthesis for the generation of isoprene in cyanobacteria was demonstrated with *Synechocystis*, entailing a process where a single host microorganism acts as both photocatalyst and processor, photosynthesizing and emitting isoprene hydrocarbons. A practical aspect of the commercial exploitation of this process in mass culture is the need to prevent invading microorganisms that might cause a culture to crash, and to provide an alternative to freshwater in scale-up applications. Growth media poised at alkaline pH are desirable in this respect, as high pH might favor the growth of the cyanobacteria, while at the same time discouraging the growth of invading predatory microbes and grazers. In addition, demonstration of salinity tolerance would enable the use of seawater for cyanobacteria cultivations. However, it is not known if *Synechocystis* growth and the isoprene-producing metabolism can be retained under such theoretically non-physiological conditions. We applied the gaseous/aqueous two-phase photobioreactor system with *Synechocystis* transformed with the isoprene synthase gene (*SkIspS*) of *Pueraria montana* (kudzu). Rates of growth and isoprene production are reported under control, and a combination of alkalinity and salinity conditions. The results showed that alkalinity and salinity do not exert a negative effect on either cell growth or isoprene production rate and yield in *Synechocystis*. The work points to a practical approach in the design of cyanobacterial growth media for applications in commercial scale-up and isoprene production.

Keywords Bioenergy · Biofuels · Cyanobacteria · Productivity · Terpenes

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Introduction

Cyanobacteria are photoautotrophic prokaryotes that use solar energy, CO_2, and H_2O to produce chemical energy and store it as biomass via oxygenic photosynthesis. The ability of cyanobacteria, like other photosynthetic systems, to carry out oxygenic photosynthesis at a high quantum yield (Ley and Mauzerall 1982; Bjorkman and Demmig 1987) makes them an excellent candidate for sunlight-driven production of renewable fuel and chemicals. Cyanobacteria can be cultivated in open racetrack ponds (Herrera et al. 1989) or closed photobioreactors (Melis 2012) on marginal land that is not suitable for agriculture, which would alleviate the issue of encroachment on land used for food crops. In the context of product generation, the cyanobacterium *Synechocystis* sp. PCC6803 has been genetically engineered with a codon-optimized *Pueraria montana* (kudzu) isoprene synthase gene (Lindberg et al. 2010; Bentley and Melis 2012), conferring upon *Synechocystis* the property of photosynthetic isoprene hydrocarbon production. Isoprene (2-methyl-1,3-butadiene; C_5H_8) is naturally produced by photosynthesis in the leaves of many species of trees, as well as other herbaceous and deciduous land plants and shrubs. Isoprene is emitted through the stomata of the leaves into the atmosphere. The yearly production of isoprene emissions by vegetation was estimated to be about 600 million tonnes (Guenther et al. 2006), with about half of that produced by tropical broadleaf trees, and the remainder originating from the photosynthesis of shrubs. Aquatic photosynthetic systems, however, including microalgae and cyanobacteria, are not endowed with the isoprene synthase gene and lack the isoprene biosynthesis process (Lichtenthaler 2007, 2010). Isoprene hydrocarbons are a useful terpenoid product, currently serving as feedstock in the synthetic chemistry industry for the production of multiple commercial commodities such as rubber, adhesives, plastics, and perfumes. It also has great potential to be used

as a renewable fuel similarly to other short-chain hydrocarbons.

The process for cyanobacterial growth, and the sequestration and harvesting of the volatile isoprene molecules has been established in gaseous/aqueous two-phase photobioreactors (Bentley and Melis 2012). In the lab, this approach utilized custom-made 1-L fed-batch bioreactors, which are fitted with a long aerator tube to enable delivery of gases (CO_2 or air), and a short tube with a septum for sampling the chemical composition of the gaseous headspace and the biomass content of the liquid phase. Typically, cultures are flushed with CO_2, sufficient to fill the headspace, and then sealed to contain the isoprene produced by photosynthesis.

In addition to the above mentioned properties of cyanobacteria that make them attractive for bio-based chemical production, many cyanobacteria strains have the capacity to tolerate extreme environmental conditions such as alkaline pH and salinity (Pikuta et al. 2007). Growth media poised at alkaline pH is desirable, as it would permit growth of the cyanobacteria, while at the same time discouraging the growth of other invading alkaline intolerant contaminants and predatory microbes and grazers (McGinn et al. 2011). An example of this premise is offered by the carbonate-requiring alkalophilic cyanobacteria *Arthrospira platensis* and *Arthrospira maxima* (*Spirulina* commercially), which thrive at high concentrations of Na-carbonate at pH values 9–11, conditions that prevent most predatory microbes and grazers from establishing themselves in the cultures (Dismukes et al. 2008). The ability of *Synechocystis* to be grown in a saline environment presents the opportunity to use seawater for cultivation instead of freshwater, which is a limited resource. In a commercial scale-up setting in which cultures are grown continuously in vast quantities of non-sterile water, these properties are potentially valuable. In this work, we further developed the gaseous/aqueous two-phase photobioreactor system with *Synechocystis* to operate under alkaline pH and salinity conditions. Rates of growth and isoprene production in *Synechocystis* transformed with the *SkIspS* gene are reported under these conditions. The results suggest a practical method by which to prevent contamination and reduce resource demand of the *Synechocystis* growth media suitable for application in commercial scale-up.

Materials and methods

The wild-type strain of *Synechocystis* sp. PCC 6803 and its *SkIspS* transformant (Lindberg et al. 2010; Bentley and Melis 2012) containing the codon-optimized kudzu isoprene synthase gene were used in this study. Both strains were maintained on 1 % agar BG-11 plates supplemented with 10 mM TES-NaOH (pH 8.2), 0.3 % sodium thiosulfate, and 5 mM glucose. The transformant strain was maintained on plates containing 25 μg mL^{-1} kanamycin. For acclimation purposes, the *SkIspS* transformant strain was also maintained on plates containing 25 mM $NaHCO_3$ (pH 10.5), and 100, 200, or 600 mM NaCl in addition to the 25 μg mL^{-1} kanamycin. Liquid cultures were grown in BG-11 media containing 25 mM of 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; pK = 7.5), Tricine (pK = 8.1), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pK = 10.5), Na_2CO_3 (pK_b = 10.2), or Na_2HPO_4 (pK_b = 7.2) depending on the target pH of each culture.

Growth conditions

Growth of *Synechocystis* wild type and *SkIspS* cultures at different pH values were conducted in 300-mL starter cultures, which were grown for 2–3 days in BG-11 media supplemented with 25 mM HEPES (pH 7.5) or 25 mM CAPS (pH 10) for each strain. Starter cultures for *SkIspS* transformants were grown in the presence of 25 μg mL^{-1} kanamycin.

Experimental cultures were then inoculated from starter cultures into pH 7, 8, 9, 10, and 11 media at an $OD_{730} \approx$ 0.05. The pH of these cultures was under the control of a buffer (HEPES, Tricine, or CAPS) selected according to the pKa of the compound, at a concentration of 25 mM. All cultures were grown at 25 °C under constant aeration and illumination at 75 μmol photons m^{-2} s^{-1} for 6–10 days.

Comparative growth analysis of *Synechocystis* wild type and *SkIspS* strains at pH 10 was conducted under defined salinity conditions in 300-mL starter cultures, which were grown for 2–3 days in BG-11 media supplemented with 25 mM Na_2CO_3 (pH 10.5). Starter cultures in media with salinity at 100, 200, or 600 mM NaCl were grown only at pH 10.5.

Batch culture growth for isoprene production

Gaseous/aqueous two-phase bioreactors (Bentley and Melis 2012) were inoculated with 700-mL cultures of *Synechocystis* cells at an OD_{730}=0.05 in BG-11 media containing 25 mM of Na_2HPO_4 at pH 7 or 25 mM of Na_2CO_3 at pH 10.5. Cultures with 100, 200, or 600 mM NaCl were grown only at pH 10.5. Cultures were grown at 25 °C under continuous illumination at 75 μmol photons m^{-2} s^{-1} and continuous aeration until an OD_{730} of 0.4–0.5 was reached. Inorganic carbon in the form of 100 % CO_2 was slowly bubbled through the bottom of the liquid culture in the reactor for a total of 5 s, delivering about

100 mL of 100 % CO_2. By varying the volume of 100 % CO_2 bubbled through the culture, we determined that 100 mL of 100 % CO_2 was necessary and sufficient to support growth without causing pH changes to the growth medium. The presence of 25 mM $NaHCO_3$/Na_2CO_3 in the growth media contributed to the inorganic carbon supply and also supported cell growth.

After flushing with 100 % CO_2, the reactor was then sealed to prevent exchange of gases with the surrounding atmosphere and also to prevent escape of the isoprene vapor accumulating in the culture headspace. The sealed reactor was incubated under continuous slow stirring and illumination of 150 μmol photons m^{-2} s^{-1} at 35 °C. Isoprene accumulation was measured every 24 h by GC analysis upon sampling the headspace of the reactor. Small aliquots from the liquid phase were also removed every 24 h for biomass accumulation measurements. Following this sampling, the reactor was flushed with 100 mL of 100 % CO_2 and sealed again for another 24-h cycle. This cycle was repeated every 24 h for up to 96 h.

Growth analysis, pigment determination, and isoprene quantification

Growth curves of *Synechocystis* wild type and *SkIspS* transformants were plotted from measurements of culture aliquots taken every 24 h, analyzing optical density of the culture at 730 nm with a Shimadzu UV-1800 UV–vis spectrophotometer. Growth was independently assessed gravimetrically from the dry cell biomass by filtering 5–10-mL culture aliquots through 0.22-μm Millipore filters, thoroughly washing the samples with 10–20 mL of distilled water in order to remove extraneous salts, then drying each sample at 90 °C for 6 h and measuring the dry cell weight (DCW). Doubling times were calculated for each growth condition by measuring the duplication rate during the exponential growth phase. Chlorophyll *a* was quantified from 1-mL culture aliquots upon pigment extraction with 100 % methanol and spectrophotometric quantification of the pigments at 665 nm according to Lichtenthaler (1987). The pH of each culture bubbled with air was measured at time zero, before aeration began, and at the end of each growth experiment (6–10 days), with a Fisher Scientific Dual Channel pH/Ion meter. The pH of each culture bubbled with 100 % CO_2 was measured every 24 h, before the repeat flushing of the culture with CO_2.

Isoprene was quantified from its GC signal amplitude upon injection of 1-mL gaseous aliquot from the reactor headspace (Fig. 1), based on a calibration curve constructed from serial dilutions of vaporized isoprene standard, measured with a Shimadzu 8A GC apparatus (Shimadzu, USA) (Fig. 2). For the calibration curve, 1-mL sample from each vaporized standard was injected in the GC and the peak amplitude of the sample was recorded (Fig. 1). Resultant peak areas (in cm^{-2}) were plotted against the known amount of isoprene injected (in μg, Fig. 2), and the slope of the standard curve was used to determine the amount of isoprene produced by *SkIspS* transformants in experimental cultures.

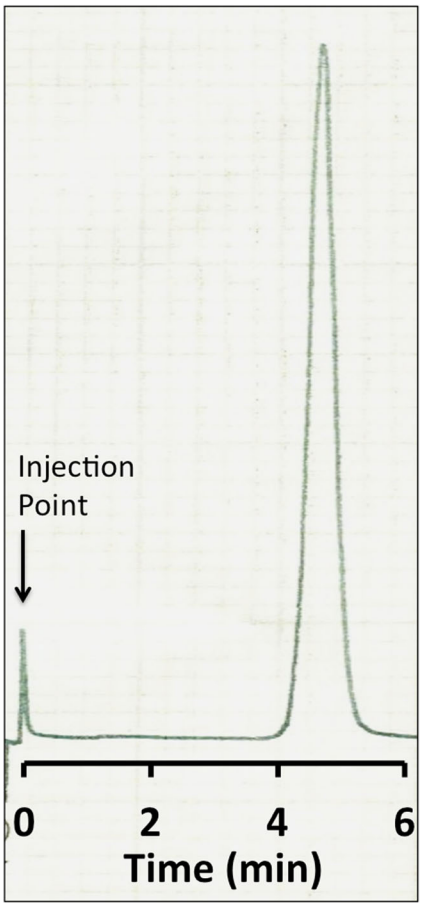

Fig. 1 Gas chromatography analysis of isoprene (2-methyl-1,3-butadiene; C_5H_8) standard. One milliliter of a vaporized isoprene sample comprising 0.087 μg pure isoprene was injected into the column inlet of the GC. The retention time for isoprene under these GC conditions was 4.3 min. Samples collected from the headspace of experimental cultures were similarly analyzed from their single peak amplitudes at 4.3 min

Spectrophotometric analysis

Cells were harvested by centrifugation at 1,000*g* for 3 min at 4 °C. Ice-cold lysis buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.2 % polyvinylpyrrolidone-40, 0.2 % sodium ascorbate, 1 mM aminocaproic acid, 1 mM aminobenzamidine, and 100 μM phenylmethylsulfonyl fluoride (PMSF) was used for suspension of the pellet. Cells were lysed in a French pressure cell operated at 4 °C, upon passing two times under 20,000 psi pressure with 30-s cooling intervals on ice. To remove unbroken cells from the thylakoid

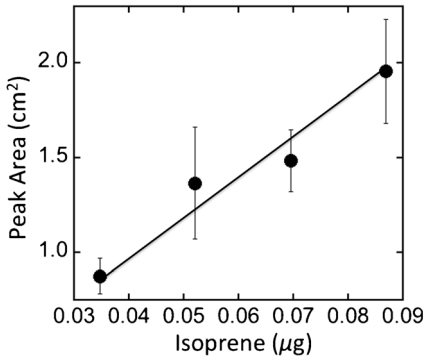

Fig. 2 Isoprene calibration curve. Serial dilutions of pure vaporized isoprene standard were prepared, and a 1-mL aliquot of each dilution was injected into the column inlet of the GC. The peak area produced by the chromatogram is plotted as a function of the amount of isoprene (μg) injected in each 1-mL sample. The slope of the straight line was used to determine the amount of isoprene produced in experimental cultures

membranes, a centrifugation at 3,000×*g* for 4 min at 4 °C was used. The membranes were collected by centrifugation of the supernatant at 75,000*g* for 45 min at 4 °C. For the spectrophotometric measurements, the thylakoid membrane pellet was resuspended in a buffer containing 50 mM Tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$.

The amplitude of the light-minus-dark absorbance difference signal at 700 nm (P700) for photosystem I (PSI), and 320 nm (Q_A) for photosystem II (PSII) was measured to determine the concentration of the photosystems in thylakoid membrane preparations (Melis 1989).

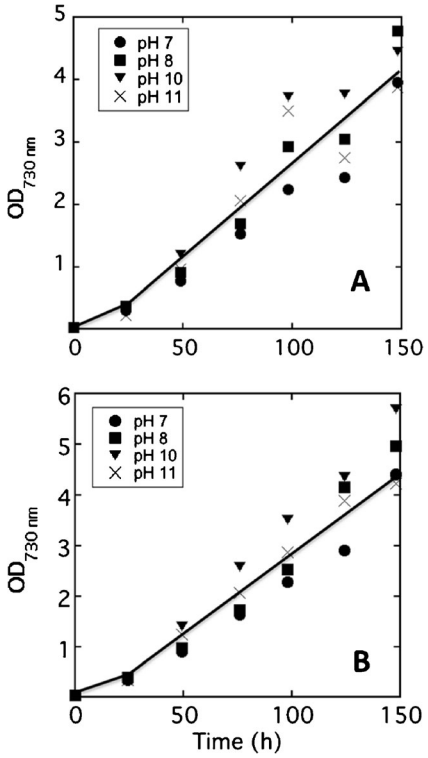

Fig. 3 Photoautotrophic growth of *Synechocystis* wild type (**a**) and *SkIspS* cell lines (**b**). Cells were grown in 300-mL liquid cultures of BG-11 supplemented with 25 mM of HEPES (pH 7 and 8), Tricine (pH 9), or CAPS (pH 10 and 11), under conditions of continuous slow aeration and illumination of 75 μmol photons m^{-2} s^{-1} for 150 h. Growth of the set of four cultures was measured in three independent experiments. Standard error of the points shown for the cultures grown at pH 7 was ±0.06, at pH 8 was ±0.08, at pH 10 was ±0.03, and at pH 11 was ±0.08

Results

Cell growth as a function of pH upon bubbling with air

The photoautotrophic rate of growth of the *Synechocystis* wild type and the *SkIspS* transformant was about the same at each pH tested—7, 8, 9, and 10—under a light intensity of 75 μmol photons m^{-2} s^{-1} at 25 °C and continuous bubbling with air in 300-mL liquid cultures (Fig. 3). Under these light-limiting conditions, the wild type registered an average doubling time of 33 h (Fig. 3a), and the *SkIspS* transformants had an average doubling time of 32 h at pH 7 and 8, and an average doubling time of 26 h at pH 10 and 11 (Fig. 3b). A systematic analysis of the average doubling times of wild type and SkIspS transformant as a function of the pH of the culture medium is shown in Fig. 4. The results showed that alkaline conditions were more favorable to growth, as they accelerated cell duplication time in both the wild type and the *SkIspS* transformants, further showing that alkalinity has no adverse effect on the fitness and growth of *Synechocystis*. It is also of interest to note that the *SkIspS* transformants appeared to grow faster, by about 15 %, relative to the wild type, regardless of the pH of the medium, an observation consistent with earlier results by Bentley and Melis (2012).

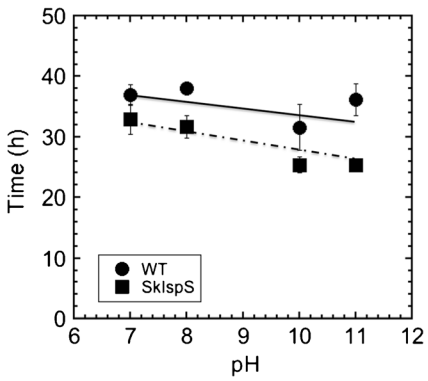

Fig. 4 Cell duplication time of *Synechocystis* as a function of pH under conditions of continuous slow aeration and illumination. Wild-type cells (*circles*) and *SkIspS* transformants (*squares*). For other conditions, please see legend of Fig. 3

Cell growth and isoprene production as a function of pH upon filling the reactor with 100 % CO_2

Rates of wild type and *SkIspS* transformant growth were determined in the sealed gaseous-aqueous two-phase 1-L reactor developed by Bentley and Melis (2012). In this case, inoculated cultures were first bubbled with air for 2 days to bring up the biomass prior to placement in the Bentley-Melis reactor. The aqueous phase of the reactor (700 mL) contained the culture, whereas the gaseous phase (500 mL) was supplemented with 100 % CO_2, slowly bubbled into the bottom of the liquid culture, so as to fill the gaseous phase (Bentley and Melis 2012). Cells were grown in such sealed reactors under a light intensity of 150 μmol photons m^{-2} s^{-1} at 35 °C. At both pH 7 and pH 10, cell mass accumulation increased linearly and by approximately sevenfold over 96 h, as measured by optical density at 730 nm (Fig. 5a). The dry cell biomass of these cultures accumulated at a rate of 6.3 mg L^{-1} h^{-1} under both pH 7 and pH 10 growth conditions (Fig. 5b), indicating that the *SkIspS* transformants were able to grow equally well under alkaline pH, when cultivated in the gaseous-aqueous two-phase reactor with 100 % CO_2. The pH of the culture in the liquid phase was measured every 24 h to determine whether there were changes due to cell nutrient uptake or CO_2 bubbling. We found that the pH of the cultures inoculated at pH 10 was maintained between 9.6 and 10.4 (Fig. 5c, squares). Cultures inoculated at pH 7 showed an increase in pH to 7.8 at time point zero after 2 days of bubbling with air. Thereafter, they showed a pH fluctuation between 7.5 and 8.2 for the majority of the experiments. Occasionally, the pH of these cultures increased to 9.2 during the last 24 h of the 96-h growth period (Fig. 5c, circles). This increase in pH is understood to originate from OH^- efflux from the cells in a process designed to balance the HCO_3^- and nutrient uptake by the cells, and is manifested when the buffering capacity of the system is not adequate (Naus and Melis 1991; Sonoda et al. 1998). Isoprene production by the *Synechocystis* cells and accumulation of these volatile hydrocarbons in the gaseous phase of the reactor occurred with a rate of about 0.63 μg L^{-1} h^{-1}, statistically indistinguishable among the samples grown at pH 7 (Fig. 5d, circles) or pH 10 (Fig. 5d, squares).

At the end of the 96-h growth period, cultures were routinely examined, both visually and microscopically to assess health and robustness of the cells (Fig. 6). Results routinely revealed that wild type and the *SkIspS* transformant strains both were able to grow equally well under neutral (Fig. 6a, b) or alkaline pH (Fig. 6c, d), when cultivated in the gaseous-

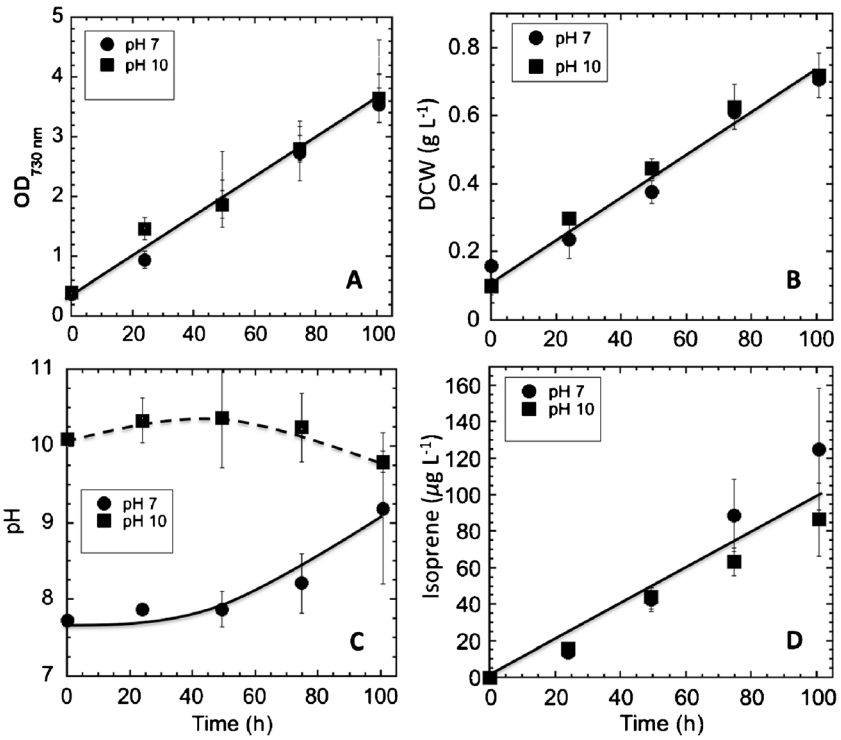

Fig. 5 Photoautotrophic growth of *Synechocystis SkIspS* transformants suspended at pH 7 (*circles*) and pH 10 (*squares*). A sealed gaseous-aqueous two-phase 1-L reactor (Bentley and Melis (2012) was used for these measurements. The liquid culture comprised 700 mL of pH 7 or pH 10 growth medium, which was bubbled with 100 mL of 100 % CO_2, sealed, and incubated for 24 h under continuous illumination of 150 μmol photons m^{-2} s^{-1}, prior to measurement. The liquid phase $OD_{730\ nm}$ (**a**), DCW (**b**), and pH (**c**), and the gaseous phase isoprene content (**d**) were measured every 24 h. Following this 24-h sampling, the reactor was flushed with 100 mL of 100 % CO_2 to purge the accumulated products of photosynthesis and to replenish the inorganic carbon supply so as to enable a resumption of photosynthesis and growth

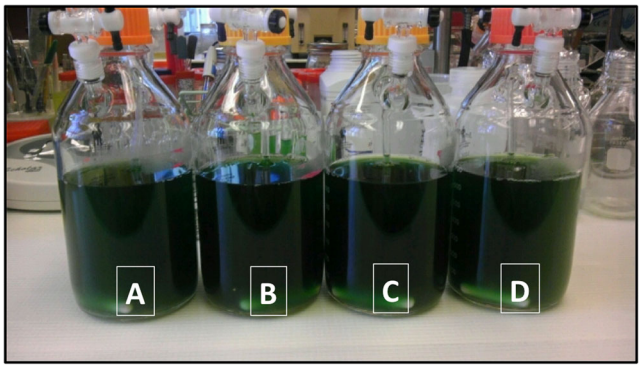

Fig. 6 Gaseous-aqueous two-phase reactors for the growth of Synechocystis *SkIspS* transformants under sealed conditions (Bentley and Melis 2012). The 500-mL headspace of the reactors was bubbled with 100 mL of 100 % CO_2 every 24 h. Isoprene accumulating in the headspace during photosynthesis of the cyanobacteria was quantified every 24 h prior to refilling the reactor with CO_2. The cultures shown were grown for 96 h at pH 7 (**a**, **b**) and pH 10 (**c**, **d**)

aqueous two-phase reactor with supplemental 100 % CO_2 bubbled through the culture.

Cell growth and isoprene production under alkaline and saline conditions

Photoautotrophic growth and isoprene production were measured at pH 10 with an additional salinity load to the growth medium. Cell growth and biomass accumulation at pH 10 in the presence of 100 or 200 mM NaCl were the same as in the control, with an average increase in $OD_{730\ nm}$ of approximately 6.6-fold over 96 h (Fig. 7a, solid symbols). The optical density $OD_{730\ nm}$ increase in the presence of 600 mM NaCl was also linear during growth (Fig. 7a, x symbols), although the slope of the growth curve at 600 mM NaCl was about half of that measured with the lower NaCl concentrations. This signifies changes in the optical properties and scattering of light by the cells at the 600 mM NaCl concentration.

Cell biomass accumulated at a rate of 6.8 mg L^{-1} h^{-1} under most salinity conditions examined, i.e., pH 10 without salinity, and pH 10 with 100 and 200 mM added NaCl (Fig. 7b). For cells grown in the presence of 600 mM NaCl, there was an ~24-h lag, after which the rate of biomass accumulation reached 7.4 mg L^{-1} h^{-1}, i.e., similar to that measured in the other cultures (Fig. 7b). These results showed that *Synechocystis* wild type and transformants have the capacity to grow normally under the combination of two altered media conditions, i.e., higher than normal pH and salinity, up to that encountered in seawater (600 mM). Absorbance spectra of these cells were measured at the exponential growth phase of the cultures (Fig. 7c). The spectra were normalized to the absorbance maximum of phycocyanin at 625 nm. It was then revealed that the chlorophyll *a* (Chl *a*) absorbance maximum at 678 nm was greater for the cells grown in the presence of NaCl than that in the control. These results suggested a greater Chl *a*/phycobilisome ratio under salinity compared to that in the control (Fig. 7c). As Chl *a* is the dominant light-harvesting pigment of PSI, and phycocyanin is the dominant light-harvesting pigment of PSII (Glazer and Melis 1987), the absorption spectra results indicate a higher PSI relative to PSII content in cells grown under salinity conditions. This hypothesis was tested upon application of direct light-minus-dark absorbance difference spectrophotometric measurements for the quantification of PSI and PSII in *Synechocystis SkIspS* transformant thylakoids (Melis 1989). Results from this analysis are shown in Table 1. A PSI/PSII ratio of 4.5:1 was measured in BG-11 pH 10 grown cells. The ratio was elevated to 5.6:1 when cells were grown in BG-11 pH 10 plus salinity. These results are consistent with previous studies, showing that salt loading in *Synechocystis* can result in an increase in PSI content to enhance cyclic electron flow, thus increasing the production of ATP (Manodori and Melis 1984) for salt extrusion (Schubert and Hagemann 1990).

Isoprene production measurements from these samples showed that, within the error of the measurement, rate and yield were not significantly changed by an increase in salinity (Fig. 7d). An average rate of isoprene accumulation of 0.48 μg L^{-1} h^{-1} was measured under most conditions, i.e., pH 10 without salinity, and pH 10 with 100 or 200 mM added NaCl. As with the respective growth curves, cells grown in the presence of 600 mM NaCl showed an ~24-h lag in isoprene production; however, at later times between 24 and 96 h, isoprene accumulation for the 600 mM NaCl cells approached that of the other cultures at 0.41 μg L^{-1} h^{-1} (Fig. 7d). The average amount of carbon partitioning to isoprene versus carbon to biomass was calculated based on the amount of isoprene produced compared to the amount of biomass (DCW) generated. The carbon-partitioning ratio was estimated to be in the range of 0.01–0.02 % at pH 10, regardless of the salinity conditions employed (Fig. 8).

At the end of the 96-h growth period, alkaline and saline *SkIspS* transformant cultures were examined visually and microscopically to assess health and robustness of the cells (Fig. 9). Results showed that the *SkIspS* transformant strains were equally healthy in the absence (Fig. 9a) or presence of 100-, 200-, or 600-mM salinity in the growth medium (Fig. 9b–d).

Discussion

The transformant *SkIspS* strain of *Synechocystis* has helped validate the notion of "photosynthetic biofuels," entailing heterologous transformation of a photosynthetic microorganism to endow the property of synthesis and release of isoprene in the aquatic environment (Lindberg et al. 2010; Bentley and

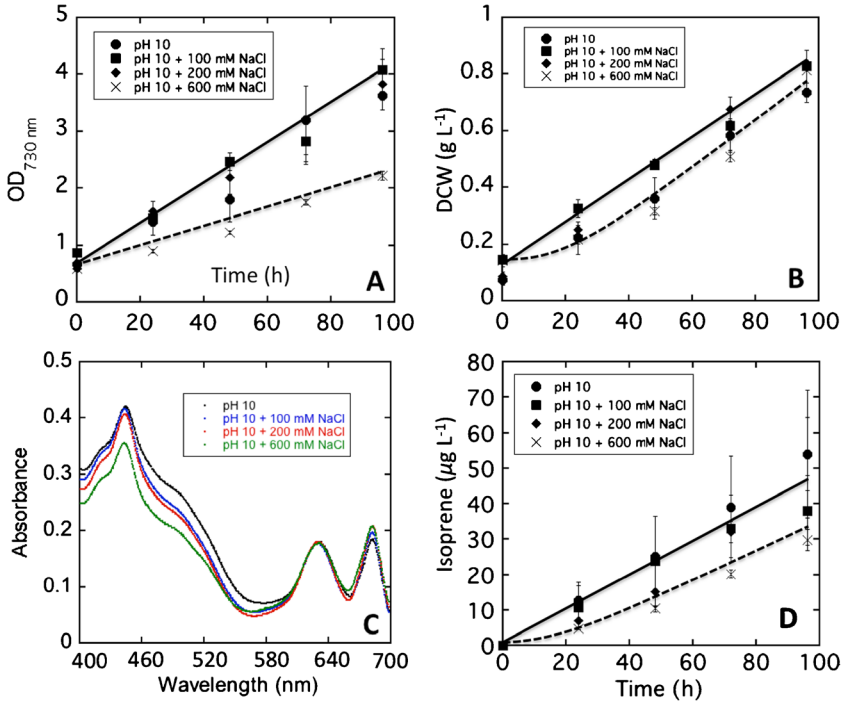

Fig. 7 Photoautotrophic growth of the *SkIspS* transformant strains at pH 10 without added salinity (*circles*), and with 100 mM NaCl (*squares*), 200 mM NaCl (*diamonds*), or 600 mM NaCl (*crosses*). A sealed gaseous-aqueous two-phase 1-L reactor was used for these measurements. The 700-mL cultures were bubbled with 100 mL of 100 % CO_2 every 24 h under continuous illumination, for a growth period of 96 h. Growth was measured as $OD_{730\ nm}$ (**a**) and dry cell weight (**b**). Pigment content (**c**) was evaluated from the absorbance spectra of intact *SkIspS* transformant strains grown at pH 10 without added salinity (*black line*), and with 100 mM NaCl (*blue line*), 200 mM NaCl (*red line*), or 600 mM NaCl (*green line*). The absorbance spectra were normalized to the 625 nm phycocyanin maximum. Isoprene accumulation was also measured as a function of time at 24-h intervals during culture incubation and cell growth (**d**)

Melis 2012. The present work contributed a practically important approach for *SkIspS* transformant growth and isoprene production under a combination of alkaline pH and salinity. A previous study has shown that *Synechocystis* is able to survive under higher than normal pH through the upregulation of many genes that help maintain the intracellular pH, including those encoding monovalent cation/anion antiporters that import protons (Summerfield and Sherman 2008). The ability to grow under alkaline pH is particularly important because high pH can substantially lower the number of contaminants, opportunistic microorganisms, and cyanobacterial grazers from invading and crashing a mass culture (McGinn et al. 2011). For example, many bacteria found in wastewater (e.g., *Escherichia coli*) have an upper pH tolerance limit of 9.2 (Parhad and Rao 1974) and thus would be unable to grow in the BG-11 pH 10 media. Wastewater is important in this respect, as it is rich in nutrients necessary and sufficient to support photosynthetic microorganism growth, and could be used as fertilizer supplement to seawater for commercial scale-up applications (Pittman et al. 2011), while at the same time providing a means of environmental remediation.

Further, the ability of the cyanobacteria to grow and produce in brackish water and saline environments, at or above seawater concentrations, provides an alternative to limited freshwater supplies for cultivation. It has been estimated that current microalgal scale-up processes could require between

Table 1 Chlorophyll and photosystem reaction center ratios in *Synechocystis SkIspS* transformant cells grown photoautotrophically in BG-11 medium (control) or in the presence of 100-, 200-, and 600-mM salinity

Parameter measured	BG11	BG-11+100 mM NaCl	BG-11+200 mM NaCl	BG-11+600 mM NaCl
Chl/P700	150±5	157±5	155±7	169±17
Chl/Q_A	672±95	908±27	863±37	924±88
PSI/PSII	4.5±0.7	5.8±0.2	5.6±0.4	5.5±0.8

The PSI/PSII photosystem stoichiometry was derived from the spectrophotometrically determined Chl/P700 (for PSI) and Chl/Q_A (for PSII) ratio of the respective samples, from which the PSI/PSII ratio was derived (Melis 1989)

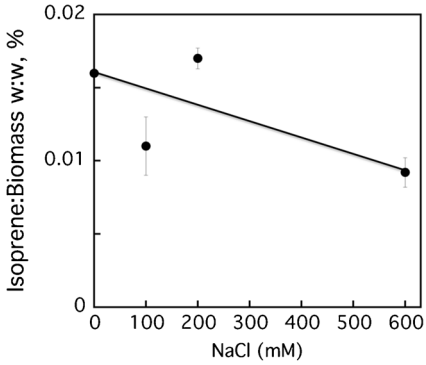

Fig. 8 Isoprene-to-biomass carbon-partitioning ratios. *SkIspS* transformant strains were grown at pH 10 without added salinity, and with 100, 200, or 600 mM NaCl for 96 h. The biomass and respective isoprene accumulation were measured and calculated for each growth condition. The amount of carbon partitioned to isoprene was calculated based on the weight of isoprene produced compared to the weight of biomass produced, assuming that approximately one half of the *Synechocystis* biomass is contributed by carbon

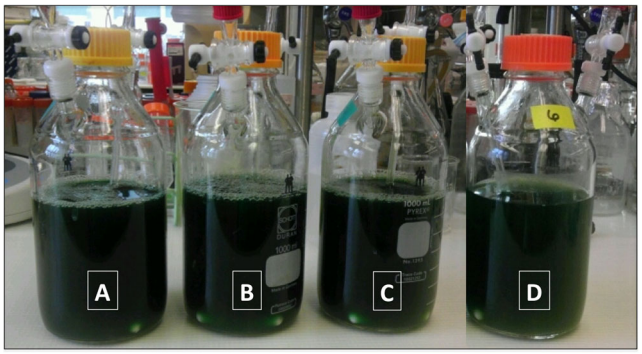

Fig. 9 Gaseous-aqueous two-phase reactors for the growth of *SkIspS* transformants under sealed conditions. The 500-mL headspace of the reactors was filled with 100 mL of 100 % CO_2 every 24 h. Isoprene accumulating in the headspace during photosynthesis of the cyanobacteria was quantified every 24 h, prior to refilling of the reactor with CO_2. The cultures shown were grown for 96 h at pH 10 without added salinity (**a**), and with 100 mM NaCl (**b**), 200 mM NaCl (**c**), or with 600 mM NaCl (**d**)

34 and 3,400 L of water per liter of biofuel produced, depending on the amount of evaporation and the water recycling methods applied (Cooney et al. 2011, Yang et al. 2011). Depending on the geographical region, cultivation of 10 billion gallons of microalgal cultures per year could potentially consume between 70 and 170 % of the total water supply destined for agricultural irrigation (Pate et al. 2011), indirectly raising the food versus fuel issue. Thus, the cultivation of cyanobacteria/microalgae for fuel and chemicals production will greatly benefit from the ability to grow these microorganisms in vastly abundant seawater.

Salinity tolerance has been shown in several strains of cyanobacteria to be due to the presence of Na^+/H^+ antiporters that work through the passive diffusion of protons under normal conditions, and active extrusion of Na^+ under high pH. The exposure to high-salinity environments has also been shown to upregulate expression of the putative gene (*sll*1864) coding for a Cl^- channel in *Synechocystis*. The synthesis under salinity stress and accumulation in the cell of compatible solutes, such as sucrose, glucosylglycerol, and glycine betaine, would help to compensate for the change in osmotic potential and to maintain cell turgor pressure (Hagemann 2011). Tolerance of *Synechocystis* to salinity would make the strain an even better candidate for commercial scale-up. The use of saline growth media, especially at NaCl concentrations equivalent to seawater (600 mM), would further minimize the need of freshwater for industrial-scale cultures, as seawater could be used in supplement with or instead of freshwater, brackish water, or wastewater.

The work also showed that a combination of alkalinity and salinity during growth did not impede the *SkIspS* strain of *Synechocystis* from their ability to generate isoprene. However, with the present state of the art, carbon partitioning to isoprene (Melis 2013) is modest and more work is needed to enhance the efficiency and yield of the isoprene production process. Bentley and Melis (2012) showed that the amount of photosynthetic carbon partitioned to isoprene production was 0.08 %, including the amount of isoprene that dissolves into the culture media (Henry's law). In this work, we estimated the isoprene-to-biomass carbon-partitioning ratio to be consistent with the earlier results from this lab. In this respect, it should be mentioned that recent studies have successfully employed metabolic engineering approaches to increase isoprene production, such as the overexpression of the rate-limiting enzymes in the methylerythritol (MEP) biosynthetic pathway in *E. coli* (Xiaomei et al. 2012; Zurbriggen et al. 2012) and the heterologous expression of the mevalonic acid biosynthetic pathway for isoprene production in cyanobacteria (Bentley et al. 2014). Further metabolic engineering approaches, in combination with growth in media optimized for contamination avoidance and materials sustainability, will provide a valuable approach for the photosynthetic production of isoprene.

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