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Heterologous β-phellandrene production by alginate immobilized *Synechocystis* sp. PCC 6803

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Abstract

The aim of the present work was the investigation of various cultivation conditions in order to provide a foundation for a sustainable, financially viable, and environmentally friendly cultivation system for the heterologous β-phellandrene production by *Synechocystis* sp. PCC 6803. *Synechocystis* cells were able to grow and form distinct colonies both at the internal and the external surfaces of calcium alginate beads and maintained their ability to produce β-phellandrene, in considerably higher amounts compared to suspension cultures, with maximum production after 6 days. Both immobilized and suspended *Synechocystis* cells exhibited a continuous and long-term ability to produce β-phellandrene, only by CO_2 addition, without renewal of the nutrients or the growth medium. However, photoheterotrophic growth of *Synechocystis*, with glucose as an alternative carbon source, had a negative impact on the heterologous production β-phellandrene. Despite the fact that cell growth and biomass accumulation were pronounced under photoheterotrophic growth conditions, β-phellandrene production was substantially decreased, indicating that this growth condition is not recommended for scale-up applications. Finally, combination of alkaline (pH 10.5) and saline (600 mM NaCl), i.e., extremophilic for *Synechocystis* growth conditions, proved to be amenable to cell growth and β-phellandrene production, albeit yields were a bit lower. The results provide new approaches for the development of larger scale, environmentally friendly, and financially viable cultivation systems for sustainable heterologous production of terpenoids by *Synechocystis*.

Keywords *Synechocystis* · β-Phellandrene · Terpenoids · Photoheterotrophic cultivation · Immobilized cells · Salinity

Introduction

Cyanobacteria are photosynthetic microorganisms that exhibit great adaptability since they inhabit in a wide range of environments, even in extreme conditions (Hitchcock et al. 2020). This property, in addition to a number of desirable features they possess, such as their versatile metabolic systems and their amenability to easy genetic modification as well as the advance in synthetic biology and metabolic engineering, has made cyanobacteria an ideal scaffold for the production of bio-based chemicals and fuels through photosynthesis and their associated metabolism (Melis 2012; Hendry et al. 2019; Lin and Pakrasi 2019). Among these products, our interest has focused on the heterologous β-phellandrene essential oil production by the cyanobacterium *Synechocystis* sp. PCC 6803. β-Phellandrene is a monoterpene, component of many plant essential oils, with a high demand in trade and industry. It can be used in pharmaceuticals, flavor, fragrance, personal-care products, cosmetics, household, and industrial supplies and has the potential to be used as an advanced biofuel (Bentley et al. 2013).

During the past few years, progress has been made through genetic and metabolic engineering in order to enhance β-phellandrene production in *Synechocystis*. More specifically, previous research has focused on improvements in photosynthetic efficiency as well as the direction of the metabolic flux towards the target terpene product (Melis 2017). Improvement of photosynthetic efficiency has been achieved by generation of *Synechocystis* transformants with a truncated light-harvesting antenna size (TLA strains), thus exhibiting enhanced sunlight utilization and productivity in high-density cultures (Kirst et al. 2014). Moreover, direction of the metabolic flux towards the target terpenoids has already been

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achieved by various ways. Firstly, the codon-optimized *Lavandula angustifolia* β-phellandrene synthase (*PHLS*) gene has been used (Bentley et al. 2013). This gene was expressed under the control of various strong promoters (e.g., the endogenous *psbA2* and *cpc* operon promoters, as well as the heterologous P*trc*) and the use of various translation initiation sites (Formighieri and Melis 2014). Furthermore, PHLS fusion constructs have been generated with highly expressed sequences in *Synechocystis*, such as the endogenous *cpcB* and the heterologous kanamycin (*nptI*) and chloramphenicol (*cmR*) sequences (Formighieri and Melis 2015, 2016; Betterle and Melis 2018, 2019). In addition, the geranyl diphosphate synthase (*GPPS*) gene as well as the enzymes of the mevalonic acid (MVA) pathway has been co-expressed along with the PHLS fusion constructs (Formighieri and Melis 2016; Betterle and Melis 2018, 2019).

However, progress that has been made in genetic engineering of *Synechocystis* is not enough for large-scale applications. This is due to the fact that large-scale systems face a variety of challenges not encountered in lab scale experiments. These challenges concern culture contamination by other microorganisms because a non-sterile medium is typically used for continuous cultivation in industrial scale applications (Chaves et al. 2015; Touloupakis et al. 2016a). Further challenges are the high biomass growth that is required for large-scale production of the desirable compounds (Yu et al. 2013), shading effects, control of biomass, and the use of substantial volumes of freshwater (Cooney et al. 2011; Pate et al. 2011; Yang et al. 2011). As a result, financially efficient and sustainable scale-up terpenoid generation requires the development of an approach where not only the genetic and metabolic engineering strategies but also the cultivation methods and environmental conditions will be taken into consideration.

Cell immobilization in calcium alginate beads possesses many advantages for large-scale cultivation over suspension cells. Alginate is a natural, nontoxic, inexpensive, transparent material that allows a better light penetration and thus alleviating shading effects. Alginate is a permeable material since it allows nutrient transport and metabolic product diffusion (Bucke and Brown 1983; Sun and Tan 2013; Maswanna et al. 2018). Immobilization protects the cells against adverse environmental factors and contaminations, facilitates biomass handling and harvesting, and leads to higher cell density cultures and enhanced productivities in smaller volumes (Duarte et al. 2013; Anjana and Kaushik 2014; Gao et al. 2016; Zur et al. 2016). Immobilized cells offer an alternative, easier, financially viable, and sustainable cultivation system for large-scale applications since they require smaller bioreactors, compared to suspension cells. Moreover, calcium alginate beads could be reused and, eventually, be dried and exploited as fertilizers (de Jesus et al. 2019; Touloupakis et al. 2016b).

It has been previously mentioned that *Synechocystis* cells are able to grow and produce isoprene under alkaline and saline growth conditions (Chaves et al. 2015). The use of water with alkaline pH and higher salinity for large-scale cultivation could prevent contaminations and alleviate concerns, since brackish and even seawater could be used instead of the limited freshwater (Chaves et al. 2015; Touloupakis et al. 2016a). Moreover, increased growth rates and higher biomass accumulation have been achieved through photoheterotrophic cultivation of *Synechocystis* that resulted in increased product yields (Chen 1996; Meireles Dos Santos et al. 2017). However, all previous work with cyanobacteria generating β-phellandrene and other similar plant essential oils was conducted in liquid-phase reactors. It is not known if cyanobacteria and the modified isoprenoid biosynthetic pathway, leading to monoterpenes production, could be viable with immobilized cells on solid substrate.

In the present work, cell growth and the long-term β-phellandrene production of suspended as well as immobilized *Synechocystis* cells in calcium alginate beads were compared. *Synechocystis* cell growth on the polymer matrix of alginate beads, as well as the cell release process, was studied by using scanning electron microscopy. In addition, the growth under photoautotrophic and photoheterotrophic cultivation conditions was examined. Finally, a combination of alkaline and saline growth conditions was applied. The aim of this study was to investigate various *Synechocystis* growth conditions in order to develop alternative, more sustainable, financially viable, and environmentally friendly cultivation systems for larger-scale applications.

Materials and methods

Strains and growth conditions

The cyanobacterium *Synechocystis* sp. PCC 6803, referred to as the wild type (WT) and its cpcB.PHLS + cpc(−cpcA), Δcpc + cpcB.PHLS + cpcA.GPPS transformants, containing the codon-optimized phellandrene synthase (*PHLS*) gene (Valsami et al. 2020) was used as experimental strains in the present study. All strains were maintained on 1% w/v agar BG-11 medium supplemented with 10 mM HEPES-NaOH buffer (pH 8.2), 0.3% w/v sodium thiosulfate, and 30 μg mL^{-1} chloramphenicol or 25 μg mL^{-1} kanamycin for the transformants. Liquid cultures were grown photoautotrophically in phosphate-buffered (25 mM KH_2PO_4, pH 7.5) BG-11 medium in a temperature-controlled chamber (28 °C), under constant aeration and at a light intensity of 50 μmol photons m^{-2} s^{-1}.

Culture preparation for β-phellandrene production

Inoculum cultures were centrifuged at 4,500 × *g* for 10 min, and the pelleted cells were resuspended in 50 mL of the desirable growth medium at an OD$_{730}$ = 0.5, in tightly capped 100 mL volume capacity glass bottles, with septa (diameter 5 cm, height

9.5 cm). Twenty milliliter of 100% carbon dioxide (CO_2) gas was flushed in the bioreactors. Cultures were incubated for 48 h at 28 °C and under 50 μmol photons m^{-2} s^{-1} and shaken at 140 rpm on a rotary shaker. β-Phellandrene was collected as described by Bentley et al. (2013). Briefly, 3 mL of hexane were added to the surface of the culture and incubated for 2 h on a rotary shaker. One microliter of the collected hexane layer was subjected to gas chromatography-mass spectrometry (GC-MS) analysis. Quantification analysis of the produced β-phellandrene was also performed by ultraviolet (UV) spectroscopy and application of the Beer-Lambert's law ($\varepsilon_{232,4\ nm}$ = 15.7 mM^{-1} cm^{-1}) (Formighieri and Melis 2014).

Cultivation of Synechocystis under photoheterotrophic conditions

Synechocystis cells were cultivated under photoautotrophic (20% CO_2) or photoheterotrophic conditions (addition of 5 mM glucose, without addition of CO_2). Experimental cultures were prepared as described in the previous section and were grown in a temperature-controlled chamber (28 °C) and light intensity of 50 μmol photons m^{-2} s^{-1}.

Cultivation of Synechocystis under different pH values and salinity conditions

In order to study growth and β-phellandrene production of Synechocystis cultures under neutral and alkaline conditions, BG-11 medium supplemented with 25 mM KH_2PO_4 (pH = 7.5) or 25 mM Na_2CO_3 (pH = 10.5) was used. Salinity conditions were created upon addition of NaCl to 600 mM in the BG-11 medium, pH = 10.5. Experimental cultures were prepared as described in the previous section and were grown in a temperature-controlled chamber (28 °C), under constant aeration and light intensity of 50 μmol photons m^{-2} s^{-1}.

Immobilization of Synechocystis in calcium alginate beads and β-phellandrene production

Calcium alginate beads with immobilized cpcB.PHLS + cpc(−cpcA) Synechocystis cells were prepared in three different alginate concentrations 3%, 4%, and 5% (w/v). Cells from the inoculum culture were collected at the late exponential phase by centrifugation at 4,500 × g for 10 min and resuspended in 5 mL BG-11 medium supplemented with HEPES-NaOH (pH 7.5) at an OD_{730} of 1. Cells were mixed with an equal volume of autoclaved sodium alginate solutions (Alginic acid sodium salt from brown algae, Fluka) with concentrations of 6%, 8%, and 10% (w/v), resulting in final alginate concentrations of 3%, 4%, and 5% (w/v), respectively, and cell concentration of an OD_{730} of 0.5. The suspensions of alginate solutions with the cells were added dropwise into a 2% (w/v) $CaCl_2$ solution, using a 1,000 μL Gilson pipette tip, to generate the calcium alginate beads. The generated beads were incubated into this solution, under gentle stirring for 1 h. Afterwards, the beads were washed twice with sterile deionized water and two more times with sterile BG-11 medium. Beads were transferred to 50 mL BG-11 medium, supplemented with HEPES-NaOH (pH 7.5), into the tightly capped glass bottles with septa. The bioreactors were then bubbled with 20 mL of 100% CO_2, sealed and incubated for 12 days at 28 °C under 50 μmol photons m^{-2} s^{-1} and shaking at 140 rpm on a rotary shaker. Experimental procedures were carried out in a laminar flow hood, in the presence of flame, previously sterilized with ethanol and UV light.

The β-phellandrene product was collected as described in the previous section, by adding a standard volume of hexane on the surface of the cultures, every 48 h. At the same time, 10 mL of 100% CO_2 were added to the bioreactors, to renew the CO_2 supply. Cultures of cpcB.PHLS + cpc(−cpcA) suspension and immobilized cells at 3% (w/v) alginate were examined, simultaneously, for a growth period of 12 days. In order to avoid adverse effects of hexane on the cultures, multiple separate bioreactors with suspended and immobilized cells were used for the β-phellandrene production, whereby each reactor was sampled once as a function of incubation time in the course of this experiment.

Scanning electron microscopy (SEM) of alginate beads

The morphology of immobilized colonies into calcium alginate beads, the mechanism of cell release, and the stability of the calcium alginate matrix during the experiment were investigated by scanning electron microscopy. Beads and suspension cells were prepared as described previously (Mavroudakis et al. 2019). Briefly, whole 3%, 4%, and 5% calcium alginate beads and thin incisions of them, immediately after their preparation (day 0) and after 12 days of their cultivation in BG-11 medium, were washed twice with 0.1 M sodium cacodylate buffer (SCB), pH = 7.4. Afterwards, beads were fixed with 2% w/v glutaraldehyde (GDA) and 2% w/v paraformaldehyde (PFA) in 0.08 M SCB for 45 min. After two washes with SCB, samples were consecutively washed with gradient concentrations of ethanol (30, 50, 70, 90, and 100% v/v) at 4 °C and finally washed with dry ethanol. Samples were then subjected to critical-point drying by using CO_2 (BALTEC, CPD 030 Critical Point Dryer) to complete the dehydration process and coated with gold (BAL-TEC, SCD 050 Sputter Coater). The samples were finally examined with a JEOL JSM-6390LV scanning electron microscope.

Determination of cell growth, biomass accumulation, and photosynthetic pigments

Cell growth and biomass accumulation were determined spectrophotometrically by measuring the optical density (OD) of the culture at 730 nm (OD_{730}) and also gravimetrically by

measuring the dry cell weight (DCW) of the cells. The quantification of chlorophyll *a* and carotenoids was performed using 100% methanol extraction according to established protocol (Lichtenthaler 1987) using a Shimadzu UV-2700 UV–vis spectrophotometer.

Photosynthetic activity measurements

Maximal photosynthetic activity (Pmax) was measured at 25 °C using a Clark type electrode system (YSI model 5300), as previously described (Valsami et al. 2020). Samples were illuminated with 500 μmol photons m^{-2} s^{-1}, and the infrared part of the applied irradiation was filtered off by using a 2% $CuSO_4$ solution in a 4 cm path length cuvette, placed between the actinic light source and the sample. Measurements were held under saturating carbon dioxide conditions, achieved by dissolving 0.034% w/v $NaHCO_3$ and 0.896% w/v tricine (pH 7.6) in the cell-containing medium (Delieu and Walker 1981). The rate of oxygen evolution was recorded continuously for a period of 1 min. Maximal photosynthetic activity was expressed in mmol O_2 (mol Chl)$^{-1}$ s^{-1} units.

GC-MS analysis

GC-MS analysis was conducted with an Agilent 6890 gas chromatograph equipped with a cool on-column injector, an Agilent 7683 automatic liquid sampler, and an Agilent 5973 inert mass selective detector. The chromatographic separation was achieved on a DB-5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies). Helium was used as a carrier gas at a flow rate of 1.2 mL min^{-1}. Oven temperature was initially maintained at 50 °C for 4 min, then increased to 150 °C at a rate of 4 °C min^{-1}, finally increased to 260 °C at a rate of 20 °C min^{-1} and held at 260 °C for 5 min. The mass spectrometer was operated in electron impact mode utilizing a 70-eV ionization energy, and the mass range scanned was 45–500 amu. Identification of the produced β-phellandrene by the *Synechocystis* cultures was based on comparison of its MS data and GC retention time with those of a commercially available standard, as previously described (Valsami et al. 2020).

Statistical analysis of the results

Each experiment was conducted at least three times comprising three independent replicates. The results reported here are the average value of all replicate measurements of each sample and their corresponding standard deviation (shown either in the tables or as error bars in the graphs). A statistical analysis by Student's *t* test was performed at each value compared to their respective control groups at control growth conditions. A $P < 0.05$ was considered statistically significant.

Results and discussion

Heterologous β-phellandrene production by *Synechocystis* sp. PCC 6803

Recent work from this and other labs (Valsami et al. 2020; Betterle and Melis 2019) has demonstrated the utility in cyanobacteria of fusion constructs as protein overexpression vectors. Case study was the fusion of the *L. angustifolia* (lavender) β-phellandrene synthase gene, as a downstream fusion with the highly expressed *cpcB* gene of *Synechocystis*, encoding the typically abundant β-subunit of phycocyanin in these photosynthetic microorganisms. The *cpcB.PHLS* gene fusion translated into substantial amounts of the corresponding CpcB.PHLS fusion protein, which retained the PHLS catalytic activity and resulted in the generation of substantial amounts of β-phellandrene hydrocarbons in these photosynthetic microorganisms.

In the present study, photoautotrophic and photoheterotrophic conditions with glucose as a supplementary carbon source were tested on WT and a *cpcB.PHLS* transformant. Cell growth and β-phellandrene productivity characteristics were measured. Biomass accumulation, measured either as OD_{730} or DCW, was comparable for WT and the cpcB.PHLS transformant (Table 1). However, biomass accumulation under photoheterotrophic conditions was remarkably higher than that of the respective photoautotrophic control in both strains. Photoheterotrophic growth caused a lowering in the photosynthetic pigment content (Table 1), measured either as chlorophyll (Chl) or carotenoid (Car). Under photoheterotrophic conditions, cells can directly assimilate carbon from glucose. Thus, the process of photosynthesis is downregulated, and the photosynthetic apparatus is diminished, including the abovementioned pigments, in order to conserve energy (Khan et al. 2016). Since the chlorophyll content is affected by the presence of glucose, the β-phellandrene yield was expressed on a DCW basis (Fig. 1). The results indicated that β-phellandrene production of photoheterotrophic *Synechocystis* cultures was lower compared to the control (Fig. 1). A decrease in productivity, down to about 40% of the control, is probably related to the fact that the heterologous production of β-phellandrene in *Synechocystis* is carried out through the methyl-erythritol-4-phosphate (MEP) pathway, the activity of which largely depends on the generation of photosynthetic compounds (carotenoids, chlorophylls, and quinones), the concentration of which was substantially diminished under photoheterotrophic conditions. In addition to the cpcB.PHLS + cpc(−cpcA) transformant, the Δcpc + cpcB.PHLS + cpcA.GPPS transformant was examined as well and exhibited similar results.

Although the use of an alternative carbon source, such as glucose, promoted cell growth and biomass accumulation, it adversely affected the production of β-phellandrene, essentially due to a substantially slower flux through the isoprenoid

Table 1 Cell growth, biomass, and photosynthesis characteristics of *Synechocystis* wild type (WT) and a *cpcB.PHLS* transformant, as well as β-phellandrene production under photoautotrophic (control) and photoheterotrophic growth conditions (5 mM glucose)

	WT	cpcB.PHLS
Photoautotrophic growth conditions		
OD_{730}	2.07 ± 0.05	1.99 ± 0.07
Biomass increase (times) (DCW_f/DCW_i)	2.49 ± 0.02	2.04 ± 0.08
Chl/OD_{730} (μg)	4.06 ± 0.03	2.80 ± 0.04
Car/OD_{730} (μg)	1.19 ± 0.01	1.63 ± 0.14
Pmax ($[mmol\ Chl]^{-1}\ s^{-1}$)	23.89 ± 1.20	32.90 ± 1.90
β-PHL (mg $[g\ DCW]^{-1}$)	ND	0.37 ± 0.03
Photoheterotrophic growth conditions		
Biomass increase (times) (DCW_f/DCW_i)	$3.12 \pm 0.03^*$	$3.31 \pm 0.25^*$
Chl/OD_{730} (μg)	$0.87 \pm 0.04^*$	$1.10 \pm 0.02^*$
Car/OD_{730} (μg)	$0.80 \pm 0.04^*$	$0.68 \pm 0.02^*$
β-PHL (mg $[g\ DCW]^{-1}$)	ND	$0.16 \pm 0.02^*$

All cultures were grown for 48 h at 28 °C, with a light intensity of 50 μmol photons m^{-2} s^{-1}. The results reported here as cpcB.PHLS are the average value of the cpcB.PHLS + cpc(−cpcA) and Δcpc + cpcB.PHLS + cpcA.GPPS transformants. Data are expressed as the average value of all replicate measurements of each sample and their corresponding standard deviation. Asterisk denotes that the values are significantly different from their respective control groups at photoautotrophic growth conditions (control growth conditions) ($^*P < 0.05$; Student's *t* test)

DCW, dry cell weight; *Chl*, chlorophyll; *Car*, carotenoids; *Pmax*, light-saturated rate of photosynthetic oxygen evolution; *β-PHL*, β-phellandrene

Fig. 1 β-Phellandrene production by photoautotrophic (control) and photoheterotrophic cultures of *Synechocystis* transformants measured as mg of β-phellandrene per g of dry cell weight that accumulated during the growth incubation period (phellandrene-to-biomass yield). The data correspond to measurements after 48 h incubation in BG-11 medium at 28 °C and under continuous illumination of 50 μmol photons m^{-2} s^{-1}. Asterisk denotes that the values are significantly different from their respective control groups at photoautotrophic growth conditions (control growth conditions) ($^*P < 0.05$; Student's *t* test)

biosynthetic pathway under these conditions. In addition to this drawback, the use of glucose or other organic carbon sources in mass cultures would increase the cost of the process as well as the risk of contamination by other microorganisms (Perez-Garcia and Bashan 2015). Thus, photoheterotrophic cultivation of *Synechocystis* for the heterologous production of β-phellandrene is not indicated for scale-up or for the calcium alginate beads experiments, which are outlined below.

Growth of *Synechocystis* immobilized on calcium alginate beads

Synechocystis cells were immobilized on calcium alginate beads. The color of the beads containing immobilized *Synechocystis* changed as a function of time from light to darker green, offering visual evidence of robust cell growth (Fig. 2a). Scanning electron microscopy images of calcium alginate beads were obtained immediately after bead formation and 12 days after cultivation in a BG-11 medium, in order to acquire information regarding cell growth and colony formation in the polymeric matrix. The bead diameter increased
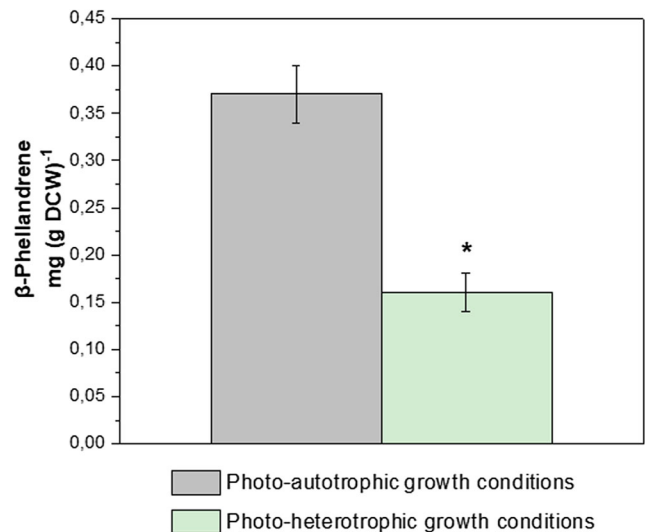
at higher alginate concentrations; it was about 2.37 and 2.66 mm for 3% and 5% (w/v) alginate, respectively (Fig. 2b).

Optical density (OD_{730}) measurements of the BG-11 medium, where immobilized cells were cultivated on alginate beads, revealed some cell release into the medium after 6 days of cultivation (Fig. 2c). Higher alginate concentrations (4% and 5%) resulted in considerably greater cell release, in contrast to 3% alginate beads that exhibited lower levels of cell release. However, even after 12 days of cell growth in a medium with 3% (w/v) alginate beads, the OD_{730} of the medium was about 0.4, namely, lower than that of the initial inoculum of OD_{730} of 0.5, suggesting that the majority of the cells remained immobilized on the alginate. This is supported by the fact that the BG-11 medium obtained a light green coloration after 6 days of cultivation, indicating the presence of *Synechocystis* cells (Fig. 2c). It is important to note that the OD_{730} measurements after the eighth experimental day were attributed not only to the released cells but to multiplication of released cells in the medium as well.

Comparative results on the effect of the alginate concentration on β-phellandrene production showed small differences in the yield of this product between the three different alginate concentrations tested (results not shown). However, after 2 days and up to 12 days of growth under the abovementioned conditions, immobilized cells exhibited particularly higher β-phellandrene production rates, almost 64% higher, compared to suspended cells in the same culture volume (Fig. 2d).
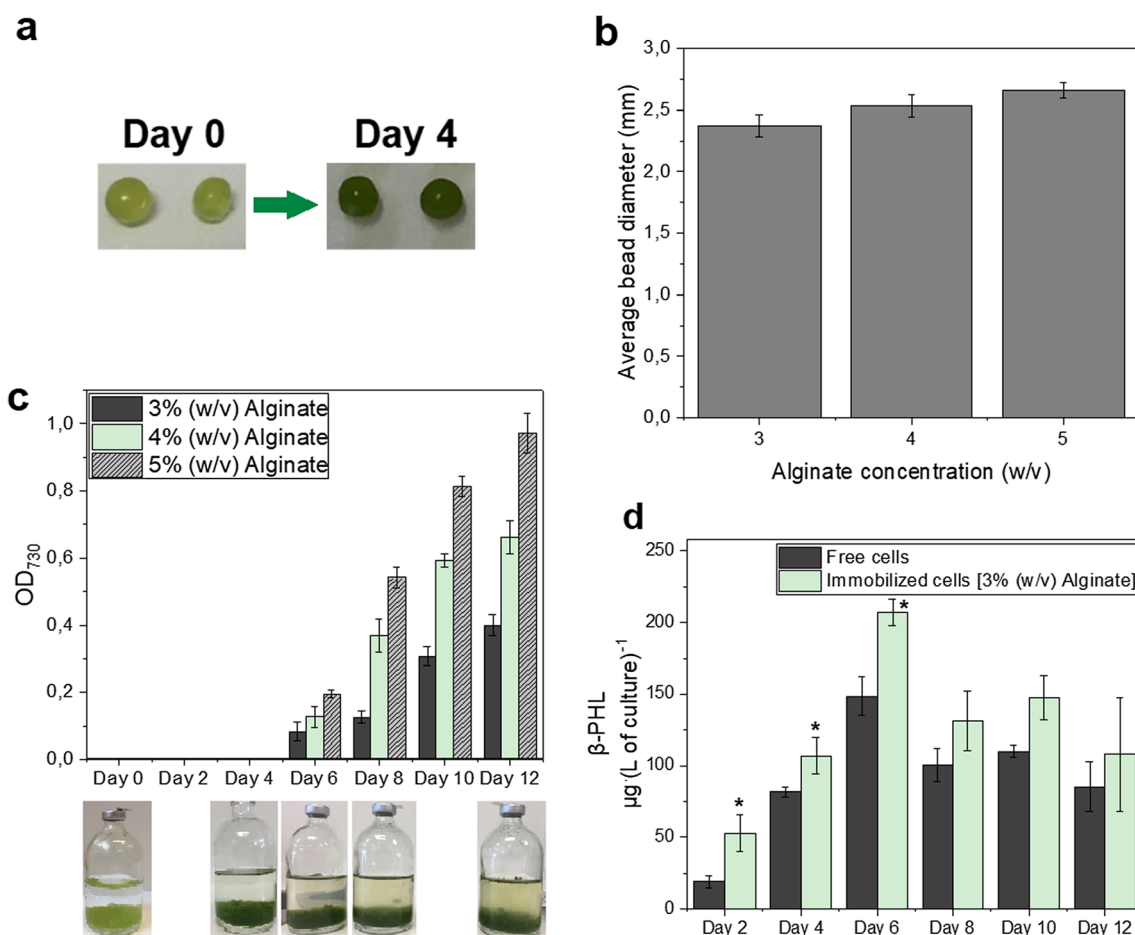
Fig. 2 (a) Calcium alginate beads with immobilized cpcB.PHLS + cpc(−cpcA) *Synechocystis* transformant cells. Beads immediately after their inoculation (experimental day 0) (left) and beads after 4 days cultivation in BG-11 medium supplemented with HEPES-NaOH (right) and 20% (v/v) CO_2, under continuous illumination of 50 μmol photons m^{-2} s^{-1}. (b) Average bead diameter of 3%, 4%, and 5% calcium alginate beads. (c) Optical density measurements at 730 nm (OD_{730}) of the BG-11 growth medium where the calcium alginate beads were cultivated, indicating the cell release from the bead surfaces to the medium. The bioreactors where the immobilized cpcB.PHLS + cpc(−cpcA) *Synechocystis* transformant cells were cultivated for 12 days are shown

below the graph. The BG-11 medium's greenish coloration at 6, 8, and 12 days of cultivation indicates the presence of suspension cells released from calcium alginate beads. (d) β-Phellandrene production by suspension and immobilized cpcB.PHLS + cpc(−cpcA) cells, after 2, 4, 6, 8, 10, and 12 days of cultivation expressed as μg β-phellandrene per liter of culture. The data correspond to measurements at the end of culture incubation in a BG-11 medium supplemented with HEPES-NaOH, at 28 °C and under continuous illumination of 50 μmol photons m^{-2} s^{-1}. Asterisk denotes that the values are significantly different from their respective control groups (free cells) at each time point ($^{*}P < 0.05$; Student's *t* test)

As shown in Fig. 3a–c, beads were spherical with a well-formed shape, regardless of the alginate concentration used. Fig. 3d–f illustrates the external surface and Fig. 3g–i the internal area of 3%, 4%, and 5% (w/v) alginate beads immediately after their preparation, at day 0. A random distribution of cpcB.PHLS + cpc(−cpcA) *Synechocystis* cells was observed on the external surface of beads after their immobilization, independent of the alginate concentration (Fig. 3d–f, cells pointed by arrows). In the beginning of the growth experiment, no cells could be observed in the interior of the beads (Fig. 3g–i). This could be attributed to the low cell concentration (OD_{730} = 0.5) used for bead inoculation. SEM images 12 days after cultivation revealed the formation of distinct colonies on the external (Fig. 4a–e), as well as the internal areas of the alginate beads (Fig. 4f). Immobilized cells on beads exhibited a similar pattern of

growth and colony formation in all three alginate concentrations used. Many protrusions were observed on the external surface of calcium alginate beads that correspond to the development of cpcB.PHLS + cpc(–cpcA) *Synechocystis* cell colonies (Fig. 4a, b). These colonies were enclosed in a thin membrane layer of the polymer matrix (Fig. 4c, d).

Colony formation on the external surface of beads was evident during cell growth. Cell division in the polymer matrix leads to colony formation. Colony formation exerted mechanical forces to the polymeric matrix as shown by a number of protrusions on the bead surface (Fig. 4b, d, and e). These lead to hole formation, resulting in cell release from the external surface of the beads to the medium (Fig. 4d, e). Furthermore, *Synechocystis* cells were able to grow and form colonies inside the beads, mainly into pores and crevices that formed within the matrix (Fig. 4f).
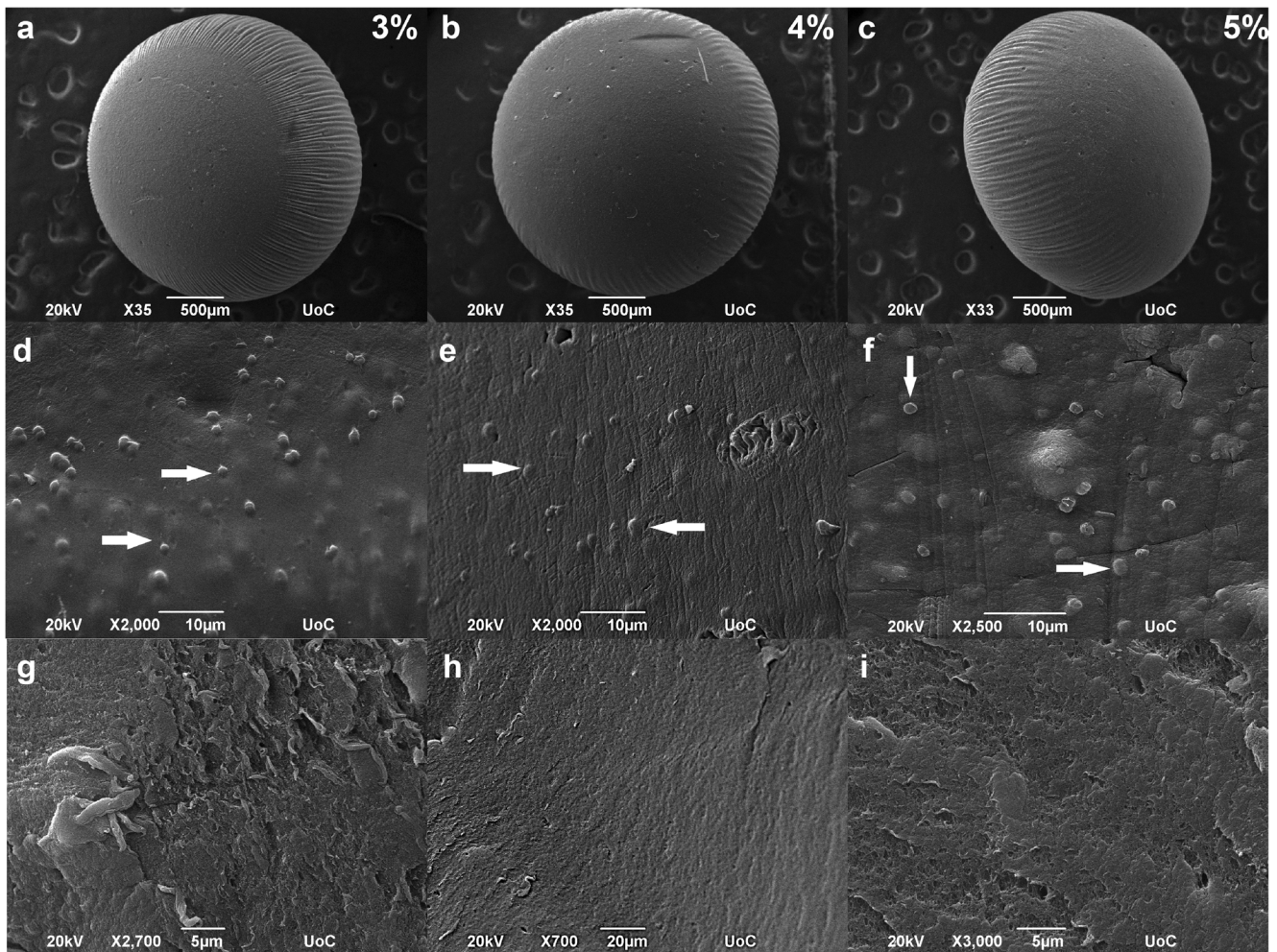
Fig. 3 Scanning electron microscopy images acquired at 20 kV electron voltage, immediately after bead preparation. Calcium alginate beads of (a) 3% alginate concentration at ×35 magnification, (b) 4% alginate concentration at ×35 magnification, and (c) 5% alginate concentration at ×33 magnification, (d–f) external surfaces of 3%, 4%, and 5% alginate beads, respectively. White arrows indicate immobilized cells. (g–i) Internal area of 3%, 4%, and 5% alginate beads, respectively

Scanning electron microscopy provided further information on the stability of calcium alginate beads in the BG-11 medium. It is known that cations such as magnesium and sodium in addition to cation chelating agents such as phosphate and citrate affect the stability of calcium alginate beads and cause polymer deterioration (Voo et al. 2011; de Jesus et al. 2019). However, in our experiments, the calcium alginate beads were not affected by the presence of low concentrations of phosphate, citric acid, and EDTA in the BG-11 medium and were stable in a BG-11 medium supplemented with HEPES-NaOH buffer, pH = 7.5, instead of KH_2PO_4, throughout the 12 day duration of the experiment.
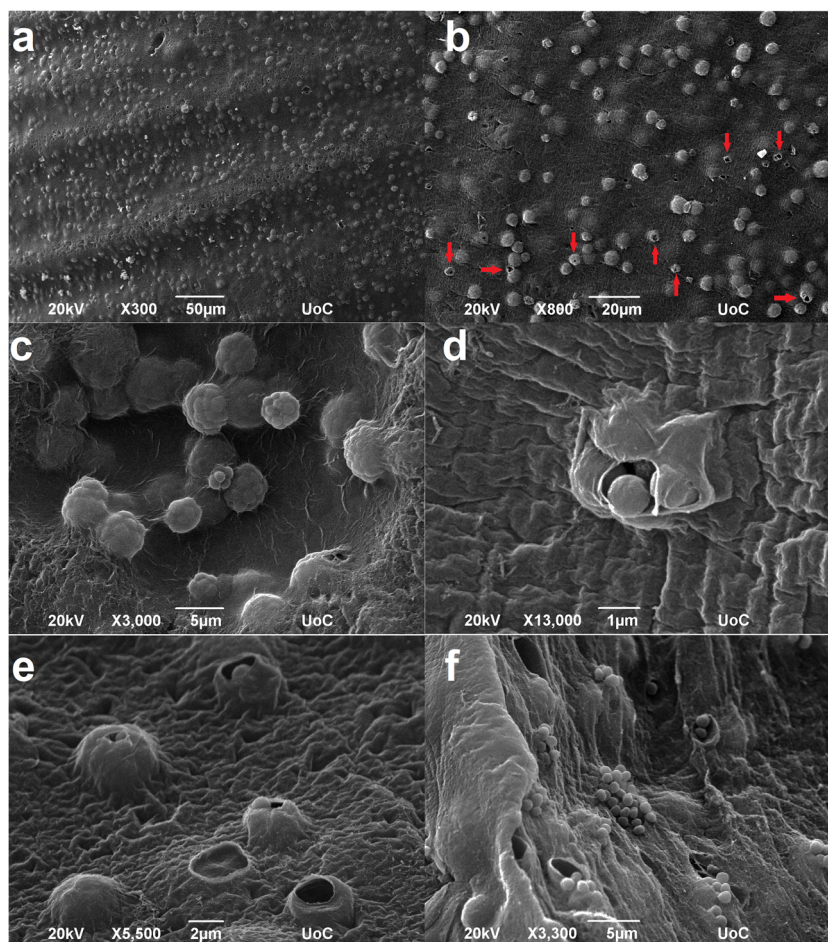
Long term β-phellandrene production by immobilized and suspension *Synechocystis* cells

Taking into account the cell release experiments, the 3% (w/v) alginate concentration was chosen for cpcB.PHLS + cpc(−cpcA)

Synechocystis immobilization in order to study the β-phellandrene production over time. The chromatographic profiles of hexane extracts of cpcB.PHLS + cpc(−cpcA) *Synechocystis* cultures showed that both suspended and immobilized cells were capable of producing β-phellandrene constitutively for 12 days (Figs. 2d and 5). The peak with a retention time of 12.10 min corresponds to β-phellandrene, as shown in the gas chromatogram of the commercial β-phellandrene standard, measured under the same GC operating conditions (see also Valsami et al. 2020). Both suspended (green colored trace) and immobilized cells (black line trace) exhibited continuous production of substantial amounts of β-phellandrene up to the sixth day of this experiment (Figs. 2d and 5).

Previous studies have focused on the β-phellandrene production by *Synechocystis* transformants measured after a fixed 48 h of cultivation (Formighieri and Melis 2014; Betterle and Melis 2018). However, Bentley et al. (2013) reported that *Synechocystis* transformants were able to produce β-

Fig. 4 Scanning electron microscopy images acquired at 20 kV electron voltage, after 12 days of beads cultivation in BG-11 medium supplemented with HEPES-NaOH, at 28 °C and under continuous illumination of 50 μmol photons m^{-2} s^{-1}. (a) External surface of 5% alginate bead at ×300 magnification. The protrusions indicate the formation of *Synechocystis* cell colonies. (b) External surface of a 3% alginate bead. Red arrows indicate the formation of bores at protrusion surfaces. (c) Presentation of the formation of *Synechocystis* cells colonies on the external surface of 4% alginate beads. (d) Presentation of cell release from protrusion bores of the external surface of 3% alginate bead to the medium. (e) External surface of 4% alginate bead. Presentation of the bores formation in protrusion surfaces and cell release to the growth medium from the external surface of 4% alginate bead. (f) Presentation of the formation of *Synechocystis* cell colonies in the internal of 3% alginate bead at ×3,300 magnification

phellandrene for 8 days, in four cycles of growth medium dilution with fresh nutrients in a bioreactor experiment. On the other hand, our study focused on the long-term β-phellandrene production by a single batch culture by addition of CO_2 only, without addition of extra nutrients or cell resuspension in fresh growth media. This is an important difference, taking into consideration that both suspended and immobilized cells have the capacity to produce β-phellandrene, by a single batch culture for a period longer than 48 h, with the maximal rate of production sustained through 6 days of growth. Interestingly, immobilized *Synechocystis* cells not only retained their capacity to produce β-phellandrene but also exhibited substantially higher production rates than suspension cultures throughout this experimental process (Fig. 2d). Many studies have previously shown that immobilization of photosynthetic microorganisms leads to increased growth rate and enhanced metabolic and photosynthetic activity. (Brouers and Hall 1986; de-Bashan et al. 2002; Singh 2003; Anjana and Kaushik 2014). A possible explanation for this are the changes in the microenvironment that the immobilized cells are subjected to that exhibit different properties compared to the growth medium where suspended cells grown (Bailliez et al. 1986; Brouers and Hall 1986). These properties are the chemical composition and the ionic strength, which probably concentrate numerous ions in the cell environment that stabilize the photosynthetic apparatus (Thomasset et al. 1984).

The use of immobilized *Synechocystis* transformant cell systems for the large-scale production of β-phellandrene or other terpenoid molecules possesses numerous advantages over suspension cell culturing systems. More specifically, substrate immobilization is a simple and economical technique (Duarte et al. 2013). Alginate is a natural polysaccharide, nontoxic for cells and stable in BG-11 medium for long periods of time. It allows cell development and β-phellandrene diffusion, while exhibiting limited cell leakage, especially in a 3% (w/v) alginate concentration. Immobilized cells in calcium alginate beads offer an easier way to handle and harvest the cell biomass, since cells grow in a controlled environment. As a result, smaller size and volume bioreactors would be required compared to that for suspension cultures. Furthermore, immobilization on a solid substrate allows reuse or the continuous utilization of the same cells, thus resulting in substantial higher product yields. Moreover, cell immobilization in calcium alginate beads offers protection from environmental factors as well as a defense against contamination by other microorganisms. Therefore, such systems could be applied to wastewater, resulting in their bioremediation and concomitant β-phellandrene production.
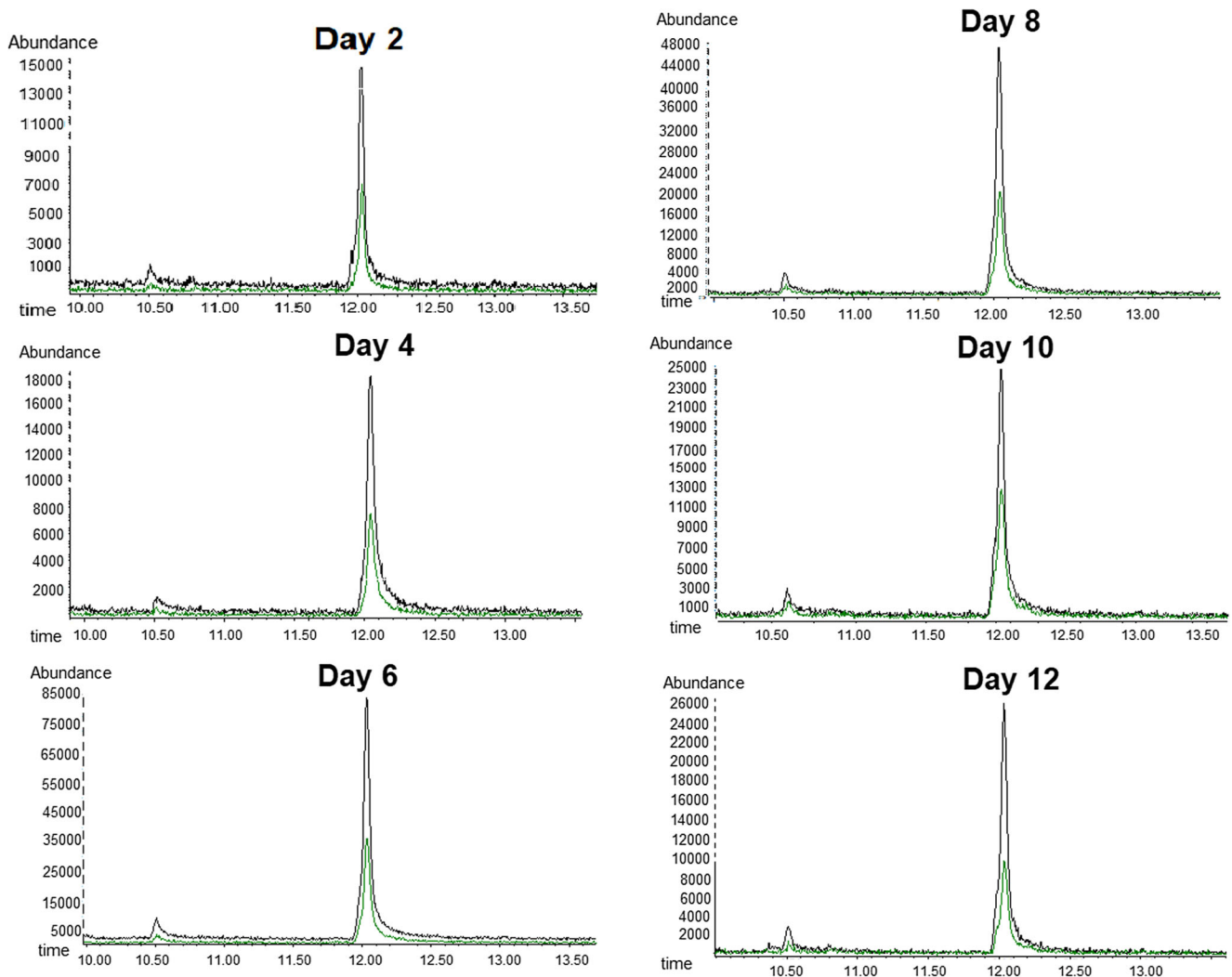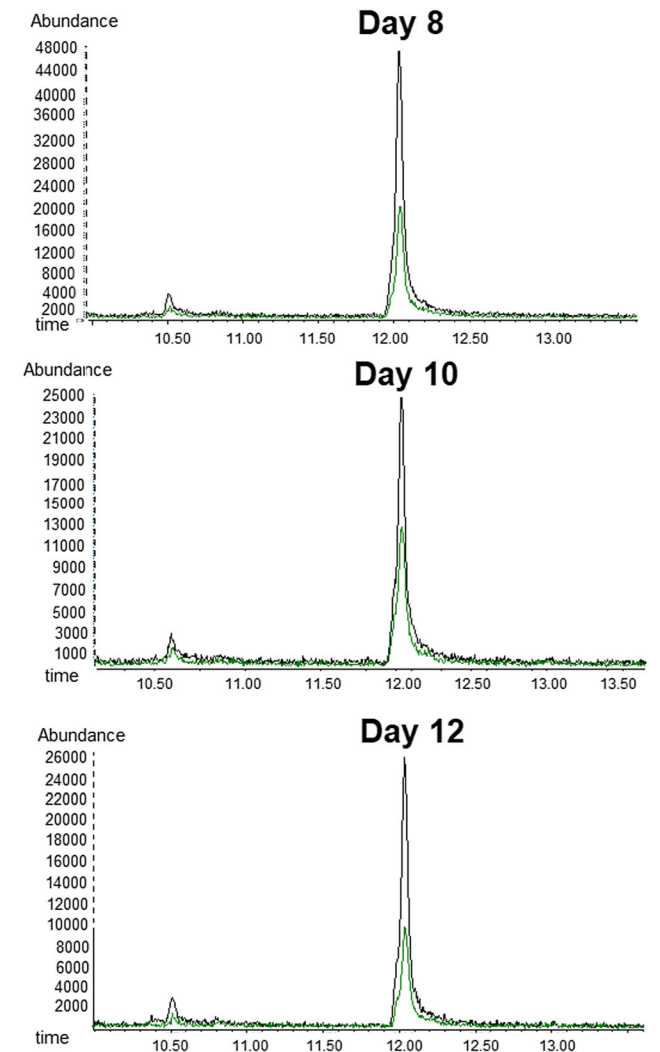
Fig. 5 Comparative gas-chromatographic profile analyses of hexane extracts from suspension (green traces) and immobilized (black traces) cpcB.PHLS + cpc(−cpcA) cells after 2, 4, 6, 8, 10, and 12 day incubation in BG-11 medium supplemented with HEPES-NaOH, at 28 °C and under continuous illumination of 50 μmol photons m^{-2} s^{-1}. The dominant peaks with a retention time of 12.10 min correspond to β-phellandrene

The polymeric matrix protects cells from possible toxic effects, while at the same time allowing the diffusion of nutrients and of the product (Zur et al. 2016). Overall, the above results offer the prospect of applying this approach to larger-scale bioreactors in order to generate products of high industrial value in an ever more renewable, financially viable, and environmentally friendly way.

Effect of alkalinity and salinity on the growth and β-phellandrene production by *Synechocystis* transformants

OD$_{730}$ measurements showed a slight decrease in *Synechocystis* WT and the cpcB.PHLS fusion transformant's growth between pH 7.5 (physiological) and pH 10.5 plus 600 mM NaCl (extremophilic condition, Table 2). The cpcB.PHLS transformant exhibited slightly slower growth compared to the WT strain, which was attributed to the subsaturating growth irradiance used (50 μmol photons m^{-2} s^{-1}), and the resulting truncated phycobilisome antenna size of the transformants, which limited the rate of photochemistry (Formighieri and Melis 2015, 2017). Cultivation under these extremophilic (salinity and alkalinity) growth conditions resulted in a slightly decreased photosynthetic pigment concentrations (chlorophyll *a* and carotenoids) compared to the control (Table 2). The β-phellandrene production yield was also lowered under these growth conditions. However, *Synechocystis* transformants maintained their ability to produce β-phellandrene, at lower yields (Table 2). In addition to the cpcB.PHLS + cpc(−cpcA) transformant, the Δcpc + cpcB.PHLS + cpcA.GPPS transformant was examined as well, yielding similar results.

The work showed that *Synechocystis* transformants were able to grow and produce β-phellandrene effectively under

Table 2 Cell growth, biomass, and photosynthesis characteristics of *Synechocystis* wild type (WT) and cpcB.PHLS fusion transformants, as well as β-phellandrene production under extremophilic growth conditions

	WT	cpcB.PHLS
Control pH 7.5 + 0 mM NaCl		
OD_{730}	2.07 ± 0.05	1.99 ± 0.07
Chl/OD_{730} (μg)	4.06 ± 0.03	2.80 ± 0.04
Car/OD_{730} (μg)	1.19 ± 0.01	1.63 ± 0.14
Pmax ([mmol Chl]$^{-1}$ s^{-1})	23.89 ± 1.20	32.90 ± 1.90
β-PHL (μg [mg Chl]$^{-1}$)	ND	22.79 ± 1.25
pH 10.5 + 600 mM NaCl		
OD_{730}	1.88 ± 0.01[*]	1.75 ± 0.01[*]
Chl/OD_{730} (μg)	3.56 ± 0.03[*]	2.62 ± 0.05[*]
Car/OD_{730} (μg)	1.07 ± 0.03[*]	1.22 ± 0.11[*]
Pmax ([mmol Chl]$^{-1}$ s^{-1})	21.25 ± 4.29	25.71 ± 1.41
β-PHL (μg [mg Chl]$^{-1}$)	ND	10.78 ± 1.76[*]

Cultures grown in BG-11 medium, pH 7.5 + 0-mM NaCl (control), and BG-11 medium pH 10.5 supplemented with 600 mM NaCl (pH 10.5 + 600-mM NaCl). All cultures were grown for 48 h at 28 °C and light intensity of 50 μmol photons m^{-2} s^{-1}. The results reported here as cpcB.PHLS are the average value of the cpcB.PHLS + cpc(−cpcA) and Δcpc + cpcB.PHLS + cpcA.GPPS transformants. Data are expressed as the average value of all replicate measurements of each sample and their corresponding standard deviation. Asterisk denotes that the values are significantly different from their respective control groups at control growth conditions (pH 7.5 + 0-mM NaCl) ([*] $P < 0.05$; Student's *t* test).

Chl, chlorophyll; *Car*, carotenoids; *P*, photosynthetic rate of oxygen evolution; *β-PHL*, β-phellandrene

physiological and extremophilic (salinity and alkalinity) conditions. *Synechocystis* fitness in alkaline pH is probably due to cellular adaptation that allows it to develop homeostasis and maintain the intracellular pH at normal levels. Such strategies are known to cause upregulation of many genes, including monovalent cation/anion antiporters (Summerfield and Sherman 2008; Chaves et al. 2015). The use of a medium with alkaline pH could prevent contaminations in mass cultures since many microorganisms do not have the capacity to tolerate higher pH (McGinn et al. 2011). Thus, the fact that *Synechocystis* growth and β-phellandrene production are not considerably affected by the alkaline pH is of great importance for their possible industrial exploitation (Touloupakis et al. 2016a). Although *Synechocystis* is a freshwater cyanobacterium, it is able to grow in saline environments (Chaves et al. 2015; Iijima et al. 2015). The mechanisms that *Synechocystis* has developed in order to acclimate in such environments and maintain the cell turgor pressure (like salt-tolerant microorganisms) include either uptake or synthesis and accumulation of compatible solutes such as glucosyl-glycerol and sucrose (Hagemann 2011; Pade and Hagemann 2015; Chaves et al. 2015; Kirsch et al. 2019). Furthermore, *Synechocystis*

possesses Na$^+$/H$^+$ antiporters that decrease intracellular Na$^+$ concentration, while at the same time increase K$^+$ concentration (Iijima et al. 2015). *Synechocystis* tolerance of high salinity would allow the use of brackish, seawater, and wastewater as alternatives for cultivation in industrial settings, where high volumes of water are required.

Overall, these results indicate that *Synechocystis* cells are able to grow and produce lower but notable amounts of β-phellandrene in a BG-11 medium at pH 10.5 in the presence of 600 mM NaCl. Despite the fact that the β-phellandrene production was slightly lower under alkaline and saline conditions, the capacity of *Synechocystis* transformants to synthesize the product is of great significance for a possible industrial exploitation. The importance stems from the fact that the entire cost of the procedure could be significantly lower by using brackish or seawater for mass cultivation of *Synechocystis*, instead of geographically limited potable water. Furthermore, the use of seawater protects cultures from certain bacterial and other microorganism contaminants. Thus, these growth conditions could be financially viable and environmentally friendly alternatives for the heterologous production of β-phellandrene by *Synechocystis* that do not raise water use and availability issues (such as competition for fresh water). By taking all these into account, it seems that the slightly lower product yield is compensated by the advantages arising from the use of brackish or seawater for large-scale *Synechocystis* cultures.

Conclusions

In the present study, the impact of various cultivation conditions on β-phellandrene production was investigated. The results of this study showed that *Synechocystis* was able to grow and form distinct colonies both at the internal and external surfaces of calcium alginate beads, producing remarkably higher β-phellandrene amounts, compared to suspension cell cultures, with maximum production after 6 days. Both immobilized and suspended cells had the capacity of a long term β-phellandrene production only by CO_2 addition, without the need of nutrient renewal or cell resuspension in a new medium. On the other hand, the photoheterotrophic growth of *Synechocystis* with glucose had a negative effect on the production of β-phellandrene, despite the fact that biomass accumulation in the culture was pronounced. Furthermore, *Synechocystis* transformants were able to grow under extremophilic (alkaline and saline) conditions, simulating those of seawater. At the same time, cells maintained their ability to produce β-phellandrene, though a decrease in β-phellandrene production was recorded. This study has demonstrated that immobilization of *Synechocystis* transformants in calcium alginate beads could provide an even more renewable, sustainable, financially viable, and environmentally

friendly alternative for the heterologous production of valuable molecules in industry. Cultivation of *Synechocystis* in a medium with higher salinity is promising, but more experiments should be carried out. On the other hand, photoheterotrophic cultivation of *Synechocystis* seems not to be suited for large-scale cultivations. The preliminary results and outcomes of the present work provide a foundation for the possible exploitation of *Synechocystis* transformants in large-scale terpenoid production.

Abbreviations *PHLS*, Phellandrene synthase; ***GPPS***, Geranyl diphosphate synthase; ***cpcB***, gene encodes the phycocyanin β-subunit; ***cpcA***, gene encodes the phycocyanin α-subunit; ***cpc* operon**, the operon that encodes the CpcB β- and CpcA α-subunits of phycocyanin and the associated linker polypeptides (CpcC2, CpcC1, and CpcD); ***cpcB.PHLS + cpc(-cpcA)*** , *PHLS* fused to the *cpcB* gene; presence of the remaining *cpc* operon genes minus the *cpcA* gene; **Δ*cpc* + *cpcB.PHLS + cpcA.GPPS***, *PHLS* fused to the *cpcB* gene and *GPPS* fused to the *cpcA* gene; absence of the remaining *cpc* operon genes

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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