Introduction of a green algal squalene synthase enhances squalene accumulation in a strain of *Synechocystis* sp. PCC 6803

Bagmi Pattanaik, Elias Englund¹, Nicholas Nolte, Pia Lindberg*

Department of Chemistry - Ångström, Uppsala University, Box 523, SE 75120, Uppsala, Sweden

**ARTICLE INFO**

**Keywords:** Cyanobacteria, *Synechocystis*, Metabolic engineering, Terpenoid biosynthesis, Squalene

**ABSTRACT**

Squalene is a triterpene which is produced as a precursor for a wide range of terpenoid compounds in many organisms. It has commercial use in food and cosmetics but could also be used as a feedstock for production of chemicals and fuels, if generated sustainably on a large scale. We have engineered a cyanobacterium, *Synechocystis* sp. PCC 6803, for production of squalene from CO₂. In this organism, squalene is produced via the methylerythritol-phosphate (MEP) pathway for terpenoid biosynthesis, and consumed by the enzyme squalene hopene cyclase (Shc) for generation of hopanoids. The gene encoding Shc in *Synechocystis* was inactivated (Δshc) by insertion of a gene encoding a squalene synthase from the green alga *Botryococcus braunii*, under control of an inducible promoter. We could demonstrate elevated squalene generation in cells where the algal enzyme was induced. Heterologous overexpression of genes upstream in the MEP pathway further enhanced the production of squalene, to a level three times higher than the Δshc background strain. During growth in flat panel bioreactors, a squalene titer of 5.1 mg/L of culture was reached.

1. Introduction

Cyanobacteria are microorganisms capable of oxygenic photosynthesis, utilizing atmospheric CO₂ as their carbon source and light as their source of energy. Biotechnological use of these organisms is attractive, as it enables sustainable production of solar fuels and renewable chemicals. In recent years, the tools and techniques available for genetic engineering and regulation of transgenic expression in cyanobacteria have developed rapidly (Vavitsas et al., 2019; Vijay et al., 2019). While the tools available, especially regarding control of gene expression, are not yet as versatile as those for *Escherichia coli*, it is now possible to do extensive engineering of several model strains of cyanobacteria. One of the most used strains is the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), which has been the focus of a number of studies aiming at metabolic engineering for photosynthetic product generation (Liu et al., 2019; Knoop et al., 2018; Angermayr et al., 2015).

In an earlier study, we generated a strain of *Synechocystis*, in which inactivation of the gene encoding squalene hopene cyclase, Shc (Δshc), resulted in accumulation of the 30-carbon oil squalene in the cells (Englund et al., 2014). Squalene is a triterpenoid molecule which in eukaryotes serves as a precursor for formation of sterols (Do et al., 2009).

In some bacteria, it is the precursor of hopanoids, complex pentaacyclic triterpenoids with a role in membrane fluidity and stability (Siedenburg and Jendrossek, 2011; Kanno and Poralla, 1999). Squalene can be produced from animal or plant sources, and has commercial use in nutrition, vaccines and cosmetics. However, if produced sustainably in large amounts, it could serve as a raw material for production of renewable chemicals and fuels, as it can be cracked to form smaller molecules suitable for different applications (Tracy et al., 2011).

The substrates for formation of all terpenoid compounds in cyanobacteria are made via the methyl-erythritol phosphate (MEP) pathway (Pattanaik and Lindberg, 2015) and references therein), which utilizes glyceraldehyde-3-phosphate (G3P) and pyruvate to form isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These two molecules are the building blocks for terpenes. Squalene is formed from IPP and DMAPP in several steps, combining them first into farnesyl diphosphate (FPP), a 15 carbon molecule. Two FPP units are then joined by the enzyme squalene synthase to form squalene (Fig. 1).

The green alga *Botryococcus braunii* has received attention due to its accumulation of hydrocarbon oils. Most of the oils produced by certain strains of *Botryococcus* are triterpenes, in the form of squalene and botryococcene (Okada et al., 1995), and squalene synthase from this...
organism may therefore be a promising enzyme to use for heterologous expression in another host strain for over-production of squalene. Several studies have addressed growth and harvest of Botryococcus oils, but such efforts are hampered by the inherent slow growth of the algae, and by the lack of genetic tools for this organism (Yoo et al., 2010; Brennan and Owende, 2010).

In the present study, we use the cyanobacterium Synechocystis for production of squalene, expressing a squalene synthase from Botryococcus braunii in combination with additional expression of genes from the MEP and terpenoid pathway in a Δshc background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for subcloning according to standard procedures. E. coli cells were grown in LB medium with addition of appropriate antibiotics (50 μg/ml kanamycin (Km), 100 μg/ml Ampicillin (Amp) or 100 μg/ml erythromycin (Em) (Sigma-Aldrich)) at 37 °C. Synechocystis cultures were grown at 30 °C under a light intensity of 20 μmol photons m−2 s−1 (except when otherwise noted) in BG11 medium (Stanier et al., 1971). Transformant strains were isolated and maintained by addition of appropriate antibiotics for selection (25 μg/ml Km, 25 μg/ml Em).

For squalene production experiments, liquid cyanobacterial cultures were grown in 30 ml of medium in 100 ml Erlenmeyer flasks on a rotary shaker under constant light. Fresh cultures were started at an OD750 of about 0.05. For induction of the PnrsB promoter, 2.5 μM NiCl2 (Merck) was added after two days at an OD750 of 0.1. For comparison of carbon and terpenoid pathway in a organism may therefore be a promising enzyme to use for heterologous expression in another host strain for over-production of squalene. Several studies have addressed growth and harvest of Botryococcus oils, but such efforts are hampered by the inherent slow growth of the algae, and by the lack of genetic tools for this organism (Yoo et al., 2010; Brennan and Owende, 2010).

In the present study, we use the cyanobacterium Synechocystis for production of squalene, expressing a squalene synthase from Botryococcus braunii in combination with additional expression of genes from the MEP and terpenoid pathway in a Δshc background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for subcloning according to standard procedures. E. coli cells were grown in LB medium with addition of appropriate antibiotics (50 μg/ml kanamycin (Km), 100 μg/ml Ampicillin (Amp) or 100 μg/ml erythromycin (Em) (Sigma-Aldrich)) at 37 °C. Synechocystis cultures were grown at 30 °C under a light intensity of 20 μmol photons m−2 s−1 (except when otherwise noted) in BG11 medium (Stanier et al., 1971). Transformant strains were isolated and maintained by addition of appropriate antibiotics for selection (25 μg/ml Km, 25 μg/ml Em).

For squalene production experiments, liquid cyanobacterial cultures were grown in 30 ml of medium in 100 ml Erlenmeyer flasks on a rotary shaker under constant light. Fresh cultures were started at an OD750 of about 0.05. For induction of the PnrsB promoter, 2.5 μM NiCl2 (Merck) was added after two days at an OD750 of 0.1. For comparison of carbon and terpenoid pathway in a organism may therefore be a promising enzyme to use for heterologous expression in another host strain for over-production of squalene. Several studies have addressed growth and harvest of Botryococcus oils, but such efforts are hampered by the inherent slow growth of the algae, and by the lack of genetic tools for this organism (Yoo et al., 2010; Brennan and Owende, 2010).

In the present study, we use the cyanobacterium Synechocystis for production of squalene, expressing a squalene synthase from Botryococcus braunii in combination with additional expression of genes from the MEP and terpenoid pathway in a Δshc background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for subcloning according to standard procedures. E. coli cells were grown in LB medium with addition of appropriate antibiotics (50 μg/ml kanamycin (Km), 100 μg/ml Ampicillin (Amp) or 100 μg/ml erythromycin (Em) (Sigma-Aldrich)) at 37 °C. Synechocystis cultures were grown at 30 °C under a light intensity of 20 μmol photons m−2 s−1 (except when otherwise noted) in BG11 medium (Stanier et al., 1971). Transformant strains were isolated and maintained by addition of appropriate antibiotics for selection (25 μg/ml Km, 25 μg/ml Em).

For squalene production experiments, liquid cyanobacterial cultures were grown in 30 ml of medium in 100 ml Erlenmeyer flasks on a rotary shaker under constant light. Fresh cultures were started at an OD750 of about 0.05. For induction of the PnrsB promoter, 2.5 μM NiCl2 (Merck) was added after two days at an OD750 of 0.1. For comparison of carbon and terpenoid pathway in a organism may therefore be a promising enzyme to use for heterologous expression in another host strain for over-production of squalene. Several studies have addressed growth and harvest of Botryococcus oils, but such efforts are hampered by the inherent slow growth of the algae, and by the lack of genetic tools for this organism (Yoo et al., 2010; Brennan and Owende, 2010).

In the present study, we use the cyanobacterium Synechocystis for production of squalene, expressing a squalene synthase from Botryococcus braunii in combination with additional expression of genes from the MEP and terpenoid pathway in a Δshc background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for subcloning according to standard procedures. E. coli cells were grown in LB medium with addition of appropriate antibiotics (50 μg/ml kanamycin (Km), 100 μg/ml Ampicillin (Amp) or 100 μg/ml erythromycin (Em) (Sigma-Aldrich)) at 37 °C. Synechocystis cultures were grown at 30 °C under a light intensity of 20 μmol photons m−2 s−1 (except when otherwise noted) in BG11 medium (Stanier et al., 1971). Transformant strains were isolated and maintained by addition of appropriate antibiotics for selection (25 μg/ml Km, 25 μg/ml Em).

For squalene production experiments, liquid cyanobacterial cultures were grown in 30 ml of medium in 100 ml Erlenmeyer flasks on a rotary shaker under constant light. Fresh cultures were started at an OD750 of about 0.05. For induction of the PnrsB promoter, 2.5 μM NiCl2 (Merck) was added after two days at an OD750 of 0.1. For comparison of carbon sources, 0.5% (wt/vol) glucose or 50 mM sodium bicarbonate (NaHCO3) with 20 mM HEPES was added to the cultures. Four days after induction with Ni2+, OD750 was recorded and cell samples were harvested by centrifugation for squalene and pigment analysis.

For the bioreactor experiment, 350 ml cyanobacterial cultures were grown in parallel in two FMT150 photobioreactors (Photon Systems Instruments) at 30 °C, with mixing and aeration supplied by compressed air and terpenoid pathway in a organism may therefore be a promising enzyme to use for heterologous expression in another host strain for over-production of squalene. Several studies have addressed growth and harvest of Botryococcus oils, but such efforts are hampered by the inherent slow growth of the algae, and by the lack of genetic tools for this organism (Yoo et al., 2010; Brennan and Owende, 2010).

In the present study, we use the cyanobacterium Synechocystis for production of squalene, expressing a squalene synthase from Botryococcus braunii in combination with additional expression of genes from the MEP and terpenoid pathway in a Δshc background.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Escherichia coli strain DH5α was used for subcloning according to standard procedures. E. coli cells were grown in LB medium with addition of appropriate antibiotics (50 μg/ml kanamycin (Km), 100 μg/ml Ampicillin (Amp) or 100 μg/ml erythromycin (Em) (Sigma-Aldrich)) at 37 °C. Synechocystis cultures were grown at 30 °C under a light intensity of 20 μmol photons m−2 s−1 (except when otherwise noted) in BG11 medium (Stanier et al., 1971). Transformant strains were isolated and maintained by addition of appropriate antibiotics for selection (25 μg/ml Km, 25 μg/ml Em).

For squalene production experiments, liquid cyanobacterial cultures were grown in 30 ml of medium in 100 ml Erlenmeyer flasks on a rotary shaker under constant light. Fresh cultures were started at an OD750 of about 0.05. For induction of the PnrsB promoter, 2.5 μM NiCl2 (Merck) was added after two days at an OD750 of 0.1. For comparison of carbon sources, 0.5% (wt/vol) glucose or 50 mM sodium bicarbonate (NaHCO3) with 20 mM HEPES was added to the cultures. Four days after induction with Ni2+, OD750 was recorded and cell samples were harvested by centrifugation for squalene and pigment analysis.

For the bioreactor experiment, 350 ml cyanobacterial cultures were grown in parallel in two FMT150 photobioreactors (Photon Systems Instruments) at 30 °C, with mixing and aeration supplied by compressed air
comparison to a squalene standard (Sigma-Aldrich) (Englund et al., 2014).

2.4. Determination of pigment content

Chlorophyll a and carotenoid contents in *Synechocystis* cells were determined using a method based on that of Chamovitz et al., (1993), as described (Englund et al., 2015). Briefly, cells were harvested by centrifugation and resuspended in 1 ml N,N-Dimethylformamide (Merck). Samples were incubated in darkness for 5 min, followed by centrifugation for 5 min at 17000 g. Pigment concentrations were calculated from the absorbance of the supernatant at 461 and 664 nm according to the following equations:

\[
\text{Chlorophyll [µg/mL]} = \frac{\text{OD}_{664}}{11.92}
\]

\[
\text{Carotenoids [µg/mL]} = \frac{\text{OD}_{461}}{0.046 \times \text{OD}_{664}}
\]

2.5. Western blotting

Total cell extracts of *Synechocystis* were separated on SDS-PAGE and blotted to PVDF membranes according to standard protocols. The membranes were incubated with primary antibodies mouse α-Flag (1:5000 dilution, Sigma-Aldrich) or rabbit α-AtpB (AS05085, 1:5000, Agrisera) followed by probing with the appropriate HRP-conjugated α-mouse or α-rabbit secondary antibodies (1:10000, Sigma-Aldrich). The probed membranes were then incubated with HRP substrate (Bio-Rad) to generate a signal detected by a ChemiDoc imaging system (BioRad).

2.6. RT-PCR

RNA was extracted from *Synechocystis* cells using TriReagent (Sigma-Aldrich) according to the manufacturer’s instructions, and contaminating DNA was removed from samples using DNAse I (ThermoFisher Scientific). cDNA was generated from 1 µg of RNA using the qScript cDNA synthesis kit (Quantabio, Beverly, MA, USA) and amplified using primers specific for BSS, CfDXS, Ecidi, EcispA, and atpA which was used as a control for equal loading of RNA (see Supplementary Table S2 for primer sequences). Each primer pair was tested on the template to determine the appropriate number of cycles to use, 28 cycles were used for each primer pairs.

2.7. Statistical analysis

Two-tailed Student’s t-test was carried out on the data accumulated from individual experiments for squalene and pigment content.

3. Results and discussion

3.1. Heterologous expression and activity of a Botryococcus braunii squalene synthase in *Synechocystis*

In previous work, we have constructed a strain of *Synechocystis* where inactivation of the gene shc encoding squalene hopene cyclase (see Fig. 1) led to accumulation of squalene in the cells (Englund et al., 2014). In order to enhance the production of squalene, we decided to investigate the effects of introducing additional genes into a Δshc strain of *Synechocystis*. In preliminary studies, over-expression of native genes including the native squalene synthase from *Synechocystis* (unpublished) did not result in increased squalene accumulation. We therefore decided to try expression of a heterologous squalene synthase, to potentially avoid regulation which may affect the native enzyme. *Botryococcus braunii* is a green alga which produces large amounts of botryococcenes, triterpene oils similar to squalene, and enzymes from *B. braunii* have also

![Fig. 2. Schematic overview of vectors used in the study. The fragment encoding Botryococcus squalene synthase (BSS) with Flag-tag was cloned in the control vector pEERM5' to generate pEERM5'_BSS. CfDXS, idi and ispA were inserted as an operon into the self-replicating plasmid pEEK to construct pEEK_dii.](image)

| Table 1 | Plasmids and strains used in this study. |
|---------|----------------------------------------|
| **Plasmid** | **Target location** | **Promoter** | **Expression of** | **Antibiotic resistance** |
| pEERM5' | shc | PnrsB | - | Em |
| pEERM5_BSS | BSS | BSS,Flag | PnrsB | Em |
| pEEK | replicating plasmid | Pvr2O | - | Km + Amp |
| pEEK_dii | replicating plasmid | Pvr2O | CfDXS, idi, ispA | Km + Amp |
| **Strains** | **Genotype** | **Antibiotic resistance** |
| *Synechocystis* WT | wild type *Synechocystis* PCC 6803 | none |
| Δshc:C | Δshc | Em |
| Δshc:BSS | Δshc:BSS,Flag | Em |
| Δshc:BSS_dii | Δshc:BSS,Flag, BSS,Flag + pEEK, CfDXS, idi, ispA | Em + Km |
been shown to form squalene (Okada et al., 2000; Niehaus et al. 2011, 2012). We selected one enzyme from *B. braunii*, the *Botryococcus* squalene synthase (BSS) (Okada et al., 2000) (accession no Q9SDW9) for expression in *Synechocystis*. The gene encoding BSS was codon optimized for *Synechocystis* and a Flag tag was added to the C-terminal end of the gene to facilitate detection of the protein. The construct was cloned in the vector pEERM5 (Fig. 2/Table 1) for insertion into the genome in the shc site. The nrsB promoter, inducible by addition of Ni²⁺ to the growth medium (Englund et al., 2016; Lopez-Maury et al., 2002), was placed in front of BSS to drive expression of the gene. The vector was transferred to the Δshc strain (Englund et al., 2014), completely replacing the inactive shc, to generate the strain Δshc:BSS. We also transformed the same strain with pEERM5, with all components except the tagged BSS, to generate the control strain Δshc:C. Transformant strains were isolated and complete segregation verified by PCR (data not shown).

Western blots were performed to verify expression of the Flag-tagged BSS in Δshc:BSS upon addition of Ni²⁺ (Fig. 3A). To determine levels of squalene, we grew cells of WT *Synechocystis*, the control strain Δshc:C, and Δshc:BSS in the presence and absence of Ni²⁺, and determined levels of squalene in the cells after 4 days (Fig. 3B). We found that the control strain accumulated 0.5 ± 0.1 mg squalene OD₅₅₀ L⁻¹, which, as expected, is similar to the levels observed in the previously investigated Δshc strain (Englund et al., 2014). There was no significant difference in squalene accumulation between cells without or with addition of Ni²⁺ in this strain. In Δshc:BSS without addition of Ni²⁺, squalene accumulation was somewhat higher compared to that of the control strain Δshc:C (p < 0.05, Fig. 3B), probably due to some leakiness of the nrsB promoter. Δshc:BSS cells grown with Ni²⁺ induction accumulated 0.95 ± 0.2 mg squalene OD₅₅₀ L⁻¹, demonstrating a highly significant increase (p < 0.001) of squalene accumulation upon induction of the inducer compared to the cells grown without inducer, as well as to the control strain Δshc:C cells (Fig. 3B).

### 3.2. Effects on carbon source and growth conditions on squalene production

Since the wildtype strain of *Synechocystis* used in this study is capable of mixotrophic growth on glucose, it was possible to investigate if there would be an effect on squalene production by growing the cells on different carbon sources. Δshc:BSS and the control strain Δshc:C were grown on BG11 medium with addition of 0.5% (wt/vol) glucose, or 50mM NaHCO₃ buffered at pH 7. Squalene accumulation was assessed 4 days after the addition of Ni²⁺ (Fig. 3B). For the control strain, addition of glucose or NaHCO₃ did not make a significant difference for the accumulation of squalene per cell. For Δshc:BSS, squalene accumulation was lower in both conditions compared to cells grown with only BG11 and induction by Ni²⁺ (Fig. 3B). However, cultures grown with addition of glucose accumulated more biomass, so that the total amount of squalene produced in these cultures was higher than in the other conditions after 4 days, reaching 2.6 ± 0.1 mg/L culture (data not shown). In cultures of Δshc:BSS grown with addition of sodium bicarbonate, the total amount of squalene was actually lower than on BG11 alone, and similar to the control strain (data not shown). In studies of production of other terpene compounds, it has been observed that the growth rate may affect the amount of terpene product generated per cell. The lower productivity towards squalene in cells grown with additional carbon sources observed here may be the result of faster growth of the cells, where more resources are directed towards formation of biomass and less towards the heterologous product. This is in agreement with an earlier study on isoprene production in *Synechococcus elongatus* (Gao et al., 2016), who found the highest rates of isoprene production in stationary phase cultures.

To further evaluate the potential for squalene accumulation in the Δshc:BSS strain, an experiment was performed where cultures of Δshc:BSS and Δshc:C were grown at higher density in lab scale photobioreactors. Squalene accumulation and growth were followed for 96 h at 30 μmol photons m⁻² s⁻¹, at which time light was increased to 50 μmol photons m⁻² s⁻¹, and squalene accumulation and growth was observed after another 96 h (Fig. 4). Growth was linear during the entire experiment for both strains, while squalene accumulation appeared to increase in the Δshc:BSS strain upon the increase in light intensity. The maximum concentration of squalene reached was 5.1 mg/L culture for the BSS expressing strain at the end of the experiment.

### 3.3. Expression of additional genes from the MEP pathway

Expressing BSS led to an increased accumulation of squalene in the cells. To enhance this further, we decided to add expression of genes from the MEP pathway. The first step of the pathway is the formation of deoxyxylulose-5-phosphate (DXP) from glyceraldehyde-3-phosphate and
pyruvate, catalyzed by the enzyme deoxyxylulose-5-phosphate synthase (DXS). In other studies, the action of DXS has been shown to be a limiting step in the generation of products from the MEP pathway (Englund et al., 2015, 2018; Harker and Bramley, 1999). Therefore, we decided to express an extra DXS in our squalene producing strain. The gene for expression was CfDXS from the plant Plectranthus barbatus (Coleus forskohlii) (GenBank ID KP899115 (Gnanasekaran et al., 2015)), truncated to remove a target peptide, as previously described (Englund et al., 2015). We also included expression of isopentenyl diphosphate isomerase from Escherichia coli (EcIDI), catalyzing the isomerization of isopentenyl diphosphate (IPP), and dimethylallyl diphosphate (DMAPP). This was based on the results from a study on isoprene production in Synechocystis, where expression of idi together with DXS lead to a combined effect on enhancing isoprene levels which was larger than that of either enzyme expressed alone (Englund et al., 2018). Furthermore, farnesyl diphosphate synthase from E. coli (EcispA) catalyzing formation of farnesyl diphosphate (FPP), the substrate for squalene synthesis, was included in the expression construct based on earlier reports identifying this enzyme as a potential bottleneck for the formation of FPP in E. coli. (Martin et al., 2003; Wang et al., 1999).

In order to be able to express these enzymes at a high level in our Synechocystis strain, we constructed an expression vector, pEEK, based on the previously reported pEEC1 but with kanamycin as selection (Englund et al., 2018). CfDXS, EcIDI, and EcispA were cloned into this vector as one operon, with expression driven by the strong promoter Prc2O to form the plasmid pEEK_dii (Fig. 2 and Table 1, see Supplementary Table S1 for exact sequence). pEEK_dii was transferred to Δshc:BSS by conjugation, to generate the strain Δshc:BSS_dii.

Expression of all heterologously introduced genes was verified by semi-quantitative RT-PCR (Fig. 5A). It was found that for BSS under control of PrnrB, expression was detectable without addition of Ni\textsuperscript{2+} in the medium, but strongly enhanced after addition of Ni\textsuperscript{2+}. For CfDXS, idi and ispA, under control of Prc2O and thus constitutively expressed, no differences in expression levels with or without Ni\textsuperscript{2+} were observed.

Squalene accumulation was determined in cells expressing the extra pathway genes in addition to the B. braunii BSS. Cells of Δshc:BSS_dii accumulated about 1.6 times as much squalene per cell compared to cells of Δshc::BSS upon induction with 2.5 μM Ni\textsuperscript{2+} (Fig. 5B), and three times as much squalene per cell as the Δshc:C strain, reaching a squalene content of 1.5 ± 0.2 μg squalene OD\textsubscript{540} L\textsuperscript{-1} 4 days after induction. While we cannot in this study separate the effect of each individual pathway enzyme on the production of squalene, the observed enhancement of squalene accumulation by over-expressing upstream MEP pathway genes is in accordance with other recent studies on enhancing the MEP pathway flux for production of terpenoids in cyanobacteria (Englund et al. 2015, 2018; Choi et al., 2016).

3.4. Effects on pigment content

Since squalene synthases use the same substrates as formation of carotenoids and the phytol tail of chlorophyll, we decided to also investigate the effect on pigments in the cells of expressing BSS and the upstream pathway genes (Fig. 6). In the strains expressing BSS, we found a significant decrease in the amount of carotenoids produced upon induction of BSS by addition of Ni\textsuperscript{2+}, compared to the Δshc:C control strain (Fig. 6A). There were no significant effects on chlorophyll content (Fig. 6B). We also looked at the combined amount of carotenoids and squalene (Fig. 6C). It is worth noting that in the Δshc:BSS strain, the amount of carotenoids and squalene combined does not increase when BSS is induced, and thus substrate appears to be shifted from carotenoids to squalene accumulation. In the Δshc:BSS_dii strain, there was no significant effect on pigment content compared to Δshc:C or Δshc:BSS strain by the constitutive overexpression of the selected MEP pathway genes (Fig. 6A and B). However, upon induction of BSS, this strain showed an overall increase of the combined amount of carotenoids and squalene compared to the same strain without induction, and an increase of about
60% compared to the Δshc:BSS strain (see Fig. 6C). Thus, the extra pathway enzymes allowed for a higher flux of substrate towards the product which was not available in the BSS strain.

4. Conclusions

In the present study, we have shown that heterologous expression of a squalene synthase from Botryococcus braunii can increase squalene accumulation in a Δshc strain of Synechocystis. Furthermore, we could attain an increased production by also expressing genes upstream in the pathway to squalene formation, reaching three times more squalene per cell compared to the Δshc:C control strain. Substrate was shuttled from generation of pigments towards production of squalene.

To improve yields of terpenoid products in cyanobacteria further, we will need to implement additional modifications to improve flux through the pathway. In a recent study, Choi et al. reported a squalene accumulation of 7.08 mg/L/OD730 in cultures of engineered Synechococcus elongatus PCC 7942. Their cells expressed dxs, idi and ispA from E. coli along two copies of overexpressed SQS from Saccharomyces cerevisiae and the cells were grown in a bioreactor with elevated CO2 levels (Choi et al., 2017). Gao et al. succeeded in attaining a yield of 1.26 g/L of isoprene from S. elongatus PCC 7942 in a long-term, continuous production experiment, by simultaneously overexpressing dxs and ispG and fusing Idi to an efficient isoprene synthase (Gao et al., 2016). Thus, there is potential for generation of terpenoid products from the MEP pathway with a high yield in cyanobacteria, however, more knowledge about the pathway and its regulation in cyanobacteria is needed. In the case of squalene, screening for squalene synthases with high intrinsic activity, and optimizing the expression constructs to achieve higher levels of the enzyme in the cell may also result in higher productivity. Engineering of the upstream carbon and co-factor supply may also be employed to enhance yields (Englund et al., 2018). Optimization of growth conditions and scale-up experiments will also be needed to assess the potential of large scale terpenoid production from cyanobacteria.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Bagmi Pattanaik: Conceptualization, Data curation, Investigation, Methodology, Validation, Visualization, Writing - original draft, Writing - review & editing.
Elias Englund: Investigation, Methodology, Writing - review & editing.
Nicholas Nolte: Investigation, Writing - review & editing.
Pia Lindberg: Conceptualization, Data curation, Supervision, Funding acquisition, Project administration, Writing - review & editing.

Acknowledgements

This work was supported by the Swedish Energy Agency [grant no 38334-1], the Carl Trygger Foundation [grant no CTS 11:266], and by the NordForsk Nordic Center of Excellence program NordAqua [project no 82845]. The authors would like to thank Christoph Howe for his help in setting up the bioreactors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00125.
References

Angermayer, S.A., Gorcs Rovira, A., Hellingswerf, K.J., 2015. Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends Biotechnol. 33 (6), 352–361. https://doi.org/10.1016/j.tibtech.2015.03.009.

Brennan, L., Owende, P., 2010. Biofuels from microalgae-A review of technologies for production, processing, and extraction of biofuels and co-products. Renew. Sustain. Energy Rev. 14 (2), 557–577. https://doi.org/10.1016/j.rser.2009.10.009.

Chamovita, D., Sandmann, G., Hirschberg, J., 1993. Molecular and biochemical characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturase is a rate-limiting step in carotenoid biosynthesis. J. Biol. Chem. 268 (23), 17348–17353.

Choi, S.Y., Lee, H.J., Choi, J., Kim, J., Sim, S.J., Kim, Y., Lee, T.S., Keasling, J.D., Wang, J.Y., Kwak, H.S., Lee, S.M., Um, Y., Kim, Y., Sim, S.J., Choi, J.I., Woo, H.M., 2016. Photosynthetic conversion of CO2 to farnesyl diphosphate-derived phytochemicals (amorpho-4,11-diene and squalene) by engineered cyanobacteria. Biotechnol. Biofuels 9, 202. https://doi.org/10.1186/s13068-016-0617-8.

Conlon, P.J., 1988. A short polypeptide marker sequence useful for recombinant protein identification and purification. Bio Technol. 6 (10), 1204–1210. https://doi.org/10.1038/3b1088-1204.

The iGEM registry of standard biological parts. http://parts.igem.org/.

Niehaus, T.D., Okada, S., Devarenne, T.P., Watt, D.S., Srivira, V., Chappell, J., 2011. Identification of unique mechanisms for triterpenoid biosynthesis in Botryococcus braunii. Proc. Natl. Acad. Sci. U. S. A. 108 (30), 12260–12265. https://doi.org/10.1073/pnas.1016223108.

Siedenburg, G., Jendrossek, D., 2011. Squalene-hopene cyclases. Appl. Environ. Microbiol. 77 (12), 3905–3915. https://doi.org/10.1128/AEM.01300-11.

The GEM registry of standard biological parts. http://parts.igem.org/.

Wang, C.W., Oh, M.K., Liao, J.C., 1999. Engineered isoprenoid pathway enhances photosynthetic carbon fixation and the effect of N-terminal modification of the green microalga Botryococcus braunii. J. Biotechnol. 72 (1–2), 171–184. https://doi.org/10.1016/S0168-1656(99)00049-7.

Yoo, C., Jun, S.Y., Lee, J.Y., Ahn, C.Y., Oh, H.M., 2010. Selection of microalgae for lipid production under high levels carbon dioxide. Bioresour. Technol. 101, S71–S74. https://doi.org/10.1016/j.biombioe.2010.11.027.