Advances in Experimental Medicine and Biology 1080

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Synthetic Biology of Cyanobacteria

Springer

10 Engineering Cyanobacteria for Photosynthetic Production of C3 Platform Chemicals and Terpenoids from CO_2

Jun Ni, Fei Tao, Ping Xu, and Chen Yang

Abstract

Recent years have witnessed a rising demand for bioproduced chemicals owing to restricted availability of petrochemical resources and increasing environmental concerns. Extensive efforts have been invested in the metabolic engineering of microorganisms for biosynthesis of chemicals and fuels. Among these, direct conversion of CO_2 to chemicals by photoautotrophic microorganism cyanobacteria represents a green route with incredibly potent. Cyanobacteria have been engineered for the production of numerous biofuels and chemicals, such as 2,3-butanediol, fatty acids, isobutyraldehyde, and *n*-butanol. Under the current condition, it might be initially wiser to produce chemicals with higher value or higher yield. Photosynthetic production of C3 platform chemicals could withdraw carbon close to fixation to maximize the pool of available carbon, thus achieving the strong production rates. Photosynthetic production of terpenoids is another good choice due to the higher value of these compounds. Here, we review recent advances in generating C3 chemicals and valuable terpenoids from cyanobacteria.

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W. Zhang, X. Song (eds.), *Synthetic Biology of Cyanobacteria*,
Advances in Experimental Medicine and Biology 1080,
https://doi.org/10.1007/978-981-13-0854-3_10

Keywords
Cyanobacteria · Photosynthetic production · C3 platform chemicals · Terpenoids

Recent years have witnessed a rising demand for bioproduced chemicals owing to restricted availability of petrochemical resources and increasing environmental concerns [1]. Extensive efforts have been invested in the metabolic engineering of microorganisms for biosynthesis of chemicals and fuels [2]. Among these, direct conversion of CO_2 to chemicals by photoautotrophic microorganism cyanobacteria represents a green route with incredibly potent [3]. Cyanobacteria have been engineered for the production of numerous biofuels and chemicals, such as 2,3-butanediol [4], fatty acids [5], isobutyraldehyde [6], and *n*-butanol [7]. Under the current condition, it might be initially wiser to produce chemicals with higher value or higher yield [8]. Photosynthetic production of C3 platform chemicals could withdraw carbon close to fixation to maximize the pool of available carbon, thus achieving the strong production rates [9]. Photosynthetic production of terpenoids is another good choice due to the higher value of these compounds [8]. Here, we review recent advances in generating C3 chemicals and valuable terpenoids from cyanobacteria.

10.1 Production of C3 Chemicals

C3 platform chemicals, such as glycerol and 1,3-propanediol (1,3-PDO), possess great potential for use as building blocks for the synthesis of numerous products including polymers, fuels, and biomaterials [10, 11]. These C3 chemicals can be synthesized from the central metabolite pyruvate or dihydroxyacetone phosphate (DHAP) in cyanobacteria (Fig. 10.1). The starting points pyruvate and DHAP are, respectively, only three steps and one step from carbon fixation, which led to the strong carbon flux to these products [9]. Moreover, perfect conservation of fixed carbon could be obtained in the photosynthetic production of C3 chemicals without decarboxylation steps. To date, C3 chemicals, including lactate, 3-hydroxypropionic acid (3HP), glycerol, 1,3-PDO, 1,2-propanediol (1,2-PDO), and dihydroxyacetone (DHA), have been successful synthesized directly from CO_2 [12, 13]. The researches that focused on the productions of C3 chemicals by engineered cyanobacteria were summarized in Table 10.1 and discussed subsequently.

10.1.1 Lactate

Lactate (LA) is a representative and versatile biochemical production; it could be used as a building block for biodegradable polylactic acid (PLA), a green alternative to petroleum-derived plastics [35]. Lactate is one of the most extensively studied chemicals produced from CO_2 by cyanobacteria. Isomeric form of LA can be

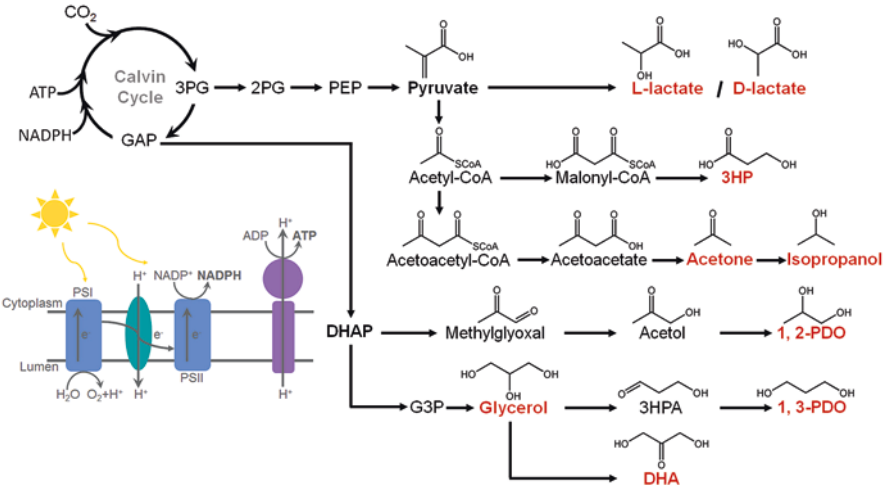

Fig. 10.1 Schematic of C3 platform chemical production pathways. These red texts in the figure are chemicals produced directly from CO_2 by engineered cyanobacteria

synthesized from the central metabolite pyruvate depending on the chiral-specific D- or L-lactate dehydrogenase (LDH) enzyme, and both isomers of lactate have been produced with high productivity by engineered cyanobacteria [13]. The first case of photosynthetic production of lactate by cyanobacteria was reported in 2010 [14]. *Synechococcus elongatus* PCC7942 was engineered to express D-LDH- and lactate transporter-encoding genes from *Escherichia coli*, and 55 mg/L D-lactate was produced and secreted by the engineered *S. elongatus* PCC7942 with the productivity of 13.8 mg/L/day [14]. In this case, the expression of lactate transporter was essential for D-lactate secretion [14]. Photosynthesis of L-lactate was first reported in an engineered *Synechocystis* sp. PCC6803 by expressing the L-LDH-encoding gene from *Bacillus subtilis*. The transhydrogenase was also expressed to convert NADPH produced by photosynthesis into available NADH, and the production of L-lactate reached 0.288 g/L [20]. Coexpression of L-LDH- and lactate transporter-encoding genes from *Lactobacillus plantarum* in *Synechocystis* sp. PCC6803 led to the secretion of L-lactate into the medium at concentration of 0.0153 g/L [21]. *Synechocystis* sp. PCC 6803 was engineered to express a mutated glycerol dehydrogenase to produce optically pure D-lactic acid from CO_2. The transhydrogenase was also expressed to improve the production of D-lactate to 1.14 g/L [15]. Moreover, the addition of acetate to the culture improved the yield of D-lactate to 2.17 g/L, which represented the highest production of lactate from cyanobacteria to date [15]. As an opposite approach, D-lactate dehydrogenase was engineered to reverse its favored cofactor from NADH to NADPH and introduced into *S. elongatus* PCC7942; thus the sufficient NADPH in cyanobacteria can be used for D-lactate formation [18]. Another strategy involves blocking two competitive pathways from the acetyl-CoA node,

Table 10.1 C3 targets, strains used, genes expressed, and titer

Chemical target	Strain	Gene(s) expressed	Gene knockout (s)	Titer (mg/L)	Productivity (mg/L/day)	Refs.
D-lactate	PCC 7942	*ldhA*, *lldP*, *udhA*		55	13.8	[14]
D-lactate	PCC 6803	*gldA101*, *sth*		1140	47.5	[15]
D-lactate	PCC 6803	*gldA101*, *sth*		1200	60	[16]
D-lactate	PCC 6803	*ldhD*	*pta*, *phaCE*	1060	265	[17]
D-lactate	PCC 7942	*ldhD*, *lldP*		829	82.9	[18]
D-lactate	PCC 7942	*mgsA*, *lldP*		1230	51.3	[19]
L-lactate	PCC 6803	*ldh/sth*		288	20.6	[20]
L-lactate	PCC 6803	*ldh*, *ldhP*		15.3	0.9	[21]
L-lactate	PCC 6803	*ldh*		1800	45	[22]
L-lactate	PCC 6803	*pk/ldh*		837	59.8	[23]
L-lactate	PCC 7002	*ldh*	*glnA*	795	199	[24]
Isopropanol	PCC 7942	*thl*, *atoAD*, *adc*, *adh*		26.5	2.9	[25]
Isopropanol	PCC 7942	*thl*, *atoAD*, *adc*, *adh*		146	9.7	[26]
Isopropanol	PCC 7942	*thl*, *atoAD*, *adc*, *adh*, *pta*		33.1	2.4	[27]
Acetone	PCC 6803	*cftAB*, *adc*	*phaCE*, *pta*	36	9	[28]
Glycerol	PCC 6803	*gpp2*		1068	62.8	[29]
Glycerol	PCC 7942	*gpp1*		1170	58.5	[30]
3-Hydroxypropionate	PCC 7942	*gpp1*, *dhaB*, *puuc*		31.7	3.2	[30]
3-Hydroxypropionate	PCC 7942	*mcr*, *msr*		665	41.6	[31]
3-Hydroxypropionate	PCC 6803	*mcr*, *accBCAD*, *birA*, *pntAB*	*phaB*, *pta*	837.2	139.5	[32]
Dihydroxyacetone	PCC 7942	*gpp1*, *dhaD*		78.6	4.9	[30]
1,2-Propanediol	PCC 7942	*sadh*, *yqhD*, *mgsA*		150	15	[33]
1,3-Propanediol	PCC 7942	*gpd1*, *hor2*, *dhaB123*, *gdrAB*, *yqhD*		288	20.6	[34]

and introducing a more efficient D-LDH was used to improve the production of D-lactate, and up to 1.06 g/L D-lactate was produced by the engineered *Synechocystis* sp. PCC 6803 [17]. The corresponding productivity (0.265 g/L/day) of D-lactate was the highest so far [17]. In addition, wastewater from anaerobic digest rich in organics, N and P, was supplemented into culture medium for photomixotrophic biosynthesis of D-lactate using engineered *Synechocystis* sp. PCC6803 [16]. Several metabolic engineering design principles were explored to improve light-driven L-lactic acid production from CO_2, including increasing the expression level of LDH, increasing the flux toward pyruvate, and decreasing the flux through the competing pathway. In the above case, 0.837 g/L of L-lactate was produced by the engineered *Synechocystis* sp. PCC6803 [23]. Recently, a novel D-lactate producing pathway was constructed in *S. elongatus* PCC7942 [19]. As shown in Fig. 10.2, D-lactate was directly produced from CO_2 using central metabolite DHAP via methylglyoxal instead of pyruvate [19].

10.1.2 Isopropanol

Isopropanol is a valuable fermentation product from certain microorganisms, which can be widely used as a solvent and dehydrated into the monomer of polypropylene [36]. Isopropanol can be synthesized from acetyl-CoA, and acetyl-CoA was derived from the central metabolite pyruvate in cyanobacteria. The production pathway for isopropanol begins with acetyl-CoA condensation to acetoacetyl-CoA by acetyl-CoA acetyl transferase (ACoAAT). Next, the acetoacetyl-CoA transferase (ACoAT) removes the CoA moiety to form acetoacetate. Acetoacetate is then irreversibly

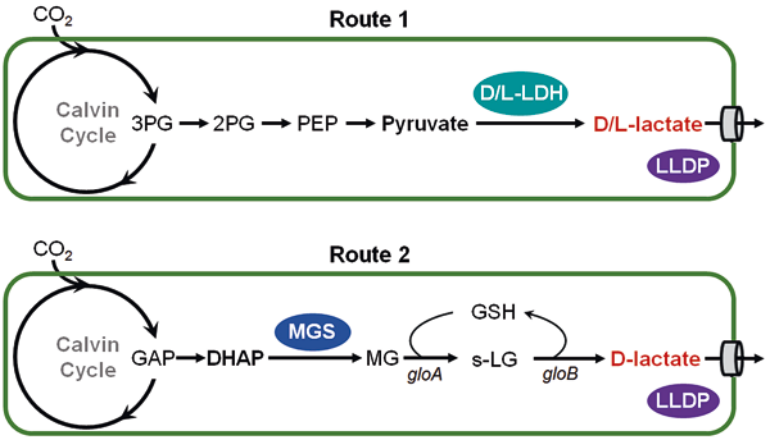

Fig. 10.2 Two pathways for synthesis of lactate from CO_2 in engineered cyanobacteria

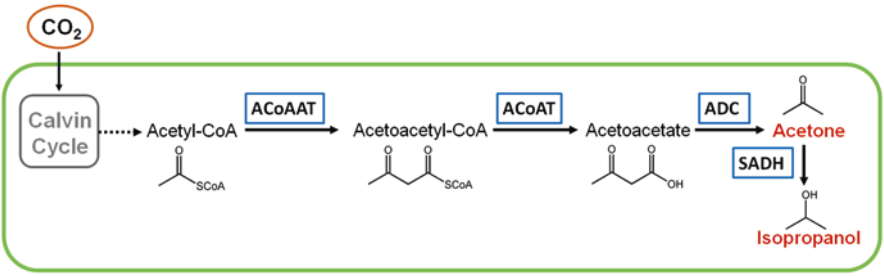

Fig. 10.3 Schematic of acetone and isopropanol production pathways in engineered cyanobacteria

decarboxylated into acetone by acetoacetate decarboxylase (ADC), which is subsequently reduced to isopropanol by the primary-secondary alcohol dehydrogenase (ADH) [37] (Fig. 10.3). In the first case of photosynthetic production of isopropanol by cyanobacteria, a synthetic pathway composed of ACoAAT, ACoAT, ADC, and ADH was constructed in *S. elongatus* PCC7942 [25]. The engineered cyanobacteria produced 26.5 mg/L of isopropanol under the optimized production conditions. After further optimization of the isopropanol-producing condition, including the use of cells in early stationary phase and buffering of the production medium to neutral pH, the titer of isopropanol was elevated to 146 mg/l [26]. In the subsequent case, the phosphate acetyltransferase (PAT)-encoding gene from *E. coli* was introduced to isopropanol-producing *S. elongatus* PCC7942 to achieve acetate production under photosynthetic conditions. And then the metabolic modified strain enabled production of 33.1 mg/l isopropanol and 12.2 mg/l acetone under photosynthetic conditions [27]. Another study uses the engineered *Synechocystis* sp. PCC 6803 to produce the precursor of isopropanol, acetone. Acetate-forming genes in *Synechocystis* sp. PCC6803 were disrupted to increase the pool of acetyl-CoA, and the titer of acetone was up to 36 mg/L [28].

10.1.3 Glycerol

As a commodity chemical, glycerol can be used as a solvent, lubricant, and humectant; moreover, it is a versatile building block in chemical synthesis and can be used as carbon source by many microorganisms [38]. Glycerol can be synthesized from the central metabolite DHAP in cyanobacteria by the endogenous glycerol-3-phosphate dehydrogenase (G3PDH) and exogenous glycerol-3-phosphatase (GPP) [39]. The expression of GPP2 from *Saccharomyces cerevisiae* in *Synechocystis* sp. PCC 6803 has yielded direct photosynthetic production of glycerol. Mild salt stress on the cells has improved the glycerol concentration to 1.068 g/L [16]. Another study also expressed the GPP1 in *S. elongatus* PCC7942, and the engineered strain YW1 accumulated glycerol to an extracellular concentration of 1.17 g/L [17]. And

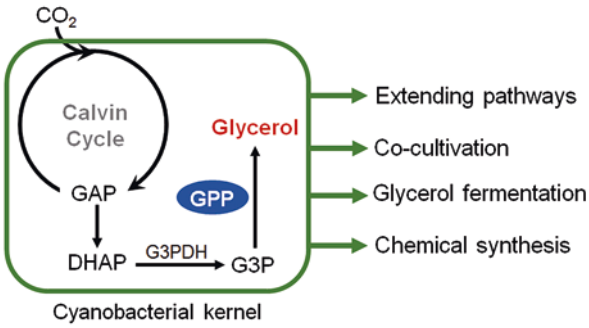

Fig. 10.4 Schematic of the production of chemicals from CO_2 with a genetically engineered cyanobacterium as the kernel

then the strain YW1 could serve as the kernel for the production of various C3 chemicals by extending heterologous pathways or co-cultivation with other microbes (Fig. 10.4). For example, the NAD^+-dependent glycerol dehydrogenase (GDH)-encoding gene was introduced to *S. elongatus* YW1 to extend the heterologous pathway, and up to 78.6 mg/L dihydroxyacetone (DHA) can be produced from CO_2 [17]. The cyanobacterial kernel displays great potential for carbon capture and storage and for sustainable production of various chemicals. The production of glycerol achieved in both studies is relatively high compared to other products. Besides the nontoxic effect of glycerol to cyanobacteria, naturally high flux to G3P and irreversible dephosphorylation contributed to the high titers.

10.1.4 3-Hydroxypropionate

3-Hydroxypropionate (3HP) is an important platform chemical with multiple applications. 3HP can be converted to several specialty chemicals, such as acrylic acid, acrylamide, 1,3-PDO, and poly-3-hydroxypropionate [40]. Glycerol can serve as a precursor to produce 3HP, and thus two enzymes were introduced to the glycerol-producing strain *S. elongatus* YW1 for the synthesis of 3HP. Glycerol dehydratase (GDHt) catalyzes the dehydration of glycerol to 3-hydroxypropionaldehyde, and aldehyde dehydrogenase (ALDH) catalyzes the oxidation of 3-HPA to 3HP [30]. Because the glycerol dehydratase used was oxygen sensitive, 3HP was only produced in dark anaerobic conditions with a titer of 31.7 mg/L in this case. *S. elongatus* PCC7942 was also engineered to synthesize 3HP from endogenous malonyl-CoA by using an alternative pathway. Expression of the malonyl-CoA reductase (mcr)- and malonate semialdehyde reductase (msr)-encoding genes enabled *S. elongatus* PCC7942 to synthesize 3HP to a final titer of 665 mg/L, which is tenfold higher than the glycerol-dependent pathway [31]. In the same study, a synthetic pathway was constructed by introduction of PEP carboxylase (Ppc), aspartate transaminase (AspC), aspartate decarboxylase (PanD), and β-alanine aminotransferase (SkPYD4) to produce 3HP via β-alanine. The engineered *S. elongatus* PCC7942 can produce 186 mg/L of 3HP [31]. These results indicated the importance of using oxygen-tolerant enzymes

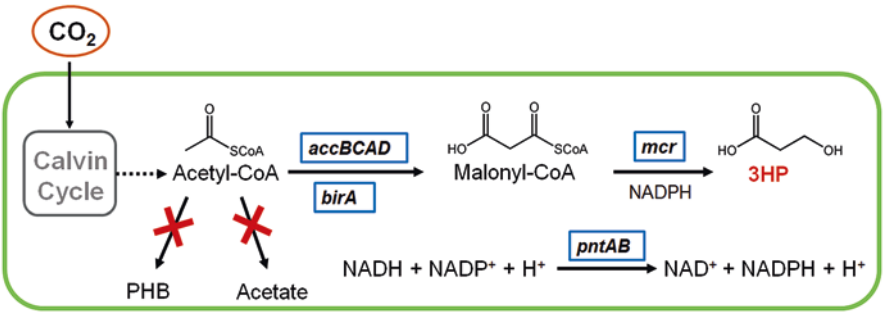

Fig. 10.5 Scheme of biosynthetic pathway of 3HP from CO_2 in engineered *Synechocystis* sp. PCC 6803 [32]. The malonyl-CoA reductase was encoded by gene *mcr*. Acetyl-CoA carboxylase was encoded by genes *accB*, *accC*, *accA*, and *accD*. Biotinilase was encoded by gene *birA*. The NAD(P) transhydrogenase was encoded by gene *pntA* and *pntB*

in cyanobacteria. Recently, the bifunctional alcohol/aldehyde dehydrogenase was introduced to *Synechocystis* sp. PCC 6803 for the production of 3HP (Fig. 10.5). After further optimization of the 3HP-producing system, including the use of different promoters, overexpression of acetyl-CoA carboxylase and biotinilase to enhance the supply of the precursor malonyl-CoA, overexpression of NAD(P) transhydrogenase to improve NADPH supply, and inactivation of the competing pathways of PHA and acetate biosynthesis, the titer of 3HP was elevated to 837.18 mg/L [32].

10.1.5 1,2-Propanediol and 1,3-Propanediol

1,2-PDO and 1,3-PDO are important chemical feedstocks. The racemic 1,2-PDO can be applied in the production of antifreeze, plasticizers, thermoset plastics, and cosmetics [41]; 1,3-PDO can be used as a monomer for polymer synthesis, such as the commercialized polytrimethylene terephthalate (PTT) [42]. By introduction of the methylglyoxal synthase (MGS), glycerol dehydrogenase (GLD), and aldehyde reductase (ADR), the engineered *S. elongatus* PCC7942 produced about 22 mg/L of 1,2-PDO [33]. Moreover, by using the NADPH-specific secondary alcohol dehydrogenases, the production of 1,2-PDO was elevated to 150 mg/L, and the accumulation of incomplete reduction product acetol was diminished [33]. 1,3-PDO can be synthesized from endogenous DHAP; a synthetic metabolic pathway comprising glycerol-3-phosphate dehydrogenase (GPD1), glycerol-3-phosphatase (HOR2), glycerol dehydratase (DhaB), and aldehyde reductase (YqhD) was constructed in *S. elongatus* PCC 7942 (Fig. 10.6). The highest titer of 1,3-PDO was up to 288 mg/L after 14 days of culture under optimized conditions [34].

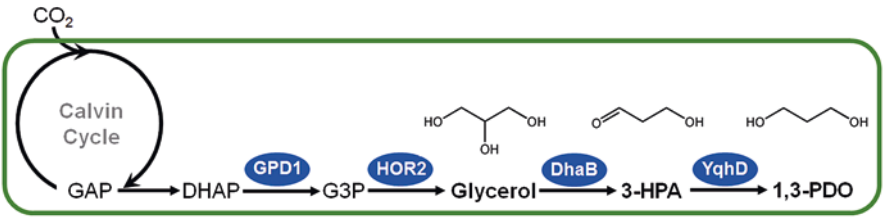

Fig. 10.6 The synthetic metabolic pathway for 1,3-PDO production in engineered *S. elongatus* PCC7942

10.2 Production of Terpenes

Terpenes are a large and diverse class of organic compounds, synthesized mainly by plants as secondary metabolites. Many terpenoids have been identified and used as natural pharmaceuticals, flavors, fragrances, agrochemicals, nutraceuticals, and, more recently, advanced biofuels. However, the current plant- and petrochemical-based supplies of terpenoids have major limitations. Cyanobacteria are an attractive host platform for terpenoid production because they streamline the solar-to-biochemical generation process. Synthetic biology and metabolic engineering have enabled the creation of cyanobacterial systems that directly convert CO_2 into various terpenoids (Table 10.2).

Terpenes are usually classified into groups according to the number of carbons: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), and tripenes (C30). Isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) are the universal building blocks of all terpenes. Two different metabolic pathways have evolved to generate IPP and DMAPP (Fig. 10.7). The first pathway is the mevalonate (MVA) pathway that is of archael/eukaryotic origin and utilizes acetyl-coenzyme A (acetyl-CoA) as the primary precursor. A second pathway is the methyl-D-erythritol 4-phosphate (MEP) pathway that is found in most bacteria, cyanobacteria, green microalgae, and plant plastids. The MEP pathway begins with the condensation of glyceraldehyde 3-phosphate (GAP) and pyruvate and undergoes a reductive isomerization reaction to form MEP. Subsequent coupling with CTP, phosphorylation, cyclization, and two reductive dehydration steps generate DMAPP and IPP, which can be interconverted through the action of IPP isomerase.

10.2.1 Hemiterpenes

The hemiterpene isoprene is an important commodity chemical used in a wide range of industrial applications, ranging from the production of synthetic rubber for tires, to use in adhesives and lubricants. Currently, isoprene is manufactured entirely from petrochemical sources. Isoprene can also be synthesized and released by a variety of plants including mosses, gymnosperms, and angiosperms, in response to

Table 10.2 Terpene synthesis from CO_2 in cyanobacteria

Terpenes	Host strain	Engineering methods	Cultivation conditions	Titer	Rate	References
Isoprene	*Synechocystis* sp. PCC 6803	Overexpressing codon-optimized IspS from *Pueraria montana*	25 °C, shift from low (10 μmol photons m^{-2} s^{-1}) to high light (500 μmol photons m^{-2} s^{-1})	NA	2.1 μg g^{-1} DW h^{-1}	[43]
	Synechocystis sp. PCC 6803	Overexpressing codon-optimized *IspS* from *Pueraria montana*	Gaseous/aqueous two-phase photobioreactor, bubbling of 500 mL 100% CO_2, 35 °C, 150 μmol photons m^{-2} s^{-1}, a diffusion-based process	350 μg L^{-1}	1.2 μg g^{-1} DW h^{-1}, 2 μg L^{-1} h^{-1}	[47]
	Synechocystis sp. PCC 6803	Overexpressing codon-optimized IspS from *Pueraria montana* and MVA pathway genes from *Enterococcus faecalis*, *E. coli*, *and Streptococcus pneumoniae*	Gaseous/aqueous two-phase photobioreactor, 35 °C, 150 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2	350 μg L^{-1}	1.3 μg g^{-1} DW h^{-1}	[43, 62]
	Synechocystis sp. PCC 6803	Overexpressing codon-optimized IspS from *Pueraria montana* and IDI from *Streptococcus pneumoniae*	Gaseous/aqueous two-phase photobioreactor, 35 °C, 150 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2 every 24 h	800 μg L^{-1}	18.8 μg g^{-1} DW h^{-1}	[46]
	Synechocystis sp. PCC 6803	Overexpressing fusion of IspS from *Pueraria montana* with CpcB protein	Gaseous/aqueous two-phase photobioreactor, 35 °C, 150 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2	2.5 mg L^{-1}	56.3 μg g^{-1} DW h^{-1}, 28.9 μg L^{-1} h^{-1}	[44]
	Synechococcus elongatus PCC 7942	Overexpressing fusion of IDI from *Saccharomyces cerevisiae* and codon-optimized IspS from *Eucalyptus globulus*, endogenous *DXS*, and *IspG* from *Thermosynechococcus elongatus*	Photobioreactor, 37 °C, 100 μmol photons m^{-2} s^{-1}, continuous aeration with 5% CO_2	1.26 g L^{-1}	1.7 mg g^{-1} DW h^{-1}, 4.3 mg L^{-1} h^{-1}	[45]

Limonene	*Synechocystis* sp. PCC 6803	Overexpressing codon-optimized LMS from *Schizonepeta tenuifolia*, endogenous DXS IDI, and GPPS	30 °C, 50 μmol photons m^{-2} s^{-1}, bubbling with 1% CO_2, gas tripping for limonene recovery	1 mg L^{-1}	2.3 μg L^{-1} h^{-1}	[48]
	Anabaena sp. PCC 7120	Overexpressing LMS from *Picea sitchensis*, DXS from *E. coli*, IDI from *Haematococcus pluvialis*, and GPPS from *Mycoplasma tuberculosis*	30 °C, 150 μmol photons m^{-2} s^{-1}, bubbling with air	521 μg L^{-1}	3.6 μg L^{-1} h^{-1}	[49]
	Synechococcus sp. PCC 7002	Overexpressing codon-optimized LMS from *Mentha spicata*	37 °C, 250 μmol photons m^{-2} s^{-1}, bubbling with 1% CO_2, dodecane overlay	4 mg L^{-1}	17.7 μg g^{-1} DW h^{-1}, 50 μg L^{-1} h^{-1}	[50]
	Synechococcus elongatus PCC 7942	Overexpressing codon-optimized LMS from *Mentha spicata*, DXS-III from *Botryococcus braunii*, IDI, and GPPS from *Abies grandis*	30 °C, 100 μmol photons m^{-2} s^{-1}, bubbling with 5% CO_2, HayeSep porous polymer absorbent for trapping limonene		92.2 μg g^{-1} DW h^{-1}	[51]
Pinene	*Synechocystis* sp. PCC 6803	Overexpressing a variant of PS from *Pinus taeda*	30 °C, 20 μmol photons m^{-2} s^{-1}, bubbling with 1% CO_2, cold trap for pinene recovery	100 μg L^{-1}	0.6 μg L^{-1} h^{-1}	[52]

(continued)

Table 10.2 (continued)

Terpenes	Host strain	Engineering methods	Cultivation conditions	Titer	Rate	References
β-Phellandrene	*Synechocystis* sp. PCC 6803	Overexpressing codon-optimized β-PHLS from *Lavandula angustifolia*	Gaseous/aqueous two-phase photobioreactor, 35 °C, 150 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2	0.2 mg L^{-1}	1.0 μg L^{-1} h^{-1}	[53]
	Synechocystis sp. PCC 6803	Overexpressing codon-optimized β-PHLS from *Lavandula angustifolia* under *P*trc promoter	Gaseous/aqueous two-phase photobioreactor, 35 °C, 170 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2	89.8 μg L^{-1}	5.2 μg g^{-1} DW h^{-1} 1.9 μg L^{-1} h^{-1}	[54]
	Synechocystis sp. PCC 6803	Overexpressing fusion of β-PHLS from *Lavandula angustifolia* with CpcB protein	Gaseous/aqueous two-phase photobioreactor, 35 °C, 170 μmol photons m^{-2} s^{-1}, bubbling 500 mL of 100% CO_2, hexane overlay	NA	66.7 μg g^{-1} DW h^{-1}	[55]
β-Caryophyllene	*Synechocystis* sp. PCC 6803	Overexpressing β-CAS from *Artemisia annua*	30 °C, 12.5 μmol photons m^{-2} s^{-1}	NA	0.3 μg L^{-1} h^{-1}	[56]
Bisabolene	*Synechococcus* sp. PCC 7002	Overexpressing codon-optimized BIS from *Abies grandis*	37 °C, 250 μmol photons m^{-2} s^{-1}, bubbling with 1% CO_2, dodecane overlay	0.6 mg L^{-1}	3.1 μg g^{-1} DW h^{-1} 6 μg L^{-1} h^{-1}	[50]
Farnesene	*Anabaena* sp. PCC 7120	Overexpressing codon-optimized FaS from *Norway spruce*	30 °C, 50 μmol photons m^{-2} s^{-1}, bubbling with 1% CO_2, resin column for farnesene recovery	305 μg L^{-1}	1.3 μg L^{-1} h^{-1}	[49]
Amorpha-4,11-diene	*Synechococcus elongatus* PCC 7942	Overexpressing ADS from *A. annua*, DXS, IDI, and FPPS from *E. coli*	30 °C, 100 μmol photons m^{-2} s^{-1}, bubbling with 5% CO_2, hexadecane overlay	19.8 mg L^{-1}	82.5 μg L^{-1} h^{-1}	[57]

13R-Manoyl oxide	*Synechocystis* sp. PCC 6803	Overexpressing diterpene synthases CfTPS2 and CfTPS3 from *Coleus forskohlii*, DXS from *C. forskohlii*	20 µmol photons m^{-2} s^{-1}	NA	4.7 µg g^{-1} DW h^{-1}	[58]
	Synechocystis sp. PCC 6803	Overexpressing diterpene synthases CfTPS2 and CfTPS3 from *Coleus forskohlii*	30 °C, bubbling with 3% CO_2, 50 µmol photons m^{-2} s^{-1}	2 mg L^{-1}	20.4 µg g^{-1} DW h^{-1}	[59]
Geranyllinalool	*Synechocystis* sp. PCC 6803	Overexpressing fusion of GLS from *Nicotiana attenuata* with CpcB protein	Gaseous/aqueous two-phase photobioreactor, 35 °C, 50 µmol photons m^{-2} s^{-1}, bubbling 100% CO_2, hexane overlay	NA	7.5 µg g^{-1} DW h^{-1}	[60]
Squalene	*Synechococcus elongatus* PCC 7942	Overexpressing SQS from *Saccharomyces cerevisiae*, DXS, IDI, and FPPS from *E. coli*	30 °C, 100 µmol photons m^{-2} s^{-1}, bubbling with 5% CO_2, hexadecane overlay	NA	64.8 µg g^{-1} DW h^{-1}	[57]
	Synechococcus elongatus PCC 7942	Overexpressing fusion of SQS *from Saccharomyces cerevisiae* with CpcB1 protein	Bag-type photobioreactor, 25 °C, 200 µmol photons s^{-1} m^{-2}, bubbling with 5% CO_2	NA	253.8 µg g^{-1} DW h^{-1}	[61]

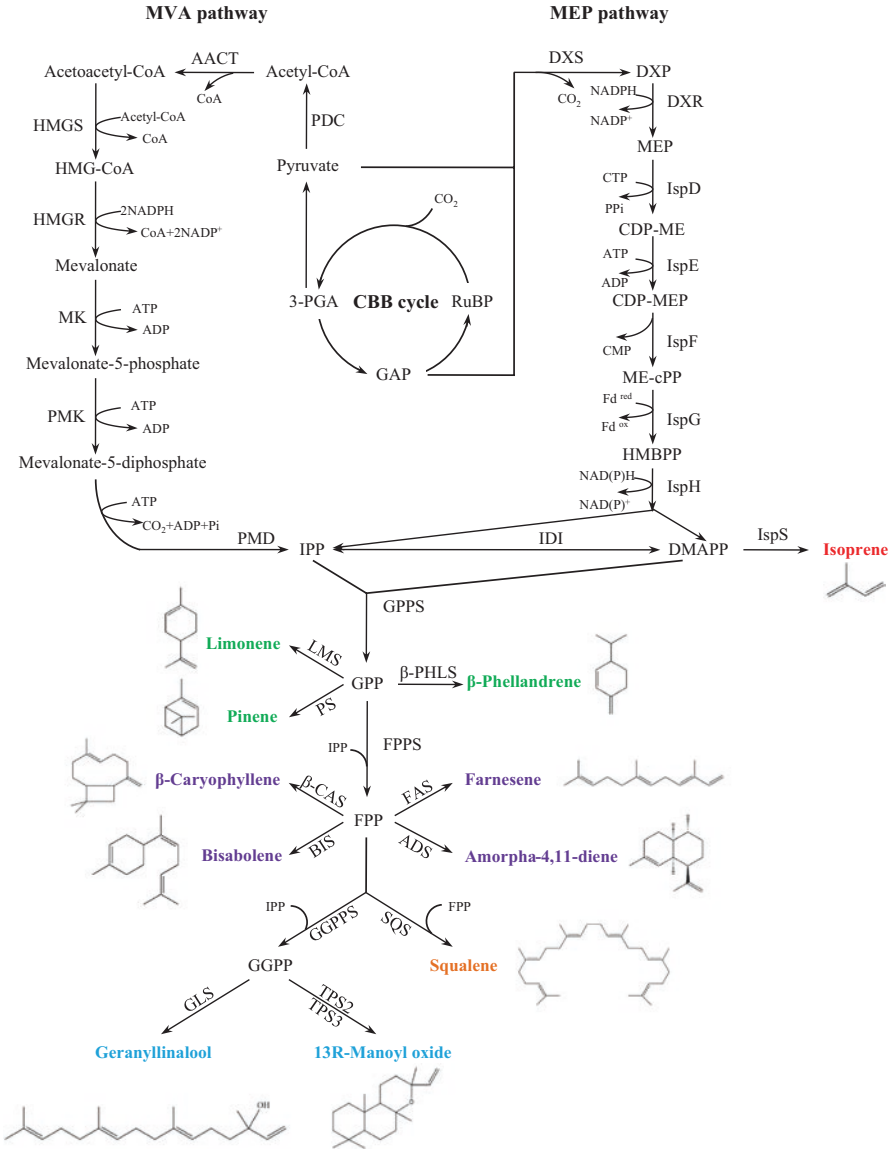

Fig. 10.7 Metabolic pathways for terpene production in cyanobacteria. Pathway intermediates: *HMG-CoA* 3-hydroxy-3-methylglutaryl-CoA, *3PGA* 3-phosphoglyceric acid, *GAP* glyceraldehyde 3-phosphate, *PYR* pyruvate, *DXP* 1-deoxy-D-xylulose 5-phosphate, *MEP* 2*C*-methyl-D-erythritol 4-phosphate, *CDP-ME* 4-diphosphocytidyl-2*C*-methyl-D-erythritol, *CDP-MEP* diphosphocytidylyl methylerythritol 2-phosphate, *MEcPP* 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate, *HMBPP* 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, *IPP* isopentenyl diphosphate, *DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate, *GGPP* geranylgeranyl pyrophosphate. Metabolic enzymes: *PDC* pyruvate dehydrogenase complex, *AACT* acetoacetyl-CoA thiolase, *HMGS* HMG-CoA synthase,

transient thermal stress. However, the collection of isoprene from plants is economically unfeasible. The need for an efficient and sustainable process prompted the efforts to develop a bio-based process for isoprene production using metabolically engineered microorganisms.

In plants, isoprene is synthesized from dimethylallyl pyrophosphate (DMAPP) by isoprene synthase. The transformation of codon-optimized isoprene synthase (IspS) gene from *Pueraria montana* (commonly known as kudzu) into *Synechocystis* sp. PCC 6803 enabled photosynthetic isoprene production at a rate of 4 μg L^{-1} h^{-1} with almost 0.1% of assimilated CO_2 partitioning as isoprene [43]. Increasing IspS expression by constructing fusion of IspS with the CpcB protein, the highly expressed β-subunit of phycocyanin, resulted in a 27-fold increase in isoprene yield [44]. Gao et al. (2016) introduced isoprene synthases from *Eucalyptus globulus* and *Populus alba* into *Synechococcus elongatus* PCC 7942. Overexpression of the isopentenyl pyrophosphate isomerase (IDI) and *P. alba* IspS enzyme fusions, especially the IDI-IspS fusion that possesses IspS at the C-terminus, increased isoprene production, which is likely due to the DMAPP channeling between the active sites of IDI and IspS [45].

The MVA and MEP pathways have been the targets of metabolic engineering efforts to increase the supply of DMAPP in cyanobacteria for improved isoprene production (Fig. 10.7). Heterologous expression of the MVA pathway in *Synechocystis* led to an increase in isoprene production by about 2.5-fold [62]. Gao et al. (2016) selected the MEP pathway for cyanobacterial isoprene synthesis. Overexpression of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in the MEP pathway had only a modest effect on isoprene production in *S. elongatus*, while overexpression of IDI markedly increased the isoprene production [45, 46]. Overexpression of IDI significantly increases the DMAPP/IPP ratio and eliminates the inhibition of isoprene synthase by IPP, thereby increasing the isoprene biosynthetic flux. These results highlight the importance of engineering a balance of DMAPP/IPP that is optimized for the synthesis of a specific terpenoid.

The 4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (IspG) was identified as a bottle enzyme of the MEP pathway by using the kinetic flux profiling approach [45]. Overexpression of IspG alleviated the flux limitation by IspG and increased isoprene production.

Isoprene is volatile and readily separated from microbial cells, thereby avoiding toxicity issues for cells and simplifying product recovery. A diffusion-based process for CO_2 uptake and product emission in gaseous/aqueous two-phase photobioreactors was used for isoprene production [47]. Long-term (21 days) continuous cultivation of *S. elongatus* strains with engineered MEP pathway resulted in the production

Fig. 10.7 (continued) *HMGR* HMG-CoA reductase, *MK* mevalonate kinase, *PMK* mevalonate-5-phosphate kinase, *PMD* mevalonate-5-diphoshate decarboxylase, *DXS* DXP synthase, *DXR* DXP reductase, *IspD* CDP-ME synthase, *IspE* CDP-ME kinase, *IspF* MEcPP synthase, *IspG* HMBPP synthase, *IspH* HMBPP reductase, *GPPS* GPP synthase, *FPPS* FPP synthase, *GGPPS* GGPP synthase, *LimS* limonene synthase, *PS* pinene synthase, *β-PHLS* β-phellandrene synthase, *β-CAS* β-caryophyllene synthase, *BIS* α-bisabolene synthase, *FAS* farnesene synthase, *ADS* amorphadiene synthase, *GLS* geranyllinalool synthase, *SQS* squalene synthase

of 1.26 g L^{-1} of isoprene from CO_2 and an average production rate of 4.3 mg L^{-1} h^{-1}, which is a significant increase for terpenoid production by photoautotrophic microorganisms [45]. The percentage of assimilated CO_2 partitioning toward isoprene synthesis reached about 40%. Interestingly, it was found that overproduction of isoprene allows enhancement in photosynthetic activity, which could be due to its continuous energy and carbon utilization (i.e., serving as a metabolic sink) that can alleviate the inhibition of photosynthesis.

10.2.2 Monoterpenes

Monoterpenes are C10 compounds built from two C5 isoprenoid units (one IPP and one DMAPP). They are currently harvested from plants and widely used in agricultural, food, pharmaceutical, and cosmetic industries. In addition, monoterpenes can be used as a supplement to liquid fuels. Cyanobacteria are usually unable to produce monoterpenes due to the lack of efficient geranyl diphosphate synthases (GPPS) and adequate monoterpene synthases.

Limonene and α-, β-pinenes are cyclic monoterpenes and widely used in fragrances and drugs and as commodity chemicals. Recently, the demand for these compounds has risen due to their suitability as renewable, high-density jet fuels. Limonene and pinene are synthesized from geranyl pyrophosphate (GPP) by plant-derived limonene synthase (LMS) and pinene synthase (PS), respectively.

Introduction of LMS from the medicinal herb *Schizonepeta tenuifolia* into *Synechocystis* sp. PCC 6803 enabled photosynthetic limonene production. Overexpression of the genes encoding DXS, IDI, and GPPS of the MEP pathway increased the limonene production by 1.4-fold and achieved a production rate of 2.3 μg L^{-1} h^{-1} [48]. The nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 was engineered to yield 521 μg L^{-1} limonene via heterologous expression of LMS from *Picea sitchensis* (Sitka spruce). The limonene production rate (3.6 μg L^{-1} h^{-1}) was increased about ninefold upon overexpression of DXS, IDI, and GPPS [49]. Heterologous expression of LMS from *Mentha spicata* in *Synechococcus* sp. PCC 7002 enabled photosynthetic limonene production at a rate of 50 μg L^{-1} h^{-1} with about 0.3% of assimilated CO_2 partitioning to limonene, resulting in a final titer of 4 mg L^{-1} limonene. A dodecane overlay on cultures enhanced limonene production [50]. By using a pea *psbA* promoter and synthetic ribosomal binding site (RBS) sequence to enhance LMS expression and overexpressing DXS, IDI, and GPPS, the limonene productivity reached 92.2 μg g^{-1} DW h^{-1} [51]. Heterologous expression of PS from *Pinus taeda* into *Synechocystis* sp. PCC 6803 led to photosynthetic pinene production. By using a PS variant that exhibited altered metal dependency, the pinene production was increased about twofold, yielding 100 μg L^{-1} of pinene from CO_2 [52].

The cyclic monoterpene β-phellandrene has commercial value as a key ingredient in medical, cosmetic, and cleaning products and potentially as a fuel. β-Phellandrene is synthesized from GPP by β-phellandrene synthase (β-PHLS) that is absent in cyanobacteria. *Synechocystis* sp. PCC 6803 was transformed with

β-PHLS from *Lavandula angustifolia*, which allowed photosynthetic production of β-phellandrene at a rate of 1.0 μg L^{-1} h^{-1} [53]. To increase the expression level of β-PHLS, the promoters *P*trc and 5′UTR of bacteriophage T7 gene 10 were used to drive gene expression of β-PHLS, leading to a 27-fold increase in β-phellandrene yield [54]. Alternatively, the expression of β-PHLS was increased by constructing fusion of β-PHLS with the CpcB protein, the highly expressed β-subunit of phycocyanin, which resulted in photosynthetic production of β-phellandrene at a rate of 66.7 μg g^{-1} DW h^{-1} [55].

10.2.3 Sesquiterpenes

Sesquiterpenes are synthesized from the condensation of one IPP monomer to the GPP molecules to form farnesyl diphosphate (FPP). The bicyclic sesquiterpene β-caryophyllene has been used in the fragrance and cosmetic industry traditionally. Extraction of β-caryophyllene often requires large amounts of plant biomass. Heterologous expression of β-caryophyllene synthase (β-CAS) from *Artemisia annua* was demonstrated in *Synechocystis* sp. PCC 6803, and photosynthetic production of β-caryophyllene from CO_2 was observed [56].

The monocyclic sesquiterpene bisabolene is used in fragrances, and its hydrogenation product has been proposed as a promising diesel replacement. *Synechococcus* sp. PCC 7002 was transformed with α-bisabolene synthase (BIS) from *Abies grandis*, yielding 0.6 mg L^{-1} of α-bisabolene from CO_2. This is equivalent to 0.06% of assimilated carbon partitioning as α-bisabolene. A dodecane overlay on cultures enhanced bisabolene production [50].

Farnesene is an acyclic sesquiterpene and used for the fragrance, flavoring, and pharmaceutical industries. It has also been proposed as potential diesel and jet fuel alternative. Introduction of farnesene synthase (FaS) from *Norway spruce* into the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 enabled photosynthetic production of 0.3 mg L^{-1} of farnesene. Farnesene production led to enhancement in photosynthetic activity [49].

Amorpha-4,11-diene is a precursor of artemisinin, an important antimalarial drug produced from the sweet wormwood *Artemisia annua*. Heterologous expression of amorphadiene synthase (ADS) in *Synechococcus elongatus* PCC 7942 enabled photosynthetic production of amorpha-4,11-diene from CO_2. Overexpression of the genes encoding DXS, IDI, and GPPS of the MEP pathway increased the amorpha-4,11-diene production by 23-fold, resulting in photoautotrophic production of 19.8 mg L^{-1} of amorpha-4,11-diene [57].

10.2.4 Diterpenes

Diterpenes are synthesized from C20 geranylgeranyl pyrophosphate (GGPP). Many diterpenoids have antimicrobial, anti-inflammatory, and anticancer activities and are used in medical applications, but they are also used in cosmetics and as food

additives or fragrances. The diterpenoid 13R-manoyl oxide is a precursor of the high-value forskolin that is used as pharmaceuticals. In *Coleus forskohlii*, the diterpene synthases TPS2 and TPS3 in tandem catalyze the formation of 13RMO from GGPP. Heterologous expression of these enzymes in *Synechocystis* sp. PCC 6803 enabled photosynthetic production of 13R-manoyl oxide from CO_2 at a rate of 20.4 μg g^{-1} DW h^{-1} [58, 59]. Overexpression of the gene encoding DXS of the MEP pathway increased the 13R-manoyl oxide production about fourfold [58].

The acyclic diterpene alcohol geranyllinalool has industrial value as fragrance in cosmetics, household cleaning supplies, and detergents. It can also be used as precursor for the chemical synthesis of the drug teprenone. *Synechocystis* sp. PCC 6803 was engineered to synthesize geranyllinalool via heterologous expression of the geranyllinalool synthase (GLS) from *Nicotiana attenuata*. The expression of GLS was increased by constructing fusion of β-PHLS with the CpcB protein, which resulted in photosynthetic production of geranyllinalool at a rate of 7.5 μg g^{-1} DW h^{-1}. The product was primarily sequestered inside the engineered cells [60].

10.2.5 Triterpenes

Squalene is a long-chain triterpene synthesized through the condensation of two molecules of FPP. It is widely used in the food, personal care, and medical industries. Since the feedstock supply for squalene production is limited and unstable because of animal protection policies on the use of shark liver oil and regional and seasonal variations of plant oils, synthetic squalene has arisen much interest. Heterologous expression of squalene synthase (SQS) from *Saccharomyces cerevisiae* in *Synechococcus elongatus* PCC 7942 enabled photosynthetic production of squalene from CO_2. Overexpression of the genes encoding DXS, IDI, and FPP synthase (FPPS) of the MEP pathway remarkably increased the squalene production by about 50,000-fold, resulting in photoautotrophic production of squalene at a rate of 64.8 μg g^{-1} DW h^{-1} [57]. Overexpression of the fusion of SQS with the CpcB protein increased the squalene production. Cultivation of the engineered strain in a scalable photobioreactor (6 L) with light optimization achieved a squalene production rate of 253.8 μg g^{-1} DW h^{-1} [61].

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