Summer 8-15-2019

Metabolic Engineering of Cyanobacteria for Production of Chemicals

Po-Cheng Lin
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

McKelvey School of Engineering
Department of Energy, Environmental & Chemical Engineering

Dissertation Examination Committee:
Himadri Pakrasi, Chair
Fuzhong Zhang, Co-Chair
Costas Maranas
Tae Seok Moon
Yinjie Tang

Metabolic Engineering of Cyanobacteria for Production of Chemicals

by
Po-Cheng Lin

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2019
St. Louis, Missouri

Table of Contents

List of Figures

List of Tables

Acknowledgments

Firstly, I would like to express my sincere gratitude to my advisor Dr. Himadri Pakrasi for his continuous support throughout my Ph.D. studies. Not only research, I also learned a lot from him regarding the leadership skills. I believe what I learned from him will become valuable assets for my future career. Secondly, I would like to thank my co-advisor Dr. Fuzhong Zhang for teaching me the knowledge of synthetic biology and metabolic engineering. Also, I thank his support through each stage of my Ph.D. studies.

Besides my advisors, I would like to thank the rest of my thesis committee: Dr. Costas Maranas, Dr. Tae Seok Moon, and Dr. Yinjie Tang, for their insightful comments and suggestions, which made my research projects successful. I thank my fellow labmates for their support and the stimulating discussions throughout my Ph.D. It has been a great pleasure to work with you all. Also, I thank my friends at Taiwanese Graduate Student Association for all the fun we have had in the last six years.

I would like to thank the Office of Science (BER), U. S. Department of Energy (DE-SC0012722) and the Consortium for Clean Coal Utilization at Washington University in St. Louis for funding my research projects. In addition, I wish to present my special thanks to the McDonnell International Scholars Academy and Taiwan Ministry of Education for providing me the fellowships. I have greatly benefited from the leadership training programs and team-building

activities in the Academy. Without all the support from the Academy, I could not win the 3 Minute Thesis Competition in the McDonnell Academy 7th International Symposium in Beijing.

Finally, I would like to thank my parents and my brother, whose love and guidance are with me in whatever I pursue. Most importantly, I wish to thank my loving and supportive wife, Fang-Tzu, and my wonderful boy, Brady, who provide unending inspiration.

Po-Cheng Lin

Washington University in St. Louis

August 2019

Dedicated to my wife, Fang-Tzu Chang.

ABSTRACT OF THE DISSERTATION

Metabolic Engineering of Cyanobacteria for Production of Chemicals

by

Po-Cheng Lin

Doctor of Philosophy in Energy, Environmental & Chemical Engineering

Washington University in St. Louis, 2019

Professor Himadri Pakrasi, Chair

Professor Fuzhong Zhang, Co-Chair

Concerns over the impact of climate change caused by CO_2 emission have driven the research and development of renewable energies. Microbial production of chemicals is being viewed as a feasible approach to reduce the use of fossil fuels and minimize the impact of climate change. With recent advances in synthetic biology, microorganisms can be engineered to synthesize petroleum-based chemicals and plant-derived compounds. Cyanobacteria are photosynthetic prokaryotes that use only sunlight, CO_2, and trace minerals for growth. Compared to other microbial hosts, cyanobacteria are attractive platforms for sustainable bioproduction, because they can directly convert CO_2 into products. However, the major challenge of using cyanobacteria for chemical production is their low productivities compared to that of conventional heterotrophic hosts. More research is needed to improve the photosynthetic conversion of CO_2 to desired compounds. In this dissertation, cyanobacteria were engineered to produce two commercially-used products, limonene and sucrose, which use distinct substrates for biosynthesis. To identify the metabolic bottlenecks for enhancing the production of limonene and sucrose, various genes and pathways were expressed in cyanobacteria, and further optimized using synthetic biology tools. Their

productivities were significantly improved compared to those reported in previous studies. The findings in this dissertation provide knowledge to improve cyanobacterial production of limonene and sucrose, and facilitate a deeper understanding of the terpene and sugar metabolism in these photosynthetic microorganisms.

Chapter 1: Introduction

This chapter was partially adapted from the following publication:

Lin PC, Pakrasi HB (2019) Engineering cyanobacteria for production of terpenoids. Planta 249 (1):145-154.

1.1 Background

1.1.1 Cyanobacterial production of chemicals

Cyanobacteria are photosynthetic microorganisms that only require sunlight, CO_2, and trace minerals for growth. In recent years, cyanobacteria are emerging hosts for production of chemicals, because these photosynthetic microorganisms directly convert CO_2 into desired compounds. By expressing heterologous pathways in cyanobacteria, CO_2 is converted to many useful products (Sengupta et al. 2018; Knoot et al. 2018), such as ethanol (Dexter and Fu 2009), 2,3-butanediol (Kanno et al. 2017), ethylene (Ungerer et al. 2012), and fatty acids (Liu et al. 2011). Several synthetic biology tools were developed for cyanobacteria in the past decade. For instance, CRISPR/Cas9 (Li et al. 2016; Wendt et al. 2016) and Cpf1 (Ungerer and Pakrasi 2016) techniques enable rapid modification of genome sequences. Development of inducible promoters (Markley et al. 2015; Oliver et al. 2013; Huang and Lindblad 2013) and CRISPR interference (Gordon et al. 2016), and characterization of endogenous promoters (Liu and Pakrasi 2018; Li et al. 2018) allow controllable and tunable expression of heterologous genes. Currently, microbial production of fuels and chemicals are mainly performed by heterotrophic microorganisms such as *Escherichia coli* and *Saccharomyces cerevisiae*, which require sugar feedstocks for growth. However, terrestrial plants that produce sugars have a low energy efficiency to convert solar energy into biomass (<0.5%) (Brenner et al. 2006), which become a major hurdle for sustainable feedstock

production. Cyanobacteria have a higher efficiency (3-9%) than land plants to utilize solar energy (Dismukes et al. 2008). Direct conversion of CO_2 to products is expected to increase the overall efficiency of production. Although cyanobacteria have been engineered to produce a variety of useful chemicals, the productivity requires to be further enhanced for commercial application. More research is needed to improve the efficiency of directing the photosynthetic carbon toward desired chemicals.

Terpenoids are a diverse group of natural products with a variety of commercial applications. In recent years, terpene synthases and the precursor pathways have been expressed in cyanobacteria for enhanced production of various terpene hydrocarbons, including isoprene, limonene, β-phellandrene, and farnesene. However, the productivities need to be further improved for commercial production. Many barriers remain to be overcome in order to efficiently convert CO_2 to terpenoids. In this Chapter, the functions and biosynthesis of terpenoids will be introduced. Recent efforts on photosynthetic production of terpenoids will be summarized. Moreover, challenges and opportunities of engineering cyanobacteria for terpenoid bioproduction will be discussed.

1.1.2 Terpenoids: functions, applications, and biosynthesis

Terpenoids (or isoprenoids) are the largest group of natural products with extreme diversity. More than 55,000 compounds have been identified (Breitmaier 2006). Some terpenoids are essential in primary metabolism, including photosynthesis (chlorophyll, carotenoids, and plastoquinone), respiration (ubiquinone), and developmental regulation in plants (gibberellins and abscisic acid) etc. Most terpenoids are produced by plants as secondary metabolites. Isoprene is a volatile

molecule emitted from numerous plant species to protect against heat stress. Isoprene enhances membrane integrity for the purpose of thermotolerance (Siwko et al. 2007), because heat can cause leakiness of thylakoid membrane and affect efficiency of photosynthesis (Sharkey et al. 2008). Some volatile terpenoids emit floral scents, which can attract pollinators and seed-dispersing animals to facilitate plant reproduction. Moreover, plants release terpenoids with toxicity or strong odor to protect against herbivores and pathogens.

Although terpenoids are extremely diverse compounds, they are all derived from the same building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) (Figure 1.1). One of the simplest type of terpenoids is isoprene, which is a five-carbon compound converted from DMAPP. Terpenoids are categorized into groups based on the number of isoprene units: hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), tetraterpenes (C40), and polyterpenes (>C40). In terms of nomenclature, terpenes are the hydrocarbon molecules, whereas terpenoids are terpenes modified with additional functional groups or structural rearrangement. However, the two terms are often used interchangeably.

Terpenoids have a variety of commercial applications, including pharmaceuticals, nutraceuticals, flavors/fragrances, and industrial chemicals (Ajikumar et al. 2008). Artemisinin is a sesquiterpene produced by the plant *Artemisia annua.* Artemisinin derivatives are the first-line antimalarial drugs recommended by World Health Organization. The price and availability of artemisinin fluctuates wildly due to unstable supply of agricultural materials. With recent improvement in synthetic biology and metabolic engineering, the yeast *Saccharomyces cerevisiae* was engineered for commercial production of artemisinic acid, the precursor of artemisinin (Paddon and Keasling 2014). Paclitaxol, a complex diterpene isolated from the Pacific yew tree

(Taxus brevifolia), is a chemotherapy drug for treatment of several types of cancer. However, the low yield of paclitaxol extracted from the bark of the yew tree (<0.01% of dry weight) is a major barrier for large-scale production (Ciddi et al. 1995). Many terpenoids are used as nutraceuticals due to their health effects to human, such as lutein, lycopene, and astaxanthin. A wide variety of monoterpenes and sesquiterpenes have characteristic fragrances which are used in the flavor and fragrance industry, such as menthol (mint odor), D-limonene (orange peel odor), α-farnesene (green apple odor), and nootkatone (grapefruit odor). Isoprene, the simplest form of terpenoid, is the main component of synthetic and natural rubber. Recently, some terpenoids (limonene, myrcene, and farnesene) or their hydrogenated forms have been determined to be compatible with diesel (Tracy et al. 2009) and aviation fuels (Chuck and Donnelly 2014), demonstrating the potential of using these terpenoids as alternative fuels.

Two distinct metabolic pathways can lead to the synthesis of IPP and DMAPP (Figure 1.1). In general, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also known as the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway or the non-mevalonate pathway) exists in bacteria, whereas the mevalonate (MVA) pathway is mainly found in archaea and eukaryotes. Plants and algae have both pathways, in which the MVA pathway operates in the cytosol and the MEP pathway functions in the plastids. The MEP pathway consists of seven enzymatic reactions. It begins with glyceraldehyde 3-phosphate (GAP) and pyruvate to form DXP. This step is catalyzed by the rate-limiting enzyme, DXP synthase (DXS). Further, DXP is converted to MEP by DXP reductase (DXR), which requires NADPH as reducing power. Subsequently, MEP is coupled with CTP to form 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDP-ME), which further undergoes phosphorylation, cyclization, and reductive dehydration to form 4-hydroxy-3-mehtyl-butenyl 1-diphosphate (HMB-PP). Finally, the HMB-PP is converted to IPP and DMAPP by HMB-PP

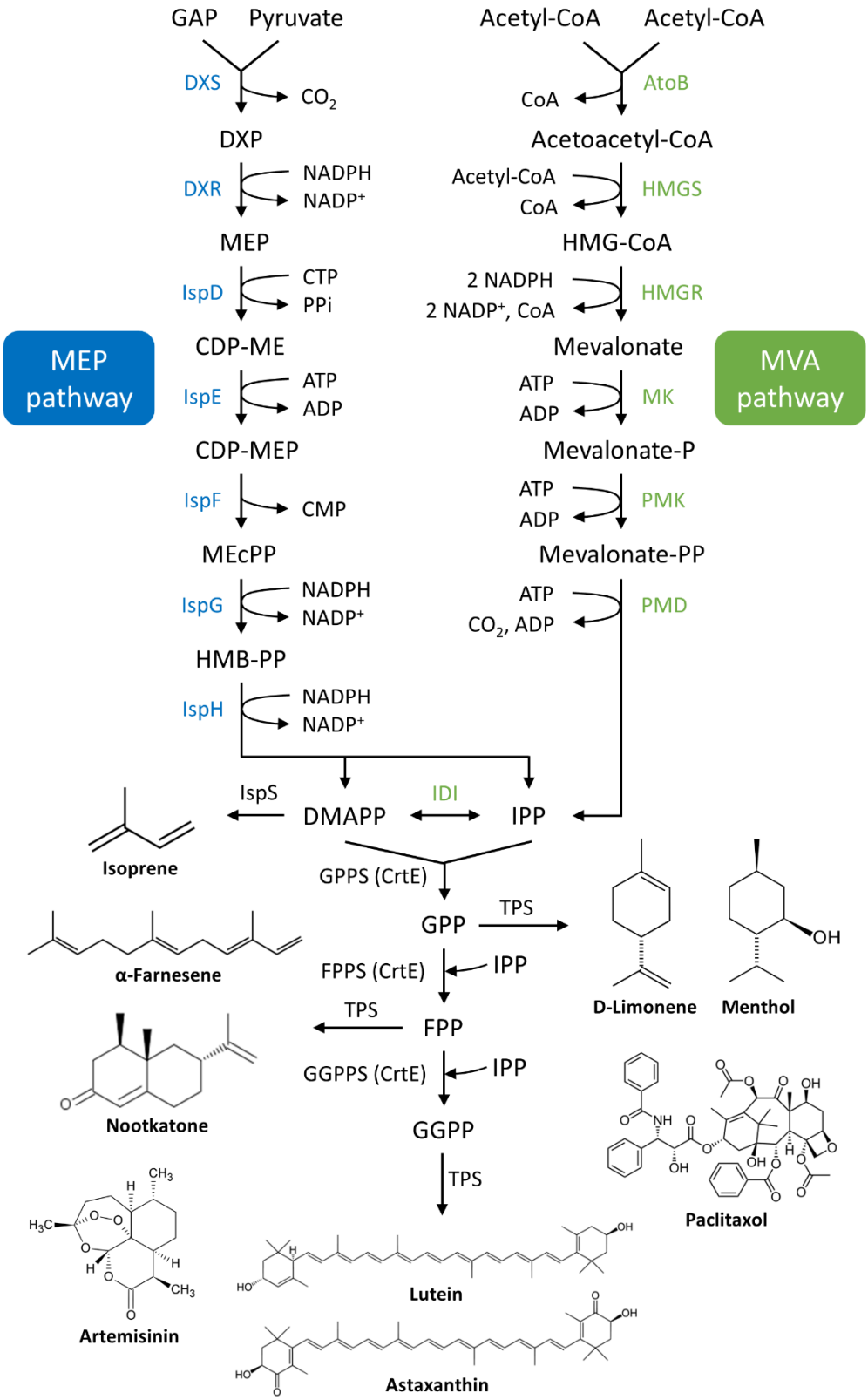

GAP
Pyruvate
DXS
CO_2
DXP
DXR
NADPH
$NADP^+$
MEP
IspD
CTP
PPi
CDP-ME
IspE
ATP
ADP
CDP-MEP
IspF
CMP
MEcPP
IspG
NADPH
$NADP^+$
HMB-PP
IspH
NADPH
$NADP^+$
MEP pathway
Acetyl-CoA
Acetyl-CoA
AtoB
CoA
Acetoacetyl-CoA
Acetyl-CoA
CoA
HMGS
HMG-CoA
2 NADPH
2 $NADP^+$, CoA
HMGR
Mevalonate
ATP
ADP
MK
Mevalonate-P
ATP
ADP
PMK
Mevalonate-PP
ATP
CO_2, ADP
PMD
MVA pathway
IspS
DMAPP
IDI
IPP
Isoprene
GPPS (CrtE)
GPP
TPS
α-Farnesene
FPPS (CrtE)
IPP
TPS
FPP
GGPPS (CrtE)
IPP
GGPP
TPS
D-Limonene
Menthol
OH
Nootkatone
Paclitaxol
Artemisinin
Lutein
Astaxanthin

Figure 1.1 Metabolic pathway of terpenoid biosynthesis.

In cyanobacteria, linear prenyl pyrophosphates (GPP, FPP, and GGPP) are sequentially synthesized by CrtE. Ternpenoid production catalyzed by terpene synthases can be from one-step or multiple-step reactions. Some of the commercially-used terpenoids are shown here. Abbreviations are as follows: GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, DXP synthase; MEP, 2-C-methyl-D-erythritol-4-phosphate; DXR, DXP reductase; CDP-ME, 4-diphosphocytidyl-2-C-methyl-D-erythritol; IspD, 2-C-methyl-D-erythritol 4-phosphate cytidyltransferase; CDP-MEP, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; IspE, 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase; MEcPP, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; IspF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; HMP-PP, 4-hydroxy-3-mehtyl-butenyl 1-diphosphate; IspG, HMB-PP synthase; IspH, HMB-PP reductase; AtoB, acetoacetyl-CoA thiolase; HMG-CoA, 3-hydroxy-3-methyl-glutaryl-CoA; HMGS, HMG-CoA synthase; HMGR, HMG-CoA reductase; MK, mevalonate kinase; PMK, mevalonate 5-phosphate kinase; PMD, mevalonate 5-pyrophosphate decarboxylase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; IDI, isopentenyl diphosphate isomerase; IspS, isoprene synthase; GPP, geranyl pyrophosphate; GPPS, GPP synthase; FPP, farnesyl pyrophosphate; FPPS, FPP synthase; GGPP, geranylgeranyl pyrophosphate; GGPPS, GGPP synthase; TPS, terpene synthase.

reductase (IspH). The enzyme isopentenyl diphosphate isomerase (IDI) interconverts IPP and DMAPP. Although it exists in the MEP pathway, it is not essential because IspH can generate both IPP and DMAPP. The MVA pathway also contains seven reaction steps. It initiates with two condensation steps, in which three molecules of acetyl-CoA are coupled to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). Afterward, HMG-CoA is converted to mevalonate, which is then phosphorylated twice to form mevalonate pyrophosphate. IPP is generated by decarboxylation of mevalonate pyrophosphate, and the IDI catalyzes isomerization of IPP to form DMAPP.

Besides their substrate requirements, the MEP and MVA pathways differ in energy utilization and carbon yield. The net reactions of both pathways for synthesizing IPP from GAP are summarized as below:

MEP: $2GAP + ADP + CTP \rightarrow IPP + CO_2 + ATP + CMP$

MVA: $3GAP + 3ADP + 4NAD(P)^+ \rightarrow IPP + 4CO_2 + 3ATP + 4NAD(P)H$

The MEP pathway utilizes carbon more efficiently, in which 83% (5/6) of the carbon substrate is used for IPP production, whereas the MVA pathway has 56% (5/9) carbon utilization. Conversely, the MVA pathway is more efficient at energy generation, in which three ATP and three NAD(P)H is produced from three molecules of GAP, whereas the MEP pathway generates one ATP from two molecules of GAP.

The downstream process of terpene synthesis begins with sequential head-to-tail condensation of DAMPP with IPP to generate linear prenyl pyrophosphates, including geranyl pyrophosphate (GPP, C10), farnesyl pyrophosphate (FPP, C15), geranylgeranyl pyrophosphate (GGPP, C20), etc. (Figure 1.1). Further, terpene synthases convert the pyrophosphate intermediates into various terpenes. Notably, the photosynthetic pigments such as carotenoids and the phytol tail on chlorophyll are synthesized from this metabolic pathway. Therefore, the terpene biosynthesis pathway is critically important for cyanobacteria.

1.1.3 Recent advances in cyanobacterial terpenoid production

Engineering the terpene synthases

To engineer cyanobacteria for terpenoid production, the choice of terpene synthase is critical. An ideal enzyme should exhibit superior catalytic rate and good solubility in the host cells. The abundance of a soluble enzyme and its activity are correlated with the production titer (Gao et al. 2016). For isoprene production, the commonly used *isoprene synthases* (*ispS*) are derived from

Populus alba and *Pueraria montana* (also known as kudzu). Recently, the *ispS* genes from various high isoprene emission plant species were expressed in *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942). The IspS from *Eucalyptus globulus* produced the highest amount of isoprene, over 5- and 25-fold higher than that from *P. alba* and *P. montana*, respectively (Gao et al. 2016). Similarly, a recent work reported that IspS from *E. globulus* and *Ipomoea batatas* increased isoprene production more than 20 fold compared to the *P. montana* IspS (Englund et al. 2018). The monoterpene limonene is widely found in plants. The limonene synthase (Lims) from *Mentha spicata* is the commonly used enzyme for limonene production. It exhibits the highest production level than that from other plant species (Table 1.1).

Another feature of terpene synthases that needs to be taken into account is enzyme promiscuity. Many terpene synthases produce multiple products. For instance, limonene synthase from *Mentha spicata* primarily produces S-limonene with trace amounts of α-pinene, β-pinene, and myrcene (Colby et al. 1993). Pinene synthase from *Citrus limon* mainly generates β-pinene but also α-pinene, sabinene, and limonene (Lucker et al. 2002). Expressing a terpene synthase with a higher product specificity prevents the formation of unwanted products (Lin et al. 2017).

To enhance expression of terpene synthase, strong promoters such as P_{cpcB} and P_{trc} promoters are widely used to drive gene expression in cyanobacteria (Lin et al. 2017; Wang et al. 2016; Kiyota et al. 2014; Davies et al. 2014). A recent work attempted stepwise metabolic engineering of *Synechococcus* 7942 for limonene production, while obtained a marginal productivity increase (Wang et al. 2016). Surprisingly, by replacing the P_{trc} promoter by the pea P_{psbA} promoter for Lims expression, the production titer increased over 100-fold (Wang et al. 2016). Proteomic analysis confirmed the increased expression of Lims by the P_{psbA} promoter in the host

Table 1.1 Selected recent literature on cyanobacterial terpenoid production

Product	Strain[a]	Titer	Time (days)	Origin of terpene synthase (TPS)	Engineering strategies	Conditions	Reference
Isoprene	7942	1.26 g L^{-1}	21	*Eucalyptus globulus*	P_{trc}-idi-ispS-dxs P_{tac}-ispG	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 50 mM $NaHCO_3$, 37°C	(Gao et al. 2016)
Isoprene	6803	2.5 mg L^{-1}	4	*Pueraria montana*	CpcB*IspS fusion protein	100 µmol photons m^{-2} s^{-1}, 25°C	(Chaves et al. 2017)
Isoprene	6803	250 µg g^{-1} DCW	8.2	*Pueraria montana*	MVA pathway	150 µmol photons m^{-2} s^{-1}, 100% CO_2[b], 35°C	(Bentley et al. 2014)
Isoprene	6803	2.8 mg g^{-1} DCW	1	*Eucalyptus globulus*	P_{trc}-ispS P_{trc}-idi-dxs	50 µmol photons m^{-2} s^{-1}, 50 mM $NaHCO_3$, 30°C	(Englund et al. 2018)
Limonene	6803	6.7 mg L^{-1}	7	*Mentha spicata*	P_{trc}-lims, P_{psbA2}-gpps, P_{rbcL}-rpi-rpe	130 µmol photons m^{-2} s^{-1}, 30°C	(Lin et al. 2017)
Limonene	7002	4 mg L^{-1}	4	*Mentha spicata*	P_{cpcB}-lims	250 µmol photons m^{-2} s^{-1}, 1% CO_2, 37°C	(Davies et al. 2014)
Limonene	7942	2.5 mg L^{-1}	4	*Mentha spicata*	P_{psbA}-lims	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 30°C	(Wang et al. 2016)
Limonene	6803	1 mg L^{-1}	30	*Schizonepeta tenuifolia*	P_{trc}-lims, P_{trc}-dxs-idi-crtE	100 µmol photons m^{-2} s^{-1}, 1% CO_2, 30°C	(Kiyota et al. 2014)
Limonene	7120	0.52 mg L^{-1}	12	*Sitka spruce*	P_{nir}-P_{psbA1}-lims-dxs-idi-gpps	150 µmol photons m^{-2} s^{-1}, 30°C	(Halfmann et al. 2014b)
Phellandrene	6803	3.2 mg g^{-1} DCW	2	*Lavandula angustifolia*	CpcB*PHLS fusion protein	50 µmol photons m^{-2} s^{-1}, 100% CO_2[b]	(Formighieri and Melis 2015)
Phellandrene	6803	5.95 mg g^{-1} DCW	2	*Lavandula angustifolia*	CpcB*PHLS, NptI*GPPS fusion proteins	100 µmol photons m^{-2} s^{-1}, 100% CO_2[b]	(Betterle and Melis 2018)
Phellandrene	6803	10 mg g^{-1} DCW	2	*Lavandula angustifolia*	MVA pathway	100% CO_2[b], light intensity not specified	(Formighieri and Melis 2016)
Amorphadiene	7942	19.8 mg L^{-1}	10	*Artemisia annua*	P_{trc}-dxs-idi-ispA P_{trc}-ads	100 µmol photons m^{-2} s^{-1}, 5% CO_2, 30°C	(Choi et al. 2016)
Farnesene	7120	0.3 mg L^{-1}	15	*Picea abies*	P_{nir}-P_{psbA1}-fas	50 µmol photons m^{-2} s^{-1}, 1% CO_2, 30°C	(Halfmann et al. 2014a)
Manoyl oxide	6803	0.45 mg g^{-1} DCW	4	*Coleus forskohlii*	P_{psbA2}-CfTPS-dxs	100 µmol photons m^{-2} s^{-1}	(Englund et al. 2015)

lims, limonene synthase; *rpi*, ribose 5-phosphate isomerase; *rpe*, ribulose 5-phosphate 3-epimerase; *crtE*, GGPP synthase; *PHLS*, phellandrene synthase; *NptI*, neomycin phosphotransferase I; *ispA*, FPP synthase; *ads*, amorphadiene synthase; *fas*, farnesene synthase; *CfTPS*, terpene synthase from *C. forskohlii*

[a] 7942, *Synechococcus elongatus* PCC 7942; 6803, *Synechocystis* sp. PCC 6803; 7002, *Synechococcus* sp. PCC 7002; 7120, *Anabaena* sp. PCC 7120.

[b] Cells were grown in a gaseous–aqueous two-phase reactor (Bentley and Melis 2012).

strain (Wang et al. 2016). In addition, protein fusion is a strategy to increase protein expression. Using the highly expressed c-phycocyanin beta subunit (cpcB) protein as a leader sequence, the CpcB-IspS fusion constructs led to a significant improvement in protein abundance and isoprene productivity (Chaves et al. 2017). Although the IspS activity was attenuated, the increased expression level compensated the reduced activity of IspS, resulting in a 27-fold increase in isoprene production (Chaves et al. 2017).

Unlike plants, cyanobacteria do not have specific prenyltransferases to generate GPP and FPP, substrates for monoterpene and sesquiterpene synthases, respectively. These diphosphate intermediates are synthesized sequentially by the enzyme CrtE, which is a GGPP synthase (Figure 1.1). A common strategy for enhancing monoterpene and sesquiterpene production is to express specific GPP and FPP synthases, respectively. Such strategy increases the amounts of GPP and FPP for terpenoid production (Lin et al. 2017; Wang et al. 2016; Choi et al. 2016; Englund et al. 2015). However, expression of these prenyltransferases reduces the carbon flux toward pigment synthesis, lowering the amount of photosynthetic pigments in cyanobacteria (Lin et al. 2017; Choi et al. 2016; Englund et al. 2015).

Engineering the precursor pathways

To enhance production of terpenoids, it is critical to increase the amount of the precursors (IPP and DMAPP), which can be achieved by engineering the MEP or MVA pathways. In *E. coli*, it is widely known that the DXS and IDI catalyze the bottleneck reactions in the MEP pathway. Overexpression of genes encoding the DXS and IDI leads to significant increase in terpene production, and this has been summarized in a recent review (Niu et al. 2017). Moreover, a

previous study extensively engineered the MEP pathway in *E. coli* to produce taxadiene, the precursor of the potent anticancer drug paclitaxol. By overexpressing and optimizing four bottleneck genes (*dxs*, *ispD*, *ispF*, and *idi*) in the MEP pathway, the optimized strain increased taxadiene production 15,000-fold, leading to 1 g L^{-1} yield in fed-batch bioreactors (Ajikumar et al. 2010).

To increase the terpene precursors in cyanobacteria, most studies focused on optimizing the native MEP pathway. A recent study engineered *Synechococcus* 7942 for isoprene production. Overexpressing the native *dxs* gene increased 20% of the isoprene yield (Gao et al. 2016). Similarly, production of limonene was enhanced by overexpressing the *dxs* gene together with other genes involved in the upstream pathway. A previous work overexpressed genes encoding the DXS (from *E. coli*), IDI (from *Haematococcus pluvialis*), and GPPS (from *Mycoplasma tuberculosis*) in *Anabaena* sp. PCC 7120, resulting in a 6.8-fold increase in limonene production (0.52 mg L^{-1} in 12 days) (Halfmann et al. 2014b). By expressing the same set of genes (*dxs*, *idi*, and *gpps* from *Synechocystis* sp. PCC 6803), the limonene yield in *Synechocystis* 6803 increased 40% to 1 mg L^{-1} in 30 days (Kiyota et al. 2014). The productivity of limonene in *Synechococcus* 7942 was increased 2.3-fold to 76.3 μg L^{-1} OD_{730}^{-1} d^{-1} by overexpressing the *dxs* gene from *Botryococcus braunii* and the *gpps* gene from *Abies grandis* (Wang et al. 2016). Moreover, increased production of the plant diterpenoid manoyl oxide was achieved by expressing the *dxs* gene from the plant *Coleus forskohlii*, which resulted in a 4.2-fold improvement in low light condition (20 μmol photons m^{-2} s^{-1}) (Englund et al. 2015). However, the *dxs*-overexpressing strain showed no improvement in production under high light condition (100 μmol photons m^{-2} s^{-1}) (Englund et al. 2015).

Compared to DXS, IDI seems to be a more important target for enhancing isoprene production in cyanobacteria. *Synechococcus* 7942 has a low ratio of DMAPP/IPP concentration (*i.e.*, 0.03 in an isoprene-producing strain) (Gao et al. 2016). Such low ratio may affect the substrate availability for isoprene production, since DMAPP is the substrate for isoprene synthase. In addition, the isoprene-emitting kudzu leaves have a high DMAPP/IPP ratio (*i.e.*, >2) (Zhou et al. 2013), indicating isoprene production is related to DMAPP availability. To increase the DMAPP/IPP ratio, the *idi* gene from *S. cerevisiae* was overexpressed in an isoprene-producing *Synechococcus* 7942, which increased the DMAPP/IPP ratio to 2.25 and resulted in a 2-fold improvement in isoprene production (Gao et al. 2016). However, co-expression of *dxs* and *idi* genes produced similar amount of isoprene compared to the *idi*-expressing strain (Gao et al. 2016), suggesting the IDI has a more critical role than DXS for enhancing isoprene production in cyanobacteria.

Besides DXS and IDI catalyzing the critical reactions for terpene synthesis, IspD and IspG were recently identified as bottleneck enzymes of the MEP pathway in cyanobacteria. Systematic overexpression of each enzyme in the MEP pathway identified IspD as the third enzyme for enhanced isoprene production in *Synechocystis* 6803 (Englund et al. 2018). Kinetic flux profiling showed that the flux through the reaction catalyzed by IspG was very low in *Synechococcus* 7942 (Gao et al. 2016). Overexpressing the *ispG* gene from *Thermosynechococcus elongatus* increased isoprene production 60% (Gao et al. 2016). The final strain in the study, with expression of *ispS*, *dxs*, *idi*, and *ispG*, produced 1.26 g L^{-1} isoprene in a photobioreactor. The productivity was 102 mg L^{-1} d^{-1} in the first nine days, which is over two orders of magnitude higher than all the reported literature on cyanobacterial terpenoid production (Table 1.1).

Another strategy to increase terpene precursors is to express the heterologous MVA pathway, which is widely used for terpene production in *E. coli*. Introduction of this pathway increases the carbon flux toward the terpene precursors, which further enhances productivity of various terpenoids such as isoprene, lycopene, limonene, and farnesene (Niu et al. 2017). With cyanobacteria, the Melis group successfully introduced the heterologous MVA pathway into *Synechocystis* 6803 for enhanced production of isoprene (Bentley et al. 2014) and β-phellandrene (Formighieri and Melis 2016). Unlike previous work in *E. coli* which expressed the MVA pathway from *S. cerevisiae*, the upper MVA pathway in their studies was derived from *Enterococcus faecalis* (HMGS, HMGR) and *E. coli* (AtoB), whereas the lower MVA pathway was obtained from *Streptococcus pneumoniae* (MK, PMK, PMD, and IDI) (Formighieri and Melis 2016; Bentley et al. 2014). Both pathways were successfully introduced into the genome of *Synechocystis* 6803, and all enzymes were actively expressed (Bentley et al. 2014). Expressing the MVA pathway increased terpene production over 2-fold (Formighieri and Melis 2016; Bentley et al. 2014), suggesting that the heterologous pathway indeed directs an extra carbon flux toward terpene synthesis. However, such improvement was less impactful than previous efforts in *E. coli*. For instance, expression of the *S. cerevisiae* MVA pathway increased artemisinic acid production 36 fold (Martin et al. 2003). Also, limonene production improved nearly 90 fold by optimizing the MVA pathway from *Staphylococcus aureus* and *S. cerevisiae* (Alonso-Gutierrez et al. 2013). The heterologous MVA pathway used in *E. coli* has been extensively studied and perhaps this optimized pathway can be expressed in cyanobacteria to see if terpene production could be further improved.

Recently, the pentose phosphate (PP) pathway in cyanobacteria was engineered for enhanced production of limonene. Lin *et al.* used the Optforce computational algorithm to identify

two genes in the PP pathway, *ribose 5-phosphate isomerase* (*rpi*) and *ribulose 5-phosphate 3-epimerase* (*rpe*), that are important for limonene synthesis. The limonene titer increased 2.3 fold to 6.7 mg L^{-1} by expressing the *rpi* and *rpe* genes from *E. coli*, and the *gpps* gene from *Abies grandis* (Lin et al. 2017).

1.1.4 Challenges in enhancing terpenoid production in cyanobacteria

Redirection of photosynthetic carbon

One of the major challenges to enhance terpenoid production by cyanobacteria is carbon partitioning in photosynthesis. Most of the fixed carbon is directed toward sugar phosphates for biomass instead of the products. The sugar biosynthetic pathways in *Synechocystis* 6803 consumes more than 80% of the fixed carbon for biomass accumulation, whereas approximately 5% of the photosynthetic carbon is allocated to terpene biosynthesis, including carotenoids, phytol groups of chlorophyll, and prenyl tails of quinone (Melis 2013). The low carbon flux to the terpene metabolic pathway results in the low yield of heterologous terpenoid production.

A common strategy for redirecting the carbon flux is to remove the competing pathway(s) of desired products. In cyanobacteria, glycogen serves as a major carbon sink for energy storage under nutrient starvation conditions, in which the carbon flux is diverted to glycogen accumulation instead of cellular growth (Hickman et al. 2013; Yoo et al. 2007). Several studies attempted to delete the glycogen biosynthetic pathway for the purpose of redirecting the fixed carbon toward desired products. A recent study reported a two-fold increase in lactic acid production using a glycogen deficient *Synechocystis* 6803 mutant (van der Woude et al. 2014). Furthermore, overexpressing a heterologous isobutanol pathway served as an alternative carbon sink that

rescued the growth phenotype of a *Synechococcus* 7942 glycogen mutant, and increased the carbon flux to isobutanol synthesis (Li et al. 2014). However, inactivation of glycogen sinks leads to metabolic imbalance under nitrogen-deplete conditions, in which organic acids such as pyruvate and α-ketoglutarate are excreted from the cells (Davies et al. 2014; Hickman et al. 2013; Carrieri et al. 2012). Unfortunately, removing the glycogen synthesis pathway did not lead to improved terpenoid production in cyanobacteria. Davies *et al.* constructed a glycogen-deficient mutant for limonene and bisabolene production. However, the production titer was extremely low due to the absence of growth under nitrogen starvation (Davies et al. 2014).

Compared to other compounds produced by engineered cyanobacteria, the productivities of terpenes are relatively low, even though many efforts have been done to increase terpene production. Presumably, the MEP pathway in cyanobacteria is highly regulated so that limited amount of the fixed carbon is directed to terpene synthesis. The terpene precursors IPP and DMAPP are distant from the central carbon metabolism. Quantitative analysis reveals that the concentrations of IPP, DMAPP, and other intermediates (MEP and HMB-PP) in the MEP pathway are three orders of magnitude lower than pyruvate in cyanobacteria (Dempo et al. 2014). This indicates that the sparse amount of precursor pools may be a limiting factor for enhancing terpene synthesis. A previous study using cell extracts of *Synechocystis* 6803 demonstrated that terpene production was increased by compounds in the PP pathway but not by substrates (pyruvate and GAP) of the MEP pathway (Ershov et al. 2002). In addition, expression of the *rpi* and *rpe* genes in the PP pathway led to increased production of limonene (Lin et al. 2017). These evidences indicate a connection between the PP pathway and terpene synthesis in cyanobacteria. Moreover, a recent study determined that the *E. coli* enzymes YajO and RibB (G108S) are able to convert ribulose 5-phosphate to DXP, the first intermediate in the MEP pathway (Kirby et al. 2015). Since

cyanobacteria has a relatively high carbon flux in the PP pathway (Abernathy et al. 2017; Young et al. 2011), this novel route can be investigated in order to avoid feedback regulation in the MEP pathway (Banerjee et al. 2013) and direct the carbon flux from the PP pathway to terpene production.

MEP vs. MVA pathways: which is better for cyanobacterial terpenoid production?

Overexpression of the heterologous MVA pathway is a common strategy to improve terpenoid production in heterotrophic microorganisms such as *E. coli* and *S. cerevisiae*. Since the native MEP pathway is highly regulated (Banerjee and Sharkey 2014), the non-native MVA pathway is able to avoid transcriptional regulation in the hosts and enable a bypass of the carbon flux to terpene synthesis. Previous studies on cyanobacterial terpenoid production were mainly focused on the MEP pathway. A few studies expressed the heterologous MVA pathway for enhanced terpene synthesis (Table 1.1). This may indicate the difficulty of introducing the MVA pathway into photosynthetic microorganisms. The MVA pathway contains seven genes, about nine kilobases in length (Bentley et al. 2014). Introducing the entire pathway into cyanobacterial genome may be challenging. Although the heterologous MVA pathway has been successfully expressed in *Synechocystis* 6803 (Formighieri and Melis 2016; Bentley et al. 2014), the production titer is similar to other studies engineering the MEP pathway (Table 1.1). The MEP and MVA pathways use different substrates. The former uses GAP and pyruvate, whereas the later requires acetyl-CoA. Isotopically nonstationary ^{13}C flux analyses showed that cyanobacteria have a relatively low flux toward acetyl-CoA compared to the sugar phosphate pathways (Abernathy et al. 2017; Young et al. 2011). Moreover, a recent study compared the precursor pools of the MEP and MVA pathways between *Synechococcus* 7942 and *E. coli* BL-21, and found that the

concentrations of GAP and pyruvate in *Synechococcus* 7942 were 5-fold and 21-fold higher than that in *E. coli*, whereas the acetyl-CoA pool was 20-fold lower (Gao et al. 2016). These data indicate that expressing the MVA pathway in cyanobacteria may be less effective than in *E. coli* for enhanced terpene production. Hence, the native MEP pathway is presumably a more attractive target for metabolic engineering, even though further research is needed to elucidate the regulation of the MEP pathway in cyanobacteria. It is known that overexpressing the enzymes in the MEP pathway can reduce terpenoid production in *E. coli* (Ajikumar et al. 2010; Kim and Keasling 2001). The expression level needs to be optimized in order to maximize the productivity. Despite many efforts to engineer the MEP pathway in cyanobacteria, optimization of the pathway is yet to be attempted. This can be achieved by modifying the ribosome binding site (RBS) (Wang et al. 2018; Oliver et al. 2014) or using inducible promoters (Markley et al. 2015; Oliver et al. 2013; Huang and Lindblad 2013) to modulate gene expression.

Balancing pigment and product synthesis

The photosynthetic pigments such as chlorophyll and carotenoids are essential for cyanobacteria. Production of terpenes will necessarily compete with pigment synthesis because the same precursor pathway is used. Although the expression of prenyltransferases such as GPP and FPP synthases is critical in enhancing terpenoid production (Lin et al. 2017; Choi et al. 2016), the carbon flux for photosynthetic pigments is diverted toward the terpene product. Previous studies reported that photosynthetic pigment levels were lowered in terpene-producing cyanobacteria (Lin et al. 2017; Choi et al. 2016; Englund et al. 2015). Decreased amount of pigments may affect the efficiency of light harvesting and photoprotection and alter the structural stability of photosynthetic complexes (Toth et al. 2015), thus leading to impaired cellular growth and terpene

production. To further increase terpenoid production in cyanobacteria, the carbon fluxes between the product and pigment synthesis should be balanced in addition to increasing the terpene precursors. Modulating the expression of prenyltransferases could presumably optimize carbon fluxes and improve terpene synthesis. A recent study reported that the expression level of GPP synthase is critically important for enhancing monoterpene production in *E. coli*. Optimization of GPP synthase expression by engineering the RBS sequence led to a sixfold improvement in geraniol production (Zhou et al. 2015).

1.1.5 Future perspectives

Cyanobacterial production of terpenoids demonstrates the potential of sustainable bioproduction using light and CO_2, although further research is needed to enhance the productivity. Challenges including the regulation of MEP pathway, carbon partitioning, and balancing pigment and limonene synthesis should be addressed collectively in order to further increase the productivity of terpenoids. With many obstacles required to be overcome, recent developments on the genetic tools for cyanobacteria (Sengupta et al. 2018) can facilitate strain construction and accelerate the design-build-test cycle, which will benefit systematic engineering of cyanobacteria. The advantage of using cyanobacteria as a production platform is direct conversion of CO_2 to desired compounds without the use of sugar feedstocks. However, the relatively slow rates of growth and CO_2 fixation of standard laboratory strains presumably lead to low productivity of chemicals. Particularly, the small fraction of fixed carbon allocated to terpene metabolism makes terpenoid productivity even lower than other compounds. To utilize cyanobacteria for commercial bioproduction, a better chassis is needed to achieve higher production titer and productivity. *Synechococcus elongatus* UTEX 2973 is a fast-growing cyanobacterium with the fastest growth rate reported to date (Yu et

al. 2015). The doubling time of this strain can be as fast as 1.5 h under high light and CO_2 conditions (Ungerer et al. 2018), which is comparable to the heterotrophic growth rate of the yeast *S. cerevisiae*. The fast growth of *Synechococcus* 2973 reflects the rapid accumulation of biomass, at a rate of over 1 g L^{-1} d^{-1} (Ungerer et al. 2018). Moreover, this strain synthesizes significant amount of glycogen as a way to reserve excess fixed carbon, with over 35% of dry cell weight after 3 days of growth (Ungerer et al. 2018). Recently, *Synechococcus* 2973 was engineered for sugar production. The productivity of sucrose reaches 0.85 g L^{-1} d^{-1} (the highest amount from photoautotrophic production), demonstrating the potential of using this strain for biotechnology applications (Song et al. 2016). With recent advances in metabolic engineering of terpene biosynthetic pathway, this fast-growing cyanobacterium offers an opportunity to improve the productivity of terpenoids.

1.2 Dissertation overview

This dissertation focuses on metabolic engineering of cyanobacteria to produce two chemicals, limonene and sucrose, which utilize distinct precursor pathways for biosynthesis (Figure 1.2). Limonene is a commercially-used terpenoid with a characteristic orange fragrance. Production of limonene requires the terpene biosynthetic pathway to provide the substrates. Sucrose is a compound sugar widely used in the food industry and biofuel production. Biosynthesis of sucrose utilizes sugar phosphates.

In Chapter 2, the model cyanobacterium *Synechocystis* sp. PCC 6803 was engineered for limonene production. In this study, we identified targets for genetic modifications that benefit the production of limonene by *Synechocystis* 6803. For that purpose, we combined synthetic biology

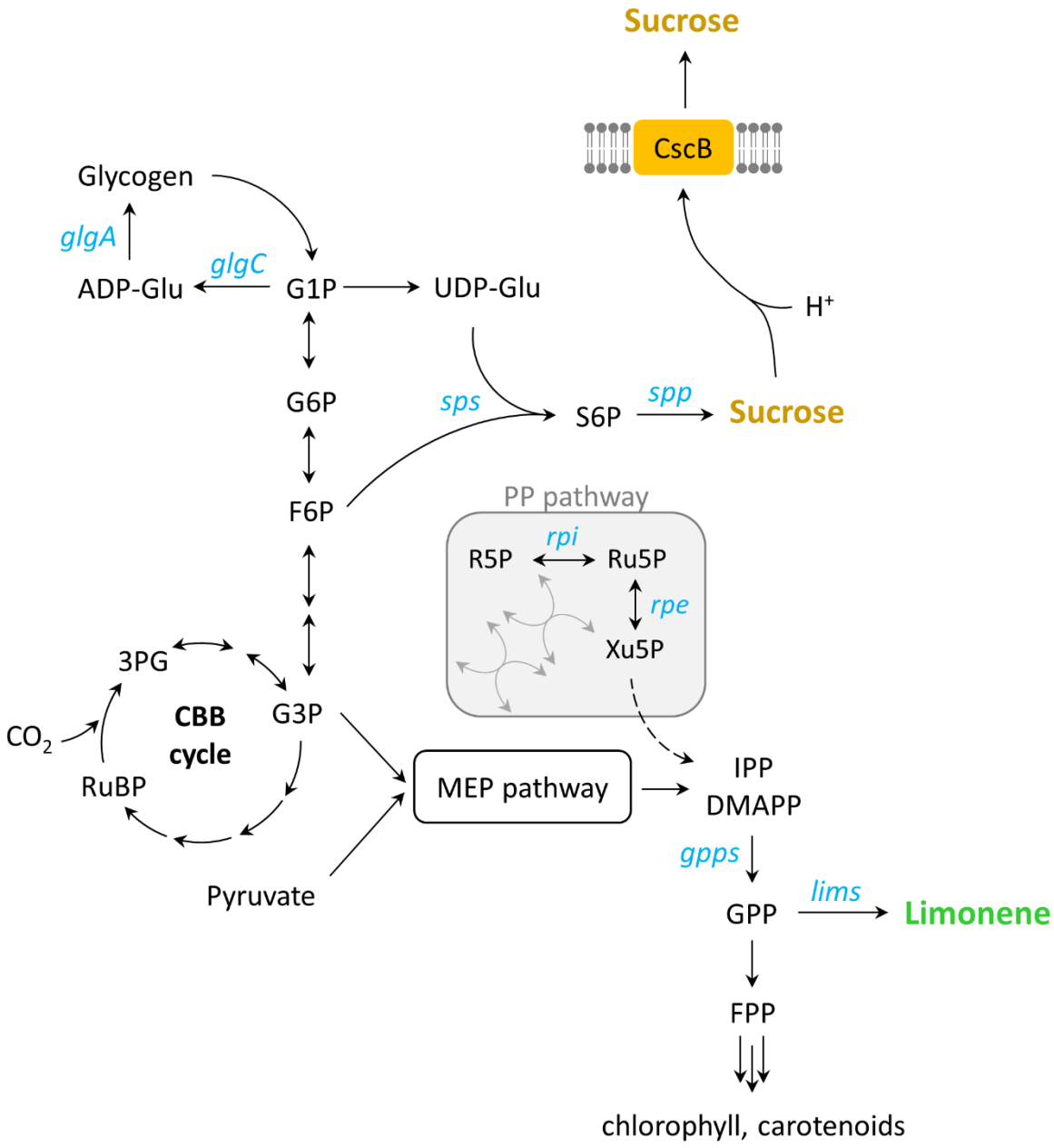


Figure 1.2 Metabolic engineering of cyanobacteria to produce useful chemicals.

In this study, cyanobacteria were engineered for sustainable production of limonene and sucrose. Important genes discussed in the dissertation were highlighted in blue. *glgA*, glycogen synthase; *glgC*, ADP-glucose pyrophosphorylase; *gpps*, geranyl pyrophosphate synthase; *lims*, limonene synthase; *rpe*, ribulose 5-phosphate 3-epimerase; *rpi*, ribose 5-phosphate isomerase; *sps*, sucrose-phosphate synthase; *spp*, sucrose-phosphate phosphatase.

and computational modeling approaches to engineer *Synechocystis* 6803 to improve the titer and productivity of limonene.

In Chapter 3, we explored the potential of engineering the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 to produce limonene. We identified a single nucleotide polymorphism (SNP) that led to a dramatic improvement of limonene productivity. Furthermore, various precursor pathways for limonene biosynthesis were overexpressed and optimized in order to improve the limonene titer. The productivity of limonene in *Synechococcus* 2973 was significantly higher than those reported in previous studies.

In Chapter 4, the fast-growing strain *Synechococcus elongatus* UTEX 2973 was engineered for sustainable production of sucrose. Using only light and CO_2, a high productivity of sucrose was achieved by overexpressing the sucrose transporter CscB in *Synechococcus* 2973 (Figure 1.2). Moreover, the sucrose biosynthesis pathway was expressed in *Synechococcus* 2973, which enabled the engineered strains to produce sucrose without salt induction.

Chapter 5 discussed the glycogen metabolism in *Synechococcus* 2973. Glycogen content and the rate of glycogen synthesis in *Synechococcus* 2973 were investigated. A glycogen-deficient mutant of *Synechococcus* 2973 was created by CRISPR/Cf1 genome editing, and its growth phenotype was scrutinized.

Finally, Chapter 6 concluded the findings in this dissertation, and discussed the types of chemicals that would be more suitable for cyanobacterial production. Moreover, future directions of engineering cyanobacteria for the production of limonene and sugar will be suggested.

References

Abernathy MH, Yu J, Ma F, Liberton M, Ungerer J, Hollinshead WD, Gopalakrishnan S, He L, Maranas CD, Pakrasi HB, Allen DK, Tang YJ (2017) Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis. Biotechnology for biofuels 10:273. doi:10.1186/s13068-017-0958-y

Ajikumar PK, Tyo K, Carlsen S, Mucha O, Phon TH, Stephanopoulos G (2008) Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Molecular pharmaceutics 5 (2):167-190

Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330 (6000):70-74. doi:10.1126/science.1191652

Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, Lee TS (2013) Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. Metabolic engineering 19:33-41. doi:10.1016/j.ymben.2013.05.004

Banerjee A, Sharkey T (2014) Methylerythritol 4-phosphate (MEP) pathway metabolic regulation. Natural product reports 31 (8):1043-1055

Banerjee A, Wu Y, Banerjee R, Li Y, Yan H, Sharkey TD (2013) Feedback inhibition of deoxy-D-xylulose-5-phosphate synthase regulates the methylerythritol 4-phosphate pathway. The Journal of biological chemistry 288 (23):16926-16936. doi:10.1074/jbc.M113.464636

Bentley FK, Melis A (2012) Diffusion-based process for carbon dioxide uptake and isoprene emission in gaseous/aqueous two-phase photobioreactors by photosynthetic microorganisms. Biotechnology and bioengineering 109 (1):100-109

Bentley FK, Zurbriggen A, Melis A (2014) Heterologous expression of the mevalonic acid pathway in cyanobacteria enhances endogenous carbon partitioning to isoprene. Mol Plant 7 (1):71-86. doi:10.1093/mp/sst134

Betterle N, Melis A (2018) Heterologous Leader Sequences in Fusion Constructs Enhance Expression of Geranyl Diphosphate Synthase and Yield of β-Phellandrene Production in Cyanobacteria (*Synechocystis*). ACS synthetic biology 7 (3):912-921

Breitmaier E (2006) Terpenes: Importance, general structure, and biosynthesis. In: Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. pp 1-9

Brenner MP, Bildsten L, Dyson F, Fortson N, Garwin R, Grober R, Hemley R, Hwa T, Joyce G, Katz J (2006) Engineering microorganisms for energy production. DTIC Document

Carrieri D, Paddock T, Maness P-C, Seibert M, Yu J (2012) Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage. Energy & Environmental Science 5 (11):9457-9461

Chaves JE, Rueda-Romero P, Kirst H, Melis A (2017) Engineering Isoprene Synthase Expression and Activity in Cyanobacteria. ACS synthetic biology 6 (12):2281-2292. doi:10.1021/acssynbio.7b00214

Choi SY, Lee HJ, Choi J, Kim J, Sim SJ, Um Y, Kim Y, Lee TS, Keasling JD, Woo HM (2016) Photosynthetic conversion of CO_2 to farnesyl diphosphate-derived phytochemicals (amorpha-4, 11-diene and squalene) by engineered cyanobacteria. Biotechnology for biofuels 9 (1):202

Chuck CJ, Donnelly J (2014) The compatibility of potential bioderived fuels with Jet A-1 aviation kerosene. Applied Energy 118:83-91

Ciddi V, Srinivasan V, Shuler M (1995) Elicitation of Taxus sp. cell cultures for production of taxol. Biotechnology letters 17 (12):1343-1346

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. The Journal of biological chemistry 268 (31):23016-23024

Davies FK, Work VH, Beliaev AS, Posewitz MC (2014) Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of *Synechococcus* sp. PCC 7002. Frontiers in bioengineering and biotechnology 2:21. doi:10.3389/fbioe.2014.00021

Dempo Y, Ohta E, Nakayama Y, Bamba T, Fukusaki E (2014) Molar-based targeted metabolic profiling of cyanobacterial strains with potential for biological production. Metabolites 4 (2):499-516. doi:10.3390/metabo4020499

Dexter J, Fu P (2009) Metabolic engineering of cyanobacteria for ethanol production. Energy & Environmental Science 2 (8):857-864

Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008) Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current opinion in biotechnology 19 (3):235-240. doi:10.1016/j.copbio.2008.05.007

Englund E, Andersen-Ranberg J, Miao R, Hamberger B, Lindberg P (2015) Metabolic Engineering of *Synechocystis* sp. PCC 6803 for Production of the Plant Diterpenoid Manoyl Oxide. ACS synthetic biology 4 (12):1270-1278. doi:10.1021/acssynbio.5b00070

Englund E, Shabestary K, Hudson EP, Lindberg P (2018) Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. Metabolic engineering 49:164-177. doi:10.1016/j.ymben.2018.07.004

Ershov YV, Gantt RR, Cunningham Jr FX, Jr., Gantt E (2002) Isoprenoid biosynthesis in *Synechocystis* sp. strain PCC6803 is stimulated by compounds of the pentose phosphate cycle but not by pyruvate or deoxyxylulose-5-phosphate. Journal of bacteriology 184 (18):5045-5051

Formighieri C, Melis A (2015) A phycocyanin· phellandrene synthase fusion enhances recombinant protein expression and β-phellandrene (monoterpene) hydrocarbons production in *Synechocystis* (cyanobacteria). Metabolic engineering 32:116-124

Formighieri C, Melis A (2016) Sustainable heterologous production of terpene hydrocarbons in cyanobacteria. Photosynthesis research 130 (1-3):123-135. doi:10.1007/s11120-016-0233-2

Gao X, Gao F, Liu D, Zhang H, Nie X, Yang C (2016) Engineering the methylerythritol phosphate pathway in cyanobacteria for photosynthetic isoprene production from CO_2. Energy & Environmental Science 9 (4):1400-1411

Gordon GC, Korosh TC, Cameron JC, Markley AL, Begemann MB, Pfleger BF (2016) CRISPR interference as a titratable, trans-acting regulatory tool for metabolic engineering in the cyanobacterium *Synechococcus* sp. strain PCC 7002. Metabolic engineering 38:170-179

Halfmann C, Gu L, Gibbons W, Zhou R (2014a) Genetically engineering cyanobacteria to convert CO_2, water, and light into the long-chain hydrocarbon farnesene. Applied microbiology and biotechnology 98 (23):9869-9877

Halfmann C, Gu L, Zhou R (2014b) Engineering cyanobacteria for the production of a cyclic hydrocarbon fuel from CO_2 and H_2O. Green Chemistry 16 (6):3175-3185

Hickman JW, Kotovic KM, Miller C, Warrener P, Kaiser B, Jurista T, Budde M, Cross F, Roberts JM, Carleton M (2013) Glycogen synthesis is a required component of the nitrogen stress response in *Synechococcus elongatus* PCC 7942. Algal Research 2 (2):98-106

Huang HH, Lindblad P (2013) Wide-dynamic-range promoters engineered for cyanobacteria. Journal of biological engineering 7 (1):10. doi:10.1186/1754-1611-7-10

Kanno M, Carroll AL, Atsumi S (2017) Global metabolic rewiring for improved CO_2 fixation and chemical production in cyanobacteria. Nat Commun 8:14724. doi:10.1038/ncomms14724

Kim SW, Keasling JD (2001) Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production. Biotechnology and bioengineering 72 (4):408-415

Kirby J, Nishimoto M, Chow RW, Baidoo EE, Wang G, Martin J, Schackwitz W, Chan R, Fortman JL, Keasling JD (2015) Enhancing Terpene yield from sugars via novel routes to 1-deoxy-d-xylulose 5-phosphate. Applied and environmental microbiology 81 (1):130-138. doi:10.1128/AEM.02920-14

Kiyota H, Okuda Y, Ito M, Hirai MY, Ikeuchi M (2014) Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. Journal of biotechnology 185:1-7. doi:10.1016/j.jbiotec.2014.05.025

Knoot CJ, Ungerer J, Wangikar PP, Pakrasi HB (2018) Cyanobacteria: promising biocatalysts for sustainable chemical production. Journal of Biological Chemistry 293 (14):5044-5052

Li H, Shen CR, Huang C-H, Sung L-Y, Wu M-Y, Hu Y-C (2016) CRISPR-Cas9 for the genome engineering of cyanobacteria and succinate production. Metabolic engineering 38:293-302

Li S, Sun T, Xu C, Chen L, Zhang W (2018) Development and optimization of genetic toolboxes for a fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Metabolic engineering

Li X, Shen CR, Liao JC (2014) Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus elongatus* PCC 7942. Photosynthesis research 120 (3):301-310. doi:10.1007/s11120-014-9987-6

Lin P-C, Saha R, Zhang F, Pakrasi HB (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. Scientific reports 7 (1):17503. doi:10.1038/s41598-017-17831-y

Liu D, Pakrasi HB (2018) Exploring native genetic elements as plug-in tools for synthetic biology in the cyanobacterium *Synechocystis* sp. PCC 6803. Microbial cell factories 17 (1):48

Liu X, Sheng J, Curtiss III R (2011) Fatty acid production in genetically modified cyanobacteria. Proceedings of the National Academy of Sciences

Lucker J, El Tamer MK, Schwab W, Verstappen FW, van der Plas LH, Bouwmeester HJ, Verhoeven HA (2002) Monoterpene biosynthesis in lemon (*Citrus limon*). cDNA isolation and functional analysis of four monoterpene synthases. European journal of biochemistry / FEBS 269 (13):3160-3171

Markley AL, Begemann MB, Clarke RE, Gordon GC, Pfleger BF (2015) Synthetic biology toolbox for controlling gene expression in the cyanobacterium *Synechococcus* sp. strain PCC 7002. ACS synthetic biology 4 (5):595-603. doi:10.1021/sb500260k

Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21 (7):796-802. doi:10.1038/nbt833

Melis A (2013) Carbon partitioning in photosynthesis. Current opinion in chemical biology 17 (3):453-456. doi:10.1016/j.cbpa.2013.03.010

Niu FX, Lu Q, Bu YF, Liu JZ (2017) Metabolic engineering for the microbial production of isoprenoids: Carotenoids and isoprenoid-based biofuels. Synth Syst Biotechnol 2 (3):167-175. doi:10.1016/j.synbio.2017.08.001

Oliver JW, Machado IM, Yoneda H, Atsumi S (2013) Cyanobacterial conversion of carbon dioxide to 2,3-butanediol. Proceedings of the National Academy of Sciences of the United States of America 110 (4):1249-1254. doi:10.1073/pnas.1213024110

Oliver JW, Machado IM, Yoneda H, Atsumi S (2014) Combinatorial optimization of cyanobacterial 2,3-butanediol production. Metabolic engineering 22:76-82. doi:10.1016/j.ymben.2014.01.001

Paddon CJ, Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nature Reviews Microbiology 12 (5):355

Sengupta A, Pakrasi HB, Wangikar PP (2018) Recent advances in synthetic biology of cyanobacteria. Applied microbiology and biotechnology:1-15

Sharkey TD, Wiberley AE, Donohue AR (2008) Isoprene emission from plants: why and how. Annals of botany 101 (1):5-18. doi:10.1093/aob/mcm240

Siwko ME, Marrink SJ, de Vries AH, Kozubek A, Uiterkamp AJS, Mark AE (2007) Does isoprene protect plant membranes from thermal shock? A molecular dynamics study. Biochimica et Biophysica Acta (BBA)-Biomembranes 1768 (2):198-206

Song K, Tan X, Liang Y, Lu X (2016) The potential of *Synechococcus elongatus* UTEX 2973 for sugar feedstock production. Applied microbiology and biotechnology 100 (18):7865-7875. doi:10.1007/s00253-016-7510-z

Toth TN, Chukhutsina V, Domonkos I, Knoppova J, Komenda J, Kis M, Lenart Z, Garab G, Kovacs L, Gombos Z, van Amerongen H (2015) Carotenoids are essential for the assembly of cyanobacterial photosynthetic complexes. Biochimica et biophysica acta 1847 (10):1153-1165. doi:10.1016/j.bbabio.2015.05.020

Tracy NI, Chen D, Crunkleton DW, Price GL (2009) Hydrogenated monoterpenes as diesel fuel additives. Fuel 88 (11):2238-2240

Ungerer J, Lin PC, Chen HY, Pakrasi HB (2018) Adjustments to Photosystem Stoichiometry and Electron Transfer Proteins Are Key to the Remarkably Fast Growth of the Cyanobacterium *Synechococcus elongatus* UTEX 2973. mBio 9 (1). doi:10.1128/mBio.02327-17

Ungerer J, Pakrasi HB (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. Scientific reports 6:39681

Ungerer J, Tao L, Davis M, Ghirardi M, Maness P-C, Yu J (2012) Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. Energy & Environmental Science 5 (10):8998-9006

van der Woude AD, Angermayr SA, Puthan Veetil V, Osnato A, Hellingwerf KJ (2014) Carbon sink removal: Increased photosynthetic production of lactic acid by *Synechocystis* sp. PCC6803 in a glycogen storage mutant. Journal of biotechnology 184:100-102. doi:10.1016/j.jbiotec.2014.04.029

Wang B, Eckert C, Maness PC, Yu J (2018) A Genetic Toolbox for Modulating the Expression of Heterologous Genes in the Cyanobacterium *Synechocystis* sp. PCC 6803. ACS synthetic biology 7 (1):276-286. doi:10.1021/acssynbio.7b00297

Wang X, Liu W, Xin C, Zheng Y, Cheng Y, Sun S, Li R, Zhu XG, Dai SY, Rentzepis PM, Yuan JS (2016) Enhanced limonene production in cyanobacteria reveals photosynthesis limitations. Proceedings of the National Academy of Sciences of the United States of America 113 (50):14225-14230. doi:10.1073/pnas.1613340113

Wendt KE, Ungerer J, Cobb RE, Zhao H, Pakrasi HB (2016) CRISPR/Cas9 mediated targeted mutagenesis of the fast growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Microbial cell factories 15 (1):115

Yoo SH, Keppel C, Spalding M, Jane JL (2007) Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. Int J Biol Macromol 40 (5):498-504. doi:10.1016/j.ijbiomac.2006.11.009

Young JD, Shastri AA, Stephanopoulos G, Morgan JA (2011) Mapping photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis. Metabolic engineering 13 (6):656-665. doi:10.1016/j.ymben.2011.08.002

Yu J, Liberton M, Cliften PF, Head RD, Jacobs JM, Smith RD, Koppenaal DW, Brand JJ, Pakrasi HB (2015) *Synechococcus elongatus* UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2. Scientific reports 5:8132. doi:10.1038/srep08132

Zhou C, Li Z, Wiberley-Bradford AE, Weise SE, Sharkey TD (2013) Isopentenyl diphosphate and dimethylallyl diphosphate/isopentenyl diphosphate ratio measured with recombinant isopentenyl diphosphate isomerase and isoprene synthase. Anal Biochem 440 (2):130-136. doi:10.1016/j.ab.2013.05.028

Zhou J, Wang C, Yang L, Choi E-S, Kim S-W (2015) Geranyl diphosphate synthase: an important regulation point in balancing a recombinant monoterpene pathway in *Escherichia coli*. Enzyme and microbial technology 68:50-55

Chapter 2: Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803

This chapter was reproduced from the following publication:

Lin P-C, Saha R, Zhang F, Pakrasi HB (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. Scientific reports 7 (1):17503. doi:10.1038/s41598-017-17831-y

Abstract

Isoprenoids are diverse natural compounds, which have various applications as pharmaceuticals, fragrances, and solvents. The low yield of isoprenoids in plants makes them difficult for cost-effective production, and chemical synthesis of complex isoprenoids is impractical. Microbial production of isoprenoids has been considered as a promising approach to increase the yield. In this study, we engineered the model cyanobacterium *Synechocystis* sp. PCC 6803 for sustainable production of a commercially valuable isoprenoid, limonene. *Limonene synthases* from the plants *Mentha spicata* and *Citrus limon* were expressed in cyanobacteria for limonene production. Production of limonene was two-fold higher with limonene synthase from *M. spicata* than that from *C. limon*. To enhance isoprenoid production, computational strain design was conducted by applying the OptForce strain design algorithm on *Synechocystis* 6803. Based on the metabolic interventions suggested by this algorithm, genes (*ribose 5-phosphate isomerase* and *ribulose 5-phosphate 3-epimerase*) in the pentose phosphate pathway were overexpressed, and a *geranyl diphosphate synthase* from the plant *Abies grandis* was expressed to optimize the limonene biosynthetic pathway. The optimized strain produced 6.7 mg/L of limonene, a 2.3-fold

improvement in productivity. Thus, this study presents a feasible strategy to engineer cyanobacteria for photosynthetic production of isoprenoids.

Keywords: limonene, *Synechocystis*, OptForce algorithm, pentose phosphate pathway

2.1 Introduction

Recent studies have demonstrated the potential of using cyanobacteria as biological platforms to produce fuels and high-value chemicals (Oliver and Atsumi 2014; Angermayr et al. 2015). Harnessing solar energy using the photosynthetic apparatus, atmospheric CO_2 is fixed into sugars, which can be further converted to desired products by engineered cyanobacteria. Due to the recent development of genetic tools for model cyanobacteria (Berla et al. 2013), expression of heterologous genes and pathways has become more feasible, thus facilitating the construction of engineered cyanobacteria for biotechnological applications. In this study, we engineered the model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis* 6803) for production of a commercially valuable isoprenoid, limonene.

Isoprenoids are one of the most diverse groups of natural products, with more than 55,000 compounds (Breitmaier 2006). Isoprenoids have multiple commercial applications, including natural pharmaceuticals, nutraceuticals, solvents, and perfume components (George et al. 2015; Wang et al. 2005). To date, commercially-used isoprenoids are mainly extracted from plants, but the low quantities of these naturally-produced chemicals have become an impediment for cost-effective production. Successful microbial production of valuable isoprenoids by engineered yeast and *E. coli* have been demonstrated (Westfall et al. 2012; Martin et al. 2003), whereas fewer

researchers have studied isoprenoid's production by cyanobacteria. To improve photosynthetic production of isoprenoids, optimization of isoprenoid biosynthetic pathways in cyanobacteria is needed using metabolic engineering coupled with computational approaches.

Limonene is a 10-carbon isoprenoid produced by plants. (R)-limonene has a characteristic fragrance of orange, and commonly exists in the rinds of citrus fruits. It is commercially used as a fragrance in perfumes or a solvent in cleaning products. (S)-Limonene is a precursor for the biosynthesis of (S)-menthol, which is the major component of mint. Recently, limonene has been evaluated as a "drop-in" replacement for diesel (Tracy et al. 2009) and jet fuels (Chuck and Donnelly 2014). The fully hydrogenated form of limonene was used as a diesel additive, exhibiting similar chemical properties compared to diesel fuel (Tracy et al. 2009). Moreover, the physical properties of limonene, such as viscosity, freezing point, and boiling point, are highly comparable to aviation fuel Jet A-1 (Chuck and Donnelly 2014).

Cyanobacteria use the methylerythritol 4-phosphate (MEP) pathway to produce isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are the building blocks for isoprenoid biosynthesis. The MEP pathway is a seven-step pathway that starts with glyceraldehyde 3-phosphate (GAP) and pyruvate, and ends with IPP and DMAPP. Further, IPP and DMAPP undergo a series of head-to-tail condensations to produce diphosphate substrates, which are then converted to isoprenoids by isoprenoid synthases. To increase isoprenoid production, the amounts of IPP and DMAPP need to be enhanced by increasing the carbon flux toward the MEP pathway.

Attempts have been made to engineer the MEP pathway for improving cyanobacterial limonene production. However, production titers are extremely low compared to other compounds

such as ethanol (Gao et al. 2012), butanol (Lan and Liao 2012), and free fatty acid (Liu et al. 2011). Genes involving in the bottlenecks of the MEP pathway were overexpressed in *Synechocystis* 6803 (Kiyota et al. 2014). The recombinant strain showed a 1.4-fold increase of limonene, and the final titer reached 1 mg/L after 30-day cultivation (Kiyota et al. 2014). In addition, researchers used similar strategies to engineer the MEP pathway in the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120 for production of limonene (Halfmann et al. 2014). The limonene yield increased up to 6.8-fold. However, the final titer remained low (0.5 mg/L over 12-day incubation) (Halfmann et al. 2014).

A previous *in vitro* study suggested that isoprenoid production in *Synechocystis* 6803 is stimulated by compounds in the pentose phosphate (PP) pathway but not by substrates in the MEP pathway (Ershov et al. 2002). Using *Synechocystis* 6803 cell extracts, isoprenoid biosynthesis was significantly improved by supplying xylulose 5-phosphate (X5P) in the PP pathway, whereas providing substrates (GAP, pyruvate, and MEP) in the MEP pathway showed lower stimulation of isoprenoid production (Ershov et al. 2002). These results indicated a connection between the PP pathway and isoprenoid production in *Synechocystis* 6803.

In addition to experimental engineering approaches, computational strain design techniques can be useful to develop non-intuitive genetic interventions to achieve the desired level of production of a particular bioproduct. To this end, the OptForce procedure (Ranganathan et al. 2010) first characterizes the wild-type strain in the form of reaction flux ranges by utilizing the ^{13}C MFA (Metabolic Flux Analysis) flux estimations as additional regulations. OptForce then contrasts the wild-type flux ranges with those in the overproducing phenotype. As a result, the algorithm identifies a set of genetic interventions (i.e., up/down-regulations and deletions) that

must happen in the metabolic reaction network for a desired level of yield. Finally, OptForce pinpoints the minimal interventions (from these changes) that are directly related to achieving the desired yield. These strategies can then be tested in an experimental setting.

In this work, we engineered *Synechocystis* 6803 for photosynthetic limonene production (Figure 2.1). To construct limonene-producing strains, genes encoding *limonene synthase* (*lims*) from *Mentha spicata* and *Citrus limon* were introduced into *Synechocystis* 6803. For generating computation-driven non-intuitive strain engineering strategies, we applied the OptForce algorithm (Ranganathan et al. 2010) on the genome-scale *Synechocystis* 6803 model *i*Syn731 (Saha et al. 2012) and also utilized ^{13}C MFA flux estimations (Young et al. 2011) under photosynthetic wild-type condition. OptForce predicted the up-regulation of two PP pathway genes, *ribose 5-phosphate isomerase* (*rpi*) and *ribulose 5-phosphate 3-epimerase* (*rpe*), in limonene-producing strains in order to divert the carbon flux toward limonene production. Furthermore, based on the prediction made by OptForce to further improve limonene production, a *geranyl diphosphate synthase* (*gpps*) from *Abies grandis* was expressed to optimize the limonene production pathway. The final recombinant strain led to a 2.3-fold improvement in yield, producing 6.7 mg/L of limonene in 7 days. The metabolic engineering strategies used in this study demonstrate the feasibility of increasing limonene production in *Synechocystis* 6803 and can be applied to phototrophic production of other high-value isoprenoids**.**

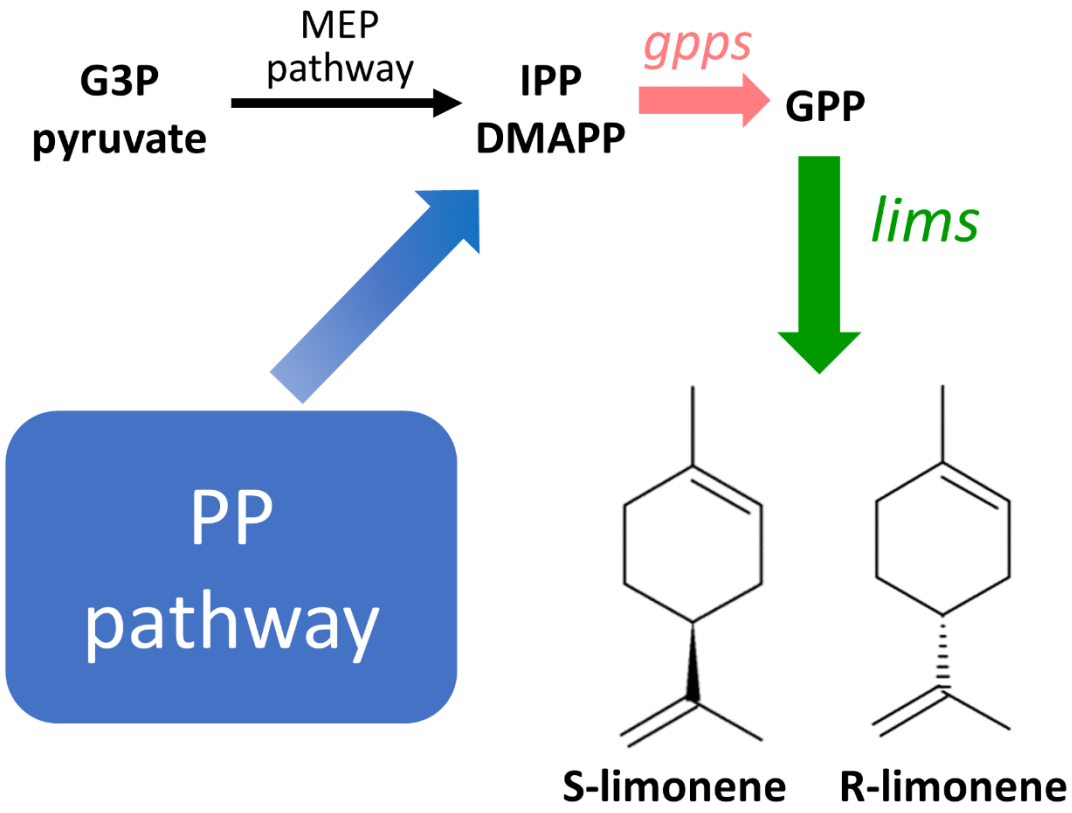


Figure 2.1 Schematic representation of engineering *Synechocystis* 6803 for production of limonene.

Codon-optimized *limonene synthases* from *Mentha spicata* and *Citrus limon* were heterologously expressed in *Synechocystis* 6803 to produce S-limonene and R-limonene, respectively. The limonene biosynthetic pathway was optimized by overexpressing genes in the pentose phosphate (PP) pathway and a *geranyl diphosphate synthase* from *Abies grandis*. G3P, glyceraldehyde 3-phosphate; MEP, methylerythritol-4-phosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; *lims*, limonene synthase; *gpps*, geranyl diphosphate synthase.

2.2 Results

2.2.1 Engineering *Synechocystis* 6803 for production of limonene

Limonene is a C10 cyclic isoprenoid converted from geranyl diphosphate (GPP). Due to the complex nature of carbocation rearrangement from GPP to limonene, limonene synthase produces not only limonene but also other monoterpenes such as bicyclic α-pinene and acyclic mycene (Bohlmann et al. 1997). To avoid the production of other unwanted byproducts, we chose limonene synthases which have the highest specificity for limonene production. Based on previous studies, limonene synthase from *Citrus limon* and *Mentha spicata* produce limonene of high purity. Expression of each of these limonene synthases in *E. coli* showed that the former produces 99% pure (R)-limonene (Lucker et al. 2002), and the latter generates 94% of (S)-limonene (Colby et al. 1993). The coding sequences of *lims* were codon optimized for *Synechocystis* 6803, and the plastid targeting sequences were removed (Davies et al. 2014; Alonso-Gutierrez et al. 2013). The truncated enzyme is known to have better catalytic activity than the native protein (Williams et al. 1998). Genes were cloned into a pCC5.2 neutral-site-targeting plasmid and driven by the trc1O promoter for higher level expression of *lims* (Figure 2.2A). Expression of an enhanced yellow fluorescent protein (EYFP) from the pCC5.2 endogenous plasmid is 8 to 14 times higher than that on the chromosome (Ng et al. 2015).

When the *lims* was cloned into a suicide plasmid and transformed into *E. coli*, we found that the gene accumulated random mutations in the *E. coli* host, leading to changes in amino acid residues or truncated proteins. This was presumably because the *lims* product is toxic to *E. coli* cells. To introduce a *lims* without mutations into *Synechocystis* 6803, we circumvented the *E. coli* cloning step by first cloning the *lims* into the suicide plasmid via Gibson assembly, and used the

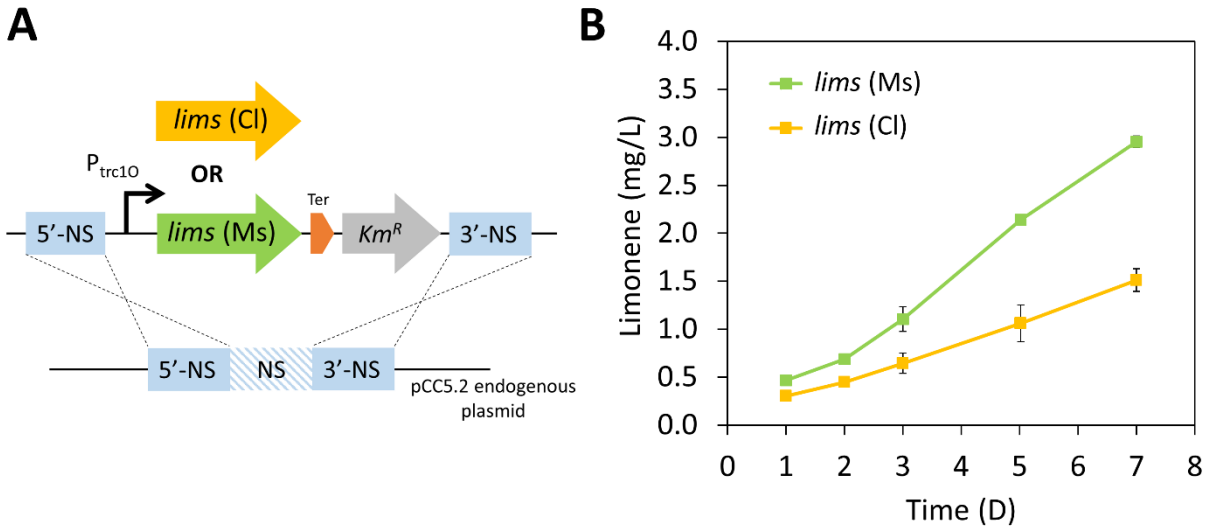


Figure 2.2 Production of limonene by engineered *Synechocystis* 6803.

(A) Introduction of *limonene synthases* into a neutral site on endogenous pCC5.2 plasmid to create limonene-producing mutants. **(B)** Time-course limonene production. Results were mean±SD of three biological replicates. *lims* (Ms), *limonene synthase* from *M. spicata*; *lims* (Cl), *limonene synthase* from *C. limon*; P_{trc1O}, trc1O promoter; NS, neutral site; Ter, terminator; Km^R, kanamycin resistance cassette.

assembled product as template for PCR to amplify the *lims* cassette flanked by upstream and downstream homologous sequences of the neutral site in pCC5.2 (Ng et al. 2015). Subsequently, the PCR product was directly used for natural transformation into *Synechocystis* 6803. The *lims* was introduced into *Synechocystis* 6803 genome via double homologous recombination (Figure 2.2A). DNA sequencing results showed that the *lims* has no mutation in *Synechocystis* 6803 (data not shown). Mutants were fully segregated after re-streaking the cells several times on BG-11 plates with antibiotics. Limonene production by engineered *Synechocystis* 6803 was tested by incubating cultures for 7 days. Because of the volatility of limonene, a dodecane overlay was applied on cultures to collect limonene in the organic layer. It has been reported that over 99% of limonene escapes from the cyanobacterial cultures (Kiyota et al. 2014), and covering an organic

overlay on cultures had little influence on growth in cyanobacteria (Davies et al. 2014). The limonene yield by the strain expressing *lims* from *M. spicata* was two-fold higher than that by the strain expressing *lims* from *C. limon* (Figure 2.2B). These results suggest that the limonene synthase from *M. spicata* exhibited better catalytic activity in *Synechocystis* 6803, and hence, the strain was used for further engineering.

2.2.2 Computational modeling

The *i*Syn731 metabolic model of *Synechocystis* 6803 (Saha et al. 2012) was used to perform the computational strain designs using the OptForce algorithm (Ranganathan et al. 2010) for overproduction of limonene. Based on the current understanding as reported in literature (Ershov et al. 2002; Poliquin et al. 2004), a connection between Calvin Benson Cycle (CBC)/PP pathway and MEP pathway (Figure 2.3) was included in the *i*Syn731 model. By superimposing the photoautotrophic flux measurements (Young et al. 2011) of 31 reactions of central carbon metabolism including the CBC and PP pathways of *Synechocystis* 6803 onto the *i*Syn731 model, the phenotypic space of the base strain was defined. All simulations were performed for a basis of 100 millimoles of CO_2 plus H_2CO_3 uptake and unlimited photon supply (Young et al. 2011). The uptake fluxes for the remaining metabolites present in the BG11 medium was set to -1,000 and the non-growth associated ATP maintenance was set at 8.39 mmole/gDW-h. In addition, the biomass flux was fixed at the optimal value subject to the experimental flux measurements (Young et al. 2011). The upper bound of the fluxes of the remaining reactions was set to 1,000 mmole/gDW-h, whereas the lower bound was set to zero and -1,000 mmole/gDW-h for irreversible and reversible reactions, respectively.

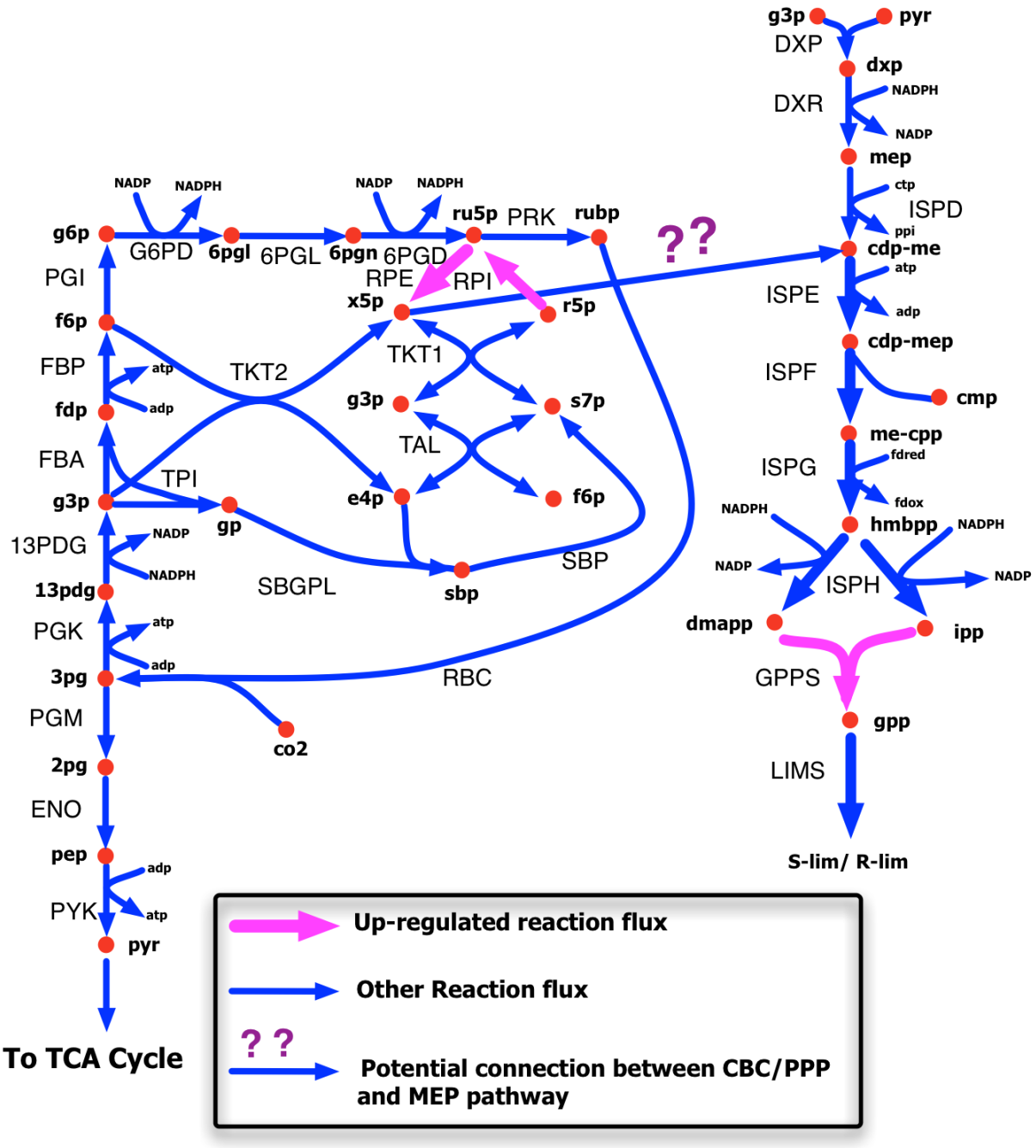


Figure 2.3 Metabolic interventions predicted by the OptForce algorithm.

Up-regulation of *rpe*, *rpi*, and *gpps* (showed with pink arrows) leads to the improved production yield of limonene.

Similarly, the limonene overproducing phenotype was obtained by maximizing and minimizing each flux of the metabolic model iteratively subject to the network stoichiometry, uptake and medium conditions, regulatory constraints, and overproduction target. In this work, a minimum production yield of 85% of the theoretical maximum of limonene (*i.e.*, 15.3 mmole/gDW-h) was set as the overproduction target, while the biomass flux was constrained to be at least 10% of its theoretical maximum (i.e., 0.021 h^{-1}) with the basis of 100 millimoles of carbon fixed (i.e., CO_2 plus H_2CO_3). The remaining parameter values including medium conditions and regulatory constraints were the same as those in the wild-type. By contrasting the maximal range of flux variability between the wild-type strain and the over-producing strain to meet the pre-specified yield of limonene, OptForce was used to identify the minimal set of genetic interventions (i.e., deletions and up-/down-regulations). In order to first explore non-intuitive interventions, reactions from the MEP and isoprenoid biosynthesis pathways were not considered as the candidates for any form of intervention. Integer cuts were used to identify alternative optimal solutions (i.e., alternative genetic intervention choices) to achieve the minimum production yield of limonene as specified earlier. The termination criterion for the OptForce procedure was set as either meeting a production yield of at least 85% of the theoretical maximum for limonene or exceeding the maximum allowable number of reaction interventions (i.e., three). Note that the OptForce procedure works at the reaction level, which is why the set of genetic manipulations can subsequently be identified by using gene-protein-reaction (GPR) associations from the *i*Syn731 model. Thus, the OptForce procedure identified up-regulation of *rpi* and *rpe* as the best possible solution, which can lead up to limonene yield at 89% of its theoretical maximum (i.e., 16.02 mmole/gDW-h). By up-regulating these two genes, OptForce suggested to force more flux from the CBC/PP pathway toward MEP pathway that can ultimately increase the production yield of

limonene (Figure 2.3). Once the set of non-intuitive interventions was obtained, as a next step, it was logical to explore if their combination with any of the intuitive one(s) from the MEP and isoprenoid biosynthesis pathways could further improve the limonene production yield that was otherwise not possible to achieve individually (*i.e.*, by the non-intuitive candidates or by the intuitive ones). With a target of a minimum production yield of 90% of the theoretical maximum of limonene, the OptForce procedure identified the up-regulation of *gpps*, *rpe*, and *rpi* that could lead the limonene production yield to 16.56 mmole/gDW-h (i.e., 92% of its theoretical maximum). Thus, the proposed interventions combined the amplification (i.e., push) of flux from the CBB/PP pathway to MEP pathway with a similar increase (i.e., pull) in the flux of the limonene synthesis. As reported in the literature (Tai and Stephanopoulos 2013), this kind of push-and-pull strategy can achieve the desired level of production yield with minimal effects caused by feedback inhibition.

2.2.3 Genetic interventions of the PP pathway to improve limonene production

Based on the prediction of the OptForce procedure, up-regulation of *rpi* and *rpe* genes in the PP pathway increases the flux toward limonene production. To test this hypothesis, the *rpi* and *rpe* genes driven by the *Synechocystis* 6803 native *rbcL* promoter were expressed on a replicating plasmid in the limonene-producing strain, resulting in 1.3-fold increase in limonene yield (3.7 mg/L) after 7 days of cultivation (Figure 2.4). Furthermore, we introduced a gene encoding a specific GPP synthase (GPPS) to optimize the limonene biosynthetic pathway. In *Synechocystis* 6803, formation of GPP is catalyzed by a farnesyl diphosphate (FPP) synthase, CrtE. It performs consecutive condensation of IPP with DMAPP, and only synthesizes GPP as an intermediate

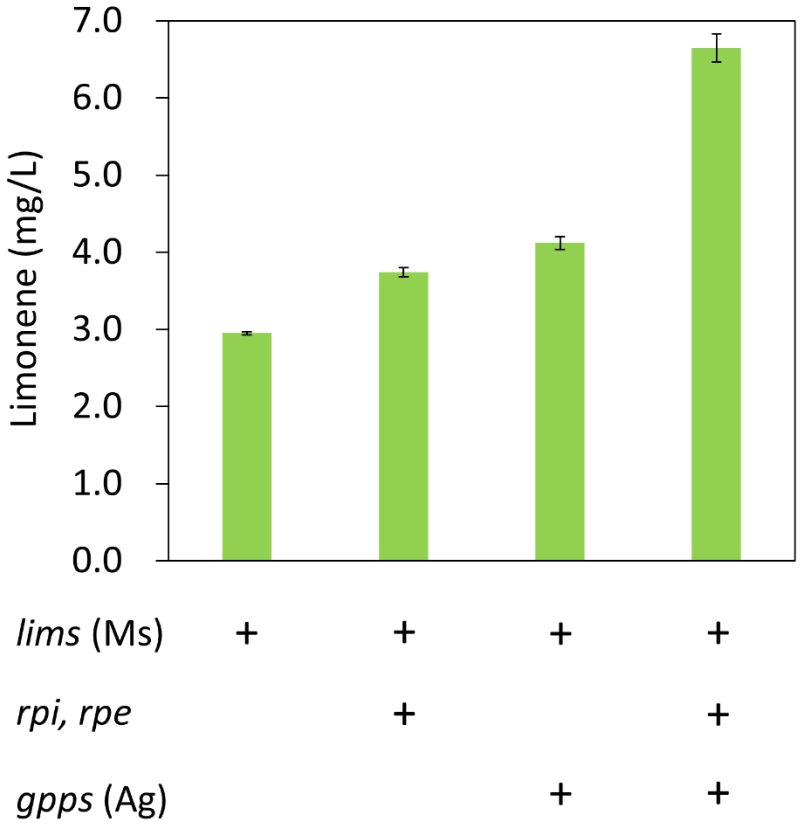


Figure 2.4 Increased limonene production by genetic modifications.

Two genes in the PP pathway (*rpi* and *rpe*) were overexpressed in *Synechocystis* 6803 to divert carbon flux toward limonene production. The *Abies grandis* GPPS 2 that specifically produce GPP was expressed to ensure sufficient GPP for limonene production. Ms, *Mentha spicata*; Ag, *Abies grandis*. Results were mean±SD of three biological replicates.

(Hosfield et al. 2004). Although the PP pathway was engineered to stimulate the limonene yield, it is possible that the native isoprenoids pathway in *Synechocystis* 6803 provides insufficient GPP for limonene production since the flux is diverted toward FPP formation for pigment synthesis. In addition, OptForce also predicted an increase (i.e., from 89% to 92% of maximum theoretical limonene yield) when up-regulation of *rpe* and *rpi* was combined with the up-regulation of *gpps*. It was reported that the GPPS 2 from *Abies grandis* specifically produces GPP (Burke and Croteau

2002). Expressing this specific *gpps* with *lims*, the limonene yield increased 1.4-fold (4.1 mg/L) (Figure 2.4). Finally, coexpression of *rpi*, *rpe*, *gpps* and *lims* resulted in a remarkable (2.3-fold) enhancement in productivity (6.7 mg/L) (Figure 2.4).

2.2.4 Pigment content in engineered *Synechocystis* 6803

Carotenoids and the phytol tail of chlorophyll, photosynthetic pigments, are derived from geranylgeranyl diphosphate (GGPP), a C20-intermediate for isoprenoid synthesis. Hence, production of limonene is expected to divert carbon flux away from pigment synthesis. To investigate the effect of limonene production on pigment content in engineered *Synechocystis* 6803, we extracted and quantified the chlorophyll and carotenoid contents. The chlorophyll content decreased over 30% in the *gpps* expression strains, whereas carotenoid levels were similar among the limonene-producing strains (Figure 2.5). These results indicate that the specific GPPS diverts the carbon flux away from pigment synthesis.

2.3 Discussion

In this study, we combined metabolic engineering with model-driven strain design strategies to engineer *Synechocystis* 6803 for enhanced limonene production. To generate limonene-producing *Synechocystis* 6803, we first constructed a suicide plasmid (Ng et al. 2015) to engineer the *lims* into the neutral site on the pCC5.2 endogenous plasmid via double homologous recombination. This is the first time that the endogenous plasmid of *Synechocystis* 6803 has been used for enhanced production for the purpose of metabolic engineering. Expression of a gene on the pCC5.2 plasmid leads to higher expression level than that on the chromosome as well as the RSF1010 self-

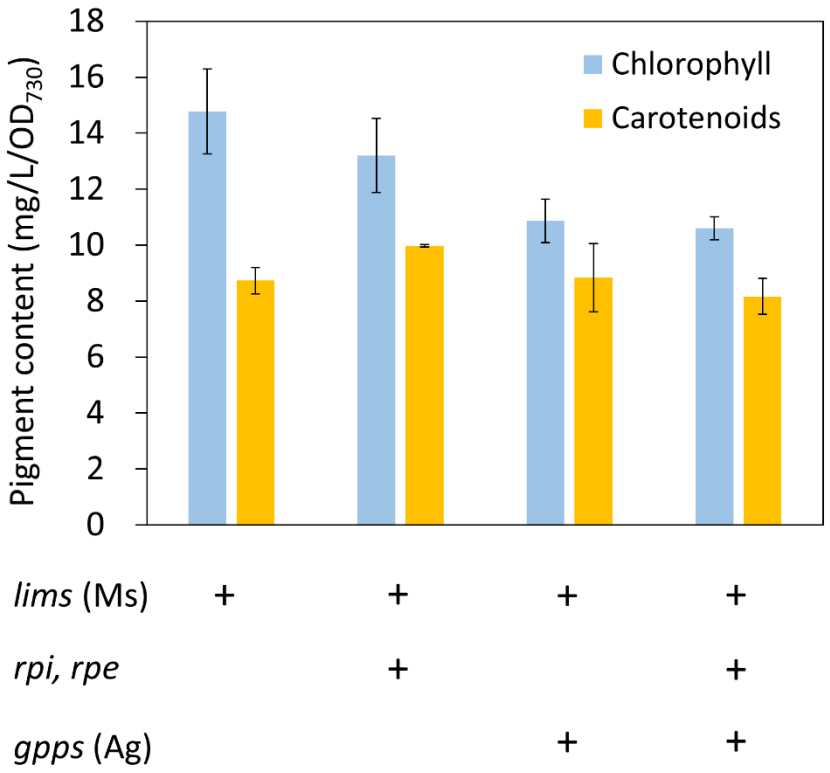


Figure 2.5 Pigment content of engineered *Synechocystis* 6803 strains.

Chlorophyll and carotenoid contents of limonene-producing strains. Ms, *Mentha spicata*; Ag, *Abies grandis*. Results were mean±SD of three biological replicates.

replicating plasmid (Ng et al. 2015). Furthermore, during the stationary phase of cell growth, the copy numbers of the endogenous plasmids (pCA2.4, pCB2.4, pCC5.2) in *Synechocystis* 6803 are 3 to 7 per chromosome (Berla and Pakrasi 2012). Using the endogenous plasmid to express the *lims* gene driven by the constitutive promoter trc1O allows high expression level at the stationary phase, decoupling growth and production, and thus leading to higher levels of production of limonene.

The higher yield with limonene synthase from *M. spicata* than that from *C. limon* may be due to the difference in enzyme kinetics of LIMS. Unfortunately, the kinetic parameters (both K_m and k_{cat}) are only available for the enzyme from *M. spicata* (Williams et al. 1998). In addition, it may be attributed to different protein expression levels. Although the same promoter was used to control the *lims* from two plant species, protein expression may vary because of different mRNA sequences and codon usage. To date, the highest reported limonene productivity in cyanobacteria was achieved by engineered *Synechococcus* sp. PCC 7002 (Davies et al. 2014). In their study, only a *lims* from *M. spicata* was overexpressed, and the yield was over 4 mg/L in 4 days (Davies et al. 2014). Our results also suggested that the LIMS from *M. spicata* performed better in limonene production (Figure 2.2B). The doubling time of *Synechococcus* 7002 is shorter than *Synechocystis* 6803 (Yu et al. 2015). Thus, the higher limonene yield from *Synechococcus* 7002 may be due to its faster growth rate. A recent study engineered *Synechococcus elongatus* PCC 7942 to produce limonene, achieving a 100-fold improvement in productivity (Wang et al. 2016). However, it should be noted that such significant increase is due to the low productivity of the original strain, which produced merely 8.5 μg/L/OD/d of limonene. The best producing strain in this study, with a *lims* (*M. spicata*) controlled by the pea plant *psbA* promoter, produced 2.5 mg/L limonene in 5 days (Wang et al. 2016).

Previously, researchers have engineered *Synechocystis* 6803 for limonene production by overexpressing genes involved in the bottleneck steps of the MEP pathway (Kiyota et al. 2014). It is known that enzymes 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and isopentenyl diphosphate isomerase (IDI) catalyze the rate-limiting reactions in the MEP pathway (Estevez et al. 2001; Wang et al. 1999). With the introduction of an additional copy of endogenous *dxs*, *idi*, and *gpps* genes, the engineered *Synechocystis* 6803 produced 1.4-fold higher yield than that of the

parent strain (Kiyota et al. 2014). However, such improvement was less effective than the strategy used in the current study. As mentioned in the Results section, the endogenous *gpps* gene may not be suitable for enhancing the production of limonene. In addition, the MEP pathway is highly regulated at genetic and metabolic levels (Banerjee and Sharkey 2014). Expressing endogenous genes in the MEP pathway may be subject to native regulations, presenting a less effective engineering approach.

Instead of manipulating the MEP pathway, we took a systematic model-driven metabolic engineering approach for finding genetic interventions in order to increase the limonene production yield. As explained in the Materials and Methods section, the OptForce procedure finds the minimal interventions to reach a desired production target. To this end, we employed OptForce on our previously developed genome-scale model *i*Syn731 in order to *'push'* more flux to MEP pathway and also to create better *'pull'* for limonene synthesis (Figure 2.3). From this *in silico* analysis, by up-regulating *rpe* and *rpi*, the metabolite pool of X5P was found to be increased that, eventually, led to increased flux through the connection between the CBC/PP pathway and the MEP pathway. In addition, up-regulation of *gpps* created an improved *'pull'* for limonene synthesis. Thus, the combination of this push-and-pull mechanism was proposed to be the best strategy to improve limonene yield by circumventing additional regulations (*e.g.* feedback inhibition). Interestingly, the same rationale could be applied to engineer cyanobacteria to produce other isoprenoid compounds.

Expression of the specific *gpps* modestly increased the limonene titer (Figure 2.4), whereas the cellular chlorophyll content was greatly influenced (Figure 2.5). Synthesis of limonene and the phytol tail of chlorophyll requires the same precursors, IPP and DMAPP. Table 2.1 compares the

Table 2.1 Comparison of chlorophyll and limonene contents

Strains	*lims*	*lims, gpps*	*lims, gpps, rpi, rpe*
Chlorophyll*	66	49	47
Limonene*	53	81	122

*The unit is presented as $\mu M/OD_{730}$ isoprene unit

changes in chlorophyll and limonene contents between the strain with *lims* only and the two *gpps*-expressing strains. Compared to the *lims*-expressing strain, additional expression of *gpps* resulted in similar level of decrease in chlorophyll content in both strains. However, expression of *rpi* and *rpe* genes further led to 1.5-fold higher limonene productivity in the *gpps*-expressing strains (122 vs. 81 $\mu M/OD_{730}$ isoprene unit). This result suggests that up-regulation of *rpi* and *rpe* enhances the carbon flux toward limonene synthesis.

Our results showed that overexpressing the genes in the PP pathway led to improved limonene production, suggesting an unidentified connection between the PP pathway and isoprenoids biosynthesis (Figure 2.4). Our observation is consistent with previous *in vitro* study using *Synechocystis* 6803 cell lysate (Ershov et al. 2002). However, the connection between the PP pathway and isoprenoids biosynthesis remains to be elucidated. It was first shown that *in vitro* isoprenoid production increased significantly by providing substrates in the PP pathway (Ershov et al. 2002), while a recent study showed that increased production of isoprenoids by PP pathway substrates does not occur through the MEP pathway (Poliquin et al. 2013). By removing the terminal enzyme of the MEP pathway in *Synechocystis* 6803 cell lysate, isoprenoid synthesis still increased by substrates in the PP pathway (Poliquin et al. 2013). Taken together, it is still unclear how the PP pathway and isoprenoid production are connected in *Synechocystis* 6803. While our

results made a strong argument for this connection, further investigation needs to be conducted to explore the details in terms of chemical conversions and genes/enzymes associated. From the modeling context, these details sometimes do not make much of a difference if they only involve aggregating linear reaction steps.

2.4 Conclusions

In this study, we engineered the model cyanobacterium *Synechocystis* 6803 to produce the isoprenoid, limonene. We applied computational strain design by using the OptForce procedure to identify minimal genetic interventions for improving limonene yield. Based on the prediction, the *rpi* and *rpe* genes in the PP pathway were overexpressed, and a specific *gpps* was introduced to optimize the limonene biosynthetic pathway. The final engineered strain produced 6.7 mg/L of limonene, which is a 2.3-fold improvement in productivity. The approach that we demonstrated can be applied to engineer cyanobacteria to produce other valuable isoprenoids.

2.5 Methods

2.5.1 Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Phusion DNA polymerase were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.5.2 Culture medium and condition

All strains were maintained in liquid BG-11 medium or on solid BG-11 plates with appropriate antibiotics at 30ºC continuous white light (50 μmoles photons m^{-2} s^{-1}).

2.5.3 DNA manipulations

Coding sequences of *lims* from *Mentha spicata* and *Citrus limon* were codon optimized for *Synechocystis* 6803 and synthesized by IDT (San Jose, CA, USA). The genes were cloned into a suicide plasmid, allowing gene insertion into the neutral site (NSP1) on the endogenous plasmid pCC5.2 in *Synechocystis* 6803 (Ng et al. 2015). The constructed plasmids were directly used as templates for PCR to amplify a fragment which contains the *lims* and a kanamycin resistance cassette flanking by upstream and downstream homologous sequences of the NSP1 (Figure 2.2A). The PCR product was then purified by DNA electrophoresis, and the linear DNA was transformed into *Synechocystis* 6803. The *rpi*, *rpe,* and *gpps* genes were cloned into a broad-host-range plasmid RSF1010 harboring a spectinomycin resistance cassette (Taton et al. 2014). All the cloning works were done by Gibson isothermal DNA assembly method (Gibson et al. 2009).

2.5.4 Strains construction and transformation

The *lims* expression cassette was transformed into *Synechocystis* 6803 through homologous recombination. Cells at mid-log phase (OD_{730} of 0.4 to 0.6) were incubated with 600 ng of linear DNA overnight at 30°C in the dark. Cells were then grown on BG-11 plates supplemented with 10 μg/mL of kanamycin for selection of transformants. Colonies were patched on BG-11 plates with 20 μg/mL of kanamycin for segregation. PCR was used to verify strain segregation. For the

construction of *rpi*, *rpe*, and *gpps* expressing strains, self-replicating plasmids (600 ng per transformation) were transformed into the strain expressing *lims*. Transformants were selected by BG-11 plates with 2 μg/mL of spectinomycin and 5 μg/mL of kanamycin.

2.5.5 Limonene production by engineered cyanobacteria

Strains were inoculated in BG-11 medium with kanamycin (10 μg/mL) and spectinomycin (4 μg/mL) to mid-log phase at 30°C with continuous white light (50 μmoles photons $m^{-2}s^{-1}$). Cells were collected by centrifugation at 7,000 x *g*, and washed by BG-11 medium to remove antibiotics. To test limonene production, the initial OD_{730} was adjusted to 0.34 (~0.5 g/L of biomass), and 50 mL of cell cultures were grown in 250-mL flasks at 30°C with continuous white light (130 μmoles photons m^{-2} s^{-1}). A 10% (v/v) dodecane overlay was covered on top of cultures to trap evaporated limonene.

2.5.6 Quantification of limonene

Limonene samples were prepared by diluting 10 μL of dodecane overlay in 990 μL of ethyl acetate, and analyzed using a gas chromatography instrument with a flame ionization detector (Hewlett-Packard model 7890A, Agilent Technologies, CA, USA) equipped with a 30 m DB5-MS column (J&W Scientific). The oven temperature program initiated at 60°C, and increased at 12°C/min to 300°C. Limonene was quantified using a (R)-limonene standard.

2.5.7 Identification of engineering interventions via OptForce

We applied the OptForce algorithm (Ranganathan et al. 2010) on the genome-scale *Synechocystis* 6803 model *i*Syn731 (Saha et al. 2012). In order to characterize the wild-type phenotype, we utilized ^{13}C MFA flux estimations (Young et al. 2011) under photosynthetic condition. Below is the step-by-step procedure that we followed:

Step 1: Identify the maximum biomass and limonene yields under photosynthetic condition.

Maximize $v_{biomass}$ or v_{ls}

Subject to

$$\sum_{j=1}^{m} s_{ij} v_j = 0 \; \forall \; \mathrm{i} \in 1, \ldots\ldots, \mathrm{n} \quad (1)$$

$$a_j v_j^{min} \leq v_j \leq a_j v_j^{max} \; \forall \; j \in 1, \ldots\ldots, \mathrm{m} \quad (2)$$

$$0 \leq v_{Nutrients} \leq v_{Nutrients}^{\max} \forall \text{ Nutrients} \in \text{Light, Carbon source(s), Micro} - \text{nutrients} \quad (3)$$

Step 2: Characterize the wild-type phenotype

Maximize/Minimize $v_j \; \forall \; j \in$ reactions without experimental flux measurements

Subject to

$$\sum_{j=1}^{m} s_{ij} v_j = 0 \; \forall \; \mathrm{i} \in 1, \ldots\ldots, \mathrm{n} \quad (1)$$

$$a_j v_j^{min} \leq v_j \leq a_j v_j^{max} \; \forall \; j \in 1, \ldots\ldots, \mathrm{m} \quad (2)$$

$$0 \leq v_{Nutrients} \leq v_{Nutrients}^{\max} \forall \text{ Nutrients} \in \text{Light, Carbon source(s), Micro} - \text{nutrients} \quad (3)$$

$$v_{biomass} \geq v_{biomass}^{max} \quad (4)$$

Step 3: Characterize the limonene over-producing phenotype

Maximize/Minimize $v_j \; \forall \; j \in 1, \ldots\ldots, m$

Subject to

$\sum_{j=1}^{m} s_{ij} v_j = 0 \; \forall \; \mathrm{i} \in 1, \ldots\ldots, \mathrm{n}$ (1)

$a_j v_j^{min} \leq v_j \leq a_j v_j^{max} \; \forall \; j \in 1, \ldots\ldots, \mathrm{m}$ (2)

$0 \leq v_{Nutrients} \leq v_{Nutrients}^{\max} \forall \; \text{Nutrients} \in \text{Light, Carbon source(s), Micro} - \text{nutrients}$ (3)

$v_{biomass} \geq 0.1 v_{biomass}^{max}$ (5)

$v_{ls} \geq 0.9 v_{ls}^{max}$ (6)

Step 4: Identify the MUST sets

In this step, fluxes ranging from step 2 and step 3 were compared to identify three different sets: reactions to be up-regulated ($MUST^U$), down-regulated ($MUST^L$), and deleted ($MUST^X$).

Step 5: Identify the minimal engineering interventions

Maximize v_j

(over *MUST* sets)

Subject to

Minimize v_j

(over *MUST* sets)

Subject to

$\sum_{j=1}^{m} s_{ij} v_j = 0 \; \forall \; \mathrm{i} \in 1, \ldots\ldots, \mathrm{n}$ (1)

$a_j v_j^{min} \leq v_j \leq a_j v_j^{max} \; \forall \; j \in 1, \ldots\ldots, \mathrm{m}$ (2)

$0 \leq v_{Nutrients} \leq v_{Nutrients}^{\max} \forall \; \text{Nutrients} \in \text{Light, Carbon source(s), Micro} - \text{nutrients}$ (3)

$v_{biomass} \geq 0.1 v_{biomass}^{max}$ (5)

MUST set conditions (7)

$$\sum \#\, of\ direct\ manipulations \leq k \quad (8)$$

Here, S_{ij} is the stoichiometric coefficient of metabolite i in reaction j and v_j is the flux value of reaction j. Parameters $v_{j,min}$ and $v_{j,max}$ denote the minimum and maximum allowable fluxes for reaction j, respectively. $V_{biomass}$ and v_{ls} represent biomass and limonene synthesis reactions under photosynthetic conditions, whereas $v^{max}{}_{biomass}$ and $v^{max}{}_{ls}$ represent the maximum theoretical yields of biomass and limonene under photosynthetic conditions. The minimal levels of biomass and the minimal target yield of limonene were set to be 10% of maximum biomass and 85% or 90% of maximum limonene yield, respectively. Finally, k represents the maximum number of interventions allowed.

2.5.8 Pigment content analysis

Cell cultures (1 mL) were collected by centrifugation at 16,000 x g for 7 min, and the supernatants were removed. To extract pigments in *Synechocystis*, pre-cooled methanol (1 mL) was added to the pellets, and mixed thoroughly by pipetting and vortexing. Samples were incubated at 4°C for 20 mins, and centrifuged at 16,000 x g for 7 min. The supernatants were removed for a spectrophotometer analysis to quantify the concentrations of carotenoids and chlorophyll. The following equations were used to calculate the pigment content: $\text{chlorophyll}\ (\mu g/mL) = (16.29 \times A_{665}) - (8.54 \times A_{552})$ (Porra et al. 1989); $\text{carotenoids}\ (\mu g/mL) = [(1000 \times A_{470}) - (2.86 \times Chl_a[\mu g/mL])]/221$ (Wellburn 1994).

References

Alonso-Gutierrez J, Chan R, Batth TS, Adams PD, Keasling JD, Petzold CJ, Lee TS (2013) Metabolic engineering of *Escherichia coli* for limonene and perillyl alcohol production. Metabolic engineering 19:33-41. doi:10.1016/j.ymben.2013.05.004

Angermayr SA, Gorchs Rovira A, Hellingwerf KJ (2015) Metabolic engineering of cyanobacteria for the synthesis of commodity products. Trends in biotechnology 33 (6):352-361. doi:10.1016/j.tibtech.2015.03.009

Banerjee A, Sharkey TD (2014) Methylerythritol 4-phosphate (MEP) pathway metabolic regulation. Natural product reports 31 (8):1043-1055. doi:10.1039/c3np70124g

Berla BM, Pakrasi HB (2012) Upregulation of plasmid genes during stationary phase in *Synechocystis* sp. strain PCC 6803, a cyanobacterium. Applied and environmental microbiology 78 (15):5448-5451. doi:10.1128/AEM.01174-12

Berla BM, Saha R, Immethun CM, Maranas CD, Moon TS, Pakrasi HB (2013) Synthetic biology of cyanobacteria: unique challenges and opportunities. Frontiers in microbiology 4:246. doi:10.3389/fmicb.2013.00246

Bohlmann J, Steele CL, Croteau R (1997) Monoterpene synthases from grand fir (Abies grandis). cDNA isolation, characterization, and functional expression of myrcene synthase, (-)-(4S)-limonene synthase, and (-)-(1S,5S)-pinene synthase. The Journal of biological chemistry 272 (35):21784-21792

Breitmaier E (2006) Terpenes: Importance, general structure, and biosynthesis. In: Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. pp 1-9

Burke C, Croteau R (2002) Geranyl diphosphate synthase from *Abies grandis*: cDNA isolation, functional expression, and characterization. Archives of biochemistry and biophysics 405 (1):130-136

Chuck CJ, Donnelly J (2014) The compatibility of potential bioderived fuels with Jet A-1 aviation kerosene. Applied Energy 118:83-91

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. The Journal of biological chemistry 268 (31):23016-23024

Davies FK, Work VH, Beliaev AS, Posewitz MC (2014) Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of *Synechococcus* sp. PCC 7002. Frontiers in bioengineering and biotechnology 2:21. doi:10.3389/fbioe.2014.00021

Ershov YV, Gantt RR, Cunningham Jr FX, Jr., Gantt E (2002) Isoprenoid biosynthesis in *Synechocystis* sp. strain PCC6803 is stimulated by compounds of the pentose phosphate cycle but not by pyruvate or deoxyxylulose-5-phosphate. Journal of bacteriology 184 (18):5045-5051

Estevez JM, Cantero A, Reindl A, Reichler S, Leon P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. The Journal of biological chemistry 276 (25):22901-22909. doi:10.1074/jbc.M100854200

Gao Z, Zhao H, Li Z, Tan X, Lu X (2012) Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. Energy & Environmental Science 5 (12):9857-9865

George KW, Alonso-Gutierrez J, Keasling JD, Lee TS (2015) Isoprenoid drugs, biofuels, and chemicals--artemisinin, farnesene, and beyond. Advances in biochemical engineering/biotechnology 148:355-389. doi:10.1007/10_2014_288

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods 6 (5):343-345. doi:10.1038/nmeth.1318

Halfmann C, Gu L, Zhou R (2014) Engineering cyanobacteria for the production of a cyclic hydrocarbon fuel from CO_2 and H_2O. Green Chemistry 16 (6):3175-3185

Hosfield DJ, Zhang Y, Dougan DR, Broun A, Tari LW, Swanson RV, Finn J (2004) Structural basis for bisphosphonate-mediated inhibition of isoprenoid biosynthesis. The Journal of biological chemistry 279 (10):8526-8529. doi:10.1074/jbc.C300511200

Kiyota H, Okuda Y, Ito M, Hirai MY, Ikeuchi M (2014) Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. Journal of biotechnology 185:1-7. doi:10.1016/j.jbiotec.2014.05.025

Lan EI, Liao JC (2012) ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 109 (16):6018-6023. doi:10.1073/pnas.1200074109

Liu X, Sheng J, Curtiss R, 3rd (2011) Fatty acid production in genetically modified cyanobacteria. Proceedings of the National Academy of Sciences of the United States of America 108 (17):6899-6904. doi:10.1073/pnas.1103014108

Lucker J, El Tamer MK, Schwab W, Verstappen FW, van der Plas LH, Bouwmeester HJ, Verhoeven HA (2002) Monoterpene biosynthesis in lemon (*Citrus limon*). cDNA isolation and functional analysis of four monoterpene synthases. European journal of biochemistry / FEBS 269 (13):3160-3171

Martin VJ, Pitera DJ, Withers ST, Newman JD, Keasling JD (2003) Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. Nat Biotechnol 21 (7):796-802. doi:10.1038/nbt833

Ng AH, Berla BM, Pakrasi HB (2015) Fine-Tuning of Photoautotrophic Protein Production by Combining Promoters and Neutral Sites in the Cyanobacterium *Synechocystis* sp. Strain PCC 6803. Applied and environmental microbiology 81 (19):6857-6863. doi:10.1128/AEM.01349-15

Oliver JW, Atsumi S (2014) Metabolic design for cyanobacterial chemical synthesis. Photosynthesis research 120 (3):249-261. doi:10.1007/s11120-014-9997-4

Poliquin K, Cunningham FX, Gantt RR, Gantt E (2013) Interactions of Isoprenoid Pathway Enzymes and Indirect Stimulation of Isoprenoid Biosynthesis by Pentose Phosphate Cycle Substrates in *Synechocystis* PCC 6803. In: Bach TJ, Rohmer M (eds) Isoprenoid Synthesis in Plants and Microorganisms: New Concepts and Experimental Approaches. Springer New York, New York, NY, pp 51-63. doi:10.1007/978-1-4614-4063-5_4

Poliquin K, Ershov YV, Cunningham FX, Jr., Woreta TT, Gantt RR, Gantt E (2004) Inactivation of *sll1556* in *Synechocystis* strain PCC 6803 impairs isoprenoid biosynthesis from pentose phosphate cycle substrates in vitro. J Bacteriol 186 (14):4685-4693. doi:10.1128/JB.186.14.4685-4693.2004

Porra R, Thompson W, Kriedemann P (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different

solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. Biochimica et Biophysica Acta (BBA)-Bioenergetics 975 (3):384-394

Ranganathan S, Suthers PF, Maranas CD (2010) OptForce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. PLoS Comput Biol 6 (4):e1000744. doi:10.1371/journal.pcbi.1000744

Saha R, Verseput AT, Berla BM, Mueller TJ, Pakrasi HB, Maranas CD (2012) Reconstruction and comparison of the metabolic potential of cyanobacteria *Cyanothece* sp. ATCC 51142 and *Synechocystis* sp. PCC 6803. PloS one 7 (10):e48285. doi:10.1371/journal.pone.0048285

Tai M, Stephanopoulos G (2013) Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production. Metab Eng 15:1-9. doi:10.1016/j.ymben.2012.08.007

Taton A, Unglaub F, Wright NE, Zeng WY, Paz-Yepes J, Brahamsha B, Palenik B, Peterson TC, Haerizadeh F, Golden SS, Golden JW (2014) Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria. Nucleic Acids Res 42 (17):e136. doi:10.1093/nar/gku673

Tracy NI, Chen D, Crunkleton DW, Price GL (2009) Hydrogenated monoterpenes as diesel fuel additives. Fuel 88 (11):2238-2240

Wang CW, Oh MK, Liao JC (1999) Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. Biotechnology and bioengineering 62 (2):235-241

Wang G, Tang W, Bidigare RR (2005) Terpenoids as therapeutic drugs and pharmaceutical agents. In: Natural products. Springer, pp 197-227

Wang X, Liu W, Xin C, Zheng Y, Cheng Y, Sun S, Li R, Zhu XG, Dai SY, Rentzepis PM, Yuan JS (2016) Enhanced limonene production in cyanobacteria reveals photosynthesis limitations. Proceedings of the National Academy of Sciences of the United States of America 113 (50):14225-14230. doi:10.1073/pnas.1613340113

Wellburn AR (1994) The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. Journal of plant physiology 144 (3):307-313

Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A, Fickes S, Diola D, Benjamin KR, Keasling JD, Leavell MD, McPhee DJ, Renninger NS, Newman JD, Paddon CJ (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proceedings of the National Academy of Sciences of the United States of America 109 (3):E111-118. doi:10.1073/pnas.1110740109

Williams DC, McGarvey DJ, Katahira EJ, Croteau R (1998) Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochemistry 37 (35):12213-12220. doi:10.1021/bi980854k

Young JD, Shastri AA, Stephanopoulos G, Morgan JA (2011) Mapping photoautotrophic metabolism with isotopically nonstationary ^{13}C flux analysis. Metabolic engineering 13 (6):656-665. doi:10.1016/j.ymben.2011.08.002

Yu J, Liberton M, Cliften PF, Head RD, Jacobs JM, Smith RD, Koppenaal DW, Brand JJ, Pakrasi HB (2015) *Synechococcus elongatus* UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2. Scientific reports 5:8132. doi:10.1038/srep08132

Chapter 3: Combinatorial optimization of terpene biosynthetic pathway for enhanced limonene production in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973

Abstract

Terpenoids are a large and diverse group of natural products with commercial applications. Microbial production of terpenes is considered as a feasible approach for the stable supply of these complex hydrocarbons. The photosynthetic prokaryotes, cyanobacteria, are potential hosts for bioproduction, because these autotrophs require only light and CO_2 for growth. Despite cyanobacteria having been engineered to produce a variety of compounds, their productivities of terpenes are mostly low. More research is needed to determine the bottleneck reactions for enhancing terpene production in cyanobacteria. In this study, we engineered the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 to produce a commercially-used terpenoid, limonene. We identified an engineered strain with a mutation in the geranylgeranyl pyrophosphate synthase CrtE, leading to a 2.5-fold increase in limonene production. Combining metabolic engineering strategies to optimize the limonene biosynthetic pathway, the engineered *Synechococcus* 2973 produced 16.4 mg L^{-1} of limonene with a rate of 8.2 mg L^{-1} day^{-1}, which is 8-fold higher than the limonene productivities reported in the literature.

3.1 Introduction

Terpenoids (or terpenes) is a diverse group of natural products with a variety of applications, including pharmaceuticals, nutraceuticals, agriculture, and flavor and fragrance (Ajikumar et al. 2008). Microbial production of terpenes offers a versatile approach compared to chemical synthesis and extraction from plant species (Niu et al. 2017). In recent years, cyanobacteria are viewed as potential hosts for microbial bioproduction, because these photosynthetic prokaryotes require only light, CO_2, and minerals for growth. In spite of many efforts to engineer cyanobacteria for terpenoid production, their titers and productivities are mostly low (Lin and Pakrasi 2019). More research is needed to identify the major hurdle for enhancing photosynthetic terpene production.

The terpene biosynthesis pathway is particularly important in photosynthetic microorganisms, because synthesis of photosynthetic pigments, including chlorophyll, and carotenoids, utilizes terpene intermediates. Cyanobacteria first use the MEP pathway to produce the 5-carbon precursors, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), for terpene synthesis. Further, the prenyltransferase CrtE performs sequential reactions to combine DMAPP with IPP to produce pyrophosphate intermediates, including GPP, FPP and GGPP (Figure 1.1). One of the major challenges of using cyanobacteria for terpene production is competition between pigment and product syntheses, in which the carbon fluxes must be balanced to maintain growth and production.

In the present work, we engineered the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 for improved production of a commercially valuable monoterpene, limonene. We discovered a high limonene-producing mutant strain with a 2.5-fold improvement

in limonene production. Whole-genome sequencing results revealed that the high limonene producer has 2 single nucleotide polymorphisms (SNPs) compared to the wild type *Synechococcus* 2973. Using the CRISPR/Cpf1 genome editing, we conducted a mutational analysis to investigate which SNP leads to enhanced limonene production. Our result showed that the mutant strain with a SNP in *crtE* drastically improved limonene productivity. This SNP mutates arginine 299 to glutamine (R299Q), which in turn alters the terpene metabolism in *Synechococcus* 2973. Furthermore, by modulating the expression of a specific GPP synthase (GPPS) from *Abies grandis*, we demonstrated that the enzyme level of GPPS is critical for controlling the carbon fluxes toward limonene synthesis. Finally, we systematically investigated various pathways for enhancing the precursor pool (IPP and DMAPP) for limonene synthesis, providing insights into the engineering of the upstream precursor pathways. This integrated approach of using a CrtE (R299Q) protein mutant, modulation of GPPS expression, and optimization of precursor pathways could be applicable for enhancing other monoterpene production in cyanobacteria.

3.2 Results

3.2.1 Identification of a high limonene-producing mutant of *Synechococcus* 2973

To engineering *Synechococcus* 2973 for production of limonene, the *limonene synthase* (*lims*) from *Mentha spicata* (Colby et al. 1993) and the specific *gpps* from *Abies grandis* (Burke and Croteau 2002) were cloned into the RSF1010 self-replicating plasmid under the control of the *trc1O* promoter (Figure 3.1). The *trc1O* promoter has a 43-fold change in expression level with induction of 1 mM IPTG (see Appendix). Since the GPPS competes with the CrtE for terpene

precursors, the expression level of GPPS should not be too strong to ensure a sufficient carbon flux for pigment synthesis. Therefore, we decided to use a synthetic ribosome binding site (RBS) with a lower translation initiation rate (TIR) to drive expression of GPPS. First, we used the RBS calculator (Salis et al. 2009) to estimate the TIR of the RBS in the trc1O promoter, and obtained a synthetic RBS with half of the TIR compared to that of the RBS in P_{trc1O} (Figure 3.1A). A Lac repressor (*lacI*) was included in the plasmid to control expression of *lims* and *gpps*. In our previous study, we reported that the *lims* gene mutated randomly in *E. coli* (Lin et al. 2017). However, the *lims* gene in plasmid lims-gpps had no mutation after introducing in the *E. coli* host. We reasoned that controlled gene expression by LacI enabled *lims* to be maintained in *E. coli* without mutations.

The plasmid lims-gpps was introduced into *Synechococcus* 2973 via bacterial conjugation. We picked 6 colonies to test limonene production. Notably, the limonene production titer from one of the mutants were 2.5-fold higher than the other strains (Figure 3.1B). To verify this result, we repeated the limonene production test, and the titer from the high-limonene producer was consistently 2.5-fold higher than the other strains (data not shown). We suspected there might be mutations in the *lims* gene or the trc1O promoter that resulted in enhanced limonene production; however, no mutation was found in the plasmid lims-gpps in the high limonene-producing strain (data not shown). These results suggest that the high-limonene producer may have mutations in the genome that benefit limonene synthesis. To test this hypothesis, we cured the plasmid lims-gpps in the high- and low-limonene producers by growing the strains in BG11 medium without the antibiotic kanamycin, and the plasmid lims-gpps was introduced back into the strains (Figure 3.1C). By testing the limonene titer in the re-constructed strains, the high producer remained to produce 2.5-fold higher of limonene than the low producers (Figure 3.1D). This result suggests that the genome in the high-limonene producer is different from the low producer.

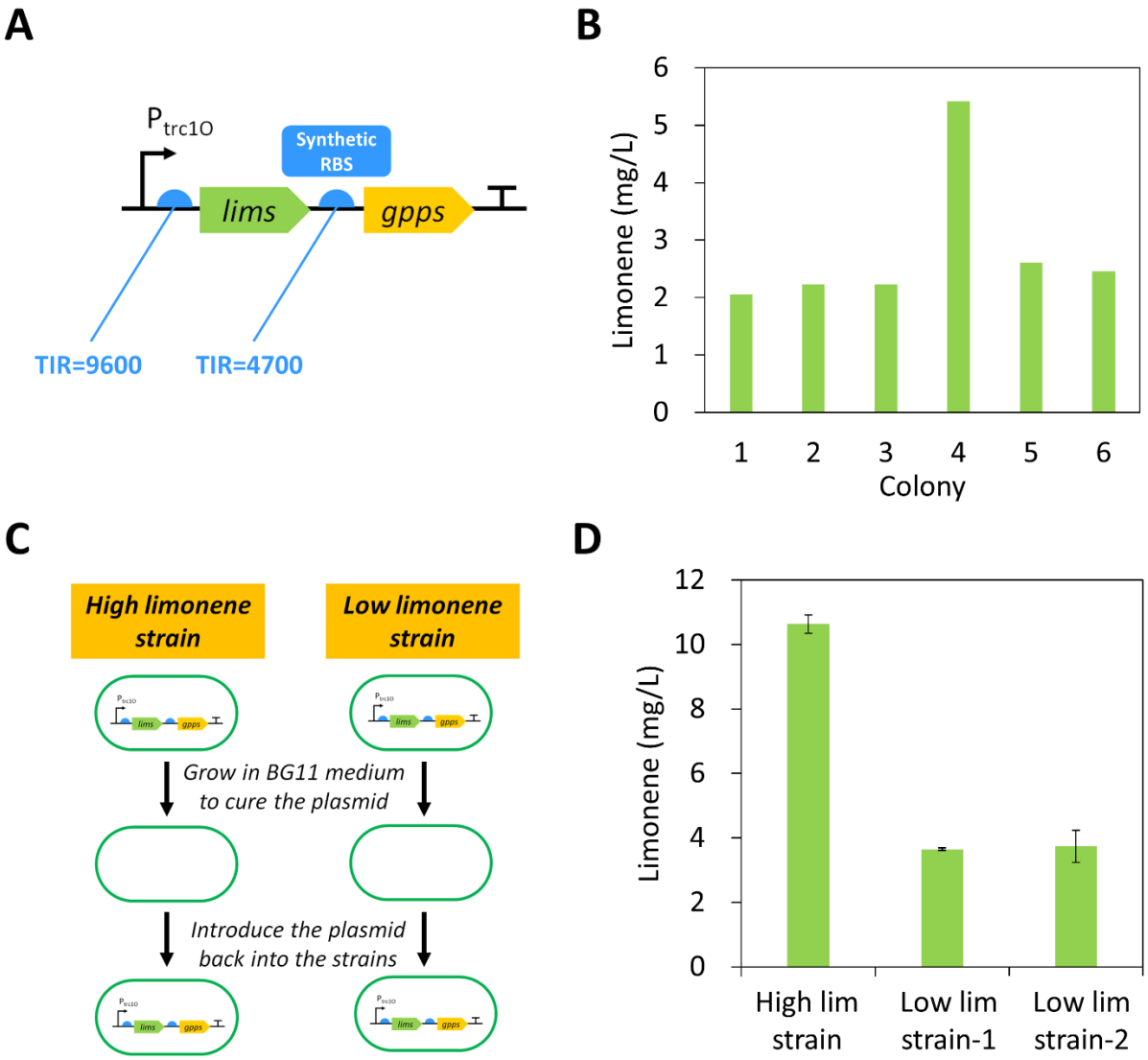


Figure 3.1 Identification of a high limonene-producing mutant of *Synechococcus* 2973.

(A) The plasmid containing *lims* from *M. spicata* and *gpps* from *A. grandis* was introduced in *Synechococcus* 2973. A synthetic RBS for the *gpps* gene was obtained from the RBS calculator, with half of the TIR compared to the RBS in *trc1O* promoter. **(B)** Limonene production in *Synechococcus* 2973 expressing the plasmid lims-gpps. One mutant produced 2.5-fold higher the amount of limonene compared to the other strains. The experiment was conducted in 1% CO_2 and 500 µmol photons m^{-2} s^{-1} light **(C)** Schematics of testing the difference in genome background in the high and low limonene producers. The plasmid lims-gpps was cured and then introduced back into the strains. **(D)** Limonene production of the re-constructed high and low limonene-producing strains. After removing the plasmid lims-gpps from the strains, the plasmid was introduced back into the strains to test limonene synthesis. The experiment was conducted in 1% CO_2 and 250 µmol photons m^{-2} s^{-1} light. RBS, ribosome biding site; TIR, translation initiation rate.

Furthermore, we conducted a time course of limonene production in the high-limonene producer. Production of limonene is associated with growth (Figure 3.2A). The titer had no increase after the cells was reaching the stationary growth phase. In cyanobacteria, the highest productivity of limonene reported in a previous study was 1 mg L^{-1} day^{-1} in *Synechococcus* sp. PCC 7002 (Davies et al. 2014). To compare the capacity of limonene production with previous studies, we cultured the high-limonene producer under the same conditions as the work of *Synechococcus* 7002 (Davies et al. 2014). The experiment was initiated at an OD_{730} of 0.4 (equivalent to 0.5 g L^{-1} cell dry weight). Cells were grown in 1% CO_2 and 250 µmol photons m^{-2}

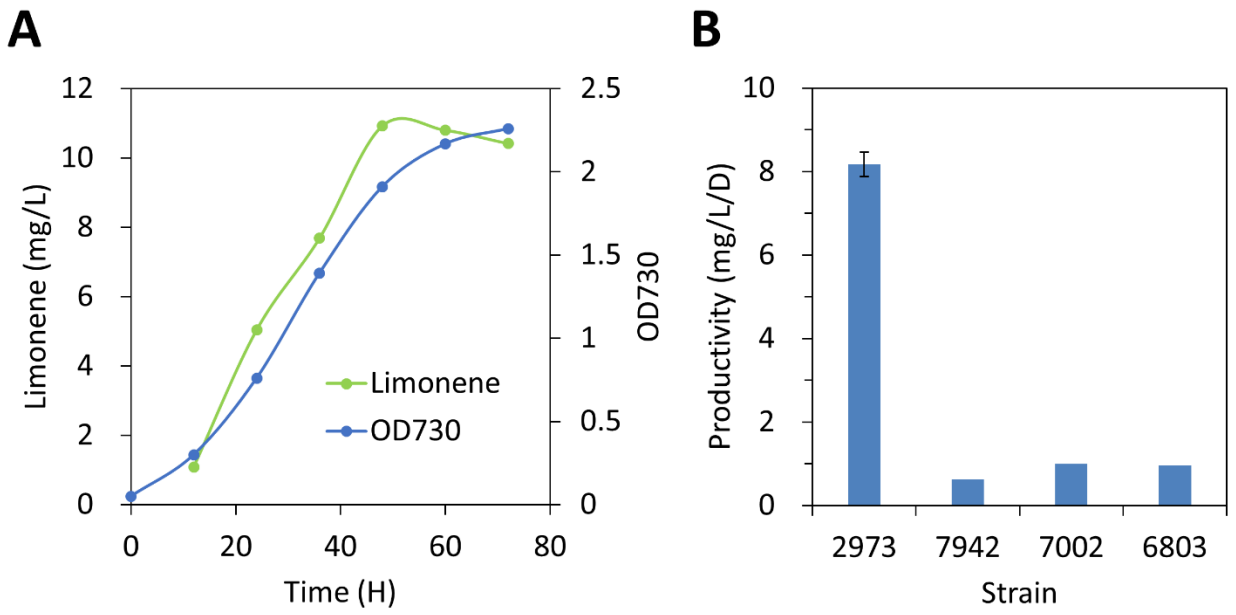


Figure 3.2 Limonene production in *Synechococcus* 2973 high limonene-producing strain.

(A) The time course of limonene production in the high limonene-producing strain. The experiment was initiated at an OD_{730} = 0.05 with 1% CO_2 and 250 µmol photons m^{-2} s^{-1} light. **(B)** Comparison of limonene productivity with previous studies. 6803, *Synechocystis* 6803 (Lin et al. 2017); 7002, *Synechococcus* 7002 (Davies et al. 2014); *Synechococcus* 7942 (Wang et al. 2016). The experiment was initiated at an OD_{730} = 0.4. The limonene titer in *Synechococcus* 2973 represents mean ± sd of three biological replicates.

s^{-1} light. Remarkably, the productivity of limonene from the high-limonene producer of *Synechococcus* 2973 was 8-fold higher than the previous work in *Synechococcus* 7002 (Figure 3.2B). The limonene titer was 16.4 mg L^{-1} after 2 days of growth. This result demonstrates the potential of using *Synechococcus* 2973 for limonene production.

3.2.2 Genome sequencing of high limonene-producing strain

To identify the mutation(s) in the high limonene producer, whole genome sequencing was conducted. A low producer (Figure 3.1B, colony 6) was included for genome sequencing to compare with the high producer. Compared to wild type of *Synechococcus* 2973, 2 single nucleotide polymorphisms (SNPs) were found in the high producer, whereas the low producer contained 1 SNP (Table 3.1). In the high producer, one SNP is on the gene encoding the *Synechococcus* outer membrane protein B (SomB), whereas the other SNP is on the GGPP synthase (CrtE). Both SNPs lead to amino acid changes (Y380H in SomB and R299Q in CrtE). As for the low producer, the SNP was also observed on the *somB* gene and caused an amino acid substitution (L234S). The SomB was discovered as a porin in the outer membrane protein in *Synechococcus* PCC 6301 (Hansel et al. 1998). The biological function of SomB is still unclear. Analysis of a transposon mutant library revealed that it is a non-essential gene in *Synechococcus* 7942 (Rubin et al. 2015). CrtE is an essential enzyme in cyanobacteria, which catalyzes the formation of pyrophosphate intermediates in terpene biosynthesis (see Chapter 1.1.3). This enzyme is critically important in cyanobacteria, because it controls the carbon flux for pigment synthesis (Figure 1.1).

Table 3.1 Genome sequencing results of *Synechococcus* 2973 high limonene-producing mutants

Nucleotide position	Locus tag	Gene function	2973 WT	High producer	Low producer	Amino acid change
1638555	M744_08675	Porin (SomB)	T	T	C	L234S
1638992	M744_08675	Porin (SomB)	T	C	T	Y380H
2496789	M744_12865	GGPPS (CrtE)	G	A	G	R299Q

3.2.3 Identification of a SNP that leads to enhanced limonene production

To identify which SNP that leads to increased limonene production in the high producer, we used CRISPR/Cpf1 genome editing to create the SNPs in *Synechococcus* 2973, generating strains with SomB (Y380H) or CrtE (R299Q) mutations. Then, the plasmid lims-gpps was further introduced into both strains to test limonene production. Notably, the SomB (Y380H) strain drastically increased its efficiency of bacterial conjugation (Figure 3.3A), indicating this porin is likely to be involved in conjugation. As shown in Figure 3.3B, the strain with CrtE (R299Q) enhanced limonene production significantly compared to that in the SomB (Y380H) strain. This result determines that the SNP in the *crtE* gene confers a high limonene productivity.

3.2.4 Modulation of GPPS expression to optimize limonene synthesis pathway

To further increase limonene production in *Synechococcus* 2973, we used the high limonene producer for the following engineering purposes. As described in Chapter 1, production of limonene will necessarily compete with pigment synthesis because both pathways use the same precursors IPP and DMAPP (Figure 1.1). In the limonene-producing strain, GPPS and CrtE competes the substrates IPP and DMAPP. Balancing the carbon fluxes between limonene and pigment syntheses could potentially improve limonene production. To achieve this goal, we

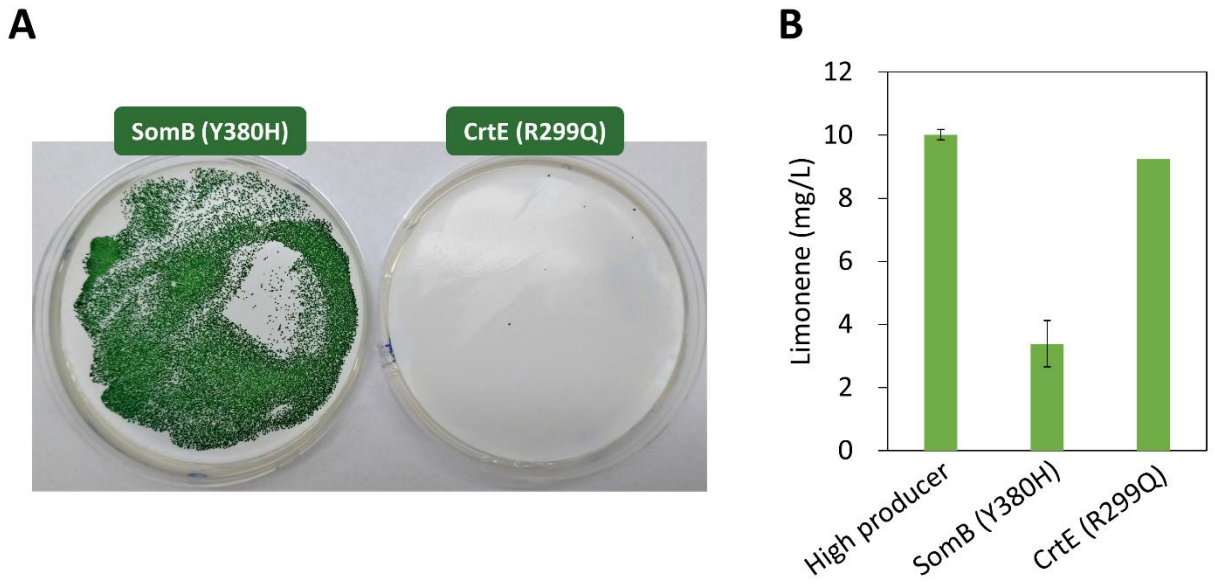


Figure 3.3 Limonene production in *Synechococcus* 2973 strains with SomB (Y380H) or CrtE (R299Q) mutations.

(A) Bacterial conjugation results. The conjugative efficiency was increased dramatically in the SomB (Y380) strain. The amount of SomB (Y380H) cells plated on the membrane was 10% of the CrtE (R299Q) cells. **(B)** Limonene production in mutant strains with SomB (Y380H) or CrtE (R299Q) mutations.

modulated the enzyme level of GPPS by using a synthetic RBS library with various translation rates to control GPPS expression (Figure 3.4A).

The RBS1 was the original sequence used for expressing the GPPS (Figure 3.1A). Since the RBS library were obtained from in silico analysis, we would like to verify if these RBS sequences can lead to different expression levels of GPPS. Therefore, the C-terminus of GPPS was fused with an enhanced yellow fluorescent protein (EYFP) using a GGGGS peptide linker (Figure 3.4B). The strength of RBS can be quantified by measuring the EYFP fluorescence in the limonene-producing mutants. As shown in Figure 3.4C, different amounts of limonene were produced in *Synechococcus* 2973 using the RBS library to drive GPPS expression. Moreover, the

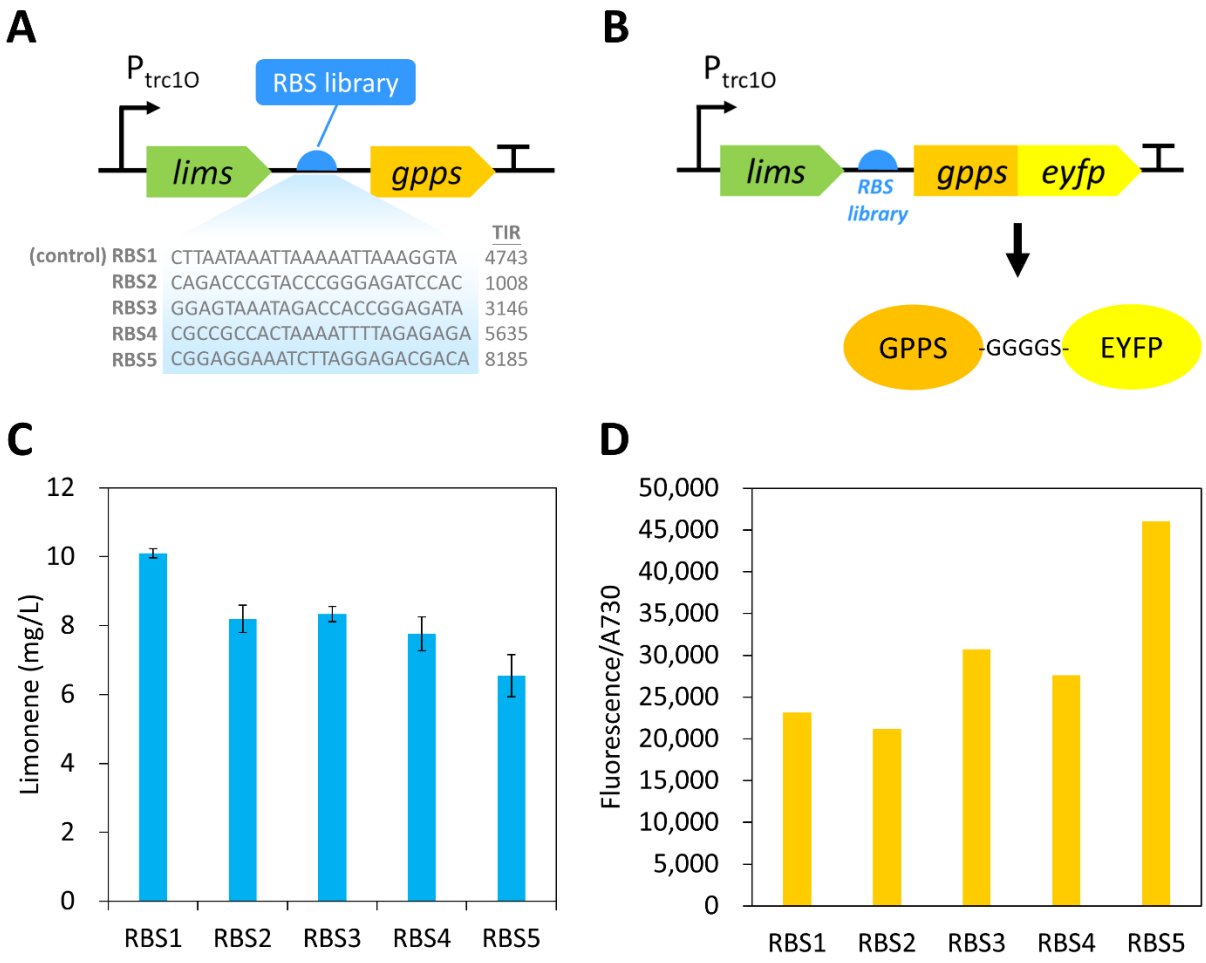


Figure 3.4 Engineering the RBS of *gpps* to optimize limonene biosynthesis.

(A) A synthetic RBS library with various translation rates was used to modulate expression of *gpps*. **(B)** GPPS was fused with an enhanced yellow fluorescent protein (EYFP) using a peptide linker to verify the strength of synthetic RBSs. **(C)** Limonene production in *Synechococcus* 2973 using a RBS library to express *gpps*. **(D)** Fluorescence level of EYFP.

result of EYFP fluorescence confirmed that the RBS sequences led to different protein levels of GPPS (Figure 3.4D). The control strain, RBS1, produced the highest amount of limonene (10 mg L^{-1}) (Figure 3.4C), whereas its GPPS expression level was the second lowest (Figure 3.4D). By contrast, the RBS5 mutant had the strongest expression of GPPS (Figure 3.4D), while the limonene titer was the lowest (Figure 3.4C), decreasing 35% compared to the control strain RBS1. It turned

out that the original RBS1 sequence is the optimized one for limonene production. Although the limonene titer could not be further improved by engineering the RBS of the *gpps* gene, these results demonstrate that optimization of GPPS expression is critical for enhancing limonene production in cyanobacteria.

3.2.5 Engineering the precursor pool to increase limonene production

The time-course of limonene production showed that limonene synthesis was associated with growth (Figure 3.2A). Cells stopped producing limonene in the stationary growth phase. It is likely the carbon flux to the MEP pathway decrease because the photosynthetic pigments are less important when cells are no longer growing. To further improve limonene production, the precursor pathway must be engineered in order to increase the substrates for limonene synthesis. Therefore, we expressed various pathways in the high limonene producer (Figure 3.5), and investigate which pathway(s) can enhance limonene production in *Synechococcus* 2973.

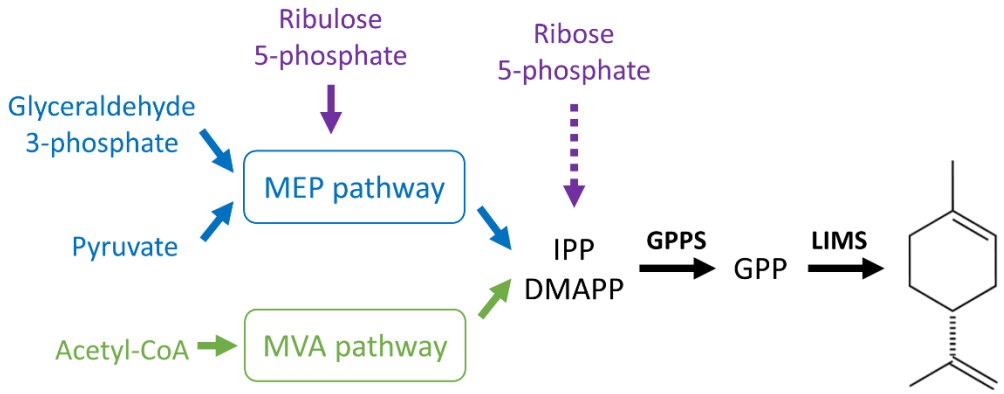


Figure 3.5 Engineering of the precursor pathways to improve limonene production.

Various terpene precursor pathways have been used to engineer cyanobacteria, *E. coli* and yeast for enhanced terpenoid production (see Chapter 1.1.3). To express these pathways in *Synechococcus* 2973, the RSF1010 plasmid was used (Huang et al. 2010). A second operon driven by the *lacUV5* promoter was cloned into the plasmid lims-gpps (Figure 3.6). Rather than expressing the genes in a neutral site, using the replicating plasmid would be more efficient to construct the mutant strains, thus accelerating the design-build-test cycle. Table 3.2 summarizes the limonene production of 17 mutant strains expressing the various terpene precursor pathways. To test production of the mutant strains, we induced the gene expression using different concentrations of IPTG (1 mM, 0.05 mM and 0.01 mM), because the levels of enzymes can greatly affect the production titer (as discussed later). The highest limonene titers in the mutant strains were listed in Table 3.2.

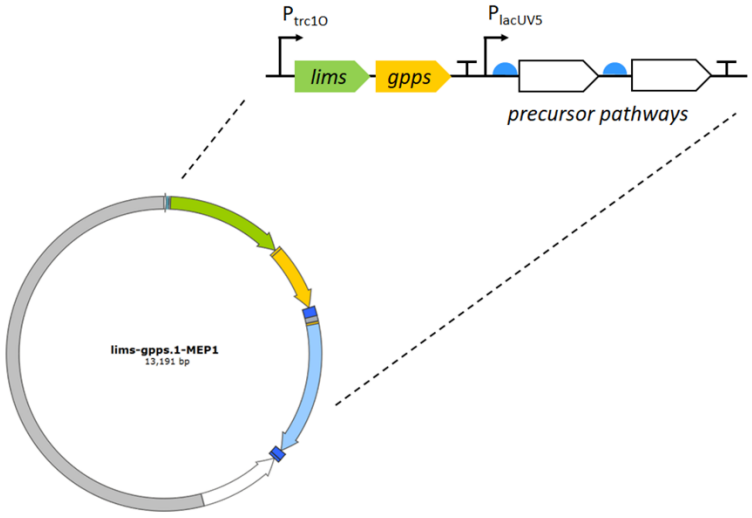


Figure 3.6 The RSF1010 plasmid for expressing terpene precursor pathways in *Synechococcus* 2973.

Table 3.2 Limonene production of mutants with overexpression of the precursor pathways

Pathway	Plasmid	Highest titer (mg L^{-1})	vs. control Strain
MEP pathway	lims-gpps-PlacUV5-dxs	4.8	-52%
	lims-gpps-PlacUV5-dxs-idi	13.0	+30%
	lims-gpps-PlacUV5-RBS(5000)-dxs	5.9	-41%
	lims-gpps-PlacUV5-RBS(5000)-idi	11.3	+13%
	lims-gpps-PlacUV5-RBS(5000)-dxs-idi	9.6	-4%
	lims-gpps-PlacUV5-RBS(5000)-dxs(A154G)	4.8	-52%
	lims-gpps-PlacUV5-RBS(5000)-dxs(A345G)	3.2	-68%
	lims-gpps-PlacUV5-RBS(5000)-dxs(A154G/A345G)	4.7	-53%
	lims-gpps-PlacUV5-RBS(5000)-dxs(A154G/A345G)-idi	5.9	-41%
	lims-gpps-PlacUV5-RBS(40000)-idi	4.6	-54%
	lims-gpps-PlacUV5-RBS(24000)-idi	6.7	-33%
	lims-gpps-PlacUV5-RBS(14000)-idi	12.8	+28%
MVA pathway	lims-gpps.1-MVA (lacI: original RBS)	plasmid unstable	
	lims-gpps.1-MVA (lacI: strong RBS)	1.0	-90%
Alternative pathway	lims-gpps.1-PlacUV5-RBS(5000)-ribB (G108S)	7.9	-21%
	lims-gpps.1-PlacUV5-RBS(5000)-yajO	7.2	-28%
	lims-gpps.1-PlacUV5-rpi-rpe	6.9	-31%

Among all the pathways expressed in *Synechococcus* 2973, only a few strains expressing genes in the MEP pathway increased limonene production (Table 3.2). DXS and IDI are known to be the rate-limiting enzymes in the MEP pathway (see Chapter 1.1.3). Our results showed that the expression level of these enzymes greatly affected limonene production in *Synechococcus* 2973. As shown in Figure 3.7, the limonene production titers varied by titrating the IPTG concentrations. The control strain, lims-gpps, produced a similar amount of limonene (10 mg L^{-1}) when IPTG was higher than 0.05 mM, whereas the titer decreased 60% to 0.4 mg L^{-1} with addition of 0.01 mM IPTG. The *dxs*-expressing strains decreased limonene production dramatically and exhibited reduced OD_{730} values, suggesting that overexpression of *dxs* may lead to cell toxicity in *Synechococcus* 2973. Surprisingly, the limonene titer in the *dxs-idi* coexpression strains increased

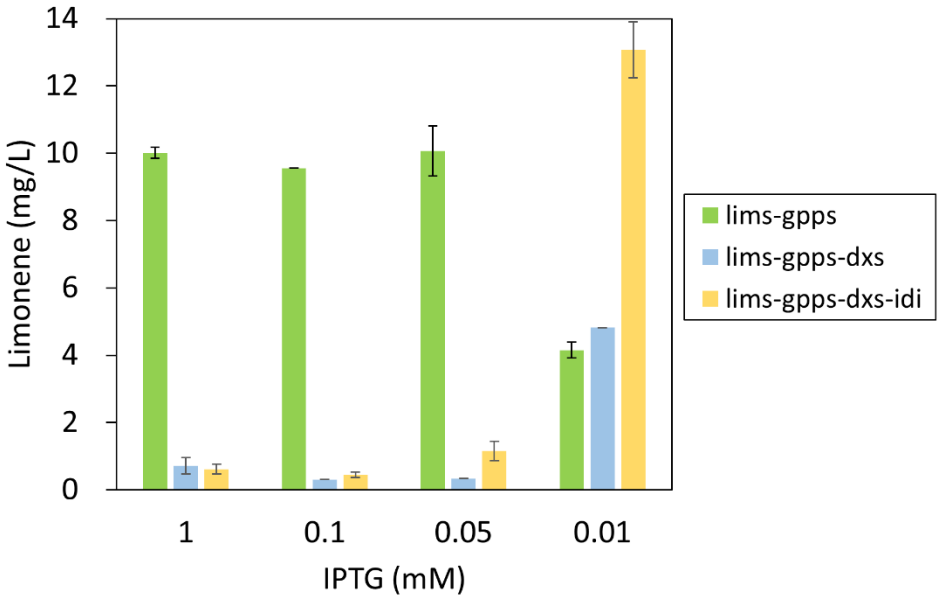


Figure 3.7 IPTG titration to modulate gene expression of the MEP pathway in *Synechococcus* 2973 limonene-producing strains.

significantly to 13 mg L^{-1} under 0.01 mM IPTG conditions (Figure 3.7). These results suggest that expression levels of DXS and IDI must be low in order to improve limonene production in *Synechococcus* 2973.

Notably, expression of the MVA pathway is considered as the most promising strategy to increase terpene production in *E. coli* and yeast, while the plasmid with the MVA pathway could not be maintained in *Synechococcus* 2973. We suspected that the leaky expression of the *lacUV5* promoter (see Appendix) resulted in the instability of this MVA plasmid. Therefore, we increased the expression level of LacI repressor using a stronger RBS obtained from the RBS calculator. This would improve the repression of *lacUV* promoter. Although the plasmid was maintained in *Synechococcus* 2973, overexpressing the MVA pathway led to impaired growth and drastically reduced limonene production (Table 3.2).

In Chapter 2, expression of genes in the pentose phosphate pathway improved limonene production in *Synechocystis* 6803 (Lin et al. 2017). Unfortunately, expressing the same genes decreased the limonene titer in *Synechococcus* 2973. In the *Synechocystis* 6803 study, the modeling results was based on the whole genome model of *Synechocystis* 6803 (Lin et al. 2017). This may indicate that the pentose phosphate metabolism in *Synechococcus* 2973 is difference from that in *Synechocystis* 6803. Therefore, the same strategy could not improve limonene synthesis in the fast-growing strain.

3.3 Discussion

In this work, we engineered the fast-growing cyanobacterium *Synechococcus* 2973 for limonene production. We identify a high limonene-producing strain that only has 2 SNPs that lead to amino acid changes in the porin SomB and the GGPP synthase CrtE. By expressing the plasmid lims-gpps in the SomB (Y380H) and CrtE (R299Q) strains, we discovered that the R299Q mutation in CrtE led to a 2.5-fold improvement in limonene production. In cyanobacteria, CrtE is an essential enzyme that catalyzes the formation of GGPP, a precursor of photosynthetic pigments including chlorophyll and carotenoids. We reasoned that the R299Q mutation in CrtE decreases its enzymatic activity, thus enabling the GPPS to convert more substrates to limonene production. Further characterization is needed to investigate the enzymatic activity of CrtE (R299Q). Additionally, the CrtE (R299Q) strain may be useful for increasing production of other terpenes which use GPP or FPP as substrates (Figure 1.1).

Interestingly, the porin SomB is likely to be involved in bacterial conjugation, because we observed that the strain with SomB (Y380H) mutation had significant increase in conjugation

efficiency (Figure 3.3A). For bacterial conjugation, the donor strain uses conjugative pili to transfer DNA into the recipient strain. It is possible that SomB participates in transfer of DNA into the cytoplasm of *Synechococcus* 2973, or interacts with conjugative pili from the donor strain. More research is required to elucidate the biological role of SomB in cyanobacteria.

Modulation of GPPS expression varied the limonene production titers in *Synechococcus* 2973 (Figure 3.4), suggesting that the enzyme level of GPPS is important to increase limonene production. In addition, this may indicate that a limited amount of fixed carbon was directed to the MEP pathway. To further increase limonene production, the precursor pool for terpene synthesis must be increased. By investigating various precursor pathways for synthesizing IPP and DMAPP, we found that most of the genetic modifications resulted in decreased limonene production (Table 3.2). Although the expression of *dxs* and *idi* in the MEP pathway slightly increased the limonene titer, our results demonstrated that the optimization of their enzyme levels is necessary for enhanced production (Figure 3.7). Biosynthesis of limonene requires 9 enzymatic reactions from the central carbon metabolism (Figure 1.1). The entire biosynthesis pathway should be systematically engineered to identify the bottleneck reactions of limonene biosynthesis (Englund et al. 2018).

This study demonstrates *Synechococcus* 2973 as a promising cyanobacterium for engineering purposes. The 17 strains listed in Table 3.2 were constructed and tested within 4 months. Using this fast-growing strain would accelerate the process of strain engineering, and streamline the design-build-test cycle to identify the most effective strategy for strain improvement.

3.4 Methods

3.4.1 Growth conditions

Synechococcus elongatus UTEX 2973 was cultured in BG11 medium at 38°C, 250 μmol photons m^{-2} s^{-1} light, and 1% CO_2 in an AlgaeTron growth chamber (Photon Systems Instruments, Czech Republic). For the mutant strains, the antibiotic kanamycin (10 μg/mL) was used in BG11 agar plates or liquid medium. To test limonene production, mutant strains were grown in BG11 medium for 24 hours, and then diluted to an OD_{730} of 0.05 (0.06 g L^{-1} cell dry weight) to initiate the experiments. Strains were grown in 10 mL of BG11 medium in 50-mL flasks with 10% (v/v) of isopropyl myristate covered as an organic overlay to trap the evaporated limonene. IPTG (1 mM) was added to induce gene expression.

3.4.2 Construction of strains

All cloning experiments were performed using the Gibson isothermal DNA assembly method (Gibson et al. 2009). The RSF1010 self-replicating plasmid was used for expression of genes in this study (Huang et al. 2010). The *lims* and *gpps* was codon optimized for *Synechocystis* 6803 and *E. coli*, respectively (Lin et al. 2017). The MVA pathway and *idi* was amplified from the plasmid JEBI2999 (Peralta-Yahya et al. 2011). The *dxs* was amplified from genomic DNA of *E. coli*. To engineer the RBS of *gpps*, synthetic RBSs with different translation rates were obtained from the RBS calculator (Salis et al. 2009). Bacterial conjugation was used to introduce plasmids into *Synechococcus* 2973 (Yu et al. 2015).

3.4.3 CRISPR/Cpf1 genome editing

CRISPR/Cpf1 was used to construct mutant strains with SomB (Y380H) or CrtE (R299Q) protein mutants. The Cpf1 plasmid cloning and genome editing procedures were based on methods reported in previous studies (Ungerer et al. 2018; Ungerer and Pakrasi 2016).

3.4.4 Whole genome sequencing

Genomic DNA (gDNA) from the high- and low-limonene producing strains was purified using genomic DNA extraction kit. The gDNA samples were sequenced at the Genome Technology Access Center (GTAC) at Washington University.

3.4.5 Limonene measurement

The limonene in the isopropyl myristate (IM) overlay was measured. The samples were prepared by diluting 100 μL of the IM overlay in 300 μL of IM, and analyzed using a gas chromatography instrument with a flame ionization detector (Hewlett-Packard model 7890 A, Agilent Technologies, CA, USA) equipped with a 30-meter DB5-MS column (J&W Scientific). The oven temperature program initiated at 70°C and held for 3 minutes, and increased at 5°C/min to 260°C and held for 3 minutes. Limonene was quantified using a (R)-limonene standard.

3.4.6 Fluorescence measurement

The engineered strains with GPPS-EYFP fusion constructs were cultured at a starting OD_{730} of 0.05 in BG11 medium with antibiotics for 24 hours. Cultures were diluted to a similar OD_{730} = 0.5

for fluorescence measurement. The fluorescence and OD_{730} of cultures were measured using a BioTek Synergy Mx plate reader (BioTek, Winooski, VT). The excitation and emission wavelengths were set to 485 and 528 nm, respectively. The fluorescence intensity was normalized by OD_{730} to compare between each strain.

References

Ajikumar PK, Tyo K, Carlsen S, Mucha O, Phon TH, Stephanopoulos G (2008) Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. Molecular pharmaceutics 5 (2):167-190

Burke C, Croteau R (2002) Geranyl diphosphate synthase from *Abies grandis*: cDNA isolation, functional expression, and characterization. Archives of biochemistry and biophysics 405 (1):130-136

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). cDNA isolation, characterization, and bacterial expression of the catalytically active monoterpene cyclase. The Journal of biological chemistry 268 (31):23016-23024

Davies FK, Work VH, Beliaev AS, Posewitz MC (2014) Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of *Synechococcus* sp. PCC 7002. Frontiers in bioengineering and biotechnology 2:21. doi:10.3389/fbioe.2014.00021

Englund E, Shabestary K, Hudson EP, Lindberg P (2018) Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. Metabolic engineering 49:164-177. doi:10.1016/j.ymben.2018.07.004

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods 6 (5):343-345. doi:10.1038/nmeth.1318

Hansel A, Pattus F, Jürgens UJ, Tadros MH (1998) Cloning and characterization of the genes coding for two porins in the unicellular cyanobacterium *Synechococcus* PCC 6301. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression 1399 (1):31-39

Huang HH, Camsund D, Lindblad P, Heidorn T (2010) Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38 (8):2577-2593. doi:10.1093/nar/gkq164

Lin P-C, Saha R, Zhang F, Pakrasi HB (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. Scientific reports 7 (1):17503. doi:10.1038/s41598-017-17831-y

Lin PC, Pakrasi HB (2019) Engineering cyanobacteria for production of terpenoids. Planta 249 (1):145-154. doi:10.1007/s00425-018-3047-y

Niu FX, Lu Q, Bu YF, Liu JZ (2017) Metabolic engineering for the microbial production of isoprenoids: Carotenoids and isoprenoid-based biofuels. Synth Syst Biotechnol 2 (3):167-175. doi:10.1016/j.synbio.2017.08.001

Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS (2011) Identification and microbial production of a terpene-based advanced biofuel. Nature communications 2:483

Rubin BE, Wetmore KM, Price MN, Diamond S, Shultzaberger RK, Lowe LC, Curtin G, Arkin AP, Deutschbauer A, Golden SS (2015) The essential gene set of a photosynthetic organism. Proceedings of the National Academy of Sciences 112 (48):E6634-E6643

Salis HM, Mirsky EA, Voigt CA (2009) Automated design of synthetic ribosome binding sites to control protein expression. Nature biotechnology 27 (10):946

Ungerer J, Pakrasi HB (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. Scientific reports 6:39681

Ungerer J, Wendt KE, Hendry JI, Maranas CD, Pakrasi HB (2018) Comparative genomics reveals the molecular determinants of rapid growth of the cyanobacterium *Synechococcus elongatus* UTEX 2973. Proceedings of the National Academy of Sciences 115 (50):E11761-E11770

Wang X, Liu W, Xin C, Zheng Y, Cheng Y, Sun S, Li R, Zhu XG, Dai SY, Rentzepis PM, Yuan JS (2016) Enhanced limonene production in cyanobacteria reveals photosynthesis limitations. Proceedings of the National Academy of Sciences of the United States of America 113 (50):14225-14230. doi:10.1073/pnas.1613340113

Yu J, Liberton M, Cliften PF, Head RD, Jacobs JM, Smith RD, Koppenaal DW, Brand JJ, Pakrasi HB (2015) *Synechococcus elongatus* UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO_2. Scientific reports 5:8132. doi:10.1038/srep08132

Chapter 4: Sustainable production of sucrose in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973

This chapter was reproduced from the following publication:

Lin PC, Zhang F, Pakrasi HB (2019) Sustainable production of sucrose in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. Biotechnology for Biofuels [*Submitted*]

Abstract

Background: Cyanobacteria are attractive microbial hosts for sustainable production of chemicals using light and CO_2. However, their low productivity of chemicals is a major challenge for commercial applications. This is mostly due to their relatively slow growth rate and carbon partitioning toward biomass rather than products. Many cyanobacterial strains synthesize sucrose as an osmoprotectant to cope with salt stress environments. In this study, we harnessed the photosynthetic machinery of the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 to produce sucrose under salt stress conditions and investigated if the high efficiency of photosynthesis can enhance the productivity of sucrose.

Results: By expressing the sucrose transporter CscB, *Synechococcus* 2973 produced 8 g L^{-1} of sucrose with a highest productivity of 1.9 g L^{-1} day^{-1} under salt stress conditions. Nearly 80% of fixed carbon was partitioned to sucrose production rather than biomass synthesis. Semi-quantitative RT-PCR showed that salt stress activated the sucrose biosynthetic pathway mostly via upregulating the *sps* gene, which encodes the rate-limiting sucrose-phosphate synthase enzyme. Notably, the strain accumulated a trivial amount of glycogen in salt stress medium. To alleviate the demand on high concentrations of salt for sucrose production, we further engineered

Synechococcus 2973 by overexpressing genes in the sucrose synthesis pathway. The engineered strain produced sucrose with a productivity of 1.1 g L^{-1} day^{-1} without the need of salt induction.

Conclusions: *Synechococcus* 2973 is a promising cyanobacterium for sustainable production of sugars from CO_2. Engineered *Synechococcus* 2973 in this study demonstrated a sucrose productivity of 1.9 g L^{-1} day^{-1}, representing the highest productivity of sucrose in cyanobacteria.

Keywords: Cyanobacteria, salt stress, sucrose, sucrose transporter CscB, *Synechococcus elongatus* UTEX 2973

4.1 Introduction

Microbial production of fuels and commodity chemicals provides alternative solutions to reduce the reliance on fossil fuel. However, the use of sugar feedstock is one of the challenges for sustainable bioproduction. Cyanobacteria are photosynthetic prokaryotes that use light, CO_2, and trace amounts of minerals for growth. Compared to terrestrial plants, cyanobacteria have higher efficiencies to utilize solar energy (Dismukes et al. 2008). In recent years, many synthetic biology tools have been developed for cyanobacteria (Sengupta et al. 2018). These tools have enabled metabolic engineering of cyanobacteria to produce various chemicals, including fuels (Gao et al. 2012), petrochemicals (Ungerer et al. 2012), sugars (Song et al. 2016), fragrances (Lin et al. 2017), and biopolymers (Wagner et al. 2016). Although cyanobacteria demonstrate the potential of converting CO_2 into desired products, most of the reported titers and productivities are still too low for commercial applications (Lin and Pakrasi 2019; Knoot et al. 2018). A more efficient

photosynthetic chassis is needed to improve CO_2 utilization and carbon partitioning toward products.

Sucrose is an important feedstock in food industry and bioethanol production. Cyanobacteria synthesize sucrose as a compatible solute to tolerate high salt environments. By synthesizing sucrose, the osmotic pressure can be maintained to avoid desiccation in salt stress conditions. Studies of various cyanobacterial strains showed that more than 60 strains accumulate sucrose under high salt conditions (Hagemann 2011). In cyanobacterial cells, sucrose is synthesized from uridine diphosphate glucose (UDP-Glu) and fructose 6-phosphate (F6P) by sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP) (Figure 4.1A).

CscB is a sucrose/H^+ symporter which belongs to the oligosaccharide:H^+ symporter family of the Major Facilitator Superfamily (Vadyvaloo et al. 2006). Bacteria with CscB are able to take up sucrose by utilizing the H^+ gradient across plasma membranes (Sahin-Toth et al. 1995). Ducat *et al.* reported the first study of using the sucrose permease CscB as a sucrose exporter in cyanobacteria (Ducat et al. 2012). Upon expression of the *E. coli* CscB in *Synechococcus elongatus* PCC 7942, the higher pH value in the extracellular medium creates a reversed proton gradient, which enables the engineered strain to export sucrose (Ducat et al. 2012). The *cscB*-expressing *Synechococcus* 7942 exported up to 2.6 g L^{-1} of sucrose with a rate of 0.9 g L^{-1} day^{-1} in high salt medium (Ducat et al. 2012).

Additionally, a sucrose biosynthetic pathway was also engineered in *Synechocystis* sp. PCC 6803 by overexpressing the *sps* and *spp* genes, leading to a 2-fold enhancement in intracellular sucrose concentration (Du et al. 2013). Importantly, this study identified that the SPS-catalyzed reaction is the rate-limiting step of sucrose biosynthesis in cyanobacteria (Du et al. 2013). Recently,

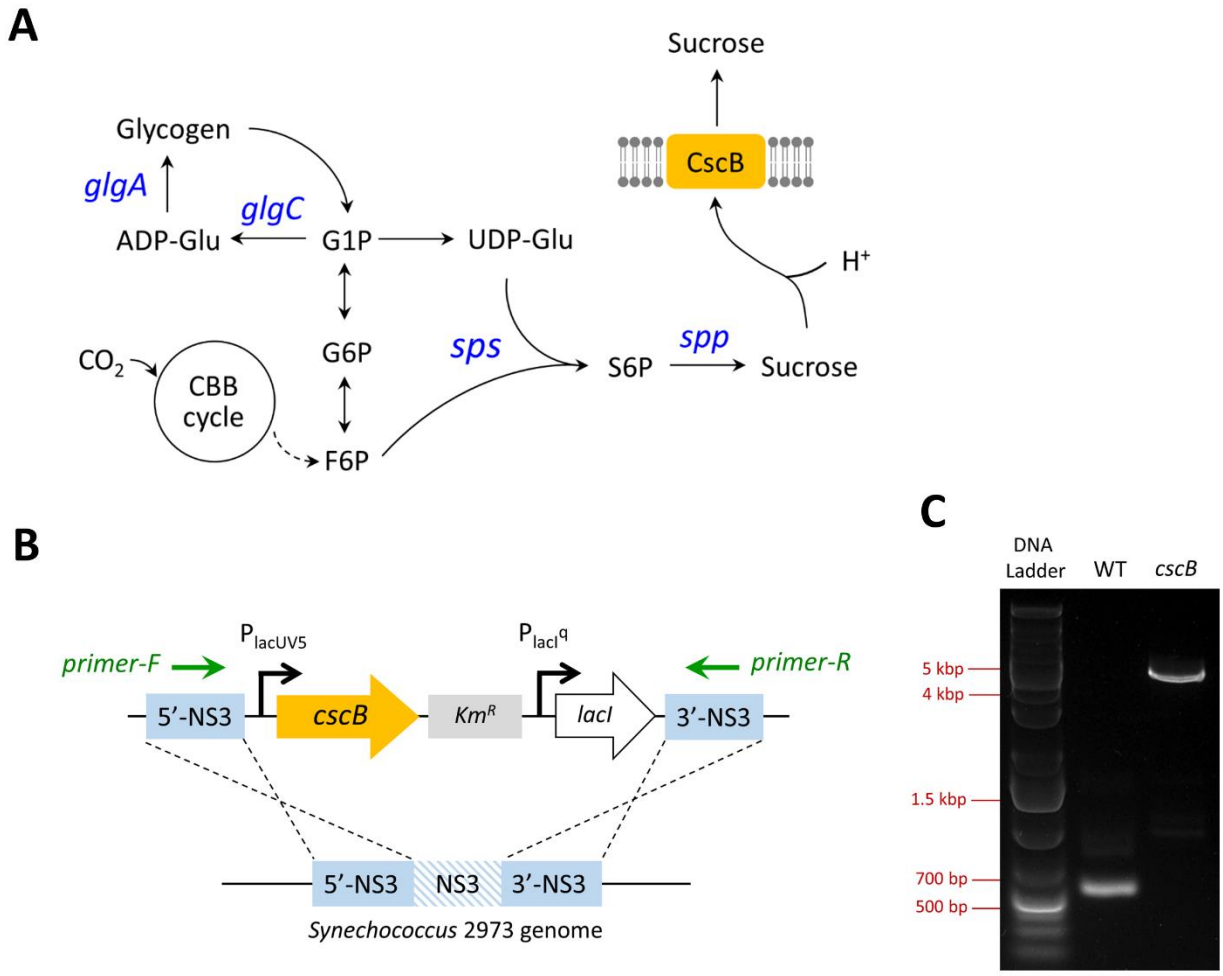


Figure 4.1 Engineering of *Synechococcus elongatus* UTEX 2973 for production of sucrose.

(A) Metabolic pathways of carbohydrate biosynthesis in *Synechococcus* 2973. The sucrose permease CscB was expressed for sucrose excretion. Genes highlighted in blue encode enzymes for glycogen and sucrose production. **(B)** Introduction of the *cscB* gene into the neutral site 3 (NS3). The primers used for PCR genotyping are highlighted in green. **(C)** PCR genotyping to confirm complete segregation of *cscB*. The wild type (WT) *Synechococcus* 2973 was used as a control. The PCR product of the WT gene is 514 base pairs (bp). No WT copy was present in the *cscB*-expressing strain.

the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973 was engineered for sucrose production by expressing the *cscB* gene (Song et al. 2016). However, the fast-growing property of *Synechococcus* 2973 did not lead to a higher sucrose titer or productivity compared to those from its closely-related strain *Synechococcus* 7942 (Song et al. 2016). These results demanded further research to uncover the potential of sugar production using this fast-growing strain.

Synechococcus 2973 has a higher carbon flux in the sugar phosphate pathway (Abernathy et al. 2017) and can accumulate a greater amount of glycogen compared to *Synechococcus* 7942 (Ungerer et al. 2018; Song et al. 2016). Inspired by these facts, we explored the capability of sucrose production in engineered *Synechococcus* 2973, and investigated whether the rapid growth rate and strong carbon fluxes in sugar metabolism can lead to a higher production titer of sucrose. In this work, we reported a high sucrose productivity to 1.9 g L^{-1} day^{-1} by engineered *Synechococcus* 2973. Moreover, we discussed the relationship between glycogen synthesis and sucrose production, providing insights on carbon partitioning of cyanobacteria in salt stress conditions. Finally, we further engineered *Synechococcus* 2973 to produce sucrose without induction by salt stress, and determined that SPS and SPP are both important enzymes in sucrose biosynthesis.

4.2 Results

4.2.1 Engineering of *Synechococcus* 2973 for sucrose production

To engineer a sucrose-exporting *Synechococcus* 2973 strain, the sucrose permease *cscB* gene was cloned into a neutral site 3 (NS3)-targeting plasmid (Niederholtmeyer et al. 2010), which was then introduced into *Synechococcus* 2973 via bacterial conjugation (Figure 4.1B). A fully segregated strain was obtained by re-patching the plates multiple times, and the corrected genome modification was confirmed by PCR genotyping (Figure 4.1C). To test sucrose production in *Synechococcus* 2973, the engineered 2973-*cscB* strain (Table 4.1) was grown in BG11 with 100-200 mM NaCl (Figure 4.2A). IPTG (1 mM) was added to induce *cscB* gene expression. The supernatants were collected for sucrose analysis. The highest sucrose titer, 8 g L^{-1} in 5 days, was observed in BG11 with 150 mM NaCl. The highest volumetric productivity was 1.9 g L^{-1} day^{-1} in 4 days, which corresponds to a sucrose yield of 3.1 $g.g^{-1}$ cell biomass. This productivity is over 2-fold higher than that from the closely-related strain *Synechococcus* 7942 (Additional file: Figure S1). Additionally, we observed that salt stress resulted in impaired growth of *Synechococcus* 2973 (Figure 4.2B), leading to decreased OD_{730} values as NaCl concentrations increased.

Table 4.1 Plasmids and strains used in this study

Plasmids		
Name	Description	Reference
pRL443	conjugal plasmid for bacterial conjugation	(Elhai et al. 1997)
pRL623	helper plasmid for bacterial conjugation	(Elhai et al. 1997)
pSL2985	P_{lacUV5}-*cscB* (*E. coli*), *lacI*, NS3-targeting [Cm^R]	(Ducat et al. 2012)
pSL3005	P_{lacUV5}-*cscB* (*E. coli*), *lacI*, NS3-targeting [Km^R]	This study
pSL3037	P_{lacUV5}-*cscB* (*E. coli*), *lacI*, bom site, NS3-targeting [Km^R]	This study
pSL3360	P_{trc1O}-*sps* (*Synechocystis* 6803), RSF1010 plasmid [Gm^R]	This study
pSL3361	P_{trc1O}-*sps-spp* (*Synechocystis* 6803), RSF1010 plasmid [Gm^R]	This study

***Synechococcus* 2973 strains**		
Name	Description	Reference
2973-*cscB*	NS3::P_{lacUV5}-*cscB* (created using pSL3037)	This study
2973-*cscB-spp*	2973-*cscB* strain with plasmid pSL3360	This study
2973-*cscB-sps-spp*	2973-*cscB* strain with plasmid pSL3361	This study

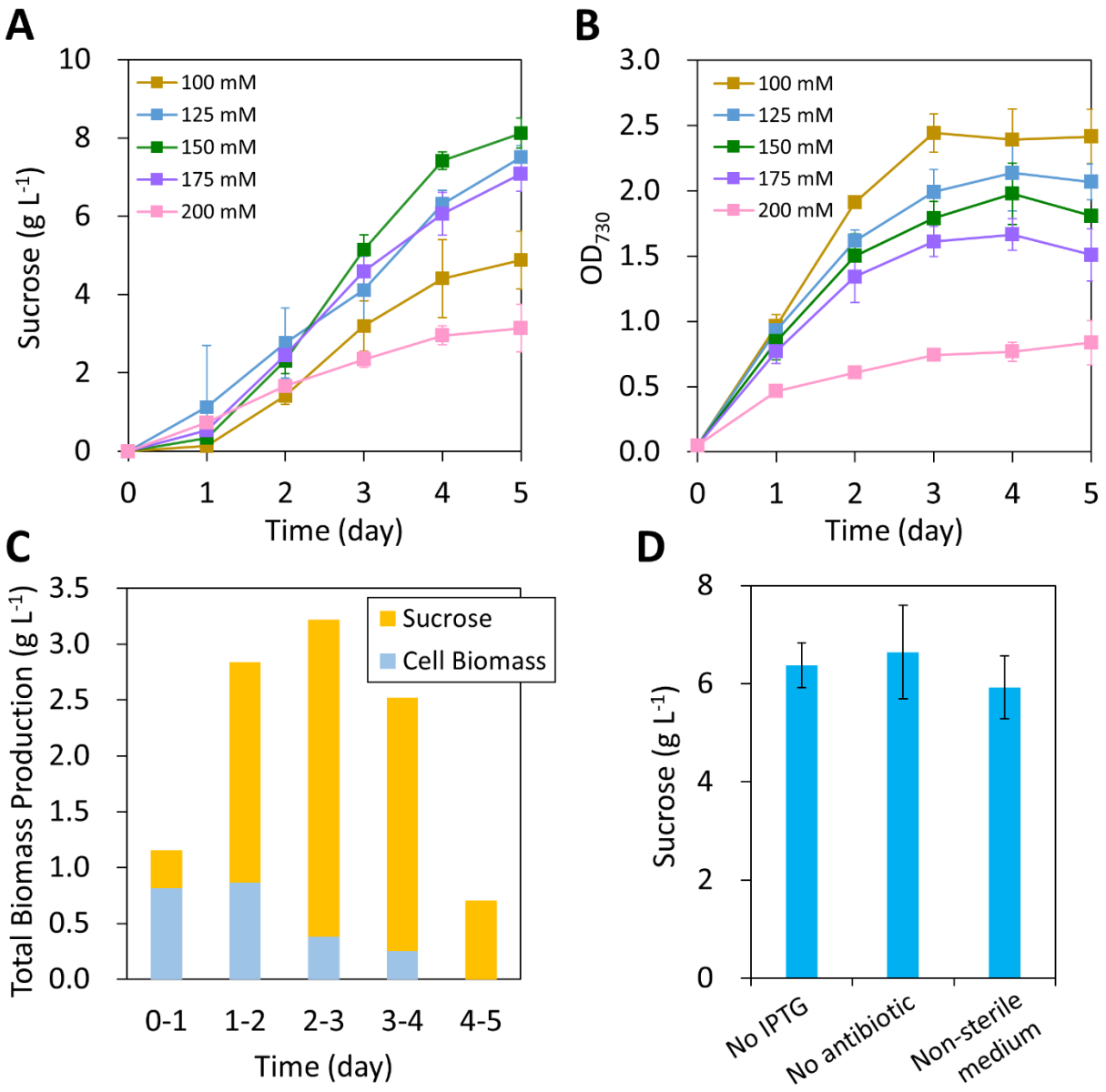


Figure 4.2 Sucrose production in *cscB*-expressing *Synechococcus* 2973.

(A) Time-course of sucrose production in the *cscB*-expressing strain in BG11 medium with various concentrations of NaCl. **(B)** OD_{730} of the *cscB*-expressing strain. **(C)** Carbon partitioning between sucrose and cell biomass (BG11 with 150 mM NaCl). **(D)** Sucrose production in different culture conditions with 150 mM NaCl (5 days). The data represents mean ± sd from three biological replicates.

To investigate how much photosynthetically fixed carbon is directed to sucrose production under salt stress conditions, we analyzed carbon partitioning between sucrose and cell biomass (Figure 4.2C). During the first 24 hours of growth, 71% of the fixed carbon (0.8 g L^{-1}) was used for cell biomass synthesis, and only 0.3 g L^{-1} of sucrose was produced in this period. From day 1 to day 4, the sucrose titer increased dramatically. During each of these days, the sucrose productivity was greater than 2 g L^{-1} day^{-1}. The highest amount of sucrose was produced between day 3-4, during which sucrose productivity reached 2.8 g L^{-1}. After day 3, over 88% of the fixed carbon was directed to sucrose. The titer decreased during day 4-5, in which only 0.7 g L^{-1} sucrose was produced.

Furthermore, we tested the capacity of sucrose production without addition of IPTG or antibiotic kanamycin, or under non-sterile BG11 medium. Without IPTG induction, the 2973-*cscB* strain produced 6.4 g L^{-1} sucrose (Figure 4.2D), which is 21% lower than that with IPTG-induced conditions (Figure 4.2A). This indicates that the *lacUV5* promoter is leaky in *Synechococcus* 2973. Similarly, the 2973-*cscB* strain produced 6.6 g L^{-1} sucrose without addition of kanamycin (Figure 4.2D), indicating the antibiotic is required to maintain stable *cscB* expression in *Synechococcus* 2973. To test the robustness of the 2973-*cscB* strain, we further tested sucrose production using non-sterile BG11 medium with 150 mM NaCl. After 5 days of cultivation, 5.9 g L^{-1} of sucrose was produced with a rate of 1.2 g L^{-1} day^{-1} (Figure 4.2D).

4.2.2 Investigation of carbohydrate metabolism in sucrose-producing UTEX 2973

After its exponential growth phase, *Synechococcus* 2973 accumulates a significant amount of glycogen to reserve excess fixed carbon (Ungerer et al. 2018). To further understand the carbon allocation between glycogen and sucrose, we analyzed the glycogen content in the sucrose-producing *Synechococcus* 2973 strain. The 2973-*cscB* cells were grown in BG11 with or without 150 mM NaCl, and the amounts of glycogen and sucrose were analyzed. The cell biomass was 2-fold higher in BG11 medium without addition of NaCl (Figure 4.3A). In the absence of NaCl, *Synechococcus* 2973 produced only 0.4 g L^{-1} of sucrose in 5 days, which is 19-fold lower than that in the presence of the salt (Figure 4.3B). In contrast, the glycogen content significantly decreased under sucrose-producing conditions. In the presence of 150 mM NaCl, the 2973-*cscB* strain accumulated glycogen less than 1% of cell dry weight (DW) during day 1 and day 3 (Figure 4.3C). At day 5, the glycogen content increased to 10% of DW, corresponding to 0.2 g L^{-1} of glycogen (40-fold lower than sucrose). Without addition of NaCl, cells accumulated glycogen to more than 40% of DW after 3 days (Figure 4.3C). These observations further confirmed that salt stress redirected carbon fluxes from glycogen accumulation to sucrose production in this engineered strain.

We next conducted a semi-quantitative RT-PCR to examine transcription of genes involved in glycogen and sucrose biosynthesis, including *glgC*, *glgA*, and *sps* (Figure 4.1A). GlgC (ADP-glucose pyrophosphorylase) and GlgA (glycogen synthase) are the enzymes responsible for glycogen biosynthesis. In *Synechococcus elongatus*, the SPS enzyme has both sucrose-phosphate synthase and sucrose-phosphate phosphatase activities (Martinez-Noel et al. 2013). Therefore, expression of the *sps* gene was monitored. The housekeeping gene *rpoA* (encoding RNA polymerase subunit α) was used as a control for this RT-PCR experiment (Kim et al. 2018). After 24 hours of growth, the transcript levels of *glgC* and *glgA* had no significant difference in the 2973-

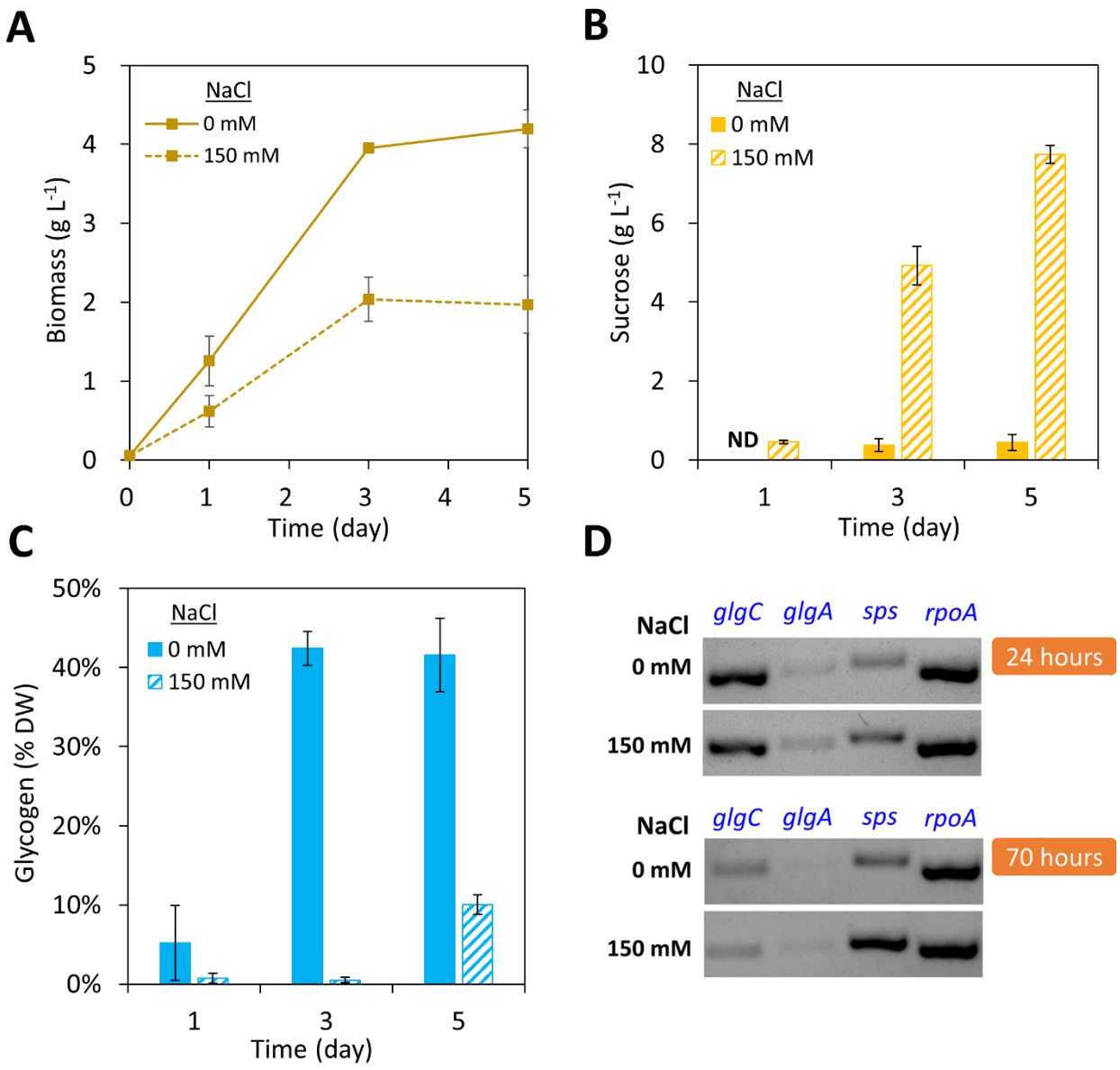


Figure 4.3 Analysis of carbohydrate metabolism in *Synechococcus* 2973 *cscB*-expressing strain.

(A) Biomass accumulation. **(B)** Sucrose production. **(C)** Glycogen content. **(D)** Semi-quantitative RT-PCR for monitoring gene expression in glycogen and sucrose syntheses. The housekeeping gene *rpoA* was used as a control. Cells were grown in BG11 medium with or without addition of 150 mM NaCl. The data in panels **A-C** represents mean ± sd from three biological replicates. ND, not detected; DW, cell dry weight.

cscB strain with or without NaCl (Figure 4.3D). Expression of *sps* was slightly higher when cells were grown in 150 mM NaCl. At 70 hours, transcription of *glgC* and *glgA* decreased compared to those at 24 hours (Figure 4.3D). The sucrose synthesis gene *sps* was highly expressed at 70 hours under salt stress (Figure 4.3D), suggesting that increased sucrose production in the salt medium is due to upregulation of the sucrose biosynthetic pathway.

4.2.3 Engineering of the sucrose biosynthetic pathway

In cyanobacteria, salt stress is required to induce sucrose production. The sucrose biosynthesis gene *sps* is highly expressed in salt BG11 medium (Figure 4.3D). To expand the applications of sucrose production in cyanobacteria, we engineered *Synechococcus* 2973 to produce sucrose in BG11 medium without added NaCl. The sucrose biosynthetic pathway from *Synechocystis* sp. PCC 6803 was expressed in the 2973-*cscB* strain (Figure 4.4A). It is known that overexpression of the *sps* and *spp* genes from *Synechocystis* 6803 leads to a 2-fold improvement in intracellular sucrose production under salt stress conditions (Du et al. 2013). To express the *Synechocystis* 6803 *sps* and *spp* genes in *Synechococcus* 2973, we cloned them into the self-replicating RSF1010 plasmid (Huang et al. 2010) under the control of an IPTG-inducible *trc1O* promoter. The plasmids were then introduced into the 2973-*cscB* strain (Table 4.1). Both *sps* and *sps-spp* overexpression strains were able to produce sucrose in BG11 medium without additional NaCl (Figure 4.4B). In the absence of IPTG, the *sps* and *sps-spp* strains produced 1.6 g L^{-1} and 3.4 g L^{-1} of sucrose in 3 days, respectively, which were 11-fold and 23-fold higher than the control 2973-*cscB* strain (Figure 4.4B). For the *sps* strain, the sucrose titers had no significant difference with or without addition of 1 mM IPTG. Interestingly, the sucrose titer decreased significantly upon addition of 1

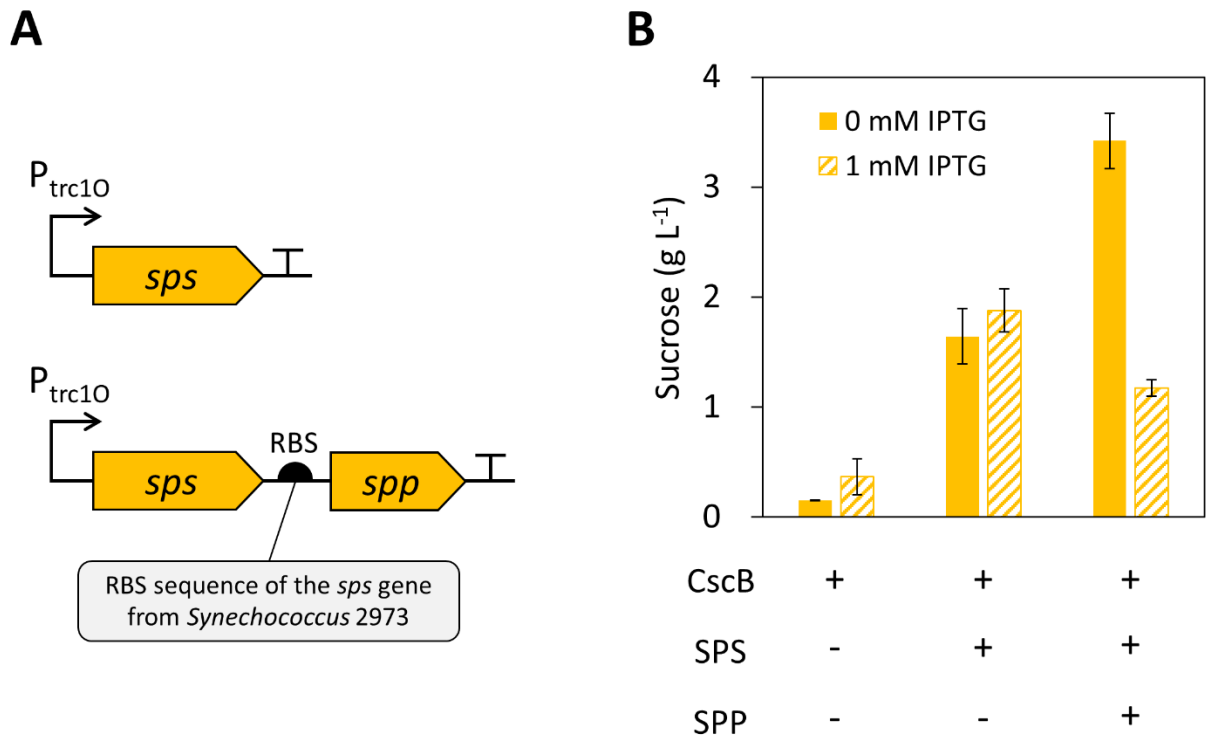


Figure 4.4 Engineering of the sucrose biosynthetic pathway in *Synechococcus* 2973.

(A) Schematics of expressing the *Synechocystis* 6803 *sps* and *spp* genes in the 2973-*cscB* strain. **(B)** Sucrose production in *sps* and *sps-spp* expression strains. Cells were cultured in BG11 medium for 3 days without addition of NaCl. The data represents mean ± sd of three biological replicates. RBS, ribosome binding site.

mM IPTG in the *sps-spp* strain (Figure 4.4B), indicating that too much enzyme synthesis may be detrimental to sucrose production.

4.3 Discussion

During this study, we engineered the fast-growing cyanobacterium *Synechococcus* UTEX 2973 to produce sucrose. By overexpressing the sucrose permease CscB, the engineered strain produced

over 8 g L^{-1} of sucrose with the highest productivity of 1.9 g L^{-1} day^{-1} in 4 days (Figure 4.2A). Remarkably, a significant amount of sucrose was produced at the stationary growth phase. From day 2 to day 5, 5.8 g L^{-1} of sucrose was produced, while the cell biomass increased only 0.7 g L^{-1} (Figure 4.2C). Nearly 90% of the photosynthetic carbon was directed to sucrose synthesis. Surprisingly, the self-shading of light in dense cell cultures had no significant impact on sucrose production. We reasoned that the high productivity of sucrose is a result of push-and-pull of carbon fluxes toward sucrose synthesis. In *Synechococcus* 2973, the high photosynthetic efficiency (Ungerer et al. 2018) and strong sugar phosphates pathways (Abernathy et al. 2017) provide a strong pushing force for sucrose production. Meanwhile, expression of the sucrose permease CscB generates a carbon sink by exporting sucrose out of the cells, thus resulting in continuous sucrose production to avoid desiccation in salt stress conditions.

To estimate the capacity of using *Synechococcus* 2973 for large-scale sucrose production, we calculated the areal production of sucrose based on assumptions of outdoor cultivation of photosynthetic microorganisms (Chisti 2008). Compared with sugar production from sugarcane, the estimated areal productivity of sucrose by *Synechococcus* 2973 is 100-fold higher (Table 4.2). Although other factors related to outdoor cultivation (*e.g.*, contamination, grazing, light availability, nutrients, and CO_2 supply) were not included in the calculation, our results demonstrate the potential of using *Synechococcus* 2973 for sugar production.

Compared to all other chemicals produced from cyanobacteria, sucrose production in this study has the highest productivity. Moreover, this is the first study demonstrating cyanobacteria can produce a chemical with a rate over 1 g L^{-1} day^{-1} using light and CO_2. A recent study reported 12.6 g L^{-1} of 2,3-butanediol production with a rate of 1.1 g L^{-1} day^{-1} from *Synechococcus* 7942

Table 4.2 Comparison of areal production of sugars

	Areal productivity (metric tons hectare^{-1} year^{-1})	Reference
Synechococcus 2973		
Sucrose	1100	This study
Sugarcane		
Feedstock	74	(Dias and Sentelhas 2018)
Sugars	11	(Somerville et al. 2010)

(Kanno et al. 2017). However, the strain was cultured under photomixotrophic conditions with 15 g L^{-1} of glucose added as an additional carbon source. The sucrose production in *Synechococcus* 2973 in the current study used CO_2 as the sole carbon source.

Previously engineered *Synechococcus* 2973 (Song et al. 2016) produced sucrose at 0.9 g L^{-1} day^{-1}, which is 2.1-fold lower than that from our 2973-*cscB* strain (Additional file: Figure S1). The discrepancy between the two studies is presumably due to differences in strain design and experimental conditions. In their study, the *cscB* was controlled by an *E. coli* trp/lac promoter (Niederholtmeyer et al. 2010) and introduced into the NS3, whereas the 2973-*cscB* strain in our study used the *lacUV5* promoter to express *cscB* (Ducat et al. 2012). Moreover, the inoculum preparation and experimental conditions are different. Their experiments were initiated by salt induction of late exponential phase cultures (cell biomass = 1.9 g L^{-1} dry weight) (Song et al. 2016). The cultures were grown in 3% CO_2 and 250 μmol photons m^{-2} s^{-1} light in a column bioreactor (Song et al. 2016). In our study, the 2973-*cscB* strain was first acclimated in BG11 medium with 150 mM NaCl for 24 hours, and the experiments were initiated at an OD_{730} of 0.05 (equivalent to 0.06 g L^{-1} dry weight) with IPTG induction. The 2973-*cscB* strain was cultured in 0.5-0.6% CO_2 and 250 μmol photons m^{-2} s^{-1} light in shaker flasks. The strain design and bioprocess development in our study have led to a higher sucrose productivity by *Synechococcus* 2973.

The levels of glycogen and sucrose are opposite when the 2973-*cscB* strain were grown with or without salt stress (Figure 4.3B and c). This suggests that glycogen and sucrose syntheses compete for carbon substrates, and the competition can be affected by culture media conditions. The semi-quantitative RT-PCR results showed that the *sps* gene was upregulated by salt induction (Figure 4.3D), which led to significant increase in sucrose production (Figure 4.3B). Surprisingly, the glycogen pathway was still expressed in salt medium (Figure 4.3D), indicating that the decreased glycogen content was not due to downregulation of glycogen synthesis genes (*glgC* and *glgA*). This also suggests that the sucrose biosynthetic pathway is more efficient than the glycogen pathway in utilizing the carbon substrates in the 2973-*cscB* strain. In fact, our results corroborate the finding that deletion of the glycogen synthesis pathway has a limited improvement on sucrose production in cyanobacteria (Ducat et al. 2012). Since a negligible amount of glycogen is produced under salt stress conditions (Figure 4.3C), the sucrose titer is unlikely to be further improved by deletion of the glycogen pathway. Moreover, a recent study reported that glycogen synthesis may serve as a carbon pool for sucrose production in cyanobacteria (Qiao et al. 2018). By using a theophylline-induced riboswitch system, reduced expression of *glgC* led to decreased sucrose titer in *Synechococcus* 7942 (Qiao et al. 2018).

By overexpressing the sucrose biosynthesis genes, sucrose production of 2973-*cscB* cells increased significantly in BG11 medium (Figure 4.4B). This is the first study to engineer cyanobacteria for sucrose production without induction of salt stress. The sucrose-producing cyanobacteria can be used as carbon feedstock for heterotrophs to produce useful chemicals (Weiss et al. 2017). However, the salt stress media may not be compatible with the culture media for heterotrophs. Engineering a sucrose-producing strain without the need of salt induction could expand the application of using cyanobacteria for co-culture systems. Expression of *sps* and *spp*

improved the sucrose titer by 23-fold with a productivity of 1.1 g L^{-1} day^{-1} (Figure 4.4B). With or without addition of 1 mM IPTG, the *sps* strain produced similar amounts of sucrose (1.6-1.8 g L^{-1}). Notably, sucrose production of the *sps-spp* coexpression strains were 1.2 g L^{-1} and 3.4 g L^{-1} with or without addition of IPTG, respectively (Figure 4.4B). The productivity increased 2.9-fold in the absence of IPTG, suggesting that the expression level of *spp* greatly affect the sucrose titer. The *cscB* and *sps-spp* genes were both controlled by IPTG-inducible promoters, while only one copy of the *lacI* repressor gene expressed in the strain (Figs. 1b and 4a). The amount of LacI repressor may be insufficient to control gene expression. Hence, leaky expression of the genes (*cscB*, *sps* and *spp*) led to a significant improvement in sucrose production. By contrast, full induction of the *spp* genes resulted in lower production of sucrose (Figure 4.4B). We also observed impaired growth of the *sps-spp* strain with 1 mM IPTG induction (Additional file: Table S1), suggesting that overexpression of the *spp* gene may cause cell toxicity and reduce sucrose productivity. It is known that SPS is the rate-limiting enzyme of sucrose biosynthesis in cyanobacteria (Du et al. 2013). Our results suggest that SPP also plays an important role in sucrose production, while its expression level is critical for enhancing sucrose production.

4.4 Conclusions

We have demonstrated the significant potential of engineered *Synechococcus* UTEX 2973 for photosynthetic sugar production. Expression of the sucrose transporter CscB led to continuous sucrose production with a rate of 1.9 g L^{-1} day^{-1} in salt stress conditions. Similar concepts can be applied to harness the strong sugars fluxes in *Synechococcus* 2973 to produce other valuable sugar compounds (Frigaard 2018). The salt stress activates the sucrose biosynthetic pathway, which

redirects a significant portion of the fixed CO_2 toward sucrose production rather than biomass and glycogen accumulation. By engineering the sucrose synthesis pathway in *Synechococcus* 2973, the mutant strains produced sucrose without salt stimulation, which could expand the versatility of using cyanobacteria for sugar production. Furthermore, we identified SPS and SPP as important enzymes for sucrose synthesis in *Synechococcus* 2973, while the expression level of SPP is particularly critical for enhancing sucrose production.

4.5 Methods

4.5.1 Growth conditions

Synechococcus elongatus UTEX 2973 was grown in BG11 liquid medium at 38°c, 250 μmol photons m^{-2} s^{-1} light, and 0.5-0.6% CO_2 in an AlgaeTron growth chamber (Photon Systems Instruments, Czech Republic). For the mutant strains, appropriate antibiotics (10 μg/mL kanamycin and 4 μg/mL gentamicin) were applied in BG11 agar plates or liquid medium. To test sucrose production, strains were first grown in BG11 medium for 24 hours, and then diluted 40-fold to BG11 with 150 mM NaCl to grow for another 24 hours. Afterwards, the salt-acclimated cultures were diluted to an OD_{730} of 0.05 (0.06 g L^{-1} cell dry weight) in 10 mL of BG11 medium in 50-mL flasks with desired concentrations of NaCl to initiate the experiments. Water was added daily to compensate for evaporation of BG11 medium. The evaporation rate is 5-7% per day.

4.5.2 Strain construction

All cloning was conducted using the Gibson isothermal DNA assembly method (Gibson et al. 2009). The sucrose permease *cscB* gene from *E. coli* (ATCC 700927) was introduced into the neutral site 3 (NS3) of *Synechococcus* UTEX 2973 (Niederholtmeyer et al. 2010). The *cscB* was controlled by the *lacUV5* promoter. A lac repressor gene (*lacI*) was included in the plasmid to control *cscB* expression. The *cscB* NS3-targeting plasmid was a gift from Prof. Daniel Ducat at Michigan State University (Ducat et al. 2012). The kanamycin resistance cassette was cloned into the plasmid to replace the chloramphenicol resistance gene. Further, the basis of mobility (bom) sequence derived from plasmid pBR322 was cloned into the plasmid. The bom site is required for gene transfer via bacterial conjugation (Finnegan and Sherratt 1982). The workflow of plasmid construction is described in Additional file: Figure S2. The sucrose biosynthesis pathway was expressed in the RSF1010 self-replicating plasmid (Huang et al. 2010). The *sps* (*sll0045*) and *spp* (*slr0953*) genes were amplified from the genomic DNA of *Synechocystis* 6803. The RBS sequence of the *sps* gene in *Synechococcus* 2973 (20 base pairs proceeding the coding sequence) was used to control translation of *spp* (Figure 4.4A). The *trc1O* promoter was used to control expression of the genes. The plasmids and mutant strains used in this study are listed in Table 4.1.

Bacterial conjugation was used to introduce the *cscB* NS3-targeting or self-replicating plasmids (Table 4.1) into *Synechococcus* 2973. *E. coli* HB101 with plasmid pRL443 was the conjugal strain, whereas *E. coli* HB101 with plasmid pRL623 and the plasmid carrying the genes of interest was the helper strain. Both *E. coli* strains and *Synechococcus* 2973 were grown in liquid cultures overnight. 100 µL of *E. coli* cultures and 400 µl of *Synechococcus* 2973 culture (OD_{730} = 0.5) were washed twice with sterilized water and BG11 medium, respectively. Centrifugation was performed at 4,000 x *g* for 5 mins. The *E. coli* cells were gently stirred during the wash step. The washed *E. coli* and *Synechococcus* 2973 strains were mixed together with 200 µL of BG11 medium

and transferred onto a nitrocellulose membrane (0.45 μm pore size, MilliporeSigma, USA) on a BG11+5% LB (v/v) agar plate. The plates were incubated at 38°C, 100 μmol photons m^{-2} s^{-1} light, and 0.5% CO_2 overnight. The membrane was transferred to new BG11 agar plates with appropriate antibiotics (50 μg/mL kanamycin and 4 μg/mL gentamicin). Colonies appeared on the membranes after 3-5 days of incubation.

4.5.3 Semi-quantitative RT-PCR

RNA was extracted from the 2973-*cscB* cultures using RNAwiz kit (Ambion/Thermo Fisher Scientific). The RNA samples were treated with DNase. Afterwards, cDNA was synthesized using a Superscript II Reverse Transcriptase kit (Thermo Fischer Scientific). PCR was performed at 24 cycles for semi-quantitative analysis of mRNA transcripts. The primers used for PCR are listed in Additional file: Table S2.

4.5.4 Measurements of sucrose, glycogen and biomass

Sucrose in the supernatant was measured using the sucrose/D-glucose assay kit (Megazyme). The glycogen assay was based on the method described in our previous study (Ungerer et al. 2018). The correlation of OD_{730} with cell dry weight was DW (g L^{-1}) = 1.3244 x OD_{730} – 0.2459 (Additional file: Figure S3).

4.5.5 Calculation of sucrose areal productivity

Under continuous light, *Synechococcus* 2973 produced 1.1 g L^{-1} day^{-1} of sucrose using non-sterile BG11 medium (Figure 4.2D). Based on this information, we calculated the areal productivity of sucrose in large-scale culture systems. We assumed the culture depth to be 0.1 meter to enable adequate light penetration (Chisti 2008), and 8 hours of available light per day. The facility is operated for 300 days per year (Richardson et al. 2010).

Abbreviations

ADP-Glu: adenosine diphosphate glucose; CBB cycle: Calvin-Benson-Bassham cycle; CscB, sucrose permease; F6P: fructose 6-phosphate; G1P: glucose 1-phosphate; G6P: glucose 6-phosphate; GlgA: glycogen synthase; GlgC: ADP-glucose pyrophosphorylase; IPTG: isopropyl β-D-1-thiogalactopyranoside; OD: optical density; RBS: ribosome binding site; RpoA: RNA polymerase subunit alpha; S6P: sucrose 6-phosphate; SPP: sucrose-phosphate phosphatase; SPS: sucrose-phosphate synthase; UDP-Glu: uridine diphosphate glucose.

References

Abernathy MH, Yu J, Ma F, Liberton M, Ungerer J, Hollinshead WD, Gopalakrishnan S, He L, Maranas CD, Pakrasi HB, Allen DK, Tang YJ (2017) Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis. Biotechnology for biofuels 10:273. doi:10.1186/s13068-017-0958-y

Chisti Y (2008) Biodiesel from microalgae beats bioethanol. Trends in biotechnology 26 (3):126-131. doi:10.1016/j.tibtech.2007.12.002

Dias HB, Sentelhas PC (2018) Sugarcane yield gap analysis in Brazil–A multi-model approach for determining magnitudes and causes. Science of the Total Environment 637:1127-1136

Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC (2008) Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Current opinion in biotechnology 19 (3):235-240. doi:10.1016/j.copbio.2008.05.007

Du W, Liang F, Duan Y, Tan X, Lu X (2013) Exploring the photosynthetic production capacity of sucrose by cyanobacteria. Metabolic engineering 19:17-25. doi:10.1016/j.ymben.2013.05.001

Ducat DC, Avelar-Rivas JA, Way JC, Silver PA (2012) Rerouting carbon flux to enhance photosynthetic productivity. Applied and environmental microbiology 78 (8):2660-2668. doi:10.1128/AEM.07901-11

Elhai J, Vepritskiy A, Muro-Pastor AM, Flores E, Wolk CP (1997) Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. Journal of bacteriology 179 (6):1998-2005

Finnegan J, Sherratt D (1982) Plasmid ColE1 conjugal mobility: the nature of bom, a region required in cis for transfer. Molecular and General Genetics MGG 185 (2):344-351

Frigaard N-U (2018) Sugar and Sugar Alcohol Production in Genetically Modified Cyanobacteria. In: Genetically Engineered Foods. Elsevier, pp 31-47

Gao Z, Zhao H, Li Z, Tan X, Lu X (2012) Photosynthetic production of ethanol from carbon dioxide in genetically engineered cyanobacteria. Energy & Environmental Science 5 (12):9857-9865

Gibson DG, Young L, Chuang RY, Venter JC, Hutchison CA, 3rd, Smith HO (2009) Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods 6 (5):343-345. doi:10.1038/nmeth.1318

Hagemann M (2011) Molecular biology of cyanobacterial salt acclimation. FEMS microbiology reviews 35 (1):87-123

Huang HH, Camsund D, Lindblad P, Heidorn T (2010) Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38 (8):2577-2593. doi:10.1093/nar/gkq164

Kanno M, Carroll AL, Atsumi S (2017) Global metabolic rewiring for improved CO_2 fixation and chemical production in cyanobacteria. Nat Commun 8:14724. doi:10.1038/ncomms14724

Kim YS, Kim JJ, Park SI, Diamond S, Boyd JS, Taton A, Kim IS, Golden JW, Yoon HS (2018) Expression of OsTPX Gene Improves Cellular Redox Homeostasis and Photosynthesis Efficiency in *Synechococcus elongatus* PCC 7942. Frontiers in plant science 9:1848. doi:10.3389/fpls.2018.01848

Knoot CJ, Ungerer J, Wangikar PP, Pakrasi HB (2018) Cyanobacteria: promising biocatalysts for sustainable chemical production. Journal of Biological Chemistry 293 (14):5044-5052

Lin P-C, Saha R, Zhang F, Pakrasi HB (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. Scientific reports 7 (1):17503. doi:10.1038/s41598-017-17831-y

Lin PC, Pakrasi HB (2019) Engineering cyanobacteria for production of terpenoids. Planta 249 (1):145-154. doi:10.1007/s00425-018-3047-y

Martinez-Noel GM, Cumino AC, Kolman Mde L, Salerno GL (2013) First evidence of sucrose biosynthesis by single cyanobacterial bimodular proteins. FEBS letters 587 (11):1669-1674. doi:10.1016/j.febslet.2013.04.012

Niederholtmeyer H, Wolfstadter BT, Savage DF, Silver PA, Way JC (2010) Engineering cyanobacteria to synthesize and export hydrophilic products. Applied and environmental microbiology 76 (11):3462-3466. doi:10.1128/AEM.00202-10

Qiao C, Duan Y, Zhang M, Hagemann M, Luo Q, Lu X (2018) Effects of Reduced and Enhanced Glycogen Pools on Salt-Induced Sucrose Production in a Sucrose-Secreting Strain of

Synechococcus elongatus PCC 7942. Applied and environmental microbiology 84 (2). doi:10.1128/AEM.02023-17

Richardson JW, Outlaw JL, Allison M (2010) The economics of microalgae oil.

Sahin-Toth M, Frillingos S, Lengeler JW, Kaback HR (1995) Active transport by the CscB permease in *Escherichia coli* K-12. Biochemical and biophysical research communications 208 (3):1116-1123

Sengupta A, Pakrasi HB, Wangikar PP (2018) Recent advances in synthetic biology of cyanobacteria. Applied microbiology and biotechnology:1-15

Somerville C, Youngs H, Taylor C, Davis SC, Long SP (2010) Feedstocks for lignocellulosic biofuels. Science 329 (5993):790-792

Song K, Tan X, Liang Y, Lu X (2016) The potential of *Synechococcus elongatus* UTEX 2973 for sugar feedstock production. Applied microbiology and biotechnology 100 (18):7865-7875. doi:10.1007/s00253-016-7510-z

Ungerer J, Lin PC, Chen HY, Pakrasi HB (2018) Adjustments to Photosystem Stoichiometry and Electron Transfer Proteins Are Key to the Remarkably Fast Growth of the Cyanobacterium *Synechococcus elongatus* UTEX 2973. mBio 9 (1). doi:10.1128/mBio.02327-17

Ungerer J, Tao L, Davis M, Ghirardi M, Maness P-C, Yu J (2012) Sustained photosynthetic conversion of CO_2 to ethylene in recombinant cyanobacterium *Synechocystis* 6803. Energy & Environmental Science 5 (10):8998-9006

Vadyvaloo V, Smirnova IN, Kasho VN, Kaback HR (2006) Conservation of residues involved in sugar/H^+ symport by the sucrose permease of *Escherichia coli* relative to lactose permease. Journal of molecular biology 358 (4):1051-1059. doi:10.1016/j.jmb.2006.02.050

Wagner J, Bransgrove R, Beacham TA, Allen MJ, Meixner K, Drosg B, Ting VP, Chuck CJ (2016) Co-production of bio-oil and propylene through the hydrothermal liquefaction of polyhydroxybutyrate producing cyanobacteria. Bioresource technology 207:166-174

Weiss TL, Young EJ, Ducat DC (2017) A synthetic, light-driven consortium of cyanobacteria and heterotrophic bacteria enables stable polyhydroxybutyrate production. Metabolic engineering 44:236-245. doi:10.1016/j.ymben.2017.10.009

Additional file for Chapter 4

Figure S1. Comparison of sucrose productivity in cyanobacteria.

Syn2973, *Synechococcus* 2973 (Song et al. 2016); Syn7942, *Synechococcus* 7942 (Ducat et al. 2012).

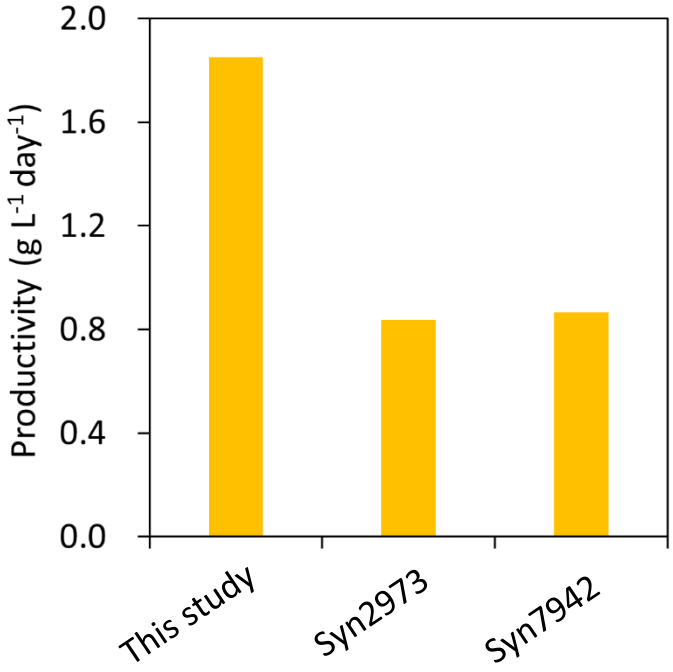
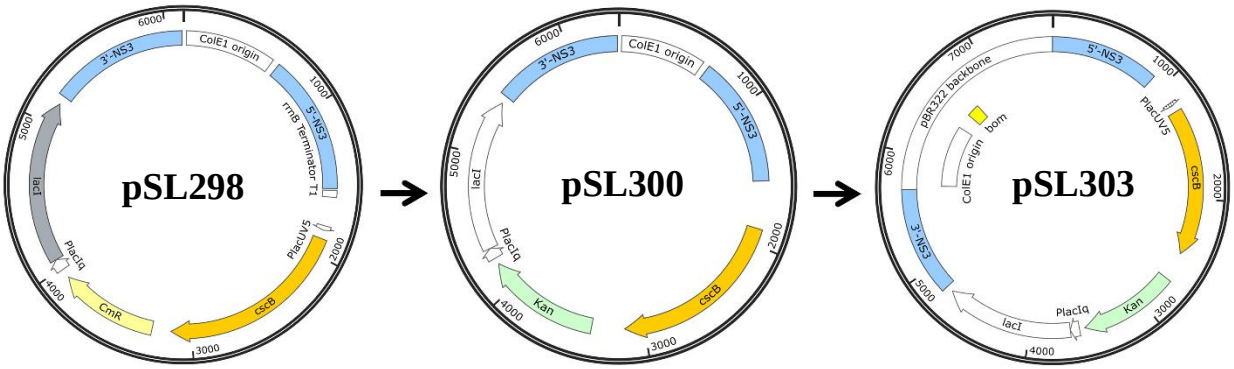


Figure S2. Workflow of plasmid construction.



Plasmid pSL2985

- This plasmid is from Dr. Daniel Ducat (Ducat et al. 2012).
- The Cm^R cassette was replaced because the helper plasmid pRL623 for bacterial conjugation also uses Cm^R cassette.

Plasmid pSL3005

- The Km^R cassette was cloned into the plasmid to replace the Cm^R cassette.

Plasmid pSL3037

- The basis of mobility (bom) sequence derived from plasmid pBR322 was cloned into the plasmid.
- This plasmid was introduced into *Synechococcus* 2973 via bacterial conjugation.

Figure S3. Correlation of cell dry weight (DW) with OD_{730} for *Synechococcus* 2973 *cscB*-expressing strain.

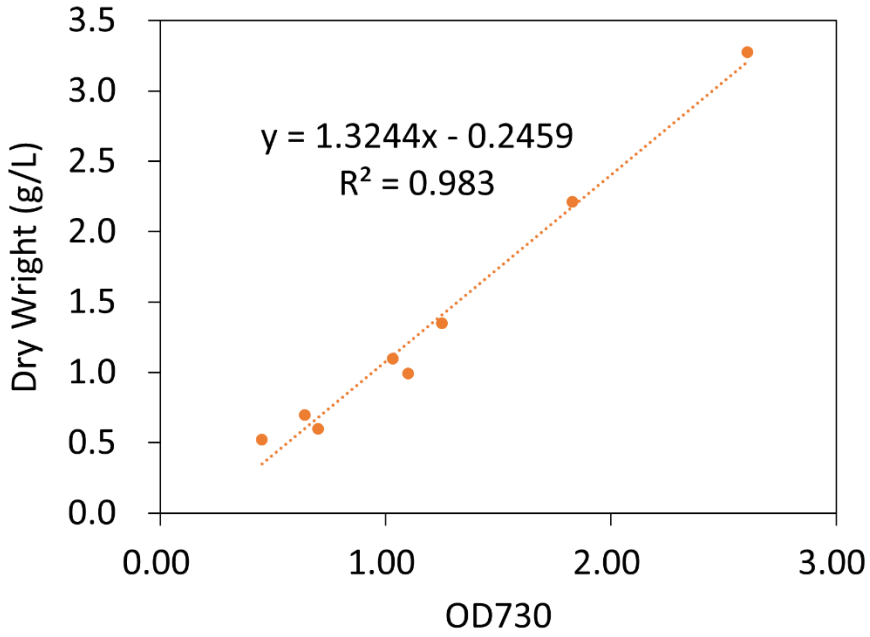


Table S1. OD_{730} of 2973 sucrose-producing strains growing in BG11 medium for 3 days

Strains	0 mM IPTG	1 mM IPTG
2973-*cscB*	3.3	3.2
2973-*cscB-sps*	2.8	0.8
2973-*cscB-sps-spp*	2.6	0.6

Table S2. Primers for semi-quantitative RT-PCR

Primer name	Sequence (5' - 3')
glgC-F	GGCCTGATGAAGCTAGATGG
glgC-R	CCCGCTTGAAGACGTAGATG
glgA-F	GGTTGGTCGATACGGTCTTC
glgA-R	CTCAGATCAACGGCCATACC
sps-F	CAGGAAGTGGAGGAGCAATAC
sps-R	CGACAGAGGCAGAGAATTTGAG
rpoA-F	GGCTGCTGATGTTGACTTTG
rpoA-R	AAGTCTAGGGCAGTCGTTTC

Chapter 5: Investigation of glycogen metabolism in *Synechococcus elongatus* UTEX 2973

This chapter contains text and figures from the following publication:

Ungerer J, Lin PC, Chen HY, Pakrasi HB (2018) Adjustments to Photosystem Stoichiometry and Electron Transfer Proteins Are Key to the Remarkably Fast Growth of the Cyanobacterium *Synechococcus elongatus* UTEX 2973. mBio 9 (1)

5.1 Introduction

Cyanobacteria synthesize glycogen as a storage compound for carbon and energy sources. Glycogen accumulation increases under conditions of nutrient limitation (Schwarz and Forchhammer 2005). Glycogen biosynthesis is initiated by the catalysis of glucose-1-phosphate adenylyltransferase (GlgC), which is the rate-limiting enzyme that converts glucose-1-phosphate to ADP-glucose. Glycogen synthase (GlgA) further converts ADP-glucose to glycogen. Since cyanobacteria synthesize glycogen as a way of carbon storage, deletion of this pathway may redirect the carbon toward production synthesis. However, it is still inconclusive whether removing the glycogen synthesis pathway would enhance production of desired products. Inactivation of the glycogen pathway causes metabolic imbalance in cyanobacteria. Previous studies showed that deletion of *glgC* alters carbon metabolism in cyanobacteria and leads to increased secretion of organic acids such as pyruvate and α-ketoglutarate (van der Woude et al. 2014; Davies et al. 2014; Carrieri et al. 2012). Interestingly, overexpression of a heterologous isobutanol pathway could rescue severe growth retardation of a *Synechococcus* 7942 Δ*glgC* mutant, and improved isobutanol production in the Δ*glgC* mutant (Li et al. 2014), suggesting that isobutanol production serves as a metabolic sink for excess carbon in *Synechococcus* 7942.

In this Chapter, glycogen accumulation in *Synechococcus* 2973 was investigated to further understand the carbon utilization in this fast-growing strain. Glycogen content and the rate of glycogen synthesis were analyzed. The closely-related strain, *Synechococcus* 7942, was included in the experiments to compare biomass and glycogen accumulation with *Synechococcus* 2973. Furthermore, CRISPR/Cpf1 genome editing was used to delete the *glgC* gene. The glycogen-deficient Δ*glgC* strain was characterized and engineered for limonene production.

5.2 Results

5.2.1 Glycogen accumulation in *Synechococcus* 2973

Synechococcus 2973 demonstrates high growth rates under high light and high CO_2 conditions. To understand more about carbon utilization by this fast-growing strain, we compared the time course of biomass and glycogen accumulation that occurs after log-phase growth, transitioning to linear growth at an optical density at 730 nm (OD_{730}) of ~0.4. *Synechococcus* 2973 undergoes a protracted period of linear growth where it reached densities much higher than our bioreactor can record. During this time, *Synechococcus* 2973 accumulates biomass steadily at a rate of 1.1 g L^{-1} day^{-1} (Figure 5.1A), which is nearly three times higher than the rate shown by *Synechococcus* 7942 (0.45 g L^{-1} day^{-1}). During the fast growth phase of *Synechococcus* 2973 (before 12 hours), the glycogen content is extremely low (1% of cell dry weight [DW]) (Figure 5.1B). After that time, cells enter a linear growth phase (Figure 5.1A). The glycogen content drastically increases between 19 and 25 hours, changing 21-fold from 0.3% to 6.3% of DW (Figure 5.1B, inset graph). The amount of glycogen increases from 6% of DW (66 mg L^{-1}) to 33% of DW (693 mg L^{-1}) within the next 24-h span (24 h to 48 h) and ultimately reaches 1.1 g L^{-1} (36% of DW) by day 3 (Figure 5.1B).

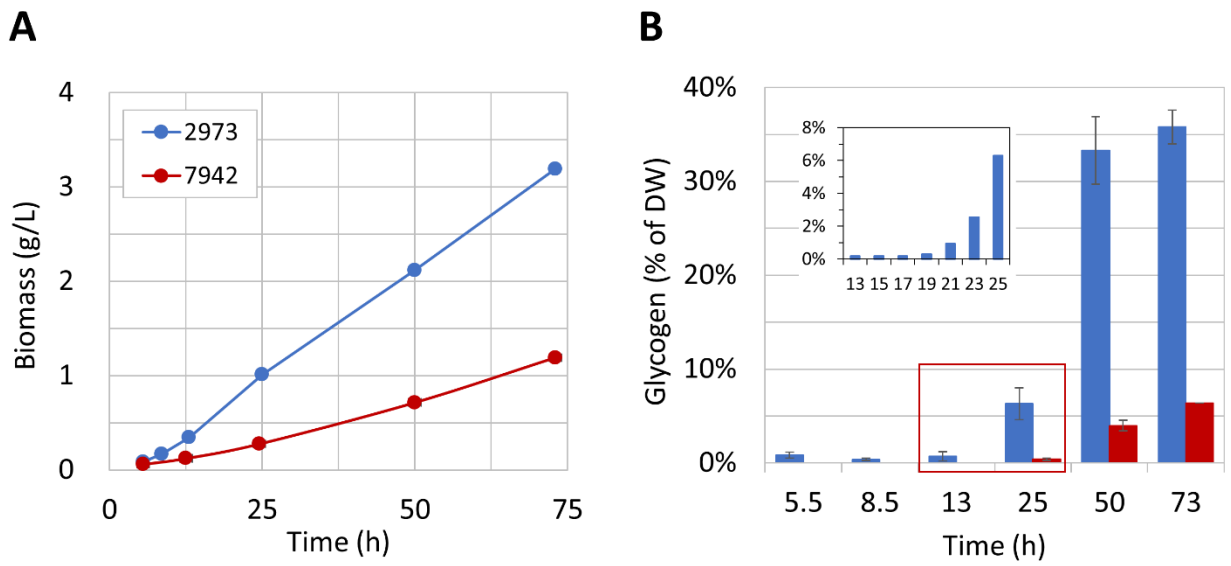


Figure 5.1 Biomass and glycogen accumulation in *Synechococcus* 2973.

(A) Biomass accumulation and **(B)** glycogen content of *Synechococcus* 2973 and *Synechococcus* 7942. The inset graph in panel (B) presents glycogen synthesis between 13 and 25 h. DW, dry weight (cell weight).

These results reveal that *Synechococcus* 2973 directs all of its fixed carbon into growth during log phase and then rapidly transitions into directing the high flux of carbon into storage during the linear growth phase. As for *Synechococcus* 7942, the biomass and glycogen contents accumulated at much lower rates. The glycogen content was negligible during the first day of growth, and it was less than 10% (75 mg L^{-1}) of DW at day 3 *(*Figure 5.1B*)*

5.2.2 Construction of a glycogen-deficient mutant of *Synechococcus* 2973

To further investigate the role of glycogen accumulation in *Synechococcus* 2973, CRISPR/Cpf1 was used to delete the *glgC* gene. *Synechococcus* 2973 has one copy of *glgC*, and it is the first gene of an operon (Figure 5.2A). A hypothetical protein is located downstream of *glgC*. Therefore,

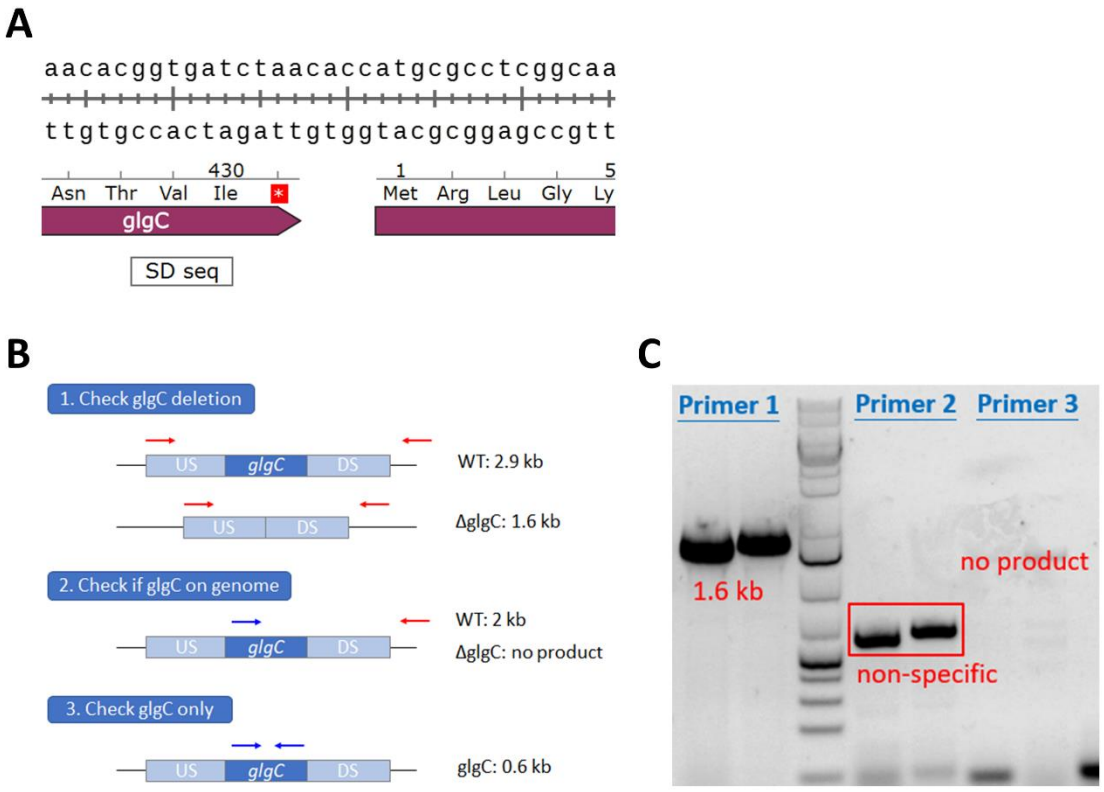


Figure 5.2 Construction of a Δ*glgC* mutant of *Synechococcus* 2973.

CRISPR/Cpf1 was used to delete *glgC*. **(A)** Genome structure depicting *glgC* (M744_13825) and the downstream gene (M744_13830) in *Synechococcus* 2973. The M744_13830 gene encodes a hypothetical protein. The intergenic region is a 4 base pair sequence. SD seq, putative Shine-Dalgarno (SD) Sequence. **(B)** Schematic representation of primers used for PCR genotyping **(C)** PCR results to confirm deletion of *glgC*.

in-frame deletion was performed to remove the coding sequence of *glgC* in order to minimize the effect of downstream gene expression (polar effects). In addition, the intergenic region between *glgC* and the downstream gene is only 4 base pair long (Figure 5.2A). This indicates that the 3' end of *glgC* coding sequence contains the ribosome binding site (RBS) of the downstream gene. It is likely that deleting the entire coding sequencing of *glgC* will interfere the translation of the downstream gene. Therefore, to avoid potential polar effects in the *glgC*-deletion strain, the

putative Shine-Dalgarno (SD) sequence at the 3' end of *glgC* was retained (Figure 5.2A). The SD sequence was predicted from an online tool EMOPEC (Empirical Model and Oligos for Protein Expression Changes; http://emopec.biosustain.dtu.dk) (Bonde et al. 2016). As shown in Figure 5.2B & C, the *glgC* gene was deleted and confirmed by PCR genotyping.

5.2.3 Characterization of a Δ*glgC* mutant of *Synechococcus* 2973

GlgC catalyzes the rate-limiting step of glycogen synthesis. The Δ*glgC* strain is expected to be glycogen deficient. To verify whether deletion of *glgC* would disable glycogen synthesis, the glycogen content of the Δ*glgC* strain was determined. As shown in Figure 5.3A, no glycogen was detected in the Δ*glgC* strain, whereas the wild type (WT) accumulated a significant amount of glycogen after 2 days of cultivation. The growth curves had no significant difference between the WT and Δ*glgC* strains (Figure 5.3B). Surprisingly, the Δ*glgC* cell cultures exhibited a darker color compared to that of the WT (Figure 5.3C). A darker culture may indicate that the Δ*glgC* cultures contained higher number of cells, or the glycogen-deficient strains had a higher chlorophyll content.

To investigate the amount of pigments and cell number in Δ*glgC* and WT cultures, the pigments were extracted and quantified, and the number of cells were quantified using a cell counter. The pigment content was normalized based on cell number in order to have a more accurate characterization. As shown in Table 5.1, the cell number of WT is about 2-fold higher than the Δ*glgC* mutant, while the OD_{730} is only 1.3-fold higher. This suggest that the OD_{730} values cannot accurately represent the number of cells while comparing the WT and Δ*glgC* strains.

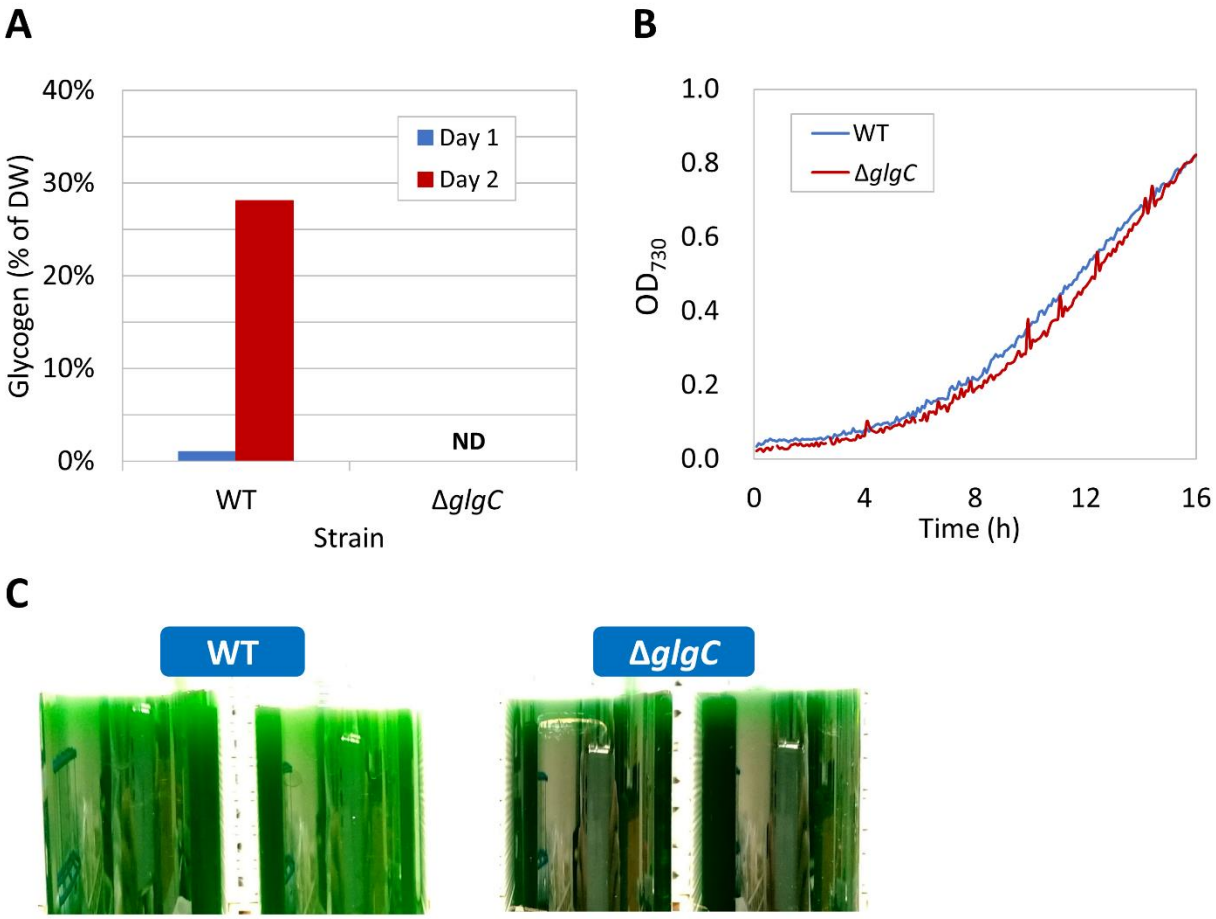


Figure 5.3 Characterization of the Δ*glgC* strain.

(A) Glycogen content in *Synechococcus* 2973 WT and Δ*glgC* strains. **(B)** Growth curve of the WT and Δ*glgC* strains. **(C)** Cell cultures after 3 days of growth at 38°C, 900 µmol photons m^{-2} s^{-1} light, and 5% CO_2. DW, cell dry weight; ND, no detection.

Table 5.1 Cell number and its corresponding OD_{730} of *Synechococcus* 2973 cultures

	Cells per mL	OD_{730}
WT	3.9 x 10^9	2.1
Δ*glgC*	2.0 x 10^9	1.6

Compared to the WT cells, the chlorophyll and carotenoids contents were 2.5-fold and 2.2-fold higher in the Δ*glgC* cells, respectively (Figure 5.4). This result confirmed our observation that the darker color in Δ*glgC* cultures was due to higher a chlorophyll content, while the mutant had less cell number than the WT.

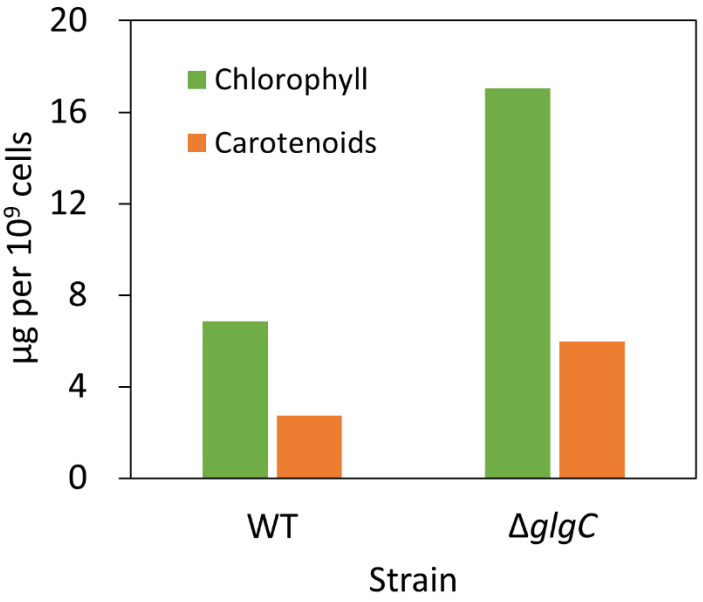


Figure 5.4 Chlorophyll and carotenoid contents of *Synechococcus* 2973 WT and Δ*glgC* mutant.

The pigment contents were normalized by cell number. Cell were grown at 38°C, 900 µmol photons m^{-2} s^{-1} light, and 5% CO_2 for 3 days.

5.2.4 Engineering of the Δ*glgC* mutant for limonene production

The Δ*glgC* mutant exhibited increased amounts of chlorophyll and carotenoids (Figure 5.4), indicating a higher carbon flux was directed toward terpene synthesis. To test if this glycogen-deficient strain could enhance limonene production, the plasmid lims-gpps (Figure 3.1A) was

introduced into the Δ*glgC* strain via bacterial conjugation. Unfortunately, the mutant strain harboring the plasmid exhibited significant growth impairment. Limonene could not be detected by gas chromatography (data not shown).

5.3 Discussion

In this Chapter, the glycogen metabolism in *Synechococcus* 2973 was investigated. In cyanobacteria, glycogen severs as a storage pool for fixed carbon under nutrient-limited conditions (Schwarz and Forchhammer 2005). Remarkably, glycogen synthesis in *Synechococcus* 2973 is not required to be induced under nutrient-limited conditions. Strain 2973 accumulated glycogen to more than 30% of DW after 50 hours of growth (Figure 5.1B), and the rate of glycogen synthesis dramatically increased after the exponential growth phase (Figure 5.1B, inset graph). If this significant amount of fixed carbon can be redirected toward production of chemical, the yields are expected to be improved significantly. However, removing the glycogen synthesis pathway for enhanced chemical production is still inconclusive in cyanobacteria. Studies have reported growth retardation that led to reduced production of terpenes in glycogen-deficient cyanobacterial strains (Lin and Pakrasi 2019). Likewise, our results showed that expression of plasmid lims-gpps lead to severe growth retardation and limonene was undetected in the engineered Δ*glgC* strain.

Interestingly, we found that the glycogen-deficient strain had higher amounts of photosynthetic pigments (Figure 5.4). Presumably, deletion of the *glgC* gene remodels the carbon metabolism in *Synechococcus* 2973, in which the photosynthetic carbon was redirected toward the pigment synthesis pathway. In fact, the higher pigment content in the Δ*glgC* strain may suggest that this strain could be a potential host for limonene production, because the photosynthetic

pigments and limonene share the same terpene precursors (Figure 1.1). However, the limonene titer is undetectable after the plasmid lims-gpps was introduced into the Δ*glgC* strain. Further research is needed (*e.g.*, flux analysis) to elucidate the carbon metabolism in the glycogen-deficient strains.

5.4 Methods

5.4.1 Growth conditions

Synechococcus 2973 was grown in BG11 medium at 38°C, 900 μmol photons m^{-2} s^{-1} light, and 5% CO_2 in an MC-1000 multicultivator (Photon Systems Instruments, Czech Republic). *Synechococcus* 7942 was grown in BG11 medium at 38°C with 400 mol m^{-2} s^{-1}light and 5% CO_2 in an MC-1000 multicultivator. To test limonene production, the *Synechococcus* 2973 limonene-producing strains were grown in BG11 liquid medium at 38°C with 1 mM IPTG, 250 μmol photons m^{-2} s^{-1} light and 1% CO_2 in an AlgaeTron growth chamber (Photon Systems Instruments, Czech Republic). Isopropyl myristate (10% of culture) was added on the cell cultures to trap evaporated limonene.

5.4.2 Calculation of biomass

The biomass was calculated based on the correlation of OD_{730} with cell dry weight: DW (g L^{-1}) = 1.0486 x OD_{730} - 0.1713.

5.4.3 CRISPR/Cpf1 genome editing and strain construction

The CRISPR/Cpf1 plasmid for *glgC* deletion was constructed based on the method reported in a previous study (Ungerer and Pakrasi 2016). Briefly, the guide RNA (gRNA) of *glgC* (5'-tcggtggaggcgcaggcagt-3') was cloned into the Cpf1 plasmid pSL2680 to create the plasmid pSL3049. Subsequently, the repair templates for *glgC* in-frame deletion were assembled into the plasmid pSL3049 to create the complete plasmid pSL3049 for knocking out *glgC*. The plasmid pSL3049 was introduced into *Synechococcus* 2973 using bacterial conjugation to delete *glgC*. The mutants were re-patched multiple times on BG11 agar plates with 10 μg/mL kanamycin. PCR genotyping was used to verify complete knock-out of *glgC*. Once the *glgC* gene was deleted completely, the Cpf1 plasmid was cured by growing the mutant in BG11 medium without kanamycin. To construct a limonene-producing Δ*glgC* strain, the plasmid lims-gpps (Figure 3.1A) was introduced in the Δ*glgC* strain via bacterial conjugation.

5.4.4 Measurement of glycogen and cell number

Cultures were collected by centrifugation and the pellets were resuspended with 300 μL KOH (30% w/v), incubated at 95℃ for 90 minutes. Glycogen was precipitated by adding 1.2 mL absolute ethanol, and samples were kept on ice for 2 hours. Glycogen was collected by centrifugation at full speed for 5 minutes. Pellets were washed twice by 1 mL absolute ethanol. The washed pellets were dried at 60℃ for 15 minutes until the remaining ethanol was evaporated. The dried samples were resuspended in 300 μL sodium acetate solution (100 mM, pH 4.75). Then, glycogen was digested to glucose by amyloglucosidase (4 U/assay) for 25 minutes at 55℃. After digestion, the insoluble pellets were removed by centrifugation, and the supernatants were used for determining the glycogen content by a glucose (HK) assay kit (Sigma, USA). Samples

untreated with amyloglucosidase were included to determine the background glucose content. By subtraction of the background glucose content, the actual glycogen concentration was determined.

To measure cell number, *Synechococcus* 2973cultures were grown in MC-1000 multicultivators at 38℃ with 5% CO_2 and 900 μmol m^{-2} s^{-1} light. Twenty microliters of culture were taken for cell counting using an automated cell counter (Cellometer Vision; Nexcelom, USA). The counted images were manually curated to improve accuracy of the counts.

5.4.5 Pigment analysis

The photosynthetic pigments were extracted and quantified based on our previous study (Lin et al. 2017) as described in Chapter 2.5.8.

References

Bonde MT, Pedersen M, Klausen MS, Jensen SI, Wulff T, Harrison S, Nielsen AT, Herrgard MJ, Sommer MO (2016) Predictable tuning of protein expression in bacteria. Nature methods 13 (3):233-236. doi:10.1038/nmeth.3727

Carrieri D, Paddock T, Maness P-C, Seibert M, Yu J (2012) Photo-catalytic conversion of carbon dioxide to organic acids by a recombinant cyanobacterium incapable of glycogen storage. Energy & Environmental Science 5 (11):9457-9461

Davies FK, Work VH, Beliaev AS, Posewitz MC (2014) Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of *Synechococcus* sp. PCC 7002. Frontiers in bioengineering and biotechnology 2:21. doi:10.3389/fbioe.2014.00021

Li X, Shen CR, Liao JC (2014) Isobutanol production as an alternative metabolic sink to rescue the growth deficiency of the glycogen mutant of *Synechococcus elongatus* PCC 7942. Photosynthesis research 120 (3):301-310. doi:10.1007/s11120-014-9987-6

Lin P-C, Saha R, Zhang F, Pakrasi HB (2017) Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. Scientific reports 7 (1):17503. doi:10.1038/s41598-017-17831-y

Lin PC, Pakrasi HB (2019) Engineering cyanobacteria for production of terpenoids. Planta 249 (1):145-154. doi:10.1007/s00425-018-3047-y

Schwarz R, Forchhammer K (2005) Acclimation of unicellular cyanobacteria to macronutrient deficiency: emergence of a complex network of cellular responses. Microbiology 151 (Pt 8):2503-2514. doi:10.1099/mic.0.27883-0

Ungerer J, Pakrasi HB (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria. Scientific reports 6:39681

van der Woude AD, Angermayr SA, Puthan Veetil V, Osnato A, Hellingwerf KJ (2014) Carbon sink removal: Increased photosynthetic production of lactic acid by *Synechocystis* sp. PCC 6803 in a glycogen storage mutant. Journal of biotechnology 184:100-102. doi:10.1016/j.jbiotec.2014.04.029

Chapter 6: Conclusions and future directions

6.1 Conclusions

Cyanobacteria are attractive hosts for production of fuels and chemicals, because these photosynthetic microorganisms directly convert CO_2 into desired products without the use of sugar feedstock. In this dissertation, cyanobacteria were engineered to explore their potential for sustainable production of limonene and sucrose, which utilize distinct precursor pools for biosynthesis. In Chapter 2, engineering of the model cyanobacterium *Synechocystis* sp. PCC 6803 enables this strain to produce limonene. To further increase the productivity of limonene, we utilized a computational modelling approach to identify targets for genetic modifications. The final engineered strains improved limonene production by 2.3 fold with a productivity of 0.96 mg L^{-1} day^{-1}, which is close to the highest productivity of limonene (1 mg L^{-1} day^{-1}) reported in a previous study (Davies et al. 2014). The engineering strategy presented in this Chapter could be useful for the metabolic engineering of *Synechocystis* 6803 to produce other terpenoids.

Since the engineered *Synechocystis* 6803 did not further enhance the productivity of limonene compared to that in previous work (Lin and Pakrasi 2019), we shifted our focus to engineering the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973, which also accumulates a significant amount of glycogen to store the photosynthetically fixed carbon (Chapter 5) (Ungerer et al. 2018). In Chapter 3, we identified a *Synechococcus* 2973 mutant strain that significantly increase limonene production for 2.5-fold, and determined that this strain has a SNP in the gene encoding the GGPP synthase CrtE. Furthermore, we systematically engineered the precursor pathways of limonene biosynthesis by modulation of GPPS expression and optimization of the MEP pathway. The limonene productivity reached 8.2 mg L^{-1} day^{-1}, which improved over

8-fold compared to previous studies. This integrated approach would be applicable for engineering of cyanobacteria to produce terpenoids.

In Chapter 4, we explored the potential of sugar production in *Synechococcus* 2973. By expressing the sucrose transporter CscB, the engineered strain exported 8 g L^{-1} of sucrose with the highest productivity of 1.9 g L^{-1} day^{-1} under salt stress conditions. This study is the first to use cyanobacteria to produce a compound with a rate higher than 1 g L^{-1} day^{-1} using only CO_2 as the carbon source. Additionally, our results showed that the salt stress activated the sucrose biosynthesis pathway, leading to a dramatic increase in sucrose production. To expand the use of sucrose-producing cyanobacteria, we engineered *Synechococcus* 2973 to produce sucrose without additional salt stress. Notably, we determined that SPS and SPP are both important enzymes for sucrose biosynthesis, while the enzyme level of SPP is particularly critical for enhancing sucrose production. This work demonstrates *Synechococcus* 2973 is a promising strain for the photosynthetic production of sugar.

The production of limonene and sucrose use distinct biosynthesis pathways (Figure 1.2). Comparing the productivities of limonene and sucrose in this dissertation, the highest rate of sucrose production is 232 fold higher than that of limonene (1.9 g L^{-1} day^{-1} vs. 0.008 g L^{-1} day^{-1}). In fact, the flux analysis results demonstrated that *Synechococcus* 2973 has strong carbon fluxes in sugar phosphates and relatively small pools of pyruvate and acetyl-CoA (Abernathy et al. 2017), suggesting its potential for sugar production. Our results verify that this fast-growing strain is an ideal host for sucrose production.

6.2 Future directions

In this dissertation, we have demonstrated that *Synechococcus* 2973 is a promising cyanobacterium for the production of limonene and sucrose compared to other cyanobacterial strains. To further improve limonene production in *Synechococcus* 2973, the entire terpene biosynthesis pathway should be systematically engineered by modulating the expression of *lims*, *gpps*, *dxs*, and *idi* (Ajikumar et al. 2010). As shown in Figure 3.7, the control strain lims-gpps produced 10 mg L^{-1} of limonene with the addition of IPTG higher than 0.05 mM. However, decreasing the concentration of IPTG (0.01 mM) improved limonene production significantly in the *dxs*-*idi* coexpression strain. These results indicate that the expression levels should be high for *lims* and *gpps* and low for *dxs* and *idi* in order to enhance the production of limonene. However, by using a weaker RBSs (TIR = 5000) for *dxs* and *idi*, the limonene titer did not improve (Table 3.2). To further optimize the limonene biosynthetic pathway, the expression of *dxs* and *idi* should be modulated by using RBS sequences with their TIR ranging between 5000-40000 in order to identify their optimal expression levels.

Besides DXS and IDI, other bottleneck enzymes in the MEP pathway should also be investigated collectively to see if the limonene titer could be increased. Previous studies reported that IspD and IspG are important enzymes in the MEP pathway of *Synechocystis* 6803 and *Synechococcus* 7942, respectively (Englund et al. 2018; Gao et al. 2016). Overexpression of these genes increased production of isoprene in cyanobacteria. We could further investigate whether IspD and/or IspG are critical enzymes for improving limonene production in *Synechococcus* 2973 by constructing the *dxs*-*idi*-*ispD* and *dxs*-*idi*-*ispG* coexpression strains.

The MEP pathway is initiated by pyruvate and GAP (Figure 1.1). The flux analysis of *Synechococcus* 2973 revealed that this strain has a smaller pool size of pyruvate compared to that of GAP (Abernathy et al. 2017). Since both substrates are utilized by DXS (the first enzyme in the MEP pathway), the smaller pool size of pyruvate may be a rate-limiting factor for improving limonene production. In the glycolytic pathways, pyruvate kinase (PK) catalyzes the conversion of phosphoenolpyruvate to pyruvate. A recent study reported that PK in *Synechococcus* 7942 is a hidden bottleneck reaction for production of isobutyraldehyde, which requires pyruvate as a precursor for biosynthesis (Jazmin et al. 2017). Overexpression of PK improved the productivity of isobutyraldehyde significantly (Jazmin et al. 2017). Coexpression of *pk* with *dxs* and *idi* may direct a higher amount of carbon flux to the MEP pathway.

The limonene produced in *Synechococcus* 2973 could potentially be toxic to the cells, although it has been reported that most of the produced limonene was released from cells without any treatments (Kiyota et al. 2014). Removal of the product can reduce toxicity and avoid feedback regulation, thus improving the production titer. Dunlop *et al.* discovered that an efflux pump from *Alcanivorax borkumensis* can serve as a limonene transporter. Expression of genes encoding the efflux pump (YP_692684) significantly increased limonene production in *E. coli* (Dunlop et al. 2011). This pump could be expressed in our limonene-producing strain to see if the limonene titer could be further improved.

The engineered *Synechococcus* 2973 encoding the CrtE (R299Q) mutant protein increased limonene productivity over 2.5-fold (Figure 3.3B). CrtE competes with GPPS for the substrates IPP and DMAPP, and controls the carbon flux to pigment synthesis (see Chapter 1.1). The R299Q mutation may alter the catalytic activity of CrtE, thus allowing a higher carbon flux being directed

toward limonene synthesis. Therefore, the enzyme activity of CrtE (R299Q) should be determined to elucidate how this amino acid change drastically improves limonene production. Furthermore, the growth phenotypes (*e.g.*, doubling time, biomass accumulation, and pigment content) of this CrtE (R299Q) mutant strain should be studied and compared with the wild type *Synechococcus* 2973. To test if the CrtE (R299Q) mutation could be used as a general approach for enhancing terpene production, this mutant strain could be engineered to produce other useful terpenes (*e.g.*, pinene and farnesene).

In Chapter 4, *Synechococcus* 2973 has demonstrated its potential for sucrose production, with a rate of 1.9 g L^{-1} day^{-1}. We could further investigate whether this strain is suitable for commercial production of sugar. To construct a robust strain for sucrose production, a constitutive promoter can be used to express *cscB*. Hence, the IPTG inducer is no longer needed to induce *cscB* expression. This could be achieved by excluding the lacI repressor in the engineered strain. The *cscB* gene will be constitutively expressed by the *lacUV5* promoter. Otherwise, the *trc* or *lac* promoters can be used to investigate which promoter leads to the highest production of sucrose.

In our sucrose production experiments, the engineered strains were cultured in 250 μmol photons m^{-2} s^{-1} light. To mimic the conditions of outdoor cultivation, the *cscB*-expressing strain could be cultured under a sinusoidal light pattern, or using the diurnal light cycle. Moreover, the strains could be grown in continuous or semi-continuous culture systems to test whether *Synechococcus* 2973 can produce sucrose continuously. This would demonstrate the feasibility of using cyanobacteria for commercial production of sugar. Besides sucrose production, we could also explore the potential of *Synechococcus* 2973 for production of other valuable sugar compounds (Frigaard 2018).

References

Abernathy MH, Yu J, Ma F, Liberton M, Ungerer J, Hollinshead WD, Gopalakrishnan S, He L, Maranas CD, Pakrasi HB, Allen DK, Tang YJ (2017) Deciphering cyanobacterial phenotypes for fast photoautotrophic growth via isotopically nonstationary metabolic flux analysis. Biotechnology for biofuels 10:273. doi:10.1186/s13068-017-0958-y

Ajikumar PK, Xiao WH, Tyo KE, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G (2010) Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. Science 330 (6000):70-74. doi:10.1126/science.1191652

Davies FK, Work VH, Beliaev AS, Posewitz MC (2014) Engineering Limonene and Bisabolene Production in Wild Type and a Glycogen-Deficient Mutant of *Synechococcus* sp. PCC 7002. Frontiers in bioengineering and biotechnology 2:21. doi:10.3389/fbioe.2014.00021

Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487. doi:10.1038/msb.2011.21

Englund E, Shabestary K, Hudson EP, Lindberg P (2018) Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. Metabolic engineering 49:164-177. doi:10.1016/j.ymben.2018.07.004

Frigaard N-U (2018) Sugar and Sugar Alcohol Production in Genetically Modified Cyanobacteria. In: Genetically Engineered Foods. Elsevier, pp 31-47

Gao X, Gao F, Liu D, Zhang H, Nie X, Yang C (2016) Engineering the methylerythritol phosphate pathway in cyanobacteria for photosynthetic isoprene production from CO_2. Energy & Environmental Science 9 (4):1400-1411

Jazmin LJ, Xu Y, Cheah YE, Adebiyi AO, Johnson CH, Young JD (2017) Isotopically nonstationary ^{13}C flux analysis of cyanobacterial isobutyraldehyde production. Metabolic engineering 42:9-18

Kiyota H, Okuda Y, Ito M, Hirai MY, Ikeuchi M (2014) Engineering of cyanobacteria for the photosynthetic production of limonene from CO_2. Journal of biotechnology 185:1-7. doi:10.1016/j.jbiotec.2014.05.025

Lin PC, Pakrasi HB (2019) Engineering cyanobacteria for production of terpenoids. Planta 249 (1):145-154. doi:10.1007/s00425-018-3047-y

Ungerer J, Lin PC, Chen HY, Pakrasi HB (2018) Adjustments to Photosystem Stoichiometry and Electron Transfer Proteins Are Key to the Remarkably Fast Growth of the Cyanobacterium *Synechococcus elongatus* UTEX 2973. mBio 9 (1). doi:10.1128/mBio.02327-17

Appendix 1: Characterization and development of IPTG-inducible promoters in *Synechococcus elongatus* UTEX 2973

To test whether the *trc1O* and *lacUV5* promoters are IPTG inducible in *Synechococcus* 2973, a gene encoding the enhanced yellow fluorescence protein (EYFP) was cloned into a RSF1010 plasmid and under the control of P_{trc1O} and P_{lacUV5} (Figure 1A). As shown in Figure 1B, both promoters were induced by IPTG. The P_{trc1O} had a 43-fold dynamic range, whereas the P_{lacUV5} had a 24-fold change by addition of 1 mM IPTG. The *lacUV5* promoter conferred a stronger expression level and was leakier than the *trc1O* promoter.

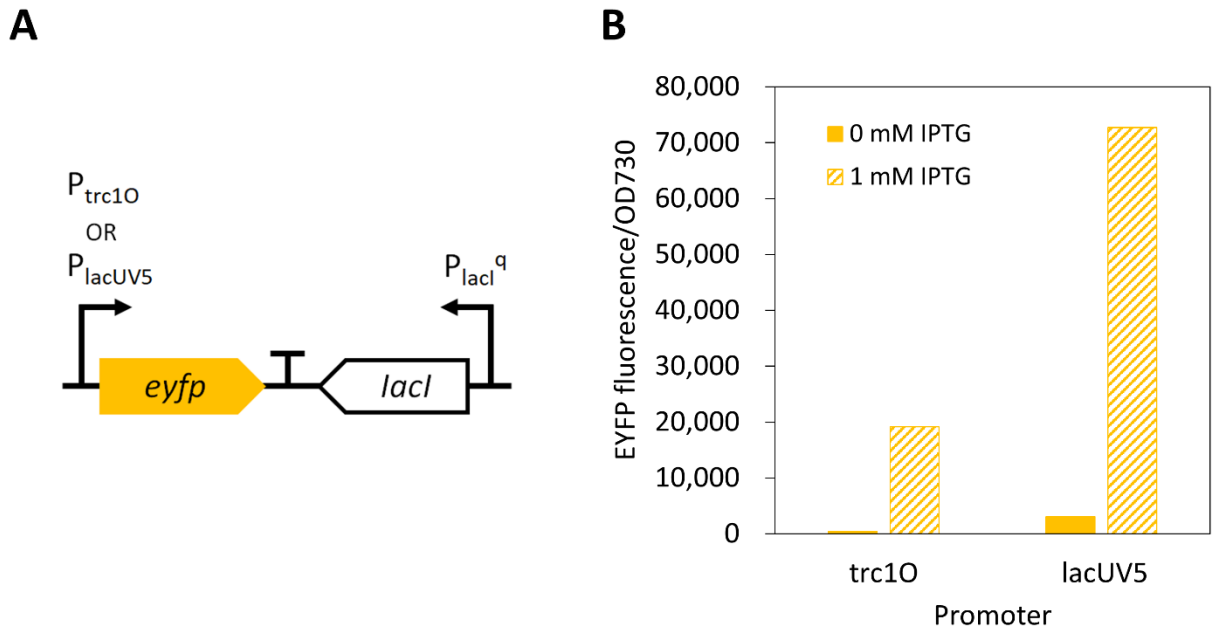


Figure 1. Characterization of IPTG-inducible promoters in *Synechococcus* 2973.

(A) Schematics of *eyfp* controlled by *trc1O* or *lacUV5* promoters. A *lacI* cassette was included to repress gene expression. **(B)** EYFP fluorescence of P_{trc1O}-*eyfp* and P_{lacUV5}-*eyfp* with or without 1 mM of IPTG. Cells were grown in 500 µmol photons m^{-2} s^{-1} light, 1% CO_2 at 38°C for 24 hours.

Currently, a limited number of inducible promoters are available for cyanobacteria. To develop genetic tools for *Synechococcus* 2973, I tried to engineer the native promoters of *Synechococcus* 2973 to make them become IPTG inducible. The strengths of various native promoters were chatacterized (Figure 2). Five native promoters (P_{0012}, P_{1479}, P_{1809}, P_{2486}, and P_{cpcB}) were chosen for engineering, by cloning the lacO1 operator after the promoter sequences (Figure 3A).

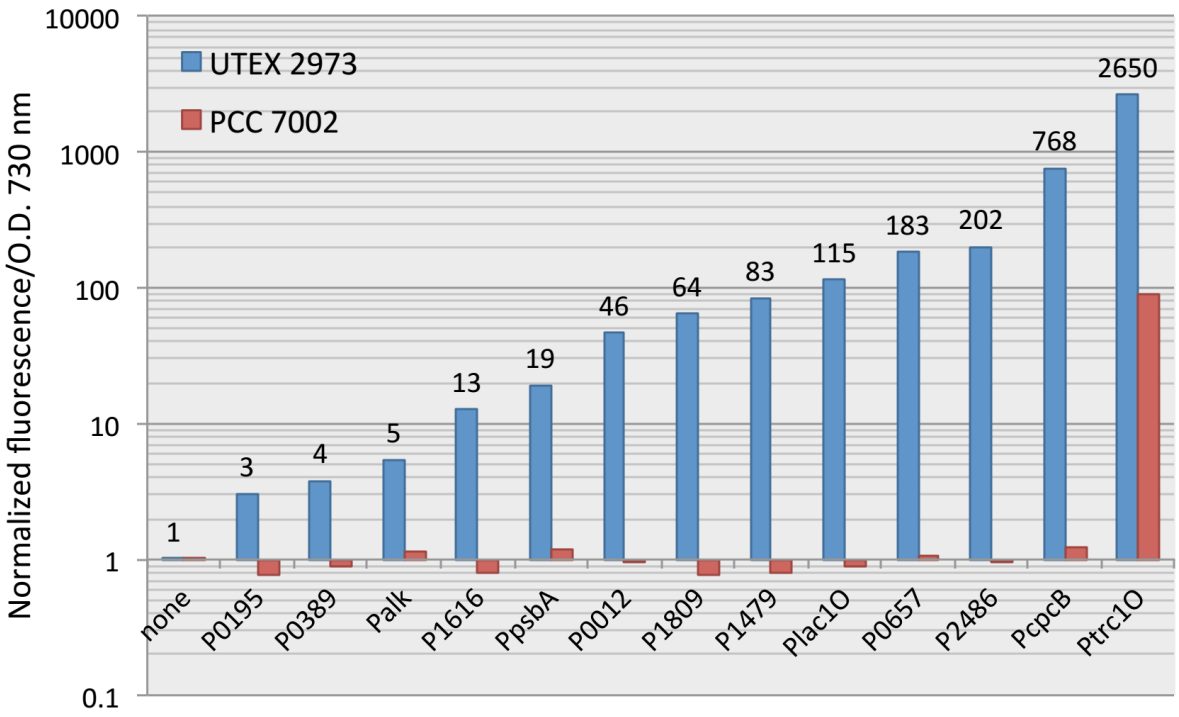


Figure 2. Characterization of *Synechococcus* 2973 native promoters (From Dr. Cory Knoot).

The strengths of 11 native promoters from *Synechococcus* 2973 were investigated using EYFP. Five promoters (P_{0012}, P_{1479}, P_{1809}, P_{2486}, and P_{cpcB}) were chosen for further engineering.

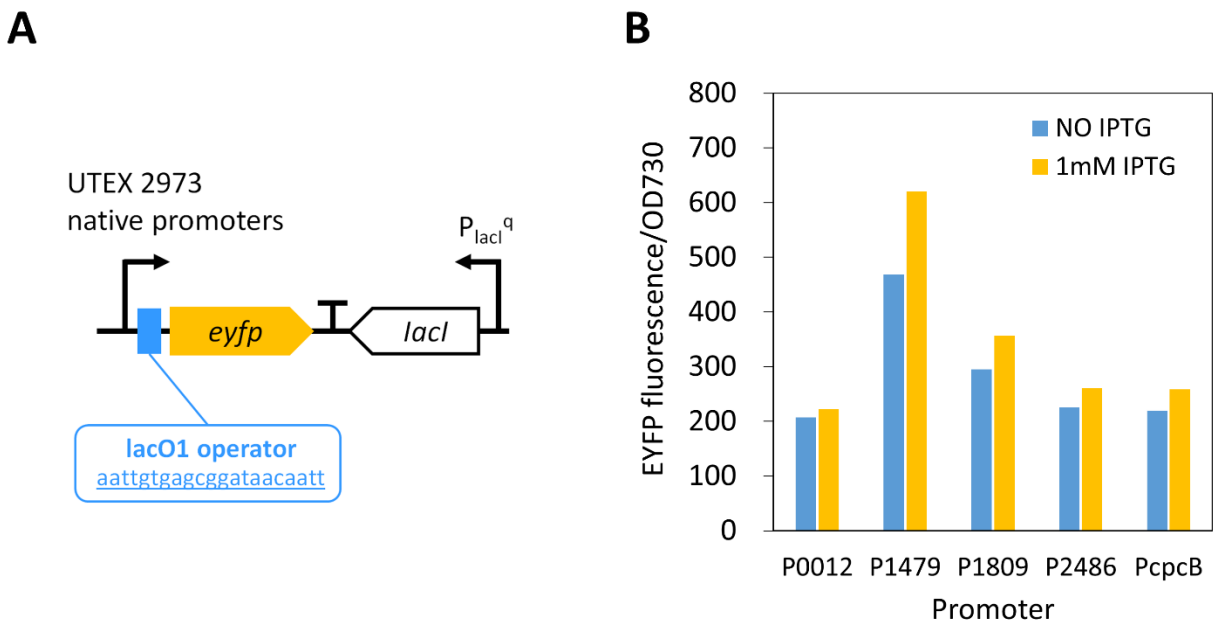


Figure 3. Engineering the native promoters in *Synehcococcus* 2973 to become IPTG inducible

(A) The lacO1 operator was cloned in the plasmid after the promoter sequences. **(B)** EYFP fluorescence of engineered promoters with or without addition of 1 mM IPTG. Cells were grown in 250 µmol photons m^{-2} s^{-1} light, 1% CO2 at 38°C for 24 hours.

As shown in Figure 3B, the engineered promoters could not be induced by IPTG. No significant difference was observed with or without addition of IPTG. Presumably, adding the lacO1 sequence abolishes the RBS in the native promoters, which in turn affects the translation of EYFP. Therefore, a strong bacterial RBS was used to replace the native RBS in the promoters (Figure 4A). By using a strong RBS to expression EYFP, the expression levels of these engineered promoters increased significantly compared to the original design (Figure 4B). The P_{0012} showed a 6.8-fold dynamic range (Figure 4B). The other promoters still could not be induced by IPTG, with dynamic ranges less than 2-fold. Further engineering is needed to make these native promoters become IPTG inducible.

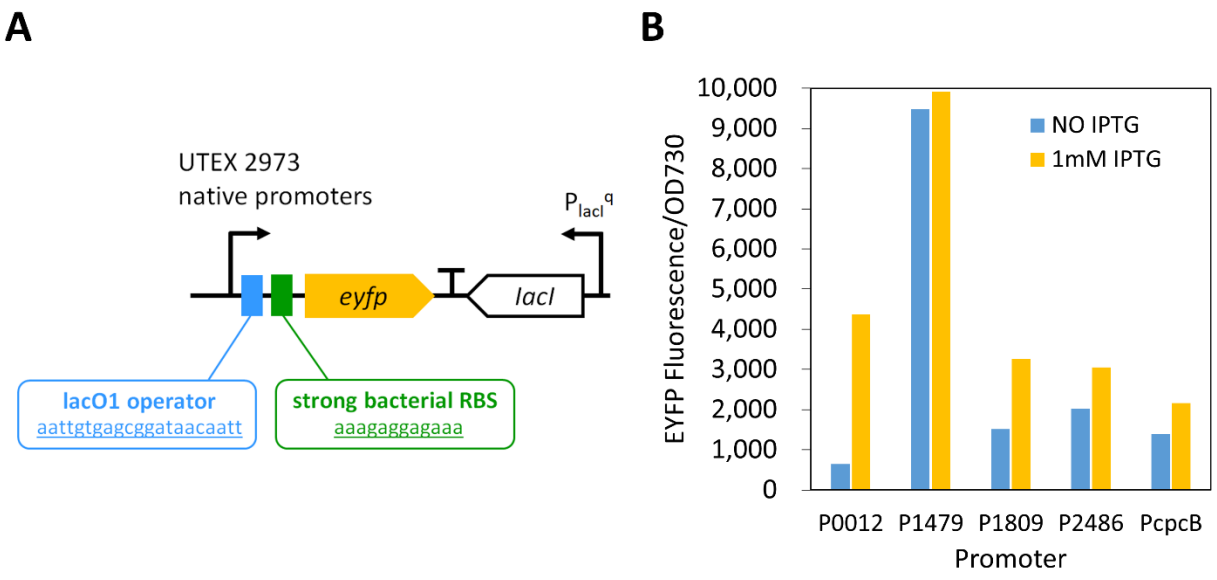


Figure 4. Improvement of the expression of engineered promoters.

(A) A strong bacterial RBS was used to replace the native RBS sequences in the promoters. **(B)** EYFP fluorescence of engineered promoters with or without addition of 1 mM IPTG. Cells were grown in 250 µmol photons m^{-2} s^{-1} light, 1% CO2 at 38°C for 24 hours.

Appendix 2: Curriculum Vitae

Po-Cheng Lin

(314)537-0914 • pclin027@gmail.com
LinkedIn: https://www.linkedin.com/in/po-cheng-lin-24020a41/

PROFESSIONAL PROFILE

Ph.D. with over 9 years of experience in **bioengineering, molecular biology, and synthetic biology.** My thesis focuses on engineering photosynthetic bacteria to produce useful chemicals. Prior to my Ph.D. studies, I worked in a microalgae laboratory to improve production of biodiesel, and in an algae company to develop processes for manufacturing lyophilized fluorescent proteins.

HIGHLIGHTS

- Engineered bacteria using synthetic biology and CRISPR genome editing techniques to improve production of a plant terpene limonene (8 fold higher than previous studies).
- Simplified a plasmid construction protocol for CRISPR genome editing to increase its efficiency by 70%.
- Won the Three Minute Thesis (3MT) Competition Judges' and People's Choice Awards at the McDonnell Academy 7th International Symposium in Beijing, China.
  (See AWARDS section for more details)

EDUCATION

Washington University in St. Louis – MO — May. 2019
Ph.D., Energy, Environmental & Chemical Engineering
- Award: McDonnell International Academy Scholarship

National Taiwan University – Taipei, Taiwan — Jan. 2010
M.S., Bioenvironmental System Engineering

National Taiwan University – Taipei, Taiwan — Jun. 2007
B.S, Public Health

RESEARCH EXPERIENCE

Washington University in St. Louis – MO — Aug. 2013 – present
Ph.D. researcher
- Engineered cyanobacteria for improved production of limonene over 8-fold.
- Simplified a plasmid construction procedure for CRISPR genome editing in cyanobacteria.
- Developed genetic tools (inducible promoters and expression vectors) for non-model cyanobacteria.

National Cheng Kung University – Tainan, Taiwan — Aug. 2012 – May. 2013
Research assistant
- Optimized bioprocess for photobioreactors to increase production of algae biofuels by 50%.
- Developed a genetic transformation system for non-model green algae.

Far-East Bio-Tec. Co., Ltd – Taipei, Taiwan — Apr. 2012 – Jun. 2012
R&D Specialist & International Sales Representative
- Developed processes for manufacturing lyophilized phycobiliproteins and relevant conjugates derived from microalgae.

PUBLICATIONS

- **P. C. Lin**, F. Zhang and H. B. Pakrasi. (2019). Sustainable production of sucrose in the fast-growing cyanobacterium *Synechococcus elongatus* UTEX 2973. *Biotechnology for Biofuels*. *Submitted*
- **P. C. Lin** and H. B. Pakrasi. (2019). Engineering cyanobacteria for production of terpenoids. *Planta*.
- J. Ungerer, **P. C. Lin**, H. Y. Chen and H. B. Pakrasi. (2018). Adjustments to photosystem stoichiometry and electron transfer proteins are key to the remarkably fast growth of the cyanobacterium *Synechococcus elongatus* UTEX 2973. *mBio*.

- **P. C. Lin**, R. Saha, F. Zhang and H. B. Pakrasi (2017). Metabolic engineering of the pentose phosphate pathway for enhanced limonene production in the cyanobacterium *Synechocystis* sp. PCC 6803. *Scientific Reports*.
- V. H. Liao, Y. J. Chu, Y. C. Su, **P. C. Lin**, Y. H. Hwang, C. W. Liu, C. M. Liao, F. J. Chang and C. W. Yu (2011). Assessing the mechanisms controlling the mobilization of arsenic in the arsenic contaminated shallow alluvial aquifer in the blackfoot disease endemic area. *J Hazard Mater*.

LEADERSHIP/TEAMWORK

Washington University, Taiwanese Graduate Student Association May 2014 – May 2015

President

- Oversaw and coordinated outreach events and tasks to assist 20+ incoming Taiwanese graduate students and postdoctoral researchers transition to life at Washington University.
- Organized 5 events (80+ attendees) to celebrate Taiwanese culture and festivals.

Washington University, McDonnell International Scholars Academy Aug. 2013 – present

Recruitment representative

- Organized and held annual information sessions (50+ attendees) to recruit perspective graduate students in Taiwan.

AWARDS

3MT Competition at McDonnell Academy 7th International Symposium (2018) – Beijing, China

- Won the Judges' and People's Choice Awards.
- Presented my research topic and its significance in just three minutes.
- Coverage from Washington University:
  - *McDonnell Scholar Wins Three Minute Thesis Competition*
  - *From Lab Bench to Stage: a McDonnell International Scholar's Journey to Winning the Three Minute Thesis Competition*

ISPR Conference on Microbial Photosynthesis (2018) – Vancouver, Canada

- Received Travel Award for oral presentation.

SKILLS

- **Microbe Transformation**
  Bacterial Conjugation, Electroporation, Chemical transformation
- **DNA Assembly**
  Gibson Assembly, Golden Gate, BioBricks
- **Genome Engineering**
  CRISPR/Cpf1 gene editing, Homologous Recombination, Lambda Red Recombineering
- **Metabolic Engineering**
  Promoter & RBS Engineering, Pathway Assembly & Optimization
- **Molecular Biology Related**
  RT-PCR, Site-Directed Mutagenesis, Transposon Mutagenesis
- **Carbohydrate Analysis**
  Glucose, Glycogen, and Sucrose measurement
- **Analytical Tools**
  Gas Chromatography (GC-FID, GC-MS), Nile Red (lipid measurement)
  Fluorescence & UV-Vis Spectroscopy
- **Protein Related**
  Recombinant Protein Expression, His-Tag Purification