

Harnessing Metabolic Engineering for the Production of Terpenoids in Cyanobacteria

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Dedication

To my mother, who always believed in me and whom I miss every day.

Declaration

Except where stated or otherwise acknowledged, the work presented in this thesis is entirely my own. No part has been submitted for another degree at the University of Nottingham or any other institution of learning.

Ryan Herold, September 2019

Abstract

Cyanobacteria are photosynthetic microorganisms that can be engineered to convert carbon dioxide into a myriad of useful chemicals. Terpenoids are the largest class of natural products and have applications in a wide variety of industries including fuels, pharmaceuticals, materials, cosmetics, and flavors and fragrances. Many of these compounds can be chemically synthesized or extracted from plants, however this is often either unsustainable or economically prohibitive. An alternative method of production relies on the heterologous expression of terpene synthase enzymes in cyanobacteria, producing the desired terpenoids directly from carbon dioxide. That strategy was explored in this work, whereby a heterologous mevalonate pathway, an enhanced methylerythritol phosphate (MEP) pathway, and a heterologous artemisinin biosynthetic pathway were all designed for expression in both *Synechocystis* and *Synechococcus*. Additionally, a heterologous patchoulol synthase from *Pogostemon cablin* (patchouli) was expressed in a cyanobacterium for the first time. A highly sensitive GC-MS quantification method was developed for the detection of patchoulol, and patchoulol production was characterized under four different growth conditions.

Keywords: Synthetic Biology, Metabolic Engineering, Cyanobacteria, *Synechocystis* sp. PCC 6803, Terpenoid, Patchoulol

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Abbreviations

°C	Degrees Celsius
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pair
Cmp	Chloramphenicol
cPCR	Colony PCR
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
E	Einstein
EDTA	Ethylene diamine-tetra-acetic acid
g	Grams
g	Gravities (relative centrifugal force)
GC	Gas Chromatography
GPP	Geranyl diphosphate
HCl	Hydrochloric Acid
HiFi	High Fidelity
IPP	Isopentyl diphosphate
Kan	Kanamycin
kb	Kilobase
L	Liter
LB	Lysogeny broth
M	Molar (moles per liter)
m	Meter
MEP pathway	Methylerythritol phosphate pathway
mL	Milliliter

MVA pathway	Mevalonate pathway
OD	Optical density
PATS	Patchoulol-producing <i>Synechocystis</i> strain
rpm	Revolutions per minute
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SANS	Santalene-producing <i>Synechocystis</i> strain
sp.	Species
μL	Microliter

1 Introduction

1.1 Introduction to Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes that are ubiquitous throughout the biosphere and are extremely important for global carbon cycling. Sometimes referred to as blue-green algae, cyanobacteria are a diverse group of organisms that exhibit a wide range of morphologies (**Figure 1**) and have evolved to thrive almost anywhere that water and light is present. Cyanobacteria can be unicellular, filamentous, or colonial,¹ are found in both tropical and polar regions, and have evolved to inhabit extreme environments including hyper-saline lakes, hot springs, and Antarctic rocks.^{2–4} Their adaptability has also allowed them to evolve symbiotic relationships with many distantly related organisms, including an interesting endosymbiotic relationship with fungi to form lichen.⁵ However, perhaps their most important symbiotic relationship was formed over a billion years ago when a cyanobacterium was phagocytized by a heterotrophic cell and managed to survive and replicate within the cell instead of being consumed. Similar events played out many times in order to form the photosynthetic plastids and mitochondria that exist as organelles in eukaryotic cells today, according to the endosymbiotic theory. The endosymbiotic theory is supported by a multitude of evidence, and explains how ancient cyanobacteria evolved to become the chloroplasts of modern plants.^{6–11}

1.1.1 Oxygenic Photosynthesis

Ancient cyanobacteria were the first organisms to evolve the ability to perform oxygenic photosynthesis,¹² allowing them to use light energy to harvest electrons from water and releasing molecular oxygen as a waste product. The ability to perform oxygenic photosynthesis was significant not

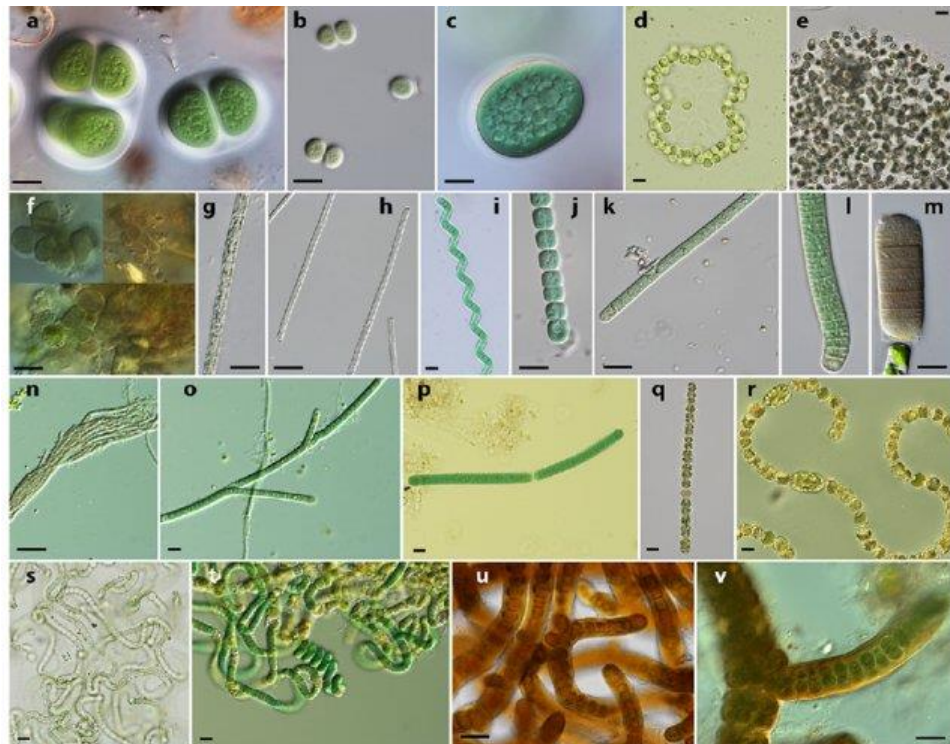


Figure 1 Morphological diversity in cyanobacteria. Groups (orders) are in bold text. **I. Chroococcales:** a *Chroococcus subnudus*, b *Ch. limneticus*, c *Cyanotheca aeruginosa*, d *Snowella litoralis*, e *Microcystis aeruginosa*. **II. Pleurocapsales:** f *Pleurocapsa minor*. **III. Oscillatoriales:** g *Planktothrix agardhii*, h *Limnothrix redekei*, i *Arthrospira jenneri*, j *Johanseninema constrictum*, k *Phormidium* sp., l, m *Oscillatoria* sp., n *Schizothrix* sp., o *Tolypothrix* sp., p *Katagnymene accurata*, **IV. Nostocales:** q *Dolichospermum planctonicum*, r *Dolichospermum* sp., s *Nostoc* sp., t *Nodularia moravica*. **V. Stigonematales:** u, v *Stigonema* sp. Scale bar a–u = 10 μ m, v = 20 μ m. Adapted from Dvořák et al., 2015.¹³

only because it made it possible to use water as an electron source but also because early earth was anoxic¹⁴ and oxygen was toxic to most living organisms. This opened an ecological niche for cyanobacteria that allowed them to proliferate in great numbers and simultaneously kill off many other microbes that might have competed with them for resources.¹⁵ Being the only prokaryotic organisms that could perform oxygenic photosynthesis, cyanobacteria are held responsible for the massive increase in atmospheric oxygen that took place 2.5–2.2 billion years ago called the Great Oxygenation Event^{12,14,16,17} (**Figure 2**). Although there is a general consensus about when the Great Oxygenation Event occurred, there is a debate around when oxygenic photosynthesis first evolved.^{12,17,18} As oxygen was toxic to most life existing at the time, cyanobacteria were responsible for the first mass-

extinction event. However, the introduction of oxygen into the atmosphere also opened up the possibility for more efficient extraction of energy from organic molecules through aerobic respiration (using oxygen as the terminal electron acceptor for the production of ATP). The ability of organisms to perform aerobic respiration allowed multicellular organisms to establish themselves and diversify to occupy new ecological niches.¹⁵

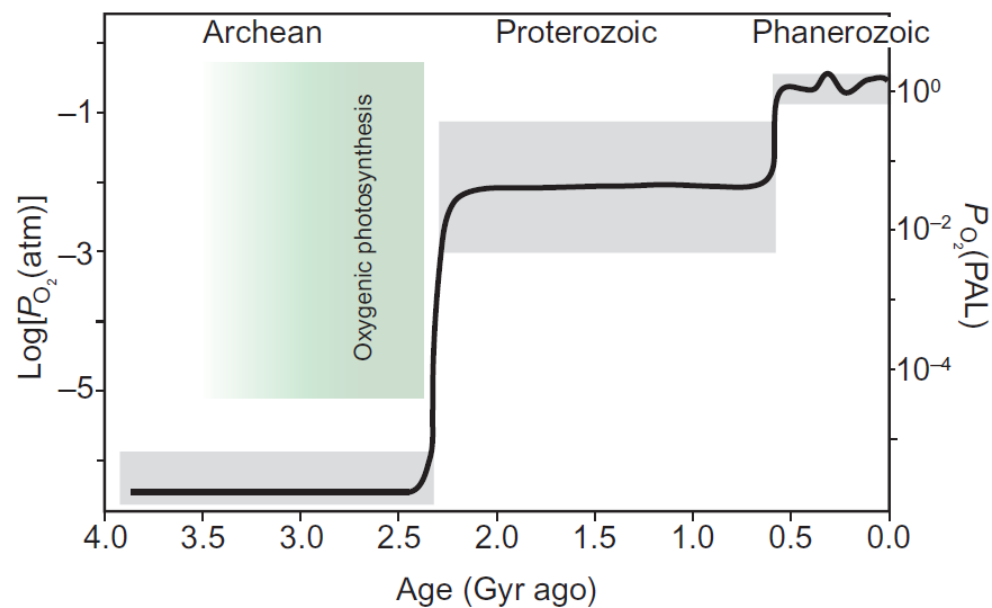


Figure 2 Levels of atmospheric oxygen throughout the history of Earth. The black line represents a view of the evolution of atmospheric oxygen over time.¹⁹ Grey bars represent the range of possible oxygen concentrations. Adapted from Hamilton *et al.*, 2016.¹⁷

Cyanobacteria are the only bacteria that have an internal thylakoid membrane, the site of the light dependent reactions of photosynthesis (**Figure 3** and **Figure 5**). Chloroplasts, the photosynthetic plastids present in plants and algae, also have thylakoid membranes, providing further support for the endosymbiotic theory.²⁰ The thylakoid membrane houses the protein complexes and accessory pigments necessary for the oxidation of water (photosystem II), the production of NADPH (ferredoxin-NADP⁺ reductase (FNR)), and the generation of ATP (ATP synthase) (**Figure 3**). Additionally, the thylakoid membrane creates a physical barrier across which a proton gradient is built up to power ATP synthesis. Carbon fixation does not occur in the thylakoid membrane but in a separate compartment called a

carboxysome. Carboxysomes are microcompartments on the order of 100–400 nm in diameter²¹ that are filled with carbonic anhydrase and the carbon fixing enzyme RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase). There is so much RuBisCO in carboxysomes that it makes up 60–70% of the total protein weight.²¹ Carboxysomes are important for efficient reduction of carbon dioxide because they simultaneously prevent photorespiration (an energy-loss pathway that occurs when RuBisCO uses oxygen as a substrate instead of carbon dioxide) by creating a barrier to oxygen. Carboxysomes also increase photosynthetic efficiency by producing carbon dioxide in situ for RuBisCO through the action of carbonic anhydrase.

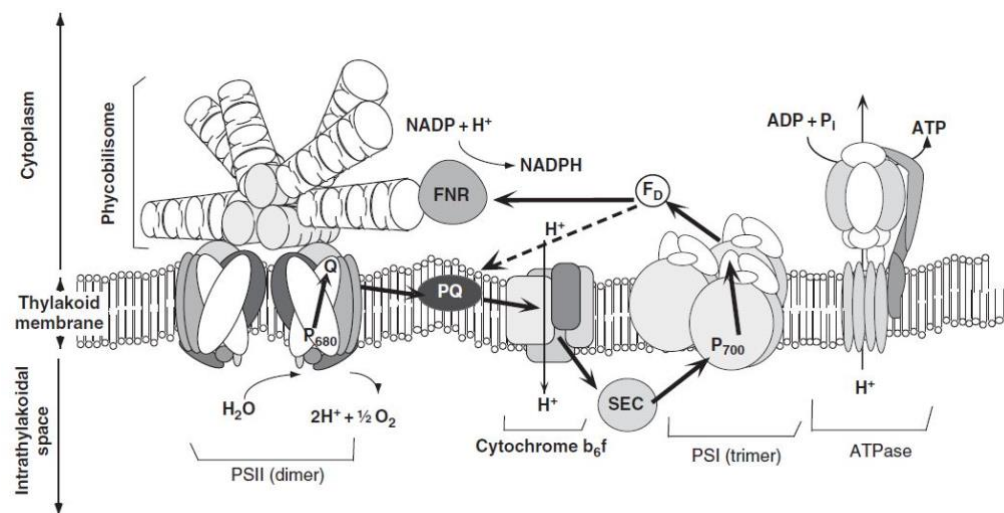


Figure 3 Photosynthetic components of a cyanobacterial thylakoid membrane. Protein complexes are labelled with brackets. Abbreviations are as follows: bound quinone (Q), plastoquinone (PQ), soluble electron carrier (SEC), ferredoxin (FD), ferredoxin-NADP⁺ reductase (FNR). Thick arrows show the direction of electron flow from water to NADPH. Dashed arrows show the electron flow under cyclic phosphorylation conditions. Thin arrows show either the transformation of chemical reactants or the transfer of protons across the thylakoid membrane. Adapted from Garcia-Pichel, 2009.²²

Similar to plants, cyanobacteria possess chlorophyll *a*, but most cyanobacteria do not contain chlorophyll *b*, with the exception of the prochlorophytes.²³ Cyanobacteria also differ from plants in their light-harvesting antennae and possess a unique structure called the phycobilisome. The phycobilisome is a protein complex consisting of multiple phycobiliproteins, which are divided into two major classes based on visual

appearance, phycocyanins and phycoerythrins. Phycocyanins are blue–green in color, and the phycoerythrins are orange–red. Phycobiliproteins are pigment-protein complexes made up of phycobilin chromophores that are covalently-bound to proteins.²⁴ The phycobilisome complex is bound to the cytoplasmic surface of the thylakoid membrane and is able to transfer light-excited electrons to both PSII and PSI.²⁵

1.1.2 Nitrogen Fixation in Cyanobacteria

The ecological diversity of cyanobacteria is mirrored by their metabolic diversity. There are a wide variety of natural products that cyanobacteria produce, and some filamentous cyanobacteria have the remarkable capacity to fix atmospheric nitrogen. This is especially impressive considering that cyanobacteria also need to fix their own carbon through photosynthesis. This dual metabolism of photoautotrophy and diazotrophy presents two major problems for cyanobacteria. One is the large metabolic burden placed on cells since fixing nitrogen requires 16 ATP per molecule of N_2 reduced. This may require changes in flux through other pathways to accommodate the high energy demand.²⁶ The second major problem is that nitrogenase enzymes are highly oxygen-sensitive, and photosystem II produces copious amounts of oxygen during photosynthesis. To simultaneously fix both CO_2 and N_2 , filamentous cyanobacteria have evolved the elegant solution of producing specialized microoxic, nitrogen-fixing cells called heterocysts. Heterocysts are produced under nitrogen-limiting conditions and are spaced out along the cyanobacterial filament with approximately 10 photosynthetic cells between each one.²⁷ As shown in **Figure 4**, heterocysts share fixed nitrogen with the carbon-fixing cells and receive fixed carbon in return. This evolved strategy is similar to many well-known symbiotic relationships between nitrogen fixing soil bacteria and terrestrial plants.

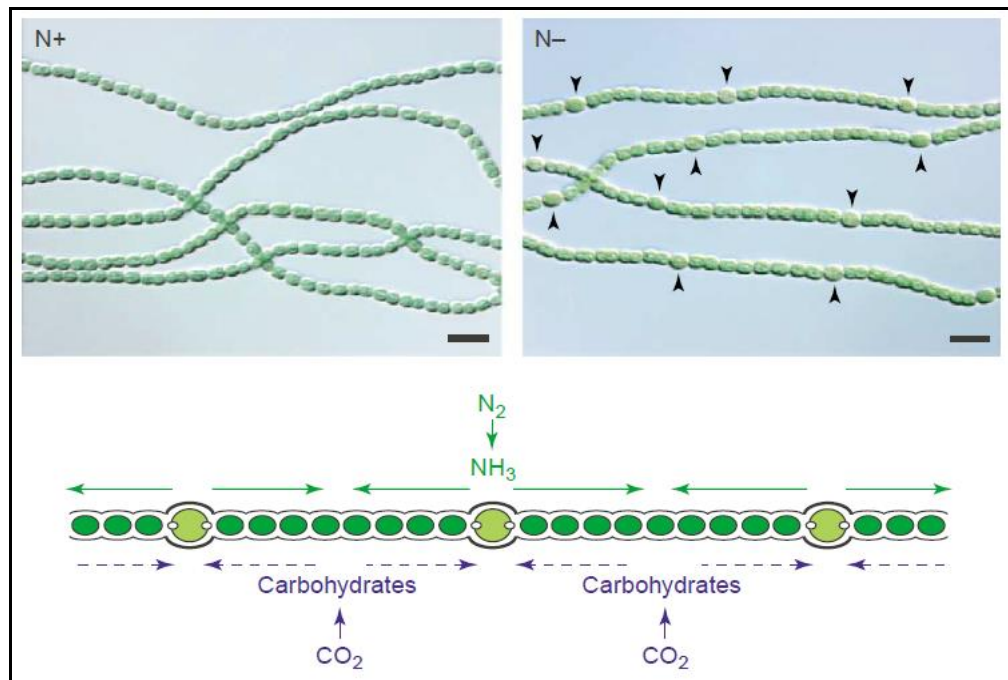


Figure 4 The Filamentous cyanobacterium *Anabaena* PCC 7120 grown with (N+) and without (N-) nitrogen. Heterocysts are indicated by arrowheads. Heterocysts supply fixed nitrogen as glutamine (as well as other amino acids) to neighboring cells, which supply heterocysts with fixed carbon in return. Filaments are made up of individual cells that each have their own plasma membrane and cell wall, all of which is enclosed within an outer membrane. Adapted from Golden and Yoon, 2003.²⁷

1.1.3 *Synechocystis* sp. PCC 6803

Synechocystis sp. PCC 6803—from here on referred to *Synechocystis*—is a unicellular model cyanobacterium that has been widely used to study photosynthesis, plastid evolution, and circadian rhythms.^{28,29} It was the first photoautotroph to have its genome fully sequenced,³⁰ which greatly improved the ability of scientists to study cyanobacterial biology. *Synechocystis* mainly grows photoautotrophically, but there are also glucose-tolerant strains that can be grown heterotrophically or mixotrophically. *Synechocystis* is naturally competent (capable of taking up exogenous DNA), making it relatively straightforward to genetically manipulate. As a model organism, many of its metabolic pathways have been well characterized, making it ideal for the introduction of novel genes and/or mutations to study their effects on cellular physiology. Additionally, its photosynthetic apparatus is fairly similar to that of terrestrial plants, making it a useful proxy for

studying the mechanisms of photosynthesis in general. **Figure 5** is an electron microscopy image of *Synechocystis* that shows the organization of the intracellular components including the thylakoid membrane (where the light dependent reactions of photosynthesis occur) and carboxysomes (where the first step of carbon fixation by RuBisCO occurs).

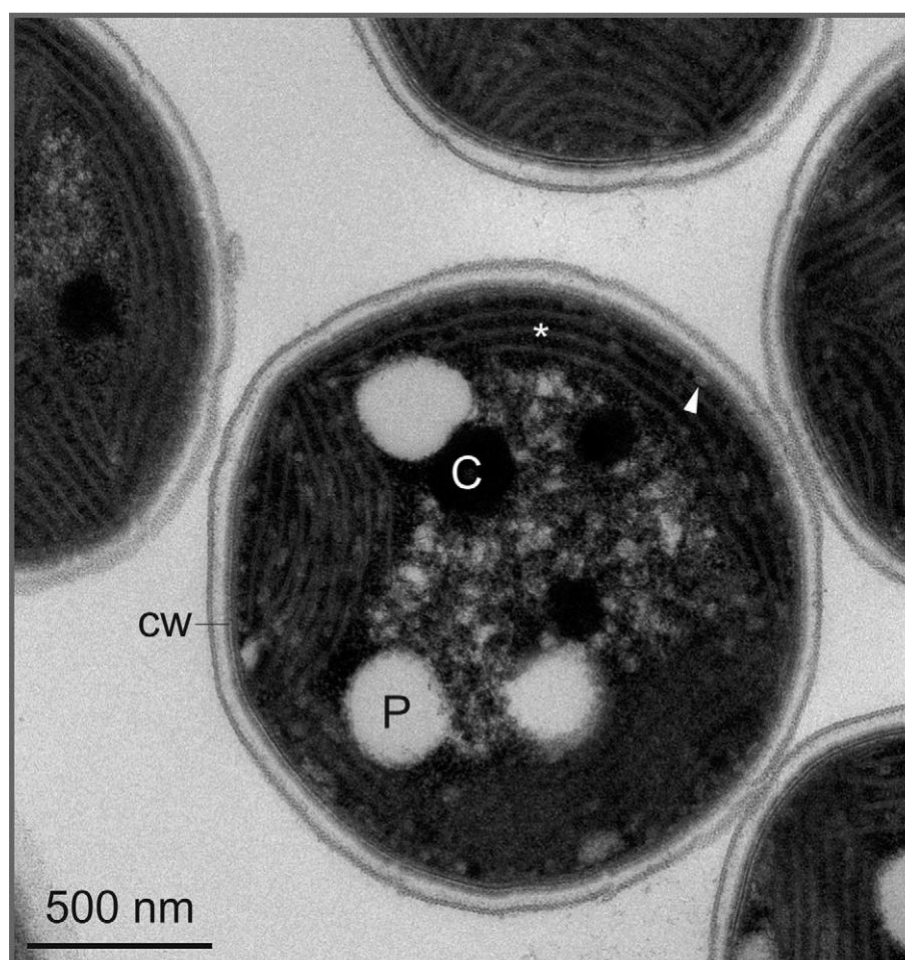


Figure 5 Electron microscopy image of *Synechocystis* sp. PCC 6803. C, carboxysome; P, PHB granules; cw, cell wall. The asterisk and the arrowhead show the thylakoid membrane and a glycogen granule, respectively. Adapted from Klemenčič *et al.*, 2017.³¹

1.2 Terpenes and Terpenoids

Terpenes are unsaturated hydrocarbons that are made up of one or more isoprene units linked together. Terpenoids (also called isoprenoids) are a class of chemical compounds that include terpenes, as well as terpene

derivatives that contain oxygen.³² Both terpenes and terpenoids are divided into several subclasses depending on the number of isoprene units comprising the molecule (**Table 1**). There are at least 30,000 terpenes³³ and over 50,000 terpenoids that are naturally produced—terpenoids being the largest class of natural products.^{34,35} This incredible molecular diversity results from both the variety of terpene synthase enzymes that exist in nature and the fact that many individual terpene synthases can produce multiple products. Terpenoids are essential for myriad cellular processes including transcription, photosynthesis, intracellular signaling, electron transport, chemical defense secretions, and protein degradation. Furthermore, terpenoids are utilized by organisms across all kingdoms of life.³⁵ In other words, terpenoids are ubiquitous in nature and are of great importance.

Table 1 Terpenoid Classes

Class	Isoprene Units	Carbon Number	Examples
Hemiterpenoids	1	C ₅	Isoprene
Monoterpenoids	2	C ₁₀	Linalool, Pinene, Limonene
Sesquiterpenoids	3	C ₁₅	Patchoulol, Santalene, Humulene
Diterpenoids	4	C ₂₀	Phytol, Retinol, Taxadiene
Triterpenoids	6	C ₃₀	Cholesterol, Squalene
Tetraterpenoids	8	C ₄₀	Carotenoids
Polyterpenoids	<i>n</i>	C _{5<i>n</i>}	Natural Rubber

Many terpenoids have a low molecular weight and high vapor pressure, making them volatile and useful for extracellular communication. In humans, terpenoids are important for many physiological processes such as vitamin A and cholesterol biosynthesis. The importance of terpenoids in human evolution can also be exemplified by our ability to detect extremely minute concentrations of certain compounds. Geosmin, the recognizable

earthy smell of soil, is one of those compounds—humans are able to detect it at a concentration of just 1 part per trillion (ppt).^{36,37} Interestingly, the ability to detect geosmin is extremely widespread among organisms, suggesting that it provides a significant fitness advantage. A study on fruit flies (*Drosophila*) revealed that they have a specific olfactory circuit that is activated exclusively by geosmin and that they use this ability to avoid food that may be contaminated with bacteria that are harmful to them or their larvae.³⁸

1.2.1 Commercial Applications of Terpenoids

The list of terpenoids with fragrances recognizable to humans is long. Examples include pinene (pine, fir), limonene (lemon, citrus), nootkatone (grapefruit), patchoulol (patchouli), and geraniol (rose). Many pleasant-smelling terpenoids constitute major fractions of plant essential oils and are used in the perfume, cosmetic, and personal care industries. Interestingly, some enantiomers of the same terpenoid smell very different when compared to each other. An example of this is linalool, which has two enantiomers: (*R*)-linalool and (*S*)-linalool (both are found in nature).³⁶ (*R*)-linalool (also known as licareol) is described as having a woody, lavender-like aroma, while (*S*)-linalool (also known as coriandrol) has a sweet, floral, citrus-like aroma.³⁹ This difference in scent perception is problematic for the cosmetic and fragrance industries, which use synthetic linalool in 60-90% of products (e.g., shampoo, lotion, soap, and antiperspirant).⁴⁰ The problem is that synthetic linalool is usually produced as a racemic mixture (containing both enantiomers and therefore both scent profiles), giving the mixture a different smell compared to either individual enantiomer. While the extraction of either linalool enantiomer from plants would solve this problem, the synthetic chemical is much less expensive to produce. An alternative method for producing enantiopure linalool and other terpenoids is through the use of biotechnology and genetic engineering. Plants possess a

variety of enantiospecific terpene synthase enzymes that can be heterologously expressed in microorganisms that are easier to manipulate and have faster growth rates.

Outside of the cosmetic and fragrance industries, terpenoids have a wide variety of applications. Some terpenoids are promising biofuel candidates to replace petroleum-derived fuels, such as farnesene, which can be used as a renewable jet fuel.⁴¹ Terpenoids also constitute a vast library of bioactive chemical structures available to biochemists for drug discovery research.³⁴ A prominent example of a terpenoid drug is the antimalarial drug, artemisinin, which earned its discoverer a Nobel Prize. **Figure 6** shows examples of terpenoids and some of their commercial applications.

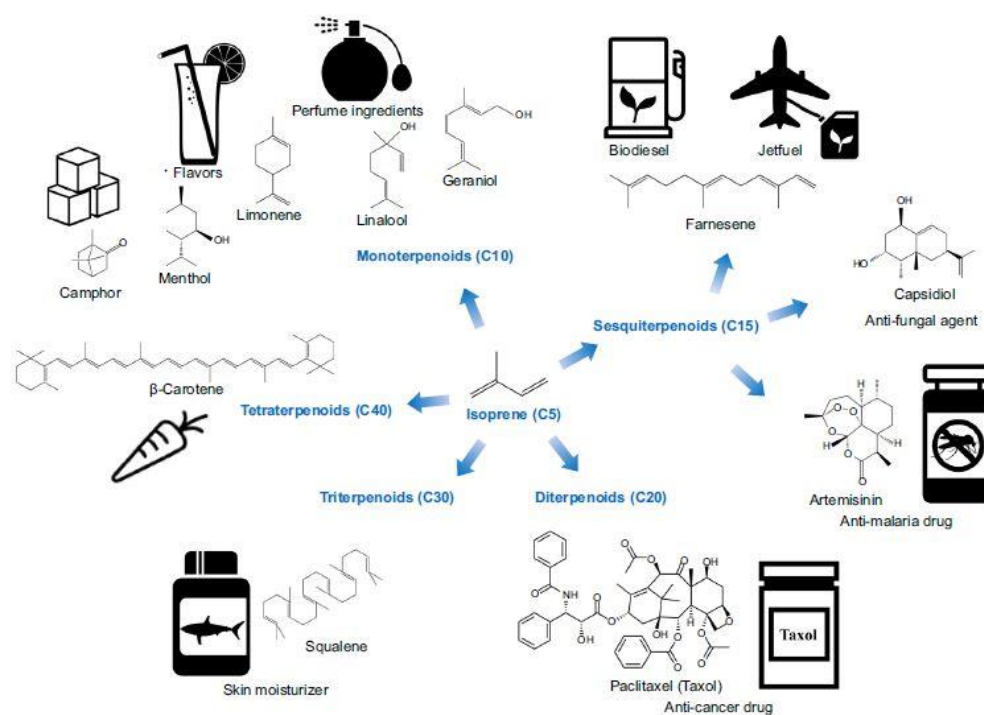


Figure 6 Examples of terpenoids and some of their commercial applications. Adapted from Zhang *et al.*, 2017.⁴²

1.2.2 Terpenoid Biosynthesis

There are two main metabolic pathways that organisms use to make terpenoids. The mevalonate (MVA) pathway is found in plants, animals, fungi,

archaea, and some bacteria. Whereas the methylerythritol phosphate (MEP) pathway is present in most bacteria (including cyanobacteria), plants, and the eukaryotic apicomplexa.³⁵ It is worth noting that plants possess both the MVA and MEP pathways and that they exist in physically separated regions of the cell; the MVA pathway is used in the cytosol and the MEP pathway in plastids.³⁵ This provides additional evidence for the endosymbiotic theory, i.e., photosynthetic plastids originating from cyanobacteria.

Both the MVA and MEP pathways produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) as five-carbon molecules for terpenoid biosynthesis (**Figure 7**). IPP and DMAPP are isomers of each other, and can be interconverted by the enzyme, isopentenyl-diphosphate delta-isomerase (IDI).³⁴ The precursors for the MEP pathway are glyceraldehyde 3-phosphate and pyruvate, which are joined together by the DXS enzyme to form 1-Deoxy-D-xylulose 5-phosphate (DXP), the first committed step in the pathway. An additional seven enzymes are then required to produce IPP and DMAPP (including the isomerase, IDI). To produce IPP and DMAPP through the mevalonate pathway, three molecules of acetyl-CoA are condensed to form HMG-CoA, and an additional five enzymes are required to convert HMG-CoA to IPP and DMAPP (including IDI). The subsequent condensation of DMAPP with either one or two molecules of IPP leads to the production of either monoterpenoids or sesquiterpenoids, respectively. Larger terpenoids (>C₁₅) are built up by additions of different combinations of IPP and DMAPP to farnesyl diphosphate (FPP).

1.3 Utilizing Cyanobacteria for Renewable Chemical Production

Cyanobacteria are promising host organisms for the production of renewable chemicals. They are genetically tractable, grow on carbon dioxide as their sole carbon source, can be cultivated using non-arable land, and grow much faster than terrestrial plants. Although genetic tools for

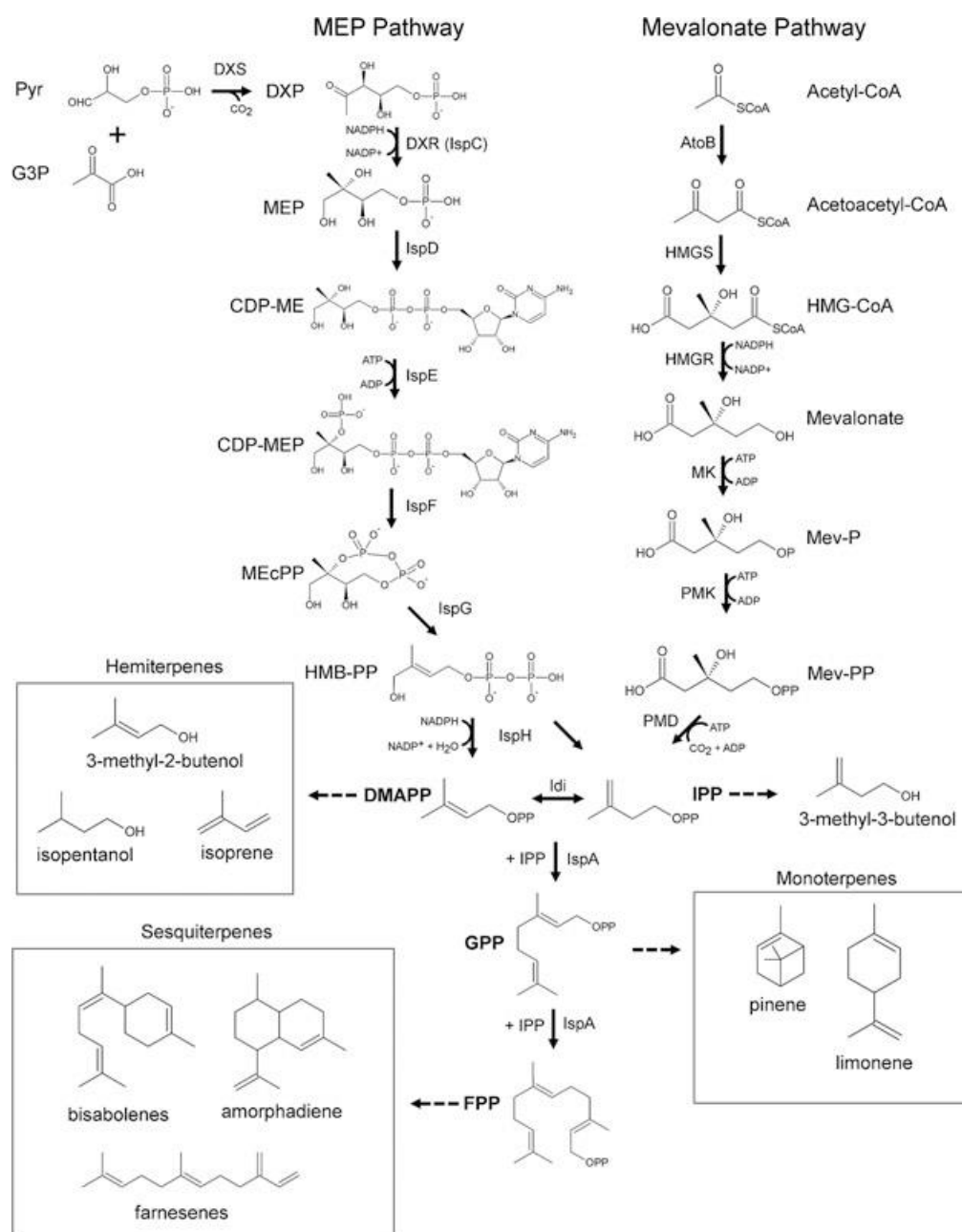


Figure 7 The two major terpenoid biosynthetic pathways, the methylerythritol phosphate (MEP) pathway and mevalonate (MVA) pathway. Both pathways produce IPP and DMAPP as five-carbon molecules for terpenoid biosynthesis. Adapted from George *et al.*, 2015.³⁴

cyanobacteria still lag behind those for heterotrophic model organisms such as yeast and *E. coli*,⁴³ significant progress has been made over the last two decades in characterizing genetic components for heterologous protein expression in cyanobacteria (e.g., promoters, ribosome binding sites, neutral sites, and homologous recombination plasmids).^{44–47} As an example, **Figure 8** shows how the pEERM vector chromosomal integration system works, which was used to generate some of the mutant strains in this work.

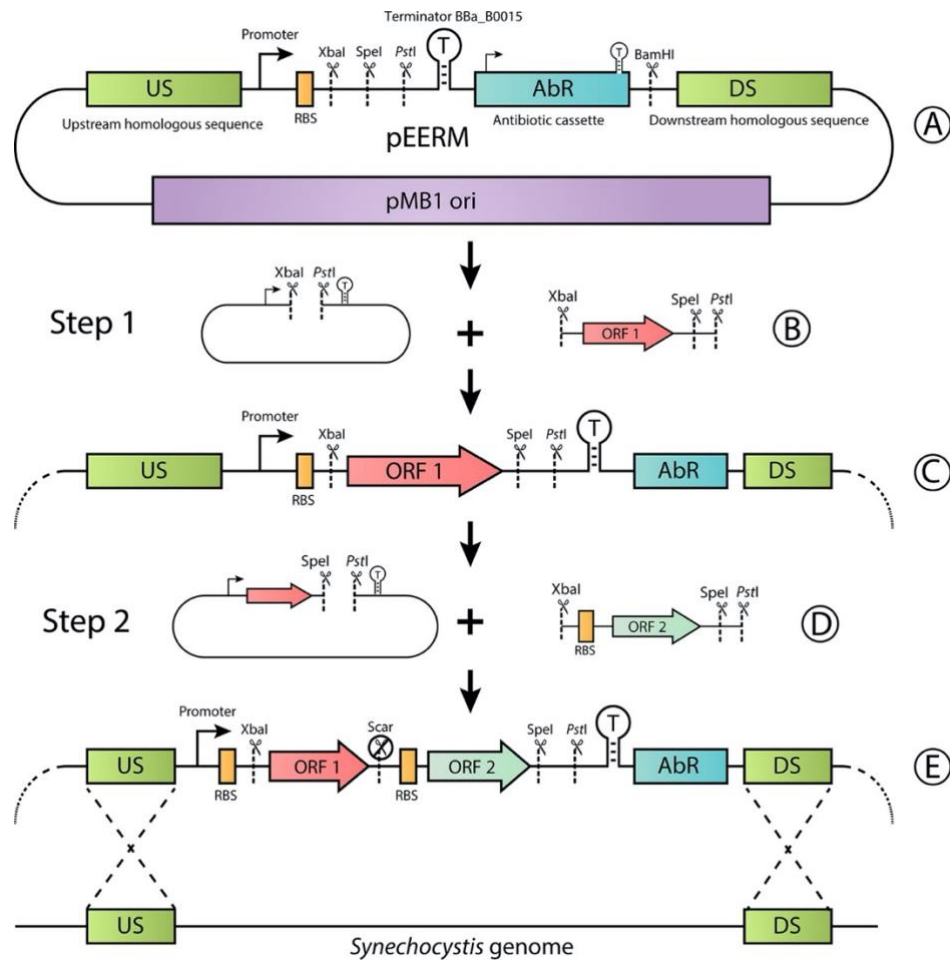


Figure 8 Schematic representation of homologous recombination in *Synechocystis* sp. 6803 using the pEERM vector system. When all genes have been inserted, the final construct can be directly inserted into the *Synechocystis* genome by homologous recombination. Adapted from Englund *et al.*, 2015.⁴⁴

By utilizing the potential of cyanobacteria, greenhouse gas emissions could be reduced by directly converting carbon dioxide to chemicals that can replace those currently derived from fossil fuels. In addition, cyanobacteria can be utilized to more sustainably produce chemicals that are derived from plants, such as patchoulol and artemisinin. Patchoulol and artemisinin are currently produced by extraction from their respective plants, *Pogostemon cablin* and *Artemisia annua*, which are mainly grown on plantations in southeast Asia and China.^{48,49} An alternative method for the production of traditionally plant-derived chemicals utilizing cyanobacteria leverages the higher photosynthetic efficiency of cyanobacteria versus traditional plants

(3–5% compared to < 0.25–3%),⁵⁰ as well as a much faster growth rate (*Synechocystis* cell doubling can occur in as little as 5.13 hours).⁵¹ These attributes of cyanobacteria speed up production because shorter periods of growth are required between harvests. In addition to the aforementioned advantages of using cyanobacteria to produce renewable chemicals, the case for producing certain compounds is even stronger in regions of the world, such as southeast Asia, where rainforest is destroyed to obtain arable land for the production of commodity chemicals such as natural rubber (polyisoprene).^{52,53} Furthermore, there are cyanobacteria that can fix their own nitrogen (see **1.1.2**), eliminating need to add nitrogen-based fertilizers which require vast amounts of energy to produce. As a final note, there are strains of cyanobacteria that are capable of growing mixotrophically (obtaining energy from both an organic carbon source and from photosynthesis), allowing for even higher chemical production rates in some cases, such as the production of isobutanol.⁵⁰

1.3.1 Cyanobacterial Terpenoid Production

Cyanobacteria naturally produce large quantities of terpenoids in the form of carotenoids and the phytol tail of chlorophyll, making them well suited for heterologous terpenoid production.⁵⁴ Recent concerns about climate change and an interest in green chemistry have driven research in the production of a wide variety of terpenoids using cyanobacteria, and there are many good review articles that have collated data from these studies.^{54–58} Of particular relevance for this thesis is a study reporting the production of 19.8 mg L⁻¹ of an artemisinin precursor, amorpha-4,11-diene, in a *Synechococcus* strain that was expressing three heterologous MEP pathway genes.⁵⁹ In that study, different combinations of heterologous MEP pathway genes were tested, and the top performing strain was used as the basis for the design of the heterologous MEP pathway in this work. Another important study for this work was the expression of a full heterologous mevalonate pathway in

Synechocystis with the goal of increasing isoprene production.⁶⁰ A major advantage of a fully heterologous mevalonate pathway in cyanobacteria is that there should not be any endogenous regulation for those genes in the host organism. This should make it possible to push more flux through the pathway to make IPP and DMAPP than may be possible using the native MEP pathway. Interestingly, the previously mentioned study only reported a 2.5-fold increase in isoprene for the strain expressing the mevalonate pathway compared to the strain containing only isoprene synthase.⁶⁰ This was a much smaller increase than the 23-fold increase reported for the top performing strain containing the three heterologous MEP pathway genes previously mentioned.⁵⁹

Though some studies have reported high cyanobacterial terpenoid yields of up to 1.26 g L⁻¹ (isoprene),⁶¹ production of commodity chemicals using cyanobacteria is generally not economical due to the low prices of chemicals derived from fossil fuels. For cyanobacterial terpenoid production to be economically viable in the current climate, products must have a sufficiently high value such that they can be produced at a lower price than the current method of production. Examples of such high-value compounds include pharmaceuticals, nutraceuticals, natural pigments, and flavor and fragrance compounds.

1.4 Objectives of this Work

The main goal of this work was to establish a cyanobacterial platform for the sustainable production of terpenoids. To achieve this, a heterologous mevalonate (MVA) pathway and an enhanced methylerythritol phosphate (MEP) pathway were designed for expression in both *Synechocystis* and *Synechococcus* to increase the production of terpenoid precursors available for heterologously expressed terpene synthases. Because the MEP and MVA pathways use different precursor molecules (MEP uses glyceraldehyde-3-phosphate (G3P) and pyruvate, MVA pathway uses acetyl-CoA), one goal of

this work was to create a strain containing both engineered pathways to see how much terpenoid yields would increase compared to strains possessing only one of the engineered pathways.

Another goal of this work was to show the production of non-native terpenoids that have not previously been produced in cyanobacteria as a proof of concept through the heterologous expression of terpene synthase enzymes. By measuring production of the target compounds, strains containing different engineered terpenoid pathways (e.g., MVA, MEP, MVA + MEP) could be assessed under different growth conditions. The ultimate goal of this was to better understand how terpenoid yields change as different pathways are overexpressed and which strategies are the most promising to pursue in future research.

1.4.1 Heterologous Mevalonate Pathway

Since cyanobacteria do not possess the mevalonate pathway, a complete heterologous pathway needed to be designed. This has been done before in *Synechocystis*,⁶⁰ but it only improved product yield by 2.5-fold. In that work, six heterologous mevalonate pathway genes needed to be expressed to produce the final isopentyl diphosphate (IPP). In this work however, a complete mevalonate pathway was designed that only required five heterologous genes to be expressed. This was possible because the *mvaE* gene from *Enterococcus faecalis* (Genbank acc. No. AF290092) is a bifunctional enzyme capable of performing both the first and third steps in the mevalonate pathway (1: joining two acetyl-CoA molecules to form acetoacetyl-CoA; 2: reducing HMG-CoA to mevalonate).⁶² By simplifying the heterologous pathway and choosing different mevalonate pathway enzymes, a goal of this work was to see if this new mevalonate pathway would give a greater fold increase in terpenoid production compared to the previous work mentioned.

1.4.2 Enhanced MEP Pathway

As previously mentioned, cyanobacteria produce terpenoids using an endogenous MEP pathway. To increase carbon flux through the MEP pathway and ultimately increase the production of terpenoid products, an enhanced MEP pathway was designed for expression in *Synechocystis* and *Synechococcus* modeled after work done in *Synechococcus* by Choi *et al.*⁵⁹ The initial plan was to obtain the assembled MEP pathway directly from Choi *et al.* and build constructs to express the pathway in both strains. However, no reply was ever received from the research group, so a similar pathway needed to be designed from the bottom up for expression in both cyanobacteria strains.

1.4.3 Terpenoid Targets for Cyanobacterial Production

A number of terpenoids were identified as candidates for cyanobacterial production. For each of the target chemicals chosen, a description is given below.

1.4.3.1 Linalool and Geraniol

Linalool and geraniol are both monoterpenoids (C₁₀), which are derived from a geranyl diphosphate (GPP) precursor (geranyl diphosphate is synthesized by the condensation of one molecule isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by the enzyme *ispA*). Geraniol is widely used as a fragrance in consumer products, including soaps and perfumes. It has a floral and recognizable rose-like odor and is a key component of the essential oils of rose, palmarosa, and citronella.⁶³ Linalool is another frequently used compound in the flavor and fragrance industry and has already been described in some detail earlier in the introduction (1.2.1). Although linalool and geraniol have both been produced in

microorganisms previously,^{64,65} neither has been produced in a photosynthetic microbe, which would offer a route of synthesis directly from CO₂.

1.4.3.2 Patchoulol and Santalene

Patchoulol and santalene are sesquiterpenoids (C₁₅) that are both produced from farnesyl diphosphate (FPP) by their respective terpene synthases and are important compounds in the cosmetic and fragrance industries. Patchoulol has a strong woody, patchouli-like odor and is the main component of patchouli essential oil, which is extracted from the patchouli plant, *Pogostemon cablin*.^{48,66} Santalene has a different wood-like odor, smelling of sandalwood, and is a component of sandalwood essential oil, extracted from the heartwood of multiple trees in the genus *Santalum*.⁶⁷

1.4.3.3 Artemisinin

The sesquiterpenoid (C₁₅), artemisinin, is a potent antimalarial drug that was discovered in 1972 by the Chinese researcher, You-You Tu, which earned her the 2015 Nobel prize. Currently, artemisinin is the preferred drug for treating malaria, a disease that sickens over 200 million people each year and caused an estimated 435,000 deaths in 2017.⁶⁸ Artemisinin is commercially harvested from the plant, *Artemisia annua* (also called sweet wormwood), which only contains 0.01–1.4% of the drug by dry weight.⁶⁹ This low productivity has resulted in a short supply of artemisinin during some years, driving up the price. Due to the issues of availability and price volatility for one of the most globally important drugs, there has been significant international effort to find a more reliable production method.⁶⁹

In one of the greatest synthetic biology success stories to date, scientists were able to discover all of the native plant genes required to produce an artemisinin precursor, artemisinic acid, and were able to engineer

yeast to produce it with titers of 25 g L⁻¹.⁷⁰ This fantastic work has recently been taken up by industry for large-scale production. However, this process still has some significant drawbacks, the main one being the need to convert artemisinic acid to artemisinin by a reaction with singlet oxygen.⁷⁰ The production of singlet oxygen required for artemisinin synthesis is generally produced by ultra violet (UV) light.^{69,71} In vitro this requires specialized photochemical equipment, but conversion in plants happens naturally and only requires growth under sunlight. In fact, it has been shown that short-term exposures of both UV-B and UV-C light (UV-B light ranges from 280–320 nm and UV-C ranges from 200–280 nm)⁷² were able to increase artemisinin yields in *A. annua* by 10.5% and 15.7%, respectively.⁷³ Based on the natural production of artemisinin in *A. annua* grown under sunlight, one hypothesis presented in this thesis is that a cyanobacterium that is expressing all of the relevant artemisinin biosynthetic genes and being grown under natural sunlight should be able to produce artemisinin in vivo directly from CO₂. This hypothesis is supported by examples of artemisinin production in engineered terrestrial plants other than *A. annua*.⁶⁹ An advantage of in vivo production of artemisinin is that it eliminates the need to purify artemisinic acid and convert it to artemisinin in vitro—something that has not yet been achieved in any model heterotroph.

Although artemisinin has been produced in small quantities in some terrestrial plants, such as tobacco,⁶⁹ this would constitute the first direct production of artemisinin in a microorganism and would provide all of the benefits of cyanobacterial production over plant production previously described (e.g., faster growth rate and higher photosynthetic efficiency). In order to produce artemisinin in cyanobacteria, the same biosynthetic genes could be used as for the production in yeast,⁷⁰ with the addition of one gene, *dbr2*, that is essential for the production of artemisinin in plants.⁶⁹ The *dbr2* gene is responsible for producing an intermediate compound, dihydroartemisinic aldehyde, which is subsequently converted to dihydroartemisinic acid by *aldh1* and then spontaneously converted to artemisinin in the presence of UV light and oxygen (**Figure 9**).

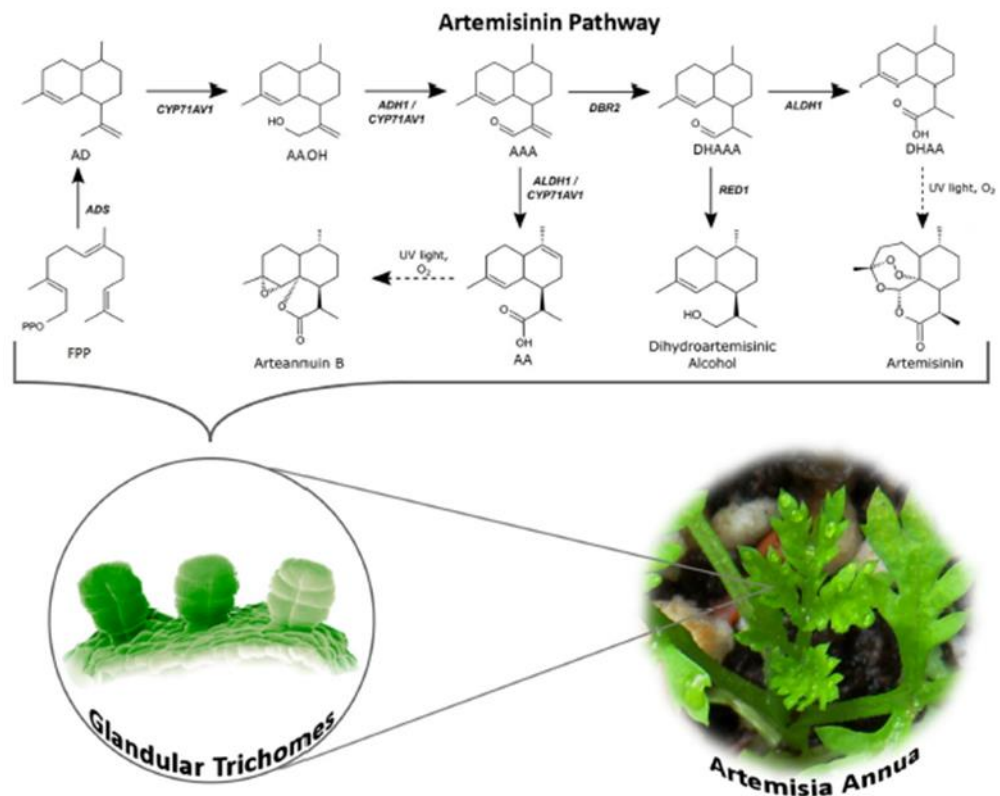


Figure 9 Artemisinin biosynthesis pathway. Biosynthesis occurs in the glandular trichomes of *Artemisia annua*. Pathway intermediates are defined as FPP, farnesyl diphosphate; AD, amorpha-4,11-diene; AAOH, artemisinic alcohol; AAA, artemisinic aldehyde; AA, artemisinic acid; DHAAA, dihydroartemisinic aldehyde; DHAA, dihydroartemisinic acid. Adapted from Ikram and Simonsen, 2017.⁶⁹

2 Materials and Methods

2.1 Chemicals

Except where otherwise stated, all chemicals used in this study were Purchased from Sigma-Aldrich.

2.2 DNA Oligonucleotides (Primers used)

Table 2 Primers designed and used in this study.

Primer Name	Sequence (5'→3')
6803AaLINS_F	CAAATACATTAGTGGAGGTTATGGCCAGTTTCAACCGG
6803AaLINS_R	TGCAGCGGCCGCTACTAGTTTTAACTGGAGGAATCATACAA CATG
6803CtGES_F	CAAATACATTAGTGGAGGTTATGGCCTTGCAAATGATCGC
6803CtGES_R	TGCAGCGGCCGCTACTAGTTTTAAGCACTGCCGCCGTC
6803PATS_F	CAAATACATTAGTGGAGGTTATGGAATTATATGCCCAATC
6803PATS_R	TGCAGCGGCCGCTACTAGTTTTAATAAGGCACGGGATG
6803psbA2_F	CAGTTGTGTCGCCCCTCTAC
6803psbA2_R	CAAATCTAACGAACCGCCCC
6803SANS_F	CAAATACATTAGTGGAGGTTATGGATTCCAGTACCGCC
6803SANS_R	TGCAGCGGCCGCTACTAGTTTTATTCTTCACCCAAGGGAATG
7942OBGES_F	CTCATCGCAAAGGTACCACAATGAGTTGCGCCCGGATTAC
7942OBGES_R	GAAGGTGAGCCGAGCTCTCATTATTGGGTAAAGAACAAGGC GTC
DOWNMEP68_F	GAACAAATAACGAGCGCAAACCTACAATG
DOWNMEP68_R	ACGCGTTGGGAGCTCTCCCATGAATTTGTATTGGGCGAC
DOWNMEP79_F	TCAGCGGAACAAATAATTTGCTCGAGTCCCTGCTCGTC

DOWNMEP79_R	GAATACTCAAGCTATGCAGGGCAGGTCGTTCAAAGACTG
DXS68_F	GCCGCGGGAATTCGATATCAGACGTCTAAGAAACCATTATT ATCATG
DXS68_R	TGCAGGCGGCCGCGAATTCATTAGGCTAACCAGGCCTTAAT C
DXS79_F	TTCGCGGCCGCTGCAGGTCGACCAGACGTCTAAGAAACCA TTATTATCATG
DXS79_R	ATCCAACGCGTTGGGAGCTCTCCATTAGGCTAACCAGGCC TTAATC
IDI68_F	TGAATTCGCGGCCGCTGCATAGAAAAGAGGAGAAATACTA GATGCAGACAGAACACGTC
IDI68_R	TCCCATATGGTCGACCTGCATTATTTTAGCTGGGTAAAGGC
IDI79_F	CTCCCAACGCGTTGGATGCATAGAAAAGAGGAGAAATACTA GATGCAGACAGAACACGTC
IDI79_R	TAGAATACTCAAGCTATGCAGGATCCTTATTTTAGCTGGGTA AAGGC
ISPA68_F	AAAATAATGCAGGTCGACCATAGAAAAGAGGAGAAATACT AGATGGATTTTCCCCAGCAAC
ISPA68_R	TTTGCCTCGTTATTTGTTCCGCTGAATGATG
ISPA79_F	CTTACCCAGCTAAAATAAGGTAGAAAAGAGGAGAAATACT AGATGGATTTTCCCCAGCAAC
ISPA79_R	AGAATACTCAAGCTATGCAGGGATCCAAATTATTTGTTCCGC TGAATGATG
LACIAUG_F	TTCAGGGTGGTGAATATGAAACCAGTAACGT
LACIAUG_R	ACGTTACTGGTTTCATATTCACCACCCTGAA
MEP5_F	GGCGAGATCACCAAGGTAGTCGGCAAATAGCACCATCGAAT GGCGCAAAAC
MEP6_R	TGTCATGATAATAATGGTTTCTTAGACGTCTCACTGCCCCGCT TTCCAG

MEPHiFi168_F	GAGTACATGCTCCCGGCCGCGGCTGACGCCGTTGGATAC
MEPHiFi168_R	TATCGAATTCCCGCGGCCGCTCACTGCCCGCTTTCCAG
MEPHiFi179_F	AGAAGCATGCTCCCGGCCGCGGCTGACGCCGTTGGATAC
MEPHiFi179_R	TATCGAATTCCCGCGGCCGCTCACTGCCCGCTTTCCAG
SeDBR2_1F	GGTACCACATATGAGAGC
SeDBR2_2R	TTTGCGATGAGTCCTTAG
SeDBR2_3F	TAAATAGTTCAACTAAGGACTCATCGCAAATGAGTGAGAA ACCGACC
SeDBR2_4R	AAGGTGAGCCGAGCTCTCATATGTGGTACCTTACAACAAAG ATCCCTTG
SsDBR2_1F	TGGAGGTTCTAGAACTAG
SsDBR2_2R	CTAATGTATTTGTCGATGTTC
SsDBR2_3F	TTCCAATCTGAACATCGACAAATACATTAGATGAGTGAGAA ACCGACC
SsDBR2_4R	CAGCGGCCGCTACTAGTTCTAGAACCTCCATTACAACAAAGA TCCCTTG
UPMEP68_F	ATTGGGCCCCGACGTCGCATGCACGGAGTCTAACTATTTAC
UPMEP68_R	CCATGGCGGCCGGGAGCATGTACTCAGCCTTCTAATGC
UPMEP79_F	ATTGGGCCCCGACGTCGCATGTGGCAGCTTAGTCCTGCG
UPMEP79_R	CCATGGCGGCCGGGAGCATGCTTCTGGAGCAGGAAGATGT C

2.3 Bacterial Strains and Growth Conditions

2.3.1 *E. coli* Strains and Growth Conditions

All *E. coli* strains used in this study are listed in **Table 3**. Cultures were grown at 37 °C with shaking at 225 rpm in Lysogeny Broth (LB) medium (purchased from Sigma-Aldrich) which was dissolved in deionized water (25 g

L⁻¹) and consisted of 5 g L⁻¹ yeast extract, 10 g L⁻¹ NaCl, and 10 g L⁻¹ tryptone. The media was autoclaved to sterilize, and 15 g L⁻¹ agar was added before autoclaving when solid medium was required. When antibiotic selection was required, LB medium was supplemented with the appropriate antibiotic at concentrations detailed in **Table 5**.

Table 3 *E. coli* strains used in this study

<i>E. coli</i> Strain	Genotype	Source
DH5α	<i>fhuA2 Δ(argF-lacZ)U169 phoA glnV44</i>	New England
	<i>Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1</i>	Biolabs, Ipswich,
	<i>endA1 thi-1 hsdR17</i>	MA
10-β	<i>Δ(ara-leu) 7697 araD139 fhuA ΔlacX74</i>	New England
	<i>galK16 galE15 e14- φ80dlacZΔM15</i>	Biolabs, Ipswich,
	<i>recA1 relA1 endA1 nupG rpsL (Str^R) rph</i>	MA
	<i>spoT1 Δ(mrr-hsdRMS-mcrBC)</i>	
MG1655	K-12 F ⁻ λ ⁻ <i>ilvG⁻ rfb-50 rph-1</i>	Dr. Samantha Bryan

2.3.2 Cyanobacteria Strains and General Growth Conditions

A glucose-tolerant (GT) strain of *Synechocystis* sp. PCC 6803 was kindly provided by Dr. Carrie Eckert and was used as the wild-type strain for this study. *Synechococcus elongatus* PCC 7942 was purchased from the Pasteur culture collection. Cyanobacterial cultures were grown in BG-11 medium that was supplemented with 10mM MOPS buffer and brought to a starting pH of 7.6. Chemical composition of the BG-11 medium is given in **Table 4**. Solid medium was obtained by the addition of 15 g L⁻¹ agar before autoclaving. For general growth of cultures and during transformation experiments, cultures were incubated at 30 °C and irradiated with LED white

light at $20 \text{ E m}^{-2} \text{ s}^{-1}$ (Einstein is one mole of photons). The concentrations of antibiotics used for selection are listed in **Table 5**.

Table 4 Composition of BG-11 medium

Component	Amount (per Liter)
NaNO_3	1.5 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.075 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.036 g
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	0.04 g
Trace Elements (1000x)	1 mL

Trace Elements (1000x)	Amount (per Liter)
Citric Acid	6 g
Ferric Ammonium Citrate	6 g
H_3BO_3	2.86 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.81 g
$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	1 g
$\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$	0.391 g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.222 g
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.079 g
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	0.049 g

Note: pH was adjusted to 7.6 prior to filter sterilization.

2.3.3 Antibiotic Concentrations

The concentrations of all antibiotics used in this study are listed in **Table 5**.

Table 5 Concentrations of Antibiotics used in this study

Antibiotic	<i>E. coli</i>	Cyanobacteria
Kanamycin	50 µg/mL	50 µg/mL
Spectinomycin	50 µg/mL	50 µg/mL
Chloramphenicol	25 µg/mL	-
Carbenicillin	100 µg/mL	-
Ampicillin	100 µg/mL	-
Hygromycin	100 µg/mL	-

2.3.4 Growth Conditions for Patchoulol Production

2.3.4.1 Photoautotrophic and Mixotrophic Growth

Cultures (70 mL) were grown in triplicate for the production of patchoulol in BG-11 medium supplemented with 10 mM MOPS buffer (starting pH = 7.6), and PATS cultures were also supplemented with 50 µg mL⁻¹ kanamycin. Cultures were grown in a Multicultivator 1000-OD (Photon Systems Instruments) photobioreactor with 0.4% CO₂ mixed with air bubbling through the culture. Photoautotrophic cultures were grown with CO₂ as their only carbon source, while mixotrophic cultures were supplemented with 10 g L⁻¹ glucose. For mixotrophic cultures, 1 mL culture samples were taken daily to track glucose consumption. Cultures were irradiated with LED white light at 100 µE m⁻² s⁻¹, and a biphasic solvent system was employed to capture the patchoulol produced by adding a 10% (v/v) overlay of n-dodecane (Acros Organics) to each culture. Optical density measurements (OD₇₅₀) were taken daily to track cell growth, and 100 µL of the dodecane overlay was sampled each day and analyzed by GC-MS at the end of the experiment for patchoulol quantification. At two time points (early and late), 2 mL of each triplicate culture was sampled for chlorophyll and carotenoid quantification.

2.3.4.2 Growth on Bicarbonate

Triplicate cultures (50 mL) were grown in 250 mL unbaffled shake flasks in BG-11 medium supplemented with 30 mM TES buffer and 50 mM sodium bicarbonate at a starting pH of 7.6. Each day, 2.5 mL of filter-sterilized 1 M NaHCO₃ in 1x BG-11 was added to each triplicate culture. To counteract the drastic pH increase resulting from growth on bicarbonate, cultures needed to have the pH adjusted to ~8 (every 8 hours for cultures grown under high light and approximately every 12 hours for cultures grown under low light) by adding concentrated HCl dropwise. If this was not done, the pH of the culture would rise above 11 and the cultures would flocculate. Cultures were grown in a reach-in lighted incubator with shaking at 100 rpm. PATS cultures were supplemented with 50 µg mL⁻¹ kanamycin. Cultures were irradiated with LED white light at either 20 µE m⁻² s⁻¹ (low light), or 100 µE m⁻² s⁻¹ (high light). To capture the patchoulol produced, a biphasic solvent system was employed by adding a 10% (v/v) overlay of n-dodecane (Acros Organics) to each culture. OD₇₅₀ measurements were taken daily to track cell growth, and 100 µL of the dodecane overlay was sampled each day and analyzed via GC-MS at the end of the experiment. At two time points (early and late), 2 mL of each triplicate culture was sampled for chlorophyll and carotenoid analysis.

2.4 Molecular Biology Procedures

2.4.1 Plasmid DNA Extraction and Purification from *E. coli*

Plasmid DNA was extracted from *E. coli* cultures using either a Qiagen Plasmid Miniprep kit or a New England Biolabs Monarch Plasmid Miniprep kit by following the manufacturer's instructions. Plasmid DNA was quantified using a µLITE spectrophotometer (BioDrop).

2.4.2 Cyanobacteria Genomic DNA Extraction and Purification

Cyanobacterial genomic DNA was extracted and purified using a *Quick-DNA Fungal/Bacterial MiniPrep Kit* (Zymo Research, Catalog No. D6005) and the manufacturer's instructions were followed. Extracted genomic DNA was then quantified using a μ LITE spectrophotometer (BioDrop).

2.4.3 DNA Quantification

DNA was quantified using a μ LITE spectrophotometer (BioDrop) by measuring absorbance at 260 nm. The spectrophotometer was first blanked with 1.5 μ L of water or elution buffer (depending on what the DNA being quantified was eluted in). A sample (1.5 μ L) was loaded onto the spectrophotometer and absorbance was measured at 230 nm, 260 nm, and 280 nm. The peak absorbance for DNA and proteins is 260 nm and 280 nm, respectively. Absorbance measurements were taken at 280 nm in order to determine if there was any protein contamination of the DNA by calculating the 260/280 absorbance ratio. A 260/280 ratio of >1.8 is generally considered acceptable and without significant protein contamination. The 230 nm absorbance was measured in order to determine if any other contaminants were present in the DNA extract, particularly guanidine that may have carried over from the column purification. A 260/230 ratio of >2 typically means there is very little to no guanidine contamination present in the DNA. DNA was only used for downstream applications if the 260/280 ratio was greater than 1.8 and the 260/230 ratio was above 2.

2.4.4 Polymerase Chain Reaction (PCR)

All Polymerase Chain Reactions (PCR) were performed in Sapphire 0.2 mL flat cap PCR tubes using a Biometra Trio (Analytik Jena, Jena, Germany)

thermocycler. Amplified DNA was then analyzed via agarose gel electrophoresis.

2.4.4.1 High-Fidelity PCR for Cloning Experiments

Q5 DNA polymerase (New England Biolabs) was used to amplify DNA for cloning experiments. **Table 6** shows the components of a typical PCR reaction using Q5, and **Table 7** shows the thermocycler conditions used to amplify DNA fragments. The Q5 polymerase was chosen for cloning

Table 6 Composition of a Q5 PCR Reaction

Component	25 μ L Reaction	50 μ L Reaction
Q5 DNA Polymerase Master Mix (2X)	12.5 μ L	25 μ L
Forward Primer (10 μ M)	1.25 μ L	2.5 μ L
Reverse Primer (10 μ M)	1.25 μ L	2.5 μ L
DNA Template (1-50 ng/ μ L)	1 μ L	1 μ L
Nuclease-Free Water	9 μ L	19 μ L
Total Reaction Volume	25 μ L	50 μ L

Table 7 Q5 PCR Thermocycler conditions

Step	Temp. ($^{\circ}$ C)	Duration
Initial Denaturation	98	1 min.
30-35 cycles	Denaturation	98
	Annealing	45-72
	Extension	72
Final Extension	72	30 sec./kb + 2 min.
Hold	4	∞

experiments because it is a high-fidelity enzyme that has 3'→5' exonuclease (proofreading) activity and is therefore less prone to introducing mutations during amplification compared to DNA polymerases without proofreading activity.

Colony PCR was performed to screen for successfully assembled plasmids from cloning experiments. To screen for successful transformants, a single *E. coli* colony was picked using a pipet tip and was patched onto a fresh agar plate containing the same antibiotic used during the transformation. The pipet tip that was used to pick the colony was then used as the DNA template for a PCR reaction and was placed in a PCR tube that contained all of the remaining components for the reaction. This method of colony PCR was used to speed up the cloning process by simultaneously patching the cells to obtain more biomass and performing a colony PCR for the same colony picked.

2.4.4.2 Colony PCR for Screening *E. coli* Cloning Experiments

GoTaq DNA polymerase (Promega) was used to amplify DNA from single colonies for screening cloning experiments. **Table 8** shows the components of a typical colony PCR reaction using GoTaq, and **Table 9** shows the thermocycler conditions used. Colonies with the correct sized band

Table 8 Composition of a GoTaq Colony PCR Reaction

Component	25 μ L Reaction
GoTaq DNA Polymerase Master Mix (2X)	12.5 μ L
Forward Primer (10 μ M)	1.25 μ L
Reverse Primer (10 μ M)	1.25 μ L
<i>E. coli</i> cells (DNA template)	-
Nuclease-Free Water	10 μ L
Total Reaction Volume	25 μ L

following PCR screening were then grown up in 10mL overnight cultures in LB medium with the appropriate antibiotic, plasmid DNA was extracted, and the DNA was sent to Eurofins Genomics for sequencing to confirm that everything was assembled correctly and that no undesired mutations were present.

Table 9 GoTaq Colony PCR Thermocycler conditions

Step		Temp. (°C)	Duration
Initial Denaturation		94	10 min.
30-35 cycles	Denaturation	94	30 sec.
	Annealing	45-72	30 sec.
	Extension	72	1 min./kb
Final Extension		72	1 min./kb + 3 min.
Hold		4	∞

2.4.4.3 Cyanobacteria Screening PCR

Cyanobacterial colonies isolated following transformation were picked using a sterile loop and were grown up in 5 mL of BG-11 with antibiotic and irradiated with $20 \mu\text{E m}^{-2} \text{s}^{-1}$ white light. Cultures were then screened for successful transformation by transferring 50 μL of culture to thin-walled PCR tubes and heating at 99 °C for 10 minutes in a thermocycler. The boiled cultures were then centrifuged to remove whole cells and cellular debris, and 1 μL of the supernatant was used as the DNA template for PCR reactions. GoTaq DNA polymerase (Promega) was used for the screening PCRs, and the reaction mixture is listed in **Table 10**. The Thermocycler conditions used were the same as those used for *E. coli* colony PCRs, but the initial denaturation step was only 2 minutes instead of 10 minutes.

Table 10 Composition of a GoTaq Cyanobacteria Screening PCR Reaction

Component	25 μ L Reaction
GoTaq DNA Polymerase Master Mix (2X)	12.5 μ L
Forward Primer (10 μ M)	1.25 μ L
Reverse Primer (10 μ M)	1.25 μ L
Cyanobacteria lysate (DNA template)	1 μ L
Nuclease-Free Water	9 μ L
Total Reaction Volume	25 μ L

2.4.4.4 *Pfu* DNA Polymerase PCR

Pfu DNA polymerase (Promega) was used to amplify DNA for site-directed mutagenesis. Components of the PCR reaction are listed in **Table 11**, and the thermocycler conditions used are shown in **Table 12**.

Table 11 Composition of *Pfu* PCR Reaction

Component	50 μ L Reaction
<i>Pfu</i> Reaction Buffer with MgSO ₄ (10X)	5 μ L
Forward Primer (10 μ M)	1.25 μ L
Reverse Primer (10 μ M)	1.25 μ L
dNTP mix (10 mM each)	1 μ L
DNA template (100 ng/ μ L)	1 μ L
<i>Pfu</i> DNA Polymerase	1 μ L
Nuclease-Free Water	39.5 μ L
Total Reaction Volume	50 μ L

Table 12 *Pfu* PCR Thermocycler conditions

Step		Temp. (°C)	Duration
Initial Denaturation		95	1 min.
30-35 cycles	Denaturation	95	30 sec.
	Annealing	45-72	30 sec.
	Extension	72	2 min./kb
Final Extension		72	2 min./kb + 3 min.
Hold		4	∞

2.4.5 Agarose Gel Electrophoresis

DNA was separated via agarose gel electrophoresis using 1% agarose in TAE buffer with SYBR Safe dye (ThermoFisher). Agarose gels were prepared by mixing 1 g of technical grade agarose (Sigma-Aldrich) in 100 mL TAE buffer (1X) (40 mM Tris base; 0.1% (v/v) glacial acetic acid; 1 mM EDTA) and melting the mixture at high heat in a microwave. SYBR Safe DNA stain (Thermo-Fisher Scientific) was added at a final concentration of 0.1 µL/mL to visualize DNA bands on the gel. DNA samples that did not already contain a loading dye buffer were supplemented with Purple Gel Loading Dye (6X) (New England Biolabs). The gel was run in 1X TAE buffer at 120 V (<200 mA) for 20-40 minutes and was visualized using a UV transilluminator (Gel Doc). A DNA ladder (NEB 1kb Plus) was run alongside samples to estimate the size of DNA bands.

2.4.6 Agarose Gel DNA Extraction

DNA bands were extracted and purified from agarose gels by excising the bands with a scalpel under UV light on a transilluminator and then purifying them using a QIAquick Gel Extraction Kit (Qiagen). The protocol

used for purification of the DNA was slightly modified from the manufacturer's instructions and is detailed below. First, DNA bands excised from the agarose gel were placed in 1.5 mL microcentrifuge tubes (Eppendorf). Instead of weighing each gel slice and adding the appropriate amount of QG buffer, 400 μ L of QG buffer was added to all excised bands. Eppendorf tubes were then incubated at 50 °C until the agarose was completely dissolved, and the protocol of the manufacturer was then followed, with the addition of two extra wash steps with PE buffer at the end prior to elution in water or the provided elution buffer. The extra steps were included because the 260/230 ratio of the DNA was poor if only one PE buffer wash was performed but improved following additional washes. DNA was eluted in 7-15 μ L of nuclease-free water depending on the concentration of DNA required and the amount of DNA initially loaded in the gel. Purified DNA was then quantified using a μ LITE spectrophotometer (BioDrop).

2.4.7 Restriction Endonuclease Digestion of DNA

All Restriction enzymes used were from the FastDigest line from Thermo-Fisher Scientific and were used with the 10X Green Buffer. Restriction digestion reactions were carried out in 0.2 mL PCR tubes and were incubated at 37 °C for 1-3 hours. One microliter of FastAP Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific) was added to remove 5' phosphate groups from digested DNA in order to prevent ligation of the plasmid backbone to itself during subsequent DNA assembly procedures. The components of a typical restriction digestion reaction are presented in **Table 13**. After the digestion reaction was complete, the DNA was analysed via agarose gel electrophoresis and was purified by gel extraction.

Table 13 Composition of a DNA Restriction Endonuclease Reaction

Component	Single Digestion	Double Digestion
Green Buffer (10X)	3 µL	3 µL
Restriction Enzyme 1	2 µL	1 µL
Restriction Enzyme 2	-	1 µL
Alkaline Phosphatase	1 µL	1 µL
DNA	1-3 µg	1-2 µg
Nuclease-Free Water	To 30 µL	To 30 µL
Total Reaction Volume	30 µL	30 µL

Note: Double digestion reactions were scaled up and 2 µL of each restriction enzyme was used if more DNA was required.

2.4.8 Preparation of *E. coli* Chemically Competent Cells

Chemically competent *E. coli* DH5α cells were prepared in-house for transformations with plasmids that did not require the high level of competency that NEB chemically competent cells provide. The competent cells were prepared by streaking out a frozen glycerol stock of *E. coli* DH5α onto an LB plate and growing it overnight at 37 °C. A single colony of *E. coli* was used to inoculate a 10 mL overnight culture in LB medium with shaking at 37 °C. The following day, 100 mL of LB media was inoculated with 1 mL of the starter culture and was again grown at 37 °C with shaking. When the OD₆₀₀ reached 0.35-0.4, the cells were put on ice for 30 min. The 100 mL culture was then divided into four by pouring 25 mL of the culture into four ice-cold 50 mL falcon tubes. The cells were then pelleted by centrifugation at 3000 g for 15 minutes at 4 °C, the supernatant was discarded, and the pellets were resuspended in 10 mL of ice-cold 100 mM MgCl₂. The cells were pelleted again by centrifugation at 2000 g for 15 minutes at 4 °C. The supernatant was again discarded, and the pellets were resuspended in 20 mL of ice-cold 100 mM CaCl₂. The cells were kept on ice for 20 minutes and then were centrifuged at 2000 g for 15 minutes at 4 °C. The supernatant was

discarded, and the pellets were resuspended in 2 mL of an ice-cold solution of 85 mM CaCl_2 with 15% glycerol by volume. The competent cells were then divided into 50 μL aliquots in sterile 1.5 mL microcentrifuge tubes and were stored at -80°C until needed.

2.4.9 *E. coli* Transformation

The same transformation protocol was used to transform both the in-house prepared chemically competent *E. coli* cells and commercially obtained chemically competent NEB DH5 α and 10- β cells (with the exception of a different outgrowth medium being used for the NEB 10- β cells). Chemically competent cells were taken from the -80°C freezer and were allowed to thaw on ice. While working under a flame, DNA was added to the cells and the tube was flicked a few times to mix. Less DNA was added when transforming already assembled plasmids ($<100\text{ ng}$), whereas more DNA was used (5 μL of in vitro plasmid assembly reaction mixture per 50 μL of cells) when the amount of correctly assembled plasmid was unknown, as is the case for HiFi assembly and site-directed mutagenesis. After the DNA was added, the cells were incubated on ice for 30 minutes. After 30 minutes, the cells were heat shocked by placing them in a 42°C water bath for 30 seconds. The cells were then allowed to recover on ice for 5 minutes. Outgrowth medium (950 mL) was then added to the cells (LB SOC medium for in-house and NEB DH5 α cells, and 10- β outgrowth medium for NEB 10- β cells), and they were placed in a 37°C shaking incubator for 1 hour. After an hour of outgrowth, 100 μL of the in-house prepared cells were plated onto a prewarmed LB plate with the appropriate antibiotic, and the plate was placed in a 37°C incubator and left overnight until colonies appeared. For the NEB cells which were transformed with an unknown amount of correctly assembled plasmid, all of the cells were plated out in case the assembly had a low efficiency. To accomplish this, the NEB cells were first pelleted by centrifugation at 13400 g for one minute. The majority of the supernatant

was then removed, and the cells were resuspended in the remaining ~100 μL of supernatant. The cell suspension was then plated onto an LB plate with the appropriate antibiotic and was incubated at 37 °C overnight until colonies appeared.

2.4.10 Cyanobacteria Transformation

Synechocystis and *Synechococcus* are both capable of taking up exogenous DNA and therefore mutant strains were obtained by natural transformation. Cultures were grown at 30 °C with 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ white light until they reached late exponential growth phase ($\text{OD}_{750} = 5-8$). Under laminar flow, 10 mL of culture was placed in a sterile 50 mL falcon tube and was centrifuged at 6000 g for 10 minutes. The supernatant was discarded, and the pellet was resuspended in 20 mL of fresh BG-11 medium to wash the cells. The cells were pelleted again by centrifugation at 6000 g for 10 minutes. The supernatant was discarded, and the cells were resuspended in 300 μL of BG-11. The cells were divided into 100 μL aliquots and placed in 1.5mL microcentrifuge tubes, and ~5 μg of DNA was added to each 100 μL aliquot. The tubes were flicked a few times to gently mix, and the cells were placed in an incubator at 30 °C under 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ white light for 5 hours. The cells were then plated onto BG-11 agar plates without any antibiotic and were allowed to recover overnight at 30 °C under 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ light. The following day, 1 mL of the working concentration of the appropriate antibiotic was dissolved in water and added underneath the agar plate to select for successfully transformed cells. Wild-type cells began to die back after a few days and desired mutant colonies were visible after about two weeks.

2.4.11 TA Cloning

TA cloning was performed using the pGEM-T Easy Vector System (Promega). The DNA fragment to be cloned into the pGEM backbone was amplified by PCR using Q5 DNA polymerase, was run on an agarose gel, and was extracted and purified from the gel. A ligation reaction was then carried out in a 0.2 mL PCR tube (**Table 14**) and was incubated at 4 °C overnight to allow the reaction to run to completion. The following day, NEB DH5 α chemically competent cells were transformed with 5 μ L of the ligation product and plated onto LB-amp agar plates supplemented with 100 μ L IPTG/X-Gal solution (ChromoMax) for blue-white screening and incubated overnight at 37 °C. White colonies were then picked and screened for the presence of the inserted DNA fragment by colony PCR. Positive colonies were then grown up in 10 mL of LB with 100 μ g/mL carbenicillin, and the plasmid DNA was extracted and sent off for sequencing to confirm the presence of the desired mutation.

Table 14 Components of TA Cloning Ligation Reaction

Component	Volume
Rapid Ligation Buffer (2X)	5 μ L
pGEM-T Easy Vector (50 ng)	1 μ L
Insert DNA (PCR Product)	1-3 μ L
T4 DNA Ligase	1 μ L
Nuclease-Free Water	0-2 μ L
Total Reaction Volume	10 μ L

2.4.12 Site-Directed Mutagenesis

Site-directed mutagenesis was performed using assembled plasmids in order to introduce mutations at desired locations. Forward and reverse

primers were designed to be 31 bp long and complementary to each other with the mutated base(s) in the middle of each primer. PCR reactions were carried out using *pfu* DNA polymerase, and the resulting PCR products were digested with the *DpnI* restriction enzyme (which only cuts methylated DNA) to remove any remaining template plasmid. NEB DH5 α chemically competent cells were then transformed with 5 μ L of the mixture and plated onto LB plates with the appropriate antibiotic. Transformant colonies were screened by colony PCR and patched onto fresh plates with antibiotic. Colonies that gave a DNA band of the correct size were grown up in 10 mL of LB overnight and the plasmid DNA was extracted and sent for sequencing to confirm that the desired mutation was introduced.

2.4.13 HiFi DNA Assembly

NEBuilder DNA HiFi Assembly (New England Biolabs) was used to join together PCR products and linearized vector DNA to form a circular plasmid. PCR products were amplified using Q5 DNA polymerase with primers that included 20-30 bp 5' tails that overlapped with the linearized vector ends to facilitate DNA annealing. Reactions consisting of one or two DNA inserts and a vector backbone were set up in a 2:1 insert:vector molar ratio, while HiFi reactions with three or more inserts were set up using a 1:1 insert:vector molar ratio. The components of a typical HiFi reaction are listed in **Table 15**. Most HiFi reactions were incubated for 1 hour at 50 °C. However, sometimes the incubation temperature was altered if the overlapping DNA fragments had a particularly high or low annealing temperature (greater than 65 °C or lower than 48 °C). In that case, the temperature of the thermal cycler was altered to allow better annealing of the overlapping 3' single stranded ends by including two 1 minute annealing steps at either higher or lower temperatures than 50 °C depending on the melting temperature of the overlapping regions (**Table 16**)

Table 15 Composition of a HiFi Assembly Reaction

Component	Volume
HiFi Assembly Master Mix (2X)	5 μ L
Linearized Vector DNA	Variable
Insert DNA (PCR Product)	Variable
Nuclease-Free Water	0-3 μ L
Total Reaction Volume	10 μ L

Table 16 Modified HiFi Reaction Conditions

Step		High Temp.	Low Temp.	Duration
4-6 cycles	Optimal HiFi Activity	50 °C	50 °C	15 min.
	Overlap Annealing	60 °C	45 °C	1 min.
	Overlap Annealing	55 °C	40 °C	1 min.
Final Extension		50 °C	50 °C	15 min.
Hold		4 °C	4 °C	∞

2.5 Optical Density Measurement

Optical density (OD) of cell cultures was measured at 750 nm using an Agilent Cary 8454 UV-Visible spectrophotometer. OD was used as a proxy for cell density because it avoids the peak absorbance of chlorophyll and other photopigments and is directly proportional to biomass dry cell weight when multiplied by a correction factor determined from an experimentally derived standard curve.

2.6 Metabolite Analyses

2.6.1 Patchoulol GC-MS Analysis

Patchoulol in dodecane was quantified by GC-MS (Agilent 7890A gas chromatograph interfaced with an Agilent 5975C inert MSD with Triple-Axis Detector). Samples (1 μ L) were injected in splitless mode at an injector temperature of 220 °C and were separated using an Agilent HP-5MS column (30 m length, 0.250 mm internal diameter, 0.25 μ m film thickness). The initial oven temperature was 100 °C for 3 min and was ramped up to 200 °C at a rate of 9 °C/min before ramping up to 300 °C at a rate of 100 °C/min, where it was held for 5 minutes for a total run time of 20.11 minutes. Helium (99.9999 %) was used as the carrier gas (1.0 mL/min. constant flow at an oven temperature of 150 °C). The ion source temperature was 220 °C. The mass spectrometer was operated at 70 eV in the electron ionization mode and the mass range scanned was 40-400 m/z. Patchoulol was identified by comparison with a primary reference standard (Sigma-Aldrich) and was quantified using a standard curve ($R^2 > 0.99$).

2.6.2 Quantification of Patchoulol from *Synechocystis*

Each day during an experiment, a sample of culture was taken to measure cell density (OD), and a 100 μ L sample of the dodecane overlay was removed and stored at -80 °C until all samples were collected. Once all samples had been collected, the concentration of patchoulol in dodecane was measured via GC-MS and was calculated using a standard curve ($R^2 > 0.99$) made using a patchoulol primary reference standard (Sigma-Aldrich) in dodecane (**Figure 10**). The amount of patchoulol produced per liter of culture was calculated by multiplying the measured patchoulol concentration in the dodecane overlay by the total volume of dodecane, and then dividing by the total culture volume. The volumes sampled from both the dodecane overlay

and cell culture were noted and subtracted from the volumes of the previous day. At the end of the experiment, both the culture and dodecane were poured into a graduated cylinder to measure the remaining volume and correct for evaporation. The corrected values were used to calculate the final production rates, but the difference was so small that it was almost negligible (less than 150 μL per day of water evaporated, while there was no measurable evaporation of the dodecane).

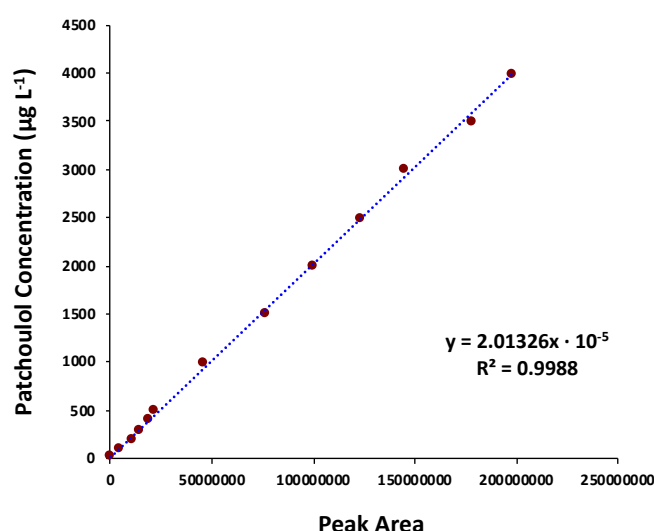


Figure 10 Standard curve used to calculate patchoulol concentration in dodecane for cultures grown on 0.4% CO_2 . A new standard curve was made each time patchoulol was quantified in order to account for any variation in the GC-MS.

2.6.3 Chlorophyll and Carotenoid Quantification

Chlorophyll and colored carotenoids were quantified based on previously established methods.^{44,74} Two 1 mL samples were taken from each triplicate culture during growth and were placed in 1.5 mL microcentrifuge tubes. The samples were pelleted by centrifugation at 16900 g for 5 minutes, the supernatant was discarded, and the cells were placed in a $-20\text{ }^{\circ}\text{C}$ freezer. The following day, the cells were allowed to thaw and were vortexed to loosen the cell pellet. The cells were resuspended in 1 mL of *N-N*-Dimethylformamide (DMF) ($\geq 99\%$, Sigma-Aldrich) and were incubated at room temperature in the dark for 5 minutes. The cells were then pelleted by

centrifugation at 16900 g for 5 minutes. The supernatant was placed in quartz cuvettes and the absorbance was measured at 461 nm and 664 nm. Concentrations of chlorophyll and colored carotenoids were calculated using the following equations: chlorophyll [$\mu\text{g/mL}$] = $\text{OD}_{664} \times 11.92$; colored carotenoids [$\mu\text{g/mL}$] = $(\text{OD}_{461} - (\text{OD}_{664} \times 0.046)) \times 4$.

2.6.4 Residual Glucose Quantification

Residual glucose was quantified from 1 mL culture supernatant samples that had been filtered through a 0.22 μm filter. Samples were loaded onto a HPLC (Agilent 1260 Infinity II LC) equipped with a refractive index detector (RID) and analyzed by isocratic elution using a 5 mM H_2SO_4 mobile phase at a constant flow rate of 0.5 mL min^{-1} on a REZEX ROA-organic acid H^+ (8%) LC column (300 mm x 7.8 mm, Phenomenex). The column temperature was maintained at 55 $^{\circ}\text{C}$ and the refractive index detector was operated at 35 $^{\circ}\text{C}$. Glucose concentration was calculated using a standard curve ($R^2 > 0.99$) (Figure 11).

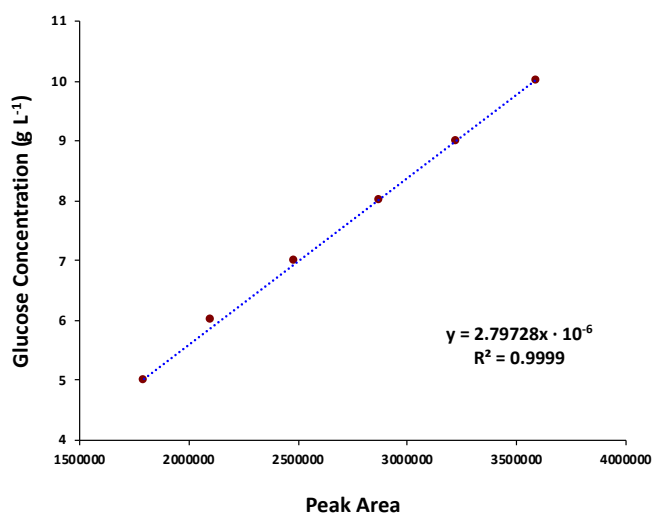


Figure 11 Standard curve used to calculate residual glucose concentration from the supernatant of mixotrophic cultures. All residual glucose samples were run on the same day with standard solutions run periodically throughout to ensure accurate quantification.

2.7 Statistical Analyses

Statistical significance was determined using a two-tailed t-test (assuming unequal variances) using the Microsoft Excel Analysis ToolPak. A difference was considered significant if it had a p-value below 0.05. Linear regression analysis was performed using Excel to generate standard curves for the quantification of patchoulol and glucose. Both standard curves achieved a coefficient of determination (R^2) greater than 0.99.

3 Terpenoid Pathway Metabolic Engineering

3.1 Design of Heterologous Mevalonate Pathway

A heterologous mevalonate pathway was designed to be expressed in both *Synechocystis* and *Synechococcus* in order to increase the production of terpenoid precursors (IPP and DMAPP) and improve overall terpenoid production (**Figure 12**). The heterologous MVA pathway was designed to be inserted into neutral site N15 of *Synechocystis*⁴⁵ and NSII of *Synechococcus*⁷⁵ (for homologous recombination and integration into the genome) with expression driven off the *trc10* promoter⁴⁷ (a strong, non-native promoter that is a hybrid between the *trp* and *lac* UV5 promoters from *E. coli*). The *trc10* promoter was used because it has been shown to drive strong gene expression in both *Synechocystis* and *Synechococcus* and because it has a *lac* operator sequence just before the ribosome binding site, allowing for the repression of transcription if the *lacI* repressor gene from *E. coli* is also heterologously expressed. Expression can then be induced by the addition of Isopropyl β -D-1 thiogalactopyranoside (IPTG). The *lacI*-IPTG induction system was chosen because it is reliable and simple to use. However, due to the high cost of IPTG, other induction systems are generally preferred for industrial-scale microbial chemical production. The N15 and NSII neutral sites were amplified from the *Synechocystis* or *Synechococcus* genomes, respectively, and the MVA pathway genes were chosen from a variety of organisms (**Table 17**) and were codon optimized and synthesized by Eurofins Genomics.

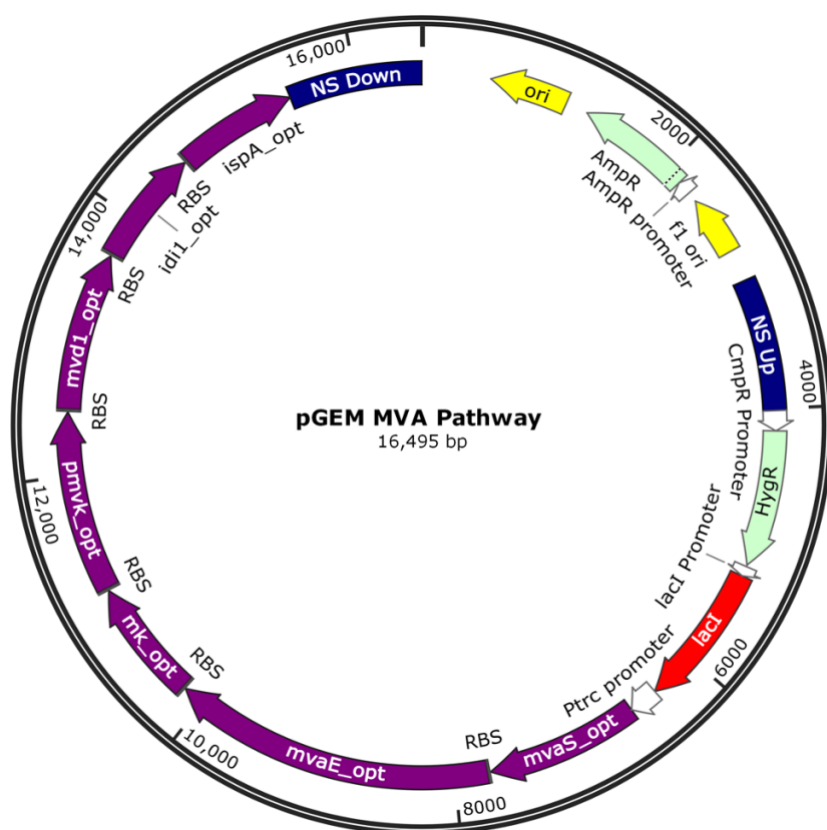


Figure 12 Plasmid map for the heterologous MVA pathway.

Table 17 List of genes used for the heterologous MVA pathway design.

Gene	Size (bp)	Native Organism	Genbank Acc. No.
<i>mvaS</i>	1152	<i>Enterococcus faecalis</i>	AF290092
<i>mvaE</i>	2409	<i>Enterococcus faecalis</i>	AF290092
<i>mk</i>	906	<i>Methanosarcina mazei</i>	NC_003901 (genome)
<i>pmvk (erg8)</i>	1356	<i>Saccharomyces cerevisiae</i>	NM_001182727.1
<i>mvd1 (erg19)</i>	1191	<i>Saccharomyces cerevisiae</i>	X97557.1
<i>idi1</i>	867	<i>Saccharomyces cerevisiae</i>	NC_001148.4
<i>ispA</i>	900	<i>Escherichia coli</i>	NP_414955.1

Note: DNA sequences were codon optimized for expression in *Synechocystis*

Unfortunately, there was not sufficient time during the MRes. to completely assemble the pathway and transform it into either

cyanobacterium. After more than ten cloning attempts to insert the *mvaS* gene into the partially assembled construct, it was decided that the limited time remaining would be better spent on assembling the more manageable enhanced MEP pathway construct. The parts of the pathway that were able to be assembled included the upstream neutral sites, chloramphenicol promoter, hygromycin resistance gene, and the mutated *lacI* gene with the start codon that was changed to the standard AUG. Work to assemble the mevalonate pathway will be continued after the completion of this MRes.

3.1.1 Site-Directed Mutagenesis of the *lacI* Start Codon

A standard site-directed mutagenesis procedure was carried out to change the alternate start codon (GUG) in the *lacI* gene to the standard methionine start codon (AUG). Briefly, the *lacI* gene was PCR amplified from a wild-type *E. coli* strain (MG1655) using Q5 DNA Polymerase and the primers MEP5_F and MEP6_R (**Table 2**), the PCR product was T/A cloned into the pGem T-easy vector system, and NEB DH5 α chemically competent cells were transformed with the product. Positive colonies were identified using blue/white screening (using IPTG and X-gal), colony PCR was performed, positive transformants were grown up in 10 mL of LB medium, and the cells were minipreped. An around-the-world PCR was performed using the primers LACIAUG_F and LACIAUG_R, and the PCR reaction mixture was digested with the *dpnI* restriction enzyme to remove any remaining template plasmid. NEB DH5 α chemically competent cells were transformed with the PCR product, colonies were screened by PCR, positive transformant colonies were grown overnight in 10 mL LB medium, a plasmid extraction was performed, and the introduced mutation was confirmed by Sanger sequencing.

3.2 Generation of Enhanced MEP Pathway Construct

3.2.1 Enhanced MEP Pathway Design

In addition to designing a heterologous MVA pathway, three MEP pathway genes were chosen to be heterologously expressed in *Synechocystis* and *Synechococcus*. A different antibiotic resistance marker (spectinomycin) and neutral sites were chosen for chromosomal integration than the mevalonate pathway design, N10 for *Synechocystis*,⁴⁵ and NSI for *Synechococcus* (neutral site sequences obtained from the pSYN_1 plasmid, Thermo-Fisher). This was done because a goal of this work was to obtain a strain possessing both the engineered MVA and MEP pathways for very high terpenoid production. The enhanced MEP pathway design included the heterologous expression of three MEP pathway genes from *E. coli*: *dxs*, *idi*, and *ispA* (**Figure 13**), that were codon optimized for expression in *Synechocystis*. Both *idi* and *ispA* were synthesized by Eurofins Genomics, but they were not able to synthesize the *dxs* gene, so it was synthesized by Genewiz. The strategy used for designing the improved MEP pathway was modeled after work done in 2016 by Choi *et al.*⁵⁹ in which they tested multiple combinations of heterologous MEP pathway genes from *E. coli* in *Synechocystis*. In that work, the top performing strain expressed the three MEP genes used in this study. The rest of the construct followed the same design as the MVA pathway, including the *trc* promoter and *lacI* repressor gene for IPTG inducible expression.

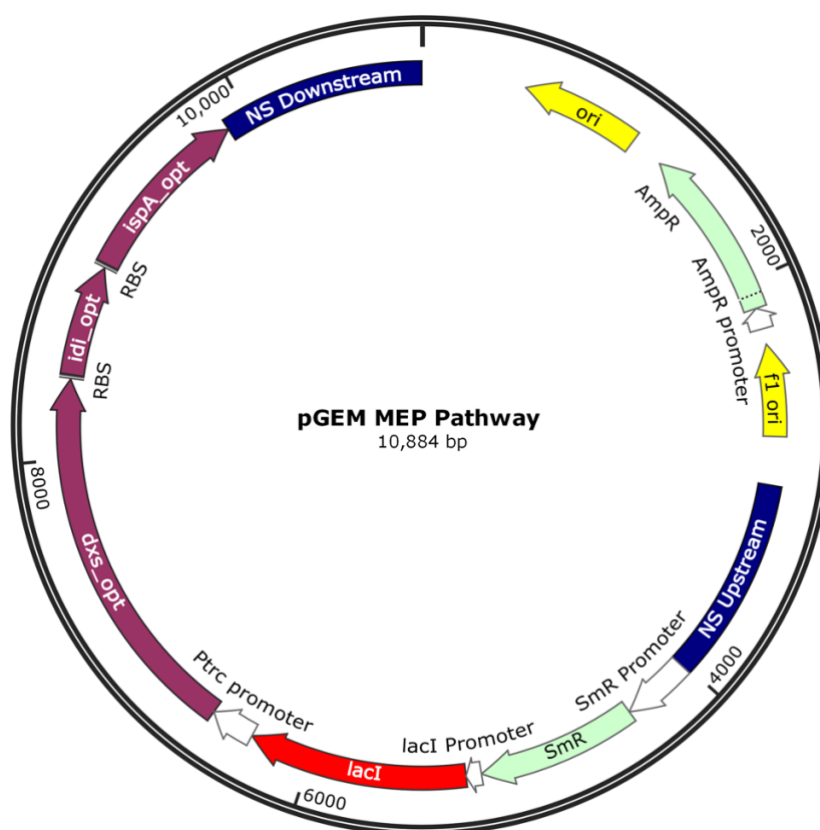


Figure 13 Plasmid map for the completed enhanced MEP pathway construct.

3.2.2 Enhanced MEP Pathway Plasmid Construction

The enhanced MEP pathway construct was initially designed to be built in fragments and then joined together; however, this turned out to be more difficult than initially assumed (despite manufacturer's claims, five-fragment HiFi reactions are extremely inefficient). The second assembly method, which ended up working, involved the stepwise cloning of each individual DNA fragment into a vector that had been linearized by restriction digestion just downstream of the previously inserted fragment. A description of the *Synechococcus* construct assembly steps is given below. The circular pGEM vector (Promega) was first linearized by digestion with *SphI*, run on an agarose gel, and gel purified. The neutral site I (NSI) was then PCR amplified from the *Synechococcus* genome using the primers UPMEP7942_F and UPMEP7942_R (**Table 2**), run on a gel, gel purified, and a HiFi assembly reaction was performed to join the two linear fragments (pGEM and NSI UP)

to form a circular plasmid. NEB DH5α chemically competent cells were then transformed with the HiFi reaction mixture, plated onto an LB-Amp plate, and incubated overnight until colonies appeared. Once colonies were obtained, they were screened via colony PCR and patched out onto fresh LB plates. Any positive colonies were grown up in 10 mL of LB-Amp, a plasmid DNA extraction was performed, and the plasmid DNA was sent to Eurofins Genomics to confirm the correct assembly by Sanger sequencing. Once the resulting construct was confirmed by sequencing, the circular plasmid was cut with *NcoI*, and the next DNA fragment was cloned in. For the sake of brevity, no further assembly steps will be described in full as above, as they follow the same procedure but with different primers, enzymes, and DNA fragments. Instead, the primers and enzymes used for the remainder of the construct assemblies are given in **Table 18** for the *Synechococcus* MEP construct assembly, and **Table 19** for the *Synechocystis* MEP construct assembly.

Table 18 *Synechococcus* Enhanced MEP Pathway Assembly Components

DNA Fragment Inserted	HiFi Primers	Restriction Enzyme to Cut Assembled Plasmid
pGEM (backbone)	N/A	<i>SphI</i>
NSI UP	UPMEP79_F & R	<i>NcoI</i>
<i>SpecR</i> + <i>lacI</i> *	MEPHiFi179_F & R	<i>NdeI</i>
<i>dxs</i>	DXS79_F & R	<i>NsiI</i>
<i>idi</i>	IDI79_F & R	<i>BamHI</i>
<i>ispA</i>	ISPA79_F & R	<i>BamHI</i>
NSI Down	DOWNMEP79_F & R	N/A

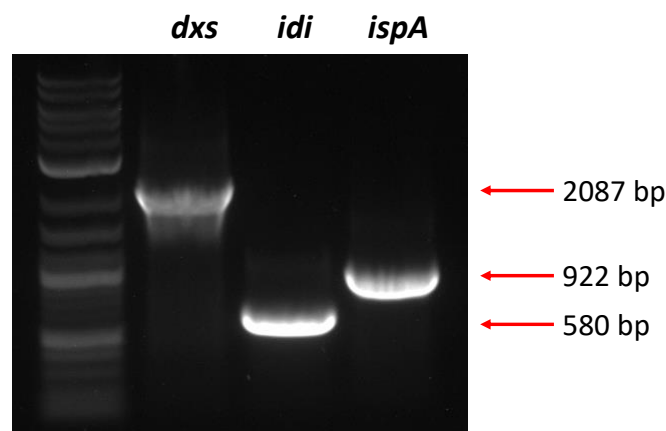
Note: The listed restriction enzyme for each DNA fragment to be inserted was used to cut the assembled circular plasmid after that fragment was correctly assembled and sequenced. *The *SpecR* + *lacI* fragment was created during the previous construct assembly attempt. A full list of primer sequences is given in **Table 2**.

Table 19 *Synechocystis* Enhanced MEP Pathway Assembly Components

DNA Fragment Inserted	HiFi Primers	Restriction Enzyme to Cut Assembled Plasmid
pGEM (backbone)	N/A	<i>SphI</i>
NSI UP	UPMEP68_F & R	<i>NcoI</i>
<i>SpecR</i> + <i>lacI</i> *	MEPHiFi168_F & R	<i>SpeI</i>
<i>dxs</i>	DXS68_F & R	<i>PstI</i>
<i>idi</i>	IDI68_F & R	<i>NdeI</i>
<i>ispA</i> and NSI Down	ISPA68_F & R and DOWNMEP68_F & R	N/A

Note: The listed restriction enzyme for each DNA fragment to be inserted was used to cut the assembled circular plasmid after that fragment was correctly assembled and sequenced.
 *The *SpecR* + *lacI* fragment was created during the previous construct assembly attempt. A full list of primer sequences is given in **Table 2**.

The *Synechocystis* MEP construct was able to be fully assembled, and **Figure 14** shows the PCR amplified genes (*dxs*, *idi*, and *ispA*) from the final construct. The *Synechococcus* MEP construct was assembled all the way through the *ispA* gene, but the downstream neutral site could not be cloned after eight attempts. Fortunately, this work will continue after this MRes and the construct will be completed. Wild-type *Synechocystis*, the *Synechocystis*

**Figure 14** Agarose gel showing the presence of all three MEP genes in the assembled *Synechocystis* construct.

PATS strain (patchoulol-producing), and *Synechocystis* SANS (santalene-producing) strain were all transformed with the completed *Synechocystis* MEP construct. Although many colonies appeared after transformation and also grew in liquid culture, there has not yet been a fully confirmed *Synechocystis* MEP engineered strain that has all three genes present (though *idi* and *ispA* have been confirmed in transformant strains). Screening of these mutant strains is ongoing.

4 Design of Heterologous Artemisinin Pathway

A heterologous artemisinin biosynthesis pathway was designed to be expressed in both *Synechocystis* and *Synechococcus*. The pathway was split up between two constructs. One construct contained all of the required genes to produce artemisinic acid (**Figure 15**), and a separate construct

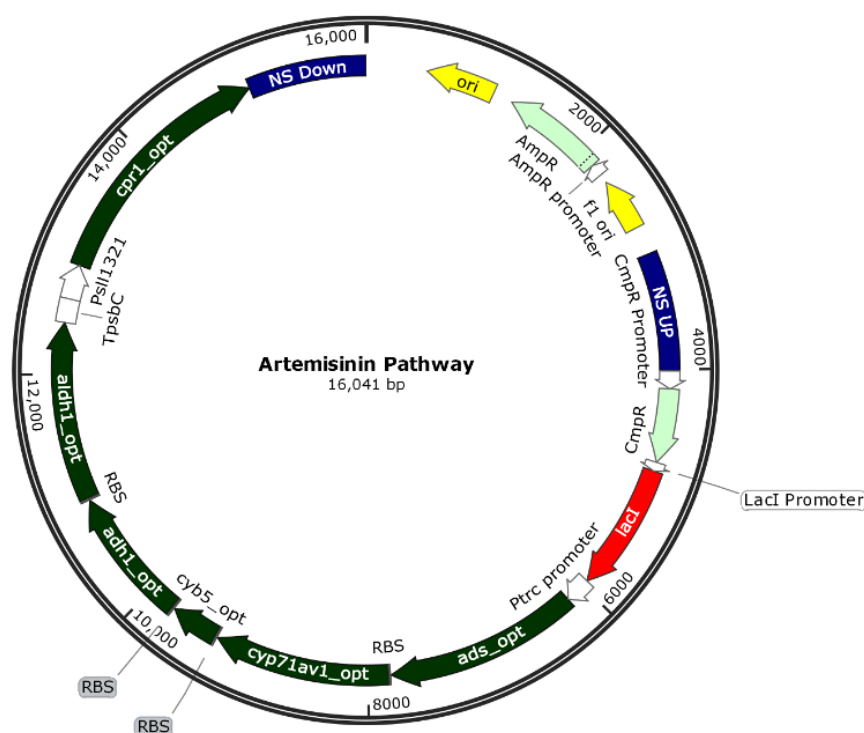


Figure 15 Heterologous artemisinin pathway plasmid map

contained only one gene, *dbp2*, which is required for the in vivo production of artemisinin⁶⁹ (*dbp2 Synechocystis* and *Synechococcus* constructs are shown in **Figure 16**). Design of the artemisinin pathway was similar to the designs previously described for the heterologous MVA pathway and enhanced MEP construct (using the *trc10* promoter⁴⁷ in combination with *lacI* for IPTG

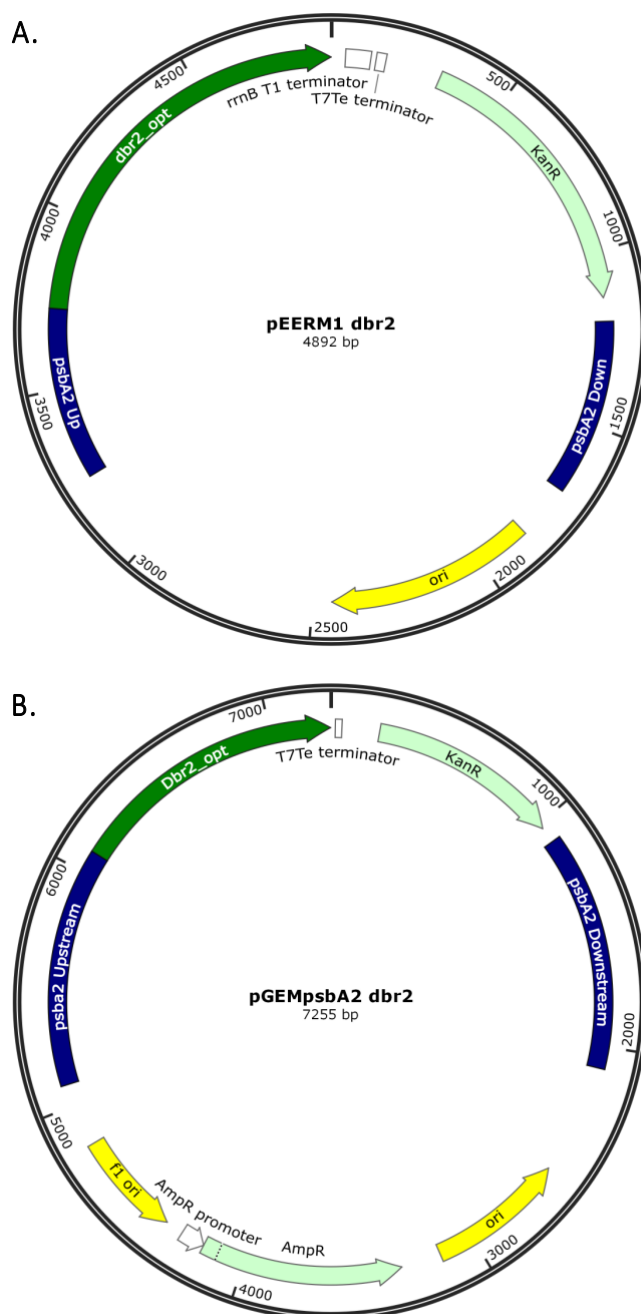


Figure 16 Plasmid maps of assembled *dbr2* constructs. **(A)** Plasmid map for the *Synechocystis* pEERM1 *dbr2* construct. **(B)** Plasmid map for the *Synechococcus* pGEMpsbA2 *dbr2* construct.

inducible gene expression); however, the *trc10* promoter was only used for the first five genes in the pathway (*ads* through *aldh1*). Expression of the last remaining gene in the construct, *cpr1*, was driven off of the weaker *psII1321* promoter⁴⁷ because it has been previously shown that cell viability (*Saccharomyces cerevisiae*) was decreased when *cpr1* was expressed at high levels but improved when expression decreased.⁷⁰ The pathway was designed to be inserted into neutral site N16 of *Synechocystis*⁴⁵ and NSIII of *Synechococcus*,⁴⁶ and a chloramphenicol resistance cassette was used (a different resistance cassette from both the MVA and MEP constructs in order for it to be possible to create strains containing all three constructs). The N16 and NSIII neutral sites were amplified from the *Synechocystis* and *Synechococcus* genomes, respectively, and the artemisinin pathway genes were codon optimized and synthesized by Eurofins Genomics (**Table 20**).

Table 20 Genes used for the heterologous artemisinin pathway design.

Gene	Size (bp)	Native Organism	Genbank Acc. No.
<i>ads</i>	1641	<i>Artemisia annua</i>	JF951730
<i>cyp71av1</i>	1488	<i>Artemisia annua</i>	JF951731
<i>cyb5</i>	411	<i>Artemisia annua</i>	JQ582841
<i>adh1</i>	1137	<i>Artemisia annua</i>	JQ582842
<i>aldh1</i>	1500	<i>Artemisia annua</i>	JQ609276
<i>cpr1</i>	2115	<i>Artemisia annua</i>	JF951732
<i>dbr2</i>	1164	<i>Artemisia annua</i>	EU704257

Note: DNA sequences were codon optimized for expression in *Synechocystis*.

Similar to the MVA and MEP construct assembly, the initial strategy of assembling DNA fragments separately and then joining them together was not very successful. Like the MEP pathway, a stepwise assembly method was later designed that proved to be more successful. Unfortunately, the artemisinin pathway was very large and required a great many cloning steps

(of which some fragments took 6–10 attempts to successfully assemble), and there was not sufficient time during the MRes to completely assemble the pathway. Because the construct was not successfully assembled, the detailed plan of assembly (all the restriction enzymes that were used and primer sequences) is not described here in detail. In a very brief summary representing months of artemisinin construct assembly, the parts of the pathway that were successfully assembled for both *Synechocystis* and *Synechococcus* include the upstream neutral sites (both N16 and NSIII), the chloramphenicol resistance cassette, the *lacI* gene, the *trc10* promoter, the *ads* gene, and the *cyp71av1* gene. The assembly of this construct will be continued and eventually completed using the cloning strategy designed during the MRes.

The *dbr2* constructs were assembled for expression in both *Synechocystis* and *Synechococcus*, each only requiring one gene to be inserted into the plasmid backbone. The assembly of the *dbr2* *Synechocystis* construct was performed by first linearizing the pEERM1 plasmid backbone (plasmid described in **Section 5.2**) by PCR amplification using the primers SsDBR2_1F and SsDBR2_2R, and PCR amplifying the codon optimized *dbr2* gene using primers SsDBR2_3F and SsDBR2_4R that had 30 bp 5' tails overlapping the pEERM1 backbone (primer sequences in **Table 2**). The two linear DNA fragments were then assembled into a circular plasmid by HiFi assembly and NEB DH5 α chemically competent cells were transformed with the plasmid. Cells were then plated onto LB-kan plates and screened for positive transformants by colony PCR. Positive transformants were analyzed by restriction digestion (digested with *EcoRI* and *HindIII*, **Figure 17**), and the correctly assembled construct (colony 2) was confirmed by Sanger sequencing (Eurofins Genomics).

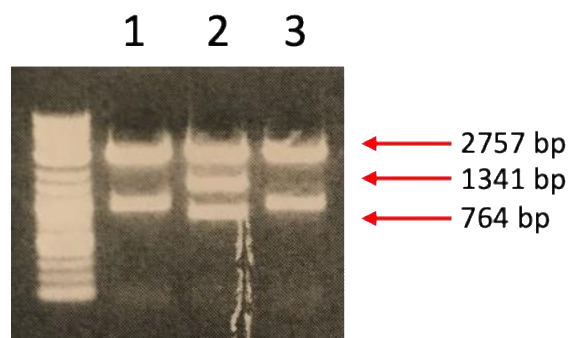


Figure 17 Restriction digestion analysis (*Eco*RI and *Hind*III) of the *Synechocystis* pEERM1 *dbr2* construct showing the incorrect assembly in colonies 1 and 3, and the correct assembly for colony 2.

The *Synechococcus dbr2* construct was assembled using a modified pGEM vector (pGEMpsbA2) with a kanamycin resistance cassette that was designed to knockout the *Synechococcus psbA2* open reading frame (obtained from Sean Craig, not published), modeled after the pEERM1 plasmid. Similar to how the *Synechocystis* vector was constructed, the pGEMpsbA2 backbone was linearized by PCR with the primers SeDBR2_1 and SeDBR2_2, and the *dbr2* gene was amplified with 30 bp 5' tails overlapping the pGEMpsbA2 backbone using the primers SeDBR2_3 and SeDBR2_4 (**Table 2**). The two linear fragments were HiFi assembled and cells were transformed with the product in the same manner as the *Synechocystis* construct. Colonies were PCR screened (**Figure 18A**), and positive mutants were confirmed by Sanger sequencing. A restriction digestion analysis of one of the constructs that had been confirmed by sequencing was performed to show the correct assembly (**Figure 18B**). Both *Synechocystis* and *Synechococcus* were transformed with their respective constructed plasmids and kanamycin resistant colonies appeared. However, positive mutants have not yet been confirmed by PCR.

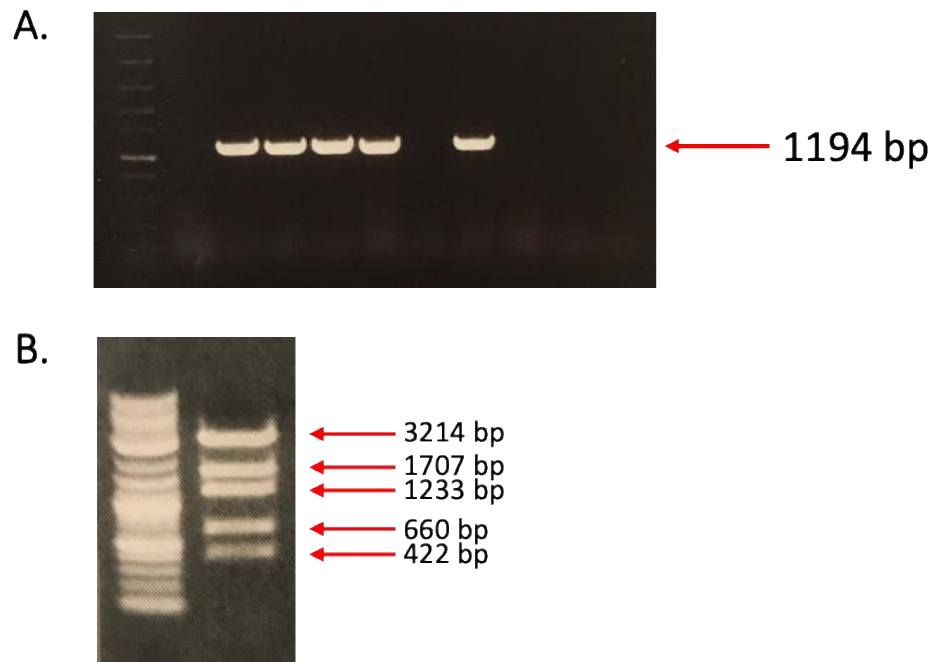


Figure 18 (A) Initial screening colony PCRs of *Synechococcus* pEERM1 *dbr2* construct in *E. coli*, showing that the correct size band was present in five of the ten colonies screened. (B) Restriction digestion analysis (*Eco*RI and *Hind*III) of the assembled (and sequenced) construct gave bands of the expected sizes.

5 Terpenoid Production in *Synechocystis*

5.1 Design of Terpene Synthase Integration Vectors

Six different terpene synthases integration vectors were designed for the expression of six terpene synthases in both *Synechocystis* and *Synechococcus*. Integration was designed in the same manner as was used for the *dbr2* gene, whereby the *psbA2* open reading frame is knocked out, leaving an intact *psbA2* promoter from which expression is driven. The pEERM1 integration vector and the pGEMpsbA2 integration vector were used for expression in *Synechocystis* and *Synechococcus*, respectively, and the only difference in the assembly design for the terpene synthases was that the backbone vector was linearized by restriction digestion instead of PCR. **Table 21** gives a list of the terpene synthases that were codon optimized and synthesized by Genewiz.

Table 21 Terpene synthase genes

Gene	Size (bp)	Native Organism	Genbank Acc. No.
<i>Aalins</i>	1725	<i>Actinidia arguta</i>	GQ338153
<i>Ctges</i>	1812	<i>Cinnamomum tenuipilum</i>	AJ457070
<i>Lalins</i>	1695	<i>Lavandula angustifolia</i>	DQ263741
<i>Obges</i>	1704	<i>Ocimum basilicum</i>	AY362553
<i>pats</i>	1659	<i>Pogostemon cablin</i>	AY508730
<i>sans</i>	1710	<i>Santalum album</i>	HQ343276

Note: DNA sequences were codon optimized for expression in *Synechocystis*.

To assemble the designed constructs, plasmid backbones were cut with *Xba*I and *Nde*I for pEERM1 and pGEMpsbA2, respectively. The genes to be inserted were then amplified using primers with 5' 20 bp tails overlapping the ends of the linearized vectors and were HiFi assembled to form circular plasmids. Only the primers for genes that were successfully cloned into their respective vectors (confirmed by sequencing) are listed in **Table 2**. Those constructs are as follows: *Synechocystis*: *pats*, *sans*, *Aalins*, and *Ctges*; *Synechococcus*: *Obges*. The primer names were designed using either 6803 or 7942 for *Synechocystis* and *Synechococcus*, respectively, followed by the gene name, followed by either F or R (e.g., 6803PATS_F, the forward primer for the *pats* gene for the *Synechocystis* (6803) construct). Both strains of cyanobacteria were transformed with all of their respective assembled constructs multiple times; however, only the *Synechocystis pats* and *sans* constructs were successfully isolated as mutant strains. Those strains are described further in the next section.

5.2 Generation of Patchoulol and Santalene

Producing Strains

Patchoulol and santalene producing strains of glucose tolerant *Synechocystis* were created by knocking out the native *psbA2* open reading frame and replacing it with a synthetic, codon optimized patchoulol synthase gene (*pats*) from *Pogostemon cablin* (patchouli) and a santalene synthase gene (*sans*) from *Santalum album* (sandalwood). Heterologous gene expression was controlled by the light-inducible native *psbA2* promoter, a commonly used technique for protein expression in cyanobacteria that was established by Lagarde *et al.*⁷⁶

The recombinant patchoulol and santalene producing strains were created by knocking out *psbA2* via double homologous recombination using the suicide vectors pEERM1-PATS and pEERM1-SANS, which were made by inserting the genes into the pEERM1 plasmid (Addgene plasmid #64024)⁴⁴

(**Figure 19**). To create the suicide vectors, the pEERM1 backbone was first linearized by restriction endonuclease digestion with *Xba*I. The synthetic *pats* gene was then PCR amplified using Q5 DNA polymerase with the primers 6803PATS_F and 6803PATS_R (**Table 2**) that were designed to have 20 bp 5' tails that overlapped with the ends of the linearized pEERM1 vector. The *sans* gene was amplified using the primers 6803SANS_F and 6803SANS_R, which were designed the same way. HiFi assembly reactions were then performed to join the PCR products with the linear pEERM1 to form the assembled circular plasmids. NEB DH5 α chemically competent cells were transformed with the assembled plasmids and plated onto LB-kan plates. After incubating overnight at 37 °C, colonies were screened by colony PCR using the same HiFi primers and were patched out onto fresh selective plates. Colonies that were positive following screening were grown up overnight in 10 mL of LB-kan, and plasmid DNA was extracted. The purified plasmid DNA was sent to Eurofins Genomics for Sanger sequencing, and all plasmids sent for sequencing were confirmed to be correctly assembled with no introduced mutations.

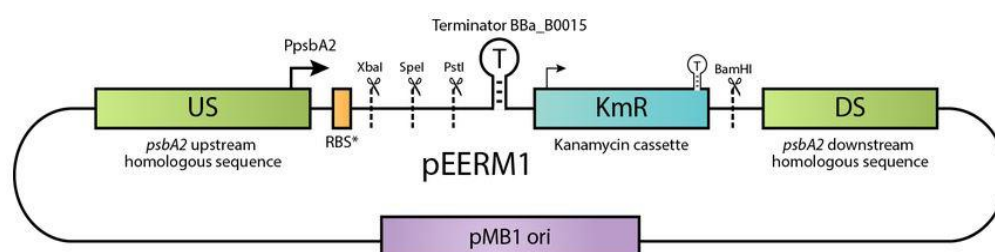


Figure 19 pEERM1 suicide vector (Addgene plasmid #64024), originally constructed by Englund *et al.*⁴⁴ Adapted from Addgene website: <http://n2t.net/addgene:64024>.

Once the plasmids had been confirmed by sequencing, wild-type glucose-tolerant *Synechocystis* sp. 6803 cells were transformed with the plasmids, and positive transformants were selected for using kanamycin. After about two weeks, resistant colonies were grown up in 5 mL of liquid BG-11 medium with 50 $\mu\text{g mL}^{-1}$ kanamycin and incubated at 30 °C under 20 $\mu\text{E m}^{-2} \text{s}^{-1}$ light. Colonies that grew were then screened by colony PCR using primers 6803psbA2_F and 6803psbA2_R (**Table 2**). Primers were designed to

anneal outside of the inserted region in the *Synechocystis* genome, producing a smaller (1721 bp) amplicon for wild-type cells and larger amplicons for the patchoulol (PATS) and santalene (SANS) strains (3480 bp for PATS and 3531 bp for SANS) (**Figure 20**). An advantage of using external primers over simply amplifying the inserted genes is that it is clear when cells are fully or only

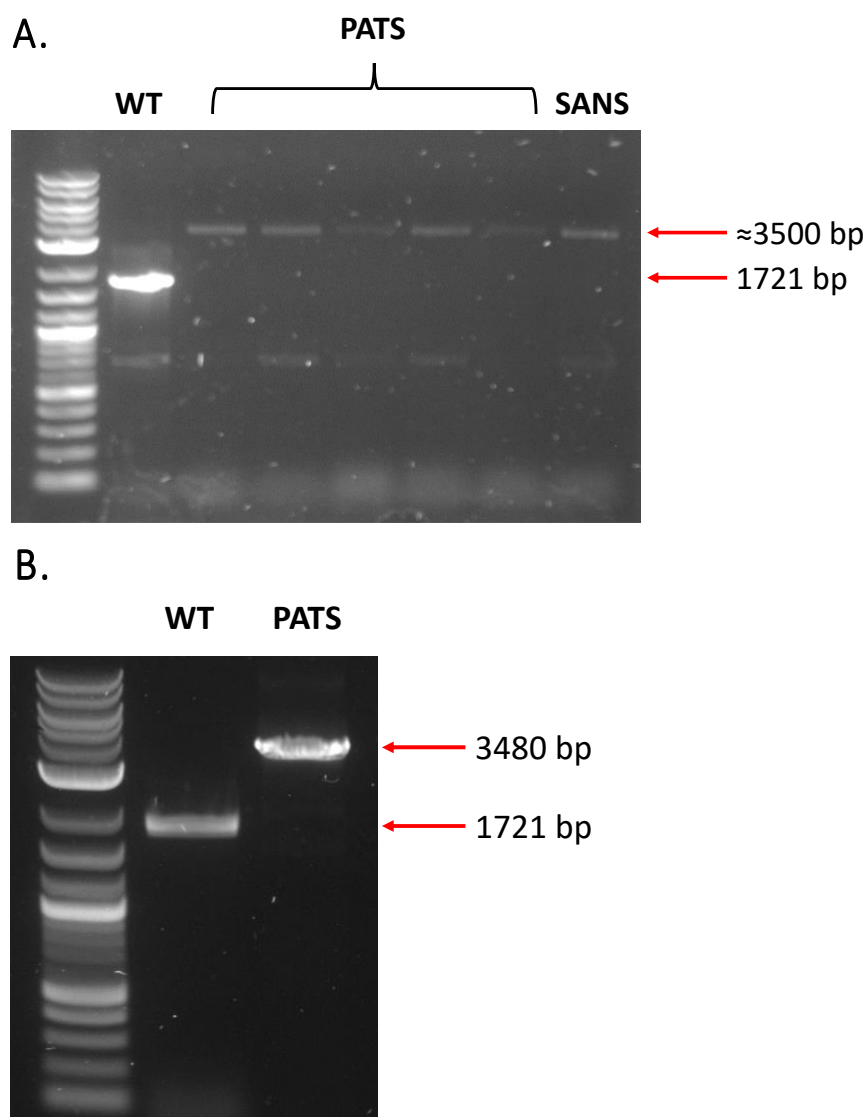


Figure 20 Agarose gel showing the fully segregated *Synechocystis* PATS and SANS strains compared to the wild type. (A) Initial screening PCR from transformant *Synechocystis* boilate showing positive mutant bands and no wild-type bands for five PATS cultures and one SANS culture. (B) Screening PCR of PATS strain seed culture prior to the inoculation of cultures for experiments. DNA was amplified from cell biolates using primers that anneal in homologous regions just outside of the *psbA2* site where recombination occurred. PATS and SANS bands include both the kanamycin resistance cassette (816 bp) and their respective genes (*pats* (1659 bp) and *sans* (1710 bp)).

partially segregated (having all copies of the genome with the desired mutation) by the presence of one or two bands. Further evidence for the generation of positive mutants was the presence of a strong, patchouli-like odor coming from the PATS strains and a sandalwood-like odor coming from the SANS strains, neither of which was present in wild-type cultures.

5.3 Photoautotrophic Patchoulol Production

In order to confirm that the PATS strain was producing patchoulol as the major product, 70 mL cultures were grown photoautotrophically with 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ white LED light and a 10% dodecane overlay (7 mL) to trap the patchoulol produced (see **Materials and Methods**). Dodecane samples (100 μL) were taken a few times throughout growth and after the culture had reached stationary phase. Samples were analyzed by GC-MS and compared to a patchoulol standard (**Figure 21**), as well as the patchoulol mass spectrum from the National Institute of Standards and Technology (NIST) MS library.⁷⁷ The retention times of the patchoulol standard and *Synechocystis*-produced patchoulol were identical (12.76 minutes, **Figure 21A**), and the mass spectrum of the patchoulol from *Synechocystis* matched with both the standard (**Figure 21B**) and the NIST mass spectrum. Wild-type *Synechocystis* did not produce any patchoulol.

To track photoautotrophic patchoulol production over time, 70 mL cultures of the patchoulol-producing *Synechocystis* strain (PATS) were grown in triplicate in a photobioreactor in BG-11 medium with 0.4% CO_2 . The media was supplemented with 50 $\mu\text{g mL}^{-1}$ kanamycin, cultures were irradiated with 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ white LED light, and 7 mL of filter-sterilized dodecane was added to the culture as a solvent overlay to trap the patchoulol produced. The recombinant PATS strain produced an average final titer of 249 $\mu\text{g L}^{-1}$ after 20 days of photoautotrophic growth on 0.4% CO_2 (**Figure 22A**) and a maximum specific production rate of 2.581 $\mu\text{g L}^{-1} \text{day}^{-1} \text{OD}_{750}^{-1}$ on day 14

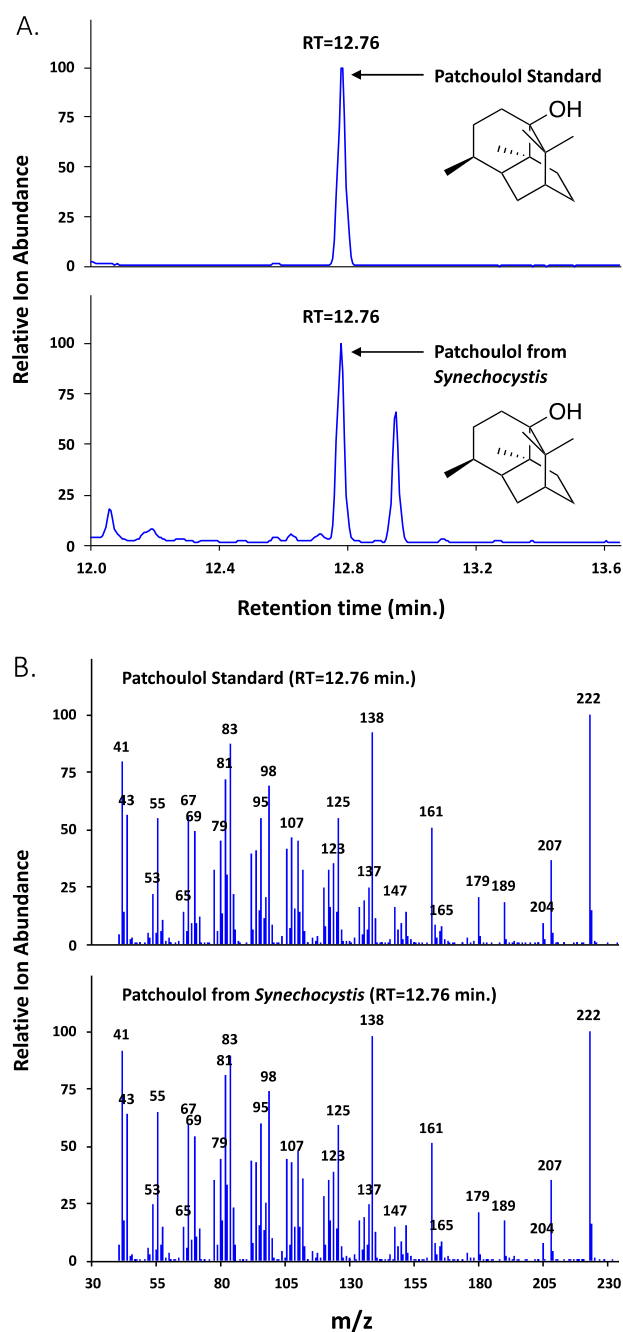


Figure 21 Gas total ion chromatograms (TIC) and mass spectra of a patchoulol standard (primary reference standard, Sigma-Aldrich) and patchoulol produced by *Synechocystis*. **(A)** Gas chromatogram of patchoulol standard (top) and patchoulol produced by the recombinant *Synechocystis* PATS strain (bottom). **(B)** Mass spectra of patchoulol standard (top) and patchoulol produced by the *Synechocystis* PATS strain (bottom). Chromatograms and mass spectra were normalized to 100 to represent relative signal intensity.

(Figure 22B). Specific patchoulol production increased each day for the first five days while the cells were in the exponential growth phase before leveling off and maintaining fairly consistent production until day 19. This trend can be seen clearly in **Figure 22B**. It is interesting that the specific production of

patchoulol was relatively unchanged between days 6 and 19, even after the cells had reached stationary phase (day 17). Since there was constant light and carbon dioxide being supplied, it is likely that the patchoulol production was limited by the depletion of a nutrient in the medium, and it may have been possible to extend production if the deficient nutrient had been added to the medium.

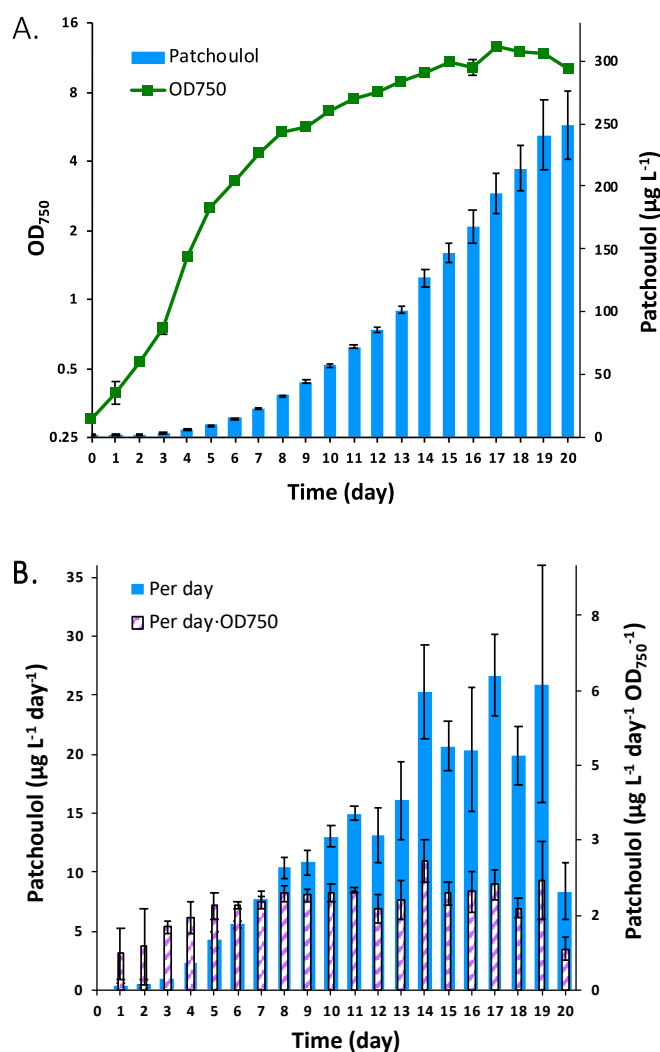


Figure 22 Production of patchoulol from triplicate *Synechocystis* cultures growing on 0.4% CO₂. **(A)** Growth curve of PATS strain and production of patchoulol per liter of culture over time. **(B)** Daily production and specific production of patchoulol. Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

5.3.1 Photoautotrophic Chlorophyll and Carotenoid Analysis

Chlorophyll and carotenoids were quantified during two timepoints during the experiment (early and late) in order to see how the expression of patchoulol synthase was affecting other metabolites that are synthesized through the MEP pathway. Carotenoids are derivatives of tetraterpenes (8 isoprene units, C_{40}) and chlorophyll is made up of a magnesium-binding tetrapyrrole ring and a phytol tail, the phytol tail being a diterpenoid (4 isoprene units, C_{20}). Because chlorophyll and carotenoids are abundant in cyanobacteria and relatively straightforward to quantify (see **Materials and Methods, 2.6.3**), they were analyzed to see if the PATS enzyme was pulling enough carbon flux away from the wild-type MEP pathway to see a noticeable difference relative to the wild-type strain. As shown in **Figure 23**, there was no significant difference in chlorophyll content between the PATS strain and wild-type during either sampling timepoint. This was relatively unsurprising considering the fact that cyanobacteria produce chlorophyll and

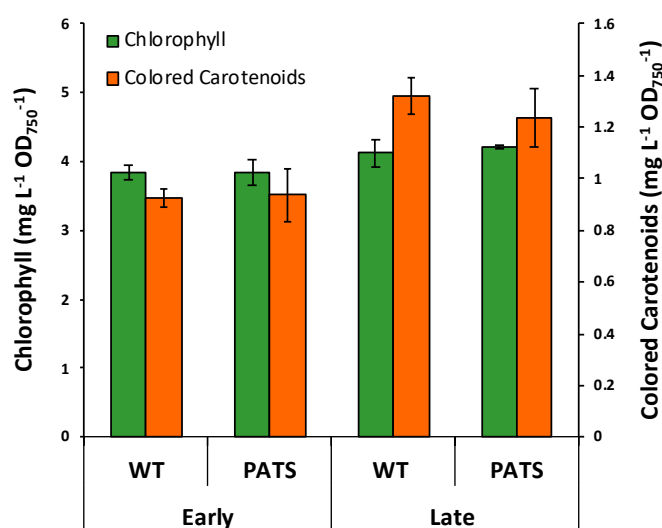


Figure 23 Chlorophyll (left axis) and carotenoid content (right axis) of *Synechocystis* wild-type (WT) and patchoulol-producing *Synechocystis* (PATS) strains grown in a photobioreactor with 0.4% CO_2 as the sole carbon source. Samples for the early and late timepoints during growth were taken on days 4 and 13, respectively. Results represent the mean of two technical replicates for each triplicate culture (6 samples total). Error bars represent the standard deviation of triplicate cultures.

carotenoids on the scale of milligrams per gram of dry cell weight, while an average of only 102 micrograms of patchoulol was produced on the day of the second timepoint (day 13). It can be seen in **Figure 24** that there was no significant difference in the growth of the PATS strain relative to wild-type. This suggests that under photoautotrophic growth conditions, the expression of patchoulol synthase and the lack of a functional *psbA2* gene had no significant effect on growth.

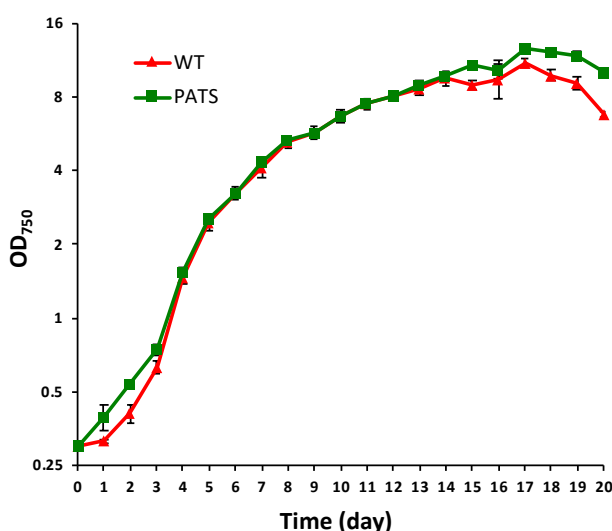


Figure 24 Growth curves of wild-type (WT) and patchoulol-producing (PATS) *Synechocystis* strains grown in a photobioreactor on 0.4% CO₂. Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

5.4 Mixotrophic Patchoulol Production

Following the production of patchoulol from CO₂, the hypothesis that mixotrophic conditions would improve patchoulol productivity was tested. The same growth conditions were used for mixotrophic growth as were used for photoautotrophic growth in the photobioreactor, with the addition of 10 g L⁻¹ glucose to the medium. Surprisingly, mixotrophic patchoulol production was much lower compared to photoautotrophic production. The mixotrophic cultures reached a maximum specific production rate of only 0.291 μg L⁻¹ day⁻¹ OD₇₅₀⁻¹ on day 4 (**Figure 25B**), compared to the maximum rate of 2.581 μg

$\text{L}^{-1} \text{ day}^{-1} \text{ OD}_{750}^{-1}$ on day 14 under photoautotrophic conditions. Furthermore, mixotrophic conditions strongly inhibited the growth of the PATS strain relative to the wild type (**Figure 26**). The growth inhibition of the PATS strain under mixotrophic conditions was quite surprising but was confirmed by a preliminary experiment (**Figure 31** given in the **Appendix**). After nine days of growth, the PATS strain produced an average final titer of $6.5 \mu\text{g L}^{-1}$ of patchouliol (**Figure 25A**), 6.8-fold less than the same strain after nine days of photoautotrophic growth. When compared to the final yield obtained from

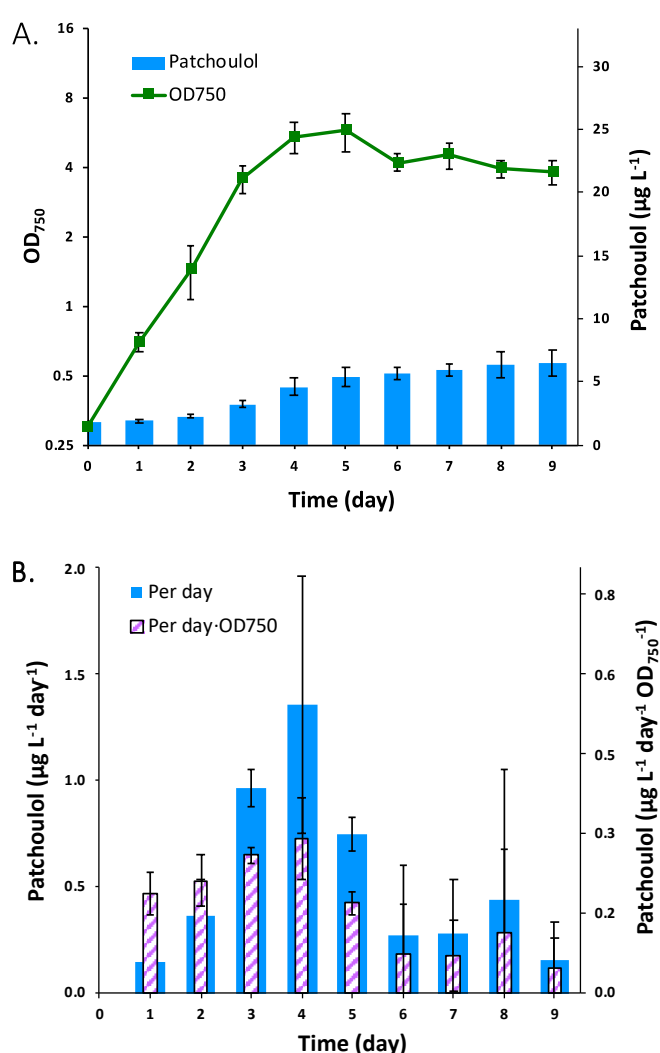


Figure 25 Mixotrophic production of patchouliol from triplicate *Synechocystis* cultures growing on 10 g L^{-1} glucose and 0.4% CO_2 . **(A)** Growth curve of PATS strain and production of patchouliol per liter of culture over time. **(B)** Daily production and specific production of patchouliol. Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

photoautotrophic growth (which took 20 days), the PATS strain under mixotrophic conditions produced 38-fold less patchoulol.

Similar to patchoulol production under photoautotrophic conditions, specific production increased each day at the beginning of the experiment (days 1-4) while the cells were in the exponential growth phase. Unlike photoautotrophic production, however, specific productivity declined after peaking on day 4, and there was not a period of consistent production that lasted multiple days after cells reached stationary phase. It is not the case that the growth of mixotrophic cultures was limited by a nutrient deficiency. This is because the wild-type cultures reached an average OD of 11.17 and were grown under the same conditions as the PATS strain, which only reached an OD of 4.53. Residual glucose was quantified to show how much each strain had consumed (**Figure 26**), the wild-type and PATS strains using 3.49 g L^{-1} and 1.46 g L^{-1} , respectively. These values agree with the much higher culture densities observed for the wild type compared to the PATS strain. Since the wild type grew to much higher cell densities under the exact same conditions as the PATS strain, the growth inhibition for PATS cultures

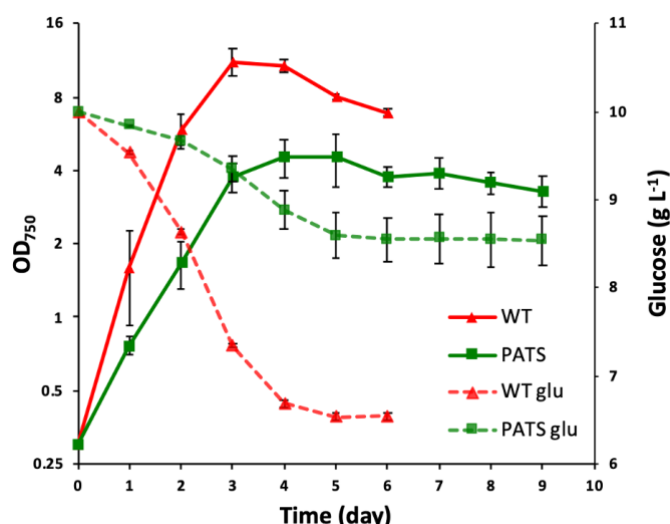


Figure 26 Growth curves of wild-type (WT) and patchoulol-producing (PATS) *Synechocystis* strains grown mixotrophically with 10 g L^{-1} glucose initial concentration and $0.4\% \text{ CO}_2$. Solid lines represent OD₇₅₀, dashed lines represent glucose concentration. Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

under mixotrophic conditions must be due to either the lack of a functional *psbA2* gene or the expression of patchoulol synthase. This is explored further in the discussion section.

5.4.1 Mixotrophic Chlorophyll and Carotenoid Analysis

Chlorophyll and carotenoid analysis of mixotrophic cultures showed larger differences between the wild-type and PATS strains compared to photoautotrophic growth, but there were still no statistically significant differences (**Figure 27**). Interestingly, the PATS strain had higher carotenoid levels at both timepoints relative to the wild type and lower chlorophyll levels at both time points, phenotypes often observed in stressed cultures and consistent with the growth inhibition observed for PATS cultures grown mixotrophically. Overall, photopigment levels were significantly lower for cultures grown mixotrophically compared to those grown under photoautotrophic conditions. Photoautotrophic wild-type cells contained 2.58-fold more chlorophyll and 3.88-fold more carotenoids than wild-type mixotrophic cells (4.13 versus 1.60 mg L⁻¹ OD₇₅₀⁻¹ chlorophyll (late growth) and 1.32 versus 0.34 mg L⁻¹ OD₇₅₀⁻¹ carotenoids (late growth)). A difference in photopigment levels was expected because photoautotrophic metabolism relies solely on harvesting light to obtain energy and therefore requires more photopigments to capture light compared to cells grown mixotrophically, which are also able to obtain energy through glycolysis.

Differences in carbon flux through the MEP pathway under different growth conditions are likely heavily linked to the biosynthesis of chlorophyll and carotenoids, since they are major terpenoid products (on the order of milligrams per gram of dry cell weight). Based on that, it logically follows that the production of patchoulol would also be linked to the biosynthesis of chlorophyll and carotenoids because more substrate (FPP) would be available to the constitutively expressed patchoulol synthase. If that is the case, then growth conditions which favor chlorophyll and carotenoid biosynthesis

should also favor patchoulol production and could help explain the disparity in patchoulol production between cells grown photoautotrophically versus mixotrophically.

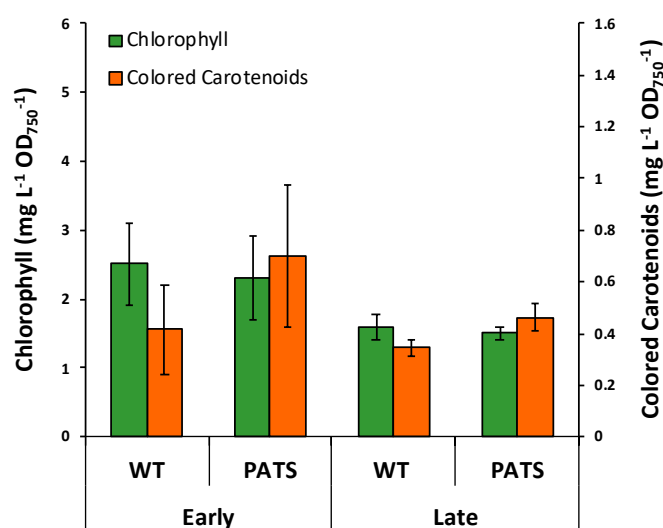


Figure 27 Chlorophyll (left axis) and carotenoid content (right axis) of *Synechocystis* wild-type (WT) and patchoulol-producing *Synechocystis* (PATS) strains grown mixotrophically with 10 g L^{-1} glucose and $0.4\% \text{ CO}_2$. Samples for the early timepoint during growth were taken on day 1, while the late timepoint samples were taken on days 3 and 4 for WT and PATS, respectively. Results represent the mean of two technical replicates for each triplicate culture (6 samples total). Error bars represent the standard deviation of triplicate cultures.

5.5 Production of Patchoulol from Bicarbonate

After investigating the effects of mixotrophic growth on patchoulol production, it was of interest to see if production could be improved by using bicarbonate (NaHCO_3) as a carbon source. In addition to the different carbon source, two light conditions were tested, low light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) and high light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$). This was done to test the hypothesis that higher light would result in higher productivity due to the higher cell densities that could be reached and also because the *psbA2* promoter is light activated (higher light should result in more expression of patchoulol synthase).⁴⁴

Cultures grown under high light produced more patchoulol than those grown under low light, confirming the initial hypothesis (**Figure 28A,B**). The final titers of patchoulol were $31.66 \mu\text{g L}^{-1}$ and $27.57 \mu\text{g L}^{-1}$ for cultures grown under high and low light, respectively. It should be pointed out, however,

that although the cultures produced similar amounts of patchoulol over the full period of sampling, the high light cultures grew much faster and were only sampled for 9 days, compared to 12 days for low light cultures. In fact, when comparing patchoulol production between the two conditions on day 5 when the high light cultures entered stationary phase and patchoulol production slowed, the high light cultures had produced significantly more patchoulol than the low light cultures ($p < 0.001$) ($27.31 \mu\text{g L}^{-1}$ patchoulol compared to only $13.45 \mu\text{g L}^{-1}$ for high and low light cultures, respectively).

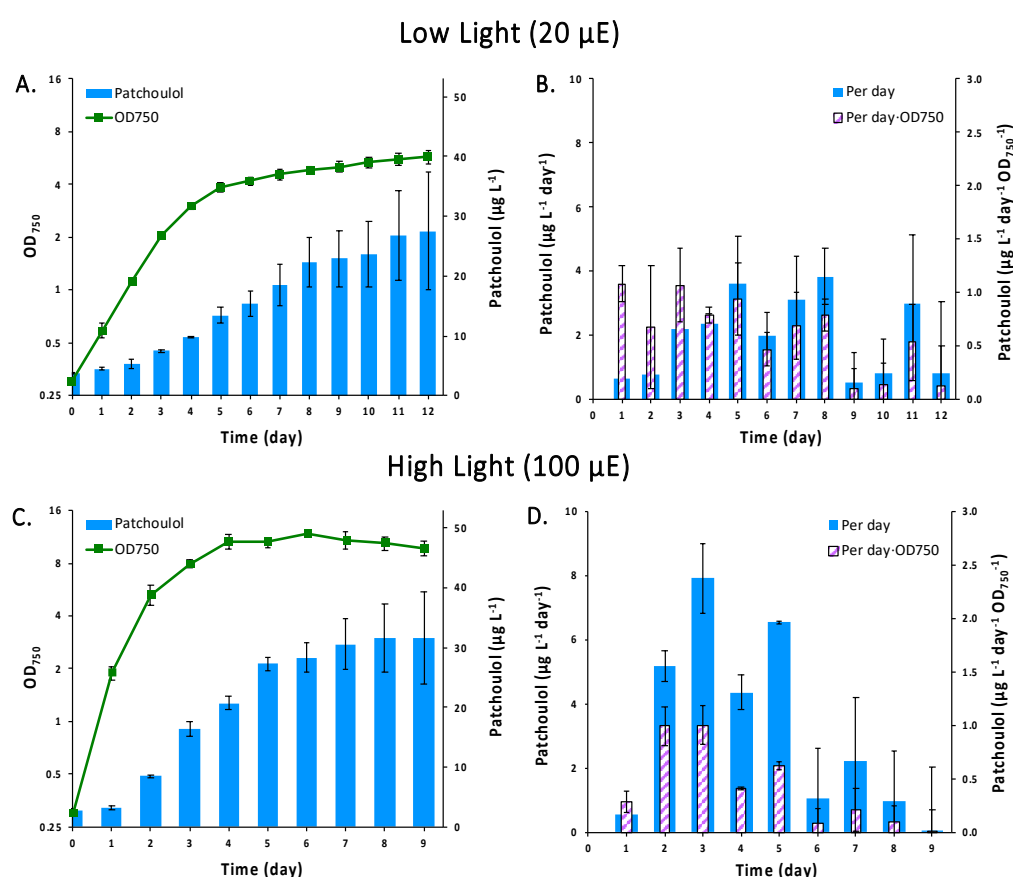


Figure 28 Patchoulol production from PATS cultures grown on bicarbonate in shake flasks. (A) Low light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) growth curve of the PATS strain and production of patchoulol over time. (B) Low light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$) daily production and specific production of patchoulol. (C) High light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$) growth curve of the PATS strain and production of patchoulol over time. (D) High light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$) daily production and specific production of patchoulol. Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

Although high light cultures produced more patchoulol than cultures grown under low, the specific productivity of low light condition was higher

than the high light specific productivity and was maintained over a longer period of time (**Figure 28C,D**). Average specific productivities were $0.60 \mu\text{g L}^{-1} \text{day}^{-1} \text{OD}_{750}^{-1}$ (over 12 days) and $0.45 \mu\text{g L}^{-1} \text{day}^{-1} \text{OD}_{750}^{-1}$ (over 9 days) for cultures grown under low and high light, respectively. This explains how the low light cultures—which reached a maximum OD of only 5.77—could produce almost as much patchoulol as the high light cultures that reached an OD of 11.76.

As expected, the high light cultures grew to higher cell densities compared to the low light cultures, showing that light was the factor limiting growth for the low light condition (**Figure 29**). Interestingly, there was no

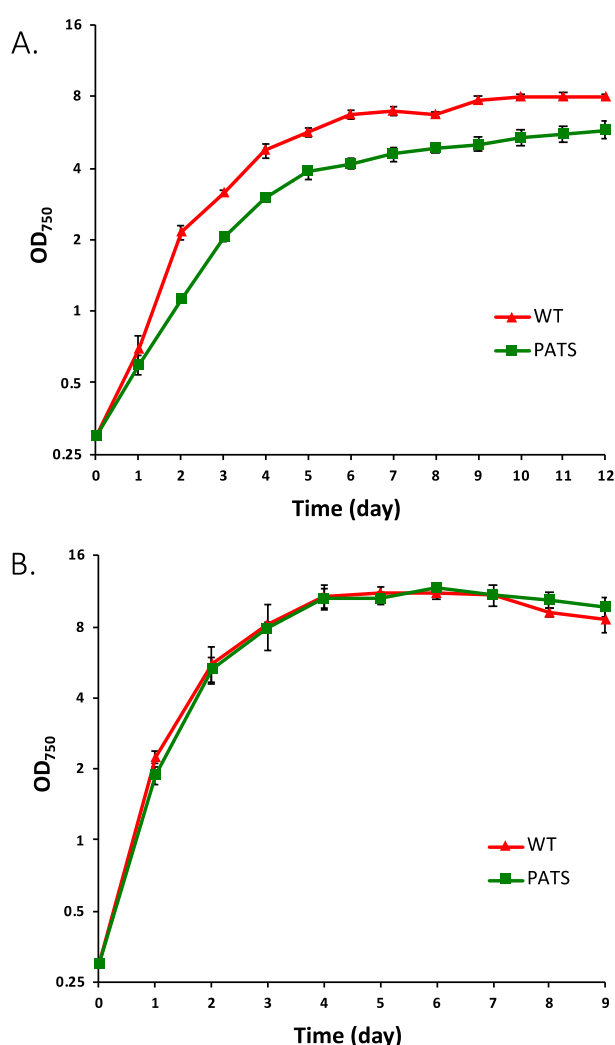


Figure 29 Growth curves of wild-type (WT) and patchoulol-producing (PATS) *Synechocystis* strains grown in shake flasks supplemented with bicarbonate. **(A)** Cultures grown under low light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$). **(B)** Cultures grown under high light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$). Error bars represent the standard deviation of triplicate cultures. Growth curves were plotted on a log base 2 scale to better represent exponential growth.

significant difference in growth between the wild-type and PATS strains when grown under high light, but the PATS strain grew slightly slower than the wild type under low light. One explanation for the slight growth inhibition could be the patchoulol synthase pulling flux away from chlorophyll and carotenoid biosynthesis. This could have slowed growth because the cultures were growing in a light-deficient environment and less efficient photopigment synthesis could have further decreased photosynthetic activity.

5.5.1 Bicarbonate Chlorophyll and Carotenoid Analysis

Similar to what was observed under photoautotrophic and mixotrophic conditions, growth under low and high light resulted in large differences in the amount of photopigments produced. The wild-type cultures grown under low light produced 2.02-fold more chlorophyll and 1.45-fold more carotenoids compared to the high light cultures at the late growth sampling timepoint (**Figure 30**). Similarly, the PATS strain contained 2.07-fold more chlorophyll but only contained 1.08-fold more carotenoids for the low light cultures compared to high light cultures. The higher production of photopigments under low light conditions was expected because light was the only factor limiting growth for those cells and increasing photopigment levels enabled them to increase their light harvesting capacity.

The differences in chlorophyll and carotenoids relative to patchoulol production were similar to what was seen previously when cultures grown photoautotrophically and mixotrophically were compared. In both cases, the chlorophyll and carotenoid levels were higher for cultures that had higher specific patchoulol production. Although in this case there was slightly more patchoulol produced under the high light condition, the pattern of specific productivity being linked to chlorophyll and carotenoid biosynthesis was maintained.

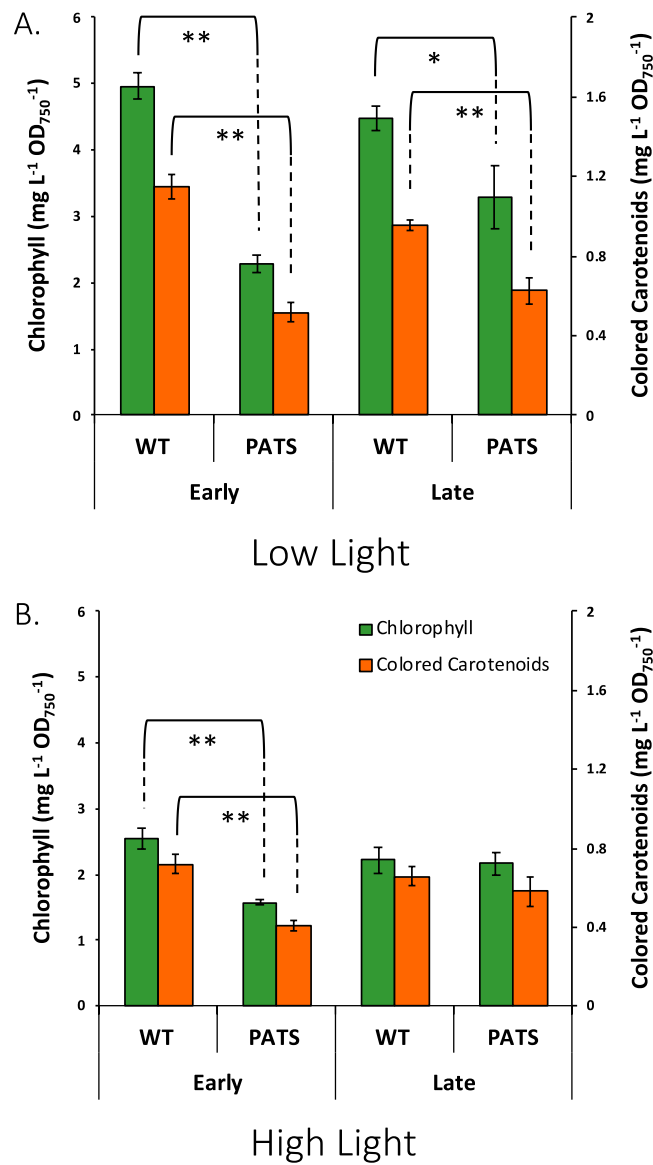


Figure 30 Chlorophyll (left axis) and carotenoid content (right axis) of wild-type (WT) and PATS strains grown on bicarbonate in shake flasks. **(A)** Cultures grown under low light ($20 \mu\text{E m}^{-2} \text{s}^{-1}$). Samples for the early timepoint were taken on day 1 for both WT and PATS. Samples taken for the late timepoint were taken on day 6 for WT and day 7 for PATS. **(B)** cultures grown under high light ($100 \mu\text{E m}^{-2} \text{s}^{-1}$). Early and late timepoint samples were taken for both strains on days 1 and 4, respectively. Results represent the mean of two technical replicates for each triplicate culture (6 samples total). Error bars represent the standard deviation of triplicate cultures. Asterisks represent statistical significance, where * = $p < 0.05$ and ** = $p < 0.01$. Statistical analysis was performed using the average values of two technical replicates for each biological replicate (3 observations).

6 Discussion and Future Work

6.1 MVA and MEP Pathway Engineering

In this work, a full heterologous MVA pathway and an enhanced MEP pathway were designed to be expressed in both *Synechocystis* and *Synechococcus* in order to increase the production of terpenoid precursors (IPP and DMAPP) for terpenoid production. The MEP pathway was able to be fully assembled and transformant colonies were isolated, but only two of the three genes were positively identified in the transformant strains by the time of the submission of this thesis. Complete assembly of the MVA was unfortunately prevented due to its large size, the number of DNA fragments needed for assembly, and the cloning strategies chosen. In addition to the final designed construct being a very large at over 16 kb, the initial strategy of trying to assemble smaller DNA fragments through stitching PCR and subsequently joining them together did not work very well. Once the strategy of successive cloning of individual fragments into a single construct using restriction enzymes was employed, progress improved but was still hampered by some DNA fragments that took 6–10 cloning attempts before being successful. As a final note, dividing the mevalonate pathway genes across multiple constructs would have probably been a better strategy than building a single construct containing all of the mevalonate pathway genes. This would have made building each construct more manageable and would have likely sped up progress. This was the strategy used by Bentley *et al.*⁶⁰ for their *Synechocystis* strain expressing the mevalonate pathway, and this strategy may be employed in future work for the assembly of the mevalonate pathway.

6.2 Artemisinin Pathway

Although some progress was made in assembling the artemisinin pathway, there is still much to do before a strain containing the full pathway will be generated. The problems encountered were similar to those for the mevalonate pathway assembly, which makes sense as the same strategies were used for both pathways. Once again, one of the main problems was the inefficiency of each cloning step, which made inserting each gene extremely time consuming. Additionally, although the artemisinin pathway was divided into two constructs, it probably should have been divided even further to make assembly more manageable.

It will be very exciting when a strain is eventually produced that contains the entire artemisinin pathway. As was discussed in the introduction, there is a lot of potential for the production of artemisinin in cyanobacteria due to their predicted ability to produce artemisinin *in vivo*. Furthermore, if a strain were to be produced that contained the heterologous mevalonate pathway, the enhanced MEP pathway, and the artemisinin pathway, it is possible that it could produce artemisinin on the scale of mg g^{-1} DCW or even higher. This hypothesis is based on the 19.8 mg L^{-1} production of the artemisinin precursor, amorpha-4,11-diene, in a *Synechococcus* strain only expressing three MEP pathway genes.⁵⁹ This would put it within the range of artemisinin produced by *Artemisia annua* (0.01–1.4% of dry weight)⁶⁹ but with a much shorter time to harvest, potentially making it a viable production method. Imagine the impact that an artemisinin-producing cyanobacterial strain could have on the treatment of malaria if it could produce the same amount of artemisinin as *A. annua* by weight percentage but only took one week to grow and could be grown anywhere in the world.

6.3 Bioproduction of Patchoulol

The results of patchoulol production under different growth conditions provided some interesting insight into how the endogenous MEP pathway may be regulated in *Synechocystis*. There seemed to be a clear relationship between conditions which favored the production of photopigments and conditions favoring the production of patchoulol that extended between growth conditions. Gaining a better understanding of this relationship will be the subject of future work.

The results of the influence of light intensity on patchoulol production presented in this work are interesting when compared to a previous study that also expressed a terpene synthase (geranylgeranyl synthase) in *Synechocystis* that was grown under low and high light (50 and 180 $\mu\text{E m}^{-2} \text{s}^{-1}$).⁷⁸ In that study, more than twice as much total geranylgeranyl was produced under the low light conditions compared to the high light. However, the “low light” condition used in that study provided more than twice amount of light than the low light condition presented in here (50 $\mu\text{E m}^{-2} \text{s}^{-1}$ compared to 20 $\mu\text{E m}^{-2} \text{s}^{-1}$). Additionally, the promoter used in that study was the *cpcB* promoter, which is the native promoter for the phycocyanin beta subunit, a strong constitutive promoter. Because the *cpcB* promoter is responsible for expressing a phycocyanin subunit, expression is induced under low light conditions in order to increase light harvesting capacity (similar to the increased photopigment levels under low light compared to high light reported in this study). Conversely, the *psbA2* promoter that was used to drive gene expression in this work is affected in an opposite manner by light, and more light leads to higher protein expression. This is because the *psbA2* promoter drives expression of the *psbA2* gene, which codes for the D1 protein, a subunit of PSII that is easily damaged by light. Under high light conditions, the D1 protein is more quickly damaged than under low light conditions, increasing its turnover rate and therefore requiring more gene expression to continuously replace the damaged

protein. In summary, the differing promoter responses to light, and the different definitions of low and high light likely account for the different production levels observed under different light conditions.

Another interesting result of this study was the observation of a significant growth inhibition for the PATS strain under mixotrophic conditions. The same growth inhibition was observed twice—first during a preliminary experiment (**Figure 31** given in the **Appendix**) and then during the experiment presented in the results section (**Figure 26**). The only difference between the conditions was the presence of glucose in the medium, and it is not clear why such a significant growth inhibition was observed. Generally, the *psbA2* open reading frame can be knocked out to drive expression of heterologous genes without having any deleterious effects on the culture.⁴⁴ In fact, it has been shown that the weakly expressed *psbA3* gene, which also codes for the D1 protein, is heavily upregulated once the *psbA2* gene becomes inactive in order to compensate for the loss.⁷⁹ If the growth inhibition was caused by light-induced stress due to the the lack of a functional *psbA2* gene, then a similar growth inhibition should have been observed under both photoautotrophic and bicarbonate high light conditions. It also seems unlikely that the growth inhibition occurred as a result of expressing patchoulol synthase, since no similar growth inhibition was observed under other conditions. A survey of the literature did not provide an explanation for the growth inhibition observed, and the mechanism responsible will need to be explored in future work.

Overall, the patchoulol production levels reached in this study under photoautotrophic conditions (249 $\mu\text{g L}^{-1}$) were within the normal range compared to other terpenoids produced in strains containing only a heterologous terpene synthase and no upstream terpenoid pathway engineering. Strains of this type producing other terpenoids range from 49 $\mu\text{g L}^{-1}$ (β -Caryophyllene) to 4 mg L^{-1} (limonene).⁸⁰ It should also be remembered that terpene synthase enzymes have a wide range of product specificity, which can directly impact product yield. In this work, multiple minor terpenoid products were detected by GC-MS (β -Caryophyllene and other

unidentified terpenoids) that were not present in wild-type cells, negatively impacting the patchoulol yield. The presence of terpenoids other than patchoulol was not surprising because patchoulol synthase has been shown to produce a wide range of products in addition to patchoulol from in vitro assays.⁸¹ In fact, *Pogostemon cablin* (patchouli) essential oil contains a complex mixture of over 24 different sesquiterpenoids but only has five known terpene synthase enzymes. Of those enzymes, four of them each produce a single sesquiterpenoid, but patchoulol synthase produces a total of 14 sesquiterpenoids including patchoulol.⁸¹ What's even more fascinating is that the same study also showed that the yield of each individual sesquiterpenoid produced by patchoulol synthase in vitro was present in the same proportion as is found in patchouli essential oil. This ability of terpene synthases to produce multiple products can be either beneficial or detrimental, depending on the application. An example of this feature being beneficial would be if the goal was to synthetically reproduce a complex mixture terpenoids to mimic the scent of an essential oil. For terpene synthases with diverse product profiles, it may only take one enzyme or a handful of enzymes to get extremely close to the characteristic odor (as is the case for patchoulol synthase and patchouli essential oil). The concept of producing synthetic essential oils from cyanobacteria has been previously explored using β -phellandrene synthase enzymes from different sources and expressing them in *Synechocystis*.⁸² On the other hand, if the goal is to produce a pure chemical, then any side reactions that occur will only reduce the final yield.

Production of patchoulol in other organisms has been quite successful in some cases, with a titer of 60 mg L⁻¹ being reached in *Corynebacterium glutamicum* that been engineered through genomic deletions and *ispA* overexpression to increase terpenoid pathway flux.⁸³ Additionally, 922 $\mu\text{g g}^{-1}$ DCW of patchoulol was produced in eukaryotic microalga, *Chlamydomonas reinhardtii*.⁸⁴ While the production of patchoulol described in this work is lower than what has been made in other organisms, it represents the first

production of patchoulol in a cyanobacterium and is a good starting point for further engineered strains that will have increased productivities.

6.4 Future Work

In this work, a heterologous mevalonate pathway, an enhanced MEP pathway, and a heterologous artemisinin biosynthetic pathway were all designed for expression in both *Synechocystis* and *Synechococcus*. In addition, six terpene synthase genes (four unique terpenoid products) were synthesized and constructs were designed for expression in both strains of cyanobacteria. By the completion of the MRes, the enhanced MEP pathway construct was fully constructed for *Synechocystis*, and five terpene synthase constructs were completed. Transformation was attempted for all completed constructs, but only patchoulol and santalene producing strains of *Synechocystis* were able to be successfully isolated. Finally, the patchoulol-producing strain (PATs) was characterized under four different growth conditions, with the best production occurring under photoautotrophic conditions.

The results presented in this thesis constitute the first work done using cyanobacteria for the production of terpenoids as part of the sustainable terpenoid platform within the Green Chemicals Beacon at the University of Nottingham. Although the goals of this thesis may have been overambitious for the timescale of a one-year MRes, much progress was made toward the ultimate goal of developing a robust cyanobacterial terpenoid production platform. Three major biosynthetic pathways were designed—two were partially assembled and one was completely assembled. Furthermore, four different growth conditions were assessed for production of the sesquiterpenoid, patchoulol, and an efficient product isolation system consisting of a dodecane solvent overlay was established. Finally, a GC-MS method was created for the detection and quantification patchoulol that should also be useful for the quantification of other terpenoids.

Future work will include the completion and expression of the mevalonate pathway designed in this work, isolation of cyanobacterial strains containing the enhanced MEP pathway, completion and expression of the artemisinin pathway, expression of the remaining individual terpene synthases, and full characterization of the santalene-producing *Synechocystis* strain. Eventually, a strain is envisaged that will possess both the enhanced MEP pathway and the heterologous mevalonate pathway, which will serve as a background strain capable of providing high carbon flux for many different terpene synthase enzymes that can be expressed to produce different products. All of this work will take time to be completed; however, there is significant potential for this work to make a real-world impact, particularly in the case of cyanobacterial artemisinin production. The work presented here lays the foundation for future research into cyanobacterial terpenoid production.

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8 Appendix

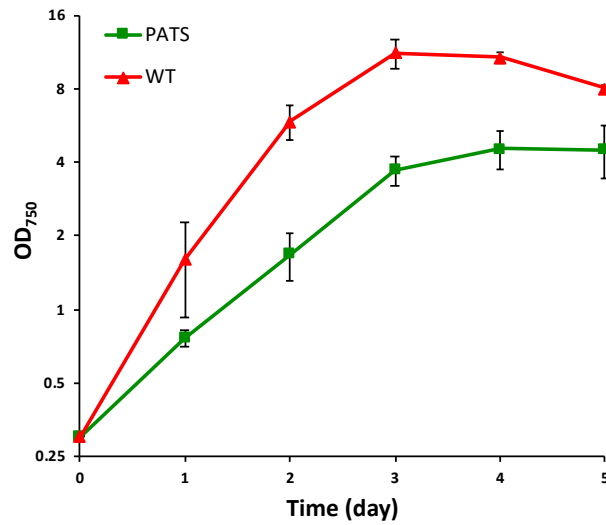


Figure 31 Preliminary experiment showing a growth inhibition for the PATS strain relative to the wild type under mixotrophic conditions. Growth curves were plotted on a log base 2 scale to better represent exponential growth.