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Metabolic Engineering of *Synechocystis* sp. PCC 6803 for Terpenoid Production

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Abstract
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In the Paris Agreement from 2015, nations agreed to limit the effects of global warming to well below 2°C. To be able to reach those goals, cheap, abundant and carbon neutral energy alternatives needs to be developed. The microorganisms that several billion years ago oxygenated the atmosphere; cyanobacteria, might hold the key for creating those energy technologies. Due to their capacity for photosynthesis, metabolic engineering of cyanobacteria can reroute the carbon dioxide they fix from the atmosphere into valuable products, thereby converting them into solar powered cell factories.

Of the many products bacteria can be engineered to make, the production of terpenoids has gained increasing attention for their attractive properties as fuels, pharmaceuticals, fragrances and food additives. In this thesis, I detail the work I have done on engineering the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 for terpenoid production. By deleting an enzyme that converts squalene into hopanoids, we could create a strain that accumulates squalene, a molecule with uses as a fuel or chemical feedstock. In another study, we integrated two terpene synthases from the traditional medical plant *Coleus forskohlii*, into the genome of *Synechocystis*. Expression of those genes led to the formation of manoyl oxide, a precursor to the pharmaceutically active compound forskolin. Production of manoyl oxide in *Synechocystis* was further enhanced by engineering in two additional genes from *C. forskohlii* that boosted the flux to the product. To learn how to increase the production of squalene, manoyl oxide or any other terpenoid, we conducted a detailed investigation of each step in the MEP biosynthesis pathway, which creates the two common building blocks for all terpenoids. Each enzymatic step in the pathway was overexpressed, and increased flux was assayed by using isoprene as a reporter and several potential targets for overexpression were identified. The final part of this thesis details the characterization of native, inducible promoters and ribosomal binding sites in *Synechocystis*.

Keywords: Metabolic engineering, Cyanobacteria, Synechocystis, Terpenoids, Genetic tools

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We can't change the world unless
we change ourselves.
- The Notorious B.I.G.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Englund**, **E.**, Pattanaik, B., Ubhayasekera, S. J. K., Stensjö, K., Bergquist, J., Lindberg, P. (2014) Production of squalene in *Synechocystis* sp. PCC 6803. *PLoS One*, 9:e90270

II **Englund**, **E.**, Andersen-Ranberg, J., Miao, R., Hamberger, B., Lindberg, P. (2015) Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide. *ACS Synthetic Biology*, 4:1270-1278

III **Englund**, **E.**, Lindberg, P. Effect of Expression of MEP Pathway Enzymes on Production of Isoprene in *Escherichia Coli* and *Synechocystis* sp. PCC 6803. *Manuscript*

IV **Englund**, **E.**, Liang, F., Lindberg, P. (2016) Evaluation of promoters and ribosome binding sites for biotechnological applications in the unicellular cyanobacterium *Synechocystis* sp. PCC 6803. *Scientific Reports*, 6:36640

Contents

Abbreviations

Synechocystis	*Synechocystis* sp. PCC 6803
E.coli	*Escherichia coli*
TCA-cycle	Tricarboxylic acid cycle
MEP pathway	Methylerythritol-4-phosphate pathway
MVA pathway	Mevalonate pathway
EYFP	Enhanced yellow fluorescent protein
Pdc	Pyruvate decarboxylase
Dxs	1-deoxy-D-xylulose 5-phosphate synthase
Idi	Isopentenyl diphosphate isomerases
Shc	Squalene hopene cyclase
Sqs	Squalene synthase
CfTPS	*Coleus forskohlii* terpene synthase
IspS	Isoprene synthase
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
P450s	Cytochrome P450 monooxygenases
3-PGA	3-phosphoglycerate
G3P	Glyceral-3-aldehyde
IDP	Isopentenyl diphosphate
DMADP	Dimethylallyl diphosphate
GPP	Geranyl diphosphate
FPP	Farnesyl diphosphate
GGPP	Geranylgeranyl diphosphate
TPP	Thiamine diphosphate
NADPH	Nicotinamide adenine dinucleotide phosphate
ATP	Adenosine triphosphate
IPTG	Isopropyl β-D-1-thiogalactopyranoside
RBS	Ribosomal binding site
BCD	Bicistronic design
5'UTR	5' untranslated region
SD	Standard deviation
µE	µmol photons m^{-2} s^{-1}
DCW	Dry cell weight

Introduction

The motivation for this work

The challenges we will be facing as a global community in the coming years are many; the failure of climate change mitigation, wars, the fresh water supply crisis and energy price shock to name a few [1]. The cause or contributor to those problems is our excessive use of fossil fuels. As the reserves of cheap oil become depleted and energy prices go up [2], economic growth will decline and hostilities between states will likely increase. Oil scarcity also leads to food price increases [3], due to the heavy reliance on fossil fuel for machines and fertilizers in our food production, which then leads to political instability and civil unrest [4].

Other than it just simply is running out, the other major problem with our use of fossil fuel is the impact it has on climate change. In the latest international climate change panel report, human activities were credited as the dominating cause of climate change, owing to our release of CO_2 from fossil fuels [5]. More droughts and heat waves, more intense weather patterns and rising sea levels are all predicted consequences with our current rate of emissions [6]. In 2015, the Paris Agreement was ratified by many nations and adopted under the United Nations Framework Convention on Climate Change, with the stated goal of limiting global warming to well below 2°C. But even at the agreed levels of reduction in carbon emissions, some studies say the agreement levels are not enough and that global mean temperatures will rise past 2°C [7].

We need alternatives to the fossil fuels we use today. Their use is not sustainable in regards to them being finite and because of their damage to our environment. To replace fossil fuels, we need renewable alternatives that do not contribute to the net carbon content in the atmosphere, can be made in large quantities cheaply and work with the infrastructure demands on fuels. The obvious choice of the source of the energy is the sun, since in one hour, the sun light hitting the earth is equivalent to a whole year's worth of energy consumption [8]. And as biologists, we like to focus on nature's way of capturing sunlight; through photosynthesis.

Cyanobacteria and their biotechnological potential

Cyanobacteria are a diverse group of gram-negative bacteria and the inventors of oxygenic photosynthesis, some 2.3 billion years ago [9]. They have played a special role in earth's history, by being the producers of the oxygen that transformed earth's atmosphere and that we all breathe today. They are important for the earth's ecosystem as primary producers in the oceans, responsible for 20-30% of overall photosynthetic production today [10]. Morphologically, they comprise a diverse group of unicellular, filamentous and colonial strains and can inhibit most ecological niches, from the frozen tundra to the scorching desert [11]. One of their most important contributions to the development of life on earth is in the endosymbiotic relationship they formed with eukaryotic organisms, creating a partnership that transcended the ages. Inside the slightly bigger cells, the cyanobacteria would morph into what is today the chloroplast, thereby creating the origin of all our plants, and made life on land possible [12].

Other than their prominent role in earth's history and their current importance as primary producers, cyanobacteria are also interesting for their potential use as fuel producers. They capture sunlight through two different protein complexes called photosystems I and II, splitting water to oxygen, electrons and protons. The electrons freed from water splitting are then used to fix CO_2 from the air, which is the basis for creating all the organic molecules that the cells are made of [9]. By altering the genetic make-up of cyanobacteria, we can hijack their light capturing ability and change them to store the chemical energy captured in photosynthesis in a form that is useful to us. This is the process that human societies have used on our edible crops since the beginning of agriculture, selecting the plants with big fruits to create genetically distinct strains that store energy from sunlight as chemical energy in the form of food. Photosynthesis is also the light capturing process we use to make biofuels, where corn and sugar cane is fermented to make ethanol by microorganisms [13].

Using cyanobacteria instead of plants to make valuable compounds such as fuels is beneficial for several reasons. Photosynthesis in cyanobacteria is several times more efficient than in plants [14], they do not spend energy making non-fermentable parts such as stems and roots, they grow year round, are easy to genetically engineer and they enable a minimum amount of steps from CO_2 fixation to end biofuel product, thereby reducing energy waste [15]. In fact, based on the product yields from cyanobacteria compared with that from the fermentation of plants, fuel production per acre could be increased 8-fold [16]. Another key benefit of cyanobacterial based production is that they do not need to be grown on arable lands and thereby compete with our food production. In fact, many species naturally grow in salt water, allowing for large scale cultivations in desert regions or along the coast in seawater, thereby decreasing our use of fresh water and the competi-

tion between food and fuel production [17]. Also, because many cyanobacteria can fix atmospheric nitrogen, the need for supplemented nutrients and fertilizers could be minimal [9].

One of the most well studied cyanobacteria and the one that has lent its name to the title of this thesis is *Synechocystis* sp. PCC 6803 (*Synechocystis*). It is a unicellular bacterium that was first isolated from a freshwater pond in Oakland, California and has since then become a model organism for cyanobacteria and for photosynthetic research [18]. Due to being highly amendable for genetic modification, naturally taking up exogenous DNA and incorporating it into its chromosome, and by being the first cyanobacterium to be sequenced [19], the popularity and wealth of knowledge about it continues to expand.

Metabolic engineering

Metabolic engineering is the rewiring of the cell metabolism to provide the ability to create new products or enhance the production of already existing ones [20]. Due to evolutionary divergence and variety of different living circumstances, there is a large diversity in the cellular metabolisms between organisms. Some grow anaerobically and need to make fermentation products to have an electron sink, others deal with grazing insects and need a way to defend themselves against them. This plethora of experiences and adaptations has led to the evolution of hundreds of thousands of different natural products made, some of which have attractive properties, such as pharmacological activities [21] or a high energy content [22]. However, extracting the intracellular product from the organism is not always feasible, due to the often only small quantities produced [23]. In those situations, the enzymes in the biosynthesis pathways of that product can be identified using new sequencing technologies, and the genes responsible isolated [24]. Then, insertion of DNA encoding those genes into a microbial host, such as a cyanobacterium, will transfer the property to make that product. In that way, production of the desired compound can be synthesized directly from CO_2, sunlight and water, in a bacterial host with superior cultivation properties, to titers that can be step wise increased by additional engineering until reaching commercially viable levels (**Fig. 1**). Also, metabolic engineering enables creating completely new products never before seen in nature, by making combinations of enzymes that normally never interact [25], or by making changes in the catalytic core of enzymes and thereby altering their function [26].

The early works of metabolic engineering involved trying to increase production of antibiotics in fungi. Because no tools for genetic engineering were available at the time, they used random mutagenesis and screened for chang-

es in phenotype. This was at times a very successful approach, improving penicillin production in *Penicillium chrysogenum* by a 10,000-fold. Later when genetic engineering became possible, most work focused on expressing recombinant protein, and not on modifying metabolisms for product formation [20]. Since then, a vast amount of different products have been made in microorganisms, such as fuels, pharmaceuticals, food additives, fragrances and bulk chemicals [27]. The most commonly used microorganisms for those biotechnological applications are *Escherichia coli* (*E.coli*) and *Saccharomyces cerevisiae* (yeast), due to the relative ease with which you can redirect their metabolism and because of the many genetic tools available [26].

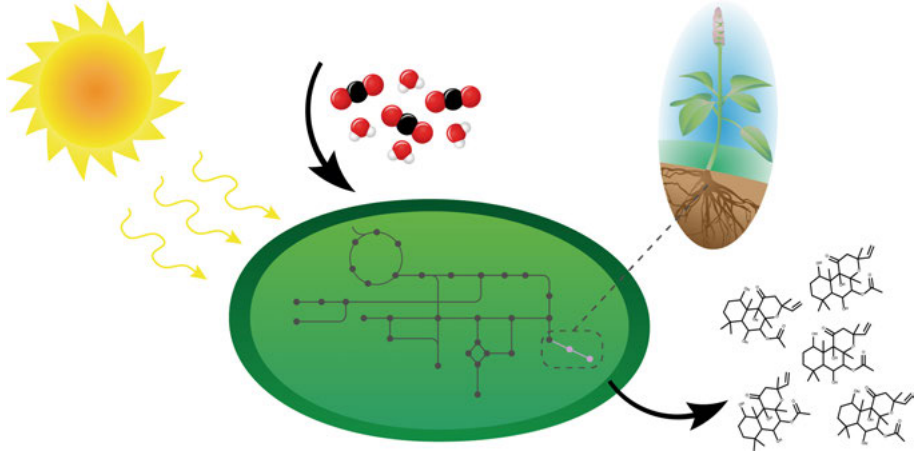

Fig. 1. The use of cyanobacteria as a production host, with manoyl oxide biosynthesis as an example.

Examples of metabolic engineering

The different possible products that can be made by metabolic engineering can be divided into high value compounds and low value compounds. High value compounds are products such as pharmaceuticals, that have high commercial value, while products such as amino acids, vitamins, flavors and fragrances are between high and low value, and bulk chemicals such as solvents and transport fuels being examples of low value compounds [28]. Production of low value compounds in microorganisms typically require a much greater efficiency due to profit margins being lower, and because of competition from making the same product inexpensively from petroleum [29]. Because the cost of the carbohydrate feedstock required for heterotrophic organisms such as *E.coli* or yeast can be more than half of the total production cost, CO_2 and light “eating“ cyanobacteria are an attractive alternative for making low value compounds [30]. Also, the solar-to-product efficiency is much higher for cyanobacterial production, 1.5% of solar energy can be

stored as a product, while the energy efficiency for heterotrophic production for ethanol is around 0.2% [16].

The cost to develop a microorganism that make a product in an economically competitive amount has been estimated to be around 50 million dollars and require 6-8 years of development. Even so, many companies are already producing compounds that have been developed using metabolic engineering, examples being the drug precursors artemisinic acid, the biofuels isobutanol and chemical building block 1,3-propanediol [20]. As a technology, metabolic engineering shows great future potential and was named as one of the top ten emerging technologies of 2016 by the World Economic Forum, together with the self-driving car and next generation batteries [31].

Engineering strategies

There are many strategies for accumulating a desired product in microorganisms. In the most basic system, only the native production capabilities of the microorganism are used and possibly enhanced by special growth conditions, such as sulfur depriving *Chlamydomonas reinhardtii* to get hydrogen production [32]. Another way of enhancing natively producing metabolites is by knocking out enzymes whose substrate you want to produce, thereby leading to an accumulation in the cells (Paper I) [33]. When the product is not natively made in the host, that metabolic ability needs to be introduced. Engineering a production capacity sometimes only requiring a single gene being expressed [34], while other times requiring a multitude of enzymes, such as for the production of opioids where twenty three metabolic steps had to be inserted to get the finished product [21].

The enzymatic properties of the heterologously expressed proteins are important to consider. All enzymes exist in several organisms but with different properties. Therefore, finding and using the most efficient enzyme for a specific catalytic step can improve production [35]. If there is no enzyme that can satisfactory catalyze the reaction with a high enough efficiency, making rationally designed mutations to an enzyme can enhance its properties [36], or even make it favor the reverse reaction [37]. Another way to improve the performance of enzymes is by using directed evolution, where you set up the conditions so that the organism requires that specific enzymatic step to grow, and then let evolution improve the enzyme for you [38].

The expression of the inserted pathway is a key parameter and has a large impact on final product formation. In some cases, getting as high amount of enzyme as possible directly improves production in a linear pattern [39], while in other cases, an inducible expression is required to prevent genetic instability of the production capabilities (Paper IV) [40]. Modulating and fine tuning the expression of individual enzymes in a multi enzyme pathway

can also have a big impact on production, by maximizing flux and minimizing superfluous synthesis of proteins [41].

Another strategy to increase flux to a desired compound is to overexpress the metabolic enzymes that are upstream of the product, to pull in carbon flux from the central metabolic reactions [42]. Alternatively, flux can be increased by deleting or down-regulating competing pathways that share the same substrate as the product, thereby channeling more substrate to that metabolite [43].

The basic properties of the microbial host can be enhanced through genetic engineering to positively affect production. How efficiently the microorganisms grow from their substrate directly correlates with yield. Therefore, increasing the efficiency of carbon fixation in autotrophic organisms is a functional strategy [44], or engineering in the capacity to use cheap and abundant substrates, such as lignocellulose, can increase the economic viability of a heterotrophic production system [45]. Another enhancement to the production strain can be to increase the tolerance of the host to the sometimes toxic product [46]. Also, introducing transporters can prevent intracellular buildup of hydrophobic products, and allow the metabolite to accumulate outside the cells to higher levels [47].

Several methods have been developed that tries to identify the underlying metabolic processes that impact the efficiency of production strains. With metabolomics, the abundance of intermediate metabolites is measured and potential accumulating bottlenecks can be identified, while in the related metabolic flux analysis method, the progress of an isotopically labeled molecule can be tracked which provides data on the flux through each metabolic steps [48]. Another way is by using metabolic models, which are increasing in quality as they are become more comprehensive and better at predicting cellular behavior, allowing non-intuitive targets for up- and downregulation to be identified [49].

Engineering of cyanobacteria

In a cyanobacterial cell, CO_2 is fixed using the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) which generates the product 3-phosphoglycerate (3-PGA) [50]. The energy the bacteria needs to build up molecules and drive cellular processes comes from the light captured by photosystem I and II. Water is split by photosystem II, releasing two electrons which eventually are used to make NADPH, and a proton gradient is built up to drive an ATP synthase [9]. The 3-PGA from carbon fixation is then the basis for every single carbon containing molecule in the cell, with the energy coming from ATP and NADPH generated by the light reactions.

Usually, low value products that need to be made in large volume to low costs are engineered into cyanobacteria, such as fuels, chemical building

blocks or food additives, but higher value compound production also occur [51]., Because cyanobacteria has a slower growth compared to heterotrophic production, rate of productions are slower. Additionally, when growing under light, they do not normally make fermentation products. Therefore, unlike *E.coli* and yeast which ferments when grown anaerobically, cyanobacteria do not have a growth condition that is optimized for making reduced molecules.

The capabilities to make many different products have been engineered into cyanobacteria, see **Fig. 2** for an extensive list. The highest productivity and titers have been reached for products such as ethanol [52], 2,3-butanediol [53] and lactic acid [39], with more than 50% of fixed carbon being redirected towards product formation [54]. What those and similar products share is being only a couple of metabolic steps from CO_2 fixation, where all carbon flux originates from, and having a high intracellular concentration of the substrate [55]. Metabolic engineering of products derived from pyruvate (ethanol, isobutyraldehyde, 2,3-butanediol) or fructose-6-phosphate (sucrose, mannitol, glycerol) usually reach g/L titers while products from the terpenoid pathway or ethylene which has a TCA-cycle metabolite as substrate, typically reach mg/L titers [54]. Of course, the abundance of substrate is not the only thing that affects product, as recently demonstrated with an engineered *Synechococcus* strain making 1.26 g/L isoprene from the terpenoid pathway [35]. Other factors such as the catalytic efficiencies of the enzymes [56], whether the product accumulates inside the cell or outside in the media and if the heterogeneous pathway contains a decarboxylation step, thereby creating favorable thermodynamic properties [57], all contribute in various degree to production. Another important factor is how much research has been performed to develop the production system for that molecule. The high producing isoprene strain could be engineered after building on the work of six previous scientific papers that described generations of isoprene producing cyanobacteria, showing the importance of persistency and the possibilities of committed metabolic engineering.

Cyanobacterial production strains are already today being tested in pilot-scale facilities, by companies such as Algenol, Sapphire Energy and Solazyme. Still, there is a long way until large scale cultivations can be economically and energy efficient, something that improved strain performances and better cultivation and product separation technologies can help realize [58].

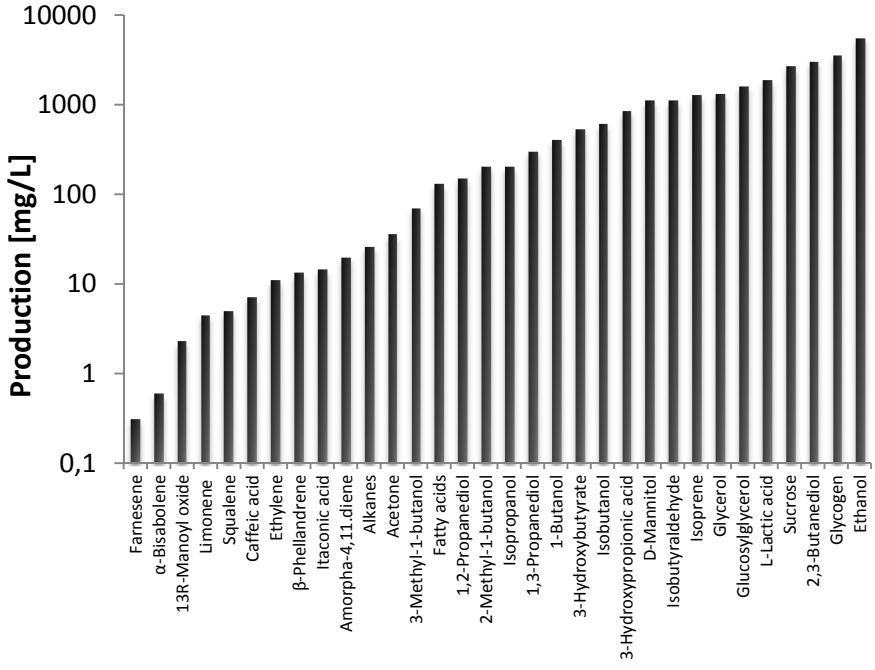

Fig 2. Highest reported production of various chemicals in cyanobacteria. Data compiled from [54, 59, 60].

Terpenoids

Terpenoids, or isoprenoids, are a large and structurally diverse class of molecules with tens of thousands of different known compounds [61]. They play vital roles in all living organisms in electron transport chains, cell wall and membrane synthesis and stability [62]. The biggest source of terpenoids comes from the plant kingdom, where they are involved in growth and development, or as secondary metabolites, fending off herbivores and interacting with the environment [63]. This has given some terpenoids properties that make them interesting for humans, for uses as fragrances, flavoring, colorants, cosmetics and pharmaceuticals. The taste of cinnamon, color of tomatoes and scent of eucalyptus are all derived from terpenoid molecules, and many can been used as drugs, such as artemisinin, one of the most potent antimalarial drugs available [62].

All terpenoids are made from the same two five-carbon (C5) precursor molecules isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) (**Fig. 3**). They are fused together to create longer and longer carbon chain length molecules, making first C10 geranyl diphosphate (GPP), then C15 farnesyl diphosphate (FPP) and finally C20 geranylgeranyl diphosphate (GGPP). Terpenoids are then synthesized from either of those precur-

sor molecules, gaining their name from how many carbons they contain. Diterpenoids are C20, triterpenoids are C30 and so on [64]. The different length precursor molecules are then converted into specific terpenoids by terpene synthases which create the structure of the compound, such as closing the molecule to create a ring structure, and then by cytochrome P450 monooxygenases (P450s) which decorate the molecule through reactions such as hydroxylations, epoxidations and deaminations [65]. Together, terpene synthases and P450s can create remarkably complex molecules which are difficult to chemically synthesize, partly explaining the interest in creating the molecules in biological systems [66].

MEP pathway and MVA pathway

The precursor molecules for all terpenoids, IDP and DMADP, can be made from two different pathways, either the methylerythritol-4-phosphate (MEP) pathway or the mevalonate (MVA) pathway. Most bacteria and plant plastids use the MEP pathway, while you and other eukaryotes, archaea and some bacteria use the MVA pathway [67]. To synthesize IDP and DMADP, the mevalonate pathway requires three acetyl-CoA while the MEP pathway uses a pyruvate and glyceral-3-aldehyde (G3P) molecule each. Due to the loss of CO_2 when acetyl-CoA is made from pyruvate, the MEP pathway is more efficient with regards to carbon utilization [68]. Having a pathway with lower loss of carbon is important for autotrophic organisms which have an energy investment in each carbon fixed. Due to the stoichiometrically higher efficiency of the MEP pathway, several studies have argued that it should be used instead of the MVA pathway [69], even though utilizing the MVA pathway for terpenoid production have been more successful so far [61].

The MEP pathway consists of seven enzymatic steps to create IDP and DMADP from pyruvate and G3P, and an eighth enzyme that interconverts IDP and DMADP (**Fig 3**). The first step is catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which is widely regarded as the bottleneck of the pathway, pulling in carbon from the central metabolism [70]. Another important enzyme in the pathway is isopentenyl diphosphate isomerases (IDI), which maintains a balance between IDP and DMADP, preventing over-accumulation or depletion of either. The enzyme has been shown to be especially important when engineering terpenoid production, by correcting an IDP:DMADP balance that becomes skewed [35]. The full regulation of the pathway is still unknown, but a key regulatory feature is the feedback inhibition that IDP and DMADP exerts on DXS, by competing with the co-factor thiamine diphosphate (TPP) for a binding site [71].

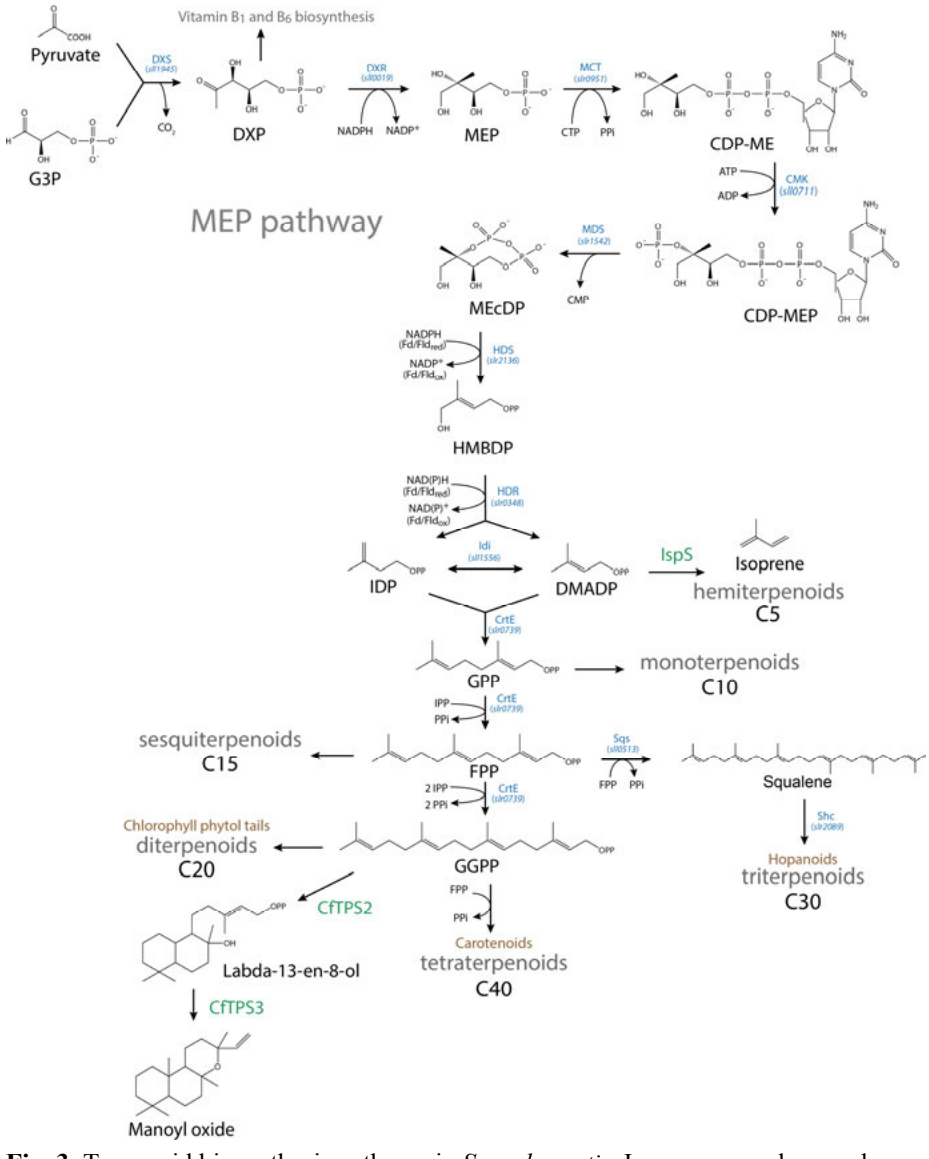

Fig. 3. Terpenoid biosynthesis pathway in *Synechocystis.* Isoprene, squalene and manoyl oxide are highlighted as they are all molecules whose production is described in this thesis. Native enzymes are marked in blue, heterologous ones in green.

Terpenoid production in cyanobacteria

There are several reasons why cyanobacteria are attractive hosts for terpenoid production. Many terpenoids have properties that make them suitable to

be used as fuels [61], which could be made cheaply in cyanobacteria, as discussed previously. Cyanobacteria also have a high natural flux towards terpenoid production [34], due to many molecules involved in photosynthesis being made from the pathway, such as carotenoids, the phytol side-chain of chlorophylls and the prenyl part of plastoquinone [72]. Less common is using cyanobacteria for production of more complex, pharmaceutical plant terpenoids. However, P450s are typically dependent on NADPH, which in cyanobacteria is readily provided by photosynthesis, but can be limiting factor for production in heterotrophic bacteria [73].

Many different types of terpenoids have been produced in cyanobacteria. Isoprene, the simplest of all terpenoids, has probably gained most attention so far [34, 35, 72, 74-76]. Other terpenoids produced in cyanobacteria are β-caryophyllene [77], β-phellandrene [78], limonene [79], farnesene [80], bisabolene [81], squalene (Paper I) [33], manoyl oxide (Paper II) [42] and amorpha-4,11-diene [82]. In this thesis, cyanobacterial production of three different terpenoids is described, the hemiterpenoid isoprene, the sesquiterpenoid squalene and the diterpenoid 13R-manoyl oxide, representing different potential uses for biotechnologically produced terpenoids; as chemical feedstock, biofuels and medicines.

Genetic tools

A typical bacterium contains several thousands of different genes that are expressed in a well-controlled manner. Expression of genes has to be done at specific conditions and with precise strengths, control of which is mediated through the actions of several genetic elements (**Fig. 4**). The most important sequence to impact gene expression is the promoter region. It is the binding site for the RNA polymerase and the initiation site of transcription. Based on their binding affinity to the RNA complex, promoters can be said to be strong if they recruit a high amount of RNA polymerases, while a weak promoter only facilitates low amount of transcription [83]. The expression of promoters can be dynamically controlled from operator sequences adjacent or inside the promoter region, where transcription factors bind to activate or inhibit expression [84]. After the RNA-polymerase have bound to the promoter and initiated transcription, it will copy the DNA strand into messenger RNA (mRNA) until it reach the hairpin structure of a terminator, where it will stop. The ribosome can bind to a ribosomal binding site (RBS) on the mRNA, which is positioned slightly upstream of the start of the gene, and initiate translation from the start codon until the stop codon, synthesizing a complete protein in the process.

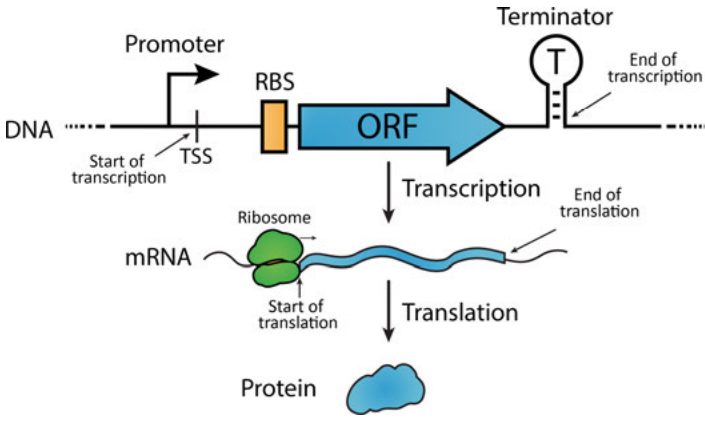

Fig. 4. The central dogma of biology and the genetic parts required for expression of a gene. Abbreviations used: TSS = transcriptional start site, ORF = open reading frame, RBS = ribosomal binding site, mRNA = messenger RNA.

Tools for heterologous expression

The same mechanisms controlling expression of native genes needs to be applied for heterologously expressed pathways. A promoter is required for transcription to occur, an RBS for each gene to be translated, and a terminator to end transcription. By characterizing promoters and RBSs to determine their strengths and properties, they can be added to a genetic toolbox, from where you can pick and combine parts to suit a specific engineering need [85].

A plasmid is usually the carrier of the expressed genes, and can have several copies per cell up to many hundreds [86]. Plasmids can also be used for inserting expression cassettes in the genome of certain organisms, usually by containing homologous regions flanking the DNA sequence to be inserted, and then using homologous recombination to integrate it to the chromosome [87].

Usually the selection of promoter has the largest impact on the result of the engineering [39]. Some promoters are inducible, meaning that they only turn on when an external stimuli is applied, usually a chemical. For engineering of heterologous pathways that are detrimental to the host, inducible promoters can be important to prevent the genes from being lost due to genetic instability, and for turning on expression only during the production phase (Paper IV) [40].

Promoters, RBSs, terminators and plasmids are not the only tools available for genetic engineering. Parts such as antibiotic cassettes to enable selection, protein tags to detect proteins and reporter genes to quantify expression are all important components [86]. More recently, advanced expression regulators such as CRISPRi [88], riboswitches [89] and TALEs [90] have come into focus, enabling more complex engineering to be done. Important to note

is that the properties of the genetic tools do not necessarily behave in the same way between species. Differences in the sigma factors of the RNA-polymerase between *Synechocystis* and *E.coli* for example make many *Synechocystis* promoters nonfunctional in *E.coli* (Paper IV) [40]. Therefore, characterization of the same parts needs to be repeated in different strains, to ensure a consistent behavior.

Another issue that complicates engineering is that using a strong promoter and strong RBS does not necessarily generate a high expression [91]. The mRNA sequence of the gene being expressed interacts in unpredictable ways with the 5' untranslated region (UTR) upstream of the gene to form secondary structures in the mRNA that can block translational initiation [92]. To circumvent that problem and increase the reliability of gene expression, "bicistronic design" (BCD) can be used, where a short coding sequence is placed upstream of the gene to be expressed [93]. When the ribosome translates the small coding sequence, it will melt any secondary structures that prevent translation of the gene. Another way to increase translational initiation is by using a self-cleaving ribozyme called RiboJ, which cleaves off the 5' untranslated region (5'UTR), leaving only a stable structure which does not block ribosomal binding [94].

Expression tools for *Synechocystis*

There are fewer genetic tools available for engineering *Synechocystis* and other cyanobacteria, than there are for more commonly used prokaryotes like *E. coli* [95]. The promoters used for metabolic engineering are usually native ones that express highly abundant proteins such as the *psbA2* promoter, expressing the D1 promoter from photosystem II, or the RuBisCO promoter P*rbcL*. There are few known, inducible promoters in *Synechocystis* that are capable of giving both high expression and low un-induced expression. While the strong, synthetic, lactose inducible promoter P*trc* has been used successfully for 2,3-butanediol production in *Synechococcus elongatus* PCC 7942 [96], only constitutive expression is possible in *Synechocystis* due to induction not functioning properly [85]. The Tet-promoter series consists of a wide variety of well-regulated promoters, but due to the light sensitivity of the inducer anhydrotetracycline, they are difficult to work with during phototrophic growth [97]. At the moment, probably the best choice for inducible expression is using native inducible promoters, such as the copper inducible *petE* promoter, or the metal inducible promoters from a gene cluster encoding metal efflux pumps, P*nrsB*, P*coaT* and P*ziaA,* which are described in Paper IV [98]. For the choice of RBS, most often a strong translation initiation is desired for *Synechocystis* expression, which RBS*, a synthetic RBS with a perfectly complimentary sequence to the ribosomal anti-Shine-Dalgarno sequence, has [11].

Expression in cyanobacteria can be done either by integrating expression construct into the genome or by keeping it on a self-replicating plasmid. Expression on a self-replicative plasmid requires a replicon that is functional in *Synechocystis*, with RSF1010 being commonly used. Those plasmids can be transferred from *E.coli* into a *Synechocystis* cell by conjugation, and after entering the cell, they will start to replicate and maintain themselves [11].

Other than the basic tools for engineering such as promoters and RBSs, advanced tools to regulate expression such as riboregulators and CRISPRi, have also been developed for *Synechocystis*. The riboregulator works by an inducible expressed RNA binding to an mRNA and thereby exposing the RBS for translation, in this way creating an inducible expression [89], while the CRISPRi system enables a multi gene repression, by using small guide RNA directing the nucleus deficient Cas9 to bind and block transcription of targeted genes [99]. The latter tool can be especially useful for inducible blockage of competing, but essential, pathways of desired products.

Aim

The aim of the work presented in this thesis and which was undertaken during my five years of PhD studies can be summaries in three points:

I. To construct and characterize terpenoid producing strains of *Synechocystis*.

II. Investigate the properties of the terpenoid biosynthesis MEP pathway and ways to increase flux through it, to enhance the production of any terpenoid.

III. To develop tools to enhance and simplify engineering of cyanobacteria, and specifically *Synechocystis*.

Results and Discussion

Construction of plasmids for *Synechocystis* engineering (Paper II & III)

To make the expression vectors used for engineering of *Synechocystis* can be a time consuming task. Typically, several pieces of DNA needs to be combined to create a functional construct, such as gene sequences, promoters, terminators, antibiotic cassettes and homologous recombination sequences. Several new DNA cloning techniques such as Gibson assembly [100] and Golden Gate cloning [101] can assemble several pieces together in a single step, but the experiences from our lab, at least of the former technique, is that the end result can be inconsistent. The traditional cloning technique, employing restriction enzymes and ligase for cloning typically generates more predictable results, but is limited in the amount of parts that can be assembled in one step.

pEERM vectors

To speed up the process of generating expression constructs, we created several plasmids that would only require a single ligation step to create a construct capable of heterologous overexpression in *Synechocystis*. The pEERM series of vectors were designed for integration of heterologous genes into the genome of *Synechocystis* at different loci and expression with different promoters. The base pEERM plasmids contain a promoter, RBS, terminator, antibiotics cassette and the homologous regions that decides at which site in the genome integration will occur. Insertion of a single or multiple genes into the plasmid is done using a method similar to BioBrick cloning, where the capacity of two restriction sites to form a scar when ligated together and thereby, move the cloning site downstream for each gene inserted, is used [102] (**Fig 5A**). The plasmids come with two different promoters, the strong *psbA2* promoter or the nickel inducible *nrsB* promoter, and with four different integration sites; in frame replacement of the *psbA2* gene, thereby "stealing" its promoter [103], in neutral site 1 which knocks out the hypothetical gene *slr1068* [104], in neutral site 2, upstream of a pseudogene [105] or in the site of squalene synthase (sqs), knocking out the cells' capacity to make triterpenoids (Paper I) [33]. The use of the pEERM vectors are detailed in Paper II.

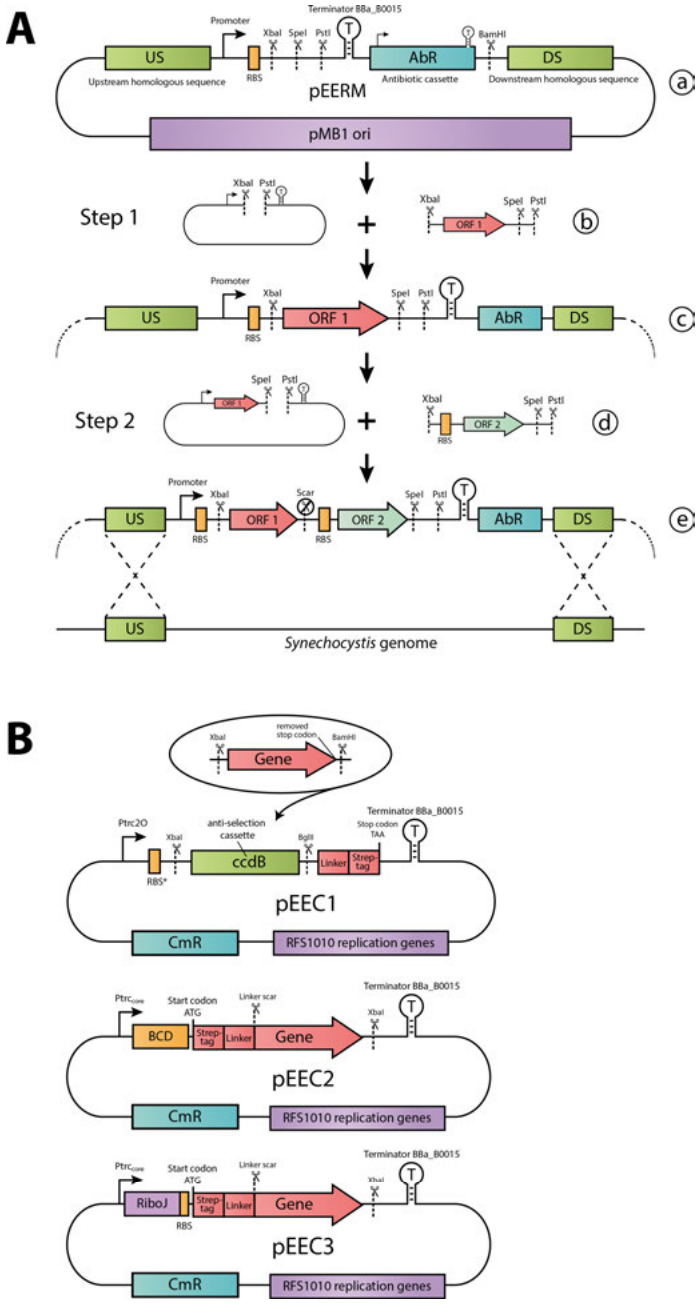

Fig. 5. Plasmid maps and cloning strategies for pEERM and pEEC. (A) Plasmid map over empty pEERM plasmid (a) which is cut (b) and an open reading frame (ORF) is inserted (c). By cutting downstream restriction sites, a second gene (d) can be ligated in and the downstream restriction sites are reformed (e). (B) The cloning strategy exemplified by pEEC1 and the plasmid maps for pEEC2 and 3 after an ORF has been inserted.

pEEC vectors

Another set of standardized expression vectors were constructed for the work described in Paper III; the pEEC series of vectors (**Fig. 5B**). Due to pEEC containing the broad host-range-replicon RSF1010 that allows the plasmid to be moved from *E.coli* to *Synechocystis* by conjugation [85], expression can be done in both organisms from exactly the same genetic sequence and context. Expression is driven by the very strong P*trc* promoter, which is inducible in *E.coli* but not in *Synechocystis*, and enhanced by the BCD [93] or RiboJ [94] genetic elements, which are meant to improve translational initiation and thereby expression. Cloning into the plasmids are based on the BglBrick format [106], where a BglII – BamHI scar forms a linker compatible sequence and attaches a strep-tag to the C- or N-terminus of the inserted gene, which allows for detection of the proteins.

The construction of the pEERM and pEEC vectors were done with specific projects in mind but almost any overexpression study in *Synechocystis* could find a use for them. Making and sharing expression vectors is a good way to minimize the amount of time spent on cloning, which is arguably the least exciting part of projects. Also, using a standardized expression system increases consistency and reliability of expression. We have, or will, upload and share these vectors on Addgene.

Production of terpenoids in *Synechocystis* (Paper I & II)

Construction and characterization of the squalene accumulating *shc* deletion strain (Paper I)

The triterpenoid squalene is a 30-carbon pure hydrocarbon that has some commercial uses in cosmetics and vaccines [61], and could be used as a biofuel if produced in high enough volumes [107]. In many bacteria, squalene is converted to hopene by the enzyme squalene hopene cyclase (Shc). Hopene is the precursor molecule to make all hopanoids (**Fig 3**), the function of which are thought to be similar to that of eukaryotic sterols; to stabilize and regulate the fluidity and permeability of membranes [108].

To determine if we could make a squalene accumulating *Synechocystis* strain, and to investigate the role of hopanoids under standard growth conditions, we constructed a *shc* deletion strain by placing a neomycin cassette in what we identified as a gene putatively encoding Shc, resulting in the Δ*shc* strain. Because of the hydrophobic properties of squalene, we reasoned that it would most likely stay in the membranes and not secrete into the media. Thus, if there was any accumulation of squalene, it would likely occur inside the cells. By creating a modified protocol for total lipid extraction, squalene from pelleted cells was extracted and detected using HPLC (**Fig. 6A**). While only small amounts of squalene were detectable in wild type, the Δ*shc* strain accumulated 72 times more, reaching 0.67 mg OD_{750}^{-1} L^{-1} (**Fig 6B**). To confirm that the deletion of *shc* was the cause of the squalene accumulation, a plasmid containing the *shc* region was conjugated into the Δ*shc* strain to complement and restore Shc functionality. The new strain Δ*shc*:pPMQ*shc* accumulated squalene much less than the Δ*shc* strain, which further indicates that the putative *shc* gene really does encode a functional squalene hopene cyclase.

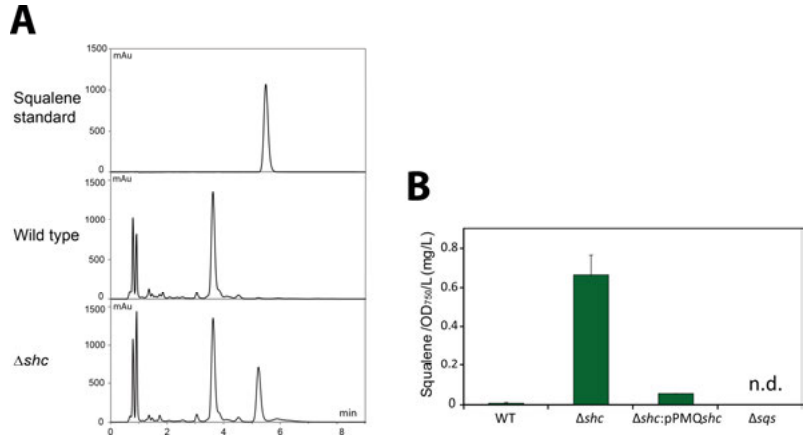

Fig. 6. Detection of squalene. (A) Total lipids were separated using HPLC and squalene was detected at 190 nm, by comparing the retention time with a pure standard. (B) Quantification of squalene in different *Synechocystis* strains, n.d. = not detected, WT = wild type. Results represent the mean of three biological replicates, error bars represent standard deviation.

We also identified a gene putatively encoding squalene synthase (*sqs*), and investigated how it affected squalene accumulation by creating a Δ*sqs* deletion strain. No squalene could be detected in that strain (**Fig. 6B**), indicating that *sqs* was correctly identified and that there are no other pathways for making squalene in *Synechocystis*.

Because the presence of hopanoids have been confirmed in the membrane of the cyanobacterium *Synechocystis* PCC 6714 [109], and squalene is the only known precursor for them, the Δ*shc* strain is likely hopanoid deficient. Also, due to a *shc* deletion strain of *Burkholderia cenocepacia* was reported to have damaged membranes [108], we reasoned that a similar phenotype in *Synechocystis* could affect the photosynthetic machinery in the thylakoid membranes. However, no reduction in growth was observed between the Δ*shc* strain and wild type *Synechocystis*, at low, medium or high light (5, 50 or 500 μmol m^{-2} s^{-1} (μE)).

Next, we tested whether the intracellular accumulation of squalene varies in different growth phases and at different light intensities. Samples were taken for squalene detection from the seed cultures (0 h), the exponential phase (40 h), late exponential phase (88 h) and stationary phase (280 h) from cultures grown at low and medium light. Squalene accumulation increased as the cells entered the later growth phases and was higher at medium light than at low light. The dilution of squalene due to cells dividing is likely the biggest source of squalene reduction, especially since the Δ*shc* strain cannot convert squalene to hopanoids. Therefore, the increase in squalene content per cell can likely be attributed to the slower growth of the cells at later growth phases and a lower dilution rate of the molecule.

In summary, this study showed that a squalene accumulating strain could be engineered by knocking out the enzyme converting squalene into hopene. We could also confirm the identity and function of *shc* and *sqs*. No phenotype was observed under standard cultivation conditions at different light intensities, indicating a nonessential role of hopanoids under standard laboratory growth. The amount of squalene accumulated was equivalent to 0.80 mg g^{-1} DCW, which requires many folds improvement before being close to a commercially viable production. Increasing flux through the MEP pathway could be a way to enhance accumulation, ways of which can be read about in Paper III.

Engineering of the plant diterpenoid manoyl oxide production in *Synechocystis* (Paper II)

Forskolin is a diterpenoid naturally found in the root cork cells of the shrub *Coleus forskohlii* [24]. The plant has been used in traditional Hindu medicine since ancient times to treat a broad range of ailments and is presently used in the treatment of glaucoma [110]. Only the first two steps of the forskolin biosynthesis pathway from *C. forskohlii* has been identified so far. The general diterpenoid precursor GGPP is converted to (13R)-manoyl oxide by the action of the two terpene synthases CfTPS2 and CfTPS3 (**Fig. 3**) [24]. Manoyl oxide is then further modified by several unknown P450s and an acyl-transferase to form forskolin. *Synechocystis* could be a good production host for forskolin due to the NADPH requirements of P450s, which can be a limiting factor in heterotrophic hosts but are readily supplied from the light reaction in phototrophic organisms [73].

In Paper II, we engineer *Synechocystis* to make the forskolin precursor manoyl oxide, which was the first reported attempt at making a complex, pharmaceutical terpenoid in a cyanobacterium. Two terpene synthases from *C. forskohlii* were cloned into three different pEERM plasmids for genomic integration into the site of *psbA2*, neutral site or *sqs*, resulting in plasmids TPS-P, TPS-N and TPS-S (see Paper II for a complete list of strains used). Expression in TPS-P were driven by the strong, light inducible *psbA2* promoter [103], while TPS-N and TPS-S used the nickel inducible *nrsB* promoter [111]. While integration in *psbA2* [112] and neutral site [104] should be silent, the *sqs* integration in TPS-S deletes squalene and hopanoid formation, which according to the results from Paper I, does not affect the viability of the cells. We reasoned that deleting *sqs* and triterpenoid production might lead to an accumulation of FPP, which then can be converted to GGPP by the native enzyme CrtE and thereby, potentially form more manoyl oxide.

The three pEERM constructs carrying the diterpene synthases from *C.forskohlii* where transformed into *Synechocystis* and positive colonies were isolated for each. All three engineered strains with CfTPS2 and 3 did make manoyl oxide, and in a stereospecific pure form. Highest manoyl oxide accumulation was produced from the P*nrsB* driven strain TPS-N, making 0.24 mg g^{-1} DCW (**Fig. 7A**). The disruption of *sqs* did not lead to a higher manoyl oxide production in TPS-S, suggesting that FPP does not become redirected towards GGPP and diterpenoid production. In bacteria, the enzyme GGPP synthase can use DMADP as substrate and make successive additions of IDP or it can use FPP, and add a single IDP to form GGPP [113]. While in yeast and mammals, the GGPP synthase selectively uses FPP to make GGPP [114], the specificity could be the opposite in *Synechocystis*, having low affinity to FPP as substrate, which would explain the results.

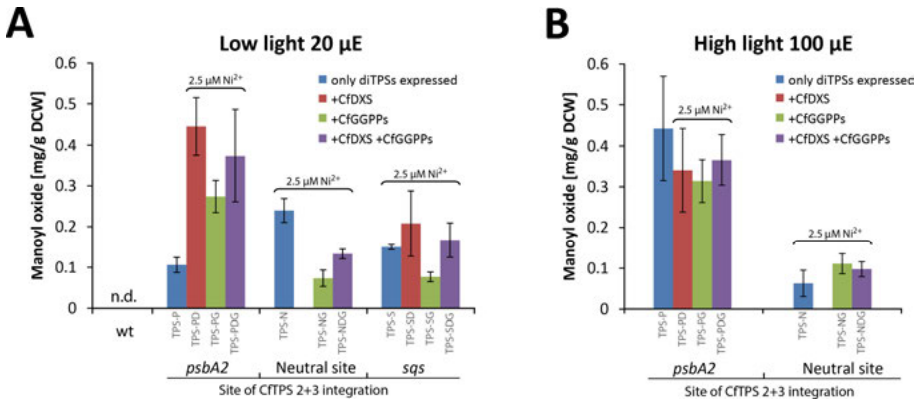

Fig. 7. Manoyl oxide production at (A) 20 µE and (B) 100 µE in engineered *Synechocystis*. Results represent the mean of six biological replicates, error bars represent standard deviation. DCW = dry cell weight, n.d. = not detected.

To boost the production of manoyl oxide, two enzymes upstream of GGPP biosynthesis were expressed; DXS (CfDXS) and GGPP synthase (CfGGPPs) from *C. forskohlii*. DXS is the first enzyme in the MEP pathway and regarded as the bottleneck [70], while GGPPs forms GGPP from DMADP and IDP. By using nonnative enzymes, we hoped to minimize any regulation that could be exerted on the enzymes. CfDXS and CfGGPPs were integrated separately, or as an operon, into neutral site II of all three manoyl oxide producing strain under the control of P*nrsB*, resulting in eight new strains.

The expression of the boosters increased manoyl oxide by up to 4.2 times in the TPS-P strain, when expressing only CfDXS. That is the highest reported terpenoid increase from expressing upstream genes in cyanobacteria, possibly due to the plant enzyme not being susceptible to native regulations (**Fig. 7A**). The same increase in production from CfDXS or CfGGPPs could not be seen in either the TPS-N or TPS-S strain, even decreasing manoyl oxide formation in some cases.

The expression of the *psbA2* promoter is induced by high light [103], and carotenoids, which manoyl oxide share the precursor with, increases with light [115]. Therefore, we wanted to investigate whether manoyl oxide accumulation would increase at high light (100 µE) for selected strains. Production in TPS-P, which expresses CfTPSs from P*psbA2* without boosters, increased 3-fold (**Fig 7B**). In contrast, P*nrsB* driven expression of CfTPSs reduced production with 5.3 times at high light compared with at low light, and boosters failed to increase accumulation in any strain. In Paper IV, we observed a reduced protein accumulation from P*nrsB* driven expression at high light, which could explain these results.

Because the biosynthesis of both carotenoids and the phytol tails of chlorophyll start from the same precursor molecule as manoyl oxide (**Fig. 3**), we investigate the effect of manoyl oxide production and expression of GGPP boosters on those pigments. When grown at low light, the non-boosted strain

that produced the most amount of manoyl oxide, TPS-N, had a significant reduction of carotenoids compared with wild type, possibly due to redirection of GGPP from carotenoids to manoyl oxide. When expressing the GGPP boosters, carotenoids increased in most strains, suggesting that pigment production also increases. The effect on chlorophyll content was less pronounced than for carotenoids, presumably because only parts of chlorophyll is made in the terpenoid pathways. At high light, because the large variation in accumulation of both pigments, we found it difficult to distinguish differences in specific pigments and across every pigment.

The strains engineered in Paper II are the first reported examples of production of high-value pharmaceuticals from complex plant pathways in cyanobacteria. The highest producing strain reached 0.45 mg g^{-1} accumulation of the forskolin precursor manoyl oxide.

Functional characterization of the MEP terpenoid biosynthesis pathway in *E.coli* and *Synechocystis* (Paper III)

The production of squalene reached 0.80 mg g^{-1} in Paper I, while in Paper II, the highest manoyl oxide producing strain accumulated 0.45 mg g^{-1}. To reach higher production titers, carbon flux needs to be diverted from the central metabolic pathways towards terpenoid production. The aim of Paper III was to investigate each enzymatic step of the MEP-pathway in *E.coli* and *Synechocystis*, to find potential bottlenecks that could increase flux through the pathway. The end goal would be to find generalized overexpression targets in the MEP pathway that can be engineered to get consistent increase of production for any terpenoid, such as squalene or manoyl oxide. Also, an engineered plug-and-play strain could be created for terpenoids, with a deregulated central metabolism that only required a terpenoid production capacity to be plugged in to get high titers, thereby speeding up the development process of new strains [20].

We started the investigation of the MEP pathway in *E.coli* and we chose isoprene as reporter of increased flux through the pathway. Isoprene is the simplest terpenoid, created directly from DMADP (**Fig. 3**). The isoprene synthase (IspS) from *Pueraria montana* [34] was codon optimized and cloned into a pET vector, creating pET IspS, and high expression of IspS in a soluble form was confirmed on a protein gel.

With an IspS expressing strain successfully engineered, we next wanted to create a second set of plasmids that would overexpress each of the eight MEP pathway enzymes from *E.coli* and *Synechocystis*. For that, we used pEEC1 which uses the strong, inducible P*trc* for expression, and includes a strep-tag, enabling detection of protein overexpression (**Fig. 5B**). Also, due to pEEC1 being capable of replication in both *E.coli* and *Synechocystis*, the same constructs could in later stage be transferred and tested in *Synechocystis*.

After each MEP gene was cloned into pEEC1, preliminary data showed some constructs not expressing measurable amounts of proteins. Because low or no expression would make us unable to assess the significance of each MEP enzyme, we created two new plasmids, pEEC2 and pEEC3, that enhanced expression of P*trc* using the genetic insulators BCD [93] and RiboJ [94] respectively. We then inserted all eight MEP genes from *E.coli* and *Synechocystis* in all three pEEC vectors to create a total of 48 constructs (see Paper III for complete list of constructs).

All 48 MEP constructs were transformed into the IspS expressing *E.coli* strain, induced with isopropyl β-D-1-thiogalactopyranoside (IPTG), and isoprene was sampled from the head space. The base production of isoprene from pET IspS with the empty vector reached 8.2 μg OD_{595}^{-1} L^{-1}, a level that expressing *E.coli* DXS increased by 14.5 times and IDI by 3.4 times (**Fig.**

8A). Both of those enzymes from *Synechocystis* also led to a higher isoprene production, but *Synechocystis* DXS did not give as high increase as the *E.coli* isoenzyme (**Fig. 8B**). Surprisingly, expression of *IspH* from *Synechocystis* increased isoprene production in *E.coli* by 3.1 times, a so far unknown target for enhanced terpenoid production. A possible explanation could be that the *Synechocystis* IspH has a higher DMADP:IDP synthesis ratio than the enzyme from *E.coli*, thereby creating more substrate for IspS.

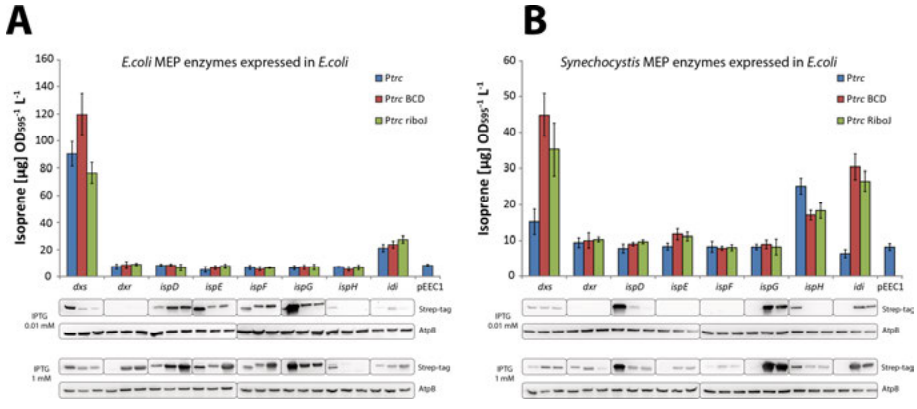

Fig.8. Isoprene production and protein accumulation from MEP enzyme overexpression in *E.coli* strains with pET IspS. MEP enzymes from (A) *E.coli* and (B) *Synechocystis* were expressed in pEEC1 (blue bars), pEEC2 (red bars) or pEEC3 (green bars). Overexpressed and strep-tagged proteins were detected on western-immunoblot with AtpB used as an equal loading control. Each data point represents the mean of six biological replicates, error bars are standard deviation.

The use of expression-enhancing BCD and RiboJ led for many constructs to significant effects on protein expression, in some cases increasing, while in others, decreasing expression (**Fig. 8**). Most striking is the expression of *Synechocystis* IDI, where using BCD or RiboJ led to detectable amounts of protein and an increase in isoprene production, while expression with only P*trc* gave neither of those effects.

DXS from *E.coli* gave the highest increase in isoprene production in our assay. To test if we could find a new bottleneck in the MEP pathway if DXS was co-expressed with IspS, we cloned *E.coli* DXS into pET IspS to form pET IspS eDxs. One construct per MEP gene with the highest protein accumulation or isoprene increase, was transferred into *E.coli* together with pET IspS eDxs. In the resulting strains, IDI from *E.coli* and *Synechocystis* increased isoprene production by 2.8 and 3.2 times respectively, while *Synechocystis ispH* increased amounts by 1.7 times (**Fig. 9**). Expressing DXS from both pET IspS eDxs and the pEEC constructs did not further enhance isoprene accumulation, indicating that step was saturated, and the bottleneck had been shifted elsewhere.

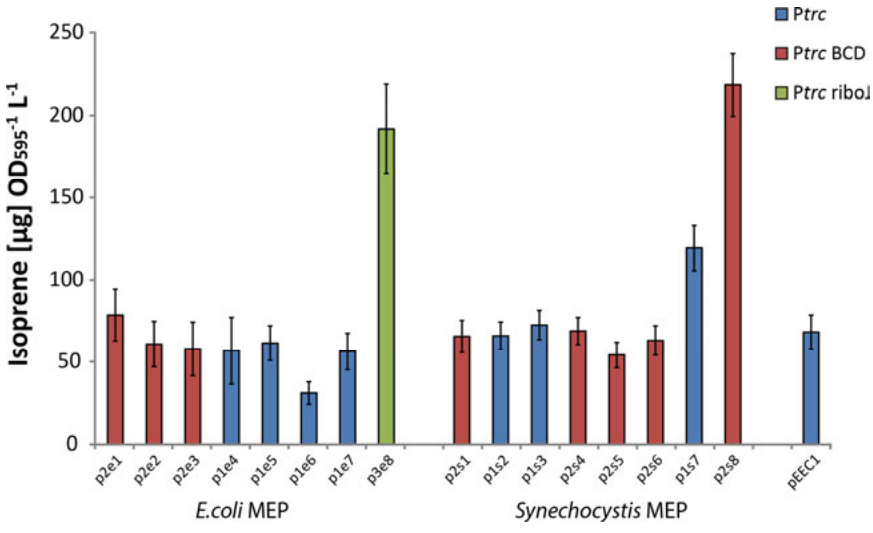

Fig. 9. Isoprene production in *E.coli* with pET IspS eDxs and one MEP enzyme from *E.coli* or *Synechocystis* overexpressed. MEP enzymes were expressed in pEEC1 (blue bars), pEEC2 (red bars) or pEEC3 (green bars). Each data point represents the mean of at least nine biological replicates, error bars are standard deviation.

The next step was to investigate MEP bottlenecks in *Synechocystis* using the same strategy. However, we first tested whether the expression pattern between different constructs expressing the same gene would be the same in *Synechocystis* as they were in *E.coli*. Therefore, we transferred constructs that had large differences in protein accumulation between expression in pEEC1 and pEEC2 in *E.coli,* into *Synechocystis*. In the resulting strains, we observed the same relative expression pattern in *Synechocystis* as in *E.coli*, indicating that a strongly expressing construct in *E.coli* has a higher probability to also have a high expression in *Synechocystis.*

The strongest expressing constructs for each MEP enzyme were conjugated into the *Synechocystis* strain SkIspS generously donated by Anastasios Melis, which have the *P. montana IspS* integrated into the *psbA2* site [74]. In the resulting strains, overexpression of DXS increased isoprene accumulation slightly, by 1.4 and 1.2 times from *E.coli* and *Synechocystis* DXS respectively (**Fig. 10**), an increase which was much lower than in *E.coli*. The native IDI had the biggest impact on isoprene production, increasing it by 1.8 times. The third enzyme in the MEP pathway, *ispD*, enhanced isoprene accumulation in all constructs expressing the different isoenzymes of it, which to our knowledge is a so far unknown target for increased terpenoid production. While *ispF* from *E.coli* also increased isoprene production per cell, this coincided with a growth reduction, making a comparison with the faster growing control strain less reliable.

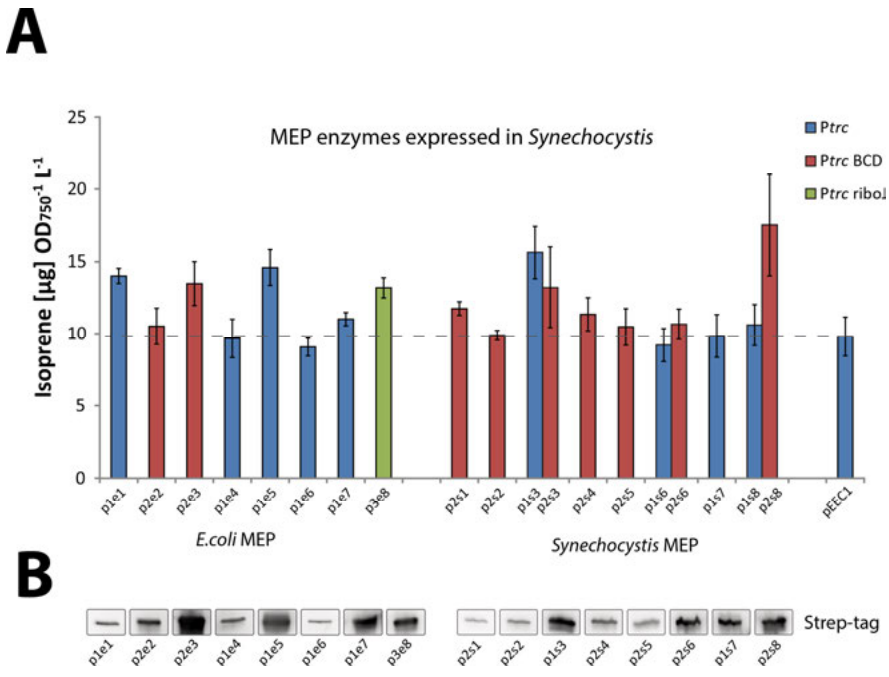

Fig.10. Isoprene production and protein accumulation in *Synechocystis* strain SkIspS, overexpressing MEP enzymes from *E.coli* or *Synechocystis*. (A) MEP enzymes were overexpressed in pEEC1 (blue bars), pEEC2 (red bars) or pEEC3 (green bars). Each data point represents the mean of three biological replicates, error bars are standard deviation. (B) Expressed proteins were detected on wester-immunoblot with a strep-tag antibody.

By overexpressing all MEP pathway enzymes individually in *E.coli* and *Synechocystis*, we could investigate which step in the pathway can contribute most to increased terpenoid production. Expression of DXS and IDI in *E.coli* increased isoprene production by 27 times while the increases in *Synechocystis* were much less dramatic. We also identified a new potential overexpression target for increased terpenoid production in *Synechocystis*; *IspD*, which can be useful in other production studies.

Another part of the study concerned the expression of proteins. The effect of using BCD or RiboJ on expression varied, sometimes improving protein accumulation, sometimes not. However, because usually at least one of the three constructs for the same enzyme expressed well, we could first test the constructs function in *E.coli*, and then transfer the best one to cyanobacteria, thereby decreasing the occurrence of non-expressing *Synechocystis* strains.

Characterization of inducible promoters in *Synechocystis* (Paper IV)

For engineering of any microorganism, having well-functioning and predictable promoters is crucial. In many cases, inducible expression is needed, such as when producing compounds toxic to the host or when knocking down essential pathways with CRISPRi [99]. Although there are several well characterized and tightly regulated promoters in commonly engineered organisms such as *E.coli* and yeast, the choices in cyanobacteria are slim. We used the Ni^{2+} inducible *nrsB* promoter in Paper II, and got higher productivity than with P*psbA2* but reduced production at high light. In Paper IV, we wanted to make a systematic study of promoters and ribosomal binding sites, which could be useful for biotechnological applications. We focused on the native, metal inducible promoters from a genetic locus in *Synechocystis* that encodes metal resistance genes [111, 116, 117]. For comparison, we also measure the expression of some native *Synechocystis* promoters which are commonly used in engineering studies.

The promoters were cloned upstream of the gene encoding enhanced yellow fluorescent protein (EYFP) and promoter activity was measured as fluorescence per cell (OD_{750}). The *nrsB* promoter showed a low leakiness and a strong induced expression, increasing by 39 times upon Ni^{2+} and Co^{2+} induction (**Fig. 11A**). None of the other metal inducible promoters reached the same expression levels as P*nrsB*, although P*nrsD* was induced by 14 times and P*nrsS* by 7 times, which could be useful for situations where lower expression levels are needed. Three different versions of P*psbA2*, differing in sequence length, resulted in large variations on expression levels, possibly due to presence or absence of regulatory sequences or slight changes to the 5'UTR.

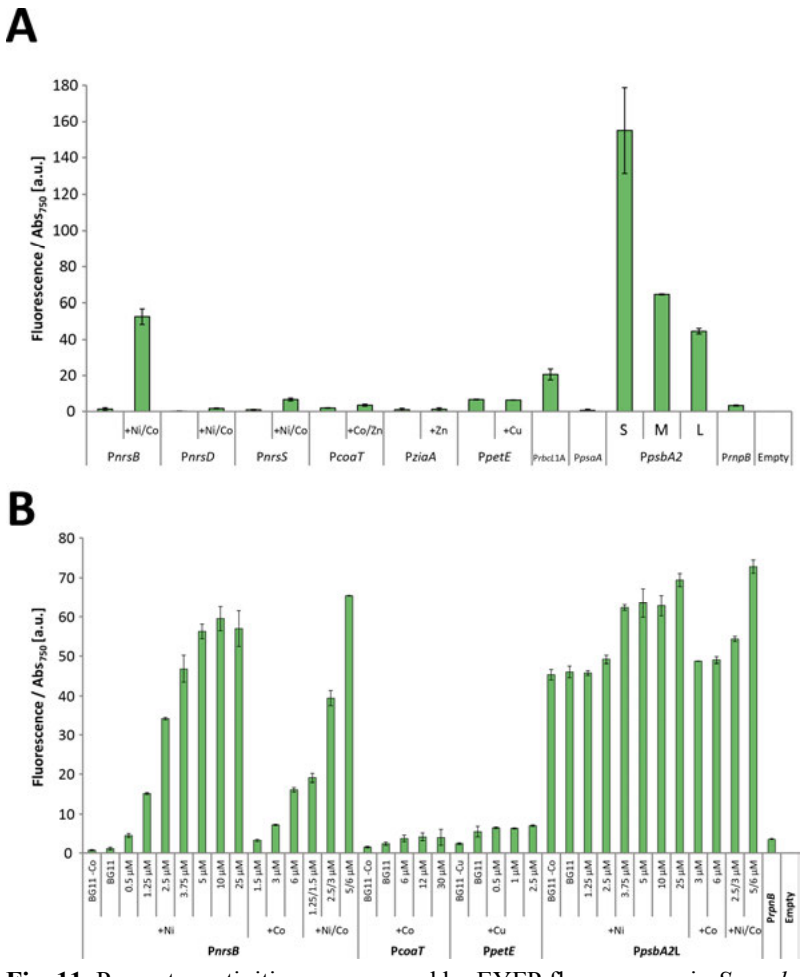

Fig. 11. Promoter activities as measured by EYFP fluorescence in *Synechocystis*. The value from the empty (vector) strain was subtracted from each other sample. (A) metal ion induction levels were 5 µM Ni^{2+}, 6 µM Co^{2+}, 4 µM Zn^{2+} and 0.5 µM Cu^{2+}. (B) Effect of varying induction concentration on promoter activity. "BG11 -metal ion" uses a modified BG11 where that trace metal has been removed. Error bars represent SD (n = 4).

Next, we investigated the induction of P*nrsB*, to see how expression was affected across a range of concentrations and to see how much Ni^{2+} and Co^{2+} contributed individually to the induction. Ni^{2+} had the largest effect on PnrsB expression, which when used in increasing concentrations led to a gradual increase in induction (**Fig. 11B**). This stepwise increase or decrease in fluorescence was equally distributed among the cells, as could be seen using a confocal microscope. For all future experiments, we decided to use 2.5 µM of Ni^{2+} for inducing P*nrsB*, because it still gave a relatively high expression at that concentration, while having only a minor effect on growth, as compared with using 5 µM Ni^{2+}.

We also tested how the *nrsB* promoter would behave if it was driving the expression of enzymes making a product. This was especially relevant, due to that P*nrsB* driven EYFP accumulation varied depending on which day and growth phase it was measured. Therefore, we made a new P*nrsB* driven ethanol producing construct, expressing pyruvate decarboxylase (*pdc*) from *Zymomonas mobilis* and the native alcohol dehydrogenase (*slr1192*) [52]. We also attempted to make the same construct driven by P*psbA2*, but the ethanol production capacity was rapidly lost in *Synechocystis*, consistent with results from a previous study [118]. This highlights the need for inducible promoter when engineering a pathway that puts a heavy burden on the cell, which would ease the metabolic load and increase genetic stability of the construct [119].

Ethanol production in the *Synechocystis* strain expressing the ethanol producing genes with the *nrsB* promoter was highest when induced with 1.25 µM Ni^{2+} (**Fig. 12A**), which differs from the EYFP results where the 2.5 µM gave twice the amount of fluorescence as 1.25 µM Ni^{2+} (**Fig. 11B**). Presence of Ni^{2+} in the media provided a clear reduction in growth for the ethanol producing strain, which was not observed for the empty vector strain. Therefore, we attributed the growth reduction to the detrimental effect of ethanol production, and not to Ni^{2+} toxicity, which also explains why we were unable to generate a constitutively expressed, ethanol producing P*psbA2* strain. At lower levels of inductions; 0 - 1 µM, the amount of ethanol produced increased with amount of induction, which was also reflected in lower growth rates (**Fig. 12B**).

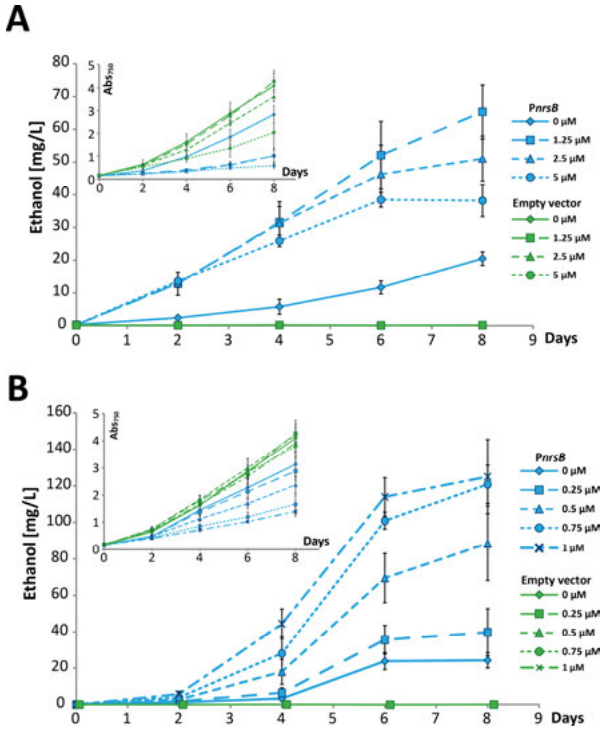

Fig. 12. P*nrsB* driven ethanol production with varying amount of Ni^{2+} induction from day 0. Error bars represent SD (n = 4).

In Paper II, high light led to lower amount of manoyl oxide produces for strains using P*nrsB*. When testing this with the EYFP expressing P*nrsB* construct, expression did go down with higher light, while a version of P*psbA2* doubled in expression from 20 µE to 100 µE (**Fig. 13A**). While the cause of the reduced expression of P*nrsB* at higher light is unknown, P*psbA2* is known to be induced at higher lights, due to a higher turnover of the D1 protein in photosystem II [103]. When growing the ethanol producing P*nrsB* strain at higher light, ethanol production increased (**Fig. 13B**). This inconsistency in P*nrsB* expression at high light when measuring fluorescence or ethanol production is unclear. However, ethanol production is not only affected by enzyme amounts but also substrate availability, which might increase with light and thereby generating more ethanol.

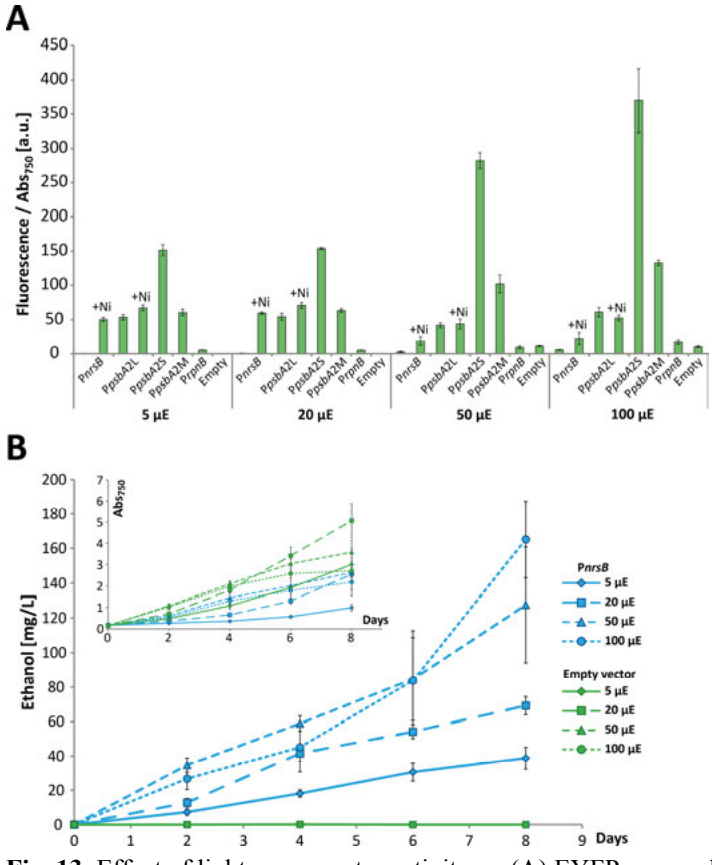

Fig. 13. Effect of light on promoter activity on (A) EYFP accumulation or (B) ethanol production. µE = µmol m^{-2} s^{-1}, error bars represent SD (n = 4).

Lastly, we wanted to characterize different RBSs in *Synechocystis*, which could be useful for applications such as constructing operons with variable expression among the genes. Therefore, we selected eight RBSs from the BioBrick registry [86], two native RBSs from highly expressed genes, and the synthetic RBS* [11], and measured their activity using two constructs with EYFP or a blue fluorescent protein (mTagBFP), designed to have high sequence dissimilarities. The strengths of the RBSs varied over a wide range of expression, which was mostly consistent between the two fluorescent reporter constructs. Although measuring the RBSs with two different genetic contexts does not conclusively determine their strength for use in all contexts, it gives a better hint than only using one construct.

In conclusion, we performed a detailed evaluation on the use of P*nrsB* for protein accumulation and product formation. It provided a relatively strong, well-regulated expression, and could be fine-tuned by varying inducer concentration. However, it also had a reduced expression at high light and the inducer is both toxic to the cells at high concentrations and is actively being pumped out, generating inconsistent expression. Nevertheless, P*nrsB* can

still be a valuable addition to the genetic engineering toolbox, especially for expression of production pathways detrimental to growth.

It is important not to assume that the expression levels in this promoter study can be generalized to be the same for any gene expression [120]. As can be seen in Paper III, the same promoter can generate different expression levels, depending on which gene sequence is expressed, even when the rest of the genetic context is the same. However, the results from the study are still valuable for understanding the induction patterns of the *nrsB* promoter, and in the relative strengths of the RBS library that was measured.

Conclusions and Future Directions

This thesis details the metabolic engineering of *Synechocystis* sp. PCC 6803 for production of terpenoids. Squalene, a molecule that can be used both as a fuel and a chemical feedstock, was produced in Paper I by deleting the native enzyme Shc. The resulting knock-out strain accumulated 0.80 mg g^{-1} DCW and showed no growth defects under standard growing conditions. Paper II detailed the production of the plant diterpenoid manoyl oxide, a precursor to the high-value pharmaceutical forskolin. By testing production from different promoters, and by boosting precursor availability with upstream enzymes, we could get production of 0.45 mg g^{-1} DCW manoyl oxide.

Cyanobacterial production of fuel and chemical feedstock terpenoids offers a promising alternative to the fossil fuels sources we use today. Based on calculations on theoretical maximal phototrophically produced terpenoids, the yield of isoprene and squalene can reach ~11 kg/m^2/year, with solar energy conversion efficiencies of ~8%, assuming all energy is redirected towards production (**Fig. 14**). Compared with ethanol production derived from sugar cane fermentation, which have a typical efficiency of 0.2% [121], the production potential of cyanobacteria are immense. Of course, reaching those yields will be a long and difficult process, and it will require a detailed knowledge of cellular processes and a tight control of the metabolism.

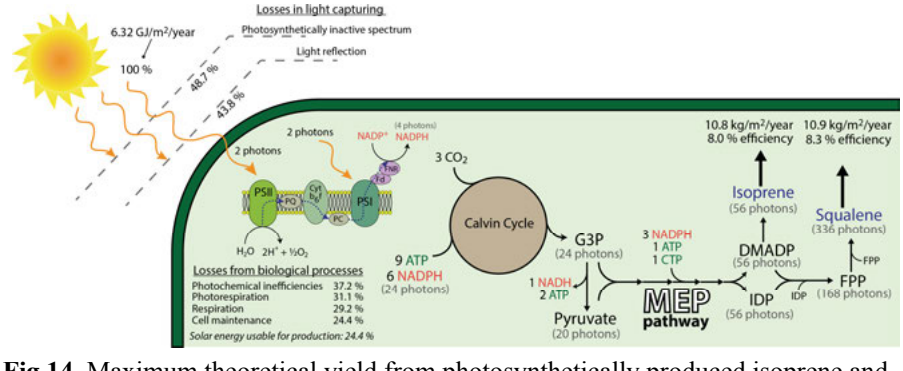

Fig 14. Maximum theoretical yield from photosynthetically produced isoprene and squalene. Efficiency is defined here as energy stored in product per total energy from sunlight. Losses from cell maintenance are calculated as 15 % of useable energy for the cell (29.2%) and assumes no cell growth [122]. Energy content values used in calculations: photons = 173.5 kJ/mol, isoprene = 3182.8 kJ/mol, squalene = 19796.4 kJ/mol. Based on figure and calculations from [57].

To contribute to the increase of that knowledge, we investigated in Paper III each metabolic step in the MEP pathway in both *E.coli* and *Synechocystis*, to find bottlenecks for terpenoid production. Some unknown targets could be identified in both *E.coli* and *Synechocystis*, expressions of which can further increase terpenoid biosynthesis.

Reaching production levels that are commercially viable, not only requires engineering of the production pathway, but also to modify other pathways, to redirect carbons from growth to product [20]. Genome wide models can help identify non-intuitive modification targets and find ways to couple production to growth [123], which enables increasing production titers by adaptive laboratory evolution [124]. For a product like squalene, the intracellular accumulation becomes limited by the volume of the bacteria, which engineering of lipid transporters may help resolve [125].

In the ideal situation, the growth of the production strain should be divided into two phases, one where they use their resources for growth, and one where they redirect all of it for making a product. For that kind of control, inducible promoters are needed. The nickel inducible promoter P*nrsB* which was characterized in Paper IV, could be a tool used for that kind of application. It had a tight regulation and a relatively high induced expression, which are attractive promoter properties. That promoter together with the ribosomal binding sites characterized can be valuable additions to the genetic toolbox of *Synechocystis*.

To summarize, the work presented in this thesis can help develop future cyanobacterial cell factories for a truly sustainable production system from CO_2, water and sunlight.

Svensk sammanfattning

Få kan ha missat de negativa konsekvenser mänsklighetens användning av fossila bränslen har skapat. Utsläppen och ackumuleringen av koldioxid i atmosfären har ökat i en oroande hastighetet, vilket värmer upp vår planet och skapar klimatförändringar. De fossila energikällorna håller dessutom på att ta slut, vilket leder till ökade energipriser och negativa konsekvenser för världens ekonomier. Tillgången till billiga energikällor har till stor del skapat välstånd och möjliggjort utveckling av industrier i rika länder, en process som länder under utveckling bör ha möjlighet att efterfölja. Därför behöver vi utveckla nya alternativa energikällor som kan skapas i stora volymer, till ett billigt pris och som inte tillför koldioxid till det naturliga kretsloppet. Solen belyser vår planet varje timma med lika mycket energi som vi förbrukar under ett helt år, vilket gör den till den naturliga energikällan. Svårigheten är hur vi ska kunna fånga solens näst intill outsinliga energi, och i vilken form den ska lagras. Solceller, vindkraftverk och vattenkraftverk är alla bra alternativ som fångar solenergin i form av elektricitet, vilket är svårt att lagra och utgör en minoritet av vår energianvändning. En annan möjlighet är att fånga solenergin i form av kemisk energi, som i ett bränsle, vilket är lättare att lagra och är den dominerande formen av energianvändning vi har idag.

Cyanobakterier, också kallade blågröna alger, har varit en av de viktigaste organismerna för utvecklingen av liv i vår jords historia. De uppfann fotosyntesen ungefär 2,3 miljarder år sedan, vilket ledde till syresättning av atmosfären, och det syre som vi alla andas idag. De är också urmodern till kloroplasten, den del av växter och alger som gör att de har fotosyntes. En av anledningarna till att cyanobakterier är intressanta för forskare idag är deras potential till att användas för att skapa bränslen direkt från solljus, luft och vatten. Naturligt använder de solljus och koldioxid från luften för att bygga upp de ämnen som de består av. Genom att använda genetisk modifieringar kan bakteriernas metabolism styras om för att istället använda koldioxiden och solljuset för att göra bränslen. Beroende på vilka modifieringar som görs och vilka gener som stoppas in, så kan man ändra bakterierna till att göra i princip vilken naturlig förekommande molekyl som helst. Eftersom cyanobakterier kan omdirigera infångad solenergi till att direkt göra en produkt utan att ödsla energi på att skapa rötter eller blad som växter behöver, och eftersom de kan växa året runt och har en ytterst effektiv fotosyntes, så skulle de kunna producera mycket högre mängd bränsle än den mängd som

man får ut från dagens sätt att göra biobränslen. De har också fördelen av att kunna växa i saltvatten, vilket gör att cyanobakterieodlingar kan förläggas till ökenområden med havsvatten som odlingsmedium, och på så sätt inte konkurera med odlingen av mat eller förbruka färskvatten.

Arbetet som beskrivs i den här avhandlingen handlar om att genmodifiera cyanobakterien *Synechocystis* sp. PCC 6803 till att göra terpenoider. Terpenoider är en stor och varierad grupp av molekyler där många mediciner, smakämnen och dofter ingår. Smaken från kanel, färgen på en tomat och doften från eukalyptus är alla exempel på terpenoidmolekyler. Artikel I handlar om att genmodifiera cyanobakterier för att göra skvalen, medan Artikel II handlar om att göra manoyloxid. Båda molekylerna är terpenoider men med olika egenskaper. Skvalen är en väldigt fet molekyl som skulle kunna användas som råvara för bensin eller till att göra plaster. *Synechocystis* innehåller naturligt den molekylen fast bara i små mängder eftersom den används för att skapa några andra ämnen. Genom att ta bort det enzymet som gör om skvalen i bakterien till andra ämnen, så fick kunde vi skapa en *Synechocystis*-stam som ackumulerade skvalen.

Manoyloxid var den andra terpenoidmolekylen vi producerade från cyanobakterier, vilket kan läses om i Artikel II. Det är en molekyl som med några fler modifieringar kan användas som en medicin mot grön starr samt för några andra åkommor. Manoyloxid görs naturligt i växten *Coleus forskohlii*, men bara i små mängder och kräver en komplicerad extraktionsprocess för att renas fram. Ett alternativt sätt att skapa medicinen vore att producera den i en bakterie, där den kan ackumuleras i högre mängder och till ett billigare pris. Vi tog därför gener från växten och stoppade in dem i *Synechocystis,* vilken då började producera manoyloxid. Genom att variera var på kromosomen vi förde in generna och med vilken ljusstyrka vi odlade bakterierna, så kunde vi optimera och öka produktionen.

Mängden skvalen och manoyloxid som våra modifierade cyanobakterier producerade var bara några få promille av totala cellmassan, vilket behöver ökas flera gånger innan det kan produceras kommersiellt. För att förstå hur terpenoider skapas naturligt i bakterier så att vi lättare kan öka produktionen, så gjorde vi en studie av terpenoid-biosyntesvägen, som beskrivs i Artikel III. Skapandet av terpenoider i bakterier börjar från en reaktionsväg bestående av åtta enzymer, som gör om substraten pyruvat och glyceraldehyd-3-fosfat till de byggstenar som bygger upp alla terpenoider. Genom att modifiera hur mycket av de åtta enzymerna som bakterierna hade, så kunde vi identifiera vilka av dem som var viktigast för att påverka terpenoidbiosyntesen, något som i framtida studier kan användas för att öka produktionen av vilken terpenoid som helst.

Vid genmodifiering av cyanobakterier för att skapa en produkt, behöver man kunna kontrollera hur starkt varje gen ska vara på. Till störst del kontrolleras styrkan av en kort DNA-sekvens framför genen som kallas för pro-

motor. Artikel IV handlar om hur vi tog flera promotorer som *Synechocystis* redan använder för några av sina gener, sedan satte in dem framför en gen som bildar ett gult, fluorescerande protein, och slutgiltigen stoppade tillbaka den nya DNA-sekvensen in i bakterien. På så sätt kunde vi bestämma hur stark varje promotorsekvens var, eftersom det var direkt proportionellt till hur mycket gult fluorescens vi fick. Vad som var speciellt för några av promotorsekvenser vi testade var att de var metall-inducerbara, d.v.s. promotorerna är av fram tills bakterien kommer i kontakt med en metalljon, varvid promotorn aktiveras och genen nedanför slås på.

Sammantaget så har resultaten från de arbeten som presenterats här lett till en ökad förståelse för hur man kan genmodifiera cyanobakterier till att producera terpenoider. Detta kan vara speciellt värdefullt för att skapa klimatneutrala energikällor.

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＼(^o^)／

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