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Engineering *Synechocystis* sp. PCC 6803 for microbial terpenoid production

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ACTA
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UPSALIENSIS
UPPSALA
2022

ISSN 1651-6214
ISBN 978-91-513-1615-4
URN urn:nbn:se:uu:diva-486274

Dissertation presented at Uppsala University to be publicly examined in Eva von Bahr, Ångströmlaboratoriet 100195, Lägerhyddsvägen 1, Uppsala, Friday, 25 November 2022 at 09:00 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Patrik R. Jones (Department of Life Sciences, Faculty of Natural Sciences, Imperial College London).

Abstract

Rodrigues, J. S. 2022. Engineering *Synechocystis* sp. PCC 6803 for microbial terpenoid production. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2197. 84 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1615-4.

As part of the efforts to develop a carbon-neutral economy, photosynthetic microorganisms such as cyanobacteria have been extensively studied as solar-powered cell factories for the production of organic compounds with industrial interest, using the atmospheric CO_2 as carbon source.

Among other metabolites, cyanobacteria produce terpenoids, a diverse group of natural products that play pivotal roles in the cell. These metabolites have industrial relevance as fragrances, colorants and biofuel precursors. The research here presented focused on the production of terpenoids in the model cyanobacterium *Synechocystis* sp. PCC 6803, with special emphasis on two compounds: bisabolene and isoprene. Bisabolene is a 15-carbon-atom molecule that can be used as feedstock for chemical synthesis and for biofuel generation. In my first two projects, I demonstrated that heterologous expression of the bisabolene synthase is enough to complete the biosynthetic pathway, and cultivation of bisabolene-producing *Synechocystis* strains in high-density conditions significantly benefits microbial production. Furthermore, overexpression of key enzymes from the native metabolism increased precursor supply. The best producing strain reached a bisabolene titre of 180 mg L^{-1} after 8 days of cultivation.

Isoprene is a small volatile terpenoid with high relevance to industry, and it plays an important role in thermotolerance in several plants. In order to map competitive pathways to terpenoid production in *Synechocystis*, I utilized a synthetic biology tool for gene knockdown of several targets and evaluated their influence on isoprene production. Six candidate genes were identified as potential targets for further modifications, to improve reallocation of the cellular resources to terpenoid production. In another study, I analysed how the production of isoprene in *Synechocystis* can affect the metabolism and cell physiology, and how isoprene production can be improved by optimizing cultivation conditions, such as light quality, light intensity and temperature. Cultivation under violet light or at higher temperatures improved significantly isoprene production. I also observed that isoprene itself modifies the physiology of the cell, contributing to a reduction in size and growth rate, and confers additional thermotolerance to *Synechocystis*.

In my last project, I participated in a collaborative work where a hybrid photobiological-photochemical approach was applied to convert photosynthetically-derived isoprene into jet fuels using light as energy source. I cultivated isoprene-producing cyanobacteria in closed vials and designed a capturing system to trap the produced isoprene. Subsequent photochemical dimerization rendered a mixture of products that, upon minor chemical modifications, fulfilled the requirements to be used as jet fuels.

Keywords: Synechocystis, metabolic engineering, bisabolene, isoprene, cyanobacteria

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ISSN 1651-6214
ISBN 978-91-513-1615-4
URN urn:nbn:se:uu:diva-486274 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-486274)

Great things have small beginnings
Sir Francis Drake

List of Papers

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

I. Dienst, D., Wichmann, J., Mantovani, O., **Rodrigues, J. S.**, Lindberg, P. (2020) High density cultivation for efficient sesquiterpenoid biosynthesis in *Synechocystis* sp. PCC 6803. *Scientific Reports*, 4, 5932

II. **Rodrigues, J. S.**, Lindberg, P. (2021) Metabolic engineering of *Synechocystis* sp. PCC 6803 for improved bisabolene production. *Metabolic Engineering Communications*, 12, e00159

III. **Rodrigues, J. S.**, Bourgade, B., Galle, K., Lindberg, P. (2022) Mapping competitive pathways to terpenoid biosynthesis in *Synechocystis* sp. PCC 6803 using a small RNA synthetic tool (manuscript).

IV. **Rodrigues, J. S.**, Kovács, L., Lukeš, M., Hoeper, R., Steuer, R., Červený, J., Lindberg, P., Zavřel, T. (2022) Characterizing isoprene production in *Synechocystis* sp. PCC 6803 – insights into effects of light, temperature, and isoprene on the cells (manuscript).

V. Rana, A., Gomes, L. C., **Rodrigues, J. S.**, Yacout, D. M. M., Arrou-Vignod, H., Sjölander, J., Vedin, N. P., El Bakouri, O., Stensjö, K, Lindblad, P., Andersson, L., Sundberg, C., Berglund, M., Lindberg, P., Ottosson, H. (2022) A Combined Photobiological-Photochemical Route to C10 Cycloalkane Jet Fuels from Carbon Dioxide via Isoprene. *Green Chemistry* (Submitted manuscript).

VI. **Rodrigues, J. S.**, Lindberg, P. / Nielsen, J., Lee, S., Stephanopoulos, G., Hudson, P.: Engineering Cyanobacteria as Host Organisms for Production of Terpenes and Terpenoids. In: Cyanobacteria Biotechnology. Chapter 9: 267 – 300 (2021). Copyright Wiley-VCH GmbH. Reproduced with permission.

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Contents

Abbreviations

A. grandis	*Abies grandis*
AgB	*Abies grandis* bisabolene synthase
asRNA	anti-sense RNA
ATP	Adenosine triphosphate
BCD	Bicistronic device
Chl a	Chlorophyll a
DCW	Dry cell weight
DMAPP	Dimethylallyl pyrophosphate
DXS	1-deoxy-D-xylulose 5-phosphate synthase
E. coli	*Escherichia coli*
E. globulus	*Eucalyptus globulus*
FPP	Farnesyl pyrophosphate
G3P	Glyceraldehyde 3-phosphate
GC	Gas chromatography
GGPP	Geranylgeranyl pyrophosphate
GPP	Geranyl pyrophosphate
HDC	high density cultivation
IDI	Isopentenyl pyrophosphate isomerase
IPP	Isopentenyl pyrophosphate
IspA	*E. coli* farnesyl pyrophosphate synthase
IspS	Isoprene synthase
MEP	Methylerythritol 4-Phosphate
MIMS	Membrane-inlet Mass Spectrometry
mRNA	messenger RNA
MVA	Mevalonate
Pyr	Pyruvate
RBS	Ribosome binding site
sRNA	small RNA
Synechocystis	*Synechocystis* sp. PCC 6803

Introduction

Terpenes & Terpenoids

Natural Occurrence & Relevance to Industry

Terpenoids[1] are a very diverse family of natural products, not only in terms of structure, but also regarding their chemical and physical properties. In fact, terpenoids constitute the largest family of secondary metabolites found in Nature, spanning over 70.000 different molecules [1]. This family of natural products can be divided into different classes according to the number of prenyl units used to generate their carbon chains: monoterpenoids have 10 carbon atoms (two prenyl units); sesquiterpenoids have 15 carbon atoms (three units); diterpenoids have 20 carbon atoms (four units); triterpenoids have 30 carbon atoms (six units); and carotenoids have 40 carbon atoms (eight units) [2]. Smaller terpenoids, such as mono- and sesquiterpenoids, tend to be volatile and often present strong aromas. Most of the compounds that give aromatic herbs and spices their characteristic scents and tastes fall into these subsets of the terpenoid family [3]. Examples of small volatiles include limonene, responsible for the citric smell of the lemon and the orange [4], sabinene, which confers the spicy flavour found in black pepper [5], and menthol and thymol, constituents of the essential oils of mint and thyme, respectively [6]. Interestingly, some of these terpenoids can present different odours depending on their stereochemistry. For example, the (*S*)-(+)-linalool presents a citric fruity odour and can be found in orange peel oil to a very high enantiomeric purity, while the (*R*)-(-)-enantiomer has the characteristic lavender smell (and it is no surprise it is found in large amounts in the essential

[1] Throughout this thesis, the reader will be exposed to an extensive discussion on a specific family of natural compounds. Although the terms *terpenes* and *terpenoids* are commonly used as synonyms to describe this family of compounds, their meanings are slightly different. The term *terpene* refers to the group of unsaturated hydrocarbons that consist of isoprene units, *i.e.* CH_2:C(CH_3)CH:CH_2. *Terpenoids*, on the other hand, are derivatives of terpenes, often modified with functional groups. To simplify the text to the reader, I decided to resort to the term *terpenoids* whenever I am generally describing the entire family of these compounds.

oil of lavender) [7]. Mono- and sesquiterpenoids, however, are not restricted to aromatic herbs; they are also present in relatively high amounts in the essential oils and olefins of many other plants, like eugenol or pinene, which are associated to the characteristic smells of eucalyptus and pine trees, respectively [8]. Plants are the major producers of these volatile terpenoids, using them as signalling molecules for intra- and interspecies communication and as protective agents against insect herbivores [8, 9].

Terpenoids with higher molecular weight tend to be less volatile and, therefore, tend to be retained inside the cells rather than escaping to the extracellular environment. Many of these, especially the carotenoids, have extended π-conjugated systems that allow these molecules to absorb and emit light from the visible range of the spectrum [10], filling nature with vibrant colours, in a diverse palette of reds (*e.g.,* lycopene), oranges (*e.g.,* β-carotene) and yellows (*e.g.,* lutein) [11]. These long chain terpenoids have several biological roles, acting as pigments in photosynthesis as well as photoprotective compounds (*e.g.,* lutein in the eye retina), as antioxidants, hormones (*e.g.,* sterols) and as structural components of the cell membranes (*e.g.,* cholesterol), to name a few [12-14].

Like Nature, humankind also found several uses in terpenoids for its day-to-day life. The strong colours and fragrances of some terpenoids make them quite useful as ingredients for perfumes and cosmetics [3, 15]. Some terpenes are extracted from resins and olefins of plants to be used as lacquers and solvents [8]. Other terpenoids are highly relevant to nutrition, as vitamins, antioxidants or food additives [3]. Some also present bioactive properties and thus, have been extensively explored by the pharmaceutical industry as precursors for drug development [16, 17] (*e.g.,* the antitumoral compound paclitaxel or the antimalarial compound artemisinin) or as adjuvants for vaccine formulae (*e.g.,* squalene emulsions) [18]. Many more examples could be enumerated of terpenoids that, in some way, influence our lifestyle. It is for this same reason that the scientific community (me included) has been focusing for several decades on deciphering how these compounds are produced in nature and, with that body of knowledge, how to make them by ourselves, either via chemical synthesis or via microbial production.

MEP & MVA pathways

All terpenoids are made from the same building blocks – two 5-carbon-atom prenyl groups called isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) [19]. There are only two pathways known to humankind that generate IPP and DMAPP – the mevalonate (MVA) and the methylerythritol 4-phosphate (MEP) pathways [20]. The MVA pathway was the first terpenoid biosynthetic pathway to be discovered [21]. Present mostly in eukaryotes and archaea (although some bacteria also possess it), this pathway makes use of acetyl coenzyme A (acetyl-CoA) to generate IPP [21].

Two molecules of acetyl-CoA are condensed into acetoacetyl-CoA, and then a third molecule of acetyl-CoA is added, resulting in 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). As HMG-CoA is also intermediate in the biosynthesis of ketone bodies, it is its reduction to mevalonate the first committed step for the synthesis of IPP (hence the name Mevalonate pathway). The order of the next steps varies with species, but essentially mevalonate is phosphorylated trice by three distinct kinases and decarboxylated by a fourth enzyme to give IPP [21]. An isomerase (IDI) then catalyses the interconversion of IPP and DMAPP. In the 1950's, contradictory results regarding the MVA pathway as sole terpenoid pathway caught the attention of the scientific community, especially regarding studies where addition of ^{13}C-labelled acetate to plants did not lead to its incorporation (or did in very minute amounts) into the carotenoids [22]. After a few decades of research, a mevalonate-independent pathway was identified in bacteria, as well as in plant and algae chloroplasts – the MEP pathway [19, 23]. In this second pathway (figure 1), pyruvate and glyceraldehyde 3-phosphate (G3P) are combined and decarboxylated into 1-deoxy-*D*-xylulose 5-phosphate (DXP), in a thiamine-assisted reaction catalysed by the enzyme DXP synthase (DXS). This sugar is also precursor for the production of pyridoxal 5-phosphate (vitamin B_6) and thiamine (vitamin B_1) [24]. It is thus the second enzymatic step of the pathway - the conversion of DXP into MEP by the enzyme DXP reductoisomerase (DXR) - the first committed step for IPP and DMAPP biosynthesis [25]. The next three steps involve, in this order, the conversion of MEP into 4-diphosphocytidyl-2-C-methyl-*D*-erythritol (CDP-ME), a phosphorylation at C2, originating 4-diphosphocytidyl-2-C-methyl-*D*-erythritol 2-phosphate (CDP-MEP), and a cyclisation of CDP-MEP, forming 2-C-methyl-*D*-erythritol 2,4-cyclodiphosphate (MEcPP). These three steps require the consumption of one molecule of CTP and 3 molecules of ATP – one for the phosphorylation of CDP-ME and two more for the regeneration of CTP [25]. This cyclic intermediate is then converted to 4-hydroxy-3-methylbut-2-enyl pyrophosphate (HMBPP) by the HMBPP synthase. The final step of the pathway involves the reduction of HMBPP into a mixture of IPP and DMAPP [19]. Similar to the MVA pathway, an isomerase (IDI) interconverts IPP and DMAPP in cells (or plastids) that use the MEP pathway [19, 23]. It is thought that the biological relevance of such isomerases is to balance the IPP:DMAPP ratio inside the cells specifically for their needs, as different classes of terpenoids require different amounts of each of the prenyl units [19]. In *Escherichia coli*, however, IDI is not essential [26], probably because the HMBPP reductase is already fine-tuned to provide the right proportion of IPP and DMAPP to the cell.

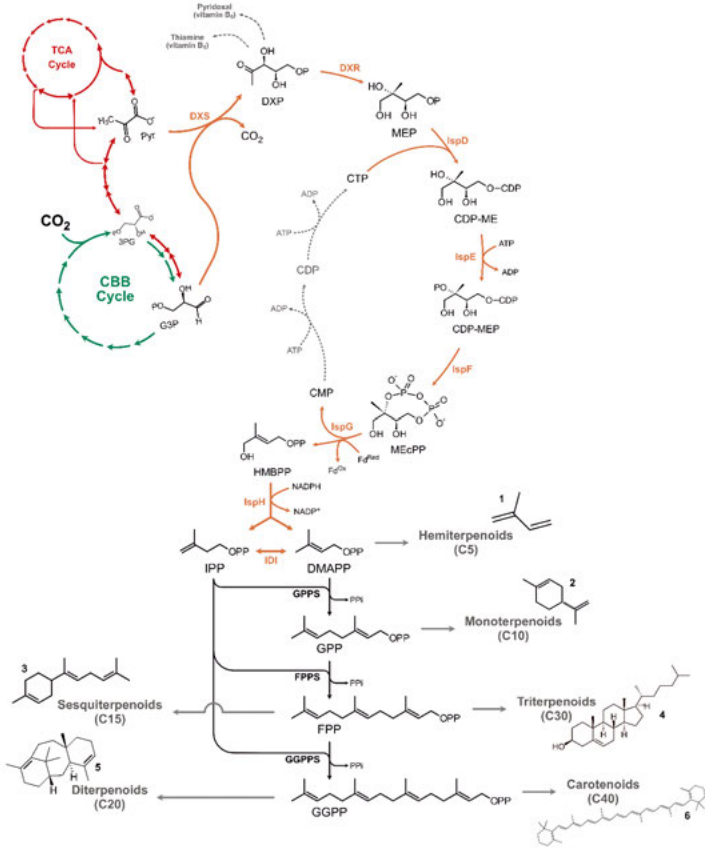

Figure 1 – Schematic representation of the methylerythritol 4-phosphate (MEP) pathway (orange arrows) and its connection to Calvin-Benson-Bassham (CBB) cycle (green arrows) and glycolysis and tricarboxylic acid (TCA) cycle (red arrows). A representative molecule was chosen for each class of terpenoids, with isoprene (1) as hemiterpenoid, limonene (2) as monoterpenoid, bisabolene (3) as sesquiterpenoid, taxadiene (5) as diterpenoid, cholesterol (4) as triterpenoid and β-carotene (6) as carotenoid. Pyr – pyruvate; 3PG – 3-phosphoglycerate; G3P – glyceraldehyde 3-phosphate; DXP – 1-Deoxy-D-xylulose 5-phosphate; MEP – methylerythritol 4-phosphate; CDP-ME – 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP – 4-diphosphocytidyl-2-C-methylerythritol 2-phosphate; MEcPP – 2-C-methyl-D-erythritol-2,4-cyclodiphosphate; HMBPP – 4-Hydroxy-3-methyl-but-2-enyl pyrophosphate; IPP – isopentenyl pyrophosphate; DMAPP – dimethylallyl pyrophosphate; GPP – geranyl pyrophosphate; FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate; CMP – cytidine monophosphate; CDP – cytidine diphosphate; CTP – cytidine triphosphate; NADPH – nicotinamide dinucleotide phosphate; PPi – pyrophosphate; DXS – DXP synthase; DXR – DXP reductoisomerase; IspD – MEP cytidylyltransferase; IspE – CDP-ME kinase; IspF – MEcPP synthase; IspG – HMBPP synthase; IspH – HMBPP reductase; IDI – IPP isomerase; Fd^{Ox} – oxidised ferredoxin; Fd^{Red} – reduced ferredoxin; GPPS – GPP synthase; FPPS – FPP synthase; GGPP synthase – GGPP synthase.

The simplest terpenes are the hemiterpenes (C_5) which are produced from a single prenyl unit (*e.g.,* isoprene). Longer terpenes are obtained by sequential condensation of these two prenyl units by polyprenyl transferases, with the release of one pyrophosphate group for each condensation reaction [27]. First, one IPP molecule and one DMAPP molecule are condensed into geranyl pyrophosphate (GPP) by a GPP synthase (GPPS). Farnesyl pyrophosphate (FPP) is then obtained by combining a second molecule of IPP to GPP by an FPP synthase (FPPS). Another IPP molecule can be added to FPP, originating geranylgeranyl pyrophosphate (GGPP), in a reaction catalysed by a GGPP synthase (GGPPS). The generated polyprenyl molecules serve then as substrate for the synthesis of terpenes by terpene synthases: GPP is used as backbone for the synthesis of monoterpenes (C_{10}); FPP originates sesquiterpenes (C_{15}); and GGPP gives rise to diterpenes (C_{20}). Head-to-head condensation of two molecules of FPP or two molecules of GGPP result in the formation of squalene or phytoene, respectively, which in turn serve as substrates for triterpenes (C_{30}) and carotenes (C_{40}) [2, 27]. These terpenes can then be further modified (*e.g.,* hydroxylations, reductions, and epoxidations) by other enzymes, such as monooxygenases or reductases, rendering many other terpenoids [19, 28].

Despite their common function, these two pathways present some differences that are worth discussing in more detail. The first differences lie in the substrate usage and the carbon loss of each pathway. The MVA pathway utilises three molecules of acetyl-CoA to generate one IPP molecule, while the MEP pathway utilises one molecule of pyruvate and one of G3P to generate one molecule of either DMAPP or IPP. In both pathways, one CO_2 molecule is lost via decarboxylation to generate a C_5-intermediate; however, each acetyl-CoA spent by the MVA pathway is, in turn, a product of the decarboxylation of a pyruvate molecule, accounting for three extra CO_2 molecules lost, compared to the MEP pathway. These pathways also present differences in terms of how much ATP and reducing power is required to drive one series of reactions. The MVA pathway uses one molecule of NAD(P)H to generate the mevalonate intermediate, and two to three molecules of ATP are required to complete the lower MVA pathway. The same amount of ATP is consumed in the MEP pathway - one in the phosphorylation of CDP-ME, and two more in the regeneration of the CTP molecule – but it requires two molecules of NAD(P)H and two electrons, provided by ferredoxin. It is interesting that a pathway that presents less carbon loss and more consumption of reducing power is highly represented in photoautotrophic microorganisms and plant plastids, while a pathway that requires less reducing power but loses more carbon is present in heterotrophs and in the cytoplasm of plant cells [19, 29].

Cyanobacteria

The microbes

Cyanobacteria, formerly called *blue-green algae* because of their characteristic blue accessory pigment, phycocyanin, are a group of Gram-negative photosynthetic prokaryotes that can be found in almost all ecosystems on Earth [30]. Their geographical distribution is remarkably wide, ranging from seawater to freshwater environments, and from frozen arctic regions to hot climates, such as deserts or hot springs [31]. Equally remarkable is their morphological variety; there are groups of unicellular, filamentous and colonial cyanobacterial strains [30]. Some filamentous strains can undergo cell differentiation upon certain environmental stimuli. For example, upon nitrogen deprivation, *Nostoc* species can develop specialised cells, heterocysts, which sacrifice their photosynthetic capability in order to provide the right conditions for the oxygen-sensitive nitrogen (N) fixation machinery [32]. This ability of many species to fix atmospheric nitrogen provides a competitive advantage when N availability is low and is one of the main reasons why cyanobacteria are present in many symbiotic associations with other organisms [32].

Although *Homo sapiens* may be the first living organism self-aware of the consequences of its lifestyle to the planet, it is most certainly not the first species to cause climate changes on a global scale. In fact, the unique ability of cyanobacteria to perform oxygenic photosynthesis is the major reason for the dramatic change that occurred roughly 2.4 billion years ago and which we now refer as *The Great Oxygenation Event* [33]. In contrast to other photosynthetic bacteria, cyanobacteria use water as electron donor to generate the required reducing power for many enzymatic reactions, as well as source of protons to build-up the gradient necessary for ATP synthesis, releasing molecular oxygen as by-product [32]. The colonisation of the oceans by cyanobacteria resulted in the oxygen-rich atmosphere we observe today, facilitating the development of aerobic respiration and complex multicellular life [34]. Besides their contribution to the development of oxygen-tolerant life, cyanobacteria are also well-known for their role in the origins of the eukaryotic plastids, as they shaped into today's chloroplasts [35].

In parallel to their highly relevant roles on the evolution of life on Earth and on the ecological dynamics of most of the ecosystems on the planet, cyanobacteria are also appealing for their potential as cell hosts for biotechnological applications. When compared to plants or algae, cyanobacteria are more efficient at harvesting solar energy [36]. Furthermore, their higher CO_2/HCO_3^- uptake and the presence of carbon concentrating mechanisms allow a faster ability to fix carbon [37]. Genetically speaking, cyanobacteria possess smaller and simpler genomes than eukaryotic photoautotrophs, making them more amenable for manipulation [36]. Finally,

cyanobacteria generally present a higher tolerance to certain abiotic stresses, namely salinity, pH, light intensity or temperature, which make the idea of cultivating engineered host cells in sea water or wastewater more feasible.

The work described in this thesis was conducted using the freshwater, non-diazotrophic, unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*), more specifically the glucose-tolerant sub-strain. Isolated from Oakland (California, USA), this cyanobacterium was the first phototrophic organism to have its genome fully sequenced [38, 39]. Furthermore, transformation of *Synechocystis* is possible via any of the common bacterial transformation techniques – natural transformation, conjugation or electroporation [40]. The function of the majority of its genes has been identified or at least predicted, which allowed the development of metabolic models [41] and extensive datasets on different omics [42], making *Synechocystis* one of the most studied cyanobacteria and a common model organism for both fundamental and applied research.

Terpenoids in Cyanobacteria

Cyanobacteria are no exception to the vast list of terpenoid-producing living organisms. They naturally produce different terpenoids, as well as some prenylated molecules important for their physiology [27]. Like other bacteria, these microorganisms resort to the MEP pathway to produce the two prenyl units necessary for terpenoid biosynthesis, utilising pyruvate and G3P derived from photosynthesis [29]. To our knowledge (which is still very sparse), cyanobacteria rely solely on one enzyme to catalyse the prenyl condensations and generate all polyprenyl backbone molecules, in opposition to plants and other microorganisms, which have different enzymes specific for each condensation step [29, 43]. The thermophilic cyanobacterium *Thermosynechococcus elongatus* is an exception, as it seems to possess as FPPS in addition to a GGPPS [44]. It is no surprise, therefore, that the major fraction of terpenoids in cyanobacteria derive from GGPP [43, 45]. This polyprenyl molecule is the substrate for the generation of phytyl pyrophosphate, crucial for the biogenesis of chlorophyll a. Carotenoids are all derived from phytoene, which is also synthesised from GGPP (resorting to two of these molecules, in this case) [46]. The major carotenoid representatives in cyanobacteria are β-carotene, β-carotene hydroxylated derivatives zeaxanthin and canthaxanthin, β-carotene keto-derivatives echinenone and nostoxanthin, and the myxol glycosides [46]. The carotenoid composition differs from species to species, and it can also vary within the same species depending on their growth stage or exposure to certain environmental stimuli (*e.g.*, light intensity) [47]. Cyanobacteria rely more on cyclic carotenoids, which are essential for both light harvest and protection against photooxidative damage [48]. Lycopene cyclases convert lycopene to γ-carotene, and then to β-carotene [46]. This later carotene is highly important

to the cell physiology, acting both as structural component and as accessory pigment in the light harvesting complexes [49]. β-carotene serves as substrate for hydroxylases and ketolases to generate the xanthophylls. In *Synechocystis*, the xanthophyll fraction is mainly comprised of zeaxanthin, echinenone, hydroxyechinenone and synechoxanthin [46]. Xanthophylls play a photoprotective role in cyanobacteria. These molecules are involved in the protection of the photosystems from excessive radiation [50]. Furthermore, they can act as scavengers of reactive oxygen species (ROS), as hydroxy carotenoids (*e.g.,* zeaxanthin) can inactivate peroxyl radicals and keto carotenoids can interact with singlet oxygen [48, 50]. Carotenoids also play crucial roles in the dissipation of excessive light as heat, being involved in a process called *non-photochemical quenching* [51]. As a response mechanism to avoid photodamage to the photosystems, cyanobacterial cells make use of small photoactive proteins, called orange carotenoids proteins (OCPs), to quench the excessive energy they are exposed to and dissipate it in the form of heat. These proteins contain one carotenoid molecule at their centre, usually echinenone or hydroxy-echinenone [51, 52]; upon excessive illumination, the carotenoid molecule suffers conformational changes, which in turn cause conformational changes to the protein. The activated OCP attaches then to the core of the phycobilisomes, allowing the exposed carotenoid molecule to quench the excessive energy [52, 53]. Another group of carotenoids that is found in abundance in cyanobacterial cells is the myxol glycosides, unique to these microorganisms [46]. The aglicone part of these carotenoids is derived from γ-carotene, and the sugar moiety is usually fucose or rhamnose, depending on the species. In *Synechocystis*, these carotenoids are myxol-fucosides [46]. Myxoxanthophylls can be found in the cytoplasm and outer membranes and are thought to have structural and protective roles on those membranes [54]. These carotenoids also contribute significantly to the stabilisation of the thylakoid membranes and play critical roles in the formation of the S-layer (a glycoproteic surface layer of the cell wall) [55].

Some cyanobacterial species (including *Synechocystis*) are capable of synthesising squalene, which is used as substrate for the biosynthesis of hopanoids [56]. These triterpenoids are similar to cholesterol and steroids, both in structure and function. A squalene hopene cyclase catalyses the conversion of squalene into hopene, which is then further modified to render the different hopanoids [57].

Cyanobacteria are rich sources of natural products, including terpenoids. Most of the studies on cyanobacterial terpenoids focused on carotenogenesis and the functions of the different carotenoids [46]. Nonetheless, there has been significant efforts to study other classes of terpenoids that naturally occur in cyanobacteria, as well as to implement heterologous pathways to expand the terpenoid repertoire in these microorganisms [29].

Metabolic engineering

The concept

The cell metabolism, in a nutshell, is an extensive collection of cascades of biochemical reactions, intertwined in a complex network and fuelled by the resources imported from the external environment by membrane transporters [58]. Some of these cascades of reactions are essential for the cell maintenance and are common to all life on earth, composing what we refer to as Primary Metabolism. Evolutionary divergence and exposure to different stresses and environmental conditions, however, contributed to the diversification of some branches of this metabolic network, fine-tuning them to operate under specific conditions [58, 59]. This second set of biochemical reactions is denoted as Secondary Metabolism, also referred to (and perhaps more accurately) as Specialised Metabolism [60]. Many of the products derived from the secondary metabolism are extremely relevant to humankind [58]. The metabolic networks, however, are optimised for the survival of the species, rather than to fulfil our needs. Metabolic engineering thus emerged as a field of research focused in the rewiring of the cellular metabolic networks to redirect the cell resources for improved production of a specific native product, or the biosynthesis of heterologous compounds [58]. Although similar to Genetic Engineering, this field of expertise envisions manipulation of the cellular metabolism as a whole system and modulation of entire metabolic pathways, instead of discreet genes [59]. The advancements in genome sequencing and DNA synthesis accelerated our learning process regarding gene expression and regulation, thus allowing us to find new combinations of biochemical reactions from different pathways (or even from different organisms) and to create novel networks that do not exist in Nature [61]. The work done by Clomburg *et al.* constitutes a good example of creation of non-natural pathways [62]. In this work, the authors designed a new terpenoid biosynthetic pathway, termed Isoprenoid Alcohol Pathway (IPA), where central carbon metabolites are first converted to C_5-alcohols, and then phosphorylated to the corresponding terpenoid precursors (*e.g.*, IPP and DMAPP). The implementation of this pathway in *E. coli* enabled the synthesis of multiple terpenoid products, including geraniol, farnesol and lycopene [62].

Computational models of the metabolic networks became another powerful tool for metabolic engineering, as they allow us to analyse the fluxes of cellular metabolites and resources inside of the cells, and learn how to improve the allocation of said resources towards specific branches of the metabolism that we are interested [63, 64]. Synthetic biology became a cornerstone of metabolic engineering, providing the *means* required for the modulation of the cellular metabolism. This field of research deals with designing and combining well defined genetic elements, by applying the ideals of modularity

and orthogonality, to generate synthetic circuits for specific functions in a cell host [59].

As once mentioned by Stephanopoulos (2012), metabolic engineering "preceded systems biology by championing the need for a systemic view of the metabolic pathways and approaches for their optimal functioning" [59]. Indeed, this dramatic evolution of Metabolic Engineering over the last decades changed our perspective on the microbial metabolic networks and set the grounds for the emergence of Systems Biology, as a more holistic approach to modify the highly dynamic metabolism of the microorganisms [65]. High-throughput measuring techniques (the so called "omics") contributed to the analysis and quantification of different components of the cell on a wider scale, paving way for systems biology to, through mathematical modelling, study how those components interact with each other and how those interactions give rise to the structure, function and dynamics of the cell [66].

Strategies to improve production

Devising a plan for the production of a compound of interest in a microbial host cell starts with learning the chemical properties of said compound, together with the search for already existing metabolic pathways capable of producing it. If there are pathways in Nature related to the biosynthesis of that compound, then one can opt to engineer the native producer, or introduce said pathway in a host organism with more advantageous metabolic properties. The availability of genetic tools also plays an important role when choosing the host organism. Many of the pathways that produce industrially relevant compounds exist in very specific organisms and its genetic manipulation can be very limiting [58]. Therefore, resorting to model microorganisms such as *Escherichia coli* or *Saccharomyces cerevisiae*, which are well-characterised and an extensive collection of tools are already available [67], is often the preferred choice. Despite the enormous diversity in biosynthetic pathways (especially among plants and microorganisms), one may not be able to find a complete pathway in Nature that fulfils the requirements needed. Furthermore, sometimes not all enzymes involved in the pathway in question are identified yet, which can compromise the heterologous reconstruction in the host cell of choice [58]. In those cases, one must then resort to a more specific and stepwise search for enzymes that can either fill in the missing pieces or be combined to generate a completely novel pathway. Enzyme engineering and enzyme evolution can also be considered, especially in cases when very few steps are missing [58]. Alternatively, one can consider a hybrid solution between biotechnology and synthetic chemistry, where instead of aiming at producing the final compound in the microorganism, one aims at generating an intermediate through means of microbial production that can then be converted to the desired product via chemical synthesis. The production of artemisinin is a good example of such hybrid approach [16, 68]. This

sesquiterpene lactone endoperoxide is highly efficient as antimalarial drug; however, extraction from the plant *Artemisia annua* renders very low yields and complete synthesis of this compound is difficult and costly, turning it unaffordable for the patients. As a more affordable strategy, Ro & Paradise *et al.* aimed at producing the intermediate artemisinic acid in yeast by engineering the mevalonate pathway and expressing an amorphadiene synthase together with a cytochrome P450 monooxygenase from *A. annua*. This organic acid can then be easily obtained from the culture medium (as the cells secrete it to the external environment) and converted to artemisinin via chemical synthesis [16, 69].

Once a suitable biosynthetic route is identified, the next logical step is to find ways to improve the performance of the strain, and several different approaches can be addressed for that purpose. Perhaps the simplest strategy lies in optimising the cultivation conditions of the strain to favour product biosynthesis [60, 70], especially when taking advantage of a native producer. The work done by Wang *et al.* is a good example of how one can improve antibiotic production by optimising the cultivation conditions of a natural producer [71]. In this study, the authors studied the effects of five parameters (initial pH, culture volume, shaking speed, temperature and inoculation volume) on the production of antibiotics by the bacterium *Xenorhabdus nematophila* YL001, by applying a combinatorial analysis strategy. Optimisation of the environmental parameters resulted in *ca.* 40% higher antibiotic productivity [71]. This strategy, however, may be more rewarding at later stages of engineering, when the production titres are more considerable. Fine-tuning the chosen pathway to maximise the cellular resources towards product formation is often priority, in order to reach more significant titres before venturing on optimisation of cultivation conditions. Precursor availability to the engineered biosynthetic pathway is a key aspect to be considered [58] and such can be improved by removing (or downregulating, at least) competitive pathways in the host cell [72]. In parallel, one can also consider improving the efficiency of the enzymes that constitute bottlenecks in the engineered pathway [72]. That can be addressed via heterologous expression of more efficient enzymes [73]. Overexpressing enzymes that catalyse irreversible reactions can have significant effects, as it forces a higher resource allocation towards the engineered pathway [58]. Reversible catalytic steps, on the other hand, can be improved by substituting the respective enzymes with isozymes that have higher inclination to catalyse the reaction in the desired direction [58]. Finding the perfect enzyme for each step of a biosynthetic pathway can be extremely difficult (if not impossible), as one should take in consideration that not only the metabolite availability matters, but also the cellular environment (temperature, pH, *etc.*). Nonetheless, one needs to try to find the candidate enzymes that, overall, present the characteristics that fit the best to the situation in question. Protein expression is another relevant parameter that should be considered when

optimising a biosynthetic pathway. In some cases, the expression level of the enzymes has a positive correlation with product formation [74]. In other cases, however, protein expression can constitute a severe burden to the cell and must be tightly regulated [74, 75]. Together with protein expression, one can also consider enzyme engineering, as strategy to improve catalytic activity, selectivity and/or stability [76, 77]. One can engineer an enzyme by directed evolution, where random mutagenesis is applied to create a library of mutants and then high-throughput methods are used to screen for variants with enhanced performance [78]. Alternatively, a more rational design strategy can be applied to perform mutations at specific residues of the protein via site-directed mutagenesis [79].

Often heterologous expression of either key enzymes or entire pathways results in a higher demand for certain cofactors, which can then become a limiting factor in the product formation [58]. Additionally, the overexpression of native enzymes or the introduction of heterologous pathways in an organism can lead to shifts in the ratios of certain intracellular metabolites, which can then impose a significant metabolic burden to the cell. For example, the work on santalene production in yeast, performed by Scalcinati *et al.* demonstrates the importance of combining precursor and cofactor supplies in microbial production [80], as the authors observed a significant increase in the titres when overexpressing simultaneously the prenyl transferase ERG20 and the NAD-dependent glutamate dehydrogenase GDH2. Modifying the ammonium assimilation pathway in yeast to rely on GDH2 instead of the NADP-dependent variant allowed a shift in the NADH:NADPH ratio in the cell towards the latter redox cofactor, counterbalancing the demands of the MVA pathway for NADH.

It is important to highlight the iterative nature of Metabolic engineering. Research conducted in this field of science (like many others) follows a Design-Build-Test-Learn (DBTL) cycle [58], where first a plan is devised to implement and/or improve the desired biosynthetic pathway, then genetic elements required to engineer the host cell are to be assembled into synthetic devices and transferred to the cells. The resulting engineered strains are tested and, finally, from the analysis of the gathered results, one can learn which other limitations should be addressed next and come up with new ideas/approaches for further improvement. Engineering a strain for significantly high titres of a given compound can be the product of a long and tedious endeavour, requiring several cycles of modifications, and it is also important to note that every case is unique with specific requirements and constraints. Nonetheless, the ever-increasing set of tools and techniques available for strain engineering covers a comfortable number of options that one can choose when improving a given biosynthetic pathway. It is also worth to mention that, over the last decade, part of the research conducted in this field has moved away from the traditional approaches of engineering native pathways and venture more into true synthetic and orthogonal systems,

ranging from *de novo* generation of biosynthetic pathways, to the rational engineering of proteins to catalyse reactions not included in the biochemistry found in Nature [81, 82]. The work done by Li and co-workers is an excellent example of such approach, as they first developed a noncanonical redox cofactor system based on nicotinamide mononucleotide (NMN^+) [83], and then engineered *E. coli* to produce this redox cofactor [84].

Cell-free systems are also worth mentioning, as they appeared as a valuable method to design and test novel biochemical pathways outside of the cellular context. For example, Schwander *et al.* combined 17 enzymes originating from nine different organisms of all three domains of life to create a completely new carbon fixation pathway, which showed to be functional *in vitro* at a rate of 5 nanomoles of CO_2 per minute per milligram of protein [85].

The emergence of this new side of Metabolic engineering is consequence of improvements in DNA synthesis and sequencing, more powerful computational models and prediction software, that resulted from a better understanding on gene expression and regulation, as well as protein function and structure.

Cyanobacteria as cell factories

Engineering cyanobacteria

More recently, the field of Metabolic engineering has expanded significantly to the photoautotrophic life, with several good examples of engineered cyanobacterial cells for the production of added-value compounds [86]. The added advantage of cyanobacteria to generate complex organic molecules from atmospheric CO_2, using an abundant energy source (*i.e.,* the Sun) and an abundant electron donor (*i.e.*, H_2O), made them appealing host microorganisms for metabolic engineering envisioning a more sustainable alternative for microbial production, especially with the increasing concerns on the consequences of anthropogenic activity. Engineering cyanobacteria requires the availability of genetic tools optimised for these microorganisms. The set of tools and techniques available for genetic manipulation of cyanobacteria is more limited than for the more traditional heterotrophic host organisms (*e.g.*, *E. coli* and *S. cerevisiae*); even so, there is a modest array of DNA and RNA-based tools available for different model cyanobacteria, including vectors, promoters, ribosome binding sites and transcription and translation regulators [87].

Vectors

In order to express heterologous genes in a cell, one requires a DNA element that allows the genes to be mobilised into the cells, maintained in those cells and properly replicated upon cell division. Plasmids, or vectors, are circular

DNA molecules used in genetic engineering for that purpose. The pPMQAK1 vector is a self-replicating vector containing an RSF1010-based replicon, which allows it to be replicated and maintained in different bacterial host cells, including *E. coli*, and is commonly used for quick transformation of *Synechocystis* via conjugation [88]. The Standard European Vector Architecture SEVA collection of self-replicating vectors constitutes another example of vectors designed for cyanobacterial metabolic engineering, which can easily be transformed into cyanobacteria via electroporation [89]. Besides self-replicating vectors, one can also resort to integrative vectors to either introduce or delete genes in the cyanobacterial genome. These vectors usually contain homology arms, which allow gene integration in specific loci via double homologous recombination [90, 91]. Compared to the self-replicating vectors, genome integration often leads to a more stable expression of the genes, but given that cyanobacteria possess several copies of their genome, it often takes longer to generate a fully segregated strain. Several integrative vectors have been generated and characterised, targeting either neutral sites or genes that are not essential, both in the chromosome and in the native self-replicating plasmids [90-92].

Promoters

One key aspect for effective genetic manipulation of a cyanobacterial cell is the control of the expression levels of the heterologous genes, being the selection of a proper promoter very important for successful production of a compound in the biological system. It is, thus, not surprising that many efforts in Synthetic Biology have been made to design and characterise promoters for defined expression in cyanobacteria [87, 93, 94]. Promoters are DNA sequences that act as binding sites for the RNA polymerase and dictate the frequency of the transcription rate. So far, most of the promoters used for metabolic engineering of cyanobacteria are native promoters, derived from genes that are highly transcribed, such the *psbA2* promoter, responsible for the expression of the gene encoding the D1 protein of the photosystem II [95], or the *cpc* promoter, which is responsible for the transcription of the phycocyanin beta subunit gene [96]. Being native to cyanobacteria, these promoters can be advantageous in the sense that the cells possess all the machinery required for them to work properly; however, this can be a double-edge sword, as they are also vulnerable to the cellular regulatory mechanisms, making them less predictable. Besides constitutive promoters, whose expression is always on, there are also several inducible promoters characterised and applicable for metabolic engineering purposes [95]. In opposition to the former, these will only be activated when in the presence of a specific stimulus (the inducer), which can be a chemical compound, a metal ion, or even specific monochromatic light or changes in light intensity [88, 97]. Inducible promoters can be advantageous when the product of interest is toxic to the cell or when transcription of the heterologous genes requires activation at a

specific cultivation period. The synthetic P_{trc} promoter has been widely used as isopropyl β-*D*-1-thiogalactopyranoside (IPTG) inducible promoter in *E. coli*. However, it can only as a strong constitutive promoter in *Synechocystis*, as this cyanobacterium does not possess the gene encoding LacI [94, 98]. Even with the introduction of the *lacI* gene in *Synechocystis*, this promoter still presents a leaky repressed activity and narrow range of regulation [94, 98]. A set of TetR-regulated promoters has been generated for expression in cyanobacteria, presenting a wide range of induction [98]; however, the light sensitivity of anhydrotetracyclin (the inducer) makes them more difficult to work in microorganisms grown photoautotrophically. Perhaps the best choice of characterised inducible promoters to engineer cyanobacteria is the endogenous promoters related to the metal homeostasis regulons [99, 100]. Examples of metal-inducible promoters include the Co^{2+}-inducible P_{coaT}, the Cu^{2+}-inducible P_{petE} and the Ni^{2+}-inducible P_{nsrB}, being the later the one presenting the best tuneability and the least leakiness when in the absence of the inducer [95]. More recently, some inducible promoters related to sugar catabolism that are well-known induction systems in *E. coli* have been introduced to the genetic toolbox for *Synechocystis*, including the arabinose-inducible *araBAD* promoter (P_{BAD}) or the rhamnose-inducible *rhaBAD* promoter [101, 102]. These promoters show good performance in cyanobacteria; however, being heterologous systems, they require the co-expression of the respective repressor proteins that respond to the respective sugar and regulate transcription.

5' Untranslated Region (5' UTR)

Once transcribed, the generated messenger RNA (mRNA) must be recognised by the ribosome and translated into the effective protein. The untranslated region upstream of the open reading frame, 5' UTR, can also be engineered to modulate protein expression in the cell. The ribosome binding site (RBS) is another important genetic element that affects gene expression. Several studies have focused on characterising different native and synthetic RBSs in *Synechocystis* [94, 95]. It is important to note that combinations of strong promoters and strong RBSs not always translates into a strong expression of the gene of interest; it has been demonstrated that the strength of the RBSs can be affected by the adjacent DNA sequence, as the resulting mRNA can form secondary structures that interfere with ribosome binding and prevent translation initiation [87]. There has been attempts to try to insulate the RBS from the nearby untranslated regions. The generation of this unwanted secondary structures can be mitigated using insulator sequences that act as self-cleaving ribozymes [103]. RiboJ is an example of these genetic insulator parts; it is a short sequence (75 nucleotides long) from the tobacco ringspot virus that, once transcribed, generates a hairpin ribozyme that cleaves itself from the mRNA. Adding this sequence between the promoter and the RBS allows the cleavage of the entire sequence at 5' of the RBS, fully exposing it

to the ribosome. This genetic insulator had been used in *Synechocystis* and, although not true for all cases, it generally improves protein expression [104, 105]. In a different approach, Mutalik *et al.* proposed a bi-cistronic design of the 5'UTR, aiming at generating a genetic part capable of melting any eventual secondary structures formed upstream the RBS [106]. It contains two RBSs – one for translation of a leader peptide, and a second, for translation of the gene of interest – and a TAATG start/stop double codon after the second RBS. The ribosome binds to the first RBS and starts translation of the leader peptide, melting any secondary structures by its helicase activity. When the ribosome reaches the start/stop codon, it stops translation of the leader peptide, facing at this stage another RBS, completely exposed. It quickly binds to this second RBS and initiates translation of the gene of interest. This bi-cistronic architecture often provides improved and more predictable expression of the proteins of interest compared to the mono-cistronic expression systems, and its applicability has been already proven successful in cyanobacteria [104, 105].

As abovementioned, when there is the need to temporally control the expression of a gene of interest, one can resort to inducible promoters to control its transcription. Riboswitches are the equivalent of inducible promoters when it comes to control translation. Riboswitches are 5'UTR elements that, once transcribed, form secondary structures that trap the RBS and prevent its recognition by the ribosome. Upon addition of the inducer, the secondary structure of the riboswitch changes in such way that leaves the RBS exposed and translation is initiated [107]. Although not many of the cyanobacterial native riboswitches have been thoroughly characterised, there are a few exceptions, such as the heat-sensitive 5'UTR of the heat-shock protein Hsp17 or the glutamine-sensitive 5'UTR of glutamine synthetase from *Synechocystis* [108, 109]. Synthetic riboswitches sensitive to theophylline have also been developed and widely used in cyanobacteria [110, 111].

Trans-acting small RNAs & translation control

Riboregulators are another set of genetic elements that can be used for gene expression control on the translation level [97]. These elements function in a similar way as the riboswitches. A cis-element present in the mRNA suffers conformational changes and forms a stem loop that prevents binding of the ribosome to the RBS. A trans-activating RNA (taRNA) interacts with the cis-repressed mRNA by base complementarity and unwinds the stem loop, exposing the RBS and promoting translation initiation [97]. A typical example of such system has been established in *E. coli* by Isaacs *et al.*, which is based on the intermolecular RNA pairing between synthetic cis-repressed (crR12) and trans-activating (taR12) elements [112]. This riboregulator was later adapted to function in *Synechocystis* by introducing the synthetic RBS [94] in the crR12 element [113].

Small RNA molecules (sRNAs) can also be used as repressible elements to silence translation. These molecules are widely used by bacteria (cyanobacteria included) to regulate gene expression [87]. However, our knowledge of sRNA-mediated translation control in cyanobacteria is still scarce. Translation control via sRNAs can be performed by expressing antisense RNA molecules (asRNAs) designed to be complementary to the translation start site of a given gene. When expressed in the cells, these asRNAs will bind to the target mRNA and create a double-stranded RNA complex that blocks translation [87]. Cheah *et al.* made use of a 350-nucleotide asRNA targeting the *pdhB* gene, which encodes the pyruvate dehydrogenase subunit B, in *Synechococcus elongatus* PCC 7942 and demonstrated a 40% reduction in the transcript levels [114]. Another example of RNA-based regulation of gene expression is the Hfq-MicC tool designed by Na *et al.* for *E. coli* [115]. This tool relies on the expression of the chaperone protein Hfq and a synthetic asRNA fused to a scaffold derived from the *micC* sRNA regulator. Once bound to the asRNA, the chaperone promotes the annealing of the asRNA to the target mRNA and recruits endoribonucleases for degradation of the target, while conferring protection to the asRNA from cleavage. This tool was later adapted for *Synechocystis* by Sun *et al.* to interfere with the competing pathway of malonyl-CoA, and the authors reported an increase in intracellular malonyl-CoA levels by 41% [116].

Microbial production in cyanobacteria

As mentioned in the previous section, cyanobacteria appeared as potential host organisms for sunlight-driven microbial production of commodity chemicals [86]. The highest productivities reached so far relate to chemicals derived from pyruvate, such as ethanol [117], butan-2,3-diol [118] or lactate [86], where the engineered strains presented more than 50% of the carbon fixed committed to the heterologous pathways. Usually, the fewer steps away a pathway is from carbon fixation, the more the energy is conserved and the higher the productivities are [86]. It is, thus, not surprising that pathways that stem from a central metabolite such as pyruvate present higher productivities. Sugar metabolism is also an active part of the metabolism in the cyanobacterial cell. Some cyanobacterial strains accumulate sugars, such as sucrose or glucosylglycerol, as protective molecules against changes in osmotic pressure [119]. Cyanobacteria also rely on sugar metabolism to synthesise glycogen, a storage polymer that, in some strains, can accumulate to very high amounts. Heterologous production of compounds derived from sugars, such as sucrose [120], glycerol [121] and mannitol [122], also present titres in the range of grams per litre of culture [86]. Products derived from acetyl-CoA or TCA cycle, on the other hand, tend to present lower productivities [86]. Nonetheless, there are some exceptions to these trends,

such as the work on butanol production in *Synechocystis*, performed by Liu *et al.* [104]. In this study, after several rounds of genetic manipulation of the cyanobacterial cell and modifications to the cultivation conditions, the authors were able to generate an engineered strain that reached a cumulative production of 4.8 g L^{-1} [104].

Heterologous production of terpenoids

Terpenoid biosynthesis in cyanobacteria is also connected to the pyruvate intracellular pool; however, the carbon partitioning towards this biosynthetic pathway is only 5% of the photosynthetically fixed CO_2, and most of it is inaccessible for heterologous production, as it is required for the biosynthesis of the light harvesting pigments [123]. Fine-tuning the metabolic network of the cyanobacterial cells is thus paramount for a better utilisation of the fixed carbon for the production of terpenoids. Nonetheless, heterologous production of different terpenoids has already been demonstrated in cyanobacteria (see figure 2), and significant efforts have been made over the last decades to strengthen our knowledge in terpenoid metabolism and regulation in these microorganisms (Paper VI) [29, 124].

Despite the diversity in terpene synthases found in Nature, many of those are promiscuous towards product formation. Furthermore, the generally slow kinetic rates of the terpene synthases constitute a bottleneck, especially when the carbon flux to this pathway is already low [29, 125]. The choice of promoters is a key feature to take in consideration for proper protein expression. A good example is the work done by Wang *et al.* on limonene production in *Synechococcus elongatus* PCC 7942, changing the promoter used to drive the expression of the limonene synthase to the pea *psbA2* promoter and integration of the synthetic device in the neutral site of the cyanobacterial chromosome resulted in a 100-fold increase in the limonene yields [126]. On a different perspective, Melis and co-workers took advantage of the highly expressed β-subunit of phycocyanin (CpcB) in *Synechocystis* to boost the protein expression of β-phellandrene synthase by generating fusion proteins of these two proteins and integrate the synthetic device in the *cpc* operon of *Synechocystis* [125, 127]. The outcome of this strategy was an increase in expression levels of the terpene synthase up to 20% of total cell protein, and an increase in β-phellandrene yields by 12-fold. In parallel to enhancing terpene expression, one should also consider to improve microbial production via pathway engineering. Several examples lie into this category, including improvements in substrate and/or cofactor availability, enhancing the native MEP pathway, and introduction of alternative pathways. Wang *et al.* and Lin *et al.* tried to improve the availability of GPP in cyanobacteria by expressing heterologous GPP synthases and observed a 2-fold and 1.6-fold increase, respectively, in limonene production [126, 128].

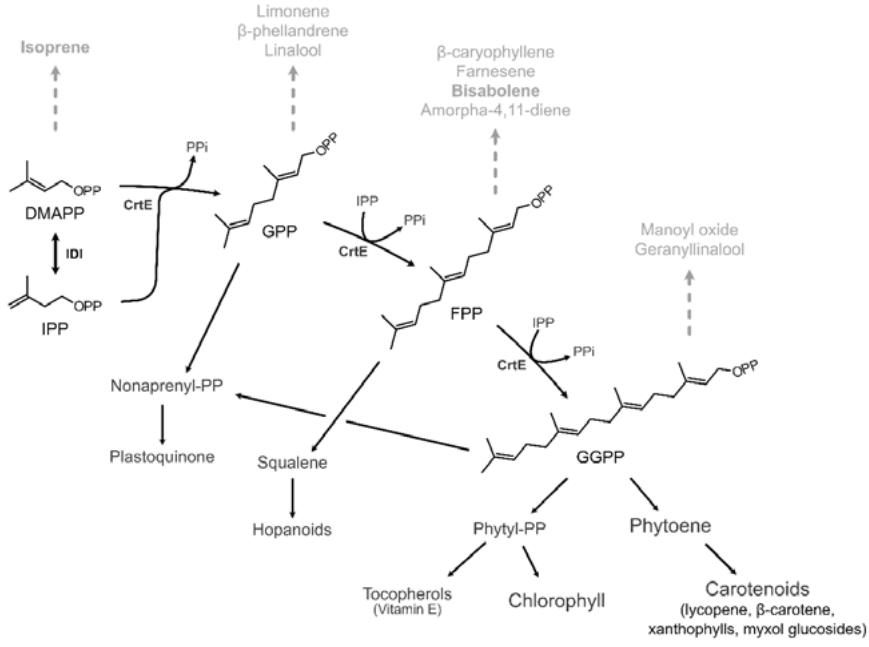

Figure 2 – Schematic representation of the terpenoid biosynthesis in cyanobacteria from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). Native terpenoids are shown in black; examples of terpenoids heterologously expressed in cyanobacteria are represented in grey and dashed lines (adapted from Paper VI, [29]). GPP – geranyl pyrophosphate; FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate; Nonaprenyl-PP – nonaprenyl pyrophosphate; Phytyl-PP – phytyl pyrophosphate; PPi – pyrophosphate; CrtE – polyprenyl transferase; IDI – isopentenyl pyrophosphate isomerase.

Heterologous expression of an FPP synthase was also the most significant step towards amorpha-4,11-diene and squalene production in the study done by Choi *et al.* [129]. On the same note, Gao *et al.* tried to overcome the high K_M of the *Populus alba* isoprene synthase for DMAPP and increase precursor availability by fusing an IDI to the terpene synthase, leading to an increase in isoprene production compared to the expression of both enzymes separated [130]. Regarding the enhancement of the native pathway, the first enzyme of the MEP pathway is known to be a limiting step for terpenoid production in cyanobacteria [131], and its overexpression had positive effects on terpenoid production in several studies [90, 129, 130]. The last enzyme, IDI, was also identified as a bottleneck in production, especially when a shift in the DMAPP:IPP ratio is required for a better availability of the polyprenyl precursors. The overexpression of this enzyme was proven fruitful for the production of isoprene, amorpha-4,11-diene and squalene [129, 130, 132]. Last, but not least, optimisation of the cultivation conditions is an important approach to improve terpenoid biosynthesis; however, this approach has been

less explored. Dienst *et al.* is a good example of the relevance of such approach (Paper I; [133]). In this study, the authors grew *Synechocystis* strains engineered for the production of three different sesquiterpenoids – bisabolene, bisabolol and patchoulol – in a high-density cultivation setup and observed an increase in the volumetric titres by more than 20-fold.

Isoprene

Isoprene (2-methylbuta-1,3-diene, according to the IUPAC nomenclature) is a 5-carbon-atom volatile organic molecule and the simplest of all terpenes found in Nature. It is a colourless liquid with a molar mass of 68 g mol^{-1} that, due to its low boiling point (*ca.* 33 to 34 ºC) and vapour pressure, readily vaporises at room temperature. This compound has an outstanding relevance to industry. Currently obtained from petrochemical sources, isoprene is mostly used for the production of synthetic rubber for tires, but its applications also include the production of adhesives and specialty elastomers [134]. Although first discovered as a product of the pyrolysis of natural rubber, this terpene is highly and widely present in Nature, being produced by many different living organisms, including bacteria, plants and animals. Its hydrophobic nature allows isoprene to easily diffuse through biological membranes and accumulate in lipophilic environments [135]. Isoprene emission by plants was identified in the 1950s [136], with estimations on global emissions ranging the 300 Tg per year [137]. At the same time, Rasmussen also identified isoprene as component of small particles present in the atmosphere that scatter light and are responsible for the blue haze observed in forested regions [138, 139].

Isoprene has been identified as part of the heat stress response mechanism of some plants, including kudzu (*Pueraria montana*), eucalyptus and aspen (*Populus* spp.) [140, 141]. This compound is synthesised directly from DMAPP in a reaction catalysed by the isoprene synthases. Several of these terpene synthases from different species have been identified and characterised, including the enzymes from several *Populus* species [142], kudzu [143], and eucalyptus [144]. Interestingly, most of the studies related to isoprene emissions were either focused on the physiological role of this terpene in the stress response of plants to temperature and light variations or on the biochemical characterisation of the isoprene synthases [145, 146]. Microbial production of isoprene was never considered until 2010, when Lindberg *et al.* presented for the first time the idea of heterologous production of isoprene in *Synechocystis* for biotechnological purposes [147]. In that study, heterologous production of isoprene was accomplished in *Synechocystis* by expressing the isoprene synthase from *Pueraria montana* (PmIspS) using the light intensity-dependent *psba2* promoter, and the authors demonstrated the importance of codon optimisation of the encoding gene. Since then, several other studies focused on improving isoprene production in cyanobacteria. In 2014, Bentley *et al.* introduced the entire MVA pathway in *Synechocystis* as an attempt to enhance the carbon partitioning to isoprene and

circumvent native regulation and the authors observed a 2.5-fold increase in the isoprene yields [148]. A few years later, Chaves *et al.* made use of the highly expressed β-subunit of phycocyanin (CpcB) to improve expression of the isoprene synthase [149]. In that study, the authors generated different fusion proteins between CpcB and PmIspS by integrating the isoprene synthase gene in the *cpc* operon of *Synechocystis* and, despite the decrease in the specific activity of the enzyme, the substantial increase in the protein amount in the cells led to an overall improvement in isoprene production by 27-fold [149]. In a subsequent study, the expression of an IDI enzyme with a bias towards DMAPP formation together with the best CpcB*PmIspS fusion protein allowed an increase in the intracellular DMAPP:IPP ration and a significant increase in the carbon utilisation for isoprene production [132]. Until today, the study that led to the highest isoprene production reported is not in *Synechocystis*, but in *S. elongatus* PCC 7942, another model cyanobacterium [130]. In this study, Gao *et al.* first tested different isoprene synthases in this cyanobacterium and identified the enzyme from *Eucalyptus globulus* (EgIspS) as one of the best. Furthermore, the authors observed an increase in isoprene production when overexpressing DXS and IDI, demonstrating a better carbon influx to the MEP pathway and a significant increase in the DMAPP:IPP ratio. To further improve isoprene production, the authors generated fusion proteins between IDI and IspS. Fusing the IspS from *Populus alba* with IDI greatly improved isoprene productivity; the same strategy, however, did not improve production when fusing IDI to EgIspS. The authors also identified IspG as limiting step in their isoprene-producing cyanobacterium and, upon expression of IDI*EgIspS fusion protein and overexpression of DXS and IspG, the resulting strain directed *ca.* 40% of the fixed carbon towards isoprene, reaching a volumetric titre of 1.26 g L^{-1} after 21 days of cultivation [130]. In 2018, Englund *et al.* conducted a systematic overexpression study of enzymes from the MEP pathway to evaluate which biochemical steps are limiting in *Synechocystis* and, using isoprene as reporter molecule, they identified DXS, IspD and IDI as the main culprits [105]. In the same study, the authors resorted to a genome-scale model to identify ten reactions from CBB cycle, lower glycolysis and cofactor synthesis as targets for enhanced carbon flux to the MEP pathway, which they then validated experimentally. In the same study, two isoprene synthases (one being EgIspS) with the highest catalytic rates reported so far were also tested. Expression of either of the isoprene synthases in *Synechocystis* was only possible when DXS and IDI were also expressed, and the final strains presented a 40-fold increase in isoprene productivity [105]. More recently, Zhou *et al.* tested whether interfering with photorespiration increases isoprene production in *Synechocystis* by deleting the *glcD1* and *glcD2* genes [150]. The resulting double knockout presented a doubling in the isoprene production, demonstrating that the energy saved by impairing photorespiration was redirected towards isoprene formation.

Bisabolene

Bisabolene (1-methyl-4-(6-methylhepta-2,5-dien-2-yl)cyclohex-1-ene, according to the IUPAC nomenclature) is a monocyclic molecule that belongs to the family of the sesquiterpenoids. This 15-carbon-atom terpene is a colourless hydrophobic liquid with a molar mass of 204.36 g mol^{-1} and can be found in Nature in the form of three distinct isomers, α-, β- and γ-bisabolene, depending on the position of their double bonds [15, 151]. The (*E*)-α-bisabolene (hereafter bisabolene) isomer can be found in the oleoresin of fir and pine trees, being synthesised by the bisabolene synthases from FPP [151, 152]. The biosynthesis of this terpenoid is wound-inducible in some species of conifers and it has been shown to play a role in the defence mechanisms against attacks by insect herbivores [152].

Like isoprene, bisabolene also presents high industrial relevance. This compound is currently used in perfumery, both as fixative agent and as fragrance [153], as well as feedstock for chemical synthesis [45]. This molecule got special attention over the last decades as Peralta-Yahya and co-workers demonstrated the applicability of its hydrogenated form – bisabolane – as biofuel for diesel engines [15]. In their study on microbial production of advanced biofuels, the authors identified the bisabolene synthase from *Abies grandis* (AgB) as the most efficient of all enzymes known to date and accomplished heterologous production of bisabolene in heterotrophic host microorganisms reaching titres over 900 mg L^{-1} upon further genetic engineering of the terpenoid biosynthetic pathway [15]. In 2014, Davies *et al.* engineered the cyanobacterium *Synechococcus* sp. PCC 7002 to produce bisabolene by expressing the bisabolene synthase from *A. grandis* both in a wild type strain and in a glycogen-deficient mutant, with the aim of understanding if disrupting glycogen could improve carbon partitioning towards terpenoid biosynthesis. Despite the lack of difference between the performance of both engineered strains, this study constituted the first successful work on microbial production of bisabolene in cyanobacteria [45]. Furthermore, Davies *et al*. demonstrated that cultivating the engineered strains with an organic overlayer allows a more efficient production of this sesquiterpenoid, as bisabolene is freed from the cells and gets trapped in the organic solvent [45]. In 2019, Sebesta and Peebles conducted the first study of bisabolene production in *Synechocystis*, where the authors tested different codon-usage sequences of AgB and different RBSs to try to maximise the expression of the bisabolene synthase [154]. Co-expression of AgB with an FPP synthase from *E. coli* improved carbon flux towards FPP, leading to bisabolene titres of 7.8 mg L^{-1} after five days of cultivation under continuous light. The authors also demonstrated a correlation between protein expression and bisabolene production [154].

Aim

The aim of the work performed throughout the last five years and described in this thesis can be summarised into three points:

I. To generate and characterise isoprene and bisabolene producing *Synechocystis* sp. PCC 6803 strains;

II. To expand the knowledge on the terpenoid biosynthetic pathway in cyanobacteria and assess ways to improve carbon partitioning through said pathway;

III. To improve the production of isoprene and bisabolene in *Synechocystis* sp. PCC 6803 via optimisation of cultivation conditions.

Results & Discussion

Photosynthetic production of (*E*)-α-bisabolene in *Synechocystis* (Papers I and II)

Heterologous (*E*)-α-bisabolene (hereafter bisabolene) production in photosynthetic microorganisms was achieved for the first time in *Synechococcus* sp. PCC 7002 by Davies *et al.* [45]. In this study, the authors raised two important aspects regarding bisabolene production in cyanobacteria: expression of the bisabolene synthase enzyme from *Abies grandis* (AgB) is enough to complete the biosynthetic pathway; and cultivating the engineered strain with an organic overlayer allows continuous removal of the product from the cells and significantly increases yields [45]. Bisabolene production had also been established in microalgae via heterologous cytosolic expression of AgB in *Chlamydomonas reinhardtii* [155]. In both studies, however, the achieved titres were very low - 0.6 mg L^{-1} (in 4 days) and 3.9 mg L^{-1} (in 7 days), respectively. In this thesis, heterologous production of bisabolene was assessed in *Synechocystis* using two different approaches: improving production by optimising cultivation conditions (Paper I); and improving precursor availability towards bisabolene production via metabolic engineering (Paper II).

Addressing cultivation conditions for efficient sesquiterpenoid biosynthesis (Paper I)

As an attempt to improve microbial production of bisabolene[2] in photoautotrophic microorganisms, a comparative analysis was conducted in *Synechocystis* using different cultivation setups, including a novel system for high-density cultivation (HDC) [156]. The gene encoding a codon-optimised version of AgB was expressed in *Synechocystis* under the control of the promoter-RBS module P_{petE}-BCD2, for strong copper-inducible expression. The resulting strain, harbouring the synthetic device in a self-replicating plasmid, was termed P_{petE}_AgBIS. In order to evaluate the performance of the expression system in *Synechocystis*, a control strain expressing the mVenus

[2] Two other sesquiterpenoids (bisabolol and patchoulol) were also used as reporters in this study; however, in line with the scope of this thesis, only the results related to bisabolene will be discussed here.

fluorescent protein under control of the same promoter-RBS module was generated.

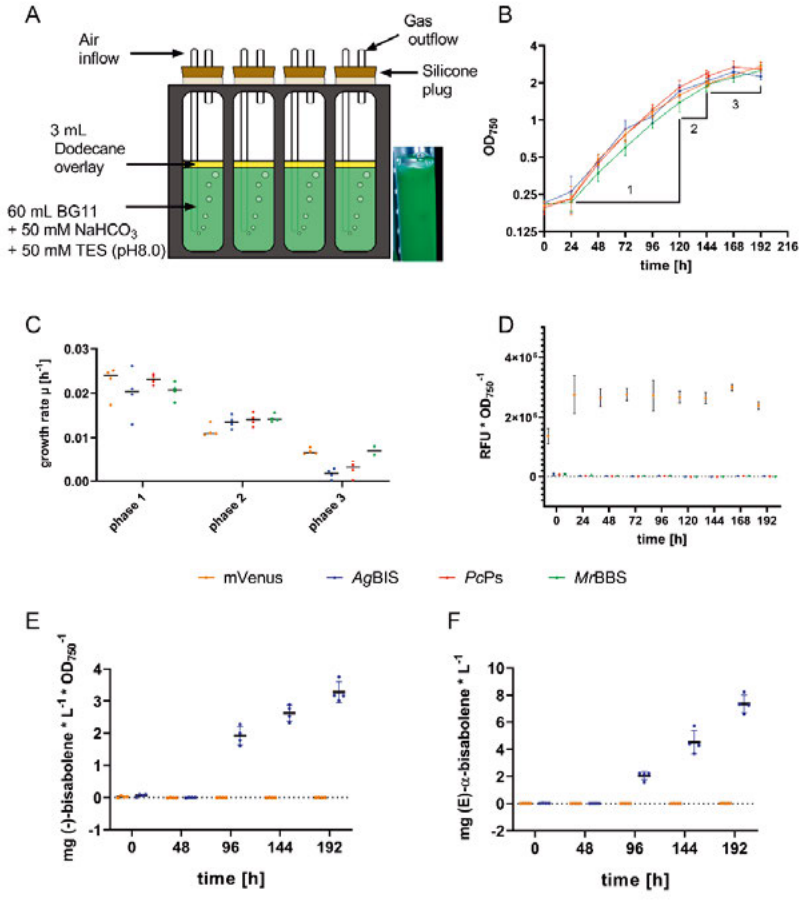

Figure 3 - Growth characteristics and heterologous gene expression in multicultivator batch cultures. Batch cultivation setup (A) in MC-1000 reactors (Photon Systems Instruments). Cell densities (OD_{750}) of all engineered strains (B) was assessed daily over 8 days of cultivation. Growth rate μ (C) was calculated from the OD_{750} data in (B). Phase 1, 2 and 3 refer to time periods 24–120 h, 120–144 h and 146–192 h, respectively. Relative fluorescence of mVenus over time (D). Volumetric (E) and specific (F) bisabolene titres, normalised to the total culture volume (mg L^{-1}) and to the OD_{750} (mg L^{-1} OD^{-1}). Figure adapted from Paper I [133].

The two engineered *Synechocystis* strains were first cultivated in 6-well culture plates for initial screening of product formation and proper copper dosage for induction, and then grown in a standard batch cultivation in a multicultivator (figure 3A), to assess the performance of the strains at different growth stages. A dodecane overlayer was applied to all cultures, for

bisabolene capture from the cells. Both strains were cultivated for 8 days in BG11 medium supplemented with bicarbonate and continuous aeration. In this setup, a lag-phase was observed on the first 24 hours. The cultures presented then a 4-day exponential growth phase, followed by 2 days of linear growth before reaching stationary phase (figure 3B and C). A constant relative expression of mVenus in the control strain was observed throughout the entire cultivation period, indicating a robust activity of the expression module and an efficient induction by 1-time addition of 2µM $CuSO_4$ (figure 3D). The bisabolene content in the dodecane layer was also assessed by periodic sampling and subsequent gas chromatography analysis. An increase in both volumetric and specific titres was observed (figure 3E and F), which suggested a dependency of cell culture productivity with growth phase and/or biomass accumulation. After 8 days of cultivation, a volumetric bisabolene titre of 7.4 ± 0.5 mg L^{-1} was reached (figure 3E).

Based on this apparent beneficial trait of cell growth on bisabolene production, the engineered strains were then cultivated in an HDC system (figure 4A), reported to facilitate fast photoautotrophic growth and stimulate biomass accumulation [157]. The cultures were grown for 4 days in a mineral rich medium and the induction was performed by adding 4 µM $CuSO_4$ at 0 and 48 hours of cultivation (to ensure proper induction when the cultures grow to higher densities). In this setup, the cultures reached optical densities one order of magnitude higher than in the previous cultivation system (figure 4B and C). They exhibited an exponential growth on the first 2 days, followed by linear growth. One of the three independent experiments was prolonged for another 4 days, with a culture dilution in a 1:1 ratio on the fourth day. These cultures presented a second linear growth phase for 2 more days, followed by a stationary phase, reaching final optical densities of *ca.* 40 (figure 4B and C). The relative fluorescence of the control strain peaked on the second day of cultivation, and started to decrease on the fourth day, indicating a dependency of the performance of the expression module on the growth stage of the cultures when in HDC conditions (figure 4D). Interestingly, the final volumetric bisabolene titre was one order of magnitude higher than the previously observed, reaching 57 ± 10 mg L^{-1} after 4 days of cultivation and 179 ± 21 mg L^{-1} after 8 days (figures 4E). Again, a correlation between a decrease in the growth rates (exponential to linear phase) and increase in the volumetric production rate was observed (figures 4G). However, when comparing the results between cultivation in HDC and in the multicultivator, a lower specific production titre was observed at days 4 and 8 (figure 4F). These results suggest that, during stages of high growth rate in HDC conditions, the intracellular carbon partitioning favours biomass accumulation over product formation.

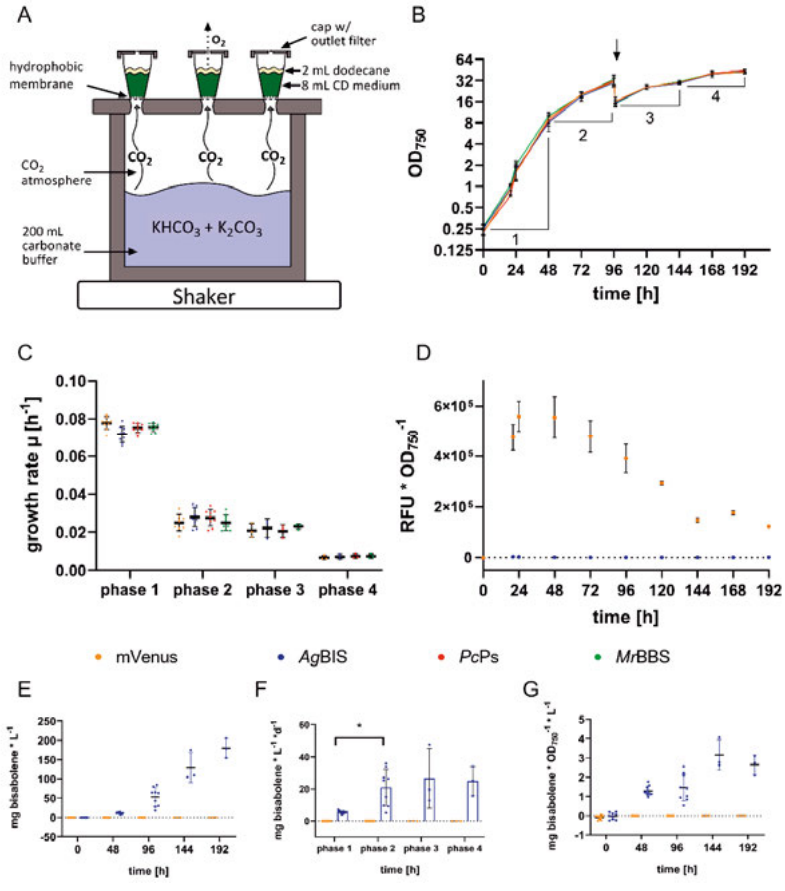

Figure 4 - Schematic representation of the HDC 6.10B system (CellDEG) (A). Cell densities (OD_{750}) of all engineered strains, recorded daily (B) over time periods of 4 days (for 2 independent experiments) and 8 days (for a third independent experiment). The arrow indicates the time point of media replenishment (96 h). Growth rate (C) μ was calculated from the OD_{750} data in (B). Phases 1-4 correspond to time periods 0–48 h, 48–96 h, 97–120 h and 120–192 h, respectively. Relative fluorescence of mVenus over time (D). Volumetric (E) bisabolene titres, normalised to the total culture volume (mg L^{-1}). Corresponding volumetric productivities (F) were calculated as the temporal yields (mg L^{-1} d^{-1}) for growth phases 1 (0–48 h), 2 (48–96 h), 3 (97–120 h) and 4 (120–192 h), respectively. Specific bisabolene titres (G), calculated as the quotient between volumetric titres and cell densities of the cultures (mg L^{-1} OD^{-1}). Figure adapted from Paper I.

The results obtained in this study indicate that HDC conditions do not improve the specific bisabolene productivity of the cell cultures, with an arrest in the specific titres when the cultures present high growth rate. Biomass accumulation seemed to be favoured over product biosynthesis in this cultivation setup; however, the dramatic increase in the general growth of the cultures compensated, leading to substantial increase in the volumetric titres.

Therefore, this analysis indicates a combination of linear growth phase and high cell density as beneficial conditions for cyanobacterial production of sesquiterpenes such as bisabolene, when coupled with *in situ* extraction by an organic layer.

Metabolic engineering of *Synechocystis* for improved bisabolene production (Paper II)

In opposition to Paper I, where the focus of the studies was on improving the cultivation to stimulate growth-dependent bisabolene productivity, this second study aimed, instead, at improving carbon flux towards bisabolene via a metabolic engineering approach.

Synechocystis relies on the MEP pathway to generate IPP and DMAPP, which are then condensed by CrtE to render the polyprenyl backbones required for the biosynthesis of hopanoids, the phytol tail of chlorophyll and tocopherols, as well as several different carotenoid molecules (figure 5A) [19]. First, a *Synechocystis* strain expressing the AgB alone (named AgBIS) was generated to serve as comparison for subsequent strains with further modifications. Contrarily to the previous study, where the bisabolene synthase was expressed under control of the copper-inducible promoter, in this study, a strong constitutive promoter was used instead to drive gene expression.

Davies *et al.* reported before that the low titres observed may be a consequence of a low availability of FPP in cyanobacteria [45]. In general, algae and plant cells possess both terpenoid biosynthetic pathways, which are localised in different cellular environments: MEP is found in the chloroplasts, while MVA is present in the cytosol [19]. These two pathways are fine-tuned to produce different DMAPP:IPP ratios, contributing to the production of different classes of terpenoids in these different environments: GPP and GGPP are more abundant in the chloroplast, being crucial for the synthesis of the light harvesting pigments; FPP is more abundant in the cytosol, being important for the production of sterols and ubiquinone [19]. Considering that *Synechocystis* only possesses one polyprenyl transferase (CrtE), it is not unreasonable that the IPP and DMAPP units may be mostly used for GGPP biosynthesis. As an attempt to evaluate if precursor (FPP) accessibility is a limiting factor in bisabolene production in *Synechocystis*, a second stain was generated, termed AgBispA, where a codon-optimised version of the FPP synthase from *Escherichia coli* (EcIspA) was co-expressed with AgB as an operon (figure 5A).

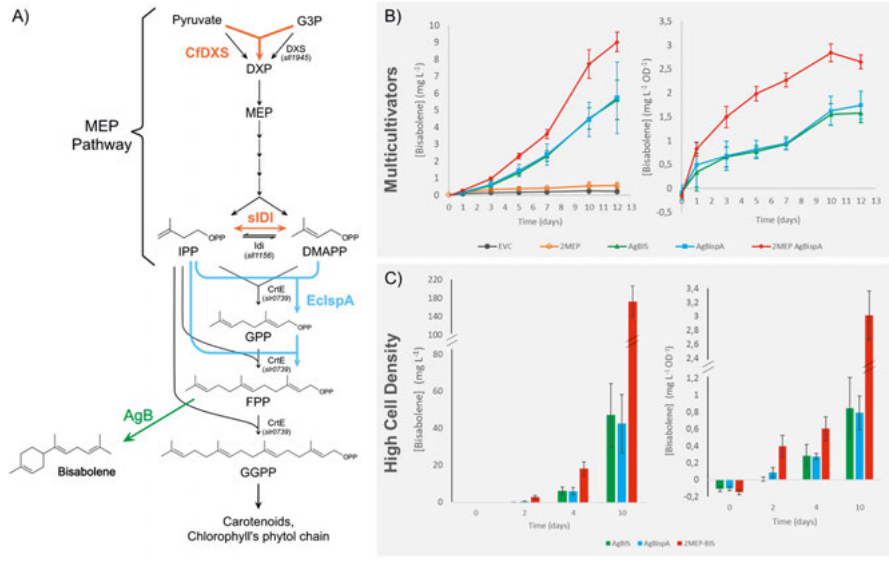

Figure 5 - Schematic representation (A) of the native MEP pathway (in black) and the metabolic engineering strategies adopted in this study for bisabolene production (green) and carbon partitioning towards terpenoid biosynthesis (orange and blue). Volumetric and specific bisabolene titres of all strains for 12-day cultivation in multicultivator (B). Volumetric and specific titres of all bisabolene-producing strains when cultivated for 10 days in HDC conditions (C). Adapted from Paper II.

Englund *et al.* conducted a study on systematic overexpression of the enzymes involved in the MEP pathway to identify limiting steps in terpenoid biosynthesis in *Synechocystis* [105]. In that study, the authors demonstrated that single overexpression of DXS, IDI or IspD significantly increased the carbon flux to isoprene formation, and expression of DXS and IDI together gave the best improvement from all combinations tested [105]. In light of those results, a third strain (2MEP-AgBispA) was generated, in which the operon with the genes encoding CfDXS and sIDI was assembled together with the AgB-EcispA operon in a convergent fashion, and then transformed into *Synechocystis* cells (figure 5A).

All synthetic devices were expressed in *Synechocystis* resorting to an RSF1010-derived self-replicating plasmid [105]. Two other *Synechocystis* strains were generated, to serve as controls: one harbouring an empty plasmid (named EVC); and another, expressing solely the DXS-IDI operon (named 2MEP).

All strains were cultivated in a multicultivator system over the course of 12 days according to the procedure described in Paper I, and growth and bisabolene accumulation in the dodecane layer were assessed periodically. Interestingly, over-expression of the EcIspA did not contribute to any improvements when co-expressed with AgB; however, enhancement of the MEP pathway via over-expression of the DXS and IDI together with EcIspA

resulted in a significant increase of both volumetric and specific bisabolene titres (figure 5B). Moreover, overexpression of DXS and IDI without AgB led to significant growth impairment, possibly due to the accumulation of one or more MEP intermediates. After 12 days of cultivation, the 2MEP-AgBispA strain reached a volumetric titre of 9 mg L^{-1} culture of bisabolene. This strain also presented a significant increase in the specific bisabolene titres, compared to AgBIS or AgBispA, suggesting a better carbon reallocation towards terpenoid biosynthesis. As a decrease in the specific bisabolene titre was observed in the previous study when high-density cultivation was applied, HDC cultivation was also carried out in this second study, to further study the carbon reallocation in the engineered strains.

Strain name	Specific yields (mg L^{-1} OD^{-1})	
	MC1000	HCD
AgB	1.45 ± 0.22 (1.55 ± 0.23)	0.8 ± 0.4 (0.9 ± 0.4)
AgBispA	1.52 ± 0.27 (1.63 ± 0.30)	0.8 ± 0.2 (0.8 ± 0.2)
2MEP-AgBispA	2.67 ± 0.18 (2.85 ± 0.19)	2.7 ± 0.7 (3.2 ± 0.3)

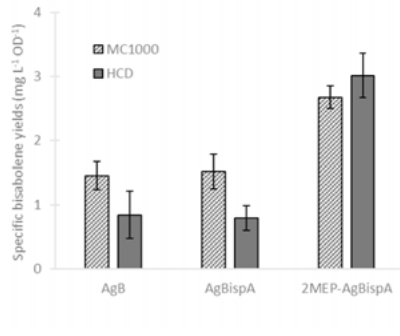

Figure 6 - Specific yields of the bisabolene-producing strains after 10 days of cultivation in multicultivator (MC) and High Cell Density (HCD) system. Values in parenthesis refer to total cumulative yields. Adapted from Paper II

All bisabolene-producing strains were cultivated in HDC over 10 days in the mineral-rich medium with a dodecane overlayer, and growth and bisabolene accumulation in the organic phase were monitored at 0, 2, 4 and 10 days of cultivation. All tested strains grew to high densities and maintained a green healthy colour until the end of the experiment. Regarding bisabolene production, cultivation of AgBIS and AgBispA in HDC conditions resulted in an increase in the volumetric titres by 9-fold when compared to the multicultivator system (figure 5C), which fits with the previous observations in Paper I. The performance of the 2MEP-AgBispA strain benefited even more from cultivation in HDC, presenting an increase in the volumetric titres by 20-fold and reaching 180 mg L^{-1} at the end of the experiment (figure 5C). Note that this titre is comparable to the results presented in Paper I. In this study, the gene encoding AgB was expressed with a constitutive promoter and a synthetic RBS [94], instead of the P_{petE}-BCD2 promoter-RBS module used in Paper I. The bi-cistronic device is known to improve translation by removing secondary structures in the mRNA and facilitating RBS exposure to the ribosome [106]. Therefore, expression protein levels between strains are not comparable, which may be the cause for the differences in volumetric titres

between the AgBIS strain (47 ± 17 mg L^{-1} in 10 days) and the bisabolene-producing strain from Paper I (179 ± 21 mg L^{-1} after 8 days). This is also in accordance with the work done by Sebesta *et al.* on bisabolene production in *Synechocystis*, as the authors demonstrated that the AgB abundance in the cell is well correlated with the product titres [154]. Nonetheless, the differences between AgBIS and 2MEP-AgBispA demonstrate the potential in enhancing carbon flux through the MEP pathway. Interestingly, the decrease in the specific titres previously observed was also noticeable in the case of AgBIS and AgBispA, but not for 2MEP-AgBispA (figure 5C and figure 6). Indeed, the specific bisabolene titre of 2MEP-AgBispA remained the same for both cultivation conditions, suggesting that enhancing the MEP pathway counteracted the natural tendency of the cell cultures to favour biomass accumulation over product formation.

In conclusion, this study applied a metabolic engineering approach for improved bisabolene production in *Synechocystis*. Overexpression of DXS, IDI and EcIspA increased the volumetric and specific titres of the engineered strain by 1.6-fold when grown in standard batch multicultivator conditions. Furthermore, overexpression of these enzymes prevented the decrease in the specific titre when the engineered strain was cultivated in high densities and contributed to a 3.5-fold increase compared to AgBIS.

Microbial production of isoprene in *Synechocystis* and its effect on metabolism and cell physiology (Papers III and IV)

Microbial production of isoprene in cyanobacteria occurs via the MEP pathway, which stems from pyruvate and G3P, two central carbon metabolites. Although cyanobacterial production of compounds derived from pyruvate often renders high productivities, only 5% of the total fixed carbon is used for terpenoid biosynthesis [123]. This is reflected in the very poor improvements observed in the heterologous isoprene production in *Synechocystis* over the last decade [105, 148-150]. Substantial modifications in the metabolic network for improved carbon fixation and/or reallocation to terpenoid biosynthesis is thus paramount for a more efficient microbial production of isoprene in cyanobacteria. The work described in paper III emerged, thus, as an attempt to find how different parts of the cyanobacterial metabolic network compete with heterologous terpenoid production and which of those parts could constitute good candidates for further engineering. In parallel, the study described in Paper IV was conducted to assess how heterologous production of isoprene affects the metabolism and cell physiology of *Synechocystis*, and how isoprene production can be improved by optimising the cultivation conditions.

Identification of competitive pathways to terpenoid biosynthesis (Paper III)

In this study, a metabolic engineering approach was applied in an isoprene-producing *Synechocystis* strain to evaluate how interfering with different pathways from the cyanobacterial metabolism affect terpenoid biosynthesis, aiming at mapping competing pathways that constitute good candidates for further engineering. Eliminating competing pathways can be very difficult, considering that the precursors for the MEP pathway are deeply connected with primary metabolism. Furthermore, as *Synechocystis* possesses several copies of its genome, gene knockout can be a very time-consuming process. Therefore, downregulation was chosen instead, as a faster screening method, with the added advantage of allowing the targeting of genes that are essential for cell survival. Twelve genes were selected, encoding enzymes related to terpenoid biosynthesis, to the central carbon metabolism and pyruvate consumption, to amino acid formation, or to general bioenergetics (see figure 7).

Genes related to the terpenoid biosynthesis were *chlP*, *crtB*, *sqs* and *crtE*, encoding enzymes involved in chlorophyll, carotenoids, squalene and general polyprenyl precursors, respectively. The gene encoding ThiG, thiazole synthase, was also chosen, as thiamine biosynthesis stems from DXP, the first intermediate of the MEP pathway. Regarding central carbon metabolism, the chosen genes were *ddH*, *pepc* and *pgi*. The first encodes lactate dehydrogenase, a pyruvate consuming enzyme; the second encodes PEP carboxylase, which was identified in a flux balance analysis performed by Englund *et al.* as an enzyme that catalyses a competing reaction to terpenoid production [105]; the last one encodes a glucose 6-phosphate isomerase, which is involved in sugar catabolism for carbon storage. Branched chain amino acids also derive from two molecules of pyruvate, which are condensed by the acetolactate synthase enzyme (AlsS). Two genes, *ilvB* and *ilvG*, were both annotated as encoding the large subunit (*i.e.*, catalytic subunit) of AlsS and, therefore, they were included in this study. The *ccmA*, encoding the first enzyme of the shikimate pathway was also included, as this pathway is used for the biosynthesis of aromatic amino acids and derives from PEP and E4P, metabolites from glycolysis and pentose phosphate pathway, respectively. Finally, concerning the bioenergetics of the cell, the gene encoding β-subunit of ATP synthase (*atpB*) was also targeted, as it was also hypothesised that a decrease in the ATP:NADPH ratio of *Synechocystis* would benefit terpenoid biosynthesis [158].

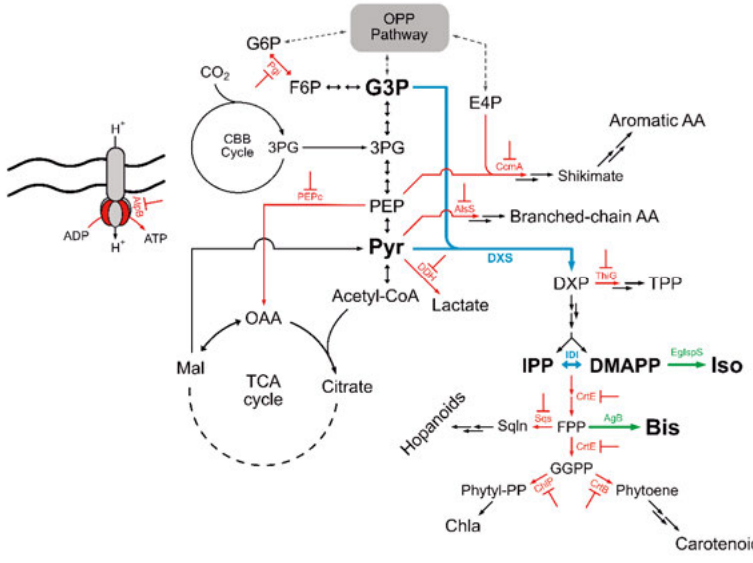

Figure 7 – Schematic representation of the metabolic pathway of a cyanobacterial cell, engineered to produced either isoprene or bisabolene, and the steps chosen as targets for gene knockdown (in red). Blue arrows represent enzymes present in the native metabolism that were overexpressed for enhanced terpenoid production. Green arrows represent heterologous expression of isoprene synthase from *Eucalyptus globulus* and bisabolene synthase from *Abies grandis* for isoprene and bisabolene production, respectively. OPP – oxidative pentose phosphate; CBB – Calvin Benson Bassham; TCA – tricarboxylic acid; G6P – glucose 6-phosphate; F6P – fructose 6-phosphate; G3P – glyceraldehyde 3-phosphate; E4P – erythrose 4-phosphate; 3PG – 3-phosphoglycerate; PEP – phosphoenolpyruvate; AA – amino acids; Pyr – pyruvate; Acetyl-CoA – acetyl coenzyme A; OAA – oxaloacetate; Mal – malate; DXP – deoxy-*D*-xylulose 5-phosphate; TPP – thiamine pyrophosphate; IPP – isopentenyl pyrophosphate; DMAPP – dimethylallyl pyrophosphate; FPP – farnesyl pyrophosphate; GGPP – geranylgeranyl pyrophosphate; Sqln – squalene; Chla – chlorophyll *a*; Iso – isoprene; Bis – bisabolene; Phytyl-PP – phytyl pyrophosphate; ADP – adenosine diphosphate; ATP – adenosine triphosphate; DXS - Deoxy-*D*-xylulose 5-phosphate synthase; IDI – IPP:DMAPP isomerase; Pgi – glucose 6-phosphate isomerase; CcmA - 3-Deoxy-*D*-arabinoheptulosonate 7-phosphate (DAHP) synthase; PEPc – PEP carboxylase; AlsS – acetolactate synthase; DDH – *D*-lactate dehydrogenase; ThiG – thiazole synthase; CrtE – polyprenyl transferase; Sqs – squalene synthase; EgIspS – isoprene synthase from *Eucalyptus globulus*; AgB – bisabolene synthase from *Abies grandis*; ChlP – geranylgeranyl pyrophosphate reductase; CrtB – phytoene synthase; AtpB – β-subunit of ATP synthase. Adapted from paper III.

The Hfq-MicC synthetic tool reported by Na *et al.* [115] and optimised for *Synechocystis* by Sun *et al.* [116] was chosen as method for individual gene knockdown of the twelve targets. This tool relies on a small RNA (sRNA), complementary to the translation start site in the mRNA, and the Hfq chaperone protein from *E. coli*. Hfq makes a complex with the asRNA, which

then binds to the mRNA, interferes with translation and promotes mRNA degradation. The expression of the chaperone was coupled with the nickel-inducible P_{nrsB} promoter, for tighter control on the activation of the tool. Additionally, isoprene, a non-native terpenoid, was selected as reporter molecule to conduct this study with the intention of insulating the effectiveness of the sRNAs from native regulation. Furthermore, isoprene is directly produced from one of the products of the MEP pathway (*i.e.*, DMAPP) and its volatile nature allows it to easily escape the cells in the form of a gas, facilitating screening.

An isoprene-producing strain with stable productivity, generated in another work (see Paper V), was used as base strain for the subsequent engineering steps. This strain, named NS1::2MEP-EgIspS, was engineered for heterologous expression of the isoprene synthase from *Eucalyptus globulus* [130] and for overexpression of DXS and IDI [105] for more efficient isoprene production, possessing all genes integrated the *slr0168* locus of the cyanobacterial chromosome [159]. The performance of this strain was assessed in closed vials over 24 hours of incubation. An exponential growth was observed in those conditions, as well as a linear time-dependent increase in the specific isoprene titres in the first 12 hours of cultivation. Once observed this linear behaviour for the first 12 hours of cultivation, the Hfq-MicC tool was first tested using *crtE* as target, as its knockdown by CRISPR interference had been previously reported to improve the production of a different non-native terpenoid [160]. The engineered strain was cultivated for 12 hours in closed vials at different nickel concentrations, and the best combination of inducer concentration and incubation period was found to be 2.5 μM $NiCl_2$ and 6 hours incubation in closed vials.

The Hfq-MicC was then used to target all other selected genes and the resulting engineered strains were cultivated for 6 hours in closed vials, both without and with 2.5 μM of $NiCl_2$. Besides *crtE*, a correlation between gene knockdown of *chlP, thiG*, *ilvG*, *ccmA* and *pgi* and increased specific isoprene titre was observed, while targeting any of the remaining genes led to similar performance as the control strains (figure 8). Real-time quantitative PCR of four target genes was performed and a decrease in relative expression was observed for all of them upon nickel induction, confirming the functionality of the Hfq-MicC system. A slight decrease in the relative expression was also observed for two of the tested targets even in the absence of inducer, indicating that the system presents some minor leakage in expression.

In *Synechocystis*, all prenyl condensations are catalysed solely by CrtE. Considering that this enzyme consumes DMAPP and IPP to build the polyprenyl precursors for the native terpenoids, it is reasonable that the knockdown of its gene translates in an increased isoprene production. Gene knockdown of *chlP* led to increase in the specific isoprene titre. GGPP is consumed both by ChlP, to build the phytol tail of chlorophyll and tocopherols, and by CrtB, for the generation of phytoene, the precursor of all

carotenoids. Chlorophyll is one of the major terpenoids in *Synechocystis*, presenting a physiological role as light-harvesting pigment in the photosystems. Chlorophyll can be recycled by re-esterification of the porphyrin ring to a new phytol tail and, despite lack of annotation in the enzymes involved in this recycling pathway, there are studies using ^{13}C labelling that highly suggest that the phytol tails can also be recycled [161]. Considering the effect of knocking down *chlP* on isoprene production, these results suggest that the recycling pathway can provide the cells with enough chlorophyll for growth, leaving more prenyl equivalents available for heterologous production.

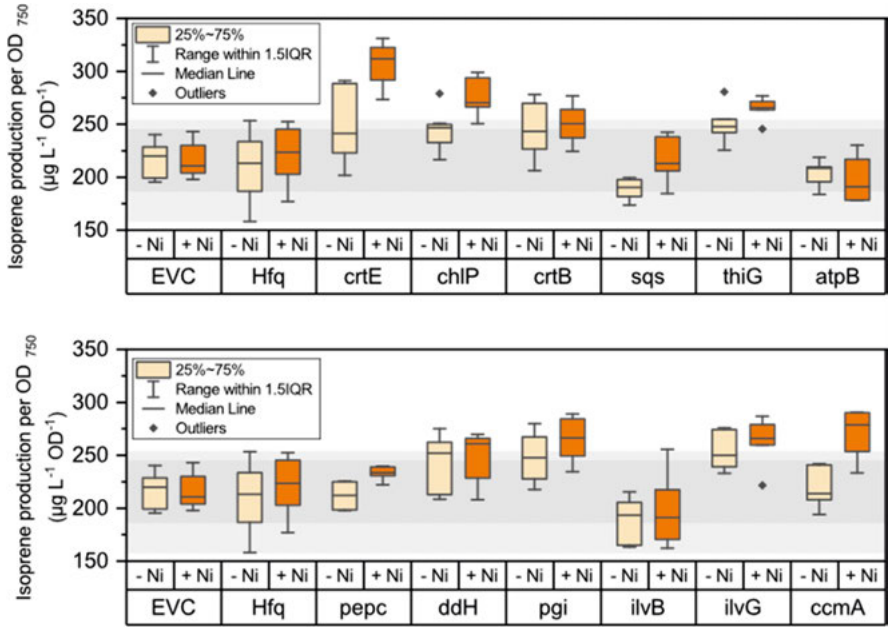

Figure 8 – Isoprene production per cell of all strains generated after 6 hours of incubation in closed vials, in the absence of nickel or in the presence of 2.5 µM $NiCl_2$. The results were split into two panels for better visualisation. In both panels, the isoprene production of both control strains was also included. Light grey area represents the production range observed for all samples of both controls, while the dark grey area represents the 25-75% range of the controls. All boxes represent values of at least six replicates from at least two independent experiments.

Targeting *thiG* also led to a positive effect on isoprene production, suggesting thiamine production as a significative competing pathway to terpenoid biosynthesis. Given that thiamine is an important cofactor for many enzymatic reactions, including the first step of the MEP pathway, one cannot simply eliminate its biosynthesis. Therefore, other strategies, *e.g.* fusing the first two enzymes of the MEP pathway for better channelling of DXP towards terpenoid production, should be considered.

Regarding amino acid biosynthesis, targeting *ilvG* translated into improved specific isoprene titres, suggesting branched chain amino acid biosynthesis as a candidate branch of the metabolic network to be engineered for improved terpenoid production. Interestingly, targeting *ilvB* did not show the same effect as targeting *ilvG*, further supporting the previous suggestions that IlvG may be the large subunit of AlsS in *Synechocystis* [162]. Engineering the shikimate pathway for higher pyruvate availability may be another option to enhance terpenoid production, as targeting *ccmA* for knockdown also gave a positive effect on the isoprene titres.

The last target that presented a positive trend towards isoprene production was *pgi*, which encodes the enzyme responsible for the interconversion of G6P and F6P. Its function is tightly connected with the carbon fluxes of glycolysis/gluconeogenesis, pentose phosphate pathway and sugar catabolism (*e.g.,* glycogen biosynthesis). The observed positive effect on isoprene production suggests sugar catabolism as another good candidate pathway to be engineered; however, a previous study on heterologous production of terpenoids in a glycogen-depleted mutant of *Synechococcus* sp. PCC 7002 did not show any improvements in the terpenoid titres when disrupting glycogen formation [45]. This suggests that either the cellular metabolism of *Synechococcus* and *Synechocystis* is different, and disrupting glycogen formation in *Synechocystis* may be beneficial for terpenoid biosynthesis, or glycogen is not the major competitor to the terpenoid biosynthetic pathway.

Finally, an experiment was conducted using bisabolene instead of isoprene as reporter molecule for knockdown of *chlP*, *crtB* and *sqs*, with the rationale that the native terpenoid biosynthesis may affect differently the production of non-native terpenoids that derive from polyprenyl precursors. A bisabolene-producing strain was generated by integrating the genes encoding DXS, IDI and AgB into the chromosome, and then engineered with the Hfq-MicC tool to target *chlP*, *crtB* and *sqs.* The engineered strains were then cultivated for 8 days with a dodecane layer and nickel induction was performed on the second day. Similarly to isoprene, targeting *chlP* for knockdown presented a positive effect in the specific bisabolene titre, strengthening the abovementioned results. Targeting *crtB*, however, presented a positive effect as well, which was not observed for isoprene. From these combined results, it seems that while chlorophyll biosynthesis is the major consumer of GGPP, the balance of GGPP in the cell is affected both by chlorophyll and carotenoids biosynthesis. Interfering with either of these pathways shifts the relative abundance of the different polyprenyl precursors, translating into a higher FPP availability for bisabolene biosynthesis. Knocking down *sqs* did not lead to any changes, possibly because the carbon flux towards hopanoids, via squalene formation, was not significant in the cultivation conditions used for this experiment. Hopanoids are important to control the fluidity of the membranes upon changes in temperature, therefore the biosynthesis of these

compounds when cultivating *Synechocystis* in standard laboratory conditions may be residual.

Characterising isoprene production in cyanobacteria – effects of light, temperature, and isoprene on the cells (Paper IV)

Isoprene is known to play important roles in plant physiology, participating in the response mechanisms against heat stress in isoprene-emitting species [146]. Thus, it would not be surprising that heterologous production of this terpenoid in *Synechocystis* may also have effects on the cells similar to those observed in plants. Additionally, stealing DMAPP from the cells to generate isoprene may affect pigment biosynthesis as well, leading to disturbances in photosynthesis and cell metabolism. It is, thus, a priority to understand how the distribution of the cellular resources changes in the isoprene producers before developing cyanobacteria as chassis for efficient terpenoid production. Therefore, this study was conducted to evaluate how the producing cells respond to specific growth conditions, which may affect the regulation of the terpenoid biosynthesis. For that, the isoprene producing strain described in Paper V was used as isoprene producer (here named "IspS") and two control strains were generated: one overexpressing DXS and IDI without expression of the isoprene synthase, named "2MEP"; and a second, expressing only the kanamycin resistance cassette, named "NS1". All strains were grown in a multicultivator system, in turbidostat regime set up to an optical density at 720 nm of *ca.* 0.5, where they were submitted to cultivation under different red, green, blue or violet monochromatic lights, as well as different light intensities and temperatures. Once the cultures were acclimated to a particular condition, isoprene production, photosynthesis efficiency and cell morphology and composition were assessed (figure 9A). Given the volatile nature of isoprene, the quantification of the isoprene was performed in closed vials. For that, samples of the acclimated cultures were taken and transferred to closed vials, which were then placed in an incubator that mimicked the cultivation conditions of the multicultivator (figure 9A). Additionally, substantial modifications in membrane-inlet mass spectrometry (MIMS) had to be made for the isoprene quantification.

When looking at the effects of cultivation under different monochromatic lights, the variations in growth rate correlate with the absorption peaks of chlorophyll and phycobilisomes: it was lowest for blue light, increased under violet and green lights, and highest under red light (figure 9B). The IspS strain presented lower growth rates for all lights tested, compared to NS1 control strain, except for blue light. These results suggest that isoprene production, which requires ATP and reduced ferredoxin, redirects energy and reducing power from biomass formation. Blue light was the condition that caused the more pronounced effects on the growth rate. It was previously reported that,

light conditions (such as blue light) that favour PSI activity over PSII cause significant imbalances in the formation of ATP and reducing equivalents, decreasing the ATP:NADPH ratio [163], which is thought to favour terpenoid biosynthesis [105, 158].

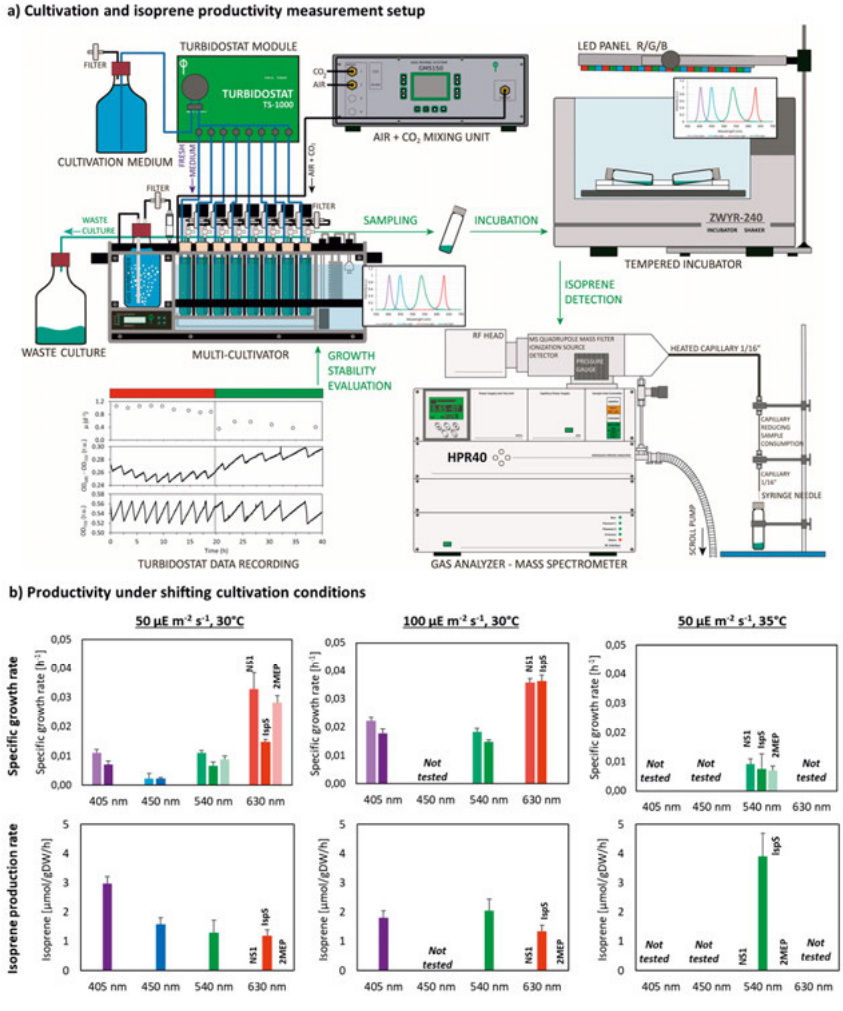

Figure 9 – Schematic representation of cultivation in a multicultivator in a turbidostat regime (A). After stabilisation under particular conditions, culture samples were transferred to closed vials for incubation in an incubator mimicking the cultivation conditions of the multicultivator. During incubations, headspace of the gas-tight vials was sampled several times for evaluation of isoprene concentration in headspace, by open inlet mass spectrometer. Specific growth rates and isoprene productivities of NS1, 2MEP and IspS strains under the tested light qualities, light intensities and temperatures (B). Adapted from Paper IV.

This could explain why the isoprene production is similar between blue light and red or green lights, despite the reduction in growth, suggesting a better carbon partitioning to terpenoid biosynthesis. An increase in the carotenoid content when the cells were grown under blue and violet light was also observed, which supports the previously mentioned hypothesis. Phycobilisome and chlorophyll content seemed to be reduced in IspS strain, regardless of the light tested, which could explain why the growth rates of this strain are lower. Concerning carotenoids, the total pool of these compounds was higher for IspS, compared to NS1, and even higher for 2MEP, which demonstrates once more the relevance of overexpressing DXS and IDI. As DXS catalyses an irreversible reaction, its overexpression forces more carbon to be reallocated to terpenoids; IDI, on the other hand, helps preventing imbalances in the IPP:DMAPP ratio.

The light intensity effect on cultivation was also addressed, by cultivating the strains under 100 µmol photons m^{-2} s^{-1} (instead of 50 µmol photons m^{-2} s^{-1}). Increasing the light intensity resulted in a general increase in the growth rate of IspS strain, but did not improve isoprene production. In fact, it even led to a decrease in the isoprene production when IspS was grown under 100 µmol photons m^{-2} s^{-1} of violet light (figure 9B). Once more, these results point out that whenever biomass accumulation is favoured, isoprene production gets compromised. The trends in pigmentation were similar to the lower light condition.

Temperature was the third parameter tested. All strains were grown under 50 µmol photons m^{-2} s^{-1} of green light at 35 ºC and the results were compared to the previous cultivation, which was carried out at 30 ºC. Pigment content was similar in this increased temperature condition, with only a variation in the amounts in xanthophylls, which was decreased in IspS, compared to NS1. Interestingly, increasing the temperature by 5 ºC resulted, roughly, in a 2-fold increase in the isoprene production. It is known that isoprene is related to response to heat stress in some plants, and that many isoprene synthases work efficiently up to 50 ºC [164]. These results indicated that cultivating at higher temperatures may be a good strategy for improved isoprene microbial production. To further address this hypothesis, the isoprene-producing strain was cultivated in small closed vials at different temperatures and isoprene content and growth were assessed over 24-hour incubation (figure 10A).

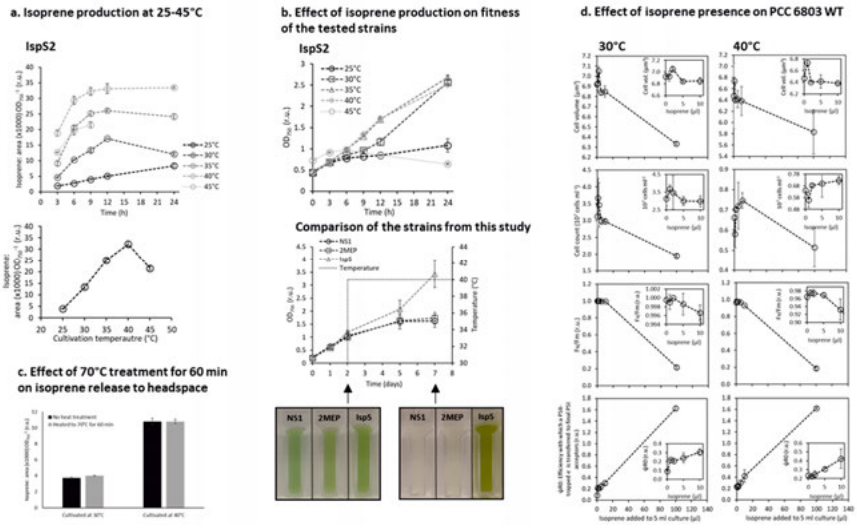

Figure 10 - Isoprene production under high temperature and the effect of isoprene on *Synechocystis* physiology. Isoprene production was evaluated in the temperature range 25 - 45 ºC (A, B). To confirm that the increased isoprene production at temperature higher than the isoprene boiling point (> 34 ºC) is a result of metabolic activity and not of simple isoprene evaporation, a heat treatment by 70 ºC for 60 min was performed (C). To address beneficial effect of isoprene presence on *Synechocystis* thermotolerance, the strains NS1, 2MEP and IspS were cultivated for 6 days at 40 ºC (B). In addition, to address physiological tolerance, 5 mL culture aliquots of *Synechocystis* WT cultures were cultivated for 24 h in the presence of 0 – 100 µL of isoprene standard (D). All cultivations were performed in glass vials sealed by PTFE/silicon rubber septa. Adapted from Paper IV.

A significant improvement in the specific isoprene titres was observed in correlation with temperature increase, with even higher productivities at 40 ºC (figure 10A). As the isoprene boiling point is *ca.* 34 ºC, heat treatment was performed to the cultures, to ensure that this effect was not just a result of a better isoprene release from the cells (figure 10C). The minor changes in isoprene content between treated and non-treated cultures could not explain alone the differences in production when the cultures were grown at 30 and 40 ºC, demonstrating that this temperature-dependent behaviour is related to metabolic changes. More interestingly, long-term cultivation at 40 ºC led to cell death for all strains, except for IspS, which survived and grew at that temperature (figure 10B). Isoprene is known to be produced by plants as response mechanism to cope with heat stress, and although not much is known about its true mechanism [150], it is thought to stabilise thylakoid membranes in plant cells exposed to elevated temperatures [145]. The findings here reported not only strengthens those previous findings, but also demonstrates that such response mechanism can be transferred to other photosynthetic organisms via heterologous expression of an isoprene synthase.

Interestingly, a reduction in cell size was observed for IspS strain regardless of the conditions tested, when compared to both controls. Given that 2MEP did not present this variation in cell size, this phenotype seemed to be tightly linked to the isoprene production. Also, considering the low isoprene yields, changes in metabolism would not be enough to account for such dramatic effects. Thus, a simple test was conducted with NS1 cells, where cultures of this strain were cultivated in closed vials in the presence of different amounts of isoprene standard. Surprisingly, a negative correlation between cell size and the amounts of isoprene added to the cultures was observed. Furthermore, adding isoprene also led to increased thermotolerance and decreased growth rates. Lea-Smith *et al.* had demonstrated before that mutants of *Synechocystis* with impaired ability to synthesise C_{15}-C_{19} hydrocarbons exhibited significant phenotypic differences, including enlarged cell size and increased division defects [165]. As isoprene is an unsaturated hydrocarbon, it is possible that small amounts of this terpenoid may be accumulating in the biological membranes and be the cause for the changes in cell morphology.

To summarise, the results observed in this study on the isoprene-producing *Synechocystis* strain can be attributed to the energy and carbon reallocation to the isoprene production only partially, since the isoprene presence likely introduced additional metabolic shifts in the IspS cells. Nonetheless, the cultivation at higher temperatures appears advantageous, not only due to the increased isoprene production rates but also due to the increased thermotolerance of the engineered strains

Hybrid approach for photobiological-photochemical production of jet fuels from isoprene (Paper V)

On a more applied perspective, a collaborative work was performed combining photosynthetic microbial production with photochemistry for a sunlight-driven production of isoprene from CO_2 and subsequent dimerization into sustainable aviation fuels (figure 11).

Jet fuels generated from petrochemical sources consist mostly of C_8 - C_{16} hydrocarbons, as a mixture of n-, iso- and cyclo-alkanes, small aromatics and alkenes. These mixtures must present a specific ratio of the different compounds in order to follow the strict requirements for jet-fuels in terms of energy density, freezing point, and viscosity. Hydrogenated mono- and sesquiterpenes have long been considered as potential jet fuels, with special focus on limonane (the hydrogenated form of limonene) [15]. There are already methods available for oligomerization of alkenes and dienes; however, these often resort to either high temperatures and pressure, or to metal catalysts. In this study, the isoprene oligomerization was addressed using arylketones for photocatalysis instead. The contributions of this thesis to this

project lie more on the photobiological side and, therefore, that part will be explored here in more detail.

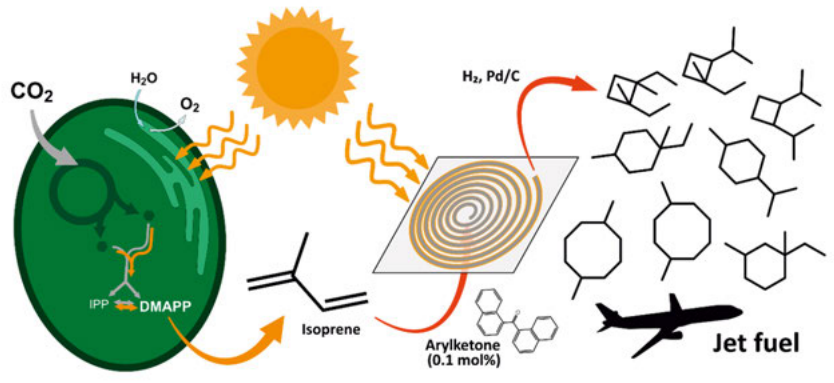

Figure 11 – Schematic representation of a combined photobiological-photochemical approach for sustainable aviation fuel generation from photosynthetically-derived isoprene and subsequent sunlight-driven dimerization into C_{10}-cycloalkanes. Adapted from Paper V [166].

As isoprene production had already been addressed before by Englund *et al.*, the already available *Synechocystis* strain harbouring the p6 EgIspS plasmid [105] was used as isoprene-producing strain to generate photosynthetically-derived isoprene for the subsequent chemical oligomerization studies. Considering the volatile nature of isoprene, the first task was to devise a cultivation setup in closed systems that would allow not only the accumulation of isoprene in the headspace, but also its capture in a deliverable way for subsequent photochemical processing. For that, liquid cultures of the isoprene-producing strain were cultivated in 60 mL closed vials, in medium supplemented with 50 mM bicarbonate and buffered with 50 mM TES at pH = 8. Cultivation was performed over 3 to 4 days with constant illumination to allow the isoprene to accumulate in the headspace. Given that isoprene is a hydrophobic compound, it can be trapped in organic solvents; a custom trapping system was thus designed for isoprene capture, where the atmosphere of the cultures was flushed through a cold solvent resorting to low pressures as driving force (figure 12). From the different n-alkanes (C6 to C12) tested as solvents, heptane was the only one that did not show peaks close to isoprene in the GC runs and remained liquid at the temperatures used for the isoprene trapping. Isoprene production was monitored by GC analysis of the headspace of the cultures before and after the isoprene capture and growth was assessed by OD_{750} measurements. Isoprene production reached 1.6 mg L^{-1} in these conditions, and capture efficiencies up to 89% were achieved using the described trapping system.

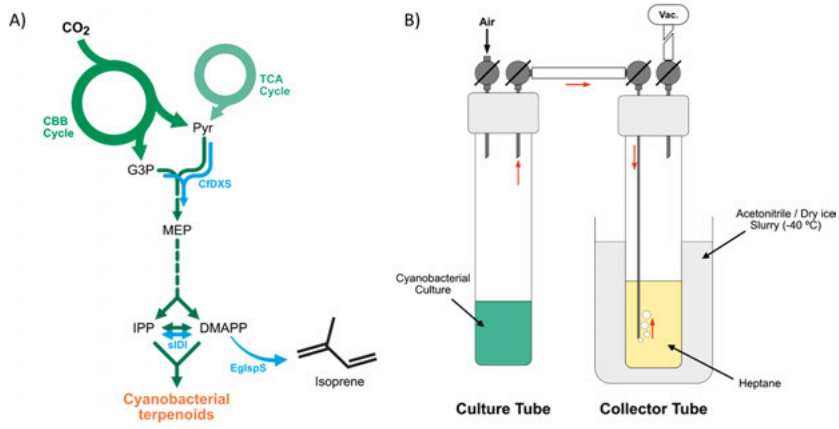

Figure 12 - Schematic representations of the cyanobacterial terpenoid pathway (green) and genetic modifications (blue arrows) in the isoprene-producing strain used in this study (A), as well as the the customised isoprene capturing system (B). CBB - Calvin-Benson-Bassham; TCA - tricarboxylic acid; Pyr - pyruvate; G3P – glyceraldehyde 3-phosphate; MEP – methylerythritol 4-phosphate; IPP – isopentenyl pyrophosphate; DMAPP – dimethylallyl pyrophosphate; CfDXS - 1-deoxy-*D*-xylulose 5-phosphate synthase from *Coleus forskohlii*; sIDI - IPP/DMAPP isomerase from *Synechocystis* sp. PCC 6803; EgIspS - isoprene synthase from *Eucalyptus globulus*. Vac – vacuum line. The red arrows indicate the flow of the isoprene vapor. Adapted from Paper V [166].

An issue that was observed in these experiments was the relatively high variability in the productivity of cultures. A second isoprene-producing strain was thus generated, with the synthetic device carrying the genes encoding DXS, IDI and EgIspS integrated in the *slr0168* locus of the cyanobacterial chromosome, for a more stable expression [159]. The resulting strain, ΔNS1::2MEP-EgIspS, presented a more consistent productivity between independent experiments.

When cultivating the isoprene-producing strains in closed vials for longer time, an increase in the pressure of the culture tubes was observed, related to the molecular oxygen build-up from photosynthetic activity and, to a minor extent, to the accumulation of isoprene. To address whether this accumulation of oxygen and isoprene in the culture tubes can be inhibitory to cell growth, a set of experiments where the atmosphere of the culture tubes was vented periodically were performed (figure 13A and B). Cultures of ΔNS1::2MEP-EgIspS were grown in closed systems for 6 days with sampling and removal of the gas phase at 12-, 24-, 48- or 72-hour intervals, and growth and isoprene production were evaluated. At every cycle, 10% of the culture was replaced by fresh medium. Cultures subjected to shorter cycles continued to grow longer (figure 13D) and kept a higher isoprene productivity for longer time. Furthermore, regardless of the cycling periodicity, the cumulative volumetric

titres were always higher than when no cycling was applied (figure 13C). These results demonstrate that semi-continuous cultivation with periodic dilution and atmospheric resetting is beneficial for isoprene production, which can be easily applicable for large scale cultivations. This trend was also observed for the production of other compounds, such as butanol [104].

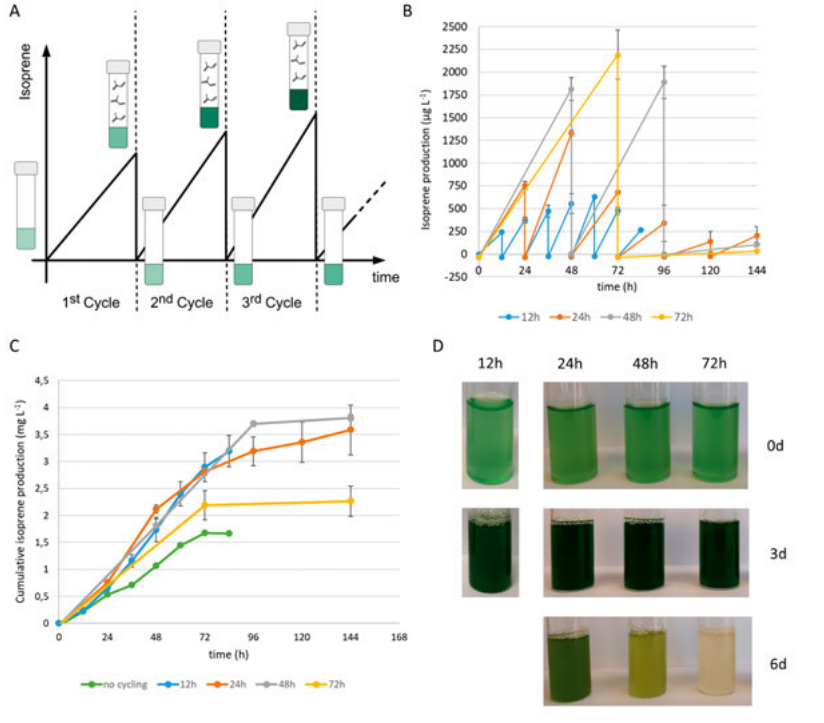

Figure 13 – Schematic representation of the cycles of isoprene production and removal in closed vials (A) and volumetric titres measured in milking experiments (B). Cumulative isoprene production (C) during the six-day experiment. Culture appearance (D) during six days of cultivation with different venting regimes. The cultures were grown in closed vials and, on every cycle, isoprene was measured, the atmosphere of the vial was reset and 10% of culture was replaced with fresh medium while maintaining sterility. In B and C, '12h', '24h', '48h' and '72h' denote the different intervals at which the respective cultures were opened for venting the gas phase. Adapted from Paper V [166].

The captured isoprene was then subjected to photochemical oligomerization by the best of the photosensitisers tested in this study and isoprene dimers could be detected, despite the low yields on photosynthetic production of isoprene. Tests with isoprene standard and simulated solar radiation were demonstrated to be possible at very high yields (*ca.* 90% conversion). Furthermore, tests were also conducted on a custom outdoors photochemical reactor to address whether the photosensitiser could work under sunlight

irradiation and, after 20 hours of irradiation, isoprene dimers were generated with yields of *ca.* 17%, proving the feasibility of the system. Heat treatment and subsequent hydrogenation of the isoprene dimers rendered a mixture of cycloalkanes whose physical properties fulfil the requirements expected for jet fuels, demonstrating that the product generated in this study can be used as fuel for the aviation sector.

Finally, and as an attempt to address whether the idea presented in this study may become an environmentally sustainable process on large scale, a life cycle assessment (LCA) was conducted. It is important to highlight that the isoprene productivities of the engineered cyanobacteria are still too low for this system to be applicable and further optimisations are required for proper scalability. The LCA was performed for the combined photobiological-photochemical system, resorting to one tonne of fuel as functional unit. The scenario described by Nilsson *et al.* for cyanobacteria production of butanol [167] was used as basis for the cultivation and production of isoprene. In this system, cultivation was assumed to take place in vertical flat panel photobioreactors covering 1 ha of land. Production phase was assumed to occur over three weeks and with a 90% carbon partitioning towards isoprene production in the cells, which would be continuously removed and transferred for photochemical processing. Further assumptions had to be made, such as cultivation using carbon from waste resources (*e.g.*, biomass combustion), 80% water recycling in the photobioreactors between production rounds and a Swedish energy mix that includes renewable energy sources. Other assumptions were made regarding energy consumption in the photochemical reactions, solar irradiation of the photochemical reactor for isoprene dimerization, and recycling of the photosensitiser and unreacted isoprene between production rounds. Overall, the climate impact was *ca.* 0.6 tonne of CO_2 equivalents per tonne of jet fuel, which is 80% less than the climate impact of fossil jet fuel. Furthermore, the LCA showed an overall positive scenario for different environmental impact categories. Nutrient composition of the cultivation medium, especially nitrates, was the dominating issue in the LCA; this, however, could be relieved by resorting to wastewater and source of nitrogen for the cyanobacteria.

In summary, this project presented a new perspective on combining microbial production in cyanobacteria with photochemical modifications to generate products with added-value to society, using solar energy as the dominating energy source. Furthermore, the product generated in this study can be used as sustainable aviation fuel, after minor modifications. Microbial production of terpenoids is still too far away to be applicable on such concepts, but the LCA conducted demonstrated a good prospect when higher productivity levels can be reached.

Conclusions & Outlook

The ability of cyanobacteria to perform oxygenic photosynthesis has inspired us to use them as cell factories for photosynthetic microbial production. This thesis was born from that dream, aiming at sunlight-driven production of terpenoids with industrial relevance in the cyanobacterium *Synechocystis*. Despite its connection to the pyruvate intracellular pool, often correlated to high productivities [86], terpenoid biosynthesis accounts for only 5% of the total fixed carbon [123]. The existence of a recycling pathway for chlorophyll [161], as well as the roles of carotenoids in these organisms in protection against photodamage [48, 168] suggest a dynamic balance between production and degradation of terpenoids, indicating that there is potential to reach higher titres for microbial terpenoid production. In the work described in this thesis, I studied how terpenoid production can be improved in *Synechocystis*, giving a special focus on two terpenoids - bisabolene and isoprene.

Bisabolene production in *Synechocystis* was first addressed, and in the two studies performed I demonstrated that growing the engineered cells to higher-densities significantly enhances the production titres. This strategy, however, has the cost of favouring biomass accumulation, which leads to decreases in the carbon allocation to terpenoid production. Applying a metabolic engineering strategy to relieve bottlenecks in the terpenoid biosynthetic pathway counteracted this effect, resulting in a strain with a 3-fold increase in the volumetric titres. From these two studies, a volumetric bisabolene titre of 180 mg L^{-1} was achieved, the highest reached in cyanobacteria, demonstrating the need to combine different approaches when enhancing microbial terpenoid production.

Regarding isoprene, the studies described in Papers III and IV culminated in several new findings that will certainly be helpful for future research. In paper III, the results suggest that interfering with amino acid biosynthesis, as well as sugar catabolism, may be beneficial for heterologous production of terpenoids. These findings are in agreement with the results from Paper IV, where a correlation between an increase in biomass accumulation and a decrease in terpenoid formation was observed. Moreover, chlorophyll biosynthesis was also indicated as potential target to be modified and the results further support the hypothesis that chlorophyll content in *Synechocystis* is a product of constant biosynthesis and recycling events [161]. While Paper

III contributed to the identification of potential targets on a metabolic engineering perspective, Paper IV demonstrated the importance of optimising cultivation conditions. From this study, I identified that lights from the visible spectrum with shorter wavelengths (*i.e.,* violet and blue) improved carbon partitioning towards terpenoid production, and violet light was the best compromise between cell growth and isoprene production. More importantly, from Paper IV, I demonstrated that not only an increase in temperature has a pronounced effect on isoprene production, I also showed that isoprene itself altered the physiology of the cells, making them more resistant to high temperatures. These findings demonstrate that the increased thermotolerance conferred to *Synechocystis* by isoprene allows the cultivation of the engineered strains at temperatures at which isoprene synthases work more efficiently. Additionally, and on a more fundamental perspective, I demonstrated that the isoprene thermotolerance mechanism can be transferred to other photosynthetic organisms via heterologous expression of an isoprene synthase, which can then be used as model organisms to study such mechanisms.

Finally, Paper V describes a more applied perspective, demonstrating that microbial production in cyanobacteria can be coupled with synthetic chemistry approaches to generate products with higher value. Furthermore, the idea presented in this study envisions the use of sunlight both for the biological and chemical sides and, despite being still on a proof-of-concept level, it constitutes the first step of a long journey towards large scale applications.

The work compiled in this thesis may not suffice for us to fully understand the complex terpenoid metabolism; however, I believe it made a significant contribution to our knowledge and has placed us one step closer to such goal.

Svensk sammanfattning

Mitt arbete som beskrivs i denna avhandling fokuserar på hur man kan förändra en specifik cyanobakterie för att generera en soldriven cellfabrik för produktion av terpenoider med hög relevans för industriell användning.

Terpenoider är den största familjen av naturligt förekommande kemiska ämnen, och den med mest diversitet. Många av de ämnen som målar landskapet i starka färger och fyller luften med dofter är terpenoider, och många av dessa kemiska ämnen har hög relevans för industriell användning för oss människor, med funktioner som pigment, dofter, vitaminer eller mediciner. Växter är en rik källa till terpenoider, men i många fall tillverkar växter så små mängder av de intressanta ämnena för att de ska kunna extraheras och användas i stor skala. Som ett mer hållbart alternativ har vi börjat använda mikroorganismer som cellfabriker för att producera kemikalier. Vi har lärt oss hur växter tillverkar dessa ämnen, och hur informationen om detta lagras i deras gener, och vi har också lärt oss hur vi kan kopiera, modifiera och överföra den informationen till mikroorganismer. Så föddes metabolisk ingenjörskonst, ett forskningsfält som fokuserar på att förändra cellers inre maskineri genom genetiska modifieringar, och på att styra om cellernas resurser till produktion av det ämne vi vill tillverka.

Cyanobakterier är fotosyntetiska mikroorganismer som funnits på vår planet i över tre miljarder år. Dessa mikroorganismer har förmågan att utnyttja solljus för att fånga koldioxid från luften, och omvandla den till alla de organiska ämnen som de behöver för att växa, och cyanobakteriers fotosyntes genererar en stor del av det syre vi andas idag. Vi insåg snart att genom att utnyttja denna fantastiska förmåga kan vi använda cyanobakterier som gröna värdorganismer för hållbar kemikalieproduktion.

Under de senaste åren har jag arbetat med att modifiera en specifik cyanobakterie, *Synechocystis*, med målet att använda den som en cellfabrik för mikrobiell produktion av två terpenoider: bisabolen och isopren. Bisabolen är en terpenoid med 15 kolatomer som produceras i vissa växter, till exempel i vissa arter av gran och tall. Det är ett ämne med industriell relevans, eftersom det kan användas som råmaterial för framställning av biobränslen. Isopren är en liten flyktig terpen som produceras av många olika organismer (inklusive hos oss människor), och används av vissa växter som försvar mot stress orsakat av höga temperaturer. Även isopren har industriella

användningsområden, eftersom det används för att framställa syntetiskt gummi till däcktillverkning, och andra material.

Jag arbetade först med att förbättra bisabolenproduktion i cyanobakterier (Paper I och II i denna avhandling). Cyanobakterier har en naturlig produktion av terpenoider, men de tillverkar inte bisabolen. Därför modifierade jag först cyanobakteriecellerna genom att introducera en gen från Kalifornisk kustgran, som kodar för det protein som tillverkar bisabolen, och jag introducerade också ytterligare gener relevanta för terpenoidsyntes från andra organismer för att förbättra bisabolenproduktionen. De modifierade cyanobakteriecellerna producerade 10 mg bisabolen per liter kultur när de odlades i tolv dagar, och efter att ha ändrat odlingsförhållandena lyckades jag nå 180 mg/L på tio dagar, den högsta bisabolenproduktion som hittills rapporterats för cyanobakterier.

Min forskning riktades därefter in på isoprenproduktion i cyanobakterier. Precis som för bisabolen så tillverkas inte isopren naturligt i cyanobakterier, men det kan göras möjligt genom att introducera det sista reaktionssteget i isopren-biosyntes i cellerna. Jag började med att introducera den gen från eukalyptus som kodar för proteinet som tillverkar isopren, tillsammans med två ytterligare gener som ökar isoprenproduktionen, i cyanobakteriens kromosom. Jag använde sedan den modifierade cyanobakterien för att studera hur isoprenproduktion konkurrerar med andra reaktioner i den naturliga metabolismen, och vilka av dessa reaktioner som kan modifieras för att förbättra produktionen av denna terpenoid (Paper III). Genom att selektivt nedreglera vissa geners funktion kunde jag observera ökad isoprenproduktion, och därmed identifiera vilka gener som kan vara lämpliga mål för ytterligare modifieringar. Jag använde samma metod även för bisabolenproduktion, och såg ett liknande mönster vilket visar att denna strategi kan användas för produktion av olika ämnen.

I ytterligare ett projekt har jag studerat hur isoprenproduktion kan påverka metabolismen och fysiologin hos cellerna under olika förhållanden. Jag odlade de isoprenproducerande cellerna med monokromatiskt ljus av olika våglängder, under olika ljusintensitet och vid olika temperaturer, och analyserade ett antal fysiologiska parametrar och koncentration av olika ämnen i cellerna (Paper IV). Jag observerade att olika våglängder på ljuset ledde till variationer i produktivitet, och att violett ljus var bäst för bildande av isopren. Temperatur var dock den parameter som hade störst effekt, då en ökning av temperaturen från 30 till 35°C ledde till tre gånger snabbare isoprenproduktion, och att öka temperaturen till 40°C gav ytterligare förbättring. Ett annat intressant resultat var att isopren i sig självt påverkar cellerna genom att göra att de blir mindre och växer långsammare. Isopren gjorde också så att cellerna kunde tåla temperaturer som var högre än de där de normaltsett kan växa, vilket bekräftar resultat från tidigare studier i växter där isopren fungerar som ett skydd mot värmestress.

I det sista projektet samarbetade vi om en metod att använda cyanobakterier för att tillverka isopren och sedan använda solljus för att på kemisk väg konvertera isoprenet till ämnen som kan användas som flygbränsle (Paper V). I detta projekt kunde jag också visa att om man odlar cellerna i slutna kärl, och med vissa intervaller öppnar kärlen och ventilerar bort bildat isopren, så kan man öka produktionen. Jag designade också ett system för att skörda isopren från kulturerna genom att fånga den i en vätska. Det infångade isoprenet kunde sedan modifieras kemiskt med hjälp av ljusenergi till flygbränslemolekyler. Detta projekt befinner sig i ett tidigt stadium, men bidrar till ett nytt perspektiv genom att visa hur man kan kombinera produktion från cyanobakterier med fotokemi för att producera industriellt relevanta kemikalier.

Mikrobiell produktion av terpenoider i cyanobakterier är en utmaning, eftersom det fortfarande finns många aspekter av cellernas metabolism och reglering som vi behöver utforska mer. Arbetet som har beskrivits i denna avhandling belyser dock några nyckelreaktioner som antingen är begränsande och behöver förbättras, eller konkurrerande reaktioner som bör förändras för att styra mer kol till terpenoid-biosyntesen. Det ökar också vår kunskap om effekter av isoprenproduktion (och isopren självt) på cellernas metabolism och fysiologi, och om vikten av att optimera odlingsförhållanden för mikrobiell produktion av denna typ av ämnen.

Sumário

O trabalho descrito nesta tese de doutoramento teve como foco a modificação de uma espécie de bactéria fotossintética, com o propósito de gerar pequenas "fábricas" capazes de utilizar a luz solar como fonte de energia para a produção de terpenóides, dado que estes apresentam alta relevância para a Indústria.

Os terpenóides são moléculas que compõem a maior e mais diversificada família de compostos naturais, incluindo compostos que dão cor às nossas paisagens e enchem o ar que respiramos com diversos aromas. Várias destas moléculas são bastante relevantes para a Indústria, servindo a humanidade como pigmentos, fragrâncias, vitaminas, ou mesmo como medicamentos. As plantas são uma fonte rica em terpenóides mas, em muitos casos, as quantidades que estas produzem são demasiado reduzidas para que a sua extracção seja viável para aplicações em larga escala. Os cientistas começaram então a utilizar microrganismos como plataformas para a produção deste tipo de compostos químicos, como alternativa mais sustentável. Começámos a aprender como é que as plantas produzem terpenóides, bem como a modificar essa informação genética e introduzi-la em microrganismos. Assim, a Engenharia Metabólica nasceu, como área de investigação que se foca na modificação da maquinaria das células via manipulação genética, e no desvio dos recursos celulares para a produção dos compostos desejados.

As cianobactérias são microrganismos fotossintéticos que habitam o nosso planeta há mais de 3000 milhões de anos. Estes organismos são capazes de utilizar a luz solar para capturar o dióxido de carbono da atmosfera e convertê-lo em vários compostos orgânicos, necessários para o seu crescimento. Estas bactérias são ainda responsáveis pela produção do oxigénio que respiramos todos os dias. Dada esta habilidade especial destes microrganismos, rapidamente começámos a utilizar as cianobactérias como "fábricas" para a produção de compostos de uma forma mais amiga do ambiente. O trabalho que realizei nos últimos anos focou-se na modificação de uma espécie de cianobactéria, *Synechocystis*, para a produção de dois terpenóides: bisaboleno e isopreno. O bisaboleno é um terpenóide produzido por diversas plantas, incluindo pinheiros e abetos. É um composto com relevância industrial, podendo ser usado quer como ingrediente em perfumes, quer como precursor para a produção de biocombustíveis. As cianobactérias produzem terpenóides,

mas não produzem bisaboleno, pelo que comecei por investigar de que forma poderia transformar *Synechocystis* para a produção deste terpenóide, assim como as estratégias que poderia adoptar para aumentar a sua produção (Artigos I e II). Para isso, primeiro modifiquei *Synechocystis* introduzindo o gene do abeto de Vancouver que codifica para a proteína que produz bisaboleno. Isto permitiu que as células passassem a produzir este terpenóide. De seguida, introduzi genes de outros organismos, que codificam para proteínas-chave no metabolismo desta cianobactéria, por forma a canalizar ainda mais os recursos celulares para a produção de bisaboleno. A expressão destas proteínas-chave permitiu duplicar a produção de bisaboleno e, uma vez alterado o método de cultivo para condições que estimulam o crescimento em densidades mais elevadas, consegui atingir uma produção de 180 mg de bisaboleno por litro de cultura, o valor mais elevado descrito na literatura.

A minha investigação voltou-se então para a produção de isopreno, destacada nos artigos III, IV e V. O isopreno é um composto pequeno e volátil, produzido por várias plantas como forma de se protegerem contra o stress térmico. Este composto é também importante para a Indústria, sendo atualmente usado na produção de borracha para pneus. Assim como no caso do bisaboleno, *Synechocystis* não produz isopreno, mas tal passa a ser possível ao introduzir o gene, por exemplo, do eucalipto, que codifica para a proteína que produz este terpenóide. No primeiro estudo, foquei-me no mapeamento de reacções do metabolismo celular que competem com a produção de terpenóides (Artigo III). Ao seleccionar diversos genes da célula e interferir com a sua função, consegui observar aumentos na produção de isopreno em alguns dos casos testados, o que permitiu a identificação de seis possíveis alvos para futura modificação genética, com a finalidade de melhorar a produção deste composto. Num segundo estudo, avaliei de que forma a produção de isopreno afeta a fisiologia das células, quando crescidas em diferentes condições de cultivo. Para tal, cultivei as células modificadas de *Synechocystis* em diferentes luzes monocromáticas, diferentes intensidades luminosas e diferentes temperaturas, e analisei diversos parâmetros fisiológicos e concentrações de vários compostos celulares (artigo IV). Neste estudo, demonstrei que o cultivo de *Synechocystis* em diferentes luzes monocromáticas afecta a produção de isopreno, sendo esta beneficiada quando as células são cultivadas com luz violeta. Mais interessante ainda é, não só a produção do isopreno ter aumentado drasticamente quando as células foram cultivadas a temperaturas mais elevadas, como a presença deste composto nas culturas permitiu-lhes sobreviver a 40 ºC. Estes resultados validam os estudos feitos em plantas e corroboram o efeito positivo do isopreno na protecção celular face à exposição a temperaturas elevadas.

O meu último projecto foi realizado numa perpectiva mais industrial, em colaboração com colegas de Química Orgânica. O objectivo deste trabalho foi utilizar cianobactérias para produzir isopreno a partir do dióxido de carbono da atmosfera, e depois usar luz solar para converter quimicamente o isopreno

em moléculas que pudessem ser usadas como combustíveis para o sector da aviação (Artigo V). Neste projecto, demonstrei que o cultivo das cianobactérias modificadas em ciclos de produção/acumulação e de captura do isopreno aumenta a produtividade das mesmas. Além disso, desenvolvi um sistema de remoção do isopreno das culturas e concomitante captura do mesmo num solvente químico. Esse isopreno capturado foi depois modificado quimicamente, usando luz como fonte de energia, resultando em moléculas que, após um passo adicional de modificação química, podem ser usadas directamente como combustível para aviões. Embora ainda se encontre numa etapa muito inicial, esta estratégia poderá vir a ser bastante importante em investigações futuras para produção de combustíveis mais ambientalmente sustentáveis.

A produção de terpenóides em cianobactérias com fins biotecnológicos não é uma tarefa fácil, uma vez que que ainda precisamos de estudar com mais detalhe o metabolismo celular e mecanismos de regulação. No entanto, o trabalho descrito nesta tese compilou informação que será importante para futuros desenvolvimentos da Engenharia Metabólica, colocando o objectivo final da produção de terpenóides em cianobactérias um passo mais próximo da realidade.

Acknowledgements

This 5-year journey has been one of the most challenging, and at the same time, one of the most rewarding moments of my life. Throughout these years, I met so many outstanding people that, not only helped me growing, professionally and personally, but also gifted me with priceless memories.

I would like to first express my profound gratitude to my supervisor **Pia Lindberg**, which demonstrated an unimaginable amount of patience to put up with my crazy ideas… as well as an unimaginable amount of trust on me, to give me the freedom to explore them. Thank you for being my mentor over the last years! Secondly, I would like to acknowledge my gratitude to my co-supervisor, **Peter Lindblad**, for welcoming me to the Cyano family and providing the informal and heart-warming environment in the group.

Karin Stensjö, thank you for being such a positive and inspiring person. I could not finish without acknowledging your trust on me whenever a problem in the lab had to be solved, or when help was needed in the courses we taught.

Thanks **Elias Englund** and **Dennis Dienst**, for your priceless help at the beginning of my PhD. You were the two people that introduced me to the work I carried out throughout these years, for which I am very grateful.

Hao Xie, thank you so much, for all of our scientific discussions, for being there whenever I needed support, for all of our lunches at Rullan, for all of the beers we drank together… for being like a brother to me!

Rui Miao, **Henrik Cam** and **Kent Cam**, I am so grateful to you! Thank you so much for all the good times we shared: the dinners, the beers, the hilarious stories… And, above all, thank you for all of the support you gave me!

Barbara Bourgade, although one of the last people I met in Sweden, you were one of the most supportive people, especially over the last months. Thank you so much for all the scientific discussions we had, for all the support you gave me in the lab, and for your friendship!

Matina Roussou and **Nikole Vogiatzi**, thank you for your emotional support, as well as for all the random chats we had in the office, which always lightened up my mood, even in the darkest days. PS: Nikole, there is still time for us to go wild with our band :P

Thanks **Kate Kukil**, for sharing this PhD experience with me since the very first day.
To all amazing people, past and present, that made the Cyano group such a wonderful place to work: **Xufeng Liu**, **Adam Wegelius** (I learnt so much from you!), **Feiyan Liang**, **Claudia Durall de la Fuente**, **Henna Mustila** (the best lab bench neighbour), **Elizabeth Söderberg**, **Johan Sjölander**, **Estelle Pignon**, **Karen Galle**, **Cecilia Blikstad**, **Amit Kugler**, and many others. More than colleagues, you were my second family throughout the last years.

I would also like to acknowledge **Johannes Messinger**, **Gustav Berggren**, **Ann Magnuson**, **Fikret Mamedov**, **Felix Ho**, **Mun Hon Cheah**, **Henrik Land**, **Orkun Aydin**, **Max Böhm**, **Princess Cabotage**, **Claudia Spallacci**, **Kaija Walter**, and many other people, past and present, from the BBC group. A special thank you note to **Manuel Boniolo**, **Luca D'Amario** and **Casper de Lichtenberg**, for all the good moments we spent together!

Marco Lorenzi, thank you for all the moments we spent together. How monotonous would be my life here in Sweden without you to talk about MtG and One Piece, to make me company in the gym, to share beers with? Thank you mate!

To **Henrik Ottosson**, **Leandro Cid Gomes** and **Anup Rana**, for the adventure we had together trying to make jets fuels out of bio-isoprene. It was indeed a long and challenging adventure, but I had a lot of fun with it.

I would like to thank **Leif Hammarström**, **Sascha Ott** and all **Fyskem** and **SMC** people, for being such great people and good neighbours. A special thanks to **Belinda Rimgard**, **Martin Axelsson**, **Astrid Nilsen-Moe**, **Anđela Brnović**, **Sigrid Berglund**, **Robert Bericat** and **Salauat Kiraev** for all the crazy party moments we had together! Thanks to the **Wine Club** and all the people that kept it running as a place for both scientific discussions and random chats about pure nonsense. Thanks **Sven Johansson**, a dear friend that help us solving so many problems in the lab. To the **(K)night club**, especially **Nidhi Kaul**, I must express my gratitude, for such amazing adventures we lived together!

Thank you **Jan Červený**, for being such a great host during my visit to Drásov, as well as all the people from the Department of Adaptive Biotechnologies, for being so friendly to me. A special thank you note to **Tomáš Zavřel**, not only for your amazing dedication to our collaborative work, but also for your friendship!

I would also like to acknowledge **Chris** and **Jason Dennis**. for your stupid jokes and crazy ideas, which helped me distracting from all stresses and problems during the last months of my PhD.

Thanks to my friends in Portugal, especially **Guilherme Pereira**, **Tiago Lopes**, **Marta Miranda**, **Ana Martins**, **Daniela Rodrigues**, **Joana Marques**, **Maria Pires**, **Bárbara Adem**, **Jéssica Lopes** and, of course, to the MAIORES **Henrique Fernandes** and **Nuno Bastos**!

Thank you to all of **my family**, for being so supportive (even when more than 3000 km away)! A special thank you note to my uncle **Luciano Ramos**, for being an inspiration to me, and for feeding my passion for science (and science fiction) with amazing conversations and experiments.

Thank you **Father**, for always being there for me, no matter what! You have been the most supportive people in my life… I would have never been able to come to Sweden and do my PhD without your support. THANK YOU!

Last, but definitely not least, I would like to thank my **Mother**. You were (and will always be) a role model to me, inspiring me to think outside of the box and to be imaginative. I would never be the person that I am now without your influence. You have my deepest gratitude… you will never be forgotten.

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