UNIVERSITY OF TURKU

EVALUATING THE POSSIBILITIES AND LIMITATIONS OF CYANOBACTERIAL HOSTS FOR FUTURE BIOTECHNOLOGICAL APPLICATIONS

Eerika Vuorio

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 626 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2020

UNIVERSITY OF TURKU

EVALUATING THE POSSIBILITIES AND LIMITATIONS OF CYANOBACTERIAL HOSTS FOR FUTURE BIOTECHNOLOGICAL APPLICATIONS

Eerika Vuorio

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA - SER. AI OSA - TOM. 626 | ASTRONOMICA - CHEMICA - PHYSICA - MATHEMATICA | TURKU 2020

University of Turku

Faculty of Science and Engineering
Department of Biochemistry
Laboratory of Molecular Plant Biology
Doctoral programme in Molecular Life Sciences

Supervised by

Academician of Science Eva-Mari Aro
Department of Biochemistry
University of Turku,
Finland

Asst. Prof. Pauli Kallio
Department of Biochemistry
University of Turku,
Finland

Reviewed by

Dr. Mervi Toivari
Industrial biotechnology and food solutions
Technical Research Center of Finland,
Finland

Jun.-Prof. Julie A. Z. Zedler
Matthias-Schleiden-Institut
Friedrich Schiller University Jena,
Germany

Opponent

Dr. David Fewer
Department of Microbiology
University of Helsinki,
Finland

ISBN 978-951-29-8079-6 (PRINT)
ISBN 978-951-29-8080-2 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)
Painosalama Oy, Turku, Finland 2020

If your dreams do not scare you,
they are not big enough.
Ellen Johnson Sirleaf

UNIVERSITY OF TURKU
Faculty of Science and Engineering
Department of Biochemistry
Laboratory of Molecular Plant Biology
EERIKA VUORIO: Evaluating the possibilities and limitations of cyanobacterial hosts for future biotechnological applications
Doctoral Dissertation, 132 pp.
Doctoral programme in Molecular Life Sciences
26.6.2020

ABSTRACT

Carbon neutral solutions and circular economy are a relevant part of a sustainable future. Solutions based on photosynthetic cyanobacteria have potential due to several facts: i) Cyanobacteria can produce target compounds directly from CO_2 and H_2O using sunlight. ii) The expanding synthetic biology toolset allows the modifications of native pathways and even the introduction of entire heterologous pathways. iii) Many strains can tolerate harsh conditions and they do not compete for prime land areas with agriculture. Cyanobacteria are more complex biotechnological production platforms compared to traditional heterotrophic bacteria, and therefore a combination of basic research and synthetic biology is required. The work presented in this thesis combines basic research of native hydrocarbon metabolism to the applied utilization of cyanobacteria for heterologous ethylene production. In addition, the host cell properties of two different cyanobacterial strains were compared and, according to the principles of circular bioeconomy, a fermentative waste product was studied as an alternative carbon source for biomass production.

The gained results highlight the unexpected importance of native hydrocarbons to cyanobacterial cells. Saturation and quantity of the aliphatic hydrocarbons is strictly controlled and membrane associated, while lack of hydrocarbons resulted in distorted growth and reduced photosynthetic activity, carbon fixation and the floating capacity of cells, which are important features for survival of photosynthetic organism in native aquatic environments. Ethylene, on the other hand, can be produced via the introduced ethylene forming enzyme. An alternative expression strategy enabled long-term ethylene production in two different host strains without the previously encountered loss of viability. Furthermore, both the original and modified gene sequence of *efe* enabled similar ethylene production in both hosts. This highlights the importance of taking into consideration strain-specific variations when designing production systems. Another important feature in production system design is the flexible use of alternative carbon sources which can function as a back-up when light is limited and increase biomass accumulation. The potential for the utilization of acetate was studied as well, and the addition of an acetate transporter was observed to increase acetate intake. This was especially beneficial for cell growth under low light conditions, despite it being accompanied by alterations in photosynthetic activity and intracellular storage compounds.

KEYWORDS: Cyanobacteria, hydrocarbons, ethylene, acetate, circular bioeconomy

TURUN YLIOPISTO
Luonnontieteiden ja tekniikan tiedekunta
Biokemian laitos
Molekulaarinen kasvibiologia
Eerika Vuorio: Syanobakteerien käyttömahdollisuuksien ja -rajoitusten arvioiminen tulevaisuuden bioteknologisten sovellusten tuotantokantoina
Väitöskirja, 132 s.
Molekulaaristen biotieteiden tohtoriohjelma
26.6.2020

TIIVISTELMÄ

Hiilineutraalit ratkaisut ja kiertotalous ovat oleellinen osa kestävää kehitystä. Yhteyttäviin sinileviin pohjautuvalla bioteknologialla on useita etuja: i) Sinilevät pystyvät tuottamaan haluttuja yhdisteitä suoraan hiilidioksidista ja vedestä auringonvalon avulla. ii) Synteettisen biologian välineistön kehittyessä luontaisia aineenvaihdunta reitistöjä on mahdollista muokata ja uusia lisätä. iii) Monet kannat kestävät karujakin olosuhteita, eivätkä sinilevät kilpaile maatalouden kanssa hyvästä kasvualasta. Sinilevät ovat paljon monimutkaisempia organismeja kuin perinteiset, pieteetillä karakterisoidut, bakteereihin ja hiivoihin pohjautuvat tuottosysteemit, joten ne edellyttävät synteettisen biologian ja perustutkimuksen yhdistelemistä. Tämä väitöskirja käsittelee luontaisten hiilivetyjen merkityksen selvittämistä perustutkimuksen menetelmin ja solujen käyttöä solutehtaina lisätyn etyleenin tuottoreitin avulla. Lisäksi tutkimuksissa vertailtiin kahta sinileväkantaa isäntäsoluina ja sovellettiin kiertotalouden periaatteita käyttämällä käymistuotteena muodostuvaa asetaattia vaihtoehtoisena energian lähteenä biomassan lisäämiseksi.

Tulokset osoittivat luontaisten alifaattisten hiilivetyjen merkityksen sinileville. Hiilivetyjen saturaatio ja määrä ovat tarkoin säädeltyjä ja yhteydessä solukalvoihin ja niiden puuttuminen vaikutti solujen kasvuominaisuuksiin sekä heikensi yhteyttämistä, hiilensidontaa ja solujen kellumista, jotka ovat tärkeitä ominaisuuksia yhteyttäville eliöille vesistöissä. Etyleenin tuotto puolestaan onnistuu lisätyn EFE–entsyymin avulla. Uudella tuottostrategialla pitkäaikainen etyleenin tuotto mahdollistettiin kahteen eri sinileväkantaan, ilman aikaisempaa isäntäsolun kärsimistä. Erilaisia *efe*-geenin variaatioita vertaamalla päästiin samoihin tuottomääriin ilman aikaisemmin käytössä olleita muokkauksia. Nämä tulokset korostivat sinileväkantojen välisiä eroja, joiden ottaminen huomioon on erityisen tärkeää tuotantostrategioita suunnitellessa. Suunnittelussa on tärkeää huomioida myös vaihtoehtoisten hiililähteiden joustava käyttö. Sekä biomassan lisäämisen että valon saatavuuden hetkellisten rajoitteiden vuoksi tutkittiin asetaatin käyttöä vaihtoehtoisena hiililähteenä. Asetaatti transportterin lisäys mahdollisti asetaatin tehokkaamman sisäänoton, joka lisäsi kasvua erityisesti vähäisessä valossa, vaikka aiheuttikin muutoksia myös solujen yhteyttämisaktiivisuuteen ja solun sisäisiin varastomolekyyleihin.

ASIASANAT: Sinilevä, hiilivedyt, etyleeni, asetaatti, kiertotalous

Table of Contents

Abbreviations

AAR	Fatty acyl-ACP reductase
ACC	1-aminocycloprone-1-carboxylate
ACP	Acyl carrier protein
ADO	Aldehyde deformylating oxygenase
AT	Acetate transporter
ATP	Adenosine triphosphate
a.u.	Arbitrary unit
b	Base
BG-11	Blue-green growth medium for cyanobacteria
BSA	Bovine serum albumin
°C	degree Celsius
cDNA	Complementary DNA
Chl *a*	Chlorophyll *a*
CBB	Calvin-Benson-Bassham cycle
Ct	Cycle threshold
DCBQ	2,6-dichloro-p-benzoquinone
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
DNA	Deoxyribonucleic acid
DUAL-PAM	DUAL-pulse amplitude modulated fluorometry
E. coli	*Escherichia coli*
EFE	Ethylene forming enzyme
EMP pathway	Embden-Meyerhof-Parnas pathway
ED pathway	Entner-Doudoroff pathway
Fd	Ferredoxin
FNR	Ferredoxin:NADPH oxidoreductase
G	Gravity
GC-MS	Gas chromatography – mass spectrometry
His-tag	Six histidine residues added to N-terminal end
i.e.	Id est, in other word
IPTG	Isopropyl-β-d-thiogalactopyranoside
KMBA	2-keto-4-methyl-thiobutyric acid

K	Kelvin
Km^R	Kanamycin resistance cassette
M	Molarity
MIMS	Membrane inlet mass spectrometry
NAD^+/NADH	Nicotinamide adenine dinucleotide
$NADP^+$/NADPH	Nicotinamide adenine dinucleotide phosphate
NORCCA	Norwegian culture collection of algae
NPP	Net primary production
m	Meter
OD	Optical density
OLS	Olefin synthase
OPP pathway	Oxidative pentose phosphate pathway
PC	Plastocyanin
PCC	Pasteur culture collection
PCR	Polymerase chain reaction
PE	Polyethylene
PET	Polyethylene terephthalate
PHB	Poly-β-hydroxybuturate
PS	Polystyrene
PsbAI	Gene encoding PS II reaction center protein D1
PS I	Photosystem I
PS II	Photosystem II
PQ	Plastoquinone
PVC	Polyvinyl chloride
RC	Reaction center
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
RTqPCR	Real-Time quantitative PCR
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Synechocystis	*Synechocystis sp.* PCC 6803
Synechococcus	*Synechococcus elongatus* PCC 7942
TCA	Tricarboxylic acid cycle
UHCC	University of Helsinki cyanobacteria collection
WT	Wild type

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Vuorio, E., Thiel, K., Fitzpatrick, D., Huokko, T., Kämäräinen, J., Dandapani, H., Aro, E-M. and Kallio, P. T. The production of alkanes and alkenes in *Synechocystis* sp. PCC 6803 is temperature-regulated and linked to photosynthetic and metabolic modulations. (manuscript)

II Carbonell, V., Vuorio, E., Aro, E-M. and Kallio, P. Sequence optimization of *efe* gene from *P. syringae* is not required for stabile ethylene production in recombinant *Synechocystis sp.* PCC 6803. *International Journal of Innovative Research in Technology & Science*, 2016; 4: 30-35.

III Carbonell, V. †, Vuorio, E. †, Aro, E-M. and Kallio, P. Enhanced stable production of ethylene in photosynthetic cyanobacterium *Synechococcus elongatus* PCC 7942. *World Journal of Microbiology and Biotechnology*, 2019; 35: 77.

IV Thiel, K., Vuorio, E., Aro, E-M. and Kallio P. T. The Effect of Enhanced Acetate Influx on *Synechocystis sp.* PCC 6803 metabolism. *Microbial Cell Factories*, 2017; 16:21.

(† equal contribution)

1 Introduction

1.1 Cyanobacteria to enable sustainability

1.1.1 Benefits of cyanobacteria based biotechnological application to respond energy demands

The initiation for the studies presented in this thesis derives from the global problems regarding the sustainability of our lifestyle with respect to the limitations set by Earth´s resources. During the next twenty years, the global economy is estimated to grow ~3.4% per year, and the human population is expected to increase from 7.4 billion to 9 billion, resulting in a +30% increase in energy demand (International Energy Agency, Fig. 1). This equals the addition of another China and India to an already challenging situation. Although many sustainable electricity production solutions are developing rapidly, *e.g.* solar, wind and water -based systems, alternative technologies that could replace fossil fuels in the production of carbon-based target compounds are still at an early phase.

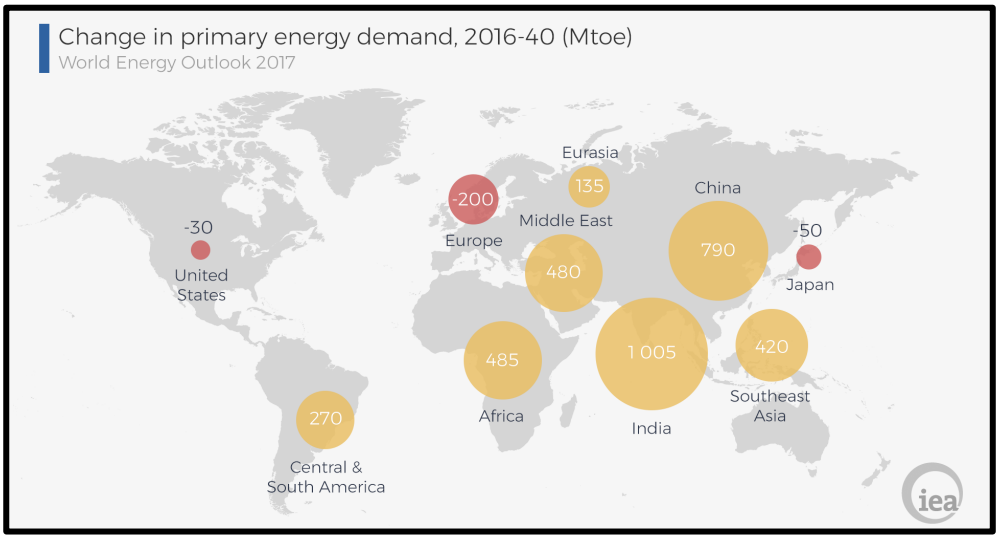


Figure 1. Overview from International Energy Agency report (IEA, 2017), where primary energy demand is expected to grow +30% by 2040.

Many parallel approaches are needed to address the issues around carbon conversion efficiency, and their simultaneous development is necessary. Due to their photosynthetic properties, fast biomass accumulation and relatively easy genetic manipulation, cyanobacteria comprise one potential solution (Khan and Fu 2020; Luan *et al.* 2020; Verma and Kuila 2020). Their utilization does not require prime land area, and up-scaled cultivation could take place in both controlled bioreactors and, in the case of wild type strains, open ponds. Co-cultures, where several species are maintained in balance and symbiosis, are also an attractive and widely studied option (Yeung *et al.* 2017; Gautam *et al.* 2019).

The cyanobacterial phylum is wide, and suitable host cells can be chosen from a broad range of cells with different native properties. The Pasteur Culture Collection (PCC), University of Helsinki Cyanobacteria Collection (UHCC) and Norwegian Culture Collection of Algae (NORCCA) are examples of large cyanobacterial strain collections containing 470, 1200 and 600 cyanobacterial strains, respectively. The species in PCC derive from all over the world, while NORCCA mostly contains Norwegian and UHCC only Finnish strains. Many of the maintained cyanobacterial strains are characterized and some have even been sequenced.

Many research groups and companies are studying multiple cyanobacterial strains with respect to their native metabolism and potential for biotechnological applications, but the available knowledge is still much more limited than for platforms based on traditional heterotrophic bacteria. Efficient production strategy planning requires a combination of well-characterized metabolism and powerful synthetic biology tools to enable the modification of the native pathways. Luckily, both of these fields are developing fast to allow targeted modification (Gale *et al.* 2019; Hagemann and Hess 2018), where a larger proportion of the harvested light energy and fixed carbon could be directed to target compounds (Thiel *et al.* 2019).

Although the complexity of cyanobacterial metabolism has its drawbacks, its unique advantage comes from low maintenance requirements – with cyanobacteria, the desired carbon-based compounds can be produced directly from CO_2, without the need to feed the cells with external reduced organic compounds such as sugars, proteins or fatty acids. This is a significant benefit, since this external energy is often derived from potential food sources, such as corn and sugarcane. Using photosynthetic hosts would release this material for food production.

Several high-demand compounds have already been produced in cyanobacteria, including ethanol (Dexter and Fu 2009), lactic acid (Angermayr *et al.* 2012), sucrose (Ducat *et al.* 2012) and ethylene (Takahama *et al.* 2003), and the industry utilizing microalgae, including cyanobacteria and algae, is growing with companies like AlgaSpring, Cellana, Algix and Algae tec. Even though the production rates are typically still low, companies like Algenol are starting to cross the threshold of making profit with a widespread product portfolio.

1.1.2 Transition from a linear economy to a circular bioeconomy

The transition from a linear "take, make, dispose" -economy to sustainable alternatives, where biobased energy and material loops are slowed down and closed, challenges biotechnological approaches as well. The somewhat overlapping terms of bioeconomy, circular economy and circular bioeconomy are presented in figure 2 and to promote circular bioeconomy, the EU has strongly advised member countries to prioritize sustainability. In fact, The Waste Framework Directive (EU, 2008, 2018a) sets targets for member states to recycle at least 55% of their municipal waste by 2025, 60% by 2030 and 65% by 2035. The climate and energy framework of Eureopean Comission sets targets for greenhouse gas emissions to be cut by at least 40% of the 1990 levels by 2030, and renewable energy forms along with energy efficiency should be increased by about one third by 2030.

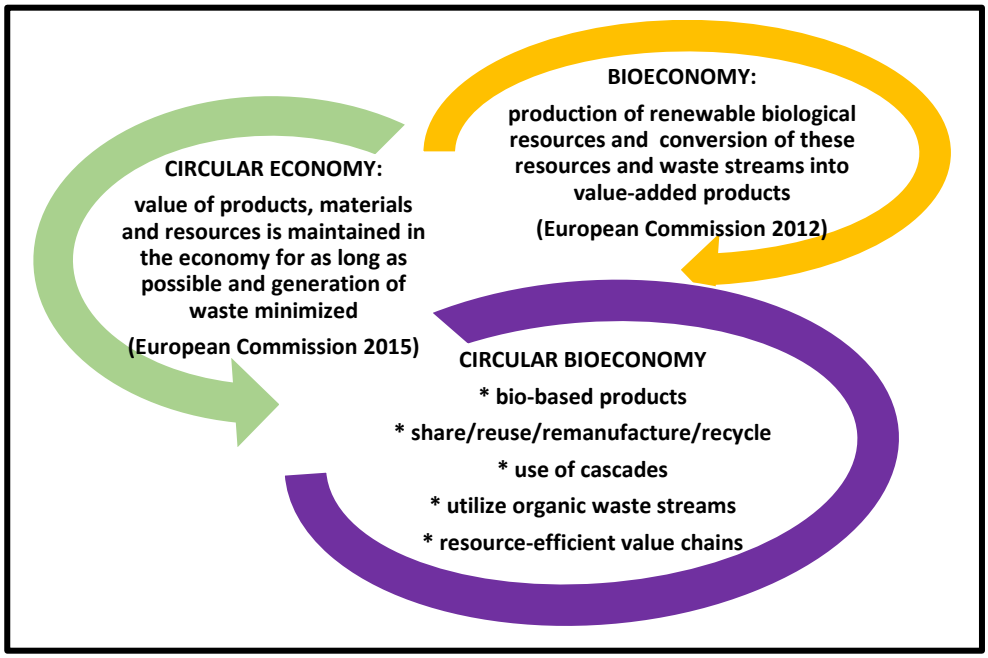


Figure 2. Defining bioeconomy, circular economy and circular bioeconomy, based on nova paper #9 on bio-based economy 2018-01

A circular bioeconomy requires novel approaches when designing biotechnological production systems. The usage of bio-based products is not enough instead, the entire life cycle needs to be taken into consideration. The recycling of the product and left-over materials should also be planned well in advance. Therefore, a circular

bioeconomy requires the formation of new complex collaborator networks and causes major changes in the entire infrastructure.

Cyanobacteria are part of blue bioeconomy, focusing on maximizing potential in aquatic resources and water expertise (EUMOFA 2018). Although the production rates of individual target compounds are still relatively low, versatility may change the game. The basic scheme of using photosynthesis to produce target compounds could be upscaled with additional starting materials and final uses of the biomass. In addition to offering sustainability advantages, widespread utilization could increase profits.

Additional starting materials should ideally consist of recyclable waste streams produced by other technologies. Cyanobacterial strains are also able to utilize nitrogen and phosphorus from municipal wastewater, which allows them to be used as a part of a water treatment process (Abdel-Raouf *et al.* 2012; Boelee *et al.* 2011). In addition, CO_2 emitting factories could reduce their carbon footprint by coupling the concentrated carbon dioxide with bioreactors used to facilitate cyanobacterial growth.

After the biotechnological production phase, the cell mass can be harvested for several purposes, as well-reviewed by Chew *et al.* (2017). Cyanobacterial cells naturally contain, for example, lipids, proteins and carbohydrates as well as small quantities of pigments, vitamins and polyunsaturated fatty acids, which can be enriched through biorefinery processes. Within biorefineries the biomass is converted to a range of fractions that function as intermediates for value-added products, similar as in oil refineries (Cherubini 2010). Potential end-products include fuels, food, fertilizers or pharmaceuticals, just to name a few potential alternatives (Chew *et al.* 2017).

1.2 Cyanobacteria as biotechnological hosts

1.2.1 Cyanobacteria are a diverse group of photosynthetic organisms

Cyanobacteria, formerly known as blue-green algae, are gram-negative photosynthetic bacteria, comprising one of the largest prokaryotic groups *(Rippka et al.* 1979; Whitton and Potts 2000). Cyanobacterial ancestors started the Great Oxygenation Event about 2.4 billion years ago (Drews 2011), and even today, the modern strains produce about 30% of oxygen annually (DeRuyter and Fromme 2008) and generate half of the biologically available nitrogen (Stal 2009). In addition, these primary producers in oceans are responsible for about half of the global net primary production (NPP), equaling 140 g of C per m^2 per year available to the first heterotrophic level in the ecosystem (Field *et al.* 1998).

Cyanobacteria are highly diverse in their morphology, physiology and habitats (Carr and Whitton 1982). Cyanobacterial strains can be either unicellular, colonial or filamentous (Rippka *et al.* 1979; Shih *et al.* 2013) and some of them can fix nitrogen (Fay 1992). They have adapted to almost all environments, ranging from deserts (Cameron 1962; Garcia-Pichel *et al.* 2001), to hot springs (Miller and Castenholz 2000) and hypersaline water reservoirs (Reed *et al.* 1984) to Antarctic meltwater bonds (Nadeau and Castenholz 2000).

Cyanobacteria are photoautotrophs capable of plant-like oxygenic photosynthesis, and many strains are also photoheterotrophs, which allows the use of other organic carbon sources. Some strains are able to grow chemoorganotrophically (Anderson and McIntosh 1991), and a few even under anaerobic conditions (Cohen *et al.* 1975). In addition to supporting the flexible use of energy sources, from a biotechnological point of view, cyanobacteria do not use resources to support other tissues.

Cyanobacteria have an unusually complex metabolism where they can carry out photosynthesis and respiration within one shared space (Mullineaux 2014) even though their cell size can be only 0,4 µm and range to over 40 µm (Whitton and Potts 2000). The distinctive features of cyanobacterial cells include multiple intracellular membrane structures, thylakoids, where photosynthesis takes place, and large protein compartments, carboxysomes, which are needed for carbon fixation.

1.2.2 Strains *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942

Due to a wide range of strain-specific properties, many cyanobacterial species are used by different laboratories. *Synechocystis* sp. PCC 6803 and *Synechococcus elongatus* PCC 7942 (*Synechocystis* and *Synechococcus* from hereon) are two well-studied cyanobacterial model organisms (Table 1). Although they have many similarities, strains specific distinct features have favored to maintain both strains as research objectives. *Synechocystis* was isolated from a Californian freshwater lake in 1968, deposited to PCC (Stanier *et al.* 1971) and became the first sequenced photosynthetic organism in 1996 (Kaneko *et al.* 1996; Kaneko and Tabata 1997). Several different laboratory versions of *Synechocystis* have evolved (compared in (Kämäräinen *et al.* 2018)), and one glucose tolerant substrain has become a particularly popular host for photosynthetic modifications (Anderson and McIntosh 1991; Williams 1988). *Synechococcus*, previously known as *Anacystis nidulans*, is also a freshwater cyanobacterium (Stanier *et al.* 1971) with a sequenced genome (Holtman *et al.* 2005). Within first publications, *Synechococcus* was reported to have a doubling time of only of 2.1 h (Kratz and Myers 1955a), which may have resulted widespread use in laboratories (Whitton and Potts 2000). Both of these strains are

naturally competent to uptake foreign DNA (Grigorieva and Shestakov 1982; Shestakov and Khyen 1970) and perform homologous recombination (Kufryk *et al.* 2002), which makes their manipulation convenient.

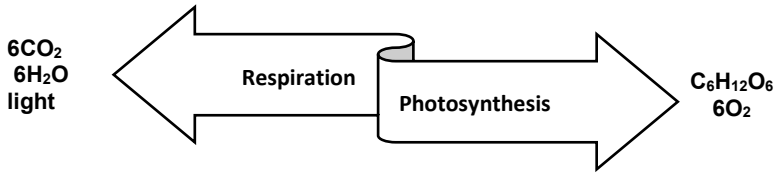
Table 1. Comparison of two widely used cyanobacterial model strains. References: [1] *(Stanier et al.* 1971); [2] (Schuergers *et al.* 2016); [3] (Sarcina and Mullineaux 2000); [4] (Kaneko *et al.* 1996; Zerulla *et al.* 2016); [5] (Holtman *et al.* 2005; Mori *et al.* 1996); [6] (Kaneko *et al.* 2003); [7] (Xu and McFadden 1997); [8] (Yang and McFadden 1993); [9] (Yang and McFadden 1994); [10] (Chen *et al.* 2008); [11] (Van der Plas *et al.* 1992); [12] (Kratz and Myers 1955b); [13] (Anderson and McIntosh 1991); [14] (Stanier *et al.* 1971); [15] (Rippka *et al.* 1979).

	Synechocystis sp. PCC 6803	*Synechococcus elongatus* PCC 7942
Kingdom Phylum Order Genus	Bacteria Cyanobacteria Chroococcales *Synechocystis*	Bacteria Cyanobacteria Chroococcales *Synechococcus*
Morphology and shape	Unicellular [1] and Circular [1]	Unicellular [1] and Rod [1]
Diameter	3 µm [2]	2x5 µm [3]
Size of circular chromosome (copy number)	3.7 Mb [4] (multiploid)	2.7 Mb [5] (multiploid)
plasmids (size)	pSYSM (120 kb) [6] pSYSX (106 kb) [6] pSYSA (103 kb) [6] pSYSG (44 kb) [6] pCC5.2 (5.2 kb) [7] pCA2.4 (2.4 kb) [8] pCB2.4 (2.4 kb) [9]	pANL (also called pUH25) (46.2 kb) [10] pANS (also called pUH24) (7.8 kb) [11]
metabolism	facultative photoautotroph [12,13]	obligate photoautotroph [12]
culture conditions	BG-11 [14, 15]	BG-11 [14,15]
ability to fix nitrogen	no [14]	no [14]

1.3 Photosynthesis and central carbon metabolism

1.3.1 Plant-like oxygenic photosynthesis

Cyanobacteria are the only prokaryotes capable of plant-like oxygenic photosynthesis, which means that they can use sunlight, carbon dioxide and water to fuel their metabolism (Cardol and Krieger-Liszkay 2017; Mamedov *et al.* 2015) (Fig. 3 a). The reaction stoichiometry is:



The light is harvested by phycobiliproteins, *i.e.* phycocyanin, allophycocyanin and phycoerythrin, which in varying combinations form antenna-like phycobilisomes, that are attached to large protein complexes called photosystem II (PS II) and photosystem I (PS I), located in thylakoid membranes. The light excites an electron to a higher orbital, and ,via resonance energy transfer, this energy is funneled through phycobilisomes to a reaction center (RC), where charge seaparation takes place and reduces a primary electron acceptor (Watanabe and Ikeuchi 2013). The membrane-bound plastoquinone (PQ) pool acts as the primary electron acceptor at PS II and the lost electrons are replaced through water splitting, which releases protons to lumen and oxygen to atmosphere. A linear transport chain transfers the electrons from PS II to PS I via the cytochrome b6-f complex. From there, the electrons are carried by soluble plastocyanin (PC) after increasing proton gradient. At PS I, another light-induced excitation takes place and cytosolic ferredoxin (Fd) functions as the primary electron acceptor. Finally, the electrons are transferred by ferredoxin:NADPH oxidoreductase (FNR) to reduce $NADP^+$ to NADPH.

To increase the proton gradient generated, the excitation energy from PS I can also be directed backwards, so that it makes another round through cyclic electron flow (Alric *et al.* 2010). The resulting proton gradient facilitates adenosine triphosphate (ATP) synthesis by ATP synthase, where a total of 14 protons is required to generate 3 ATP molecules (Seelert *et al.* 2000). Generally, the ratio between the ATP and NADPH molecules produced is 3:2, in order to facilitate carbon fixation, and cyclic electron flow is necessary to balance this ratio (Allen 2002; Alric *et al.* 2010). On the otherhand, ATP is needed by the cell also for other purposes.

1.3.2 Carbon fixation and central carbon metabolism

The produced ATP and NADPH are directed to carbon assimilation reactions in a Calvin-Benson-Bassham cycle to produce organic compounds from atmospheric CO_2 (Janasch *et al.* 2018) (Fig. 3 b). This carbon fixation process takes place in the cytoplasm and carboxysomes, which are specialized polyhedral microcompartments containing ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Kerfeld and Melnicki 2016; Shively *et al.* 1973). Carbon dioxide is accumulated into the carboxysomes by a carbon concentrating mechanism (CCM) through active transportation (Badger and Price 2003). Finally, the fixed carbon is in the form of glyceraldehyde-3-phosphate, which functions as a precursor to all other organic compounds the cell needs.

Glucose is an important organic compound that can be stored as glycogen, which can be used as a universal feedstock molecule for cells (Yoo *et al.* 2007). Glycogen storage is especially important for cyanobacteria when light is scarce and

photosynthesis is limited (Monshupanee and Incharoensakdi 2014). The stored energy is retrieved through three alternative routes: the Embden-Meyerhof-Parnas (EMP) pathway (produces 2* NADH and 2* ATP per glucose), the Entner-Doudoroff (ED) pathway (produces 1* NADPH, 1* NADH and 1* ATP per glucose) or the oxidative pentose phosphate (OPP) pathway (produces 2* NADPH per glucose) (Fig. 3 c) (Chen *et al.* 2016). After glycolysis, generated pyruvate continues through the tricarboxylic acid (TCA) cycle under aerobic conditions or through fermentative metabolism under anaerobic conditions (Fig. 3 c). The cyanobacterial TCA cycle produces reducing equivalents and precursors through unusual steps where 2-oxoglutarate dehydrogenase is replaced by 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase (Zhang and Bryant 2011).

Cyanobacterial species may encounter regular anoxic conditions when oxygen consumption exceeds production during dark night time or due to shadowing in dense cultures (Stal and Moezelaar 1997). Common fermentation end products include acetate, ethanol, lactate, formate, glycolate and oxalate (Heyer and Krumbein 1991). Acetate is an especially interesting product, since after conversion to acetyl-CoA it can serve as a building block to produce glutamate, glutamine, proline and arginine for protein synthesis or secondary metabolites, such as terpenoids, flavonoids and polyketides. Some cyanobacterial species also have a glyoxylate cycle, which allows acetate utilization to fuel even the entire metabolism (Zhang and Bryant 2015). The glyoxylate cycle is a shortcut in the TCA-cycle where two decarboxylation steps are bypassed, enabling gluconeogenesis.

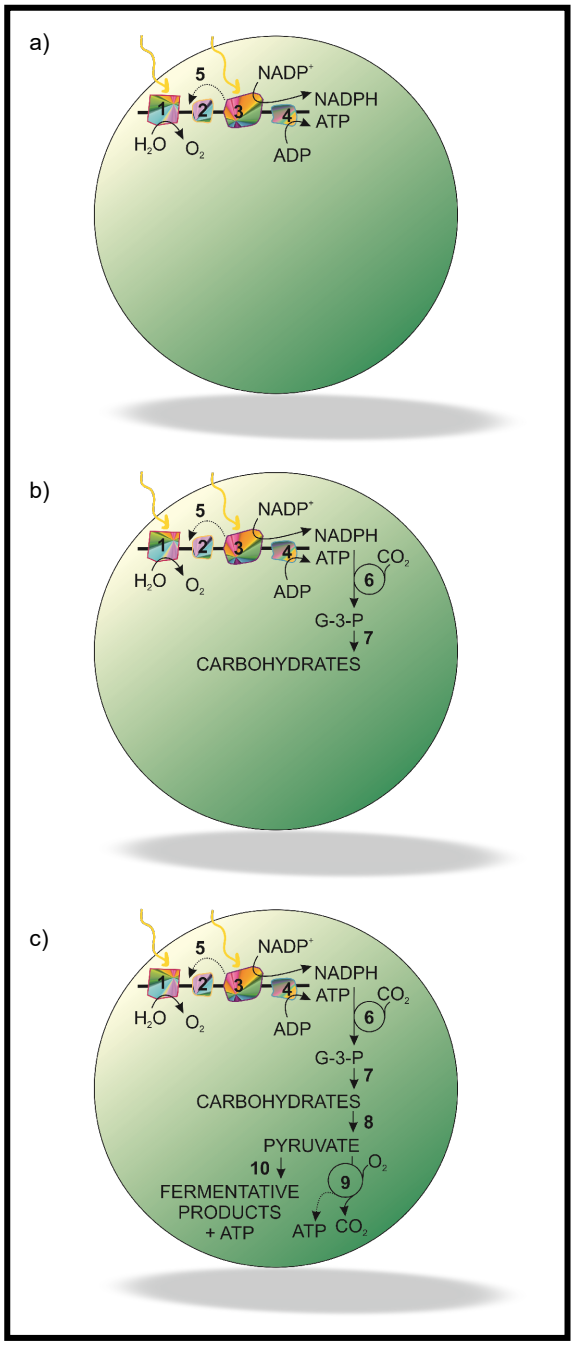


Figure 3. Simplified presentation of light and dark reactions as well as central carbon metabolism in cyanobacteria.

a) Light reactions take place in the thylakoid membranes, where a linear electron transport chain transfers electrons from water to $NADP^+$. Electrons are excited at (1) photosystem II, where they facilitate the splitting of H_2O and the release of O_2. The electrons are transferred through the plastoquinone pool to (2) the cytochrome b_6f complex, where the energy is used to transfer protons into the lumen. Soluble plastocyanin transfers the electrons from the cytochrome b_6f complex to (3) photosystem I where the electrons are excited by light a second time before being transferred by ferredoxin and ferredoxin:NADPH reductase to reduce $NADP^+$ to NADPH in the cytoplasm. As the electrons move from PS II to PS I the energy is used to transfer several protons to the lumen side of the thylakoid membranes. (4) F_1F_0 ATP synthase uses this proton gradient to power the production of ATP. In (5) cyclic electron flow, the electrons circle from photosystem I back to the PQ pool to increase the proton gradient and allow the adjustment of the intracellular ATP:NADPH ratio.

b) The NADPH and ATP produced are used in (6) a Calvin-Benson-Bassham cycle, where atmospheric CO_2 is bound and glyceraldehyde-3-phosphate generated and directed towards the production of other organic compounds, such as (7) sugars.

c) Carbohydrates, such as glucose, can be used to maintain metabolism. Glucose is broken down through (8) glycolysis into pyruvate, which can be used for multiple purposes. Under aerobic conditions, it can be broken down further through (9) a tricarboxylic acid cycle or, under anaerobic conditions, through (10) fermentative metabolism.

1.3.3 Strain-specific variations in the photosynthetic steps

Based on the widely recognized endosymbiosis theory, the photosynthesis of higher plants originates from cyanobacteria, since the responsible organelles, chloroplasts, trace back to engulfed cyanobacterial ancestors (Yoon *et al.* 2004). Therefore, the photosynthetic steps are similar among different organisms. However, several differences have also evolved between higher plants and cyanobacteria, as well as between cyanobacterial strains. One example of these differences that is relevant to the current topic can be found in the repair cycle of the PS II protein complex, where the D1 and D2 proteins function as docking sites for many redox-active components involved in water oxidation. D1, in particular, is easily damaged by light and needs to be replaced with another copy frequently (Mulo *et al.* 2009; Tyystjärvi *et al.* 1994). D1 is encoded by a *psbA* gene family in different ways among the cyanobacterial species: In *Synechococcus*, the D1:1 isoform is encoded by *psbAI* under low light conditions, while the production of D1:2, encoded by *psbAII* and *psbAIII*, increases under high light conditions (Schaefer and Golden 1989). *Synechocystis* produces only one type of D1 with two different genes, i.e. *psbA2* and *psbA3*, which can compensate for each other (Vass *et al.* 2000). Thus, if in biotechnological applications heterologous genes are introduced to a locus coding D1 proteins, the distortion of parallel genes influences the viability of the host cell in different extent and such strain specific variations should be taken into consideration when designing modifications.

1.3.4 Studying photosynthesis

Many photosynthetic processes can be measured, and different strains can be compared based on their photosynthetic efficiencies. This is especially useful when assessing the consequences of genetic modifications or experimental conditions. From a biotechnological viewpoint, the desired goal is, usually, efficient photosynthesis and the straightforward transference of the gathered energy into target compounds with minimal energy loss.

In phycobilisomes, the pigment molecules absorb most efficiently in 500 to 650 nm range and absorbed energy is transferred to two chlorophyll *a* (Chl *a*) reaction center molecules in PS I and PS II (Liu et al. 2005). Absorption maximum of PS I RC is at 700 nm, and chl *a* is called P700, while absorption maximum of PS II RC is at 680 nm, and respectively the chl *a* is called P680 (Ishikita *et al*. 2006). After exication of RC Chl *a* the charge separation takes place and the primary electron acceptor captures the lectron from RC Chl *a* and forwards it to an electron transfer chain (Watanabe and Ikeuchi 2013) The phycobilisomes are able to move between the PS II and PS I complexes and the efficiency of the light-harvesting properties of PS II and PS I can be evaluated based on the different

absorption properties of P700 and P680, using for example, DUAL-pulse amplitude modulated fluorometry (Dual-PAM) (Campbell *et al.* 1998; Kalaji *et al.* 2014; Schuurmans *et al.* 2015).

Once the primary electron acceptor of P680, plastoquinone, has accuired the excited electrons from Chl *a*, the replacing electrons are withdrawn from two water molecules, simultaneously releasing protons to increase the proton gradient and releasing O_2 into the atmosphere as a side product. Oxygen evolution can be measured with, for example, an oxygen electrode (González *et al.* 2001). Although the oxygen atoms in the P680 reaction center are well stabilized by a manganese-four-cluster, harmful singlet oxygen and reactive oxygen species are released throughout the photosynthetic processes. The singlet oxygen can be detected through His-mediated oxygen uptake and the reactive oxygen species, i.e. hydrogen peroxide, hydroxyl radicals, peroxyl radicals and peroxynitrite, through fluorescence measurement after adding 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA). (Mattila *et al.* 2015)

Membrane inlet mass spectrometry (MIMS) allows a measurement of gas fluxes based on the stable isotope distribution of gases. The quantification takes place in a mass spectrometer, where gases enter through a gas-permeable membrane. The method can be used to measure, for example, gross and net oxygen evolution as well as carbon fixation with supplemented isotopes. (Beckmann *et al.* 2009; Schuurmans *et al.* 2015).

Several other methods can also be used to characterize the photosynthetic properties of different strains. Light absorption properties can be measured by illuminating cells with a wide light spectrum (Wojtasiewicz and Stoń-Egiert 2016). When cells are frozen to 77 kelvin and illuminated with 580 nm or 440 nm light, PS II and PS I peaks with phycobilisomes absorb light. The quantitation of associated proteins can be made with a traditional western blot based on well-studied antibodies or with more modern methods, such as selected reaction monitoring (SRM) quantitative proteomics with mass spectrometry (Vuorijoki *et al.* 2016).

1.4 Hydrocarbons

1.4.1 Native hydrocarbons

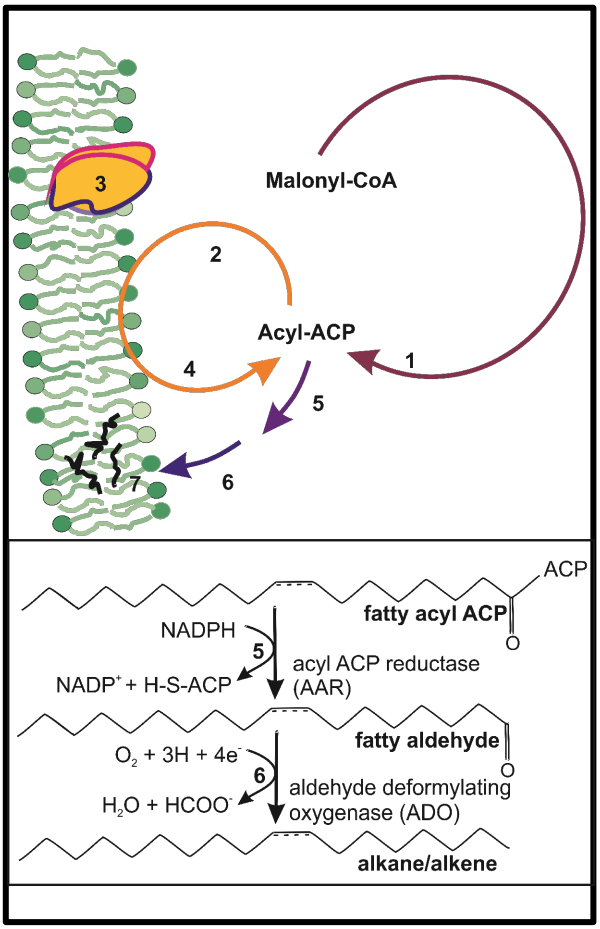
Aliphatic hydrocarbons, i.e. alkanes, alkenes and alkynes, are components in petroleum-based fuels used in traditional combustion engines. They are currently mainly produced from fossil crude oil, and therefore alternative sustainable sources are widely sought-after. Cyanobacterial native hydrocarbons were already reported 50 years ago (Han *et al.* 1968: Han *et al.* 1969; Winters *et al.* 1969), and more

recent studies confirm the aliphatic hydrocarbon production as a unique feature among all studied cyanobacterial species (Coates *et al.* 2014). The conserved production of these inert and hydrophobic compounds, without any known function, seems peculiar, especially since all the other micro-organisms appear to lack this property.

The most common cyanobacterial hydrocarbons i.e. heptadecane, heptadecene, pentadecane and 7-methylheptadecane, all fit into the "diesel-range" (Coates *et al.* 2014). Combustion quality in diesel fuels is described with a cetane number (CN), which quantifies the elapsed time between injection and combustion. The CN numbers for these cyanobacterial hydrocarbons are $CN_{heptadecane}$ =105, $CN_{pentadecane}$=96 and $CN_{7\text{-}methylheptadecane}$=79-80 (Creton *et al.* 2010), which makes them excellent fuel candidates for existing combustion engines.

The biological purpose of native hydrocarbons is still elusive, and hypotheses include participation in chemical signaling, buoyancy, preventing desiccation and maintaining membrane fluidity (Coates *et al.* 2014). Recent findings suggest that native hydrocarbons may be important for cyanobacterial photosynthesis, and more specifically to the maintenance of proper ATP:NADPH ratio by influencing cyclic electron flow (Berla *et al.* 2015) and proper PS II to PS I ratio (Lea-Smith *et al.* 2016). In addition, hydrocarbons appear to be associated with cyanobacterial membrane curvature, which in turn influences the growth and morphology of the cells (Lea-Smith et al. 2016). In plants and insects, native hydrocarbons are known to function as surface waxes, water barriers, antipathogens (Bernard *et al.* 2012; Bourdenx *et al.* 2011; Kosma *et al.* 2009) and even pheromones (Blomquist and Jackson 1979; Howard and Blomquist 2004).

Cyanobacteria have two mutually exclusive pathways for producing hydrocarbons (Coates *et al.* 2014; Lea-Smith *et al.* 2015; Lea-Smith *et al.* 2016), i.e. the AAR/ADO pathway (Schirmer *et al.* 2010) and the Olefin synthase (OLS) pathway (Mendez-Perez *et al.* 2011). The majority of studied cyanobacterial species, including *Synechocystis*, contain the AAR/ADO pathway where 1) *fatty acyl-ACP reductase* (AAR encoded by *sll0209*: *aar*) releases and reduces an aldehyde from an acyl carrier protein (ACP) and 2) *aldehyde deformylating oxygenase* (ADO encoded



by *sll0208*; *ado*) cleaves a formate in an oxygen-dependent reaction resulting in final C_{n-1} hydrocarbon (Li *et al.* 2011; Li *et al.* 2012; Schirmer *et al.* 2010; Warui *et al.* 2011) (Fig. 4). Fatty acyls that derive directly from fatty acid biosynthesis, are converted into saturated alkanes, while fatty acyls that have been detached from lipid acyl chains may be unsaturated by membrane-bound desaturases (Murata and Wada 1995; Wada and Murata 1990) and converted into alkenes.

Figure 4. Hydrocarbon biosynthesis in the AAR/ADO pathway: 1. Newly synthesized Acyl-ACP from fatty acid synthesis; 2. Acyl-chain synthesis for membrane lipids; 3. Membrane-bound desaturases; 4. Recycling of acyl chains; 5. Acyl ACP reductase (AAR); 6. Aldehyde deformylating oxygenase (ADO)

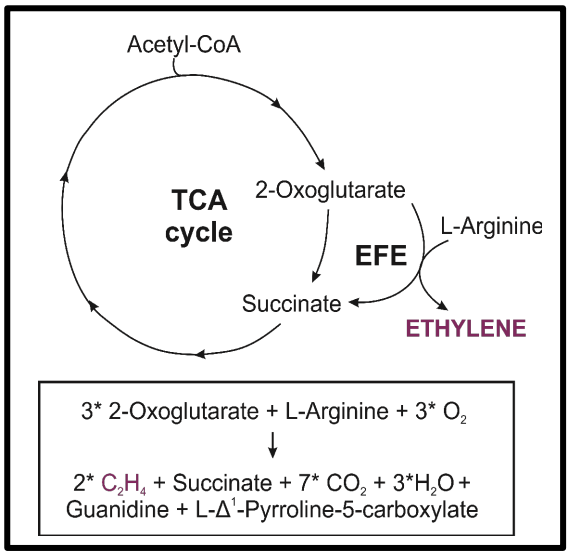
The natural hydrocarbon content in cyanobacteria is only ~ 0.1 % of the dry cell weight. Interestingly, cyanobacteria with the OLS pathway contain, on average, more than twice the amount of hydrocarbons compared to the strains with the AAR/ADO pathway (~0.17% vs. ~0.07%) (Coates *et al.* 2014). Many researchers have attempted to boost or modify the native hydrocarbon production to achieve higher levels with various strategies, including 1) the overexpression of AAR and ADO combined with 2) increasing substrate flux from the membrane acyl chains through the overexpression of lipolytic enzyme (lipA encoded by *sll1969*) and acyl-ACP synthetase (aas encoded by *slr1609*), 3) overexpressing acetyl-CoA carboxylase to increase fatty acid biosynthesis and 4) inhibiting poly-β-hydroxybuturate (PHB) formation by deleting betaketothiolase (encoded by *slr1993*) (Gao *et al.* 2012; Kaiser *et al.* 2013; Wang *et al.* 2013). By combining several modifications, the hydrocarbon content was, at its highest, increased almost tenfold, equaling about 1.3% of dry cell weight (Wang *et al.* 2013).

Clearly, the native hydrocarbon content of cyanobacterial cells is strictly controlled. In fact, the native expression of ADO is controlled by two different promoters (Klähn *et al.* 2014) and the hydrocarbon production can also be controlled post-translation (Kageyama *et al.* 2015). The structure of ADO itself has been well characterized (Jia *et al.* 2015) and successfully modified to favor shorter substrates (Khara *et al.* 2013). The reaction appears to follow first-order kinetics (Paul *et al.* 2013) with a low enzymatic turnover (Andre *et al.* 2013). In addition to tightly regulated expression, low catalytic activity and substrate affinity, also precursor supply is thought to be a potential bottleneck (Kageyama *et al.* 2015), although an excess of fatty aldehydes has not increased hydrocarbon production in the same ratio as supplied (Kaiser *et al.* 2013).

1.4.2 EFE-mediated hydrocarbon production

Ethylene (C_2H_4) is a volatile hydrocarbon with a high industrial value, since it is a starting material in the plastic industry for polystyrene (PS), polyethylene terephthalate (PET), polyvinyl chloride (PVC) and polyethylene (PE). Ethylene is also a widely used plant hormone that promotes crop growth and induces fruit ripening in the food industry. In addition, it has the potential to replace gasoline in combustion engines if sustainable production can be upscaled. Based on The Ethylene Technology Report (2016), the global ethylene production exceeded 150 million tons in 2015, and demand is increasing. Currently, ethylene is produced from fossil fuels through conventional steam cracking, releasing 1.5-3 tons of CO_2 per one ton of ethylene, which requires a substantial amount of energy and releases significant amounts of toxic chemicals (Ungerer *et al.* 2012; Worrell 2000). Therefore, the production of ethylene is one of the largest individual sources of CO_2 emissions in the chemical industry.

In nature, ethylene functions in the abscission of leaves, fruit ripening (Dong *et al.* 1992), non-specific defense signaling (Gottwald *et al.* 2012) and mediation of virulence (Weingart *et al.* 2001). In plants, the ACC pathway converts methionine to ethylene via the S-adenosyl-L-methionine and 1-aminocycloprone-1-carboxylate (ACC) steps (Dong *et al.* 1992; Rodrigues *et al.* 2014). Micro-organisms have at least two pathways for the production of ethylene: In most species, like *Cryptococcus albidus*, it is produced through the KMBA pathway from methionine via the 2-keto-4-methyl-thiobutyric acid (KMBA) step (Ogawa *et al.* 1990). In many plant pathogens, such as several *Psesudomonas* species, ethylene forming enzyme (EFE)



directly converts 2-oxoglutarate (i.e. α-ketoglutarate) and L-arginine into ethylene (Nagahama *et al.* 1991) (Fig. 5).

Figure 5. Ethylene formation by ethylene forming enzyme (EFE) from 2-oxoglutarate and L-arginine.

Ethylene production by photosynthetic cyanobacterial hosts has been studied using several different approaches (Table 2). Due to the simple one-step reaction, most studies rely on *efe* from *Pseudomonas syringae* (Fig. 5) accompanied by additional changes in the host strain, such as modifications in the TCA cycle (*ogcd/ssadh*) (Mo *et al.* 2017; Veetil *et al.* 2017; Zhu *et al.* 2015), in the substrate supply through an introduced transporter (*kgtP*) (Zhu *et al.* 2015) or in the native transcription factor (*ntcA*) (Mo *et al.* 2017). Due to difficulties encountered in maintaining long-term production at stable rate without compromising the viability of the host, the native gene sequence has been modified in many ways and a wide range of expression strategies have been tested. *Synechococcus*, in particular, has been sensitive to higher rates of ethylene production when *PsbAI*-derived elements and/or loci have been utilized. These experiments have resulted in a yellowish phenotype and mutations in specific CGATG-areas of the *efe* gene, known as “mutational hot spots”, causing frameshift and inactivation of the ethylene production (Sakai *et al.* 1997; Takahama *et al.* 2003). In the end, combination of high yields and long term production, without compromizing viability of the cells, is necessity for biotechnological application in the future.

Table 2. A literature review of the overproduction of ethylene based on the ethylene forming enzyme. The results have been divided into two groups based on host cells (*Synechococcus* and *Synechocystis*) and, within each table, categorized according to production levels. Different modifications that have produced similar ethylene amounts within one publication have been grouped and separated with a dash (-). If stabilitiy of etyhylene production (+/-) or the phenotype of the mutant strain (bluegreen-green or yellowgreen) was mentioned, it is presented in the table.

Synecho-coccus	*efe*	promoter	plasmid/site (copy number)	productivity ($\mu L*L^{-1}*h^{-1}*OD^{-1}$)	stability (+/-) phenotype	reference
R2-SPcΔpUH25	original	Pefe	pUC303-EFE03	<50	N.R.	(Fukuda *et al.* 1994)
R2-SPcΔpUH24	original	PlacZ	-pUC303-EFE10 -pUC303-EFE30	<50	+	(Sakai *et al.* 1997)
R2-SPcΔpUH24	original	PpsbA1	pUC303-pEXE3-I	<50	-	(Sakai *et al.* 1997)
R2-SPcΔpUH24	original	PpsbA1 shrunken	pUC303-pEXEΔ9	<50	+	(Sakai *et al.* 1997)
R2-SPc	original	PpsbA1	*psbA1* (1*efe)	<50	N.R.	(Wang *et al.* 2000)
R2-SPcΔpUH24	original	Pefe	pUC303-EFE03	50−100	+	(Sakai *et al.* 1997)
WT	-original -optimized (b)	Ptrc	*NSI* (1*efe)	100−200	+	(Carbonell *et al.* 2019)
R2-SPcΔpUH24	original	PpsbA1	pUC303-pEXE3 Δ1-Δ8 [PpsbA1 variations]	100−400	-	(Sakai *et al.* 1997)
WT	original	PpsbA1	*psbA1* (1*efe)	200−500	-	(Takahama *et al.* 2003)

Synecho-cystis	*efe*	promoter	plasmid/site (copy number)	productivity $\mu L*L^{-1}*h^{-1}*OD^{-1}$	stability (+/-) phenotype	reference
WT	optimized (b) His-tag	-Psmt -PpetE -Pcoa	-pDF-smt-EFEh -pDF-pet-EFEh -pDF-coa-EFEh	<50	N.R.	(Guerrero *et al.* 2012)
WT	optimized (e) TT BB0014 -RBS-H -RBS-C9 -RBSC10 -RBSC11	Ptrc	*slr0168* (1*efe)	<50	N.R.	(Veetil *et al.* 2017)
Δ*ssadh*	optimized (c) His-tag	PcpcB	*slr0370* (1**efe*)	50−100	N.R.	(Zhu *et al.* 2015)
WT	optimized (e) TT BB0014 RBS-30	Ptrc	pVZ321-Ptrc-Rbs30-efe	50−100	N.R.	(Veetil *et al.* 2017)
WT	optimized (b) His-tag	-Ptrc -PA1lacO-1	-pDF-trc-EFEh -pDF-lac-EFEh	100−200	N.R.	(Guerrero *et al.* 2012)
Δ*ogdc*	optimized (c) His-tag	PcpcB	*sll1981* (1**efe*)	100−200	N.R.	(Zhu *et al.* 2015)
Δ*psbAII*	optimized (a) 3X-FLAG tag	PpsbA	*slr0427* (1**efe*)	100−200	N.R.	(Lee *et al.* 2015)
WT	optimized (b)	Ptrc	pDF-trc-sy-EFE	100−200	+	(Carbonell *et al.* 2016)

WT	optimized (e) TT BB0014 -RBS-0 -RBS-34 -RBS-30	Ptrc	slr0168 (1*efe)	100–200	N.R.	(Veetil *et al.* 2017)
WT	optimized (e) TT BB0014 RBS-30	-3*Ptrc -5*Ptrc	slr0168 (1*efe)	100–200	N.R.	(Veetil *et al.* 2017)
Δ*ogdc*	optimized (e) TT BB0014 RBS-30	Ptrc	-slr0168 (1*efe) -pVZ321-Ptrc-Rbs30-efe	100–200	N.R.	(Veetil *et al.* 2017)
WT	original	Ptrc	pDF-trc-o-EFE	200–500	+	(Carbonell *et al.* 2016)
WT	optimized (a) 3X-FLAG tag	PpsbA	slr0168 (1*efe)	200–500	+	(Ungerer *et al.* 2012)
WT	optimized (c) His-tag	-PpsbA2 (7942) -PpsbA1 -PpsbD -PgroESL -PglnA -PcI -PcpcB	-slr0168 (1*efe)	200–500	N.R.	(Zhu *et al.* 2015)
-Δ*ogdc* -Δ*ogdc/* Δ*ssadh*	optimized (c) His-tag	PcpcB	-slr0168, *sll1981* (2*efe) -slr0168, *sll1981, slr0370* (3*efe)	200–500	N.R.	(Zhu *et al.* 2015)
WT (motile)	optimized (d) lambda *oop* TT	Ptac	pVZ325-Ptac-efe	500–1,000	N.R.	(Kuchmina *et al.* 2017)
NtcA OE (Δ*phaAB*)	optimized (c) His-tag	PcpcB	slr0168 (1*efe)	500–1,000	N.R.	(Mo *et al.* 2017)
Δ*psbAII*	optimized (a) 3X-FLAG tag	PpsbA	slr0168, slr0427 (2*efe)	500–1,000	+	(Ungerer *et al.* 2012)
-WT -Δ*psbAII*	optimized (a) 3X-FLAG tag T7 terminator rbsV4	PpsbA	-slr0168 (1*efe) -slr0168, *slr0427* (3*efe)	500–1,000	N.R.	(Xiong *et al.* 2015)
-WT -Δ*ogdc,* Δ*ssadh kgtP* (ΔphaAB)	optimized (c) His-tag	-PpsbA2 (6803 mod.) -PcpcB	-slr0168 (1*efe) -slr0168, *sll1981, slr0370* (3*efe)	500–1,000	+	(Zhu *et al.* 2015)
WT	optimized (c) His-tag	-PcpcB -PpsbA2 (6803) -Prbc	-slr0168 (1*efe)	500–1,000	N.R.	(Zhu *et al.* 2015)
-Δ*ntcA* (unseg.) -Δ*ogdc/* Δ*ssadh/* Δ*ntcA* (unseg.)	optimized (c) His-tag	PcpcB	-slr0168 (1*efe) -slr0168, *sll1981, slr0370* (3*efe) -slr0168, *sll1981, slr0370, sll1423* (4*efe)	>1,000	N.R.	(Mo *et al.* 2017)

1.5 Aims of the Study

In order to compare the possibilities and limitations of cyanobacterial platforms in biotechnological applications, the host cell has to be characterized in detail. The native metabolism of the host cell, the tools to make targeted changes in the native pathways and the knowledge on how to manipulate the entire energy flux towards the desired end-products are all equally important pieces of the puzzle. In addition, cyanobacteria have the potential to play a significant part in the circular bioeconomy through utilization of waste streams as additional energy sources that can be used to produce valuable compounds.

The key objectives of this Thesis were:

(i) Elucidate the importance of native hydrocarbons for cyanobacterial cells under variations in temperature and osmotic pressure.

(ii) Compare different production strategies and cyanobacterial host strains for stable long-term ethylene production.

(iii) Find out if acetate influx can be boosted by introducing an acetate transporter and how this affects the cyanobacterial host.

2 Materials and methods

2.1 Cyanobacterial strains

A glucose tolerant substrain of *Synechocystis sp.* PCC 6803 (Kaplan) and *Synechococcus elongatus* PCC 7942 were studied and modified via transformation. Required DNA fragments, including a kanamycin resistance cassette (Km^R) designed to disrupt *sll0208* gene, an ethylene forming enzyme (*efe*) gene from *Pseudomonas syringae* pv. *phaseolicola* (Pirkov *et al.* 2008) and an acetate transporter gene *yjcG* (*actP*) from *Escherichia coli* (*E. coli*) K-12 substrain MG1655 (Gimenez *et al.* 2003), were generated with a polymerase chain reaction (PCR), cloned into self-replicating or integrating plasmids and used for a transformation. Two modified versions of the original *efe* (*o-efe*) were generated, with optimized codon usage, avoidance of repeated cgatg –sequences (*sy-efe*) and addition of six histidine residues to the N-terminal end (*sy-efeH*) (Guerrero *et al.* 2012). *E. coli* strain DH5α was used as a host cell during plasmid propagation and the constructs were transformed via natural transformation (Eaton-Rye 2011). Positive colonies were selected with antibiotic resistance and verified with a colony PCR. The resulting mutant strains are listed in table 3.

Table 3. List of studied cyanobacterial strains. All the strains were generated to these studies, except ID 3 and 4 have been generated previously by Guerrero *et al.* (2012).

ID	Strain	Modification/vector	Host	Publication (publication/ gene id.)
1	Δ*ado*	*sll0208*::Km^R	*Synechocystis*	I
2	S. 6803:*o-efe*	pDF-trc-*o-efe*_Sp	*Synechocystis*	II
3	S. 6803:*sy-efeH*	pDF-trc-*sy-efeH*_Sp	*Synechocystis*	II (Guerrero *et al.* 2012)
4	S. 6803:*sy-efe*	pDF-trc-*sy-efe*_Sp	*Synechocystis*	II (Guerrero *et al.* 2012)
5	S. 7942:NSI_ *o-efe*	pUC19_7942_NSI_trc_ *o-efe*_Sp	*Synechococcus*	III (D13182.1)
6	S. 7942:NSI_ *sy-efeH*	pUC19_7942_NSI_trc_ *sy-efeH*_Sp	*Synechococcus*	III (KX184731)
7	AT	pDF-lac-yjcG	*Synechocystis*	IV
8	CS	pDF-trc	*Synechocystis*	IV

2.2 Growth conditions

Cyanobacterial strains were grown under antibiotic selection in pH buffered BG-11 medium with 17 mM $NaNO_3$ (J. B. Baker) (Rippka *et al.* 1979) and solid plates with added 1.5% agar (w/v) and 0.3% (w/v) sodium thiosulfate. Cultures were maintained in a Sanyo growth chamber (SanyoElectric Co. Ltd) with supplemented CO_2 or in Algaetron 230 growth chambers (Photon Systems Instruments) with atmospheric CO_2. Unless stated otherwise, the cultures were grown at 30 °C, under constant light of about 20−50 µmol photons m^{-2} s^{-1} and with ~120 rpm orbital shaking. Erlenmeyer flasks contained ~40% culture and ~60% head phase.

Experiments were executed in Erlenmeyer flasks or in sealed serum bottles in the Sanyo growth chamber or in a MC1000 photobioreactor (Photon Systems Instruments), equipped with a Hailea cooling system. In the multicultivator, 80 mL cultures were grown under atmospheric CO_2 with adjusted aeration by bubbling. LED lights were calibrated with Li-COR (Li-250A Light Meter, Biosciences) and the temperature was set to 15−38 °C. Used supplements included 0.5−1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.5 M sorbitol, 0−30 mM sodium acetate, 5 mM L-arginine, 1 mM 2-oxoglutarate and 50 mM bicarbonate. In sealed serum bottles, air phase was replaced with 99% (/v) gaseous ethylene or nitrogen (AGA) to study potential toxicity.

2.3 Spectrophotometric measurements

Growth rate of the cyanobacterial cultures was determined based on optical properties, either using a spectrophotometer (Thermo Scientific Genesys 10S UV-Vis Spectrophotometer) at 750 nm or using the integrated densitometer at 730 nm within the MC1000 multicultivator. The spectrophotometer was also used to quantify a chlorophyll *a* content of the samples after methanol extraction. An absorption spectrum of cellular pigments was determined either with a plate reader (TECAN infinite M200 PRO) between 400−750 nm or with an OLIS CLARiTY 17 UV/Vis spectrophotometer equipped with 8 mL ICAM cuvette (On Line Instrument Systems, Inc.). The results from the OLIS (370−750 nm) were converted to real absorbance values (AU/cm) using Fry's method (Fry *et al.* 2010).

2.4 Microscopic measurements

A light microscope (Leitz Orthoplan Large Field Research Microscope) was used to count cells and measure their relative sizes from parallel cultures transferred into a 0.1 mm Bürker cuvette. The cells were photographed (Leica DFC420C/Leica Application Suite V 4.1) and measured with the Adobe Photoshop CS4 Ruler Tool.

2.5 Determination of dry cell weight

Dry cell weight was analyzed from samples adjusted to $OD_{750\ nm} = 1$ with volume = 5 mL. The culture samples were filtered through preweighted microfiber membranes (VWR), washed and dried at 98 °C before final weighting with an analytical scale (Sartorius MC1 Research RC 210P).

2.6 Quantification of storage compounds

Poly-β-hydroxybutyrate was quantitated from samples adjusted to $OD_{750\ nm} = 0.1$ and V = 50 mL. The cells were collected and suspended in 0.5 M NaOH, incubated at 85 °C and neutralized with 1 M HCl. The hydrolyzed 3-hydroxybutyrate was quantitated with a commercial D-3-Hydroxybutyric Acid Assay Kit (Megazyme) according to the manufacturer´s instructions. The measurement was based on the stoichiometric production of NADH by 3-hydroxybutyrate dehydrogenase and a subsequent reaction where NADPH-dependent diaphorase generated a colored end-product measured with a plate reader (Tecan infinite 200 PRO).

Glycogen was quantitated from 1 mL culture samples with a commercial Total Starch Assay kit (Megazyme) (Yoo *et al.* 2007). The cells were lysed with acid-washed glass beads (Sigma) and vortexed in a solution containing ethanol. The supernatant was used for chlorophyll *a* quantitation, required for normalization, and glycogen contained in the cell debris was broken to glucose with α-amylase and amyloglucosidase. A colorimetric reaction was induced with a glucose oxidase/ peroxidase -reagent and glucose was quantified against a glucose standard with a plate reader (Tecan infinite 200 PRO).

2.7 Determination of cellular buoyancy

A sedimentation of cultures adjusted to $OD_{750\ nm} = 2{,}0$ and 2.5 was monitored in clear 15 mL polypropene falcon tubes with a scale on one side. The cultures were left still and the changes were recorded with photographs daily.

2.8 Quantification of acetate consumption

To verify acetate consumption, cultures were set to $OD_{750\ nm} = 0.05$ and supplemented with 15 mM acetate. Samples were collected every other day for ten days and quantification was performed with the Acetate Colorimetric Assay kit (Sigma-Aldrich) according to the manufacturer´s instructions. After the cells were broken down in the assay buffer, the enzymatic reaction was carried out on a 96-well microtiter plate and measured with a plate reader (Tecan infinite 200 PRO).

2.9 Preparation of cell fractions

2.9.1 Preparation of supernatant fraction and cellular fraction for GC-MS analysis

To allow a localization of native hydrocarbons, the *supernatant* and *cellular fraction* were separated from 1 mL culture aliquots. After centrifugation, the *supernatant* was transferred into a new tube, mixed well with 2:3 volume of methanol (HiPerSolv CHROMANORM, VWR Chemicals) and transferred into 2 mL GC-MS vials (Agilent) for analysis. The cell pellet was broken down with strong vortexing after adding approximately equal volume of acid-washed glass beads (Sigma) and 800 mL of methanol. The beads and debris were removed by centrifuging and filtering the mixture (Costar 8169 spin-X centrifuge filter 0.22 µm), and the *cellular fraction* was transferred to GC-MS vials for analysis.

2.9.2 Preparation of total cell extract, soluble fraction and membrane fraction

In order to generate the *total cell extract, soluble fraction* and *membrane fraction*, cells were collected and frozen with liquid nitrogen. The thawed pellets were transferred into Eppendorf Safe-Lock Tubes™ and resuspended (50 mM Hepes-NaOH pH 7.5, 30 mM $CaCl_2$, 800 mM Sorbitol, 1 mM ε-amino-n-caproic-acid and Protease Inhibitor Mini Tablets (Pierce)). The cells were broken with Bullet Blender® Storm 24 (NEXT ADVANCE) after the addition of approximately 250 mg of 0.15 mm Zirconium oxide beads (NEXT ADVANCE) and the gained *total cell extracts* were transferred into new microcentrifuge tubes.

In addition, some of *the total cell extract* was used to prepare membrane fractions and soluble fractions, which were separated by centrifuging for 40 minutes at 16 000 G (Sigma 1-14). *The soluble fraction* was transferred into a new microcentrifuge tube and *the membrane fraction* was resuspended in a storage buffer (50 mM Hepes-NaOH pH 7.5, 30 mM $CaCl_2$, 600 mM Sorbitol, 1 M betaine monohydrate and Protease Inhibitor Mini Tablets). Later, the fractions were used for western blot analysis and GC-MS analysis, where they were mixed with methanol and transferred into GC-MS vials.

2.10 Transcript analysis

In order to quantify messenger ribonucleic acid (mRNA), cells were collected after reaching $OD_{750\ nm} = 1$. RNA was isolated using TRIsure™ (Bioline) according to the manufacturer´s instructions and quantified (NanoDrop) before and after Turbo

DNase (Ambion) treatment. cDNA synthesis with QuantiTect® Reverse Transcription kit (Qiagen) was applied for 1 µg of RNA. Finally, the samples were diluted to 1:5 with water and used for real-time quantitative PCR (RTqPCR) (iQ5 RT-PCR, Bio-Rad Laboratories). RTqPCR samples contained cDNA, primers and iQ™ SYBR® Green supermix (Bio-Rad Laboratories). The expression levels were calculated and normalized from recorded cycle threshold (Ct) values (Livak and Schmittgen 2001; Ramakers *et al.* 2003).

2.11 Protein analysis

2.11.1 Generation of antibody

In order to prepare a polyclonal antibody against ADO, an overexpression vector pACYC-*sll0208* containing the *ado* gene (*sll0208*) with an N-terminal His-tag was generated, sequenced (MWG) and expressed in *E. coli* BL21 (DE3). After IPTG-induced protein production, the cells were lysed with Cell Disrupter (Constant Systems Ltd) and the His-tagged ADO was purified with a TALON metal affinity resin. Finally, the ADO was size separated with SDS-PAGE and the correct ~ 27 kDa protein band was excised from the gel and sent to Agrisera as a template for antibody preparation.

2.11.2 Protein quantitation, SDS-PAGE and Western blot analysis

Protein content was measured with the Bradford dye-binding method (Bradford 1976) against a bovine serum albumin (BSA) standard. The protein quantity was adjusted, and the final samples were diluted 1:1 with a buffer containing 138 mM Tris-HCl pH 6.8, 6 M Urea, 22.2% glycerol, 4.3% SDS, 10% β-mercaptoethanol and a small quantity of Bromophenol blue. After incubation, the samples were centrifuged and separated according to size with 12% SDS-PAGE containing 6 M urea.

The separated proteins were electro-transferred to Immobilon PVDF membrane (Millipore), which was blocked with 5% milk (BIO-RAD blotting grade blocker) and treated with primary antibodies (Agrisera) and secondary Rabbit IgG HRP from donkey (VWR GE Healthcare). Finally, the membrane was incubated with ECL™ Western blotting Detection Reagent (Amersham™ GE Healthcare), the signal was detected with a film and quantitated with FluorChem 80000 (Alpha Ease FC, Alpha Innotech). As a final step the membranes were dyed using the Coomassie blue staining method.

2.12 Hydrocarbon analysis

2.12.1 GC-MS analysis for longer hydrocarbons

Native hydrocarbons were identified and quantitated with an Agilent 7890C gas chromatography equipped with an autosampler, a 5975C inert mass spectrophotometer (Agilent) and an Innowax capillary column (260 °C, 30 m * 0.32 mm * 0.5 µm). The samples were injected with splitless mode for analysis. Helium was used as a carrier gas (1.9 ml min^{-1}) and injector temperature was 250 °C. The separation of hydrocarbons was carried out with a temperature program: 2 min at 55 °C, followed by 20 °C min^{-1} steps up to 155 °C, 8 °C min^{-1} steps up to 180 °C, 20 °C min^{-1} steps up to 250 °C and finally 10 min at 250 °C. The hydrocarbons were identified and quantified against a dilution series of commercial analytical standards (Sigma-Aldrich). The selected ions for the MS peak area filtering were m/z = 57 for heptadecane; m/z = 55 for heptadecene and pentadecane; m/z = 83 for pentadecene.

2.12.2 Quantification of ethylene

In order to measure ethylene production, 1 mL of culture was supplemented with 1 mM IPTG, transferred into 10 mL serum bottles and sealed. Ethylene was quantified from the headspace with a GC connected to a flame ionization detector (Perkin Elmer) with a CP-CarboBOND fused silica capillary column (50 m * 0.53 mm * 0.74 mm) from Varian. The temperature in the injector and oven was 80 °C and in the detector 200 °C. Helium was used as the carrier gas (7 mL min^{-1}). Integrated MS peak areas were analyzed against a commercial standard and normalized against $OD_{750\ nm}$ and time of incubation.

2.13 Measurement of reactive oxygen species

2.13.1 Quantification of singlet oxygen

Singlet oxygen was quantitated based on His-mediated oxygen uptake, where oxygen is removed through the oxidation of histidine by $^1O_2$. The samples were washed, concentrated and the rate of singlet oxygen-induced oxygen uptake was measured with a Hansatech DW2 O_2 electrode. Parallel samples were measured under saturated light with and without supplemented 5 mM His.

2.13.2 Determination of reactive oxygen species

Reactive oxygen species (ROS), *i.e.* hydrogen peroxide, hydroxyl radicals, peroxyl radicals and peroxynitrite, were measured from parallel sample sets incubated with and without supplemented 25 µM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) for 90 minutes under darkness. Fluorescence (excitation=485 nm and emission=535 nm) and autofluorescence (680 nm from chl *a*) were measured with a plate reader (Tecan infinite 200 PRO) using a 96-well microtiter plate (Perkin Elmer Isoplate™ -96 F, Black frame and Clear well) from washed and concentrated samples. Next, the samples were incubated under growth light for 45 minutes and the fluorescence measurement was repeated. Autofluorescence was used for normalization.

2.14 Measurement of photosynthesis and respiration

2.14.1 Analysis of cellular gas fluxes

Steady-state cellular gas flux rates (O_2 and CO_2) were measured with MIMS under increasing light intensities of 0, 25, 50, 75, 100, 200 and 500 µmol photons m^{-2} s^{-1}. The culture samples were adjusted to Chl *a* = 15 µg ml^{-1} and supplemented with 1 mM HCO_3^- and 50 µg mL^{-1} carbonic anhydrase (Sigma). Gross O_2 evolution rates were calculated based on changes in supplemented $^{18}O_2$ gas (98%, CK Isotopes) according to Beckmann *et al.* (2009).

2.14.2 Fluorescence measurements and P700 absorbance

Photosynthetic properties were measured from intact cells with a pulse amplitude modulated fluorometer Dual-PAM-100 (Walz). The samples were adjusted to Chl *a* = 15 µg mL^{-1}, supplemented with 1 mM total HCO_3^-, dark-acclimated for 10 min and treated with saturating pulses of 5,000 µmol photons m^{-2} s^{-1} (300 ms) and a strong far-red (FR) light (720 nm, 75 W m^{-2}). Thereafter the samples were treated with red actinic light (620 nm) of either 20 µmol photons m^{-2} s^{-1} or 50 µmol photons m^{-2} s^{-1} or with 60-s illumination periods of gradually increasing actinic light intensities from 20 to 350 µmol photons m^{-2} s^{-1}. The light curves were recorded and the effective yield of PS II, [Y(II)], was calculated as $(F_{m'}-F_s)/F_{m'}$ and of PSI, [Y(I)], as $(P_{m'} – P)/P_m$.

2.14.3 Induction of state transitions

In order to record state transitions, the samples were adjusted to Chl *a* = 15 µg ml^{-1} and supplemented with 1 mM HCO_3^-. The transitions were induced after 10 minutes of dark-acclimation with blue actinic light (460 nm, 44 µmol photons m^{-2} s^{-1}) to induce State I. After 135 s, the blue actinic light was replaced with red actinic light (620 nm, 44 µmol photons m^{-2} s^{-1}) to induce transition to State II. Finally, after 215 s, the light was changed back to blue and State I was induced. During illumination, the maximal fluorescence in light ($F_{m'}$) was measured by applying saturating pulses (5,000 µmol photons m^{-2} s^{-1}, 300 ms) and the effective yield of PS II, [Y(II)], was calculated as $(F_{m'}-F_s)/F_{m'}$.

3 Results

3.1 Native hydrocarbons influence on several properties within cyanobacterial cells

3.1.1 Hydrocarbon metabolism is closely associated to membranes

In order to clarify the largely elusive subject of hydrocarbons in cyanobacteria, the native alkanes and alkenes of model strain *Synechocystis* were identified. The results clearly demonstrate that *Synechocystis* produces both saturated and unsaturated C17 hydrocarbons, while C15 could not be detected (Publication I: Fig. S4). The presence of both heptadecane and heptadecene indicates that at least some parts of the substrates originate from the detached fatty acyl chains of membrane lipids, since all four desaturases (DesA-D) are membrane-bound (Murata and Wada 1995; Wada and Murata 1990), and lack of C15 implies substrated specificity most likely by AAR, which in fresh water species appears to prefer C18 substrates over C16 (Kudo *et al.* 2016).

The localization of detected alkanes and alkenes demonstrates a close membrane association, since neither the external supernatant nor the soluble fraction of the cells contained any of the detected hydrocarbons (Publication I: Fig. S3). This also raised interest towards verifing the location of the corresponding ADO enzyme, which, as a default, is a soluble cytoplasmic protein without any known signal peptide. Interestingly, ADO could be found in both the soluble and membrane fractions, indicating a close association with membrane proteins (Publication I: Fig. 3). Moreover, the results from samples grown at two temperatures suggest that the relative amount of the membrane-bound and soluble forms of the enzyme is not stable, but instead ADO is more abundant at 22 °C than at 30 °C and supplemented sorbitol slightly reduced the amount of ADO. (Publication I: Fig. 3 and S8.)

3.1.2 Saturation of hydrocarbons is influenced by environmental conditions

After analyzing the effects of a wide range of temperatures with and without sorbitol-induced osmotic stress, the results clearly indicated environment-dependent

variations in the hydrocarbon saturation. While the amount of heptadecane remained relatively stable, the amount of unsaturated heptadecene was clearly reduced in response to an increase in temperature (Publication I: Fig. 2.). Since the cultures had already adapted to a temperature of 30 °C at the preculture stage, the relative hydrocarbon content remained almost unchanged in this condition while acclimation was clear in other tested conditions. In contrast, sorbitol-induced osmotic stress had only a minor influence on native hydrocarbon content. (Publication I: Fig. 2.)

3.1.3 Lack of native hydrocarbons affects growth and chlorophyll *a* content

In order to determine how important hydrocarbons are for cyanobacterial cells, a hydrocarbon-deficient mutant strain was generated and compared with WT *Synechocystis* in terms of several parameters, as presented in table 4. The mutant strain appears to contain 33−42 % less chlorophyll *a*, depending on the temperature, than the WT. In addition, based on optical density and cell count, the growth of the hydrocarbon-deficient mutant strain is retarded, while cell diameter and therefore the volume of the Δ*ado* cells is clearly increased. The dry cell weight (DCW) depends on both the cell number and the cell size, which is why the DCW of the mutant strain is lower at 22 °C and higher at 30 °C than the DCW of the WT. This difference in cell size appears to distort the comparability of strains based on optical density measurements.

Table 4. Comparison of WT *Synechocystis sp.* PCC 6803 and Δ*ado* mutant strains in terms of optical density (OD), chlorophyll *a* (Chl *a*), cell count, dry cell weight (DCW), the diameter of cells and the calculated cell volume. The cells were adjusted to $OD_{750\ nm}$=0.5 and grown for four days at 22 °C or 30 °C in the MC1000 multicultivator, and the averages and standard deviations were determined from three biological replicates.

Strain	OD	Chl *a*	(cells L^{-1}) Cell count	(g L^{-1}) DCW	(a.u.) Diameter	(a.u.) Cell volume
WT 22°C	1.22 ±0.0436	7.76 ±0.513	3.77 *10^{12} ±0.540 *10^{12}	0.324 ±0.0269	0.28 ±0.0120	1.15 *10^{-2}
	100%	100%	100%	100%	100%	100%
Δ*ado* 22°C	0.95 ±0.0486	4.50 ±0.426	3.07 *10^{12} ±0.515 *10^{12}	0.281 ±0,0188	0.31 ±0.0170	1.56 *10^{-2}
	78%	58%	81%	84%	111%	136%
WT 30°C	1.23 ±0.0798	8.57 ±0.365	4.36 *10^{12} ±0.974 *10^{12}	0.285 ±0.0233	0.28 ±0.0140	1.15 *10^{-2}
	100%	100%	100%	100%	100%	100%
Δ*ado* 30°C	1.11 ±0.0381	5.73 ±0.00970	3.87 *10^{12} ±0.588 *10^{12}	0.332 ±0.0311	0.32 ±0.0170	1.72 *10^{-2}
	90%	67%	89%	113%	114%	149%

3.1.4 The hydrocarbon-deficient mutant strain has reduced buoyancy and an increased amount of intracellular storage compounds

The WT *Synechocystis* and Δ*ado* mutant strains behaved differently when the cultures were maintained in a laboratory, which gave an idea to observe their buoyancy in stationary cultures for several days. Surprisingly, the Δ*ado* mutant cells sank down significantly faster and the resulting pellet was more compact compared to WT *Synechocystis* (Publication I: Fig. 4). To determine possible reasons behind the decreased buoyancy and increased dry cell weight of the strain (Table 4), the amount of intracellular storage compounds was analyzed. As it turned out, the hydrocarbon-deficient mutant cell contained 31−66 % more poly-β-hydroxybutyrate and two to four times more glycogen, depending on the temperature, than the WT *Synechocystis* cells (Publication I: Table 2).

3.1.5 Hydrocarbon-deficient mutant strain had alterations in pigment profile

To find out more about the energy harvesting capacity of the mutant strain, its light-absorbing properties were studied and compared against the WT. As already presented in table 4, the chlorophyll *a* content of the mutant strain is significantly reduced. Based on an absorbance scan (370−750 nm) performed using OLIS, in addition to the chlorophyll *a* (680 nm; 430 nm), also relative amounts of carotenoids (at 485 nm) and phycobilisomes (at 620 nm) were reduced (Publication I: Fig. 5) which may result unoptimal light-harvesting capacity.

3.1.6 Maximal photosynthetic rate and carbon fixation of hydrocarbon mutant strain is reduced

In order to find out how the Δ*ado* strain and the WT *Synechocystis* compare when photosynthetic gas fluxes are evaluated, MIMS was used to measure oxygen and carbon exchange. When strain specific difference in chlorophyll *a* content was taken into account (Table 4), all measured parameters including the net oxygen evolution and the total carbon fixation were reduced in the Δ*ado* strain (Publication I: Fig. 6). Therefore the photosynthetic activity of the mutant strain appears to be impaired and the phenomenon increases at lower temperatures.

To study light reactions in detail, the Δ*ado* and WT *Synechocystis* strains were analyzed using Dual-PAM. Because all measured parameters are calculated functional values, differences in the Chl *a* content do not influence on these results. Overall, no major differences were observed in the performance of the strains. The Δ*ado* PSII yield [Y(II)] was slightly higher, especially at lower actinic light, while

thereafter the differences between the strains diminished (Publication I: Fig. S11). The cultivation temperature and higher actinic light intensities up to to 350 µmol photons m^{-2} s^{-1} seemed irrelevant for the function of photosynthetic reactions in studied strains.

When light reactions were studied, the first measurements of Δ*ado* are higher after dark adaptation of PS II (Publication I: Fig. S11 e, i, q, u), which implies that the phycobilisomes have not been transferred to PS I for respiratory purposes. The same phenomenom was observed with light-induced state transitions (Publication I: Fig. S12), where changes in blue and red actinic light induce state transitions. The results supported a higher phycobilisome association around PS II, especially at 22 °C, for the Δ*ado* strain.

3.2 As an alternative for native hydrocarbon production, ethylene can be produced via an introduced ethylene-forming enzyme

3.2.1 Achieving stable ethylene production in *Synechococcus*

Most recent studies on photoautotrophic ethylene production have used *Synechocystis sp.* PCC 6803 as a host cell due to previously encountered problems with *Synechococcus elongatus* PCC 7942 cells at higher production rates (Table 2). In the first phase, the goal was to rule out the potential toxicity of ethylene to *Synechococcus* cells by transferring the cultures into sealed serum bottles and replacing air with commercial ethylene gas. Supplemented ethylene did not cause any observable changes in the cultures when compared to parallel batches grown under air or nitrogen conditions (Publication III: Fig. 5). Therefore, it appears that the previously encountered complications were not caused by ethylene itself.

As the next step, an alternative engineering strategy for introducing *efe* into *Synechococcus* was designed, where any association with the previously used *psbAI* was avoided. In this strategy, the gene encoding for ethylene-forming enzyme was successfully integrated into proposed neutral site I (NSI, GenBank: U30252.3) locus of the genome (Ditty *et al.* 2005; Mackey *et al.* 2007), and ethylene production was confirmed with GC-MS. The ethylene production rate was higher than in any other stable production systems expressed in *Synechococcus* strain (Table 2, Publication III: Fig. 2) and remained relatively stable even for long-term experiments, without any indication of inactivation or loss of viability (Publication III: Fig. 6).

3.2.2 Comparison of ethylene production by different variants of the *efe* gene

To overcome earlier difficulties in achieving stable ethylene production, the native *efe* gene has been modified in several ways (Table 2). In order to test the efficiency and stability of different variants of ethylene-forming enzyme, two parallel studies were made with alternative host strains, *i.e. Synechocystis* (publication II) and *Synechococcus* (publication III). The expression strategy in *Synechocystis* was based on earlier study by Guerrero *et al.* (2012) and utilized a RSF1010-derived, self-replicating plasmid with a constitutive P_{trc} promoter to compare the original sequence (Table 3: strain 2) and two codon-optimized versions with and without a His-tag (Table 3: strain 3 and 4, respectively). The expression strategy in *Synechococcus* was based on the genomic integration of *efe* genes into the NSI locus (Ditty *et al.* 2005; Mackey *et al.* 2007) under a constitutive P_{trc} promoter and comparing the original sequence (Table 3: strain 5) to the optimized version with a His-tag (Table 3: strain 6).

The results showed similar ethylene production in all tested constructs (Publication II: Fig. 1 and Publication III: Fig 2.), which was supported by the similar mRNA levels of the *efe* variants (Publication II: Fig. 2). In addition, the ethylene production levels also remained stable during extended patch cultures (Publication II: Fig. 1 and Publication III: Fig. 6), without any signs of instability or alterations in phenotype (Publication II: Fig. 1 and Publication III: Fig. 3, 4 and 6). The ethylene production rate in *Synechocystis* varied between 150−250 µL of ethylene per $L*h*OD_{750\ nm,}$ and the total production of the original sequence was even slightly higher than that of the optimized genes (Publication II: Fig. 1 b). The ethylene production rates of both *efe* gene variants in the *Synechococcus* genome were slightly lower, averaging approximately 100 µL of ethylene per $L*h*OD_{750\ nm}$ (Publication III: Fig. 2 b).

3.2.3 Substrate supplementation to boost ethylene production rates

Ethylene-forming enzyme requires L-arginine and 2-oxoglutarate as primary substrates for ethylene production, which may cause a bottleneck. To find out if the substrate availability limits ethylene production, the cultures were supplemented with these substrates. While L-arginine only had a minor boosting effect on ethylene production, 2-oxoglutarate caused a moderate yet significant improvement and the best results were achieved by co-supplementing both of the substrates simultaneously (Publication III: Fig. 7). Unfortunately, the total induction remained relatively modest even at its best, which may be partially caused by the limited native

ability to uptake L-arginine and 2-oxoglutarate from the medium (Montesinos *et al.* 1997; Vázquez-Bermúdez *et al*. 2000).

3.3 Acetate can boost cell growth under low-light conditions

3.3.1 Facilitated acetate intake

To find out how additional carbon and energy sources influence *Synechocystis*, acetate was evaluated as a potential supplement. The genome of *Synechocystis* seems to lack genes homologous to known acetate transporters (Cyanobase) and therefore the native intake capacity appears to rely on spontaneous diffusion through membrane barriers (Gibson 1981; Ihlenfeldt and Gibson 1977). To improve the influx of acetate, a gene encoding the acetate transporter ActP from *E. coli* was successfully introduced as a heterologous over-expression target (Gimenez *et al*. 2003; Stenberg *et al.* 2005). The *yjcG* gene was expressed under an inducible lac promoter in the generated acetate transporter strain (AT), and phenotypic alterations were compared against a control strain with an empty plasmid (CS) under different conditions.

The growth rate of the AT strain expressing the heterologous transporter increased when acetate was supplemented into the growth media (Publication IV: Fig. 1 c, d and e). Without supplementation, the growth rate was similar between the AT strain and the control strain (Publication IV: Fig. 1f). Moreover, there was a clear correlation between the enhanced growth rate and the amount of supplemented acetate (Publication IV: Fig. 1g), and the acetate consumption of the AT mutant strain could be verified by quantifying the diminishing acetate from culture samples taken throughout the experimental period (Publication IV: Fig. 2).

3.3.2 Availability of excess acetate improves growth rate when light is limited

The increased availability of acetate had the most dramatic influence under low light intensities. The supplemented acetate increased the growth rate of the AT strain about ten-fold under 20 µmol photons m^{-2} s^{-1} (Publication IV: Fig. 1 e), while under 50 µmol photons m^{-2} s^{-1}, the difference was only moderate (Publication IV: Fig. 1 c), and under 250 µmol photons m^{-2} s^{-1} no difference was observed (Publication IV: Fig. 1 h). It appears that the cells benefit more from the additional carbon and energy sources when photosynthesis is limited due to low light intensities.

In order to find out if the change in the growth rate is influenced by the morphological differences between the studied strains, the cell size and dry cell

weight were determined. Both of these parameters were highly similar between the AT and CS strains when the samples were normalized based on optical density (Publication IV: Table 2 and 3), and therefore the variations in growth rate are expected to reflect the actual number of the cells and not to be distorted by morphological variations. Thus, the enhanced acetate influx appears to increase cell division rate, not cell size.

3.3.3 AT mutant strains has decreased glycogen storages, while PHB content resembles WT

To find out if facilitated acetate influx influences cyanobacterial storage compounds, the strains´intracellular glycogen and PHB storages were quantitated. While the studied strains had similar PHB contents (Publication IV: Table 1), the acetate transporter mutant strain and the control strain utilized glycogen storages in a different manner, which resulted in a decreased glycogen content in the mutant strain (Publication IV: Fig. 3). In general, the glycogen storages and the growth rate are connected, since glycogen storages are consumed when cell division is fast (Yoo et al. 2007) and this may offer at least a partial explanation for the observed difference (Publication IV: Fig. 1 e).

3.3.4 Enhanced acetate utilization of the mutant strain has a negative effect on photosynthetic activity

Even though the variations in glycogen content may be explained by differences in growth rates, the strains were also evaluated in terms of their photosynthetic activity. Interestingly, enhanced acetate influx decreased photosynthetic activity in the AT mutant strain. Both the light-saturated oxygen evolution in PS II (Publication IV: Fig. 4 a) and the net oxygen evolution for the entire reaction chain from water to CO_2 (Publication IV: Fig. 4 b) were significantly reduced. Additionally, the amount of released reactive oxygen species (i.e. hydrogen peroxide, hydroxyl radicals, peroxyl radicals and peroxynitrite) was decreased in the AT mutant strain (Publication IV: Fig. 6 a), while the amount of singlet oxygen was similar between the compared strains (Publication IV: Fig. 6 b).

Finally, the light-harvesting properties of the studied strains were characterized and the resulting spectrum revealed distinctive variations in their light-absorbing properties: the Chl *a* amount (~680 nm) of the AT mutant strain was decreased, while its carotenoid content (~485 nm) was substantially increased. Interestingly, phycobilisome content (~620 nm) remained similar in both strains. (Publication IV: Fig. 5.) Therefore, the impaired photosynthetic activity of the mutant strains may relate to distortion in their light-harvesting properties.

4 Discussion

4.1 Inactivation of native hydrocarbon biosynthesis influences cyanobacterial energy metabolism

4.1.1 Basis for defining the research strategy

Hydrocarbon production is a conserved feature among cyanobacteria, but the purpose of the hydrocarbons remains elusive. They must be important, because all studied cyanobacterial strains contain one of the two alternative pathways, AAR/ADO or Ols, that allow them to synthesize these hydrophobic and inert end products (Coates *et al.* 2014; Lea-Smith *et al.* 2015; Lea-Smith *et al.* 2016). The native aliphatic hydrocarbons are typically 15−19 carbons long and they constitute 5.63 % of plasma membrane and 17.41 % of thylakoid membrane lipid fractions (Lea-Smith *et al.* 2016). Due to the possibility of using such hydrocarbons as a replacement for fossil fuels, many attempts to enhance their production have been made, with limited success. Therefore, these hydrocarbons are expected to have an important and tightly regulated role in the metabolism of cyanobacterial cells, and revealing this native role is a necessity for the evaluation of their biotechnological feasibility.

Since the studied model organism, *Synechocystis sp.* PCC 6803, contains the AAR/ADO pathway (Fig. 4), an initiation for publication I was to choose specific culture conditions where the expression of the ADO is altered. After carefully going through the microarray studies combined by Singh *et al.,* a wide temperature range and sorbitol-induced osmotic stress were selected, based on the increase in *ado* expression levels (Paithoonrangsarid *et al.* 2004; Singh *et al.* 2006; Singh *et al.* 2010; Suzuki *et al.* 2001). In addition to the expected influence on hydrocarbon production, both temperature and osmotic stress are known to influence cell membranes by triggering changes in lipid saturation to maintain stable fluidity (Los *et al.* 2013; Mikami and Murata 2003; Huflejt *et al.* 1990; Khomutov *et al*. 1990).

4.1.2 Hydrocarbon metabolism is highly associated with cellular membranes

Our results clearly support a close connection between the membranes and hydrocarbon metabolism, which has also been suggested previously (Berla *et al*. 2015; Lea-Smith *et al.* 2016). None of the native hydrocarbons were excreted out of the cells, and the intracellular soluble fraction was hydrocarbon-free as well (Publication I: Fig. S3). All of the hydrocarbons were located in the membrane fraction, and, to be more precise, only C17 heptadecane and heptadecene were abundant enough to be quantified (Publication I: Fig. S3 and S4.). The presence of both saturated and unsaturated hydrocarbons reveals that a significant amount of substrates originates from detached membrane lipid moieties (Publication I: Fig. 2), since the double bonds in *Synechocystis* are generated solely by membrane-bound desaturases (Murata and Wada 1995).

The unsaturation of hydrocarbons correlates well with the known temperature-dependent changes in membrane lipids, *i.e.* at higher temperatures, the membrane lipids contain more saturated moieties, while at lower temperatures, unsaturation is more prominent (Los and Murata 2004; Los *et al.* 2013; Mikami and Murata 2003; Wada and Murata 1990). Similarly, heptadecene was more abundant at lower temperatures, while the relative heptadecane content increased with temperature (Publication I: Fig. 2 and S3). Similar trend has also been previously observed in level of unsaturation in *Synechococcus sp.* PCC 7002 expressing the alternative Ols pathway and producing solely unsaturated hydrocarbons (Mendez-Perez *et al.* 2014). Even though the saturation of the membrane lipids is also known to buffer against osmotic stress (Huflejt *et al.* 1990; Khomutov *et al*. 1990), the influence on hydrocarbon saturation or content was only minor (Publication I: Fig. 4).

In addition to the substrates and the end-products of the hydrocarbon metabolism, the enzymatic pathway also appears to function, at least partially, in close relation to the cell membranes. The location of the ADO enzyme was studied, and while it is known to be a soluble protein, it was detected in both the soluble fraction and the membrane fraction (Publication I: Fig. 3). The interaction is strong enough to keep the enzyme attached even after mechanically disrupting the cell. Therefore, ADO appears to work at the membrane interface, where the end-products can be easily deposited into the lipid layers. Based on previous yeast two-hybrid experiment, the ADO interacts with seven proteins, of which two have known transmembrane domains (Sato *et al.* 2007). In addition, a similar localization distribution was observed with both ADO and AAR, when they were expressed in *E. coli* (Kudo *et al.* 2016) and therefore this is expected to be important for their proper function.

4.1.3 Hydrocarbon-deficient mutant has many defects that would decrease the competitiveness of a photosynthetic organism in aquatic environment

As the next step, a hydrocarbon-deficient mutant strain was generated by interrupting the gene encoding ADO with the kanamycin resistance cassette. This resulted in a clear phenotype with respect to a wide range of metabolic activities, without compromising viability. Unfortunately, it is still difficult to say whether many of the observed changes are directly caused by the lack of hydrocarbons or created as a result of some indirect changes.

When comparing the strains, which were grown for four days after adjustment to the same optical density, the Δ*ado* mutant strain showed reduced growth, based on optical density and cell count, in all tested conditions (Table 4 and Publication I: Fig. S10), which has previously also been observed in strains missing the AAR enzyme (Berla *et al.* 2015; Lea-Smith *et al.* 2016). However, due to the bigger cell size of the mutant strain, also reported previously with the Δ*aar* strain (Lea-Smith *et al.* 2016), the dry cell weight of the Δ*ado* ended up being even higher than that of WT in cultures grown at 30 °C (Table 4). Therefore, under certain growth conditions, the Δ*ado* mutant strain is able to accumulate more biomass than WT *Synechocystis*.

One of the most dramatic differences between the mutant strain and the WT was related to their floating properties. The mutant strain sunk to the bottom of the test tube significantly faster and resulted more compact and defined pellet (Publication I: Fig. 4). Therefore, either the floating properties of the mutant strain are defective, or the cell composition is altered by the lack of hydrocarbons. The observed differences in intracellular storage compounds are expected to influence the composition, since the mutant strain contained several times more glycogen than WT *Synechocystis* (Publication I: Table 2), which is known to function as a carbon storage, when optimal growth cannot be achieved (Luan *et al.* 2019).

Previously only minor variations in the pigment content of hydrocarbon mutant strains lacking AAR have been reported (Lea-Smith *et al.* 2016), while in this case, one evident difference between the Δ*ado* and WT strains was in their chlorophyll *a* content. In fact, the mutant strain had only two thirds of the Chl *a* content of the WT (Table 4). When the absorption properties of Δ*ado* were studied in detail, in addition to Chl *a*, the absorption properties of both the phycobilisomes and the carotenoids seem to be distorted (Publication I: Fig. 5). This suggests that the harvested light may simply not provide enough energy to maintain WT-like photosynthesis and, due that, also growth.

To evaluate how reduced pigment content effects to photosynthetic properties, the carbon fixation and net oxygen evolution efficiencies were determined by measuring the gas fluxes with labeled isotopes. As it turned out, both the carbon uptake (Publication I: Fig. 6 e, f) and net oxygen evolution (Publication I: Fig. 6 g,

h) in the mutant strain were decreased compared to the WT. In earlier studies, a larger cell size has been associated with increased respiration (Tang and Peters 1995), which could also explain the reduced net oxygen evolution in the Δ*ado* strain.

When light-harvesting reactions were analysed in detail, the PS II capacity of the mutant strain was slightly increased and thereafter the electron flow performed at similar efficiency in the studied strains (Publication I: Fig. S11). The first measurement points of PS II after dark adaption (Publication I: Fig. S11 a, e, i, m, q and u) and the hindered movement when state transitions were induced (Publication I: Fig. S12) suggest that the phycobilisomes could be more abundant around PS II, while the previously reported uncoupling of phycobilisomes in the hydrocarbon-deficient Δ*aar* mutant (Lea-Smith *et al.* 2016) was not observed with the Δ*ado* mutant.

Our results suggest that native hydrocarbons have tightly membrane associated function, which influences cell growth, carbon metabolism, photosynthesis and floating properties. Hydrocarbons are known to influence on membrane fluidity (McIntosh *et al.* 1980), but due to small native quantity, this most likely cannot extend to all membranes. The restricted movement of phycobilisomes, especially at lower temperatures where also the membranes are more rigid, suggest a potential local function for hydrocarbons to maintain proper fluidity around photosynthetic complexes.

Many of the phenotypic changes caused by the deletion of the hydrocarbons could decrease the viability of the cells in natural environments. Optimal cell growth, floating properties and photosynthesis are crucially important for aquatic organisms that need to float at the proper depth to gather enough light to survive. Therefore, the highly impaired buoyancy of the mutant strain would be a major disadvantage. The reduced growth rate and storing the excess energy in storage compound reserves are also disadvantages in the long run, not to mention observed defects in photosynthesis and carbon fixation.

4.2 Production of ethylene via alternative expression strategies

4.2.1 Heterologous ethylene production in photosynthetic cyanobacteria

Ethylene – a short gaseous aliphatic hydrocarbon – is one of the most produced compounds in the chemical industry. Heterologous ethylene production within cyanobacteria was studied in publications II and III through transformation of an ethylene-forming enzyme that catalyzes a one-step reaction where ethylene is produced from L-arginine and 2-oxoglutarate (Fig. 5). Previously, long-term

ethylene production at higher production rates has been difficult to achieve due to instability and, sometimes, even the loss of viability (Takahama *et al.* 2003; Sakai *et al.* 1997). Table 2 presents a wide literature review of the different approaches that have been used to overcome these problems, which have been encountered especially when using *Synechococcus* as a host cell. The cause of these problems has never been clarified, which served as a starting point for this research.

To verify that higher levels of ethylene itself do not reduce the viability of the cells, an experiment where air phase was replaced with ethylene was made. Excess ethylene content did not cause any observable harm to the growth or visible phenotype of *Synechococcus elongatus* PCC 7942, and should therefore not be a limiting factor for ethylene production (Publication III: Fig. 5). Similar experiments have been made with other cyanobacterial species, including *Synechocystis sp.* PCC 6803, with the same conclusion (Le Henry *et al.* 2017), but since the cyanobacterial strains are a highly heterologous group, the properties cannot be automatically extrapolated from one strain to another.

4.2.2 Previously encountered problems appear to originate from strain-specific properties

In order to find out if sequence modifications benefit ethylene production, the original *efe* gene and the synthetic *efe* genes with host-specific codon optimization and the avoidance of "mutational hotspots" were compared in two different cyanobacterial hosts, i.e. *Synechocystis* and *Synechococcus*. The hotspots refer to repetitive CGATG sequences, that have been associated with the inactivation of ethylene production (Sakai *et al.* 1997; Takahama *et al.* 2003). In addition, a common feature in previous unsuccessful ethylene production studies using a *Synechococcus* host has been the association of the used expression strategy with the *PsbAI* gene. Thus, an alternative expression strategy in *Synechococcus* was used where the *efe* gene, accompanied by a trc-promoter, was inserted into the NSI site (GenBank: U30252.3), which had been previosly used successfully in another context (Ditty *et al.* 2005; Mackey *et al.* 2007). The expression strategy in *Synechocystis*, on the other hand, was based on earlier study by Guerrero *et al.* (2012) and utilized self-replicating plasmid.

The results of this experiment showed no significant differences in the ethylene production rate or long-term activity of the original sequence and the synthetic codon-optimized ones (Publication II: Fig. 1 and III: Fig. 2 and 6). In addition, though *Synechocystis* allowed higher production efficiencies, *Synechococcus* was not far behind (Publication II: Fig. 1 and III: Fig. 2). This suggests that all previous problems are related to the use of the *psbAI* gene, which appears to be an excellent example of strain-specific evolutionary differences (Mulo *et al.* 2009). *PsbAI*

encodes photosystem II reaction center protein D1 (Aro *et al.* 1993; Golden *et al*. 1986), which is essential for *Synechococcus* under normal growth conditions, while in *Synechocystis*, it can be disrupted due to alternative gene encoding compatible replacement protein (Varman *et al.* 2013a; Yu *et al.* 2013). Noteworthy is, that although our results do not allow a direct comparison between plasmid and integration based production systems, it is generally thought, that, due to higher copy number, plasmid based systems are more efficient while integration based systems are more stabile.

4.2.3 Increasing production of ethylene via substrate supply

Ethylene production rates have been up-scaled through different *efe* gene expression strategies (listed in Table 2) and the optimization of cultivation conditions. One potential bottleneck that could limit the production is the availability of substrates due to poor metabolic flux towards the TCA cycle from which the 2-oxoglutarate derives (You *et al.* 2014). To verify whether the availability of the substrates creates a bottleneck, L-arginine and/or 2-oxoglutarate were supplemented into the culture medium. This resulted in a slight positive effect in ethylene production rates, and the co-supplementation of both substrates together was the most successful at stimulating ethylene production (Publication III: Fig. 7). Higher induction may be achievable via introduced heterologous transporters that facilitate the influx of L-arginine and 2-oxoglutarate, since passive diffusion through membranes is expected to be limited (Montesinos *et al.* 1997; Vázquez-Bermúdez *et al.* 2000). However, even if the cell has abundant substrates available, the activity of EFE may become the next limiting factor.

4.3 Acetate can boost cell growth under specific conditions

4.3.1 Driving force to increase utilization of additional carbon sources

In order to generate efficient cell factories, versatility is always regarded as a bonus. For example, alternative carbon sources can enable efficient biomass accumulation and production of target compounds even when the primary energy source, light in the case of cyanobacterial hosts, is scarce. Therefore, in the focus of publication IV was to continue evaluating the biotechnological potential of cyanobacteria by monitoring how acetate supplementation influences *Synechocystis*. Acetate is a natural waste product synthesized through anaerobic fermentation. Many organisms can also direct it back into glucose through the glyoxylate cycle (Zhang and Bryant

2015), but even the strains, such as *Synechocystis*, which lack this pathway can utilize acetate in lipid biosynthesis or direct it into the oxidative TCA cycle to produce several amino acids, ATP and reducing compounds. Previously, acetate influx has not been considered as a limiting step in *Synechocystis,* and to verify this, a heterologous acetate transporter was introduced into the cells to enable active transport in addition to passive diffusion.

4.3.2 Passive acetate influx appears to limit use of supplemented acetate

The growth of WT *Synechocystis* appears not to benefit from supplemented acetate under the conditions tested (Publication IV: Fig. 1 a and b), which is in agreement with previous publications (Varman *et al.* 2013b). However, supplemented acetate boosted the growth of the mutant strain expressing the acetate transporter (Publication IV: Fig. 1 c-e), with a clear correlation between the quantity of supplemented acetate and the increase in growth rate (Publication IV: Fig. 1 g). Furthermore, the consumption of the supplemented acetate from the growth medium was faster in the mutant cultures than the control strain, which depended solely on passive diffusion (Publication IV: Fig. 2). These findings indicate that acetate influx is a limiting step in *Syncechocystis* cultures, and therefore contradict previous publications where uncharged acetate was expected to diffuse freely at the pH ranges used (Ihlenfeldt and Gibson 1977) and the conversion of acetate to acetyl-CoA was thought to comprise the actual bottleneck (Gibson 1981).

4.3.3 Acetate improves biomass formation when availability of light is a limiting factor

After enabling the influx of acetate through the heterologous acetate transporter, the increase in growth was greater at lower light intensities (Publication IV: Fig. 1 e), while the differences diminished as light levels increased (Publication IV: Fig. 1 h). This suggests that *Synechocystis* benefits from the additional carbon source most when the light energy available for photosynthesis is limited. Under these conditions, the cells profit from flexible mixotrophic conditions and adjust the metabolic flux accordingly (Kämäräinen *et al.* 2017). On the other hand, increased light intensity allows plentiful energy, and the advantages provided by supplemented acetate and the heterologous transporter fades away.

To find out whether the increased growth rate was caused by a larger cell size or a faster cell division, the cultures were examined under a microscope (Publication IV: Table 3) and dry cell weight was determined (Publication IV: Table 2). Both parameters supported a similar size of the cells, and therefore the enhanced acetate

utilization appears to increase cell division. Intracellular storage compounds were also determined and, while the intracellular poly-β-hydroxybutyrate content was similar between the strains, the AT mutant strain contained decreased amounts of glycogen (Publication IV: Fig. 3). Intracellular glycogen storages are known to fluctuate depending on the growth rate and culture conditions: at higher growth rates, the glycogen storages are consumed faster and when environmental conditions limit growth, the excess carbon and energy are converted to glycogen (Gründel *et al*. 2012). However, even without changes in growth rate, supplemented acetate has been shown to reduce glycogen storages in WT *Synechocystis* (Wu *et al.* 2002).

Small carboxylate anions, such as acetate, have the potential to act as direct inhibitors and compete with bicarbonate/carbonate when binding to PS II (Shevela *et al.* 2007), which is suspected to alter charge recombination properties at least in *Chlamydomonas reinhardtii* (Roach *et al.* 2013). Therefore, the photosynthetic properties of the acetate transporter mutant strain were measured, and indeed, the increased acetate influx had a negative effect on the photosynthetic properties of the cells. The oxygen evolution rate of PS II decreased to 50 % (Publication IV: Fig. 4 a) and was accompanied by a significant reduction in the light-saturated photosynthesis of the whole chain (Publication IV: Fig. 4 b). In addition, the chlorophyll *a* content of the cells was reduced, while carotenoid content was increased (Publication IV: Fig. 5). The carotenoids protect the DNA, molecules and lipids against intracellular reactive oxygen species, which were alse decreased in the mutant strain (Publication IV: Fig. 6).

5 Conclusions

The research focus within this thesis included several objectives, from investigating the basic metabolism of cyanobacteria around native hydrocarbons to biotechnological approaches for ethylene production and from comparing alternative host cells and production strategies to studying alternative carbon sources. The common feature in this research is in understanding biotechnological possibilities and limitations introduced by a more complex host organism compared to traditional heterotrophic bacteria. As a benefit, using the photosynthetic cyanobacteria for biotechnological applications, instead of yeast and bacteria, would allow a direct conversion of carbon dioxide to target end-products, with energy derived only from sunlight. However, as the results show, cyanobacteria are considerably more complicated organisms, and many of their features are still unknown.

For instance, the cyanobacterial native hydrocarbons would be an ideal replacement for fossil fuels. Unfortunately, the results revealed a high importance of these inert and hydrophobic chemicals to several cellular properties and their absence altered the entire cell metabolism, from growth to buoyancy and from photosynthetic properties to storage compounds. Thus, the potential for significantly upscaling the production of these hydrocarbons for biofuel production may be limited, while the unexpected sinking of the mutant strain may be a beneficial feature for cell harvesting in other applications.

Heterologous ethylene production in cyanobacteria has several advantages, including easy collection from the head-space. Yet maintaining closed cultures also has many disadvantages. While studying ethylene production, an additional objective was to evaluate alternative host strains and expression strategies for biotechnological purposes by introducing different gene variants in *Synechocystis* and *Synechococcus* strains either as self-replicating plasmids or as a part of the genome. The results clearly demonstrate that the original *efe* performed as well as the modified versions, and with proper expression strategies, both the *Synechocystis* and the *Synechococus* hosts supported stable long-term production without any signs of previously reported reduced viability. These results highlight the strain-specific variations and the importance of addressing these differences when designing expression strategies in the future.

To enable flexible production systems, the principles of circular bioeconomy should be taken into consideration. Therefore, the influence of supplemented acetate was studied to evaluate the potential to increase the production of target compounds and biomass even when light is scarce. Acetate was chosen for the proof of concept, since it is a fermentative waste product in one context and a building block in another. Introduction of acetate transporter enabled more efficient acetate usage and boosted growth rate, although photosynthetic activity was simultaneously reduced. In addition, the extra source of carbon and energy was only beneficial in specific conditions, and not a feature that could be extrapolated without verification. These features highlight the urgent need to connect the basic research to the biotechnological goals in order to design the best expression systems for a sustainable future.

While the presented results support the idea that there is potential in using cyanobacteria as carbon-neutral cell factories, they clearly highlight of the need to collect more information to achieve this goal. Cyanobacterial cells are not yet characterized in sufficient detail to allow the changes caused by modifications to be predicted well. However, through a combination of basic and applied research, the necessary information will be collected allowing the establishing an optimal guide for successful biotechnological production strategies.

Acknowledgements

The work for this thesis was carried out under the supervision of Academician of Science Eva-Mari Aro and Assistant Professor Pauli Kallio in the Synthetic Biology of Cyanobacteria group, which is part of the Molecular Plant Biology Unit, University of Turku, Finland. I am grateful to the Department of Biochemistry for enabling my thesis work and the University of Turku Doctoral Programme in Molecular Life Sciences for enabling my doctoral studies.

When I started to look for a summer job in 2011, my goal was to find an established group with excellent scientific integrity and an important research topic. I got the opportunity to become one of Eva-Mari´s summer employees, and after a few anxious days, the welcoming atmosphere of BioCity started to feel like a second home. I am extremely grateful to Eva-Mari for giving me an opportunity to grow as a scientist. I admire her leadership with a clear vision and even though her schedule is busy, she has a welcoming presence and an understanding attitude toward her employees. I am also grateful for her braveness to speak about the challenges and obstacles within scientific life.

Pauli started as my supervisor in 2012, and he has been a driving force for my maturation as a researcher ever since. We have experienced many ups and downs during these years, but through mutual respect, and equally strong heads, difficulties have been conquered with a smile. I am extremely grateful to Pauli for challenging me to come out of my comfort zone, for giving me time when needed, for giving me the freedom to take responsibility and for supporting me when necessery.

I am grateful for the scientific hub in the Molecular Plant Biology Unit. There have been always people to ask advice from and troubleshoot with. A special gratitude goes to all my co-authors and to our little entity, the Synthetic Biology of Cyanobacteria group, where so many good things come together. During these years, since 2011, many of my colleagues have become dear friends with whom I hope to share the next decades as well.

I am thankful to Dr. David Fewer for agreeing to be my learned Opponent. I would like to acknowledge Dr. Mervi Toivari and Jun.-Prof. Julie A. Z. Zedler for critically reviewing my thesis and Henna Rauduskoski for language revision. In

addition, I would like to warmly thank my advisory committee and Prof. Eevi Rintamäki for guidance throughout the Ph.D. project.

In the end, my biggest gratitude goes to all my loved ones, especially to my princess trio <3

May 2020
Eerika Vuorio

List of References

Abdel-Raouf, N., Al-Homaidan A. A. & Ibraheem I. B. M. (2012) Microalgae and wastewater treatment. *Saudi Journal of Biological Sciences,* 19**,** 257-275.

Allen, J. F. (2002) Photosynthesis of ATP—Electrons, Proton Pumps, Rotors, and Poise. *Cell,* 110**,** 273-276.

Alric, J., Lavergne J. & Rappaport F. (2010) Redox and ATP control of photosynthetic cyclic electron flow in Chlamydomonas reinhardtii (I) aerobic conditions. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1797**,** 44-51.

Anderson, S. L. & McIntosh L. (1991) Light-activated heterotrophic growth of the cyanobacterium *Synechocystis* sp. strain PCC 6803: a blue-light-requiring process. *Journal of bacteriology,* 173**,** 2761-2767.

Andre, C., Kim, S. W., Yu X.-H. & Shanklin J. (2013) Fusing catalase to an alkane-producing enzyme maintains enzymatic activity by converting the inhibitory byproduct H_2O_2 to the cosubstrate O_2. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 3191-3196.

Angermayr, S., Paszota M. & Hellingwerf K. (2012) Engineering a cyanobacterial cell factory for production of lactic acid. *Appl Environ Microbiol,* 78.

Aro, E. M., Virgin I. & Andersson B. (1993) Photoinhibition of Photosystem II. Inactivation, protein damage and turnover. *Biochim Biophys Acta,* 1143**,** 113-34.

Badger, M. R. & Price G. D. (2003) CO_2 concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. *Journal of Experimental Botany,* 54**,** 609-622.

Beckmann, K., Messinger, J., Badger, M. R., Wydrzynski T. & Hillier W. (2009) On-line mass spectrometry: membrane inlet sampling. *Photosynthesis Research,* 102**,** 511-522.

Berla, B. M., Saha, R., Maranas C. D. & Pakrasi H. B. (2015) Cyanobacterial Alkanes Modulate Photosynthetic Cyclic Electron Flow to Assist Growth under Cold Stress. *Sci Rep,* 5**,** 14894.

Bernard, A., Domergue, F., Pascal, S., Jetter, R., Renne, C., Faure, J.-D., Haslam, R. P., Napier, J. A., Lessire R. & Joubès J. (2012) Reconstitution of Plant Alkane Biosynthesis in Yeast Demonstrates That Arabidopsis ECERIFERUM1 and ECERIFERUM3 Are Core Components of a Very-Long-Chain Alkane Synthesis Complex. *The Plant Cell,* 24**,** 3106.

Blomquist, G. J. & Jackson L. L. (1979) Chemistry and biochemistry of insect waxes. *Progress in Lipid Research,* 17**,** 319-345.

Boelee, N. C., Temmink, H., Janssen, M., Buisman C. J. N. & Wijffels R. H. (2011) Nitrogen and phosphorus removal from municipal wastewater effluent using microalgal biofilms. *Water Research,* 45**,** 5925-5933.

Bourdenx, B., Bernard, A., Domergue, F., Pascal, S., Léger, A., Roby, D., Pervent, M., Vile, D., Haslam, R. P., Napier, J. A., Lessire R. & Joubès J. (2011) Overexpression of Arabidopsis ECERIFERUM1 Promotes Wax Very-Long-Chain Alkane Biosynthesis and Influences Plant Response to Biotic and Abiotic Stresses. *Plant Physiology,* 156**,** 29.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry,* 72**,** 248-254.

Cameron, R. E. (1962) Species of Nostoc Vaucher Occurring in the Sonoran Desert in Arizona. *Transactions of the American Microscopical Society,* 81**,** 379-384.

Campbell, D., Hurry, V., Clarke, A. K., Gustafsson P. & Öquist G. (1998) Chlorophyll Fluorescence Analysis of Cyanobacterial Photosynthesis and Acclimation. *Microbiology and Molecular Biology Reviews,* 62**,** 667.

Carbonell, V., Vuorio, E., Aro, E.-M. & Kallio P. (2016) Sequence Optimization of Efe Gene from *P. Syringae* is Not Required for Stable Ethylene Production in Recombinant *Synechocystis* sp. PCC 6803. *International Journal of Innovative Research in Techonology and Science,* 4**,** 30-35.

Carbonell, V., Vuorio, E., Aro, E.-M. & Kallio P. (2019) Enhanced stable production of ethylene in photosynthetic cyanobacterium *Synechococcus elongatus* PCC 7942. *World Journal of Microbiology and Biotechnology,* 35**,** 77.

Cardol, P. & Krieger-Liszkay A. (2017) From light capture to metabolic needs, oxygenic photosynthesis is an ever-expanding field of study in plants, algae and cyanobacteria. *Physiologia Plantarum,* 161**,** 2-5.

Carr, N. G. & Whitton. B. A. (1982) *The Biology of Cyanobacteria*. University of California Press.

Chen, X., Schreiber, K., Appel, J., Makowka, A., Fahnrich, B., Roettger, M., Hajirezaei, M. R., Sonnichsen, F. D., Schonheit, P., Martin, W. F. & Gutekunst, K. (2016) The Entner-Doudoroff pathway is an overlooked glycolytic route in cyanobacteria and plants. *Proc Natl Acad Sci U S A,* 113**,** 5441-6.

Chen, Y., Kay Holtman, C., Magnuson, R. D., Youderian, P. A. & Golden, S. S. (2008) The complete sequence and functional analysis of pANL, the large plasmid of the unicellular freshwater cyanobacterium *Synechococcus elongatus* PCC 7942. *Plasmid,* 59**,** 176-192.

Cherubini, F. (2010) The biorefinery concept: Using biomass instead of oil for producing energy and chemicals. *Energy Conversion and Management*, 51, 1412-1421.

Chew, K. W., Yap, J. Y., Show, P. L., Suan, N. H., Juan, J. C., Ling, T. C., Lee, D.-J. & Chang, J.-S. (2017) Microalgae biorefinery: High value products perspectives. *Bioresource Technology,* 229**,** 53-62.

Coates, R. C., Podell, S., Korobeynikov, A., Lapidus, A., Pevzner, P., Sherman, D. H., Allen, E. E., Gerwick, L. & Gerwick, W. H. (2014) Characterization of cyanobacterial hydrocarbon composition and distribution of biosynthetic pathways. *PLoS One,* 9**,** e85140.

Cohen, Y., Padan, E. & Shilo, M. (1975) Facultative anoxygenic photosynthesis in the cyanobacterium *Oscillatoria limnetica*. *Journal of Bacteriology,* 123**,** 855.

Creton, B., Dartiguelongue, C., de Bruin, T. & Toulhoat, H. (2010) Prediction of the Cetane Number of Diesel Compounds Using the Quantitative Structure Property Relationship. *Energy & Fuels,* 24**,** 5396-5403.

DeRuyter, Y. S. & Fromme, P. (2008) *Molecular structure of the photosynthetic apparatus*. United Kingdom: Caister Academic Press.

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

Ditty, J. L., Canales, S. R., Anderson, B. E., Williams, S. B.& Golden, S. S. (2005) Stability of the *Synechococcus elongatus* PCC 7942 circadian clock under directed anti-phase expression of the kai genes. *Microbiology,* 151**,** 2605-13.

Dong, J. G., Fernández-Maculet, J. C.& Yang, S. F. (1992) Purification and characterization of 1-aminocyclopropane-1-carboxylate oxidase from apple fruit. *Proceedings of the National Academy of Sciences,* 89**,** 9789.

Drews, G. (2011) The Evolution of Cyanobacteria and Photosynthesis. In *Bioenergetic Processes of Cyanobacteria: From Evolutionary Singularity to Ecological Diversity,* eds. Peschek, G. A., Obinger, C. & Renger, G. 265-284. Dordrecht: Springer Netherlands.

Ducat, D. C., Avelar-Rivas, J. A., Way, J. C. & Silver, P. A. (2012) Rerouting carbon flux to enhance photosynthetic productivity. *Appl Environ Microbiol,* 78.

Eaton-Rye, J. J. (2011) Construction of gene interruptions and gene deletions in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Methods Mol Biol,* 684**,** 295-312.

EUMOFA, European Market Observatory for Fisheries and Aquaculture Products (2018) BLUE BIOECONOMY Situation report and perspectives. *European Commission*

Fay, P. (1992) Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiological Reviews,* 56**,** 340.

Field, C. B., Behrenfeld, M. J., Randerson, J. T.& Falkowski, P. (1998) Primary production of the biosphere: integrating terrestrial and oceanic components. *Science,* 281**,** 237-40.

Fry, E. S., Kattawar, G. W., Strycker, B. D. & Zhai, P.-W. (2010) Equivalent path lengths in an integrating cavity. *Applied Optics,* 49**,** 575-577.

Fukuda, H., Sakai, M., Nagahama, K., Fujii, T., Matsuoka, M., Inoue, Y. & Ogawa, T. (1994) Heterologous expression of the gene for the ethylene-forming enzyme from *Pseudomonas syringae* in the cyanobacterium *Synechococcus*. *Biotechnology Letters,* 16**,** 1-6.

Gale, G. A. R., Schiavon Osorio, A. A., Mills, L. A., Wang, B., Lea-Smith, D. J. & McCormick, A. J. (2019) Emerging Species and Genome Editing Tools: Future Prospects in Cyanobacterial Synthetic Biology. Microorganisms, 7 doi: 10.3390/microorganisms7100409.

Gao, Q., Wang, W., Zhao, H. & Lu, X. (2012) Effects of fatty acid activation on photosynthetic production of fatty acid-based biofuels in *Synechocystis* sp. PCC6803. *Biotechnol Biofuels,* 5**,** 17.

Garcia-Pichel, F., López-Cortés, A. & Nübel, U. (2001) Phylogenetic and Morphological Diversity of Cyanobacteria in Soil Desert Crusts from the Colorado Plateau. *Applied and Environmental Microbiology,* 67**,** 1902.

Gautam, K., Tripathi, J. K., Pareek, A. & Sharma, D. K. (2019) Growth and secretome analysis of possible synergistic interaction between green algae and cyanobacteria. 127, 213-221.

Gibson, J. (1981) Movement of acetate across the cytoplasmic membrane of the unicellular cyanobacteria *Synechococcus* and *Aphanocapsa. Archives of Microbiology,* 130**,** 175-179.

Gimenez, R., Nuñez, M. F., Badia, J., Aguilar, J.& Baldoma, L. (2003) The gene *yjcG*, cotranscribed with the gene acs, encodes an acetate permease in *Escherichia coli*. *J Bacteriol,* 185**,** 6448-55.

Golden, S. S., Brusslan, J. & Haselkorn, R. (1986) Expression of a family of *psbA* genes encoding a photosystem II polypeptide in the cyanobacterium *Anacystis nidulans* R2. *Embo j,* 5**,** 2789-98.

González, L., Bolaño, C.& Pellissier, F. (2001) Use of Oxygen Electrode in Measurements of Photosynthesis and Respiration. In *Handbook of Plant Ecophysiology Techniques,* ed. Reigosa Roger, M. J.141-153. Dordrecht: Springer Netherlands.

Gottwald, S., Samans, B., Lück, S. & Friedt, W. (2012) Jasmonate and ethylene dependent defence gene expression and suppression of fungal virulence factors: two essential mechanisms of *Fusarium* head blight resistance in wheat? *BMC Genomics,* 13**,** 369.

Grigorieva, G. &Shestakov, S. (1982) Transformation in the cyanobacterium *Synechocystis* sp. 6803. *FEMS Microbiology Letters,* 13**,** 367-370.

Gründel, M., Scheunemann, R., Lockau, W. & Zilliges, Y. (2012) Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium *Synechocystis s*p. PCC 6803. *Microbiology,* 158.

Guerrero, F., Carbonell, V., Cossu, M., Correddu, D. & Jones, P. R. (2012) Ethylene synthesis and regulated expression of recombinant protein in *Synechocystis* sp. PCC 6803. *PLoS One,* 7**,** e50470.

Hagemann, M. & Hess, W. R. (2018) Systems and synthetic biology for the biotechnological application of cyanobacteria. *Current Opinion in Biotechnology*, 49, (94-99).

Han, J., Chan, H. W. S. & Calvin, M. (1969) Biosynthesis of alkanes in *Nostoc muscorum*. *Journal of the American Chemical Society,* 91**,** 5156-5159.

Han, J., McCarthy, E. D., Hoeven, W. V., Calvin,, M.& Bradley, W. H. (1968) Organic geochemical studies, ii. A preliminary report on the distribution of aliphatic hydrocarbons in algae, in bacteria, and in a recent lake sediment. *Proc Natl Acad Sci U S A,* 59**,** 29-33.

Heyer, H. & Krumbein, W. E. (1991) Excretion of fermentation products in dark and anaerobically incubated cyanobacteria. *Archives of Microbiology,* 155**,** 284-287.

Holtman, C. K., Chen, Y., Sandoval, P., Gonzales, A., Nalty, M. S., Thomas, T. L., Youderian, P. & Golden, S. S. (2005) High-Throughput Functional Analysis of the *Synechococcus elongatus* PCC 7942 Genome. *DNA Research,* 12**,** 103-115.

Howard, R. W. & Blomquist, G. J. (2004) ECOLOGICAL, BEHAVIORAL, AND BIOCHEMICAL ASPECTS OF INSECT HYDROCARBONS. *Annual Review of Entomology,* 50**,** 371-393.

Huflejt, M. E., Tremolieres, A., Pineau, B., Lang, J. K., Hatheway, J. & Packer, L. (1990) Changes in membrane lipid composition during saline growth of the fresh water cyanobacterium *Synechococcus* 6311. *Plant physiology,* 94**,** 1512-1521.

Ihlenfeldt, M. J. & Gibson, J. (1977) Acetate uptake by the unicellular cyanobacteria *Synechococcus* and *Aphanocapsa*. *Arch Microbiol,* 113**,** 231-41.

Ishikita, H., Saenger, W., Biesiadka, J., Loll, B.& Knapp, E.-W. (2006) How photosynthetic reaction centers control oxidation power in chlorophyll pairs P680, P700, and P870. *Proceedings of the National Academy of Sciences,* 103**,** 9855.

Janasch, M., Asplund-Samuelsson, J., Steuer, R. & Hudson, E. P. (2018) Kinetic modeling of the Calvin cycle identifies flux control and stable metabolomes in *Synechocystis* carbon fixation. *Journal of Experimental Botany,* 70**,** 973-983.

Jia, C., Li, M., Li, J., Zhang, J., Zhang, H., Cao, P., Pan, X., Lu, X.& Chang, W. (2015) Structural insights into the catalytic mechanism of aldehyde-deformylating oxygenases. *Protein & cell,* 6**,** 55-67.

Kageyama, H., Waditee-Sirisattha, R., Sirisattha, S., Tanaka, Y., Mahakhant, A. & Takabe, T. (2015) Improved Alkane Production in Nitrogen-Fixing and Halotolerant Cyanobacteria via Abiotic Stresses and Genetic Manipulation of Alkane Synthetic Genes. *Curr Microbiol,* 71**,** 115-20.

Kaiser, B. K., Carleton, M., Hickman, J. W., Miller, C., Lawson, D., Budde, M., Warrener, P., Paredes, A., Mullapudi, S., Navarro, P., Cross, F. & Roberts, J. M. (2013) Fatty Aldehydes in Cyanobacteria Are a Metabolically Flexible Precursor for a Diversity of Biofuel Products. *PLOS ONE,* 8**,** e58307.

Kalaji, H. M., Schansker, G., Ladle, R. J., Goltsev, V., Bosa, K., Allakhverdiev, S. I., Brestic, M., Bussotti, F., Calatayud, A., Dąbrowski, P., Elsheery, N. I., Ferroni, L., Guidi, L., Hogewoning, S. W., Jajoo, A., Misra, A. N., Nebauer, S. G., Pancaldi, S., Penella, C., Poli, D., Pollastrini, M., Romanowska-Duda, Z. B., Rutkowska, B., Serôdio, J., Suresh, K., Szulc, W., Tambussi, E., Yanniccari, M. & Zivcak, M. (2014) Frequently asked questions about in vivo chlorophyll fluorescence: practical issues. *Photosynthesis Research,* 122**,** 121-158.

Kaneko, T., Nakamura, Y., Sasamoto, S., Watanabe, A., Kohara, M., Matsumoto, M., Shimpo, S., Yamada, M. & Tabata, S. (2003) Structural Analysis of Four Large Plasmids Harboring in a Unicellular Cyanobacterium, *Synechocystis* sp. PCC 6803. *DNA Research,* 10**,** 221-228.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C. & Tabata, S. (1996) Sequence Analysis of the Genome of the Unicellular Cyanobacterium *Synechocystis* sp. Strain PCC6803. II. Sequence Determination of the Entire Genome and Assignment of Potential Protein-coding Regions. *DNA research,* 3**,** 109-136.

Kaneko, T. & Tabata, S. (1997) Complete Genome Structure of the Unicellular Cyanobacterium *Synechocystis* sp. PCC6803. *Plant and Cell Physiology,* 38**,** 1171-1176.

Kerfeld, C. A. & Melnicki, M. R. (2016) Assembly, function and evolution of cyanobacterial carboxysomes. *Current Opinion in Plant Biology,* 31**,** 66-75.

Khan, S. & Fu, S. (2020) Biotechnological perspectives on algae: a viable option for next generation biofuels. *Current Opinion in Biotechnology*, 62, 146-152.

Khara, B., Menon, N., Levy, C., Mansell, D., Das, D., Marsh, E. N. G., Leys, D.& Scrutton, N. S. (2013) Production of Propane and Other Short-Chain Alkanes by Structure-Based Engineering of Ligand Specificity in Aldehyde-Deformylating Oxygenase. *ChemBioChem,* 14**,** 1204-1208.

Khomutov, G., Fry, I. V., Huflejt, M. E. & Packer, L. (1990) Membrane lipid composition, fluidity, and surface charge changes in response to growth of the fresh water cyanobacterium *Synechococcus* 6311 under high salinity. *Arch Biochem Biophys,* 277**,** 263-7.

Klähn, S., Baumgartner, D., Pfreundt, U., Voigt, K., Schön, V., Steglich, C. & Hess, W. R. (2014) Alkane Biosynthesis Genes in Cyanobacteria and Their Transcriptional Organization. *Front Bioeng Biotechnol,* 2**,** 24.

Kosma, D. K., Bourdenx, B., Bernard, A., Parsons, E. P., Lü, S., Joubès, J. & Jenks, M. A. (2009) The Impact of Water Deficiency on Leaf Cuticle Lipids of *Arabidopsis*. *Plant Physiology,* 151**,** 1918.

Kratz, W. A. & Myers, J. (1955a) Nutrition and Growth of Several Blue-Green Algae. *American Journal of Botany,* 42**,** 282-287.

Kratz, W. A. & Myers, J. (1955b) Photosynthesis and Respiration of Three Blue-Green Algae. *Plant physiology,* 30**,** 275-280.

Kuchmina, E., Klähn, S., Jakob, A., Bigott, W., Enke, H., Dühring, U. & Wilde, A. (2017) Ethylene production in *Synechocystis* sp. PCC 6803 promotes phototactic movement. *Microbiology*.

Kudo, H., Nawa, R., Hayashi, Y. & Arai, M. (2016) Comparison of aldehyde-producing activities of cyanobacteria acyl-(acyl carrier protein) reductases. *Biotechnology for Biofuels*, 9, 234.

Kufryk, G. I., Sachet, M., Schmetterer, G. & Vermaas, W. F. J. (2002) Transformation of the cyanobacterium *Synechocystis* sp. PCC 6803 as a tool for genetic mapping: optimization of efficiency. *FEMS Microbiology Letters,* 206**,** 215-219.

Kämäräinen, J., Huokko, T., Kreula, S., Jones, P. R., Aro, E. M. & Kallio, P. (2017) Pyridine nucleotide transhydrogenase PntAB is essential for optimal growth and photosynthetic integrity under low-light mixotrophic conditions in *Synechocystis* sp. PCC 6803. *New Phytol,* 214**,** 194-204.

Kämäräinen, J., Nylund, M., Aro, E.-M. & Kallio, P. (2018) Comparison of ethanol tolerance between potential cyanobacterial production hosts. *Journal of Biotechnology,* 283**,** 140-145.

Le Henry, M., Charton, M., Alignan, M., Maury, P., Luniov, A., Pelletier, I., Pontalier, P.-Y., Binder, B. M., Vaca-Garcia, C. & Chervin, C. (2017) Ethylene stimulates growth and affects fatty acid content of *Synechocystis* sp. PCC 6803. *Algal Research,* 26**,** 234-239.

Lea-Smith, D. J., Biller, S. J., Davey, M. P., Cotton, C. A., Perez Sepulveda, B. M., Turchyn, A. V., Scanlan, D. J., Smith, A. G., Chisholm, S. W. & Howe, C. J. (2015) Contribution of cyanobacterial alkane production to the ocean hydrocarbon cycle. *Proc Natl Acad Sci U S A,* 112**,** 13591-6.

Lea-Smith, D. J., Ortiz-Suarez, M. L., Lenn, T., Nürnberg, D. J., Baers, L. L., Davey, M. P., Parolini, L., Huber, R. G., Cotton, C. A. R., Mastroianni, G., Bombelli, P., Ungerer, P., Stevens, T. J., Smith, A. G., Bond, P. J., Mullineaux, C. W. & Howe, C. J. (2016) Hydrocarbons Are Essential for Optimal Cell Size, Division, and Growth of Cyanobacteria. *Plant Physiology,* 172**,** 1928.

Lee, T. C., Xiong, W., Paddock, T., Carrieri, D., Chang, I. F., Chiu, H. F., Ungerer, J., Hank Juo, S. H., Maness, P. C.& Yu, J. (2015) Engineered xylose utilization enhances bio-products productivity in the cyanobacterium *Synechocystis* sp. PCC 6803. *Metab Eng,* 30**,** 179-189.

Li, N., Chang, W. C., Warui, D. M., Booker, S. J., Krebs, C.& Bollinger ,J. M. (2012) Evidence for only oxygenative cleavage of aldehydes to alk(a/e)nes and formate by cyanobacterial aldehyde decarbonylases. *Biochemistry,* 51**,** 7908-16.

Li, N., Nørgaard, H., Warui, D. M., Booker, S. J., Krebs, C.& Bollinger, J. M. (2011) Conversion of Fatty Aldehydes to Alka(e)nes and Formate by a Cyanobacterial Aldehyde Decarbonylase: Cryptic Redox by an Unusual Dimetal Oxygenase. *Journal of the American Chemical Society,* 133**,** 6158-6161.

Liu, L.-N., Chen, X.-L., Zhang, Y.-Z. & Zhou, B.-C. (2005) Characterization, structure and function of linker polypeptides in phycobilisomes of cyanobacteria and red algae: An overview. *Biochimica et Biophysica Acta - Bioenergetics*, 1708, 133-142.

Livak, K. J. & Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods,* 25**,** 402-8.

Los, D. A., Mironov, K. S.& Allakhverdiev, S. I. (2013) Regulatory role of membrane fluidity in gene expression and physiological functions. *Photosynth Res,* 116**,** 489-509.

Los, D. A. & Murata, N. (2004) Membrane fluidity and its roles in the perception of environmental signals. *Biochim Biophys Acta,* 1666**,** 142-57.

Luan, G., Zhang, S. & Lu, X. (2020) Engineering cyanobacteria chassis cells toward more efficient photosynthesis. *Current Opinion in Biotechnology*, 62, 1-6.

Luang, G., Zhang, S., Wang, M. & Lu, X. (2019) Progress and perspective on cyanobacterial glycogen metabolism engineering. *Biotechnology Advances*, 37, 771-786.

Mackey, S. R., Ditty, J. L., Clerico, E. M. & Golden, S. S. (2007) Detection of rhythmic bioluminescence from luciferase reporters in cyanobacteria. *Methods Mol Biol,* 362**,** 115-29.

Mamedov, M., Govindjee, V. Nadtochenko, V. & Semenov, A. (2015) Primary electron transfer processes in photosynthetic reaction centers from oxygenic organisms. *Photosynthesis Research,* 125**,** 51-63.

Mattila, H., Khorobrykh, S., Havurinne, V.& Tyystjärvi, E. (2015) Reactive oxygen species: Reactions and detection from photosynthetic tissues. *Journal of Photochemistry and Photobiology B: Biology,* 152**,** 176-214.

McIntosh, T. J., Simon, S. A. & MacDonald, R. C. (1980) The organization of n-alkanes in lipid bilayers. *Biochim Biophys Acta,* 597**,** 445-63.

Mendez-Perez, D., Begemann, M. B. & Pfleger, B. F. (2011) Modular Synthase-Encoding Gene Involved in α-Olefin Biosynthesis in *Synechococcus* sp. Strain PCC 7002. *Applied and Environmental Microbiology,* 77**,** 4264.

Mendez-Perez, D., Herman, N. A. & Pfleger, B. F. (2014) A desaturase gene involved in the formation of 1,14-nonadecadiene in *Synechococcus* sp. strain PCC 7002. *Applied and environmental microbiology,* 80**,** 6073-6079.

Mikami, K. & Murata N. (2003) Membrane fluidity and the perception of environmental signals in cyanobacteria and plants. *Prog Lipid Res,* 42**,** 527-43.

Miller, S. R. & Castenholz, R. W. (2000) Evolution of Thermotolerance in Hot Spring Cyanobacteria of the Genus *Synechococcus*. *Applied and Environmental Microbiology,* 66**,** 4222.

Mo, H., Xie, X., Zhu, T.& Lu, X. (2017) Effects of global transcription factor NtcA on photosynthetic production of ethylene in recombinant. *Biotechnol Biofuels,* 10**,** 145.

Monshupanee, T. & Incharoensakdi, A. (2014) Enhanced accumulation of glycogen, lipids and polyhydroxybutyrate under optimal nutrients and light intensities in the cyanobacterium *Synechocystis* sp. PCC 6803. *Journal of Applied Microbiology,* 116**,** 830-838.

Montesinos, M. L., Herrero, A.& Flores, E. (1997) Amino acid transport in taxonomically diverse cyanobacteria and identification of two genes encoding elements of a neutral amino acid permease putatively involved in recapture of leaked hydrophobic amino acids. *Journal of Bacteriology,* 179**,** 853.

Mori, T., Binder, B. & Johnson, C. H. (1996) Circadian gating of cell division in cyanobacteria growing with average doubling times of less than 24 hours. *Proceedings of the National Academy of Sciences,* 93**,** 10183.

Mullineaux, C. W. (2014) Co-existence of photosynthetic and respiratory activities in cyanobacterial thylakoid membranes. *Biochimica et Biophysica Acta (BBA) - Bioenergetics,* 1837**,** 503-511.

Mulo, P., Sicora, C. & Aro, E.-M. (2009) Cyanobacterial *psbA* gene family: optimization of oxygenic photosynthesis. *Cellular and Molecular Life Sciences,* 66**,** 3697.

Murata, N. & Wada, H. (1995) Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria. *The Biochemical journal,* 308 (Pt 1)**,** 1-8.

Nadeau, T.-L. & Castenholz, R. W. (2000) CHARACTERIZATION OF PSYCHROPHILIC OSCILLATORIANS (CYANOBACTERIA) FROM ANTARCTIC MELTWATER PONDS. *Journal of Phycology,* 36**,** 914-923.

Nagahama, K., Ogawa, T., Fujii, T., Tazaki, M., Tanase, S., Morino, Y. & Fukuda, H. (1991) Purification and properties of an ethylene-forming enzyme from *Pseudomonas syringae* pv. phaseolicola PK2. *J Gen Microbiol,* 137**,** 2281-6.

Ogawa, T., Takahashi, M., Fujii, T., Tazaki, M. & Fukuda, H. (1990) The Role of NADH:Fe(III)EDTA Oxidoreductase in Ethylene Formation from 2-Keto-4-Methylthiobutyrate. *Journal of Fermentation and Bioengineering,* 69**,** 287-291.

Paithoonrangsarid, K., Shoumskaya, M. A., Kanesaki, Y., Satoh, S., Tabata, S., Los, D. A., Zinchenko, V. V., Hayashi, H., Tanticharoen, M., Suzuki, I. & Murata, N. (2004) Five histidine kinases perceive osmotic stress and regulate distinct sets of genes in Synechocystis. *J Biol Chem,* 279**,** 53078-86.

Paul, B., Das, D., Ellington, B. & Marsh, E. N. G. (2013) Probing the Mechanism of Cyanobacterial Aldehyde Decarbonylase Using a Cyclopropyl Aldehyde. *Journal of the American Chemical Society,* 135**,** 5234-5237.

Pirkov, I., Albers, E., Norbeck, J. & Larsson, C. (2008) Ethylene production by metabolic engineering of the yeast *Saccharomyces cerevisiae. Metab Eng,* 10**,** 276-80.

Ramakers, C., Ruijter, J. M., Deprez, R. H.& Moorman, A. F. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neurosci Lett,* 339**,** 62-6.

Reed, R. H., Chudek, J. A., Foster, R.& Stewart, W. D. P. (1984) Osmotic adjustment in cyanobacteria from hypersaline environments. *Archives of Microbiology,* 138**,** 333-337.

Rippka, R., DeRuelles, J., Waterbury, J. B., Herdman, M.& Stanier, R. Y. (1979) Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Microbiology,* 111**,** 1-61.

Roach, T., Sedoud, A. &Krieger-Liszkay, A. (2013) Acetate in mixotrophic growth medium affects photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition. *Biochim Biophys Acta,* 1827**,** 1183-90.

Rodrigues, M. A., Bianchetti, R. E.& Freschi, L. (2014) Shedding light on ethylene metabolism in higher plants. *Frontiers in plant science,* 5**,** 665-665.

Sakai, M., Ogawa, T., Matsuoka, M.& Fukuda, H. (1997) Photosynthetic conversion of carbon dioxide to ethylene by the recombinant cyanobacterium, *Synechococcus* sp. PCC 7942, which harbors a gene for the ethylene-forming enzyme of Pseudomonas syringae. *Journal of Fermentation and Bioengineering,* 84**,** 434-443.

Sarcina, M. & Mullineaux, C. W. (2000) Effects of tubulin assembly inhibitors on cell division in prokaryotes in vivo. *FEMS Microbiology Letters,* 191**,** 25-29.

Sato, S., Shimoda, Y., Muraki, A., Kohara, M., Nakamura, Y. & Tabata, S. (2007) A large-scale protein protein interaction analysis in *Synechocystis* sp. PCC6803. *DNA Res,* 14**,** 207-16.

Schaefer, M. R. & Golden, S. S. (1989) Differential expression of members of a cyanobacterial *psbA* gene family in response to light. *Journal of Bacteriology,* 171**,** 3973.

Schirmer, A., Rude, M. A., Li, X., Popova, E. & del Cardayre, S. B. (2010) Microbial biosynthesis of alkanes. *Science,* 329**,** 559-62.

Schuurmans, R. M., van Alphen, P., Schuurmans, J. M., Matthijs, H. C. P. & Hellingwerf, K. J. (2015) Comparison of the Photosynthetic Yield of Cyanobacteria and Green Algae: Different Methods Give Different Answers. *PLOS ONE,* 10**,** e0139061.

Seelert, H., Poetsch, A., Dencher, N. A., Engel, A., Stahlberg, H. & Müller, D. J. (2000) Proton-powered turbine of a plant motor. *Nature,* 405**,** 418-419.

Shestakov, S. V. & Khyen, N. T. (1970) Evidence for genetic transformation in blue-green alga *Anacystis nidulans*. *Molecular and General Genetics MGG,* 107**,** 372-375.

Shevela, D., Klimov, V. & Messinger, J. (2007) Interactions of photosystem II with bicarbonate, formate and acetate. *Photosynth Res,* 94**,** 247-64.

Shih, P. M., Wu, D., Latifi, A., Axen, S. D., Fewer, D. P., Talla, E., Calteau, A., Cai, F., Tandeau de Marsac, N., Rippka, R., Herdman, M., Sivonen, K.. Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K. W., Han, C. S., Rubin, E. M., Eisen, J. A., Woyke, T., Gugger, M. & Kerfeld, C. A. (2013) Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. *Proceedings of the National Academy of Sciences,* 110**,** 1053.

Shively, J. M., Ball, F., Brown, D. H. & Saunders, R. E. (1973) Functional Organelles in Prokaryotes: Polyhedral Inclusions (Carboxysomes) of *Thiobacillus neapolitanus. Science,* 182**,** 584.

Singh, A. K., Elvitigala, T., Cameron, J. C., Ghosh, B. K., Bhattacharyya-Pakrasi, M. & Pakrasi, H. B. (2010) Integrative analysis of large scale expression profiles reveals core transcriptional response and coordination between multiple cellular processes in a cyanobacterium. *BMC Syst Biol,* 4**,** 105.

Singh, A. K., Summerfield, T. C., Li, H.& Sherman, L. A. (2006) The heat shock response in the cyanobacterium *Synechocystis* sp. Strain PCC 6803 and regulation of gene expression by HrcA and SigB. *Arch Microbiol,* 186**,** 273-86.

Stal, L. J. (2009) Is the distribution of nitrogen-fixing cyanobacteria in the oceans related to temperature? *Environmental Microbiology,* 11**,** 1632-1645.

Stal, L. J. & Moezelaar, R. (1997) Fermentation in cyanobacteria. *FEMS Microbiol Reviews,* 21.

Stanier, R. Y., Kunisawa, R., Mandel, M. & Cohen-Bazire, G. (1971) Purification and properties of unicellular blue-green algae (order *Chroococcales*). *Bacteriological Reviews,* 35**,** 171.

Stenberg, F., Chovanec, P., Maslen, S. L., Robinson, C. V., Ilag, L. L., von Heijne, G. & Daley, D. O. (2005) Protein complexes of the *Escherichia coli* cell envelope. *J Biol Chem,* 280**,** 34409-19.

Suzuki, I., Kanesaki, Y., Mikami, K., Kanehisa, M.& Murata, N. (2001) Cold-regulated genes under control of the cold sensor Hik33 in *Synechocystis. Mol Microbiol,* 40**,** 235-44.

Takahama, K., Matsuoka, M., Nagahama, K.& Ogawa, T. (2003) Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the *psbAI* locus. *Journal of Bioscience and Bioengineering,* 95**,** 302-305.

Tang, E. P. Y. & Peters, R. H. (1995) The allometry of algal respiration. *Journal of Plankton Research,* 17**,** 303-315.

The Ethylene Technology Report (2016) *Research and markets*, report ID: 3643672

Thiel, K., Patrikainen, P., Nagy, C., Fitzpatrick, D., Pope, N., Aro, E-M. & Kallio, P. (2019) Redirecting photosynthetic electron flux in the cyanobacterium *Synechocystis* sp. PCC 6803 by the deletion of flavodiiron protein Flv3. *Microbial Cell Factories*, 18, 189.

Tyystjärvi, T., Aro, E.-M., Jansson, C. & Mäenpää, P. (1994) Changes of amino acid sequence in PEST-like area and QEEET motif affect degradation rate of D1 polypeptide in photosystem II. *Plant Molecular Biology,* 25**,** 517-526.

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

Van der Plas, J., Oosterhoff-Teertstra, R., Borrias, M.& Weisbeek, P. (1992) Identification of replication and stability functions in the complete nucleotide sequence of plasmid pUH24 from the cyanobacterium *Synechococcus* sp. PCC 7942. *Molecular Microbiology,* 6**,** 653-664.

Varman, A. M., Xiao, Y., Pakrasi, H. B. & Tang, Y. J. (2013a) Metabolic Engineering of *Synechocystis* sp. Strain PCC 6803 for Isobutanol Production. *Applied and Environmental Microbiology,* 79**,** 908.

Varman, A. M., Yu, Y., You, L.& Tang, Y. J. (2013b) Photoautotrophic production of D-lactic acid in an engineered cyanobacterium. *Microb Cell Fact,* 12**,** 117.

Vass, I., Kirilovsky, D., Perewoska, I,. Máté, Z., Nagy, F.& Etienne A.-L. (2000) UV-B radiation induced exchange of the D1 reaction centre subunits produced from the *psbA2* and *psbA3* genes in the cyanobacterium *Synechocystis* sp. PCC 6803. *European Journal of Biochemistry,* 267**,** 2640-2648.

Veetil, V. P., Angermayr, S. A. & Hellingwerf, K. J. (2017) Ethylene production with engineered *Synechocystis* sp PCC 6803 strains. *Microb Cell Fact,* 16**,** 34.

Verma, S.& Kuila A. (2020) Involvement of green technology in microalgal biodiesel production. Reviews on Environmental Health, doi: 10.1515/reveh-2019-0061.

Vuorijoki, L., Isojärvi, J., Kallio, P., Kouvonen, P., Aro, E.-M., Corthals, G. L., Jones, P. R. & Muth-Pawlak, D. (2016) Development of a Quantitative SRM-Based Proteomics Method to Study Iron Metabolism of *Synechocystis* sp. PCC 6803. *Journal of Proteome Research,* 15**,** 266-279.

Vázquez-Bermúdez, M. F., Herrero, A. & Flores, E. (2000) Uptake of 2-Oxoglutarate in *Synechococcus* Strains Transformed with the *Escherichia coli kgtP* Gene. *Journal of Bacteriology,* 182**,** 211.

Wada, H. & Murata, N. (1990) Temperature-Induced Changes in the Fatty Acid Composition of the Cyanobacterium, *Synechocystis* PCC6803. *Plant Physiol,* 92**,** 1062-9.

Wang, J.-S., Araki, T., Matsuoka, M.& Ogawa, T. (2000) A Model of Photoinhibition Related to mRNA Instability in Ethylene Production by a Recombinant Cyanobacterium. *Journal of Theoretical Biology,* 202**,** 205-211.

Wang, W., Liu, X. & Lu, X. (2013) Engineering cyanobacteria to improve photosynthetic production of alka(e)nes. *Biotechnol Biofuels,* 6**,** 69.

Warui, D. M., Li, N., Nørgaard, H., Krebs, C., Bollinger, J. M. & Booker, S. J. (2011) Detection of Formate, Rather than Carbon Monoxide, As the Stoichiometric Coproduct in Conversion of Fatty Aldehydes to Alkanes by a Cyanobacterial Aldehyde Decarbonylase. *Journal of the American Chemical Society,* 133**,** 3316-3319.

Watanabe, M. & Ikeuchi, M. (2013) Phycobilisome: architecture of a light-harvesting supercomplex. *Photosynthesis Research,* 116**,** 265-276.

Weingart, H., Ullrich, H., Geider, K.& Völksch, B. (2001) The Role of Ethylene Production in Virulence of *Pseudomonas syringae* pvs. glycinea and phaseolicola. *Phytopathology,* 91**,** 511-518.

Whitton, B. A. & Potts, M. 2000. *Introduction to the cyanobacteria*. Netherlands: Kluwer Academic Publisher.

Williams, J. G. K. (1988) [85] Construction of specific mutations in photosystem II photosynthetic reaction center by genetic engineering methods in Synechocystis 6803. In *Methods in Enzymology*, 766-778. Academic Press.

Winters, K., Parker, P. L. & Van Baalen, C. (1969) Hydrocarbons of Blue-Green Algae: Geochemical Signfficance. *Science,* 163**,** 467.

Wojtasiewicz, B. & Stoń-Egiert, J. (2016) Bio-optical characterization of selected cyanobacteria strains present in marine and freshwater ecosystems. *Journal of applied phycology,* 28**,** 2299-2314.

Worrell, E., Phylipsen, D., Einstein, D. & Martin, N. 2000. *Energy use and energy intensity of the U.S. chemical industry*. LBNL-44314.

Wu, G. F., Shen, Z. Y.& Wu, Q. Y. (2002) Modification of carbon partitioning to enhance PHB production in *Synechocystis* sp. PCC6803. *Enzyme and Microbial Technology,* 30**,** 710-715.

Xiong, W., Morgan, J. A., Ungerer, J., Wang, B., Maness, P. &Yu, J. (2015) The plasticity of cyanobacterial metabolism supports direct CO_2 conversion to ethylene. *Nature Plants.,* 1.

Xu, W. & McFadden, B. A. (1997) Sequence Analysis of Plasmid pCC5.2 from Cyanobacterium *Synechocystis* PCC 6803 That Replicates by a Rolling Circle Mechanism. *Plasmid,* 37**,** 95-104.

Yang, X. & McFadden, B. A. (1993) A small plasmid, pCA2.4, from the cyanobacterium *Synechocystis* sp. strain PCC 6803 encodes a rep protein and replicates by a rolling circle mechanism. *Journal of Bacteriology,* 175**,** 3981.

Yang, X. & McFadden, B. A. (1994) The Complete DNA Sequence and Replication Analysis of the Plasmid pCB2.4 from the Cyanobacterium *Synechocystis* PCC 6803. *Plasmid,* 31**,** 131-137.

Yeung, T., Kwan, M., Adler, L., Mills, T. J., Neilan, B. A. Conibeer, G. & Patterson, R. (2017) Increased methane production in cyanobacteria and methanogenic microbe co-cultures. *Bioresource Technology*, 243, 686-692.

Yoo, S. H., Keppel, C., Spalding, M. & Jane, J. L. (2007) Effects of growth condition on the structure of glycogen produced in cyanobacterium *Synechocystis* sp. PCC6803. *Int J Biol Macromol,* 40**,** 498-504.

Yoon, H. S., Hackett, J. D., Ciniglia, C., Pinto, G. & Bhattacharya, D. (2004) A Molecular Timeline for the Origin of Photosynthetic Eukaryotes. *Molecular Biology and Evolution,* 21**,** 809-818.

You, L., Berla, B., He, L., Pakrasi, H. B.& Tang, Y. J. (2014) ^{13}C-MFA delineates the photomixotrophic metabolism of *Synechocystis* sp. PCC 6803 under light- and carbon-sufficient conditions. *Biotechnol J,* 9**,** 684-92.

Yu, Y., You, L., Liu, D., Hollinshead, W., Tang, Y. J. & Zhang, F. (2013) Development of *Synechocystis* sp. PCC 6803 as a phototrophic cell factory. *Mar Drugs,* 11**,** 2894-916.

Zerulla, K., Ludt, K.& Soppa, J. (2016) The ploidy level of *Synechocystis* sp.PCC 6803 is highly variable and is influenced by growth phase and by chemical and physical external parameters. *Microbiology,* 162**,** 730–739.

Zhang, S. & Bryant, D. A. (2011) The Tricarboxylic Acid Cycle in Cyanobacteria. *Science,* 334**,** 1551.

Zhang, S. & Bryant, D. A. (2015) Biochemical validation of the glyoxylate cycle in the cyanobacterium *Chlorogloeopsis fritschii* strain PCC 9212. *J Biol Chem,* 290, 14019-30.

Zhu, T., Xie, X., Li, Z., Tan, X.& Lu, X. (2015) Enhancing photosynthetic production of ethylene in genetically engineered *Synechocystis* sp. PCC 6803. *Green Chemistry,* 17**,** 421-434.

UNIVERSITY OF TURKU

Painosalama Oy, Turku, Finland 2020

ISBN 978-951-29-8079-6 (PRINT)
ISBN 978-951-29-8080-2 (PDF)
ISSN 0082-7002 (Print)
ISSN 2343-3175 (Online)