PHOTOSYNTHETIC CYANOBACTERIA AS FUTURE BIOTECHNOLOGICAL HOSTS; CONSIDERATIONS IN REGARDS TO METABOLITE TOXICITY AND COFACTOR REDOX BALANCE

Jari Kämäräinen
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Jari Kämäräinen
LIST OF ORIGINAL PUBLICATIONS

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<th>Abbreviation</th>
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<tbody>
<tr>
<td>ABE</td>
<td>ABE (Acetone–butanol–ethanol) fermentation</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BG-11</td>
<td>Blue-green growth medium for cyanobacteria</td>
</tr>
<tr>
<td>CBB</td>
<td>Calvin-Benson-Bassham cycle</td>
</tr>
<tr>
<td>Chl a</td>
<td>Chlorophyll a</td>
</tr>
<tr>
<td>Cyt</td>
<td>Cytochrome</td>
</tr>
<tr>
<td>Cyt b6f</td>
<td>Cytochrome b6f complex</td>
</tr>
<tr>
<td>DCW</td>
<td>Dry cell weight</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthesis</td>
</tr>
<tr>
<td>FD</td>
<td>Ferredoxin</td>
</tr>
<tr>
<td>FNR</td>
<td>Ferredoxin NADP⁺ oxidoreductase</td>
</tr>
<tr>
<td>G3P</td>
<td>Glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>KmR</td>
<td>Kanamycin resistance cassette</td>
</tr>
<tr>
<td>LTC</td>
<td>Linear electron transfer chain</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt, unit for electrical potential</td>
</tr>
<tr>
<td>NADP⁺</td>
<td>Nicotinamide adenine dinucleotide phosphate (oxidized)</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NDH</td>
<td>NADH dehydrogenase</td>
</tr>
<tr>
<td>NDH-1</td>
<td>NAD(P)H dehydrogenase complex type 1</td>
</tr>
<tr>
<td>OD₇₃₀nm</td>
<td>Optical density of at absorption wavelength 730 nanometers</td>
</tr>
<tr>
<td>OD₇₅₀nm</td>
<td>Optical density of at absorption wavelength 750 nanometers</td>
</tr>
<tr>
<td>OEC</td>
<td>Oxygen evolving complex</td>
</tr>
<tr>
<td>P680</td>
<td>Reaction center chlorophyll a, primary electron donor of PS II</td>
</tr>
<tr>
<td>P700</td>
<td>Reaction center chlorophyll a, primary electron donor of PS I</td>
</tr>
<tr>
<td>PBS</td>
<td>Phycobilisome</td>
</tr>
<tr>
<td>PC</td>
<td>Plastocyanin</td>
</tr>
<tr>
<td>PCC</td>
<td>Pasteur culture collection</td>
</tr>
<tr>
<td>PCE</td>
<td>Photon conversion efficiency</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>pH</td>
<td>Negative of the base 10 logarithm of the activity of the protons</td>
</tr>
<tr>
<td>PQ</td>
<td>Plastoquinone</td>
</tr>
<tr>
<td>PS</td>
<td>Photosystem</td>
</tr>
<tr>
<td>RC</td>
<td>Reaction center</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>sp.</td>
<td>Species</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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ABSTRACT

There is an increasing global need for developing new sustainable alternatives and technological solutions for replacing oil-based products currently in use. In response to this demand, photosynthetic cyanobacteria have been extensively studied as next-generation biotechnological hosts for the large-scale production of different carbon-based chemicals of interest directly from CO$_2$. The strategy is based on the intrinsic capacity of cyanobacterial cells to utilize solar energy for fixing CO$_2$ into complex organic molecules, together with our ability to engineer the cells for increased product range and improved performance. This Thesis is an expansion to the long-term basic research carried out at the Molecular Plant Biology (Department of Biochemistry, University of Turku, Finland), which aims to understand the molecular mechanisms of photosynthesis and associated regulatory networks. The key focus of the Thesis is on i) the toxicity of a range of end-products and related biosynthetic intermediates, and ii) enzymatic factors associated with the native regulation of intracellular cofactor redox homeostasis in cyanobacteria.

The first part of the Thesis concentrated on evaluating the growth-inhibitory effects of several chemicals and metabolites associated with hydrocarbon biosynthesis, towards a range of cyanobacterial species. The objective was to gain information which could be used for rational selection of the most appropriate target chemicals, engineering strategies and suitable hosts for biotechnological production trials and further photosynthetic cell factory. The comparison revealed significant differences between the apparent toxicity effects that decreased in the series from aldehydes to alcohols to alkanes, while ethanol and propane appeared as the most prominent candidates for continuous production. The length of the carbon chain was shown to have a clear impact in regards to the physiological tolerance, which expectedly reflected the solubility and capacity to penetrate through the cell membrane. The work also underlined the strain-specific features between different cyanobacteria, and complications in direct quantitative comparison between alternative hosts.

The second part of the Thesis concentrated on elucidating the biological role of the enzyme pyridine nucleotide transhydrogenase PntAB as part of the regulation of NAD(H)/NADP(H) homeostasis in cyanobacteria. Generation and characterization of Synechocystis sp. PCC 6803 deletion mutant ∆pntA devoid of the native transhydrogenase activity revealed that the enzyme plays a central role in the maintenance of sufficient NADPH supply under mixotrophic growth conditions when the photosynthetic activity of the cell is limited. The activity of PntAB was also linked with ATP metabolism and the photosynthetic repair mechanisms, which together are important considerations for the design of any engineered cyanobacterial systems.
Ihmiskunta on vähitellen siirtymässä fossiilisten raaka-aineiden aikakaudesta kestävän kehityksen talouteen, ja tämän onnistuminen vaatii systeemisen tason muutoksia nykyisiin tuotantostrategioihin. Osana tätä kehitystä, syanobakteereita on tutkittu mahdollisina tuotto-organismeina tulevaisuuden bioteknologisiin sovelluksiin. Idea perustuu siihen, että syanobakteerit kykenevät luonnostaan sitomaan auringon energian avulla ilialiidioksidia suoraan erilaisiksi orgaanisiksi yhdisteiksi, mikä yhdistettyynä yhä kehittyviin geneettisiin muokkausmenetelmiin, avaisi mahdollisuuden moninaisten uusiutuvien kemikaalien teolliseen valmistukseen. Tämä väitöskirjatyö pohjaa Turun yliopiston molekulaarisen kasvibiologian yksikössä tehtyyn uraauurtavaan perustutkimukseen, jonka tavoitteena on ymmärtää fotosynteesiin liittyviä molekyylitason vuorovaikutuksia ja säätelymekanismia. Väitöskirjan keskeisinä teemoina ovat i) erilaisten kemikaalien syanobakteerisoluille aiheuttamat toksisuusvaikutukset, sekä ii) solunsisäiseen kofaktorien hapetus- pelkistystasapainon ja yhteyttämiseen liittyvät entsymaattiset reaktiot.

Väitöskirjan ensimmäinen osa käsittelee hiilivetyjen biosynteesiin liittyvien erilaisten yhdisteiden liittävävaikutuksia syanobakteerisoluille. Tavoitteena oli saada kattavampi käsitöys eri yhdisteryhmien välisistä eduista ja rajoitteista, sekä käytössä olevien syanobakteerikantojen ominaisuuksista, jotka tulisi huomioida mahdollisen tuottoosysteemin suunnittelussa. Vertailu osoitti, että kemikaalien vaikutus solujen kasvuun vähensi siirryttäessä aldehydeistä, alkoholeihin ja siitä edelleen hiilivetyihin, samalla kun hiiliketjun pituus ja kantakohtaiset ominaisuuuden vaikuttivat merkittävästi lopputulokseen. Tulokset korostivat myös eri syanobakteerikantojen luontaisia fenotyyppisiä eroja, jotka vaikeuttavat eri kantojen suoraa kvantitatiivista vertailua.

1 INTRODUCTION

1.1 Welfare and regulation

Throughout history, the search for better and carefree life has led people to strive for material benefits, and to strongly invest on consumer goods at individual level. Together with growing world population, this has led to the overconsumption of natural resources, and to environmental consequences which affect all residents of the Earth (for a review, see Singh et al., 2014). Continuously growing global consumption, increasing need for food and energy, and associated imbalance has given rise to growing concern and disagreement, which especially affect the developing countries. This is a question of equality and justice, which is also recognized in the Universal Declaration of Human Rights (United Nations). Who has the right to impose on someone else's well-being? Do Western countries have the right to demand developing countries to use sustainable solutions, or vice versa, even if it means their slower economic development? Equality and fairness are being pursued by politics and law, but also by scientific knowledge, when deciding on issues related to the future of mankind.

Changes in attitudes and consumer behavior are realizing slowly because global environmental effects do not necessarily touch people at personal level. At the same time, the required operational changes are not profitable investments for the oil industry or individual commercial enterprises measuring success in short-term quartiles, that would directly encourage taking responsibility for the mitigations. For these reasons, outlining the future measures on energy and food economics should be based on a long-term vision as part of governmental and corporate strategic planning, involving political leaders, international associations and research scientists from different specific fields (REN21 Secretariat 2017). This transition from fossil-fuel based economy towards artificial-oriented strategies started from the Kyoto Protocol 2005, which was the first legally binding international agreement to tackle climate change by setting restrictions on industrialized countries. The following 2015 Paris Agreement prepared within the United Nations Framework Convention on Climate Change (UNFCCC) involving 195 countries has the common objective to reduce greenhouse gas emissions. Central aims of the agreement are to maintain the increase in global average temperature below 2°C relative to the levels of the pre-industrial era, and to enhance adaption of climate change tolerance towards harmful impacts without compromising food production. Ministry of economic affairs and employment in Finland has launched the road map for 2050 for a strategic level guidance towards carbon neutral society (Energia- ja ilmastotiekartta 2050, 2014), which in practical terms means that energy-
related emissions should be almost zero by 2050. The share of renewable energy in the final energy consumption in the EU is to be increased from 8.5% in 2005 to 20% by 2020 (European Commission) and in the share of biofuels in transport fuels should be at least 10% by 2020.

1.2 Production of renewable compounds; replacement of oil

Around 80% of mankind’s energy needs are currently satisfied by fossil fuels, and the social infrastructure and industrial production rely primarily on oil. Oil is used for transportation, energy for industry and households, and at the primary material for different chemicals, solvents, and plastics in large global scale. The great challenge is to shift from the use of these non-renewable resources to sustainable alternatives to meet the international requirements in reducing carbon emissions (Energy Roadmap 2050, 2011). In practice, this transition would require dramatically increasing the use of resources which are replenished in a short time-span, thus not adding to the increase in the atmospheric CO₂ levels. This will invariably require the development of new strategies and industrial solutions which would be both sustainable, offering long-term solutions, and which are integratable into the current network without relying on the total renewal of global infrastructures in the oil-based economy. As part of the transition towards sustainable circular bioeconomy, significant breakthroughs have been achieved in the recent years in the direct conversion of solar energy into electricity using solar panels (photovoltaics) (Ferroni & Hopkirk, 2016; Hammarström, 2012), which in parallel to the other accessible technologies for generating renewable energy (wind and hydropower), now provide commercially competitive large-scale alternatives for energy production (Halme et al., 2015). These systems, however, are limited by inadequate means of storing the energy, and will only partially complement the current needs now covered by the use of fossil resources – especially as to the need for carbon-based products.

The generation of biomass is based on photosynthetic fixation of carbon dioxide from air by photoautotrophic organisms, plants and cyanobacteria, which provide all the organic matter (food and carbon based raw-material) on Earth. Although the fossil reserves currently in use also initially derive from the same (ancient) photosynthetic reactions, the critical difference in using oil versus the biomass generated now is the turnover rate of carbon release and fixation. In addition to providing energy and building blocks for all nonphotosynthetic heterotrophic lifeforms, biomass derived from photosynthesis can be used for generating basically all the products that are currently manufactured from oil. Besides fuels used for heating and transportation this includes multitude of different products including organic solvents, household
chemical, plastics, textiles and other materials needed globally in enormous scale to meet the current scale of demand. Primarily, biomass can be used as the starting material for a variety of different industrial processes including different combinations of physical and thermochemical treatments to extract, modify and reorganize the organic molecules into desired end-products. For example, any biomass can be broken down in controlled oxygen-limited combustion process (gasification) into a mixture of \( \text{CO}_2 \), \( \text{CO} \) and \( \text{H}_2 \) (Synthetic gas; syngas), which can then be used for large-scale chemical conversion process (Fischer–Tropsch) into different hydrocarbon end products – as direct drop-in replacements for petroleum-derived chemicals. In parallel to the energy-consuming thermochemical conversions, wood can also be used directly as a building material, or as a precursor for a range of cellulose-derivatives including paper and fabrics, in addition to more sophisticated products which are currently in development (Lilja & Loukola-Ruskeeniemi, 2017). Biomass-derived lipids on the other hand can be converted via chemical transesterification and esterification reactions into renewable diesel, as exemplified by the industrial processes used by NesteOil corporation (Engman et al., 2016) and UPM corporation (Peters & Stojcheva 2017), which use a range of waste materials and pine oil as the starting materials, respectively. There are also a range of biotechnological applications (for a review, see Kalluri et al., 2014), systems which rely on the use of living cells as biological catalysts, which enable the conversion of biomass-based starting materials such as carbohydrates into specified target products (Scaife et al., 2015). The best large-scale examples include ethanol fermentation by yeast and other microorganisms (Humphrey & Lee, 1992) to produce bioethanol, anaerobic digestion of biomass by microbial colonies into methane, as well as ABE fermentation producing acetic acid, butanol and ethanol by different Clostridium species. In addition, corresponding biotechnology applications are also used for the commercial production of industrial enzymes (for a review, see Singh et al., 2016), a variety of pharmaceuticals including antibiotics and cancer drugs (for a review, see Shu, 1998), as well as in food processing (Potter & Hotchkiss, 1995).

Biomass is available in different forms and from a multitude of different sources, but for the development of sustainable production platforms for chemicals and fuels, the utilization of biomass should not compete with food production (Aro, 2016). The focus of developing long-term strategies must thus rely principally on the use of waste materials such as industrial side-streams and municipal waste, left-over material from agriculture in addition to rational use of forest resources (EASAC32, 2017). Although the parallel approaches provide an economical way of recycling available resources, the overall energy-efficiency is limited by the investments needed for harvesting, transport and multi-step processing. As for substituting fossil materials in the current global infrastructure, however, the most critical constraint is that the amount of available biomass (or derived materials) is not sufficient to meet the current demand. Thus, in
addition to the readjustment of consumer behavior and efficient integration of existing sustainable strategies, new complementary industrial solutions will need to be developed to reduce our global dependence on fossil resources.

As an alternative to the systems which rely on the use of photosynthesis-derived starting materials for processing, a range of different methodologies are being developed to more directly capture CO₂ into the final target products (for a review, see Rabaey & Rozendal, 2010). These strategies include the use of photosynthetic microorganisms as biotechnological production hosts for the solar-driven conversion of atmospheric CO₂ into desired target products, and different hybrid systems, which base on the use of electricity to generate the chemical reductive power (e.g. H₂ via electrolysis) for heterotrophic microbial hosts. Associated mature technologies do not yet exist, but the proof of concept has already been established, and the strategies have been recognized as prominent technologies for future investment and development (for a review, see Larkum et al., 2012). This research is intimately interlinked with the development and use of new synthetic biology strategies (Penttilä, 2017), which would contribute to the merging of biochemistry, engineering, in silico modeling and different fields of biotechnology towards actual practical advance in the field (for a review, see Georgianna & Mayfield, 2012; Maurino & Weber, 2012).

1.3 Cyanobacteria as photosynthetic production host

1.3.1 Cyanobacterium as a research organism

Cyanobacteria are genetically and morphologically diverse group of ancient photoautotrophic bacteria which possess the distinct capacity to perform oxygen-producing photosynthesis by oxidation of water (see section 1.3.2). Besides being the original source of oxygen in the atmosphere (for a review, see Reski, 2009), cyanobacteria are expected to be the primordial form of chloroplasts (for a review, see Komenda, Sobotka & Nixon, 2012; Reski, 2009) in eukaryotic photosynthetic organisms which currently, together with cyanobacteria, are responsible for the global carbon cycling (for a review, see Ting et al., 2002). In addition, the ability to cope under modest environmental conditions and use of simple nutrients, the autotrophic metabolism has provided cyanobacteria the opportunity to spread widely in different aquatic habitats and ecological niches on Earth. Different cyanobacterial species exist as individual single cells or as multicellular filaments with different specialized functions, with the common cellular architecture comprising of six separate compartments: Three membrane structures (outer membrane, cytoplasmic membrane and thylakoid membrane) confining three corresponding soluble compartments (periplasmic space,
INTRODUCTION

cytoplasm and thylakoid lumen) (Fig. 1) (Giddings, Withers & Staehelin, 1980; Liberton et al., 2006; Rajalahti et al., 2007; van de Meene et al., 2006). Due to the relative simplicity of the cells in comparison to eukaryotic organisms, cyanobacteria have become established model organisms in fundamental photosynthesis research that aim at understanding the complex structural/molecular mechanisms and regulatory interactions. Currently, the complete genome sequences are available for over 300 cyanobacterial strains (Ramos, Morais & Vasconcelos, 2017), and the function of different genes and proteins have been studied at molecular level in various contexts (Schirmer et al., 2010; Wang, Postier & Burnap, 2004). *Synechocystis* sp. PCC 6803 (later *Synechocystis* 6803) is one of the most extensively studied model cyanobacterial species (for a review, see Berla et al., 2013) for which a range of engineering tools (Huang & Lindblad, 2013), analytical strategies (Vuorijoki et al., 2016) and metabolic models (Joshi, Peebles & Prasad, 2017; Knoop et al., 2013) have been developed. Besides serving as a convenient test platform, this also provides a foundation for developing more elaborate engineering approaches also for other cyanobacterial strains.

![Figure 1. Transmission electron microscopy images of cyanobacteria. a) Synechocystis sp. PCC 6803, and b) Synechococcus sp. PCC 7002. Different cellular compartments labelled in the image: 1) Outer membrane 2) Cytoplasmic membrane 3) Thylakoid membrane 4) Periplasmic space, 5) Cytoplasm and 6) Thylakoid lumen. [Image: Kämäräinen et al.]](image)

### 1.3.2 Photosynthesis and carbon assimilation

Photosynthesis is a complex multi-step process in which the energy from the sun is converted into chemical form. In oxygenic photosynthesis the cells utilize solar radiation as energy, water as the primary electron donor, and CO$_2$ as carbon source to produce energy-rich reduced organic compounds, while molecular oxygen is released as a side-product (Fig 2). This process can be divided into *light reactions* in which the
electrons derived from solar-driven water splitting are passed on to the soluble electron carrier NADP⁺ to produce NADPH, and the subsequent dark reactions which use NADPH for the reduction of CO₂. The protons which accumulate in the thylakoid lumen upon photosynthetic light reactions are utilized for the synthesis of ATP, which is also required for carbon fixation.

In cyanobacteria, the components required for the photosynthetic light reactions are located on specific structures called thylakoid membranes, and include enzyme complexes photosystem II (PSII) (Komenda, Sobotka & Nixon, 2012), cytochrome b₆f, and photosystem I (PSI) (Fromme, Jordan & Krauss, 2001; Fromme et al., 2006), in addition to a range of soluble electron carriers (for a review, see Mulo, Sicora & Aro, 2009). The light energy is first harvested by light-collecting antennae, phycobilisome (PBS) complexes, associated with PSII and PSI. In PBSs the pigments responsible for the energy (photon) absorption are phycocerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin, which together with chlorophyll pigments in photosystems allow the cells to take advantage of different wavelengths of the solar spectrum in the range of 400-700 nm (MacColl, 1998). The excitation energy from the light harvesting complexes is subsequently transferred to the PSII reaction center (RC) chlorophyll P680, which triggers the primary charge separation and subsequent step-wise electron transfer through the photosynthetic electron transfer chain. The electron from PSII is transferred via the plastoquinone pool (PQ) to cytochrome b₆f, and further through plastocyanin (PC) to PSI (Cramer et al., 2006; Renger, 2012; Umena et al., 2011). The electron is re-excited by the reaction center chlorophyll P700 in PSI, after which it is passed by ferredoxin and ferredoxin NADP⁺ oxidoreductase to the oxidized soluble redox carrier nicotinamide adenine dinucleotide phosphate (NADP⁺) forming NADPH. At the PS II side, the electron hole generated as a result of the primary charge separation is filled by electrons from molecular water, resulting in water oxidation and release of molecular oxygen.

In the subsequent dark reactions, the NADPH is used to drive the reduction of CO₂ in the Calvin-Benson-Bassham (CBB) cycle in specific proteinaceous organelles, carboxysomes, in the cytoplasm (Badger & Price, 2003). The CBB cycle can be divided into three phases; CO₂ fixation, reduction reactions, and regeneration of the starting materials. The primary end-product is glyceraldehyde-3-phosphate (G3P), which is essentially the precursor for downstream biosynthetic reactions to generate the organic molecules required by the cell. The carbon fixation requires the input of energy in the form of ATP, which is generated as part of the photosynthetic electron transfer reactions. In this process the protons originating from the water-oxidation reactions are transferred into the thylakoid lumen, thus forming a transmembrane proton gradient, which is used by the ATP synthase to produce ATP.
Besides the solar-driven primary autotrophic metabolism (Knoop et al., 2010), cyanobacteria are also capable of heterotrophic growth and the use of carbohydrates or other reduced organic substrates as source of carbon and energy (Singh & Sherman, 2005). Metabolically, this requires complex regulatory networks to adjust the cell’s needs according to different growth conditions, access to light and to carbon-based energy precursors. For example, *Synechocystis* 6803 stores glucose in the form of intracellular glycogen under autotrophic growth when CO$_2$ and light are abundant (Hihara et al., 2001; Lehmann & Wöber, 1976; Omata et al., 1999; Singh & Sherman, 2005), whilst in darkness glycogen is mobilized and used for heterotrophic metabolism. In a similar manner, certain strains can take up extracellular glucose from the surroundings and utilize it for mixotrophic growth to support cellular functions when light is inadequate to maintain optimal photoautotrophic metabolism. Many of the factors associated with the transition between the different metabolic modes are still poorly understood. Such a regulation of the intracellular redox balance at the interface of autotrophic and heterotrophic growth is one of the areas of interest in this Thesis.

![Diagram of photosynthetic apparatus](image)

**Figure 2. Simplified representation of the photosynthetic apparatus and the components associated with photosynthetic light-reactions and CO$_2$ fixation in cyanobacteria.** Solar energy captured by the light-harvesting complex (LHC) is transferred to the photosystem II (PSII) reaction center P680 (RC P680) to generate P680$^+$. Water molecules serve as electron donor to RC680 and molecular oxygen is released as side product. Electrons released from P680 are passed down the electron transfer chain via the plastoquinone pool (PQ), the cytochrome b$_6$f complex (b$_6$f) and plastocyanin (PC) to PSI. The electrons re-exited at PSI are transferred via ferredoxin (FD) to ferredoxin NADP$^+$ oxidoreductase (FNR) to generate NADPH, which is subsequently used for reducing CO$_2$ in the Calvin-Benson-Bassham cycle, with glyceraldehyde-3-phosphate (G3P) as output. The proton gradient formed across the thylakoid membrane is used by the ATPase complex to drive the synthesis of ATP, which is also required in the process.
1.3.3 Design of autotrophic production platforms

Cyanobacteria have been studied in various contexts as prominent autotrophic production hosts to be used in future biotechnological applications such as high-value products (for a review, see Ducat, Way & Silver, 2011; Hays & Ducat, 2015) and biofuels (Jansson, 2012). The idea of these applications is usually to take advantage of the native photosynthetic machinery of the cyanobacterial cells, and engineer the metabolism for the production of desired chemical compounds directly from CO₂ and water, using solar radiation as energy. In comparison to the conventional heterotrophic biotechnological applications now in use (e.g. bioethanol), the critical advantage of this direct approach is that the production would not be limited by the availability of biomass-derived carbohydrates as starting materials, which is perhaps the most important single obstacle in current large-scale systems. So far cyanobacteria have been engineered for the overproduction of numerous native end-products as well as chemicals not naturally produced by the cells, (including different alcohols, organic acids and their derivatives, hydrocarbons, and carbohydrates (for a review, see Angermayr et al., 2015; Gorchs Rovira & Hellingwerf, 2015). Although different engineering strategies have expanded the end-product scope and improved the titers (Wijffels, Kruse & Hellingwerf, 2013), significant increase in the overall performance must be achieved before commercially competitive industrial applications can be established. The identified challenges include improving the strategies for enhanced strain engineering, improved pathway design, end-product tolerance, and product separation to allow the development of efficient continuous cultivation systems (Chisti, 2008; Lau, Matsui & Amirul, 2015; Li et al., 2008; Melis, 2009).

The foundation to economically competitive autotrophic production platforms is sufficiently high photon conversion efficiency (PCE), capture of the harnessed light energy in the specified target products, which is currently inadequate in all engineered systems (Long et al., 2006; Melis, 2009). This means that the cell metabolism must be more rigorously rerouted to channel the electron and carbon flux toward the desired target metabolite, instead using it for other functions such as storage or growth, and without losing general performance and cell viability. In practice this starts from rational pathway design in which (i) the limiting endogenous biosynthetic steps are enforced by overexpression of appropriate native or heterologous enzymes, accompanied by (ii) generation of metabolic detours around stringently regulated enzyme-catalyzed steps, and (iii) introduction of entirely new functions which allow the production of non-native products. In parallel, competing endogenous pathways which are not necessary for the production strain must be blocked, to prevent the distribution of the energy into unwanted cellular functions. These engineering approaches must effectively take into consideration the metabolic context of the cell, and possible
interference with vital cellular equilibria such as those related to cofactor redox balance (Paper III) which have to be re-established for the designed pathways to function (Angermayr, Paszota & Hellingwerf, 2012). In order to design systems which are compatible with continuous cultivation applications, focal considerations also include product transport from the interior of the cell into the surrounding medium, followed by product removal without harming the cells, which are closely interlinked with the product toxicity to the host (Paper I, II). Besides the target product physicochemical properties considered at the primary design phase, product transport may be enhanced by the introduction of specific heterologous transporter proteins into the system (Robinson, Woolhead & Edwards, 2000). Altogether, the current objective is to generate a more comprehensive understanding of the metabolic interactions, limitations as well as associated adverse effects, and together with computer-aided modeling and new synthetic biology strategies, allow the design of more effective production strains for future applications.
2 AIMS OF THE STUDY

This Thesis focuses on photosynthetic cyanobacteria, in context with the development of future biotechnological applications for the conversion of solar energy into renewable carbon-based commodities directly from CO₂. The work carried out at the Molecular Plant Biology (University of Turku) is a part of a wider international research entity, which collectively aims at understanding different molecular and cellular functions and restrictions that must be considered in the rational design of novel cyanobacterial production platforms.

The main objectives of the Thesis were to:

i) Evaluate the toxicity effects of different supplemented chemicals to different cyanobacterial hosts, in order to obtain a more comprehensive view of the potential and limitations associated with the autotrophic production of intermediates and alternative end-products.

ii) Study factors regulating the intracellular cofactor redox-balance in the cyanobacterium *Synechocystis* 6803. Specifically, the aim was to elucidate the biological function of transmembrane pyridine dinucleotide transhydrogenase PntAB in the interconversion of NAD(H)/NADP(H) at the interface of photoautotrophic and mixotrophic metabolism.
3 OUTLINE OF THE EXPERIMENTAL PROCEDURES

A brief general overview of the key preparative and analytical methods used in the Thesis is provided below. More comprehensive descriptions of the procedures can be found in the corresponding paper I, II, III.

Cell strains

The cyanobacteria used in the Thesis included five different laboratory substrains of *Synechocystis* sp PCC 6803, in addition to, *Synechococcus elongatus* PCC 7942 (later *Synechococcus* 7942), *Synechococcus* sp. PCC 7002 (later *Synechococcus* 7002) and two mutant strains; ethanol-producing *Synechocystis* 6803 variant SAA012 (later *Synechocystis* SAA012) and *Synechocystis* 6803 SigE over-expression strain (later *Synechocystis* SigE) (see Paper II, Table 1). *Escherichia coli* (later *E. coli*) strain DH5α was applied for all preparative molecular biology steps.

Culture media and default growth conditions

Cyanobacteria were cultured in BG11 medium (Rippka et al., 1979) buffered with TES-KOH to pH 7.5-8.0, with the exception of *Synechococcus* 7942 which was grown in A+ medium (Stevens, Patterson & Myers, 1973) supplemented with 4 µg/l vitamin B12. The main cultures were typically grown at 30°C under atmospheric CO₂ or with supplied sodium bicarbonate (10 – 50 mM) in the case of sealed cultivations under white light illumination in continuous mode or in diurnal rhythm at intensities ranging from 20 µmol photons m⁻² s⁻¹ to 200 µmol photons m⁻² s⁻¹ depending on the experimental set-up. Kanamycin (up to 50 µg ml⁻¹) was added to maintain selection pressure and glucose (5.5 mM) to induce mixotrophic growth when necessary. Other additives included alcohols (ethanol, butanol, heptanol, docodecanol), aldehydes (butanal), organic acids (butyrate, laurate), and hydrocarbons (propane, hexane and undecane) supplemented in different concentrations in the toxicity trials. The *E. coli* strains were routinely cultured in Luria-Bertani medium (Tryptone 10 g L⁻¹, NaCl, 10 g L⁻¹, yeast extract, 5 g L⁻¹) with supplemented antibiotics (ampicillin 100 mg L⁻¹ and kanamycin 50 mg L⁻¹) for transformant selection at 37°C in 250 rpm shaking.

Main cultivation methods for cyanobacteria

The cyanobacterial batch cultures were carried out in Erlenmeyer flasks or sealed serum bottles in a cultivation cabinet AlgaeTron AG230-ECO (Photon Systems Instruments, Brno, Czech Republic) or Versatile Environmental Test Chamber MLR-351 (SANYO Electric Co., Ltd. Osaka, Japan) in 150 rpm shaking. Alternatively, the batch cultivations were conducted in 70 ml volume in a photobioreactor Multi-Cultivator MC
1000 (Photon Systems Instruments, Brno, Czech Republic). The continuous cultivations were performed in 400 ml FMT 150 flat-panel photobioreactors (Photon Systems Instruments, Brno, Czech Republic).

**Molecular biology procedures**

Standard molecular biology procedures were applied for plasmid preparation and analysis. Available commercial kits were used for plasmid extraction and purification (NucleoSpin Extract, Duren, Germany II and QIAprep Spin Miniprep Kit, Hilden, Germany). Other enzymes and reagents were purchased from New England Biolabs (Ipswitch, MA, USA), Thermo Fisher Scientific or Fermentas (Vilnius, Lithuania). Oligonucleotides (Table 1) were ordered from Eurofins MWG operon (Ebersberg, Germany).

**Preparation of Synechocystis sp. PCC 6803 gene disruption mutants**

Inactivation of selected target genes in *Synechocystis* sp. PCC 6803 was performed by inserting a Kanamycin resistance cassette in the middle of the ORFs by homologous recombination. PCR was used to amplify 500bp fragments at each side of the *Synechocystis* sp. PCC 6803 integration sites and corresponding Kanamycin resistance cassettes using the primers specified in Table 1, followed by assembly into the backbone plasmid pUC19. The resulting integration plasmids were transformed into *Synechocystis* sp. PCC 6803 (Eaton-Rye, 2011), selected on BG-11 plates under gradually increasing concentrations of kanamycin (up to 50 µg ml⁻¹), and verified for segregation using colony PCR with the original amplification primers (Table 1).
Table 1. The PCR primers used for amplifying the target gene regions for homologous recombination (denoted by the ID codes), and for cloning the corresponding kanamycin resistance cassettes in *Synechocystis* sp. PCC 6803. Complementary sequences are shown in capitals and overhangs in small letters, with restriction sites underlined.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’&gt;3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>slr2072-FOR</td>
<td>taatgagctcatcaaatgacatttaatcattcgct</td>
</tr>
<tr>
<td>slr2072-REV</td>
<td>attaggatccATCATCCCATATTGCTGACAGAGGG</td>
</tr>
<tr>
<td>slr0752-FOR</td>
<td>taatgagctgtcacagacgcaactttccc</td>
</tr>
<tr>
<td>slr0752-REV</td>
<td>attaggatcccAACGTCTCTCGTGCAACCCGTC</td>
</tr>
<tr>
<td>slr0891-FOR</td>
<td>taatgagctccggactactctttctatagcaaggg</td>
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<tr>
<td>slr0891-REV</td>
<td>attaggatccAGGGCAGCTCCAAGGAAATGTCTTTC</td>
</tr>
<tr>
<td>slr1239-FOR</td>
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</tr>
<tr>
<td>slr1176-FOR</td>
<td>taatgagctcTCAAGAAGGGATTGGAAGGCTCCG</td>
</tr>
<tr>
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</tr>
<tr>
<td>slr0208-FOR</td>
<td>taatgagctctgcagatagtgtgcaacctcaattg</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>slr0721-REV</td>
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<td>slr1981-REV</td>
<td>attaggatccGCGGAGAATTGTTGGtagttgacctg</td>
</tr>
<tr>
<td>kan_AvrII-FOR (slr1239, slr2072)</td>
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</tr>
<tr>
<td>kan_AvrII-REV (slr1239, slr2072)</td>
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</tr>
<tr>
<td>kan_Nhel-FOR (slr0752)</td>
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</tr>
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<tr>
<td>kan_PspOmI-FOR (sll0891)</td>
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<tr>
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<tr>
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<tr>
<td>kan_Btgl-FOR (sll0209)</td>
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<tr>
<td>kan_Btgl-REV (sll0209)</td>
<td>atattCCGCGCTGAGCAGCGAGGCATAACTGAGCATAACCCCTT</td>
</tr>
</tbody>
</table>

**Spectrophotometric analysis**

Growth of the cyanobacterial strains was monitored spectrophotometrically at 730 nm or 750 nm using Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, Waltham, MA, USA), or the optical measurement system integrated as part of the photobioreactor MC1000 (Photon Systems Instruments, Czech Republic). Absorption spectra (375-750nm) were recorded using Olis CLARiTY 17 UV/VIS/NIR
spectrophotometer (OnLine Instrument Systems, Inc., Winter Park, USA) for cultures adjusted to OD _750nm_ 0.25.

**Dry cell weight determination**

To determine cyanobacterial dry cell weight (DCW) at a specified OD, 2ml of cultures was filtered through a pre-weighted 1 x 0.45 µm, 25 mm diameter Durapore® PVDF filters (Millipore, Billerica, MA, USA), dried at 110°C for 24 h in an oven, and weighted using an analytic scale (Mettler ToledoXA105 DualRange, Zurich, Switzerland).

**Western plot analysis**

For Western blot analysis whole-cell samples were solubilized in Laemmli SDS buffer containing 5% β-mercaptoethanol and 6 M urea, and separated in 12% SDS-PAGE containing 6 M urea. Samples normalized to total protein content (Bradford) were transferred to PVDF membranes (Immobilon-P, Millipore), followed by immunodetection with protein-specific antibodies (Agrisera, Vännäs, Sweden).

**Measurement of PSII and PSI photosynthetic properties**

The Chl a fluorescence and P700 absorbance were analyzed simultaneously with Dual-PAM-100 (Walz, Germany) to determine the efficient yield of PSII, Y(II), and PSI, Y(I), donor side limitation of PSI, Y(ND), and maximal photochemical efficiency of PSII (F_v/F_m) measured in the presence of DCMU. In addition, the system was used for monitoring the maximal amount of photo-oxidizable P700 (P_m), and ability to perform state transitions. Fluorescence emission spectra of intact cells at 77 K were determined with QE Pro-FL spectrofluorometer (Ocean Optics Inc.) equipped with a monochromator (Applied Photophysics Ltd.; f/ 3.4 grating).

**Ethanol quantitation**

Ethanol concentration in cyanobacterial culture samples was measured using a commercial K-ETOH Ethanol Assay Kit (Megazyme International, Bray, Ireland) which allows quantitative analysis of NADH formed in successive stoichiometric enzymatic reactions, according to the manufacturer’s instructions.

**Determining the GI50 values**

A numeric value of the growth inhibition parameter GI50 for ethanol toxicity for cyanobacterial strains was evaluated based on the ethanol concentration at which the recorded cell growth (OD _750nm_ ) was decreased to half over a 24 h batch cultivation period under increasing concentrations (0 - 27.6 gL⁻¹) of supplemented ethanol into growth media.
Transmission electron microscopy and estimation of relative cell sizes

Transmission electron microscopy (TEM) imaging of cyanobacteria cell thin sections was performed using JEM-1400Plus Transmission Electron Microscope (JEOL Inc., Peabody, MA, USA) at 80 kV. Cells were fixed with glutaraldehyde s-collidine buffer (5%), postfixed with osmium tetroxide (OsO₄) (2%) containing potassium (3%) ferrocyanide, dehydrated with ethanol, and flat embedded in a 45359 Fluka Epoxy Embedding Medium kit. Prepared sections (ultra microtome, 70 nm) were stained using uranyl acetate and lead citrate. Images were analyzed with Fiji image processing software, Fiji open-source platform for biological image analysis (Schneider, Rasband & Eliceiri, 2012). Relative sizes of the cyanobacterial cells were estimated based on the TEM images and Fiji software analyzing, by measuring the cross sectional circle of several hundreds of cells, and averaging the maximum values at the end of the linear area (n=50).

Bioinformatic analysis

PntAB homology model was assembled using Modeller (Sali & Blundell, 1993) based on the crystal structures available for homologous enzymes in Protein Data Base (PDB) (Altschul et al., 2005). Transmembrane helix prediction was carried out with TMHMM Schnerver 2.0 (Krogh et al., 2001), and the best scoring models from Modeller were further evaluated using MolProbity (Chen et al., 2010). Phylogenetic analysis of the homologous sequences found in BLAST were performed with CLUSTAL OMEGA (Sievers et al., 2011) and iTOL (Letunic & Bork, 2007). Computational analysis was also used to predict the potential yields of specified end-products using flux-balance-analysis (FBA). The evaluation was based on the metabolic model for Synechocystis 6803 (Knoop et al., 2013; Knoop et al., 2010) using MATLAB (version 2011a, The MathWorks Inc.) and COBRA Toolbox 2.0 (Schellenberger et al., 2011).
4 RESULTS

4.1 Physiological tolerance of cyanobacteria towards supplemented chemicals

4.1.1 Sensitivity of cyanobacterial strains to chemicals estimated based on growth

One of the central themes in the Thesis was to evaluate the toxicity effects of various supplemented chemicals on cyanobacteria, in order to assess associated limitations for potential future biotechnological applications. Cyanobacteria selected for the comparative analyses included *Synechococcus* 7942, *Synechococcus* 7002, and a number of commonly used *Synechocystis* sp. PCC 6803 laboratory substrains (Paper II, Table 1). In addition to the wild-type strains, two *Synechocystis* sp. PCC 6803 mutants, ethanol-producing *Synechocystis* SAA012 (Savakis, Angermayr & Hellingwerf, 2013) and *Synechocystis* SigE over-expression strain as described in Osanai’s article (Osanai et al., 2011), were chosen for the study. The toxicity of selected chemicals was studied using sealed serum bottle batch cultivations under white light illumination (60, 100 or 130 μmol photons m\(^{-2}\) s\(^{-1}\)) or continuous photobioreactor cultures (200 μmol photons m\(^{-2}\) s\(^{-1}\)). The analysis was carried out by evaluating the impact of different concentrations of the supplemented chemicals on cell growth, which is a prominent indicator of product toxicity as well as strain tolerance. The studied compounds represented some of the main classes of potentially interesting products including alcohols and hydrocarbons, or associated metabolic intermediates such as fatty acids and aldehydes of different lengths.

The compounds analyzed for toxicity (Paper I) were selected primarily by the specific interest towards engineered (hypothetical) hydrocarbon biosynthetic pathways which produce alternative end-products, alcohols (C4-C12) or the corresponding C\(_{n-1}\) alkanes (C3-C11) in cyanobacteria. In addition, the primary pathway precursors (fatty acids) as well as the common biosynthetic intermediates (aldehydes) were included in the comparison. In regards to the alternate end-products, the results showed that alkanes are likely to be significantly less toxic to the cell than the alcohols formed in parallel, as seen for hexane vs. heptanol (Paper I, Fig. 3). Supplementation of aldehydes resulted in a more pronounced inhibitory effect on cell growth in comparison to the corresponding organic acids and alcohols, as observed for butanal vs. butyrate and butanol (Paper I, Fig. 2b). As exemplified by the comparison between ethanol and butanol (Paper I, Fig. 2a), and butyrate and laurate (Paper I, Fig. 2b, 4b), the toxicity of alcohols and fatty acids increased with carbon chain length. In the case of alkanes, however, hexane (Paper I, Fig. 3) had a clearly more severe effect than longer chain
length undecane (Paper I, Fig. 4a). The findings also underlined the strain-specific differences as seen in the higher sensitivity of *Synchococcus* 7942 towards ethanol, hexane, undecane and laurate, in comparison to *Synechocystis* 6803.

Bioethanol is currently the most extensively used renewable transport fuel (REN21 Secretariat 2017), and represents the most mature form of the existing autotrophic microbial production technologies (for a review, see Ho et al., 2014). For these reasons, biological tolerance to ethanol was evaluated in more detail with several selected cyanobacterial strains, in regards to cell growth, cell size, and pigment content (Paper II). Growth inhibition parameter GI50 used as an indicator of toxicity showed that two strains belonging to the species of *Synchococcus*, *Synchococcus* 7002 and *Synchococcus* 7942, respectively, were the most but also the least tolerant to supplemented ethanol of cyanobacterial strains involved in the study (Paper II, Fig. 1 and 2). The most tolerant appeared to be *Synechococcus* 7002 strain followed by *Synechocystis* SAA012, and various *Synechocystis* 6803 substrains. Ethanol tolerance of the motile *Synechocystis* 6803C strain (Moscow, 6803C) was distinctively low compared to the other *Synechocystis* strains.

### 4.1.2 Strain-specific responses to culture conditions

Based on the initial comparison, spectrophotometric analysis, transmission electron microscopy and evaluation of dry cell weight were carried out to further study of the ethanol-induced stress effects in respect to cellular morphology and growth. The analysis was conducted on the most tolerant wild type strains, *Synechococcus* 7002 and *Synechocystis* 6803 (Kaplan strain), which were grown under the respective strain-specific optimal conditions at 37 °C under 130 μmol photons m⁻² s⁻¹ (condition I) and at 30 °C under 60 μmol photons m⁻² s⁻¹ (condition II), respectively. After 48 hour cultivation *Synechococcus* 7002 and *Synechocystis* 6803 strains were compared for OD750 and dry cell weight (Paper II, Table 2), and it appeared that the total cell mass per absorbance was significantly lower for *Synechocystis* 6803 than for *Synechococcus* 7002.

The divergence between the strains was further investigated, under the growth conditions I and II, with cell imaging by transmission electron microscopy (TEM) and spectrophotometric cell pigment profiling. The microscopic analysis indicated that while the *Synechocystis* 6803 cell size (3.01 and 2.58 arbitrary units) was systematically larger in comparison to *Synechococcus* 7002 (2.00 and 2.24 arbitrary units), the cells were always smaller when grown under the strain-specific un-optimal conditions (89 % and 83 %, respectively) (Paper II, Table 3). Chance in temperature and light intensity changed also cellular pigment profiles of the cyanobacterial strains by increasing overall pigment content in *Synechococcus* 7002 (Paper II, Fig. 4a) and by changing the balance
of the phycobilisome: chlorophyll ratio in *Synechocystis* 6803 (Paper II, Fig. 4b). In addition, after 48 h culturing under strain specific optimal growth condition for these two strains, the size of *Synechococcus* 7002 cells gradually decreased under increasing ethanol concentration (77 % in 36.8 gL⁻¹ EtOH), while for *Synechocystis* 6803 no correlation between the ethanol concentration and the cell size could be observed (Paper II, Table 4). As for the tolerance, the continuous photobioreactor cultures showed that the tolerance for ethanol declined steadily with both strains within three weeks cultivation period under gradually increasing ethanol concentration, however so that there were no effect under 9.2 gL⁻¹ of ethanol concentration for growth but under higher ethanol concentrations *Synechocystis* 6803 appeared to be more tolerant compared to *Synechococcus* 7002.

### 4.2 Inactivation of specific target genes and functional characterization in *Synechocystis* 6803

#### 4.2.1 Generation of a series of knock-out mutants in *Synechocystis*

As part of the Thesis, eight *Synechocystis* 6803 knock-out mutants were designed to study different metabolic interactions and effects *in vivo* (unpublished apart from ΔpntA; see Paper III). The target genes (Table 2) were selected based on various criteria as part of the ongoing projects, covering a range of metabolic entities of interest. The main focus was on the pyridine nucleotide transhydrogenase subunit alpha (encoded by slr1239; pntA), an indispensable functional component of an integral membrane protein complex PntAB, with proposed role in the maintenance of NADP(H) / NAD(H) redox balance in the cell (see section 4.2.2). Aldehyde deformylating oxygenase (encoded by sll0208; ado), a unique enzyme found only in cyanobacteria, was selected as a target due to its role as a key catalytic component in the biosynthesis of hydrocarbons (Klahn *et al.*, 2014) – a process which has drawn significant scientific interest in the field of biofuel research over the last few years (Kallio *et al.*, 2014; Patrikainen *et al.*, 2017). Glucose-1-phosphate adenylyltransferase (encoded by slr1176; glgC) catalyzes the first committed step of the biosynthesis of glycogen which is the central carbohydrate storage compound in cyanobacteria (Carrieri *et al.*, 2015; Grundel *et al.*, 2012). Inactivation of this route has been previously shown potentially enhance the productivity of specific end-products in *Synechocystis* 6803 (van der Woude *et al.*, 2014) making glgC a prominent knock-out target as a starting point for engineering production strains towards various metabolites. The selected targets also included genes encoding malate dehydrogenase (sll0891; citH) and malic enzyme (slr0721; me) which generate a potential bypass with phosphoenolpyruvate carboxylase from phosphoenolpyruvate to pyruvate in *Synechocystis* 6803 (Bricker *et al.*, 2004; Knoop *et
RESULTS

This route has been postulated to enhance the flux to pyruvate under certain conditions, making the genes interesting research subjects in elucidating the associated metabolic interactions and regulation in more detail. The remaining three inactivation targets were selected for directed evolution studies. The idea was to evaluate the capacity of the organism to adapt to potentially lethal mutations via native enzyme-catalyzed detours, which were predicted based on the existing metabolic model of Synechocystis 6803 (Knoop et al 2010) to evaluate the capacity of the organism to adapt to potentially lethal mutations. In the case of threonine deaminase (encoded by slr2072; ilvA) and acetolactate synthase (encoded by sll1981; ilvB) involved in the biosynthesis of branched-chain amino acids, the objective was to explore an existing detour from pyruvate to acetolactate via citramalate. In the case of enolase (encoded by slr0752; eno) catalyzing a central step in glycolysis, the conversion of 2-phosphoglycerate to phosphoenolpyruvate, the aim was to investigate a potential biosynthetic bypass from glyceraldehyde-3-phosphate to pyruvate via dihydroxyacetone phosphate.

The genes selected as knock-out targets (Table 2) were inactivated by introducing a kanamycin expression cassette in the middle of the coding regions using homologous recombination. The plasmid-based disruption constructs designed for the purpose were transformed into Synechocystis 6803, followed by several rounds of selection on kanamycin-containing plates. The segregation of the knock-out mutants was studied by colony PCR (Fig. 3), which confirmed that the disruption of pntA (slr1239), ado (sll0208), citH (sll0891), me (slr0721), ilvA (slr2072) and ilvB (sll1981) was successful in all the copies of the host chromosome. In contrast, the glgC (slr1176) and eno (slr0752) could not be segregated despite of repeated replating under increasing antibiotic concentrations, as seen in the presence of a band corresponding to the undisrupted WT gene on SDS-PAGE (Fig. 3).

**Table 2. Selected knock-out target genes in Synechocystis sp. PCC 6803.** The enzyme functions and the associated pathways of interest are listed in the vertical columns.

<table>
<thead>
<tr>
<th>Gene</th>
<th>EC class</th>
<th>Annotation</th>
<th>Pathway/function of interest</th>
<th>Segregation</th>
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<tr>
<td>slr1239, pntA</td>
<td>EC 1.6.1.2</td>
<td>Pyrimidine nucleotide transhydrogenase α</td>
<td>NAD(P)H redox homeostasis</td>
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<td>sll0208, ado</td>
<td>EC 4.1.99.5</td>
<td>Aldehyde deformylating oxygenase</td>
<td>Hydrocarbon biosynthesis</td>
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<td>slr1176, glgC</td>
<td>EC 2.7.7.27</td>
<td>Glucose-1-phosphate adenylyltransferase</td>
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<td>sll0891, citH</td>
<td>EC 1.1.1.37</td>
<td>Malate dehydrogenase</td>
<td>TCA-cycle (pyruvate metabolism)</td>
<td>Confirmed</td>
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<tr>
<td>slr0721, me</td>
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<td>EC 4.2.1.11</td>
<td>Enolase</td>
<td>Glycolysis</td>
<td>-</td>
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</table>
RESULTS

Figure 3. Colony PCR verification of the disruption of the selected target genes in *Synechocystis* sp. PCC 6803 (see Table 2) visualized by agarose gel electrophoresis. The expected PCR fragment sizes were in the range 2-3 Kb (target gene with integrated kanamycin cassette) versus 1 Kb (native WT control denoted as WT C). *GlgC* (*slr1176*) and *eno* (*slr0752*) could not be segregated.

4.2.2 PntA is required for native transhydrogenase PntAB activity in *Synechocystis* 6803

Out of the generated *Synechocystis* 6803 mutants, the Δ*pntA* strain was selected for a comprehensive study to elucidate the function and biological role of the transhydrogenase PntAB in respect to the maintenance of NAD(H)/NADP(H) equilibria in this specific host. The homology model generated for the enzyme on the basis of existing crystal structures from other organisms (Paper III) revealed that PntAB is a heterodimeric transmembrane protein composed of two interacting polypeptides α (PntA) and β (PntB), which together form three functional domains dI, dII, and dIII needed for the catalytic activity. While the domains dI and dIII are associated with the binding of NAD+/NADH and NADP+/NADPH, respectively, the dII domain forms an integral transmembrane proton channel, which is used to power the hydride transfer between the nicotinamide dinucleotide cofactors. The 3D model clearly demonstrated that PntA is involved in both the substrate binding (dI) and the proton channel formation (dII), implicating that the deletion of *pntA* would invariably prevent the native activity of PntAB. As a starting point for the functional studies, besides the colony PCR performed for the Δ*pntA* mutant, the absence of PntA was also confirmed by Western blot analysis using a PntA-specific antibody (Paper III, Fig. 3b).
4.2.3 Deletion of pntA results in growth defects under low-light mixotrophic conditions

*Synechocystis* 6803 WT and ΔpntA mutant were cultivated under different growth conditions to find out when and under which conditions the transhydrogenase PntAB would be specifically needed by the cell for maintaining the native redox balance between NAD(H) and NADP(H). The phenotypic growth characterization was carried out using a commercial photobioreactor MC1000 which allows the comparison of eight parallel replicates with automatic optical density measurement under controlled light conditions (Fig. 4 and 5). Growth of the strains was compared under constant illumination (5, 20 and 50 μmol photons m⁻² s⁻¹) or under diurnal rhythm (0-20, 0-50 or 0-200 μmol photons m⁻² s⁻¹), and in the presence and absence of supplemented glucose (Paper III Fig. 4 and 5). Prior this work, MC1000 had not been reportedly used for studying cyanobacteria (at least to our knowledge), so in order to validate the set-up and to evaluate the reproducibility of the acquired data, all experiments were carried out several times, typically with three parallel biological replicates in each case.

Under standard autotrophic culture conditions the WT *Synechocystis* 6803 and the ΔpntA deletion strain grew in an identical way, and no phenotypic features could be linked with the absence of the native transhydrogenase PntAB activity (Paper III Fig. 4 and 5). However, shift to mixotrophic growth conditions, induced by the supplementation of glucose in the medium and simultaneous decrease of the light intensity, resulted in clear growth defects in the ΔpntA mutant in comparison to the WT. As seen in the successive runs, the growth rate of the mutant strain gradually decreased upon the step-wise transition towards heterotrophic conditions, until finally the growth was completely arrested at the lowest light intensities – unlike recorded for the WT (Paper III, Fig. 5c). This trend was consistent and repeatable throughout all the culture trials, and was observed under both continuous light, as well as under sinusoidal diurnal rhythm in the presence of glucose.

From the viewpoint of the technical execution of the work, the repeated cultivations and alteration of the culture parameters allowed rather extensive evaluation of the potential and limitations of the MC1000 photobioreactor system. While the equipment proved to provide an advanced alternative for the conventional flask cultivations with the possibilities for automated growth monitoring and versatile light settings, the platform was shown to have several clear shortcomings, which limit or complicate its use. The light settings and adjusting the aeration of the parallel replicate culture tubes proved to be sometimes difficult to reproduce in a precise manner, and sampling was relatively laborious as it required system disassembly and transfer of the tubes into the laminar hood to ensure sterility. In addition, the user interphase of the system appeared not to be very user-friendly, and there were several complications with both
the software and the hardware, which forced many of the cultivation trials to be repeated.

Figure 4. Example of eight parallel cultures. Growth curves (OD$_{730\text{nm}}$) of *Synechocystis* sp. PCC 6803. Wild type strain (black line) and ΔpntA mutant strain (red line) with added glucose (solid line) and without glucose (dashed line). Growth under diurnal light a) 0-50 μmol photons m$^{-2}$ s$^{-1}$, and b) 0-500 μmol photons m$^{-2}$ s$^{-1}$. The cultivations were performed in photobioreactor MC1000, which allows simultaneous cultivation of eight parallel batch cultures.

Figure 5. Bioreactor MC1000 photographed after 6 days of cultivation of *Synechocystis* sp. PCC 6803 under continuous light 50 μmol photons m$^{-2}$ s$^{-1}$. Two tubes (1, 2) on the left are wild type without supplemental glucose; tubes 3, 4 are wild type with supplemental glucose; tubes 5, 6 are PntAB mutant without supplemental glucose and tubes 7, 8 on the right are PntAB mutant with supplemental glucose.
4.2.4 Biophysical characterization revealed defects in the photosynthetic apparatus of the ΔpntA mutant

The pntA-deficient Synechocystis 6803 mutant strain was subjected to a series of biophysical analyses to evaluate the underlying mechanisms associated with the growth defects observed under low-light mixotrophic conditions. The measurements were carried out using Dual-PAM-100 fluorometer system on the ΔpntA and WT cells cultivated under diurnal conditions (0-50 μmol photons m⁻² s⁻¹ light) in the presence of 5.5 mM supplemented glucose. Primarily, the analysis revealed a clear reduction of the maximal fluorescence ($F_m^{FR}$) and significantly lowered effective yield of PSII, Y(II) in the mutant in comparison to the WT (Paper III; Fig. 6a and b). This was accompanied by increased donor side limitation of PSI, Y(ND), while the overall effective yield of PSI, Y(I), was not significantly affected (Paper III, Fig. 6c). The mutant cells also exhibited impaired capacity to perform state transitions, to regulate the distribution of the phycobilisomes between PSII and PSI for balancing the absorbed light (Paper III, Fig. 7b). The possible reasons behind the observed reduction in the PSII capacity of the ΔpntA mutant were further investigated 77 K fluorescence emission spectroscopy and semi-quantitative Western analysis. The fluorescence analysis showed lowered PS II – specific signals (685 nm and 695 nm) in response to Chl $a$ excitation (440 nm), and implied overall decrease in the PS II: PS I ratio in the mutant strain in comparison to the WT. This was supported by the antibody-based evaluation of selected reaction center proteins, D1 (for a review, see Mulo, Sicora & Aro, 2009) and PsaB (Fromme et al., 2006) specific for PS II and PS I, respectively; the relative amount of D1 was decreased in comparison to PsaB in response to the low-light mixotrophic conditions in the ΔpntA mutant (Paper III, Fig. 8). In contrast, no differences were observed under autotrophic conditions between the ΔpntA strain and the WT control in any of the analyzed marker proteins.

4.2.5 Stage of characterization of other constructed Synechocystis 6803 mutants

Besides PntAB, the aldehyde deformulating oxygenase (ADO) has been under specific interest in the group, and has formed the foundation for generating engineered pathways for propane (Kallio et al., 2014; Menon et al., 2015) and other volatile alkanes (Patrikainen et al., 2017). The ADO deficient mutant generated in this study has recently been applied to study the function of ADO in Synechocystis 6803 (Vuorio et al. under preparation), in order to elucidate the biological role of naturally produced hydrocarbons in cyanobacteria – which currently remains unclear. The other four segregated Synechocystis 6803 mutants (slr2072, sll1981, sll0891 and slr0721) are ready for further investigation as part of other projects.
4.3 Theoretical production efficiency of specific target products in *Synechocystis 6803*

Maximal theoretical potential of different pathways for fuel production was evaluated with stoichiometric analysis of reconstructed pathways by using computational modeling. The objective was to estimate the relative theoretical capacity of *Synechocystis 6803* of converting CO₂ into selected carbon-based end products including ethanol, propane, ethylene, butanol, octadecanoic acid, heptadecane and octadecanol determined as the energy yield per mol photons (kJ g DW⁻¹ h⁻¹). The analysis revealed that the theoretical conversion efficiencies between the different target metabolites were rather similar, yet the highest energy yield per photon were calculated for ethanol, butanol and octanoid acid (Paper I, Table 3a). For ethylene, another product of interest in our research, the obtained conversion efficiency was especially poor, which can be explained by the unoptimal ATP/NADPH ratio needed for the process in comparison to other biofuels (Paper I, Table 3a).
5 DISCUSSION

5.1 Cyanobacteria as a cell factory

Cyanobacteria have been extensively studied as engineering targets for the autotrophic production of a range of industrially interesting chemicals directly from CO₂ (for a review, see Angermayr et al., 2015), and based on this concept, were recently recognized as potential biotechnological hosts for future industrial applications – provided that various biological and technological bottlenecks can be solved (Chartier et al, 2016; Purchase et al., 2015). Key considerations include the production efficiency and yield, compatibility of the chemicals with the host, techniques for continuous harvesting of the end-product, possibilities for upscale, in addition to matters such as legislation and biosafety. This Thesis focused specifically on several biological aspects, toxicity of chemical compounds to the cyanobacterial host (Paper I, II), and factors associated with the regulation of intracellular redox balance (Paper III), which must be understood at the molecular level in order to circumvent obvious metabolic constraints already at the design phase. The work underlined the importance of fundamental research in acquiring a comprehensive view to the underlying interactions and mechanisms which are needed for rational engineering of the strains to direct the metabolic flux from the photosynthetic reactions towards a specified end-product. In addition, the work provided insight to the limitations associated with photobioreactor cultivation systems as well as challenges faced with the comparison of different cyanobacterial strains, in context with the different set-ups and culture conditions used in the field (Paper II).

5.2 PntAB has a significant role in the maintenance of redox balance under mixotrophic conditions

In autotrophic metabolism, the electrons derived from the photosynthetic water cleavage at PSII are ultimately captured in reduced high-energy organic compounds, which represent the chemical form of the stored solar energy that runs heterotrophic life on Earth. From biotechnological perspective, when the photosynthetic capacity of cyanobacteria is harnessed for the capture of CO₂, the objective is to direct the water-derived electrons into the specified target products as efficiently as possible, instead of allowing them to be used for other metabolic purposes such as cell growth or storage in some other form. It is thus essential to understand how the native electron transfer reactions are regulated, and which processes may become limiting factors upon introduction of non-native enzymatic reactions, or the modulation of the endogenous
reactions by enzyme over-expression or inactivation. In this context, one part of the Thesis (Paper III) focused on the native regulation of the redox balance of nicotinamide cofactors, soluble electron carriers that are required for numerous different enzyme-catalyzed redox reaction as part of catabolic and metabolic reaction cascades, in the cyanobacterial host Synechocystis 6803. Specifically, the study aimed at understanding the biological role of the integral membrane protein pyridine nucleotide transhydrogenase PntAB in regards to the redox equilibrium between NADP(H) and NAD(H) under different metabolic modes (Fig. 6).

PntAB is a heteromultimeric integral enzyme complex found in various heterotrophic and autotrophic organisms, which couples the hydride transfer between NAD(H) and NADP(H) with the translocation of protons down the proton gradient through the membrane. Although the function of PntAB has been established in heterotrophic model organisms like E. coli, where acts to sustain the NADPH/NAD+ ratio sufficiently high as part of the glycolytic redox regulation, the role of the enzyme as part of autotrophic redox regulation in cyanobacteria had not been previously studied. From this starting point, the physiological conditions under which PntAB is important for Synechocystis 6803 was investigated by inactivating the gene coding for one of the two essential enzyme subunits, PntA, followed by analysis of cell growth under autotrophic and mixotrophic growth modes under varying light intensities.

The results invariably showed that PntAB is required specifically under heterotrophic growth conditions, under low light and in the presence of glucose, while it is redundant under fully autotrophic conditions. As primary product of photosynthesis is NADPH, while the reducing equivalents derived from the breakdown of glucose are mainly in the form of NADH, the results confirmed that PntAB is specifically needed in Synechocystis for reducing NADP+ at the expense of NADH under conditions when photosynthesis is unable to sustain optimal levels of NADPH.

To understand the associated interactions, the study was further expanded to evaluate the physiological effects caused by the lack of PntAB, and consequent reduction in the intracellular NADPH/NAD+ ratio under low-light mixotrophic conditions in Synechocystis. A series of biophysical measurements using DUAL-PAM-100 fluorometric analytical system revealed that the retarded growth of the ΔpntA strain was accompanied by reduction in the overall photosynthetic capacity. This was seen in the decrease in maximal fluorescence and PSII effective yield in parallel to PSI donor side limitation in the mutant strain in comparison to the WT. The absence of pntA also resulted in the detachment of phycobilisomes and reduced capacity to perform state transitions when the cells were grown under limiting light and in the presence of glucose. Furthermore, 77 K fluorescence emission characterization and Western
analysis supported the findings and revealed reduced PSII/PSI ratio and overall downregulation of PSII.

Taken together, the obtained results clearly showed that the inability of the Δ*pntA* *Synechocystis* 6803 cells to sustain sufficient levels of NADPH under low light resulted in the malfunction of the photosynthetic machinery and consequent reduction of growth as compared to the WT cells. It is assumed that this interconnection may be at least partly linked with the intracellular ratios of NADPH/NADP⁺ and the ATP/ADP. First, the ATP production via the NDH-1 pathway is expected to be compromised when sufficient NADPH is not available (Peltier, Aro & Shikanai, 2016; Shikanai & Aro, 2016), while at the same time the production of ATP through glycolytic metabolism is downregulated under the mixotrophic conditions (You, He & Tang, 2015). This results in decreased ATP/ADP ratio inside the cell, which subsequently may affect the PSII repair cycle which is dependent on ATP and constantly needed even under low-light conditions. As a consequence, the damage inflicted on the photosynthetic machinery would further reduce the concentrations of NADPH and ATP, thus amplifying the effect and further diminishing the capacity of the cell to control the imbalance (Kramer & Evans, 2011).
Figure 6. Simplified representation of the photosynthetic apparatus in operation and the role of PntAB a) under photoautotrophic conditions, and b) under mixotrophic conditions. Under photoautotrophic conditions photosynthetic apparatus maintains the intracellular ratios of NADPH/NADP\(^+\) and ATP/ADP, and electron flow needed to produce precursors for carbon products is sufficient (bold arrows). Under mixotrophic conditions, when photosynthetic activity is low, NADPH is generated by PntAB using NADH produced in glycolysis, restoring optimal ratios of NADPH/NADP\(^+\) and ADP/ATP (bold arrows).

5.3 Physiological tolerance of cyanobacteria for chemicals

Introduction of heterologous biosynthetic pathways or enforcing native metabolic reactions may significantly alter the concentration of associated metabolites,
biosynthetic intermediates or end-products inside the cell as well as in the surrounding extracellular medium. This is an important biotechnological consideration, as an excess of a given metabolite and interlinked metabolic imbalance are expected to result in undesired effects which negatively affect the well-being of the host, and thus reduce the efficacy of the production system. Such adverse effects may span from minor growth defects to general stress response and ultimately to cell death, and depend on the chemical structure and concentration of the compound, in addition to the growth phase and time of exposure. The encountered direct toxic effects may be caused by spontaneous reactions between the over-produced chemical and different cellular components, in parallel to alterations in intercellular concentration or related metabolites which interfere with native enzymatic housekeeping functions. In addition to the toxin, also the host selection plays an important role, as different organisms have varying protective mechanisms for inactivating or excreting the toxic product, together with active cellular repair mechanisms. From this premise, the other focus of this Thesis (Paper I, II) was on the toxicity effects of selected compounds on various cyanobacterial species, with the objective of expanding our understanding on the potential and limitations associated with alternative production platforms as future applications.

All cyanobacteria studied up to date share a unique intrinsic capacity to generate hydrocarbons, alkanes and alkenes, in their metabolism (Lea-Smith et al., 2016). This has spurred wide-ranging research interest towards the corresponding biosynthetic pathways, as they could provide access to biotechnological applications for the production of renewable hydrocarbon products to replace petroleum-derived commodities currently in use. Besides pathways which have been engineered to produce, for example, propane (Kallio et al., 2014) or other C7-C17 alkanes in heterologous hosts [recently summarized in (Patrikainen et al., 2017)], this sets the basis for evaluating the relative toxicity of the alkane-pathway intermediates and end-products toward cyanobacteria in this work (Paper I). Specifically, the hydrocarbon pathway of interest proceeds from (i) the release of fatty acid precursors from fatty acid synthesis (FAS) by a specific thioesterase, followed by (ii) the conversion into corresponding aldehyde by carboxylic acid reductase (Akhtar, Turner & Jones, 2013), and finally (iii) the formation of C_{n-1} hydrocarbons by the activity of the key enzyme aldehyde deformylating oxygenase (ADO) (Coates et al., 2014; Pandelia et al., 2013). Hydrocarbon biosynthesis in heterologous hosts is typically accompanied by the formation of the corresponding alcohols as side-products via the action of endogenous alcohol dehydrogenases (Patrikainen et al., 2017), and while these reactions compete with ADO for the aldehyde precursors, alcohols of different chain-lengths themselves serve as interesting end-products and potential fuels. Based on these reaction sequences, the primary target compounds in this study were saturated linear alkanes propane (C3), hexane (C6) and undecane (C11), and selected representatives of the C_{n-1}
precursors, carboxylic acids (butyrate, laurate), aldehydes (butanal), as well as corresponding alcohols (butanol, heptanol, dodecanol, in addition to ethanol).

The study was conducted by supplementing cyanobacterial cultures with different concentrations of the selected chemicals, followed by the comparison of the effect on growth. Comparison of the C4 pathway products revealed that aldehyde is clearly more toxic than the corresponding carboxylic acid or alcohol, as anticipated based on the general electrophilic reactivity of the carbonyl group with biological nucleophilic targets such as lipids (Paper I, Figure 2b). This also reflects the typical detoxification strategy in biological systems where reactive alkanes are actively converted into less reactive alcohols by the action of different alcohol dehydrogenases, as seen in, for example, the presence of parallel alcohol dehydrogenases in *Synechocystis* 6803 (encoded by slr1192 and slr0942 based on homology comparison), or 13 homologs found in *E. coli* (Rodriguez & Atsumi, 2014). At the other extreme, alkanes are generally least toxic and less harmful than, for example, alcohols in the same chain-length range (Paper I, Figure 3) due to the inert chemical nature of saturated hydrocarbons. While this makes alkanes prominent biotechnological targets, the work also emphasizes the effect of the product carbon chain length, as it affects the physicochemical interactions with the cell. This is especially the case with propane (C3) which is expected to freely penetrate through the cell membrane in contrast to undecane (C11) which has poor permeability that could restrict the development of continuous production systems. Notably, hexane (C6) appeared to be significantly more toxic to the cells than either propane or undecane, which is expected to reflect a combination of different physicochemical properties such as volatility, viscosity and solubility. The effect of the chain length was also observed in the increasing relative toxicity of C4 versus C12 carboxylic acids, as well as the C2 and C4 alcohols. This may be linked with the increased ability of the longer molecules to disrupt hydrophobic membrane structures, as previously also reported for toxicity effects in *E. coli* (Wilbanks and Trinh, 2017).

The experiments also underlined the differences between alternative cyanobacterial host strains (Berla et al., 2015; Kageyama et al., 2015; Lea-Smith et al., 2016), as revealed by the comparison between *Synechocystis* 6803 and *Synechococcus* 7942 in respect to a range of chemicals (Paper I), and the comparison of the tolerance of a number of alternative cyanobacterial strains towards ethanol (Paper II). Systematic strain-specific characteristics were very obvious in the relative sensitivity of *Synechococcus* 7942 towards ethanol, hexane, undecane and laurate in comparison to *Synechocystis* 6803, as evaluated based on the impact on cell growth. As both of these strains have been extensively studied cyanobacterial model species in the field of metabolic engineering, each having their own pros and cons (Jablonsky, Papacek & Hagemann, 2016), these toxicity effects are an important consideration when designing
a production chassis for a particular metabolite of interest. Ultimately, the work carried out in this Thesis focused largely on ethanol, which currently appears to be most prominent biofuel target in cyanobacteria due to relatively high production titer of 5.5 gL⁻¹ (Gao et al., 2012) and 7.1 gL⁻¹ (Dehring et al., 2012), and the existing status in the global fuel markets.

Comparison of the growth inhibition parameter GI₅₀ for alternate cyanobacterial species cultivated in the presence of up to 27.6 g/L (3.5 % V/V) supplemented ethanol also emphasized the importance of experimental validation for rational species and strain selection; *Synechococcus* 7002, *Synechocystis* 6803 (substrain Kaplan) and *Synechocystis* SAA012 were amongst the most tolerant strains, whereas the GI₅₀ value for the most sensitive strain *Synechococcus* 7942, were considerably lower (Paper II, fig. 2). Besides the growth defects, ethanol also induced other phenotypic changes including alteration of cell size (Paper II, Table 4). Notably, also these associated effects were strain-specific, making the direct comparison of culture optical density, chlorophyll content or dry cell weight between alternative strains challenging. For example, the calculated differences in ethanol tolerance between *Synechocystis* 6803 and *Synechococcus* 7002 in various growth conditions, was significantly affected by parameters used in the comparison. The results demonstrated that this also applies for the quantitative analysis of any single strain cultured under varying conditions, which call for caution whenever normalizing data to either optical density or chlorophyll content as routinely is the custom.
In the transition towards circular bioeconomy, there is an undeniable need for developing new strategies for generating energy, biofuels and renewable materials which would not compromise food production or other critical aspects of human well-being, while promoting the efficient use of local resources and infrastructure. The associated challenges are not trivial and require uniform strategies and collaboration between multidisciplinary players in research, industry and politics, in the socioeconomic framework of national policies and international regulations. It is clear that there will be no single solution to fulfill these demands, and consequently, the current fields of research involve various perspectives and interest towards different emerging future technologies and industrial solutions. For example, electricity-based innovations and applications have dramatically improved over the last decades (for a review, see Halme et al., 2015; Rubin et al., 2015), but will not be able to globally cover all the needs of the petroleum-based consumer society in the upcoming years. At the other extreme, the strategies which solely base on the use of biomass as the starting material can never meet the scale of current demand (REN21 Secretariat 2017), even if the technological processes were operating at maximal efficiency. In parallel to the other lines of development, there has been increasing interest towards biotechnological production platforms which do not rely on biomass-derived substrates, and can expand the repertoire of potential products beyond the physicochemical methods (Penttilä, 2017). These include different hybrid systems which combine the use of renewable electricity and microbial hosts capable of complex bioconversion reactions (Purchase et al., 2015), or the utilization of photosynthetic microorganisms which are able to directly fix CO₂ into carbon-based metabolites with the energy from the sun. Although majority of the research associated with the emerging biotechnological strategies is still at the level of proof-of-concept (Chartier et al, 2016), advances in the field will continue to improve the prospects for evaluating more realistically the future industrial scale feasibility and true commercial potential of the systems.

The study of autotrophic microorganisms at the Molecular Plant Biology, University of Turku, is founded on long-term research dedicated towards understanding the function and regulation of photosynthetic machinery. As the key motivation also to this Thesis work, the research is systematically expanding towards more applied fields of study, as an integral part of the University strategic research profiling which is setting the course for the upcoming years as recently summarized in Academy of Finland (BioFuture2025 programme). From a broader perspective, also the Finnish industry has addressed
potential interest towards emerging biotechnology applications (Penttilä, 2017), and the development of associated synthetic biology tools for more advanced engineering strategies, higher throughput and overall efficiency. This is at least in part promoted by the international agreements for decreasing CO$_2$ emissions, as well as customer’s active interest towards using renewable materials in consumer goods. As for the autotrophic biotechnology applications, the most advanced technologies currently focus on the production of bioethanol, which despite the highest obtained titers (Gao et al., 2012; Dehring et al., 2012) and existing up-scaled pilot systems (Angermayr et al., 2015) has severe limitations due to the low relative market price of fuels. However, significant amount of resources in the research field is now being placed on the development of more efficient engineering strategies for enhancing photon conversion efficiency and expanding the product profile to increase the compatibility of the systems at commercial level. While the emerging technologies have recently been recognized as potential future strategies at EU-level, also a number of key information and technology gaps have been identified, which are closely interlinked with understanding the biological systems at molecular level to allow rational engineering and technical solutions for improved output. Despite the advances, this strongly underlines the importance of continued fundamental research on cellular functions and interactions, which is still needed for any relevant technological breakthroughs.
Cyanobacteria have become widely used model organisms for basic photosynthetic research as well as targets for metabolic engineering, due to their relative simplicity and malleability in comparison to photoautotrophic eukaryotes. This work spanned from fundamental research and looked at cyanobacteria from a potential applied perspective, focusing on strain sensitivity towards different target products of interest, and possible metabolic constraints with regards to redox cofactor balance. The results emphasized the importance of understanding cyanobacteria both at the macro scale and at the level of individual gene functions, as all metabolic processes are interlinked and dependent on the environmental conditions. The study provided new knowledge on the regulation of NAD(P)H cofactor redox homeostasis in *Synechocystis* sp. PCC 6803, demonstrating the close connection of the energy balance and the maintenance of the integrity of the photosynthetic apparatus in the cell at the interphase of autotrophic and heterotrophic metabolic modes. In addition, the work underlined the diversity between different cyanobacterial strains in relative sensitivity to chemicals, the cellular responses triggered by culture conditions, and the strain-specific features that need to be considered when comparing or selecting a strain for a specific use.
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Jari
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PHOTOSYNTHETIC CYANOBACTERIA AS FUTURE BIOTECHNOLOGICAL HOSTS; CONSIDERATIONS IN REGARDS TO METABOLITE TOXICITY AND COFACTOR REDOX BALANCE