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DEVELOPING SYNTHETIC BIOLOGY STRATEGIES FOR ENHANCING THE EFFICIENCY OF ENGINEERED CYANOBACTERIAL EXPRESSION SYSTEMS

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EXPRESSION SYSTEMS**

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ABSTRACT

Cyanobacteria are unique prokaryotic microbes performing oxygenic photosynthesis. They have inherent capacity of the direct conversion of solar energy, CO₂ and water into carbon-based compounds. Scientists' increasing abilities to engineer cyanobacterial cells makes them an attractive next-generation biotechnological host for sustainable production systems, independent of biomass-derived substrates. However, in order to develop commercially viable systems, the strategies for rational strain engineering and enhanced electron and carbon fluxes towards the specified target compounds should be improved by synthetic biology approaches. To date, the development of robust engineering tools for solar-driven cyanobacterial production systems has lagged considerably behind compared to more common heterotrophic microbial production platforms such as *Escherichia coli* and *Saccharomyces cerevisiae*. The focus of this thesis was to develop synthetic biology tools and strategies for cyanobacterium *Synechocystis* sp. PCC 6803 for efficient and predictable strain engineering and pathway design.

The new synthetic biology tools established in this study include the characterisation of thirteen different *ribosome binding sites* (RBS) instrumental in adjusting the translational efficiency of synthetic pathways in *Synechocystis*. Nine different *integration sites*, both in the chromosome and endogenous plasmids, were validated for parallel and alternative expression of the gene of interest. Two *expression vector backbones* were designed and constructed for parallel and alternative expression strategies. A set of *quantitative reporter markers* were deployed as analytical tools which allowed the comparative evaluation of the expression of *Synechocystis*. An *assembly system* was adapted in *Synechocystis* for an accelerated pathway design and for a construction platform for expression cassettes or operons. In addition, a series of engineered *Synechocystis* strains were constructed to investigate whether the redirection of electron and carbon fluxes could enhance the photosynthetic electron flux towards the target end-product. This was accomplished by deleting the competing native flavodiiron protein-dependent electron transfer pathway and introducing an alternative sink for the excited electrons using sucrose as an end-product for electrons. This investigation revealed that a significant proportion of the electrons could be rescued and redirected towards the downstream biosynthetic reactions resulting in improved production efficiency. Furthermore, the potential use of acetate as supplementary carbon source for improving the cyanobacterial production system was addressed, and revealed to have a clear advantage for growth, especially under low light conditions.

In summary, the results presented in this thesis provide new synthetic biology tools and engineering strategies for promoting the development of cyanobacteria-based cell factories; they also provide a better understanding of the endogenous regulatory systems in *Synechocystis*, as a part of the progress towards future biotechnological solutions that will generate sustainable carbon-based products directly from CO₂.

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TIIVISTELMÄ

Syanobakteerit ovat ainoita tumattomia mikrobeja, jotka kykenevät happea vedestä vapauttavaan yhteyttämiseen. Syanobakteereiden luonnollinen kyky sitoa epäorgaaninen hiilidioksidi ja vesi valoenergian avulla erilaisiksi orgaanisiksi hiiliyhdisteiksi sekä niiden metabolin muokkailtavuus tekevät syanobakteereista kiinnostavia tulevaisuuden tuottosäntiä bioteknologiin sovellutuksiin, jotka olisivat täysin riippumattomia biomassaperäisistä lähtöaineista. Jotta voitaisiin kehittää taloudellisesti kannattavia tuottosysteemejä, pitää ensin kehittää parempia työkaluja syanobakteerien muokkaukseen ja aineenvaihdunnan elektroni- ja hiilivirtojen valjastamiseen kohti haluttuja yhdisteitä. Tehokkaiden muokkaustyökalujen kehittäminen syanobakteereille on kuitenkin jäänyt jälkeen yleisempien toisenvaraisten bakteri- ja hiivasolusäntien kehityksestä. Tämä väitöskirjatyö keskittyi kehittämään *Synechocystis* sp. PCC 6803 -syanobakteerille uusia synteettisen biologian työkaluja ja -tapoja, joilla parannetaan aineenvaihduntareittien suunnittelua ja muokkausta.

Tässä väitöskirjatyössä kehitettyihin uusiin synteettisen biologian työkaluihin kuuluu kolmentoista erilaisen *ribosomin sitoutumiskohdan* määrittely, mikä mahdollistaa translaatiotehokkuuden säätämisen synteettisissä aineenvaihduntareiteissä. Yhdeksän erilaista kromosomissa ja plasmidiaalisessa genomissa sijaitsevaa *integraatiokohtaa* määriteltiin, mikä mahdollistaa haluttujen geenien vaihtoehdoisen ja/tai samanaikaisen ilmentämisen *Synechocystis*-bakteerin genomissa. Kaksi erilaista *plasmidivektorityökalua* kehitettiin lisäämään geenien ilmentämisen vaihtoehtoja. *Synechocystiksellä* testattiin erilaisia kvantitatiivisia *markkereita*, joita voidaan käyttää geenii-ilmentymisen vertailuun. Synteettisten aineenvaihduntareittien suunnittelua sekä geenien ja operonien yhteenliittämistä helpottamaan otettiin käyttöön *Synechocystikselle* muokattu *koontisysteemi*. Lisäksi tutkittiin, voidaanko aineenvaihdunnan elektroni- ja hiilivirtojen uudelleenohjauksella parantaa yhteyttämässä syntyvien elektronien kohdistamista haluttuihin lopputuotteisiin. Tämä tapahtui poistamalla elektroneista kilpaileva flavoproteiinivälitteinen aineenvaihduntareitti ja lisäämällä soluihin vaihtoehtoinen elektroninielu. Elektroni- ja hiilivirtojen uudelleenohjausta muokatuissa *Synechocystis*-soluissa arvioitiin käyttämällä sakkaroosia ja luontaisia varastopolymeereja markkereina. Tulokset osoittivat, että merkittävä osuus elektroneista voitiin uudelleenohjata aineenvaihdunnan lopputuotteisiin sekä halutun lopputuotteen tuottoa voitiin parantaa. Edelleen tutkittiin, voidaanko *Synechocystiksen* tuottosysteemejä parantaa käyttämällä asetaattia lisähiililähteenä. Asetaatin havaittiinkin parantavan *Synechocystiksen* kasvua erityisesti himmeämmässä valossa.

Tämän väitöskirjatyön myötä saatuja synteettisen biologian työkaluja ja solujen muokkaustapoja voidaan käyttää syanobakteerituottosysteemien kehittämiseen. Tämä tutkimus on tuottanut myös tärkeää perustietoa syanobakteerien valoreaktioista ja metaboliareiteistä solutehtaiden suunnittelun parantamiseksi ja siten edistää siirtymistä fossiilipohjaisiin polttoaineisiin nojaavasta energiataloudesta kestävien energiaratkaisujen bionalouteen.

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Abbreviations

ATP	adenosine triphosphate
bp	base pair
BG-11	blue-green growth medium for cyanobacteria
CBB	Calvin-Benson-Basham
CmR	chloramphenicol resistance cassette
CO ₂	carbon dioxide
CoA	coenzyme A
CRISPR	clustered regularly-interspaced short palindromic repeats
GMO	genetically modified organism
DNA	deoxyribonucleic acid
FID	flame ionization detector
Flv	flavodiiron protein
GC	gas chromatography
GFP	green fluorescence protein
GOI	gene-of-interest
MIMS	membrane inlet mass spectrometry
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenide dinucleotide phosphate
NADP ⁺	nicotinamide adenide dinucleotide phosphate (oxidized)
NADPH	nicotinamide adenide dinucleotide phosphate (reduced)
ORF	open reading frame
PCC	Pasteur culture collection
PCR	polymerase chain reaction
pH	negative logarithm of proton concentration
PHB	polyhydroxybutyrate
PS	photosystem
RBS	ribosome binding site
RNA	ribonucleic acid
ROS	reactive oxygen species
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme
sp.	species

TCA	tricarboxylic acid
TES	2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino] ethanesulfonic acid
YFP	yellow fluorescence protein
WT	wild type

List of Original Publications

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

- I** Thiel K., Mulaku E., Dandapani H., Nagy C. Aro E.-M. and Kallio P. (2018) Translation efficiency of heterologous proteins is significantly affected by the genetic context of RBS sequences in engineered cyanobacterium *Synechocystis* sp. PCC 6803. *Microb Cell Fact*, 17(1), 34.
- II** Nagy C.*, Thiel K.*, Mulaku E., Aro E.-M. and Kallio P. Quantitative characterization of new genomic integration sites in the chromosome and the native plasmids for engineering the cyanobacterium *Synechocystis* sp. PCC 6803. Manuscript.
- III** Thiel K., Vuorio E., Aro E.-M. and Kallio P. (2017) The Effect of Enhanced Acetate Influx on *Synechocystis* sp. PCC 6803 metabolism. *Microb Cell Fact* 16(21): 1–12.
- IV** Thiel K., Patrikainen P., Nagy C., Fitzpatrick D., Pope N., Aro E.-M. and Kallio P. (2019) Redirecting photosynthetic electron flux in the cyanobacterium *Synechocystis* sp. PCC 6803 by the deletion of flavodiiron protein Flv3. *Microb Cell Fact* 18(189).

* Equal contribution.

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1 Introduction

1.1 Significance of cyanobacteria

1.1.1 Cyanobacteria as photosynthetic cell factories

Cyanobacteria are a diverse group of Gram negative, autotrophic prokaryotes harbouring an intrinsic capacity to perform oxygenic photosynthesis. As they have already existed on earth for about 2.4 billion years, cyanobacteria have an established role as primary producers and responsible for 20–30 % of overall photosynthetic production (DeRuyter and Fromme, 2008) as well as concomitantly producing nearly 30 % of the global biomass (Bryant, 2003). Their inherently versatile and flexible carbon metabolism allows growth under photoautotrophic, mixotrophic and heterotrophic conditions, thus enabling cyanobacteria to inhabit various ecological niches both in marine and terrestrial environments (Whitton, 2012). A model organism among cyanobacteria is *Synechocystis* sp. PCC 6803 (Williams, 1988) (*Synechocystis*). *Synechocystis* has been widely studied as an autotrophic host for the photosynthetic production of the desired compounds directly from abundant raw materials: sunlight, CO₂ and water. Through intricate photosynthetic processes, *Synechocystis* harvests solar energy to split water into oxygen, electrons and protons, and channels the electrons further to incorporate CO₂ into eventually more complex carbon compounds, which preserve the chemical energy in the carbon-carbon bonds, which originate from the capture of solar energy. The key advantage of the direct conversion of solar energy into commercially interesting renewable carbon-based compounds over the existing heterotrophic microbial production platforms is their independence from biomass-derived substrates; thus they are without the need for the production, storage and breakdown of sugars or cellulose. Therefore, this direct solar conversion technology would make solar-driven biomanufacturing a truly sustainable production system for renewable compounds, thus promoting the way towards a sustainable bioeconomy. Indeed, the enormous potential of photosynthetic cyanobacterial cell factories has already been recognised among the scientific commodity and business fields as well as at the EU-level as part of emerging future technologies (Chartier et al., 2016).

Cyanobacteria have been engineered to produce a wide range of native and non-native end-products, which can be regarded as benchmarks for potential industrial applications. The compounds, which have been produced on a *Synechocystis* platform so far include, for example, alcohols (Gao et al., 2012) (Luan et al., 2015); hydrocarbons as alka/(e)nes (Wang et al., 2013) (Ungerer et al., 2012) and isoprenes (Bentley et al., 2014); sugars (Ducat et al., 2012); organic acids as lactate (Angermayr et al., 2014) (Zhou et al., 2014) and 3-hydroxypropionic acid (Wang et al., 2016) as well as fatty acids (Tan et al., 2011). More comprehensive lists of the compounds produced in *Synechocystis* can be found in several reviews (Lau et al., 2015) (Berla et al., 2013) (Angermayr et al., 2015) (Oliver et al., 2016) (Savakis and Hellingwerf, 2015). Moreover, cyanobacteria have been employed in commercial pilot scale production systems for producing ethanol, and have been tested by a number of companies (e.g. Solazyme; Sapphire Energy). However, the titers obtained ranged from between milligrams per liter to several grams per liter and took up 50 % of the organic carbon fixed through the photosynthesis, and this is below the possibility of a economical break-even point of such low-price compounds, even though they are needed in high volume (Luan and Lu, 2018) (Oliver et al., 2016). Thus, the cyanobacterial production systems need to be improved in order to reach economically competitive levels compared to the current petroleum-derived production or the current commercial renewable production systems. In addition, cyanobacterial cells can be engineered to also produce relatively complex and valuable low-titer-high-price -compounds (see above).

One of the main reasons for the non-optimal performance of the autotrophic production platforms is that the current engineering strategies fail to utilise the entire photosynthetic capacity to a sufficient degree in order to efficiently convert the captured photons into chemically stored energy. Therefore, it would be important to enhance the flux of excited electrons and fixed carbon from the photosynthetic reactions towards those target compounds of interest for production. Improvement efforts demand robust engineering tools and optimal expression strategies as well as comprehensive information of the genetic elements of species-specific host. Consequently, there is an apparent need to develop more robust cyanobacterial engineering tools as well as improve the engineering strategies in order to elevate the predictability and end-product yields of autotrophic cyanobacterial production platforms. However, to move closer to these goals requires an in-depth molecular-level understanding of photosynthetic light reactions and carbon fixation reactions, a knowledge of the native cell metabolism and associated regulatory mechanism as well as an inclusive understanding of the downstream biosynthetic pathways and reactions with regard to the desired compound of interest.

1.1.2 Photosynthesis of cyanobacteria

The photosynthetic machinery, the engine of the autotrophic cell factory, is conserved among the photosynthetic organisms as plants, eukaryotic algae and cyanobacteria. In general, photosynthetic reactions can be divided into light reactions and carbon fixation reactions. Through intricate light dependent processes in the thylakoid membrane, harvested solar energy is used to split water into oxygen, electrons and protons by the photosystem II (PSII) complex. Simultaneously, oxygen is released as a by-product and protons are harnessed via a proton motive force for ATP synthesis by ATP synthase. Electrons, in turn, are transferred from PSII through a plastoquinone pool to a Cytochrome *b₆f*-complex, and further via plastocyanin to a photosystem I (PSI) and finally through the membrane into an NADP⁺ for NADPH formation. Eventually, the obtained NADPH and ATP, the energy carrying molecules, are used to reducing power of all the energy-requiring reactions in the cell, including the carbon fixation reactions. Ribulose-1,5-bisphosphate carboxylase/oxygenase enzyme (Rubisco), encapsulated in carboxysomes, catalyses the incorporation of inorganic CO₂ from the air to form two 3-phosphoglycerate molecules in the first reaction of the Calvin-Benson-Basham (CBB) cycle. Subsequently, glyceraldehyde-3-phosphate is generated to serve as a precursor for the biosynthesis of more complex molecules via different metabolic pathways. Excess of carbon and energy reserves are stored in the form of storage compounds, such as glycogen and polyhydroxybutyrate (PHB), which can be catabolised back to carbon skeletons and reducing power according to the cell's needs. Indeed, the great plasticity of sugar metabolism in cyanobacteria allows them to adjust the intracellular energy level and redox balance to respond to the variable growth conditions, such as dark periods. Cyanobacteria fix atmospheric CO₂ into biomass and growth (i.e. to normal cell proliferation, maintenance and acclimation), but through engineering strategies the carbon flow can be redirected to the sustainable production of the desired target compounds.

Although different photosynthetic organisms have similarities in their light and dark reactions, there are also some distinct species-specific differences in photosynthetic machineries, especially related to the photoprotection and regulatory mechanisms of light reactions. For example, characteristic to cyanobacteria is that the photosynthetic and respiratory processes occur concomitantly in the thylakoid membranes (Mullineaux, 2014) (Liu 2016). Thus, cyanobacteria have a relatively complex electron transport network that enables energy provision, a fine-tuned regulation of the flow of electrons, regulation of the redox state of the cells, and safety valves as protective electron sinks. In *Synechocystis*, one of these safety mechanisms involves a heterodimeric flavodiiron protein Flv1/3, which has been shown to prevent over-reduction of the photosynthetic electron transfer chain and consequent PSI damage by directing electrons back to O₂ (Allahverdiyeva et al.,

2011) (Mustila et al., 2016). This is biologically important especially under stress conditions such as fluctuating light and low CO₂ (Allahverdiyeva et al., 2013). Although the reactions represent an important native protective function, from the viewpoint of biotechnological applications, the Flv1/3 might compete with the target pathway for the electrons, thus reducing the overall production efficiency.

1.1.3 *Synechocystis* sp. PCC 6803 – a model cyanobacterium for development of cell factories

Cyanobacteria are promising microorganism for the development of autotrophic cell factories. As aquatic microbes, they are endowed with the ability to grow with minimal nutrient requirements, both photoautotrophically and mixotrophically, on cheap carbon sources such as atmospheric CO₂, allowing cultivation in places that do not compete with arable land. Cyanobacteria are a diverse group of bacteria, and it has been estimated that there are over 6000 species within the cyanobacteria group (Nabout et al., 2013). *Synechocystis*, which is a unicellular, non-toxic, freshwater cyanobacterium, has been used as a model organism for versatile cyanobacterial research areas, like photosynthesis, circadian rhythm, stress reactions and applied biotechnological research. In addition, the glucose-tolerance of the *Synechocystis* substrain enables studies of heterotrophic metabolisms in darkness or studies of photosynthesis (Williams, 1988) (Anderson and McIntosh, 1991). Despite some shortcomings as relatively long duplication time, multiple chromosomes or potential genetic instability, *Synechocystis* is an attractive host candidate among cyanobacteria for a tailored cell factory. Accumulated species-specific information, the recent advancements in preparative and analytical tools and engineering techniques, as well as better understanding of biological networks improve industrial properties of *Synechocystis*. Importantly, detailed understanding of individual model organisms also provide tools and know-how for the rational modification of the other cyanobacteria of interest. *Synechocystis* was the first phototrophic organism to be fully sequenced (Kaneko et al., 1996), making it accessible for various computational analysis in basic and applied sciences. Accordingly, *Synechocystis* is the host organism used also in the experimental work of this thesis.

1.2 Engineered genetic modifications in the cyanobacterial host

1.2.1 Targeted modifications can be realised by a variety of different methods

Optimised solar-driven cyanobacterial production systems require engineering of the metabolic capacities in order to increase the overall performance and productivity as well as to alleviate the most critical bottlenecks of the production pathways. For production of a target compound in a heterologous host, it is essential to introduce modifications in the host cell genome by bringing in new functions, deleting competing pathways or altering the regulation of the genes, preferably in a controlled manner. Often a native function of an endogenous gene needs to be enhanced by means of overexpression. Heterologous overexpression strategies, in turn, involve the import of exogenous genes from other organisms to the production host in order to introduce a novel function, which does not natively exist in the host. Overexpression strategies include insertion of the appropriate (set of) heterologous gene(s), that can further function in one or several biosynthetic pathways. Generally, in engineered bacterial systems multiple genes can be introduced as an operon, which is a functional cluster of desired genes under the control of a single promoter. In addition to overexpress the primary enzyme responsible for a target compound expression, there might be a need, for instance, to overexpress a potential auxiliary enzyme assisting the transport of a target compound out from the cell or to support the proper function of the primary enzyme. Concomitantly, the enzymes responsible for unwanted or competing reactions can be knocked out or downregulated to minimize the competing side-streams and effectively funneling the excess of cellular resources towards the specific target pathway.

Modifications can be introduced into the cell either by an autonomously replicating plasmid or by genome editing approaches, both taking advantage of capability of *Synechocystis* for natural transformation. For both common *Synechocystis* engineering approaches, *E. coli* is normally used as the host for plasmid construction due to the inconvenience and longer growth times of *Synechocystis*. Broad-host-range expression vectors are needed when proceeding to *Synechocystis* transformation for expressing the target genes. However, the number of available shuttle vectors has been very limited in *Synechocystis*, comprising mainly of the RSF1010 replicon based self-replicative expression plasmids. Examples of such autonomously replicating plasmids include pDF-lac (Guerrero et al., 2012), pFC1 (Mermat-Bouvier and Chauvat, 1994), pPMQAK1 (Huang et al., 2010) and pSEVA (Ferreira et al., 2018). Recently, tools for plasmid-based engineering have increased as new expression vectors derived from the native

Synechocystis plasmids pCA2.4 and pCB2.4 have been generated (Liu and Pakrasi, 2018). In genomic engineering strategy, DNA fragments or plasmids endowed by specific homologous flanking regions are used for inserting an expression cassette by a homologous recombination mechanism into the target locus of the genome (Berla et al., 2013). In addition to the chromosome, the endogenous plasmids of *Synechocystis* may be potential sites for the expression of heterologous genes (Armshaw et al., 2015). Moreover, chromosome modifications have been considered to be relatively stable after segregation has been established; although, recently, autonomously replicating plasmids were shown to stay rather stable in the *Synechocystis* cells even without continuous antibiotic pressure (Ferreira et al., 2018).

1.2.2 Control points of expression

In addition to maximizing the expression level of a specific target protein, balanced expression levels of the enzymes in multi-gene pathways must be tuned and optimised to respond to the particular requirements for the production of a target compound under specific conditions. This requires tight and predictable control of the expressed genes with a proper expression efficiency mediated through actions of several endogenous regulatory elements. Basically, the same mechanisms controlling expression of native genes are also applied for heterologously expressed pathways. Cyanobacteria have developed transcriptional, post-transcriptional, translational and post-translational regulatory systems to respond to intracellular and extracellular changes, which can be exploited for engineering purposes as well. Figure 1 shows the central dogma of molecular biology illustrating how the information endowed in DNA is transferred through a two-step process; from DNA to RNA via transcription and further from RNA to protein via translation. In addition, Figure 1 shows the essential genetic elements of DNA needed for expression of a gene and concurrently serving as alternative and parallel valves for controlling the transcriptional and translational expression of the gene-of-interest. One of the main regulatory switches is the promoter region controlling the binding of RNA polymerase and thus the initiation of transcription. From an engineering perspective, the most desired promoters may be well-controlled, either constitutive promoters with no extra load from the expression of repressors or tunable inducible promoters with a broad dynamic range. However, the selected induction strategy should not induce any detrimental side effects on the native metabolism e.g. acute toxicity or induction of unwanted native promoters). Terminators, in turn, are additional control elements for ending the transcription thus ensuring that expression of a gene does not affect the transcription of an adjacent downstream gene. Thus far, only the Rho-independent transcriptional termination mechanism, which is based on the formation

of hairpin structures in the messenger RNAs (mRNA) resulting in the release of RNA-polymerase from the DNA strand, has been suggested to exist in *Synechocystis*; this is based on the absence of genes coding homologous variants of *E. coli* Rho protein in *Synechocystis* genome (Ramey et al., 2015). Until recently, the terminators used for engineering purposes in *Synechocystis* have included the endogenous Rubisco terminator *TrbcS* (Lin et al., 2017) and the *E. coli* terminator *TrrnB* (Wang et al., 2013), but a new set of terminators have also been evaluated (Liu and Pakrasi, 2018). The possibility of using multiple terminator variants in construct design diminishes the risk of a homologues recombination between the identical sequences which can lead to an elimination of the fragment in between the sequences (Viola et al., 2014). RBS sequences, which recruit the ribosome to bind to the correct position on the mRNA, can be used to control the translation efficiency of individual ORFs. Given that the functions of regulatory elements may be clearly species-specific, as observed for example between *E. coli* and *Synechocystis* (Heidorn et al., 2011) (Huang et al., 2010) (Ma et al., 2002), information concerning the regulatory elements is crucial for biotechnological engineering purposes. RiboJ elements (Englund et al., 2018) (Patrikainen et al., unpublished) have currently been used in *Synechocystis* for enhancing the translational initiation and thereby expression. The RiboJ is a self-cleaving ribozyme, which cleaves out the 5' untranslated region on mRNA uncovering a stable RBS structure, which promotes the ribosome to bind (Lou et al., 2012), thus, potentially enhancing the translational initiation.

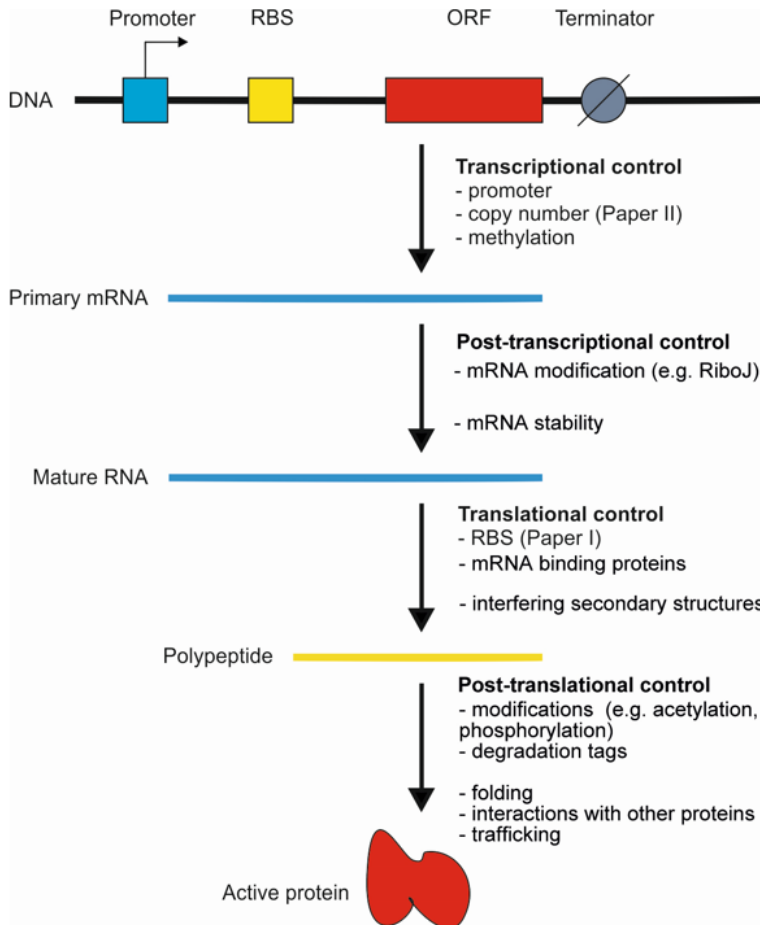


Figure 1. A schematic illustration of the essential genetic elements of DNA needed for expression of a gene and showing the different transcriptional and translational control points for alternative stages to adjust the gene expression in *Synechocystis* sp. PCC 6803.

1.2.3 The expression construct assembly strategies

Improved cyanobacterial production systems require engineering of the cells including high-throughput approaches for construct assembly. Some modular construct assembly methods have been developed to accelerate the plasmid and pathway construction phases. BioBrick™ standard (Shetty et al., 2008) is one of the most known assembly strategies for piecing together various functional DNA elements like promoters, RBSs, ORFs, terminators and antibiotic cassettes on a plasmid platform. BioBricks™ is based on recorded interchangeable parts flanked by standardised restriction enzyme sites, enabling a number of genetic elements to be iteratively added into the same operon. Compatibility between the BioBrick parts are ensured by prefix

and suffix regions on the plasmid backbone that flank the beginning and the end of the main element, respectively. In the BioBrick Assembly Standard 10, the prefix contains EcoRI and XbaI cutting sites and the suffix contains SpeI and PstI cutting sites. The insert part of choice is cut out of its plasmid with the enzymes EcoRI and SpeI, while the recipient plasmid containing the other BioBrick part, is opened using EcoRI and XbaI. The insert and vector parts are then handled using normal cloning techniques and assembled together since both EcoRI sticky ends are compatible with each other and the SpeI sticky end comes together with the XbaI sticky end. The resulting new part, located now in the recipient plasmid, contains both the original unchangeable parts as well as the same prefix and suffix regions as its "parents" enabling subsequent assembly steps with more BioBrick parts. Worth noticing is, however, that stitching together the XbaI and SpeI restriction sites leaves behind a scar, which is a remnant of the original restriction sites, thus no longer recognisable for the restriction enzymes. Re-use of standardized parts accelerate construct assembly as there is no need to go through the whole cycle of design and manipulation every time. In addition, BioBrick parts are registered and available to all. However, majority of the standardized parts are initially studied and characterized in *E. coli* or yeast, which are the most commonly used microbial work horses in biotechnology. Thus, feasibility of the parts for example to cyanobacteria is not yet so well understood in many cases. In addition, use of only a certain number of restriction enzymes may set a limitation when trying to assemble multiple parts in more complex system.

Another strategy for the construct assembly is the isothermal Gibson assembly® method (Gibson et al., 2009). The Gibson assembly® method allows the assembly of multiple desired DNA elements simultaneously in a single step. As the Gibson assembly® is based on the sequence identity, the desired DNA parts should contain overhang sequences of 20–40 bp on both sides of the DNA parts so that the overhangs are overlapping with the targeted adjacent DNA fragments. First, designed overhangs are created via PCR primers between the adjacent DNA elements in a PCR reaction. Then, these DNA parts with the overhangs are mixed with a set of three enzymes; exonuclease, DNA polymerase and ligase. The T5 exonuclease is allowed to be attached to the 5' ends of the sequences, generating single-stranded DNA ends that contain an overlapping region with the adjacent element. The generated overlapping sequences are let to anneal together, and the DNA polymerase finishes the formation of double stranded DNA by filling the remaining single stranded gaps. Finally, Taq ligase seals the strands. The resulting single, scarless DNA product contains all the desired DNA fragments, either in a linear or closed form. As several DNA parts may be combined at the same time, less cloning steps, reagents and time is needed. However, end of the parts may be slightly susceptible to mutations when DNA polymerase is involved in closing the gaps, and the commercial kits are relative expensive.

1.2.4 Expanding the strain engineering strategies

Besides more efficient assembly strategies for putting the genetic elements together, better engineering strategies for modifying the *Synechocystis* genome are also needed to enhance the production systems. Moreover, other newly developed tools include: advanced expression regulators such as CRISPRi (Yao et al., 2016) enabling a multi-gene-repression (knock-down); CRISPR technology with the alternative nuclease Cpf1 for efficient and precise modifications of *Synechocystis* genome (Ungerer and Pakrasi, 2016), riboswitches creating an inducible translation (Ohbayashi et al., 2016); riboregulators controlling gene expression at the post-transcriptional level based on self-binding RNA-RNA interactions (Ueno et al., 2018) (Sakamoto et al., 2018) and degradation tags for controlling cellular enzyme levels that affect the flux of metabolic pathways (Huang et al., 2010) (Landry et al., 2013). Recently, Sun et al 2018 reported the potential for new artificial RNA tools for down-regulation of both endogenous and exogenous gene(s) in *Synechocystis* (Sun et al., 2018). Furthermore, manipulation of cellular regulatory systems, such as sigma factors and response regulators, may be one possible approach for carbon flux optimisation in the engineered *Synechocystis* cell factory (Osanai et al., 2011) (Osanai et al., 2014).

1.2.5 Host optimization

In accordance with the cell factory concept, novel metabolic modifications may be integrated as a part of the host metabolism. However, also improving the host compatibility may assess the optimisation of the synthetic pathways. For example, the endogenous fluxes of electrons and carbon in the host can be re-allocated through the chosen pathways for product generation. Engineering the photosynthetic electron transfer reactions (auxiliary electron transfer reactions), more solar energy, in the form of excited electrons, can be targeted to the end-product. The carbon flux optimisation can be mediated for example via enhancing supporting pathways for the key precursors (like acetyl-CoA, pyruvate or malonyl-CoA) for increased productivity (Wang et al., 2016) (Anfelt et al., 2015) (Osanai et al., 2015) (Varman et al., 2013). Another approach for optimising the cellular resources to the desired end-product includes deletion of the competing native carbon sinks for storage compounds (Gao et al., 2012) (Lopes da Silva et al., 2018) (van der Woude et al., 2014) (Zhou et al., 2012). As a photosynthetic host, efficiency of innate light reactions in photosynthetic machineries as well as carbon fixation processes in *Synechocystis* can be improved, for example, by introducing an additional bicarbonate uptake transporter for improved C_i uptake (Kamennaya et al., 2015), or by overexpressing RuBisCO for increased photosynthesis and a faster growth rate (Liang and Lindblad, 2017), or by modification of the light harvesting antenna

structures for enhanced photosynthetic efficiency (Joseph et al., 2014) (Kirst et al., 2014). Supplementing the host with fixed carbon source can may provide additional carbon building blocks and thus promote end-product formation (Varman et al., 2013) (Matson and Atsumi, 2018) (Wu et al., 2001) (Khetkorn et al., 2016) (Lee et al., 2015). Transport engineering is a part of host optimization addressing process improvement of a cell factory. Substrate uptake can be enhanced by introducing a transporter for importing the substrate inside the cell (Thiel 2017). Efficient product-removal by an exporter, in turn, may be essential for preventing reaction equilibrium formation and for enhancing the product tolerance, accompanied by the fact that the export of the product into the extracellular matrix may abolish the potential cost of purification since the end-product does not need to be mechanically separated from the cell mass (Luan and Lu, 2018). Modifying the cofactor supply in *Synechocystis* may also be one approach for optimising the living cell factories. The pool of innate cofactors includes NADP or NAD, and their reduction supply reducing power for heterologous overexpression in synthetic pathways. Microbial production in general requires reducing power, which is normally generated through photosynthesis in the form of NADPH, the predominant form of the reducing equivalents in cyanobacteria (Tamoi et al., 2005). The desired genes can be selected based on their cofactor preferences, as in some cases the heterologous enzymes are NADH-dependent. For example, Angermayr and the coworkers improved lactate production rate and yield in *Synechocystis* by co-expressing a heterologous soluble transhydrogenase in order to increase the cellular NADH / NAD⁺ ratio preferred by L-lactate dehydrogenase responsible for lactate production (Angermayr et al., 2012).

1.3 From basic research towards biotechnological applications

As a technology, metabolic engineering has been globally recognised as one of the top ten emerging technologies (World Economic Forum, EU Horizon 2020 Sunrise). Via metabolic engineering, genetic and regulatory processes in the cell can be optimized (Kumar and Prasad 2011). Synthetic biology is a new prominent approach which brings the metabolic engineering possibilities of the living cell factories to the next level. Still, conventional genetic manipulations serve as the basis for synthetic biology. Synthetic biology can be defined as sophisticated design and construction of novel biologically based parts, devices, and circuits of cellular machinery (i.e. new-to-nature), as well as the redesigning of existing natural biological systems (Huang et al., 2010) (Berla et al., 2013) (Sengupta et al., 2018). Synthetic biology includes the use of modular standardised parts, systematically validated for improved specifications of the system that result in more tailored and predictable metabolic pathways which together with reprogramming of the cellular behaviour lead to next-

generation production platforms (Huang et al., 2010). To meet the requirements of the increased complexity of the advanced production systems, synthetic biology takes advantage of inclusive computational design and technological engineering sciences (Santos-Merino et al., 2019). Synthetic biology is suggested to be the enabling technology for development of synthetic living factories and designer microorganisms for efficient and direct conversion of solar energy to carbon-based products, as a part of the transition from fossil-based economy to a sustainable bioeconomy. Concomitant exploitation of synthetic biology and metabolic engineering approaches would help to alleviate the most critical bottlenecks and enhance the performance of synthetic metabolic pathways of the production systems.

As part of the progress towards future biotechnological solutions to generate carbon-based products directly from CO₂, intensive basic and applied research has been done in the Molecular Plant Biology unit at University of Turku contributing to the development of synthetic biology strategies and tools for advanced applications of solar-driven cyanobacterial production systems. The background of this thesis relies on decades of profound work in basic research on photosynthesis at the Molecular Plant Biology unit, University of Turku, Finland. The generated in-depth understanding of photosynthetic light and atmospheric carbon reduction reactions, the electron fluxes as well as identifying the factors influencing the direct photon conversion efficiencies are all crucial for developing autotrophic production platforms.

2 Aims of the Study

Increasing global demand for consumer goods as well as progressive consciousness of environmental concerns call for the discovery of sustainable alternatives to produce various carbon-based chemicals. Photosynthetic biomanufacturing is an attractive future biotechnological solution for sustainable and renewable production of carbon-based commodities. Cyanobacteria are one of the most promising microbial cell factories due to their innate ability to fix greenhouse gas CO₂ into carbon-based chemicals powered by sun light. Optimisation of this production platform still needs different rational engineering strategies systematically applied to enhance the cyanobacterial production systems.

This thesis work responds to the apparent call for better engineering tools and knowledge of solar-driven cyanobacterial production systems by exploring new synthetic biology approaches in *Synechocystis* aiming at improving the performance of direct conversion of solar energy into sustainable carbon-based products. More specifically, the scope of this thesis work was to develop and validate a new synthetic biology toolbox and cyanobacterial expression strategies in the engineered *Synechocystis* host. The work was carried out in the Molecular Plant Biology unit at the University of Turku, as a part of the broader international research which collectively contributes to exploring the photosynthesis and potential photosynthesis-derived applications.

The specific objectives of the thesis were to:

- i) Develop a new synthetic biology toolkit for *Synechocystis* (e.g. RBSs, integration sites, assembly method, expression and integration vectors).
- ii) Study ribosomal binding sites as control elements for translational tuning.
- iii) Evaluate new alternative expression loci in *Synechocystis*.
- iv) Test the effect of removal of an alternative electron sink in *Synechocystis* light reactions and further evaluate the consequences on product formation, using sucrose, PHB and glycogen as reporters.
- v) Study the effect of acetate on growth in an engineered acetate transporter strain of *Synechocystis*.

3 Brief Description of Materials and Methods

Here, a brief overview is given of the strains, growth conditions, molecular biology tools and analytical methods used in this thesis. More detailed descriptions are presented in the corresponding original **Papers I–IV**.

3.1 Microbiology

A glucose-tolerant substrain of *Synechocystis* sp. PCC 6803 (Williams, 1988) was used as the background for generating all the cyanobacterial strains characterised in this research. *Escherichia coli* strain DH5 α was used as a generic host for plasmid propagation.

The cyanobacterial cells were grown in a BG-11 medium buffered with 20 mM TES-KOH (pH 8.0) (Rippka et al., 1979) with supplemented antibiotics when needed to maintain the selection pressure in the cultivations. Depending on the specific application, the batch cultures were incubated at 30 °C under continuous light of 20–200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at either atmospheric CO₂ or 1 % CO₂ in a growth chamber MLR-351 (Sanyo, Japan) or Algaetron 230 (Photon Systems Instruments, Czech Republic) or alternatively in a photobioreactor MC1000 (Photon Systems Instrument, Czech Republic).

3.2 Molecular biology

Standard molecular biology techniques and commercial kits (Qiagen, Germany) and enzymes (New England BioLabs, USA or Thermo Fisher Scientific, USA) were used for DNA manipulation, using a cloning strategy described in Paper I. Oligonucleotides were purchased from Eurofins MWG Operon (Germany), and larger gene fragments from GenScript (USA).

3.3 Analytical methods

Measuring cell growth. Cell growth was monitored using GENESYS 10S UV–Vis spectrophotometer (Thermo Fisher Scientific, USA) or a photobioreactor MC1000 (Photon Systems Instrument, Czech Republic).

Quantitation of chemicals using commercial kits. Commercial kits were used for analysing sucrose (Sucrose/D-Glucose Assay Kit; Megazyme), glycogen (Total Starch Assay Kit; Megazyme), PHB (D-3-Hydroxybutyric Acid Assay Kit; Megazyme), acetate (Acetate Colorimetric Assay kit; Sigma-Aldrich) and ROS (CM-H₂DCFDA; Invitrogen) according to manufacturer’s instructions.

Fluorescence analysis. Quantitative fluorescent analysis of the intact *Synechocystis* strains expressing sYFP2 and GFPmut3b was carried out using a Tecan microplate reader (Tecan infinite 200 PRO, Switzerland) with 495 nm (ex)/535 nm (em) and 485 nm (ex)/525 nm (em), respectively.

Gas flux analysis. Measurements of ¹⁶O₂ (mass 36) and ¹⁸O₂ (mass 44) exchange were monitored by MIMS directly from suspension cultures under increasing light intensities of 0, 25, 50, 75, 100, 200 and 500 μmol photons m⁻² s⁻¹. Gross O₂ evolution rates were calculated from the addition of ¹⁸O₂ gas (98 %, CK Isotopes) based on equations and methods of (Beckmann et al., 2009).

Absorption Spectroscopy. Pigment analysis (370 nm - 700 nm) of the strains was carried out with the OLIS CLARiTY 17 UV/Vis spectrophotometer (On Line Instrument Systems, USA) and processed using Fry's method (Fry et al., 2010). Alternatively, absorption scans were recorded using a Tecan microplate reader.

Dry cell weight. Aliquots of the *Synechocystis* cultures were concentrated to the same optical density, and an equivalent volume, then the samples were filtered through pre-weighted microfiber membranes (VWR). The membranes were oven-dried and weighted using an analytical scale (Sartorius MC1 Research RC 210P).

Gas chromatography. Ethylene production efficiency of the engineered *Synechocystis* strains was monitored by quantitating ethylene from the headspace of sealed culture vials by GC against a commercial gas standard using GC-FID (Perkin Elmer AutoSystem, USA) with CP-CarboBOND fused silica capillary column (Varian, 50 m × 0.53 mm) under isothermal conditions (oven and injector 80 °C, and detector 200 °C with H₂ carrier gas at a flow rate 7 ml min⁻¹).

In silico analysis. For statistical comparison of the different datasets Pearson’s correlation coefficient (Pearson and Friedrich, 1894) and Spearman’s rank correlation (Spearman, 1987) (Spearman, 2010) were used. Nucleotide sequence analysis was performed using the Salis RBS calculator (Salis et al., 2009) and the UTR Designer (Seo et al., 2014) for the prediction of the translation initiation rate. To predict the most stable mRNA secondary structures with the minimum free energy RNAfold Server hosted by ViennaRNA web service (Smith et al., 2010) and mfold web server (Zuker, 2003) were used.

The light-saturated oxygen evolution using a Clark-type oxygen electrode.

Oxygen evolution capacity of PSII was measured from the *Synechocystis* samples concentrated to the same optical density in the presence and absence of an electron acceptor 2,6-dichloro-p-benzoquinone (DCBQ) and ferricyanide to maintain DCBQ in oxidised form using a Clark-type oxygen electrode (Hansatech Ltd) under saturating constant light. The light-saturated whole chain net photosynthesis was measured similarly in the presence of NaHCO₃ as the carbon source.

Singlet oxygen detection. The rate of histidine-mediated singlet oxygen-induced oxygen uptake was determined from the *Synechocystis* samples concentrated to the same optical density using a Clark-type oxygen electrode. Measurements were performed in the presence and absence of histidine under saturating constant light.

Microscopy. For the microscopic evaluation of cell size and morphology, the *Synechocystis* strains were examined under a light microscope (Leitz Orthoplan Large Field Research Microscope, Germany) and photographed with a digital microscope camera (Leica DFC420C). The cells were visualised at 100-fold magnification with a Leica Application Suite V 4.1.

4 Main Results

4.1 New synthetic biology strategies and tools adapted for engineering *Synechocystis*

As cyanobacterial engineering has traditionally relied on conventional cloning strategies, which are relatively rigid for efficient construction of intricate microbial cell factories, the underlying objective in this thesis was to facilitate the process of design, and the assembly of expression constructs and pathways by adapting a new BioBrick–inspired strategy (Zelcbuch et al., 2013) (Shetty et al., 2011) for *Synechocystis* (**Paper I**) (Figure 2). In this modular “plug-and-play” approach compatible genetic elements could be fused in any order via iterative cloning procedures based on restriction site recycling (Zelcbuch et al., 2013) (Figure 2). The gene of interest for expression was designed to form a fusion with a chloramphenicol resistance cassette (CmR), which enables the selection, and is flanked by restriction sites NsiI and XhoI. According to the assembly system, a synthetic gene-of-interest (GOI) linked to a CmR was first subcloned directly downstream of one of the RBS sequences selected from the RBS library in the pNiv carrier plasmids (NsiI-XhoI). To form a multi-gene-operon in pNiv assembly plasmid, the vector part was cut using NheI-XhoI sites and the insert part using SpeI-SalI sites. Eventually, the GOIs were then directly subcloned into a specifically designed expression plasmid pDF-lac2 (SpeI-SalI) under the control of the promoter PA1lacO-1. If the genes were planned to be integrated into the genome, the GOIs from pNiv were subcloned into an integration vector pSI1b (SpeI-SalI), which provides a pair of sequences for targeted homologous recombination. Finally, pDF-lac2 and pSI1b harboring the GOIs were transformed into *Synechocystis*. As a result, the new cloning strategy generated in this research was shown to successfully work as planned, allowing any combinations of the desired genetic elements from the library. Consequently, the new assembly system eases and accelerates the construct and pathway design and assembling process. The work establishes for *Synechocystis* a new cloning system in which each RBS and GOI combination can be chosen freely. As an additional result, a new shuttle vector pDF-lac2 and the integration plasmid pSI1b, both compatible with the generated cloning strategy, were prepared and were verified to be functional in *Synechocystis* (**Papers I, II and IV**).

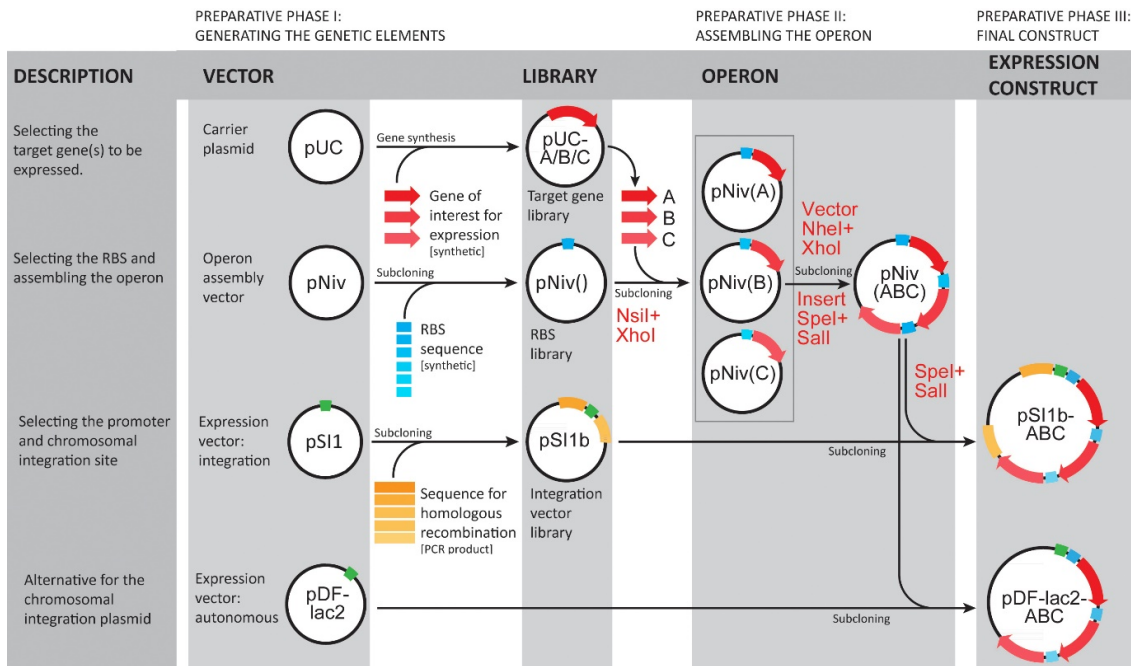


Figure 2. The new assembly system used in this study. The approach allows a combination of the genes of interest (red) with the chosen ribosome binding sites (blue) into functional operons. Assembly of the genetic elements into multi-gene operons relies on iterative subcloning steps using particular restriction sites at definite steps in pNiv-vector based platform. The final operons can be subcloned to either integration vectors (pSI1b) combined with specified homologous recombination sites (orange) and a selected promoter (green) or an autonomously replicating plasmid (pDF-lac2) for expression in *Synechocystis* sp. PCC 6803. All the preparative phases are carried out in *E. coli* enabling efficient and a relatively easy construction of the desired pathways. Furthermore, the genetic elements and gene expression can be evaluated *in vivo* based on reporter genes (Table 2) selected for *Synechocystis* in this study.

Applying synthetic biology approaches to generate better solar-to-chemicals production systems requires characterised tools to allow precise control of gene expression. In this study, different RBS sequences (**Paper I**) and different integration sites (**Paper II**) were systematically evaluated over the course of time, thus expanding the synthetic biology toolbox of genetic parts for *Synechocystis* and increasing the valuable molecular-level knowledge of these genetic elements. All the new elements generated and evaluated in this study are presented in Table 1a. It is important to note that the constructed libraries together with the generated cloning strategy and the new vectors provide the means for pathway optimisation and engineering in *Synechocystis*, as the expression of each protein can be modulated at a translational level by selecting appropriate combinations of RBSs with genes of interest and by selecting carefully evaluated loci for over-expression of gene targets. The specific gene targets for modifications in this thesis are listed in Table 1b.

Table 1a: Elements of the synthetic biology toolbox generated or adapted for *Synechocystis* sp. PCC 6803 as a part of this research.

Element	Name	Number of different elements	Function / purpose	Reference
RBS	S1-S7, A-E, Z	13	Control elements for translational tuning	Paper I
Integration site	ISC1-9, ISP1-7	16	Genomic integration sites for expression cassettes	Paper II
Expression vector backbone	pDF-lac2 pSI1b	2	Expression strategies	Paper I Paper II
Reporter gene	Ethylene YFP GFP	3	Reporters for comparative analysis of expression	Paper I Paper I-II Paper I-II
Assembly system		1	Construction platform for expression cassettes or operons	Paper I

Table 1b: Introduced genetic modifications in *Synechocystis* sp. PCC 6803 used in this research.

Gene	Gene product	Origin	Type of modification	Function / purpose in this work	Reference
<i>efe</i>	Ethylene forming enzyme EFE	<i>Pseudomonas syringae</i>	Over-expression	Marker protein; conversion of 2-oxoglutarate into ethylene	Paper I
<i>GFPmut3b</i>	Fluorescence protein variant GFP	<i>Aequorea victoria</i>	Over-expression	Marker protein; fluorescence signal	Paper I-II
<i>sYFP2</i>	Fluorescence protein variant YFP	<i>Aequorea victoria</i>	Over-expression	Marker protein; fluorescence reporter	Paper I-II
<i>yjcG</i>	Acetate transporter ActP	<i>Escherichia coli</i>	Over-expression	Substrate uptake; facilitate intake of acetate	Paper III
<i>cscB</i>	Sucrose permease CscB	<i>Escherichia coli</i>	Over-expression	Product removal; export of sucrose	Paper IV
<i>flv3</i>	Flavodiiron protein Flv3	<i>Synechocystis</i>	Over-expression	Complementation of $\Delta flv3$	Paper IV
<i>flv3</i>	Flavodiiron protein Flv3	<i>Synechocystis</i>	Knock-out	Inactivation of the functional Flv1/3 heterodimer	Paper IV
<i>gppS</i>	Glucosylglycerol phosphate synthase GGPS	<i>Synechocystis</i>	Knock-out	Metabolic modification; inactivation of the competing pathway for sucrose synthesis	Paper IV
<i>sps</i>	Sucrose phosphate synthase SPS	<i>Synechocystis</i>	Over-expression	Metabolic modification; alleviating the limiting step for sucrose synthesis	Paper IV

4.2 Evaluating the expression efficiencies and the effects of introduced changes to the cell

Optimal evaluation systems utilize pre-established procedures as well as easy and rapid analytical strategies. Likewise, the reporter markers must possess adequate sensitivity and specificity, be essentially non-toxic to the host and allow continuous monitoring directly from the cells avoiding many disadvantages associated with the breakage of cells. For the evaluation of the genetic and metabolic modifications introduced *in vivo* in the engineered *Synechocystis* strains, multiple different reporter systems were used (Table 2). Fluorescent proteins, possessing the fluorophore which exhibits fluorescence when exposed to light in the appropriate excitation range, were used as reporters of expression in living systems using optical instruments. Two colour-shifted genetic derivatives of fluorescent reporter proteins were applied in this research work: *yellow fluorescence protein* (sYFP2) (Nagai et al., 2002) and a codon-optimized *green fluorescence protein* (GFPmut3b) (Cormack et al., 1996). The YFP and GFP served as indicators of the RBS strength i.e. translation efficiency and expression from the integrated multi-gene-operons at different loci. The fluorescence measurements were carried out directly from the intact cells in a well-plate format, allowing relatively high throughput. Accordingly, based on the acquired fluorescence data from multiple parallel replicates and independently produced datasets, the results were highly reproducible, and could be further verified with the statistical comparison; thus allowing direct quantitative comparison of the translation and expression efficiencies of the various RBS sequences and integration sites (**Papers I–II**).

The native carbon storage compounds, *glycogen* and *PHB*, were also used as reporters for evaluation of the introduced genetic modifications (**Paper IV**). The small molecule compounds *sucrose* and *ethylene* were used in the study for quantitative evaluation of the upstream level and downstream level modifications, respectively; this was done directly from the intact cells as the sucrose export was facilitated into the media by the heterologous transporter and the ethylene passes freely through the cell membrane into the headspace. Sucrose, which is naturally synthesized by *Synechocystis* as a cellular response to salt stress (Klähn and Hagemann, 2011), accompanied by its proposed additional role as a signal molecule (Desplats et al., 2005), provided a marker compound for overall autotrophic production efficiency. Overall autotrophic production efficiency was performed in order to study the effects of the competitive sink removal for electron and carbon distribution in the engineered *Synechocystis* strains. A heterologous ethylene-forming enzyme (Efe), in turn, was used for evaluating the connection between gene sequence and translation efficiency (**Paper I**). All the reporters used in this research work are listed in Table 2.

Table 2: Alternative reporter systems used in this thesis for quantitative evaluation of different modifications in *Synechocystis* sp. PCC 6803.

Reporter system	Use/Purpose	Reference
YFP/GFP	Translational efficiency	Paper I
Ethylene	Translational efficiency	Paper I
YFP	Copy number and construct genetic stability	Paper II
Sucrose	Effect of genetic modification and cultivation conditions	Paper IV
Glycogen/PHB	Effect of competing alternative metabolic fluxes, alternative native sinks	Paper IV

Other non-invasive methods to evaluate the effects of the introduced modifications on the overall fitness of the cells in the engineered strains included analyses of the growth rate, pigment content, reactive oxygen species (ROS) as well as cell size and morphology (**Papers I–IV**). As *Synechocystis* is a photosynthetic organism, measuring oxygen evolution and gas flux rates are apparent means to evaluate possible changes produced by the introduced genetic modifications to the host (**Papers III–IV**). In contrast, analyses of some reporter compounds may need disruption of the cells as in case of determining the intracellular storage compounds, glycogen and PHB. As the introduced genetic modifications might have led to the re-allocation of cellular resources towards different storage sinks, glycogen and PHB were systematically quantified for any indication of carbon and electrons fluxes (**Paper III–IV**).

4.3 The RBS library generated as a part of synthetic biology toolbox provides adjustable translational control

The capacity to control and tune gene expression at different levels (transcriptional, translational and post-translational level) is crucial for engineering advanced production systems with a predictable and adjustable outcome. In an operon based-structure, RBS sequences are instrumental for tuning translational efficiency in the synthetic pathways. In this study, thirteen different RBS sequences were selected, and subjected to systematic quantitative evaluation of translation efficiencies for the first time in *Synechocystis* (**Paper I**). Six of the sequences had previously been evaluated in *E. coli*, with a range of expression level (Zelcbuch et al., 2013) (Salis et al., 2009). The other seven RBS sequences were derived either directly from the native, highly expressed cyanobacterial genes or from the expression constructs designed for *Synechocystis*, and were now first time systematically compared for efficiency.

The generated 26 *Synechocystis* strains with 13 distinct RBS sequences that regulate the translation of either YFP or GFP were analysed for quantitative comparison of the expression levels in two different analytical trials. First, through *full dataset* analysis each of the strains were evaluated individually on separate days using six biological replicates with three technical replicates ($n = 18$). Second, through *one-day dataset* analysis all the 13 strains were analysed in parallel on the same day but with only three technical replicates ($n = 3$) for YFP and GFP. There was no difference in growth of the strains. As seen in Figure 3, both approaches resulted in coherent fluorescence profiles between the full dataset and one-day dataset for YFP (Fig. 3a) and GFP (Fig. 3b). The observed high correlation between the parallel datasets was further verified with statistical analysis with notable confidence (p values under 0.001), all emphasising the repeatability and reliability of the system. The studied 13 RBS sequences resulted in a relatively broad overall expression level range for both fluorescent reporters in *Synechocystis* (Figure 3), thus offering a reliable regulatory tool for controlling protein expression at a translational level in engineered cyanobacteria. The work increased valuable information of limitations and possibilities, and can help to select appropriate RBSs for over-expression constructs or multicistronic pathways in *Synechocystis*. Moreover, the generated RBS library offers the required parts and possibilities for optimizing the expression at translational level in *Synechocystis*.

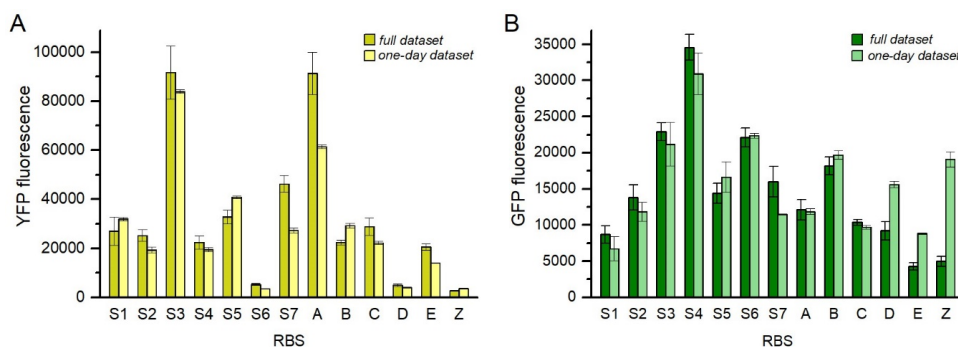


Figure 3. Fluorescence representing translational efficiencies of the 13 RBSs in engineered *Synechocystis* sp. PCC 6803 strains. The RBSs were evaluated in respect to **(A)** YFP and **(B)** GFP, measured from intact cells at 6 h after induction. In A and B, fluorescence from the *full data set* (six biological replicates with three technical replicates) of YFP and GFP is shown in dark yellow and green, and fluorescence from the *one-day dataset* (one representative clone with three technical replicates) of YFP and GFP is shown in light yellow and green, respectively.

4.4 Target gene sequence may have a significant impact on translational efficiency

As the YFP and GFP genes did not show relevant nucleotide sequence similarity due to the GFP codon optimisation (**Paper I**), these reporters were considered as representatives of unrelated target genes for studying the effect of the downstream coding region on the RBS-specific expression. Although some of the RBS strains resulted in analogous translation patterns with both fluorescence markers, the overall fluorescence profiles between YFP and GFP datasets were divergent (Figure 3a compared to Figure 3b), which was further verified by the low statistical correlation between the YFP and GFP datasets. As the only difference between the individual comparable strains was the downstream gene sequence, the results indicated that translational efficiency was not determined by the specific RBS sequences alone but was also affected by the following downstream region. This observed gene-specific difference was further supported by an additional analysis of ethylene production with a selected set of RBSs. To illustrate complicity and unpredictability of the translational efficiency of the selected RBSs in different target gene contexts, Figure 4a shows RBSs which exhibit the greatest divergence while Figure 4b shows those with the greatest similarity.

The recorded data was complemented with several *in silico* analyses in order to uncover potential nucleotide-sequence -based factors, as well as putative mRNA secondary structure interactions between the RBS and the coding region that would have explained the observed RBS-specific and gene-specific variations. However, the existing *in silico* prediction tools failed to predict the performance of the studied sequences which would correlate with the experimental results in *Synechocystis*.

In general, these experiments revealed the frequent and extensive effects of the downstream sequences on the level of translation in cyanobacteria. Consequently, the gene-specific interactions, which may potentially reduce the translational efficiency for a given RBS-gene combinations, were shown to be difficult to predict. Moreover, experimental validation for each case separately is not convenient for future engineering approaches. Thus, there is a need to alleviate the effect of unwanted secondary interactions, for instance, by incorporating an additional insulator sequence element which would break the interaction between the RBS and the coding region in order to overcome the issue (Levin-Karp et al., 2013).

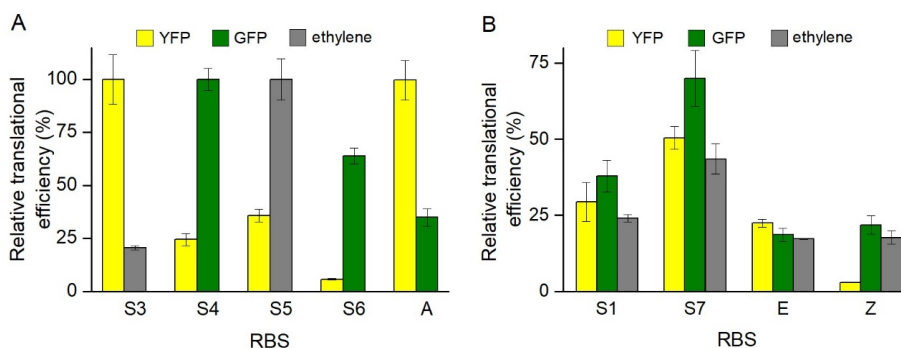


Figure 4. Translational efficiencies of selected RBS sequences in the engineered *Synechocystis* sp. PCC 6803 strains measured from intact cells at 6 h after induction. The relative translational efficiencies of the RBSs measured by means of YFP (yellow bars), GFP (green bars) and ethylene (grey bars) expression which differ the most from each other (A) and which exhibit the greatest similarity (B).

4.5 A characterised integration site library expands the choice of expression loci

The success of cyanobacterial biotechnological applications provide a stable expression of the introduced recombinant genes and metabolic pathways. Operons-of-interests can be integrated into cyanobacterial genome via a homologous recombination process using integration plasmids. The genome of *Synechocystis* (3.6 Mbp) encompasses a chromosome with 3200 open reading frames (ORF) (Mitschke et al., 2011), and seven endogenous plasmids containing altogether roughly 400 ORFs (Labarre et al., 1989), 4 large megaplasmids (pSYSM, pSYSX, pSYSA and pSYSG) (Kaneko et al., 2003) and 3 small plasmids (pCC5.2, pCA2.4, pCB2.4) (Xu and McFadden, 1997). Given that the genome is highly polyploid, the copy number of the circular chromosome and the endogenous plasmids varies depending on the growth phase and some external factors (Zerulla et al., 2016) (Berla and Pakrasi, 2012). Indeed, the existence of multiple chromosomes in the cell is also a unique engineering challenge specific to cyanobacteria chassis. Although many chromosomal sites have been used for integration purposes in *Synechocystis*, expression and stability of the integration sites among chromosomal and endogenous plasmids have not been systematically studied thus far in *Synechocystis*. To find new integration sites for efficient and stable expression in *Synechocystis*, nine chromosomal integration sites and an integration site for each of the seven inherent plasmid were selected either from the transcriptomic data or from the literature for the systematic comparison using a fluorescent protein YFP as a quantitative reporter (Paper II). In addition, a new and functional integration plasmid called pSI1B,

which endows the same modular assembly capacity as described in **Paper I**, was constructed (**Paper II**).

Eventually, four strains with YFP integrated at chromosomal loci (*slr0944*, *sll0058*, *slr1311* and *slr0168*), one strain with YFP integrated in the native miniplasmid (pCA2.4) and four strains with YFP integrated in the native megaplasmid (pSYSM *slr5037-slr5038*, pSYSX *slr6037*, pSYSA *slr7023* and pSYSG *slr8030*) were successfully generated. The strains did not show any differences with respect to host visual phenotype or growth.

The fluorescence of the integrated YFP was measured in three independent trials. Through *full dataset* analysis each of the strains were evaluated individually on separate days using six biological replicates with three technical replicates collected every second hour at 0, 2 h, 4 h, 6 h, 24 h and 48 h after induction. Through *one-day dataset* analysis all the 10 strains were analysed in parallel on the same day but with only three biological replicates with three technical replicates or alternatively with only one biological replicate with three technical replicates.

According to the resulting fluorescence profiles of the integration mutant strains the 24 h time point was selected for evaluating the maximum fluorescence levels (Figure 5), as at the 48 h time point the expression of most of the strains had already evened out. The results showed that both the maximum expression capacity and initial expression rates were at relatively similar levels between the chromosomal and native megaplasmid sites. However, the expression efficiencies of the small native plasmid (pCA2.4) and the RSF1010-derived expression plasmid pDF-lac2, which were used as controls, were notably higher (Figure 5).

Stability of the integrated expression constructs of the generated mutant strains was tested. The results indicated that the integration into the selected genomic sites were actually very stable as the measured fluorescence values did not decline during a cultivation period of several weeks despite the lack of antibiotic selection. Notably, although the stability of the autonomously replicating plasmids has generally been imputed to be relatively unstable and considered to require continuous antibiotic pressure, this study supports the recent evidence by Ferreira and the coworkers showing that replicative plasmids can be stable as well (Ferreira et al., 2018). In this study, pDFlac2 plasmid was demonstrated to be as stable as any of the integration sites, when expressing a gene product which is not harmful for the cell (**Paper II**).

The novel research in **Paper II**, presents new, conditionally neutral integration sites in the four native megaplasmids (pSYSM, pSYSX, pSYSA and pSYSG) that have not previously been used for introducing expression cassettes in *Synechocystis*. Moreover, the work expands the set of alternative expression strategies for expressing heterologous genes and provides for the first time comprehensive, quantitative information on the comparison of the parallel integration sites and their long term stability in *Synechocystis*. In addition, the work shows that the expression

levels between all chromosomal integration sites and the four native megaplasmiids are relatively similar.

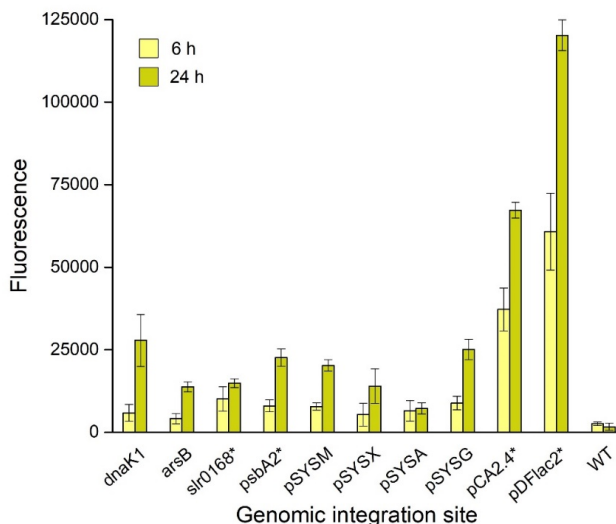


Figure 5. YFP fluorescence from the studied genomic integration sites representing translational efficiency after 6 h (light yellow) and 24 h (dark yellow) from induction (n= 18). *DnaK1*, *arsB*, *slr0168* and *psbA2* are the chromosomal integration sites, pSYSM, pSYSX, pSYSA and pSYSG are the integration sites in the megaplasmiids and pCA2.4 is the integration site in miniplasmid. The asterisks indicate that the corresponding genomic integration site and the autonomously replicating expression plasmid pDF-lac2 has been also studied previously in literature and representing as controls in this comparison (Angermayr et al., 2012) (Dexter and Fu, 2009) (Armshaw et al., 2015) (Guerrero et al., 2012).

4.6 Electron flux distribution from photosynthetic light reactions is dependent on light intensity and the genetic context of the cell

The development of sufficiently efficient cyanobacterial platforms for industrial purposes requires robust electron and carbon fluxes from the solar-driven, water splitting reactions through the carbon fixation processes towards a target product. However, as some portion of the energy may be lost throughout the native metabolism of the cell, targeted modifications may diminish the energy loss by deleting the unwanted routes and thus increasing the flux in the desired direction. In addition, introducing a sturdy sink which draws strongly electron and carbon fluxes from the intermediary metabolism increases the fluxes towards an end-product. Inactivation of Flv3 (*flv3*, *slr0550*), inherently functioning as an alternative electron sink in *Synechocystis* light reactions, particularly when the light reactions and carbon fixation are not in equilibrium, resulted in an increased electron flux towards the

introduced sucrose reporter, which concurrently acts as a strong carbon sink (**Paper IV**). Here, the native sucrose production capacity of *Synechocystis* was triggered by salt stress and accompanied by the introduction of a heterologous *sucrose permease* (CscB from *E. coli*) facilitating the export of the produced sucrose (**Paper IV**). Under this experimental set up, deletion of *flv3* in *Synechocystis* improved the sucrose production under low growth light compared to corresponding control strains, without differences in growth or pigment content. In parallel, the gas flux analyses showed an increase in the net oxygen evolution and total CO₂ uptake for the $\Delta flv3$ strain and a concomitant abolishment of the oxygen uptake with respect to the control strain. The gas flux data suggested that inactivating the competing electron route of Flv3 increased the availability of reducing equivalents in the $\Delta flv3$ mutant that could further be diverted to enhance CO₂ fixation, thus eventually facilitating the downstream metabolic processes. Altogether, both rerouting the electrons and introducing a strong sink potentially enhance the overall biosynthetic efficiency of the photoautotrophic system as shown in elevated electron flux derived from water-splitting PSII and improved levels of sucrose end-product (**Paper IV**).

Under higher light conditions the observed benefits in the $\Delta flv3$ mutant strain vanished as demonstrated by the lowered sucrose productivity and the slight growth recession in comparison to the control strain, indicating that the protective function of Flv3-dependent endogenous regulatory system is required under higher light condition. However, the potential issues related to higher light were overcome by strengthening the sink allure even further via introducing some auxiliary modifications. These included over-expression of *sucrose phosphate synthase* (*sps*; *sll0045*), a limiting step in the sucrose biosynthesis (Du et al., 2013), in combination with the inactivation of the *glucosylglycerol phosphate synthase* (*ggps*; *sll1566*) that is required for the formation of a parallel competing osmoprotectant glucosylglycerol in *Synechocystis* (Du et al., 2013). These additional modifications resulted in an enhanced flux towards sucrose when compared to the respective $\Delta flv3$ strain without these additional modifications.

These experiments showed that the excited electrons that originate from the water splitting PSII can be used more efficiently for production of target chemicals; instead of wasting the reducing capacity in re-reduction of oxygen to form water by the Flv1/3 heterodimer. Importantly, the results emphasised that the distribution of carbon and electron fluxes is very complex in the cell (**Paper IV**). The electron flux distribution was shown to be dependent on the light conditions and the genetic context of the $\Delta flv3$ mutants, through favouring the production of either sucrose (Figure 6a) or one of the two main storage compounds, glycogen (Figure 6b) or polyhydroxybutyrate (Figure 6c). The *flv3* deletion was shown to increase the productivity of sucrose under low and moderate-light conditions but this effect was lost when additional modifications were introduced as well as under higher light

conditions. The additional genetic modifications strengthening the overall sink favoured the accumulation of glycogen instead of sucrose under moderate light conditions. Elevated light conditions, in turn, led to the accumulation of PHB in the *flv3* deletion strains both with and without additional modifications, and no longer allocated the fixed carbon to either sucrose or glycogen. The results might propose that flavodiiron protein Flv3 may have a possible role in determining the ATP/NADPH –ratio in the cell, and thus affect the synthesis of the end-products. Understanding these complex interactions is critical for rational modulation of the metabolic electron flux distribution.

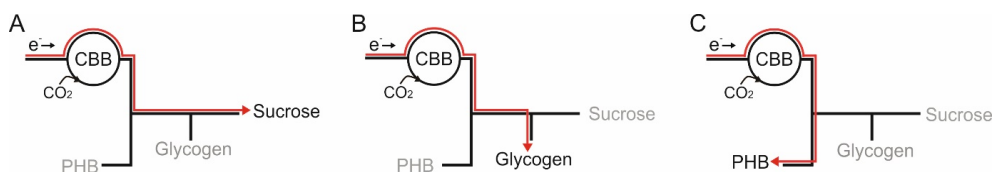


Figure 6. Different options of electron flux distribution to sucrose, glycogen & PHB in the engineered *Synechocystis* sp. PCC 6803 strains. Electron flux distribution is dependent on light and genetic context; the figure illustrates possible routes towards sucrose (A), glycogen (B) and PHB (C).

4.7 Acetate is a potential supplementary carbon source in cyanobacterial production systems

One promising approach to enhance the yields in cyanobacterial production platforms is to supplement the cultures with a non-cost and ubiquitous nutrient source like by-products or chemical feedstocks – given that the host endows the required catabolic pathways for the carbon supplement natively or engineered. An alternative additional carbon source may provide auxiliary energy e.g. by promoting productivity under dark phases in diurnal conditions or helping to mitigate the cell-shading effects in dense cultures. Acetate is a carbon supplement which has potential to increase the intracellular pool of acetyl-CoA and pyruvate available for biosynthetic reactions (Ihlenfeldt and Gibson, 1977) (Varman et al., 2013) (Summers et al., 1999). In addition, acetate has shown to have the added benefit of being a wastewater and industrial process polisher of the discharge (Kim et al., 2016) (Osman, 2014). Despite the disability to exploit acetate as a sole carbon source (Varman et al., 2013) (Knoop et al., 2013), *Synechocystis* can still assimilate acetate by conversion into acetyl-CoA (Summers 1999), and further to pyruvate, which is the most prominent precursor for the engineered heterologous pathways in cyanobacterial chassis (Angermayr et al., 2015). Indeed, acetate has been reported to be an attractive carbon feedstock to promote production of the desired end-

products in *Synechocystis* (Varman et al., 2013), other cyanobacterial species (Kusakabe et al., 2013) as well as in microalgae (Sorigué et al., 2016).

In the study, the first barrier for acetate utilisation in *Synechocystis* was addressed (**Paper III**) by investigating whether the introduction of an acetate transporter could facilitate the uptake of supplemented acetate in mixotrophic conditions. This could be beneficial when developing more efficient *Synechocystis* cell factories. A heterologous acetate transporter ActP from *E. coli* (Gimenez et al., 2003) was introduced to assist the transport of added acetate from the medium into the cell (**Paper III**). The results showed that improved acetate intake can stimulate the growth of the cyanobacterial host (Figure 7). This emerged particularly under low-light conditions, because then the photosynthetic activity is weaker and thus non-optimal for pure autotrophic growth. The decrease of acetate concentration in the medium was inversely proportional to growth (Figure 7), while phenotypic differences were not observed between the mutant and the control strain in the absence of acetate. Moreover, increased growth due to the auxiliary acetate carbon source was associated with rewiring of the glycogen metabolism, meaning that more resources were allocated to growth rather than to glycogen accumulation.

These observations may be associated with increased acetyl-CoA pool and enhanced biosynthesis of acetyl-CoA derived metabolites, which together lead to an enhancement of growth by redirecting the carbon fluxes. The results of **Paper III** suggest that a supplementation of cyanobacterial production system with an economically and environmentally sustainable auxiliary carbon source with a concomitant expression of a corresponding transporter may enhance the overall output of biotechnological production systems. Acetate supplementation has the potential to advance biomass evolution and intracellular flux towards the initial starting precursor of a synthetic pathway.

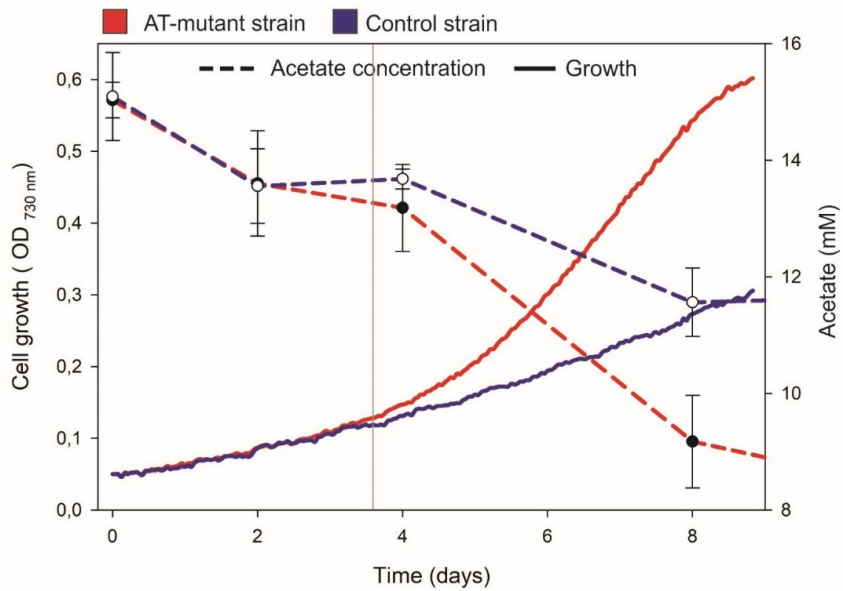


Figure 7. Relative growth and use of acetate. Acetate consumption (dashed line) for the acetate transporter mutant (red) and the control strain (blue) illustrated together with the growth (solid line) of the strains monitored over an eight-day cultivation under continuous light of $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. The grey vertical line emphasizes the point when the acetate transporter begins to utilise the supplemented acetate more than the control strain resulting in enhanced growth.

5 Discussion

5.1 More robust synthetic biology tools are needed for creating biotechnological applications

There is an imperative need to develop new technologies for producing biofuels and other industrially relevant compounds in a sustainable manner. Currently many strategies for sustainable bioeconomy rely on the use of biomass-related technologies. However, biomass production efficiency is too poor to substitute fossil-based fuels and energy production globally (Aro, 2016). Moreover, exploiting the arable land for fuel and chemical production instead of food contains many ethical and societal issues. A dependence on fuel molecules in modern societies will remain despite the recent massive advances in sustainable energy production technologies such as photovoltaic and wind power technologies.

Direct conversion of solar energy into the desired chemicals in cyanobacteria is one of the emerging approaches for sustaining the supply of fuels and chemicals, and is independent of plant-derived sugars. Indeed, biofuels produced by direct solar conversion technology are referred to as fourth generation biofuels in the biofuel innovation progression maintaining a better sustainability than the previous generations of biofuels (Aro, 2016). Harnessing the natural capacity of capturing clean solar energy and converting non-cost CO₂ into the desired compounds by engineered cell factories can contribute to the transition towards a carbon neutral bioeconomy, which is crucial in climate change mitigation.

The key bottlenecks, which have restricted the solar-driven production systems from reaching a commercially competitive biomanufacturing level, are the deficiency of robust metabolic engineering tools and the overall relatively low productivity. Synthetic and systems biology can provide the novel and more efficient tools needed for rational engineering of cyanobacterial expression strategies accompanied by the means of easily introducing flexible modifications into the host. This would accelerate the engineering processes and improve the desired outcome. Accordingly, the more robust engineering tools do not only alleviate the barrier of the main bottlenecks, but also avoid the time-consuming trial and error approach, enabling the predictable plug-in selection of the most suitable and proper genetic elements for the specific purpose. Efficient synthetic biology tools are crucial when

taking advantage of basic scientific research for developing specific applications as well as for the high throughput production systems for industrial biotechnological purposes. Evaluation and utilisation of these genetic tools result in expansion of knowledge on complex metabolic interactions in cyanobacteria. Moreover, synthetic biology tools help to identify and diminish the bottlenecks thus promoting the design of more advanced production platforms and processes. Host-specifically validated and optimised synthetic biology tools enable predictable control and regulation on the expression systems introduced, which is a cutting-edge advancement over the conventional metabolic engineering approaches, enabling implementation of more complex engineering and manipulation strategies for solar-driven cyanobacterial production systems. Additionally, synthetic biology allows expansion of a more diverse range of target compounds, further complemented by novel combinatorial pathways to produce completely new-to-nature products. Synthetic biology approaches can also be used to rationally reallocate photosynthetic carbon fluxes throughout primary metabolism to direct more carbon and electrons towards a target product.

This research work focused on systematic development and evaluation of synthetic biology tools in *Synechocystis*. Here, the synthetic biology tools comprise the design strategy, assembly platforms, integration and expression vectors as well as regulatory and monitoring tools. The deployed design and assembly strategy was based on the use of standardised interchangeable genetic elements following iterative cloning steps. A palette of new genetic engineering tools such as translational control elements and integration sites were generated and quantitatively evaluated *in vivo* to be ready for the rational utilisation when constructing new synthetic pathways and introducing modifications into the host. These strategies attempt to improve predictability and diminish time consumed to trial and error processes. In addition, the study has also provided insights into the effect of adjacent genetic contexts in the expression level, thus increasing the understanding of the potential limitations and opportunities when designing synthetic pathways in cyanobacterial cell factories. Furthermore, research work also studied how to take the best advantage of the photosynthetic machinery of the host by redirecting the electron and carbon fluxes.

5.2 Transport of compounds in and out of the cell

Import and export of compounds is important in microbial production host. Despite the fact that some small metabolites can freely diffuse in and out of the cell in cyanobacteria, some industrially desired products cannot be directly secreted or transported out of the cell because of e.g. the existence of a hydrophobic cellular envelop barrier, the deficiency of a suitable membrane-crossing transporter or the size-based limitations of the target compound (Luan and Lu, 2018) (Stebegg et al.,

2019). Importantly, efficient secretion of a product from the cell drives the dynamic equilibrium towards the final products. This is especially the case in toxic products or intermediates that easily result in a general negative feedback regulation in the metabolism if they remain inside the cell. Accordingly, secretion of the product from the cells reduces the associated inhibitory effects and improves the tolerance, thus increasing the production capacity (Luan and Lu, 2018). As harvesting and lysing the cells in order to release and separate the target compound from the biomass require time and energy, cyanobacteria can be engineered to conveniently transport the target product out of the cell after they are synthesized, which improves the economic and environmental feasibility of photosynthetic biomanufacturing (Gao et al., 2016). For example, a specific strategy to improve the membrane crossing secretion in the case of sucrose, a CscB-transporter assisted sucrose secretion strategy, was applied in the **Paper IV**. The same sucrose permease was also earlier successfully expressed in *Synechococcus elongatus* by Ducat and the coworkers (Ducat et al., 2012). In contrast to extracellular secretion for export, a transporter-mediated import can also be applied, for instance to advance the precursor supply for improving the desired outcome of a higher productivity in the production chassis. In **Paper III**, a heterologous YjcG-transporter was introduced to enhance the acetate influx into the cell, leading to enhanced/improved growth and rewiring of the cellular resources.

5.3 Metabolic engineering at the interface of switching the mode of metabolism

Cyanobacteria inherently possess a tremendous metabolic plasticity, rooted in their evolutionary adaptability, which enabled the phylum to cope with environmental variations (Xiong et al., 2017). The metabolic flux alterations, carefully regulated to maintain the balance of resources in the cell, is exploited in different ways to respond to variable physiological conditions such as differences in energy input from the sunlight or variations in the carbon substrates, both in short- and long-term processes. The metabolic network, in which multiple routes vary in the reaction scheme but are still linked together through the joint actions, includes, in some cases, multiple routes that are operated in parallel and even result in the same outcome, as in case of co-existence of both linear and cyclic electron transfer routes or the co-existence of three glycolytic pathways in *Synechocystis*. A motive to invest in these apparently costly overlapping pathways is that the multiple options endow bioenergetic versatility, which the cell can use to control the supply of ATP and NADPH, allowing prompt tuning of the energy demands. Moreover, cyanobacteria have storage compounds such as glycogen and PHB, which are used as buffering and storage systems for carbon metabolism, as an important response to nutrient

deficiency and under diurnal conditions. Notably, these two carbon sinks, deriving from different nodes on the metabolic network, offer distinct control points for tuning the metabolic flux according to the cell's need.

The amenability for alterations in carbon metabolism and bioenergetics is an essential foundation for engineering the cyanobacterial production systems. Thus, the rational rewiring of metabolic fluxes is one of the engineering approaches to enhance productivity (Xiong et al., 2017). Cyanobacterial engineering approaches for redirecting endogenous fluxes towards target products may include: the deletion of the competing sinks (e.g. **Paper IV**), introduction of new sinks in form of the target pathway(s) (e.g. **Paper IV**), rewiring the pathways to enhance intermediate pools (e.g. **Paper III**; (Xiong et al., 2015) (Anfelt et al., 2015)), or coupling together photosynthetic activity and carbon metabolism (**Paper IV**; (Oliver et al., 2016)). Rewiring may appear in the cell in many ways, for example, an introduced synthetic pathway for a product (as sucrose) can serve as an alternative metabolic sink, which can be used as a driving force for the biosynthesis of desired compounds and restored carbon fixation (**Paper IV**). From a metabolic engineering perspective, metabolic plasticity is realised through the observation that targeted modification at one node may provoke plasticity and metabolic flux at other nodes. For example, **Paper III** and **Paper IV** describe examples of differently modulated cellular resources. In **Paper III**, additional carbon source rewired the carbon flux in preference to enhanced growth instead of glycogen accumulation. Indeed, photomixotrophic production using engineered cyanobacteria via rewiring the metabolic pathways may be beneficial for the outcome in some cultivation conditions, as suggested in **Paper III** and other contexts. For example, the production of isobutanol, an attractive biofuel candidate, was highest under mixotrophic conditions compared to that produced photoautotrophically or heterotrophically in *Synechocystis* (Matson and Atsumi, 2018). In addition, mixotrophic conditions in the presence of acetate doubled the production of D-lactate in *Synechocystis* compared to photosynthetically produced yield; even though ^{13}C labelings indicated that the end-product was mainly derived from CO_2 , the supplemented acetate still increased the acetyl-CoA pool and thus subsequently the end-product formation (Varman et al., 2013). Photomixotrophic conditions in the presence of acetate have also been reported to enhance the production of PHB, a biodegradable polymer which can be used to replace petroleum-based plastics, compared to photosynthetic production in *Synechocystis* (Wu et al., 2001) (Khetkorn et al., 2016). Supplementation of a carbon source into the *Synechocystis* strains engineered to utilise xylene improved the productivity of ethylene compared to the sole photosynthetic production capacity due to the fact that the mixotrophic conditions increased the flux through the TCA cycle towards ethylene production (Lee et al., 2015). In **Paper IV**, allocation of the autotrophically fixed carbon to different alternative sinks (sucrose, glycogen or PHB) was strongly

dependent on the light conditions and the genetic background of the sucrose producing strain, highlighting the extremely complicated interactions of the metabolic plasticity. The location of glycogen and PHB sinks on the interface of photoautotrophic and heterotrophic metabolism also emphasizes their role in diurnal conditions. Indeed, the day-night regime associated with the endogenous regulatory network due to metabolic plasticity should be taken into account when designing large-scale industrial operations e.g. in open-pond systems.

The cyanobacterial metabolic network is apparently highly intricate and plastic, yet well-orchestrated. Engineering design for rewiring the carbon fluxes must take into account the context in question, as different target compounds, growth conditions and genetic backgrounds may need a unique set of modifications, potentially complemented with experimental *in vivo* validations. More fundamental research is needed to enhance the understanding of the complex interactions and dynamic regulations of the endogenous metabolic fluxes, eventually aiming at maximising the production of target products in engineered cyanobacterial hosts. In addition, system biology should be exploited more routinely to develop cell factories.

6 Concluding Remarks and Future Perspectives

The direct conversion of solar to fuels and other chemical compounds is an attractive alternative solution to creating a truly sustainable bioeconomy along with the other clean technology concepts. Synthetic biology can be the enabling technology for the development of the next-generation living cell factories, allowing carbon neutral biomanufacturing for the compounds inevitably needed for modern societies. Strong, fundamental, basic research of the photosynthetic organisms will provide the pivotal foundation for the applications taking advantage of the enormous potential of photosynthetic machineries. In the future, direct solar conversion innovations using cyanobacteria as a catalyst may contribute worldwide to CO₂-aware energy security, environmental sustainability, nutritional security and industrial production for renewable commodity chemicals.

The ability of engineered cyanobacterial strains to fix CO₂ into industrially relevant target chemicals is essential for true commercialisation. Engineering based on efficient new tools, characterised genetic elements, installation of exogenous metabolic pathways, host optimisation as well as enhanced bioreactor design and culturing techniques is necessary for scale up processes in large-scale open environments instead of laboratory conditions. Different strategies should be systematically and in parallel applied to the development of more effective cyanobacterial production platforms with maximised productivity and metabolic pathway flux. Understanding the associated processes, metabolic effects, and the species-specific limitations is of great scientific and biotechnological interest for the design of future applications in autotrophic production platforms such as *Synechocystis*.

The efficiency of the cyanobacterial production systems still need to be improved. Synthetic biology approaches developed during this thesis may serve as a starting point for engineering optimised multi-gene pathways in cyanobacteria (**Paper I–IV**). The RBS evaluation (**Paper I**) enables better control for expression of individual proteins from a multicistronic operon. The importance and complexity of targeted flux towards end-products should be taken into account when engineering cell factories (**Paper IV**). The interplay of possible additional carbon sources for the

cell dynamics must be examined carefully as regards engineering efforts (**Paper III**). Further work on the development of synthetic biology methods for cyanobacteria is necessary. For example, the context dependence of RBS sequence and adjacent coding region should be diminished by an insulator in order to improve the translational control.

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