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Towards renewable chemicals production in cyanobacteria

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Abstract

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The intensive fossil fuel combustion by humanity resulted in the increase of atmospheric CO_2 concentration creating the greenhouse effect, which in turn causes severe environmental problems. Thus, humanity should find solutions to decrease dependency on fossil hydrocarbons in order to lower CO_2 emission into the atmosphere. Photosynthetic microorganisms, including cyanobacteria, exhibit great potential for this purpose, being able to fix and directly convert CO_2 into organic chemicals using solar irradiation as an energy source. Establishing cyanobacteria as microbial cell factories enables the sustainable production of bulk chemicals and biofuels.

In this thesis, the production of industrially relevant chemicals, i.e. succinate and aromatic compounds by the cyanobacterium *Synechocystis* sp. PCC 6803 was explored. Succinate is naturally formed via the tricarboxylic acid cycle (TCA cycle) in cyanobacteria. Phosphoenolpyruvate carboxylase (PEPc) is an essential enzyme in replenishing the oxaloacetate intermediate of the TCA cycle. Succinate production was investigated by introducing a new capacity for its synthesis via overexpression of heterologous glyoxylate shunt genes together with overexpression of native PEPc. The implementation of the glyoxylate shunt proved successful as succinate productivity was enhanced in certain conditions. Moreover, the formation of succinate during anaerobic darkness was explored. The enzyme L-aspartate oxidase was studied and the in vitro ability of this enzyme to reduce fumarate to succinate anaerobically was demonstrated, which contributes to the understanding of the cyanobacterial TCA cycle for future engineering purposes.

The production of the first intermediates of the plant phenylpropanoid pathway, *trans*-cinnamic and *p*-coumaric acid, which derive from the aromatic amino acids phenylalanine and tyrosine, was implemented by overexpression of phenylalanine- and tyrosine ammonia lyases in *Synechocystis*. The subsequent metabolic engineering, such as the elimination of competing pathways of tocopherol synthesis, demonstrated increased productivity for both target molecules. Moreover, laboratory evolution of *Synechocystis* was performed and several metabolic mutants were selected for their ability to secrete phenylalanine in the growth medium. The laboratory-evolved mutants provide an important basis for investigating pathway regulation of aromatic amino acid synthesis. In summary, the findings in this thesis contribute to the development of cyanobacteria as microbial cell factories for the sustainable production of renewable chemicals.

Keywords: Synechocystis PCC 6803, metabolic engineering, succinate, phenylpropanoids, laboratory evolution

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List of Papers

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

I. Durall, C.*, Kukil, K.*, Hawkes, J. A., Albergati, A., Lindblad, P., & Lindberg, P. (2021). Production of succinate by engineered strains of *Synechocystis* PCC 6803 overexpressing phosphoenolpyruvate carboxylase and a glyoxylate shunt. *Microbial cell factories*, 20(1), 1-14.

II. Kukil, K., & Lindberg, P. (2022). Expression of phenylalanine ammonia lyases in *Synechocystis* sp. PCC 6803 and subsequent improvements of sustainable production of phenylpropanoids. *Microbial cell factories*, 21(1), 1-16.

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V. Kukil, K., Lindberg, P. (2022) Metabolic engineering of *Synechocystis* PCC6803 for the improved production of phenylpropanoids. *Manuscript*

VI. Xie, H., Kukil, K., Lindberg, P., Miao, R., & Lindblad, P. (2022). Metabolic Rewiring and Cultivation Optimization for Photosynthetic Biofuel Production in Cyanobacteria. In *Bio-Clean Energy Technologies: Volume* 1 (pp. 45-80). Springer, Singapore.

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Contents

Abbreviations

2-OG	2-oxoglutarate
AAA	Aromatic amino acids
C4H	Cinnamate-4-hydroxylase
CBB cycle	Calvin-Benson-Bassham cycle
CM	chorismate mutase
DAHP	3-deoxy-d-arabinoheptulosonate-7-phosphate synthase
DAHPS	DAHP synthase
E4P	Erythrose-4-phosphate
GABA	γ-aminobutyric acid
HPP	4-hydroxyphenylpyruvate
HPPD	4-hydroxyphenylpyruvate dioxygenase
ICL	Isocitrate lyase
Laspo	L-aspartate oxidase
MS	Malate synthase
PAL	Phenylalanine ammonia lyase
*p*Cou	*p*-coumaric acid
PEP	Phosphoenolpyruvate
PEPc	Phosphoenolpyruvate carboxylase
Phe	L-phenylalanine
PRM	phenylalanine resistant mutants
SDH	Succinate dehydrogenase
TAL	Tyrosine ammonia lyase
TCA cycle	Tricarboxylic acids cycle
*t*CA	*trans*-cinnamic acid
Trp	L-tryptophan
TTA	2-thenoyltriluoroacetone
Tyr	L-tyrosine
WT	Wild type
E. coli	*Escherichia coli*
Synechocystis	*Synechocystis* sp. PCC 6803

Introduction

Motivation of the study

Climate change in the twenty-first century caused by increase of atmospheric carbon dioxide (CO_2) levels affects many aspects of the natural world [1]. Human activities, mainly the burning of fossil hydrocarbon, have caused an approximately 1.5 ^{o}C increase of average temperature compared to the pre-industrial levels [2]. The consequences of global warming lead to the rising of sea levels due to thawing of ice sheets and glaciers. Even a small further increase of average temperature will have drastic and catastrophic outcome. Therefore, society is required to take immense measures to minimize the ongoing climate changes. Introducing and maintaining environmental sustainability of human activities rise in relevance on the political agenda [1].

Since CO_2 emission is the driving force of global warming, efforts to decrease our dependence on fossil fuels are intensified. On the other hand, the solar irradiation that reaches the atmosphere every day delivers a gigantic amount of energy. The efficient harvest and storage of the solar energy could also decrease the consumption of fossil hydrocarbon. Moreover, one of the promising option would be to transform solar irradiation and carbon dioxide directly into bulk chemicals and/or biofuels with the use of photosynthetic microorganisms, such as cyanobacteria or microalgae. Employing these organisms in an environmentally sustainable and economically effective manner could potentially decrease a substantial fraction of our society's use of fossil fuels. Among photosynthetic microorganisms cyanobacteria show superior photosynthesis capabilities and can convert up to 10% of the sun's energy into biomass, compared to the 5% achieved by algae and 1% recorded by crops such as corn or sugarcane [3]. As my small contribution to the establishment of the abovementioned approach, I have investigated the production of industrially relevant chemicals, succinate and aromatic amino acids and their derivatives, by cyanobacteria.

Cyanobacteria as hosts for production of industrially relevant chemicals

Cyanobacteria are Gram-negative bacteria that have evolved in the biosphere of the Earth during billions of years. These organisms are responsible for the increase of oxygen concentration in the Earth's atmosphere and were progenitors of chloroplasts in eukaryotes [4]. Cyanobacteria are the largest and most diverse group of photosynthetic prokaryotes, they differ morphologically (unicellular, filamentous or colonial) and can be found in diverse types of environments. In marine ecosystems, cyanobacteria are the main producers of primary biomass, whereas the diazotrophic strains are vital in the biogeochemical nitrogen cycle. Due to their broad spreading in distinct ecosystems, cyanobacteria have evolved a wide range of adaptive systems and sensory mechanisms [5].

In cyanobacteria, oxygenic photosynthesis occurs in thylakoids, whereas respiratory electron flow takes place in both the thylakoid and cytoplasmic membrane systems. Therefore, in cyanobacteria both photosynthetic electron transport and respiration are performed in the same compartment sharing membrane complexes for these two opposite reactions allowing multiple interconnected pathways of electron transport. This is considered to be a quite unique feature of cyanobacteria [6]. Similarly to plants, Photosystem II and Photosystem I catalyse the central reactions of solar energy conversion in cyanobacteria. The light harvesting antenna of both photosystems consists of chlorophylls and carotenoids that capture light. Additionally, both are functionally connected to the large membrane associated phycobilisomes, whose function is to increase the absorption capacity of the light-capturing system [7]. The generated strong reducing equivalents and energy rich molecules, NADPH and ATP, are used to assimilate inorganic minerals, such as carbon dioxide, nitrate or even molecular nitrogen in nitrogen-fixing cyanobacteria. Via the Calvin-Benson-Bassham (CBB) cycle, the carbon dioxide is fixed to produce glyceraldehyde-3-phosphate, which is further incorporated into primary nutrient biomass in photosynthetic organisms. The CBB cycle is the primary carbon fixation pathway in nature and the major electron "sink" of photosynthesis.

Although cyanobacteria have been known since early 19th century [8], they have attracted attention as catalysts for the direct conversion of carbon dioxide into reduced fuels and other valuable compounds only recently. Their photoautotrophic growth allows truly sustainable production of target compounds, where CO_2 is acquired from the atmosphere and sunlight provides the source of energy, whereas conventional heterotrophic systems (*Saccharomyces cerevisiae*, *Escherichia coli*, etc.) require a supply of an organic carbon feedstock for growth.

As photosynthetic microorganisms, cyanobacteria have a number of superior properties. They require minimal nutrient supply, and their cultivation does not require arable land. Some strains can even be cultivated in

wastewater, desert regions or seawater [9-11]. Compared to plants that also perform oxygenic photosynthesis, cyanobacteria have faster growth rates, and the newly isolated fast-growing strains can reach the doubling time of heterotrophs [12-15]. Additionally, cyanobacteria exhibit higher photosynthetic efficiencies than microalgae and plants and are easier to genetically engineer [16, 17]. However, titers of desirable target molecules generated by cyanobacteria are still too low for commercial use for most products.

The goal of my thesis is to engineer cyanobacteria as a biotechnological platform for renewable, solar-powered production of chemicals. In this study, a unicellular, glucose-tolerant strain *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) was used. *Synechocystis* is a model strain isolated from freshwater, its full genomic sequence was available already in 1997 [18]. Despite the long history of intensive research on this strain, the understanding of many key features of physiology and biochemistry is still far from completion. For instance, less than ~30% of the genome is annotated, which is less than half compared to *Escherichia coli (E. coli*) [19].

The engineering of *Synechocystis* in this project is focused on over-production of molecules of industrial interest: succinate and aromatic compounds. The selected targets are products of central metabolic pathways and are important precursors for a wide range of different industrially relevant chemicals. These molecules originate from central carbon compounds, such as phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P), which are derived from CO_2 fixation in cyanobacteria. However, their synthesis occurs via different pathways, which is why this thesis consists of two main blocks, where each part explores the production of succinate or aromatic compounds by *Synechocystis*.

Metabolic engineering of cyanobacteria

Metabolic engineering of an organism refers to a directed alteration of the cellular activities, which is achieved by modifying the flux or features of existing biochemical reactions, or by introducing new reactions into an organism. The accelerated development of recombinant DNA technology allowed the establishment of metabolic engineering as an approach for rapid introduction of directed genetic changes and subsequent analysis of the results at the cellular level, unlike classical approach of mutagenesis and screening for the desired phenotype [20]. Metabolic engineering consists of iterative steps of construction of recombinant strains, analysis of their performance in respect to the parental strains and designing of a next target for genetic engineering.

Clearly, for successful metabolic engineering of any organism, a large variety of tools for genetic manipulation that allows controlled gene expression and metabolic reconstruction should be available. In case of the model-strain *Synechocystis*, multiple tools for gene expression and regulation, such as vectors, promotors, transcription factors etc. have been developed and this

toolbox is expanding every year. Due to the different cyanobacterial holopolymerases, well-characterized promoter systems from *E. coli* such as the lactose-induced P_{lac} promoter, are often not compatible and demonstrate poor functionality [21, 22]. Many native, synthetic and semisynthetic promoters have been characterized recently, including the strong promotors P_{psbA2}, P_{rbcL}, P_{cpcB} [22, 23]; metal inducible promoters P_{nrsB}, P_{coaT}, P_{petE} and P_{ziaA} [24, 25]; rhamnose, vanillate and arabinose inducible promoters P_{rhaBAD}, P_{vanCC}, P_{BAD} [26-28]. However, the issues of promoter leakiness and low expression strength are not completely solved. Standardized insulator sequences between 5'UTR and CDS such as bicistronic design or RiboJ adapted from *E. coli* studies were successfully applied in *Synechocystis* and allow efficient translation of desired proteins [29-32].

The genetic elements combined together in a DNA construct, should be transfered into the cell to subject an organism to genetic modification. Foreign DNA elements can be delivered inside of the cyanobacterial cell via natural uptake, conjugation, or electroporation. The natural competence, i.e. ability to take up DNA from the environment and insert it into their own genome by homologous recombination, is widespread within prokaryotes including cyanobacteria [33], and is the simplest way of delivering DNA into the cell. Heterologous DNA sequences can be introduced into the chromosome or endogenous plasmids via homologous recombination to create a deletion, insertion, or replacement. For the model strain *Synechocystis*, several *loci* of neutral sites where heterologous DNA insertion seems not to affect metabolism are identified and used [34, 35]. *Synechocystis* as well as many cyanobacterial strains is polyploid, and thereby more time and effort is required to reach full segregation after mutagenesis. Nonetheless, even after full segregation is reached, heterologous genes can be lost as was reported in some studies [36, 37], which is considered to relate to genomic instability. The issues of genomic instability potentially take part as the adaptive evolution to stresses and have not been fully elucidated in cyanobacteria yet [38-40].

The extrachromosomal gene expression on self-replicating plasmid is also a common approach in synthetic biology of cyanobacteria. Self-replicating plasmids are maintained in the host cell using selective marker genes, which usually are the antibiotic resistance genes.

Both approaches, the use of self-replicating plasmids or homologous recombination, are widely used in synthetic biology of cyanobacteria, and each has certain limitations and advantages. The general disadvantage for both is the small number of available selection markers in case extensive strain engineering is needed. In contrast, markerless genetic modification of chromosomes is the next step in cyanobacterial strain development. The most recent state-of-art technique in genome engineering, CRISPR, has been successfully applied in cyanobacteria as well [41]. This technology allows one-step precise and efficient markerless modification of DNA sequences, which is an alternative to traditionally used homologous recombination. Another tool adapted

from the CRISPR-Cas9 systems, CRISPR interference (CRISPRi), allows simultaneous knockdown of multiple genes in cyanobacteria [42].

Pathway engineering for enhanced productivity

One of the key aspects in metabolic engineering is analysing the metabolic network structure of a certain pathway of interest. This includes identification of the presence of specific enzymes activities and their biochemical characteristics, such as cofactor requirements, which in summary gives an overview of a metabolic route [20]. In general, the metabolic network of a cell consists of a myriad of reactions catalysed by similar number of enzymes, whose expression is tightly regulated in response to cellular needs. Additionally, in many cases a complete metabolic structure is still unknown even for heavily studied model strains. The amount of flux carried through a certain pathway is what we are interested in metabolic engineering, as the increase of flux towards the molecule of interest that we aim to produce by cyanobacteria theoretically should lead to an increase in its concentration. However, in practice introduction of functional enzymes affecting the central cellular pathway processes creates a cascade of alterations, which may lead to unpredictable results due to the metabolic crosstalk. The major part of cellular energy and reductive power derived from photosynthetic light reactions is directed towards the cell proliferation and maintenance of the viability. Therefore, only by careful engineering of metabolic pathways, the energy and carbon can be redirected into the synthesis of molecules of interest allowing their sustainable production by cyanobacteria.

Quite often, the overexpression of native or heterologous enzymes is not enough to enhance the productivity, and thereby the metabolic flux should be rerouted from the potential competing pathways. This can be achieved by eliminating the competing enzymatic activities by knocking out (or down) the corresponding genes using conventional homologous recombination, or newer methods, such as riboswitches, antisense RNA or CRISPRi [42-45].

One of the approaches to redirect the metabolic flux into desired product is to create a ‘sink’ effect. It is based on the idea, that the carbon consuming reactions associated with growth are slower than carbon fixation, and thereby the excess of cellular carbon prevents any greater fixation. This existing bottleneck could be relieved by introduction of ‘carbon sink’, which is represented by a high flux pathway that converts cellular carbon into a molecule that is secreted from the cell and is not consumed by central metabolism. In this way, the decoupling of growth by a carbon sink can potentially maximise the turnover of the CBB cycle reactions and thereby increase both carbon fixation and light capture [46]. Indeed several papers have demonstrated that additional carbon sink results in a boost of the photosynthetic activity and growth in cyanobacteria [47, 48]

Another important aspect of pathway engineering is the catalytic efficiency of enzymes within the pathway, which in sum defines the catalytic efficiency of a whole metabolic route. Biochemical characteristics of a protein may include slow turnover rates, catalysis of a reversible reaction, or broad substrate specificity, resulting in different products at the same time that in the end decreases the formation efficiency of a desired metabolite. In order to overcome this issue, enzyme engineering is applied. There are two general strategies for obtaining a protein molecule with improved characteristics: rational design and directed evolution, which are very powerful when combined. Rational design is based on the three-dimensional structure of the protein and aims to resolve the relationship between the protein structure and function, and thus design knowledge-based targets for modification. This method usually includes resolving of a crystal structure of an enzyme in order to create a 3D structure model. With accumulation of the structure-function knowledge, more precise design for a mutant protein can be achieved [49]. Recently, a powerful tool for protein structure prediction based on the amino acids sequence was developed, which greatly facilitates the rational design of proteins, whose crystal structure is not yet resolved [50].

The second approach, protein-directed evolution, is based on generating random mutations in the protein and screening the generated library for desired specific functionality. For this method, the screening procedures to identify a target protein of interest are the most laborious step. However, recently developed screening techniques, such as display technologies, protein reporter biosensors or *in vivo* continuous evolution have greatly facilitated and enabled a high-throughput protein engineering via directed evolution [51].

Succinate metabolism and production in cyanobacteria

Succinate, like the other four-carbon organic acids fumarate and malate, are important precursors in pharmaceutical and food industries [52, 53]. As most organic acids, they are natural metabolites in microorganisms. Succinate is a central metabolite of the tricarboxylic acids (TCA) cycle, which is present in all living organisms. Succinate can be formed via three routes, oxidative TCA cycle, the reductive branch of TCA cycle and finally, the glyoxylate pathway.

The cyanobacterial TCA cycle (Figure 1) is considered unique, since it lacks the 2-oxoglutarate dehydrogenase complex. Instead, two alternative bypasses of succinate synthesis from 2-oxoglutarate (2-OG) were identified in *Synechocystis*: via the γ-aminobutyric (GABA) shunt [54] or via 2-oxoglutarate decarboxylase and succinic semialdehyde dehydrogenase [55]. These bypasses are closing the cyanobacterial TCA cycle, which was considered to be incomplete for a long time. In light conditions, the TCA cycle in cyanobacteria functions as a branched chain pathway rather than a cycle, synthetizing carbon skeletons for nitrogen assimilation via 2-OG and providing substrates for synthesis of amino acids of the aspartate family from oxaloacetate. The enzymatic activity of phosphoenolpyruvate carboxylase (PEPc) is essential in providing oxaloacetate into TCA cycle. In dark conditions and in the presence of oxygen, the oxidative branch of TCA cycle is most active. This is due to the membrane associated enzyme complex succinate dehydrogenase (SDH), which is the key component in the reduction of the respiratory electron transport chain as it catalyses the oxidation of succinate to fumarate [56].

Many microorganisms including cyanobacteria are able to secrete succinate during anaerobic fermentation via the reductive branch of the TCA cycle [57]. In this pathway, oxaloacetate is reduced via the intermediates malate and fumarate to succinate (Figure 1). In cyanobacteria, based on similarity with other bacteria, such as *E. coli*, succinate formation has been assumed to occur via fumarate reduction by SDH. However, detailed biochemical information on this enzyme complex is not available in cyanobacteria. The studies on SDH were carried out by deletion of open reading frames of SDH subunits in *Synechocystis* and subsequent measurements of succinate or fumarate accumulation and no fumarate reductase activity has been demonstrated [58, 59]. Possibly, cyanobacteria possess another route to synthesize succinate from fumarate. This is indicated by the fact that a mutant of *Synechococcus* PCC 7002 lacking all enzymes known to form succinate (SDH, succinyl-CoA synthase, succinic semialdehyde dehydrogenase and 2-oxoglutarate decarboxylase) accumulated similar levels of succinate as the wild type (WT) strain [60], while the formation of succinate from fumarate is supported by data from metabolite turnover flux analysis [61].

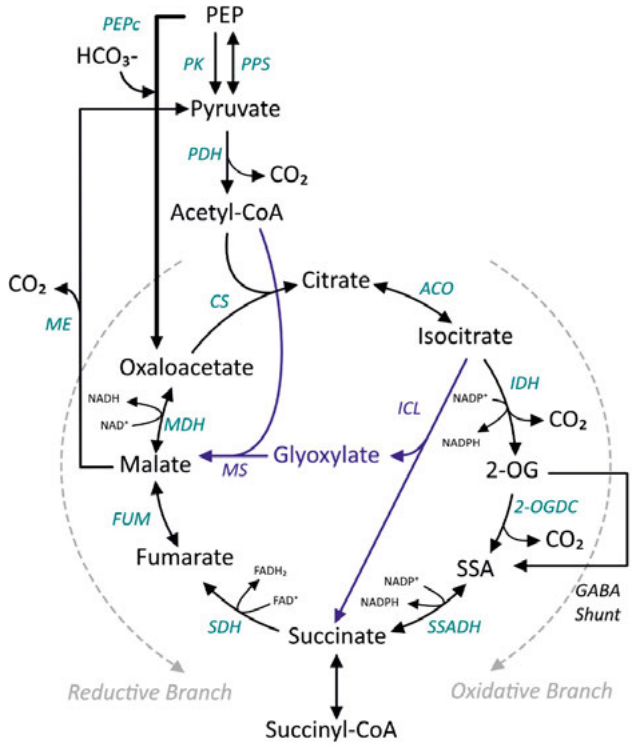

Figure. 1 TCA cycle of *Synechocystis* PCC 6803. Glyoxylate shunt is absent in *Synechocystis* and indicated in blue color. Abbreviations: PEP, phosphoenolpyruvate; 2-OG, 2-oxoglutarate; 2-OGDC, 2-oxoglutarate decarboxylase; ACO, aconitase; CS, citrate synthase; GABA, γ-aminobutyrate; ICL, isocitrate lyase; IDH, isocitrate dehydrogenase; MDH, malate dehydrogenase; ME, malic enzyme; MS, malate synthase; PDH, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPc, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; PPS, phosphoenolpyruvate synthase; SDH, succinate dehydrogenase; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase.

A third route of succinate synthesis, the glyoxylate shunt, exists in many bacteria including a few diazotrophic species of cyanobacteria [62, 63]. The shunt consists of two enzymes: the first enzyme, isocitrate lyase (ICL), cleaves isocitrate to succinate and glyoxylate, whereas the second enzyme, malate synthase (MS) condenses the glyoxylate with acetyl-CoA to form malate. The glyoxylate pathway is essential for growth on substrates that degrade solely to acetyl moieties (acetate, fatty acids), since the two decarboxylation steps are omitted and the preserved carbon can be redirected to biomass formation through gluconeogenesis [63]. However, in *Synechocystis*, the first enzyme of the gluconeogenesis pathway, PEP carboxykinase (PEPCK), that converts oxaloacetate into PEP is absent [64]. The glyoxylate shunt that is naturally present in some cyanobacteria may play a supporting role in nitrogen fixation during the night phase [62].

Engineering strategies for succinate production in cyanobacteria have been based on exploiting the oxidative [59, 65-68] or the reductive branches of the TCA cycle [61, 69-72]. For instance, overexpression of *citH*, encoding malate dehydrogenase combined with product stripping by medium exchange, enhanced production of succinate, malate and fumarate through the reductive branch of TCA cycle during anaerobic fermentation [72]. It was also demonstrated that deletion of *ackA*, which encodes acetate kinase that catalyses the production of acetate from acetyl phosphate, decreases acetate level and increases succinate levels in *Synechocystis* under the same conditions [70].

The strategies to enhance the succinate production under aerobic conditions include downregulation or knocking out SDH activity [43, 59], knocking out *glgC* to abolish glycogen synthesis together with PEPc and citrate synthase overexpression [73], as well as overexpression of the ethylene forming enzyme (*efe*) [66, 74]. Overexpressing seven genes (encoding 2-oxoglutarate decarboxylase, succinate semialdehyde dehydrogenase, PEPc, sedoheptulose-1,7-bisphosphatase, citrate synthase and succinate transporters) and knocking out SDH and glycogen synthase A resulted in photoautotrophic production of 0.93 $g \cdot L^{-1}$ succinate in 5 days from the fast growing *Synechococcus elongatus* PCC 11801 [67].

Metabolism of aromatic amino acids and derivatives in cyanobacteria

Aromatic amino acids (AAA) and aromatics-derived compounds are a class of commercially relevant chemicals with applications in chemical, food and pharmaceutical industries as fragrances, cosmetics, nutraceuticals and pharmaceuticals [75, 76]. Aromatic molecules within plant secondary metabolites/natural products derive from three AAA, L-phenylalanine (Phe), L-tyrosine (Tyr) and L-tryptophan (Trp), which are synthesized via the shikimate pathway (Figure 2).

The shikimate pathway is a central metabolic route and exists in plants, fungi and microorganisms, but not in animals [77]. It starts from condensation of E4P and PEP into the first compound 3-deoxy-d-arabinoheptulosonate-7-phosphate synthase (DAHP) by the enzyme DAHP synthase (DAHPS). Through six consecutive reactions, DAHP undergoes cyclisation to form chorismate. Chorismate is the last shared precursor in the synthesis of the three AAA as well as other pathway products, such as folic acid and plastoquinones, which makes chorismate a main metabolic hub before branching of the shikimate pathway [78, 79].

Chorismate can be utilised by anthranilate synthase to form Trp or undergo isomerization to prephenate by chorismate mutase (CM) leading to Phe and Tyr synthesis. Prephenate dehydratase forms phenylpyruvate from prephenate by oxidative decarboxylation, whereas phenylpyruvate is eventually transaminated to form Phe. In cyanobacteria, Tyr synthesis occurs via the arogenate route, where prephenate is aminated to arogenate and converted to Tyr [80, 81]. Alternatively to the arogenate route, tyrosine can be formed from prephenate via 4-hydroxyphenylpyruvate (HPP), which is common among microorganisms including *E. coli* [82].

In cyanobacteria, based on available data in KEGG (https://www.kegg.jp), 4-hydroxyphenylpyruvate dioxygenase (HPPD) oxidizes HPP to form homogentisate, which is the starting intermediate in the synthesis of tocopherols and tocotrienols (vitamin E). In plants, homogentisate is a common precursor for both plastoquinone and vitamin E. However, it was demonstrated that *Synechocystis* does not require HPPD for plastoquinone synthesis and the hydrophilic head groups of plastoquinone derive from chorismate [78, 83].

Central carbon metabolism
E4P
PEP
DAHPS
Pi
DAHP
DHQS
Pi
DHQ
DHQD
H2O
DHS
SDH ?
NADPH
NADP+
SHK
SK
ATP
ADP
S3P
EPSPS
PEP
EPSP
CS
Pi
CHA
CM
Prephenate
PDT
H2O + CO2
PAT ?
AA
KA
AS
Gln
Glu
Pyr
PQ
Folate
ANT
TRP
PHE
PPY
ARG
TYR
AT
KA
AA
ADH
NADPH + CO2
NADP+
HPPD
O2
CO2
HPP
HGA
α-Toc
PAL
TAL
C4H
tCA
pCou
Phenylpropanoid pathway

Figure 2. Schematic overview of Shikimate pathway for biosynthesis of aromatic amino acids in cyanobacteria. Compounds and enzymes not present in *Synechocystis* are highlighted in red and grey colours. Abbreviations: AGN, arogenate; ANT, anthranilate; CHA, chorismate; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; E4P, erythrose-4-phosphate; EPSP, 5-enolpyruvylshikimate 3-phosphate; HGA, homogentisate; HPP, 4-hydroxyphenylpyruvate; *p*Cou, *para*-coumaric acid; PEP, phosphoenolpyruvate; PHE, L-phenylalanine; PPY, phenylpyruvate; PQ, plastoquinone; S3P, shikimate-3-phosphate; SHK, shikimate; *t*CA, *trans*-cinnamic acid; TRP, L-tryptophan; TYR, L-tyrosine. Enzymes: ADH, arogenate dehydrogenase; AS, anthranilate synthase; AT, aminotransferase; C4H, cinnamate-4-hydroxylase; CM, chorismate mutase; CS, chorismate synthase; DAHPS, 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase; DHQD, 3-dehydroquinate dehydrogenase; DHQS, 3-dehydroquinate synthase; EPSPS, 5-enolpyruvylshikimate 3-phosphate synthase; HPPD, 4-hydroxyphenylpyruvate dioxygenase; PAL, phenylalanine ammonia lyase; PAT, prephenate aminotransferase; PDT, prephenate dehydratase; SDH, shikimate dehydrogenase; SK, shikimate kinase; TAL, tyrosine ammonia lyase.

Phenylpropanoids, a diverse group of secondary metabolites with unique biological functions involved in stress response and protection in plants, originate from Phe and Tyr as the initial substrates. The phenylpropanoid pathway starts from non-oxidative deamination of Phe by phenylalanine ammonia lyase (PAL) to yield *trans*-cinnamic acid (*t*CA). This reaction plays a key role in synthesis of phenylpropanoids, since it links primary metabolism with secondary metabolites synthesis. In plants, *t*CA can be further converted to *p*-coumaric acid (*p*Cou) by the membrane bound enzyme cinnamate-4-hydroxylase (C4H) [84]. Alternatively, a bifunctional phenylalanine/tyrosine ammonia lyase creates a shortcut to *p*Cou synthesis by using Tyr as substrate and eliminating the step performed by C4H. In bacteria, a Tyrosine ammonia lyase (TAL) has been discovered, which catalyses deamination of Tyr to *p*Cou. Bacterial TAL activity is connected to the synthesis of antibiotics and the chromophore of photoactive yellow protein [85-87] unlike the role in synthesis of flavonoids and coumarins in plants [88, 89]. PAL was also discovered in filamentous cyanobacteria, however its role in metabolism in these organisms is not determined yet [90].

Under natural conditions, cyanobacterial cells produce moderate amounts of aromatic amino acids [91]. The allosteric control for aromatic biosynthesis in cyanobacteria is considered endo-oriented [92]. For endo-oriented organisms the early-pathway regulation has the major control for the entire branched pathway, unlike exo-oriented organisms, such as *Bacillus subtilis,* for which the exogenous availability of the end-pathway products has a major emphasis on the regulation of peripheral pathway branches [92]. An early study of Hall et al (1982) [81] on 48 cyanobacterial strains demonstrated that regulation of AAA synthesis differs greatly from *E. coli* or *Corynebacterium glutamicum.* In *E. coli*, three isoforms of DAHPS exist that are selectively feedback-inhibited by Phe, Tyr or by Trp [93], whereas among cyanobacterial

species inhibition of DAHPS was observed predominantly by Phe [81], or by Tyr in case of *Synechococcus* sp. strain PCC6301 [94]. Only in the filamentous cyanobacterium *Anabaena* sp. ATCC 29151 presence of two regulatory DAHPS isoenzymes sensitive to Phe or Tyr inhibition was reported [95]. Interestingly, the DAHPS in *Synechocystis*, encoded by the *sll0934* locus (KEGG https://www.genome.jp/kegg/), was also annotated as a carboxysome formation protein and the fully segregated knock out of this *loci* was demonstrated [96]. It is unclear how the cell can survive the knock out of this supposedly essential gene, unless some alternative DAHPS exists in *Synechocystis*.

In cyanobacteria, only in few species the CM was found to be feedback inhibited by Phe, Tyr, or Trp [81]. In *E. coli,* the bifunctional CM/phephanate dehydrogenase enzyme TyrA, which forms Tyr via chorismate and HPP, is sensitive to Tyr inhibition [97], whereas the bifunctional CM/prephenate dehydratase complex is inhibited by Phe [98]. Although the Tyr synthesis in cyanobacteria occurs via formation of arogenate, the enzyme arogenate dehydrogenase demonstrated no inhibition by Tyr [99], whereas the enzyme prephenate dehydratase is inhibited by Phe and activated by Tyr [81]. In *Synechocystis*, the overexpression of feedback-resistant TyrA together with feedback-resistant DAHPS from *E. coli* lead to the accumulation of nearly 15 times more Phe than Tyr, whereas in *E. coli* the overexpression of same genes resulted in Tyr overproduction over Phe [100, 101]. A summary of all observed regulation of the AAA biosynthesis in all cyanobacteria species that were investigated is presented in figure 3.

The production of phenylpropanoid compounds and AAA in cyanobacteria exhibit high potential, despite the shikimate pathway is understudied in these organisms. Compared to heterotrophic organisms, where the requirement for high levels of reduced equivalents for high-yield phenylpropanid synthesis becomes a principal limitation [102], phototrophic cyanobacteria create a large pool of NADPH during photosynthetic light reactions. Additionally, the fact that the shikimate pathway is essential for viability could potentially lead to high flux of precursors for synthesis of native and non-native aromatic metabolites in cyanobacteria [103].

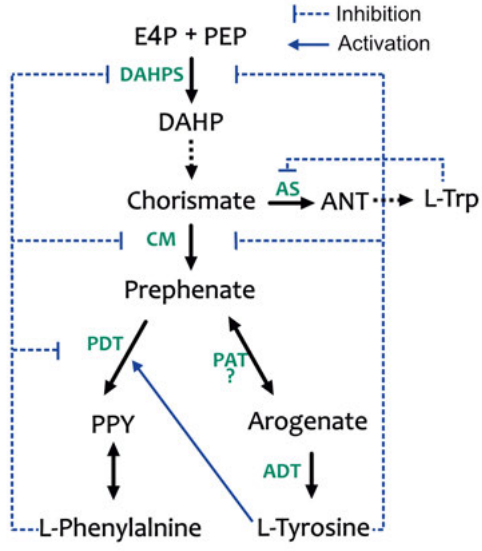

Figure 3. Enzyme regulation of AAA synthesis observed in cyanobacteria. Summarized and adapted from references [80, 81, 92, 94]. Note that not all of the presented regulation exists simultaneously in each cyanobacterial species. Abbreviations: ADT, arogenate dehydrogenase; ANT, anthranilate; AS, anthranilate synthase; CM, chorismate synthase; DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate; DAHPS, DAHP synthase; E4P, erythrose-4-phosphate; L-Trp, L-Tryptophane;PAT, prephenate aminotransferase; PDT, prephenate dehydratase; PEP, phosphoenolpyruvate; PPY, phenylpyruvate.

In general, for the microbial overproduction of aromatic compounds, the first step is the removal of the allosteric feedback inhibition of DAHPS to increase flux into the shikimate pathway and then further deregulation of branch point controlling enzymes is the pathway [98, 104]. However, unlike in other microbial hosts, the shikimate pathway in cyanobacteria is still understudied, which makes rational pathway engineering challenging. To date, only a few reports on production of phenylethanol [105], caffeic acid, *t*CA, *p*Cou, and AAA in cyanobacteria are available [48, 100, 105-107].

The strategies implemented so far in cyanobacteria to improve the productivity were enhancing the carbon flux into the shikimate pathway by relieving the feedback inhibition of key enzymes in the pathway, such as overexpression of feedback-resistant DAHPS and feedback-resistant chorismate mutase/prephenate dehydratase from *E. coli* [100]. Similarly, random mutagenesis to obtain Trp overproducing strain was combined with overexpression of feedback-resistant DAHPS and feedback-resistant anthranilate synthase to enhance Trp synthesis [48]. Another strategy for productivity enhancement consisted in eliminating consumption by knocking out competing pathways. The deletion of a putative laccase in *Synechocystis* encoded by the *slr1573* gene

led to increased *p*Cou titers, due to the presumable role of laccase in the oxidation of phenolic compounds in cyanobacteria [107]. Two-step synthesis of *p*Cou via Phe and *t*CA was enhanced by arogenate dehydrogenase deletion [106]. However, unless other Tyr synthesis routes exist in *Synechocystis*, it is unclear how cells are able to survive a knockout of this supposedly essential enzyme [108].

Aim

The aim of this PhD thesis can be summarised in the following parts:

1. Enhance succinate production by introducing a glyoxylate shunt in the cyanobacterium *Synechocystis* PCC 6803.

2. Explore succinate formation under specific conditions of anaerobic fermentation.

3. Establish production of primary intermediates of plant phenylpropanoids in *Synechocystis* PCC 6803.

4. Enhance the production of aromatic amino acids and derived phenylpropanoids by engineering new strains.

Results and discussion

Succinate production in *Synechocystis* sp. PCC 6803 (Papers I, III)

Expression of glyoxylate shunt in *Synechocystis* (Paper I)

To explore a new engineering strategy of succinate production in *Synechocystis* we introduced the glyoxylate shunt, which consists of enzymes isocitrate lyase (ICL) and malate synthase (MS), and investigated its effect on the extracellular succinate accumulation. Overexpression of PEPc, as previously demonstrated, facilitates growth and photosynthetic activity, which is in line with the main role of the anaplerotic PEPc reaction that directs carbon into the TCA cycle [109, 110]. Additionally, the PEPc overexpression resulted in the increased succinate secretion under conditions of anaerobic darkness [61]. Thereby, we decided to a introduce partial (ICL) and complete glyoxylate shunt (ICL and MS) in both WT and a strain overexpressing PEPc obtained in a previous study [109].

The glyoxylate shunt genes were amplified from *E. coli*, *aceAB* as an operon and *aceA* alone were integrated into *Synechocystis* under the control of the Ni^{2+}-inducible promoter P_{nrsB} [24]. In addition, two control strains were created, WT_C, based on WT and 2P_C, based on WT+2xPEPc, where only a resistance cassette was integrated instead of the *aceAB* expression construct [111] (see Table 1 for the list of engineered strains obtained).

Table 1. Engineered strains of *Synechocystis* PCC 6803 used/created in Paper I

Engineered strain	Genotype	Reference
WT+Km^r	*Synechocystis* 6803 Δ*psbA2*::*Km*^{r}	[109]
WT+2xPEPc	*Synechocystis* 6803 Δ*psbA2*::*pepC-Km*^{r} *pepC-pepC*	[109]
WT_C	*Synechocystis* 6803 Δ*psbA2*::*Km*^{r} *slr1068*::*Cm*^{r}	This study
2P_C	*Synechocystis* 6803 Δ*psbA2*::*pepC-Km*^{r} *pepC-pepC slr1068*::*Cm*^{r}	This study
2P_I	*Synechocystis* 6803 Δ*psbA2*::*pepC-Km*^{r} *pepC-pepC slr1068*:: P*nrsB-aceA*$_{Ec}$*-Cm*^{r}	This study
2P_IM	*Synechocystis* 6803 Δ*psbA2*::*pepC-Km*^{r} *pepC-pepC slr1068*:: P*nrsB-aceAB*$_{Ec}$*-Cm*^{r}	This study

The partial and complete glyoxylate shunt was successfully overexpressed when *Synechocystis* WT+2xPEPc was used as a background and the strains 2P_I (expressing ICL) and 2P_IM (expressing ICL and MS) were obtained.

The transformation of the glyoxylate shunt genes *aceAB* as an operon in WT *Synechocystis* was not accomplished, since no colonies appeared after transformation. This might be related to the minimal expression of these genes, since the P_{nrsB} promoter has a basal level of expression in BG11 medium, without addition of extra Ni^{2+} for induction [24]. Possibly, the activity of MS may interfere with glyoxylate and 2-phosphoglycolate metabolism in the cell because of glyoxylate consumption by this enzyme. The overexpression of PEPc seemed to relieve this effect on cell metabolism due to elevated oxaloacetate levels, enabling the partitioning of isocitrate towards glyoxylate and malate and thus reducing the effect of MS expression. Only ICL alone was successfully introduced in the WT+Km^r, however, this strain was not further evaluated in the project.

The resulting four strains (Table 1) were compared with respect to extracellular succinate accumulation. For this, different conditions such as standard growth conditions, darkness and anoxic darkness were used. For each condition, different treatments were applied, such as addition of an inhibitor of aerobic respiration, 2-thenoyltriluoroacetone (TTA), and addition of acetate together with TTA. Then, nitrogen depleted media was tested at each treatment together with standard BG11 media. The cells were pre-cultivated in bubbling flasks until OD_{750}~1 and were then concentrated nearly 27 times in BG11 or $BG11_0$, supplemented with inducer (Ni^{2+}) and respective additives (TTA and TTA+Acetate). The obtained results are presented in Figure 4.

Our main findings of this work are the following. Expression of the glyoxylate shunt in *Synechocystis* seems to have a strong impact on cellular metabolism, since it was not possible to obtain WT *Synechocystis* expressing both ICL and MS genes, but only the partial shunt expressing ICL. The highest succinate titer in this study was obtained in darkness with TTA with the strain expressing only ICL, probably due to created shortcut towards succinate, which could function as a carbon sink. In light and standard media, heterologous expression of the glyoxylate shunt genes did not improve extracellular succinate accumulation, when comparing control strain 2P_C to 2P_I and to 2P_IM. Addition of TTA during anoxic darkness conditions resulted in decreased succinate accumulation compared to standard conditions. A possible reason could be the inhibition of succinate formation via fumarate reduction due to either decreased SDH activity or by a possible, yet unknown candidate for fumarate reduction to succinate. Supplementation with acetate increased succinate titers for the strains with extra copies of PEPc compared to the control strain WT_C. However, in this case, there was no difference in succinate production between the control strain 2P_C and the partial or complete glyoxylate shunt strains 2P_I or 2P_IM. Thereby, the overexpression of glyoxylate shunt did not seem to improve the assimilation of acetate in *Synechocystis*, probably due to the absence of PEPCK, the first enzyme of gluconeogenesis [112]. As indicated previously, the acetate uptake is restricted by its transport

inside the cell [113], therefore the overexpression of an acetate transporter could lead to better acetate incorporation.

Altogether, PEPc plays an important role in directing carbon flux towards the TCA cycle, whereas the introduced glyoxylate shunt provides insight in the coordination of the carbon metabolism in the cell.

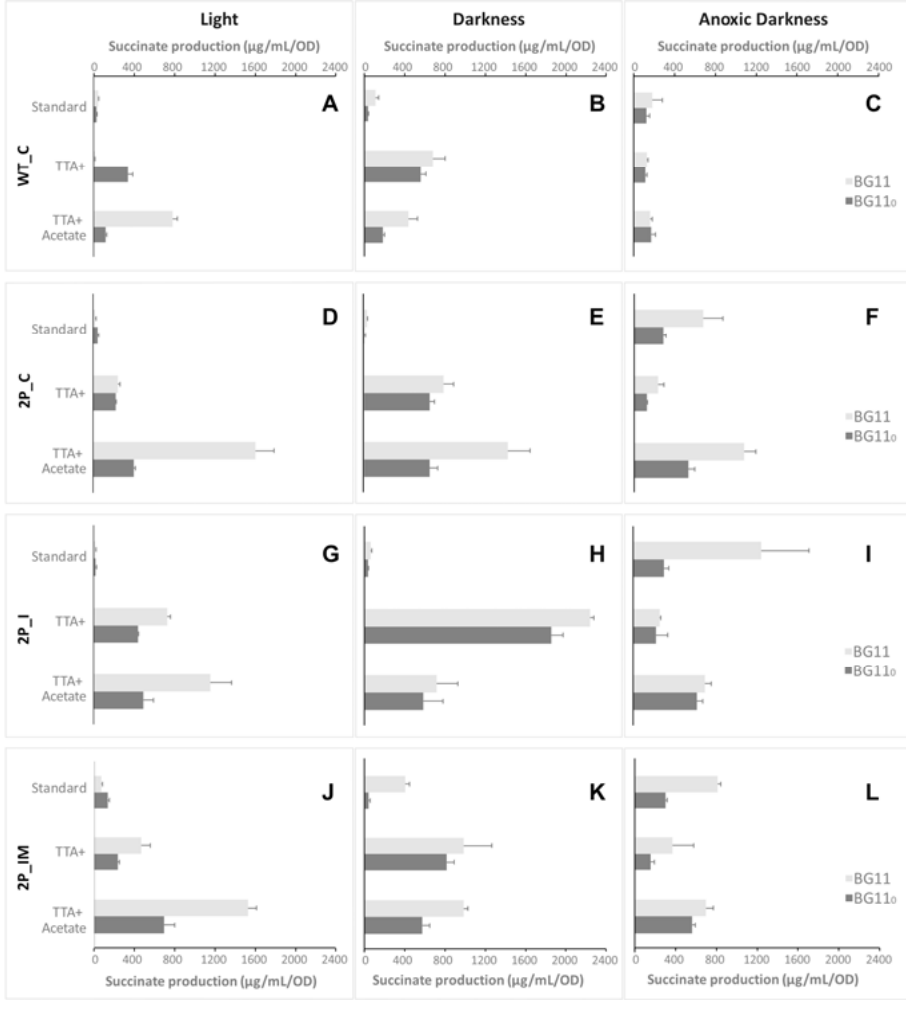

Figure 4. Succinate titres in the growth medium of *Synechocystis* strains created in this study under all tested conditions. BG11 corresponds to media with nitrate, $BG11_0$ corresponds to media without any nitrogen source. Standard corresponds to BG11 or $BG11_0$ and 5 µM of $NiCl_2$ incubated for 4 days. +TTA corresponds to BG11 or $BG11_0$, 5 µM of $NiCl_2$ and 1mM of 2-thenoyltrifluoroacetone (TTA) incubated for 4 days. TTA+Acetate corresponds to BG11 or $BG11_0$, 5 µM of $NiCl_2$, 1mM of TTA, 50 mM of Tris pH 7.5 and 0.2% Acetic acid glacial incubated for 4 days. Reprinted from Paper I.

The role of succinate dehydrogenase and L-Aspartate oxidase in succinate formation in anoxic darkness (Paper III)

The idea of this project originated from Paper I. In the previous study, we observed less secreted succinate after addition of the SDH inhibitor TTA to the media during anoxic darkness. This could further mean that SDH reduces fumarate to succinate, and while the TTA inhibits SDH, less succinate is accumulated. This data was in agreement with the results of metabolite turnover analysis [61], where succinate formation from fumarate in dark anoxic conditions was supported. However, detailed biochemical information of the SDH enzyme complex is absent in cyanobacteria. Additionally, a quadruple knock out mutant of *Synechococcus* PCC 7002 lacking all enzymes known to form succinate accumulated similar succinate levels as the WT strain [60].

These reports in the literature suggest the existence of another route to synthesize succinate from fumarate. Similarly to *E. coli*, the reduction of fumarate to succinate could occur by activity of the flavoenzyme L-aspartate oxidase (Laspo, EC 1.4.3.16) in cyanobacteria. In *E. coli*, specifically, Laspo (Uniprot entry P10902) oxidizes L-aspartate using oxygen or fumarate as electron acceptors forming iminoaspartate and peroxide or succinate, respectively (Figure 5). The choice of substrate is based on the intracellular concentration of oxygen [114]. The Laspo enzymatic reaction is the first step of *de novo* NAD cofactors synthesis and the orthologous genes of this pathway are present in *Synechocystis* [115].

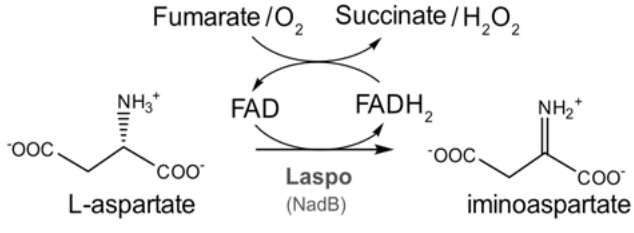

Figure 5. Scheme of the reaction catalysed by Laspo enzyme. Laspo (encoded by *nadB* gene) oxidazes L-aspartate to iminoaspartate using either oxygen or fumarate as electron acceptor for FAD reoxidation and forming oxygen peroxide or succinate correspondingly. Adapted from Paper III

Several *Synechocystis* strains with *nadB* and SDH open reading frames deletions were constructed (see Figure 6A) in order to clarify the contribution of Laspo and SDH to succinate formation in *Synechocystis* by comparing their succinate accumulation titers in anaerobic darkness.

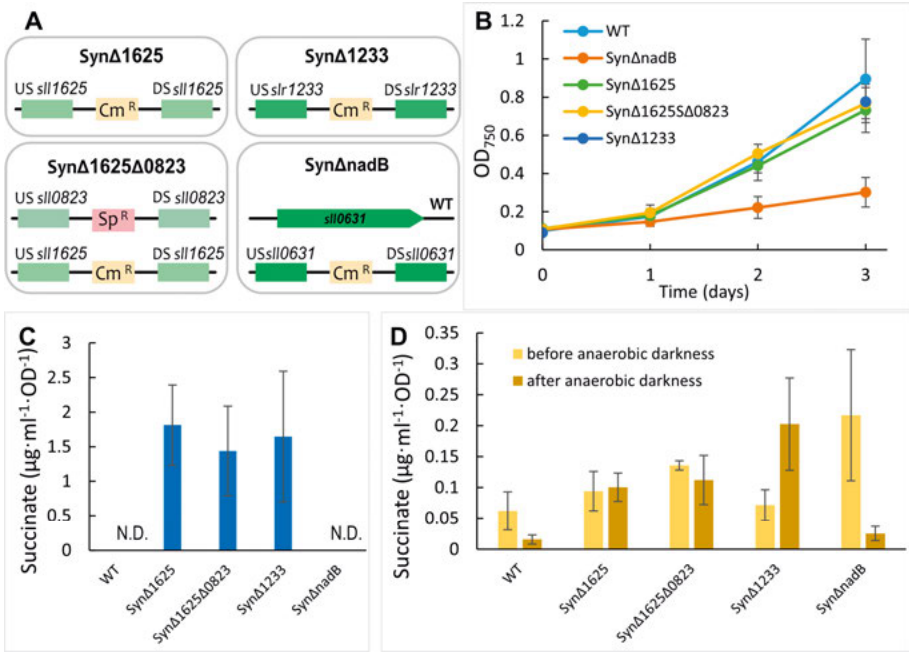

Figure 6. Schematic overview of engineered *Synechocystis* strains, their growth and succinate levels. **A** Illustration of knockout strains of *Synechocystis* created in this study. **B** Growth curves of knockout strains during three days of cultivation. **C** Succinate accumulated in the growth medium after two days of anoxic fermentation. **D** Intracellular succinate detected before and after anoxic fermentation. OD_{750} refers to optical density at 750 nm. N.D. not detected. Error bars represent the standard deviation of three biological replicates. Reprinted from Paper III.

Three ORFs of the SDH complex have been identified in *Synechocystis*, where *sll1625* and *sll0823* encode two homologous iron-sulfur cluster proteins (subunit B, *sdhB*s) and *slr1233* encodes a flavoprotein (subunit A, *sdhA*) [58, 59]. The obtained knock out strains were deficient in both B subunits (SynΔ1625Δ0823), one B subunit (SynΔ1625) or A subunit (SynΔ1233). The inactivation of *nadB* (*sll0631*) encoding Laspo in *Synechocystis* did not result in fully segregated strains and the partial knock out strain SynΔnadB was further used in this study. The SynΔnadB strain exhibited slower growth compared to other strains created in this study (Figure 6B) possibly due to altered TCA cycle metabolites flux or an inability to synthesize required pool of co factors as a result of lower *nadB* gene dosage.

After anaerobic fermentation, strains SynΔ1625Δ0823, SynΔ1625 and SynΔ1233 showed similar levels of secreted succinate, which were higher than in WT and SynΔnadB strains, indicating a succinate dehydrogenase activity of SDH rather than fumarate reduction under these conditions.

Recombinant *Syn*Laspo enzyme was purified in *E. coli* using Strep-Tactin affinity chromatography (the expected size of 62 kDa (including strep-tag) was confirmed by SDS-PAGE, see Figure 3 in Paper III). The activity of purified *Syn*Laspo was determined using an assay based on measuring oxaloacetate levels, formed from spontaneous hydrolysis of iminoaspartate, which is not stable in aqueous solutions [116, 117]. The L-aspartate oxidase activity was determined by steady-state kinetics by varying L-aspartate concentration. The apparent dissociation constant and the turnover number were measured to be K_M 10.2 ± 1 mM and k_{cat} 19.2 ± 1 min^{-1} respectively. Similarly to plant Laspo [118], substrate inhibition occurred with non-physiological L-aspartate concentrations above 30 mM (Figure 7A). The ability of *Syn*Laspo to use fumarate as electron acceptor during oxidation of L-aspartate (L-aspartate-fumarate oxidoreductase activity) was assayed in the anaerobic conditions with the same experimental setup. The results in Figure 7B show the increase of product formation with the addition of fumarate to the reaction mix, and the appearance of a succinate mass peak was confirmed by LC-MS (Paper III supplementary material).

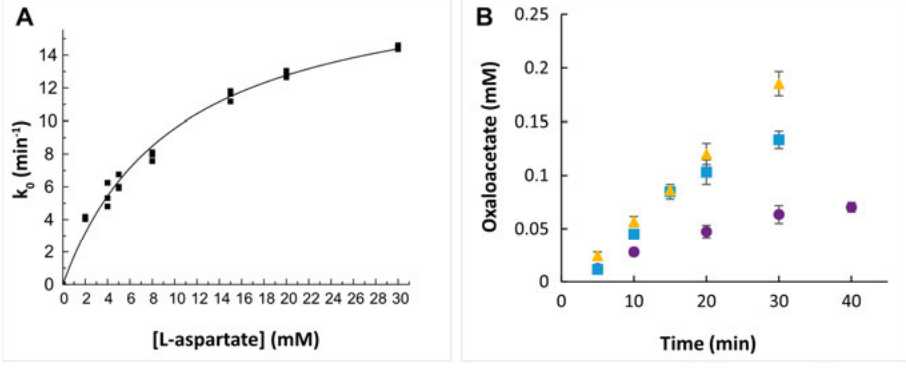

Figure 7. Activity of *Syn*Laspo under aerobic (**A**) and anaerobic conditions (**B**), detecting oxaloacetate formation. **A** Michaelis-Menten fitting of L-aspartate oxidase activity, assayed with varying concentrations of L-aspartate in the reaction mix. Fitting was done using OriginLab software. **B** L-aspartate-fumarate oxidoreductase activity, measured with 20 mM L-aspartate without fumarate (●), with 0.1 mM fumarate (■) and with 0.5 mM fumarate (▲) at 30 ºC. Reprinted from Paper III

Taken together, we were able to demonstrate the L-aspartate-fumarate oxidoreductase activity of *Syn*Laspo, in which fumarate can replace oxygen as an electron acceptor. Given that *Syn*Laspo is functional under anaerobic conditions, we suggest that the reduction of fumarate to succinate during anoxic darkness can be a byproduct of the Laspo reaction, which is the first step in biosynthesis of NAD cofactors. These findings contribute to the understanding of cyanobacterial TCA cycle metabolism and to the future engineering projects of sustainable production of succinate and other dicarboxylic acids.

Production of AAAs and their derivatives in *Synechocystis* (Papers II, IV, V)

Expression of phenylalanine ammonia lyases in *Synechocystis* for phenylpropanoid production (Paper II)

To enable the production of *t*CA, the starting intermediate of phenylpropanoid pathway in plants (Figure 2), we have selected several candidate PAL proteins for expression in *Synechocystis*. Since PAL enzymes are present in all plant species and some bacteria, we have selected a set of genes with different characteristics and origin, which included two *pal* genes from plants, two from filamentous cyanobacteria and one from bacteria [119].

For the bacterial PAL from *Treponema socranskii*, the kinetic data was not available in the literature. During the initial cloning procedures, we discovered that *Ts pal* encodes a Tyrosine ammonia lyase, producing *p*Cou from Tyr. However, by performing directed mutagenesis in a single position, which was shown to correspond to the substrate selectivity (residues 86 and 87 from phenylalanine-histidine were changed to phenylalanine-leucine that confer PAL activity [120]), the substrate selectivity was changed from Tyr to Phe.

Six genes including the modified *Ts pal*, were cloned into two nearly identical self-replication vectors, which differ only by the position of Strep-tag sequence: either N- or C-terminal. This was done in order to evaluate heterologous protein expression and production of *t*CA. After conjugation, 13 strains of *Synechocystis* were obtained including a control strain that only contained an antibiotic resistance cassette (see Table 2).

Producing strains were evaluated compared to each other in order to determine a best-performing candidate. The results after six days of photosynthetic growth showed that highest specific titer of *t*CA (40.2 ± 5.6 mg $L^{-1}OD^{-1}$) was achieved by the AtC strain, which carries *pal* from *Arabidopsis thaliana* with a C-terminal Strep-tag sequence transcribed from the pEEKC plasmid (Figure 8A). We also noted a pronounced difference in the PAL expression levels, where C-terminally tagged PAL proteins showed stronger bands than the ones, which were tagged N-terminally (Figure 8C). This correlated with higher titers of the product in most of the cases. The observed differences in expression levels of the same PAL might be possibly due to the different stability of PAL proteins when expressing an N- or C-terminal tag, as well as mRNA transcript stability. The extra bands of smaller molecular size than the PALs may be due to degradation of the detected proteins. The production of *p*Cou by strains TsC and TsN showed similar values, although TsC protein levels were significantly higher than those of the TsN strain (Figure 8B and C).

Table 2. List of *Synechocystis* strains used in Paper II

Strains	Comment	Antibiotics resistance
EvC	Control strain of *Synechocystis* WT harboring pEEK* plasmid for Km resistance	Km
PcC	*Petroselinum crispum* PAL expressed from Ptrc RBS* in pEEK	Km
AtC	*Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEK	Km
NpC	*Nostoc punctiforme* PAL expressed from Ptrc RBS* in pEEK	Km
AvC	*Anabaena variabilis* PAL expressed from Ptrc RBS* in pEEK	Km
TsC	*Treponema socranskii* PAL expressed from Ptrc RBS* in pEEK	Km
TsC-H87L	*Treponema socranskii* PAL with mutation H87L expressed from Ptrc RBS* in pEEK	Km
PcN	*Petroselinum crispum* PAL expressed from Ptrc RBS* in pEEKN	Km
AtN	*Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEKN	Km
NpN	*Nostoc punctiforme* PAL expressed from Ptrc RBS* in pEEKN	Km
AvN	*Anabaena variabilis* PAL expressed from Ptrc RBS* in pEEKN	Km
TsN	*Treponema socranskii* PAL expressed from Ptrc RBS* in pEEKN	Km
TsN-H87L	*Treponema socranskii* PAL with mutation H87L expressed from Ptrc RBS* in pEEKN	Km
Pc3C	*Petroselinum crispum* PAL expressed from Ptrc RiboJ in pEEK3C	Km
At3C	*Arabidopsis thaliana* PAL expressed from Ptrc RiboJ in pEEK3C	Km
Np3C	*Nostoc punctiforme* PAL expressed from Ptrc RiboJ in pEEK3C	Km
Av3C	*Anabaena variabilis* PAL expressed from Ptrc RiboJ in pEEK3C	Km
Ts3C	*Treponema socranskii* PAL expressed from Ptrc RiboJ in pEEK3C	Km
Ts3C-H87L	*Treponema socranskii* PAL with mutation H87L expressed from Ptrc RiboJ in pEEK3C	Km
Pc3N	*Petroselinum crispum* PAL expressed from Ptrc RiboJ in pEEK3	Km
At3N	*Arabidopsis thaliana* PAL expressed from Ptrc RiboJ in pEEK3	Km
Np3N	*Nostoc punctiforme* PAL expressed from Ptrc RiboJ in pEEK3	Km
Av3N	*Anabaena variabilis* PAL expressed from Ptrc RiboJ in pEEK3	Km
Ts3N	*Treponema socranskii* PAL expressed from Ptrc RiboJ in pEEK3	Km
Ts3N-H87L	*Treponema socranskii* PAL with mutation H87L expressed from Ptrc RiboJ in pEEK3	Km
EvC*Δslr1573*	Control strain of *Synechocystis* WT harboring pEEK* plasmid for Km resistance with knocked-out *slr1573*	Km, Cm
AtC*Δslr1573*	*Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEK with knocked-out *slr1573*	Km, Cm
AtN*Δslr1573*	*Arabidopsis thaliana* PAL expressed from Ptrc RBS* in pEEKN with knocked-out *slr1573*	Km, Cm
TsCΔ*slr1573*	*Treponema socranskii* PAL expressed from Ptrc RBS* in pEEK with knocked-out *slr1573*	Km, Cm
TsN*Δslr1573*	*Treponema socranskii* PAL expressed from Ptrc RBS* in pEEKN with knocked-out *slr1573*	Km, Cm

As the next step to increase the titer of target compounds, we evaluated the use of stronger expression cassettes for increasing PAL protein levels. For this, *pal* genes were cloned into the vectors pEEK3 and pEEK3C, which as before, are differing from one another only by the Strep-tag sequence position. Additionally, in these vectors the promotor sequence is followed by the sequence of self-cleaving ribozyme RiboJ. Applying RiboJ facilitates ribosome binding to mRNA transcripts, which in turn, enables strong and predictable gene expression [32]. As a result of use of these stronger expression cassettes, the variation between C- and N-tagged PAL proteins expression decreased, however, the *t*CA productivity was not improved (see Figure 5 in Paper II). With this, we could conclude that the PAL expression level does not seem to be the bottleneck of *t*CA production in *Synechocystis*.

To further improve the production levels, we knocked out the laccase gene *slr1573* in some of the producing strains, as the discovery of this gene was connected to the decomposition of *p*Cou inside the cell [107]. However, in contrast to the previous study, where authors detected 25-fold increase of *p*Cou titers after the laccase enzyme elimination in *Synechocystis*, in our case the production of target compounds in the *Δslr1573* background was lower when compared to WT background strains (see Figure 8 in Paper II).

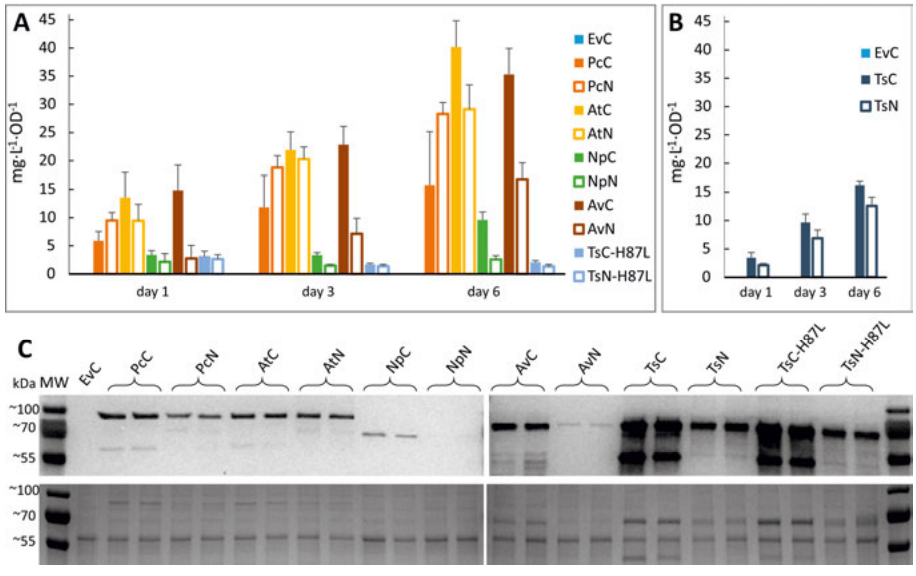

Figure 8. Productivity and Western Blot of *Synechocystis* strains overexpressing PAL or TAL from the pEEK vector. **A** *t*CA production in strains EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN,TsC-H87L and TsN-H87L. **B** *p*Cou production in strains TsC and TsN. Samples of growth medium were taken on day one, three and six. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. **C** Western Blot and SDS-PAGE of extracts from engineered *Synechocystis* strains (EvC, PcC, PcN, AtC, AtN, NpC, NpN, AvC, AvN, TsC-H87L,TsN-H87L,TsC and TsN) analyzed for presence of PAL proteins. Upper panel displays the Western Blot using anti-Strep antibody; bottom panel shows the SDS-PAGE loaded with 5µg of soluble fraction from protein crude extract of the different strains. Reprinted from Paper II.

We also noted that all of the *Δslr1573* strains exhibited a strong phenotype of slower growth rate and lower pigment content, which might be a direct or a secondary effect caused by the deletion of *slr1573* (see figures 9 and 10 in Paper II).

As the final step, we aimed to test whether it will be possible to reach high volumetric titers of molecules of interest by improving the growth conditions. For this purpose, we used a commercial kit for small-scale high-density cultivation CellDEG (www.celldeg.com), which was shown to successfully overcome high-density cultivation limitations [121-123]. The application of a CellDEG platform for growth of the engineered strains resulted in remarkably high volumetric titers for target compounds reaching 797.8 ±153.3 mg L^{-1} of *t*CA by strain AvC and 411.6 ± 94.9 mg L^{-1} of *p*Cou by strain TsC after 4 days of cultivation (Table 3 of paper II). This work contributes to the capability of exploiting the primary metabolism of AAA synthesis in cyanobacteria for sustainable production of plant phenylpropanoids.

Laboratory evolved *Synechocystis* for AAA and phenylpropanoid production (Paper IV)

In this project, instead of performing the genetic manipulations to promote AAA secretion [100], we have applied selective pressure of Phe, which inhibits the growth of some cyanobacteria [80, 81] in order to obtain spontaneous resistant mutants as was noted previously [124].

Growth inhibition by externally provided Phe is attributed to the inhibition of the first enzyme in the pathway, DAHPS, which leads to the starvation for Tyr and Trp and subsequently causes the decrease of phycocyanin content and yellow coloration of culture similarly to chlorosis [80]. During the cultivation of *Synechocystis* EvC control strain (see Table 3 for the list of strains used in this study) with different Phe concentrations in the medium, we demonstrated that the Phe consumption during growth inhibition appears to be minimal (see Figure 9C). The growth recovers due to the appearance of resistant cells, whereas their emergence frequency seems to depend on the concentration of provided Phe (Figure 9A). Possibly, the growth inhibition is a result of the "intake-only" operation mode of the amino acid transporters [124, 125], because of which the externally provided Phe will be captured by cells despite that it leads to growth impairment.

Table 3. List of *Synechocystis* strains used in Paper IV

Strain	Comment	Antibiotic resistance	Reference
EvC	Empty vector control strain of *Synechocystis* WT harboring pEEK* plasmid for Km resistance	Km	[119]
AtC	Based *Synechocystis* WT harboring *Arabidopsis thaliana* PAL expressed from P*trc* RBS* in pEEK	Km	[119]
TsC	Based *Synechocystis* WT harboring *Treponema socranskii* TAL expressed from P*trc* RBS* in pEEK	Km	[119]

Strains based on spontaneous resistant mutants obtained in this study

Name	Comment	Name	Comment	Antibiotic resistance
AtPRM1	Same as PRM1, but carries *At-pal*-pEEK	**TsPRM1**	Same as PRM1, but carries *Ts-tal*-pEEK	Km
AtPRM3	Same as PRM3, but carries *At-pal*-pEEK	**TsPRM3**	Same as PRM3, but carries *Ts-tal*-pEEK	Km
AtPRM4	Same as PRM4, but carries *At-pal*-pEEK	**TsPRM4**	Same as PRM4, but carries *Ts-tal*-pEEK	Km
AtPRM5	Same as PRM5, but carries *At-pal*-pEEK	**TsPRM5**	Same as PRM5, but carries *Ts-tal*-pEEK	Km
AtPRM6	Same as PRM6, but carries *At-pal*-pEEK	**TsPRM6**	Same as PRM6, but carries *Ts-tal*-pEEK	Km
AtPRM7	Same as PRM7, but carries *At-pal*-pEEK	**TsPRM7**	Same as PRM7, but carries *Ts-tal*-pEEK	Km
AtPRM8	Same as PRM8, but carries *At-pal*-pEEK	**TsPRM8**	Same as PRM8, but carries *Ts-tal*-pEEK	Km
AtPRM10	Same as PRM10, but carries *At-pal*-pEEK	**TsPRM10**	Same as PRM10, but carries *Ts-tal*-pEEK	Km
AtPRM11	Same as PRM11, but carries *At-pal*-pEEK	**TsPRM11**	Same as PRM11, but carries *Ts-tal*-pEEK	Km
AtPRM12	Same as PRM12, but carries *At-pal*-pEEK	**TsPRM12**	Same as PRM12, but carries *Ts-tal*-pEEK	Km

The individual Phe resistant colonies were obtained after spreading *Synechocystis* WT cells on BG11 agar plates supplemented with Phe and re-inoculated into liquid BG11 medium with increased Phe concentration several times. This way ten phenylalanine resistant mutants (PRM1-12) were collected, which were resistant to 12 mM Phe in the medium.

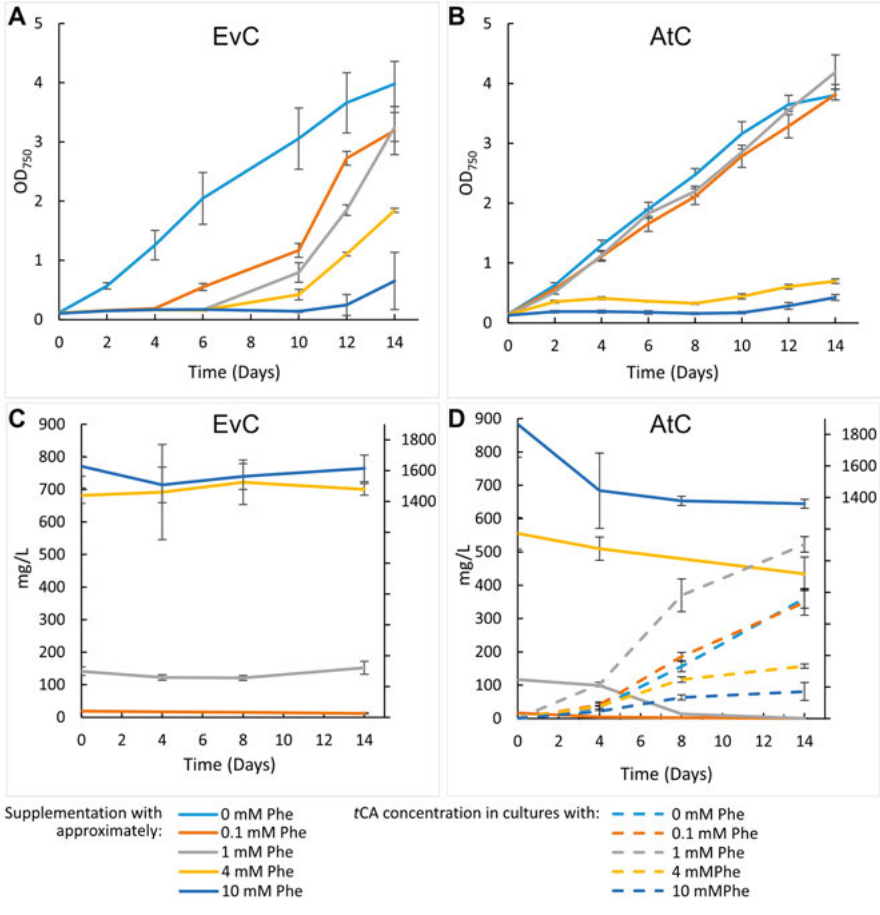

Figure 9. Growth of of EvC (**A**) and AtC (**B**) with addition of various Phe concentrations during 14 days under constant 45 µmol photons m^{-2} s^{-1} light intensity. Concentration of Phe in the growth medium of EvC (**C**) and AtC (**D**) cultures. Note that data from growth experiments of cultures with 10 mM Phe in panels **C** and **D** are displayed on the secondary axes on the right, for better visualization of the values. Error bars represent standard deviation of the biological replicates. Reprinted from Paper IV.

The resulting mutants were able to secrete the aromatic amino acids Phe and Tyr into the growth medium. Comparative growth and production experiments showed that PRMs had similar specific production of Phe, with a maximum obtained level of 9.06 ±0.65 mg $L^{-1}OD^{-1}$ for PRM10 after eight days of photoautotrophic growth (Figure 10A). To test the potential of Phe secretion by PRMs, high-density cultivation was used, which resulted in lower specific production than in shake flasks, with the exception of one mutant, PRM8, which reached the highest specific production of 24.9 ±7.2 mg $L^{-1}OD^{-1}$ or 610 ± 195.6 mg L^{-1} after four days (Figure 10B).

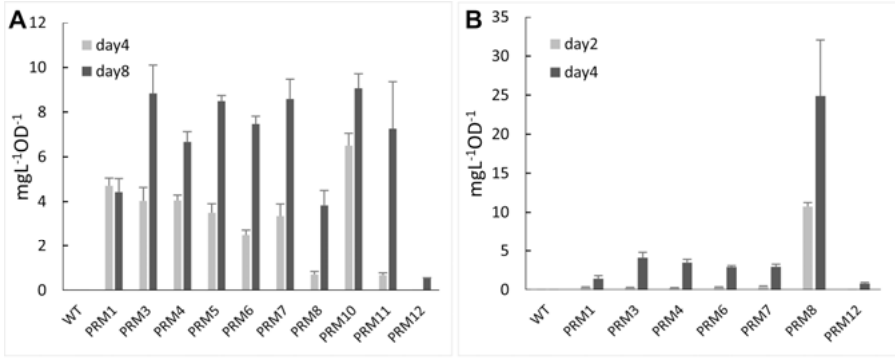

Figure 10. Comparison of Phe production in PRMs during **A** standard shake flask cultivation **B** high-density cultivation. Samples were taken from the growth medium at day four and eight of shake flaks experiment and on days two and four of HDC. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation of three biological replicates. Adapted from paper IV.

We aimed to investigate, whether the lower productivity of HDC is related to the reuptake of Phe during rapid growth. We pereformed another experiment, where HDC cultures on day two were supplemented with approximately 10mM of Phe as the final concentration. Indeed, the supplemented Phe was partly incorporated in WT and PRM1 cultures, whereas in case of PRM8, the Phe concentration in the medium increased (see figure 5A in Paper IV), which may indicate transporter mutation(s). The secretion of AAAs in the medium is likely to be attributed to the high rate of Phe synthesis due to overflow of precursors to the end products caused by pathway deregulation [100], and the slower rate of intake by transport systems.

In order to determine whether PRMs can reach higher productivity of *t*CA and *p*Cou compared to the AtC and TsC with WT *Synechocystis* background (Paper II), we overexpressed PAL and TAL in the mutant strains, generating two groups of mutant strains, AtPRMs and TsPRMs (Table 3). However, the *t*CA productivity decreased nearly four times for some AtPRMs compared to control AtC strain, whereas the *p*Cou production remained in a similar range between TsPRMs and TsC (Figure 11A and C). The decrease of *t*CA production in AtPRMs compared to the WT background seems very puzzling and indicates that the shikimate pathway regulation is more complex in these mutants and cannot solely be explained by the loss of feedback-inhibition sensitivity of DAHPS.

To further examine the potential of PRMs for phenylapropanoid production, we cultivated AtPRMs and TsPRMs in high-density conditions. The results demonstrated same production patterns relative to the corresponding WT background strain as in shake flasks experiment, except for AtPRM8 and TsPRM8 (Figure 11B an C). Both these strains reached higher specific production than the corresponding control strains with 52.7±14 mg $L^{-1}OD^{-1}$ and

a final titer 1116.7±314 mg L^{-1} of *t*CA for AtPRM8 and 47.1±6 mg L-*1* OD^{-1} with a final titer of 1108.8±149.7 mg L^{-1} *p*Cou for TsPRM8 after four days of high-density cultivation. However, unlike Phe, the secreted *t*CA and *p*Cou by AtPRMs and TsPRMs are not metabolized. Thus, the high productivity of PRM8 is not only connected to the transport system mutation(s).

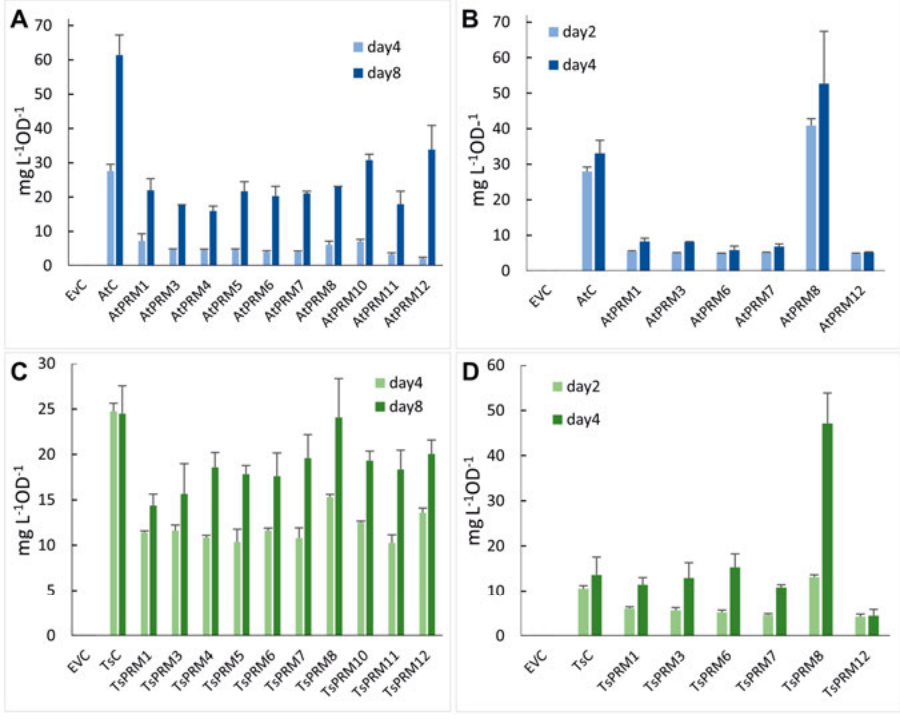

Figure 11. Comparison of *t*CA production in AtPRMs during **A** standard shake flask cultivation **B** high-density cultivation; comparison of *p*Cou production in TsPRMs during **C** standard shake flask cultivation and **D** high-density cultivation. Samples were taken from the growth medium at day four and eight of shake flaks experiment and on days two and four of HDC. The values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation of three biological replicates. Adapted from paper IV.

Altogether, the different productivity of Phe and reporter molecules by different PRM variants indicate diverse mutations that were acquired as an adaptive mechanism against Phe inhibition. Whole genome sequencing would be necessary to reveal which mutations appeared in the mutants. This information is of high importance for understanding cyanobacterial metabolism and regulation of AAA synthesis, and will help to identify novel targets and strategies for the enhancement of production rates in *Synechocystis*.

Further enhancement of phenypropanoid production in *Synechocystis* (Paper V)

In Paper II, *t*CA and *p*Cou production in *Synechocystis* was reported to reach 49.6±8.7mg L^{-1} of *t*CA and 25.6±3.9 mg L^{-1} of *p*Cou at day six, which is still below the highest reported values [100], although due to different growth conditions and normalisation used, the comparison of production rates is problematic. Therefore, in Paper V we focused on further enhancement of *Synechocystis* productivity for these compounds via evaluating novel targets in the shikimate pathway (Figure 2).

Firstly, we knocked out the HPPD that oxidases HPP to homogentisate, which may result in an increase of AAA precursors inside the cells and subsequently enhance the *t*CA and *p*Cou productivity. We also intended to test the effect of overexpression of several heterologous aromatic transaminases, since this metabolic step of the shikimate pathway is poorly understood. The tyrosine aminotransferase *tyrB* from *E.coli*, the hypothetical AAA transaminase *tyrB* from *Nostoc* sp. 3335mG and the modified plant prephanate aminotransferase PAA-AT (gene name abbreviated as *pat* in this study) from *Arabidopsis thaliana* with two amino acid substitutions T84V and K169V, which change the enzyme activity to HPP transamination into Tyr, were used in this study. It is plausible, that the heterologous transaminases insensitive to native regulation mechanisms will facilitate the turnover of their substrates. In order to increase the carbon flux from chorismate to the Phe and Tyr terminal branch, the CM domain ($pheA_{CM}$) of the fused chorismate mutase/prephenate dehydratase enzyme from *E. coli* was overexpressed. It was demonstrated previously, that when the CM domain is expressed alone, it retains catalytic activity and becomes insensitive to L-Phe inhibition [126]. To enhance the *p*Cou productivity, the cyclohexadienyl dehydrogenase from *Zymomonas mobilis*, encoded by *tyrC* was overexpressed [126]. In combination with HPDD knockout and heterologous transaminases and/or the CM domain, the created metabolic route from prephenate into HPP and Tyr might be favourable for *p*Cou production.

The series of pPDD integrative plasmids were built to recombine with the *pdd* locus encoding HPPD. Each target gene either was cloned alone into the pPDD plasmid, or was assembled in an operon. The resulting constructs were transformed into three *Synechocystis* strains: WT, AtC, TsC [119], generating a series of strains that overproduce AAA, *t*CA or *p*Cou respectively. Plasmids and strains created in this study are listed in Table 4.

Engineered *Synechocystis* strains were cultivated under constant light intensity for seven days and sampled on days four and seven for LC-MS measurements of growth medium in order to compare the productivity of the obtained strains. No Phe or Tyr was detected in the culture medium of strains with WT background. It appears, that the overexpression of abovementioned genes in an operon or alone, were not sufficient to promote secretion of Phe

or Tyr from the cell. However, the Western-blot analysis is needed to clarify the protein expression in the obtained strains.

The specific production titer of accumulated *t*CA and *p*Cou in the growth medium during photoautotrophic growth is presented in Figure 12. The *t*CA production of the ΔpddAt strain was nearly 1.5 times higher than of the parental strain AtC accumulating 138±3.5 mg L^{-1} or 77.2±3.7 mg $L^{-1}OD^{-1}$ of *t*CA.

Table 4. List of plasmids and strains used in Paper V

Construct/Strain	Comment	Antibiotic resistance	Reference
pEERM3	Empty backbone based on pJ344 vector backbone for integration into neutral site 1 (*slr1068*) of *Synechocystis* 6803, carrying P*nrsB* promotor and RBS*	Cm	[127]
pPDD	Same as pEERM3 but with P*trc2O* promotor, RBS* and N-terminal strep-tag sequence and homologous recombination regions to *slr0090*	Em	This study
AtC	*Synechocystis* strain harboring pEEK shuttle vector expressing *Arabidopsis thaliana* PAL from *Ptrc* RBS*	Km	[119]
TsC	*Synechocystis* strain harboring pEEK shuttle vector expressing *Treponema socranskii* TAL from *Ptrc* RBS*	Km	[119]

Construct	Genes expressed	*Synechocystis* background used for transformation		
		WT	AtC	TsC
		Strain name		
pPDD		ΔpddWT	ΔpddAt	ΔpddTs
pPDD-BE	*tyrB* from *E. coli*	ΔpddE	ΔpddAtE	ΔpddTsE
pPDD-BN	*tyrB* from *Nostoc*	ΔpddN	ΔpddAtN	ΔpddTsN
pPDD-P	*pat*	ΔpddP	ΔpddAtP	ΔpddTsP
pPDD-C	*tyrC*	ΔpddC (not fully segregated)	ΔpddAtC	ΔpddTsC (not fully segregated)
pPDD-CM	$pheA_{CM}$	ΔpddCM	ΔpddAtCM	ΔpddTsCM
pPDD-BE-C	*tyrB* from *E. coli, tyrC*	-	ΔpddAtE-C	ΔpddTsE-C
pPDD-BN-C	*tyrB* from *Nostoc, tyrC*	ΔpddN-C	ΔpddAtN-C	ΔpddTsN-C
pPDD-P-C	*PAT, tyrC*	ΔpddP-C	-	-
pPDD-BE-CM	*tyrB* from *E. coli,* $pheA_{CM}$	ΔpddE-CM	ΔpddAtE-CM	ΔpddTsE-CM
pPDD-BN-CM	*tyrB* from *Nostoc,* $pheA_{CM}$	ΔpddN-CM	ΔpddAtN-CM	ΔpddTsN-CM
pPDD-P-CM	*PAT,* $pheA_{CM}$	ΔpddP-CM	ΔpddAtP-CM	ΔpddTsP-CM
pPDD-C-CM	*tyrC,* $pheA_{CM}$	-	-	ΔpddTsC-CM
pPDD-BE-C-CM	*tyrB* from *E. coli, tyrC,* $pheA_{CM}$	ΔpddE-C-CM (not fully segregated)	ΔpddAtE-C-CM	ΔpddTsE-C-CM (not fully segregated)
pPDD-BN-C-CM	*tyrB* from *Nostoc, tyrC,* $pheA_{CM}$	ΔpddN-C-CM	ΔpddAtN-C-CM	ΔpddTsN-C-CM
pPDD-P-C-CM	*PAT, tyrC,* $pheA_{CM}$	-	-	-

'-'indicates that no colonies appeared after transformation.

The overexpression of transaminases individually as well as the *tyrC* gene alone showed no production improvements compared to ΔpddAt, suggesting that the titer increase was due to *pdd* deletion. In the case of overexpression of the CM domain insensitive to Phe inhibition, the *t*CA accumulation decreased compared to AtC, possibly due to the inhibition of prephenate dehydratase by the end product Phe (Figures 2 and 3). The overexpression of transaminases in combination with *tyrC* and/or $pheA_{CM}$ also did not increase the *t*CA synthesis. Similarly to what was observed with *t*CA production, there was no significant difference of *p*Cou titers among the strains with a Δpdd knockout. The productivity of *p*Cou in almost all engineered strains (except

ΔpddTsN-CM) was increased nearly 1.5 times comparing to the control strain TsC, while the ΔpddTs strain reached 32.0±4.3 mg L^{-1} OD^{-1} or 72.3±10.3 mg L^{-1} of *p*Cou after 7 days of photoautotrophic growth (Figure 12B).

The obtained results demonstrate that only the HPPD inactivation was sufficient to promote the *t*CA and *p*Cou synthesis. Cyanobacteria exhibit endo-oriented control of the multibranched aromatic biosynthetic pathway as suggested previously, where the pathway regulation is tuned to the endogenous formation of the initial pathway substrates [92]. Thus, the earliest branching point enzyme monitoring flow has the major role on the end-product regulation [92]. As was observed previously by overexpression of feedback-insensitive DAHPS [100], the loss of early-pathway regulation provides sufficient elevation of substrate levels, and eliminates the flow restriction by terminal branch enzymes leading to extracellular accumulation of Phe and Tyr. Similarly, in our study, there was no Phe or Tyr secretion observed with overexpression of the terminal enzymatic steps in Phe and Tyr synthesis.

Thereby, the overexpression of a feedback resistant version of DAHP synthase, the first enzyme in the shikimate pathway, will be the next step in continuation of this project. The results of the whole genome sequencing of PRM variants in Paper IV, would possibly suggest new targets for pathway engineering in order to enhance production of AAAs and their derivatives in *Synechocystis*.

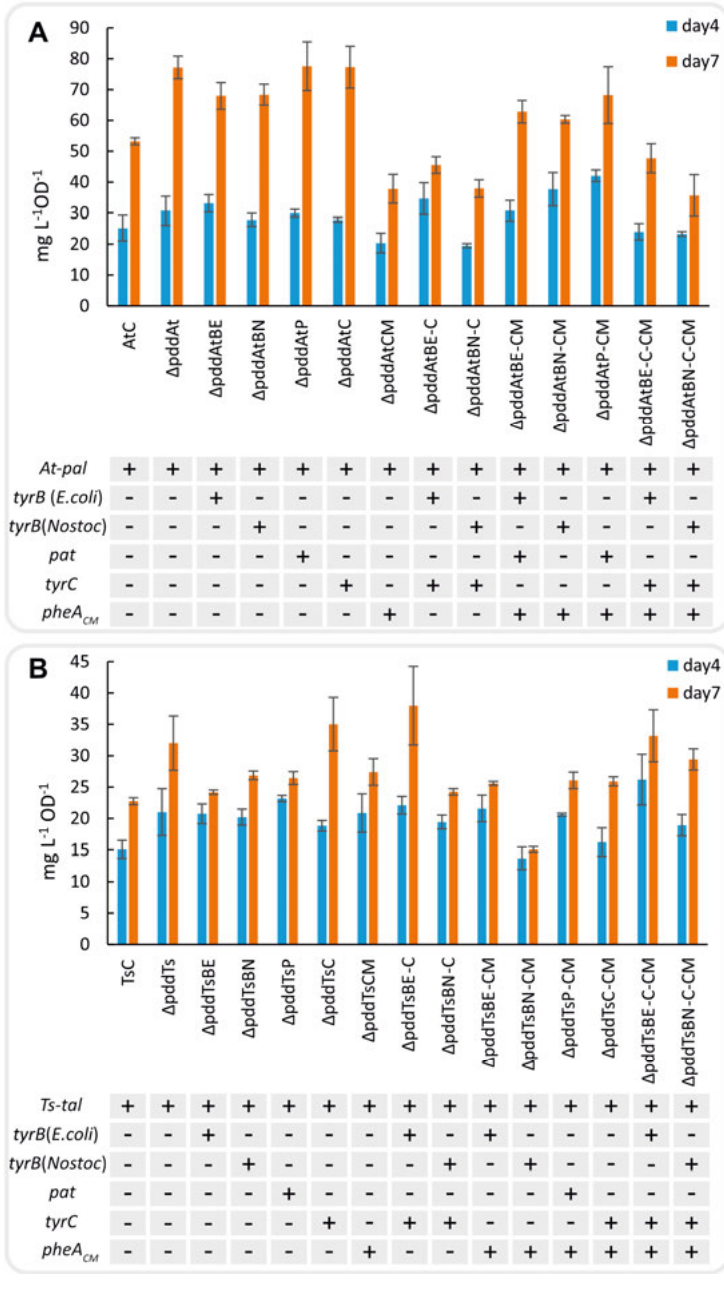

Figure 12. Specific production titer of *t*CA (**A**) and *p*Cou (**B**) of *Synechocystis* strains engineered in this study during photoautotrophic growth. Values are the means of three biological replicates and two technical replicates, error bars represent the standard deviation. Reprinted from Paper V.

Conclusions and outlook

The data obtained in my doctoral thesis expands the knowledge of cyanobacterial metabolism and helps developing cyanobacteria as a platform to produce renewable chemicals in a sustainable manner. I have demonstrated the effect of glyoxylate shunt on succinate secretion in *Synechocystis* PCC 6803. Furthermore, I investigated the succinate formation from fumarate during anaerobic darkness conditions. I was able to demonstrate *in vitro* that the enzyme L-aspartate oxidase reduces fumarate to succinate as a by-product of L-aspartate oxidation. It is likely that in anaerobic darkness the cells are secreting succinate due to this activity and not due to succinate dehydrogenase. These findings advance our understanding of cyanobacterial TCA cycle metabolism and will be helpful in designing strategies for succinate production using anaerobic fermentation. Nonetheless, biochemical characterization of the succinate dehydrogenase complex would be needed to fully address its catalytic properties and potential fumarate reductase activity. So far, this membrane-associated enzymatic complex was never studied *in vitro* in cyanobacteria.

The use of *Synechocystis* for production of aromatic compounds was also detailed in this thesis. I have demonstrated the production of *trans*-cinnamic (*t*CA) and *p*-coumaric (*p*Cou) acids, the first intermediates of plant phenylpropanoids pathway after overexpression of phenylalanine- and tyrosine ammonia lyases (PAL and TAL, respectively). Aiming to enhance the productivity, I found out that the elimination of a competing pathway, the tocopherols biosynthesis, increases the *t*CA and *p*Cou production. Moreover, I performed the laboratory evolution of *Synechocystis* and have obtained several metabolic mutants that were able to secrete phenylalanine in the growth medium. After PAL and TAL overexpression, the obtained mutant strains demonstrated different *t*CA and *p*Cou productivity in respect to control strains. Identifying which mutations caused the phenotype would provide important information and will help designing novel targets for improved aromatic compounds production. The combination of resistant mutants, particularly PRM8 with the overexpression of genes from the terminal steps of the pathway, would be a next goal, since the elevated flux through shikimate pathway in the mutant may promote higher productivity of the target aromatic molecules. It would be also interesting to use the process of the growth inhibition by phenylalanine and appearance of resistant cells as a model for studying the molecular mechanisms that are responsible for mutagenesis. So far, the mechanisms behind

genetic instability in cyanobacteria, such as loss of expression constructs, have not been completely resolved.

Svensk sammanfattning

Cyanobakterier är kända för att orsaka algblomning i sjöar och hav. Algblomning orsakar fiskdöd och förgiftningar som påverkar miljön negativt. Detta fenomen uppstår vanligtvis på grund av ökade nivåer av kväve och fosfor som sköljs ner i vattendrag efter gödsling av grödor. Cyanobakterier är fotosyntetiserande bakterier som har utvecklats på jorden under miljoner år. De kan precis som växter utföra fotosyntes, där vattenmolekyler klyvs med hjälp av solenergi för att skapa elektroner och en protongradient över cellernas lipidmembran. Energin som skapas med denna gradient används för att producera kemiska energibärare som i sin tur används för att fixera atmosfärens koldioxid till organiska föreningar. Klyvningen av vattenmolekylen leder till att en syremolekyl skapas och släpps ut i miljön. Fotosyntetiserande cyanobakterier var det som från början gav upphov till syret i atmosfären, och i förlängningen, livet på jorden som det ser ut idag. Cyanobakterierna är en spridd och framgångsrik organismgrupp som hittas i de flesta olika miljöer, i allt från öknar till glaciärer.

På senare tid har man upptäckt att cyanobakterier, precis som de mer välkända heterotroferna Escherichia coli och Saccharomyces cerevisiae, kan användas för att producera industrirelevanta kemikalier. Men till skillnad mot heterotrofa organismer som behöver en organisk kolkälla, vanligtvis i form av socker, kan cyanobakterier använda koldioxid från atmosfären för sin tillväxt. Med modern rekombinant DNA-teknologi och genteknik kan cyanobakterier modifieras för att producera industriellt intressanta molekyler.

Genteknik och metabolisk ingenjörskonst bygger på förståelse för molekylärbiologins centrala dogm. Metabolismen kan beskrivas som alla kemiska reaktioner som sker i en levande organism, och dessa reaktioner katalyseras av enzymer som accelererar bildandet av nya molekyler. Varje enzym består av kedjor av olika aminosyror bundna i sekvens till varandra med peptidbindningar, och aminosyrasekvensen definierar strukturen och egenskaperna hos enzymet. Informationen om enzymens aminosyrasekvens finns i cellens tanskriptom i form av mRNA, där en aminosyra representeras av ett kodon bestående av tre av de fyra kvävebaserna. Kodonen översätts till aminosyror i translationsprocessen som katalyseras av cellens ribosomer. Informationen om vilka kodon som ska finnas i cellens mRNA finns lagrad cellens gener i form av DNA. Ett enzym kodas alltså av ett mRNA som i sin tur kodas av en gen i cellens kromosom. När man vill modifiera en organism genetiskt för att

ge den en ny funktion, till exempel kunna skapa en viss kemisk förening, så kopierar man genen som kodar för enzymet som katalyserar skapandet av föreningen. Denna genkopia sätt sedan in i cellens DNA med hjälp av rekombinant teknologi.

I denna avhandling har jag modifierat cyanobakterien Synechocystis PCC 6803 för att kunna producera succinat, aromatiska aminosyror och andra liknande molekyler som är intressanta för kemikalieindustrin. För att åstadkomma detta riktade jag in mig på de metaboliska reaktionsvägar där dessa molekyler skapas. Succinat är en naturlig förekommande metabolit i cytronsyracykeln. Denna reaktionsväg är en primär energikälla hos heterotrofer. Men i cyanobakterier, där fotosyntesen är den dominerande energikällan, är citronsyrecykelns funktion närmare kopplad till kväveupptag och aminosyrasyntes. Genom att införa nya reaktionsvägar i anslutning till citronsyracykeln har jag lyckats förändra metabolitflödena runt det centrala enzymet PEPc. Med dessa förändringar har jag lyckats öka cellens utsöndring av succinat. I ett annat projekt har jag undersökt vilket enzym som ligger bakom succinatproduktionen under anaerobisk fermentation, när syre saknas och fotosyntes inte är möjlig. Jag upptäckte ett möjligt alternativ till enzymet succinatdehydrogenase (som normalt förknippas med succinatproduktione) som verkar kunna ligga bakom produktionen under dessa förhållanden. Jag har lyckats visa att det enzymet, som kallas L-aspartatoxidas, kan skapa succinat in vitro och troligen även in vivo. Denna upptäckt ökar vår förståelse för hur citronsyracykeln fungerar i cyanobakterier, och bäddar för fler intressanta och användbara genetiska modifieringar i framtiden.

Aromatiska aminosyror, fenylalanin, tyrosin och tryptofan, syntetiseras i cyanobakterier i det så kallade skikamatflödet som är en metabolitisk reaktionsväg med sju steg. Hos växter är de aromatiska aminosyrorna startmetaboliter i lång och komplicerade reaktionsväg där många olika aromatiska ämnen skapas. Dessa ämnen fyller en mängd olika funktioner i växten men vissa är även intressanta för människan. I ett av mina forskningsprojekt modifierade jag Synechocystis för att skapa fenylpropandioler som är metaboliter i denna reaktionsväg. Jag förde in de första enzymerna i denna reaktionskedja, som kallas PAL och TAL och katalyserar reaktionerna där metaboliterna tCA och pCou skapas från sina substrat, i Synechocystis. Med dessa modifieringar, tillsammans med genteknisk eliminering av närliggande reaktionsvägar, har jag lyckats skapa en stam av Synechocystis som producerar betydande halter av tCA och pCou, metaboliter som alltså inte naturligt förekommer i denna organism. I ett andra steg applicerade jag dessa genetiska modifikationer på laboratorieevolverade stammar av Synechocystis som jag utvecklat för att utsöndra fenylalanin. De resulterande stammarna visade signifikant ökad produktion jämfört med kontrollsammar. Sammanfattningsvis så har forskningen i denna avhandling ökat kunskapen om cyanobakteriers metabolism och bidragit med nya cyanobakteriestammar som kan producera industriellt intressanta kemikalier från förnyelsebara källor.

Summary in English

Cyanobacteria are widely known for causing the "algal blooms" in open waters during summer. Algal blooms are accompanied with the accumulation of toxins in water and fish death because of low oxygen levels due to oxygen consumption when the biomass is degraded. These events usually appear as a result of increased nitrogen and phosphorous concentration in waters, while these minerals get into the water from crops fertilizers. Now, cyanobacteria are photosynthetic bacteria that appeared and evolved on Earth billions of years ago. Just like plants, they can perform oxygenic photosynthesis, meaning that cyanobacteria can split water molecules, using solar energy to create an electron and proton gradient across the membrane for synthesizing the energy rich molecules that in turn will be used to assimilate carbon dioxide CO_2 into organic compounds. As the result of water splitting molecular oxygen is released into the environment. This feature of oxygenic photosynthesis in cyanobacteria was the cause for the gradual increase of oxygen concentration in the early Earth atmosphere creating the life as we see it now. Cyanobacteria are the most diverse and most successful group of microbes, which can be found in all the types of environments, from deserts to glaciers.

Recently, cyanobacteria were considered as attractive organisms, which can be used to produce industrially relevant chemicals, similarly to other well-known heterotrophic organisms such as *Escherichia coli* or *Saccharomyces cerevisiae.* However, unlike heterotrophs, which require an organic carbon source, usually in a form of sugars, cyanobacteria use CO_2 from the atmosphere, water and sunlight for growth. With the use of recombinant DNA technologies, cyanobacteria can be genetically engineered to make them produce desired natural or heterologous chemical compounds.

The principal of genetic engineering is based on the central dogma of molecular biology. The metabolism can be represented as all chemical reactions that are happening in a living organism performed by proteins, which accelerate reactions and chemical rearrangements. Each enzyme consists of the sequence of amino acids bound to each other with a peptide bound and arranged in a certain sequence. The amino acid sequence, called polypepide, defines the structure and properties of a mature folded enzyme. The polypeptide sequence is synthesized based on messenger RNA molecule during translation process on ribosomes in a code manner, where one amino acid is represented by a three-letter code from four ribonucleic acids. The mRNA is synthesized on

DNA molecule during transcription, and each ribonucleotide monomer in a sequence of mRNA correspond to a deoxyribonucleotide monomer in sequence of DNA on the chromosome. Briefly, one protein is synthesized on one mRNA polymer molecule, which is encoded by one DNA gene sequence. Thereby, when we want to engineer an organism (host) to give it a function, for instance, of synthesizing a new metabolite, we copy the gene sequence that encodes a protein, which catalyses the desired reaction from another organism and introduce it into the host using recombinant DNA technologies.

In this thesis, I aimed to engineer the cyanobacterial strain *Synechocystis* PCC 6803 to produce industrially relevant molecules, succinate and aromatic amino acids and their derivatives. For this, I looked into the pathways where these molecules are formed. Succinate is a natural metabolite which is synthesized in the tricarboxylic acids cycle (TCA cycle). The TCA cycle in oxygenic heterotrophs is a main source of energy for cells, however in cyanobacteria, where photosynthesis is the main energy obtaining process, TCA cycle functionality is connected to the synthesis of carbon skeletons for nitrogen assimilation and amino acid synthesis. The enzymatic activity of phosphoenolpyruvate (PEPc) is essential in replenishing oxaloacetate, an intermediate of the TCA cycle. I wanted to explore if more succinate can be secreted out by cells after introducing alternative pathway for succinate synthesis, the glyoxylate shunt, into a previously created strain with extra copies of PEPc. As a result, some obtained strains showed improved succinate secretion.

In the next project, I questioned what enzyme is responsible for the succinate formation from fumarate by fumarate reduction during conditions of dark anaerobic fermentation, when oxygen is absent and photosynthesis is not possible. I found out that there is an alternative potential candidate L-aspartate oxidase, apart from succinate dehydrogenase complex that is suggested to perform this reaction. I purified L-aspartate oxidase enzyme and after checking its biochemical activity, found out that it is able to reduce fumarate to succinate during the L-aspartate oxidation *in vitro*, and possibly, in the cell *in vivo*. These findings contribute to the understanding of the cyanobacterial TCA cycle for future engineering purposes.

Aromatic amino acids, such as phenylalanine, tyrosine and tryptophan are synthesized in the cyanobacteria in the shikimate pathway. In plants, they are the staring intermediates of the phenylpropanoid pathway, where a myriad of aromatic molecules are synthesized, which have various functions in plants and also have broad applications for human use. I aimed to engineer *Synechocystis* to produce phenylpropanoids. Therefore, the first enzymes of this pathway, phenylalanine- and tyrosine ammonia lyases (PAL and TAL respectively), which produce *trans*-cinnamic (*t*CA) and *p*-coumaric (*p*Cou) acids from phenylalanine and tyrosine respectively, were introduced into *Synechocystis*. The *t*CA and *p*Cou compounds are not native to *Synechocystis* and not metabolized inside of the cell, which makes them good reporter molecules for the pathway engineering. Aiming to enhance their formation, I found that the

elimination of a competing pathway, the tocopherols biosynthesis, increases the *t*CA and *p*Cou productivity. Moreover, I performed laboratory evolution of *Synechocystis* and have obtained several metabolic mutants that are able to secrete phenylalanine in the growth medium. After overexpressing PAL or TAL in the mutants, the resulting strains based on one mutant showed superior productivity than the control strains. The laboratory evolved *Synechocystis* mutants represent an important material for studying the regulation of the pathway for aromatic amino acids synthesis.

In summary, the findings in my doctoral thesis expand the knowledge of cyanobacterial metabolism and help developing cyanobacteria as a platform to produce renewable chemicals in a sustainable manner.

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References

1. AR, I. W. (2014) Climate change 2014: Impacts, adaptation, and vulnerability, *Summary for Policymakers*, 1-44.
2. Hoegh-Guldberg, O., Jacob, D., Bindi, M., Brown, S., Camilloni, I., Diedhiou, A., Djalante, R., Ebi, K., Engelbrecht, F. & Guiot, J. (2018) Impacts of 1.5 C global warming on natural and human systems, *Global warming of 15° C*.
3. Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E. & Madamwar, D. (2011) Cyanobacteria and microalgae: a positive prospect for biofuels, *Bioresource technology*. **102**, 10163-10172.
4. Brocks, J. J., Logan, G. A., Buick, R. & Summons, R. E. (1999) Archean molecular fossils and the early rise of eukaryotes, *science*. **285**, 1033-1036.
5. Knoll, A. H. (2008) Cyanobacteria and earth history, *The cyanobacteria: molecular biology, genomics, and evolution*. **484**.
6. Vermaas, W. F. (2001) Photosynthesis and respiration in cyanobacteria, *e LS*.
7. Lea-Smith, D. J., Bombelli, P., Vasudevan, R. & Howe, C. J. (2016) Photosynthetic, respiratory and extracellular electron transport pathways in cyanobacteria, *Biochimica et Biophysica Acta (BBA)-Bioenergetics*. **1857**, 247-255.
8. Stanier, R. & Cohen-Bazire, G. (1977) Phototrophic prokaryotes: the cyanobacteria, *Annual review of microbiology*. **31**, 225-274.
9. Markou, G. & Georgakakis, D. (2011) Cultivation of filamentous cyanobacteria (blue-green algae) in agro-industrial wastes and wastewaters: a review, *Applied Energy*. **88**, 3389-3401.
10. Wu, L., Zhu, Q., Yang, L., Li, B., Hu, C. & Lan, S. (2018) Nutrient transferring from wastewater to desert through artificial cultivation of desert cyanobacteria, *Bioresource technology*. **247**, 947-953.
11. Cui, J., Sun, T., Chen, L. & Zhang, W. (2020) Engineering salt tolerance of photosynthetic cyanobacteria for seawater utilization, *Biotechnology Advances*. **43**, 107578.
12. Włodarczyk, A., Selão, T. T., Norling, B. & Nixon, P. J. (2020) Newly discovered *Synechococcus* sp. PCC 11901 is a robust cyanobacterial strain for high biomass production, *Communications biology*. **3**, 1-14.
13. Jaiswal, D., Sengupta, A., Sohoni, S., Sengupta, S., Phadnavis, A. G., Pakrasi, H. B. & Wangikar, P. P. (2018) Genome features and biochemical characteristics of a robust, fast growing and naturally transformable cyanobacterium *Synechococcus elongatus* PCC 11801 isolated from India, *Scientific reports*. **8**, 1-13.
14. Jaiswal, D., Sengupta, A., Sengupta, S., Madhu, S., Pakrasi, H. B. & Wangikar, P. P. (2020) A novel cyanobacterium *Synechococcus elongatus* PCC 11802 has distinct genomic and metabolomic characteristics compared to its neighbor PCC 11801, *Scientific reports*. **10**, 1-15.
15. Yu, J., Liberton, M., Cliften, P. F., Head, R. D., Jacobs, J. M., Smith, R. D., Koppenaal, D. W., Brand, J. J. & Pakrasi, H. B. (2015) *Synechococcus*

elongatus UTEX 2973, a fast growing cyanobacterial chassis for biosynthesis using light and CO2, *Scientific reports.* **5**, 1-10.
16. Branco dos Santos, F., Du, W. & Hellingwerf, K. J. (2014) *Synechocystis*: not just a plug-bug for CO_2, but a green *E. coli*, *Frontiers in bioengineering and biotechnology.* **2**, 36.
17. Mur, L., Gons, H. & Van Liere, L. (1978) Competition of the green alga *Scenedesmus* and the blue-green alga *Oscillatoria* in light limited environments, *FEMS Microbiol Lett.* **1**, 335-338.
18. Kaneko, T. & Tabata, S. (1997) Complete genome structure of the unicellular cyanobacterium *Synechocystis* sp. PCC6803, *Plant and Cell Physiology.* **38**, 1171-1176.
19. Mills, L. A., McCormick, A. J. & Lea-Smith, D. J. (2020) Current knowledge and recent advances in understanding metabolism of the model cyanobacterium *Synechocystis* sp. PCC 6803, *Bioscience reports.* **40**.
20. Nielsen, J. (2001) Metabolic engineering, *Applied microbiology and biotechnology.* **55**, 263-283.
21. Heidorn, T., Camsund, D., Huang, H.-H., Lindberg, P., Oliveira, P., Stensjö, K. & Lindblad, P. (2011) Synthetic biology in cyanobacteria: engineering and analyzing novel functions in *Methods in enzymology* pp. 539-579, Elsevier.
22. Huang, H.-H., Camsund, D., Lindblad, P. & Heidorn, T. (2010) Design and characterization of molecular tools for a synthetic biology approach towards developing cyanobacterial biotechnology, *Nucleic acids research.* **38**, 2577-2593.
23. Wang, B., Eckert, C., Maness, P.-C. & Yu, J. (2018) A genetic toolbox for modulating the expression of heterologous genes in the cyanobacterium *Synechocystis* sp. PCC 6803, *ACS synthetic biology.* **7**, 276-286.
24. Englund, E., Liang, F. & Lindberg, P. (2016) Evaluation of promoters and ribosome binding sites for biotechnological applications in the unicellular cyanobacterium Synechocystis sp. PCC 6803, *Scientific reports.* **6**, 36640.
25. Peca, L., Kos, P. & Vass, I. (2007) Characterization of the activity of heavy metal-responsive promoters in the cyanobacterium *Synechocystis* PCC 6803, *Acta Biologica Hungarica.* **58**, 11-22.
26. Kelly, C. L., Taylor, G. M., Hitchcock, A., Torres-Mendez, A. & Heap, J. T. (2018) A rhamnose-inducible system for precise and temporal control of gene expression in cyanobacteria, *ACS synthetic biology.* **7**, 1056-1066.
27. Behle, A., Saake, P., Germann, A. T., Dienst, D. & Axmann, I. M. (2020) Comparative dose–response analysis of inducible promoters in cyanobacteria, *ACS Synthetic Biology.* **9**, 843-855.
28. Ferreira, E. A., Pacheco, C. C., Pinto, F., Pereira, J., Lamosa, P., Oliveira, P., Kirov, B., Jaramillo, A. & Tamagnini, P. (2018) Expanding the toolbox for *Synechocystis* sp. PCC 6803: validation of replicative vectors and characterization of a novel set of promoters, *Synthetic Biology.* **3**, ysy014.
29. Mutalik, V. K., Guimaraes, J. C., Cambray, G., Lam, C., Christoffersen, M. J., Mai, Q.-A., Tran, A. B., Paull, M., Keasling, J. D. & Arkin, A. P. (2013) Precise and reliable gene expression via standard transcription and translation initiation elements, *Nature methods.* **10**, 354-360.
30. Wegelius, A., Li, X., Turco, F. & Stensjö, K. (2018) Design and characterization of a synthetic minimal promoter for heterocyst-specific expression in filamentous cyanobacteria, *PloS one.* **13**, e0203898.
31. Englund, E., Shabestary, K., Hudson, E. P. & Lindberg, P. (2018) Systematic overexpression study to find target enzymes enhancing production of terpenes

in *Synechocystis* PCC 6803, using isoprene as a model compound, *Metabolic engineering.* **49**, 164-177.
32. Lou, C., Stanton, B., Chen, Y.-J., Munsky, B. & Voigt, C. A. (2012) Ribozyme-based insulator parts buffer synthetic circuits from genetic context, *Nature biotechnology.* **30**, 1137-1142.
33. Schirmacher, A. M., Hanamghar, S. S. & Zedler, J. A. (2020) Function and benefits of natural competence in cyanobacteria: From ecology to targeted manipulation, *Life.* **10**, 249.
34. Kunert, A., Hagemann, M. & Erdmann, N. (2000) Construction of promoter probe vectors for *Synechocystis* sp. PCC 6803 using the light-emitting reporter systems Gfp and LuxAB, *Journal of microbiological methods.* **41**, 185-194.
35. Satoh, S., Ikeuchi, M., Mimuro, M. & Tanaka, A. (2001) Chlorophyll b expressed in cyanobacteria functions as a light-harvesting antenna in photosystem I through flexibility of the proteins, *Journal of Biological Chemistry.* **276**, 4293-4297.
36. Jacobsen, J. H. & Frigaard, N.-U. (2014) Engineering of photosynthetic mannitol biosynthesis from CO_2 in a cyanobacterium, *Metabolic engineering.* **21**, 60-70.
37. Takahama, K., Matsuoka, M., Nagahama, K. & Ogawa, T. (2003) Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the *psbAI* locus, *Journal of bioscience and bioengineering.* **95**, 302-305.
38. Jones, P. R. (2014) Genetic instability in cyanobacteria–an elephant in the room?, *Frontiers in bioengineering and biotechnology.* **2**, 12.
39. Lim, G. (2020) *Investigation of the Genes sll1354, sll1165, and sll1520 in Genomic Instability in Cyanobacterium Synechocystis sp. PCC 6803*, University of Otago.
40. Wang, B., Xu, Y., Wang, X., Yuan, J. S., Johnson, C. H., Young, J. D. & Yu, J. (2021) A guanidine-degrading enzyme controls genomic stability of ethylene-producing cyanobacteria, *Nature communications.* **12**, 1-13.
41. Ungerer, J. & Pakrasi, H. B. (2016) Cpf1 is a versatile tool for CRISPR genome editing across diverse species of cyanobacteria, *Scientific reports.* **6**, 1-9.
42. Yao, L., Cengic, I., Anfelt, J. & Hudson, E. P. (2016) Multiple gene repression in cyanobacteria using CRISPRi, *ACS synthetic biology.* **5**, 207-212.
43. Huang, C. H., Shen, C. R., Li, H., Sung, L. Y., Wu, M. Y. & Hu, Y. C. (2016) CRISPR interference (CRISPRi) for gene regulation and succinate production in cyanobacterium S. elongatus PCC 7942, *Microb Cell Fact.* **15**, 196.
44. Higo, A., Isu, A., Fukaya, Y. & Hisabori, T. (2016) Efficient gene induction and endogenous gene repression systems for the filamentous cyanobacterium Anabaena sp. PCC 7120, *Plant and Cell Physiology.* **57**, 387-396.
45. Higo, A., Isu, A., Fukaya, Y. & Hisabori, T. (2017) Designing synthetic flexible gene regulation networks using RNA devices in cyanobacteria, *ACS Synthetic Biology.* **6**, 55-61.
46. Oliver, J. W. & Atsumi, S. (2015) A carbon sink pathway increases carbon productivity in cyanobacteria, *Metabolic engineering.* **29**, 106-112.
47. Ducat, D. C., Avelar-Rivas, J. A., Way, J. C. & Silver, P. A. (2012) Rerouting carbon flux to enhance photosynthetic productivity, *Applied and environmental microbiology.* **78**, 2660-2668.
48. Deshpande, A., Vue, J. & Morgan, J. (2020) Combining random mutagenesis and metabolic engineering for enhanced tryptophan production in Synechocystis sp. strain PCC 6803, *Applied and environmental microbiology.* **86**, e02816-19.

49. Woodley, J. M. (2022) Integrating protein engineering into biocatalytic process scale-up, *Trends in Chemistry*.
50. Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Žídek, A. & Potapenko, A. (2021) Highly accurate protein structure prediction with AlphaFold, *Nature.* **596**, 583-589.
51. McLure, R. J., Radford, S. E. & Brockwell, D. J. (2022) High-throughput directed evolution: a golden era for protein science, *Trends in Chemistry*.
52. Cukalovic, A. & Stevens, C. V. (2008) Feasibility of production methods for succinic acid derivatives: a marriage of renewable resources and chemical technology, *Biofuels, Bioproducts and Biorefining*. **2**, 505-529.
53. Yin, X., Li, J., Shin, H.-d., Du, G., Liu, L. & Chen, J. (2015) Metabolic engineering in the biotechnological production of organic acids in the tricarboxylic acid cycle of microorganisms: advances and prospects, *Biotechnology advances.* **33**, 830-841.
54. Xiong, W., Brune, D. & Vermaas, W. F. (2014) The γ□aminobu tyric acid shunt contributes to closing the tricarboxylic acid cycle in S ynechocystis sp. PCC 6803, *Molecular microbiology.* **93**, 786-796.
55. Zhang, S. & Bryant, D. A. (2011) The tricarboxylic acid cycle in cyanobacteria, *Science.* **334**, 1551-1553.
56. Cooley, J. W. & Vermaas, W. F. (2001) Succinate dehydrogenase and other respiratory pathways in thylakoid membranes of Synechocystis sp. strain PCC 6803: capacity comparisons and physiological function, *J Bacteriol.* **183**, 4251-8.
57. Song, H. & Lee, S. Y. (2006) Production of succinic acid by bacterial fermentation, *Enzyme and microbial technology.* **39**, 352-361.
58. Cooley, J. W., Howitt, C. A. & Vermaas, W. F. (2000) Succinate: quinol oxidoreductases in the cyanobacterium Synechocystis sp. strain PCC 6803: presence and function in metabolism and electron transport, *Journal of Bacteriology.* **182**, 714-722.
59. Mock, M., Schmid, A. & Bühler, K. (2019) Photoautotrophic production of succinate via the oxidative branch of the tricarboxylic acid cycle influences glycogen accumulation in Synechocystis sp. PCC 6803, *Algal Research.* **43**, 101645.
60. Zhang, S., Qian, X., Chang, S., Dismukes, G. C. & Bryant, D. A. (2016) Natural and Synthetic Variants of the Tricarboxylic Acid Cycle in Cyanobacteria: Introduction of the GABA Shunt into Synechococcus sp. PCC 7002, *Front Microbiol.* **7**, 1972.
61. Hasunuma, T., Matsuda, M. & Kondo, A. (2016) Improved sugar-free succinate production by Synechocystis sp. PCC 6803 following identification of the limiting steps in glycogen catabolism, *Metab Eng Commun.* **3**, 130-141.
62. Zhang, S. & Bryant, D. A. (2015) Biochemical Validation of the Glyoxylate Cycle in the Cyanobacterium Chlorogloeopsis fritschii Strain PCC 9212, *J Biol Chem.* **290**, 14019-30.
63. Cronan Jr, J. E. & Laporte, D. (2005) Tricarboxylic acid cycle and glyoxylate bypass, *EcoSal Plus.* **1**.
64. Gruendel, M., Knoop, H. & Steuer, R. (2017) Activity and functional properties of the isocitrate lyase in the cyanobacterium Cyanothece sp. PCC 7424, *Microbiology.* **163**, 731-744.
65. Mock, M., Schmid, A. & Bühler, K. (2020) Directed reaction engineering boosts succinate formation of Synechocystis sp. PCC 6803_Δsll1625, *Biotechnology Journal*, 2000127.

66. Sengupta, A., Pritam, P., Jaiswal, D., Bandyopadhyay, A., Pakrasi, H. B. & Wangikar, P. P. (2020) Photosynthetic Co-Production of Succinate and Ethylene in A Fast-Growing Cyanobacterium, Synechococcus elongatus PCC 11801, *Metabolites.* **10**.
67. Sengupta, S., Jaiswal, D., Sengupta, A., Shah, S., Gadagkar, S. & Wangikar, P. P. (2020) Metabolic engineering of a fast-growing cyanobacterium Synechococcus elongatus PCC 11801 for photoautotrophic production of succinic acid, *Biotechnol Biofuels.* **13**, 89.
68. Lan, E. I. & Wei, C. T. (2016) Metabolic engineering of cyanobacteria for the photosynthetic production of succinate, *Metab Eng.* **38**, 483-493.
69. Hasunuma, T., Matsuda, M., Kato, Y., Vavricka, C. J. & Kondo, A. (2018) Temperature enhanced succinate production concurrent with increased central metabolism turnover in the cyanobacterium Synechocystis sp. PCC 6803, *Metab Eng.* **48**, 109-120.
70. Osanai, T., Shirai, T., Iijima, H., Nakaya, Y., Okamoto, M., Kondo, A. & Hirai, M. Y. (2015) Genetic manipulation of a metabolic enzyme and a transcriptional regulator increasing succinate excretion from unicellular cyanobacterium, *Front Microbiol.* **6**, 1064.
71. Ueda, S., Kawamura, Y., Iijima, H., Nakajima, M., Shirai, T., Okamoto, M., Kondo, A., Hirai, M. Y. & Osanai, T. (2016) Anionic metabolite biosynthesis enhanced by potassium under dark, anaerobic conditions in cyanobacteria, *Sci Rep.* **6**, 32354.
72. Iijima, H., Watanabe, A., Sukigara, H., Iwazumi, K., Shirai, T., Kondo, A. & Osanai, T. (2021) Four-carbon dicarboxylic acid production through the reductive branch of the open cyanobacterial tricarboxylic acid cycle in *Synechocystis* sp. PCC 6803, *Metabolic Engineering.* **65**, 88-98.
73. Li, H., Shen, C. R., Huang, C. H., Sung, L. Y., Wu, M. Y. & Hu, Y. C. (2016) CRISPR-Cas9 for the genome engineering of cyanobacteria and succinate production, *Metab Eng.* **38**, 293-302.
74. Mo, H., Xie, X., Zhu, T. & Lu, X. (2017) Effects of global transcription factor *NtcA* on photosynthetic production of ethylene in recombinant *Synechocystis* sp. PCC 6803, *Biotechnology for biofuels.* **10**, 1-13.
75. Averesch, N. J. H. & Kromer, J. O. (2018) Metabolic Engineering of the Shikimate Pathway for Production of Aromatics and Derived Compounds-Present and Future Strain Construction Strategies, *Front Bioeng Biotechnol.* **6**, 32.
76. Marienhagen, J. & Bott, M. (2013) Metabolic engineering of microorganisms for the synthesis of plant natural products, *Journal of biotechnology.* **163**, 166-178.
77. Sprenger, G. A. (2006) Aromatic amino acids in *Amino acid biosynthesis~ pathways, regulation and metabolic engineering* pp. 93-127, Springer.
78. Nowicka, B. & Kruk, J. (2016) Cyanobacteria use both *p*-hydroxybenozate and homogentisate as a precursor of plastoquinone head group, *Acta Physiologiae Plantarum.* **38**, 1-12.
79. Riccardi, G., de Rossi, E. & Milano, A. (1989) Amino acid biosynthesis and its regulation in cyanobacteria, *Plant Science.* **64**, 135-151.
80. Hall, G. C. & Jensen, R. A. (1980) Enzymological basis for growth inhibition by L-phenylalanine in the cyanobacterium *Synechocystis* sp. 29108, *Journal of Bacteriology.* **144**, 1034-1042.
81. Hall, G., Flick, M., Gherna, R. & Jensen, R. (1982) Biochemical diversity for biosynthesis of aromatic amino acids among the cyanobacteria, *Journal of bacteriology.* **149**, 65-78.

82. Schenck, C. A., Holland, C. K., Schneider, M. R., Men, Y., Lee, S. G., Jez, J. M. & Maeda, H. A. (2017) Molecular basis of the evolution of alternative tyrosine biosynthetic routes in plants, *Nature Chemical Biology.* **13**, 1029-1035.
83. Dähnhardt, D., Falk, J., Appel, J., van der Kooij, T. A., Schulz-Friedrich, R. & Krupinska, K. (2002) The hydroxyphenylpyruvate dioxygenase from *Synechocystis* sp. PCC 6803 is not required for plastoquinone biosynthesis, *FEBS letters.* **523**, 177-181.
84. Yadav, V., Wang, Z., Wei, C., Amo, A., Ahmed, B., Yang, X. & Zhang, X. (2020) Phenylpropanoid pathway engineering: An emerging approach towards plant defense, *Pathogens.* **9**, 312.
85. Williams, J. S., Thomas, M. & Clarke, D. J. (2005) The gene *stlA* encodes a phenylalanine ammonia-lyase that is involved in the production of a stilbene antibiotic in *Photorhabdus luminescens* TT01, *Microbiology.* **151**, 2543-50.
86. Zhu, L., Cui, W., Fang, Y., Liu, Y., Gao, X. & Zhou, Z. (2013) Cloning, expression and characterization of phenylalanine ammonia-lyase from *Rhodotorula glutinis*, *Biotechnol Lett.* **35**, 751-6.
87. Kyndt, J., Meyer, T., Cusanovich, M. & Van Beeumen, J. (2002) Characterization of a bacterial tyrosine ammonia lyase, a biosynthetic enzyme for the photoactive yellow protein, *FEBS letters.* **512**, 240-244.
88. Nishiyama, Y., Yun, C.-S., Matsuda, F., Sasaki, T., Saito, K. & Tozawa, Y. (2010) Expression of bacterial tyrosine ammonia-lyase creates a novel *p*-coumaric acid pathway in the biosynthesis of phenylpropanoids in *Arabidopsis*, *Planta.* **232**, 209-218.
89. Winkel-Shirley, B. (2001) Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology, *Plant physiology.* **126**, 485-493.
90. Moffitt, M. C., Louie, G. V., Bowman, M. E., Pence, J., Noel, J. P. & Moore, B. S. (2007) Discovery of two cyanobacterial phenylalanine ammonia lyases: kinetic and structural characterization, *Biochemistry.* **46**, 1004-1012.
91. Dempo, Y., Ohta, E., Nakayama, Y., Bamba, T. & Fukusaki, E. (2014) Molar-based targeted metabolic profiling of cyanobacterial strains with potential for biological production, *Metabolites.* **4**, 499-516.
92. Jensen, R. A. & Hall, G. C. (1982) Endo-oriented control of pyramidally arranged metabolic branchpoints, *Trends in Biochemical Sciences.* **7**, 177-180.
93. Brown, K. & Doy, C. (1966) Control of three isoenzymic 7-phospho-2-oxo-3-deoxy-d-arabino-heptonate-d-erythrose-4-phosphate lyases of *Escherichia coli* W and derived mutants by repressive and "inductive" effects of the aromatic amino acids, *Biochimica et Biophysica Acta (BBA)-Enzymology and Biological Oxidation.* **118**, 157-172.
94. Hall, G. C., Flick, M. & Jensen, R. (1983) Regulation of the aromatic pathway in the cyanobacterium *Synechococcus* sp. strain PCC6301 (*Anacystis nidulans*), *Journal of bacteriology.* **153**, 423-428.
95. Hall, G. C. & Jensen, R. (1981) Regulatory isozymes of 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase in the cyanobacterium *Anabaena* sp. strain ATCC 29151, *Journal of Bacteriology.* **148**, 361-364.
96. Ogawa, T., Marco, E. & Orus, M. I. (1994) A gene (*ccmA*) required for carboxysome formation in the cyanobacterium *Synechocystis* sp. strain PCC6803, *Journal of bacteriology.* **176**, 2374-2378.
97. GETHING, M. J. H. & DAVIDSON, B. E. (1976) Chorismatemutase/prephenate dehydratase from *Escherichia coli* K12: 2. evidence for identical subunits catalysing the two activities, *European Journal of Biochemistry.* **71**, 327-336.

98. Rodriguez, A., Martnez, J. A., Flores, N., Escalante, A., Gosset, G. & Bolivar, F. J. M. c. f. (2014) Engineering *Escherichia coli* to overproduce aromatic amino acids and derived compounds. **13**, 126.
99. Legrand, P., Dumas, R., Seux, M., Rippert, P., Ravelli, R., Ferrer, J. L. & Matringe, M. (2006) Biochemical characterization and crystal structure of *Synechocystis* arogenate dehydrogenase provide insights into catalytic reaction, *Structure.* **14**, 767-76.
100. Brey, L. F., Włodarczyk, A. J., Thøfner, J. F. B., Burow, M., Crocoll, C., Nielsen, I., Nielsen, A. J. Z. & Jensen, P. E. (2020) Metabolic engineering of *Synechocystis* sp. PCC 6803 for the production of aromatic amino acids and derived phenylpropanoids, *Metabolic engineering.* **57**, 129-139.
101. Lütke-Eversloh, T. & Stephanopoulos, G. (2005) Feedback inhibition of chorismate mutase/prephenate dehydrogenase (TyrA) of *Escherichia coli*: generation and characterization of tyrosine-insensitive mutants, *Applied and Environmental Microbiology.* **71**, 7224-7228.
102. Chemler, J. A., Fowler, Z. L., McHugh, K. P. & Koffas, M. A. (2010) Improving NADPH availability for natural product biosynthesis in *Escherichia coli* by metabolic engineering, *Metabolic engineering.* **12**, 96-104.
103. Xiong, W., Cano, M., Wang, B., Douchi, D. & Yu, J. (2017) The plasticity of cyanobacterial carbon metabolism, *Current Opinion in Chemical Biology.* **41**, 12-19.
104. Ikeda, M. (2006) Towards bacterial strains overproducing L-tryptophan and other aromatics by metabolic engineering, *Applied microbiology and biotechnology.* **69**, 615-626.
105. Ni, J., Liu, H. Y., Tao, F., Wu, Y. T. & Xu, P. (2018) Remodeling of the Photosynthetic Chain Promotes Direct CO2 Conversion into Valuable Aromatic Compounds, *Angew Chem Int Ed Engl.* **57**, 15990-15994.
106. Gao, E.-B., Kyere-Yeboah, K., Wu, J. & Qiu, H. (2021) Photoautotrophic production of *p*-Coumaric acid using genetically engineered *Synechocystis* sp. Pasteur Culture Collection 6803, *Algal Research.* **54**, 102180.
107. Xue, Y., Zhang, Y., Cheng, D., Daddy, S. & He, Q. (2014) Genetically engineering *Synechocystis* sp. Pasteur Culture Collection 6803 for the sustainable production of the plant secondary metabolite *p*-coumaric acid, *Proc Natl Acad Sci U S A.* **111**, 9449-54.
108. Tiruveedula, G. S. S. & Wangikar, P. P. (2017) Gene essentiality, conservation index and co-evolution of genes in cyanobacteria, *PLoS One.* **12**, e0178565.
109. Durall, C., Rukminasari, N. & Lindblad, P. (2016) Enhanced growth at low light intensity in the cyanobacterium *Synechocystis* PCC 6803 by overexpressing phosphoenolpyruvate carboxylase, *Algal Research.* **16**, 275-281.
110. Scholl, J., Dengler, L., Bader, L. & Forchhammer, K. (2020) Phosphoenolpyruvate carboxylase from the cyanobacterium *Synechocystis* sp. PCC 6803 is under global metabolic control by PII signaling, *Mol Microbiol*.
111. Durall, C., Kukil, K., Hawkes, J. A., Albergati, A., Lindblad, P. & Lindberg, P. (2021) Production of succinate by engineered strains of *Synechocystis* PCC 6803 overexpressing phosphoenolpyruvate carboxylase and a glyoxylate shunt, *Microbial cell factories.* **20**, 1-14.
112. Gründel, M., Knoop, H. & Steuer, R. (2017) Activity and functional properties of the isocitrate lyase in the cyanobacterium *Cyanothece* sp. PCC 7424, *Microbiology.* **163**, 731-744.
113. Thiel, K., Vuorio, E., Aro, E. M. & Kallio, P. T. (2017) The effect of enhanced acetate influx on *Synechocystis* sp. PCC 6803 metabolism, *Microb Cell Fact.* **16**, 21.

114. Tedeschi, G., Negri, A., Mortarino, M., Ceciliani, F., Simonic, T., Faotto, L. & Ronchi, S. (1996) L□Aspartate Oxidase from *Escherichia coli*: II. Interaction with C4 Dicarboxylic Acids and Identification of a Novel L□Aspa rtate: Fumarate Oxidoreductase Activity, *European journal of biochemistry.* **239**, 427-433.
115. Gerdes, S. Y., Kurnasov, O. V., Shatalin, K., Polanuyer, B., Sloutsky, R., Vonstein, V., Overbeek, R. & Osterman, A. L. (2006) Comparative genomics of NAD biosynthesis in cyanobacteria, *J Bacteriol.* **188**, 3012-23.
116. Yamada, R.-h., Nagasaki, H., Wakabayashi, Y. & Iwashima, A. (1988) Presence of D-aspartate oxidase in rat liver and mouse tissues, *Biochimica et Biophysica Acta (BBA)-General Subjects.* **965**, 202-205.
117. Marinoni, I., Nonnis, S., Monteferrante, C., Heathcote, P., Härtig, E., Böttger, L. H., Trautwein, A. X., Negri, A., Albertini, A. M. & Tedeschi, G. (2008) Characterization of L□aspartate o xidase and quinolinate synthase from Bacillus subtilis, *The FEBS journal.* **275**, 5090-5107.
118. Hao, J., Petriacq, P., de Bont, L., Hodges, M. & Gakiere, B. (2018) Characterization of l-aspartate oxidase from Arabidopsis thaliana, *Plant Sci.* **271**, 133-142.
119. Kukil, K. & Lindberg, P. (2022) Expression of phenylalanine ammonia lyases in Synechocystis sp. PCC 6803 and subsequent improvements of sustainable production of phenylpropanoids, *Microbial cell factories.* **21**, 1-16.
120. Watts, K. T., Mijts, B. N., Lee, P. C., Manning, A. J. & Schmidt-Dannert, C. (2006) Discovery of a substrate selectivity switch in tyrosine ammonia-lyase, a member of the aromatic amino acid lyase family, *Chem Biol.* **13**, 1317-26.
121. Rodrigues, J. S. & Lindberg, P. (2021) Metabolic engineering of *Synechocystis* sp. PCC 6803 for improved bisabolene production, *Metabolic engineering communications.* **12**, e00159.
122. Dienst, D., Wichmann, J., Mantovani, O., Rodrigues, J. S. & Lindberg, P. (2020) High density cultivation for efficient sesquiterpenoid biosynthesis in *Synechocystis* sp. PCC 6803, *Scientific reports.* **10**, 1-16.
123. Lippi, L., Bähr, L., Wüstenberg, A., Wilde, A. & Steuer, R. (2018) Exploring the potential of high-density cultivation of cyanobacteria for the production of cyanophycin, *Algal research.* **31**, 363-366.
124. Labarre, J., Thuriaux, P. & Chauvat, F. (1987) Genetic analysis of amino acid transport in the facultatively heterotrophic cyanobacterium *Synechocystis* sp. strain 6803, *Journal of bacteriology.* **169**, 4668-4673.
125. Quintero, M. J., Montesinos, M. L., Herrero, A. & Flores, E. (2001) Identification of genes encoding amino acid permeases by inactivation of selected ORFs from the *Synechocystis* genomic sequence, *Genome Res.* **11**, 2034-40.
126. Chávez-Béjar, M. I., Lara, A. R., López, H., Hernández-Chávez, G., Martinez, A., Ramírez, O. T., Bolívar, F. & Gosset, G. (2008) Metabolic engineering of *Escherichia coli* for L-tyrosine production by expression of genes coding for the chorismate mutase domain of the native chorismate mutase-prephenate dehydratase and a cyclohexadienyl dehydrogenase from *Zymomonas mobilis*, *Applied and Environmental Microbiology.* **74**, 3284-3290.
127. Englund, E., Andersen-Ranberg, J., Miao, R., Hamberger, B. r. & Lindberg, P. (2015) Metabolic engineering of *Synechocystis* sp. PCC 6803 for production of the plant diterpenoid manoyl oxide, *ACS synthetic biology.* **4**, 1270-1278.

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