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Increased Carbon Fixation for Chemical Production in Cyanobacteria

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Abstract
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The combustion of fossil fuels has created many environmental problems, the major one, the greenhouse effect. Thus, we need solutions in order to replace fossil fuels and recycle the CO_2 in the atmosphere. Renewable energies have created attention the last decades but electricity is the main energy form obtained. Photosynthetic organisms (including cyanobacteria) can be used as cell factories since they can convert solar energy to chemical energy. In addition, the requisites to grow them are few; light water, CO_2 and inorganic nutrients. Cyanobacteria have been genetically engineered in order to produce numerous chemicals and fuels of human interest in direct processes. However, the amount of product obtained is still low. Increased carbon fixation in cyanobacteria results in higher production of carbon-based substances. This thesis focuses on the effects of overexpressing the native phosphoenolpyruvate carboxylase (PEPc) in the model cyanobacterium *Synechocystis* PCC 6803. PEPc is an essential enzyme and provides oxaloacetate, an intermediate of the tricarboxylic acid cycle (TCA cycle). The TCA cycle is involved in connecting the carbon and nitrogen metabolism in cyanobacteria. The strains were further engineered to produce ethylene and succinate, two examples of interests for the chemical and fuel industry. Strains with additional PEPc produced significantly more ethylene and succinate. Moreover, an *in vitro* characterization of PEPc from the cyanobacterium *Synechococcus* PCC 7002 was performed. The focus was on oligomerization state, kinetics and the structure of the carboxylase. This thesis demonstrates that increasing carbon fixation and discovering the bottlenecks in chemical production can lead to higher yields and gives us hope that cyanobacteria can be commercialized.

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Contents

Abbreviations

ATP	Adenosine triphosphate
BCD	Bicistronic design
Calvin cycle	Calvin-Benson-Bassham cycle
CEF	Cyclic electron flow
CETCH	Crotonyl-coenzyme A (CoA)/ethylmanonyl-CoA/hyrodxybutyryl-CoA
Cm	Chloramphenicol
C. peniocystis	*Coccochoris peniocystis*
CRISPR	Clustered regularly interspaced short palindromic repeats
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECR	Enoyl-CoA carboxylase reductase
EFE	Ethylene-forming enzyme
ED	Entner-Doudoroff
EMP	Embden-Meyerhof-Parnas
GABA	Gamma aminobutyrate shunt
gltA	Citrate synthase
HCO_3^-	Bicarbonate
ICL	Isocitrate lyase
Km	Kanamycin
MDH	Malate dehydrogenase
MOG pathways	Malonyl-CoA-oxaloacetate-glyoxylate pathways
mRNA	Messenger ribonucleic acid
MS	Malate synthase
NADPH	Nicotinamide adenine dinucleotide phosphate
OPP	Oxidative pentose pathway
O_2	Oxygen
PEP	Phosphoenolpyruvate
PEPc	Phosphoenolpyruvate carboxylase
PPSA	Phosphoenolpyruvate synthase
PSI	Photosystem I
PSII	Photosystem II
RBS	Ribosomal binding site
RNA	Ribonucleic acid

RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose-1,5-bisphosphate
SEC	Size exclusion chromatography
Sp	Spectinomycin
Strep tag	Streptavidin tag
PEPc	Phosphoenolpyruvate carboxylase
PEPc PCC 7002	Purified PEPc from the cyanobacterium *Synechococcus* PCC 7002 with a strep tag attached to the N-terminus
PPSA	Phosphoenolpyruvate synthase
S. volcanus	*Synechococcus volcanus*
Syn PCC 6803	*Synechocystis* PCC 6803
TCA cycle	Tricarboxylic acid cycle
WT	Wild type
2PGA	2-Phosphoglycerate
3PGA	3-Phosphoglycerate
3'UTR	3' Untranslated region
5'UTR	5' Untranslated region

Introduction

The motivation of this work

Since the industrial revolution and the combustion of fossil fuels started in the middle of the 18th century, the levels of CO_2 in the atmosphere have increased dramatically. This increase has been exponential during the last centuries being a major contributor to the increase in greenhouse effect. If CO_2 levels continue increasing, the world's temperature will be more elevated causing catastrophic effects. These effects will include extinction of some species or the increase of the level of seawater (McCarty 2001, Vermeer and Rahmstorf 2009, Höök 2013, Clark et al 2016).

As a result, we need solutions in order to reduce, or at least, not increase the current CO_2 levels in the atmosphere. Biofuels may contribute to recycle the CO_2 gas emitted when fuels are burned since photosynthetic organisms fix CO_2 and convert it to biomass. The idea of growing excessively photosynthetic organisms to capture CO_2 and/or GMMs with increased carbon fixation in order to help to reduce the CO_2 levels are being tested (Puppan 2002, Packer 2009).

The last decades, investigation has focused on five primary renewable energy sources, wind, hydraulic, geothermal, sun and biomass. Most of those renewable energies produce electricity but there is a current (and future) high demand on fuels and chemicals (Manzano-Agugliaro 2013). It is predicted that the oil reserves are going to be exhausted in few decades so new strategies to produce fuel are needed. One appealing approach is to produce products by using waste or photosynthetic organisms. According to the Cambridge Dictionary, Biofuel is “fuel that is made from living things or their waste and is less harmful to the environment than other types of fuel”. Biofuels have already been produced but generations that are more modern are being investigated.

The first generation of biofuels were made by conversion of food-vegetable, starch/sugars, cellulose or vegetable oil (Aro 2016). It was considered economically and environmentally friendly, but the main disadvantages are the competition for land with food production and the increment of deforestation (Porqueras et al 2012).

Second generation of biofuels were based on the same technology as the first generation, but no food-vegetables were used. Instead, lignocellulosic material and organic waste were the sources (Porqueras et al 2012, Aro 2016). The advantages of this generation were that food agriculture is not used and it is environmental friendly.

Third generation of biofuels would be produced from microalgae and seaweeds. The organisms can be used to produce biofuels from their biomass or they can naturally produce biofuel precursors. The cells could be genetically engineered and seawater and/or waste could be used for cultivation. The advantage is that there was not competition with e.g. agricultural land. The main disadvantages were slow production and large cultivation areas (Porqueras et al 2012, Aro 2016).

The fourth generation of biofuels will use synthetic biology to genetically engineer cyanobacteria and algae. In addition, it involves the combination of photovoltaics and microbes. In this generation, the aim is to produce the biofuels and chemicals in direct processes and create green synthetic cell factories (Demribas 2011, Lü et al 2011, Aro 2016).

In this thesis, I engineered cyanobacteria with molecular biology tools in order to enhance the carbon fixation process and increase biofuel and biochemical production (fourth biofuel generation).

Cyanobacteria and biotechnology

Cyanobacteria are gram-negative prokaryotes with the capacity to perform oxygenic photosynthesis. It is believed that these organisms were responsible for rising the O_2 levels in the atmosphere 2.3 billion years ago (Stainer and Cohen Bazire 1997, Kasting and Siefert 2002, Dvornyk et al 2003). Cyanobacteria are widely distributed in many habitats, from extreme environments to aquatic systems, and they are present in different forms, from unicellular to filamentous. One of the most interesting features that some cyanobacteria have is that they are capable of fixing nitrogen from the atmosphere. This fact makes them important in habitats where usable nitrogen is a limiting component (Kasting and Siefiert 2002).

Using cyanobacteria or microalgae to produce substances of human interest or to reduce CO_2 levels in the atmosphere has gained attention. The fact that growing these microorganisms do not need to compete for fertile land and require few compounds makes them good candidates. In addition, many strains grow in seawater making the technology more sustainable (Karube et al 1992, Stephens et al 2010, Kumar et al 2011).

Cyanobacteria have a high value because they can naturally produce substances of human interest such as H_2, toxins, biomass, fatty acids, etc (Belay et al 1993, Chaiklahan et al 2008, Dutta et al 2015). In addition, they can be genetically engineered to produce valuable substances such as sugars and biofuels and other chemicals in direct processes (Rosgaard et al 2012, Paper VI). Prokaryotes do not (in general) have compartments and are simpler organisms compared to eukaryotes and therefore they are easier to genetically engineer. In this thesis, all the experiments were performed in the model cyanobacterium *Synechocystis* PCC 6803 (*Syn* PCC 6803). *Syn* PCC 6803 is a unicellular cyanobacterium isolated from a freshwater lake in California (USA) in 1968 (Stanier et al 1971). The genome was sequenced in 1996 (Kaneko et al 1996) and the cells can be naturally transformed (Grigorieva 1982).

Metabolic engineering

Metabolic engineering involves the modification of genetic elements and regulation in order to produce a substance or make the process more efficient (Bailey 1991). This idea raised since during many centuries, microorganisms were used to produce substances such as cheese, alcohol, etc. (Alam et al 1988, Caplice and Fitzgerald 1999, Tamang et al 2016).

Metabolism is all the chemical and physical reactions that happen in a living organism. The chemical reactions are performed by enzymes and enzymes are proteins which have the capacity to accelerate reactions, thus they are catalysts. How enzymes are encoded and expressed is a regulated complex process.

The central dogma of biology (Crick 1970) is represented in Figure 1. DNA is transcribed into RNA. Promoters and transcription factors regulate transcription. Promoters are DNA sequences upstream of the gene-coding region where the transcription factors and the RNA polymerase bind. Once the polymerase is attached to the DNA, it recognizes the transcription starting site (TSS) and it starts to "read" the DNA creating the mRNA. The transcription is done when the RNA polymerase reads a DNA sequence that makes the RNA polymerase to drop from the DNA, called terminator (Figure 1). The released mRNA has different regions; the 5´UTR region, the ribosomal binding site (RBS), the coding sequence of the enzyme, the stop codon and the 3´UTR (Figure 1). The RBS in the mRNA is recognized by the ribosomes and the translation process starts. The starting of the translation process may be controlled by the 5´UTR sequence. The ribosome reads 3 nucleotides (one codon) of the mRNA and adds an amino acid making a chain called peptide. When the last codon is read (stop codon), the ribosome dissociates from the mRNA and the translation is finished. The peptide formed while translation happens, is folded into the active structure (enzyme) by different interactions

of the side chains of the peptide- in some cases this process can be assisted by other proteins, called chaperones.

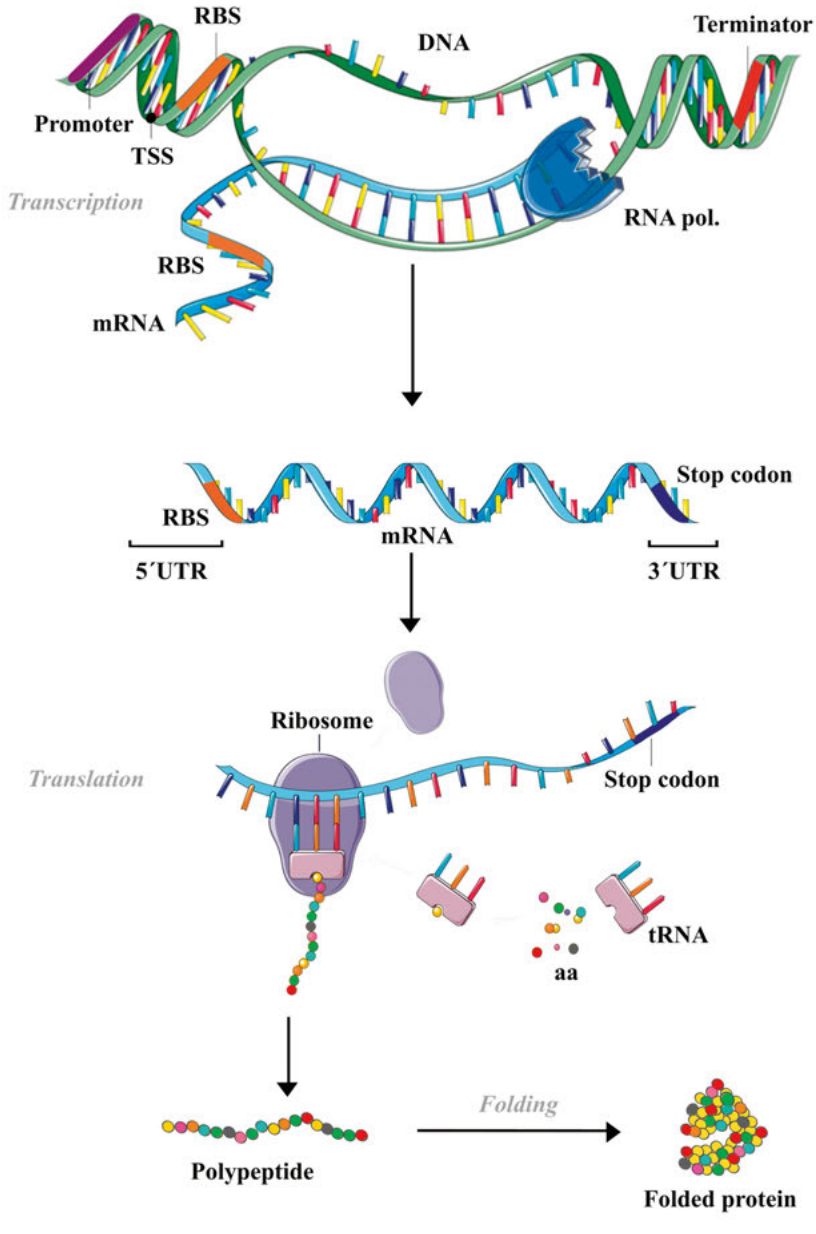

Figure 1. The central dogma of biology. aa = amino acids, mRNA = messenger RNA, RBS = ribosomal binding site, RNA pol = RNA polymerase, tRNA = transfer RNA, TSS = transcription starting site, 3´UTR = 3´ untranslated region, 5´ UTR = 5´ untranslated region.

The knowledge of all these processes and elements allowed to us to develop synthetic biology. Synthetic biology is the combination of biology and engineering to create novel biological functions and systems (Jouhten 2012). One of the troubles of using cyanobacteria for production of substances of human interest is that the yield is very low. However, we and others have found several ways to increase the production of substances.

Promoters can be constitutively active or induced by substances. Several different native promoters have been discovered in *Syn* PCC 6803 (Mohamed et al 1993, Abe et al 2014, Zhou et al 2014, Englund et al 2016). Few of these native promoters have been modified showing an increased or decreased expression of the downstream gene (Huang et al 2010, Qi et al 2013). In addition, heterologous promoters have been tested and shown to be active in *Syn* PCC 6803 (Ferino and Chauvat 1989, Huang and Lindblad 2013, Camsund et al 2014, Camsund and Lindblad 2014). Usually strong promoters favours the production of substances but in some cases a weak and/or inducible promoter can be better for production, especially when the product is toxic (Gold 1990, Giacalone et al 2006).

RBS have an effect on the translation process and therefore on the expression of proteins. Different native RBS have been identified in *Syn* PCC 6803 and modified showing different protein levels (Thiel et al 2018). In addition, synthetic RBS have been designed and implemented in *Syn* PCC 6803 leading to different expression levels (Heidorn et al 2011). We have the hypothesis that heterologous expression increases the productivity of substances because the non-native protein is not regulated by the host organism. However, our lab experienced that when the hydrogenase from *Chlamydomonas reinhardtii* was introduced into *Syn* PCC 6803 the protein was transcribed but not translated (Lindblad et al 2019). Mutalik et al 2013 developed a bicistronic design (BCD) in *Escherichia coli* that has two RBS and makes sure that any secondary structures around the second RBS are melted by the translation of the leader peptide. Thus, the ribosome can bind to the RBS and translation can occur. Lindblad et al 2019, implemented this strategy in cyanobacteria showing the translation of HydA using the BCD construct.

Riboswitches and riboJ are other ways to control translation. In riboswitches, molecules can bind to the 5´UTR of the mRNA and block or initiate translation. Several riboswitches have been discovered in bacteria (including cyanobacteria), filamentous fungi, green algae, and higher plants (Nudler and Mironov 2004, Winkler and Breaker 2005, Thore et al 2006, Perez et al 2016). However, only few have been characterized in cyanobacteria (Wagner et al 2015, Perez et al 2016), even though a large number of genes are predicted to be regulated by riboswitches (Singh et al 2018). Again, several heterologous riboswitches have been successfully tested in cyanobacteria (Nakahira et al 2013, Ma et al 2014, Ohbayashi et al 2016). RiboJ are “RNA leaders” that

help to standardize translation. It consists of 75 nucleotide sequence including the satellite RNA of tobacco ringspot virus and followed by a 23 nucleotide hairpin. During the posttranscriptional process, the ribozyme cleave the RNA resulting in a hairpin just upstream of the RBS. Thus, after cleavage of the RiboJ, the 5'UTR region is the same in all mRNAs transcribed containing the RiboJ sequence and the translation process is predicted to be standardized (Lou et al 2012, Clifton et al 2018). RiboJ has been used in cyanobacteria for several applications including for biofuel production (Taton et al 2014, Englund et al 2018, Miao et al 2018).

Antisense RNA can control the translation process too. In this case, an antisense RNA is synthesized and binds complementary to the mRNA. Consequently, the ribosome cannot bind or cannot elongate the peptide chain since there is a physical block impeding the translation. Antisense RNAs have been discovered in all three kingdoms of life (Wagner and Simons 1994, Brantl 2002) including cyanobacteria (Georg et al 2009, Mitschke et al 2011). Nonetheless, there are no studies where antisense RNAs are used to down regulate genes for higher production of substances in cyanobacteria.

Another way to increase heterologous expression is optimizing the codon usage. The majority of amino acids are encoded by different codons. Each organism has a different abundance of tRNA (tRNAs is a RNA that carry an amino acid (Figure 1). Changing rare codons (without changing the amino acid) for more common ones in the host organism, has shown to have positive effects on protein expression in different organisms such as bacteria (cyanobacteria), green algae, plants, mammalian cells and others (Rouwendal et al 1997, Patterson et al 2005, Burgess-Brown et al 2008, Lindberg et al 2010, Lindblad et al 2012, Wang et al 2012).

The amount of product can be enhanced by different strategies, for instance, by fusing two proteins (scaffold) (Dueber et al 2009, Moon et al 2010). It is believed that scaffolds help the production of substances when the intermediate is toxic (the product of the first enzyme and the substrate of the second one in the pathway) (Dueber et al 2009). The secretion of toxic desired products by transporters can also help (Doshi et al 2013, Peralta-Yahya et al 2012) as well as deletion of competitive pathways (Peralta-Yahya et al 2012). However, it is important to know if the pathway is essential for the cell. Thus, knocking down pathways may be a more suitable solution.

A few decades ago, Stern et al discovered extra genic palindromic sequences (Stern et al 1984), that lately were identified to be an "immune" system of different organisms (Makarova et al 2002, Mojica et al 2005, Pourcel et al 2005, Ishino et al 2018). Several clustered regularly interspaced short palindromic repeats (CRISPR) systems have been found in nature and it has been estimated that 90% of archaea and 40% of bacteria have a CRISPR system

(Makarova et al 2011, Qi et al 2013). Much information about CRISPR systems is available, for a review, see Barrangou 2014. The simplest CRISPR system discovered is when the cells are infected by a foreign DNA and the DNA is stored into the CRISPR loci. Then, the cells transcribe the palindromic repeats stored in the CRISPR locus and the RNA produced is cleaved into small fragments. The fragmented RNA has an antisense sequence complementary to the pathogen DNA and a sequence that can be recognized by Cas9 endonuclease protein. When the cells are infected again with the same foreign DNA, the RNA binds together with the Cas9 to the foreign DNA. Lastly, the Cas9 cleaves the pathogen DNA avoiding the replication of it (Barrangou 2014).

Qi et al 2013, engineered the Cas9 protein in order to block translation but avoid the cleavage of DNA and therefore use it as a reversible strategy for downregulation of gene expression (CRISPRi). They showed that the best DNA strand to target in order to silence the gene expression is the non-template strand. They also demonstrated that the repression was better when the CRISPRi system was close to the TSS. The advantage with CRISPRi is that multiple gene expressions can be controlled in a reversible way, making the process very efficient. The CRISPRi has been implemented in cyanobacteria showing that the system can knock down the expression of several genes at the same time and therefore increase production of substances of human interest (Yao et al 2015, Huang et al 2016, Li et al 2016).

Bacteria have two different forms of DNA, both circular. The chromosome (Carins 1963) and in some bacteria, a DNA molecule called plasmid which replicates independently from the chromosome (Kado 1998). With the current knowledge of molecular biology, the genetic engineering in cyanobacteria can be done in both DNAs. So, with all the knowledge mentioned above, we have the possibility to knock out and/or knock down genes, overexpress proteins or express heterologous proteins in more efficient ways. Nowadays, we can challenge nature by expressing heterologous or synthetic pathways in cyanobacteria and other organisms in order to increase/decrease processes or produce substances of human interest.

Photosynthesis and carbon fixation in cyanobacteria

Photosynthesis is divided into two group of reactions; the light dependent and the light non-dependent reactions. During light the protein complex located in the thylakoid membrane, Photosystem II (PSII), splits water into O_2 and H^+ obtaining an electron that is excited and transferred to the plastoquinone complex (PQ). The PQ is then oxidized by transferring the excited electron to the cytochrome b_6f (cyto b_6f). The electron is then passed to the soluble protein plastocyanin (PC) which transfers the electron to the Photosystem I (PSI). In

the PSI complex, the electron is excited again by light and transferred to the ferredoxin (Fd), then to the Ferredoxin-NADPH-reductase (FNR) which reduces $NADP^+$ into NADPH. When water is split, H^+ accumulate in the thylakoid lumen. In addition, during the transfer of the electron from the PQ to the cyto b_6f, H^+ are transported in to the lumen of the thylakoid. The accumulation of H^+ in the thylakoid lumen makes a differential pH between the lumen of the thylakoid and the cytoplasm (Figure 2A). These H^+ are then used to drive the ATP synthase. The products of the light dependent reactions are NADPH (reducing power) and ATP (energy). They are important for the light non-dependent reactions as well as other reactions in the metabolism of oxygenic photosynthetic organisms (Figure 2A) (Lambers et al 2008).

Light non-dependent reactions are those reactions that use the NADPH and the ATP synthetized during the light dependent reactions. These products are used by the Calvin-Benson-Bassham cycle (Calvin Cycle) in order to fix inorganic carbon (CO_2) and synthesize 3-phosphoglycerate (3PGA) (Figure 3) (Lambers et al 2008). The enzyme responsible to fix carbon in the Calvin cycle is ribulose 1,5-biphosphate carboxylase/oxygenase (RuBisCO) and it is the most abundant protein on earth (Raven 2013). There are four RuBisCO forms found in nature. Form I is the most abundant one and it is present in chemoautotrophic bacteria, purple bacteria, cyanobacteria, red and brown algae and all higher plants (Andersson 2008, Watson and Tabita 1997). RuBisCO has a hexadecameric structure composed by 8 large and 8 small subunits (Watson and Tabita 1997). In cyanobacteria, RuBisCO is assembled into the hexadecameric structure by the assistance of the GroEL/GroES chaperonin and chaperone X (Liu et al 2010). RuBisCO can take either CO_2 or O_2 as a substrate leading to two different processes (Figure 3) (Eisenhut et al 2008).

RuBisCO has more specificity to O_2 than CO_2 and when O_2 binds to RuBisCO, 2-phosphoglycerate (2PGA) and 3PGA are formed. 2PGA is metabolized in a toxic pathway and may lead to loss of carbon (Photorespiration) (Figure 3) (Eisenhut et al 2008). Interestingly, it is unknown why this process is essential for the cells (Eisenhut et al 2008*). In order to overcome the high specificity of RuBisCO towards O_2, cyanobacteria and other photosynthetic organisms have evolved the carbon concentrating mechanism (CCM) consisting of adaptations made to increase CO_2 levels around RuBisCO (Price et al 2007). In cyanobacteria, the CCM consists of the inorganic carbon transporters, the carboxysome and the carbonic anhydrase.

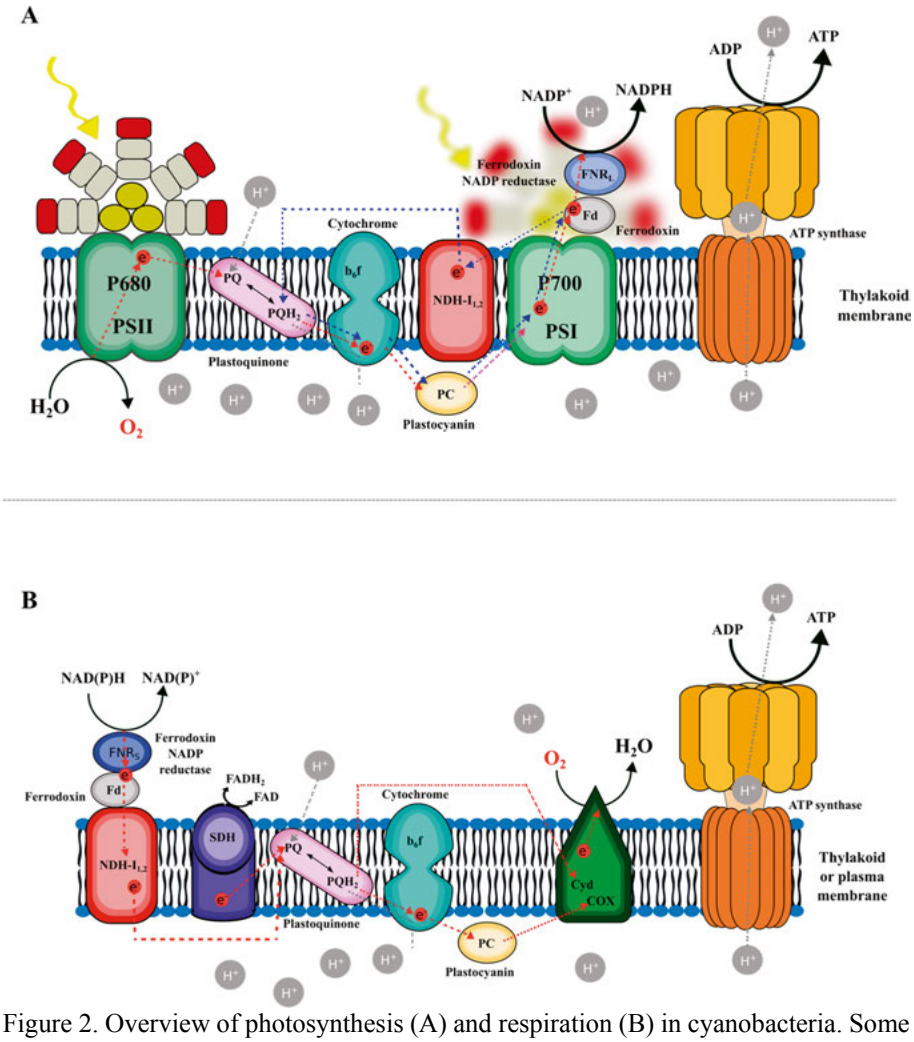

Figure 2. Overview of photosynthesis (A) and respiration (B) in cyanobacteria. Some components are shared in these two processes on the thylakoid and plasma membrane. Abbreviations: PSII - Photosystem II, PQ - Plastoquinone, PC - Plastocyanine, PSI - Photosystem I, SDH - Succinate dehydrogenase, NDH - NADH dehydrogenase-like , NADP - Nicotinamide adenine dinucleotide phosphate, ATP - Adenosine Triphosphate and Cyd COX - cytochrome *bd*-quinol oxidase - cytochrome *c* oxidase.

There are five inorganic carbon transporters in cyanobacteria, three take up bicarbonate and two CO_2 (Figure 4) (Price 2011). The bicarbonate transporters are located in the plasma membrane and it is suggested that bicarbonate passes through the outer membrane by porins. In contrast, CO_2 transporters are based on plastoquinone oxidoreductase NADPH dehydrogenase respiratory complexes (NDH-I) (Figure 4) and therefore they are suggested to be located in the thylakoid membrane. CO_2 can pass passively through the membrane so the main activity of these two transporters is the hydration of CO_2 by two proteins

which perform the opposite reaction of carbonic anhydrase (Figure 4) (Mi et al 1995, Price et al 2002, Price 2011).

BicA is a Na^+ dependent transporter and it has a low affinity for bicarbonate. The genes encoding this transporter are constitutively expressed. BCT1 is an ATP dependent transporter that has high affinity for bicarbonate. It is induced by high light as well as low levels of inorganic carbon. StbA is another Na^+ dependent transporter, induced under low inorganic carbon levels and with a relatively high affinity for bicarbonate (Price 2011). The CO_2 transporters, NDH-I_3 and NDH-I_4 complex are induced under low inorganic carbon level and constitutively expressed, respectively (Price 2011).

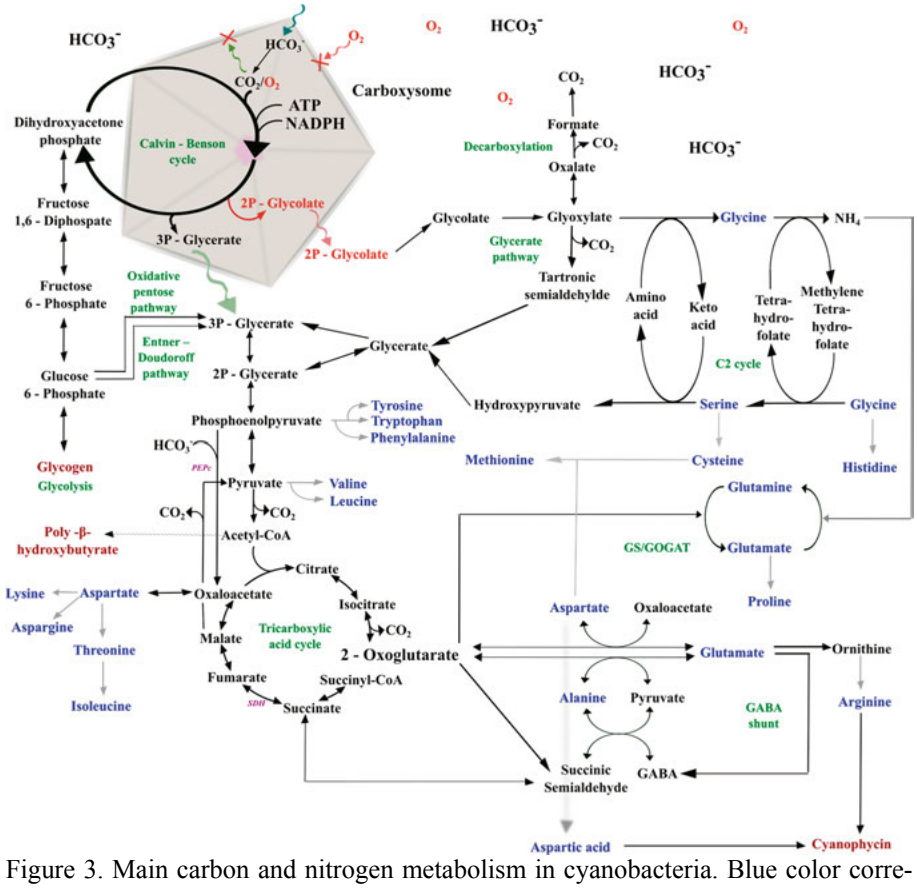

Figure 3. Main carbon and nitrogen metabolism in cyanobacteria. Blue color corresponds to amino acids, green color to the name of the pathways, red color correspond to photorespiration and maroon to storage molecules.

Carboxysomes are polyhedral cytosolic inclusion bodies composed by several proteins. Two different carboxysomes are found in cyanobacteria depending on the RuBisCO type encapsulated and their respective structural proteins. 1,5- biphosphate (RuBP) produced in the Calvin cycle and bicarbonate diffuse

through the carboxysome shell where carbonic anhydrase and RuBisCO are grouped together (Figure 3). Once bicarbonate is inside the carboxysome, carbonic anhydrase converts it into CO_2. RuBisCO is then surrounded by high levels of CO_2 since the later cannot diffuse trough the carboxysome shell (So et al 2002). Thereafter, RuBisCO converts RuBP and CO_2 into 3PGA. 3PGA diffuses the carboxysome shell and can be either further metabolized or used to regenerate RuBisCO's substrate, RuBP.

The excess of carbon fixed in the Calvin cycle is stored as glycogen. During the darkness, glycogen is broken down to glucose (Smith 1983). Glucose is metabolized through glycolysis (Embden-Meyerhof-Parnas (EMP), oxidative pentose pathway (OPP) and the Entner-Doudoroff (ED) pathway (Figure 3, Chen et al 2016) and further downstream by the tricarboxylic acid cycle (TCA cycle) (Zhang and Bryant 2011). During these processes abundant reducing power are generated but only small amount of ATP is obtained. The NADPH produced is oxidized in the NDH-I_4 complex and the electrons in the succinate dehydrogenase complex (SDH) (Figure 2B and 3), both located in the thylakoid and plasma membrane, contribute to reduce the PQ. The reduction of the PQ accepts also H^+, which is transferred into the thylakoid membrane and the final electron acceptor, is O_2. The H^+ gradient created is used to drive the ATP synthase and ATP is produced for essential reactions. In cyanobacteria, photosynthesis and respiration share machinery in the thylakoid membrane but respiration mostly happens in the cytoplasmic membrane (Peschek 1999). When O_2 is not present, the cells produce other substances (lactate, acetate, succinate, etc in order to get rid of excess electrons and this process is called fermentation (Binder 1982, Vermaas 2001).

In addition of RuBisCO, there are other carbon fixing enzymes in plants and cyanobacteria. Phosphoenolpyruvate carboxylase (PEPc) is an important carbon fixation enzyme for C4 and CAM plants and it is also present in bacteria (including cyanobacteria), fungi and C3 plants (Svensson 2003). PEPc catalyzes the conversion of phosphoenolpyruvate and bicarbonate, in the presence of Mg^{2+}, into oxaloacetate and inorganic phosphorus. It is a very important carboxylase in C4 and CAM plants since PEPc fixes carbon producing oxaloacetate which is converted into malate in the mesophyll cells present in leaves. After that, the malate is transported to the bundle sheath cells (in C4 plants) where it is decarboxylated forming pyruvate and CO_2. The former is then transported again to the mesophyll cells in order to restart the cycle while the CO_2 is used by RuBisCO (Ehleringer 2002).

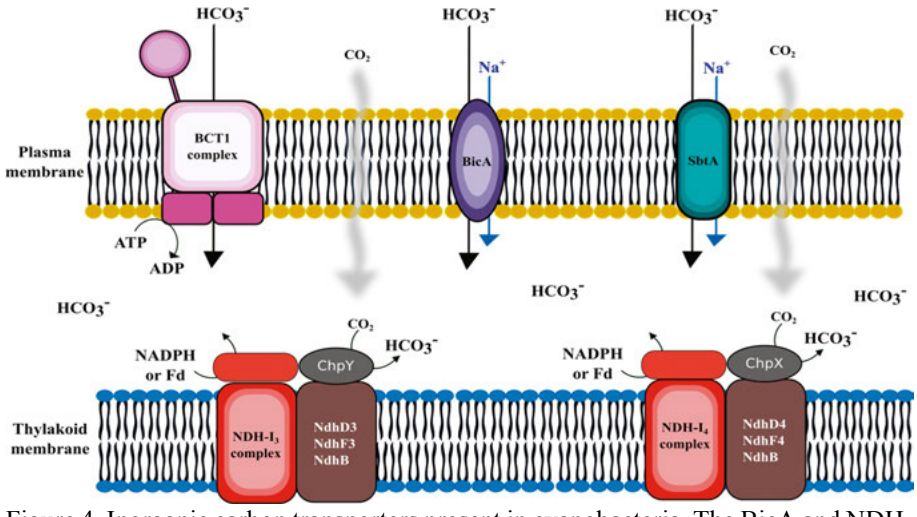

Figure 4. Inorganic carbon transporters present in cyanobacteria. The BicA and NDH-I_4 are constitutively expressed while BCT1, SbtA and NDH-I_3 are induced in low levels of inorganic carbon. The transporters located in the plasma membrane transport bicarbonate while the ones located in the thylakoid membrane transport CO_2.

There are several PEPc isoforms found in nature, some related with photosynthesis and others involved in C/N partitioning, ripening fruit, seed formation and more (Lepiniec et al 1993, Chollet et al 2002, Oleary et al 2011), but all subunit forms are around 100 kDa. The cyanobacterial crystal structure of PEPcs have not been determined yet but all the amino acid sequences of PEPc discovered so far have the key residues to form the homotetramer structure, glutamic acid (E528) and arginine (R533) (*Syn* PCC 6803 numbering) (Smith and Plazas 2011).

In cyanobacteria (C3 metabolism), PEPc plays an anaplerotic role providing carbon skeletons for the nitrogen metabolism since the reaction that PEPc catalyzes produces an intermediate of the TCA cycle (Figure 3). It was believed that cyanobacteria had an incomplete TCA cycle because the enzyme 2-oxoglutarate dehydrogenase is lacking and therefore succinyl coenzyme A cannot be synthesized. In 2011, Zhang and Bryant discovered that *Synechococcus* PCC 7002 has two enzymes, 2-oxoglutarate carboxylase and succinic semialdehyde dehydrogenase, which convert 2-oxoglutarate to succinate semialdehyde and the succinate semialdehyde to succinate, respectively (Zhang and Bryant 2011) (Figure 3). In addition, they concluded that these enzymes are present in all cyanobacteria except in *Prochlorococcus* and marine *Synechococcus* species. Xiong et al 2014, demonstrated that an intact gamma amino butyrate shunt (GABA shunt) is present in the cyanobacterium *Syn* PCC 6803 and that this shunt has a major contribution to succinate production compared to the pathway described by Zhang and Bryant (Figure 3).

It was suggested that in cyanobacteria, only 5% of the photosynthetic carboxylation rate is present in darkness and that PEPc may fix up to 20% of the total carbon fixed when the cells are in the steady state (Owttrim and Coleman 1986). PEPc is essential for cyanobacteria (Luinenburg and Coleman 1990) and several PEPcs from different organisms have been characterized (Table 1).

In 2014, Jia et al overexpressed and knocked down *pepc* in the cyanobacterium *Anabaena* sp. PCC 7120. Both strains showed higher photosynthesis compared to the wild type (WT). However, decreased dark respiration was only observed in the strain with down regulated PEPc. In addition, the former strain showed higher photosynthesis when the cells were exposed to environmental stresses like low temperature and high salinity (Jia et al 2014).

Yan et al 2015, downregulated the PEPc activity and they demonstrated a higher and lower carbohydrate and protein content, respectively in the *Syn* PCC 6803 cells. However, a lower growth rate was not observed in this engineered strain compared to the WT. It was also observed that PEPc activity was stable during growth except for the stationary phase. This in agreement with what it was observed in the cyanobacterium *Coccochoris peniocystis* (*C. peniocystis*) back in 1986 (Owttrim and Colman 1986).

It has been shown that several substances can inhibit PEPc activity. In green algae, higher plants and the in cyanobacteria *Synechococcus volcanus* (*S. volcanus*) and *C. peniocystis,* PEPc is repressed by malate and aspartate (Owttrim and Colman 1986, Lepiniec et al 1993, Rivoal et al 1998, Chen et al 2002). In the cyanobacterium *C. peniocystis*, the carboxylase is also inhibited by oxaloacetate and citrate (Owttrim and Colman 1986). Recently, Takeya et al showed that PEPc activity from *Syn* PCC 6803 was not inhibited by malate and aspartate at pH 7.0, while a clear inhibition could be seen at pH 9.0 (Takeya et al 2017). It has been suggested that the pH in cyanobacteria fluctuates during light and darkness (Coleman and Colman 1981). The combination of less bicarbonate in the cells (due to lower CO_2 pumped during darkness), low PEP levels and lower pH with the production of inhibitors could contribute to the reduced PEPc activity in darkness (Coleman and Colman 1981, Owttrim and Coleman 1986, Iwaki et al 2006).

In addition to RuBisCO and PEPc, there are at least two other carboxylases present in cyanobacteria. Acetyl-CoA carboxylase is involved in the fatty acid pathway providing lipids for the cell membrane (Gornicki et al 1993) and carbamoyl phosphatase synthetase is involved in the production of pyrimidine and arginine (Cunin et al 1986).

Table 1. Characterized PEPc from different organisms. Abbreviations: ACoa- Acetyl-CoA, ATP- Adenosine Triphosphate, C-Citrate, D-Aspartate, DAP- Dihydroxyacetone phosphate, E-Glutamate, F- Fumarate, G6P- Glucose-6-Phosphate, IC-Isocitrate, M-Malate, ML- Manolate, NADPH-Nicotinamide adenine dinucleotide phosphate, OAA-Oxaloacetate, O-2 oxoglutarate, P-Phosphate, Pyr- Pyruvate, Q-Glutamine, RT-Room temperature, S-succinate, 3PGA- 3-phosphoglyceraldehyde, n.t. no tested. (?) temperature used for the activity assay but the optimal is not specified, * optimal temperature but the temperature between brackets is the one used for the *in vitro* activity assay.

Organism	T (°C)	pH	V max (units/ mg)	Km (PEP) mM	Km (HCO_3^-) mM	Inhibitor/ Activator	Ref
Anabaena PCC 7120	35	8	2.6	1.1	0.24	A, M	Takeya et al 2017
Coccochloris peniocystis	40	8	8.84	0.6	0.8	O, M lesser extent: C, IC, O, ATP, D, ML, P/ Pyr, 3PGA, NADPH	Owttrim and Colman 1986
Synechococcus PCC 7002	35-35	7.5-8	14.43-20.74	1.06-0.77	0.97-0.24	n.t.-Q/S	(IV)
Synechococcus vulcanus	42* (30)	9 7.5	25.3 17.3	0.53 0.58	nd 0.48	D	Chen et al 2002
Synechocystis PCC 6803	30	7.3	1.74	0.34	0.8	S, M, F, C, A	Takeya et al 2017
Oceanimonas smirnovii	20 (RT)	10	21.8	1.22	0.139		Park et al 2015
Chlamydomonas reinhardtii	25 (?)	8.8 8.1	22 18			E,D,O,M/Q DAP	Rivoal et al 1998
Selenastrum minutum	25 (?)	9 9	 5.29 5.71	(S50) 2.23 0.32		Q	Schuller et al 1990
Zea Mays	30 (?)	7.3 8	18.2 23	1.48 0.59	0.12 0.1	M, D/ G6P	Chen et al 2002, Takeya et al 2017, Willeford et al 1992

Metabolic engineering for increased carbon fixation and subsequent higher biofuel production

It is known that carbon fixation is one of the bottlenecks in the production of substances in photoautotrophic organisms. Since prokaryotes have a simpler gene distribution compared to plants, most studies have been performed on bacterial or cyanobacterial RuBisCOs. Most research have focused on trying to increase the carboxylase activity and the specificity towards CO_2 (Whitney et al 2011), but a great significant improvement has never been achieved. In 2015, we summarized the knowledge about engineering for increased carbon fixation in cyanobacteria (Paper I).

Numerous amino acid substitutions have been made in loop 6 of RuBisCO since the loop is involved in the specificity of the enzyme. Leucine 332 was substituted by different amino acids (alanine, isoleucine, methionine, threonine and valine) but all of these substitutions resulted in a diminution of specificity (Lee et al 1993). Alanine 340 was replaced by histidine and asparagine, and a slight increase of specificity was observed in both substitutions (13% and 9%, respectively), while the carboxylation rate decreased (25-33%) or increased (19%), respectively (Madgwick et at 1998). Other substitutions have been made in the loop 6, but no significant improvement was achieved (Madgwick et at 1998, Parry et al 2003).

In *Synechococcus* PCC 6301, the replacement of methionine 259 to threonine improved the carboxylation catalytic efficiency by 12% and the affinity by 15% (Greene et al 2007). In addition, the single substitution of different amino acids increased the affinity for RuBP (Muller-Cajar et al 2008). Several modifications of the small subunit in *Synechococcocus* and *Anabaena* affected the structure and decreased the carboxylation rate (Lee et al 1991, Read et al 1992, Kostov et al 1997). Many other substitutions have been done in the large subunit of RuBisCO from different organisms leading, in most cases, to repressed catalytic activity or increased specificity towards O_2 (Reviewed in Kellogg and Juliano 1997). Another approach tested was the combination of large and small subunits of RuBisCO from different organisms. This approach resulted in incorrect assembly or increased specificity towards O_2 (Wang et al 2001). The combination, for instance, of the small subunit of *Cylindrotheca* sp.N1 or *Olisthodiscus luteus* with the large subunit of *Synechococcus* PCC 6803 led to an improvement of the specificity towards CO_2 (Read and Tabita 1992). All these results suggest that the RuBisCO's small subunit is involved in the specificity and stability of the enzyme.

In cyanobacteria, few studies with increased carbon fixation and higher production of carbon based compounds have been published. Higher *in vitro* RuBisCO activity was detected in the cyanobacterium *A. nidulans* when both the native RuBisCO's subunits were introduced under the *lac* promoter (Daniell

et al 1989). In *Synechococcus elongatus* PCC 7942, the insertion of a gene, which encodes a protein for sucrose export, increased the PSII activity, chlorophyll content, carbon fixation and biomass (Ducat et al 2012). The same cyanobacterium, *S. elongatus*, was genetically engineered in order to produce isobutyraldehyde. The overexpression of RuBisCO in the already engineered strain resulted in higher isobutyraldehyde production (Atsumi et al 2009). In another study, where *Synechococcus* was engineered to produce 2,3-butanediol, the overexpression of some enzymes between the 3PGA and pyruvate resulted in higher production of the biofuel (Oliver et al 2015). Kanno et al 2017, engineered *S. elongatus* PCC 7942 to improve glucose metabolism, by downregulating the native carbon fixation regulation and enhancing the bottlenecks of carbon fixation resulting in increased of 2-3 butanediol production.

Liang and Lindblad 2017, overexpressed RuBisCO and three other enzymes of the Calvin cycle in *Syn* PCC 6803. The engineered strains showed higher growth rate as well as biomass accumulation. These four engineered strains were coupled with the pathway to produce ethanol and the production of the alcohol was enhanced compared to the control strain (Liang et al 2018). In addition, Liang and Lindblad 2017 overexpressed RuBisCO tagged with the Flag tag in both the N-terminus (FL50) and the C-terminus (FL52). The strains with tagged RuBisCO showed higher protein level, increased growth, photosynthetic activity and *in vitro* RuBisCO activity. The tagged RuBisCO seemed to be more transcribed and may have stabilized translation (Liang and Lindblad 2017).

Kamennaya et al 2015 overexpressed the BicA bicarbonate transporter in *Syn* PCC 6803 under the control of the inducible *PnirP* promoter. When the engineered cells were induced and cultivated with bubbling flasks without supplemented CO_2 higher growth rate, optical density and higher biomass compared to WT was observed. However, if the cells were supplemented with CO_2, the engineered strain decreased doubling time but produced more biomass compared to the WT. Several experiments showed that the extra carbon incorporated was directed towards saccharide-rich exopolymeric substances (Kamennaya et al 2015).

A synthetic pathway based on the 3-hydroxypropionate bicycle was introduced in the cyanobacterium *S. elongatus* PCC 7942. The synthetic pathway bypasses photorespiration by re-assimilating glyoxylate, which is a toxic product in photosynthetic organisms (reviewed in Dellero et al 2016) (Figure 3). The result of this study was increased carbon fixation in the cyanobacterium (Shih et al 2014).

Some scientists have tried to design and introduce a synthetic carbon fixation pathway in heterotrophic organisms since autotrophic organisms grow slower and seems to be more difficult to engineer (Gong and Li 2016, Schwander et

al 2016). The carboxylase chosen in this pathway (crotonyl-coenzyme A (CoA)/ethylmanonyl-CoA/hyrodxybutyryl-CoA (CETCH) is the enoyl-CoA carboxylase reductase (ECR) which has a carboxylation activity 37 times higher than RuBisCO. The CETCH pathway is shorter and requires less ATP and NADPH than the existing aerobic carbon fixation pathways and the product released from this pathway is glyoxylate. Unfortunately, this pathway has not been yet implemented in any living organism but it has been tested *in vitro* (Schwander et al 2016).

The synthetic carbon fixation malonyl-CoA-oxaloacetate-glyoxylate (MOG) pathways were created in 2010 by Bar-Even and colleagues. These synthetic metabolic pathways were designed based on 5000 enzymes, five existing carbon fixation pathways and their carboxylases. Four different criteria were applied including the affinity of the enzymes, the energetic cost for the cell, the favorable thermodynamic reactions and how the synthetic pathway would affect the host organism. Several synthetic pathways were created with at least one carboxylase. The results showed that the most efficient carboxylase is PEPc. Shorter pathways were created but they concluded that the most efficient ones were the C4 glyoxylate/alanine and the C4 glyoxylate/lactate. These two pathways share the first six enzymes and differ only in the last reactions (Bar-Even et al 2010).

Only four studies about overexpression of PEPc in cyanobacteria are available. In 2014, Jia et al overexpressed PEPc in *Anabaena* sp. PCC 7120 showing higher photosynthetic rates. In 2016, we published a paper which is described in the next section (Paper II). In the same year, Li et al published a paper where *S. elongatus* PCC 7942 was engineered with the CRISPR-Cas9 system. Obtained strain showed increased succinate production (11 fold) compared to WT by knocking down one of the enzymes for glycogen synthesis and knocking in two enzymes in the TCA cycle, *gltA* and PEPc (Li et al 2016). In addition, Hasunuma et al 2016, showed increased succinate production when PEPc was overexpressed in *Syn* PCC 6803 (Hasunuma et al 2016).

Aim

The aim of my thesis can be summarized in four points:

1. Overview and understanding of photosynthesis with a focus on carbon fixation and subsequent downstream metabolism in cyanobacteria
2. Overexpression of native enzymes of the synthetic MOG pathway in cyanobacteria and analysis of the effects
3. Characterization of phosphoenolpyruvate carboxylase (PEPc) from cyanobacteria
4. Increased production of selected chemicals by genetic engineering of cyanobacteria with additional PEPc

Results and Discussion

Partial introduction of the MOG pathway in *Synechocystis* PCC 6803 (Paper II)

After studying the MOG pathways, we identified that the enzymes, 1-3 (Phosphoenolpyruvate synthase (PPSA), PEPc and Malate dehydrogenase (MDH) are present in the genome of *Syn* PCC 6803. Thus, we decided to overexpress the carboxylase individually and the three first enzymes of the pathways in an operon under the control of the native light regulated promoter, *PpsbA2*. The introduced genes were designed to homologous recombine with the *psbA2* in the chromosome of the cyanobacterium and therefore replace the native *psbA2*. The *psbA2* codes for the protein D1 involved in the PSII. Mohamed et al 1993, showed that the gene encoding this protein has two copies in the genome. Thus, when the *psbA2* is knocked out the *psbA3* is activated providing the D1 protein needed (Mohamed et al 1993).

After confirming the introduction of the additional genes in both engineered strains, we observed that full segregation in all the chromosomes could not be achieved. The recombination sites (designed to recombine with the *psbA2*) were 500 bp and the introduced *ppsa, pepc* and *mdh* were 2500 bp, 3000 bp and 1000 bp, respectively. Thus, the extra copies of the native genes were much larger than the designed homologous recombination site.

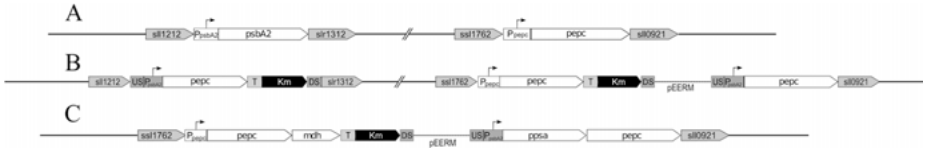

Figure 5. Location of the genetic constructs in the engineered strains. A corresponds to the WT genome, B to the engineered strain with two extra copies of *pepc* (WT+2xPEPc) and C to the engineered strain containing one extra copy of *ppsa*, *pepc* and *mdh* (WT+PPM) (Paper II).

As a result, in the engineered strain overexpressing only the native *pepc* (WT+2xPEPc), the extra copy of the *pepc* recombined with the native *pepc* and the *psbA2* and therefore the engineered strain contained two extra copies of the *pepc*. The engineered strain overexpressing the three genes of the MOG

pathway (WT+PPM) showed that the native *pepc* and the extra copy of *pepc* recombined but in this case, it did not recombine with the *psbA2* (Figure 5).

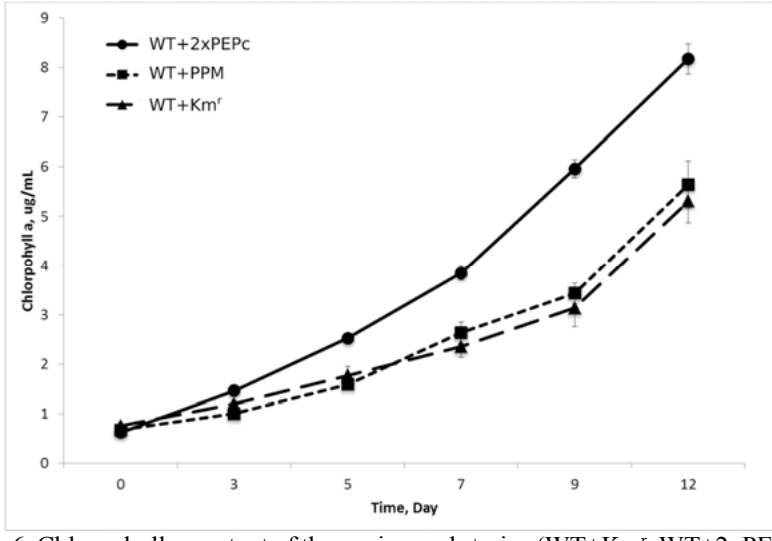

Figure 6. Chlorophyll *a* content of the engineered strains (WT+Km^r, WT+2xPEPc and WT+PPM) for 12 days under low light intensity (3 µmol photons·m^{-2}·s^{-1}). Mean ± SE (Paper II).

When the engineered strains were grown under normal light (45 µE·m^{-2}·s^{-1}), no differences were observed in chlorophyll *a* content. Interestingly, when they were grown in low light (3 µE·m^{-2}·s^{-1}), the engineered strain with two extra copies of *pepc* (WT+2xPEPc) showed a higher chlorophyll *a* content compared to the control strain (WT+Km^r). Nevertheless, no differences were observed between the control strain (WT+Km^r) and the engineered strain containing the three first genes of the MOG pathways (WT+PPM) (Figure 6).

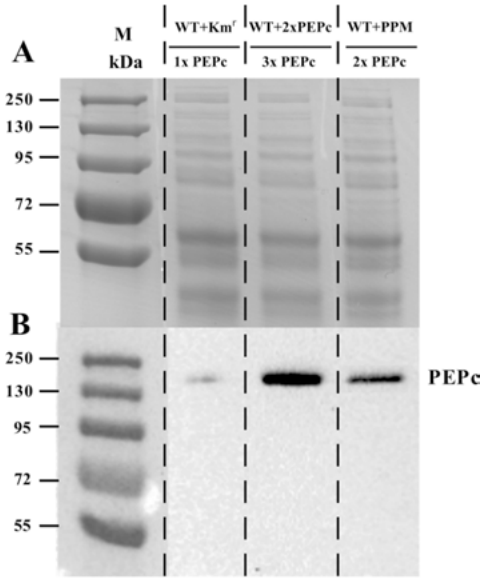

Figure 7. PEPc protein level in the engineered strains (day 9 of Figure 6). A corresponds to the SDS-PAGE where PEPc cannot be detected and B corresponds to the Western immunoblot using an antibody against PEPc. M corresponds to the marker in kDa (Paper II).

Firstly, the WT-PEPc level (WT+Km^r) was barely detectable while the engineered strains showed higher levels of PEPc (Figure 7). The PEPc protein level increased by increasing the number of *pepc* copies present in the genome (Figure 7). Secondly, in agreement with the higher PEPc protein level, the *in vitro* PEPc activity assays showed higher activity in the engineered strains, WT+2xPEPc and WT+PPM, compared to the control strain (WT+Km^r) (Table 2).

Table 2. *In vitro* PEPc activities in the engineered strains (day 9 of Figure 6).

Engineered strain of *Synechocystis* PCC 6803	Number of copies of *pepc*	*In vitro* PEPc activity (nmol of malate·mg of protein^{-1}·min^{-1})
WT+Km^r	1	35.4 ± 5.1
WT+2xPEPc	3	80.3 ± 12.0
WT+PPM	2	42.2 ± 7.6

Characterization of PEPc from the cyanobacterium *Synechococcus* PCC 7002 (Paper III)

PEPc from the cyanobacterium *Synechococcus* PCC 7002 was chosen to be kinetically characterized because the PEPc from *Syn* PCC 6803 was already characterized (Takeya et al 2017). In addition, this cyanobacterium has a higher growth rate compared to *Syn* PCC 6803 (Yu et al 2015) and the PEPc from another *Synechococcus* showed the highest Vmax and a low Km for bicarbonate compared to other cyanobacterial strains (Table 1, Chen et al 2002). The carboxylase has conserved amino acids typical for freshwater strains, while it is a marine strain (Smith and Plazas 2011) and a predicted amino acid model structure showed that the PEPc from *Synechococcus* PCC 7002 has two extra barrels compared to other PEPcs (Smith and Plazas 2011). This makes the *Synechococcus* PCC 7002 enzyme interesting to examine.

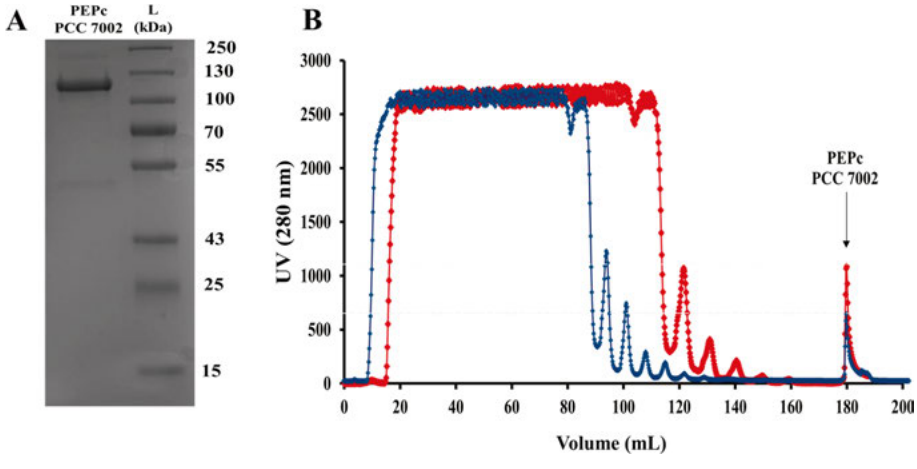

Figure 8. Purified Phosphoenolpyruvate carboxylase (PEPc) from *Synechococcus* PCC 7002. Blue color corresponds to the purification of PEPc PCC 7002 in TBS pH 8.0. Red color corresponds to the purification of PEPc PCC 7002 in TBS+25 mM $MgCl_2$, pH 8.0. A SDS-PAGE showing the purified PEPc PCC 7002. L corresponds to marker (kDa). B Chromatograms showing the influence of Mg^{2+} on the yield of purified PEPc (Paper III).

The PEPc from *Synechococcus* PCC 7002 (PEPc PCC 7002) was successfully purified by adding a Strep-tag on the N-terminus (Figure 8A). Interestingly, when the protein was purified in the presence of Mg^{2+} (25 mM $MgCl_2$), the yield of the purification was almost doubled compared to when the process was lacking the divalent cation (Figure 8B).

PEPc has been reported to be present in both as a dimer and a tetramer, the former being the inactive and the latter the active form. The stabilization of the tetramer is due to an interaction between a glutamic acid (E498) and an

arginine (R503) (*Synechococcus* PCC 7002 numbering) and it is conserved among all PEPcs (Smith and Plazas 2011). The tetramer dissociates into dimer in excess of dilution, in agreement with our data (Paper III, Willeford and Wedding 1992).

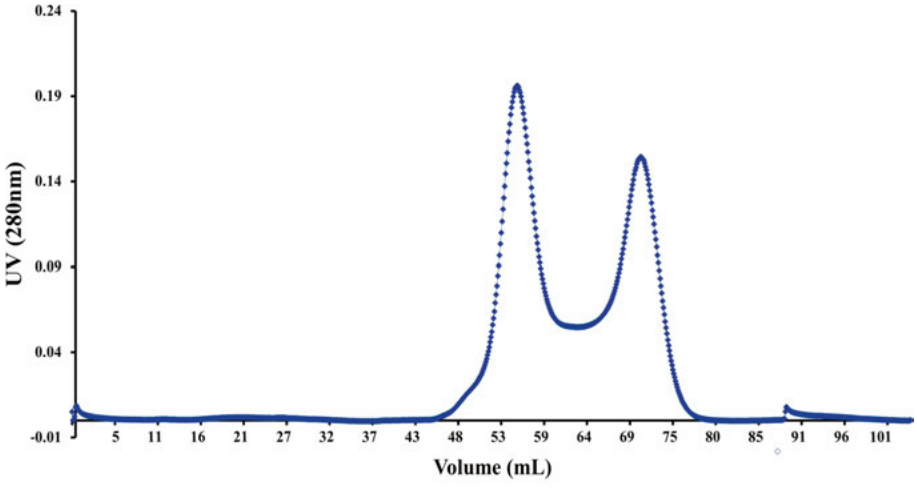

Figure 9. Chromatogram of PEPc PCC 7002 using size exclusion chromatography (Superdex) when PEPc PCC 7002 was run with TBS+25 mM $MgCl_2$, pH 8.0. The fraction collected between 50-60 mL corresponds to the tetramer while the fraction between 66-76 corresponds to the dimer (Paper III).

The purified PEPc PCC 7002 showed two different levels of activities and therefore we suspected that at least two oligomers were present. Size exclusion chromatography (SEC-Superdex column) was performed in order to verify the number of isoforms. The volume of the protein and buffer loaded into the column (570 µL) was very small compared to the total volume of the column (100 mL). The high dilution may result in the dissociation of the protein into the dimer form and that is why the small peak form (dimer) was always eluted in all the conditions tested (Table 3). Since, at that stage, we did not know if the large and the small peaks corresponded to the tetramer and dimer, size exclusion chromatography-small scattering angle X ray (SEC-SAXS) (using the column Shodex) was performed. The molecular weight of the proteins corresponding to the small and the large peaks were estimated to be 231 kDa and 462 kDa for the dimer and tetramer, respectively and this is agreement with the theoretical molecular calculated 115.4 kDa/monomer. In the Shodex column (4.6 mL) coupled to the SAXS, the dilution was much smaller and therefore the main peak observed in all the conditions tested was the tetramer (Figure 10). In addition, when the protein concentration was lowered to the same as used in the Superdex column (0.87 mg/mL), the peak of the dimer was

larger. With all these data, together with the data from the Batch-SAXS we can conclude that there is a higher proportion of dimer when the concentration is lower.

When the purified PEPc PCC 7002 with TBS+25 mM $MgCl_2$, pH 8.0 (Mg_25) was run in the SEC-Superdex, two isoforms were eluted (Figure 9), but when the protein was run with only TBS, pH 8.0, only the dimer form eluted (Table 3). When SEC-Shodex was used, the dimer form was never observed alone but the tetramer peak increased when $MgCl_2$ was used (Figure 11). Thus, 25 mM of $MgCl_2$ has an influence on the formation of the tetramer in PEPc PCC 7002 and this fact might explain the higher yield of purified PEPc PCC 7002 compared to when the divalent cation was not present (Figure 8B). A similar effect was observed in the PEPc from *Crassula argentea* but with lower concentration of Mg^{2+} (Wu and Wedding 1985).

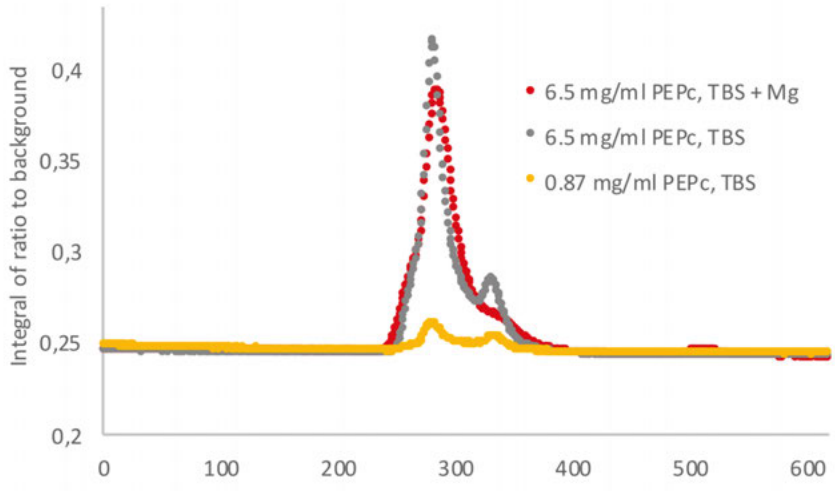

Figure 10. Overlay of signal plots from SEC-SAXS (Shodex) of PEPc PCC 7002 in TBS at two different concentrations (red, grey) and in TBS with 25 mM $MgCl_2$ (yellow). The main peak corresponds to the tetramer, the small peak corresponds to the dimer (Paper III).

After that, we decided to investigate if the substrates and/or the cofactor of PEPc induced the tetramerization form. The concentrations used were based on the *in vitro* PEPc assay that we used in our previous assays (Paper II). When low concentration of $MgCl_2$ (10 mM $MgCl_2$, Mg_10) or $NaHCO_3$ (5 mM) were used, the dimer was the only isoform present (Table 3). Interestingly, 5 mM of PEP induced the tetramerization form (Table 3). We suspect that the low concentration of Mg^{2+} did not induce the tetramerization form due to the dilution excess during the SEC-Superdex. However, it has not been observed before that PEP induces the tetramerization form of PEPc.

When we started to combine the substrates with/without cofactor, we could observe that the tetramer was eluted when 5 mM $NaHCO_3$ and 10 mM of $MgCl_2$ were mixed. It was proposed that, in the PEPc reaction, Mg^{2+} binds first, then PEP and the last HCO_3^- (Kai et al 2003), so it is difficult to explain why $MgCl_2$ (10 mM) and $NaHCO_3$ (5 mM) condition induced the tetramerization.

Table 3. Conditions used in the size exclusion chromatography-Superdex. All the conditions contained TBS pH 8.0.

Buffer	Addition to the TBS buffer	Tetramer	Dimer	ratio (tetramer:dimer)
TBS	-	-	+	0 : 1
Mg_25	25 mM $MgCl_2$	+	+	1.4 : 1
Mg_10	10 mM $MgCl_2$	-	+	0 : 1
HCO_3^-	5 mM $NaHCO_3^-$	-	+	0 : 1
PEP	5 mM PEP	+	+	1.06 : 1
Mg_10+ HCO_3^-	10 mM $MgCl_2$ and 5 mM $NaHCO_3^-$	+	+	0.85 : 1
Mg_10+PEP	10 mM $MgCl_2$ and 5 mM PEP	+	+	1.3 : 1
HCO_3^-+ PEP	5 mM $NaHCO_3^-$and 5 mM PEP	+	+	0.85 : 1
Mg_10+ HCO_3^-+PEP	10 mM $MgCl_2$, 5 mM $NaHCO_3^-$ and 5 mM PEP	+	+	Unknown (higher:lower)

Not surprisingly, since individually PEP induced the tetramer form, when either $NaHCO_3$ (5 mM) or $MgCl_2$ (10 mM) were mixed with PEP (5 mM), the tetramer was also eluted (Table 3). The PEPc from maize may be induced when PEP and Mg^{2+} are present forming a complex (Willeford et al 1990), and this is somehow in agreement with our results since this condition showed

the higher ratio towards tetramer when PEP, or low concentration of Mg^{2+} and HCO_3^-, was present (Table 3).

Since PEP interfered with the reading at A_{280}, when the PEPc reaction was performed, no peaks were observed. Instead, the tubes where the dimer and tetramer eluted in the previous runs, were collected and an SDS-PAGE was run. The tetramer form was the main isoform present in the reaction (Table 3, Paper III), in agreement with other studies showing that the tetramer is the active form of PEPc (Wu and Wedding 1985).

The tetramer form eluted from the experiment Mg_25 (Table 3, Figure 9) was kinetically characterized (T-PEPc PCC 7002). Even though the high concentration of Mg^{2+} can influence the kinetics of the carboxylase, we considered that in the other experiments, the substrates were incubated with the carboxylase and it could have more influence on the kinetics of the enzyme. In addition, we were not in control of the dimer fraction since we concentrated the protein after elution (and therefore the dimer might be transformed to tetramer) and the dimer converted to tetramer during the reaction. The dimer fraction showed less activity compared to the tetramer, 9.5 ± 1.1 units·mg^{-1} and 20.74 ± 1.8 units·mg^{-1}, respectively.

The optimal pH of T-PEPc PCC 7002 is 7.5-8 (Figure 11A) and this is in the range of all cyanobacterial PEPc characterized so far (Table 1). The optimal temperature is 35°C being the same as the PEPc from *Anabaena* (Figure 11B, Table 1, Takeya et al 2017). The carboxylase obeyed Michaelis - Menten kinetics for both substrates (Figure 11C and D) and this concurred with the same enzyme from the cyanobacterium *C. peniocystis* (Owttrim and Coleman 1986). The Km for PEP was 0.77 mM being one of the highest among all the cyanobacterial PEPc characterized so far (Table 1). PEP is abundant during the day (Eisenhut et al 2008) and lower in darkness but still present in the cells (Owttrim and Coleman 1988, Hanai et al 2014). The Km for HCO_3^- is 0.24 mM being the lowest Km, together with that of *Anabaena* PEPc, of all cyanobacterial PEPcs (Table 1). During the day, the inorganic carbon transporters are active and it has been estimated that the level of HCO_3^- is 3-4 mM in *C. peniocystis,* with lower levels during the night (Coleman and Colman 1981). The Vmax of the tetramer at optimum conditions was 20.74 ± 1.8 units·mg^{-1} (Table 1).

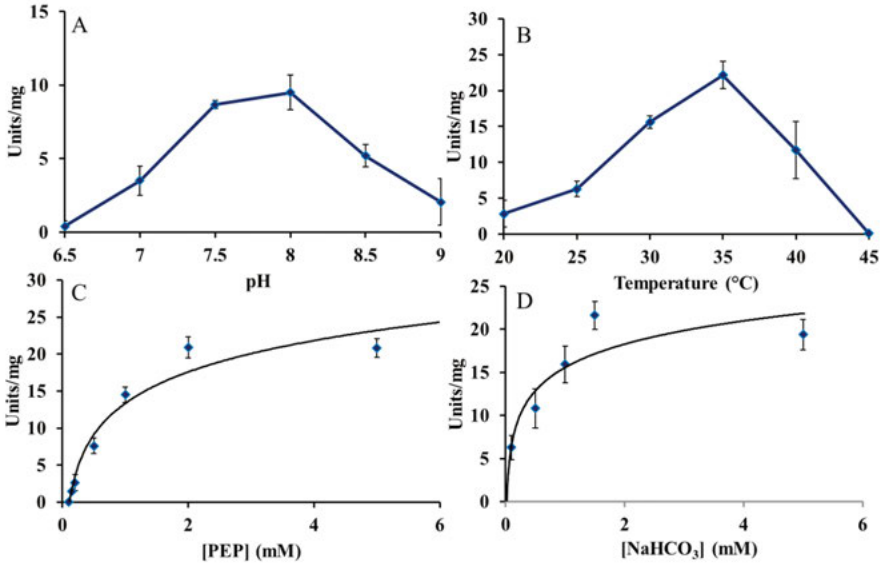

Figure 11. Specific activity of purified tetramer of PEPc PCC 7002 obtained in Figure 10. A Specific activity of T-PEPc PCC 7002 using different pHs (23°C). B Specific activity of T-PEPc PCC 7002 using different temperatures (pH 7.5). C Specific activity of T-PEPc PCC 7002 using different concentrations of PEP (pH 7.5, 35°C). D Specific activity of T-PEPc PCC 7002 using different concentrations of $NaHCO_3^-$ (pH 7.5, 35°C). One unit is defined as 1 µmol of NAD^+ produced per minute. Mean ± SD. (Paper III)

It is well known that PEPcs are well conserved on the C-terminus but not on the N-terminus where the residues for regulation are located. Malate is a known inhibitor of PEPc and in photosynthetic PEPcs, malate seems to induce the dimer form and therefore "inactivate" the enzyme (Wu and Wedding 1985). When PEPc PCC 7002 was run in the Superdex-SEC together with Mg_25, the tetramer form eluted, meaning that malate did not dissociate the enzyme under the conditions tested. It can either be that the malate does not affect the oligomerization of PEPc PCC 7002 or that the oligomerization towards the tetramer with high concentrations of Mg^{2+} is too strong to be affected by the concentration of malate that we tested. In addition, when the *in vitro* PEPc activity assay was performed, 2 mM of malate did not affect the Vmax of the protein (Figure 12). Thus, malate is not an allosteric inhibitor of PEPc PCC 7002 under the conditions tested.

In order to observe if potential activators and inhibitors may affect the Vmax of the enzyme, the concentration of the $NaHCO_3$ and PEP were reduced to 1 mM in the *in vitro* PEPc activity assays. 5 mM of 2-oxoglutarate, 5 mM of glycine, 2 mM of aspartic acid and 2 mM of citric acid did not affect the Vmax of the PEPc PCC 7002 (Figure 12). Different pH-values been reported to enhance the effect of some of these compounds (Takeya et al 2017). The fact

that the cyanobacterial pH changes during day and night and that these compounds may change the levels during light and darkness could have a different effect on the carboxylase *in vivo* (Hatch 1979, Coleman and Colman 1981, Kenyon et al 1987).

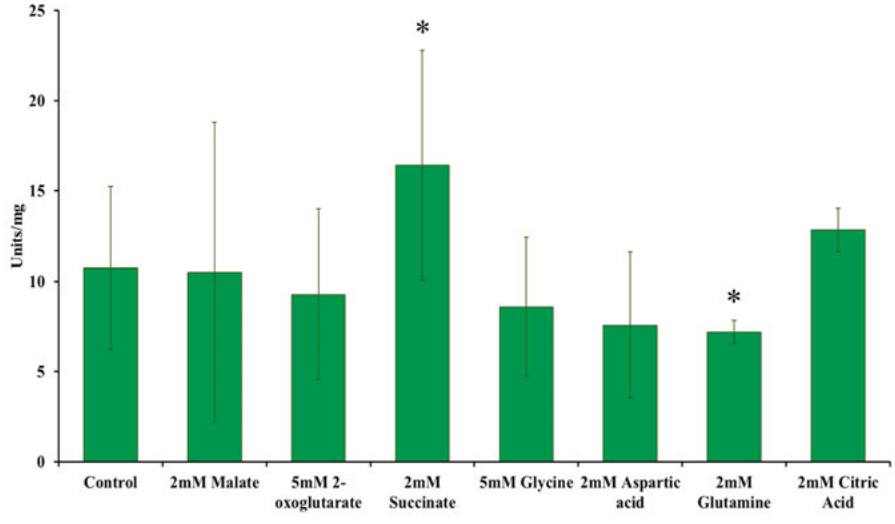

Figure 12. Specific activity of the tetramer form of PEPc PCC 7002 eluted in Figure 10 in the presence of different compounds. The amount of PEP and HCO_3^- was 1 mM. One unit is defined as 1 µmol of NAD^+ produced per minute. Mean ± SD. Asterisks correspond to significant differences compared to the control (Paper III).

While none of the compounds discussed above affected T-PEPc PCC 7002, 2 mM of succinate and 2 mM of glutamine increased and decreased the Vmax, respectively (Figure 12). Succinate has been reported to be an inhibitor (Ozaki and Shiio 1969, Wong and Davis 1973) but in agreement with our results (T-PEPc 7002), it seems to also be an activator of PEPc from *Syn* PCC 6803 (Takeya et al 2017). Against expectations, glutamine showed to be an inhibitor of T-PEPc PCC 7002 while it has been reported to be an activator in green algae and to *S. volcanus* (Schuller et al 1990, Rivoal et al 1998, Chen et al 2002).

The scattering curves obtained with SEC-SAXS were used to compare the calculated scattering curves from crystallized PEPc. The PEPc from *E. coli* was the most similar structure among all the scattering curves compared. This is not surprising since the *E. coli* PEPc was crystalized with the presence of Mn^{2+} and this organism contains a PEPc involved in the TCA cycle, similar function of PEPc in cyanobacteria.

Increased ethylene production by overexpressing PEPc (Paper IV)

Ethylene is an important hormone in plants and it is involved in germination, fruit ripening and senescence (Aharoni and Lieberman 1979, Boller et al 1983, Abeles and Takeda, 1990). There are several pathways found in nature (Kende 1993, Weingart 1999). *Pseudomonas syringae* has a simple pathway in order to produce ethylene before the infection of its host. The pathway expresses the ethylene-forming enzyme (*efe*) which uses 2-oxoglutarate and arginine as substrates and produce ethylene and succinate. Both products are precursors of biofuels and plastics.

The *efe* was successfully introduced in two cyanobacteria, *Syn* PCC 6803 and *S. elongatus* PCC 7942, giving rise to ethylene production. It was believed that the *efe* was unstable but a recent study showed that the instability may be associated to the expression strategy used (Carbonell et al 2016). Many studies have been done in order to increase the ethylene expression and therefore the production. Different promoters, RBS and number of copies have been tested in order to increase ethylene production (Guerrero et al 2012, Ungerer et al 2012, Veetil et al 2017, Xiong et al 2015 and Zhu et al 2015).

Since we discovered that our previous strain WT+2xPEPc showed higher chlorophyll *a* level, and chlorophyll *a* is produced from glutamate and the latter is synthesized from 2-oxoglutarate, we decided to introduce the *efe* in *Synechocystis* WT+2xPEPc and WT+Km^r (Paper II) under the control of the inducible promoter, *PnrsB*. Interestingly, the engineered strain overexpressing *pepc* and expressing *efe* (CD-P, Table 4) showed significant higher ethylene production (10.5 ± 3.1 µg · ml^{-1} · OD^{-1}· day^{-1}) compared to the control strain CD-C (6.4 ± 1.4 µg · ml^{-1}· OD^{-1}· day^{-1}) ($p=0.002$) (Figure 13). The Efe protein level was similar in both strains (Figure 14), while the PEPc protein level was higher in the CD-P (Figure 15) leading to the conclusion that PEPc is a bottleneck in the production of ethylene in *Syn* PCC 6803.

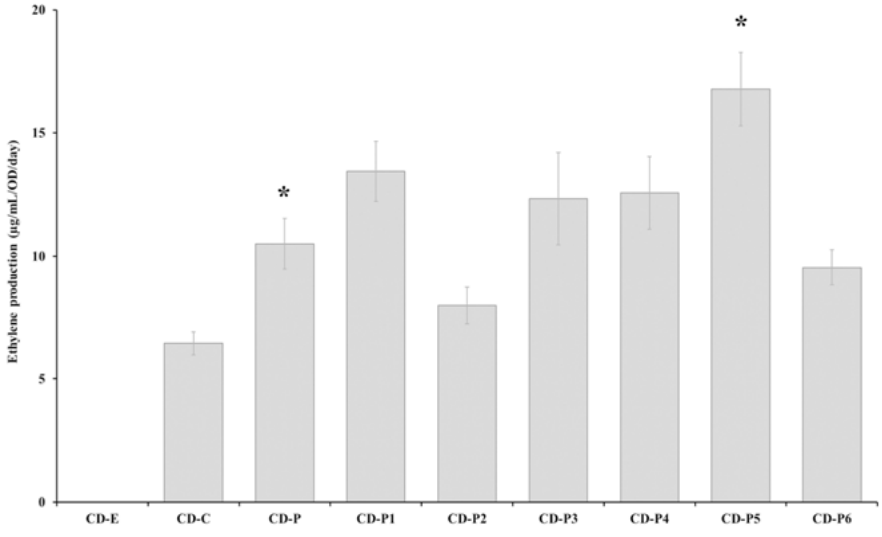

Figure 13. Ethylene production of the engineered strains created in this study (Table 4). Mean ± SE. Asterisks correspond to significant differences compared to the control (Paper IV).

In order to further improve the ethylene production, another copy of *pepc* and/or phosphoenolpyruvate synthase, *ppsa* (from *Syn* PCC 6803 or *Synechococcus* PCC 7002) were introduced into CD-P. The created engineered strains are summarized in Table 4 and the Efe, PPSA and PEPc protein levels are shown in Figure 14 and 15. The engineered strains expressing either an extra copy of the native PPSA (CD-P2) and/or PEPc (CD-P3 and CD-P1) or a copy of the PEPc (CD-P4) or PPSA+PEPc from *Synechococcus* (CD-P6) did not further increase the ethylene production (Figure 14). The engineered strain with the heterologous expression of PPSA from *Synechococcus* (CD-P5) however, showed significant higher ethylene production (16.8 ± 4.5 $\mu g \cdot ml^{-1} \cdot OD^{-1} \cdot day^{-1}$) compared to the control engineered strain overexpressing PEPc, CD-P ($p=0.003$) (Figure 13). The DNA and amino acid identity between PPSA from *Syn* PCC 6803 and *Synechococcus* proteins is 70.5 and 76.6%, respectively. Since not much is known about PPSA, it might be that some differences can be attributed to the amino acids, located at the N-terminus or on the insertion between 145-160 amino acid sequence PPSA PCC 7002 (Paper IV). This may explain the different kinetics and regulation of the enzymes in the metabolism of *Syn* PCC 6803.

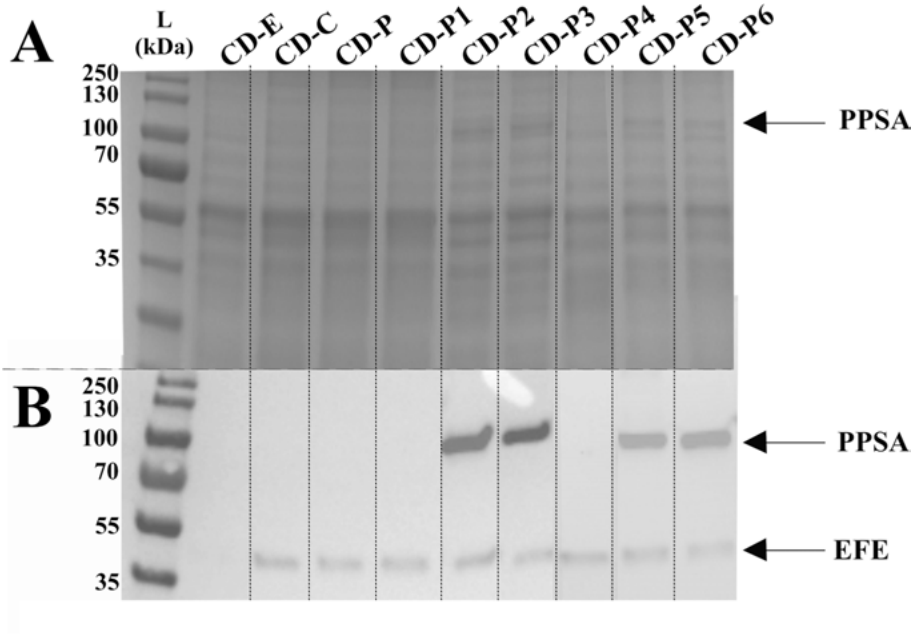

Figure 14. Expression of the Flag tagged proteins, ethylene-forming enzyme (Efe) and phosphoenolpyruvate synthase (PPSA). A SDS-PAGE loaded with 4 µg of crude extract of the engineered strains (Table 4). B Western immunoblot using the anti-flag antibody. The upper band correspond to PPSA (approximate molecular weight 91.17 and 92.56 KDa for $PPSA_{6803}$ and $PPSA_{7002}$, respectively) and the lower band to EFE (approximate molecular weight 42.16 kDa) (Paper IV).

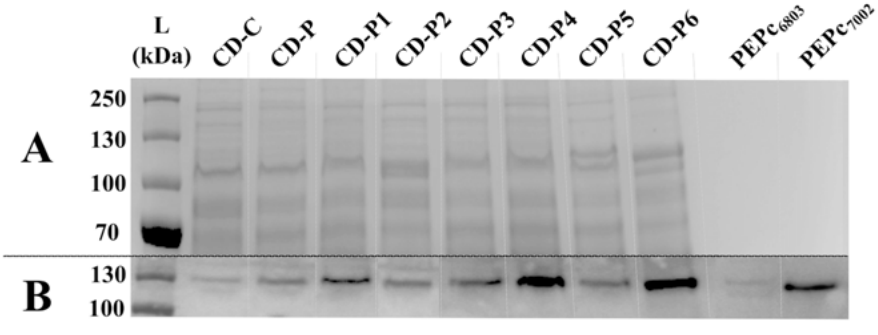

Figure 15. Expression of phosphoenolpyruvate carboxylase (PEPc) of the engineered strains (Table 4). A SDS-PAGE loaded with 31 µg of crude extract of the engineered strains (Table 4). B Western immunoblot using the anti-PEPc antibody. The PEPc approximate molecular weight is 110 KDa. $PEPc_{6803}$ and $PEPc_{7002}$ corresponds to purified protein (100 ng loaded) of PEPc from *Synechocystis* PCC 6803 and *Synechococcus* PCC 7002, respectively (Paper IV).

Further bottlenecks for ethylene production were investigated by the addition of several substances to the engineered strain CD_P. When arginine was added

to the medium in our standard condition (50 mM Tris pH 8.0, 50 mM $NaHCO_3$, Km (25 μg · mL^{-1}), Cm (20 μg · mL^{-1}), 5 μM of $NiCl_2$ and 20 $\mu E \cdot m^{-2} \cdot s^{-1}$), the ethylene production did not increase (BG11+Arg, Figure 16) agreeing with Carbonell et al 2019. However, higher Efe protein concentration was observed. We conclude that arginine and Efe are not a limiting the ethylene production. It is known that nitrogen starvation increases 2-oxoglutarate levels and therefore may increase ethylene production. However, in a previous study the opposite effect was observed (Ungerer et al 2012). We did not see a decrease in ethylene production in 24h (Figure 16) in agreement with Carbonell et al 2019, when 2-oxoglutarate was added. The addition of arginine into the nitrogen starvation condition ($BG11_0$+Arg) did not increase or decrease ethylene production compared to the standard condition (Figure 16). When nitrogen starvation and arginine are present, the cyanophycin (the nitrogen storage compound synthesized from arginine and aspartic acid) synthesis is upregulated (Stephan et al 2000) and arginine may not be available for the Efe reaction. Another strategy in order to increase 2-oxoglutarate levels was the addition of azaserine which is an inhibitor of the glutamine amino transferase in the GS-GOGAT cycle (Merida et al 1991). However, the ethylene production was decreased instead (BG11+Aza and BG11+Arg+Aza+Cyclo, Figure 16) while the Efe protein level was similar to the standard condition.

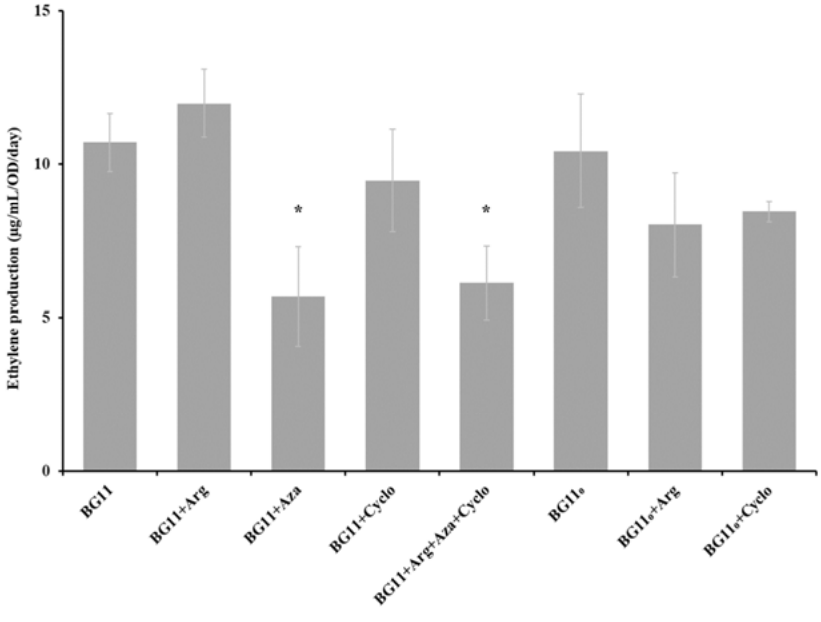

Figure 16. Ethylene production when the engineered strain CD-P was incubated with different conditions. Mean ± SE. Asterisks correspond to significant differences compared to the control. All the conditions contained 50 mM Tris pH 8.0, 50 mM $NaHCO_3$, Km (25 μg · mL^{-1}), Cm (20 μg · mL^{-1}), 5 μM of $NiCl_2$ and 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 24h. Arg corresponds to 50 μM of arginine, Aza corresponds to 100 μM azaserine,

BG11 corresponds to media with nitrate, $BG11_0$ corresponds to media without nitrate, Cyclo corresponds to 100 µM cyclodyxim (Paper IV).

Acetyl-CoA may be an activator of PEPc and since the overexpression of PEPc can lead to lower acetyl-CoA and lower activity of citrate synthase (reaction between acetyl-CoA and citrate, Figure 3), the Acetyl-CoA carboxylase was inhibited by cycloxydim. Interestingly, the cells did not increase ethylene production (Figure 16) meaning that acetyl-CoA is not a limited substrate for the TCA cycle in the engineered strain CD-P. Acetyl-CoA may be replaced by the "C4 like metabolism". When cycloxydim was added the CD-P1 and CD-P4, the engineered strains increased ethylene production (Figure 17) meaning that acetyl-CoA is a limiting compound in those strains.

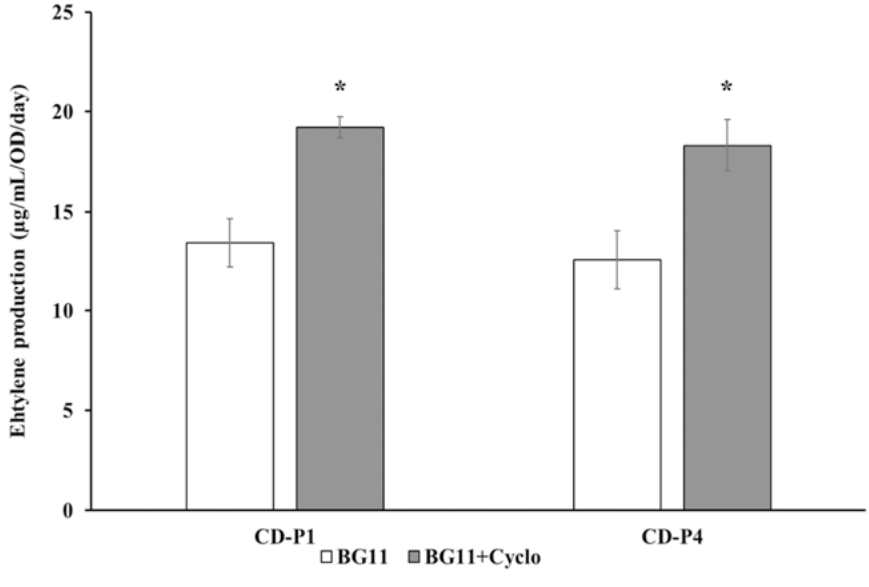

Figure 17. Ethylene production when the engineered strains CD-P1 and CD_P4 were incubated in standard (BG11) or addition of 100 µM cyclodyxim (BG11+Cyclo) Mean ± SE. Asterisks correspond to significant differences compared to the BG11 (Paper IV).

Table 4. Engineered strains used/constructed in this study.

Engineered strains	Genetic modification
CD-E	Resistant to Km, Sp and Cm
CD-C	Resistant to Km and Cm and expressing the *efe* from *P.syringae* (resistant to Sp)
CD-P	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *P.syringae* (Sp) and resistant to Cm
CD-P1	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *P.syringae* (Sp) and another extra copy of the native *pepc* (Cm)
CD-P2	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *P syringae* (Sp) and an extra copy of the native *ppsa* (Cm)
CD-P3	Overexpressed *pepc* from Syn PCC 6803 (Km), expressing the *efe* from *P.syringae* (Sp), an extra copy of the native *ppsa* and another extra copy of the native *pepc* (Cm)
CD-P4	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *P.syringae*(Sp) and another extra copy of *pepc* from *Synechococcus* (Cm)
CD-P5	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *Psyringae* (Sp) and another extra copy of the *ppsa* from *Synechococcus* (Cm)
CD-P6	Overexpressed *pepc* from *Syn* PCC 6803 (Km), expressing the *efe* from *P.syringae* (Sp) and another extra copy of the *ppsa* and *pepc* from *Synechococcus* (Cm)

Increased succinate production by introducing a glyoxylate shunt and overexpressing PEPc (Paper V)

Succinate is an important industrial precursor since 1,4-butanediol, adipic acid and some pharmaceuticals including antibiotics and amino acids (Zeikus et al 1999) can be synthesized from it. Succinate is a key metabolite in the TCA cycle (Figure 3) and secrete naturally during fermentation (Nghiem et al 1997, Guettler et al 1999, Lee et al 2002, Song and Lee 2006, Dashko et al 2014). It is known that during light, the oxidative branch of the TCA cycle is the most operative, since 2-oxoglutarate is used for the nitrogen metabolism (Hasunuma et al 2016). In darkness, SDH plays an important role by reducing the PQ and therefore contributing to the synthesis of ATP and the oxidative branch is the most active (Hasunuma et al 2016). In anoxic darkness, a very reductive environment and no final electron acceptor for the electron transport chain, the cells need to get rid of electrons. Consequently, some substances are secreted (succinate, acetate…), and under this condition, the reductive branch of the TCA cycle is the most active (Hasunuma et al 2016).

The glyoxylate shunt is present in some bacteria and in some nitrogen fixing cyanobacteria and it is connected to the TCA cycle (Maloy et al 1980, McKinlay et al 2015, Zhang and Bryant 2015). The advantage of this cycle is that bacteria are capable to grow with acetate or fatty acids as a sole carbon source. It also avoids the carbon loss of the isocitrate dehydrogenase and in bacteria the 2-oxoglutarate dehydrogenase. The first enzyme of the glyoxylate pathway isocitrate lyase (ICL) consumes the TCA cycle intermediate isocitrate and produces two products, glyoxylate and succinate. Glyoxylate is further metabolized by malate synthase (MS) (the second enzyme of the glyoxylate shunt) or by the photorespiration metabolism (Eisenhut et al 2008).

The overexpression of PEPc has been shown to increase succinate secretion in cyanobacteria under dark anoxic conditions (Hasunuma et al 2016). Thus, we decided to introduce the partial (ICL) and the complete glyoxylate shunt (ICL+MS) in the engineered strains WT+Km^r and WT+2xPEPc (Paper II) (Table 5). Interestingly, the introduction of the complete glyoxylate shunt into the WT+Km^r resulted in no colonies leading to the conclusion that the leakage of the *PnrsB* expressing ICL and MS affects the viability of the cells. However, the partial and the complete glyoxylate shunt was successfully introduced in the WT+2xPEPc (2P_C) creating 2P_I and 2P_IM, respectively. The engineered strains are summarized in Table 5.

Table 5. Engineered strains created/used in this study. Km corresponds to kanamycin resistant cassette, Cm corresponds to chloramphenicol resistant cassette, ICL corresponds to isocitrate lyase from *E. coli* BL21, and MS to malate synthase from *E. coli* BL21.

Engineered strain	Genetic modification
WT_C	WT+Km^r (Paper II) resistant to Cm
2P_C	WT+2xPEPc (Paper II) resistant to Cm
2P_I	WT+2xPEPc expressing ICL (Cm)
2P_IM	WT+2xPEPc expressing ICL and MS (Cm)

In light and darkness, the 2P_IM showed the highest succinate secretion in the presence of nitrogen (Figure 18J and K). In anoxic darkness, 2P_I was the strain secreting most succinate (Figure 18I). In nitrogen starvation, 2P_IM showed the highest succinate secretion in light and darkness (Figure 18J and K) while in nitrogen starvation, anoxic darkness the highest secretion was observed 2P_I (Figure 18I).

Interestingly, when SDH was inhibited the succinate secretion increased compared to the non-blocked SDH treatment in all conditions tested with the exception of dark anoxic and nitrogen starvation (Figure 18). In the presence of nitrogen, in light or darkness, the 2P_IM showed the highest succinate secretion (Figure 18J and K) while in anoxic darkness 2P_C had the highest succinate secretion (Figure 18F). The 2P_I was the strain with secreting most succinate when SDH was inhibited, nitrogen starvation in light, darkness and anoxic darkness (Figure 18G, H and I).

With these data we can conclude that SDH is active in light, darkness and in fermentation and thus contributing to reduce the PQ in the electron transport chain.

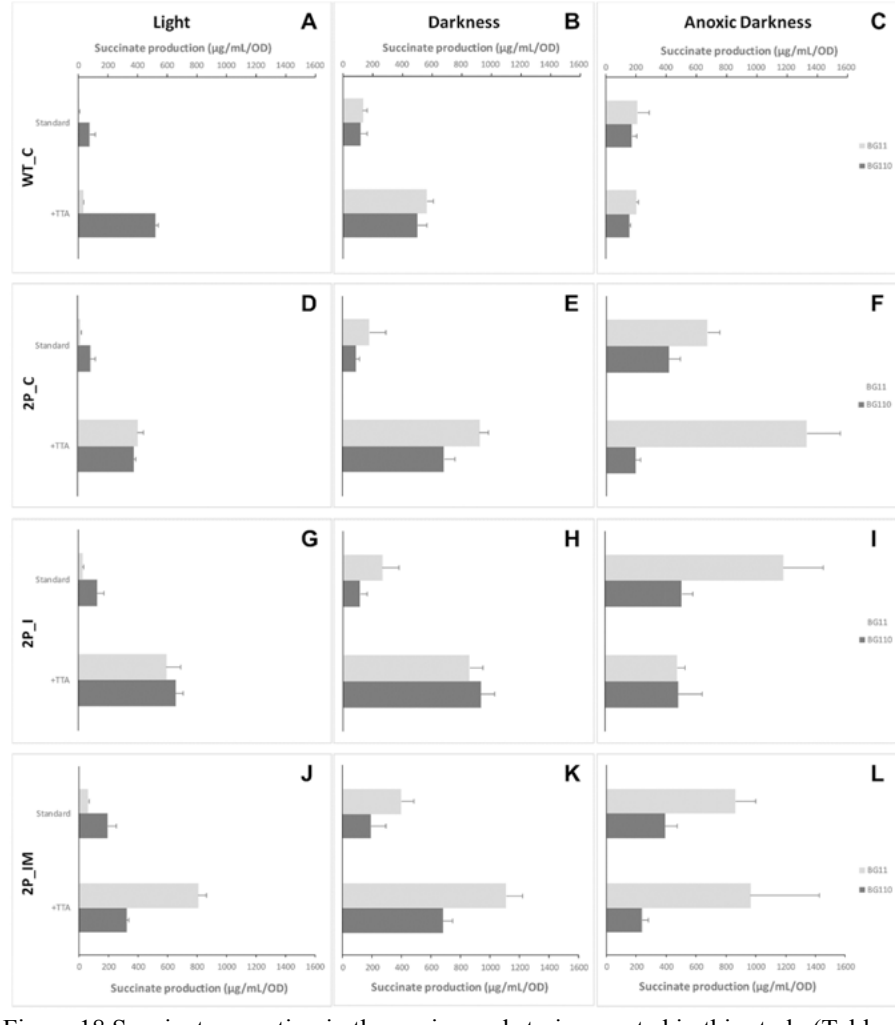

Figure 18.Succinate secretion in the engineered strains created in this study (Table 5). BG11 corresponds to the standard media, $BG11_0$ corresponds to the standard media without nitrogen source. Light condition was performed at 20 $\mu E \cdot m^{-2} \cdot s^{-1}$ for 4 days, Dark condition was performed in darkness for 4 days and anoxic darkness was performed in darkness and anaerobic conditions for 4 days. Standard corresponds to BG11 or $BG11_0$, with 5 µM of $NiCl_2$ and +TTA corresponds to BG11 or $BG11_0$ + 5 µM of $NiCl_2$ and 1 mM of 2-thenoyltrifluoroacetone.

Western Blots showed higher PEPc levels in 2P_C, 2P_I and 2P_IM compared to the WT_C (Paper V). The ICL was expressed in the 2P_I and 2P_IM strains while the MS was only expressed in the 2P_IM strain (Paper V). The expression levels of ICL and MS was different depending on the conditions used in the 2P_IM strain (Figure 19). Higher expression of ICL and MS was present in light conditions compared to dark or anoxic darkness (Figure 19). It has been reported that the *PnrsB* has different levels of activity depending on the

light condition and that may explain the differences observed in the levels of ICL and MS, even though darkness condition was not tested in those studies (Englund et al 2015, Englund et al 2016). Nitrogen starvation increased ICL and MS protein levels in light (Figure 19). It may be that the *PnrsB* has a different activity during nitrogen stress or that secondary effects in this condition affects the metal homeostasis of the cells resulting in higher expression of the promoter.

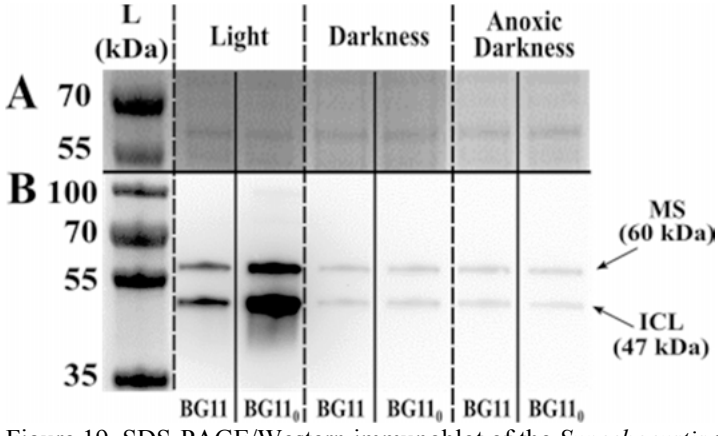

Figure 19. SDS-PAGE/Western immunoblot of the *Synechocystis* strain 2P_IM, analyzed for the presence of isocitrate lyase (ICL) and malate synthase (MS). *A* SDS-PAGE of crude protein from the 2P_IM strain in light, darkness and anoxic darkness. *B*Western immunoblot using anti-Strep antibody for the 2P_IM strain in light, darkness and anoxic darkness.

The overexpression of PEPc (2P_C) had a positive effect on growth in light, light+acetate and darkness+acetate (Paper V). However, the engineered strain 2P_I showed decreased growth rate compared to the 2P_C because most likely the cells metabolized the toxic compound, glyoxylate (Dellero et al 2016). Interestingly, the growth was recovered to the 2P_C levels in the 2P_IM strain since the complete glyoxylate shunt was introduced and it connected with the TCA cycle.

In closing, it would be interesting to further engineer the 2P_IM strain by knocking out or down SDH. The resulting strain could be cultivated in diurnal light cycles. The advantage is that succinate could be produced in the same high yield constantly during light and darkness periods.

Conclusions and Future Directions

This thesis demonstrates that increased carbon fixation, by other means than overexpressing RuBisCO, can enhance production of carbon-based compounds of human interest such as ethylene and succinate. In addition, the cyanobacterial PEPc has been demonstrated to be active in the tetramer form and have similar profile to other non-photosynthetic C3 PEPcs from *E. coli*.

With all the metabolic engineering tools that researchers have been developed for cyanobacteria, future engineered cyanobacterial strains containing or overexpressing multiple pathways may be feasible for large scale biochemical or biofuel production.

For instance, the inhibition of SDH in the strain expressing the glyoxylate shunt and overexpressing PEPc (2P_IM-Paper V) showed the highest succinate secretion in light and darkness. The 2P_IM could be further engineered by introducing the ethylene-forming enzyme and knocking out SDH. If the resulting strain would be grown in diurnal cycles, the cells would produce ethylene and succinate simultaneously in both light and darkness given the innovation of producing higher rates in dark periods. The advantage of these two compounds is that ethylene is a gas and succinate is secreted into the media, so no complex downstream processes would be required which would make the process more economic. In addition, if bottlenecks would be identified and optimized cultivation would be achieved it could result in improved productivity.

Svensk sammanfattning

Cyanobakterier är intressanta organismer då de är de enda kända bakterier som genomför syre-bildande fotosyntes. Cyanobakterier, till skillnad från de flesta bakterier, kräver endast ljus vatten, koldioxid, och vissa icke-organiska näringsämnen för att växa. Eftersom dessa mikroorganismer är relativt enkla att modifiera genetiskt har flera metaboliska reaktionsvägar för att producera biobränsle introducerats. Tack vare forskning är det i dag möjligt att framställa biobränsle genom att odla modifierade cyanobakterier men mängden biobränsle som kan utvinnas är fortfarande låg och framställnings- och extraktionsprocessen kostsam.

Cyanobakterier tar framförallt in koldioxid genom ribulosbisfosfatkarboxylas-oxygenas (RuBisCO), världens vanligaste enzym, och den berömda Calvin-Benson-Bassam-cykeln. När koldioxid är inne i cellerna används det för att bygga socker, fetter, och proteiner. Emellertid är RuBisCO ett ineffektivt enzym eftersom det förutom koldioxid även tar in syre som inte kan användas för detta syfte. Om vi betraktar reaktionsvägarna i cellen som produktionskedjor så kan vi öka produktionen genom att tillföra mer substrat. Om vi således ökar mängden koldioxid som tas upp av cellerna kan detta leda till ökad produktion av de ämnen vi intresserar oss för. Det har visat sig möjligt att öka produktionen av flera biobränslemolekyler på detta sätt (t.ex. etanol, isobutanol och succinat). Merparten av forskningen som gjorts på detta område har fokuserat på att introducera fler kopior av RuBisCO, eller andra proteiner som naturligt förekommer i Calvin cykeln, för att öka kolfixeringen och därmed produktionen av biobränsle.

Bar-Even med kollegor (2010) designade flera nya reaktionsvägar som teoretiskt skulle förbättrar koldioxidupptagningen i fotosyntetiska organismer genom att kombinera delar av de sex kolfixeringsreaktioner som finns i naturen.

Genom att kombinera delar av de sex kolfixeringsreaktionerna som finns i naturen har Bar-Even med kollegor (2010) designat flera nya reaktionsvägar som teoretiskt skulle förbättra koldioxidupptagningen i fotosyntetiserande organismer. Enzymet som fixerar kol i dessa nya reaktioner är fosfoenolpyruvatkarboxylas (PEPc). Detta enzym finns representerat i många metaboliska processer (både i fotosyntetisk och icke-fotosyntetisk metabolism) och är mer effektivt än RuBisCO. Växter som finns i varma klimat har utvecklat

mer effektiva processer för att bibehålla vatten och ta upp koldioxid och enzymet som möjliggör den förbättrade koldioxidupptagningen är PEPc.

Cyanobakterier innehåller PEPc naturligt men det är inte deras huvudsakliga kolfixeringsenzym. Utifrån idén från Bar-Even med kollegor har vi ökat antalet kopior av det naturligt förekommande PEPc-enzymet i cyanobakterien *Synechocystis sp.* PCC 6803. De nya genetiskt modifierade cellerna med fler kopior av PEPc har högre kolfixeringsaktivitet och snabbare tillväxt.

Etylen är en grundkomponent i bioplaster samt biobränsle och den industriella produktionen av eten orsakar stora mängder koldioxidutsläpp. Det är således viktigt att hitta alternativa, mer miljövänliga, sätt att producera etylen. Genom att ta en gen från bakterien *Pseudomonas syringae* och introducera den i cyanobakterier har forskare lyckats producera etylen. Dessa genetiskt modifierade celler som producerar etylen har optimerats genom olika strategier men aldrig kombinerats med genetiskt modifierade celler som tar upp mer koldioxid. I min studie har vi kombinerat genetiskt modifierade cyanobakterier med högre halter PEPc med genen som producerar etylen. Dessa genetiskt dubbelmodifierade cyanobakterier producerar mer etylen än de cyanobakterier som tidigare designats för att producera etylen. Vidare har ytterligare genetiska modifikationer introducerats för att producera ännu högre halter etylen.

Succinat är en grundkomponent i bioplaster och vissa biologiskt nedbrytbara polymerer men används även som en livsmedelstillsats. Cyanobakterier producerar succinat naturligt och olika förhållanden kan förbättra utsöndrandet av denna organiska syra. Vissa organismer, t.ex. bakterien *Escherichia coli (E. coli*), har flera olika reaktionsvägar att producera succinat. I mina studier har vi introducerat en alternativ reaktionsväg från *E. coli* till mina modifierade cyanobakterier med högre halt av PEPc. De genetiskt modifierade cellerna med mer PEPc visade högre succinatnivåer än icke-modifierade celler, och när vi kombinerade dessa med reaktionsvägen från *E.coli* gick succinatproduktionen upp ytterligare.

Under mina doktorandstudier ville jag lära mig mer om biokemi. Vi bestämde mig därför för att karaktärisera PEPc från en annan cyanobakterie, *Synechococcus sp.* PCC 7002. Genom detta experiment visade det sig att PEPc fanns två olika former. Proteinet bestod av antingen två eller fyra likadana subenheter (homodimer eller homotertramer), beroende på under vilka förhållanden experimentet utfördes. När proteinet bestod av fyra subenheter var det mer aktivt än när det bestod av två. Det visade sig också att PEPc-reaktionen från *Synechococcus* var snabbare än reaktionen hos *Synechocystis.* Eftersom PEPc-enzymets proteinstruktur är okänd i cyanobakterier använde

vi en teknik som tillät oss att observera formen av PEPc i låg upplösning. Efter en jämförelse med alla kända strukturer av PEPc kunde vi avgöra att PEPc från *Synechococcus* är mycket lik PEPc från *E. coli*.

Det krävs fortfarande mycket forskning innan cyanobakterier kan börja användas för storskalig produktion av biokemiska produkter och biobränslen. Mina framsteg visar att genmodifiering av kolfixieringsmekanismen kan öka produktionen av efterfrågade ämnen och substanser från cyanobakterier.

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