Physiological and genetic studies towards biofuel production in cyanobacteria
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Citation for published version (APA):

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Chapter 7

General Discussion

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This thesis describes a range of studies on cyanobacteria and *Synechocystis* sp. PCC 6803 in particular. All studies were aimed at optimizing a cyanobacterial cell factory for biofuel production, but in each chapter a different approach is used. In chapters 2, 3 and 4 aspects of the photo physiology of *Synechocystis* sp. PCC 6803 are explored to further our understanding of photosynthesis and cell growth and to aid in the optimization of these processes. Chapters 5 and 6 describe a more direct approach to optimize the production of cellulose in cyanobacteria, as a potential biofuel precursor by manipulating physiological conditions and introducing genes for cellulose synthesis. Together this has resulted in a thesis in which each chapter is a story in its own right. To avoid repetition, this general discussion only pertains to findings that transcend the individual chapters and which require further discussion; furthermore, it is combined with some general conclusions from, and implications of the results presented in this thesis.

The redox state of the PQ pool

With the adaptation of the quinone/quinol detection techniques described by Bekker [124] and Kruk [123] for the determination of the *in vivo* redox state of the PQ pool in cyanobacteria a new piece of the photosynthesis puzzle was revealed. This new information confirmed that the redox state of the PQ pool cannot directly be inferred from PAM fluorimetry data and showed that inhibitors of photosynthetic electron transfer and changes in the light and nutrient conditions have different effects on the overall redox state of the PQ pool than expected. The data also contradicts the theory that state transitions are regulated by the redox state of the PQ pool [9,10,144,325]. In fact, the data described in chapters 2 and 4 show that the PQ pool redox state is set by the state of the light harvesting antenna and subsequent PSII activity, rather than the other way around: Illumination with phycobilisome-specific light, carbon limitation and saturating intensities of white light all resulted in a state II with poor energy transfer to PSII and an oxidized PQ pool. Concomitantly, preferential excitation of PSI with far-red light, high carbon availability and light limitation, all resulted in a state I and a more reduced PQ pool. Furthermore, the photosynthetic inhibitor DBMIB prevents outflow of electrons out of the PQ pool by blocking the Qo pocket of the cytochrome b6f complex and accordingly prevents transfer of electrons to PSI. Because of this, DBMIB was expected to cause a reduction of the PQ pool, and Mullineaux and Allen showed that this indeed led to a state II transition [9]. However,
we found that DBMIB results in an oxidation of the PQ pool (Chapter 2), which is most likely caused by increased quinol oxidase activity [326]. This finding, in combination with the reported state II transition in the presence of DBMIB further supports the notion that state transitions are not regulated by the redox state of the PQ pool.

To ensure optimal growth in any given physiological condition and to prevent accumulation of reduced electron transfer chain intermediates (because of the associated increased risk of formation of reactive oxygen species), energy production in the form of ATP and NADPH by photosynthetic electron transfer, and the consumption of these two cellular free-energy currencies by carbon fixation and metabolism, must remain balanced. State transitions and other regulatory and/or dissipative mechanisms associated with the photosynthetic electron transfer chain, for instance modulation of the rate and nature of cyclic electron transfer, allow cyanobacteria to rapidly and dynamically maintain this (free) energy balance. To properly regulate these processes the cell would need to detect aspects of both energy production and energy consumption to maintain the balance. Since the data described in this thesis show that the redox state of the PQ pool is mainly determined by PSII activity and therefore energy production, it would be a poor indicator of the cell's (free) energy balance. Many studies in the literature report on the involvement of the cytochrome $b_{6}f$ complex in the regulation of state transitions in higher plants, algae and cyanobacteria [154,327-329]. Involvement of this complex is further confirmed since blocking the Q$_{0}$ site of the cytochrome $b_{6}f$ complex with DBMIB results in a state II transition [9] without reducing the PQ pool [Chapter 2]. The fore-mentioned studies show that binding of PQH$_{2}$ in the Q$_{0}$ site of the cytochrome $b_{6}f$ complex triggers a conformational change in the cytochrome which triggers thylakoid phosphorylation and a state II transition in plants and algae [154,329]. Because quinol turnover in the Q$_{0}$ site occurs on a much shorter time scale than antenna migration [329], a stable state II of the light harvesting antenna requires semi-permanent occupation of the Q$_{0}$ site by PQH$_{2}$ and a stable state I requires a frequently vacant Q$_{0}$ site. This can be achieved both by the supply of plastoquinol molecules and by the rate of oxidation of the quinol molecules mediated by PSI activity. With the plastoquinone redox state detection technique described in this thesis fully oxidized PQ pools were very rarely detected, and presumably only because the amount of PQH$_{2}$ present dropped below the detection limit. So even with a relatively oxidized PQ pool, quinol
molecules will still be available to bind to the cytochrome \( b_{6f} \) complex. Since it has been demonstrated in this thesis that cells in state I have an oxidized PQ pool and cells in state II have a reduced PQ pool, regulation of the state transitions could be mediated by the turnover rate of the cytochrome \( b_{6f} \) complex rather than the redox state of the system and this turnover rate will be largely determined by the plastoquinol oxidation rate at the \( Q_0 \) site, fueled by PSI activity and NADP\(^+\) availability rather than by the amount of plastoquinol molecules present in the PQ pool [Fig. 1].

Alternatively, regulation could occur at the \( Q_A \) binding site of PSII or the \( Q_i \) binding site of cytochrome \( b \) in the cytochrome \( b_{6f} \) complex. These binding sites are located at the stromal side and semi-quinone formation at these sites could allow for electrostatic interaction which could directly affect PBS binding. However, addition of DCMU results in full reduction of \( Q_A \) by preventing PSII turnover and electron transfer from PSII to the PQ pool. This then results in a state I transition with increased PBS binding to PSII [111]. If regulation occurred at the level of \( Q_A \) full reduction of this quinone should drive PBS away from PSII. It has been theorized that under strongly reducing conditions such as DCMU and high light \( Q_A \) can be reduced twice to form a quinol molecule which could lose its charge due to protonation [330]. This would then give off an uncharged or oxidized signal, resulting in a state I transition. This would explain the DCMU response when assuming regulation at the level of \( Q_A \), however a state I transition in high light could be quite detrimental for the cell. In the experiments conducted by Vener et al. [154] on the cytochrome \( b_{6f} \) complex of spinach thylakoids the oxidation rate at the \( Q_i \) site was described as very rapid causing cytochrome \( b \) to appear oxidized throughout the experiment in which both state I and state II could be observed and was most likely induced by changes in the plastoquinol occupancy of the \( Q_0 \) site. With the current knowledge regulation of state transitions by the turnover rate of the cytochrome \( b_{6f} \) complex at the \( Q_0 \) site seems most plausible.
Figure 7.1. Proposed regulation of state transitions. The thickness of the arrows indicates the electron flow rate from slow (thin arrow) to fast (thick arrow). The state of the PQ binding sites at the cytochrome and PSII is color coded from white (mostly oxidized) to dark grey (mostly reduced). The font size depicts the size of the different pools of oxidized and reduced electron carriers, ADP and ATP and loose and bound PBS. When NADP⁺ and ADP are readily available (A) turnover of PSI will be fast, resulting in a rapid oxidation of the Q₀ site. The cytochrome complex will signal an oxidized state (go sign) and trigger a state I with strong PBS binding. This will increase turnover of PSII, resulting in a relatively reduced (< 50 %) PQ pool. When NADP⁺ and ADP are limiting (B) turnover of PSI will be slow, resulting in slow oxidation of the Q₀ site. The cytochrome complex will signal a reduced state (stop sign) and trigger a state II with weak PBS binding. This will decrease turnover of PSII, resulting in an oxidized, but not fully oxidized, PQ pool.

‘Direct conversion’ and the regulation of CO₂ fixation in cyanobacteria

In recent years a large research effort has been spent on introducing fermentative pathways into cyanobacteria for the direct production of fermentative fuel products. Increasing the knowledge about the physiology of an organism of interest can help in optimizing both growth and product formation conditions. Also, it can help explain unforeseen changes in the physiology of the transgenic strains. For instance, several of these studies observed an increase in total carbon fixation rate in biofuel producing cyanobacterial strains, as compared to the corresponding WT under the same growth
This can only be explained by the presence of a higher capacity for CO$_2$ fixation and energy production in the wild-type than is used even when growing at the maximal growth rate. The study described in Chapter 4 on the photo-physiology of *Synechocystis* under light saturated- and light limited growth conditions shows that this may very well be the case. When growing exponentially under light saturated conditions, energy consumption by carbon fixation and overall growth is limiting, and the cells are in a state II, with a large fraction of the phycobilisome light harvesting antennae that are not transferring their excitation energy to either of the two photosystems. When light became limiting, so did energy production and the cells gradually shifted to state I and phycobilisome binding increased, although unbound phycobilisome proteins were still detected. Under these conditions protein expression of components of the light reactions and of the light harvesting antenna showed no apparent increase or decrease (see Chapter 4), as was also reported in the literature [234,235]. This indicates that *Synechocystis* has the capacity to harvest and utilize more light energy than it does under light saturated conditions. Presumably this additional capacity exists to allow the cell to rapidly adapt to changing light conditions. Borirak et al. [332] showed a clear up-regulation of proteins involved in CO$_2$ fixation in an ethanol producing strain of *Synechocystis* sp. PCC 6803, compared to the corresponding wild-type. Together this demonstrates that the bottleneck for energy harvesting and biomass production is downstream of the light- and dark reactions of photosynthesis. Behrenfeld et al. [97] discuss the principle of co-limitation, in which multiple factors can have a minor or major influence on growth (rate). They state that energy production and consumption is necessary for more than photosynthesis and carbon fixation, and that nutrient acquisition and reduction, for instance when nitrate is used as the nitrogen source, and general metabolism such as amino acid and carbohydrate metabolism also play a major role. Not in the least because all these different processes require a different ratio of ATP:NADPH for balanced growth, necessitating dynamic changes in how energy is converted to maintain the energy balance in the cell. By introducing a pathway that requires carbon and energy in the form of ATP and/or NAD(P)H, the natural energy balance of the cell is distorted. To obtain a new balance the cell has the capacity to simply increase energy production by increasing light energy transfer to the photosystems to accommodate the increase in energy consumption and increase expression of proteins
involved in CO₂ fixation, thus minimizing the effect of the heterologous pathway on overall growth rate. This can be done throughout all growth phases, so both under light saturated- and light limited conditions. The ratio of supply of ATP over NADPH can be adjusted by in/decreasing the activity of any of the many pathways for cyclic electron transfer [333].

The different growth phases of phototrophic organisms and their corresponding physiology also hold implications for product formation. Increasing the available knowledge on the differences between the different growth phases can help in selecting the appropriate growth phase for optimal rates of product formation. The rate of production of lactate and butanediol by Synechocystis showed variation between growth phases, with higher production rates in the linear phase of growth [18,236]. When light is limiting growth, more carbon may be available for non-growth related processes such as biofuel production and/or the change in energy balance and the ATP:NADPH ratio required for biofuel production may be easier for the cell to accommodate during the linear phase of growth. Other factors, such as the amount of ATP and NAD(P)H required for any particular biofuel compound, and any regulatory mechanisms in play, may further influence growth-phase dependency of product formation and further research into this could prove very useful.

Increasing photosynthetic efficiency
To further increase biofuel production, biomass accumulation and even crop yields in plants, researchers are looking for ways to optimize photosynthesis [334]. Especially under conditions with saturating amounts of white light, light harvesting in photosynthetic organisms is highly dissipative. In addition, in dense cultures and canopies, cells close to the surface capture most of the incoming light and lose it to dissipation, while cells residing at the bottom reside in the dark. In recent years several attempts have been made to truncate or decrease expression of light harvesting antenna to reduce exciton dissipation and increase light penetration depth in an attempt to improve overall efficiency of growth in dense cultures. These attempts were successful in green algae were cells with truncated light-harvesting chlorophyll antenna (tla) showed enhanced photosynthetic productivity and improved light utilization efficiency under mass culture conditions compared to wild type strains [335,336], be it that tla mutants of Chlamydomonas reinhardtii did not exhibit higher productivity compared to wild-type in mass culture conditions and instead showed
decreased fitness and a higher susceptibility to photodamage [337]. This may be due to changes in photoprotection mechanisms caused by the changes in the antenna system. These results indicate that antenna truncation may not be such a straightforward way to increase photosynthetic efficiency. This is also reflected in the efforts made to increase photosynthetic productivity in cyanobacteria by truncating or even deleting the phycobilisome (PBS) antenna [192,193,216]. In light saturating conditions large amounts of harvested light energy are lost via PBS uncoupling (Chapter 4) and other dissipation pathways that are linked to the phycobilisomes, such as OCP-mediated uncoupling [122] and the flavodiiron proteins 2 and 4 [338]. Yet cells with truncated PBS antenna only showed increased productivity in low carbon and high light conditions [193]. Experiments with different light colors have already shown that *Synechocystis* grows poorly in chl *a* specific blue light, mainly due to the bacterial phytochrome cph2 [339], which inhibits phototaxis towards blue light [340]. However, experiments with red light with different spectral properties show that *Synechocystis* also grows slightly better when illuminated with PBS specific 635 nm light as opposed to chl *a* specific 670 nm light (P. van Alphen et al., in preparation). This shows the importance of phycobilisome driven light harvesting in cyanobacteria and more research may be required to develop more effective ways to lower energy dissipation and simultaneously increase photosynthetic efficiency. In the green algae *Chlamydomonas reinhardtii* photosynthetic productivity in dense mass cultures could be increased by illumination with high intensity, but minimally absorbed, yellow light, which has a greater penetration depth into the culture and causes much less photodamage [341]. This strategy could be interpreted as an alternative to antenna truncation. Another alternative could be the introduction of other light harvesting compounds such as bacteriochlorophyll *b* and chlorophyll *d*, which absorb in the far-red region, could provide an interesting alternative for or supplement to phycobilisomes and increase the bandwidth of the light that can be used for photosynthesis [334].

Another interesting line of research to increase photosynthetic efficiency is to increase expression of, or improve turnover capacity, of proteins involved in CO₂ fixation. This can be done by increasing the expression of carbon transporters and carbonic anhydrases [27], alter Rubisco to increase turnover and/or carboxylation efficiency [254] or increase expression of Calvin cycle enzymes. Overexpression of fructose-1,6-bisphophatase
yielded good results in transgenic plants [342] but had detrimental effects on growth in *Chlamydomonas reinhardtii* [343] demonstrating that increasing expression of Calvin cycle enzymes can be beneficial, but it can also disturb this crucial pathway by depleting a certain metabolite in the cycle, like G3P (glyceraldehyde-3-phosphate) or DHAP (dihydroxyacetone-phosphate) or by creating new bottlenecks elsewhere in the cycle [343]. Mutation of Rubisco resulted in a ~55% increase in photosynthesis rate in *Synechocystis* sp. PCC 6803, as measured by the oxygen exchange rate, but had no significant effect on growth rate [254]. However, the growth experiment described in the study was of a batch culture with a short exponential phase and a long light limited growth phase [254]. To fully reveal the effect of alterations in carbon fixation, the transgenic strains should be studied under conditions under which carbon fixation is the only rate limiting factor, such as the exponential phase of growth and ideally in the presence of a carbon sink. As was done in the study where a carbon transporter and carbonic anhydrase were introduced into *Synechococcus* PCC 7942 alongside a cellulose synthase, resulting in increased cellulose production [27].

A way to avoid biomass formation in direct biofuel production systems is to uncouple product formation from growth and produce biofuels from static stationary phase cultures. By producing large amounts of biofuel without significant cell growth, the total nutrient requirement for biofuel production, especially with regard to nitrogen and micronutrients, can be lowered. This can be achieved by using inducible promoters [344] or by making use of existing regulatory mechanisms that are induced in the stationary phase of growth. One such regulatory mechanism is cyclic diguanylate mono phosphate (c-di-GMP). In bacteria the cytoplasmic c-di-GMP concentration regulates cell aggregation, biofilm formation, motility and virulence [73,308,311] and c-di-GMP signaling may also play a role in the stationary phase [345]. (Over)production of exopolysaccharides like cellulose, which is also regulated by c-di-GMP, could be an excellent candidate for growth uncoupled production by inducing sessility in the stationary growth phase, which in cyanobacteria can also be achieved by blue light illumination [308,311]. We have already observed this in *C. epipsammum* where cellulose content and cell aggregation naturally increased in late growth phase cultures (Chapter 5).
Cellulose production

Initially the aim of this project was to produce cellulose as a pure, crystalline product from cyanobacteria, to be used in the cellulose industry as an alternative for cellulose derived from wood. This proved to be rather challenging and has not yet been achieved. Most reports on cellulose production in cyanobacteria do not provide statements on the crystallinity of the cellulose produced, or they report formation of semi-crystalline or non-crystalline cellulose [23,25,28,41]. Furthermore, cyanobacterial exopolysaccharide layers are the most complex of all reported EPS structures [263,268] and any cellulose produced in the EPS will be mixed with a wide variety of other polysaccharide structures. Recently, methods have been developed to derive cellulose from agricultural waste [346] and biofuel from the cellulosic biomass of agricultural waste [347]. At current cellulose production rates from cyanobacteria and because of its non-crystalline form, it is not very likely that cyanobacteria-derived cellulose will be able to compete economically with cellulose produced from wood and plant waste.

This, however, does not mean that cellulose produced by cyanobacteria cannot be economically interesting. Cellulose is structurally one of the simplest polysaccharides, consisting entirely of glucose monomers. Producing large amounts of poly-glucose shifts the cells C:N balance towards carbon. Such low nitrogen biomass would be very suitable as a feedstock for fermentative processes. Cyanobacteria generally have a rather high N content, and when fermented this will result in formation of considerable amounts of alkalizing ammonium and toxic ammonia [262]. Cellulose-producing cyanobacteria and/or algae can be grown to high cell densities, starved for nitrogen to increase the carbohydrate (cellulose) content and lower the nitrogen content, and then be grinded into a lysate broth that can be used as a feedstock for fermentative applications [348]. Starvation for nitrogen can also help make the lysis process easier [262]. *C. epipsammum* grows in nitrogen poor environments and as a result has a very low N-content for a cyanobacterium, combined with cellulose production levels of 20 to 40% of dry weight. This cyanobacterium would be an excellent natural (i.e. not genetically engineered) candidate for such a process.

Although *C. epipsammum* grows very well in liquid culture, its ecological niche is a biofilm [23]. Cyanobacterial- and algal biofilms are generally held together by the exopolysaccharides produced by the cyanobacteria and algae from which they are
composed [263,349]. For the production of cellulose and other exopolysaccharides, large scale cultivation of biofilms of cyanobacteria may be a viable alternative to growth in liquid culture. Although such commercial biofilm systems would produce considerably less biomass, they would also require less water and nutrients per kg of biomass. And by aiming at products whose formation can be uncoupled from growth, such as cellulose and other exopolysaccharides, much less biomass formation would be required than for products that are only produced by growing cells. Biofilms in nature mostly are complex consortia of a wide array of phototrophic and heterotrophic bacteria. Reports are available that show that the presence of certain heterotrophic bacteria stimulates biofilm formation and cell aggregation [261,350]. This suggests that the use of mono-cultures may not be advisable for this kind of application. For water purification and/or general mixed exopolysaccharide production one could try to grow naturally occurring biofilms on a larger scale.

Waste water treatment
In waste-water treatment, biofilm forming bacteria are used to purify the water from excessive nutrients and heavy metals [271,272,351]. To achieve this, these bacteria are grown in so-called rotating biological contactors [269]. These reactors often contain large spirals on which the bacteria can grow. These spirals are then suspended just above a water column. When the spiral rotates the blades containing the biofilm are alternatingly exposed to water and air [269]. Alternatively, biofilm reactors can be based on a trickle down system, in which biofilms are grown on large angular or vertically placed surfaces from which water can trickle down [352,353]. Both of these systems can be designed in such a way that biofilms would also be exposed to light. For instance, a design based on a biological contactor that includes a flexible membrane strung around rotating bars allowing alternate exposure to water and air and light [270]. An application of this system in a raceway pond photobioreactor is also described and harvesting of the biomass occurs by scraping the top layer of the biofilm [270]. A combination of the spiral column or flexible membrane bioreactor with a horizontal tubular photobioreactor [354,355] could also be very useful and increase productivity and lower the risk of infection compared to the raceway pond design. The trickle down system could be designed resembling solar panels.
Transgenic cellulose production

Currently the highest cellulose content in a transgenic cyanobacterium has been recorded in *Synechococcus* PCC 7002 at 13 % (w/w) of dry weight [28]. This is still lower than the amount of cellulose produced by *C. epipsammum*, but with further physiological and genetic optimization the cellulose content of the transgenic strains may very well come to exceed that of *C. epipsammum* in the future. Although introduction of cellulose synthase genes in *Synechococcus*, and in some other cyanobacteria, resulted in the production of detectable amounts of cellulose [25-28], the efforts described in Chapter 6 on *Synechocystis* did not yield similar results, despite taking a similar approach. An explanation for this may lie in the fact that *Synechocystis* does not naturally encode the components required for cellulose synthesis and *Synechococcus* does. The crystalline nature of the cellulose is achieved by the collaborative functioning of a wide array of enzymes. In bacteria, specifically in *G. xylinus*, the β-1,4-glucan is produced and transported across the periplasmic space by BcsAB [55], which is activated by the second messenger c-di-GMP [356]. BcsC and BcsD then facilitate cellulose transport across the outer membrane and play a role in the crystallization process [57-59]. The cellulose synthase complexes are arranged in a distinctive manner in cellulose producing organisms. In bacteria they are arranged in straight, evenly spaced, lines across the membrane. This allows the individual protruding fibrils to meet and interact with one another in an organized fashion, which then results in a crystalline structure of the cellulose produced. Because *Synechocystis* does not encode the enzymes required for cellulose synthesis itself, it will most likely also not possess the regulatory mechanisms and auxiliary proteins required to properly produce and export cellulose fibrils, *Synechococcus* on the other hand probably does. This would explain why introducing only the *bcsAB* genes from *G. xylinus* into cyanobacteria led to detectable amounts of extracellular cellulose in *Synechococcus*, but not in *Synechocystis*.

This does not mean that cellulose production by *Synechocystis* is not possible. It will just require addition of more genes involved in cellulose production. In *Synechocystis* increased binding of the fluorescent cellulose-binding dye Calcofluor White was observed in strains containing the *bcsAB* genes from *G. xylinus* in combination with an UDP-glucose pyrophosphorylase (UGPase) gene. Also, a partial rescue of the ΔglgC (ADP-glucose pyrophosphorylase (AGPase) deletion) phenotype was observed [Chapter 6] which suggests
that cellulose was produced. For cellulose production in *Synechococcus* it proved beneficial to delete the native cellulose synthase gene [28]. It appeared that the regulation of cellulose production was disrupted by deletion of the native cellulose synthase gene. This led to higher and more uncontrolled production of cellulose by the transgenic cellulose synthase proteins. *Synechocystis* should also be unable to regulate cellulose production and introduction of all genes required for cellulose production may lead to high levels of poorly controlled production, especially in combination with an UGPase in a ΔAGPase background. However, this raises questions of how genetically stable such a construct will be, because mutants that manage to block the uncontrolled synthesis of cellulose will have a growth advantage. Also, not all aspects of cellulose synthesis, and all genes involved, are currently known.

In summary: Continuing this project further, to successfully produce cellulose in *Synechocystis*, may be too difficult and consume too much time, especially because cellulose can already be produced in considerable amounts in *Synechococcus*. Beyond that, producing cellulose in *C. epipsammum* is a competitive alternative.