Physiological studies to optimize growth of the prototype biosolar cell factory
Synechocystis sp. PCC6803
van Alphen, P.

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References


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culturati

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Summary

The oxygenic photoautotroph *Synechocystis* sp. PCC6803 is a present-day cyanobacterium, a lineage with billions of years of history. Its ancestors first evolved the ability to perform oxygenic photosynthesis: using sunlight to extract electrons from water to form sugars out of CO₂. This unbounded success has seen them spread all over Earth, including in the chloroplasts of algae and plants. They are responsible for the high percentage of oxygen in the atmosphere in the so-called Great Oxygenation Event that shapes our current climate.

The cyanobacteria are extensively researched organisms, of which *Synechocystis* is a well-characterized model organism for various types of research including fundamental research of photosynthesis itself. Recently, in an effort to stop climate change and move society to a sustainable future, *Synechocystis* and other photoautotrophic microalgae are being engineered to produce fuels and other chemicals directly from CO₂. This requires a thorough understanding of the inner workings of the organism in order to meet demand, now and in the future.

The work presented in this thesis aimed to increase our understanding of metabolism and regulation thereof in *Synechocystis*. To this end, state of the art photobioreactors were used to precisely control and measure all parameters of growth.

**Chapter 1** is a review of the current state of achieving a bio-based society with the use of microalgae. One of the biggest challenges remains to efficiently grow microalgae in high density mass-cultures. Wild type microalgae thrive on minimal light intensities and evolved methods of dissipating energy when it is in excess in order to deprive their competition of light. In an industrial setting, this leads to excess light energy causing damage at the outer layers while the innermost layer remains in darkness. In the chapter, we discuss the strategies proposed so far to overcome this challenge with a special emphasis on mass-culturing technology and optimizing strains for use in such large-scale facilities.

In **Chapter 2**, we explore the limitations of growth of *Synechocystis* by investigating the commonly used growth medium, BG-11, and growth conditions. We present an improved medium suited for prolonged cultivation, BG-11-PC, that dissolves the precipitation issues that plague BG-11 in continuous cultivation systems. We show that, unexpectedly, it is the sulfate concentration that limits growth in batch cultivation and causes entry into the stationary phase. Importantly, sulfate limitation was shown to cause rapid bleaching and death of *Synechocystis* which is linked to the formation of reactive oxygen species.

By applying these results, we report on high growth rates of up to 4.1 h doubling time that can be sustained in photobioreactors operated in chemostat mode. This represents a considerable increase in the maximum growth rate from the traditionally considered maximum of 7-8 h doubling time for continuous growth.

**Chapter 3** and **4** show the results of a recently developed technique to analyze time-resolved fluorescence emission spectra in vivo in *Synechocystis*. This technique allows the total spectrum to be decomposed into the spectra and concentrations of the individual
fluorescing species. Using this technique, we show that in a mutant of *Synechocystis* that lacks photosystem I (PSI), an unknown quencher acts on photosystem II (PSII) in an oxygen-dependent way when subjected to a dark-to-light transition. Interestingly, the amount of time spent in the dark prior to the transition has a considerable effect on the timing of the quenching observed. The obtained data were used to create a minimal model to describe the system.

We expand on this in Chapter 4 and extend the application of the method to a *Synechocystis* mutant that lacks a functioning type I NDH complex as well as the wild type itself. The data obtained in this chapter lead us to conclude that the quencher constitutes a pool of alternative electron sinks that bypasses the PQ pool. We propose that this oxygen-dependent quencher may be the flavodiiron proteins Flv4/2. Furthermore, we present evidence for the PSII-PSI-PBS megacomplex that has recently been postulated to exist.

**Chapters 5, 6 and 7** delve into the circadian clock of *Synechocystis*. The clock is an essential mechanism for many organisms to keep the time in order to deal with fluctuating presence of light/nutrients and to separate mutually incompatible processes in time. In Chapter 5, the clock was definitively shown to be present in *Synechocystis* and is surprisingly robust. The highly controlled and stable growth conditions as a result of Chapter 2 were used to show sustained oscillations without measurable damping for weeks. Importantly, the clock was mainly found to have an impact on growth rate, which varied periodically to much greater extent than cellular composition.

This was further explored in Chapter 6, which dissects proteomics, transcriptomics and metabolomics in a large investigative study. *Synechocystis* was subjected to a diel cycle of 12 h dark / 12 h light in a low-oxygen environment to characterize its physiology. We show that growth rate is a function of time in the light and completely halts in the dark. The dry weight is remarkably constant throughout the day while the composition varies. Glycogen is assimilated during the day and is slowly consumed during the night until shortly before light when it is rapidly degraded. The very slow consumption, despite fermentative conditions, indicates that the maintenance requirements are extremely low.

In Chapter 7, we explore the physiology of *Synechocystis* mutants lacking the core clock components. Deletion of the *kaiAB1C1* operon led to the complete absence of circadian rhythms. Interestingly, this mutant displayed a severe impaired growth phenotype in the photobioreactors, even in continuous light. However, this phenotype could be rescued by what appears to be a suppressor mutation, the identity of which remains to be elucidated.

In an effort to construct a so-called ‘hourglass’ clock, which requires sequential periods of light/dark to reset the clock every day, *kaiB1* was deleted without disrupting the remaining genes of the operon. This mutant displayed circadian rhythms in day/night, but not in continuous light. The growth rate of this mutant is reversibly decreased in continuous light. The putative additional *kai* genes present in *Synechocystis*, i.e. *kaiC2B2*, *kaiB3* and *kaiC3* could not rescue the wild type phenotype of either mutant, indicating they are not essential to clock function.
Finally, Chapter 8 puts the results in a broader context and discusses the implications for future research.
Samenvatting

De oxxgiene fotoautotroof Synechocystis sp. PCC6803 is een hedendaagse cyanobacterie, een microalg met miljarden jaren geschiedenis. Hun voorouders evolueerden voor het eerst de mogelijkheid om oxxgiene fotosynthese toe te passen: zonlicht gebruiken om elektronen los te maken uit water om daar vervolgens CO₂ mee om te zetten in suikers. Door dit ongekende succes zijn ze overal op Aarde terug te vinden, zelfs in andere organismen, namelijk de chloroplasten van algen en planten. Ze zijn verantwoordelijk voor het hoge percentage zuurstof in de atmosfeer sinds het zogenaamde ‘Great Oxygenation Event’ dat ons huidige klimaat geschapen heeft.

De cyanobacteriën zijn uitgebreid onderzochte organismen, waarvan Synechocystis een goed gekarakteriseerd modelorganisme is voor verscheidene soorten onderzoek, waaronder fundamenteel onderzoek aan fotosynthese zelf. Tegenwoordig worden Synechocystis en andere fotoautotrofe organismen gemoeficeerd om biobrandstoffen en andere chemische stoffen te produceren in een poging klimaatverandering een halt toe te roepen en een duurzame samenleving te realiseren. Hiervoor is het nodig om zo’n organisme volledig te doorgronden.

Het werk dat gepresenteerd wordt in dit proefschrift heeft als doel om onze kennis van het metabolisme van Synechocystis te vergroten. Om dat doel te bereiken zijn zeer geavanceerde fotobioreactoren gebruikt om Synechocystis precies gecontroleerd te laten groeien en daaraan te kunnen meten.

Hoofdstuk 1 is een overzicht van de huidige staat van het realiseren van een duurzame samenleving met behulp van microalgen. Een van de grootste uitdagingen blijft het efficiënt cultiveren van microalgen bij hoge dichtheden. In de natuur gedijen microalgen zoals cyanobacteriën juist bij minimale lichtintensiteiten en hebben ze manieren ontwikkeld om overbodige energie te kunnen lozen en zo de concurrentie geen licht te gunnen. In een industriële omgeving is dit echter contraproductief en leidt het tot schade aan de buitenste lagen terwijl de binnenste lagen in het donker zitten. In het hoofdstuk bespreken we de strategieën die tot nu toe voorgesteld zijn om dit probleem op te lossen, met nadruk op cultivatie bij hoge dichtheden en het optimaliseren van stammen voor zulke toepassingen op grote schaal.

In Hoofdstuk 2 verkennen we de grenzen van de groei van Synechocystis door te kijken naar het standaard groeimedium, BG-11, en de groeicondities. We presenteren een verbeterd medium (BG-11-PC) dat geschikt is voor langdurige cultivatie en de neerslag oplost van BG-11. Deze neerslag bemoedigde het gebruik van fotobioreactoren voor langdurige groei. Verder laten we zien dat, tegen de verwachting in, gebrek aan sulfaat de groei doet stoppen en leidt tot de stationaire fase. Tevens zorgt gebrek aan sulfaat ervoor dat de cellen abrupt bleken en afsterven, wat we in verband konen brengen met de vorming van reactieve zuurstofvormen. Door deze resultaten toe te passen komen we tot zeer hoge groeisnelheden, tot wel 4.1 uur verdubbelingstijd, die ook op de lange termijn
vol te houden zijn in fotobioreactoren. Dit is een significante verhoging van de maximale groeisnelheid van *Synechocystis*, traditioneel gezien rond de 7-8 uur bij continue groei.

**Hoofdstukken 3 en 4** bevatten de resultaten van een recent ontwikkelde techniek om tijd-opgeloste fluorescentie-emissiespectra van levende *Synechocystis* cellen te analyseren. Deze techniek geeft de mogelijkheid om het gehele spectrum te analyseren voor de spectra van verschillende fluorescerende entiteiten en deze los van elkaar te zien. Met deze techniek hebben we een tot nog toe onbekend, zuurstof-afhankelijk eiwit gevonden dat elektronen kan accepteren van fotosysteem II (FSII) in een mutant van *Synechocystis* dat geen fotosysteem I (FSI) meer heeft en blootgesteld werd aan een donker-naar-licht overgang. Opmerkelijk is dat de hoeveelheid tijd in het donker voor de overgang naar licht een sterk effect heeft op het tijdsverloop van de werking van dit eiwit. De data werd gebruikt voor het maken van een klein model dat dit systeem beschrijft.

We borduren hierop voort in **Hoofdstuk 4** en gaan een stap verder met de toepassing van de techniek op zowel wild type *Synechocystis* als een mutant zonder werkend type I NDH-complex. De data uit dit hoofdstuk leidt ons tot de conclusie dat het onbekende eiwit een alternatieve elektronacceptor is die om de plastoquinonen heen kan. We stellen dat deze zuurstof-afhankelijke eiwitten de flavodiiron eiwitten Flv4/2 kunnen zijn. Daarnaast presenteren we bewijs voor het FSII-FSI-PBS megacomplex waar recent het bestaan van gesuggereerd is.

**Hoofdstukken 5, 6 en 7** duiken in de circadiene klok van *Synechocystis*. Deze klok is een cruciaal mechanisme in vele organismen om de tijd bij te houden zodat ze kunnen omgaan met fluctuerende aanwezigheid van licht of voedingsstoffen. In **Hoofdstuk 5** laten we zien dat *Synechocystis* daadwerkelijk een robuuste klok heeft. De zeer gecontroleerde en stabiele groeicondities zoals beschreven in **Hoofdstuk 2** werden gebruikt om te laten zien dat er nauwelijks sprake is van het dempen van de oscillatie van de klok. Van belang is dat we aangetoond hebben dat de klok een sterk effect heeft op de groeisnelheid, sterker dan op de samenstelling van de cel zelf.

Dit is verder uitgediept in **Hoofdstuk 6** door te kijken naar alle eiwitten, metaboolieten en afgeschreven genen van *Synechocystis* in een groot, verkennend onderzoek. De cyanobacterie werd blootgesteld aan een dag/nachtritme van 12 uur donker / 12 uur licht in condities met weinig zuurstof. We laten zien dat de groeisnelheid een functie van tijd is in het licht en volledig stopt in het donker. Het drooggewicht van de culture blijft verbazingwekkend gelijk over de gehele dag terwijl de samenstelling verschilt. Glycogeen wordt gedurende de dag verzameld en langzaam verbruikt in de nacht tot kort voor het aanbreken van de dag, wanneer het in versneld tempo wordt opgemaakt. De zeer trage consumptie onder fermentatiecondities laat zien dat *Synechocystis* zeer weinig energie voor onderhoud nodig heeft.

In **Hoofdstuk 7** kijken we naar mutanten van *Synechocystis* waarin de klok is verstoord. De klok volledig verwijderen door het *kaiAB1C1*-operon weg te halen leidt tot het geheel afwezig zijn van circadiene ritmes. Interessant is dat deze mutant in eerste instantie zeer slecht groeide in fotobioreactoren, zelfs in continu licht. Dit bleef echter niet zo, door wat
lijkt op een onderdrukkende mutatie die het mogelijk maakt weer normaal te groeien. Wat deze mutatie precies is, is nog onbekend.


Tenslotte worden in Hoofdstuk 8 alle resultaten in een bredere context geplaatst en de implicaties voor toekomstig onderzoek besproken.
Publications


Patent:
Klaas Jan Hellingwerf, Theo van Lieshout, Paul Koblens, Wilmar van Grondelle, Pascal van Alphen
Arrangement of a photobioreactor or a microbiological reactor

* Authors contributed equally to this work
Abbreviations

ATP  Adenosine Triphosphate
ANOVA  Analysis of variance
CBB cycle  Calvin-Benson-Bassham cycle
CCM  Carbon-Concentrating Mechanism
CEF  Cyclic Electron Flow
COX  Cytochrome c oxidase
DA  Dark Adaptation
DCMU  Dichlorophenyl-dimethylurea
dCO₂  concentration of dissolved carbon dioxide
dO₂  concentration of dissolved oxygen
DW  Dry Weight
EET  Excitation Energy Transfer
F6P  Fructose-6-phosphate
FBP  Fructose-1,6-bisphosphate
FDP  Flavodiiron proteins
FNR  Ferredoxin–NADP+ Reductase
Flv1/3 (2/4)  Flavodiiron protein 1/3 (2/4)
Fₘ  Maximum fluorescence during a saturating pulse
Fₛ  Steady-state fluorescence at the end of background illumination
Fᵥ  Variable fluorescence
HliP  High-light inducible protein
LEF  Linear Electron Flow
NADPH  Nicotinamide Adenine Dinucleotide Phosphate
NDH-I (-II)  type-I (-II) NAD(P)H Dehydrogenase
NMR  Nuclear Magnetic Resonance
MS  Mass Spectrometry
LC-MS  Liquid chromatography–mass spectrometry
LED  Light Emitting Diode
LOS norm.  Least oscillatory set normalization
ODₜₙ  Optical Density measured at λ nm
PB  Phycobilisome
PBR  Photobioreactor
PC  Plastocyanin
PLS-DA  Partial Least Square Discriminant Analysis
PMF  Proton-Motive Force
PPP  Pentose Phosphate Pathway
ppm  parts per million
PQ(H₂)  Plastoquinone(plastoquinol)
PS  Photosystem
<table>
<thead>
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<tr>
<td>RT</td>
<td>Room Temperature</td>
</tr>
<tr>
<td>RuBisCO</td>
<td>Ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
<td>SAS</td>
<td>Species Associated Spectrum</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate Dehydrogenase</td>
</tr>
<tr>
<td>SVD</td>
<td>Singular Value Decomposition</td>
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<tr>
<td>TCA cycle</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TP</td>
<td>Time Point</td>
</tr>
<tr>
<td>VIP</td>
<td>The variable’s importance on projections</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
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Acknowledgements

Suddenly, a period of 5 years and a bit comes to a frantic end and then all there is left to do is to thank all of those who have contributed to the work described in this thesis and to who I am today. Even though not all of them can be individually named, some definitely will be and who better to start with other than my promotor, Klaas Hellingwerf. I am most grateful for the opportunity to be an ‘AIO’ in MMP. I remember well the day that I unexpectedly received an email from you, simply saying that “we need to talk”, when I thought there was no hope for a position at MMP anymore. Thank you, also, for reminding me – frequently – that your door is open and that I can come to you with any problem. I greatly appreciated our discussions even though we certainly didn’t agree on everything and I arguably didn’t come to you as often as I should have.

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Working with these fancy photobioreactors has some advantages and disadvantages. An advantage is that because they’re a very limited resource, I didn’t have quite as many students as some of my colleagues. A disadvantage is that when I did have one, they’d take a lot of my time. Fortunately, I’ve had the pleasure of supervising various students who were well worth that time: Laura, Boudewijn, Marian, Martijn and Pimmy. Thank you for putting up with my less than optimal educational skills.

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Ja, mam, ik ben nu écht afgestudeerd.

Pascal
Physiological studies to optimize growth of the prototype biosolar cell factory Synechocystis sp. PCC6803

Pascal van Alphen