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8 General discussion

Pascal van Alphen

The aim of the overall Toolbox project, of which this work is but a small part, has been to improve our understanding of *Synechocystis* metabolism. This was done in order to establish a 'toolbox' of methods to expand our ability to use *Synechocystis* for realizing a sustainable society that does not rely on fossil reserves for fuel and chemical feedstocks. The need for this is urgent and clear due to the ever-increasing demand for energy, whereas the majority of our current supply is increasingly unstable, finite and a cause of climate change, leading to further problems such as our food supply. The idea is as simple as it is elegant: recycle CO_2 into energy carriers and chemical feedstock using the abundant energy provided by the sun by a process that has been ongoing for billions of years on Earth (23). Even though everything is technically possible – the scientific literature is filled with reports of production of a wide range of compounds from CO_2 using photosynthetic organisms such as *Synechocystis* (258) – and the sun provides enough energy to the Earth in an hour to satisfy our energy demand for a year (29), we still rely on fossil reserves. The reason is that the current biocatalysts (i.e. engineered photosynthetic organisms such as *Synechocystis*) are not yet capable of providing the necessary quantities at a sufficient rate and yield due to, in part, a lack of understanding of metabolism and its regulation in cyanobacteria like *Synechocystis*. In this thesis, we have looked into growth optimization (**Chapter 2**), light harvesting (**Chapters 3** and **4**) and circadian regulation (**Chapters 5** to **7**). An alternative approach to increase the productivity of mass-culturing is discussed in **Chapter 1**.

In this chapter, the thesis chapters will be summarized and discussed in context of each other and expanded on with as yet unpublished data which may provide leads for future research.

8.1 Rational medium design

A, if not the, critical component of cultivating a microorganism at any scale is the medium. The medium *Synechocystis* is typically grown in, BG-11, was developed for growing many species of cyanobacteria (called blue-green algae at the time) rather than *Synechocystis* specifically (135-137, 145). As described in **Chapter 2**, this medium is suboptimal for use with *Synechocystis* for several reasons. The formation of a precipitate containing iron and calcium (both as a salt with phosphate) at the preferred physiological pH (roughly 8 to 9) and the lack of proper pH buffering led us to develop a modified version, named BG-11-PC for its ability to facilitate prolonged cultivation in continuous cultivation systems. Because BG-11-PC does not form a precipitate at pH 8, it allows for using $NaHCO_3$/pCO_2 as a buffer in continuous culturing systems or a non-metal complexing buffer such as PIPPS in flask experiments.

Beyond the need for reproducible conditions, **Chapter 2** shows that growth is limited by different components depending on the mode of growth. In batch cultivation, sulfate becomes limiting and initiates entry into the stationary phase, while in continuous cultivation (e.g. chemostat) phosphate is the limiting nutrient. Additionally, entry of the stationary phase due to sulfate limitation leads to ROS formation and the irreversible

bleaching of pigments. In the chapter, we do not go into further optimizations of the medium, save a few recommended changes based on the aim of a given experiment, i.e. lifting the sulfur limitation in batch cultivation when high densities are required and increasing phosphate in continuous culture. However, a critical look at other nutrients is warranted as well. From the biomass composition, it is clear that potassium drops to suboptimal levels well before the final OD is reached in BG-11. The measured content of 12.86 mg K gDW^{-1} yields a theoretical maximum biomass density of 1.40 gDW L^{-1} (OD_{730} ≈ 7.8). In batch, this is likely not to be the case due to the usage of KOH-adjusted buffers, but in continuous cultures it should not be assumed that potassium is in great excess. Even so, the fact that the base used to set the pH of the buffer might matter, is troublesome.

Kratz & Myers (1955) remarked that the concentration of micronutrients could be varied considerably without observable effects on growth and that no attempt was made to define actual requirements. The biomass composition reported in **Chapter 2** may be used to give an indication of trace metal requirements, despite that only manganese could be quantified. Its cellular content is calculated to allow a final density of 37 gDW L^{-1}, indicating it is present in great (> 10-fold) excess. Due to the low detection limit for most elements that make up the micronutrients, this leads to a projected maximum achievable density of orders of magnitude greater than actually achieved, clearly indicating that BG-11 is in very great excess in regard to these elements. In the case of boron, it may actually not even be required as to the best of our knowledge, no use for it in cyanobacteria is known and cultivating *Synechocystis* in BG-11-PC without boron led to no observable phenotype (data not shown). Nickel may be an example of the reverse. It was shown to be required for growth (151) and there are various known nickel metalloproteins such as urease (287) and hydrogenase (288), yet it is not explicitly added to BG-11 and is present only in the nM range. Addition of nickel did not appear to make a difference, however (**Chapter 2**), though it cannot help reproducibility to depend on impurities in other chemicals to supply sufficient nickel.

For copper and zinc, it is less clear. The detection limit for copper was relatively high (0.022 mg L^{-1}), leading to a theoretical maximum density of only 2.14 gDW L^{-1} even though it is undetected. Zinc, on the other hand, was the most abundant micronutrient in the biomass with a projected maximum yield of 0.49 gDW L^{-1}. Even though this may indicate that zinc is not present in excess, the control contained the same order of magnitude of zinc, making it difficult to accurately determine the actual biomass content. Remarkably, this was true for calcium and iron as well, despite the far greater known requirement for these elements than zinc. For calcium, its requirement was verified and reduction of the calcium concentration 48-fold to 5 µM led to a final OD_{730} of 10, remarkably close to the expected value based on the projected maximum biomass density calculated from the biomass composition (OD_{730} ≈ 7, data not shown). The magnesium content in the biomass is close to that of sulfur and is another example of a nutrient that is present in less-than-optimal concentrations over the course of a batch experiment, allowing a theoretical

density of only 1.63 gDW L^{-1} (OD_{730} ≈ 9.0), which is lower than sulfur due to its lower molecular mass. As described in **Chapter 2**, no phenotype appears up to approximately 5 times lower magnesium content. At 8 times lower content, the stationary phase is entered around OD_{730} 6 and is accompanied by a pale green, transparent phenotype.

One can conclude that while most nutrients are present in (great) excess, some are not and should be increased accordingly to ensure constant conditions over the course of the whole growth curve until a clearly defined stationary phase is entered due to exhaustion of a single nutrient. Nitrogen limitation is an extensively researched nutrient limitation with a clear and reversible phenotype (158), lending itself well as limiting nutrient. Conversely, magnesium and sulfur are both shown to be required in significant quantities, the latter leading to an irreversible stationary phase in BG-11, making magnesium sulfate a logical choice to increase to great excess. This is possible without precipitation issues as a comparable medium used with cyanobacteria, A^+ (156), actually contains nearly 100-fold more magnesium sulfate than BG-11, around 1.5-fold more phosphate, 10-fold more calcium and comparable amounts of iron, yet does not precipitate, (presumably) due to a slightly higher concentration of EDTA (80 µM) than in BG-11-PC (50 µM).

In **Chapter 2**, we have shown that high concentrations of EDTA causes a long lag phase, though it is not clear why. It is interesting that pre-culturing at high concentrations does not prevent this lag phase upon sub-culturing (**Chapter 2**, **Fig. 2.1**). The fact that EDTA chelates many metal ions may indicate that a particular metal becomes sufficiently unavailable at high EDTA concentrations as to halt growth. The more of this metal gets taken up, the less of it will be available in uncomplexed form, suggesting active uptake is required to keep up with decreasing supply in the face of increased demand. An explanation for the increasing growth rate observed in these cultures is that this metal is acquired by an uptake mechanism involving excreted chelators. These putative excreted chelators would be diluted significantly when sub-culturing, requiring their re-synthesis, leading to a lag phase. Iron, a metal taken up by some organisms using dedicated chelators called siderophores, is a possible candidate, however, extensive work by Keren and coworkers has shown that *Synechocystis* does not produce siderophores nor take up complexed iron (157). Other metals that have known chelators are copper, cadmium, zinc, nickel and cobalt (289-291), though except for cobalt, this appears mainly to be used to detoxify rather than to facilitate uptake. In the case of cobalt, a 'cobalophore' has been described for *Prochlorococcus* MED4-Ax, a marine cyanobacterium (292). While *Synechocystis* has a strict requirement for cobalt in photoautotrophic conditions (Vitamin B_{12}, or cobalamin, is a co-factor of methionine synthase), cobalt uptake was shown to depend on *hupE* (293), a membrane protein which is singly able to convey nickel and/or cobalt uptake when heterologously expressed in *E. coli* (294). Furthermore, when high EDTA was tested in turbidostat, no observable lag phase was caused by approximately 4 % dilution per cycle in BG-11 containing 200 µM EDTA at sub-maximal growth rate of around 0.07 h^{-1} (data not shown).

In conclusion, while BG-11-PC is a step forward in developing a chemically defined medium in which all nutrients required for optimal growth are present in sufficient quantities over the whole, defined, growth curve, it is not there yet and further research into the mechanism of the EDTA-induced lag phase and nutrient requirements is necessary.

8.2 Maximum growth rate

The issue of maximum growth rate (μ_{max}) has been a hotly contested topic during the work described in this thesis. For biotechnological applications, the μ_{max} is an important parameter because it directly translates to the capacity to fix carbon. At the start of this work, growth of *Synechocystis* sp. PCC6803 was considered slow and suboptimal with a doubling time (Dt) of around 8 h, almost twice as long as that of another model cyanobacterium, *Synechococcus elongatus* PCC7942 (~ 4.9 h, (295)). Despite that, *Synechocystis* is a commonly chosen organism for various biotechnological applications due to the wealth of information and genetic tools available. Another *Synechococcus* that is even faster than 7942 is *Synechococcus* PCC7002 (Dt ≈ 3.5 h when grown on a reduced nitrogen source, (270)). However, its growth is dependent on external addition of vitamin B_{12} which is an undesirable trait from a biotechnological point of view. Even 7002 at 3.5 h Dt is still slow compared to commonly used heterotrophs such as *Escherichia coli* (20 m) and *Saccharomyces cerevisiae* (70 m).

The number of publications on production of a certain compound of interest in these cyanobacteria has increased dramatically in recent years (57, 258, 297) and to further increase production levels, even faster growing cyanobacteria are sought. In the race to find the fastest growing cyanobacterium the current frontrunner is *Synechococcus* UTEX 2973, formerly known as *Anacystis nidulans*, which is reported to reach growth rates up to 0.4 h^{-1} (Dt ≈ 1.7 h) (135, 295). Most (bio)chemical reactions have a strong temperature dependence (the circadian clock being a significant exception) with a Q_{10} of 2-3, i.e. with an increase of 10 °C, the rate increases by a factor 2-3. It is therefore not surprising that the highest growth rates reported were obtained in the 38-41 °C range, whereas *Synechocystis* is routinely grown at 30 °C. A significant effort was made by Zavřel et al. (2015) to characterize various 'wild types' of *Synechocystis* PCC6803 in regard to optimal temperature and light intensity and quality. It was shown that 30 °C is indeed not optimal and that higher growth rates can be obtained at higher temperatures up to 35 °C if provided with sufficient CO_2 and light. The highest growth rate obtained at this temperature (Dt ≈ 5.1 h) is remarkably close to that of *Synechococcus* 7942 at 38 °C (Dt ≈ 4.9 h). An aspect that should not be overlooked is under which conditions these results were obtained: a photobioreactor operated in turbidostat mode, facilitating prolonged exponential growth under precisely controlled conditions. In the work described in this thesis, conducting experiments in precisely controlled conditions has been strongly emphasized and is reflected in the first report of steady-state growth rates

up to 0.16 h^{-1} (Dt ≈ 4.1 h) in *Synechocystis* when grown in chemostat mode at 32 °C (**Chapter 2**).

Something that is rarely covered in studies aiming to maximize growth rate is how this translates to higher biomass densities. High growth rates are generally achieved at low biomass density with high light intensities that penetrate the culture easily. In **Chapter 2**, we have used orange-red light (635 nm) which is maximally absorbed by the main light harvesting complex (the phycobilisome) of *Synechocystis*. This has been shown to be energetically the most effective light source at low densities but drops in efficiency quickly with increasing density (**Chapter 2**, (298). In an attempt to achieve high growth rates (0.08 h^{-1}) at high density, we found that even with all nutrient limitations lifted by using 5x BG-11-PC, we could not achieve a density greater than slightly over OD_{730} = 3 in chemostats (data not shown). Increasing light further actually decreased the OD. This problem is described in **Chapter 1** and may be solved in two different ways, one of which (reducing the light harvesting capacity per cell) is described in **Chapter 1**. However, in cyanobacteria, the antenna truncation approach appears less effective than in green algae (121). Alternatively, the light source may be changed to one that is less efficiently absorbed (298, 299). This approach has not been explored in this thesis though it clearly shows promise for mass culturing cyanobacteria.

It is remarkable that in a relatively short period of time, the textbook value of 8 h Dt has been cut in half, with no absolute maximum found yet, without any genetic engineering required. This raises the question of why it was generally accepted that *Synechocystis* could grow no faster. A possible reason is the lack of specialized lab equipment to deliver high light intensities and elevated levels of CO_2 as well as properly controlling other conditions (e.g. pH and temperature). Dedicated cultivation systems such as the photobioreactors used in this work (**Chapter 2**) allow such conditions to be fulfilled and are starting to become more widely adopted. Another reason may be biological rather than simply providing sufficient nutrients. Even though 30 °C was shown to be suboptimal, a Dt far below the textbook 8 h is possible at this temperature in chemostat mode (**Fig. 2.6**). Interestingly, the textbook μ_{max} could also be exceeded in turbidostat mode, but only when the turbidostat range (i.e. the thresholds between which the biomass density is kept) is reduced in otherwise unchanged conditions (see **Fig. S2.2**). It appears that the dilution event itself causes the growth rate to settle at a lower than maximal value. This seems similar to what was observed with high EDTA cultivation, though there are some inconsistencies, the main one being that in turbidostat with high (200 µM) EDTA, individual dilution events of approximately 4 % do not appear to cause any lag phase such as that induced by shear stress (**Chapter 2**). Nevertheless, the observation of increased growth rate with a smaller turbidostat range indicates that there is much left to be discovered.

Growth rate has also been shown to be influenced by the circadian clock, which appears to facilitate higher peak growth rates during the light period of a diel cycle compared to continuous light (**Chapter 5** and **6**). However, a clock deletion mutant

without circadian regulation appears to grow slightly faster in continuous light (**Chapter 7**), possibly because of getting 'stuck' at peak growth rate metabolism (254). Even though knocking out the clock will have a large impact on all aspects of metabolism (**Chapter 6**), it may simplify culturing in continuous light substantially and ultimately be the more efficient option in 3D reactors with artificial lighting as described in **Chapter 1**.

8.3 Light harvesting

The process of photosynthesis is a delicate balance of input and output of energy, yet has to cope with fluctuations on multiple levels due to its dependence on energy input from the sun. Beyond slow and repetitive changes caused by seasonal differences and the daily cycle, something as simple as a cloud passing by can cause light intensity to drop orders of magnitude and vice versa on very a short time scale. On the other end of photosynthesis, CO_2 availability may decrease to near zero, leading to the possibility of reactive oxygen species being formed due to removing the terminal electron acceptor for the photosynthetic apparatus. The 'dark' reactions, as the Calvin-Benson-Bassham cycle in which inorganic carbon is assimilated is often referred to, therefore seems a misnomer as they are inextricably linked to photosynthesis.

Cyanobacteria are well-equipped to deal with fluctuating lighting and inorganic carbon conditions, however. Excess energy can be released at various junctions of photosynthesis. For example, at the very start of light harvesting, state transitions are used regulate the distribution of excitation energy (178). Additionally, the principal light-harvesting complex of cyanobacteria, the phycobilisome, can be directly quenched by the orange carotenoid protein (300). Further down the electron transport chain, if the primary electron acceptor (Q_A) is not available, chlorophyll *a* in the RC of PSII can release its energy by fluorescence emission (301). Lastly, even at the end of the chain, energy can be dissipated by driving futile cycles with the generated energy (ATP) and redox (NADPH) carriers (194, 302). Due to the non-invasive nature of measuring fluorescence, pulse amplitude modulated fluorometry (PAM) has been widely adopted as measure of efficiency of photosynthesis in algae and plants by estimating the quantum yield of PSII (303). Despite its widespread abundance, photosynthesis in cyanobacteria is sometimes considered less efficient than in eukaryotic algae due to a lower apparent quantum yield (304). This misconception stems from some properties unique to cyanobacteria, namely their intertwined respiratory and photosynthetic electron flow causing a more reduced PQ pool in the dark, the interfering background fluorescence of the phycobilisomes (PBS) and a high PSI/PSII ratio, rather than an inherently lower photon conversion efficiency (38, 305).

In an effort to dissect how energy is distributed over the photosystems (via so-called state transitions) in response to a dark-to-light transition, a method was developed in Acuña et al. (2016) to decompose the fluorescence emission spectrum of whole *Synechocystis* cells. The application of this method on wild type *Synechocystis* and mutant derivatives is described in **Chapters 3** and **4**, and has led to evidence for the existence of a

PBS-PSII-PBI megacomplex, the identification of an oxygen-dependent quencher much like Flv2/4 and a minimal model to describe the interplay of involved species (**Chapters 3** and **4**). A rather puzzling conclusion is that the quencher described in **Chapter 3** and **4** appears to behave much like Flv2/4, except that it should not be expressed under the tested high carbon conditions in wild type cells. To shed light on this matter, an Flv2/4 deletion mutant will have to be tested under the same conditions. Furthermore, we show that dark adaptation for longer than 20 min leads to substantial differences in state-transitions upon re-illumination. In plants, the chloroplasts are separate from the mitochondria and dark adaptation removes all input of the electron transport chain. In cyanobacteria, the cross-talk between photosynthesis and respiration is likely to cause a difference in redox state at the onset of illumination, depending on the length of the dark adaptation. The presented method (50) may also be used to probe processes at the interface of photosynthesis and respiration.

8.4 The circadian clock

Except for photobioreactors which are designed with the use of artificial lighting powered by renewable energy in mind, as discussed in **Chapter 1**, using solar energy in many other instances requires dealing with the periodic availability of sunlight. Many organisms have a way to do this in the circadian clock, including *Synechocystis*, as shown in **Chapter 5** and **6**. The main result from these chapters is that metabolism is significantly influenced by the clock and should not be ignored. Unfortunately, in many cases, it is. Not many studies with *Synechocystis* are designed with the clock in mind and how this may influence the parameters of interest. For transcriptome studies, for example, one should carefully sample at the same time of the day before and after the tested stimulus, otherwise the rhythmic expression of the gene(s) of interest may be misleading. This becomes even more problematic when different strains are compared that may not be in the same phase of their circadian cycle. As emphasized in **Chapter 2**, growth rate can be of greater influence than the subject/topic of interest, and so may the clock.

On the other hand, the extensive regulation that appears to be exerted by the clock can be harnessed to stimulate heterologous production of compounds of interest. In **Chapter 6**, the substantial upregulation of genes in the night or day may provide a framework to express genes at opportune moments. Alternatively, removal of the clock may be of similar interest to biotechnological applications as shown in **Chapter 7**. In **Chapter 6** we note the switch to fermentation in the anoxic night. This is reflected in the slight increase in fermentation products such as acetate, but mainly in the strong upregulation of the Ni-Fe hydrogenase (*hoxEFUYH*) and polyhydroxybutyrate (PHB) synthesis genes (slr1828 and slr1829). Even though hydrogen could not be measured in our experimental setup, PHB was verified to be synthesized exclusively in the night (Carpine et al. unpublished results). Fermentation in cyanobacteria may also be exploited for biotechnological applications. One of the pitfalls of the kind of metabolic engineering employed to allow *Synechocystis* to produce these compounds of interest is that the

introduced pathway is not part of the evolutionary stable strategy (ESS) (306) and thus subject to elimination, also known as genetic instability (307, 308). For a fermenting organism such as *E. coli*, it was shown that production could be increased by eliminating all other fermentation pathways through which redox balance is achieved, leaving only the pathway that leads to formation of the product of interest (309). Something similar has been proposed for cyanobacteria, i.e. to remove alternative electron flow pathways of photosynthesis to modulate the NADPH/ATP ratio and force the use of an introduced pathway (310). However, because there are many possible ways for cyanobacteria to modulate this ratio in the light, knocking them all out would remove robustness to the point that such a cell could not survive in any but the most strictly controlled conditions, which will be hard, if not impossible, in large-scale reactors. Instead, a potentially valuable strategy may be to eliminate competing fermentation pathways and force the production of a fermentation product in the night under anoxic conditions or in a terminal oxidase deletion background in aerobic conditions where the many AEF pathways cannot be used. While we have shown in **Chapter 6** that maintenance requirements in the night are very low and thus the expected productivity in the dark is not very high, the actual goal is to make the heterologous pathway part of the ESS and ensure productivity in the light will not be lost.

An alternative approach to the ESS problem is coupling growth directly to production of a compound of interest that is part of the native metabolism (311). By knocking out the pathway for re-assimilation of acetate (a by-product of amino acid metabolism), growth becomes strictly coupled to production of acetate, forcing the cells to produce acetate. While this is a clear solution to the ESS problem, the production levels are fairly low compared to the amount of biomass that is produced. However, if the biomass itself is valuable this is not necessarily a downside (57, 312).

8.5 Population heterogeneity in photobioreactors

A primary interest of this work has been to investigate population heterogeneity of cultures grown in a photobioreactor. The common assumption is that a 'well-stirred' reactor is homogeneous and thus only one population with a normal distribution is present. A way to assess this assumption is to have an easy to measure parameter that is sensitive to cellular differences, such as the expression of a fluorescent protein. To this end, *Synechocystis* strain UL018 (313) which has been genetically modified to express *yfp* under the strong Ptrc promoter from the pCA2.4 native plasmid, was cultivated in a photobioreactor under turbidostat control and inspected for heterogeneity of YFP fluorescence. The obtained steady-state at 25 µmol photons m^{-2} s^{-1} orange-red light resulted in a growth rate of 0.026 h^{-1} (~27 h Dt) after approximately 10 days of culturing with little light passing through the culture, suggesting a clear light gradient was present in the reactor (not further quantified).

The obtained steady-state was perturbed by increasing light intensity to 37 µmol photons m^{-2} s^{-1}, resulting in an increase in growth rate to 0.036 h^{-1} (~19 h Dt). Samples

taken directly before and after increasing light intensity were analyzed using a flow cytometer (**Fig. 8.1**). The wild type *Synechocystis* parent of UL018 was sampled from another reactor as a negative control. At t = 0 h, a slight shoulder is visible at the high fluorescence part of the histogram.

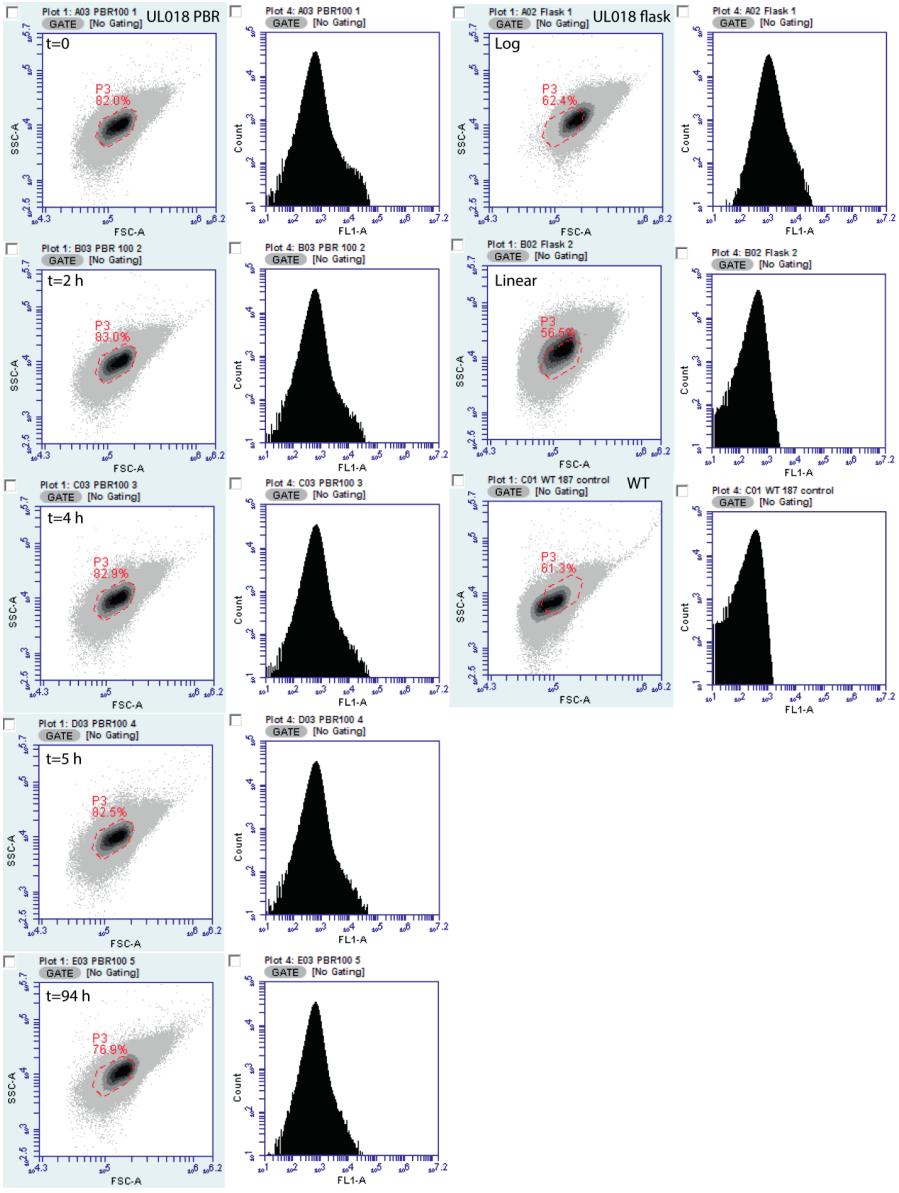

Fig. 8.1. Flow cytometer analysis of UL018 in a controlled reactor (left series) and a flask (right series) with a wild type control (bottom right). Parameters shown are side scatter (SSC), forward scatter (FSC) and fluorescence near 530 nm (FL1). P3 is drawn around the dense dot cloud of t=0 h of the reactor samples.

This is in accordance with observations using a fluorescence microscope, which showed a generally higher level of fluorescence than in WT, but with some cells being particularly bright.

The increase in light intensity reduced this heterogeneity to what appears to be a slightly skewed normal distribution (**Fig. 8.1**, PBR t = 94 h). Cell size, reflected in side and forward scatter, increases with growth rate as expected. In the flask culture, the exponential phase sample appears similar to t = 94 h of the reactor, but fluorescence drops to almost wild type levels in the linear phase. Despite culturing conditions with a clear light gradient, little heterogeneity was found and was even further reduced after an increase in light intensity in the culture in the photobioreactor. Similarly, during exponential, unlimited growth in a batch culture in a flask, no evidence of multiple subpopulations could be found. YFP fluorescence dropped with growth rate and onset of nutrient limitation during batch growth in a flask but remained fairly constant overall in the PBR.

8.6 Concluding remarks

In the work presented in this thesis, concrete steps forward have been made in realizing a bio-based society. The promise of a bio-based society rests on our ability to generate the required amounts of energy carriers and chemical feedstocks through microorganisms such as *Synechocystis*. Strategies to achieve this were discussed in **Chapter 1**. In **Chapter 2**, we have shown that metabolism can be accelerated simply by optimizing (or at least improving) the growth conditions the organism is subjected to and it will be exciting to see to what limits we can go. The method described and applied in **Chapters 3** and **4** is still in its infancy and holds great promise for future research. Even though the circadian clock is not a popular subject of research in *Synechocystis*, **Chapters 5 – 7** have shown that it is of critical importance to metabolism in a solar-powered world and deserves further attention.

The aim was to increase our understanding of the regulation and parameters of growth in *Synechocystis* and while that has been accomplished, arguably, more questions have been raised than have been answered. Indeed, nothing is ever easy.