Towards stable cyanobacterial cell factories

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Epilogue: Towards stable cyanobacterial cell factories

During the doctoral studies that have culminated in this thesis, we took clear steps to enable cyanobacteria to convert CO_2 into commodity products while maintaining sustained productivity. This was accomplished by aligning product formation with cell growth under continuous light conditions. At the industrial scale, cyanobacteria often grow in large outdoor ponds and in closed photobioreactors, *e.g.* in a greenhouse [4], where natural solar radiation is their primary source of light and energy. This means that these cultures are subject to an oscillating light-dark cycle. So then the question arises: "how can we achieve stable production under darkness, when cyanobacteria do not grow?" Beyond that, cyanobacteria have to effectively respond to harsh and dynamically changing conditions at the industrial scale, like *e.g.* nutrient limitation and extreme cultivation temperatures. It is therefore also important to know how we can make use of such industrial settings for efficient and stable production. As an extension of the concept developed in **Chapter 4** (and implemented in **Chapters 4** and **5**), we here propose to use the strategy of aligning the genetically engineered product formation with microbial fitness; that is to say, to couple product formation to the intrinsic abilities of cyanobacteria to adjust their metabolism to suit their surrounding conditions. Hence, the fitter ones carrying product formation favored by Darwinian selection would not be outcompeted, such that productivity can be maintained during extended time periods.

To implement this concept, we have to first understand how cyanobacteria adjust their metabolism to the relevant industrial conditions, and then carefully incorporate the target product pathway(s) into their native metabolic regulation network. Below we illustrate this approach with one of the important industrial settings – darkness, as a case study for fumarate production.

Stable fumarate production under darkness

Together with the 2017 Amsterdam team of IGEM (International Genetically Engineered Machine), which I co-supervised, we explored stable fumarate production in *Synechocystis* under darkness. During the night, *Synechocystis* derives energy by catabolizing its glycogen storage [281]. The TCA cycle is often associated with this process as it can provide the electron donors for ATP production via respiration. As fumarate is an intermediate metabolite in the TCA cycle, increasing the carbon flux through the TCA cycle could be exploited to increase fumarate production during the night. However, a recent publication shows that *Synechocystis* does not favor the TCA cycle at night as anticipated, but has rather evolved to prefer the Pentose Phosphate Pathway (PPP) for

hexose catabolism [282]. During the night, the PPP also acts as an electron-donor producing pathway, and apparently this pathway is preferred over the TCA cycle in *Synechocystis* for this function [282], potentially because it releases only a small part of the energy stored in the glycogen: The end-product of PPP will be a pentose (5 carbon skeleton), while the TCA cycle produces CO_2, which was so costly to fix in the first place.

To increase the flux through the TCA cycle, the gene encoding glucose-6-phosphate dehydrogenase enzyme (*zwf*, encoded by *slr1843*), catalyzing the first step in the PPP, was deleted. The underlying assumption is that cells are then forced to display a higher flux towards the TCA cycle, to reconcile the loss in the production of reducing equivalents for respiration-coupled ATP production. When combined with the *fumC* deletion, this is predicted to lead to increased production of fumarate, which we have tested (and confirmed!) in a batch cultivation experiment (Figure 7.1).

The results of this experiment show that the *ΔfumC* and the *ΔfumCΔzwf* are both capable of producing fumarate during the day, using the growth-coupling strategy. During the night, the *ΔfumCΔzwf* strain produces significantly more fumarate than the *ΔfumC* strain. These results furthermore confirm that at night the *ΔfumCΔzwf* strain is forced to direct more carbon from glycogen catabolism towards the TCA cycle, thereby forming fumarate. We thus engineered a *Synechocystis* cell factory that is able to produce fumarate around the circadian clock, using two different production strategies - both stable - one for the daylight period and another for the night. On top of this, since we used for the engineering only gene knock-outs and did not resort to the cloning of heterologous genes, the *ΔfumCΔzwf* strain will be a stable production strain for many generations to come. As a bonus, the higher nighttime production in the *ΔfumCΔzwf* strain, as compared to the *ΔfumC* strain, does imply that by knocking out the *zwf*, we force flux through the TCA cycle. This is an important finding, as it opens up opportunities for the nighttime production of valuable TCA cycle intermediates in *Synechocystis*. To our knowledge such a diurnal, dual strategy, photoautotrophic cell factory has not been reported before.

This approach of fumarate production is a typical example of hijacking the intrinsic abilities of a cyanobacterial metabolic adjustment (*i.e.* respiration) to an important industrial setting – darkness – for stable product formation. Other industrial settings, such as nutrient limitation, *e.g.* nitrogen, phosphorus, *etc.,* or extreme cultivation conditions, *e.g.* high pH, high/low light intensity, toxicity of product accumulation *etc.*, can also be explored, using the same strategy. But of course, the possibility to use this strategy successfully depends heavily on a thorough understanding of how cells respond to those industrial settings.

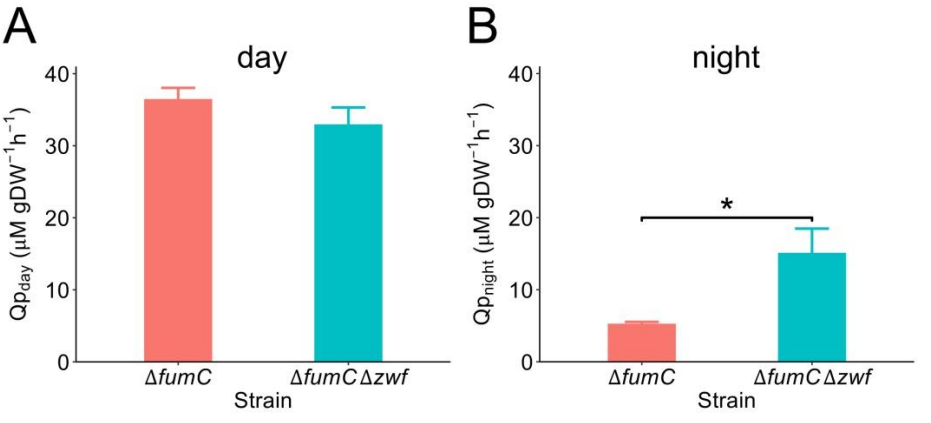

Figure 7.1 Fumarate productivity during the day (A) and the night (B) in a batch cultivation experiment in a Multi-Cultivator under the 8-h-day/16-h-night cycle. OD_{730} and fumarate concentrations were measured at every perceived dawn and dusk. Fumarate productivity was calculated separately for each day and night period, using the fumarate concentration differences, while dividing by both the average OD_{730} and the time interval between dawn and dusk. Cell dry weight was calculated based on a conversion of OD_{730} into 148 mg L^{-1} OD_{730}^{-1} [164]. Results here only represent the 4th day/night cycle (*i.e.* from 72 h to 96 h), when fumarate concentrations can be reliably measured. The error bars indicate the standard deviations of 4 replicates for the *ΔfumC* and 3 replicates for the *ΔfumCΔzwf* strain (* represents the p-value <0.05, from a two-tailed Student's t-test)

Understanding cyanobacterial metabolic regulation

Fluxomics studies have played a fundamental role in clarifying cyanobacterial metabolic adaptation to varying conditions. That is because fluxomics studies allow a systems-level analysis of cellular metabolic fluxes [283]. With the information from such studies, we could know how exactly cells re-distribute their metabolic fluxes to respond to a specific condition. Such understanding would be of great benefit to facilitate selection of target pathway(s) that can be incorporated into cyanobacterial metabolism, to couple to their native adaptability, *i.e.* microbial fitness, for efficient and stable production. As illustrated above, we have successfully coupled cyanobacterial respiration to fumarate production in the dark. This would not have been easy to accomplish without the results of a recent fluxomics study [282]. This example clearly shows the importance of fluxomics studies for target gene selection for the construction of stable production strains. By saying this, it certainly does not mean that the importance of other studies, *e.g.* genome-scale modelling, physiological analyses, *etc*., can be underestimated. From a fundamental point

of view, it would be very interesting to further validate the results described above with the corresponding fluxomics data.

^{13}C metabolic flux analysis is a common approach to trace the flow of carbon from labeled substrates into cellular metabolites [284]. From the labeling pattern, the magnitude of all cellular metabolic fluxes can then be computationally estimated using a metabolic network model. ^{13}C metabolic flux analysis has been widely used in *Synechocystis* to estimate its flux distribution under photoautotrophic- [285], heterotrophic- and mixotrophic growth conditions [286]. Furthermore, transcriptomic- and metabolomic data have also been integrated into metabolic flux analysis, such that deep insights of cyanobacterial metabolic regulation can be derived [287]. FBA has also been extensively used *in silico*, to simulate the metabolic flux distribution, with/without further constraints from other omics data [281]. In summary, together with genome-scale modelling and physiological studies, metabolic flux analysis can help us to better understand cyanobacterial metabolism, and its adjustment under different stress/industrial conditions.

Personal remarks

I got involved in the metabolic engineering field nearly a decade ago. It started with mammalian cells (*e.g.* rCHO) for antibody production, then *E. coli* for the manipulation of its respiration chain, and now cyanobacteria for the development of stable photosynthetic cell factories. During these 10 years of study and work, I have had the opportunity to witness a flood of exciting proof-of-concept studies, employing different kinds of microbial systems for various types of product formation with multiple engineering tools and methods. Now I am about to finish my Ph.D., and I cannot help to wonder what I have learnt from those studies and from my own experiences. If I may, I want to summarize two of them as my key take home messages.

Let EVOLUTION be our friend. Evolvability is an intrinsic ability of microbes. Our efforts to transform microbes into workhorses can be regard as an endless battle against their ability to evolve. Eventually, evolution will win the battle and our “efficient” cell factories will become “inefficient”. Instead of constantly fighting against microbial evolvability, how about making friends with them? Next time when an attempt is made to engineer a microbe for the production of a specific compound, I suggest that we first ask ourselves the following questions: *What is the function of the target compound in this microbe? Can we make the production of this compound beneficial or mandatory for this microbe? How can we make the production of this compound stable?*

Let NATURE be our guidance. Our ability (scientifically and technically) to genetically modify microbes to develop cell factories, originates from nature. Nature provides us numerous excellent examples of how microbes with immensely complicated regulation systems can precisely adjust themselves in response to surrounding perturbations. That is the reason why microbes are so robust, which is a key criterion for stable and efficient microbial cell factories as well. Understanding the abilities of a microbe to effectively manage its own regulation systems will shed light on how stable and efficient microbial cell factories can be devised. Hence, whenever we are not sure on how to proceed with cell-factory design, how about looking deep into nature itself for inspiration?