Towards stable cyanobacterial cell factories
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Summary

Microbial bioengineering has the potential to contribute to the further development of human society by providing sustainable, novel, and cost-effective production pipelines. Cyanobacteria, the photosynthetic bacteria that are capable of directly converting CO_2 to chemicals, fueled only by (sun)light, are particularly attractive for such applications. However, as is common to other microbial systems too, cyanobacteria are no exception when it comes to the challenges presented by maintaining sustained productivity of heterologous products. This is due to the growth-rate impairment caused by the burdensome product formation. Selected spontaneous non-producing mutants tend to grow faster than the producing strains, so that they are gradually taking over the population. Consequently, the total productivity of the culture will be impaired. This unstable productivity has been reported extensively in other microbial systems; however, for cyanobacteria not much focus has been placed on investigating it. The latter would be particularly pertinent, as cyanobacteria have been extensively genetically modified for the formation of a variety of chemical products. In this thesis, this is exactly what we studied, *i.e.* the instability of the cyanobacterial 'direct conversion' process, with the aim of developing stable cyanobacterial cell factories.

Chapter 1 of this thesis provides an overview of the key research questions associated with the development and application of cyanobacterial cell factories. Those questions include: (i) the basic fundaments and advantages of the cyanobacterial 'direct conversion' process; and (ii) how this process can be brought to an industrial scale, facilitated by use of the synthetic-biology toolkit for cyanobacteria and by mathematical modelling.

In **Chapter 2**, we take a well-characterized cyanobacterial cell factory for the production of lactate, and modulated its productivity without changing the expression level of the heterologous lactate dehydrogenases (derived from different lactic acid bacteria) that catalyze the crucial step in product formation. This was achieved by using a nonmetabolizable analogue of fructose 1, 6-bisphosphate that allosterically activates the target pathway enzyme (*i.e.* lactate dehydrogenase). Using this strategy, we could show that the instability of product formation is mainly caused by channeling the fixed carbon away from biomass formation, rather than by any other effect, such as a protein expression burden due to synthesis of the lactate dehydrogenase.

In **Chapter 3**, we describe a new method of phototrophic batch cultivation for cyanobacteria. This method, the photonfluxostat, is based on dynamically dosing the amount of incident light, relative to the culture's cell density (*i.e.* OD), to ensure that light intensity per OD remains constant. When applied to *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*), different, yet constant and truly exponential growth rates can be reliably obtained by varying the biomass-specific light flux. This cultivation method allows a convenient and better characterization of the growth rate and its associated physiological

parameters of the cells. This method has been adopted (in following Chapters) to study the relationship between growth rate and product formation.

In **Chapter 4**, we propose a novel strategy to stabilize production in engineered cell factories. This can be done by aligning the production of physiological metabolites to the formation of biomass, by targeted gene deletion(s). Formation of such a metabolite is then 'coupled to growth'. In order to identify which compounds are suitable to be produced in this fashion, we developed an *in silico* tool that 'Finds Reactions Usable in Tapping Side-products' (FRUITS), based on the genome-scale metabolic model of the host organism. When applied to *Synechocystis*, a total of nine target metabolites were identified, when allowing a maximum of four gene deletions. We validated this approach experimentally for acetate production, thereby creating the first growth-coupled photoautotrophic cell factory.

As a further confirmation of this strategy, **in Chapter 5** we test in *Synechocystis* the stable production of fumarate, a compound with a variety of potential commercial applications. Upon deletion of the gene, predicted by the algorithm we developed (see Chapter 4) to block intracellular fumarate re-utilization, indeed we observed that fumarate was produced as a side-product of anabolism, which then accumulated and was exported out of the cells. When the stability of this production was tested in turbidostat cultures it turned out to be stable for over 25 days. In contrast, a strain engineered using the classical heterologous pathway expression strategy that initially displayed the same degree of carbon partitioning into product, lost its ability to form product within 5 to 10 days.

In **Chapter 6**, we discuss genetic instability as one of the main challenges for the application of synthetic biology in the synthesis of commodity products by cyanobacteria. This challenge is general to any 'cell factory' approach, in which the cells grow for multiple generations. Based on studies carried out in different microbial hosts, we could identify three distinct strategies that have been proposed to tackle this problem: (i) reduce microbial evolvability by decreasing the spontaneous mutation rate; (ii) align product formation with cell growth/fitness; and paradoxically, (iii) efficiently re-allocate cellular resources to formation of product by uncoupling this process from growth of the cells. The implementation of either of these strategies requires an advanced synthetic biology toolkit. We therefore reviewed the current methods available for cyanobacteria, and identify areas of focus in which further developments are urgently needed. Furthermore, we discuss how these potentially stabilizing strategies may be combined, to further increase productivity, while maintaining the stability of the cyanobacteria-based 'direct conversion' process.