Towards stable cyanobacterial cell factories

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The research reported in this thesis was carried out in the Molecular Microbial Physiology Group of the Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam. Wei Du was supported by a PhD scholarship from China Scholarship Council.

Cover: The cover, showing a stable cyanobacterial cell factory fueled by sunlight, is inspired by Chinese philosophy - yin and yang. The conflicting aspects of *(i)* cell growth (yin) and *(ii)* product formation (yang) can be harmonized by balanced feeding of the production system (represented by the tree), so that sustainable and stable production (indicated by the apples) can be achieved. The metabolic pathways in the background provide an overview of the potential targets from which a specific product can be tapped off in such cell factories.

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prof. dr. ir. K.I.J. Maex

ten overstaan van een door het College voor Promoties ingestelde

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All we have to decide is what to do with the time that is given us.

— J.R.R. Tolkien

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Preface

Irrespective of where we come from, we all share the global responsibility of ensuring that our societies are sustainable. We have been depleting the world's resources and filling the atmosphere with abnormal levels of CO_2 for too long. But CO_2 can also be used as a resource - plants and certain microbes have been doing so for billions of years. This is the foundation for the bio-based economy – reducing and eventually replacing the use of fossil fuel. Up to now, the focus in bio-based production has been on producing biofuels using plants to produce sugars, which are then used as substrate in microbial fermentation processes. But there is an alternative way - using cyanobacteria to take up CO_2 from the atmosphere and directly converting it into useful products.

Cyanobacteria are prokaryotes including a vast array of different species, most capable of (oxygenic) photosynthesis [1]. With fast photoautotrophic growth in comparison to other photosynthetic eukaryotes (*e.g.* plants), cyanobacteria also have the advantages of not competing for arable land and being more easily genetically accessible. Additionally, cyanobacterial direct conversion of CO_2 into products relieves us from the need of sugar extraction processes from plants, a burdensome necessary step for microbial fermentation using chemoheterotrophs. Hence, cyanobacterial direct conversion to product is expected to have a higher overall efficiency per surface area. Altogether, these advantageous traits have made the concept of cyanobacterial direct conversion (*i.e.* cyanobacterial cell factories) draw much attention recently.

Promising as it sounds, the development of efficient cyanobacterial cell factories is still challenging. One of the technical hurdles, common to all genetically engineered microbial cell factories, is to maintain sustained productivity. This is because traditional genetic engineering strategies for microbial product formation, *i.e.* through introduction of heterologous pathways encoded by multiple genes with constitutive expression levels, are burdensome for cell growth. Consequently, spontaneous non-producing mutants tend to grow faster, thereby gradually taking over the population and undermining the total productivity of the culture. In general, this technical hurdle is common to any microbial system that was genetically engineered for product formation. Yet, for cyanobacteria not much attention had been paid to this aspect before this project commenced. In this thesis, we decided to specifically study the important issue of the instability of cyanobacterial direct conversion processes, with the aim of conceiving novel strategies to prevent this problem.

Thesis overview:

This thesis starts by providing an overview of the key research questions in the development and application of cyanobacterial cell factories (**Chapter 1**). Those questions include the basic fundaments and advantages of the cyanobacterial direct conversion process; how synthetic toolkits and mathematic modelling could help during the scale-up of these processes to an industrial scale. In **Chapter 2**, we take well-characterized cyanobacterial factories of lactate, and modulate production without changing the expression level of the heterologously expressed lactate dehydrogenase, taken from various lactic acid bacteria. This was achieved by using a nonmetabolizable analogue to allosterically activate the production pathway. Using this strategy, we could show that the observed instability in production is mainly caused by channeling the fixed carbon away from biomass formation, rather than by any other effect, such as the protein expression burden. **Chapter 3** describes the design and application of a new cultivation method – the photonfluxostat. This was accomplished through dynamically adjusting incident light intensity based on cell density, such that the culture is irradiated by a constant biomass-specific light flux. This cultivation method allows us to easily characterize the relationship between growth rate and productivity, a key criterion for evaluating the extent to which growth and product formation are coupled. In **Chapter 4**, we propose a novel strategy to stabilize production in engineered cells. This is done by aligning the production of native metabolites to the formation of biomass in a so-called growth-coupled manner. 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. We validated this approach experimentally in this chapter for acetate production through engineering of the first growth-coupled photoautotrophic cell factory. This concept was further explored in **Chapter 5**, but now focusing on the growth-coupled production of fumarate, a compound with a variety of potential utilizations and that was predicted to lead to a higher carbon partitioning according to our simulations reported in chapter 4. We were able to show that fumarate productivity is stable during prolonged cultivation for over 600 hours, with a carbon partitioning well above 20%. In **Chapter 6**, we summarize different strategies applied in other microbial production systems, to shed new light on strategies of stabilizing product formation in cyanobacterial cell factories. To make the application of cyanobacterial cell factories more efficient, other aspects of technical hurdles in the production process are also covered. A brief epilogue with reflections on the main body of work described in this thesis, along with past experiences and current developments, is then presented with the goal of distilling some general wisdom from the studies reported in this thesis.