# 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.

## Samenvatting

Microbiële biotechnologie heeft de potentie om bij te dragen aan de ontwikkeling van nieuwe, duurzame, en economisch competitieve manieren om 'commodity' verbindingen te produceren. Cyanobacteriën, fotosynthetiserende bacteriën die in staat zijn $CO_2$ direct om te zetten in nuttige verbindingen, op basis van de energie uit (zon)licht, zijn bijzonder aantrekkelijk om gebruikt te worden als basis voor zulke toepassingen. Zoals ook wordt waargenomen in veel andere microbiële systemen, zijn cyanobacteriën geen uitzondering op de regel dat deze duurzame productie op de lange termijn nog vaak instabiel is. Dit is een gevolg van de last die de productie van de gewenste stoffen oplevert voor het producerende organisme, en daardoor haar groeisnelheid negatief beïnvloedt. Celdeling en groei gaan gepaard met het ontstan van spontane mutaties, die niet-producerende mutanten opleveren, doordat zulke mutanten over het algemeen sneller kunnen groeien dan de producerende stam, en dus de populatie overnemen. Dit leidt dan uiteindelijk tot een culture waarin de totale productiviteit sterk verminderd is. Over dergelijke instabiele productiviteit is uitgebreid gerapporteerd in uiteenlopende microbiële systemen, maar voor cyanobacteriën echter nog nagenoeg niet onderzocht. Deze instabiliteit is zeer belangrijk aangezien cyanobacteriën breed ingezet kunnen worden voor het produceren van een breed spectrum aan verbindingen. De nadruk in het onderzoek wat in dit proefschrift wordt beschreven ligt op de genetische instabiliteit van de directe omzetting van $CO_2$ met behulp van cyanobacteriën, met als doel stabiele cyanobacteriële cellulaire fabrieken te ontwikkelen.

**Hoofdstuk 1** geeft een overzicht van de belangrijkste onderzoeksvragen op het gebied van de ontwikkeling en toepassing van cyanobacteriële 'cell factories', zoals bijvoorbeeld (i) de fundamenten en voordelen van de directe omzetting van $CO_2$ in commodity producten door cyanobacteriën, en (ii) hoe deze productie opgeschaald kan worden tot een industrieel proces, met behulp van synthetisch-biologische gereedschappen en wiskundige modellen.

In **Hoofdstuk 2** hebben we de goed-gekarakteriseerde cyanobacteriële fabriek voor de productie van lactaat bestudeerd, door de productiviteit daarvan te variëren zonder de expressie van het geïntroduceerde lactaat-dehydrogenase te veranderen. Dit is gedaan door een metabool inactieve analoog van fructose-1,6-bisfosfaat toe te voegen, die door allostere regulatie de activiteit van het gebruikte lactaat-dehydrogenase kan veranderen. Dankzij deze strategie konden we laten zien dat de instabiliteit van de lactaat productie voornamelijk terug te voeren is op de hoeveelheid koolstof die afgetapt wordt van het intermediair metabolisme, ten faveure van vorming van het eindproduct lactaat (en daardoor niet voor de groei van het organisme beschikbaar is). Dit impliceert dat de last die de synthese van het lactaat-dehydrogenase zelf

oplevert voor de cyanobacterie niet de oorzaak was van de waargenomen instabiliteit.

In **Hoofdstuk 3** beschrijven we een nieuwe methode om cellen in een batchculture te kweken. Deze methode, de fotonfluxostat, is gebaseerd op het dynamisch doseren van de hoeveelheid invallend licht, relatief ten opzichte van de cel-dichtheid (hier gemeten als de optische dichtheid (OD)), en wel zo dat de lichtintensiteit per OD constant blijft. Deze aanpak zorgt ervoor dat *Synechocystis* sp. PCC6803 (*Synechocystis*) binnen ruime grenzen exponentieël kan groeien met een vooraf instelbare, constante groeisnelheid. Deze nieuwe methode maakt het mogelijk om de afhankelijkheid van diverse fysiologische karakteristieken van de cyanobacterie te bepalen als functie van de groeisnelheid. In hoofdstukken 4 en 5 is deze methode toegepast om de relatie tussen groeisnelheid en productvorming te bestuderen.

**Hoofdstuk 4** introduceert een nieuwe strategie voor stabiele product-vorming in genetisch gemodificeerde cellen. Dit is gedaan door cel-eigen producten uit het intermediair metabolisme te kiezen, en door deletie van specifieke genen er voor te zorgen dat deze producten niet meer geassimileerd kunnen worden. Hierdoor is de productie van deze stoffen intrinsiek gekoppeld aan de vorming van biomassa, waardoor groei alleen mogelijk is als de gewenste stof ook geproduceerd wordt. De stoffen die geschikt zijn voor deze aanpak zijn geïdentificeerd door een *in silico* analyse die gebruik maakt van in-house ontwikkelde software (Vind Reacties Bruikbaar voor het Aftappen van Bijproducten (VRBVAB)), gebaseerd op een genoom-breed model voor het intermediair metabolisme van het te gebruiken organisme. Voor *Synechocystis* zijn met deze aanpak negen metabolieten geïdentificeerd, als er tot maximaal vier genen uitgeschakeld mogen worden. Deze aanpak is experimenteel gevalideerd voor acetaat productie en daarmee is de eerste groei-gekoppelde fotoautotrofe ‘cell factory’ gemaakt.

Als verdere bevestiging van het succes van de ontwikkelde strategie hebben we in **Hoofdstuk 5** de stabiele productie van fumaraat getest in *Synechocystis*. Deze verbinding heeft een aantal belangrijke toepassingen, o.a. in de polymeer synthese. Na verwijdering van het gen waarvan door ons algoritme voorspeld werd dat het essentieel zou zijn voor assimilatie van fumaraat, bleek er inderdaad met de voorspelde snelheid fumaraat gevormd te worden, als bijproduct van de groei van het organisme, terwijl het meeste hiervan geëxporteerd wordt uit de cellen. De stabiliteit van deze vorm van fumaraat productie werd in turbidostaat experimenten verder getest; zij bleef stabiel gedurende tenminste 25 dagen. Een klassiek ge-engineerde stam die in eerste instantie een vergelijkbaar productieniveau liet zien, verloor deze productie-

capaciteit binnen 5 tot 10 dagen door het spontaan ontstaan van niet-producerende mutante cellen.

In **Hoofdstuk 6** wordt de genetische (in)stabiliteit besproken als één van de grootste uitdagingen voor de toepassing van synthetische biologie in de synthese van bulkproducten door cyanobacteriën. Deze uitdaging is echter van toepassing op elke 'cellulaire fabriek' waarin de cellen – tijdens de vorming van het product - meerdere generaties groeien. Gebaseerd op onderzoek in verschillende micro-organismen hebben we drie unieke strategieën geïdentificeerd die getest kunnen worden om dit probleem van genetische instabiliteit te verkleinen. Dit zijn respectievelijk: (i) de kans op het optreden van mutaties verminderen door de intrinsieke mutatie-snelheid te verlagen; (ii) de vorming van het product te koppelen aan cellulaire groei; en (iii) het efficiënt herverdelen van cellulaire hulpbronnen, door de vorming van het product los te koppelen van de groei. Voor de implementatie van deze strategieën zijn geavanceerde synthetisch-biologische gereedschappen nodig. In dit hoofdstuk wordt ook een overzicht gegeven van de bestaande methodes op dit gebied voor cyanobacteriën en worden gebieden geïdentificeerd waar de focus op gelegd zou moeten worden om specifieke ontwikkelingen te stimuleren. Daarnaast bespreken we hoe potentiële stabiliserende strategieën gecombineerd kunnen worden om de productiviteit van de 'cell factories' verder te verhogen, terwijl daarbij hun genetische stabiliteit gewaarborgd blijft.

# 摘　要

微生物生物工程可以为人类社会提供新型、可持续、经济有效的产品合成路线，从而蕴含着支撑未来社会发展所需的可能性。作为光合细菌，蓝细菌因其可以利用太阳能将二氧化碳直接转化为化学产品而尤其受到关注。但和其它微生物一样，蓝细菌光合系统也很难维持稳定的化学品合成能力，所以其实际应用也备受质疑。一般而言，化学产品的合成对于微生物细胞自身是一个沉重的负担，其正常生长必然受到损害。当有自发的随机基因突变发生从而使得某些微生物摆脱了这类负担，这些突变的特定微生物与原来相比生长速率变快进而逐渐占据整个微生物群体，导致了整个微生物群体生产能力的降低。这种生产能力不稳定的问题在其它微生物系统已有广泛的研究和报道，但在蓝细菌领域却少有研究。因蓝细菌已经并正在被大量的基因工程改造去合成各种各样的产品，其生产能力的稳定性研究则显得愈发重要。鉴于此，本论文将重点研究蓝细菌光合转化过程的不稳定性，以期构建稳定的蓝细菌细胞工厂。

本论文**第一章**概述了构建和应用蓝细菌细胞工厂过程中的关键科学问题。这些问题包括蓝细菌光合定向转化过程中的理论基础及其优势，以及相关遗传工具和数学模型怎样更好的促进蓝细菌转化过程的规模化放大直至将来的产业化。

**第二章**中，在之前已充分研究的蓝细菌异源表达来源于不同乳酸菌的乳酸脱氢酶从而合成乳酸的工作基础上，我们采取了一种新型的策略去调控乳酸的合成但不改变乳酸脱氢酶的表达水平。该策略通过加入果糖 1, 6-二磷酸的一种不可代谢的结构类似物来别构激活目标代谢途径。通过使用该策略，我们发现蓝细菌化学品合成能力的不稳定性主要归结于其光合作用固定的碳源没有被有效的用于细胞生物质的合成，而非其它的作用，比如过量表达目的蛋白对细胞生长造成的负担等。

**第三章**描述了一种新型的批式培养方法。该培养方式——“恒光流培养”，基于细胞密度实时动态调节入射光的强度，使得每单位细胞群体接受的光流量恒定。当该培养方式应用于培养集胞藻 PCC6803 时，通过改变每单位细胞群体接受的光流量，我们获得了不同组别的细胞对数生长期恒定且可靠的细胞生长速率。这种培养方式可被方便的用于解析细胞生长速率及其相关生理学参数之间的关系，比如产品的合成能力。我们接下来通过该培养方式研究了细胞生长速率与产物合成能力之间的关系。

在**第四章**，我们提出了一种在工程细胞系中稳定产品合成能力的新型策略。该策略通过敲除相关基因，从而将细胞自身特定代谢物的合成与细胞生长相偶联。为鉴定哪些代谢物适用于该策略，我们根据宿主系统的基因组代谢模型开发了一个 *in silico* 计算机模拟工具来寻找相应产物合成的反应。当应用于集胞藻 PCC6803

时，在允许最多敲除 4 个基因的前提下，我们鉴定到了 9 个目标代谢物。我们对其中的乙酸合成进行试验验证，首次成功构建了基于生长偶联方式合成化学品的光合细胞工厂。

作为以上策略的进一步验证和延伸，在**第五章**中，我们测试了在集胞藻 PCC6803 中稳定生产一种具有多种应用前景的化学品——延胡索酸。根据第四章的预测结果，我们敲除了细胞胞内回收利用延胡索酸的反应，然后观测到作为合成代谢副产物的延胡索酸的累积及其胞外分泌。在恒浊器培养过程中，细胞合成延胡索酸的能力在 25 天后仍然维持稳定。而运用常规策略构建的对照菌株与延胡索酸合成菌株相比，两者虽生产能力类似，但其产物合成能力在 5 到 10 天内彻底丢失。

**第六章**，在运用合成生物学手段改造蓝细菌生产化学品过程遇到的诸多挑战中，我们讨论了其中主要的挑战之一——生产能力的不稳定性。该挑战其实广泛存在于各类细胞工厂中，但凡细胞需要生长至相应的代数。根据已报道的不同种类微生物宿主遗传不稳定性的研究，我们概括了三种策略以应对：1）通过降低微生物自发突变的几率来削弱微生物的进化能力；2）将产物的合成与细胞生长或进化优势相偶联；3）通过将产物的合成与细胞生长解偶联来有效的重新分配细胞资源。当然，无论哪一种策略的实施都需要先进的合成生物学工具。我们综述了现阶段已应用于蓝细菌的各类工具及方法，并提出了部分急需改进的方面。最后，在确保蓝细菌定向转化过程稳定的前提下，我们讨论了稳定的生产策略如何与其它策略相结合从而进一步提高蓝细菌的化学品生产能力。

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杜伟 @ Amsterdam
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## List of publications

**Du, W**[#]., Liang, F[#]., Duan, Y., Tan, X., and Lu, X. 2013. Exploring the photosynthetic production capacity of sucrose by cyanobacteria. *Metabolic Engineering. 19*, 17–25 ([#] equal contribution).

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