Photoautotrophic overproduction of mannitol in Synechocystis sp. PCC6803 using osmotic pressure as a driving force

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Summary

Microbial cell factories have a huge potential to meet the requirements of rapidly developing human societies by providing sustainable, novel and low-cost production systems. Cyanobacteria, well characterized photosynthetic bacteria that are capable of the direct conversion of CO_2 into valuable chemicals by using sunlight, are particularly attractive for such applications. However, as found in other microbial cell factories from different phylogenetic clades, unstable yield of chemicals from cyanobacteria is becoming a big challenge for industrial application in the future. This is often due to the heterologous expression of pathways towards compounds that lead to growth-inhibiting phenotypes. Under this background, producer cells tend to rapidly accumulate spontaneous mutations that increase their growth rate but hamper product formation. In this thesis, we propose a new strategy for solving these instability problems, based on associating resistance to stress to the production of target compounds. To prove this idea, we successfully stabilize and improve mannitol production under salt stress in *Synechocystis* sp. PCC6803.

In **chapter I (General Introduction)**. We described the background and main questions that this research work attempts to address. This chapter gives an overview of the molecular tools developed for constructing a photosynthetic factory from CO_2 in cyanobacteria, and provides several solutions for solving unstable productivity in cyanobacteria. Moreover, we introduce the new idea of using salt stress to stabilize and improve mannitol production in *Synechocystis* sp. PCC6803.

In **chapter II**. The sustainable production of mannitol in *Synechocystis* sp. PCC6803 from CO_2 was first achieved by the heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*), but this proved to be unstable. We validated that mannitol could function as a compatible solute in *Synechocystis* sp. PCC6803, and its derivative strains in which the ability to produce the native compatible solutes was impaired. We tested the genetic stability of all these strains with and without salt stress, in respect to their mannitol productivity during prolonged turbidostat cultivations.

The obtained results show that mannitol production under salt stress conditions in the *Synechocystis* sp. PCC6803 strain that cannot synthesize its endogenous compatible solutes (ΔCS_M) is remarkably stable, while the control strain (WT_M) completely loses this ability in only 6 days. Subsequent DNA sequencing of isolates that had lost the ability to synthesize mannitol revealed that multiple types of mutation occurred in the *mtlD* gene that can explain the disruption of mannitol production.

In **chapter III**, we demonstrated that mannitol production in *Synechocystis* sp. PCC6803 is restricted by the formation of a toxic intermediate, mannitol-1-phosphate. To achieve relatively higher mannitol production in cells, we used an inducible promoter, Pnrsb, in the presence of 5 μM nickel as activator to control the expression level of *mtlD*, while overexpressing *m1p* using the strongest known promoter for these cells, Ptrc1. Under this optimized expression system (SWW011), the concentrations of the toxic mannitol-1-phosphate cannot impose lethal effect on cells, and more mannitol-1-phosphate is transformed into mannitol with higher efficiency. Compared with the first engineered mannitol producer *Synechocystis* sp. PCC6803 of chapter II, mannitol production from SWW011 is increased roughly 8 times.

In **chapter IV**, we used adaptive laboratory evolution strategies with increased salt pressure to improve mannitol production in the ΔCS_M engineered strain. Mannitol production from evolved strains is increased 24 times under this strategy compared with the original mannitol producer from chapter II. In addition, several candidate genes that might (in)directly influence mannitol expression in cells were discovered using whole genomic sequencing technology on the evolved strains in comparison with the original mannitol producers. Among these genes, *pnp* encoding polyribonucleotide nucleotidyltransferase was proved to negatively affect mannitol production in cells via reverse engineering methods.

In **chapter V**, we tried a structurally different way to synthesize mannitol in the cells such that the production of toxic intermediates (i.e. mannitol-1-phosphate)

could be avoided altogether. The new biosynthetic mannitol pathway consists of mannitol dehydrogenase (*mdh*) from *Lactobacillus reuteri* and sucrose synthase (*susA*) from *Anabaena*. However, it can only help *Synechocystis* sp. PCC6803 to produce 0.058 mg/l/OD of extracellular mannitol in 3 days under 300 mM of salt stress conditions. The mannitol productivity using this pathway is, for now, far less than the one obtained using the one of chapters II, III and IV.

In **chapter VI (General Discussion)**, the many methods for improving mannitol production in *Synechocystis* sp. PCC6803 are discussed, including maximizing photosynthetic efficiency and optimization of the mannitol synthesis pathway. Moreover, we summarized the characteristics and biosynthetic pathways of other non-native compatible solutes which could be achieved in the future and stabilized using the strategy advance here, i.e. associating resistance to osmotic stress to the production of target compounds in cyanobacteria in the future.

Samenvatting

Microbiële celfabrieken hebben een enorme potentie om snel ontwikkelende samenleving van duurzame, innovatieve, en goedkope productiesystemen te voorzien. Cyanobacterien, uitgebreid gekarakteriseerde photosynthetische bacteriën die in staat zijn om CO_2 direct te converteren naar waardevolle chemicalien door gebruik te maken van zonlicht, zijn voor dergelijke toepassingen bijzonder interessant. Echter vormt de instabiele opbrengst van chemicaliën door cyanobacteriën, zoals ook in andere phylogenetische clades wordt opgemerkt, een grote uitdaging. Dit is vaak te wijten aan het feit dat heterologe expressie van metabole productieroutes tot fenotypes leiden met verminderde groei. In dit geval hebben producerende cellen namelijk sterk de neiging om spontane mutaties op te bouwen die hun groeisnelheid verhogen maar de productvorming belemmeren. In dit proefschrift stellen we een nieuwe strategie voor om deze instabiliteitsproblemen op te lossen, gebaseerd op het koppelen van stressresistentie met de productie van gewenste verbindingen. Om dit aan te tonen, hebben we succesvol de productie van mannitol onder zoutstress in *Synechocystis* sp. PCC6803 gestabiliseerd en verbeterd.

In hoofdstuk I (algemene inleiding) hebben we de achtergrond en hoofdvragen die dit onderzoek probeert te beantwoorden beschreven. Dit hoofdstuk geeft een overzicht van de moleculaire technieken die zijn ontwikkeld voor het construeren van een photosynthetische fabriek met CO_2 in cyanobacteriën, en biedt verschillende manieren om de instabiele productiviteit in cyanobacteriën te verbeteren. Bovendien introduceren we nieuwe ideeën om zoutstress te gebruiken om mannitolproductie in *Synechocystis* sp. PCC6803 te stabiliseren en verbeteren.

In hoofdstuk II is de duurzame productie van mannitol van CO_2 in *Synechocystis* sp. PCC6803 eerst bereikt door de heterologe expressie van mannitol-1-fosfaat-5-dehydronase (*mtlD*) en mannitol-1-fosfatase (*m1p*), maar dit bleek instabiel te zijn. We hebben gevalideerd dat mannitol zou kunnen functioneren als een compatibele oplosstof in *Synechocystis* sp. PCC6803 en in afgeleide stammen waarin het vermogen om osmolieten te produceren is aangetast. We hebben de genetische stabiliteit van al deze stammen met en zonder zoustress getest, met betrekking tot mannitol productiviteit tijdens een langdurige turbidostat-kweek. De verkregen resultaten laten zien dat mannitolproductie onder zoutstressomstandigheden opmerkelijk stabiel is in stammen van *Synechocystis*

sp. PCC6803 die geen compatibele oplosstoffen (ΔCS_M) kunnen synthetiseren, terwijl de controlestam (WT_M) dit vermogen volledig verliest in slechts 6 dagen. Daaropvolgende DNA-sequentiebepaling van isolaten, die het vermogen hebben verloren om mannitol te synthesiseren, laat zien dat er meerdere soorten mutaties in het *mtlD* gen optraden die de verstoring van mannitolproductie kunnen verklaren.

In hoofdstukk III hebben we aangetoond dat mannitolproductie in *Synechocystis* sp. PCC6803 wordt beperkt door de vorming van een toxisch tussenproduct, mannitol-1-fosfaat. Om een relatief hogere mannitol productie in cellen te bereiken, hebben we een induceerbare promoter, Pnrsb, gebruikt in aanwezigheid van 5μM nikkel om het expressieniveau van *mtlD* te activeren, terwijl *m1p* tot overexpressie is gebracht onder de sterkste bekende promoter voor cellen, *Ptrc1*. In dit geoptimaliseerde expressiesysteem (SWW011) zijn de concentraties van het toxische mannitol-1-fosfaat niet staat om een letaal effect te hebben op cellen en wordt meer mannitol-1-fosfaat met hogere efficiëntie omgezet in mannitol. Vergeleken met de eerste onwikkelde mannitolproducent *Synechocystis* sp. PCC6803 van hoofdstuk II, wordt de mannitolproductie in SWW011 ruwweg 8 keer verhoogd.

In hoofdstuk IV hebben we adaptieve laboratorium evolutie-strategiën met verhoogde zoutdruk toegepast om mannitolproductie van de ΔCS_M-stam te verbeteren. De productie van mannitol uit geëvolueerde stammen wordt in deze strategie 24 keer verhoogd in vergelijking met de oorspronkelijke mannitolproducent uit hoofdstuk II. Bovendien werden verschillende potentiële genen ontdekt die een directe invloed zouden kunnen hebben op de expressie van mannitol in cellen met behulp van de techniek waarbij het volledige genoom van de geëvolueerde stammen wordt gesequenced en wordt vergeleken met de originele mannitolproducenten. Van deze genen is via de *reverse engeering* methode bewezen dat *pnp*, coderend voor polyribonucleotide nucleotidyltransferase, de mannitolproductie in cellen negatief beïnvloedt.

In hoofdstuk V hebben we een structureel andere manier gebruikt om mannitol in cellen te synthetiseren, zodat de productie van toxische tussenproducten (d.w.z. manntiol-1-fosfaat) volledig vermeden kan worden. De nieuwe biosynthetische mannitolroute bestaat uit mannitol dehydrogenase (*mdh*) uit *Lactobacillus reuteri* en sucrose synthase (*susA*) uit *Anabaena*. Het kan *Synechocystis* sp. PCC6803

echter alleen helpen om 0.057 mg/l/OD extracellulair manntiol te produceren in 3 dagen onder 300mM zoutstress. De mannitol productiviteit in deze route is tot dusver veel lager dan de producitiviteit die werd verkregen in hoodfstuk II, III en IV.

In hoofdstuk VI (algemene discussie) worden de vele methoden voor het verbeteren van de mannitolproductie in *Synechocystis* sp. PCC6803 besproken, inclusief het maximaliseren van fotosynthetische efficiëntie en optimalisatie van de synthese route naar mannitol. Bovendien hebben we de kenmerken en biosynthetische routes van andere heterologe oplosstoffen samengevat die in de toekomst zouden kunnen worden bereikt en die kunnen worden gestabiliseerd met de beschreven methode, namelijk het koppelen van resistentie tegen osmotische stress met de productie van gewenste verbindingen in cyanobactiëren.

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List of Publications

Wu, W., Du, W., Gallego, R.P. et al. Using osmotic stress to stabilize mannitol production in *Synechocystis* sp. PCC6803. ***Biotechnol Biofuels*** 13, 117 (2020).

Wu, W., Anne, J. et al. Improvement in Mannitol Production of Engineered *Synechocystis* sp. PCC6803 via Adaptive Laboratory Evolution. (Manuscript in preparation)

Wu, W., Du, W. et al. Enhancement of Mannitol Production by Fine-Tuned Expression of Mannitol-1-phosphate-5-dehydrogenase in *Synechocystis* sp. PCC6803. (Manuscript in preparation)