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

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General Discussion

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In this thesis, all studies are aimed at stabilizing and improving mannitol production in *Synechocystis* sp. PCC6803, but in each chapter a different approach was used. In **chapter II**, mannitol production was achieved in *Synechocystis* sp. PCC6803 by heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*; in short: 'mannitol cassette'). However, this production system turned out to be genetically unstable, because heterologous mannitol expression directly competed for metabolic intermediates with biomass formation, which imposed a huge burden for cell growth. To stabilize mannitol production in *Synechocystis* sp. PCC6803 in this chapter, we first validated that mannitol production can work as well as the native compatible solutes (e.g., sucrose and glucosyl-glycerol) for salt resistance. Under this background, the mannitol production system was proven to be stable under salt stress condition in the mutants which lacked sucrose and glucosyl-glycerol in their arsenal against salt stress. However, even with this stabilized mannitol production system, mannitol production in *Synechocystis* sp. PCC6803 is still very low, and therefore, it is unable to meet the requirements of industrial scale. This is partly explained in **chapter II**, where a mutated *mtlD* which misses a methionine at position 332 was accidently expressed under control of promoter Ptrc1 for mannitol synthesis in cells. This mutated *mtlD* definitely lowered the activity level of the 'mannitol cassette'. To optimize the expression level in **chapter III**, we chose different promoters such as Ptrc1, Psba2 and Pnrsb to control the expression of two versions of *mtlD*, respectively and eventually found that using the weakest promoter Pnrsb with 5 μM nickel as inducer to control the unmutated *mtlD* in combination with overexpression of *m1p* can achieve roughly 8 times higher mannitol production. Meanwhile, we discovered that other relatively strong promoters are unable to sustain unmutated *mtlD* expression. These results indicated that overexpression of *mtlD* using strong promoters causes over-accumulation of the intermediate product mannitol-1-phosphate, which imposes a big burden for cells. Consequently, it became clear that fine-tuned expression of *mtlD* is a bottleneck to achieve a high yield of mannitol in *Synechocystis* sp. PCC6803. In **chapter IV**, we used an adaptive evolution experiment to acclimate mannitol producers with higher mannitol productivity under increased salt pressure. This strategy helped mutant to increase mannitol production 24 times over 164 generations. Genome comparisons after sequencing of the evolved cells and their parental population, we found several mutations on

mainly *pnp* and these might be involved in the regulation of mannitol production in the producer cells. In the last experimental, **chapter V**, we used a fundamentally different way to synthesize mannitol from intracellular sucrose under salt shock conditions by heterologously expressing mannitol dehydrogenase (*mdh*) and sucrose synthase (*susA*). However, the limited pool of substrate results in a very low mannitol productivity in *Synechocystis* sp. PCC6803 using this strategy.

In this chapter, the main findings of this doctoral project will be summarized and discussed in context of each other and expanded on how to further increase mannitol production in the future (Fig.1).

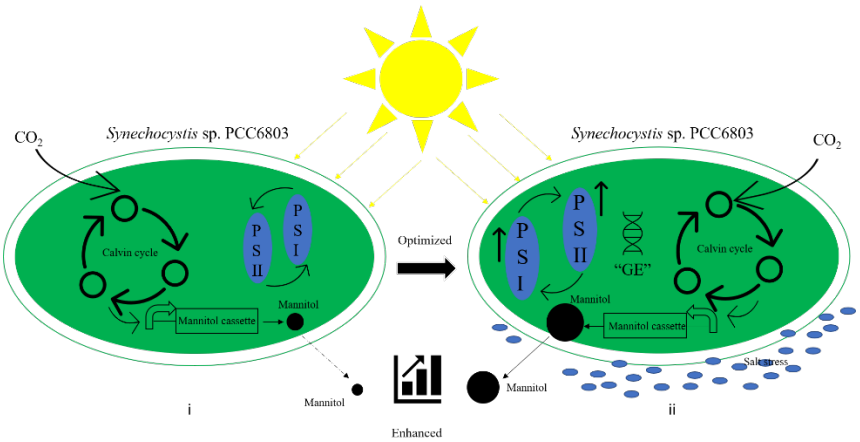

Fig.1 A photosynthetic factory for mannitol production in *Synechocystis* sp. PCC6803. (i) low mannitol production from naive cell factory. **(ii)** high mannitol production from highly efficient cell factory with optimized photosynthesis and mannitol synthetic pathway which are achieved by "GE" (gene editing) under salt stress.

Maximizing photosynthetic efficiency

To further increase mannitol production in *Synechocystis* sp. PCC6803, the highest possible photosynthetic efficiency is required by cells. In recent years, multiple approaches have already been explored to increase the efficiency of photosynthesis, which are (i) the expansion the photosynthetically active radiation spectrum, (ii) reducing antenna size, (iii) expression an improved

ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and/or (iv) modulation the ratio of formation of ATP and NADPH.

As the first step of photosynthesis, collection of the light energy from the sun by light-harvesting complexes directly determines how much sunlight can be harvested and transferred into the photosynthetic systems. In *Synechocystis* sp. PCC6803, cells have already evolved light-harvesting antennas, such as the phycobilisomes, to absorb in a spectral range within 700 nm where the radiant energy is available (1). It is well recognized that spectral expansion can capture more available light, and meanwhile, antenna size reduction can prevent losses due to the light saturation effect (2). Both are important considerations to judge the efficiency of light harvesting systems (2). Except for this energy conversion side to improve photosynthetic efficiency, increasing carbon fixation by overexpression of RuBisCO is also considered in previous sustainable applications, such as improving isobutyraldehyde production by this way (3).

Photosynthetic reactions are generally divided into light and dark reactions. In light reactions, light energy is converted into ATP and NADPH as chemical energy stored in cells (4). During dark conditions, this chemical energy is consumed for generating organic compounds from CO_2. Thus, the generation of ATP and NADPH from light and its consumption in dark is the key for coupling the light and dark reactions. Based on theoretical calculations, the ratio of generated ATP/NADPH is lower than its consumption (5). This indicates that ATP generation from light cannot meet the requirement for CO_2 fixation. So, modulation of the ratio of formation of ATP and NADPH should therefore solve this imbalanced energy supply/requirement and increase photosynthetic efficiency.

Optimization mannitol synthetic pathway

In this thesis, we have narrowed on two different biosynthetic mannitol production pathways in *Synechocystis* sp. PCC6803. In **chapters II, III and IV**, we achieved mannitol production in cells by heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*) to convert (part of) the endogenous metabolite fructose-6-phosphate through the accumulation of the intermediate mannitol-1-phosphate. In **chapter V**, we explored the possibility of another mannitol biosynthetic pathway from

sucrose via accumulation of the intracellular intermediate fructose by overexpression of mannitol dehydrogenase (*mdh*) and sucrose synthase (*susA*). Although, both pathways can realize mannitol production in *Synechocystis* sp. PCC6803, optimization for them is still needed by cells in term of improving and stabilizing mannitol production yields.

Mannitol production achieved by the expression genes *mtlD* and *m1p* shows genetic instability in both *Synechococcus* sp. PCC7002 and *Synechocystis* sp. PCC6803. This is because the overaccumulation of the intermediate mannitol-1-phosphate is harmful for cells (as the results of **chapter III** suggest). To decrease the concentration of mannitol-1-phosphate in cells, a fusion protein, generated from previously separate genes has been expressed in *Synechococcus* sp. PCC7002 (6). This artificial metabolic pathway not only reduced the number of genes required for mannitol synthesis, but also limited the accumulation level of toxic intermediate in cells. Under this strategy, genetic instability of mannitol production in *Synechococcus* sp. PCC7002 was solved although production levels remained modest (6). In microbial cell factory, this fusion construct strategy has been widely used for improving production. For example, fusion constructs enhance heterologous β-phellandrene production in *Synechocystis* sp. PCC 6803 (7) and a translational fusion protein joining 4-coumarate CoA-ligase and stilbene synthase help yeast produce 15-fold more resveratrol than the co-transformed cells (8). Therefore, we speculate that the fusion engineering approach can be used to further enhance mannitol production in *Synechocystis* sp. PCC6803.

The biosynthesis of glycogen in living organisms is one of the main strategies for the intracellular storage of carbon and energy (9). In cyanobacteria, glycogen can be only accumulated from carbon fixation under light conditions during photosynthesis and is then used to maintain the basic physiological activity in dark period (9). In *Synechococcus* sp. PCC7002 cells, genetic inactivation of glycogen biosynthesis by knocking out *glgA1*/*glgA2* redirects the metabolic flux to mannitol, which helps mannitol producing cells to synthesize higher yields of mannitol (10). This methods has also been used in *Synechocystis* sp. PCC6803 for increasing lactic acid production (11). Thus, we speculate that impairment of glycogen synthesis might also improve mannitol production in *Synechocystis* sp. PCC6803 in a short time but results in a genetic

instability phenomenon during long-term cultivation, since the function of glycogen appears essential to cells (9).

In **chapter V**, mannitol production was achieved from intracellular sucrose in *Synechocystis* sp. PCC6803 by expression of *mdh* and *susA*. However, the extremely low productivity, probably due to insufficient substrate pools, limits the application of this route. In order to increase the concentration of substrate, glucosyl-glycerol phosphate synthase (*ggpS*) was knocked out by the replacement with a chloramphenicol resistance cassette. However, although sucrose is increased by this method, mannitol production is then completely blocked. We speculated the reason is that knocking out *ggpS* results in ADP-glucose overaccumulation (12) and such high level of ADP-glucose interferes with *susA* activity, resulting in no intermediate fructose generation. In contrast to knocking out *ggpS*, overexpression of the sucrose phosphate synthase (*sps*) is also helpful for enhanced sucrose, but unable to result in ADP-glucose overaccumulation (12). This strategy will be used to improve mannitol production in our future studies. ADP-glucose, as the key metabolite in *Synechocystis* sp. PCC6803, is originally generated by expression of *glgc* and involved in the pathway towards intracellular glycogen (13). Here, ADP-glucose is also synthesized as a side product by heterologous expression *susA*. In order to achieve the growth-coupled production of mannitol in cells, we plan to delete the native gene *glgc* from cells and only rely on *susA* for the synthesis of ADP-glucose. However, it never worked in our strain to achieve a fully segregation of *glgc* knockout mutants after many attempts, so we speculate that *glgc* is essential to cells under the conditions we applied.

Application of promising salt inducible promoters for mannitol expression in *Synechocystis* sp. PCC6803

Inducible systems with low leakiness and high expression levels are needed for genetic engineering in all microbial cell factories, especially when the recombinant proteins or metabolic pathways of interest are toxic to the host cells, such as the ‘mannitol cassette’ appears to be in *Synechocystis* sp. PCC6803.

In **chapter II** of this thesis, a stable mannitol production system was established when mannitol producers were cultivated under specific salt stress at lab-scale.

However, in the industrial scale, a suitable salt concentration is hard to be selected to stabilize mannitol production, since this salt concentration should inhibit cells growth, only allowing cells to replicate at a reduced rate. As cultivation time goes by, cells will increasingly adapt to constant salt pressures, and mannitol production becomes ultimately useless for them in term of salt resistance. Consequently, mannitol production cannot be considered stable at that time. To tackle this possible problem, the salt inducible promoter is considered as a useful strategy to precisely control the level of mannitol production based on salt stress in the environment. In a previous study, genes whose salt-induced expression is enhanced by salt treatment with 684 mM NaCl were identified by DNA-microarray technique (14). Excluding genes with unknow function, *ggpS* shows the highest induction level there, so we chose the native promoter of *ggpS* to control the expression of the mannitol cassette and acclimated this mutant by adaptive laboratory experiment under increased salt stress in **chapter IV** (Table 1). However, compared with mannitol production from the 'mannitol cassette' under control of the strong promoter Ptrc1, the native promoter of *ggpS* had a very low expression level, which only synthesized roughly 3.75-fold less mannitol under the same cultivation conditions.

Table 1. Top 10 genes that show the highest induction factors in *Synechocystis* sp. PCC6803 after salt treatment with 684 mM NaCl (14)**.**

ORF[a]	Gene	Function	Ind-Factor[b]
sll1862		Unknown	265.2
sll1863		Unknown	231.5
sll0939		Unknown	86.1
sll1566	*ggpS*	Glucosyl-glycerol phosphate synthase	84.5
sll0938		Asp transaminase	71.7
ssr2595	*hliB*	High light-inducible polypeptide	50.5
sll0528		Probable metallo-protease	50.3
slr0895		Transcriptional regulator	44.8
sll1653	*menG*	2-Phytyl-1,4-benzoquinone methyltransferase	40.0
slr0959		Probable metal dependent protease	39.8

[a]Open reading frame (ORF). [b]Induction factor for each gene in a typical experiment.

To achieve mannitol production by salt inducible promoters in industrial scale, non-native promoters with strong expression level originated from other microorganisms, especially from extremely halophilic species should be considered to apply in *Synechocystis* sp. PCC6803. Examples are for instance

the fragment P23423 isolated from *Bacillus subtilis* (15), *promA* from *Halomonas elongata* DSM 2581T (16), *katB* promoter from *Anabaena* sp. PCC7120 (17) and CrGPDH3 from *Chlamydomonas reinhardtii* (18), to list but a few.

Outlook

One of the keys for further development and optimization of 'direct conversion' from CO_2 into valuable compounds via photosynthesis in *Synechocystis* sp. PCC6803 is to overcome unstable yield of target compounds. To solve this, we proposed the idea of associating resistance to a stress to production of compounds. Mannitol production is here shown as precisely a successful example of this strategy by stabilizing by salt stress in purposely engineered cells. As mentioned in the '**general introduction**', except for mannitol, many compatible solutes have also been proved to help host cells to survive osmotic stress. So, we propose that achieving the production of these compounds in *Synechocystis* sp. PCC6803 under specific osmotic stress might also prevent potential instability problems. Here, we summarize the information of some compatible solutes including their biosynthetic pathway and the type of potentially useful osmotic pressures in Table 2.

Table 2. Non-native compatible solutes that might be stabilized by specific osmotic pressures in *Synechocystis* sp. PCC6803

Name of compounds	Biosynthetic pathway	Precursor	Type of osmotic pressures	Ref
Glycine betaine	*Glycine betaine aldehyde dehydrogenase (GsbA) and type-III alcohol dehydrogenase (GbsB)*	Choline	Salt/temperature	(19)
Trehalose	Trehalose-6-phosphate phosphatase (*TPP*) and trehalose 6-phosphate synthase (*TPS*)	Glucose-6-phosthate	Salt	(20)
Ectoine	L-2,4-diaminobutyric acid Nγ-acetyltransferase (*ectA*), L-2,4-diaminobutyric acid transaminase (*ectB*) and L-ectoine synthase (*ectC*)	L-aspartyl phosphate	Salt	(21)
Dimethylsulfoniopropionate	Methyltransferase (*dsyB*)[a]	Methionine	Temperature	(22)
Proline	pyrroline-5-carboxylate reductase (*proH*), glutamate-5-kinase (*proJ*) and glutamate-5-semialdehyde dehydrogenase (*proA*)	Glutamate	Salt	(23)

[a]*dsyB* is the only discovered gene involved in dimethylsulfoniopropionate synthesis

Concluding remarks

In the work presented in this thesis, many strategies have been made to stabilize and improve mannitol production in *Synechocystis* sp. PCC6803. After 4 years of hard work, although a stable mannitol production system has been established at a laboratory scale and a super mannitol producer has been selected from adaptive evolutionary experiments, there is still a big gap to reach the actual large-scale application. I hope this thesis can contribute a solid theoretical basis for achieving mannitol production in *Synechocystis* sp. PCC6803 at a commercial scale, and possibly even inspire researchers to develop highly efficient photosynthetic cell factories for other valuable compounds with similar properties.

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