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General Introduction

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Introduction: a photosynthetic cell factory in cyanobacteria

The development of science and technology initially aiming to make life more convenient has already caused high emissions of carbon dioxide (CO_2), which is a new threaten for the living environment of human beings (1,2). The increasing global temperature on our planet resulted from rising CO_2 is leading to extreme weather and related catastrophes (3). It is generally considered that reducing the atmospheric CO_2 is necessary to alleviate global warming effects (4). Therefore, the recycling of CO_2 into useful fuel and chemicals by photosynthetic organisms has received high interests in recent years (5,6). The first-generation biofuels including bioethanol, biodiesel and biogas are achieved under this background from crops (7), however, this approach presents several drawbacks. 1) it competes with edible crops over land use. 2) it leads to a huge amount of biowaste, which exerts pressures on environment (8). In an attempt to solve these drawbacks, a new approach has been proposed. This approach uses photosynthetic organism such as cyanobacteria which has been engineered to convert CO_2 directly into high value compounds or biofuels, without need for arable land and high amounts of biomass (9–12).

Cyanobacteria are historically called as “blue-green algae”, but they are now classified in a independent phylum within bacterial clades (13–15). As organisms that emerged a long time ago, cyanobacteria are photosynthetic prokaryotes which exist almost everywhere on the planet including some extreme environments such as rocks, desert sand, hot springs and salt marshes. These hosts rely on photosynthesis and Calvin-Benson Cycle for biomass production using sunlight and CO_2 as energy and carbon source, respectively (13). Some subspecies of cyanobacteria have also been discovered to fix nitrogen directly into ammonium for growth (16). This means that cyanobacteria are important in controlling biological productivity and carbon flux in many habitats (17). In the last two decades, direct conversion of CO_2 into valuable compounds by cyanobacteria as cell factory has already attracted great interest, since they appear superior over other photosynthetic organisms such as plants and algae, which are shown below: 1) around 55% of anthropogenic carbon emissions is fixed and reintroduced in the global carbon cycles and cyanobacteria, themselves constitute 25% of the global carbon fixation (11,18). Such high levels of global carbon fixation in cyanobacteria are 10 times higher than plants and twice the conversion

from algae (19); 2) for an organism to be used as chassis for metabolic engineering, it must be amenable to genetic modification to allow efficient gene editing. Compared with plants and algae, modifying the genome of cyanobacteria is simple (20). The DNA fragments are much easier to introduce in target organisms by natural transformation, conjugation or electroporation (20). Many cyanobacteria meet these criteria such as *Synechocystis* sp. PCC6803, *Synechococcus* sp. PCC7002, *Synechococcus* elongatus PCC7942, *Anabaena* sp. PCC7120 and *Synechococcus elongatus* UTEX2973 (21,22).

Genetic modification

Cyanobacterial transformation

The ability of transformation in bacteria is regarded as the basis of biotechnology applications (23). In recent years, transformation in cyanobacteria has been studied and extensively reviewed. Several cyanobacterial strains can be transformed with linear DNA fragments or constructed plasmids, which are first edited in *E. coli*. In the 1970s, natural transformation was first described in *Synechococcus* sp. PCC7942 and subsequently, a few species of *Synechocystis* and *Synechococcus* were found to be naturally transformable with exogenous DNA (21,24). The efficiency of natural transformation in cyanobacteria can be influenced by many factors including the length and concentration of the DNA fragments, transformation methods and the (state of the) host species (25). The optimum conditions for natural transformation in *Synechocystis* sp. PCC6803 have been well studied and characterized (25). Although, natural transformation is regarded as the most efficiency way to accept DNA for genetic modification in cyanobacteria, little is known about why natural transformation only takes place in certain unicellular cyanobacteria. This may be due to the activity of some nuclease in cells unable to carry out natural transformation, which degrades exogenous DNA and results in the inability of the host to incorporate it via natural transformation (26).

Conjugation is also one of the most common methods used for incorporation of DNA in the host, which is successfully used in many species of cyanobacteria (27,28). However, compared with natural transformation methods, conjugation

requires more steps for molecular construction. To implement conjugation process, we have to firstly construct replicative plasmids in donor strains and then transform the plasmids into the host cells (29). This process of plasmid construction may result in genetic toxicity in donor strains due to the overexpression of exogenous DNA. Another drawback of conjugal transformation in cyanobacteria is that constructed plasmids should be replicative in the donor strain (29). In *Synechocystis* sp. PCC6803, a triparental mating method is used for conjugation which requires a helper strain to assist the transfer of the target plasmids from donor strains into *Synechocystis* sp. PCC6803 (30). The conjugation efficiency is therefore highly improved. However, unfortunately, this method is only useful in specific cyanobacteria (31,32).

Although, electroporation has been proven with extremely high efficiency as a suitable method of transformation in *E. coli* (33), past studies suggest that transfection of cyanobacteria by electroporation is inefficient and requires a large amount of donor DNA (34). Furthermore, the extracellular layers of some cyanobacteria set up a hard barrier that stop DNA from entering into the cells (34). However, even with a lot of drawbacks, a small number of cyanobacteria have been proven to be capable of withstanding transformation by electroporation (35).

Selectable Markers

It is essential to be able to select for a rare transformant from a large population of treated cyanobacterial cells during the process of genetic engineering. Usually, host cells that contain the exogenous DNA are identified by transferring genes with specific genetic information. When the host cells are cultured in the presence of markers, such as antibiotics, only those rare transformants which have incorporated for example an antibiotic resistance cassette, will have the ability to grow. In *Synechocystis* sp. PCC6803 cells, kanamycin, spectinomycin, nourseothricin and chloramphenicol have proven efficiency (36–39). This somewhat limited number of available antibiotics suitable to be used restricts genetic modification in *Synechocystis* sp. PCC6803. Under this background, different markerless strategies for knocking in/out genes have emerged for these

cells. *SacB-aphX* method is commonly used in glucose tolerant *Synechocystis* sp. PCC6803 (40). It first introduces both the genes with an antibiotic marker (kanamycin resistance, *aphX*) and a negative marker (sucrose sensitivity, *sacB*) into the chromosome. Then a second-round of homologous recombination replaces the first-round insertion with a sequence of interest, which is selected by sucrose. These two rounds of genetic modification, leaves the mutants free of antibiotic selection markers. However, this method is not useful in glucose sensitive *Synechocystis* sp. PCC6803. In order to get over this usage limitation, a similar method has been established in wild type *Synechocystis* sp. PCC6803, which uses *mazF* to replace *sacB*, selected by nickel in the last round of transformation (41). This *mazF-aphX* method might be also useful in *Synechococcus*. However, these traditional recombination methods for construction of markerless mutants are not suitable for multiple site-directed mutations. In recent years, CRISPR/*cpf1* genome editing technology is rapidly developing for markerless knock-ins, knock-outs or multiple specific point mutations in *Synechococcus, Synechocystis* and *Anabaena* (42). But, the latter method is not widely used yet.

Regulation of exogenous gene expression

To achieve photosynthetic factories from CO_2 and sunlight in cyanobacteria, fine-tuning of exogenous gene expression under the control of a stable and robust synthetic biology tool is very important (43). Although compared with the most studied model microorganism, such as *E. coli* and yeast, the development of gene expression in cyanobacteria still lagging behind, various promoters, ribosome binding sites and neutral sites used for engineering cyanobacteria have already been characterized in recent years (44).

In the model engineered strain *Synechocystis* sp. PCC6803, many native promoters have already been characterized, including strong promoters such as Psba2, PcpcB, PrbcL and their derivatives, as well as inducible promoters such as Pnrsb, PcoaT, PpetE, PziaA and PcpcG2. Except for these native promoters, the function of many promoters originated from *E. coli* such as PterR, PlacO, Ptrc and their derivatives have also been tested in *Synechocystis* sp. PCC6803 (45).

The strength of constitutive promoters is compared by measuring fluorescence of a report protein in a standardized setting. The promoter Ptrc is regarded as the strongest one in *Synechocystis* sp. PCC6803 and often used for heterologous gene expression (46). However, engineering of cyanobacteria for specific compounds often requires the introduction of multiple genes in a well-controlled manner. This indicates that the strongest promoter might be not suitable for expression of all the exogenous genes, especially for some toxic genes. So well controlled expression is also very important in the case where engineered pathways are producing toxic intermediate products. In a previous study in which most inducible promoters were compared in terms of strength and leakiness, the promoter Pnrsb stood out in the comparison since it showed relatively high expression level with inducer usage and has a very low leakiness without induction (47). Under this background, this inducible promoter is often used for expression of toxic genes in *Synechocystis* sp. PCC6803, such as the previously mentioned *mazF* (41). In addition to inducible promoters, riboswitches are also used to regulate gene expression in cyanobacteria. This strategy may include two parts: a repressed mRNA and a transactivating RNA. Both working together can achieve roughly 13-fold differences in expression levels (48).

Ribosome binding sites (rbs) are another effective control element for synthetic biology applications, which initiate protein expression in cyanobacteria. Unlike promoters, only a few rbs regions are identified in cyanobacteria, such as RBSv4 and its derivatives (43). Many rbs regions discovered in *E. coli* shows no function in cyanobacteria. This might be because cyanobacteria have relatively complicated post-transcriptional gene regulation. In addition, the traits of cyanobacteria such as polyploidy, circadian rhythms and other features also result in rbs differences between cyanobacteria and *E. coli*.

Neutral sites are genome locations where genetic modification causes no noticeable phenotype changes under standard cultivation conditions. Incorporation of target genes in neutral sites of the chromosome of cyanobacteria has been widely used to achieve stable production in recent years (49). Many studies predict possible neutral sites based on model and randomly mutations on these regions to check their availability by observation of the phenotypic changes.

Up to now, several neutral sites have been identified for gene expression in *Synechocystis* sp. PCC6803 such as *slr0168*, *slr1495-sll1397* and *slr1362-sll1274* (43,50,51).

Benefiting from the fast development of synthetic biology tools in cyanobacteria, a great variety of industrially useful chemicals have already been achieved in this photosynthetic platform (Fig.1). Together, they are considered as the most sustainable chemical production known from CO_2. However, there are still some limitations associated with the use of cyanobacteria in the production of chemicals at a commercial level. Here, we highlight some key limitations that must be overcome to realize the biotechnological potential of cyanobacteria.

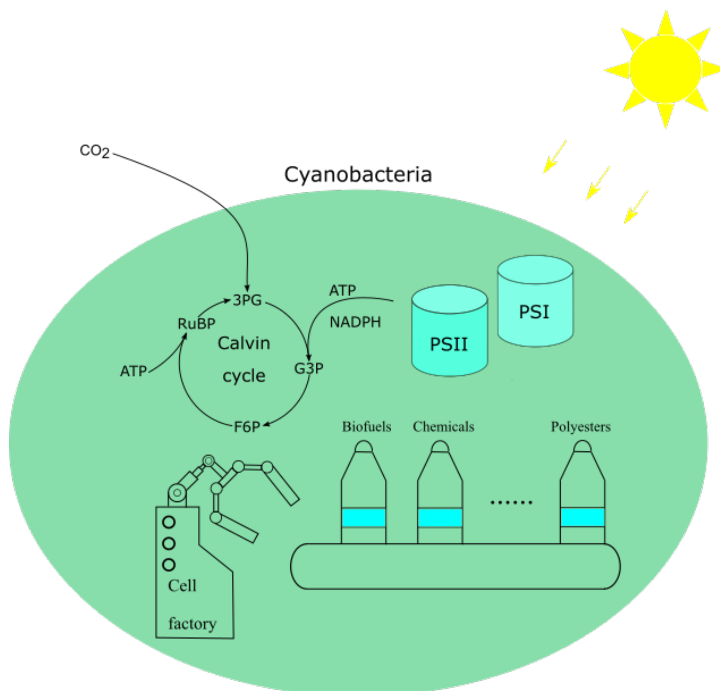

Fig.1 Overview of cyanobacterial cell factories for producing biofuels, chemicals, polyesters, etc. Abbreviation: 3PG, 3-phosphoglycerate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; RuBP, ribulose-1,5-bisphosphate.

Limitations to achieve a stable photosynthetic factory in cyanobacteria

The robustness of a stable photosynthetic factory in cyanobacteria refers to the ability of maintaining a given phenotype and keeping relatively high efficiency of production during the cultivation process (52). However, this is often not the case and a production trait can be negatively affected by genetic instability, leading to decreased productivity (53). In recent years, genetic instability of production strains has been reported in *Synechocystis* sp. PCC6803, *Synechococcus elongatus* PCC7942 and *Synechococcus* sp. PCC7002, which are mainly caused by the redirection of too much carbon flux from necessary biomass into production synthesis and/or toxicity of (intermediate) product. This genetic instability has been observed in the production of many different compounds (54,55).

In engineered strains of *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7002, ethanol production was achieved by overexpression of pyruvate decarboxylase (*pdc*) from *Zymomonas mobilis* and alcohol dehydrogenase (*adh*) from *Synechocystis* sp. PCC6803 (54). However, due to the fixed carbon being directed mainly into ethanol, the amount of phycocyanin in the ethanol producers is severely decreased. The latter causes lethal effects on the cells, and as a consequence, the stability of ethanol production is compromised. Sequencing data revealed that lots of mutations including point mutations, insertions and deletions on the ethanol cassette result in the inactivation of ethanol synthesis in revertants (54).

In 2012, a higher lactic acid production was obtained by co-expression L-lactate dehydrogenase (*ldh*) and transhydrogenase (*sth*) into the chromosome of *Synechocystis* sp. PCC6803. However, genetic instability with a duplication of ~ 160 bp in the *sth* gene generates premature stop codons and a lactic acid producer with such a gene is quickly outcompeted by emerging revertant mutants within the population (55).

Similar genetic instability was also found in mannitol production from *Synechococcus* sp. PCC7002. There, the single base deletion in *mtlD* of the mannitol cassette caused a frame-shift, resulting in truncated protein and impairs production synthesis (56). A subsequent study proved that the intermediate product of mannitol is toxic for cell, and used a fusion protein strategy, bypassing the accumulation of intermediate product that can increase the stability of production in *Synechococcus* sp. PCC7002 (57).

The ethylene production on both engineered strains of *Synechocystis* sp. PCC6803 and *Synechococcus* elongatus PCC7942 is also unstable due to genetic instability (58,59). In essence, it has been found that the acquisition of mutations on the pathways leading to products (not needed by the cell) can help the host to decrease the burden of product formation, and consequently to regain the fitness. This causes a big challenge for industrial application (44).

The solutions to solve phenotypic instability in cyanobacteria

The coupling of bacterial growth and target compound production is an effective solution to solve phenotypic instability in cyanobacteria (44). Bacterial growth can be defined as a proliferation of a bacterium into daughter cells, which is the result of complicated metabolic interactions and reflects a high degree of (bio)chemical and physicochemical synchronization in bacteria. On a first approximation, metabolism in a living cell is divided into two opposing streams of interconversions: (i) catabolism, the degradation of energy-rich nutrients and macromolecules into smaller units, leading to the formation of molecules such as ATP, NADH and NADPH, which serve as energy donors for other cellular process; and (ii) anabolism, the production of new macromolecules through process, that require the energy donors from catabolism (60). Product synthesis coupled with either catabolism or anabolism maintains a closed redox/energy balance, which improves the efficiency of production in cyanobacteria (60). In the actual applications of this principle, this redox/energy coupled strategy tends to rely on interventions designed by computational models. Erdrich *et al* (2014) blocked *in silico* cyclic and other alternative electron flows to increase the ATP/NADPH ratio and eventually achieved an improved synthesis of ethanol in

Synechocystis sp. PCC6803 (61). In addition, computational model-derived knockout strategies to couple ATP/NADPH and biofuels have also been shown in *Synechocystis* sp. PCC6803, where lowering ATP/NADPH ratio by the knockouts of specific genes is used for coupling alcohols, alkanes and limonene, and therefore, improve productivity (62). Such successful examples show that redox/energy coupled production strategies, mainly based on computational model prediction, are a promising method to solve instability in cyanobacterial cell factories.

Du et al in a subsequent study, reported a novel strategy called 'FRUITS' to tackle the instability issue in *Synechocystis* sp. PCC6803. There, the production of native metabolites is aligned to the formation of biomass rather than using energy or redox regeneration (63). It directly couples the production of target compounds to unique pathways required for biomass synthesis, by deleting the native alternative metabolic routes. Under this strategy, the side products of anabolic pathways become stoichiometrically linked to bacterial growth ensuring growth coupled production. The method has been demonstrated in practice for acetate and fumarate as two successful examples in which this strategy can be used for stabilizing production in *Synechocystis* sp. PCC6803 (63,64).

Despite the great progress achieved in cyanobacteria to solve the genetic instability problem, engineering industrial microbial factories still remains a challenge, since the number of compounds using redox/energy coupled or FRUITS strategy is limited. For instance, in some specific target compounds, their own toxicity requires the host to be extremely robust for keeping stable production. Therefore, it is urgently necessary to explore alternative methods to solve instability issues in these cases. In this chapter, we come up with a "fitness coupled" strategy by using heterologous compounds to mitigate the stress from specific cultivation conditions for the hosts. Under this background, these heterologous compounds are needed by cells to fight against the adverse environment and their productions are therefore stabilized (Fig.2).

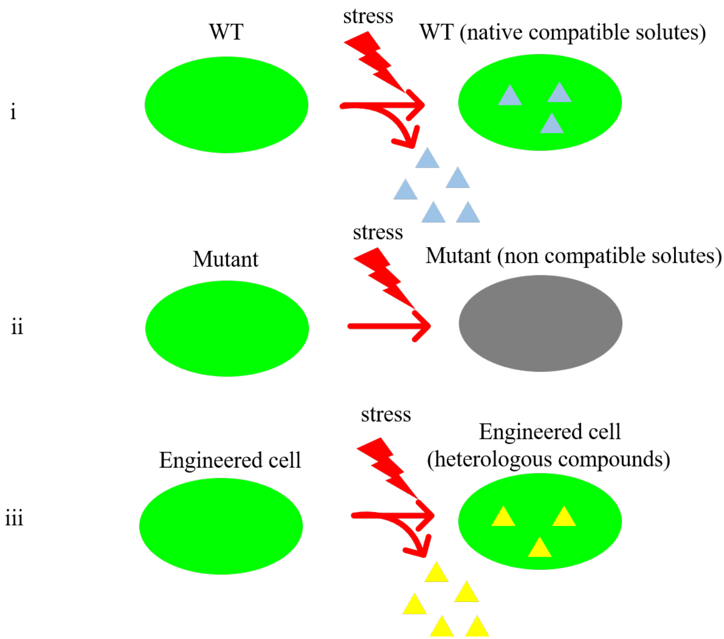

Fig.2 Schematic representation of fitness coupled strategy in cyanobacteria for producing heterologous compounds (non-native compatible solutes) under osmotic stress. (i) mechanism of wild type (WT) in response to osmotic stress. (ii) mutant with inactivated production of native compatible solutes is not viable under osmotic stress. (iii) heterologous compounds (non-native compatible solutes) from engineered cell alleviate the osmotic stress for the host. Green ellipse represented living cyanobacterial cell; grey ellipse represented dead cyanobacterial cell; blue triangles represented native compatible solutes; yellow triangles represented heterologous compounds (non-native compatible solutes).

Compatible solutes biosynthesis in response to salt stress in cyanobacteria

Many cyanobacteria strains are found in water with different salinities. They have evolved clear mechanisms to adapt to different concentrations of salt pressure by natural selection. In general, high salt pressure generates two main problems for organisms. First, salinity results in a loss of water and turgor pressure. Second, the higher concentration of inorganic ions from living environment results in a continuous influx of inorganic ions (65). Unlike in halophilic bacteria, cyanobacteria do not have the capacity to contain high internal concentration of

ions. Therefore, limiting cytoplasmic ionic concentration at low level is the main strategy for salt resistance in cyanobacteria (66). Under this background, activation of inorganic efflux channels during salt pressure is found in cyanobacteria to maintain a favorable ion concentration. In addition, these cyanobacteria also accumulate compatible solutes, which allow water uptake and help to keep balance of cellular osmotic pressure (66).

The concept of compatible solutes is associated with small molecules that act as osmolytes and help organisms to survive under osmotic stress (67). In cyanobacteria, a close connection between the minimum lethal concentration of salt and the primary native compatible solutes have been discovered (66). Sucrose, trehalose, glycosyl-glycerol and glycine betaine are four native compatible solutes found in cyanobacteria. The accumulation of sucrose as the main compatible solute in association with low salt tolerance was first discovered in *Nostoc muscorum*, *Synechococcus* sp. PCC6301 and *Anabaena variabilis* (68–70). In addition to sucrose, trehalose is also an osmolyte found in freshwater cyanobacteria *Nostoc* sp. PCC7120 (71). Glycosyl-glycerol is regarded as a compatible solute in strains of moderate salt tolerance, which help cyanobacteria survive in higher salt pressure and is needed in marine habitats. However, not all cyanobacteria that can synthesize glycosyl-glycerol are originally from marine environments, such as *Synechocystis* sp. PCC6803 (72). In addition to *Synechocystis* sp. PCC6803, glycosyl-glycerol has been found in roughly 200 additional species, including a marine *Synechococcus* strain (66). Glycine betaine is used as a major compatible solute in strains of highest salt tolerance such as *Synechococcus* sp. PCC7418, *Synechococcus* strains WH7803 and *Prochlorococcus* MIT9313 (73,74). With the help of these different native compatible solutes, cyanobacteria can resist different levels of salt pressure. Clarification of the pathways producing compatible solutes and their regulation, gives us a better understanding of the mechanism triggered in response to salt stress in cyanobacteria.

Synechocystis sp. PCC6803 is the cyanobacterium in which the molecular mechanism of salt stress has been studied in more detail. The genes involved in the production of its native compatible solutes (i.e., sucrose and glycosyl-glycerol)

under salt stress have been clarified. Knocking out sucrose phosphate synthase (*sps*) and glycosyl-glycerol phosphate synthase (*ggpS*) results in impaired biosynthesis of sucrose and glycosyl-glycerol, and therefore, make this compatible solutes free cell more sensitive to salt pressure (75).

In this thesis, we synthesize non-native compatible solutes in compatible solutes free cyanobacterial cell under salt pressure and hope to solve the genetic instability problem under this production system. Beyond the native compatible solutes (i.e., sucrose, trehalose, glycosyl-glycerol and glycine betaine) in cyanobacteria, other compatible solutes such as D-pinitol, inositol, D-ononitol and mannitol, have proved their functionality on the regulation of osmosis pressure in many species of bacteria (76). Here, we chose to only focus on mannitol production in *Synechocystis* sp. PCC6803 for a few reasons. First, mannitol production has been achieved in *Synechococcus* sp. PCC7002 but proved genetic instability (56), so it appears as scientifically challenging. Second, the synthesis pathway of the native compatible solutes in *Synechocystis* sp. PCC6803 has been clearly identified so that achieving a compatible solutes free mutant in *Synechocystis* sp. PCC6803 is achievable. Furthermore, *Synechocystis* sp. PCC6803 is originally from fresh water, which strongly suggests it to be of higher sensitivity to salt stress than for instance a marine *Synechococcus* sp. PCC7002. During high salt acclimation, we therefore would expect that more mannitol has to be produced in *Synechocystis* sp. PCC6803 than in *Synechococcus* sp. PCC7002 in order to achieve the same respective biomass production rates. Given these reasons, we chose *Synechocystis* sp. PCC6803 as a host to achieve a stable high production of mannitol.

Mannitol characteristics

Mannitol is a six-carbon sugar alcohol with a pleasant taste and stability, which is widely used in the chemical and pharmaceutical fields as a potent osmotic diuretic and a well-known antioxidant. Its addition to food is also presumed to be health promoting for human beings (77).

The production of mannitol is mainly based on the hydrogenation of glucose and fructose at a ratio of 1:1 from inverted sugar or starch (78). Recently, bacteria have gained more attention to be developed as fermentation factories to biosynthesize mannitol. There are two distinct pathways of mannitol biosynthesis identified in heterofermentative and homofermentative lactic acid bacteria (77). In heterofermentative bacteria, mannitol can be obtained from fructose by expression of mannitol dehydrogenase (*mdh*) (77). This biocatalysis process requires fructose as substrate in the medium, which cannot be used in cyanobacteria, since fructose uptake from the medium is toxic for most of cyanobacteria. The pathway identified in homofermentative lactic acid bacteria which transforms fructose-6-phosphate via accumulation of the intermediate mannitol-1-phosphate into mannitol appears more promising. This process is achieved by expression of both phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*) (79). Compared with fructose, fructose-6-phosphate is more abundant in cyanobacteria, since it is one of the main metabolites in the Pentose Phosphate Pathway (80). In 2014, the first engineered mannitol producer cyanobacterial strain was achieved by this strategy in *Synechococcus* sp. PCC7002, however, it proved unstable (56). Subsequently, an optimized strategy aiming to solve this problem was applied, which used the fusion the genes previously used to simplify the biological engineering process in *Synechococcus* sp. PCC7002, but lowered mannitol productivity (57).

In **chapter II**, we first achieved mannitol production in *Synechocystis* sp. PCC6803 by heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*). Then, we proved mannitol production can confer cells with higher salt tolerance. Then, using this production cassette, we were able to further stabilize mannitol production under salt pressure in *Synechocystis* sp. PCC6803 mutant that have the main native compatible solutes deleted (ΔCS_M).

In **chapter III**, we tried to increase production by modulating the strength of expression of the mannitol cassette. Our data showed that the intermediate product mannitol-1-phosphate is toxic for *Synechocystis* sp. PCC6803. It is a big challenge to achieve a balance point between growth and mannitol production,

since high expression of *mtlD* is lethal to the cells, but deficient expression leads to low productivity. Under this background, we achieved roughly 8 times higher mannitol production under control of Pnrsb with the presence of 5 μM nickel in the medium as inducer, which reached 93 mg/l after 7 days of induction.

In **chapter IV**, we used an adaptive laboratory evolution strategy with increased salt pressure to improve mannitol production in ΔCS_M. Mannitol production was therefore improved 24 times in *Synechocystis* sp. PCC6803 under this strategy compared with the used parent ΔCS_M from chapter II. In addition, candidate genes that might be involved in the regulation of mannitol production in the cells were selected based on comparative genome resequencing of evolved and original mannitol producers. Among these genes, *pnp* encoding polyribonucleotide nucleotidyltransferase was found to negatively affect mannitol production in cells via reverse engineering methods.

In **chapter V**, we tried a structurally different way (from intracellular fructose) to synthesize mannitol in the cell such that the production of toxic intermediates could be avoided. In order to finalize this idea, codon optimized sucrose synthase (*susA*) and mannitol dehydrogenase (*mdh*) were expressed in *Synechocystis* sp. PCC6803. As a result, an extracellular mannitol yield to 0.058 mg/l/OD730 in 3 days under 300 mM salt shock condition was obtained.

Concluding remarks

In this study, the first engineered mannitol producer was achieved in *Synechocystis* sp. PCC6803 by overexpression *mtlD* and *m1p*. The obtained mannitol production can confer cell with higher salt resistance. Subsequently, salt pressure was successfully used to stabilize and increase mannitol production in the producing cells. This new fitness coupled strategy based on associating salt resistance to the production of mannitol was identified and might be easily extended to any other model microbial production system in the future.

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