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Chapter V

Exploring a new pathway of mannitol production by expressing mannitol dehydrogenase and sucrose synthase in *Synechocystis* sp. PCC6803

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Abstract

Mannitol production in cyanobacteria has been achieved via heterologous expression of mannitol-1-phosphate-5-dehydrogenase and mannitol-1-phosphatase, to convert part of the endogenous metabolite fructose-6-phosphate into mannitol. However, the intermediate product of this pathway, mannitol-1-phosphate is toxic for cell, which inhibits mannitol production in cyanobacteria. Here, two codon-optimized genes, mannitol dehydrogenase (*mdh*) from *Lactobacillus reuteri* and sucrose synthase (*susA*) from *Anabaena* sp. PCC 7119, together comprising a biosynthetic pathway from sucrose into mannitol, were expressed in *Synechocystis* sp. PCC6803 resulting in accumulation of mannitol in cultures under salt condition. As a result, an extracellular mannitol yield to 0.058 mg/l/OD_{730} in 3 days under 300 mM salt was obtained. In addition, glucosyl-glycerol phosphate synthase was knocked out from this mannitol producer cell to increase sucrose concentration up to 1.51 mg/l/OD_{730} in 3 days under 900 mM salt. Contrary to expectation, the high accumulation of sucrose by this way, instead of boosting mannitol production by increasing the supply of substrate for its synthesis, it blocked the synthesis of the intermediate product fructose, and therefore, impaired mannitol production. In this study, we explored the possibility of using a new pathway for biosynthesis of mannitol from CO_2 in *Synechocystis* sp. PCC6803.

Introduction

Mannitol is a 6 carbon sugar alcohol, which has been widely used in the food, medical, pharmaceutical and chemical fields (1–3). The great potential value of mannitol motivates us to conceive a strategy for its sustainable production. In previous studies, mannitol production in cyanobacteria has been achieved via heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*), to convert part of the endogenous metabolite fructose-6-phosphate into mannitol (4–6). This two-step pathway of mannitol synthesis has been successfully applied in both *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*) and *Synechococcus* sp. PCC7002 (hereafter *Synechococcus*) (4–6). However, toxicity of the intermediate product, mannitol-1-phosphate, has been found to incur this pathway with genetic instability (4). In addition to this two-step strategy, direct conversion from fructose into mannitol via mannitol dehydrogenase (*mdh*) has also been well studied in lactic acid bacteria, such as *Leuconostoc* (7), but has not been attempted in cyanobacteria yet. In this study, we explored the possibility of using this direct conversion to synthesize mannitol from CO_2 in cyanobacteria (Fig.1).

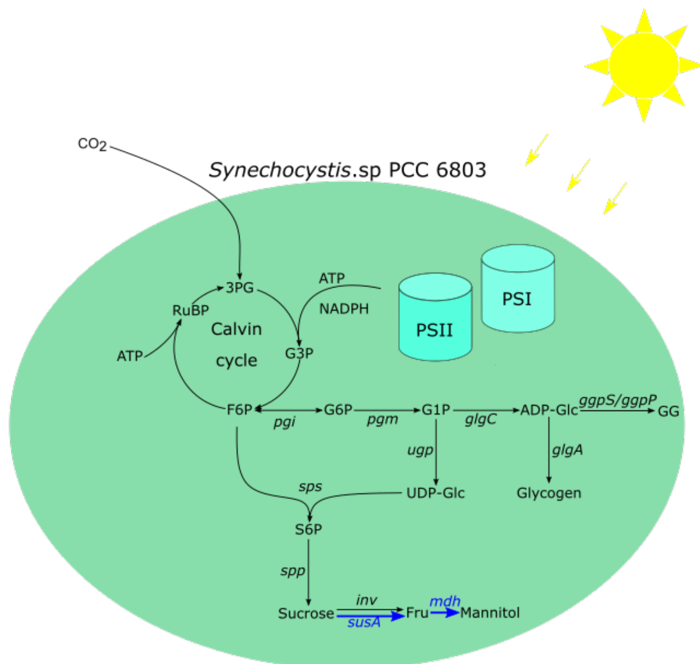
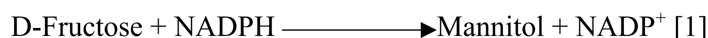


Fig.1: Overview of the engineered biosynthetic pathway to mannitol in the cyanobacterium *Synechocystis* sp. PCC6803. Black solid arrows represent native steps in cell; Blue solid arrows represent introduced pathway of mannitol synthesis; engineered genes are shown in blue. 3PG, 3-phosphoglycerate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; G1P, glucose-1-phosphate; ADP-Glc, ADP-glucose; UDP-Glc, UDP-glucose; S6P, sucrose-6-phosphate; Fru, Fructose; *pgi*, glucose-6-phosphate isomerase; *pgm*, phosphoglucomutase; *glgC*, ADP-glucose pyrophosphorylase; *ugp*, UDP-glucose pyrophosphorylase; *glgA*, glycogen synthase; *sps*, sucrose phosphate synthase; *spp*, sucrose phosphate phosphatase; *inv*, invertase; *susA*, sucrose synthase; *mdh*, mannitol dehydrogenase; RuBP, ribulose-1,5-bisphosphate; *ggpS*, glucosyl-glycerol phosphate synthase; *ggpP*, glucosyl-glycerol phosphate synthase; GG, glucosyl-glycerol.

Synechocystis is a model freshwater cyanobacteria. Its genome has been sequenced and many molecular biology tools are available to study this cyanobacterium. Recently, *Synechocystis* has gained much attention to convert CO_2 directly into many valuable compounds, such as ethanol, sorbitol and fumarate (8–10). Here, a one-step mannitol biosynthetic pathway was introduced into *Synechocystis* by heterologous expression of codon optimized *mdh* from *Lactobacillus reuteri* encoding mannitol dehydrogenase (see reaction [1]) (11).



D-Fructose + NADPH $\longrightarrow$ Mannitol + $NADP^+$ [1]

This pathway converts fructose into mannitol. However, intracellular fructose is not an abundant metabolite in *Synechocystis*, as it is only produced during sucrose breakdown via invertase (*sll0626*) (12). In order to cover the shortage of intracellular fructose, external fructose supply as a supplement to BG-11 medium has been used for producing compounds such as H_2 and poly-β-hydroxybutyrate in *Synechocystis* (13,14). This addition of fructose not only impairs the growth of *Synechocystis*, but also increases the cost of cultivation (15). Therefore, in this study, we plan to increase the native production of intracellular fructose directly from CO_2, aiming to achieve increased mannitol production via *mdh*.

Many cyanobacteria can naturally synthesize sucrose and use it for resistance to salt pressure (12). In N_2-fixing strain, such as *Anabaena sp.* PCC 7119 (hereafter *Anabaena*), sucrose breakdown can be performed by sucrose synthase (*susA*), which uses (A/U)DP to split sucrose into (A/U)DP-glucose and fructose (reaction [2]) (16). Here, the codon-optimized gene, *susA* from *Anabaena* encoding sucrose synthase was incorporated in *Synechocystis* to increase the concentration of intracellular fructose under high salt conditions.

Sucrose + A/UDP $\longleftrightarrow$ A/UDP-glucose + Fructose [2]

Our data showed that a mannitol yield of 0.058 mg/l/OD_{730} in 3 days was achieved in the *Synechocystis* cells with heterologous co-expression of *mdh* and *susA* (sww016) under 300 mM salt. In addition, as shown in the reaction [2], this reversible reaction is dependent on the concentration of substrate, therefore, we

aimed to improve fructose concentration by increasing intracellular sucrose concentration, and thereby, further increasing mannitol production. However, although high sucrose accumulation was achieved in this study by knocking out glucosyl-glycerol phosphate synthase (*ggpS*) in the background of sww016 (sww026), mannitol production was also unfortunately impaired. We speculated that *ggpS* deletion resulted in ADP-glucose overaccumulation, which blocked the direction of fructose formation and led to insufficient fructose concentration in the cell (17).

Methods and materials

Strains and culture conditions

Strains of *E. coli* were grown in LB liquid medium under 37ºC in a shaking incubator at 200 rpm, or on a solid LB plate with 1.5 % agar. Antibiotics were added to LB liquid medium or LB solid plate as follows: kanamycin (50 µg mL^{-1}) or Ampicillin (100 µg mL^{-1}), either separately or in combination.

Strains of *Synechocystis* were cultivated in a modified BG-11 liquid medium (18) with 25 mM PIPPS buffer or on solid modified BG-11 plate supplementary with 1.5% agar and 0.3% (w/v) sodium thiosulphate at 30ºC in a shaking incubator with red light of moderate intensity (~ 50 µmol photons m^{-2} s^{-1}) and 120 rpm shaking speed. To construct *Synechocystis* mutants, kanamycin (50 µg mL^{-1}), chloramphenicol (35 µg mL^{-1}) or spectinomycin (50 µg mL^{-1}) was used in either liquid BG-11 medium or solid BG-11 plates. The culture density was monitored by determining the optical density at a wavelength of 730 nm (OD_{730}).

Gene synthesis with codon optimization

The gene sequence of mannitol dehydrogenase (*mdh*) from *Lactobacillus reuteri* (NCBI Reference Sequence: AAS55855.1) and sucrose synthase (*susA*) from *Anabaena* sp. PCC 7119 (NCBI Reference Sequence: AJ010639.1) were directly taken from NCBI database. Codon optimization was performed based on the codon usage table compiled for *Synechocystis* (https://www.kazusa.or.jp/codon/).

The genes were synthesized by Integrated DNA Technologies and were separately ligated to the pUC plasmids resulting in pUCmdh and pUCsusA, respectively.

Plasmids and *Synechocystis* mutant construction

To express optimized *mdh* in the chromosome of *Synechocystis* at the neutral site *slr0168*, the optimized *mdh* was cut by restriction enzyme NdeI and BamHI from plasmid pUCmdh and then cloned into the plasmid pHKH020 (19), which contains ~ 1kb upstream and downstream of *slr0168*, promoter Ptrc1 and kanamycin resistance cassette. The new plasmid used for transformation *mdh* in the *Synechocystis* at *slr0168* was named as pWW009. In addition, restriction enzyme NdeI and BamHI were also used to clone optimized *susA* from pUCsusA into pHKH020 at the same site to generate plasmid pWW008, which was used to insert *susA* at locus of *slr0168* in the chromosome of *Synechocystis*. The optimized *susA* togethering with promoter Ptrc1 was subsequently amplified from pWW008 and fused with ~ 1kb upstream and downstream of neutral site *n15* (20,21) and spectinomycin resistance cassette, via overlap PCR. The fused fragment was sequenced to check for the absence of mutations and then used directly for transformation of the chromosome of *Synechocystis* at the neutral site present in locus *n15*. To knock out *ggpS* from *Synechocystis*, the chloramphenicol resistance cassette was amplified from SAW011 (22) and fused with ~ 1 kb upstream and downstream of *ggpS* by using overlap PCR method, the fused fragment was then used for transformation in *Synechocystis* to replace the native gene *ggpS*, selecting by chloramphenicol. All the mutants were confirmed by PCR and the primers used were listed in the Table 1. The confirmed cells were stored at -80ºC in BG-11 medium with 20 % (V/V) glycerol.

Table 1 Primers used in the study

Primer name	Sequence	Purpose
Hom1n15_F	5’-GACGGAATGTTGGCAGGTAGC-3’	Amplification of homologous region upstream the *n15* gene
Hom1n15_R	5’-GACGAACAGTTTAGCCTCCT-3’	Amplification of homologous region upstream the *n15* gene

susA_F	5'-AGGAGGCTAAACTGTTCGTCTTGACAATTAATCATCCGGCT-3'	Amplification of optimized *susA* and *ptrc1* and fused with homologous region upstream the *n15* gene
susA_R	5'-ATGAATCCTTAATCGGTACC-3'	Amplification of optimized *susA* and *ptrc1* and fused with spectinomycin resistance cassette
Omega_F	5'-GGTACCGATTAAGGATTCATAATTCCGGGGATCCTCTAGA-3'	Amplification of spectinomycin resistance cassette and fused with optimized *susA*
Omega_R	5'-ATGAATCCTTAATCGGTACC-3'	Amplification of spectinomycin resistance
Hom2n15_F	5'-GGTACCGATTAAGGATTCATCCGCAAATTCACATTCGCTGT -3'	Amplification of homologous region downstream the *n15* gene and fused with spectinomycin resistance cassette
Hom2n15_R	5'-GAGTCTGATTCTGTGGGGCT-3'	Amplification of homologous region downstream the *n15* gene
Seq1susA_R	5'-AAGACGATTCTGAAAATC-3'	Sequencing optimized *susA* gene
Seq2susA _F	5'-GTGAGCGATCGCCCCAATG-3'	Sequencing optimized *susA* gene
Seq3susA _F	5'-ATGAATGCTGCTAACTTCG-3'	Sequencing optimized *susA* gene
Seq4susA _F	5'-AGATTATTCAGGATAAGAT-3'	Sequencing optimized *susA* gene
Seq1mdh_F	5'-GATGCTGTTGGTGTTACTC-3'	Sequencing optimized *mdh* gene
Seq2mdh _R	5'-TTAGTAGTGTCGATAGT-3'	Sequencing optimized *mdh* gene
Hom1ggps_F	5'-TCCTTTCCCAACGAAACAAG-3'	Amplification of homologous region upstream the *ggpS* gene
Hom1ggps_R	5'-TTCTAGAGGATCCCCGGAATTAAAATCAGCGGTCTCCAAAA-3'	Amplification of homologous region upstream the *ggpS* gene and fused with chloramphenicol resistance cassette
Chloramphenicol_F	5'-TTTTGGAGACCGCTGATTTTAATTCCGGGGATCCTCTAGAA-3'	Amplification of chloramphenicol resistance cassette and fused with homologous region upstream the *ggpS* gene
Chloramphenicol_R	5'-ACAACTGGCATTGGCGATCGCATGAATCCTTAATCGGTACC-3'	Amplification of chloramphenicol resistance cassette and fused with homologous region downstream the *ggpS* gene
Hom2ggps_F	5'-GGTACCGATTAAGGATTCATGCGATCGCCAATGCCAGTTGT-3'	Amplification of homologous region downstream the *ggpS* gene and fused with chloramphenicol resistance cassette
Hom2ggps_R	5'- TATCCACAAACGCTTCCACA-3'	Amplification of homologous region downstream the *ggpS* gene

Crude protein extraction

Wild type (WT) *Synechocystis* and mutant sww003 were separately cultivated under previously mentioned conditions with illumination at the red-light intensity of approximately ~ 50 μE m^{-2} s^{-1}. Cells were harvested by centrifugation under 4,500 rpm at 4ºC and resuspended into a 100 mM Hepes buffer at pH 8.0 and a 100 mM Mops buffer at pH 6.5, respectively. To this, 60% (w/v) 0.1-mm glass beads (Sigma-Aldrich) were added. The cells were subsequently disrupted in a Precellys 24 bead-beater (Bertin Technologies) by 20 s of beating at 6000 rpm, followed by 120 s on ice, which was repeated a total of three times. Insoluble material and glass beads were removed by centrifugation at 15,000 rpm and 4 °C for 30 min. The protein concentration in the supernatant was determined with the Pierce BCA Protein Assay (Thermo Scientific) according to the manufacturer's protocol (23).

Assay for sucrose synthase in vitro

For determination of sucrose synthase (SusA) in crude protein of WT and sww003 under either pH 6.5 or pH 8.0 condition, the reaction mixture contained 40 mM sucrose, 10 mM UTP/ATP and the crude protein with Hepes buffer (pH 8.0) or Mops buffer (pH 6.5) in a total volume of 20 μl was used. The reaction was allowed to proceed for 30 min incubation under 30ºC to form ATP/UTP-glucose, and then was immediately used for determination of glucose by D-Fructose D-Glucose Assay Kit (Megazyme) based on manufacturer's protocol (24). The specific SusA activity was calculated based on synthesis of ATP/UTP-glucose from sucrose and expressed as percent of total crude protein rate.

Determination of extracellular mannitol in *Synechocystis*

Extracellular mannitol in WT, sww002, sww016 and sww024 was determined in the supernatant of their cultures using a D-Mannitol-L-Arabitol Assay Kit (Megazyme). In brief, 1 ml of cell cultures were removed by centrifugation at 12,000 rpm for 1 min. Then, 100 μl of the supernatant was used for extracellular mannitol measurement according to previous study (4).

Determination of sucrose in *Synechocystis*

2-mL aliquots of WT, sww016 and sww024 cultures after induction by 0, 300 mM, 600 mM or 900 mM salt were centrifuged at 12000×g for 5 min to separate cell pellets and supernatants. For determination of sucrose concentration, the cell pellets were broken according to previous study, with a minor modification (25). In brief, the cell pellets were suspended in 1 mL of 80 % of ethanol (v/v) and then heated under 65ºC for 5 hours, leading to release intracellular sucrose. After centrifugation at 12000×g for 5 min, the supernatant was transferred into a new tube and volatilized by heating under 65ºC over night. The dry residues were dissolved by MQ water and analyzed by Sucrose/Fructose/D-Glucose Assay Kit (Megazyme) according to manufacturer's protocol for determination of sucrose content (26).

Detection the function of Mdh in *Synechocystis*

To determine the function of Mdh, 0.05%, 0.1%, 0.2% and 0.5% fructose (v/v) with liquid BG-11 medium was used for cultivation of WT and sww002. The growth curve was determined by observation on optical density at 730 nm and extracellular mannitol content was measured by previous methods in the process of cultivation.

Results and discussion

Genetic overexpression of *mdh* in *Synechocystis* strains

In order to express mannitol in *Synechocystis*, *mdh* and *susA* genes, each under control of the Ptrc1 promoter, were inserted at the neutral sites *slr0168* or *n15*, either separately or in combination (Table 2). Growth behavior of wild type (WT) and *mdh* overexpression strains was compared and showed no significant difference (Fig.2). In addition, no mannitol production was detected in WT, sww002 (overexpression of *mdh*) or sww016 (overexpression of *mdh* and *susA*) under normal conditions (Figs 3 and 4). This showed that heterologous expression *mdh* in *Synechocystis* did not inhibit growth of cells and the expression of single *mdh* was not sufficient to obtain mannitol production in normal BG-11. We

speculated that a small pool size of substrate, such as fructose, limits mannitol production under these standard growth conditions.

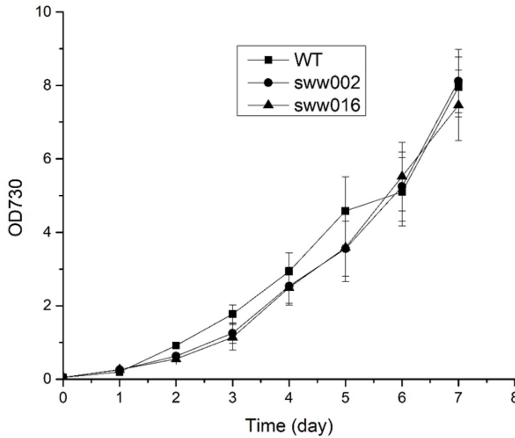


Fig.2 Growth curve in cultures of WT, sww002 and sww016 in normal BG-11 medium. Symbols: solid squares, WT; solid circles, swww002; solid up triangles, sww016. Values represented the average of at least three biological replicates (error bars represent standard deviation).

Table 2 Plasmids and strains used in this study

Plasmid and strains	Description	Reference
pHKH020	pBluescript II SK+ Δslr0168:: Ptrc1::ldh::tt::Kan^R	(19)
pUCmdh	plasmid containing optimized *mdh*	In this work
pUCsusA	plasmid containing optimized *susA*	In this work
pWW008	pBluescript II SK+ Δslr0168:: Ptrc1::susA::tt::Kan^R	In this work
pWW009	pBluescript II SK+ Δslr0168:: Ptrc1::mdh::tt::Kan^R	In this work
WT	*Synechocystis* sp. PCC6803 wild type	(4)
sww002	*Δslr0168::Ptrc1::mdh::tt::*Kan^R on WT background	In this work
sww003	*Δslr0168::Ptrc1::susA::tt::*Kan^R on WT background	In this work

sww016	*Δn15::Ptrc1::susA::tt::SpeΩ^R* on sww002 background	In this work
sww024	*ΔggpS::Cam^R* on sww016 background	In this work

Kan^R indicates kanamycin resistance gene, *Spe*Ω^R indicates spectinomycin resistance gene, *Cam*^{R} indicates chloramphenicol resistance gene, *tt* indicates terminator

In previous studies, a mannitol biosynthetic pathway was genetically engineered by expressing NAD^+-dependent Mdh from *Leuconostoc pseudomesenteroides* in both *E. coli* and *Bacillus megaterium*, which converts exogenous fructose to mannitol (27,28). However, here we used $NADP^+$-dependent Mdh from *Lactobacillus reuteri* instead of the NAD^+-dependent Mdh for converting fructose into mannitol in *Synechocystis*. This, mainly because NADPH is the primary reductant produced in *Synechocystis* under photoautotrophic conditions, with the ratio of NADP(H) to NAD(H) being approximately 10 (29) Some successful $NADP^+$-dependent enzymes have already been applied in *Synechocystis* for achieving lactate, sorbitol and alcohol production, such as $NADP^+$-lactate dehydrogenase, sorbitol-6-phosphate dehydrogenase and alcohol dehydrogenases (9,30,31). So, we assumed the reason why $NADP^+$-dependent Mdh does not achieve mannitol production in *Synechocystis* is strictly due to the limited fructose rather than lack of NADPH. To test this hypothesis, 0.05%, 0.1%, 0.2% and 0.5% fructose (v/v) was separately mixed in BG-11 liquid medium for cultivation of both WT and SWW002. Exogenous fructose can cause fatal growth inhibition of *Synechocystis* cells, and surely, we did not observe growth in cultures with 0.5 % fructose in the medium, which is in concordance with previous studies (Fig.3) (15). In addition, when fructose lower than 0.5% was used in BG-11 liquid medium, an extracellular yield of 8.2 mg L^{-1} OD_{730}^{-1} mannitol in 7 days could be detected from culture of sww002 and growth was partially impaired (Fig.3). Altogether, this experiment indicated that (i) WT is incapable of producing mannitol in BG-11 supplemented with fructose; (ii) overexpression of $NADP^+$-dependent Mdh can indeed synthesize mannitol by adding low concentration of fructose (lower than 0.5% fructose); and (iii) lack of intracellular fructose accumulation is the limiting factor for SWW002 to produce mannitol in standard BG-11 medium.

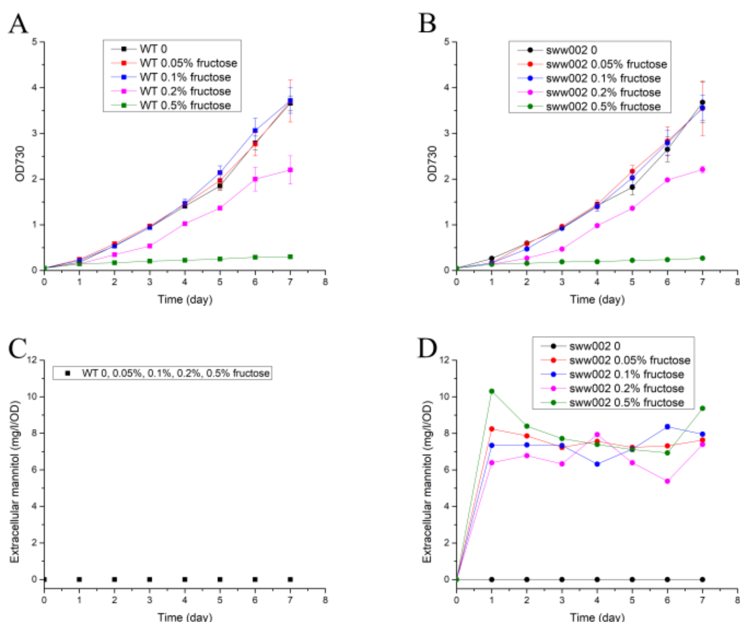


Fig.3: Growth curve (A and B) and extracellular mannitol production (C and D) in cultures of WT and sww002 in normal BG-11 medium supplemented with 0 (black), 0.05% (red), 0.1% (blue), 0.2% (pink) and 0.5% (olive) of fructose. Symbols: solid squares, WT; solid circles, swww002; Black solid squares in **C** represented no extracellular mannitol production in normal BG-11 with 0, 0.05%, 0.1%,0.2% and 0.5% of fructose. Values represented the average of at least three biological replicates (error bars represent standard deviation).

Although mannitol production was successfully achieved by heterologous expression of $NADP^+$-dependent *mdh* in *Synechocystis* by adding fructose, it is not a good strategy for mannitol synthesis in future applications because of two main reasons: 1) Although exogenous fructose increases the extracellular mannitol concentration, this increased production is not high enough to offset the increased cost of adding fructose to the media. Furthermore, if fructose is a necessary substrate that we have to use for mannitol production, lactic acid bacteria as a host to produce mannitol would be much better than *Synechocystis* in terms of high productivity, since mannitol production from lactic acid bacteria can reach 150 g L^{-1}, close to its solubility limit of 180 g L^{-1} at 25°C, which is

much higher than 8.2 mg L^{-1} OD_{730}^{-1} from *Synechocystis* (7). 2) Fructose usage for cultivation of *Synechocystis* would have to be kept low such that it does not introduce a growth inhibition, which could result in genetic instability and further restrict mannitol productivity. Therefore, improving intracellular fructose content via biosynthesis from CO_2 is an attractive alternative for achieving mannitol production in *Synechocystis* overexpressing $NADP^+$-dependent *mdh*, instead of using external fructose.

In 2018, F. Kirsch et al. identified one invertase gene (*sll0626*), which can degrade intracellular sucrose into fructose and glucose. This appears to be the only native gene found in *Synechocystis* directly involved in fructose synthesis (12). In this study, sww002 in which *mdh* is overexpressed was cultivated in normal BG-11 liquid medium followed by salt shock. There no mannitol was produced, even though, the sucrose content did accumulate to 0.15 mg L^{-1} OD_{730}^{-1} in one day. However, this accumulated sucrose was completely degraded in three days (Figs.4 and 5), most likely leading to the formation of fructose, but oddly no mannitol. We hypothesized the reason for this was that the native gene *sll0626* in *Synechocystis* was unable to convert enough fructose, which strongly restricted mannitol production during the cultivation under salt stress.

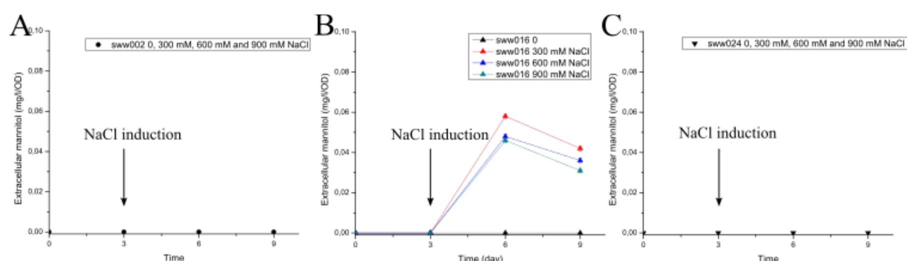


Fig.4 Extracellular mannitol production in cultures of sww002 (A), sww016 (B) and sww024 (C) in normal BG-11 medium inducted by 0 (black), 300 mM (red), 600 mM (blue) and 900 mM (pink) of NaCl. Symbols: Symbols: solid circles, sww002; solid up triangles, swww016; solid down triangles, sww024. Solid squares and down triangles in **A** and **C** represented no extracellular mannitol production shocked by 0, 300 mM, 600 mM and 900 mM

of NaCl. Values represented the average of at least three biological replicates (error bars represent standard deviation).

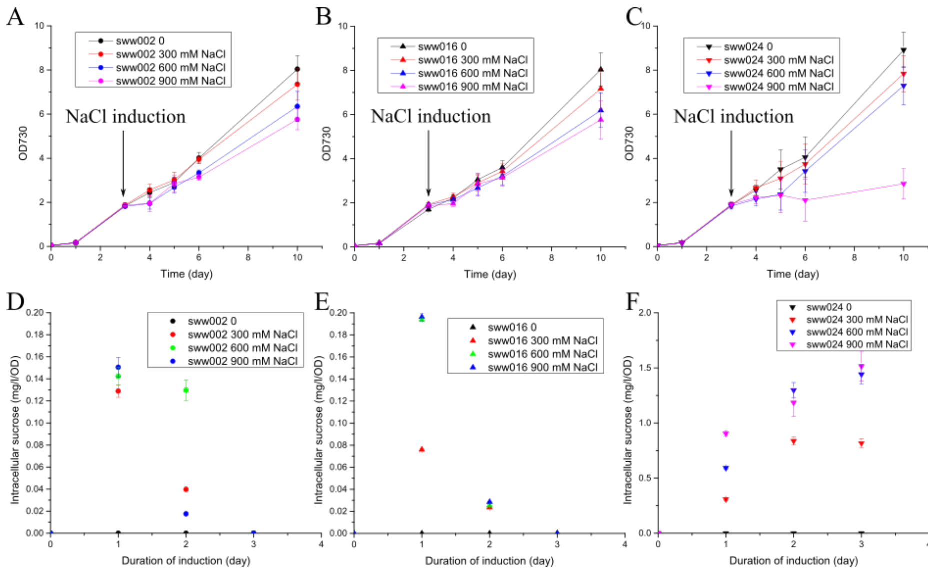


Fig.5 Growth curve (A, B and C) and intracellular sucrose production (D, E and F) in cultures of sww002, sww016 and sww024 in normal BG-11 medium inducted by 0 (black), 300mM (red), 600mM (blue) and 900mM (pink) of NaCl. Symbols: solid circles, sww002; solid up triangles, swww016; solid down triangles, sww024. Values represented the average of at least three biological replicates (error bars represent standard deviation).

SusA specific activity determination in vitro

To enhance fructose synthesis from sucrose, optimized *susA* from *Anabaena* encoding sucrose synthase was introduced into *Synechocystis* (sww003). As shown in Fig.6, the crude protein from both WT and sww003 can synthesize fructose from sucrose *in vitro* using ATP/UTP as substrate under both at pH 6.5 and pH 8. Compared with WT, sww003 did show significantly higher activity of fructose synthesis. This indicated that *susA* expression might help solve the problem of fructose deficiency in sww002.

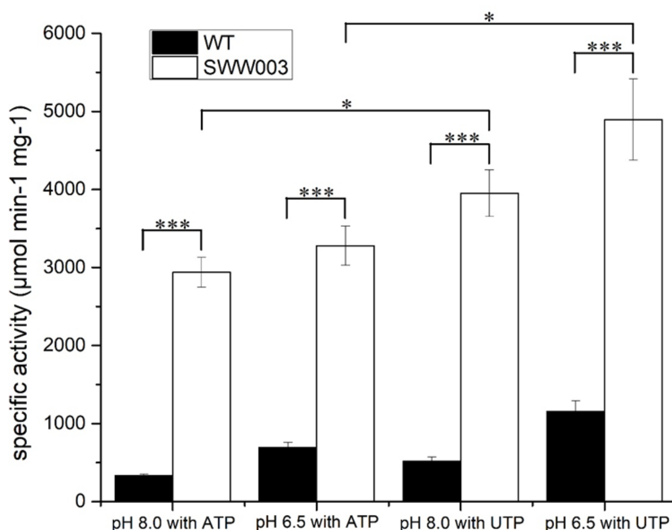


Fig.6: Specific catalytic efficiency of SusA in absence of 5mM ATP or UTP from WT (Black bars) or sww003 (white bars) cell free extracts. P value analysis symbol: *, P≤0.05; ***≤0.001. Values represented the average of at least three biological replicates.

Determination of extracellular mannitol production in *Synechocystis* cells overexpressing *mdh* and *susA*

Mannitol production was indeed achieved in cells coexpressing *mdh* and *susA* (sww016) under salt pressure, which showed roughly 0.055 mg L^{-1} $OD_{730}{}^{-1}$ in 3 days (Fig.4). Compared with sww002, sww016 did not show significant improvement of intracellular sucrose concentration but successfully synthesized mannitol, which corroborated our hypothesis that the native *sll0626* cannot make enough fructose available for mannitol production under salt shock conditions. Compared with another mannitol producing *Synechocystis* strain, which co-expresses *mtlD* and *m1p*, the mannitol production achieved by *mdh* and *susA* was roughly 10 times lower (4). We speculated that lack of substrate might restrict

mannitol accumulation, since intracellular sucrose in both SWW002 and SWW016 was completely degraded in 3 days after salt shock, and no mannitol was produced afterwards.

Therefore, increasing sucrose production might be a good solution for further improvement of mannitol production in *Synechocystis* via the NADP+ dependent *mdh*. A previous study reported that sucrose content could be increased by the mutation of *ggpS* for blocking glycosyl-glycerol (GG) synthesis and thereby enabling increased carbon flux towards sucrose (17). In this study, *ggpS* was successfully knocked out from sww016 (sww024) to increase sucrose content in *Synechocystis*. The intracellular concentration of sucrose was indeed increased to 1.59, 1.41 and 0.84 mg L^{-1} OD_{730}^{-1}, in 3 days after 900 mM, 600 mM and 300 mM salt was used, respectively (Fig.5). In contrast, the sucrose from sww002 and sww016 was completely degraded in 3 days (Fig.5). It seems that the deletion of *ggpS* not only increased sucrose expression, but also blocked sucrose breakdown. However, the elevated sucrose did not result in increased mannitol production in sww024 (Fig.4). The previous study reported that protein GgpS can consume ADP-glucose and convert it into GG-phosphate, the key intermediate product during GG synthesis (17). We therefore speculated that the *ggpS* deletion in sww024 resulted in an overaccumulation of ADP-glucose, which inhibited the formation of fructose (reaction [2]).

In WT *Synechocystis*, ADP-glucose is synthesized by ADP-glucose pyrophosphorylase (*glgC*) using glucose-1-phosphate and ATP as substrates. The glucose moiety of ADP-glucose is then transferred into glycogen as the major carbon storage source in *Synechocystis*. In this study, gene *susA* also synthesizes ADP-glucose using ATP and sucrose as substrate. Thus, *glgC* replacement by *susA* was attempted here as well to closely connect mannitol production with glycogen. Unfortunately, we were unable to obtain a fully segregated *glgC* knockout despite multiple attempts (data not shown). This negative result suggests that *glgC* is essential for *Synechocystis* and the implementation of *susA* alone was insufficient for replacing the functionality of *glgC*.

Conclusions

Here we designed an alternative strategy for mannitol production in *Synechocystis*. We wanted to avoid relying on the overexpression of *mtlD* and *m1p*, to convert fructose-6-phosphate into mannitol, which is known to lead to the toxic accumulation of the intermediate mannitol-1-phosphate. Instead, we envisioned another pathway for mannitol synthesis in which the intracellular sucrose pool accumulated during salt stress would be used instead. It consists of a two-step conversion of sucrose to fructose catalyzed by *susA*, followed by the activity of *mdh* (Fig.1). This was successfully implemented in *Synechocystis* sp. PCC6803, and as a result, lead to an extracellular mannitol yield of 0.058 mg/l/OD730 in 3 days under 300 mM salt. Finally, additional strategies were employed to try to improve productivity further, which unfortunately, were not deemed successful. This paper explored a new pathway of mannitol expression in *Synechocystis* sp. PCC6803 and provided theoretical basis for future application.

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