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

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Chapter III

Enhancement of mannitol production by fine-tuned expression of mannitol-1-phosphate-5-dehydrogenase in *Synechocystis* sp. PCC6803

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Abstract

Mannitol production was previously achieved in freshwater *Synechocystis* sp. PCC6803 via heterologously expressing mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*) under control of the strong promoter Ptrc1. However, low extracellular mannitol production, which is only 5.54 mg/l after 7 days of cultivation, cannot meet the requirement of commercial-scale production. This is probably caused by insufficient expression level of the mutated *mtlD*, which has a methionine missing at position 332. In this study, we compared mannitol production levels by using different promoters including Ptrc1, Psba2 and Pnrsb to control the expression level of (un)mutated *mtlD* in *Synechocystis* with co-expression of *m1p*. Our data suggests that only the weakest promoter, Pnrsb, can support the expression of unmutated *mtlD* in *Synechocystis*, which lead to 18.2 mg/l mannitol titer in 7 days without induction. The latter was already much higher than the first engineered mannitol-producer *Synechocystis*, and the usage of 5 µM nickel as inducer in the medium could significantly further increase mannitol production for this mutant up to 92.9 mg/l after 7 days induction, but partially inhibited growth. In contrast, attempts with the other somewhat stronger promoters always failed to express the unmutated *mtlD* probably due to toxicity of over-accumulated intermediate product, mannitol-1-phosphate. These results clearly suggest that the expression level of *mtlD* is the bottleneck of achieving high yield of mannitol in *Synechocystis* and mannitol production can be enhanced by fine-tuned expression of *mtlD*.

Key words

Mannitol; *Synechocystis*; Mannitol-1-phosphate-5-dehydrogenase; Pnrsb;

Introduction

Mannitol is a naturally occurring six-carbon sugar polyol found in many bacteria, yeasts, algae and several plants like celery and onion (1). The health-promoting functions of mannitol provide these with added-value in the medicine, pharmaceutical, food and chemical fields (2). Therefore, achieving mannitol production with a low-cost and environmentally friendly way has gained much attention to be developed. Compared with using *E. coli*, *yeast* or plants to synthesize mannitol, cyanobacterium presents several advantages: 1) *E. coli* and *yeast* rely on nutrients including glucose and fructose to survive, but cyanobacterium only absorbs sunlight with basic inorganic compounds to maintain fundamental metabolic requirements, which drastically lowers the cost needed by cyanobacterium for chemical production; 2) the cultivation of cyanobacteria has the potential to occupy non-arable land, which, unlike plants, can refrain from exerting further pressure on natural resources (3). Above all, achieving mannitol production in cyanobacteria shows a huge potential for large-scale cultivation in the future.

In our previous study reported in Chapter II, the sustainable production of mannitol via the direct conversion of CO_2 in the freshwater cyanobacterium, *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*) was achieved by heterologous expression of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*). Although stable, it unfortunately displayed a very low yield, only 5.54 mg/l in 7 days of cultivation with red light of moderate intensity (~50 μmol photons m^{-2} s^{-1}) (4). This low efficiency of mannitol production definitely limits its commercial value in the industrial field. One possibility leading to such low yield was due to insufficient expression level of the mutated *mtlD*. In the previous chapter, a fragment containing the fused mannitol cassette (*mtlD* and *m1p*) was introduced into neutral site *slr0168* in *Synechocystis*, but a methionine was stubbornly missing at position 332 of *mtlD*. Nonetheless, even with this mutated *mtlD*, mannitol production could still be synthesized by cells, albeit at low level (4).

In this study, several plasmids used for expression of (un)mutated *mtlD* (Supplementary table S1) under control of relatively weak promoters including Psba2 and Pnrsb and/or *m1p* under control of Ptrc1 was respectively constructed

in *E. coli*, and subsequently, used to try to be integrated in the chromosome of *Synechocystis*. The data showed that only the weakest promoter Pnrsb can be used to control the expression of the intact *mtlD* (i.e. without any sequence deletions and/or substitutions) for producing mannitol in *Synechocystis.* Furthermore, upon induction by nickel, mannitol production can be increased, but ultimately it becomes lethal to the cells. To balance cell growth with mannitol productivity, 5 µM was determined as the most suitable inducer concentration for mannitol expression, which results in the mannitol production of 92.9 mg/l in 7 days induction.

In conclusion, the average mannitol production in *Synechocystis* was increased nearly 8 times after fine-tuned expression of *mtlD,* as its overexpression was proved to be lethal for cells. Thus, using inducible promoter Pnrsb with a moderate inducer concentration to control the level of *mtlD* expression is a good solution to tackle the issue of low production of mannitol in engineered *Synechocystis* strains (Fig.1).

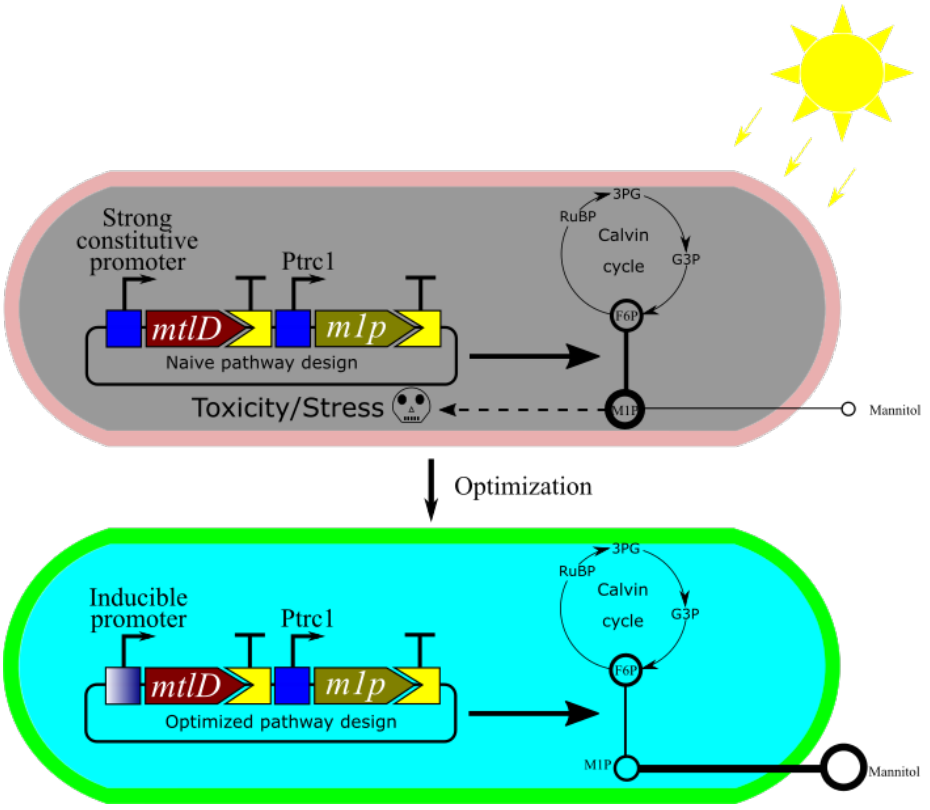


Fig.1 Overview of the engineered biosynthetic pathway to mannitol in *Synechocystis* under optimized pathway design by fine-tuned expression of *mtlD*. Engineered genes are shown in white. Abbreviations: RuBP, ribulose-1,5-bisphosphate; 3PG, 3-phosphoglycerate; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; M1P, mannitol-1-phosphate; *mtlD*, mannitol-1-phosphate dehydrogenase from *E. coli*; *m1p*, Mannitol-1-phosphatase from *E.tenella*.

Methods and Materials

Strains and culture conditions

Strains of *E. coli* were grown in LB liquid medium at 37ºC in a shaking incubator with 200 rpm, or on solid plates containing 1.5% agar. Kanamycin (50 µg/ml), spectinomycin (20 µg/ml) or ampicillin (100 µg/ml) were added to LB liquid, or solid medium, either separately or in combination, to select mutants when necessary.

Synechocystis, a glucose-tolerant wild type obtained from D. Bhaya, University of Stanford, Stanford CA, was cultivated in a modified BG11 medium (5) with 25 mM PIPPS buffer (PH 8.0) at 30ºC, either in a shaking incubator with 120 rpm under continuously red light (~50 µmol photons m^{-2} s^{-1}), or on a solid plate, supplemented with 1.5% agar and 0.3% (w/v) sodium thiosulphate under continuous red light (~50 µmol photons m^{-2} s^{-1}). Antibiotics were used in both liquid and solid BG11 medium, either separately or in combination, to select mutants with appropriate concentrations as follows: kanamycin (50 µg/ml) and/or spectinomycin (20 µg/ml). Growth was measured by monitoring optical density (OD) using a spectrometer at 730 nm.

Plasmid construction

In order to express *m1p* under control of promoter Ptrc1 in *Synechocystis* (Supplementary table S2), the plasmid pWW001 (*Ptrc1_m1p_Spe*Ω), a derivative of pHKH020, containing the Ptrc1-driven codon optimized *L-ldh* cassette of L. lactis sub sp. *cremoris* MG1363 was modified (6) and used to introduce *m1p* in the neutral site, *slr0168* of *Synechocystis*. The codon-optimized *m1p* gene with NdeI and AvrII restriction sites was PCR amplified from pUCm1p (4) and used to replace *L-ldh* cassette of plasmid pHKH020 according to standard cloning techniques (7), creating plasmid pWW002 (*Ptrc1_m1p_Kan*). The spectinomycin resistance cassette was amplified from plasmid pHSH with primers (Supplementary table S3) introducing flanking cloning sites for SalI to replace the kanamycin cassette of pWW002 (8), the new plasmid containing *m1p* gene under promoter Ptrc1 with spectinomycin resistance cassette was named as pWW001 (*Ptrc1_m1p_ Spe*Ω).

To obtain plasmids pWW003 (*Psba2_unmutated mtlD_Kan*) and pWW004 (*Ptrc1_mutated mtlD_Kan*), which was used to express either the native or mutated *mtlD* under promoter Psba2 (Supplementary table S2), respectively in *Synechocystis*, the plasmid pHKH009 carrying a gene encoding a reporter-enzyme (*lacZ*) under promoter Psba2 was modified (6). Briefly, native and mutated *mtlD* was PCR amplified from pHKHmtlD and WT_M (Δ*slr0168*::*Ptrc1*::*mutated mtlD*::*m1p*::*Kan*) (4), respectively, meanwhile, two restriction enzyme sites, NdeI and BamHI were introduced at ends for both

genes via the same primers during amplification (Supplementary table S3). Then, either the native or mutated *mtlD* were cloned to replace *lacZ* of plasmid pHKH009 according to standard cloning techniques, resulting in pWW003 and pWW004, respectively.

To construct plasmids used for expression either native or mutated *mtlD* under inducible promoter Pnrsb, the nickel inducible operon of *nrsS*, *nrsR* and *nrsB* was first amplified by PCR using *Synechocystis* wild type as template (9) (Supplementary table S2). The native or mutated *mtlD* fragment was fused with nickel inducible promoter, respectively, by overlap PCR and the restriction enzyme sites XbaI and AvrII at two ends of fused fragment were also introduced via primers (Supplementary table S3). Subsequently, *L-ldh* cassette with promoter Ptrc1 of plasmid pHKH020 was replaced by the fused fragments according to standard cloning techniques (7). The new plasmid contains expression of native or mutated *mtlD* under Pnrsb was named as pWW005 (*Pnrsb_unmutated mtlD_Kan*) and pWW006 (*Pnrsb_mutated mtlD_Kan*), respectively. Then, the fragment of Pnrsb and native *mtlD* including transcriptional terminator were PCR amplified from pWW005 (*Pnrsb_unmutated mtlD_Kan*) and XbaI was inserted at both ends via primers (Supplementary table S3). Meanwhile, the plasmid pWW002 (*Ptrc1_m1p_Kan*) was made linear via digestion by XbaI and native *mtlD* under Pnrsb with terminator was inserted by ligation to create a new plasmid which contains native *mtlD* under control of Pnrsb and *m1p* under control of Ptrc1, named as pWW007 (*Pnrsb_unmutated mtlD_Ptrc1_m1p_Kan*). All the plasmids were sequenced before integration in the chromosome of *Synechocystis* to confirm the absence of mutations. The primers used are listed in the supplementary file (Supplementary table S3).

Natural transformation for *synechocystis*

Natural transformation for genomic integration in *Synechocystis* was performed as in the previous chapter (10), using kanamycin (50 µg/ml) and/or spectinomycin (20 µg/ml) as selection pressure to drive full segregation. Briefly, 10 µg of specific plasmid DNA was mixed with 200 µl concentrated *Synechocystis* cells from an exponentially growth (OD_{730}=10) and incubated in the white light incubator with ~50 µmol photons m^{-2} s^{-1} for 5 hours. Then,

mixed cells were spread on a commercial membrane (Pall Corporation, USA) which was placed on the BG11 solid plate. After 24 hours incubation under white light incubator, the membrane was transferred onto a new solid BG11 plate with specific antibiotics until individual colonies isolation. The segregation status of mutants was achieved by propagations in the presence of antibiotics and confirmed by colony PCR, using appropriate primers (Supplementary table S3). The sequencing results were used to confirm the correct insertion.

Mannitol determination by kit

Extracellular mannitol concentrations were determined in supernatant of *Synechocystis* cultures using a D-Mannitol-L-Arabitol Assay Kit (Megazyme). Cells from different cultures were collected by centrifugation for 5 min under 14000 rpm and 100 µl of supernatant was used for extracellular mannitol measurement based on instruction from the kit (11).

Growth curve determination

The *Synechocystis* mutants used in this study were revived from glycerol stock in BG11 in red light incubator with 50 µmol photons m^{-2} s^{-1} until OD_{730} reaches 1. Then, the equivalent to an initial OD 0.05 of cells were transferred from precultures and cultivated in fresh BG11. The growth curves were determined by spectrometer at OD_{730}.

To study mannitol production from mutants influenced by nickel as inducer, mutants were first revived from a glycerol stock and cultivated in the flask in the red light incubator with 50 µmol photons m^{-2} s^{-1} until OD_{730} reaches roughly 0.5. Then 0, 5, 10, 15 and 20 µM nickel was added in the BG11 medium, respectively. The growth curves were determined by spectrometer at OD_{730}.

Results and discussion

Expression of native *mtlD* in the absence of M1p is not viable in *Synechocystis*

The viability of mannitol producing mutants in cyanobacteria is well documented in the scientific literature (4,11). In fact, the mannitol cassette

consisting of mannitol-1-phosphate-5-dehydrogenase (*mtlD*) and mannitol-1-phosphatease (*m1p*) has been used to produce mannitol in both the freshwater cyanobacterium *Synechocystis* (4) and the marine cyanobacterium *Synechococcus* sp. PCC7002 (hereafter, *Synechococcus*) (11). However, a methionine stubbornly missing at position 332 of *mtlD* in *Synechocystis* and a not completely segregated mannitol cassette in the chromosome of *Synechococcus,* proved that metabolic engineering for mannitol production in cyanobacteria remained to a certain extent difficult. In this study, we used different types of promoters to precisely control expression of *mtlD* for optimization of the pathway of mannitol synthesis in *Synechocystis*.

To implement this idea, the plasmids carrying expression of either native or mutated *mtlD* (Supplementary figure S1 and table S1) and/or *m1p* (Supplementary table S1) were constructed in *E. coli* (Fig.2, Table 1). In the previous study, the native *mtlD* under Ptrc1 was failed to be cloned in *E. coli*, so fusion fragments which contained expression system of native *mtlD* and *m1p* under Ptrc1 instead of plasmids were introduced in the chromosome of *Synechocystis* at neutral site *slr0168*. However, a methionine was accidently, but stubbornly missing at position 332 of *mtlD* in *Synechocystis.* Even constructs expressing this mutated *mtlD* were still found to produce mannitol (4). We speculated that the overexpression *mtlD* is stressful for both *E. coli* and *Synechocystis*. Therefore, weaker promoters of *Synechocystis* were used to control expression level of (un)mutated *mtlD* in this study.

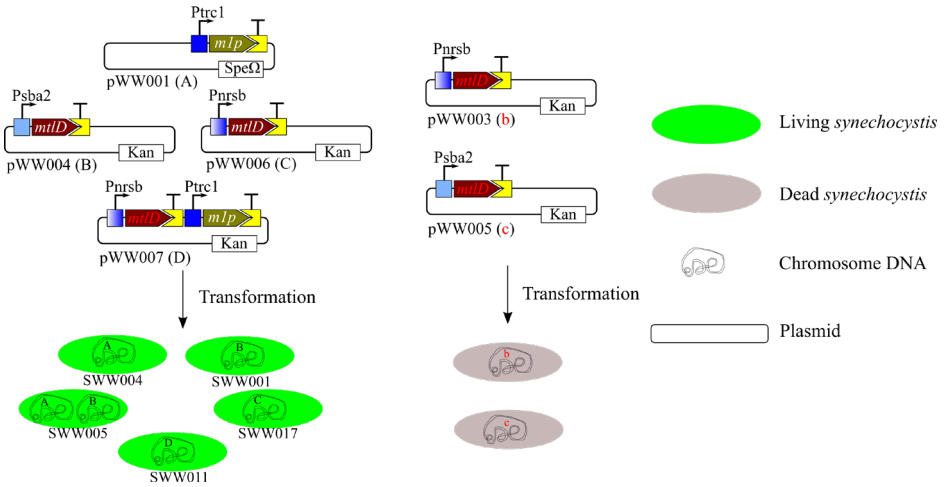


Fig.2 Integration (un)mutated *mtlD* and/or *m1p* in the chromosome of *Synechocystis*. Mutated *mtlD* is shown in white; native *mtlD* is shown in red; the plasmids unsuccessfully introduced in *Synechocystis* are shown in lowercase characters with red color; the plasmids successfully introduced in *Synechocystis* are shown in capitalized characters with black color; ellipses in green represent living *Synechocystis*; ellipses in grey represent dead *Synechocystis*; twisted-pair lines represent chromosome in *Synechocystis*. Abbreviations: *mtlD*, mannitol-1-phosphate dehydrogenase from *E. coli*; *m1p*, Mannitol-1-phosphatase from *E.tenella*; Kan, kanamycin resistance gene; SpeΩ, spectinomycin resistance gene.

Table 1. Plasmids and strains used in this study.

Plasmid and strains	Description	Reference
pHSH	$Ap^rSpe\Omega^r$, Omega cassette	(8)
pHKHmtlD	Ap^r, containing optimized native *mtlD*	(4)
pUC57m1p	Ap^r, containing optimized *m1p*	(4)
pHKH020	Ap^rKan^r, Ptrc1-driven codon optimized L-ldh cassette of *L. lactis* sub sp. cremoris MG1363 containing upstream and downstream fragments of *slr0168*	(6)
pWW001	$Ap^rSpe\Omega^r$, pHKH020 derivate used to express *m1p* under control of promoter Ptrc1 at *slr0168* gene locus	In this work
pWW002	Ap^rKan^r, pHKH020 derivate used to express *m1p* under control of promoter Ptrc1 at *slr0168* gene locus	In this work
pHKH009	Ap^rKan^r, Psba2-driven lacZ at *slr1311*	(6)
pWW003	Ap^rKan^r, pHKH009 derivate used to express *native mtlD* under control of promoter Psba2 at *slr1311* gene locus	In this work
pWW004	Ap^rKan^r, pHKH009 derivate used to express mutated *mtlD* under control of promoter Psba2 at *slr1311* gene locus	In this work
pWW005	Ap^rKan^r, pHKH020 derivate used to express native *mtlD* under control of promoter Pnrsb at *slr0168* gene locus	In this work
pWW006	Ap^rKan^r, pHKH020 derivate used to express mutated *mtlD* under control of promoter Pnrsb at *slr0168* gene locus	In this work
pWW007	Ap^rKan^r, pHKH002 derivate used to express native *mtlD* under control of promoter Pnrsb and *m1p* under control of promoter Ptrc1 at *slr0168* gene locus	In this work
WT	*Synechocystis* sp. PCC6803 wild type	D. Bhaya
WT_M	Δ*slr0168::Ptrc1::mutated mtlD::m1p::Kan*	(4)
SWW001	*slr1311::Psba2::mutated mtlD::Kan*	In this work
SWW004	Δ*slr0168::Ptrc1::m1p::Spe*Ω	In this work
SWW005	Δ*slr0168::Ptrc1::m1p::Spe*Ω, *slr1311::Psba2::mutated mtlD::Kan*	In this work
SWW011	Δ*slr0168::Pnrsb::unmutated mtlD::Ptrc1::m1p::Kan*	In this work
SWW017	Δ*slr0168::Pnrsb::mutated mtlD::Kan*	In this work

Ap^r, ampicillin resistance cassette; $Spe\Omega^r$, spectinomycin resistance cassette; Kan^r, kanamycin resistance cassette.

Psba2 promoter has been widely used in photosynthetic production of isoprene and ethanol in *Synechocystis* (12,13) and the transcript of Psba2 was concluded to be approximately ten times lower than Ptrc1 (14). In this study, the plasmids used for (un)mutated *mtlD* expression controlled by native Psba2 (*slr1311*) (15) were constructed in *E. coli*, named as pWW003 (*Psba2_unmutated mtlD_Kan*) and pWW004 (*Psba2_mutated mtlD_Kan*), respectively. However, we were not able to transform pWW003 in neither WT nor SWW004 (Δ*slr0168*::*Ptrc1*::*m1p*::*SpΩ*), but Psba2 with mutated *mtlD* was successfully

integrated in both of these strains and named as SWW001 (*slr1311*::*Psba2*::*mutated mtlD*::*Kan*) and SWW005 (Δ*slr0168*::*Ptrc1*::*m1p*::*SpΩ*, *slr1311*::*Psba2*::*mutated mtlD*::*Kan*). This suggested that native *mtlD* under Psba2 showed higher expression level resulting in higher toxicity than mutated *mtlD* for *Synechocystis*. To confirm this, we tried to express a functional native *mtlD* under control of inducible promoter Pnrsb in *Synechocystis*, since Pnrsb promoter is recognized as a nickel inducible promoter in *Synechocystis*, the expression level under this promoter is active only by the presence of nickel in the medium (16). In this case, if correct, *mtlD* can be expressed under control of Pnrsb with absence of nickel in *Synechocystis* and cell growth is inhibited only by nickel usage, it would prove expression of native *mtlD* is a big burden for cells. However, native *mtlD* controlled by Pnrsb is still unable to be integrated in the chromosome of *Synechocystis* with absence of nickel in WT unless with co-expression of M1p. This indicated that promoter Pnrsb is leaky since single expression of *mtlD* by this promoter under no nickel condition still causes too much pressure to be tolerated by cells. Moreover, the protein M1p can help cells release pressure from over-accumulation of mannitol-1-phosphate via degradation mannitol-1-phosphate to less stressful mannitol and therefore survives cell.

Mannitol-1-phosphate is not the first sugar phosphate that has been suspected as a burden to cells. In previous study, accumulation of non-metabolizable glucose-phosphate in *E. coli* is growth inhibitory and induces a specific stress response, which is coordinated by a transcription factor SgrR (17). The similar toxicity phenomenon from fructose-1-phosphate and galactose-1-phosphate in *yeast* has also been reported (18). However, the underlying cause of stress from sugar phosphates is not clear. One hypothesis is that sugar phosphate could present osmotic problems for the cells and the growth inhibition could partially be removed by addition of native compatible solutes such as sucrose and/or glucosyl-glycerol (17). In this study, expression *m1p* can decrease accumulation of mannitol-1-phosphate and produce mannitol, which has been proved its function as a compatible solute in *Synechocystis*, such that osmotic pressure is therefore relieved from cells (4).

To further detect the effect of *mtlD* expression on cell growth, WT_M (Δ*slr0168*::*Ptrc1*::*mutated mtlD*::*m1p*::*Kan*), SWW005

(Δ*slr0168*::*Ptrc1*::*m1p*::*SpΩ*, *slr1311*::*Psba2*::*mutated mtlD*::*Kan*) and SWW011 (Δ*slr0168*::*Pnrsb*::*unmutated mtlD*::*Ptrc1*::*m1p*::*Kan*) were cultivated in nickel depleted BG11 medium, where they showed similar growth phenotype (Fig.3A). The addition of nickel in the medium halted growth for WT, WT_M and SWW011 (Fig.4). This phenotype is attributable to a toxic effect of nickel, since it activated undesirable redox reactions in *Synechocystis* such that cells growth was negatively affected (19). Despite all this, the restraint level of growth in SWW011 was significantly highest due to increasing expression level of MtlD controlled by Pnrsb with presence of nickel (Fig.4). These data proved our hypothesis that overexpression *mtlD* in *Synechocystis* is highly stressful to cells and further explained the phenomenon that expression of native MtlD under control of promoters Ptrc1 or Psba2 is not achievable, since both promoters have been shown to be too strong in *Synechocystis* (8). In addition, we speculated one methionine missing on *mtlD* should likely decrease its expression activity, since this mutation is located on the non-functional linker region between the mannitol dehydrogenase C-terminal domain (pfam08125) and mannitol dehydrogenase Rossmann domain (pfam01232) (4).

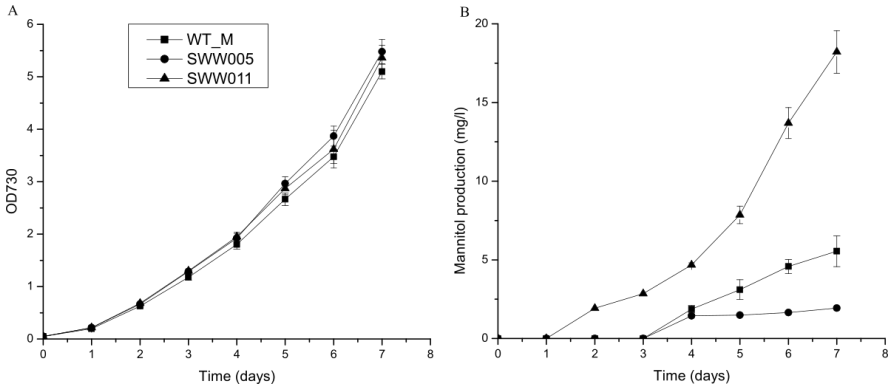


Fig.3 Growth curve and mannitol production of *Synechocystis* mutants in BG-11 medium. (A) Growth curve in cultures of WT_M, SWW005 and SWW011. **(B)** Mannitol accumulation in three different mannitol-producing strains WT_M, SWW005 and SWW011. Symbols: squares, WT_M; circles, SWW005; triangles, SWW011. Values represent the average of at least three biological replicates (error bars represent standard deviation).

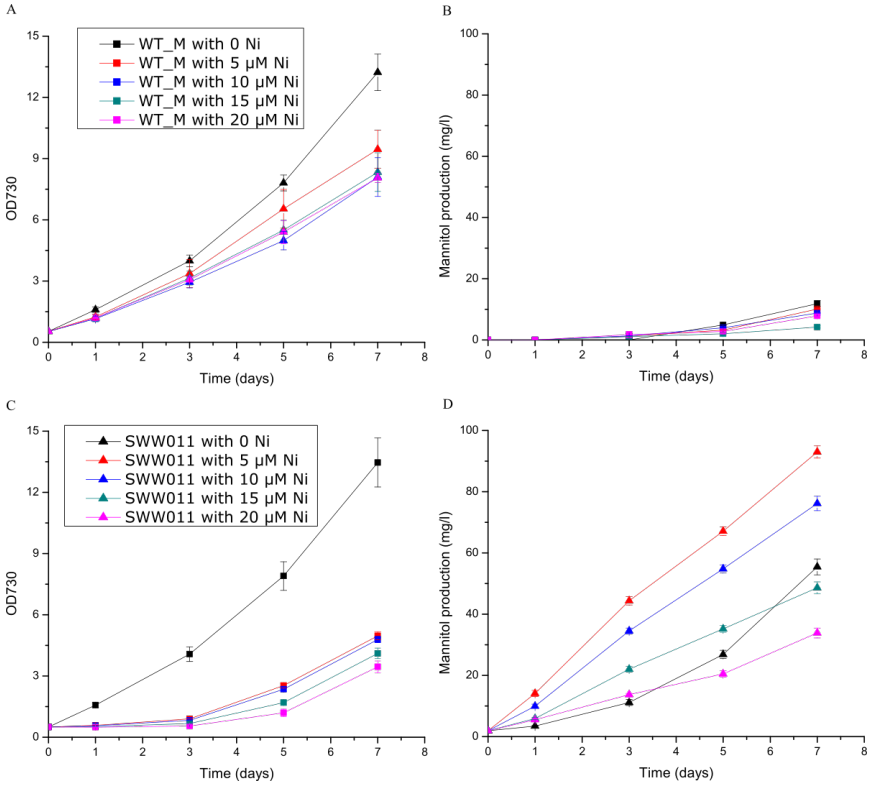


Fig.4 Growth curve and mannitol production of WT_M (A and B) and SWW011 (C and D) after induction by nickel in BG11 medium. Symbols: squares, WT_M; triangles, SWW011. Colors: black, no induction, red, induction by 5 µM nickel; blue, induction by 10 µM nickel; teal, induction by 15 µM nickel; fuchsia, induction by 20 µM nickel. Values represent the average of at least three biological replicates (error bars represent standard deviation).

Enhancement of extracellular mannitol production by fine-tuned expression *mtlD*

Mannitol production from each constructed mutant has been measured using an enzymatic kit. SWW011 showed the highest mannitol production in 7 days with absence of nickel, which reached 18.2 mg/l, while WT_M and SWW005 only produced 5.5 mg/l and 1.9 mg/l, respectively (Fig.3B). These results corroborated our previous conclusion that both the Pnrsb promoter is leaky, and that the mutation on *mtlD* has a negative impact on its activity. We also show that fine-tuned expression of *mtlD* is a potential bottleneck to increase mannitol

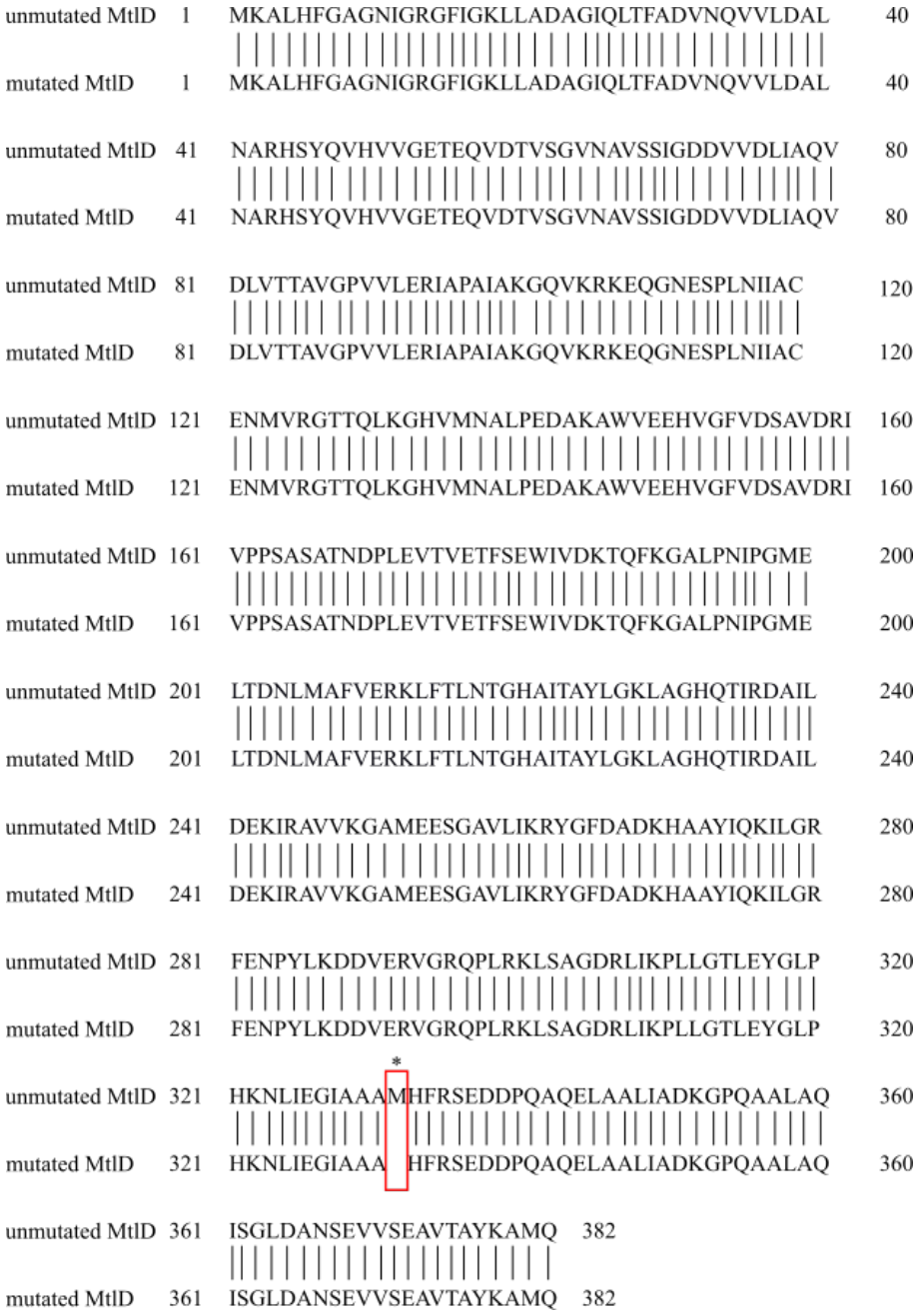
production. These results inspired us to find out the way to balance cells growth with mannitol production. In order to find a solution, different concentration of nickel was used to control promoter Pnrsb for cultivation of SWW011. We found that 5 μM nickel is suitable for mannitol production in this strain, since side-effect on cell physiology was within an acceptable range and mannitol concentration was accumulated up to 92.9 mg/l after 7-day inductions, which was the highest titer compared with WT_M and SWW011 under other concentration of nickel usage (Fig.4).

Conclusions

Our results demonstrate that mannitol production in *Synechocystis* is restricted by the formation of a toxic intermediate product – mannitol-1-phosphate. It is a big challenge to find a balance point between growth and mannitol production, since highly expression of *mtlD* is lethal to the cells, but deficient expression leads to low productivity. In this study, we tackled this problem by the usage of an inducible promoter, Pnrsb, in the presence of 5 μM nickel in the medium. Our optimized-mannitol producer was able to generate 92.9 mg/l mannitol after 7-days of induction. This represents an 8-fold increase in comparison with the engineered mannitol-producer *Synechocystis* from our previous chapter, which displayed 11.8 mg/l mannitol within the same conditions and during the same time period.

Supplementary data

Supplementary figure S1. Amino acid sequence of both native and mutated MtlD used for mannitol expression under various promoters. Red square with asterisk indicates one mutation.


unmutated MtlD 1 MKALHFGAGNIGRGFIGKLLADAGIQLTFADVNQVVLDAL 40
mutated MtlD 1 MKALHFGAGNIGRGFIGKLLADAGIQLTFADVNQVVLDAL 40
unmutated MtlD 41 NARHSYQVHVVGETEQVDTVSGVNAVSSIGDDVVDLIAQV 80
mutated MtlD 41 NARHSYQVHVVGETEQVDTVSGVNAVSSIGDDVVDLIAQV 80
unmutated MtlD 81 DLVTTAVGPVVLERIAPAIAKGQVKRKEQGNESPLNIIAC 120
mutated MtlD 81 DLVTTAVGPVVLERIAPAIAKGQVKRKEQGNESPLNIIAC 120
unmutated MtlD 121 ENMVRGTTQLKGHVMNALPEDAKAWVEEHVGFVDSAVDRI 160
mutated MtlD 121 ENMVRGTTQLKGHVMNALPEDAKAWVEEHVGFVDSAVDRI 160
unmutated MtlD 161 VPPSASATNDPLEVTVETFSEWIVDKTQFKGALPNIPGME 200
mutated MtlD 161 VPPSASATNDPLEVTVETFSEWIVDKTQFKGALPNIPGME 200
unmutated MtlD 201 LTDNLMAFVERKLFTLNTGHAITAYLGKLAGHQTIRDAIL 240
mutated MtlD 201 LTDNLMAFVERKLFTLNTGHAITAYLGKLAGHQTIRDAIL 240
unmutated MtlD 241 DEKIRAVVKGAMEESGAVLIKRYGFDADKHAAYIQKILGR 280
mutated MtlD 241 DEKIRAVVKGAMEESGAVLIKRYGFDADKHAAYIQKILGR 280
unmutated MtlD 281 FENPYLKDDVERVGRQPLRKLSAGDRLIKPLLGTLEYGLP 320
mutated MtlD 281 FENPYLKDDVERVGRQPLRKLSAGDRLIKPLLGTLEYGLP 320
*
unmutated MtlD 321 HKNLIEGIAAAMHFRSEDDPQAQELAALIADKGPQAALAQ 360
mutated MtlD 321 HKNLIEGIAAA HFRSEDDPQAQELAALIADKGPQAALAQ 360
unmutated MtlD 361 ISGLDANSEVVSEAVTAYKAMQ 382
mutated MtlD 361 ISGLDANSEVVSEAVTAYKAMQ 382

Supplementary table S1. Sequence of optimized (un)mutated *mtlD* and *m1p* used in this study for mannitol expression.

Gene	Gene sequence
Unmutated *mtlD*	ATGAAAGCTTTGCACTTTGGCGCGGGTAATATTGGACGGGGGTT TATTGGCAAGTTATTAGCGGACGCCGGAATCCAATTGACCTTTG CCGACGTTAACCAGGTAGTCTTAGATGCCTTGAATGCCCGTCAC TCCTATCAGGTGCACGTGGTGGGGGAAACCGAACAAGTTGACAC AGTATCCGGAGTAAACGCCGTGTCCTCGATTGGCGACGATGTTG TGGATCTTATCGCTCAAGTGGATTTGGTAACCACGGCTGTTGGG CCGGTGGTCTTAGAAAGAATTGCTCCGGCCATCGCTAAGGGCCA AGTGAAACGCAAAGAACAAGGAAATGAATCTCCCTTGAACATC ATTGCGTGCGAAAATATGGTGCGTGGAACTACTCAACTGAAAGG GCATGTAATGAACGCGTTGCCCGAAGACGCCAAAGCGTGGGTA GAGGAACACGTGGGCTTTGTTGATTCCGCCGTTGATCGCATTGTT CCCCCTAGTGCCTCTGCCACTAATGACCCCTTAGAAGTGACTGTT GAAACGTTCTCCGAATGGATTGTGGATAAAACGCAATTTAAAGG CGCTTTACCCAACATTCCCGGCATGGAGTTGACTGACAATCTGA TGGCCTTTGTGGAACGTAAACTCTTCACCTTAAATACTGGCCATG CCATTACCGCATACCTTGGTAAACTGGCCGGTCATCAAACCATC CGCGATGCTATTTTAGATGAAAAAATTCGCGCAGTTGTAAAGGG CGCGATGGAGGAATCCGGAGCGGTATTGATCAAGCGGTATGGCT TCGACGCAGATAAGCATGCTGCCTACATCCAAAAAATTTTGGGA CGCTTTGAAAATCCGTATTTGAAGGACGACGTTGAACGGGTGGG GCGTCAACCCCTGCGAAAATTAAGTGCGGGGGATCGTTTAATCA AACCATTACTGGGTACCTTAGAGTACGGCTTGCCCCACAAAAAT TTGATCGAAGGGATTGCCGCCGCTATGCACTTCCGGTCCGAAGA CGATCCACAAGCTCAAGAGTTGGCCGCTCTGATCGCCGATAAAG GCCCACAGGCCGCCCTAGCACAGATTAGCGGCTTGGACGCTAAT AGCGAGGTAGTATCCGAAGCTGTTACGGCCTATAAGGCCATGCA A
Mutated *mtlD*	ATGAAAGCTTTGCACTTTGGCGCGGGTAATATTGGACGGGGGTT TATTGGCAAGTTATTAGCGGACGCCGGAATCCAATTGACCTTTG CCGACGTTAACCAGGTAGTCTTAGATGCCTTGAATGCCCGTCAC TCCTATCAGGTGCACGTGGTGGGGGAAACCGAACAAGTTGACAC AGTATCCGGAGTAAACGCCGTGTCCTCGATTGGCGACGATGTTG TGGATCTTATCGCTCAAGTGGATTTGGTAACCACGGCTGTTGGG CCGGTGGTCTTAGAAAGAATTGCTCCGGCCATCGCTAAGGGCCA AGTGAAACGCAAAGAACAAGGAAATGAATCTCCCTTGAACATC ATTGCGTGCGAAAATATGGTGCGTGGAACTACTCAACTGAAAGG GCATGTAATGAACGCGTTGCCCGAAGACGCCAAAGCGTGGGTA GAGGAACACGTGGGCTTTGTTGATTCCGCCGTTGATCGCATTGTT CCCCCTAGTGCCTCTGCCACTAATGACCCCTTAGAAGTGACTGTT GAAACGTTCTCCGAATGGATTGTGGATAAAACGCAATTTAAAGG CGCTTTACCCAACATTCCCGGCATGGAGTTGACTGACAATCTGA TGGCCTTTGTGGAACGTAAACTCTTCACCTTAAATACTGGCCATG CCATTACCGCATACCTTGGTAAACTGGCCGGTCATCAAACCATC CGCGATGCTATTTTAGATGAAAAAATTCGCGCAGTTGTAAAGGG CGCGATGGAGGAATCCGGAGCGGTATTGATCAAGCGGTATGGCT TCGACGCAGATAAGCATGCTGCCTACATCCAAAAAATTTTGGGA CGCTTTGAAAATCCGTATTTGAAGGACGACGTTGAACGGGTGGG GCGTCAACCCCTGCGAAAATTAAGTGCGGGGGATCGTTTAATCA AACCATTACTGGGTACCTTAGAGTACGGCTTGCCCCACAAAAAT TTGATCGAAGGGATTGCCGCCGCTCACTTCCGGTCCGAAGACGA TCCACAAGCTCAAGAGTTGGCCGCTCTGATCGCCGATAAAGGCC CACAGGCCGCCCTAGCACAGATTAGCGGCTTGGACGCTAATAGC GAGGTAGTATCCGAAGCTGTTACGGCCTATAAGGCCATGCAA

m1p	ATGGCTGAGACCGAATGGACCCCCGAAGCCCTGTCTGGCCGCTA CGAAGAAATTAAGTCCTGCATTCCGCAACAATTGGAAGCTTATG CCCGGTTCCTACGGGAAGCAGCACCTGAAGACTTGCGTCGGTGG CAGCAGATTGCCCAGGATTTAAAGCTTGAGCTGAACTTGGAAAA CGGCAGAATTAAATACAAAAAAGAGTTTAAACCGCTAGAATTA CCCGTTGATATTTGCTATATAAGGCATGGCAAAACCCAGGGGAA CACGGAACCCCGGGTTTTCCAAGGTCAGGTTGATTATGCGAATA ACCAACTCACTCAGCAGGGACAACAGCAGGCCGCCGCTGCTGCT ACTAAGTTAGAAGCCATGGCGGCTGCCAAAGAGTTTATCCCCGA TCTCCTGCTCTCCTCTCCCTTACTGCGCGCGGTACATACCGCACA ACCCTTCGTTGACGCCAATCCCAAACCCTTATTCAGAGTGTTGCC CGAATTAGCTGAGATGGCTTTTGGCGAATGGGATAACCGTAAGG TAGCCGAATTGGAAAAAGATGACCCCGCCCACCTGTTTTACCTC CAGCAGAACGCTGTGATCAAAGCTAAAGGTCCCCACCGCATCTG CTGTCAATTATGGCAGTCGCCAGAGTGGCTTGAAGGTAAAAAGG AACTGCCGGCCGAAAATTTTTTGGAATGTTTGGACCGGCAACGT AAAGCTTTGATCAAAGTTGGCGAGATCGCTAAAGAACTATGTGG ACCCAGCTGTGGCGAACGTAAGCCCCGGGTCGCAGTTTACGGCC ATTCTATGGCCGGCGCCGCCGTATCCGTACTTTTGGGATTCGGTA AAGAAGACCAGTTGGGCTTCTTGGGTTTTGATGGCAATTATATT ATGCCCAATGCCACTCCGACCATCTTGATTCCCAACGCGAAACC G

Supplementary table S2. Sequence of promoters Ptrc1, Psba2 and Pnrsb used in this study for mannitol expression. The sequence following Ptrc1 promoter was GCTAGCATTAAAGAGGAGAAATGACAT (RBS underlined).

Promoter	Promoter sequence
Ptrc1	TTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAA CAATTTCACACA
Psba2	ATGACAACGACTCTCCAACAGCGCGAAAGCGCTTCCTTGTGGGAACAGTTT TGTCAGTGGGTGACCTCTACCAACAACCGGATTTATGTCGGTTGGTTCGGT ACCTTGATGATCCCCACCCTCTTAACTGCCACCACTTGCTTCATCATTGCCT TCATCGCCGCTCCCCCCGTTGACATCGACGGTATCCGTGAGCCCGTTGCTG GTTCTTTGCTTTACGGTAACAACATCATCTCTGGTGCTGTTGTACCTTCTTC CAACGCTATCGGTTTGCACTTCTACCCCATCTGGGAAGCCGCTTCCTTAGAT GAGTGGTTGTACAACGGTGGTCCTTACCAGTTGGTAGTATTCCACTTCCTC ATCGGCATTTTCTGCTACATGGGTCGTCAGTGGGAACTTTCCTACCGCTTAG GTATGCGTCCTTGGATTTGTGTGGCTTACTCTGCCCCCGTATCCGCTGCCAC CGCCGTATTCTTGATCTACCCCATTGGTCAAGGCTCCTTCTCTGATGGTATG CCCTTGGGTATTTCTGGTACCTTCAACTTCATGATCGTGTTCCAAGCTGAGC ACAACATCCTGATGCACCCCTTCCACATGTTAGGTGTGGCTGGTGTATTCG GTGGTAGCTTGTTCTCCGCCATGCACGGTTCCTTGGTAACCTCCTCCTTGGT GCGTGAAACCACCGAAGTTGAATCCCAGAACTACGGTTACAAATTCGGTC AAGAAGAAGAAACCTACAACATCGTTGCCGCCCACGGCTACTTTGGTCGGT TGATCTTCCAATATGCTTCTTTCAACAACAGCCGTTCCTTGCACTTCTTCTT GGGTGCTTGGCCTGTAATCGGCATCTGGTTCACTGCTATGGGTGTAAGCAC CATGGCGTTCAACCTGAACGGTTTCAACTTCAACCAGTCCATCTTGGATAG CCAAGGCCGGGTAATCGGCACCTGGGCTGATGTATTGAACCGAGCCAACA TCGGTTTTGAAGTAATGCACGAACGCAATGCCCACAACTTCCCCCTCGACT TAGCGTCTGGGGAGCAAGCTCCTGTGGCTTTGACCGCTCCTGCTGTCAACG GTTAA

Pnrsb	TTAAGAATGGACTATCGGTACAGAAAAAATGGGTAACTGGATGGTGAATA AACTTCCCTTACCCAATGCACTCTCCACCGTTAAAGACCCCCTATGCTTAAC GGTGATCACCTGGGCAATGGCGAGTCCCAACCCTGTCCCCCCCGTTTTGCG CGAACGATCTCGATTAACTCGGTAAAAACGCTCAAAAATGTGTTCCTGTTG GTCGGGGGCAATGCCGATGCCGGTATCTTGCACGGTGATGATAGCCATCTG TTCATGGGATGTCAGGGTAATATCAACACGTCCCCCAGCAGTTGTGTATTG AATGGCGTTGGCAATTAGGTTTGAGACCAGTCGATAGAGTTGGGATTCATT ACCCCAGGCGTAAACTTCCCCTGAACTCAGATCACTGCTGAGATCAATGTG GGCGGCGATCGCTAATTCTAAAAACTCTTCGGTGAGGTCACTGACTAAATC ATTTAAACAACAAAGCCGCCAATCTTCGGCGGTGGTTTCCTGCTCTAAGCG ACTTAGTAGCAATAAATCCGTAATCAATTGGCTTAATCGCCTTCCCTGTCGT TCAACGGTATGTAGCATGGTGTTAATTTCTGGGGAATGGCTTGAGTCGATG CGTAATACCGCTTCCACCGTGGCCAACAGACTAGCCAATGGCGATCGTAAT TCATGGGCTGCATTCGCGGTGAATTGTTGTTGTTGTTGGTAGGACTGGTAA ATGGGACGCATGGCTAACCCCGCTAAGCCCCAACTGGAGAAGGCGACCAA ACCCAGGGCAATGGGAAAACTAAGCCCTAAAATCCAAAGAATACGTTTAT TTTCGGCATCAAAGGCTGCCAGGCTCCGGCCAATTTGTAGATAGCCCCAGG AAGATTTGTCTGTATTACCGGCGCTATGCAAAATGGTGGTGAATTGTCGAT ACCGATCGCCGGTTGGGGGGTGAATAGTCTGCCAAGTTTCCTGGTTAAAAA TGGAGGATAGGGAAGCCGGTTGATTAGGCGAAAAAGCCAGCAGGTTGCCT TGATAATCAAATAAACGAATGTAATATAAACTGCGATCACTAATGCCCAAC GTGTGACGTTCAATCAGGGTGGGGTTGACCTGGCAGGGTTGGTTGACCAAA CACAGATCGGGCAACATTTTTTGTAATACTCCGGTGGGACTAGCATTACTC GGCAACATCGGCTCTAAACTGTCATGCAACGTCCCGGCGATCGACTCCACT TCTCGCTCCAACGCCATCCAGTTGGCCTGCACAATGGCACGATAAACCCCC AACCCCAACAGGGTAAGAATTCCCCCCATTACTAGGGCATACCAGAAAGC CAATTGCAGACGACTACGGGCAAAGAGGCGACGGGTATTCATGGCGATAG GGTGAACCGATAGCCTTGACCGGGAACTGTTTTAATTGGGCAAGGACAATT TTGTTGAGCTAGCTTGCGTCGTATCAAACGCATTTGGGCCGCCACCACATT ACTCATGGGCTCCTCATCAAGATCCCACAGTTGTTGCCGGATCTTGCTACC GGAAATGATCCGCTCTGGGTTTTGCATCAGATATTGAAAAATTTGAAATTC TCTTACGGTTAAAGCAATTTCCTGTCTTTCTAGGTTTAGTGGCTCCGAGATA GTTACCGATAACAGATTATTACTGGGATCAAGGCTGAAGTTGCCCAAAGTT AAAATTTGCGGTTGGAATTGTGGCGATCGCCGTTGTAGTGCCCGCAGTCTT GCTAATAGCTCTGCCATCACAAACGGTTTTGTTAGATAGTCATCTGCCCCG GCATCTAGTCCTTCGACACGGTTTTCCGGTTCTCCTAACGCTGTTAACATCA ACACCGGCAAGGAATTACCCTGGGTTCTCAGTTTTTGACAGAGTTCCAAAC CCGATAATCCCGGCAGTAACCAATCCACAATGGCAAGGGTGTATTCCGTCC ATTGATTTTCCAAATAATCCCAAGCTTGGGAGCCATCCGTCACCCAATCCA CCACATACTTTTCACTAACTAGCACTTTCTTAATAGCCATTCCCAAATCCGT CTCATCTTCCACCAGCAAAATTCGCATCGCCTCTGCCTTTTTTATAACGGTC TGATCTTAGCGGGGGAAGGAGATTTTCACCTGAATTTCATACCCCCTTTGG CAGACTGGGAAAATCTTGGACAAATTCCCAATTTGAGGT

Supplementary table S3. Primers used in this study. Restriction enzyme sites are shown as underlined.

Primer name	Sequence	Purpose
m1p_F	5'-CATATGGCTGAGACCGAATGGACCCC-3'	Amplification of optimized *m1p* and addition of a NdeI restriction site at the 5′
m1p_R	5'-CCTAGGTTATTACGGTTTCGCGTTGGGAATC-3'	Amplification of optimized *m1p* and addition of an AvrII restriction site at the 3′
Omega_F	5'-GTCGACAATTCCGGGGATCCTCTAGA-3'	Amplification of streptomycin resistance cassette and addition of a SalI restriction site at the 5'
Omega_R	5'-GTCGACATGAATCCTTAATCGGTACC-3'	Amplification of streptomycin resistance cassette and addition of a SalI restriction site at the 3'
mtlD_F1	5'-CATATGAAAGCTTTGCACTTTGGCGC-3'	Amplification of optimized (un)mutated *mtlD* and addition of a NdeI restriction site at the 5'
mtlD_R1	5'-GGATCCTTGCATGGCCTTATAGGCCG-3'	Amplification of optimized (un)mutated *mtlD* and addition of a BamHI restriction site at the 3'
pNrsB_F1	5'-TCTAGATTAAGAATGGACTATCGGTA-3'	Amplification of Pnrsb and addition of a XbaI restriction site at the 5′
pNrsB_R1	5'-AAGTGCAAAGCTTTCATATGACCTCAAATTGGGAATTTGT-3'	Amplification of Pnrsb and fused with (un)mutated *mtlD*
mtlD_F2	5'-ACAAATTCCCAATTTGAGGTCATATGAAAGCTTTGCACTT-3'	Amplification of (un)mutated *mtlD* and fused with Pnrsb
mtlD_R2	5'-CCTAGGTTGCATGGCCTTATAGGCCG-3'	Amplification of (un)mutated *mtlD* and addition of an AvrII restriction site at the 3'
mtlD_R3	5'-TCTAGATAGTAAAATAATAAAAAAGCCG-3'	Amplification of (un)mutated *mtlD* and addition of a XbaI restriction site at the 3'

Reference

1. Wisselink H., Weusthuis R., Eggink G, Hugenholtz J, Grobben G. Mannitol production by lactic acid bacteria: a review. Int Dairy J. 2002 Jan 1;12(2–3):151–61.
2. Dai Y, Meng Q, Mu W, Zhang T. Recent advances in the applications and biotechnological production of mannitol. J Funct Foods. 2017;36:404–9.
3. Branco dos Santos F, Du W, Hellingwerf KJ. *Synechocystis*: Not Just a Plug-Bug for CO_2, but a Green E. coli. Vol. 2, Frontiers in Bioengineering and Biotechnology . 2014. p. 36.
4. Wu W, Du W, Gallego RP, Hellingwerf KJ, van der Woude AD, Branco dos Santos F. Using osmotic stress to stabilize mannitol production in *Synechocystis* sp. PCC6803. Biotechnol Biofuels. 2020;13(1):117.
5. van Alphen P, Abedini Najafabadi H, Branco dos Santos F, Hellingwerf KJ. Increasing the photoautotrophic growth rate of *Synechocystis* sp. PCC 6803 by identifying the limitations of its cultivation. Biotechnol J. 2018;13(8):1700764.
6. Angermayr SA, Hellingwerf KJ. On the use of metabolic control analysis in the optimization of cyanobacterial biosolar cell factories. J Phys Chem B. 2013;117(38):11169–75.
7. Russell DW, Sambrook J. Molecular cloning: a laboratory manual. Vol. 1. Cold Spring Harbor Laboratory Cold Spring Harbor, NY; 2001.
8. Angermayr SA, Van der Woude AD, Correddu D, Vreugdenhil A, Verrone V, Hellingwerf KJ. Exploring metabolic engineering design principles for the photosynthetic production of lactic acid by *Synechocystis* sp. PCC6803. Biotechnol Biofuels. 2014;7(1):99.
9. Cheah YE, Albers SC, Peebles CAM. A novel counter-selection method for markerless genetic modification in *Synechocystis* sp. PCC 6803. Biotechnol Prog. 2013;29(1):23–30.
10. Heidorn T, Camsund D, Huang H-H, Lindberg P, Oliveira P, Stensjö K, et al. Chapter Twenty-Four - Synthetic Biology in Cyanobacteria: Engineering and Analyzing Novel Functions. In: Voigt CBT-M in E, editor. Synthetic Biology, Part A. Academic Press; 2011. p. 539–79.
11. Jacobsen JH, Frigaard N-U. Engineering of photosynthetic mannitol biosynthesis from CO_2 in a cyanobacterium. Metab Eng. 2014 Jan

1;21:60–70.
12. Pade N, Erdmann S, Enke H, Dethloff F, Dühring U, Georg J, et al. Insights into isoprene production using the cyanobacterium *Synechocystis* sp. PCC 6803. Biotechnol Biofuels. 2016;9(1):89.
13. Yoshikawa K, Hirasawa T, Shimizu H. Effect of malic enzyme on ethanol production by *Synechocystis* sp. PCC 6803. J Biosci Bioeng. 2015;119(1):82–4.
14. Ferreira EMA. Evaluation and improvement of *Synechocystis* sp. PCC 6803 tolerance to temperature and development of Synthetic Biology tools. 2014;
15. Eriksson J, Salih GF, Ghebramedhin H, Jansson C. Deletion Mutagenesis of the 5′ psbA2 Region in *Synechocystis* 6803: Identification of a Putative cis Element Involved in Photoregulation. Mol Cell Biol Res Commun. 2000;3(5):292–8.
16. Dühring U, Baier K, Germer F, Shi T. Genetically enhanced Cyanobacteria for the production of a first chemical compound harbouring zn^{2+}, co^{2+} or ni^{2+}-inducible promoters. Google Patents; 2017.
17. Vanderpool CK. Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress. Curr Opin Microbiol. 2007 Apr 1;10(2):146–51.
18. Gibney PA, Schieler A, Chen JC, Bacha-Hummel JM, Botstein M, Volpe M, et al. Common and divergent features of galactose-1-phosphate and fructose-1-phosphate toxicity in yeast. Mol Biol Cell. 2018;29(8):897–910.
19. Küpper H, Kroneck PMH. Nickel in the environment and its role in the metabolism of plants and cyanobacteria. Met Ions Life Sci. 2007;2:31–62.