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RESEARCH ARTICLE

Applied Cellular Physiology and Metabolic Engineering

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

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Abstract

D-Mannitol production was achieved in freshwater *Synechocystis* sp. PCC6803 via the heterologous expression of mannitol-1-phosphate dehydrogenase (*mtID*) and mannitol-1-phosphatase (*m1p*) under control of the strong promoter P_{trc1} . However, only 5.54 mg L^{-1} of mannitol was found extracellularly after 7 days of cultivation, likely due to insufficient expression of a mutated *mtID* lacking a methionine at position 332. This study compared mannitol levels using different promoters (P_{trc1} , P_{psbA2} and P_{nrsB}) to control the expression of (un)mutated versions of *mtID* in *Synechocystis* with co-expression of *m1p*. Our data suggest that even without the inducer, the weakest promoter, P_{nrsB} , can support the expression of an unmutated *mtID* in *Synechocystis*, which leads to 18.2 mg L^{-1} of mannitol in 7 days without induction. Such titer is already much higher than the first engineered mannitol-producing *Synechocystis*. When $5 \mu\text{M}$ nickel sulfate was added to the medium as an inducer, mannitol production could significantly be increased further, up to 92.9 mg L^{-1} after 7 days of induction, but it partially inhibited growth. Attempts with the other increasingly stronger promoters always failed to express the unmutated *mtID*, probably due to the toxicity caused by the accumulation of the intermediate product, mannitol-1-phosphate. These results clearly suggest that the expression level of *mtID* is the bottleneck in achieving a high yield of mannitol in *Synechocystis*, and consequently, that mannitol production can be enhanced by fine-tuning its expression. Future research is needed to identify bottlenecks that hinder mannitol productivity and long-term stability, facilitating the engineering of more efficient mannitol-producing cyanobacterial strains.

KEYWORDS

(D-)mannitol, Mannitol-1-phosphate dehydrogenase, P_{nrsB} , sustainability, *Synechocystis* sp. PCC6803

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1 | INTRODUCTION

D-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 properties of mannitol add significant value across the medical, pharmaceutical, food, and chemical industries.² Therefore, the development of low-cost mannitol production in an environmentally friendly way has gained much attention. Compared with either using *Escherichia coli*, yeast, or plants to synthesize mannitol, cyanobacteria present several advantages: (i) *E. coli* and yeast rely on organic nutrients, like for example glucose and fructose, to survive, but a cyanobacterium only requires (sun)light with basic inorganic compounds to maintain fundamental metabolic requirements, which drastically lowers costs; (ii) the cultivation of cyanobacteria has the potential to be carried out on non-arable land, which, unlike plants, can refrain from exerting further pressure on natural resources³; and (iii) achieving mannitol production in cyanobacteria shows a huge potential for large-scale cultivation in the future under different cultivation set-ups.^{4,5}

In our previous study,⁶ the sustainable production of mannitol via the direct conversion of CO₂ in the freshwater cyanobacterium, *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis*) was achieved by heterologous expression of mannitol-1-phosphate dehydrogenase (*mtlD*) and mannitol-1-phosphatase (*m1p*). Although stable, it unfortunately displayed both a very low yield and productivity. Mannitol production was only 5.54 mg L⁻¹ in 7 days of cultivation with red light of moderate intensity (~50 μmol photons m⁻² s⁻¹).⁶ This low efficiency of mannitol production definitely limits its applicability at an industrial scale. One of the culprits leading to such a low yield is due to the insufficient expression level of the mutated *mtlD*. In the previous study,⁶ a fragment containing the fused mannitol cassette (*mtlD* and *m1p*) was introduced into the neutral site *slr0168* in *Synechocystis*, but a methionine was stubbornly missing at position 332 of *mtlD*. Nonetheless, even with this mutated *mtlD*, mannitol could still be synthesized by cells, albeit at the low level indicated above.⁶

In this study, we engineered several plasmids for the expression of (un)mutated *mtlD* under the control of different promoters with varying strength (Supplementary Table S2). The different constructs were engineered in *E. coli*, and subsequently transferred to *Synechocystis* for integration into the chromosome. The data showed that only the weakest promoter P_{nrsB} can be used to control the expression of the intact *mtlD* (i.e., without any sequence deletions and/or substitutions) for production of mannitol in *Synechocystis*. Moreover, induction by nickel using the nickel-inducible promoter P_{nrsB} led to increased mannitol production. However, under these conditions, excessive mannitol accumulation eventually became lethal to the cells. To balance cell growth with mannitol productivity, 5 μM Ni²⁺ was determined as the most suitable inducer concentration for mannitol expression, which results in the production of 92.9 mg L⁻¹ in 7 days. Mannitol production in *Synechocystis* was therefore increased nearly 8 times after fine-tuned expression of *mtlD*. Thus, using the inducible promoter P_{nrsB} 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 (Figure 1a).

2 | METHODS AND MATERIALS

2.1 | Strains and culture conditions

Strains of *E. coli* were grown in LB liquid medium at 37 °C in a shaking incubator at 200 rpm, or on solid plates containing 1.5% agar. Kanamycin (50 mg L⁻¹), spectinomycin (50 mg L⁻¹) or ampicillin (100 mg L⁻¹) were added to LB media, either separately or in combination, to select mutants when necessary.

Synechocystis, a glucose-tolerant wild type obtained from D. Bhaya, Stanford University, Stanford CA, was cultivated in a modified BG11 medium⁷ with 25 mM PIPPS buffer (PH = 8.0) at 30 °C, either in a shaking incubator at 120 rpm under continuous 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 media, either separately or in combination, to select mutants with appropriate concentrations as follows: kanamycin (50 mg L⁻¹) and/or spectinomycin (20 mg L⁻¹). Growth was measured by monitoring optical density (OD) at 730 nm using a standard laboratory spectrometer.

2.2 | Plasmid construction

In order to express *m1p* under the control of promoter P_{trc1} in *Synechocystis* (Supplementary Table S2), the plasmid pWW001 (P_{trc1-m1p-SpeΩ}), a derivative of pKH020, containing the P_{trc1}-driven codon optimized *L-ldh* cassette of *L. lactis subsp. cremoris* MG1363 was modified⁸ 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 pUCm1p⁶ and used to replace the *L-ldh* cassette of plasmid pKH020 according to standard cloning techniques,⁹ creating plasmid pWW002 (P_{trc1-m1p-Kan}). The spectinomycin resistance cassette was amplified from plasmid pSHH with primers (Supplementary Table S3) introducing flanking cloning sites for Sall to replace the kanamycin cassette of pWW002¹⁰; the new plasmid containing the *m1p* gene under promoter P_{trc1} with the spectinomycin resistance cassette was named pWW001 (P_{trc1-m1p-SpeΩ}).

To obtain plasmids pWW003 (P_{psbA2-unmutated mtlD-Kan}) and pWW004 (P_{psbA2-mutated mtlD-Kan}), which were used to express either the native or mutated *mtlD* under promoter P_{psbA2} (Supplementary Table S2), respectively, in *Synechocystis*, the plasmid pKH009 carrying a gene encoding a reporter enzyme (*lacZ*) under promoter P_{psbA2} was modified.¹⁰ Briefly, native and mutated *mtlD* were PCR amplified from pKHmtlD and WT_M (Δ*slr0168*::P_{trc1}::mutated *mtlD*::*m1p*::*Kan*),⁶ respectively; meanwhile, two restriction enzyme sites, NdeI and BamHI, were introduced at the ends for both

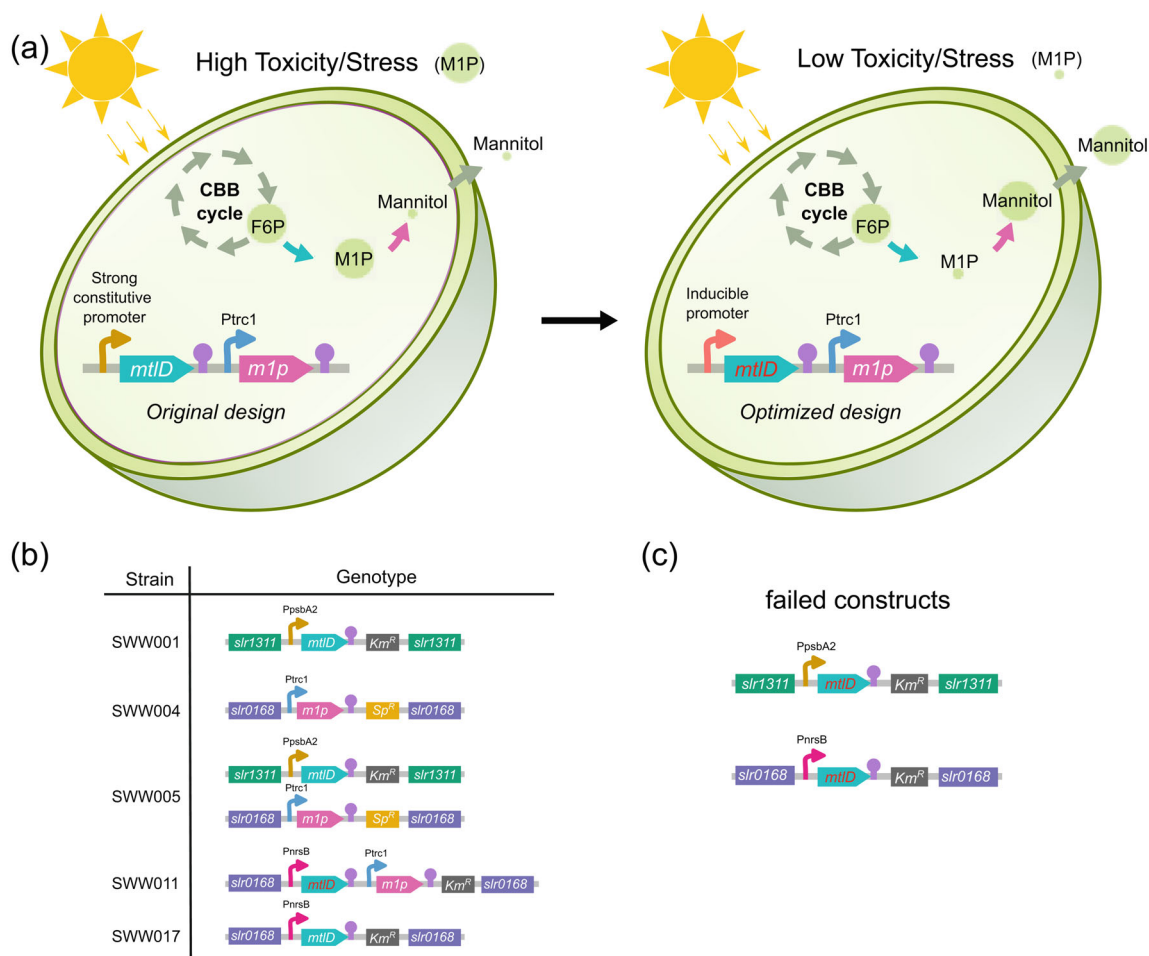


FIGURE 1 Overview of the Engineered Mannitol Biosynthetic Pathway in *Synechocystis*. (a) overall pathway design before/after optimization by fine-tuned expression of *mtlD* via an inducible promoter system. (b) constructs successfully developed in this study. (c) constructs that consistently failed despite multiple attempts. Mutated *mtlD* is shown in white; native *mtlD* is shown in red. F6P, fructose-6-phosphate; M1P, mannitol-1-phosphate; *mtlD*, mannitol-1-phosphate dehydrogenase from *E. coli*; *m1p*, Mannitol-1-phosphatase from *Eimeria tenella*; *Km^R*, kanamycin resistance gene; *Sp^R*, spectinomycin resistance gene.

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

To construct plasmids used for the expression of either native or mutated *mtlD* under the inducible promoter *P_{nrsB}*, the nickel-inducible operon composed of *nrsS*, *nrsR*, and *PnrsB* was first amplified by PCR using *Synechocystis* wild type as the template¹¹ (Supplementary Table S2). The native or mutated *mtlD* fragment was fused with the nickel-inducible promoter, respectively, by overlap PCR, and the restriction enzyme sites *XbaI* and *AvrII* at the two ends of the fused fragment were also introduced via the primers (Supplementary Table S3). Subsequently, the *L-Idh* cassette with the promoter *P_{trc1}* of plasmid pHKH020 was replaced by the fused fragments according to standard cloning techniques.⁹ The new plasmids that can drive the expression of native or mutated *mtlD* under *P_{nrsB}* were named pWW005 (*P_{nrsB}*-unmutated *mtlD*-*Kan*) and pWW006 (*P_{nrsB}*-mutated *mtlD*-*Kan*), respectively. Then, the fragment of *P_{nrsB}* and native *mtlD*,

including the transcriptional terminator, were PCR amplified from pWW005 (*P_{nrsB}*-unmutated *mtlD*-*Kan*) and an *XbaI* restriction site was inserted at both ends via primers (Supplementary Table S3). Meanwhile, the plasmid pWW002 (*P_{trc1}*-*m1p*-*Kan*) was linearized via digestion by *XbaI*, and native *mtlD* under *P_{nrsB}* with the terminator was inserted by ligation to create a new plasmid that contains native *mtlD* under the control of *P_{nrsB}* and *m1p* under the control of *P_{trc1}*, named pWW007 (*P_{nrsB}*-unmutated *mtlD*-*P_{trc1}*-*m1p*-*Kan*). All the plasmids were sequenced before integration into the chromosome of *Synechocystis* to confirm the absence of mutations. All primers used are listed in the supplementary file (Supplementary Table S3).

2.3 | Natural transformation of *Synechocystis*

Natural transformation for genomic integration in *Synechocystis* was performed as in the previous study,¹² using kanamycin (50 mg L⁻¹) and/or spectinomycin (20 mg L⁻¹) as selection pressure to drive full

segregation. Briefly, 2 µg of specific plasmid DNA was mixed with 200 µL concentrated *Synechocystis* cells harvested during the exponential phase of growth (1 mL, OD₇₃₀ = 1) and incubated in a white light incubator with ~50 µmol photons m⁻² s⁻¹ for 5 h. Then, mixed cells were spread on a commercial membrane (Pall Corporation, USA) which was placed on a BG11 solid plate. After incubation for 24 h under white light, the membrane was transferred onto a new solid BG11 plate with specific antibiotics until individual colonies could be isolated. The full segregation status of mutants was achieved by propagation in the presence of antibiotics and confirmed by colony PCR, using appropriate primers (Supplementary Table S3). Sequencing results were used to confirm the correct insertion.

2.4 | D-Mannitol assay

Extracellular mannitol concentrations were determined in the supernatant of *Synechocystis* cultures using a D-Mannitol-L-Arabitol Assay Kit (Megazyme). Cells from the selected culture were collected by centrifugation for 5 min under 14,000 rpm, and 100 µL of supernatant was used for extracellular mannitol measurement according to the instructions from the kit.

2.5 | Growth curve determination

The *Synechocystis* mutants used in this study were revived from a glycerol stock (~16%) in BG11 in a red light incubator with 50 µmol photons m⁻² s⁻¹ until OD₇₃₀ reached 1. Then, the equivalent of an initial OD 0.05 of cells was transferred from precultures and cultivated in 20 mL fresh BG11 in 100 mL shake flasks. The growth curves were determined by monitoring OD₇₃₀ over time.

To study mannitol production from mutants influenced by nickel as an 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 OD₇₃₀ reaches roughly 0.5. Then 0, 3, 5, 10, 15, and 20 µM nickel were added to the BG11 medium, respectively. The growth curves were again determined by measuring OD₇₃₀ over time using a spectrophotometer.

2.6 | Prolonged serial cultivation of SWW011

To study the stability of mannitol production in SWW011 under the optimized induction condition, SWW011 was cultivated in 10 mL liquid BG-11 medium with 5 µM nickel in a 50 mL shake flask under the previously mentioned cultivation conditions. Cells were diluted and inoculated into fresh medium with 5 µM nickel at OD₇₃₀ of 0.5 after 4 days of cultivation to maintain the growth phase in three parallel cultures. The final OD₇₃₀ and mannitol titer were measured every 4 days as previously described above. The mannitol titer (mg L⁻¹ OD⁻¹) of SWW011 with 5 µM nickel induction at the 4th day prior to dilution was defined as 100%.

TABLE 1 Plasmids and strains used in this study.

Plasmid and strains	Description	Reference
pHSH	Ap ^r SpeΩ ^r , Omega cassette	10
pHKHmtID	Ap ^r , containing optimized native <i>mtID</i>	6
pUC57m1p	Ap ^r , containing optimized <i>m1p</i>	6
pHKH020	Ap ^r Kan ^r , P _{trc1} -driven codon optimized <i>L-lidh</i> cassette of <i>L. lactis</i> sub sp. <i>cremoris</i> MG1363 containing upstream and downstream fragments of <i>slr0168</i>	8
pWW001	Ap ^r SpeΩ ^r , pHKH020 derivative used to express <i>m1p</i> under control of promoter P _{trc1} at <i>slr0168</i> gene locus	In this work
pWW002	Ap ^r Kan ^r , pHKH020 derivative used to express <i>m1p</i> under control of promoter P _{trc1} at <i>slr0168</i> gene locus	In this work
pHKH009	Ap ^r Kan ^r , P _{psbA2} -driven lacZ at <i>slr1311</i>	8
pWW003	Ap ^r Kan ^r , pHKH009 derivative used to express native <i>mtID</i> under control of promoter P _{psbA2} at <i>slr1311</i> gene locus	In this work
pWW004	Ap ^r Kan ^r , pHKH009 derivative used to express mutated <i>mtID</i> under control of promoter P _{psbA2} at <i>slr1311</i> gene locus	In this work
pWW005	Ap ^r Kan ^r , pHKH020 derivative used to express native <i>mtID</i> under control of promoter P _{nrsB} at <i>slr0168</i> gene locus	In this work
pWW006	Ap ^r Kan ^r , pHKH020 derivative used to express mutated <i>mtID</i> under control of promoter P _{nrsB} at <i>slr0168</i> gene locus	In this work
pWW007	Ap ^r Kan ^r , pHKH002 derivative used to express native <i>mtID</i> under control of promoter P _{nrsB} and <i>m1p</i> under control of promoter P _{trc1} at <i>slr0168</i> gene locus	In this work
WT	<i>Synechocystis</i> sp. PCC6803 wild type	D. Bhaya
WT_M	Δ <i>slr0168</i> ::P _{trc1} ::mutated <i>mtID</i> :: <i>m1p</i> ::Kan	6
SWW001	<i>slr1311</i> ::P _{psbA2} ::mutated <i>mtID</i> ::Kan	In this work
SWW004	Δ <i>slr0168</i> ::P _{trc1} :: <i>m1p</i> ::SpeΩ	In this work
SWW005	Δ <i>slr0168</i> ::P _{trc1} :: <i>m1p</i> ::SpeΩ, <i>slr1311</i> ::P _{psbA2} ::mutated <i>mtID</i> ::Kan	In this work
SWW011	Δ <i>slr0168</i> ::P _{nrsB} ::unmutated <i>mtID</i> ::P _{trc1} :: <i>m1p</i> ::Kan	In this work
SWW017	Δ <i>slr0168</i> ::P _{nrsB} ::mutated <i>mtID</i> ::Kan	In this work

Note: Ap^r, ampicillin resistance cassette; SpeΩ^r, spectinomycin resistance cassette; Kan^r, kanamycin resistance cassette.

3 | RESULTS AND DISCUSSION

3.1 | Repeated attempts to express the native *mtID* in the absence of M1p in *Synechocystis* consistently failed

The viability of mannitol-producing mutants of cyanobacteria is well documented in the scientific literature,^{6,13,14} as summarized in the Supplementary Table S4. In fact, a synthetic mannitol cassette

consisting of mannitol-1-phosphate dehydrogenase (*mtlD*) and mannitol-1-phosphatease (*m1p*) has been used to produce mannitol in the freshwater cyanobacterium *Synechocystis*,⁶ *Synechococcus elongatus*,¹⁴ and the marine cyanobacterium *Synechococcus* sp. PCC7002 (hereafter, *Synechococcus*).¹³ However, a methionine insistently remained missing at position 332 of *mtlD* in *Synechocystis*⁶ and an incompletely segregated mannitol cassette in the chromosome of *Synechococcus*¹³ proved that metabolic engineering for mannitol production in cyanobacteria remained, to a certain extent, challenging. In this study, we used different types of promoters to precisely control the expression level of *mtlD* for optimization of the pathway of mannitol synthesis in *Synechocystis*.

To implement this idea, the plasmids carrying either the native or the mutated *mtlD* (Supplementary Figure S1 and Table S1) and/or *m1p* (Supplementary Table S1) were constructed in *E. coli* (Figure 1b,c, Table 1). In the previous study, the native *mtlD* under P_{trc1} could not be cloned in *E. coli*, so fusion fragments that contained an expression system of native *mtlD* and *m1p* under P_{trc1} , instead of plasmids, were introduced into the chromosome of *Synechocystis* at the neutral site *slr0168*. However, inadvertently, a methionine was stubbornly left missing at position 332 of *mtlD* in *Synechocystis*. Nevertheless, constructs expressing this mutated *mtlD* were still found to produce mannitol.⁶ We hypothesized then that the overexpression of *mtlD* is stressful for both *E. coli* and *Synechocystis*. Therefore, here, weaker promoters of *Synechocystis* were used to control the expression level of (un)mutated *mtlD*.

The P_{psbA2} promoter has been widely used in light-driven production of isoprene and ethanol in *Synechocystis*^{15,16} and the transcripts from P_{psbA2} were concluded to be approximately 10 times less abundant than those originating from P_{trc1} .¹⁷ In this study, the plasmids used for (un)mutated *mtlD* expression controlled by native P_{psbA2} (*slr1311*)¹⁸ were constructed in *E. coli*, named as pWW003 (P_{psbA2} _unmutated *mtlD*_Kan) and pWW004 (P_{psbA2} _mutated *mtlD*_Kan), respectively. However, we were not able to transform pWW003 in neither WT nor SWW004 (Δ *slr0168*:: P_{trc1} ::*m1p*::*Sp* Ω), but P_{psbA2} with mutated *mtlD* was successfully integrated in both of these strains and named as SWW001 (*slr1311*:: P_{psbA2} ::mutated *mtlD*::Kan) and SWW005 (Δ *slr0168*:: P_{trc1} ::*m1p*::*Spe* Ω , *slr1311*:: P_{psbA2} ::mutated *mtlD*::Kan). This suggested that native *mtlD* under P_{psbA2} showed a higher enzymatic activity 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 P_{nrsB} in *Synechocystis*. The P_{nrsB} promoter has been characterized as a nickel inducible promoter in this genus, with its expression level being dependent on the presence of nickel ions in the medium.¹⁹ In this case, if correct, *mtlD* can be expressed in *Synechocystis* under control of P_{nrsB} in the absence of nickel, and cell growth will be increasingly inhibited as the concentration of nickel increases. This would prove that the expression of the native *mtlD* itself is a big burden for the *Synechocystis* cells. However, the native *mtlD* controlled by P_{nrsB} is still unable to be integrated into the chromosome of *Synechocystis* in the absence of nickel, unless accompanied by the co-expression of M1p. This strongly suggests that the promoter P_{nrsB} is leaky in this strain,

since the remaining expression of *mtlD* in the absence of nickel is sufficient to impose too much of a fitness burden such that it cannot be tolerated by the cells. The protein M1p, when co-expressed, is sufficient to release the pressure caused by the over-accumulation of mannitol-1-phosphate via its degradation to less stressful mannitol, and therefore, cells then thrive.

Mannitol-1-phosphate is not the first sugar phosphate that has been found (when accumulating in the cytosol) to be a burden to cells. In a previous study, accumulation of a non-metabolizable glucose-phosphate in *E. coli* was found to inhibit growth and induce a specific stress response, which is coordinated by the transcription factor SgrR.²⁰ A similar toxicity phenomenon has also been reported for fructose-1-phosphate and galactose-1-phosphate in yeast,²¹ and also in *Synechocystis*.²² 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 the addition of native compatible solutes such as sucrose and/or glucosyl-glycerol.²⁰ In this study, *m1p* expression can decrease accumulation of mannitol-1-phosphate and produce mannitol, which has been proven to function as a compatible solute in *Synechocystis*, such that osmotic pressure is therefore relieved from cells.⁶

To further detect the effect of *mtlD* expression on cell growth, WT_M (Δ *slr0168*:: P_{trc1} ::mutated *mtlD*::*m1p*::Kan), SWW005 (Δ *slr0168*:: P_{trc1} ::*m1p*::*Spe* Ω , *slr1311*:: P_{psbA2} ::mutated *mtlD*::Kan) and SWW011 (Δ *slr0168*:: P_{nrsB} ::unmutated *mtlD*:: P_{trc1} ::*m1p*::Kan) were cultivated in nickel-depleted BG11 medium, where they showed a similar a growth phenotype (Figure 2a). The addition of nickel to the medium halted growth for both strains WT_M and SWW011 (Figure 3a,c). This phenotype has been attributed to a toxic effect of nickel, since it activates undesirable redox reactions in *Synechocystis*.²³ Irrespective of the latter, the inhibitory effect of nickel on the growth of SWW011 was significantly higher, assumedly due to the increased expression level of MtlD controlled by P_{nrsB} (Figure 3c). These data support our hypothesis that the overexpression of *mtlD* in *Synechocystis* is highly stressful to cells. Furthermore, it also sheds light on why the expression of only the native MtlD under the control of P_{trc1} or P_{psbA2} is not achievable, since both promoters have been shown to be strong promoters in *Synechocystis*.¹⁰ The toxicity of high MtlD expression is decreased when a methionine located on the presumably non-functional linker region between the mannitol dehydrogenase C-terminal domain (pfam08125) and the mannitol dehydrogenase's Rossmann domain (pfam01232) is missing from the sequence of MtlD, likely decreasing its activity.⁶

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

Mannitol production from each constructed mutant has been measured using an enzymatic kit. SWW011 showed the highest mannitol production in 7 days with the absence of nickel, which reached 18.2 mg L⁻¹, while WT_M and SWW005 only produced 5.5 and

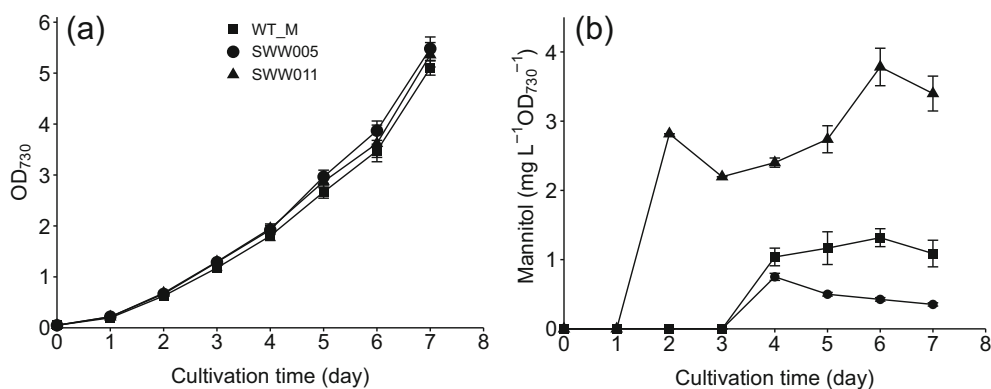


FIGURE 2 Growth curve and mannitol production of *Synechocystis* mutants in BG-11 medium. (a) Growth curves of WT_M, SWW005, and SWW011 cultures. (b) Mannitol accumulation in three different mannitol-producing strains WT_M, SWW005, and SWW011. Symbols: Squares, WT_M; circles, SWW005; triangles, SWW011. Values represent the average of at least three biological replicates (error bars represent standard deviation).

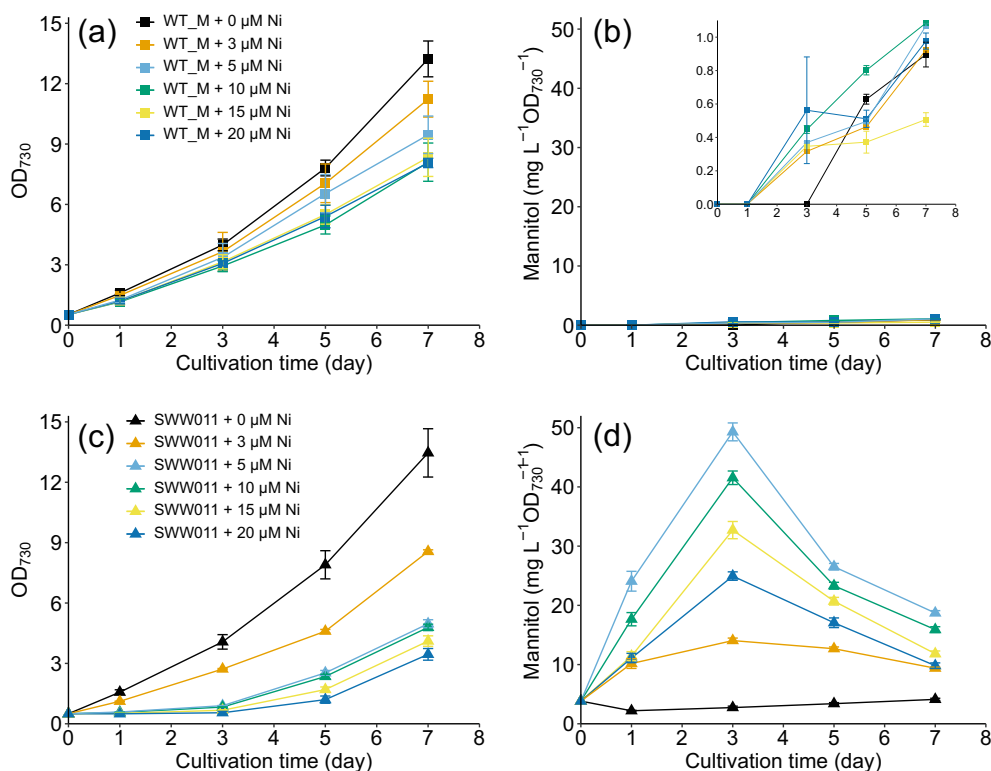


FIGURE 3 Growth curve and mannitol production of WT_M (a and b) and SWW011 (c and d) after induction by different doses of nickel in BG11 medium. The same Y-axis scale was applied to (b) and (d) to enable a direct comparison of mannitol production across different strains. Additionally, an inset plot was added to (b) for clearer visualization of mannitol production. Values represent the average of at least three biological replicates (error bars represent standard deviation).

1.9 mg L⁻¹, respectively (Figure 2b). These results corroborated our previous conclusion that both the P_{nrsB} promoter is leaky and that the mutation in *mtID* has a negative impact on its activity. We also show here that the expression level of *mtID* is a potential bottleneck to increase mannitol production. These results inspired us to find a way to balance cell growth with mannitol production. In order to find a solution, different concentrations of nickel were used to control promoter P_{nrsB} for the cultivation of SWW011. We found that 5 μM nickel is suitable for achieving high mannitol production in this strain while the side effects on cell physiology were kept within an acceptable range (Figure 3b,d). Under such conditions, mannitol

concentration reached 92.9 mg L⁻¹ after 7 days of induction, which is the highest titer in comparison with any other strain under any other growth condition (Supplementary Figure S2).

3.3 | Stability of mannitol production under optimized induction conditions

We checked the stability of mannitol production in SWW011 under optimal induction conditions, that is, upon the addition of 5 μM nickel, by performing serial cultivation-dilution cycles. By approximately the

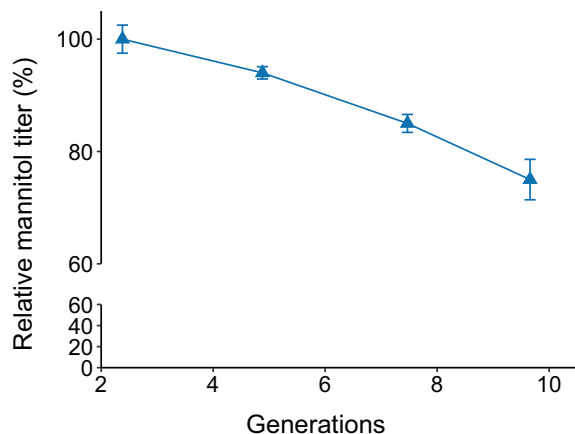


FIGURE 4 Relative mannitol titer changes of SWW011 with 5 μ M nickel induction every 4 days during the “growth-dilution” cycles. Values represent the average of at least three biological replicates (error bars represent standard deviation).

10th generation, the mannitol titer observed was already lowered to roughly 75% that of the initial population (Figure 4). We found that the growth rate of the strain appeared faster as its mannitol production was lowered during the process of “growth-dilution” cycles. These data indicate that the mannitol producers of SWW011 with 5 μ M nickel induction are quickly outcompeted by emerging revertant mutants within the population. The instability of mannitol production observed is very much in line with previous reports^{6,13} and greatly restricts its application in large scale processes in the future, which should be considered. The latter can be overcome by additionally implementing (i) fitness-coupling strategies, such as the conferral of osmotic-stress resistance⁶; (ii) or the introduction of growth-coupled metabolic engineering strategies^{24,25}; and (iii) introduction of two-stage growth uncoupled production strategies reliant on induction systems suitable to be applied in industrial settings.²⁶

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 high 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, P_{nrsB} . In the presence of 5 μ M nickel in the medium, our optimized mannitol producer was able to produce 92.9 mg L⁻¹ mannitol after 7 days of induction. This represents an 8-fold increase in comparison with the previously reported best mannitol-producing *Synechocystis*. Mannitol itself is still not expected to be toxic for *Synechocystis* at these concentrations. However, it has been found to be toxic for a specific strain, NIES-298, of *Microcystis aeruginosa*. This inhibitory effect has been proposed to provide a non-toxic basis to control biomass blooms.²⁷ A more systematic test of

potential mannitol inhibition in different cyanobacterial strains, including the ones developed in this study, would be very pertinent. Also, future work focusing on stabilizing mannitol productivity, namely within the production set-ups and timelines used at an industrial scale, will still be needed to move the strains engineered here to full blown production settings.

AUTHOR CONTRIBUTIONS

Wenyang Wu: Writing – original draft; methodology; investigation; validation. **Wei Du:** Writing – original draft; conceptualization; data curation; supervision; methodology; investigation; visualization. **Klaas J. Hellingwerf:** Writing – review and editing; supervision. **Filipe dos Branco dos Santos:** Writing – review and editing; funding acquisition; conceptualization; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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REFERENCES

- Wisselink HW, Weusthuis RA, Eggink G, Hugenholtz J, Grobden GJ. Mannitol production by lactic acid bacteria: a review. *Int Dairy J*. 2002;12(2):151-161. doi:10.1016/S0958-6946(01)00153-4
- Dai Y, Meng Q, Mu W, Zhang T. Recent advances in the applications and biotechnological production of mannitol. *J Funct Foods*. 2017;36:404-409. doi:10.1016/j.jff.2017.07.022
- dos Santos F, Du W, Hellingwerf KJ. *Synechocystis*: not just a plug-bug for CO₂, but a Green E. Coli. *Front Bioeng Biotechnol*. 2014;2:36. doi:10.3389/fbioe.2014.00036
- Novoveská L, Nielsen SL, Eroldogan OT, et al. Overview and challenges of large-scale cultivation of photosynthetic microalgae and cyanobacteria. *Mar Drugs*. 2023;21(8). doi:10.3390/md21080445
- Madsen MA, Semerdzhiev S, Amtmann A, Tonon T. Engineering mannitol biosynthesis in *Escherichia coli* and *Synechococcus* sp. PCC 7002 using a green algal fusion protein. *ACS Synth Biol*. 2018;7(12):2833-2840. doi:10.1021/acssynbio.8b00238

6. Wu W, Du W, Gallego RP, et al. Using osmotic stress to stabilize mannitol production in *Synechocystis* sp. PCC6803. *Biotechnol Biofuels*. 2020;13(1):117. doi:[10.1186/s13068-020-01755-3](https://doi.org/10.1186/s13068-020-01755-3)
7. van Alphen P, Abedini Najafabadi H, dos Branco 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. doi:[10.1002/biot.201700764](https://doi.org/10.1002/biot.201700764)
8. 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):11169-11175. doi:[10.1021/jp4013152](https://doi.org/10.1021/jp4013152)
9. Russell DW, Sambrook J. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Vol 1; 2001.
10. 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;7:99. doi:[10.1186/1754-6834-7-99](https://doi.org/10.1186/1754-6834-7-99)
11. 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. doi:[10.1002/btpr.1661](https://doi.org/10.1002/btpr.1661)
12. Heidorn T, Camsund D, Huang H-H, et al. Chapter twenty-four - synthetic biology in cyanobacteria: engineering and analyzing novel functions. In: Voigt C, ed. *Methods in Enzymology*. Vol 497. Academic Press; 2011:539-579. doi:[10.1016/B978-0-12-385075-1.00024-X](https://doi.org/10.1016/B978-0-12-385075-1.00024-X)
13. Jacobsen JH, Frigaard N-U. Engineering of photosynthetic mannitol biosynthesis from CO₂ in a cyanobacterium. *Metab Eng*. 2014;21:60-70. doi:[10.1016/j.ymben.2013.11.004](https://doi.org/10.1016/j.ymben.2013.11.004)
14. Pritam P, Sarnaik AP, Wangikar PP. Metabolic engineering of *Synechococcus elongatus* for photoautotrophic production of mannitol. *Biotechnol Bioeng*. 2023;120(8):2363-2370. doi:[10.1002/bit.28479](https://doi.org/10.1002/bit.28479)
15. Pade N, Erdmann S, Enke H, et al. Insights into isoprene production using the cyanobacterium *Synechocystis* sp. PCC 6803. *Biotechnol Biofuels*. 2016;9(1):89. doi:[10.1186/s13068-016-0503-4](https://doi.org/10.1186/s13068-016-0503-4)
16. Yoshikawa K, Hirasawa T, Shimizu H. Effect of malic enzyme on ethanol production by *Synechocystis* sp. PCC 6803. *J Biosci Bioeng*. 2015; 119(1):82-84. doi:[10.1016/j.jbiosc.2014.06.001](https://doi.org/10.1016/j.jbiosc.2014.06.001)
17. Ferreira E. Evaluation and improvement of *Synechocystis* sp PCC 6803 tolerance to temperature and development of Synthetic Biology tools. 2014.
18. 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):292-298. doi:[10.1006/mcbr.2000.0227](https://doi.org/10.1006/mcbr.2000.0227)
19. Dühring U, Baier K, Germer F, Shi T. Genetically Enhanced Cyanobacteria for the Production of a First Chemical Compound Harbouring Zn₂₊, Co₂₊ or Ni₂₊-Inducible Promoters. 2017.
20. Vanderpool CK. Physiological consequences of small RNA-mediated regulation of glucose-phosphate stress. *Curr Opin Microbiol*. 2007; 10(2):146-151. doi:[10.1016/j.mib.2007.03.011](https://doi.org/10.1016/j.mib.2007.03.011)
21. Gibney PA, Schieler A, Chen JC, 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. doi:[10.1091/mbc.E17-11-0666](https://doi.org/10.1091/mbc.E17-11-0666)
22. Chin T, Okuda Y, Ikeuchi M. Improved sorbitol production and growth in cyanobacteria using promiscuous haloacid dehalogenase-like hydrolase. *J Biotechnol*. 2019;306:100002. doi:[10.1016/j.btecx.2019.100002](https://doi.org/10.1016/j.btecx.2019.100002)
23. Küpper H, Kroneck PMH. Nickel in the environment and its role in the metabolism of plants and cyanobacteria. *Nickel and its Surprising Impact in Nature*; Wiley. 2007:31-62. doi:[10.1002/9780470028131.ch2](https://doi.org/10.1002/9780470028131.ch2)
24. Du W, Jongbloets JA, van Boxtel C, et al. Alignment of microbial fitness with engineered product formation: obligatory coupling between acetate production and photoautotrophic growth. *Biotechnol Biofuels*. 2018;11(1):38. doi:[10.1186/s13068-018-1037-8](https://doi.org/10.1186/s13068-018-1037-8)
25. Guillaume MC, Branco dos Santos F. Assessing and reducing phenotypic instability in cyanobacteria. *Curr Opin Biotechnol*. 2023;80: 102899. doi:[10.1016/j.copbio.2023.102899](https://doi.org/10.1016/j.copbio.2023.102899)
26. Shabestary K, Klamt S, Link H, Mahadevan R, Steuer R, Hudson EP. Design of microbial catalysts for two-stage processes. *Nature Rev Bioeng*. 2024;2(12):1039-1055. doi:[10.1038/s44222-024-00225-x](https://doi.org/10.1038/s44222-024-00225-x)
27. Jung J, Seo YL, Jeong SE, Baek JH, Park HY, Jeon CO. Linear six-carbon sugar alcohols induce lysis of *Microcystis aeruginosa* NIES-298 cells. *Front Microbiol*. 2022;13. doi:[10.3389/fmicb.2022.834370](https://doi.org/10.3389/fmicb.2022.834370)

SUPPORTING INFORMATION

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