Supplementary Material

Title

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

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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	HKNLIEGIAAA*MHFRSEDDPQAQELAALIADKGPQAALAQ	360
mutated MtlD	321	HKNLIEGIAAA HFRSEDDPQAQELAALIADKGPQAALAQ	360
unmutated MtlD	361	ISGLDANSEVVSEAVTAYKAMQ	382
mutated MtlD	361	ISGLDANSEVVSEAVTAYKAMQ	382

Figure S2. Total mannitol production of SWW011 after induction by different doses of nickel in BG11 medium. Values represent the average of at least three biological replicates (error bars represent standard deviation).

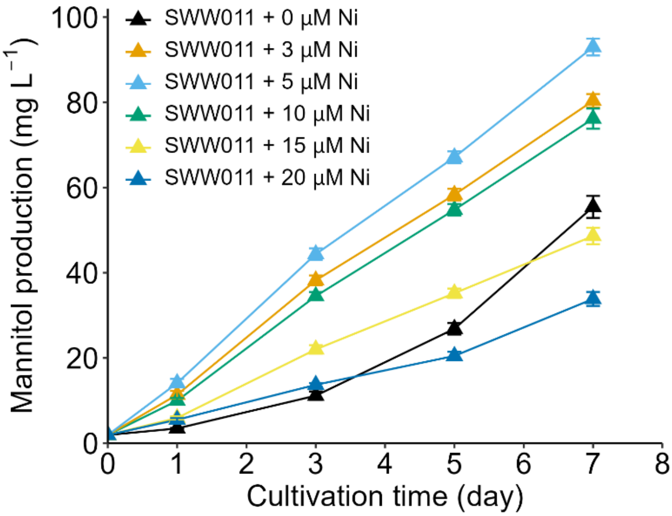

Table S1. Sequence of optimized (un)mutated *mtlD* and *m1p* used in this study for mannitol expression.

Gene	Gene sequence
Unmutated *mtlD*	ATGAAAGCTTTGCACTTTGGCGCGGGTAATATTGGACGGGGGT TTATTGGCAAGTTATTAGCGGACGCCGGAATCCAATTGACCTTT GCCGACGTTAACCAGGTAGTCTTAGATGCCTTGAATGCCCGTC ACTCCTATCAGGTGCACGTGGTGGGGGAAACCGAACAAGTTGA CACAGTATCCGGAGTAAACGCCGTGTCCTCGATTGGCGACGAT GTTGTGGATCTTATCGCTCAAGTGGATTTGGTAACCACGGCTG TTGGGCCGGTGGTCTTAGAAAGAATTGCTCCGGCCATCGCTAA GGGCCAAGTGAAACGCAAAGAACAAGGAAATGAATCTCCCTTG AACATCATTGCGTGCGAAAATATGGTGCGTGGAACTACTCAACT GAAAGGGCATGTAATGAACGCGTTGCCCGAAGACGCCAAAGC GTGGGTAGAGGAACACGTGGGCTTTGTTGATTCCGCCGTTGAT CGCATTGTTCCCCCTAGTGCCTCTGCCACTAATGACCCCTTAG AAGTGACTGTTGAAACGTTCTCCGAATGGATTGTGGATAAAACG CAATTTAAAGGCGCTTTACCCAACATTCCCGGCATGGAGTTGA CTGACAATCTGATGGCCTTTGTGGAACGTAAACTCTTCACCTTA AATACTGGCCATGCCATTACCGCATACCTTGGTAAACTGGCCG GTCATCAAACCATCCGCGATGCTATTTTAGATGAAAAAATTCGC GCAGTTGTAAAGGGCGCGATGGAGGAATCCGGAGCGGTATTG ATCAAGCGGTATGGCTTCGACGCAGATAAGCATGCTGCCTACA TCCAAAAAAATTTTGGGACGCTTTGAAAATCCGTATTTGAAGGAC GACGTTGAACGGGTGGGGCGTCAACCCCTGCGAAAATTAAGT GCGGGGGATCGTTTAATCAAACCATTACTGGGTACCTTAGAGT ACGGCTTGCCCCACAAAAATTTGATCGAAGGGATTGCCGCCGC TATGCACTTCCGGTCCGAAGACGATCCACAAGCTCAAGAGTTG GCCGCTCTGATCGCCGATAAAGGCCCACAGGCCGCCCTAGCA CAGATTAGCGGCTTGGACGCTAATAGCGAGGTAGTATCCGAAG CTGTTACGGCCTATAAGGCCATGCAA
Mutated *mtlD*	ATGAAAGCTTTGCACTTTGGCGCGGGTAATATTGGACGGGGGT TTATTGGCAAGTTATTAGCGGACGCCGGAATCCAATTGACCTTT GCCGACGTTAACCAGGTAGTCTTAGATGCCTTGAATGCCCGTC ACTCCTATCAGGTGCACGTGGTGGGGGAAACCGAACAAGTTGA CACAGTATCCGGAGTAAACGCCGTGTCCTCGATTGGCGACGAT GTTGTGGATCTTATCGCTCAAGTGGATTTGGTAACCACGGCTG TTGGGCCGGTGGTCTTAGAAAGAATTGCTCCGGCCATCGCTAA GGGCCAAGTGAAACGCAAAGAACAAGGAAATGAATCTCCCTTG AACATCATTGCGTGCGAAAATATGGTGCGTGGAACTACTCAACT GAAAGGGCATGTAATGAACGCGTTGCCCGAAGACGCCAAAGC GTGGGTAGAGGAACACGTGGGCTTTGTTGATTCCGCCGTTGAT CGCATTGTTCCCCCTAGTGCCTCTGCCACTAATGACCCCTTAG AAGTGACTGTTGAAACGTTCTCCGAATGGATTGTGGATAAAACG CAATTTAAAGGCGCTTTACCCAACATTCCCGGCATGGAGTTGA CTGACAATCTGATGGCCTTTGTGGAACGTAAACTCTTCACCTTA AATACTGGCCATGCCATTACCGCATACCTTGGTAAACTGGCCG GTCATCAAACCATCCGCGATGCTATTTTAGATGAAAAAATTCGC GCAGTTGTAAAGGGCGCGATGGAGGAATCCGGAGCGGTATTG ATCAAGCGGTATGGCTTCGACGCAGATAAGCATGCTGCCTACA TCCAAAAAAATTTTGGGACGCTTTGAAAATCCGTATTTGAAGGAC GACGTTGAACGGGTGGGGCGTCAACCCCTGCGAAAATTAAGT GCGGGGGATCGTTTAATCAAACCATTACTGGGTACCTTAGAGT ACGGCTTGCCCCACAAAAATTTGATCGAAGGGATTGCCGCCGC TCACTTCCGGTCCGAAGACGATCCACAAGCTCAAGAGTTGGCC GCTCTGATCGCCGATAAAGGCCCACAGGCCGCCCTAGCACAG ATTAGCGGCTTGGACGCTAATAGCGAGGTAGTATCCGAAGCTG TTACGGCCTATAAGGCCATGCAA

m1p	ATGGCTGAGACCGAATGGACCCCCGAAGCCCTGTCTGGCCGC TACGAAGAAATTAAGTCCTGCATTCCGCAACAATTGGAAGCTTA TGCCCGGTTCCTACGGGAAGCAGCACCTGAAGACTTGCGTCG GTGGCAGCAGATTGCCCAGGATTTAAAGCTTGAGCTGAACTTG GAAAACGGCAGAATTAAATACAAAAAAGAGTTTAAACCGCTAGA ATTACCCGTTGATATTTGCTATATAAGGCATGGCAAAACCCAGG GGAACACGGAACCCCGGGTTTTCCAAGGTCAGGTTGATTATGC GAATAACCAACTCACTCAGCAGGGACAACAGCAGGCCGCCGC TGCTGCTACTAAGTTAGAAGCCATGGCGGCTGCCAAAGAGTTT ATCCCCGATCTCCTGCTCTCCTCTCCCTTACTGCGCGCGGTAC ATACCGCACAACCCTTCGTTGACGCCAATCCCAAACCCTTATTC AGAGTGTTGCCCGAATTAGCTGAGATGGCTTTTGGCGAATGGG ATAACCGTAAGGTAGCCGAATTGGAAAAAGATGACCCCGCCCA CCTGTTTTACCTCCAGCAGAACGCTGTGATCAAAGCTAAAGGT CCCCACCGCATCTGCTGTCAATTATGGCAGTCGCCAGAGTGGC TTGAAGGTAAAAAGGAACTGCCGGCCGAAAATTTTTTGGAATGT TTGGACCGGCAACGTAAAGCTTTGATCAAAGTTGGCGAGATCG CTAAAGAACTATGTGGACCCAGCTGTGGCGAACGTAAGCCCCG GGTCGCAGTTTACGGCCATTCTATGGCCGGCGCCGCCGTATC CGTACTTTTGGGATTCGGTAAAGAAGACCAGTTGGGCTTCTTG GGTTTTGATGGCAATTATATTATGCCCAATGCCACTCCGACCAT CTTGATTCCCAACGCGAAACCG

Table S2. Sequence of promoters P_{trc1}, P_{psbA2} and P_{nrsB} used in this study for mannitol expression. The sequence following P_{trc1} promoter was GCTAGCATTAAAG<u>AGGAGA</u>AATGACAT (RBS underlined).

Promoter	Promoter sequence
P_{trc1}	TTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGA TAACAATTTCACACA
P_{psbA2}	ATGACAACGACTCTCCAACAGCGCGAAAGCGCTTCCTTGTGGGAACA GTTTTGTCAGTGGGTGACCTCTACCAACAACCGGATTTATGTCGGTT GGTTCGGTACCTTGATGATCCCCACCCTCTTAACTGCCACCACTTGC TTCATCATTGCCTTCATCGCCGCTCCCCCCGTTGACATCGACGGTAT CCGTGAGCCCGTTGCTGGTTCTTTGCTTTACGGTAACAACATCATCTC TGGTGCTGTTGTACCTTCTTCCAACGCTATCGGTTTGCACTTCTACCC CATCTGGGAAGCCGCTTCCTTAGATGAGTGGTTGTACAACGGTGGTC CTTACCAGTTGGTAGTATTCCACTTCCTCATCGGCATTTTCTGCTACA TGGGTCGTCAGTGGGAACTTTCCTACCGCTTAGGTATGCGTCCTTGG ATTTGTGTGGCTTACTCTGCCCCCGTATCCGCTGCCACCGCCGTATT CTTGATCTACCCCATTGGTCAAGGCTCCTTCTCTGATGGTATGCCCTT GGGTATTTCTGGTACCTTCAACTTCATGATCGTGTTCCAAGCTGAGCA CAACATCCTGATGCACCCCTTCCACATGTTAGGTGTGGCTGGTGTAT TCGGTGGTAGCTTGTTCTCCGCCATGCACGGTTCCTTGGTAACCTCC TCCTTGGTGCGTGAAACCACCGAAGTTGAATCCCAGAACTACGGTTA CAAATTCGGTCAAGAAGAAGAAACCTACAACATCGTTGCCGCCCACG GCTACTTTGGTCGGTTGATCTTCCAATATGCTTCTTTCAACAACAGCC GTTCCTTGCACTTCTTCTTGGGTGCTTGGCCTGTAATCGGCATCTGGT TCACTGCTATGGGTGTAAGCACCATGGCGTTCAACCTGAACGGTTTC AACTTCAACCAGTCCATCTTGGATAGCCAAGGCCGGGTAATCGGCAC CTGGGCTGATGTATTGAACCGAGCCAACATCGGTTTTGAAGTAATGC ACGAACGCAATGCCCACAACTTCCCCCTCGACTTAGCGTCTGGGGAG CAAGCTCCTGTGGCTTTGACCGCTCCTGCTGTCAACGGTTAA

P_{nrsB}	TTAAGAATGGACTATCGGTACAGAAAAAATGGGTAACTGGATGGTGA ATAAACTTCCCTTACCCAATGCACTCTCCACCGTTAAAGACCCCCTAT GCTTAACGGTGATCACCTGGGCAATGGCGAGTCCCAACCCTGTCCC CCCCGTTTTGCGCGAACGATCTCGATTAACTCGGTAAAAACGCTCAA AAATGTGTTCCTGTTGGTCGGGGGCAATGCCGATGCCGGTATCTTGC ACGGTGATGATAGCCATCTGTTCATGGGATGTCAGGGTAATATCAAC ACGTCCCCCAGCAGTTGTGTATTGAATGGCGTTGGCAATTAGGTTTG AGACCAGTCGATAGAGTTGGGATTCATTACCCCAGGCGTAAACTTCC CCTGAACTCAGATCACTGCTGAGATCAATGTGGGCGGCGATCGCTAA TTCTAAAAACTCTTCGGTGAGGTCACTGACTAAATCATTTAAACAACA AAGCCGCCAATCTTCGGCGGTGGTTTCCTGCTCTAAGCGACTTAGTA GCAATAAATCCGTAATCAATTGGCTTAATCGCCTTCCCTGTCGTTCAA CGGTATGTAGCATGGTGTTAATTTCTGGGGAATGGCTTGAGTCGATG CGTAATACCGCTTCCACCGTGGCCAACAGACTAGCCAATGGCGATCG TAATTCATGGGCTGCATTCGCGGTGAATTGTTGTTGTTGTTGGTAGGA CTGGTAAATGGGACGCATGGCTAACCCCGCTAAGCCCCAACTGGAG AAGGCGACCAAACCCAGGGCAATGGGAAAACTAAGCCCTAAAATCCA AAGAATACGTTTATTTTCGGCATCAAAGGCTGCCAGGCTCCGGCCAA TTTGTAGATAGCCCCAGGAAGATTTGTCTGTATTACCGGCGCTATGCA AAATGGTGGTGAATTGTCGATACCGATCGCCGGTTGGGGGGTGAATA GTCTGCCAAGTTTCCTGGTTAAAAATGGAGGATAGGGAAGCCGGTTG ATTAGGCGAAAAAGCCAGCAGGTTGCCTTGATAATCAAATAAACGAAT GTAATATAAACTGCGATCACTAATGCCCAACGTGTGACGTTCAATCAG GGTGGGGTTGACCTGGCAGGGTTGGTTGACCAAACACAGATCGGGC AACATTTTTTGTAATACTCCGGTGGGACTAGCATTACTCGGCAACATC GGCTCTAAACTGTCATGCAACGTCCCGGCGATCGACTCCACTTCTCG CTCCAACGCCATCCAGTTGGCCTGCACAATGGCACGATAAACCCCCA ACCCCAACAGGGTAAGAATTCCCCCCATTACTAGGGCATACCAGAAA GCCAATTGCAGACGACTACGGGCAAAGAGGCGACGGGTATTCATGG CGATAGGGTGAACCGATAGCCTTGACCGGGAACTGTTTTAATTGGGC AAGGACAATTTTGTTGAGCTAGCTTGCGTCGTATCAAACGCATTTGGG CCGCCACCACATTACTCATGGGCTCCTCATCAAGATCCCACAGTTGT TGCCGGATCTTGCTACCGGAAATGATCCGCTCTGGGTTTTGCATCAG ATATTGAAAAATTTGAAATTCTCTTACGGTTAAAGCAATTTCCTGTCTT TCTAGGTTTAGTGGCTCCGAGATAGTTACCGATAACAGATTATTACTG GGATCAAGGCTGAAGTTGCCCAAAGTTAAAATTTGCGGTTGGAATTG TGGCGATCGCCGTTGTAGTGCCCGCAGTCTTGCTAATAGCTCTGCCA TCACAAACGGTTTTGTTAGATAGTCATCTGCCCCGGCATCTAGTCCTT CGACACGGTTTTCCGGTTCTCCTAACGCTGTTAACATCAACACCGGC AAGGAATTACCCTGGGTTCTCAGTTTTTGACAGAGTTCCAAACCCGAT AATCCCGGCAGTAACCAATCCACAATGGCAAGGGTGTATTCCGTCCA TTGATTTTCCAAATAATCCCAAGCTTGGGAGCCATCCGTCACCCAATC CACCACATACTTTTCACTAACTAGCACTTTCTTAATAGCCATTCCCAAA TCCGTCTCATCTTCCACCAGCAAAATTCGCATCGCCTCTGCCTTTTTT ATAACGGTCTGATCTTAGCGGGGGAAGGAGATTTTCACCTGAATTTC ATACCCCCTTTGGCAGACTGGGAAAATCTTGGACAAATTCCCAATTTG AGGT

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

Primer name	Sequence	Purpose
m1p_F	5'-<u>CATATG</u>GCTGAGACCGAATGGACCCC-3'	Amplification of optimized *m1p* and addition of a NdeI restriction site at the 5´
m1p_R	5'-<u>CCTAGG</u>TTATTACGGTTTCGCGTTGGGAATC-3'	Amplification of optimized *m1p* and addition of an AvrII restriction site at the 3´
Omega_F	5'-<u>GTCGAC</u>AATTCCGGGGATCCTCTAGA-3'	Amplification of streptomycin resistance cassette and addition of a SalI restriction site at the 5'
Omega_R	5'-<u>GTCGAC</u>ATGAATCCTTAATCGGTACC-3'	Amplification of streptomycin resistance cassette and addition of a SalI restriction site at the 3'
mtlD_F1	5'-<u>CATATG</u>AAAGCTTTGCACTTTGGCGC-3'	Amplification of optimized (un)mutated *mtlD* and addition of a NdeI restriction site at the 5'
mtlD_R1	5'-<u>GGATCC</u>TTGCATGGCCTTATAGGCCG-3'	Amplification of optimized (un)mutated *mtlD* and addition of a BamHI restriction site at the 3'
pNrsB_F1	5'-<u>TCTAGA</u>TTAAGAATGGACTATCGGTA-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'-<u>CCTAGG</u>TTGCATGGCCTTATAGGCCG-3'	Amplification of (un)mutated *mtlD* and addition of an AvrII restriction site at the 3'
mtlD_R3	5'-<u>TCTAGA</u>TAGTAAAATAATAAAAAAGCCG-3'	Amplification of (un)mutated *mtlD* and addition of a XbaI restriction site at the 3'

Table S4. A summary of studies on mannitol production in cyanobacteria.

Cyanobacterial species	Experimental implementation	Mannitol titre	References
Synechococcus sp. PCC 7002	heterologous expression of *mtlD* and *m1p*, with additional inactivation of glycogen synthesis. mutations occurred and complete segregation not achieved.	1.1 g mannitol L^{-1} (after 12 days at OD_{730} of 13)	(Jacobsen & Frigaard, 2014)
Synechocystis sp. PCC6803	heterologous expression of *mtlD* and *m1p*, with additional knockout of *sps* and *ggpS*, in combination with salt stress. methionine missing at position 332 of *mtlD*	~ 8 μM OD_{730}^{-1} in a turbidostat at OD_{730} of 1, under 200 mM NaCl salt stress	(Wu et al., 2020)
Synechocystis sp. PCC6803	heterologous expression of *mtlD* and *m1p*, with *mtlD* regulated under the nickel-inducible promoter	92.9 mg mannitol L^{-1} after 7 days	This study
Synechococcus elongatus PCC 7942	heterologous expression of *mtlD* and *m1p*, with expression of *mtlD* under different promoters	701 ± 15 mg mannitol L^{-1} (after 12 days at OD_{730} of ~5)	(Pritam et al., 2023)
Synechococcus elongatus PCC 11801	heterologous expression of *mtlD* and *m1p*, with expression of *mtlD* under different promoters	401 ± 18 mg mannitol L^{-1} (after 12 days at OD_{730} of 10)	(Pritam et al., 2023)
Synechococcus elongatus PCC 11802	heterologous expression of *mtlD* and *m1p*, with expression of *mtlD* under different promoters	537 ± 18mg mannitol L^{-1} (after 12 days at OD_{730} of ~8)	(Pritam et al., 2023)

References

Jacobsen, J. H., & Frigaard, N.-U. (2014). Engineering of photosynthetic mannitol biosynthesis from CO2 in a cyanobacterium. *Metabolic Engineering*, *21*, 60–70. https://doi.org/https://doi.org/10.1016/j.ymben.2013.11.004

Pritam, P., Sarnaik, A. P., & Wangikar, P. P. (2023). Metabolic engineering of Synechococcus elongatus for photoautotrophic production of mannitol. *Biotechnology and Bioengineering*, *120*(8), 2363–2370. https://doi.org/https://doi.org/10.1002/bit.28479

Wu, W., Du, W., Gallego, R. P., Hellingwerf, K. J., van der Woude, A. D., & Branco dos Santos, F. (2020). Using osmotic stress to stabilize mannitol production in Synechocystis sp. PCC6803. *Biotechnology for Biofuels*, *13*(1), 117. https://doi.org/10.1186/s13068-020-01755-3