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Sorbitol production and optimization of photosynthetic supply in the cyanobacterium *Synechocystis* PCC 6803

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ABSTRACT

Biochemicals production is a major theme in the application of photosynthesis to address global warming and organic-resource problems. Among biochemicals, sugar alcohols have attracted research attention because they are directly derived from two photosynthetic products, sugars and reductants. Here, we produced sorbitol photosynthetically by using cyanobacteria and modified the supply of its substrates through genetic engineering. Expression of an NADPH-dependent enzyme that generates sorbitol-6-phosphate, S6PDH, was highly toxic to cyanobacteria likely due to the sorbitol production, whereas expression of an NADH-dependent enzyme, SrlD2, yielded no sorbitol. The toxicity was partly overcome by introducing a theophylline-inducible riboswitch for S6PDH expression and optimizing induction, but sorbitol production was still low and severely inhibited growth. Co-expression of fructose-1,6-bisphosphatase drastically alleviated the growth inhibition, but did not increase short-term sorbitol production. The NADPH/$NADP^+$ ratio decreased during sorbitol production. Overexpression of a membrane-bound transhydrogenase for NADPH generation from NADH elevated the short-term sorbitol production, but only partly alleviated the growth inhibition. Notably, a strain overexpressing all three enzymes exhibited sustainable sorbitol production at 312 mg/L, which was nearly 27-fold higher than the yield of the initial S6PDH-overexpressing strain. We discuss these results in relation to the optimization of photosynthetic supply for sorbitol production in cyanobacteria.

1. Introduction

Global warming and a depletion of fossil resources have emerged in recent years due to the increase in global population and in carbon dioxide (CO_2) emission. Photosynthetic production of biochemicals from CO_2 is a promising solution for overcoming these environmental and organic-resource problems because CO_2 emission cannot be readily reduced in a society that depends on fossil resources. For the concept, cyanobacteria, which are autotrophic prokaryotes that perform oxygenic photosynthesis, have been studied for their application in the production of various organic materials (Case and Atsumi, 2016; Gao et al., 2016), including sugars (Du et al., 2013; Niederholtmeyer et al., 2010), organic acids (Chin et al., 2015; Wang et al., 2015), and isoprenoids (Kiyota et al., 2014; Lindberg et al., 2010); this is because genetic manipulation and efficient phototrophic cultivation have been established for cyanobacterial strains. To further develop and improve photosynthetic production in cyanobacteria, it is crucial to fine-tune the underlying photosynthetic metabolism toward target production.

For the fine-tuning of photosynthetic biochemicals production, sugar alcohols are highly suitable target compounds because they are produced from sugars directly derived from the Calvin cycle and from the excess reducing equivalents generated through the light reaction in oxygenic phototrophs. Various sugar alcohols (mannitol, sorbitol, xylitol, etc.) are produced naturally in addition to sucrose in certain plants for the translocation of carbons and energy between source and sink organs (Noiraud et al., 2001), and some sugar alcohols are also produced in algae, fungi, bacteria, and yeast for storage, osmoregulation, and scavenging of active oxygen species (Iwamoto and Shiraiwa, 2005; Kobayashi et al., 2015). Coupled with the progressive increase in demand for products such as sweeteners and humectants, the industrial manufacture of various sugar alcohols has been established in the form of chemical hydrogenation of sugars or fermentative processes (Grembecka, 2015). However, these production systems require sugars that are eventually derived from photosynthesis, and thus it is challenging to directly produce sugar alcohols through photosynthesis by using combinatorial genetic engineering.

Abbreviations: Synechocystis, *Synechocystis* sp. PCC 6803; SrlD2, NAD-dependent sorbitol-6-phosphate dehydrogenase; S6PDH, NADP-dependent sorbitol-6-phosphate dehydrogenase; FBPase, fructose-1,6-bisphosphatase; PntAB, membrane-bound transhydrogenase

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Engineered production of two sugar alcohols by using cyanobacteria has been reported recently: mannitol production in *Synechococcus* sp. PCC 7002 (Jacobsen and Frigaard, 2014) and erythritol production in *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) (van der Woude et al., 2016). These systems achieved photosynthetic production in cyanobacteria, although certain critical points remain to be resolved: One, because the carbons of these sugar alcohols are derived directly or closely from Calvin cycle metabolites, their production might be unfavorable, via depletion of critical metabolite(s) such as fructose-6-phosphate (F6P), which is essential for regeneration of the cycle, as suggested by Jacobsen and Frigaard (2014); two, reductants, which are mainly supplied from water by oxygenic photosynthesis, are additionally required to convert sugars to sugar alcohols, and thus the balance between CO_2 fixation and NADPH supply might be readjusted; and three, the export of sugar alcohols into the culture medium might also be limited in these systems. These hurdles are likely to be universally encountered in photosynthetic production involving the use of engineered cyanobacteria.

Here, we focused on the sugar alcohol, sorbitol, as the target product in engineered cyanobacteria. In nature, sorbitol is produced by NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH) in leaves and translocated to fruits as a major product in *Rosaceae* plants such as apple (Zhou et al., 2001). Moreover, in heterotrophic microorganisms such as lactic acid bacteria, sorbitol is produced by NAD-dependent sorbitol-6-phosphate dehydrogenase (SrlD2) through fermentation as a waste product from excess reductants (Ladero et al., 2007). To evaluate the suitable enzyme in the photosynthetic production of sorbitol based on metabolic engineering, we introduced the gene encoding S6PDH or SrlD2 into cyanobacteria, respectively. To avoid growth inhibition during sorbitol production, supply of carbons and reductants was modified in sorbitol-producing cyanobacteria. Our approach in sorbitol production would provide insights into future application of photosynthetic productions.

2. Materials and methods

2.1. Strains and plasmid construction

The strains and plasmids used in this study are shown in Table 1. The glucose-tolerant strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001) was used as a platform in this study. Codon-optimized DNA fragments (Fig. S1) were synthesized by FASMAC (Atsugi, Japan). The nucleotide sequences of plasmids and primers are shown in Fig. S2 and Table S1, respectively. Plasmids were constructed using PrimeSTAR Max DNA Polymerase (Takara Bio, Otsu, Japan), the In-Fusion HD Cloning Kit (Takara Bio), and *Escherichia coli* strain JM109.

The sequence of SrlD2 was derived from *Lactobacillus plantarum* WCFS1 (GenBank accession no. NC_004567 REGION: complement (3277908..3278711)) (Kleerebezem et al., 2003), and the S6PDH sequence was from *Malus domestica* (GenBank accession no. D11080) (Kanayama et al., 1992). For constitutive expression, the synthesized DNA of *srlD2* or *s6pdh* was PCR-amplified using the primer set of srlD2-3Ftrc and srlD2-4Rrrn or s6pdh-1Ftrc and s6pdh-2Rrrn, respectively. The amplified fragment was cloned into a plasmid vector harboring a *trc* promoter, an *rrnB* terminator, a kanamycin-resistance cassette, and a neutral site near *slr0846* for homologous recombination; this generated pB46KTsrlD2T or pS46KTs6pdhT (Fig. S2). For inducible expression of *s6pdh*, the *trc*::theophylline-riboswitch promoter (*trc*::riboswitch-E*; Nakahira et al., 2013) was PCR-amplified from pVZR-GFP (Ohbayashi et al., 2016) by using the primer set TR-1Fcom and TR-6R, and incorporated into pS46KTs6pdhT-derived DNA amplified using the primer set s6pdh-3FTR and Km-36Rcom; this yielded pS46KRs6pdhT (Fig. S2C). *Synechocystis* wild-type cells (WT) were transformed with these DNAs, and complete segregation of the integrated DNAs on the chromosome was confirmed using PCR. Hereafter, the strain harboring *s6pdh* with the *trc*::theophylline-riboswitch promoter is abbreviated as the strain "s6pdh."

The sequence of spinach (*Spinacia oleracea*) fructose-1,6-bisphosphatase gene (*fbp*) was derived from GenBank accession no. L76555 (Martin et al., 1996). The *fbp* coding region without the chloroplast-targeting sequence was synthesized with codon optimization, PCR-amplified using the primer set fbp-1Ftrc and fbp-4Rrrn, and cloned into a plasmid vector harboring a *trc* promoter, an *rrnB* terminator, a chloramphenicol-resistance cassette, and a neutral site near *IS203c*; this generated pSISCTfbpT (Fig. S2D). The endogenous *fbp* genes of *Synechocystis*, *fbp-I* (*slr2094*) and *fbp-II* (*slr0952*), were PCR-amplified using the genomic DNA of *Synechocystis* and gene-specific primers (Fbp1-1Ftrc and Fbp1-4Rrrn for *fbp-I*, and Fbp2-1Ftrc and Fbp2-2Rrrn for *fbp-II*) and cloned into a plasmid, which yielded pBISCTsfbp1T and pBISCTsfbp2T, respectively (Figs. S2E and S2F). These plasmids were introduced into the strain s6pdh, and complete segregation of the integrated genes was confirmed using PCR. These three overexpression strains are abbreviated as s6pdh/fbp, s6pdh/fbp-I, and s6pdh/fbp-II, respectively.

Genes encoding the endogenous transhydrogenase subunits, *pntA*

Table 1
Strains and plasmids used in this study.

	Description[a,b]	Reference
Strain		
WT	Wild type *Synechocystis* sp. PCC 6803, glucose tolerant	Ikeuchi and Tabata (2001)
srlD2	WT harboring the genome-integrated lactic acid bacteria *srlD2* by pB46KTsrlD2T	This study
s6pdh	WT harboring the genome-integrated apple *s6pdh* by pS46KRs6pdhT	This study
s6pdh/fbp	s6pdh harboring the genome-integrated spinach *fbp* by pSISCTfbpT	This study
s6pdh/fbp-I	s6pdh harboring the genome-integrated *Synechocystis fbp-I* by pSISCTsfbp1T	This study
s6pdh/fbp-II	s6pdh harboring the genome-integrated *Synechocystis fbp-II* by pSISCTsfbp2T	This study
s6pdh/pnt	s6pdh harboring the genome-integrated *Synechocystis pntAB* by pBcaSTpntAT-PpntBP	This study
s6pdh/fbp/pnt	s6pdh/fbp harboring the genome-integrated *Synechocystis pntAB* by pBcaSTpntAT-PpntBP	This study
Plasmid		
pVZR-GFP	P_{trc}::riboswitch-*lacO-GFP*^{mut2}-T_{rrnB}; Kan^r	Ohbayashi et al. (2016)
pB46KTsrlD2T	P_{trc}-*srlD2*-T_{rrnB}; Kan^r; near *slr0846* homologous recombination region	This study
pS46KTs6pdhT	P_{trc}-*s6pdh*-T_{rrnB}; Kan^r; near *slr0846* homologous recombination region	This study
pS46KRs6pdhT	P_{trc}::riboswitch-*lacO-s6pdh*-T_{rrnB}; Kan^r; near *slr0846* homologous recombination region	This study
pSISCTfbpT	P_{trc}-*fbp*-T_{rrnB}; Cm^r; near *IS203c* homologous recombination region	This study
pBISCTsfbp1T	P_{trc}-*fbp-I*-T_{rrnB}; Cm^r; near *IS203c* homologous recombination region	This study
pBISCTsfbp2T	P_{trc}-*fbp-II*-T_{rrnB}; Cm^r; near *IS203c* homologous recombination region	This study
pBcaSTpntAT-PpntBP	P_{trc}-*pntA*-T_{rrnB}; P_{psbA2}-*pntB*- T_{psbA2}; Spc^r; near *carA* homologous recombination region	This study

[a] The flanking regions of *slr0846*, *IS203c*, and *carA* were used for neutral sites in the genomic DNA of *Synechocystis*.
[b] Kan^r, kanamycin-resistance cassette; Cm^r, chloramphenicol-resistance cassette; Spc^r, spectinomycin-resistance cassette.