博士論文（要約）

Sorbitol production by cyanobacteria toward application of photosynthesis

(光合成の応用に向けたシアノバクテリアによるソルビトール生産)

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Abstract

Photosynthetic production of valuable products from carbon dioxide can be leveraged to address problems of global warming and depletion of resources. Cyanobacteria, which are photoautotrophic prokaryotes, have been studied to produce chemicals and biofuels via photosynthesis. Sugar alcohols are attractive compounds for the photosynthetic production, since they are produced from sugars via Calvin cycle and from reducing power via light reaction in photosynthesis.

I focused on photosynthetic production of sorbitol by using a model cyanobacterium *Synechocystis* sp. PCC 6803 through metabolic engineering. In Chapter 1, I found that expression of an NADPH-dependent enzyme that generates sorbitol-6-phosphate successfully led to production of sorbitol but was highly toxic to cyanobacteria. Overexpression of a fructose-1,6-bisphosphatase alleviated the growth inhibition. Overexpression of a membrane-bound transhydrogenase for NADPH generation elevated the short-term sorbitol production rate, but only partly alleviated growth inhibition. Further engineering was evaluated for the enhancement of NADPH supply in sorbitol-producing cyanobacteria.

To further improve the sorbitol productivity, I attempted to screen an enzyme for dephosphorylation of sorbitol-6-phosphate among some of haloacid dehalogenase-like hydrolase superfamily (Chapter 2). Active enzymes of *Escherichia coli* for sorbitol-6-phosphatase were detected by enzymatic assay.

※近い将来において刊行される期待がある内容（5 年以内に出版予定）

Finally, I discuss about further optimization for engineering of cyanobacteria toward application of photosynthesis.

Contents

Abbreviations

ATP	Adenosine tri-phosphate
BPG	1,3-Bisphosphoglycerate
CO_2	Carbon dioxide
F6P	Fructose-6-phosphate
FBP	Fructose-1,6-bisphosphate
FBPase	Fructose-1,6-bisphosphatase
FLV	Flavodiiron protein
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GAP	Glyceraldehyde-3-phosphate
HAD	Haloacid dehalogenase-like hydrolase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
NAD(H)	Nicotinamide dinucleotide, (reduced form)
NADP(H)	Nicotinamide dinucleotide phosphate, (reduced form)
NDH	Type-I NADPH dehydrogenase complex
OD	Optical density
PCR	Polymerase chain reaction
2PG	2-Phosphoglycerate
3PG	3-Phosphoglycerate
*p*NPP	*p*-nitrophenyl phosphate
PntAB	Membrane-bound transhydrogenase consisting of PntA and PntB proteins
Ru5P	Ribulose-5-phosphate
RuBP	Ribulose-1,5-bisphosphate

RubisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase
S6PDH	NADP-dependent sorbitol-6-phosphate dehydrogenase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOT	Sorbitol transporter
TCA cycle	Tricarboxylic acid cycle
Tris	Tris (hydroxymethyl) aminomethane
WT	Wild-type

General introduction

Photosynthesis toward sustainability

Global warming and depletion of fossil resources have emerged as urgent issues in recent years due to the increase in global population and in carbon dioxide (CO_2) emission. Photosynthetic production of commodities from CO_2 is a promising solution for overcoming these environmental and organic-resource problems given that CO_2 emission cannot be readily reduced in a society that heavily depends on fossil resources. In oxygenic photosynthesis, the energy from sunlight is captured to generate high-energy intermediates ATP and NADPH (Shikanai and Yamamoto, 2017), and they are utilized for the assimilation of CO_2 to essential cellular components (Baroukh et al., 2015). While terrestrial oxygenic photosynthesis is dominated by plants, cyanobacteria and eukaryotic algae are main photosynthetic organisms in marine photosynthesis, and play an important role in global carbon cycling via the oxygenic photosynthesis (Dismukes et al., 2008). Cyanobacteria, which are photoautotrophic prokaryotes and are formerly known as blue-green algae, are especially promising candidates for production of various commodities, because they grow faster than plants and algae. Moreover, their genetic manipulation methods and efficient photoautotrophic cultivation system have been established (Berla et al., 2013; Kumar et al., 2011; Ruffing, 2011). Therefore, the development and application of engineered cyanobacteria offers the promise of renewable and more carbon-neutral processes for the sustainability.

Biotechnology for Cyanobacterial production

Engineering of cyanobacteria for photosynthetic production

Photosynthetic production by engineered cyanobacteria was pioneered from a few compounds such as ethylene (Sakai et al., 1997) and ethanol (Deng and Coleman, 1999). Recent advances in the ability to genetically manipulate and characterize cyanobacterial metabolism have opened the gateway for metabolic engineering of cyanobacteria toward promising production. Accordingly, cyanobacteria have been studied for their application in the production of various commodities such as chemicals and biofuels (Angermayr et al., 2015; Case and Atsumi, 2016; Ducat et al., 2011; Gao et al., 2016; Oliver and Atsumi, 2014). **Table 0_1** summarizes the typical photosynthetic productions by engineered cyanobacteria showing the highest titer of products. These studies led me to find the key concepts of cyanobacterial production: carbon flow to product, reducing power, expression of suitable enzymes, and export of intracellular product.

Carbon flow to product

Carbon flow is critical for the productivity in photosynthetic organisms. Specifically, the titer of product seem to be high when the substrate of the target product is closely related to the chemical compounds in Calvin cycle, which involves CO_2 fixation reaction catalyzed by ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO). Sugar phosphates are promising candidates of substrates, since they are abundant intermediates in Calvin cycle and other cellular metabolisms, and indeed their usage have led productivities to the high titer (Ducat et al., 2012; Jacobsen and Frigaard, 2014; van der Woude et al., 2016). Pyruvate is also a favorable substrate for various products

such as ethanol (Gao et al., 2012), lactic acid (Angermayr and Hellingwerf, 2013; Varman et al., 2013), and some biofuels (Atsumi et al., 2009; Li et al., 2014; Oliver and Atsumi, 2015), because this compound holds a central position in carbon metabolism and is separated by the only four reactions from the point of CO_2 fixation. In contrast, the productivity of beneficial commodities of terpenoids such as isoprene (Bentley et al., 2014) and limonene (Davies et al., 2014; Kiyota et al., 2014) is limited likely because the substrates isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which are the final compounds in the methylerythritol-phosphate pathway, are distant from the central carbon flow. The limited carbon flow toward the terpenoids biosynthesis could be overcame by the overexpression of a rate-limiting enzyme, 1-deoxy-D-xylulose-5-phosphate synthase, leading to improvement of productivity (Kiyota et al., 2014; Kudoh et al., 2014).

More fundamentally, it is desirable to enhance the CO_2 fixation step. To achieve the concept, enhancement of RubisCO, which is well known as a rate-limiting enzyme of the Calvin cycle, by the overexpression of its subunits successfully increased the productivity of isobutyraldehyde (Atsumi et al., 2009). However, it is rare to improve the production titer by the enhanced RubisCO possibly due to the difficulty of effective expression of RubisCO in engineered organisms. In literature, the level of CO_2 fixation and sugar compounds could be increased by the overexpression of fructose-1,6-bisphosphatase and/or sedoheptulose-1,7-bisphosphatase, which are the rate-limiting enzymes in Calvin cycle, in engineered phototrophs (Lefebvre et al., 2005; Miyagawa et al., 2001; Serrato et al., 2009; Yabuta et al., 2008). The utilization of the central carbon compounds as a substrate and the overexpression of a rate-limiting enzyme to enhance the carbon flow are promising approaches to improve the

photosynthetic productions.

Reducing power

Reducing cofactors (e.g., NAD(P)H) are requisite driving forces of cellular metabolisms, and thus are significantly relevant to the high productivity. In cyanobacteria, NADP(H) is more abundant than NAD(H) (Cooley and Vermaas, 2001; Ishikawa et al., 2016; Takahashi et al., 2008; Tamoi et al., 2005), since NADPH is generated by the light reaction and utilized after CO_2 fixation. Accordingly, high titer of products is often represented in the NADPH-dependent processes than in the NADH-dependent ones, as shown in **Table 0_1**. Notably, D-lactate dehydrogenase that was altered its favored cofactor from NADH to NADPH could produce more D-lactate than canonical NADH-dependent enzyme in engineered cyanobacteria (Li et al., 2015). The use of an NADPH-dependent acetoin reductase also drove the generation of the final product 2,3-butanediol (Savakis et al., 2013). These studies suggest that NADPH is the preferred reducing power for effective productions via photosynthesis.

The NADPH-dependent productivity is generally improved by the enhancement of NADPH supply. NADPH-dependent production of 3-hydroxypropionic acid, for instance, could be remarkably increased by overexpression of a membrane-bound transhydrogenase that mainly catalyzes NADPH generation from NADH (Wang et al., 2015). Enhancement of the linear electron transport for $NADP^+$ reduction could also improve the productivity of ethanol via increase in NADPH (Yoshikawa et al., 2017). Further, in NADH-dependent production, overexpression of a soluble transhydrogenase that mediates the reversible conversion between NADH and NADPH has increased the production of lactic acid (Niederholtmeyer et al., 2010; Varman et al., 2013), implying

the effective reinforcement of NADH supply. Otherwise, deletion of other competed NADH-consuming pathways, mainly that of lactate biosynthesis, led to enhancement of NADH generation and thereby hydrogen production catalyzed by the NADH-dependent hydrogenase in cyanobacteria (McNeely et al., 2010). These researches indicate that the selection and the reinforcement of reducing power are crucial points for improvement of photosynthetic production associated with carbon metabolisms.

Expression of suitable enzymes

To promote cyanobacterial biotechnology, heterologous expression of foreign enzymes should be carefully selected and expressed in the host cells. The first heterologous production of commodities (i.e., ethylene) in cyanobacteria was achieved by the expression of the ethylene-forming enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2 in *Synechococcus elongatus* PCC 7942 (Sakai et al., 1997). However, this system was unstable and easily lost during maintenance (Sakai et al., 1997). The problem was improved by stable expression of the codon-optimized enzyme in a different cyanobacterium *Synechocystis* sp. 6803, leading to stable and higher expression of the enzyme, resulting in much higher production (Ungerer et al., 2012). Screening of heterologous enzymes is also helpful for optimization of productivity, because the enzymes catalyzing a same reaction show diverse enzymatic kinetics in various organisms. Actually, the expression of key enzymes from several organisms affected the titer and quality of 2,3-butanediol (Oliver et al., 2013) and 1-butanol (Lan et al., 2013). However, expression of a specific enzyme is sometimes difficult because coding gene and/or enzymatic properties of certain enzymes still remain uncharacterized. In this case, an enzyme of similar enzyme activity may be borrowed for a catalyst to

proceed a desired reaction. For example, van der Woude et al. (2016) introduced two foreign enzymes into *Synechococcus* sp. PCC 7002 to convert erythrose-4-phosphate to erythritol and they successfully borrowed a member (TM1254) of haloacid dehalogenase-like hydrolases (HADs) of *Thermotoga maritima* for the erythrose 4-phosphatase (van der Woude et al., 2016). This was because a specific enzyme erythrose 4-phosphatase is not known in the database even now but the substrate screening of HADs revealed that TM1254 possessed a high hydrolase activity on erythrose-4-phosphate (Kuznetsova et al., 2005). Heterologous production of sorbitol is even a worse case, as an enzyme sorbitol-6-phosphatase essential for the production was not known. Instead, I screened several nonspecific hydrolases of *Escherichia coli* and *Synechocystis* sp. PCC 6803 and found some indeed exhibited sorbitol-6-phosphatase activity (Chapter 2). It is an important approach to explore novel or better enzymes for application to the metabolic engineering of cyanobacterial productions.

Export of intracellular product

Although products are often or sometimes excreted from recombinant cells to the culture medium, the efflux mechanism is usually passive diffusion, which may limit the productivity. Sugar alcohols such as glycerol, erythritol, and mannitol can be excreted to the culture medium to some extent likely in a way of passive diffusion. However, excretion of even glycerol can be facilitated by action of a facilitated transporter as shown in yeast (Luyten et al., 1995). Excretion of larger or charged molecules is even more difficult. Lactate was excreted by introduction of lactate/H^+ cotransporter, and glucose was exported by introduction of glucose/fructose facilitator (Niederholtmeyer et al., 2010). Sucrose was successfully produced and exported by co-expression of sucrose

permease (sucrose/H^+ symporter) (Ducat et al., 2012). The care of hydrophobic compounds may be analogous but slightly different from hydrophilic ones as mentioned above. Free fatty acids can be excreted to the culture medium even without modification, but their export was enhanced by expression of an RND-type efflux system involved in the export (Kato et al., 2015). Thus, excretion of products should be enhanced by introduction of their transporter. Facilitated diffusion may allow transport in both directions irrespective of the integration of a transporter in the membrane. Symporter with proton may require correct orientation of the protein with regard to the proton gradient across the membrane. Moreover, the facilitated excretion should lower the level of intracellular accumulation of products, leading to alleviation of potential feedback down-regulation of their production, which has been established in the chloroplast system in the history of endosymbiosis.

Sugar alcohols

Sugar alcohols as attractive product

It is crucial to fine-tune the underlying photosynthetic metabolism toward target production to further develop and improve photosynthetic production in cyanobacteria. For the concept, 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 photosynthesis. Various sugar alcohols (sorbitol, mannitol, xylitol, etc.) in addition to sucrose are produced naturally in certain plants for the translocation of carbons and energy between source and sink organs (Noiraud et al., 2001). 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 originally derived from photosynthesis, and thus it is desirable to directly produce sugar alcohols through photosynthesis by using combinatorial genetic engineering.

Sorbitol biosynthesis and production

D-Sorbitol, which is also systematically called as D-glucitol, is one of the major sugar alcohols and has numerous uses such as sweetener and humectant (Jonas and Silveira, 2004). Industrial production of sorbitol is performed by hydrogenation of glucose with

hydrogen gas and chemical catalysts (Zhang et al., 2013), and a few heterotrophic microorganisms such as *Zymomonas mobilis* likely possess the applied biological production of sorbitol via fermentation (He et al., 2014). However, sorbitol production using chemical or sugar-consuming process should be avoided in a sense of the problem of environments and resources. Biologically, sorbitol is a primary photosynthate and translocated carbohydrate found in *Rosacea* plants (Loescher, 1987) and a waste product of anaerobic metabolism in lactic acid bacteria (Monedero et al., 2010). In these organisms, sorbitol is produced from glucose-6-phosphate or fructose-6-phosphate by a two-step reactions mediated by NAD(P)-dependent sorbitol-6-phosphate dehydrogenase and sorbitol-6-phosphatase (**Fig. 0_1**): NADP-dependent sorbitol-6-phosphate dehydrogenase (EC 1.1.1.200) is encoded by the *s6pdh* gene in apple *Malus domestica* (Kanayama et al., 1992) and NAD-dependent sorbitol-6-phosphate dehydrogenase (EC 1.1.1.140) is encoded by *srlD1* or *srlD2* in lactic acid bacterium *Lactobacillus plantarum* (Kleerebezem et al., 2003). A gene for the sorbitol-6-phosphatase has not yet been identified in any organism, although its enzyme activity has been detected in plant extracts (Grant and Rees, 1981; Zhou et al., 2003). In metabolic engineering, heterologous biosynthesis of sorbitol has been reported following the introduction of only *s6pdh* into persimmon (Gao et al., 2001) and yeast (Shen et al., 1999). In these cases, some intrinsic enzyme(s) must hydrolyze the phosphate ester of the intermediate, sorbitol-6-phosphate, which is a non-natural metabolite. Sorbitol production in lactic acid bacteria is also enhanced by the overexpression of endogenous sorbitol-6-phosphate dehydrogenase and by repression of other NADH-consuming biosynthesis (De Boeck et al., 2010; Ladero et al., 2007). The sorbitol production could

still be improved by the introduction of an additional sorbitol-6-phosphatase gene, although whether the dephosphorylation step is rate-limited is unclear.

Production of sugar alcohols by cyanobacteria

Although sorbitol has not been produced in cyanobacteria, engineered production of the two sugar alcohols by using cyanobacteria has been reported recently: D-mannitol production in *Synechococcus* sp. PCC 7002 (Jacobsen and Frigaard, 2014) and erythritol production in *Synechocystis* sp. PCC 6803 (van der Woude et al., 2016). These systems achieved photosynthetic production by heterologous expressions of biosynthesis enzymes (i.e., reductase and phosphatase), although certain critical points remain to be resolved: (i) Because the carbons of these sugar alcohols are derived directly or closely from Calvin cycle metabolites, their production might affect the balance between metabolic flows; (ii) reductants, which are mainly supplied from 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 (iii) the export of sugar alcohols to the culture medium might also be limited in these systems. These hurdles are likely to be universally encountered in photosynthetic production utilizing engineered cyanobacteria.

Aim of this thesis

In this thesis, I aim to produce the sorbitol using a model cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis*) toward application of photosynthetic productions. Since cyanobacteria do not naturally biosynthesize sorbitol, I have constructed the recombinant pathway of sorbitol biosynthesis based on metabolic engineering in *Synechocystis* (**Fig. 0_2**). To improve the sorbitol productivity by engineered cyanobacteria, I have attempted to fine-tune carbon and reductants supply, which are key factors in cyanobacterial productions as mentioned before. Furthermore, active enzymes for sorbitol-6-phosphatase have been investigated to promote heterologous production of sorbitol in any organisms. This thesis shows a promising approach to apply the engineering of photosynthesis for sustainable productions.

Chapter 1

Sorbitol production and optimization of photosynthetic supply in cyanobacteria

Introduction

I focused on the sugar alcohol sorbitol as the target product in engineered cyanobacteria. In nature, sorbitol is produced 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 through fermentation as a waste product from excess reductants (Ladero et al., 2007). I introduced into cyanobacteria the gene encoding the sorbitol-biosynthesis enzyme from apple, NADP-dependent sorbitol-6-phosphate dehydrogenase (hereafter S6PDH), or from anaerobic lactic acid bacteria, NAD-dependent sorbitol-6-phosphate dehydrogenase (SrlD2). I found that the NADPH-dependent enzyme was effective in sorbitol production but was toxic to cyanobacterial cells, whereas the NADH-dependent enzyme did not support sorbitol production. Thus, I further fine-tuned the production system by employing approaches such as using riboswitch induction of S6PDH, suppressing toxicity, and enhancing the supply of NADPH. My engineering strategy could be expanded to several biomaterials for production from near the core of the carbon-fixation cycle in cyanobacteria.

Results

Sorbitol-6-phosphate dehydrogenase expression in cyanobacteria

I attempted to produce sorbitol through photosynthesis in *Synechocystis* by introducing one of the two distinct sorbitol-6-phosphate dehydrogenases, NADH-dependent SrlD2 or NADPH-dependent S6PDH. Whereas SrlD2 catalyzes the reduction of fructose-6-phosphate (F6P) with NADH to produce sorbitol-6-phosphate, S6PDH catalyzes the reduction of glucose-6-phosphate (G6P) with NADPH to produce sorbitol-6-phosphate (**Fig. 0_1**). The strain constitutively expressing the gene *srlD2* grew normally, like *Synechocystis* wild-type cells (WT), but no sorbitol production was detected (**Fig. 1_1**). By contrast, introduction of the gene *s6pdh* under the strong constitutive promoter, P_{trc}, yielded no transformants, probably due to the toxicity of S6PDH in *Synechocystis*. To overcome this problem, *s6pdh* was expressed with a theophylline-inducible riboswitch for regulation of translation under the *trc* promoter, P_{trc}::riboswitch (Nakahira et al., 2013). Transformants were successfully isolated and complete segregation of genome-integrated *s6pdh* was achieved under the non-inducing condition (i.e., without theophylline), and thus the strain "s6pdh" was obtained.

The strain s6pdh was cultivated with or without 2 mM theophylline to evaluate S6PDH expression in *Synechocystis*. As expected, theophylline addition strongly inhibited the growth of the strain s6pdh, which grew normally, like WT, when theophylline was not added (**Fig. 1_1A**). WT growth was not inhibited by 2 mM theophylline (**Fig. 1_1A**). Production of sorbitol (11.7 mg/L at 201 h) was detected in the culture medium of the strain s6pdh treated with theophylline, whereas no sorbitol was detected either in the strain s6pdh cultured without theophylline or in WT (**Fig.**

1_1B). By contrast, expression of SrlD2 or S6PDH led to sorbitol production in *E. coli* with little growth retardation. These results indicate that (i) WT is incapable of producing sorbitol; (ii) NADH-dependent SrlD2 does not support sorbitol production in *Synechocystis*; (iii) NADPH-dependent S6PDH can produce sorbitol in *Synechocystis*; and (iv) sorbitol production or expression of S6PDH causes fatal growth inhibition of *Synechocystis* cells. Hereafter, I focused on the NADPH-dependent sorbitol enzyme, S6PDH.

Optimization of S6PDH expression with theophylline induction

To improve the sorbitol productivity of the strain s6pdh, I investigated the theophylline-induction condition that is optimal for alleviating growth inhibition and enhancing sorbitol production. First, the induction time for the addition of 2 mM theophylline was examined. Typically, growth inhibition was observed at 2 days after theophylline addition. The inhibition was less pronounced when the cell density at the theophylline addition was higher (**Fig. 1_2A**). The cells that were induced at 50 h produced more sorbitol than did the cells that were induced at 0 or 26 h (**Fig. 1_2B**); however, the cells that were induced at 50 or 74 h produced comparable amounts of sorbitol when the induced period was same. These results suggest that an elevated cell density does not necessarily support increased sorbitol production. Thus, theophylline addition was fixed at 48 h of cultivation, which corresponded to a cell density at a 730 nm optical density (OD_{730}) of about 2.0.

Next, the concentration of added theophylline was evaluated for the fixed induction time at 48 h of cultivation (**Fig. 1_3**). Growth inhibition became increasingly severe with an elevation of theophylline concentration. However, sorbitol accumulation did not

show a clear dependence on theophylline concentration except at 0.1 mM during the initial phase of induction (48–96 h). During the late phase (120–266 h), maximal accumulation was recorded with 0.5 mM theophylline (the yield was 111 ± 8.79 mg/L of sorbitol for 266 h of cultivation), probably due to lesser inhibition than at relatively higher concentrations. For the remainder of the study, the induction was fixed at 0.5 mM theophylline addition at 48 h of cultivation. Notably, higher concentrations of theophylline did not further enhance sorbitol production even in the initial phase where growth was not markedly inhibited. The rate of sorbitol production might have been limited by supply of the substrates or other factor(s). Moreover, the survival of cells appeared to be critical for sustainable production of sorbitol.

FBPase overexpression in sorbitol-producing cyanobacteria

Regarding growth inhibition during sorbitol production, I postulated that a critical shortage of photosynthetic products such as F6P or NADPH might cause the inhibition, because S6PDH consumes G6P and NADPH. G6P is directly converted from F6P, which is supplied from fructose-1,6-bisphosphate by frucrose-1,6-bisphosphatase (FBPase) that is one of the rate-limiting steps in the Calvin cycle. I introduced three FBPase genes coupled with a strong *trc* promoter into a neutral site of the strain s6pdh: spinach *fbp* and *Synechocystis fbp-I* and *fbp-II*. FBPase overexpression apparently alleviated the cell death, and spinach *fbp* was the most effective among the examined FBPases (**Fig. 1_4A**). Consequently, sorbitol production of the strain s6pdh/fbp was enhanced particularly at the late phase as compared with that in the parent strain s6pdh (**Fig. 1_4B**). Enzyme assays performed using crude extracts revealed that total FBPase activity was approximately 5-, 3-, and 1.5-fold higher, respectively, in the strains

s6pdh/fbp-I, s6pdh/fbp-II, and s6pdh/fbp than in the parent strain (**Fig. 1_4C**). I also introduced a second copy of spinach *fbp* into the strain s6pdh/fbp, but this did not further improve growth or sorbitol production (**Fig. 1_5**), which suggested that FBPase activity was not the limiting factor in the strain s6pdh/fbp. Regulation of FBPase or the balance relative to other enzymes might be critical for the alleviation of cell death or for sustained sorbitol production.

Engineering for enhancement of NADPH supply

To evaluate NADPH consumption during sorbitol production, I measured the amounts of NADPH and $NADP^+$ in the crude extracts of cells grown with or without theophylline induction. The NADPH level and the NADPH/$NADP^+$ ratio decreased substantially during the sorbitol production after induction in the strains s6pdh and s6pdh/fbp (**Figs. 1_6A**–**C**), but the measurements without induction showed little change between 48 and 96 h (**Figs. 1_6D**–**F**). These results confirmed the notion that the NADPH level was decreased due to its consumption for the induced sorbitol production.

To enhance the NADPH supply, I overexpressed the endogenous genes for membrane-bound pyridine nucleotide transhydrogenase, *pntA* and *pntB*, which encode the peripheral and the membrane-bound subunits of the transhydrogenase, respectively. The complex formed by the gene products catalyzes electron transfer directly from NADH to $NADP^+$ at the expense of membrane potential (Kämäräinen et al., 2016). I introduced another copy of *Synechocystis pntA* and *pntB* genes with strong promoters into a neutral site of the strains s6pdh and s6pdh/fbp. The overexpression of *pntAB* only slightly alleviated the growth inhibition in the strain s6pdh/pnt (**Fig. 1_7A**), but

noticeably improved sorbitol production at the initial phase where growth was not yet markedly inhibited (**Fig. 1_7C**). Similar effects of *pntAB* overexpression were also observed in the strain s6pdh/fbp/pnt, and thus the sustainable production of sorbitol was further improved in the triple-overexpression strain s6pdh/fbp/pnt after the induction (**Fig. 1_7B**; 202 ± 9.70 mg/L of sorbitol for 264 h of cultivation). These results indicate that overexpression of the membrane-bound transhydrogenase (PntAB) effectively accelerated sorbitol production, but, in contrast to FBPase overexpression, was not adequately effective in rescuing the growth inhibition. I also introduced a homologous *pntAB* derived from *E. coli* into the strain s6pdh/fbp, but this triple-overexpression strain showed neither alleviation of growth inhibition nor enhanced sorbitol production (**Fig. 1_8**). These results suggest that the endogenous transhydrogenase, but not the *E. coli* enzyme, is suitable for integration into the thylakoid membrane in *Synechocystis*. To further evaluate the overexpression of *Synechocystis pntAB*, the NADPH/$NADP^+$ ratio was assayed using the crude extracts of the strains s6pdh/pnt and s6pdh/fbp/pnt. However, the overexpression of *pntAB* led to no increase in the NADPH level or NADPH/$NADP^+$ ratio (**Figs. 1_6A and 1_6C**), although the sorbitol production was improved (**Figs. 1_7B and 1_7C**). The overexpression of transhydrogenase might be insufficient for the recovery of the NADPH/$NADP^+$ ratio, but be effective for enhancement of the turnover of NADPH/$NADP^+$ to support sorbitol production.

I attempted to further engineer the strain s6pdh/fbp to enhance NADPH supply for the improvement of sustainable sorbitol production. First, I overexpressed glucose-6-phosphate dehydrogenase (G6PDH), which catalyzes the first-step and rate-limiting reaction in the oxidative pentose phosphate pathway and generates NADPH from the substrate G6P, derived from *Synechocystis* (*szwf*) and *E. coli* (*ezwf*).

NADPH level was likely recovered by overexpression of endogenous G6PDH (*szwf*) (**Figs. 1_9D**–**F**), whereas both the strains overexpressed G6PDH produced less sorbitol than the parent strain s6pdh/fbp (**Figs. 1_9B and 1_9C**). Thus, engineering of NADPH supply by modulating the core of oxidative pentose phosphate pathway may be inappropriate to apply in sorbitol-producing cyanobacteria.

To enhance NADPH generation without interfering with carbon metabolisms, I attempted to repress electron flow that may compete with the reduction of $NADP^+$ in light reaction. One strategy was disruption of flavodiiron proteins (FLVs) that mediate direct reduction of oxygen to water (Allahverdiyeva et al., 2015). Among the four genes of FLVs in *Synechocystis*, I deleted an *flv3* gene and the mutant was cultivated under normal and high light. Sorbitol productivity and NADPH supply, however, were not improved by deletion of *flv3* (**Fig. 1_10**).

Another engineering for enhancement of NADPH supply was the inactivation of type-I NADPH dehydrogenase complex (NDH) that is engaged in the cyclic electron transport (Ogawa and Mi, 2007). I attempted to create *⊿ndhI* mutant strain because the subunit is essential for optimal complex formation and function of NDH in cyanobacteria (Dai et al., 2013). The deletion of an *ndhI* gene was not completed in the strain s6pdh/fbp (**Fig. 1_11A**), as described in the previous study by Dai et al. (2013). The partial deficient *ndhI* strain (ndhI-less) was assayed for sorbitol production. Notably, $NADPH/NADP^+$ ratio was recovered after induction (**Fig. 1_11G**), and the sorbitol production rate was slightly improved at initial phase (**Fig. 1_11D**). However, the sustainable productivity of sorbitol was lower than that of the parent strain **(Fig. 1_11C**), because the cell integrity of ndhI-less strains might be affected (**Fig. 1_11B**).

These results of NADPH engineering suggest that (i) NADPH supply is a

rate-limiting factor in cyanobacterial production of sorbitol; (ii) overexpression of *Synechocystis* PntAB is most effective for the sorbitol production among examined NADPH engineering; and (iii) the proteins that are involved in the carbon metabolism, FLV-mediated electron transport or NdhI-based cyclic electron transport are not effective for the NADPH-dependent production of sorbitol. Therefore, I have utilized the endogenous PntAB for sorbitol-producing cyanobacteria in the following chapter.

S6PDH activity in engineered cyanobacteria

S6PDH activity was measured in the crude extracts from sorbitol-producing cells at 168 h of cultivation with theophylline induction. The activity was comparable among the single-, double-, and triple-overexpression strains, whereas no activity was detected in WT (**Fig. 1_12**). This result suggests that S6PDH is expressed under theophylline induction and that the improvement of sorbitol productivity in the double- and triple-overexpression strains was not due to a modification of S6PDH expression.

Sorbitol productivity under high CO_2 and high light

To directly increase photosynthetic supply, the sorbitol-producing strains expressing S6PDH, FBPase, and/or PntAB were cultivated under conditions with high CO_2 for increasing carbon source or with high light for increasing NADPH. In the high CO_2 conditions (4% (v/v) CO_2), the growth inhibition with induction was alleviated even in the strain s6pdh (**Fig. 1_13A**), whereas the amounts of sorbitol were comparable or less than those of cells cultivated under 1% (v/v) CO_2 (**Figs. 1_7B and 1_13B**). This suggests that increasing carbon is slightly effective for alleviation of the growth

inhibition but not for enhancement of the sorbitol production, like overexpression of FBPase.

In the high light conditions (100 μmol photons $\cdot m^{-2} \cdot s^{-1}$), the cells rapidly grew before induction but died soon after the induction (**Fig. 1_14A**). Sorbitol production rate at the initial phase was improved in the strains s6pdh and s6pdh/fbp but not in the strains that overexpressed endogenous PntAB, comparing to the normal light conditions (**Figs. 1_7C and 1_14B**). NADPH/$NADP^+$ ratio was likely recovered after induction in all strains (**Fig. 1_14E**). The enhancement of NADPH supply by high light might be effective to accelerate the sorbitol production, but other rate-limiting step should be improved for the further acceleration of sorbitol production in cyanobacteria.

Intracellular sorbitol

One possible reason for the growth inhibition and for the limited sorbitol productivity could be the accumulation of excess amounts of sorbitol in cells. Therefore, I determined the intracellular sorbitol concentration after induction with theophylline (**Fig. 1_15**). The four sorbitol-producing strains accumulated approximately 8–9 mM sorbitol within the cells after 96 h cultivation (48 h induction). After cultivation for 168 h (120 h induction), the double- and triple-overexpression strains accumulated sorbitol at amounts comparable to or a little more than that after 48 h induction, but the intracellular sorbitol level in the strain s6pdh decreased, probably due to loss of cellular integrity. These results suggest that sorbitol-producing cells can accumulate ~13 mM sorbitol without exhibiting severe growth inhibition, and that the cell death in the strain s6pdh is not resulted from excessive accumulation of intracellular sorbitol.

It should be noted that cells with higher intracellular sorbitol concentrations released

more sorbitol in the culture medium somehow (**Figs. 1_7B and 1_15**). Sorbitol may be excreted through passive diffusion, although the molecular mechanism of this excretion in *Synechocystis* is currently unknown. To accelerate the efflux of intracellular sorbitol, I introduced and expressed two genes encoding the apple sorbitol transporter (SOT), which uptakes sorbitol for the translocation in tissues, in the strain s6pdh/fbp/pnt. However, expression of *sot* genes could not accelerate to excrete the intracellular sorbitol, and rather impaired the growth and decreased the titer of sorbitol production (**Fig. 1_16**). SOT of apple might not be suitable for the improvement of sorbitol productivity in cyanobacteria, although these transporters have high specificity to sorbitol.

Discussion

I succeeded in photosynthetic production of sorbitol and in its improvement by using a model cyanobacterium, *Synechocystis* sp. PCC 6803. Two types of sorbitol-6-phosphate dehydrogenase, NADH-dependent SrlD2 and NADPH-dependent S6PDH, were introduced into *Synechocystis*, and only S6PDH enabled sorbitol production (**Fig. 1_1**). This finding might indicate that NADPH is fully available but NADH is scarce under the phototrophic condition in cyanobacterial cells as noted in McNeely et al. (2010) (**Fig. 1_6**). However, the sorbitol production by S6PDH was coupled with severe growth inhibition in *Synechocystis* cells. I found that FBPase overexpression was effective to alleviate the growth inhibition (**Fig. 1_4**). This finding indicates that the depletion of G6P or F6P in the central metabolism near the Calvin cycle by massive consumption via the S6PDH reaction may be the major reason for the severe growth inhibition during sorbitol production.

FBPase is one of the rate-limiting enzymes of the Calvin cycle, although the type of this enzyme and its regulation have diverged between plants and cyanobacteria. Typically, the activity of chloroplast FBPase in higher plants is redox-regulated through the ferredoxin/thioredoxin system in a light-dependent manner (Reichert et al., 2003; Villeret et al., 1995). Several cyanobacteria possess FBPase-I and FBPase-II: FBPase-I exhibits both FBPase and sedoheptulose-1,7-bisphosphatase activities, being essential for the Calvin cycle, whereas FBPase-II is specific to fructose-1,6-bisphosphate but cryptic for photosynthesis (Cotton et al., 2015; Feng et al., 2014; Tamoi et al., 1998; Tamoi et al., 1999). When cyanobacterial FBPase-I and FBPase-II were introduced into higher plants, photosynthesis and plant growth were improved (Ichikawa et al., 2010;

Miyagawa et al., 2001; Tamoi et al., 2006). Conversely, overexpression of wheat FBPase in a cyanobacterium, *Anabaena* PCC 7120, enhanced biomass production (Ma et al., 2005). Heterologous or endogenous overexpression of cyanobacterial FBPase-I and FBPase-II in *Synechocystis* also effectively increased the biomass and slightly increased growth (Liang and Lindblad, 2016). These effects on biomass production in cyanobacteria and plants might reflect upregulation of the rate-limiting step in photosynthetic carbon fixation. Thus, FBPase overexpression would be expected to alleviate the cell death occurring during sorbitol production, as shown in this study. Toxicity similar to that reported here was also observed in the constitutive production of mannitol in *Synechococcus* sp. PCC 7002 (Jacobsen and Frigaard, 2014). This toxicity might also be mitigated by co-expressing excess FBPase. I found that growth inhibition was alleviated more effectively following the overexpression of spinach FBPase (s6pdh/fbp) than that of endogenous FBPase-I or FBPase-II, although FBPase enzyme activity itself was lower in the strain s6pdh/fbp than in the strains s6pdh/fbp-I and s6pdh/fbp-II (**Fig. 1_4**). These results suggest that FBPase activity was no longer limited in these overexpression strains. Photosynthetic regulation of the endogenous metabolic network might be affected by cyanobacterial FBPase during sorbitol production. Thus, for further fine-tuning the sorbitol production, it would be crucial to carefully evaluate the side effects and direct effects of each overexpression.

NAD(P)H is essential as reducing power in metabolism in all organisms, and is balanced by several enzymes (Spaans et al., 2015). Pyridine nucleotide transhydrogenases catalyze reversible conversion between NADPH/$NADP^+$ and NADH/NAD^+ and frequently play critical roles in the optimal growth of organisms such as *E. coli* (Sauer et al., 2004) and *Synechocystis* (Kämäräinen et al., 2016). Two distinct

isoforms of the enzyme exist: the energy-independent soluble transhydrogenase (UdhA) and the energy-dependent, or proton-translocating, membrane-bound transhydrogenase (PntAB). Despite its mediation of reversible reactions in vitro, UdhA physiologically catalyzes the hydride transfer reaction toward NADH generation because of the relatively higher concentration of NADPH in aerobic cells. By contrast, PntAB catalyzes the generation of NADPH by using the electrochemical proton gradient across the membrane under physiological conditions (Fuhrer and Sauer, 2009; Jackson, 2012; Leung et al., 2015; Sauer et al., 2004). Accordingly, overexpression of *E. coli* UdhA in cyanobacteria, for example, improved NADH-dependent production of lactate but reduced phototrophic growth, probably due to NADPH consumption by UdhA (Niederholtmeyer et al., 2010). Conversely, overexpression of the endogenous PntAB in *Synechocystis* increased the NADPH-dependent production of 3-hydroxypropionic acid without inhibition of phototrophic growth (Wang et al., 2015). Here, sorbitol production clearly decreased the NADPH level and NADPH/$NADP^+$ ratio (**Figs. 1_6A**–**C**). Thus, I overexpressed the endogenous PntAB in sorbitol-producing cells together with or without FBPase, and found that in both cases, PntAB overexpression substantially improved the sorbitol production at the initial induction phase, but did not markedly rescue the growth inhibition as compared with FBPase overexpression (**Fig. 1_7**). Moreover, PntAB overexpression failed to mitigate the decline of the NADPH/$NADP^+$ ratio; this was probably because the supply of reducing equivalents from NADH was not substantial as compared with the consumption of NADPH by S6PDH.

I have further sought to increase NADPH generation by engineering: overexpression of G6PDH and enhancement of $NADP^+$ reduction in light reaction of photosynthesis. G6PDH encoded by a *zwf* gene plays a major role in NADPH generation and reducing

balance through the oxidative pentose phosphate pathway in heterotrophs (Fuhrer and Sauer, 2009). This enzyme is repressed by redox modulation in photoautotrophs such as plants (Wenderoth et al., 1997) and cyanobacteria (Sundaram et al., 1998; Udvardy et al., 1984). Both types of G6PDH could not only enhance the NADPH generation but also improve the NADPH-dependent productions of poly-hydroxybutyrate in *E. coli* (Lim et al., 2002) and ethanol in *Synechocystis* (Choi and Park, 2015). Accordingly, to improve sorbitol productivity with enhancement of NADPH supply in cyanobacteria, I overexpressed G6PDH from *E. coli* and *Synechocystis*. Sorbitol production and NADPH supply, however, were not improved (**Fig. 1_9**). Since NADPH consumption is related to the sorbitol production (**Fig. 1_6**), the recovery of NADPH by endogenous G6PDH was probably due to decreasing of sorbitol production. NADPH level should be increased without induction if G6PDH overexpression could generate more NADPH, but the data did not support that (**Figs. 1_9D**–**F**). Further study will be needed for the effect of overexpression of G6PDH.

Reduction of $NADP^+$ is coupled to the linear electron transport of light reaction in photosynthesis, and thus repression of other competing electron flows may enhance the generation of NADPH. NDH functions as the receptor of cyclic electron transport and modulates CO_2 uptake and respiration for optimal growth. *Synechocystis* possesses the genes for 15 subunits of NDH (Ogawa and Mi, 2007) in addition to the assembly factors of efficient NDH (Dai et al., 2013; Wang et al., 2016). The inactivation of NDH apparently gives critical phenotypes in cyanobacteria; core subunits are essential for the cells. NdhI localizes at the terminal part of NDH and plays a crucial role in the electron transfer process from unknown electron donor (likely NAD(P)H or ferredoxin), and thus the inactivation of NdhI inhibits the optimal photosynthesis even with partial

segregation of the mutant (Dai et al., 2013). Although my ndhI-less strain was also not segregated, sorbitol production and NADPH recover were likely improved (**Fig. 1_11**). In the mutant deficient in NdhB, which is one of the core subunits (Ogawa, 1991), NADP is mostly in the reduced form (Cooley and Vermaas, 2001) and the growth are drastically inhibited (Dai et al., 2013; Ohkawa et al., 2000). These observations suggest that NDH-deficient mutant might be somewhat effective for NADPH-dependent sorbitol production in cyanobacteria, whereas it is difficult to construct the stable *ndhI*-less. Only ndhB-deficient mutant would be suitable for this idea. I obtained the ndhB-deficient mutant and tried to introduce *s6pdh*, but failed at this moment.

FLV mediates direct reduction of oxygen to water using the electron likely from NAD(P)H or ferredoxin (Allahverdiyeva et al., 2015). *Synechocystis* has four genes of FLV, and the FLV1/FLV3 heterodimer is crucial for optimal growth of the cells (Allahverdiyeva et al., 2013; Allahverdiyeva et al., 2011). Accordingly, *flv3* gene was deleted in the strain s6pdh/fbp, but the sorbitol productivity was hardly improved under cultivation with normal and high light (**Fig. 1_10**). This may suggest that FLVs do not function significantly under conditions for sorbitol production.

In sorbitol-producing cyanobacteria, two critical processes require improvement: export of sorbitol and dephosphorylation of sorbitol-6-phosphate. However, the genes responsible for these steps are not yet known in any organisms. The process of sorbitol excretion might be limited in *Synechocystis*, because the intracellular sorbitol concentration was ≥20-fold higher than that in the culture medium (**Figs. 1_7B and 1_15**). In apple, several genes have been cloned as sorbitol-uptake transporters for phloem loading (Watari et al., 2004) and unloading (Gao et al., 2005). These genes are expressed in source leaves and certain sink organs and confer proton/sorbitol

cotransporter activity in heterologous expression systems. I postulated that these enzymes might exhibit transport activity in both directions, and thus I introduced the genes into my strains. The excretion of sorbitol, however, was not clearly accelerated (**Fig. 1_16**), probably because these transporters might act as a sorbitol-uptake transporter in cyanobacterial cells.

Sorbitol-6-phosphate, the product of S6PDH, should be dephosphorylated by an unidentified enzyme(s) that exhibit broad specificity because sorbitol-6-phosphate and sorbitol are not naturally occurring metabolites in *Synechocystis*. The intracellular concentration of this metabolite is unknown, but the dephosphorylating activity in the cells might be insufficient for massive production. Previous studies have reported that several organisms possess cytoplasmic hydrolases belonging to a family of haloacid dehalogenase-like hydrolases (HADs) showing dephosphorylation activities to a wide range of metabolites. For instance, *E. coli* possesses 23 members of this family, which present distinct specificity spectra (Kuznetsova et al., 2006). Some of these enzymes might support the sorbitol production in the sorbitol-6-phosphate dehydrogenase-expressing *E. coli* (**Fig. 1_17**). The *Synechocystis* genome harbors potential candidate genes which may be responsible for sorbitol-6-phosphatase activity. Further characterization of these proteins and their overexpression might improve the production of sorbitol. These ideas will be investigated in the following chapter.

Materials and methods

Strains and plasmid construction

The cyanobacterial strains, plasmids, and primers used in Chapter 1 are shown in **Tables 1_1, 1_2, and 1_3**, respectively. The glucose-tolerant strain of the unicellular cyanobacterium *Synechocystis* sp. PCC 6803 (Ikeuchi and Tabata, 2001) was used as a platform in this study. 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. Codon-optimized DNA fragments (**Fig. 1_18**) were synthesized by FASMAC (Atsugi, Japan).

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. 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. *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. 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. 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* (*slr1239*) and *pntB* (*slr1434*), were PCR-amplified from the genomic DNA by using these primers: SpntA-3Ftrc and SpntA-2Rrrn for *pntA*, and SpntB-1FA2P and SpntB-2RA2T for *pntB*. The *pntA* product was cloned into a plasmid vector harboring a *trc* promoter, an *rrnB* terminator, a spectinomycin-resistance cassette, and a neutral site near *carA*; this produced pBcaSTpntAT. The *pntB* product was cloned into a plasmid harboring the

endogenous *psbA2* promoter, a *psbA2* terminator, a chloramphenicol-resistance cassette, and the neutral site near *IS203c*, which yielded pBISCPpntBP. These plasmids were combined by performing PCR and infusion cloning to generate pBcaSTpntAT-PpntBP by using specific primer sets (carA-16FpsaA and rrn-2R for pBcaSTpntAT, and psbA2-52Frrn and psbA2-16RpsaA for pBISCPpntBP). The final plasmid was introduced into the strain s6pdh or s6pdh/fbp, and complete segregation of the integrated genes was confirmed using PCR. These overexpression strains are abbreviated s6pdh/pnt and s6pdh/fbp/pnt, respectively.

Genes encoding the *g6pdh* of *Synechocystis* (*slr1843*: *szwf*) and *E. coli* (*ezwf*) were PCR-amplified from the genomic DNA by using the primer set szwf-1Ftrc and szwf-2Rrrn for *szwf* and ezwf-1Ftrc and ezwf-2Rrrn for *ezwf*, and were cloned into a plasmid vector harboring a *trc* promoter, an *rrnB* terminator, a spectinomycin-resistance cassette, and a neutral site near *carA*, resulting in pBcaSTszwfT and pBcaSTezwfT, respectively. The plasmid was introduced into the strain s6pdh/fbp, and complete segregation of the integrated genes was confirmed using PCR. These overexpressing strains are abbreviated s6pdh/fbp/szwf and s6pdh/fbp/ezwf, respectively.

The *flv3* and *ndhI* genes were inactivated by replacement with an erythromycin resistance cassette in plasmid DNA of pTflv3-Er and pSndhI-Er, respectively. Mutants were generated by natural transformation of the cells of strain s6pdh/fbp with the plasmid DNAs and selected on BG11 plate containing erythromycin. The segregation was examined by PCR with primers used for the construction of plasmid.

The sequences of sorbitol transporter (SOT) were derived from *Malus domestica* for SOT2 (GenBank accession no. **NC_024250.1** REGION: complement (8921007..8924133)) (Gao et al., 2005) and SOT3 (GenBank accession no.

NC_024250.1 REGION: complement (2403616..2406135)) (Watari et al., 2004). The coding region was synthesized with codon optimization, and was PCR-amplified using the primer set msot2-3FTR and msot2-2Rrrn for *sot2* and msot3-3FTR and msot3-2Rrrn for *sot3*, respectively. The *sot* products and the *trc*::theophylline-riboswitch promoter amplified with TR-1Fcom and TR-6R were cloned into a plasmid vector harboring an *rrnB* terminator, an erythromycin-resistance cassette, and a neutral site near *slr2031*, resulting in pB31ERsot2T and pB31ERsot3T. The plasmid was introduced into the strain s6pdh/fbp/pnt, and complete segregation of the integrated genes was confirmed using PCR. These strains are abbreviated s6pdh/fbp/pnt/sot2 and s6pdh/fbp/pnt/sot3, respectively.

Culture conditions

Cyanobacterial cells were cultivated using 50 mL of BG11 liquid medium (Rippka et al., 1979) containing 20 mM HEPES-KOH buffer (pH 7.8) plus antibiotics: kanamycin, chloramphenicol, and/or spectinomycin (each at 20 μg/mL). Cultures were grown at 31°C with bubbling of 1% (v/v) CO_2 under continuous illumination with white fluorescent lamps of 30 μmol photons$\cdot m^{-2}\cdot s^{-1}$. At high cultivation conditions, 4% CO_2 or 100 μmol photons$\cdot m^{-2}\cdot s^{-1}$ was used, respectively. For sorbitol production, 50 mL of fresh medium was inoculated with late-log-phase cells at OD_{730} of 0.2; OD_{730} was monitored using a UV-2600 spectrophotometer (Shimadzu, Japan). Theophylline in dimethyl sulfoxide (prepared as a 200 mM stock solution) was used for induction.

Sorbitol quantification

Aliquots (typically 1.5 mL) of the culture were harvested by centrifugation (5,000×*g*, 5 min, 4°C) to separate the culture supernatant and cells. The cells were washed with 1 mL of BG11 medium and resuspended in 300 µL of distilled water. After mixing with 1 g of 0.1-mm zirconia/silica beads (Biospec Products, Fisher scientific), cells were disrupted using a bead beater (Micro Smash MS-100R, TOMY) by agitating the suspension for 5 cycles at 3,000 rpm for 30 sec with 1-min intervals with cooling. The crude extract was collected through centrifugation. The culture supernatant and crude extract were heat-treated (80°C, 15 min) and clarified by centrifugation (20,000×*g*, 5 min, 4°C), and sorbitol amounts were determined by using a D-sorbitol/xylitol assay kit according to the manufacturer's instructions (R-Biopharm, Germany). Sorbitol was measured as absorbance at 492 nm (A_{492}). Intracellular concentration of sorbitol was calculated based on the packed cell volume of WT (9.2×10^{-4} OD_{730}^{-1}).

Enzyme activity assay

For assaying S6PDH activity, cells in 20 mL of culture at 168 h of cultivation were harvested by centrifugation (5,000×*g*, 10 min, 4°C) and resuspended in 2 mL of a solution containing 100 mM Tris-HCl buffer (pH 9.0) and 1 mM DTT. Cells were disrupted as described in Section "Sorbitol quantification", and the crude extract was collected by brief centrifugation and then clarified by centrifugation at 20,000×*g* for 5 min at 4°C. S6PDH activity was measured as NADPH consumption as described previously (Kanayama and Yamaki, 1993), with minor modifications. Briefly, a reaction mixture was prepared containing 100 µL of 1 M Tris-HCl buffer (pH 9.0), 20 µL of 10 mM NADPH, and 20 µg of crude-extract protein in a volume of 900 µL. After pre-incubation for 5 min at 30°C, 100 µL of 500 mM G6P was added to start the

reaction, and the decrease of A_{340} was monitored at 30°C for 2 min. One unit (U) of S6PDH was defined as the amount of enzyme that catalyzed the oxidation of 1 μmol of NADPH per min.

For the FBPase activity assay, cells in 1.5 mL of culture at 96 h of cultivation were harvested by centrifugation and disrupted as described in Section "Sorbitol quantification", and then the crude extract was collected and clarified as described in the preceding paragraph. FBPase activity was assayed as enzyme-coupled $NADP^+$ reduction as previously described (Kelly et al., 1982), with minor modifications. Briefly, a reaction mixture was prepared containing 100 mM Tris-HCl buffer (pH 9.0), 10 mM $MgCl_2$, 0.5 mM EDTA, 0.3 mM $NADP^+$, phosphoglucose isomerase (1.4 U; Sigma-Aldrich), glucose-6-phosphate dehydrogenase (0.7 U; Sigma-Aldrich), and 20 μg of crude-extract protein in 1 mL. After preincubation for 5 min at 30°C, 50 μL of 12 mM fructose-1,6-bisphosphate was added to the reaction mixture, and the A_{340} increase was monitored at 30°C for 5 min; 1 U of FBPase was defined as the amount of enzyme that catalyzed the reduction of 1 μmol of $NADP^+$ per min.

Protein concentrations were measured by using the Bradford (Bradford, 1976) with bovine serum albumin as the standard.

NADP(H) quantification

NADP(H) was quantified using an NADP/NADPH quantification colorimetric kit (BioVision, USA). Cells in 1.5 mL of culture were harvested by centrifugation (5,000×*g*, 5 min, 4°C), washed with 1 mL of cold 100 mM sodium phosphate buffer (pH 8.2), and disrupted by agitation the suspension for 3 cycles at 3,000 rpm for 30 sec with 1-min intervals with cooling, and then the crude extract was collected and clarified by

centrifugation at 20,000×*g* for 5 min at 4°C. $NADP^+$ was selectively removed by heat treatment (15 min, 60°C) as per the manufacturer's instructions, whereas total NADP(H) was quantitated without heat treatment. NADPH and/or $NADP^+$ concentrations were quantitated by performing a cycling color-development reaction for 2 h at room temperature and then recording the A_{450}. The amounts of NADP(H) were calculated according to the standard curve and expressed as pM per OD_{730} in the culture.

Chapter 2

Detection of active sorbitol-6-phosphatase

Introduction

Sorbitol-6-phosphatase (EC 3.1.3.50) catalyzes sorbitol production from sorbitol-6-phosphate in certain organisms but has not been identified unequivocally. The active phosphatase for sorbitol-6-phosphate is required to further improve heterologous production of sorbitol. Since *E. coli* and *Synechocystis* cells produced sorbitol when the sorbitol-6-phosphate dehydrogenase is overexpressed (**Figs. 1_1 and 1_17**), potential phosphatase genes may be identified from the genomes.

In this chapter, I focused on the haloacid dehalogenase-like hydrolase (HAD) superfamily, which includes phosphatase, phosphonatase, dehalogenase, phosphoglucomutase, and ATPase activities and shows a fairly broad specificity (Burroughs et al., 2006; Koonin and Tatusov, 1994). These proteins are widely distributed in almost all organisms, although their physiological roles have remained unclear. There are 28 and 45 genes for the HAD enzymes in the genomes of *E. coli* and the yeast *Saccharomyces cerevisiae*, respectively. In particular, the specificity and kinetic parameters of the phosphatase activity of the soluble HADs of these organisms have been extensively characterized (Kuznetsova et al., 2006; Kuznetsova et al., 2015). These HADs have been grouped into several categories, depending on their substrate specificity. However, the activity of *E. coli* HADs for sorbitol-6-phosphate has not been assayed.

I picked up six *E. coli* HADs, HAD1 (YniC), HAD2 (YfbT), HAD4 (YihX), HAD6 (YqaB), HAD12 (YbiV), and HAD13 (YidA) for screening of the sorbitol-6-phosphatase activity, because these enzymes exhibited dephosphorylation activity preferentially against some sugar phosphates that are similar to

sorbitol-6-phosphate (Kuznetsova et al., 2006). I also prepared a single HAD-like protein (Slr0953) of a cyanobacterium *Synechocystis*, which was reported to be a sucrose-phosphatase (Lunn, 2002). I found that some of these HADs exhibited sorbitol-6-phosphatase.

Results

Screening of HADs for sorbitol-6-phosphatase

Six HADs of *E. coli* and a single HAD of *Synechocystis* (Slr0953) were purified into homogeneity (**Fig. 2_1**) and were assayed. Interestingly, HAD1, HAD2, HAD12, and HAD13 but not HAD4 and HAD6 showed phosphatase activity for the fixed concentration (0.25 mM) of sorbitol-6-phosphate (**Table 2_1**). The phosphatase activities against *p*NPP, which is a general phosphatase substrate, were also confirmed in all purified *E. coli* HADs at levels comparable to those reported in a previous study (Kuznetsova et al., 2006). To further evaluate the sorbitol-6-phosphatase activity, the enzyme kinetic parameters of HAD1, HAD2, HAD12, and HAD13 were determined using Lineweaver-Burk plots (**Fig. 2_2**). The K_m values and the maximal activities (V_{max}) of the four HADs were in the range of 1.8–4.5 mM and 24–158 μmol·min^{-1}·mg^{-1} of protein, respectively. It is also noteworthy that no phosphatase activity was detected for HAD4 and HAD6 even at higher concentrations of sorbitol-6-phosphate, which attributed the near maximal activity to the other *E. coli* HADs (**Fig. 2_2**). The single HAD member of *Synechocystis* (Slr0953) exhibited low activity for *p*NPP but showed no activity for sorbitol-6-phosphate (**Table 2_1**). This finding eliminates the possibility that the sorbitol production was assisted by Slr0953 in the *s6pdh*-expressing cyanobacteria.

Table 2_2 summarizes their kinetic parameters for sorbitol-6-phosphate as well as the "preferred" substrates that showed the highest catalytic efficiencies (k_{cat}/K_m) in the previous reports (Kuznetsova et al., 2006; Lunn, 2002). The K_m value of HAD1 was lowest but somehow comparable to those of HAD12, HAD2, and HAD13. The k_{cat}

value of HAD2 was highest, followed by that of HAD1, which was low, and those of HAD12 and HAD13 were even lower. The results indicated that the k_{cat}/K_m values of HAD2 and HAD1 were high while those of HAD12 and HAD13 were low. When these values were compared with those in the literature, the k_{cat}/K_m value of HAD2 for sorbitol-6-phosphate was higher than that for glucose-6-phosphate while the values of HAD1, HAD12, and HAD13 were lower than those for the preferred substrates. The k_{cat}/K_m value of HAD13 for the preferred substrate (erythrose-4-phosphate) was much higher than the value for sorbitol-6-phosphate, showing the high specificity for the preferred substrate.

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Discussion

I identified the following four HADs of *E. coli* as sorbitol-6-phosphatases: HAD1 (YniC), HAD2 (YfbT), HAD12 (YbiV), and HAD13 (YidA). The sorbitol-6-phosphatase activity was also detected for yeast HADs, but the best k_{cat}/K_m value exhibited by YNL010W was only 2.7×10^3 (Kuznetsova et al., 2015), which is lower than those of the four HADs of *E. coli* (**Table 2_2**). Therefore, HAD1 and HAD2 are currently the most plausible candidates for overexpression to enhance the dephosphorylation of sorbitol-6-phosphate in the engineered organisms for sorbitol production. It might also be worth expressing HAD12 and HAD13 to improve the sorbitol production, although they may also exhibit other promiscuous activities (Kuznetsova et al., 2006). Of course, it would be still possible to find better genes for sorbitol-6-phosphatase than HAD1 or HAD2 from *E. coli*, because it possesses many HAD genes and other phosphatase genes. Alternatively, disruption of endogenous HAD1 or HAD2 in the sorbitol-producing *E. coli* would give us simple answer to this possibility. On the other hand, Slr0953, the only HAD-like protein in *Synechocystis*, which is currently reported as sucrose-phosphatase (Lunn, 2002), did not show the sorbitol-6-phosphatase activity, despite that the *s6pdh*-expressing cells produce sorbitol as shown in Chapter 1. According to the genome information, *Synechocystis* possesses at least 12 genes of potential phosphatase (Kaneko et al., 1996). Four genes tentatively assigned as putative inositol phosphatase-like proteins, for example, may be responsible for dephosphorylation of sorbitol-6-phosphate in vivo, because the phosphate ester in inositol-phosphate could be similar to sorbitol-6-phosphate. Moreover, inositol metabolism has not been characterized well in *Synechocystis*.

The physiological roles of numerous HADs that have phosphatase activity with broad specificity have not yet been fully elucidated. These include HAD1 of *E. coli* and DOG1 and DOG2 of yeast, which are both known to be essential for acquiring resistance against the toxic substance 2-deoxyglucose (Kuznetsova et al., 2006; Randez-Gil et al., 1995; Sanz et al., 1994). The discovery that these HADs exhibit phosphatase activity with 2-deoxyglucose-6-phosphate as the preferred substrate strongly suggest that the generation or accumulation of non-natural 2-deoxyglucose-6-phosphate is similarly toxic to *E. coli* and yeast. The broad substrate specificity of such HADs could be profitable if they are exposed to certain non-natural compounds.

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In this context, it was strange that *E. coli* HAD2 acts preferentially on the important metabolite, glucose-6-phosphate. However, my data demonstrated that HAD2 could serve as sorbitol-6-phosphatase rather than a glucose-6-phosphatase, which is consistent with the presumed dogma of scavenging toxic non-natural compounds. Moreover, I observed that the growth of *E. coli* was not markedly retarded by the overexpression of HAD2. These results suggest that HAD2 may also serve as a scavenger without severely affecting the intrinsic metabolisms. This implies that the overexpression of HAD2 might also facilitate the detoxification of the side effects of sorbitol production in recombinant cyanobacteria.

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Materials and methods

Strains and plasmid construction

The strains, plasmids, and primers used in Chapter 2 are shown in **Tables 2_4 and 2_5**, respectively. Genes of the target HADs were amplified using PCR with the PrimeSTAR Max DNA polymerase (TaKaRa Bio, Japan), the genome DNA of *E. coli* JM109 or *Synechocystis*, and the primer set; HAD1-1F/2R, HAD2-1F/2R, HAD4-1F/2R, HAD6-1F/2R, HAD12-1F/2R, and HAD13-1F/2R for *E. coli* or slr0953-1F/2R for *Synechocystis*. The PCR products were cloned into the expression vector pET-28a (+) (Merck, Germany), which harbors N-terminal His-tag to proteins, using the In-Fusion HD cloning kit reagents (TaKaRa Bio, Japan). These pET-28a-HAD plasmids were introduced into *E. coli* C41 (DE3) (Merck, Germany) for the overexpression of protein.

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Protein expression, purification, and SDS-PAGE analysis

E. coli C41 (DE3) harboring pET-28a-HAD was cultured in 1 liter of LB medium at 37°C. When each culture reached an OD_{600} of 0.4 to 0.8, 1 mM isopropyl β-D-1-thiogalactopyranoside was added (final concentration, 100 μM), and the cells were grown at 37°C for 3 h to achieve induction. The cells from each culture were then collected by centrifugation at 4,220×g for 15 min, suspended in 20 mM HEPES (pH 7.5) containing 100 mM NaCl and 10% (wt/vol) glycerol, and homogenized three times with a French press at 1,500 kg/cm^2. The soluble proteins were collected by centrifugation at 50,000×g for 30 min. His-tagged HADs were purified by Ni-affinity column chromatography (HisTrap HP; GE Healthcare, Little Chalfont, United

Kingdom), with the eluent consisting of a gradient of 30 to 430 mM imidazole in the aforementioned buffer system. Proteins in each fraction were subjected to SDS-PAGE, followed by Coomassie brilliant blue R-250 staining. Low-molecular-mass calibration kit standards (GE Healthcare) served as the molecular mass markers. The fraction enriched in each targeted protein was dialyzed against the aforementioned buffer to remove the imidazole. Protein concentration was assayed using the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

Phosphatase activity assay

The phosphatase activity was assayed as follows: 200 μL of the reaction mixture containing 50 mM HEPES-KOH (pH 7.0), 2 mM $MgCl_2$, 1 μg of each purified protein, and a predetermined concentration of D-sorbitol-6-phosphate barium salt (Sigma-Aldrich, Germany) or 10 mM *p*-nitrophenylphosphoric acid disodium salt (*p*NPP) (Nacalai Tesque, Japan) was incubated at 30°C for 10 min. The liberated inorganic phosphate was determined using a Malachite Green phosphate assay kit (BioAssay Systems, USA), which is based on color development at 620 nm. Phosphatase activities of HADs were defined as the amount of enzyme catalyzing the generation of 1 μmol phosphate from substrates per 1 min.

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General discussion

Achievement in this thesis

This thesis has opened the gateway for photosynthetic production of sorbitol in cyanobacteria by introduction of the NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH). Cellular toxicity was avoided by inducible expression of S6PDH using theophylline riboswitch. The production of sorbitol was improved by the enhanced supply of the substrates for S6PDH reaction (G6P and NADPH). The production was further enhanced by introduction of the newly identified sorbitol-6-phosphatase (HAD1). These results would be a first example that photosynthetic production was systematically optimized at the induced expression, Calvin cycle, supply of NADPH, and dephosphorylation of the intermediate, although more improvements should be needed.

In Chapter 1, I reported that overexpression of FBPase and membrane-bound transhydrogenase (PntAB) was effective for the sorbitol production, which requires an additional supply of hexose and reductants via photosynthesis. The enhancement of carbon and NADPH supply is reasonable because these substrates are unusually consumed with heterologous production of sorbitol. However, dephosphorylation of sorbitol-6-phosphate was also critical in the sorbitol production as shown in Chapter 2. The limiting steps for the sorbitol production might have been changed when HAD1 was overexpressed. The engineering of Calvin cycle and NADPH supply should be reevaluated on the background of the strain s6pdh/had1, even though overexpression of FBPase and PntAB apparently improved the productivity of sorbitol in the strain.

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Ongoing: export of sorbitol

The last critical point to be done is improvement of the sorbitol excretion. There are no report for the exporter of sugar alcohols such as sorbitol and mannitol, whereas various uptake transporters for sorbitol were reported for apple and related plants. I expressed two of the apple transporters into sorbitol-producing cyanobacteria, but they did not clearly work for the efficient export of sorbitol at the moment. Another promising candidate may be one of sugar exporters. For instance, eukaryotic SWEET and prokaryotic SemiSWEET are known as the sugar transporters that mainly mediate the facilitative diffusion of sucrose and hexoses depending on the concentration gradient (Feng and Frommer, 2015), although transport of sorbitol has not yet been examined. Heterologous expression of the *Arabidopsis* SWEET protein and bacterial SemiSWEET protein enhanced efflux of sucrose from *Synechococcus* cells (Xuan et al., 2013). I am now trying to express the bacterial SemiSWEET protein in the sorbitol-producing strains to assess whether sorbitol production is facilitated or not.

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Theoretical yields for photosynthetic production of sorbitol

To evaluate the sorbitol production by the engineered cyanobacteria, I estimated theoretical yields of the sorbitol production under photoautotrophic conditions in two ways: one is the theoretical yield based on the assumption that total input energy is exclusively directed to sorbitol and another is the practical maximal yield of the cells growing at a linear phase in the experiments. The former input energy-based yield can

be calculated based on shape of the culture tube as follows: white light of 30 μmol photons·m^{-2}·s^{-1}, optical section area of 27 cm^2 for 50 mL culture, and cultivation time of 216 h, giving rise to 0.0630 mol photons per 50 mL or 1.26 mol photons·L^{-1}. The cell suspension above OD_{730} = 2 for the sorbitol production experiments means almost all photons are absorbed by cells. In the light reaction, 8 photons are utilized to generate 3 molecules of ATP and 2 molecules of NADPH, which are consumed for fixation of one molecule of CO_2 or 1/6 molecule of glucose. Thereby, the total input photons can be converted to 0.1575 mol fixed CO_2·L^{-1} or 0.0263 mol glucose·L^{-1}. Although the source of additional NADPH for reduction of glucose-6-P to sorbitol-6-P is not known, its demand is only 1/12 of NADPH for production of glucose-6-P, being negligible at the moment. Therefore, the theoretical maximal value (yield) of sorbitol in my experimental system will be 0.0263 mol·L^{-1} or 4.79 g sorbitol·L^{-1}.

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However, some of the input light may be reflected on the tube surface, some absorbed energy may be dissipated into heat instead of photochemical reactions, and some of photochemical energy may be used for side reactions (e.g. photoinhibition) other than photosynthesis. Efficiency of the real photosynthesis may be changed during the growth (from the end of the exponential phase to the linear phase). Therefore, the net biomass increase can be regarded as the practical maximal yield of photosynthesis in the cells of the sorbitol production experiments. In the laboratory, the dry mass of *Synechocystis* cells is 220 mg OD_{730}^{-1}·L^{-1} (Kiyota, H. and Ikeuchi, M., unpublished data).

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Finally, I would like to emphasize again that the tuning of many metabolic pathways is essential for better production system of chemicals and biofuels by engineering of phototrophic cyanobacteria. I believe that my work in this thesis will contribute to the beginning part of the extensive tuning.

Tables and Figures

Table 0_1

Photosynthetic productions by engineered cyanobacteria.

Product	Titer	Rate	Substrate [a]	Cofactor	Host [b]	Genetic manipulation [c]	Reference
Acetone	36 mg/L	—	Acetyl-CoA	—	6803	*phaA*/*adc*/*ctfAB*-ox, *phaCE*/*pta*-del	Zhou et al. (2012)
2,3-Butanediol	2380 mg/L	5 mg/L/OD/h	Pyruvate	NADPH	7942	*adh*/*alsD*/*alsS*-ox	Oliver et al. (2013)
1-Butanol	404 mg/L	2 mg/L/h	Malonyl-CoA	NAD(P)H	7942	*nphT7*/*pduP*/*phaB*/*phaJ*/*ter*/*yqhD*-ox	Lan et al. (2013)
Erythritol	256 mg/L	0.4 mg/L/h	Erythrose-4-P	NADPH	6803	*gld1*/*tm1254*-ox	van der Woude et al. (2016)
Ethanol	5500 mg/L	9 mg/L/h	Pyruvate	NADPH	7942	*pdc*/*adh*-ox, *phaAB*-del	Gao et al. (2012)
Ethylene	—	7 mg/L/h	2-Oxoglutarate	—	6803	*efe*/*efe*-ox	Ungerer et al. (2012)
Fatty acids	197 mg/L	8 mg/L/h	Malonyl-CoA	NADPH	6803	*tesA*-ox, *pta*-del	Liu et al. (2011)
Fatty alcohols	3 mg/gDCW	—	Malonyl-CoA	NAD(P)H	6803	*far*-ox, *ado*/*acp*-del	Yao et al. (2014)
Glucose/Fructose	45 mg/L	—	Glucose/Fructose	—	7942	*galU*/*invA*/*glf*-ox	Niederholtmeyer et al. (2010)
Glycerol	1320 mg/L	—	Glycerol-3-P	—	6803	*gpp*-ox	Savakis et al. (2015)
3-Hydroxybutyric acid	533 mg/L	1 mg/L/h	Acetyl-CoA	NADPH	6803	*hdb*/*phaAB*-ox, *phaEC*-del	Wang et al. (2013)
3-Hydroxypropionic acid	837 mg/L	—	Malonyl-CoA	NADPH	6803	*mcr*/*accBCAD*/*birA*/*pntAB*-ox	Wang et al. (2015)
Isobutanol	550 mg/L	—	Pyruvate	NADPH	7942	*alsS*/*ilvCD*/*kivD*/*yqhD*-ox, *glgC*-del	Li et al. (2014)
Isobutyraldehyde	1100 mg/L	6 mg/L/h	Pyruvate	NADPH	7942	*alsS*/*ilvCD*/*kivD*/*rbcLS*-ox	Atsumi et al. (2009)
Isoprene	0.2 mg/L	—	IPP/DMAPP	—	6803	*ispS*/*atoB*/*hmgSR*/*mk*/*pmk*/*pmd*/*fni*-ox	Bentley et al. (2014)
Isopropanol	146 mg/L	—	Acetyl-CoA	NADPH	7942	*sadh*/*thl*/*atoAD*/*adc*-ox	Hirokawa et al. (2015)
D-Lactic acid	1310 mg/L	9 mg/L/h	Pyruvate	NADPH	7942	$ldhDn^{ARSdR}$/*lldP*-ox	Li et al. (2015)
L-Lactic acid	1840 mg/L	3 mg/L/h	Pyruvate	NADH	6803	*ldh*-ox	Angermayr and Hellingwerf (2013)
Limonene	4 mg/L	—	IPP/DMAPP	—	7002	*msLS*-ox	Davies et al. (2014)
D-Mannitol	1100 mg/L	6 mg/L/h	Fructose-6-P	NADH	7002	*mtlD*/*mlp*-ox, *glgA1A2*-del	Jacobsen and Frigaard (2014)
Sucrose	2690 mg/L	36 mg/L/h	Sucrose	—	7942	*cscB*-ox, *invA*/*glgC*-del	Ducat et al. (2012)

[a] The starting substrate from natural metabolites for the biosynthesis of product.

[b] Producing host of cyanobacterial strain: 6803, *Synechocystis* sp. PCC 6803; 7942, *Synechococcus elongatus* PCC 7942; 7002, *Synechococcus* sp. PCC 7002.

[c] Overexpression (-ox) and deletion (-del) of exogenous or endogenous genes for cyanobacterial production.

Table 1_1

Cyanobacterial strains used in Chapter 1.

Strain	Description	Reference
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/fbp/fbp	s6pdh/fbp harboring the genome-integrated second copy of spinach *fbp* by pScaSTfbpT	This study
s6pdh/pnt	s6pdh harboring the genome-integrated *Synechocystis pntAB* by pBcaSTpntAT-PpntBP	This study
s6pdh/epnt	s6pdh harboring the genome-integrated *E. coli pntAB* by pBcaSTepntABT	This study
s6pdh/fbp/pnt	s6pdh/fbp harboring the genome-integrated *Synechocystis pntAB* by pBcaSTpntAPsB	This study
s6pdh/fbp/szwf	s6pdh/fbp harboring the genome-integrated *Synechocystis g6pdh* by pBcaSTszwfT	This study
s6pdh/fbp/ezwf	s6pdh/fbp harboring the genome-integrated *E. coli g6pdh* by pBcaSTezwfT	This study
s6pdh/fbp/*Δ*flv3	s6pdh/fbp completely deleted a *flv3* on genome by pTflv3-Er	This study
ndhI-less	s6pdh/fbp partially deleted a *ndhI* on genome by pSndhI-Er	This study
s6pdh/fbp/pnt/sot2	s6pdh/fbp/pnt harboring the genome-integrated apple *sot2* by pB31ERsot2T	This study
s6pdh/fbp/pnt/sot3	s6pdh/fbp/pnt harboring the genome-integrated apple *sot3* by pB31ERsot3T	This study

Table 1_2

Plasmids used in Chapter 1.

Plasmid	Description [a, b]	Reference
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
pScaSTfbpT	P_{trc}-*fbp*-T_{rrnB}; Spc^r; near *carA* 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
pBcaSTepntABT	P_{trc}-e*pntA-epntB*-T_{rrnB}; Spc^r; near *carA* homologous recombination region	This study
pBcaSTszwfT	P_{trc}-*szwf* -T_{rrnB}; Spc^r; near *carA* homologous recombination region	This study
pBcaSTezwfT	P_{trc}-*ezwf* -T_{rrnB}; Spc^r; near *carA* homologous recombination region	This study
pTflv3-Er	Replacement of *flv3* to Er^r by homologous recombination between *sll0549* and *slr0565*	from Ms. Okuda
pSndhI-Er	Replacement of *ndhI* to Er^r by homologous recombination between *ndhA* and *ndhG*	from Ms. Okuda
pB31ERsot2T	P_{trc}::riboswitch-*lacO-sot2*-T_{rrnB}; Er^r; near *slr2031* homologous recombination region	This study
pB31ERsot3T	P_{trc}::riboswitch-*lacO-sot3*-T_{rrnB}; Er^r; near *slr2031* homologous recombination region	This study

[a] The near regions of *slr0846, IS203c, carA*, and *slr2031* are neutral sites in the genomic DNA of *Synechocystis*.

[b] Kan^r, kanamycin-resistance cassette; Cm^r, chloramphenicol-resistance cassette; Spc^r, spectinomycin-resistance cassette; Er^r, erythromycin cassette.

Table 1_3

Primers used in Chapter 1.

Name	Sequence
srlD2-3Ftrc	GAGGAATAAACCATGAATAATAGTTGGATCAACATC
srlD2-4Rrrn	CAGACCGCTTCTGCGCTAGCCTCGGCTTTTCCCGCCCGCCAC
s6pdh-1Ftrc	GAGGAATAAACCATGAGTACCGTCACCCTATC
s6pdh-2Rrrn	CAGACCGCTTCTGCGCTAGGCGTACACGTCCAAGCCCC
rrn-1F	CGCAGAAGCGGTCTGATAAA
trc-14R	CATGGTTTATTCCTCCTTATTT
TR-1Fcom	CCGCTTCGCAATATTCAAATATTCTGAAATGAGC
TR-6R	CATCTTGTTGCCTCCTTAGCAGG
s6pdh-3FTR	TAAGGAGGCAACAAGATGAGTACCGTCACCCTATC
Km-36Rcom	AATATTGCGAAGCGGCCAACCTTTCATAGAAGGCG
fbp-1Ftrc	AAGGAGGAATAAACCATGGCGGCCGTAGGGGAGGC
fbp-4Rrrn	CAGACCGCTTCTGCGCTATGCTAGGTATTTCTCTA
Fbp1-1Ftrc	GAGGAATAAACCATGGACAGCACCCTCGGTTTA
Fbp1-2Rrrn	CAGACCGCTTCTGCGTTAATGCAGTTGGATTACTTTG
Fbp2-1Ftrc	GAGGAATAAACCATGACCGTTAGTGAGATTCA
Fbp2-2Rrrn	CAGACCGCTTCTGCGCTAATTACCCTGCCGTTGTT
SpntA-3Ftrc	AAGGAGGAATAAACCATGACCATTGCCGCCCCTAA
SpntA-2Rrrn	CAGACCGCTTCTGCGTTACTTCCGAAACATTTTCAGC
SpntB-1FA2P	AAGGAATTATAACCAAATGTCTAACAGTTTACAAACCG
SpntB-2RA2T	CATTACACCAAGGAACTAAACGGAAACTAGAACCT
carA-16FpsaA	ATTTTTACCCCATTGGATAACGTTAAATTTTTTAG
rrn-2R	GCGTTCACCGACAAACAACA
psbA2-52Frrn	TTTGTCGGTGAACGCTGCAAGGCCCAGTGATCAAT
psbA2-16RpsaA	CAATGGGGTAAAAATCACAGGTTCTTCCCCCATAA
szwf-1Ftrc	AAGGAGGAATAAACCATGGTAACGCTACTCGAAAA
szwf-2Rrrn	CAGACCGCTTCTGCGCTAAAGTCGGCGCCAGCGAC
ezwf-1Ftrc	AAGGAGGAATAAACCATGGCGGTAACGCAAACAGC
ezwf-2Rrrn	CAGACCGCTTCTGCGTTACTCAAACTCATTCCAGG
msot2-3FTR	GGAGGCAACAAGATGACAAGTATTCTATTAGG
msot2-2Rrrn	CAGACCGCTTCTGCGCTAGTGAACTTGTCCTTTGT
most-3FTR	GGAGGCAACAAGATGGCTGATCGGCGAGCTGA
msot3-2Rrrn	CAGACCGCTTCTGCGCTAGAACACCTGTGCGTTAT

Table 2_1

Screening of phosphatase activities of several HADs from *E. coli* and *Synechocystis* for sorbitol-6-phosphate and *p*NPP.

		*p*NPP	
HADs	Sorbitol-6-P	This study	Kuznetsova et al. 2006
HAD1 (YniC)	6.1	0.48	0.60
HAD2 (YfbT)	9.3	0.86	1.1
HAD4 (YihX)	ND	0.50	0.22
HAD6 (YqaB)	ND	0.65	0.84
HAD12 (YbiV)	2.9	3.8	2.4
HAD13 (YidA)	2.0	0.38	0.70
Slr0953 (SPP)	ND	0.12	—

Phosphatase activities (μmol·min^{-1}·mg^{-1} of protein) are shown. Assays were performed in the presence of substrates (0.25 mM sorbitol-6-phosphate or 10 mM *p*NPP). Reference activities of *p*NPP (Kuznetsova et al. (2006)) are also shown. Slr0953 is sucrose-6-phosphate phosphatase (SPP), which is a single protein of the HAD superfamily in the cyanobacterium *Synechocystis* sp. PCC 6803. ND indicates that activity was not detected even at higher concentrations of sorbitol-6-phosphate (0.25–10 mM).

Table 2_2

Kinetic parameters of selected HADs for sorbitol-6-phosphate and the reported preferred substrates.

	Sorbitol-6-P (present study)			Preferred substrate (previous studies)			
HADs	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Compound	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
HAD1 (YniC)	1.8	21	1.2×10^4	2-Deoxyglucose-6-P	0.61	33	5.4×10^4
HAD2 (YfbT)	3.9	63	1.6×10^4	Glucose-6-P	1.8	13	7.1×10^3
HAD4 (YihX)	—	ND	—	Glucose-1-P	0.24	1.4	5.9×10^3
HAD6 (YqaB)	—	ND	—	Fructose-1-P	1.7	20	2.0×10^4
HAD12 (YbiV)	2.3	12	5.3×10^3	Fructose-1-P	1.4	111	8.0×10^4
HAD13 (YidA)	4.5	14	3.2×10^3	Erythrose-4-P	0.019	19	1.0×10^6
Slr0953	—	ND	—	Sucrose-6-P	0.0075	21	2.8×10^6

Kinetic parameters against sorbitol-6-phosphate were estimated from the data in Fig. 2. Parameters of *E. coli* HADs for the preferred substrates are from Kuznetsova et al. (2006). Slr0953 is sucrose-6-phosphate phosphatase, which is a single protein of the HAD superfamily in *Synechocystis*, and the kinetic parameters for sucrose-6-phosphate are from Lunn, (2002). P, phosphate. ND, not detected.

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Table 2_5
Primers used in Chapter 2.

Name	Sequence
HAD1-1F28a	CGCGGCAGCCATATGTCAACCCCGCGTCAGAT
HAD1-2R28a	CTCGAATTCGGATCCCTAACCGAGAAGGTCTTTTG
HAD2-1F28a	CGCGGCAGCCATATGCGGTGCAAAGGTTTTCT
HAD2-2R28a	CTCGAATTCGGATCCTTACTGAATAATAACATCGC
HAD4-1F28a	CGCGGCAGCCATATGCTCTATATCTTTGATTT
HAD4-2R28a	CTCGAATTCGGATCCTTAGCATAACACCTTCGCGA
HAD6-1F28a	CGCGGCAGCCATATGTACGAGCGTTATGCAGG
HAD6-2R28a	CTCGAATTCGGATCCTTACAGCAAGCGAACATCCA
HAD12-1F28a	CGCGGCAGCCATATGAGCGTAAAAGTTATCGT
HAD12-2R28a	CTCGAATTCGGATCCTTAGCTGTTAAAAGGGGATG
HAD13-1F28a	CGCGGCAGCCATATGGCTATTAAACTCATTGC
HAD13-2R28a	CTCGAATTCGGATCCTTAATTCAGCACATACTTCT
slr0953-1F28a	CGCGGCAGCCATATGCGACAGTTATTGCTAAT
slr0953-2R28a	CTCGAATTCGGATCCTCAGCTCAAAAAATCGAAAT
HAD1-3Ftrc	GAGGAATAAACCATGTCAACCCCGCGTCAGAT
HAD1-5Rrrn	CAGACCGCTTCTGCGCTAACCGAGAAGGTCTTTTG
HAD6-3Ftrc	GAGGAATAAACCATGTACGAGCGTTATGCAGG
HAD6-4Rrrn	CAGACCGCTTCTGCGTTACAGCAAGCGAACATCCA

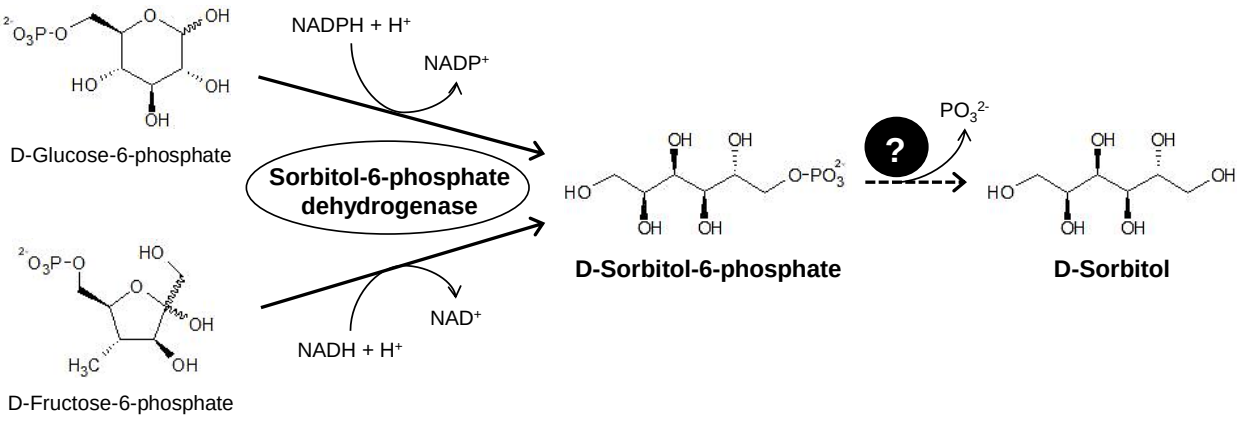

Fig. 0_1. Sorbitol biosynthesis by the two-step enzymatic reactions.
Sorbitol is produced by yet unidentified phosphatase from sorbitol-6-phosphate, which is supplied from glucose-6-phosphate or fructose-6-phosphate by NAD(P)-dependent sorbitol-6-phosphate dehydrogenase.

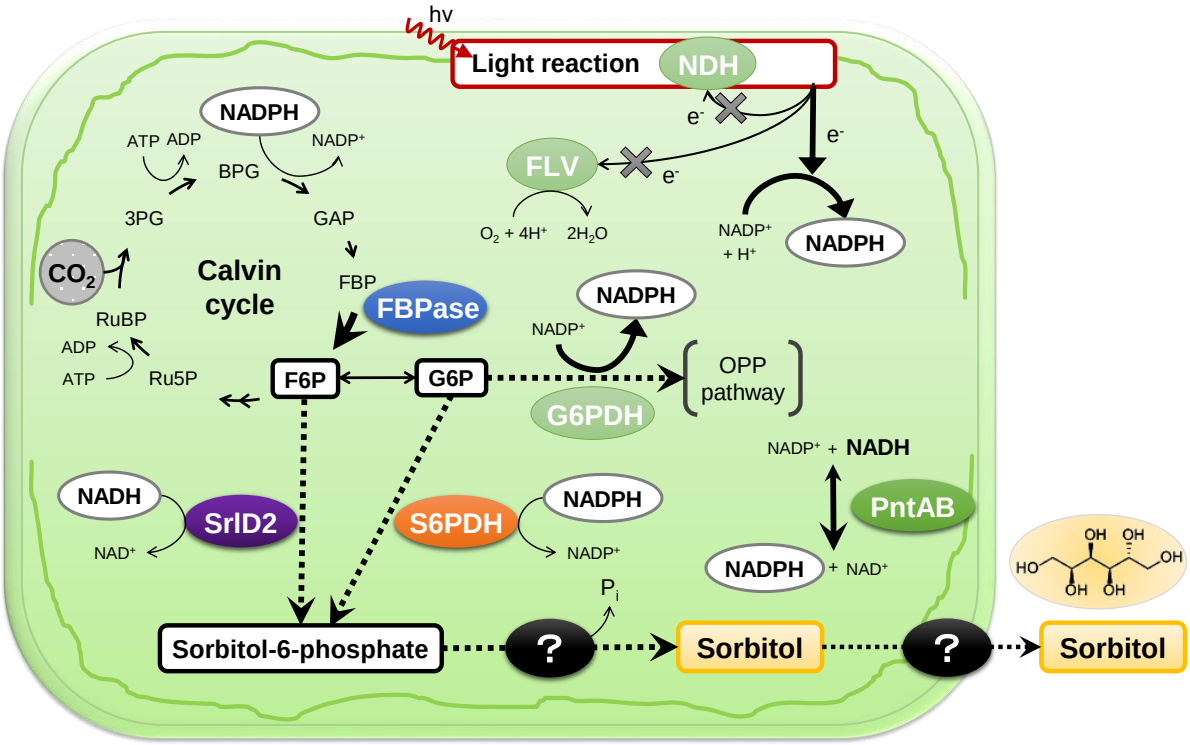

Fig. 0_2. Strategy of sorbitol production in cyanobacteria.

The sorbitol biosynthetic pathway was constructed by introducing specific enzymes: NADP-dependent sorbitol-6-phosphate dehydrogenase (S6PDH), which catalyzes the reduction of G6P to sorbitol-6-phosphate, and NAD-dependent sorbitol-6-phosphate dehydrogenase (SrlD2), which catalyzes the reduction of F6P to sorbitol-6-phosphate. Fructose-1,6-bisphosphatase (FBPase) was overexpressed to enhance Calvin cycle. To fine-tune the NADPH supply, membrane-bound transhydrogenase (PntAB) and glucose-6-phosphate dehydrogenase (G6PDH) were overexpressed and flavodiiron protein (FLV) and NADPH dehydrogenase complex (NDH) were inactivated. Some enzymes for dephosphorylation of sorbitol-6-phosphate and for export of intracellular sorbitol were examined, whereas these specific enzymes were not yet identified.

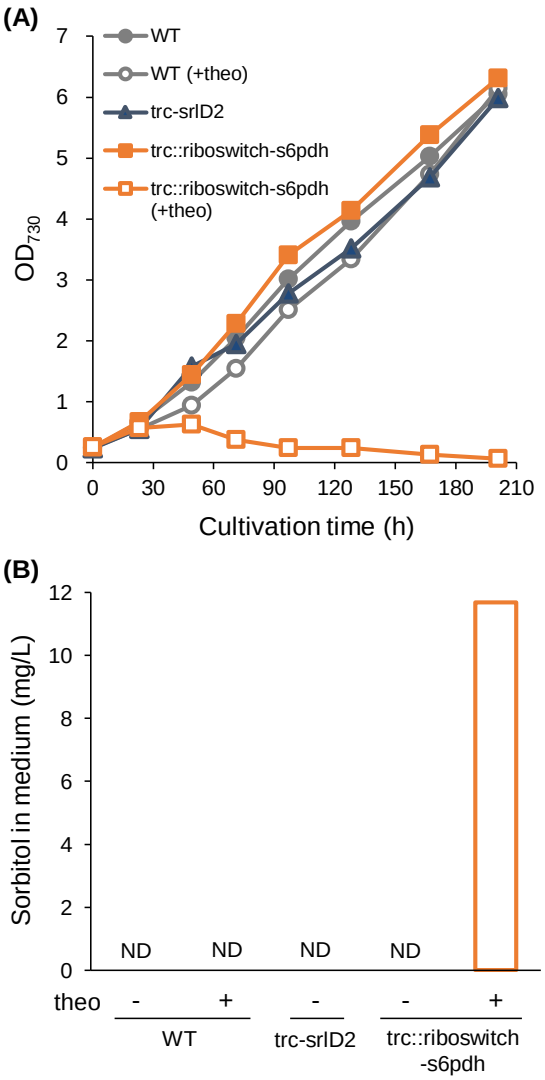

Fig. 1_1. Expression of sorbitol-6-phosphate dehydrogenase in cyanobacteria.
Cyanobacterial cells were cultured with (open symbols) or without (closed symbols) theophylline. Growth (A) was assayed at each cultivation time. Theophylline (2 mM) was added at the start of cultivation. Sorbitol in the medium supernatant (B) was quantified at the end of cultivation. ND, not detected. WT, wild-type strain; trc-srlD2, strain harboring *srlD2* with *trc* promoter; trc::riboswitch-s6pdh, strain harboring *s6pdh* with *trc* promoter incorporated theophylline-inducible riboswitch; theo, 2 mM theophylline.

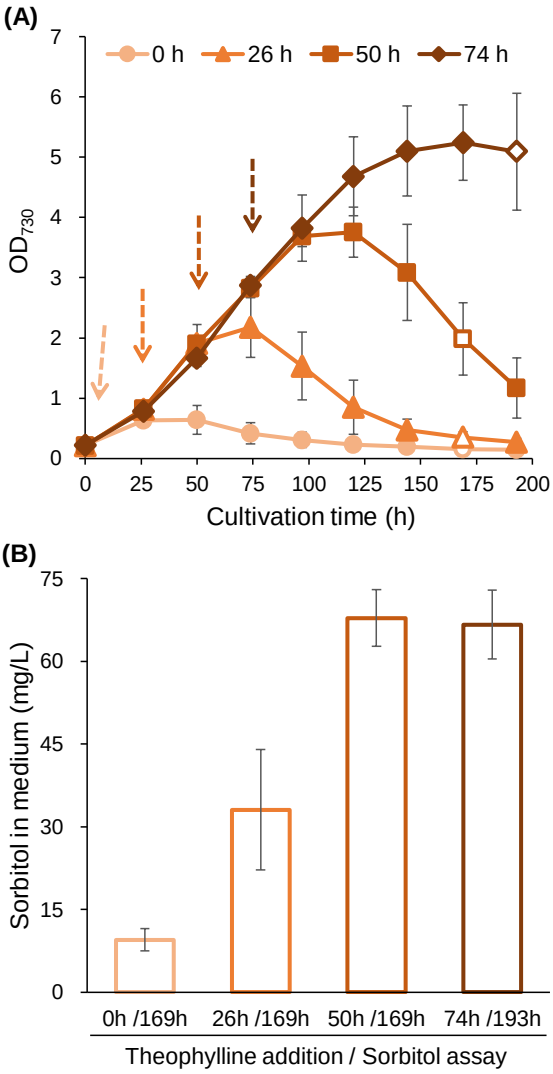

Fig. 1_2. Effect of time of theophylline addition on growth (A) and sorbitol production (B) in the strain s6pdh.

Sorbitol in the medium supernatant was quantified at the sampling points, shown using open symbols, after cultivation for 169 or 193 h. Dashed arrows denote 2 mM theophylline addition at cultivation times of 0, 26, 50, and 74 h. Error bars: standard deviation (*n*=3).

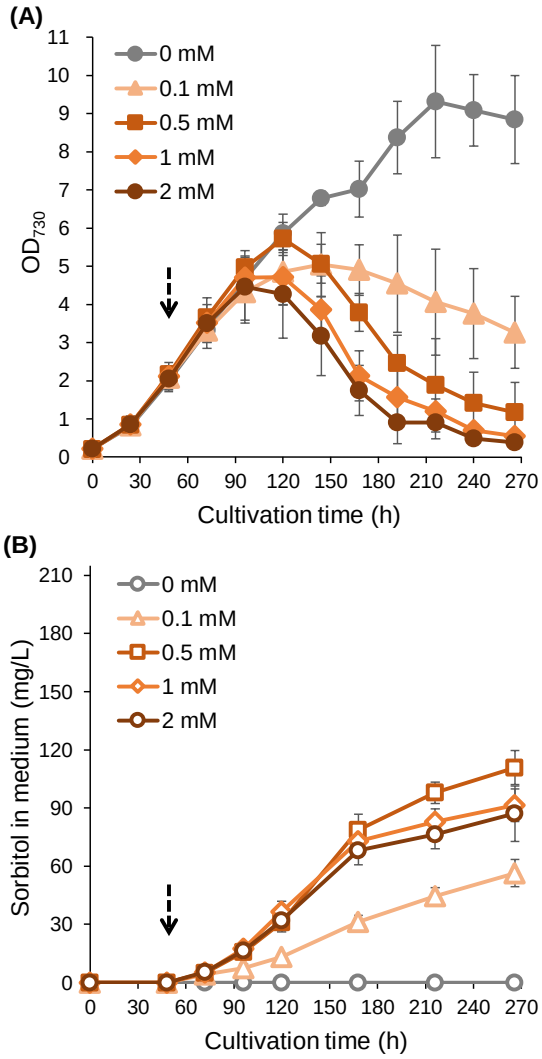

Fig. 1_3. Effect of theophylline concentration on growth (A) and sorbitol production (B) in the strain s6pdh.

Sorbitol in the medium supernatant was quantified at each indicated cultivation time. Dashed arrows denote addition of theophylline at various concentrations (0.1–2 mM). Error bars: standard deviation (*n*=3).

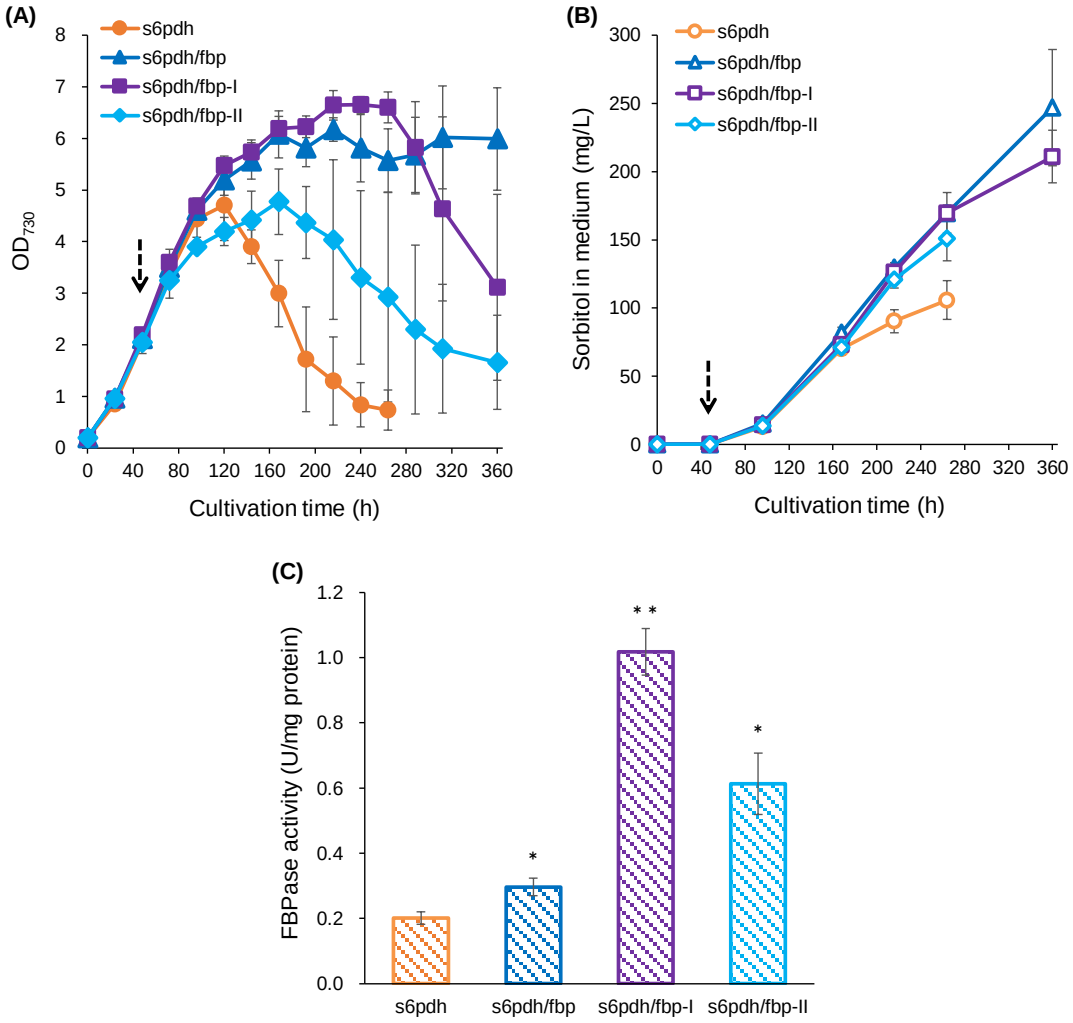

Fig. 1_4. Overexpression of FBPase derived from spinach or cyanobacteria in the strain s6pdh.

Growth (A) and sorbitol production (B) were assayed at each cultivation time. FBPase activity (C) was assayed using the crude extract of cells harvested at 96 h of cultivation. Asterisks: significantly different from s6pdh, according to *t* tests (**P* < 0.05, ***P* < 0.01). Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (*n*=3). s6pdh, strain harboring *s6pdh*; s6pdh/fbp, strain harboring *s6pdh* and spinach *fbp*; s6pdh/fbp-I, strain harboring *s6pdh* and *Synechocystis fbp-I*; s6pdh/fbp-II, strain harboring *s6pdh* and *Synechocystis fbp-II*.

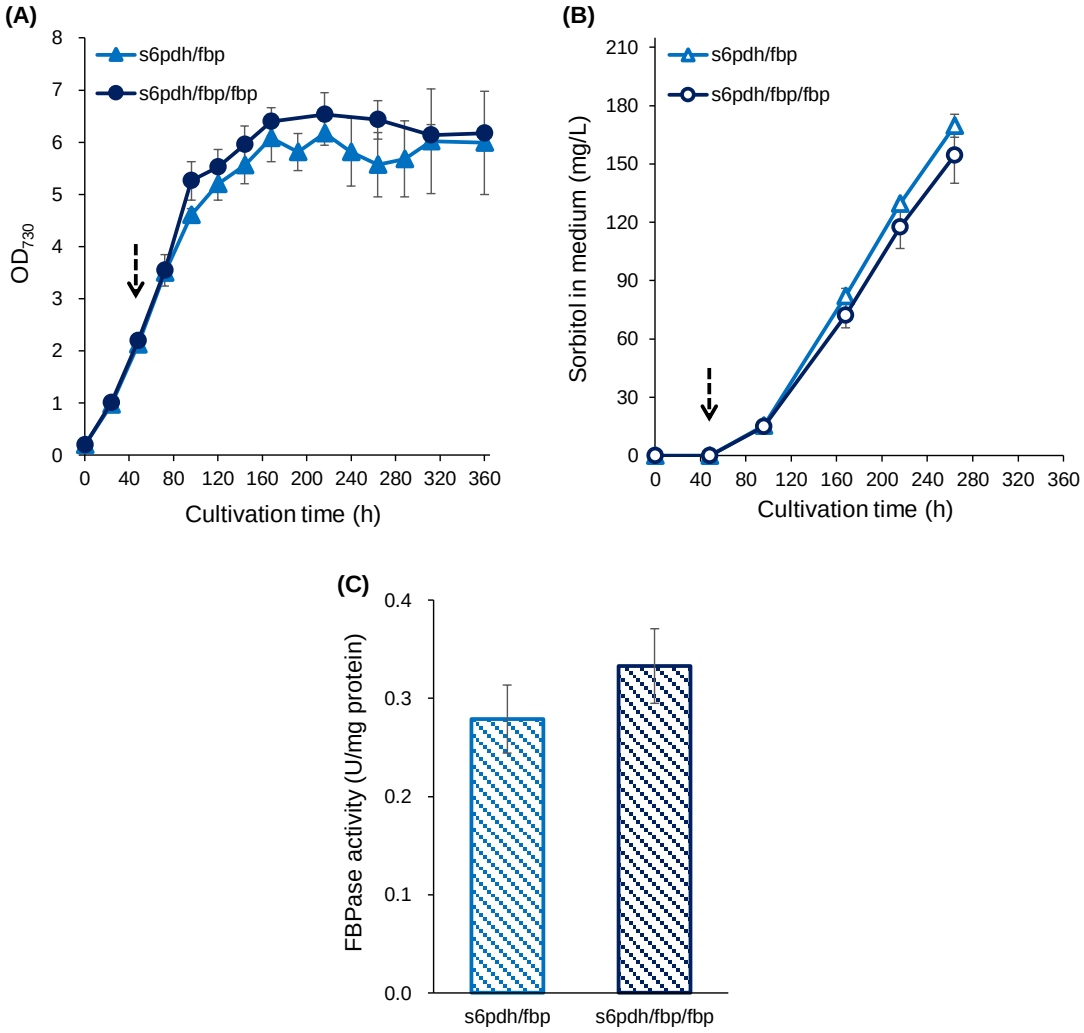

Fig. 1_5. Double overexpression of spinach FBPase.

Growth (A) and sorbitol production (B) were assayed at each cultivation time. FBPase activity (C) was assayed using the crude extract of cells harvested at 96 h of cultivation. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (*n*=3). s6pdh/fbp, strain harboring *s6pdh* and *fbp*; s6pdh/fbp/fbp, strain harboring *s6pdh* and two copy of *fbp*.

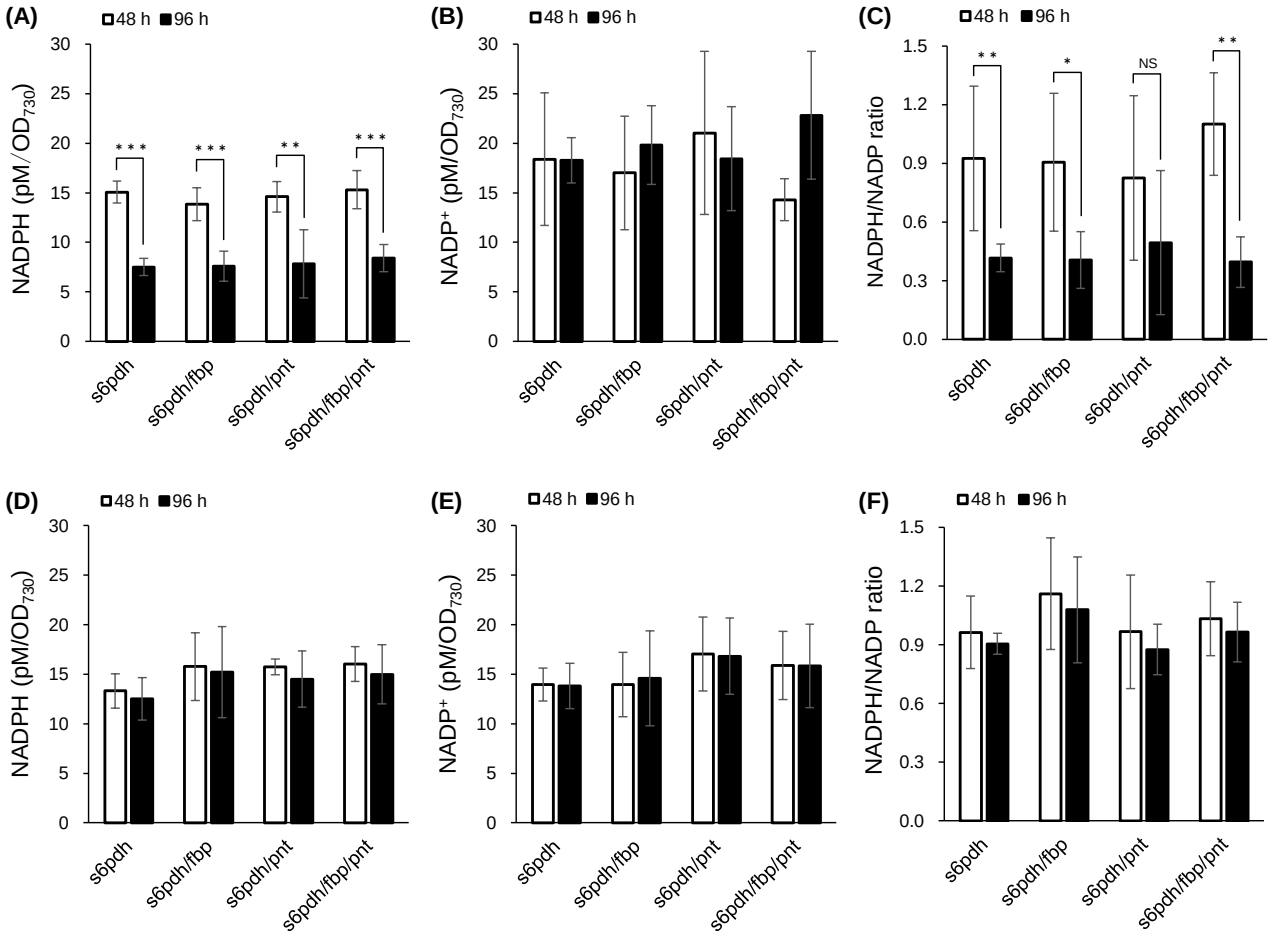

Fig. 1_6. NADP(H) amounts and ratio in cells cultured with (A, B, and C) or without (D, E, and F) theophylline.

NADPH (A and D) and $NADP^+$ (B and E) were quantified using cells harvested at 48 and 96 h of cultivation. NADPH/$NADP^+$ ratio (C and F) was calculated from those amounts. The cells were induced with 0.5 mM theophylline after 48 h cultivation. Asterisks: significantly different, according to *t* tests (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$); NS: not significant. Error bars: standard deviation ($n \geq 3$).

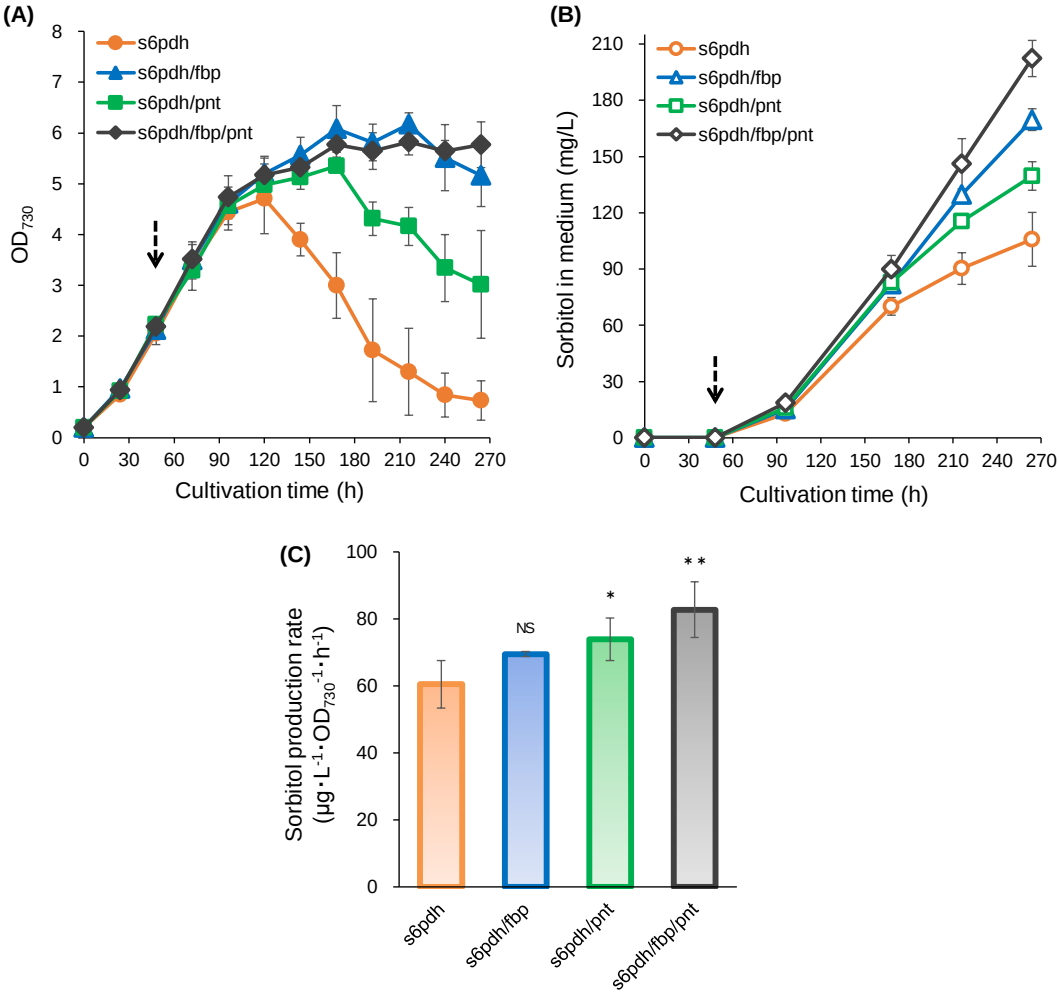

Fig. 1_7. Improvement of sorbitol productivity by overexpression of FBPase and/or PntAB in the strain s6pdh.

Growth (A) and sorbitol production (B) were assayed at each cultivation time. Sorbitol production rate (C) was calculated using the values of OD_{730} and sorbitol measured in the medium at 96 h of cultivation. Asterisks: significantly different from s6pdh, according to *t* tests (*$P < 0.05$, **$P < 0.01$); NS: not significant. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (n=4). s6pdh, strain harboring *s6pdh*; s6pdh/fbp, strain harboring *s6pdh* and *fbp*; s6pdh/pnt, strain harboring *s6pdh* and *pntAB*; s6pdh/fbp/pnt, strain harboring *s6pdh*, *fbp*, and *pntAB*.

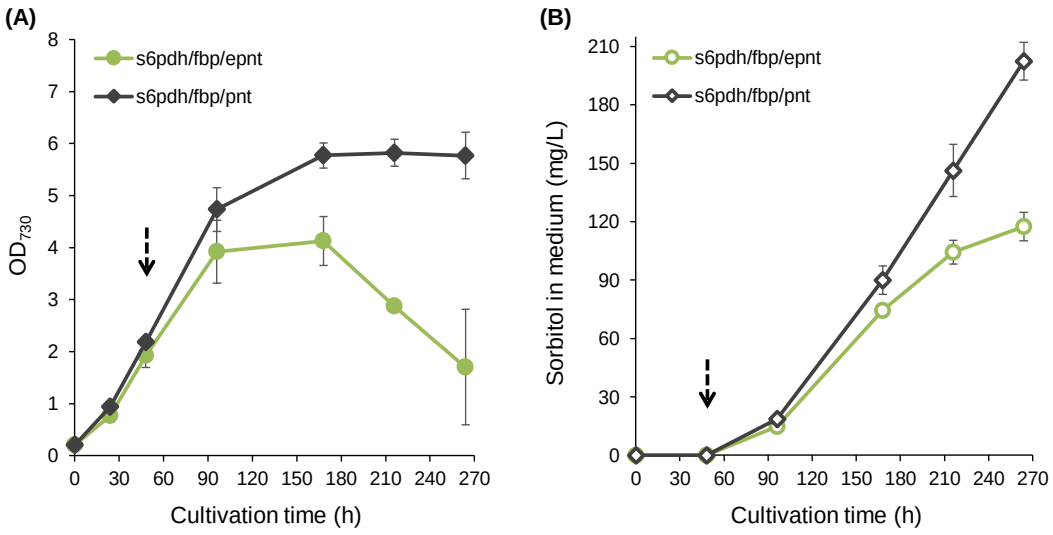

Fig. 1_8. Overexpression of membrane-bound transhydrogenase derived from *E. coli* or *Synechocystis* in the strain s6pdh/fbp.

Growth (A) and sorbitol production (B) were assayed at each cultivation time. Dashed arrows represent 0.5 mM theophylline addition. Error bars mean standard deviation (*n*=3). s6pdh/fbp/epnt, strain harboring *s6pdh*, *fbp*, and *E. coli pntAB*; s6pdh/fbp/pnt, strain harboring *s6pdh*, *fbp*, and *Synechocystis pntAB*.

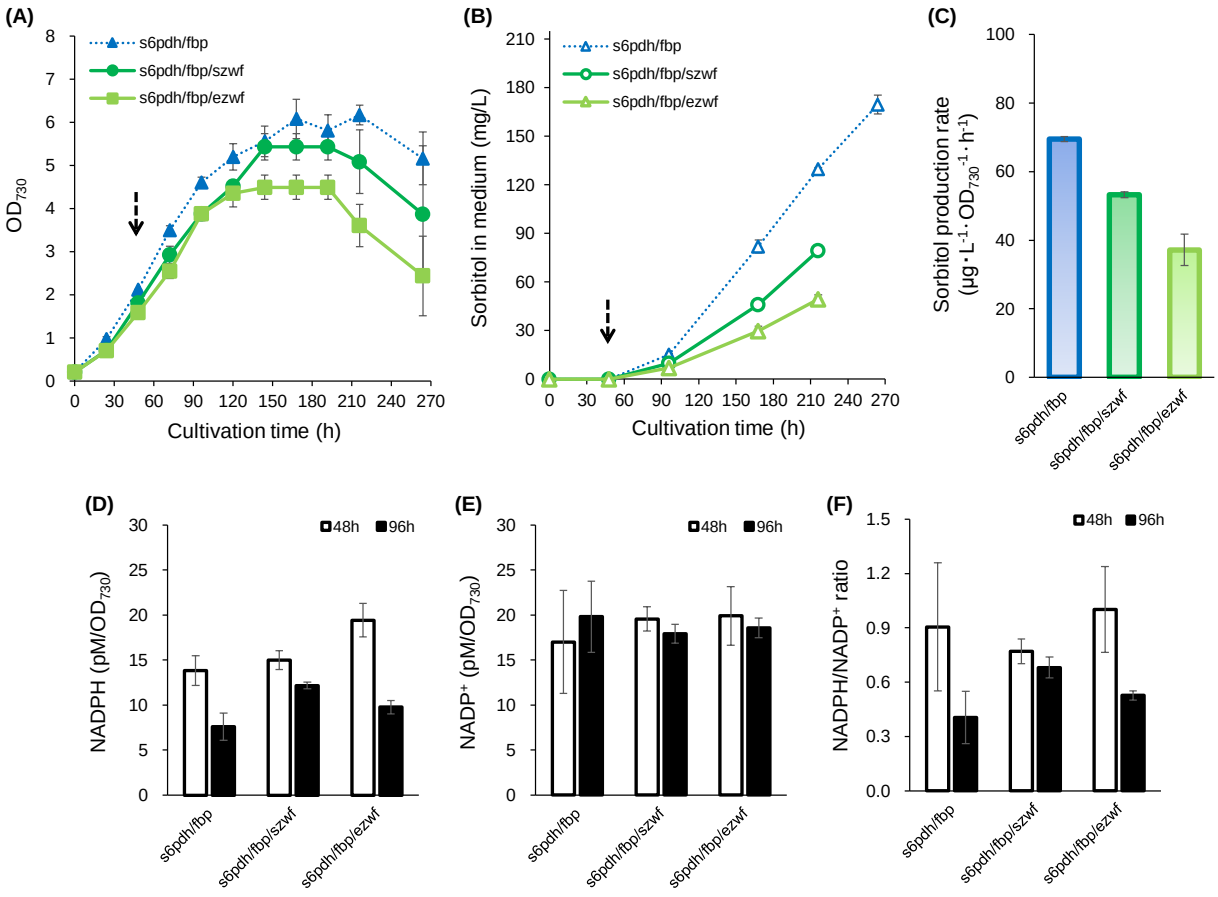

Fig. 1_9. Overexpression of G6PDH derived from *Synechocystis* (*szwf*) and *E. coli* (*ezwf*) in the strain s6pdh/fbp.

Growth (A) and sorbitol production (B) were assayed at each cultivation time. Sorbitol production rate (C) was calculated using the values of OD_{730} and sorbitol measured in the medium at 96 h of cultivation. NADPH (D) and $NADP^+$ (E) were quantified using cells harvested at 48 and 96 h of cultivation. NADPH/$NADP^+$ ratio (F) was calculated from those amounts. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (n=3).

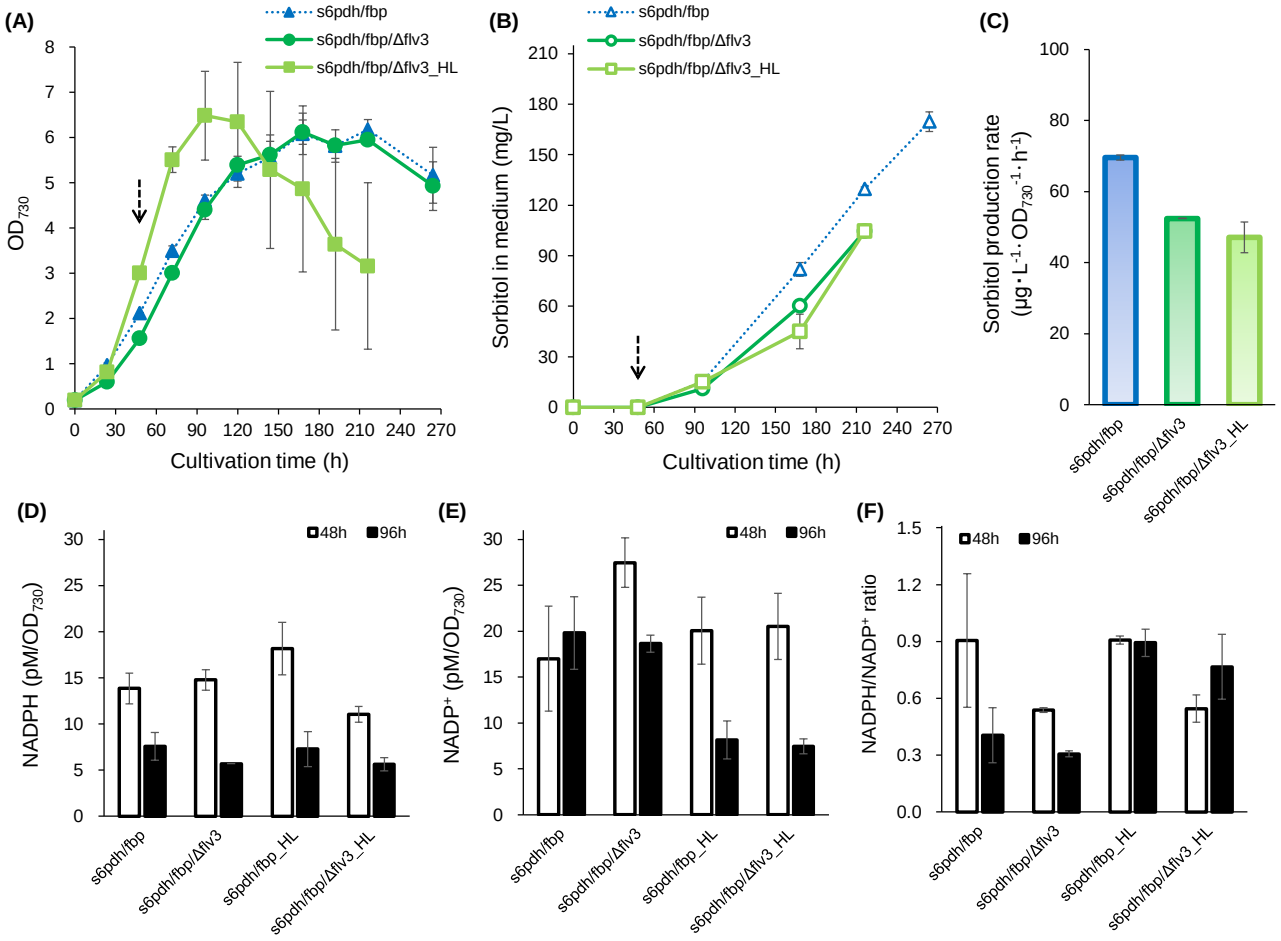

Fig. 1_10. Effects of *Δflv3* in the strain s6pdh/fbp.

The strains were cultivated under normal growth light or high light (HL: 100 μmol photons·m^{-2}). Growth (A) and sorbitol production (B) were assayed at each cultivation time. Sorbitol production rate (C) was calculated using the values of OD_{730} and sorbitol measured in the medium at 96 h of cultivation. NADPH (D) and $NADP^+$ (E) were quantified using cells harvested at 48 and 96 h of cultivation. NADPH/$NADP^+$ ratio (F) was calculated from those amounts. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (n=3).

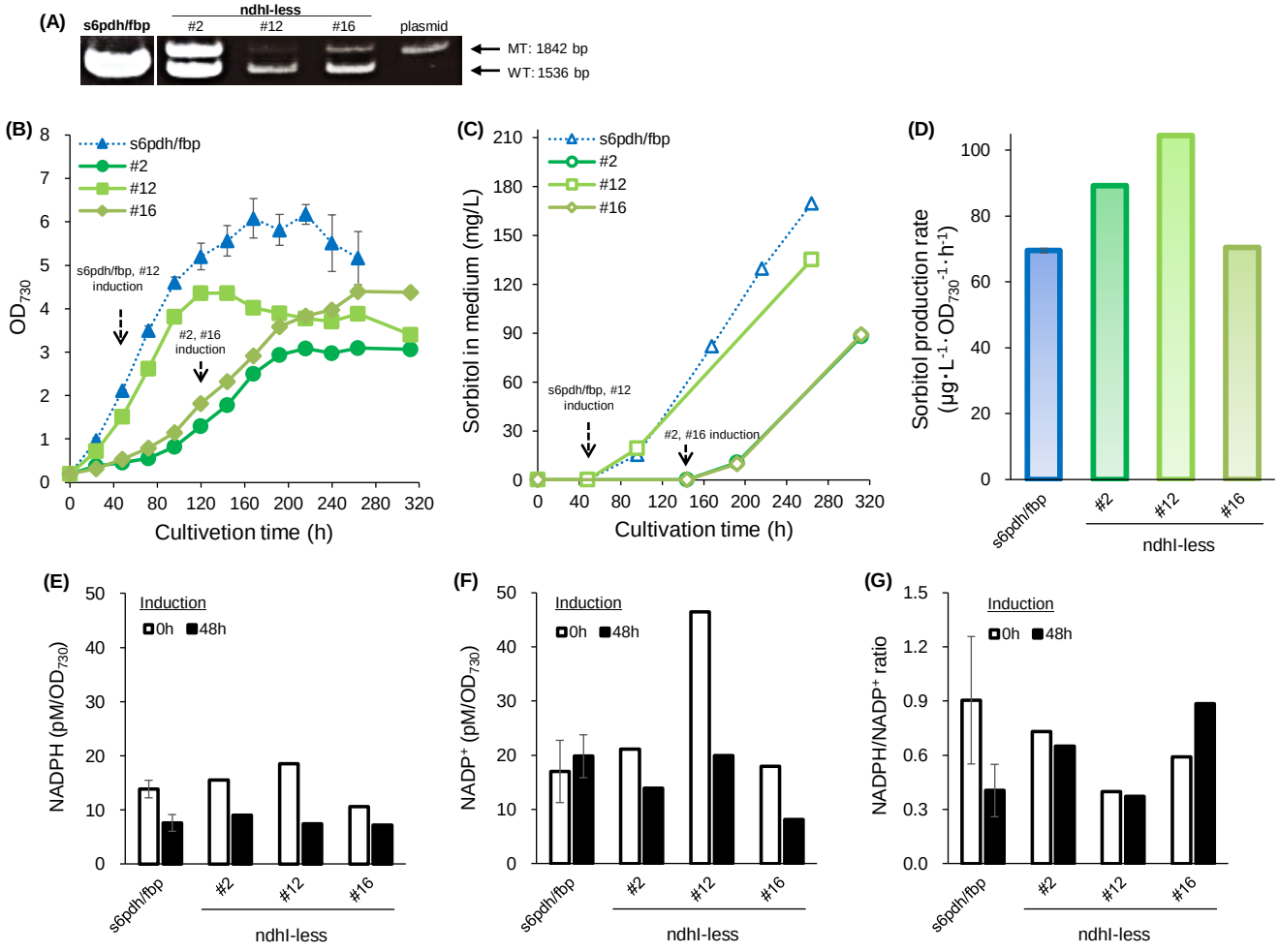

Fig. 1_11. Effects of partial deficient *ndhI* in the strain s6pdh/fbp.

Segregation of *ndhI* deletion (A) was checked by PCR; the pSndhI-Er plasmid was used as a positive control. Growth (B) and sorbitol production (C) were assayed at each cultivation time. Sorbitol production rate (D) was calculated using the values of OD_{730} and sorbitol measured in the medium after 48 h induction. NADPH (E) and $NADP^+$ (F) were quantified using cells before (0 h) and after (48 h) induction. NADPH/$NADP^+$ ratio (G) was calculated from those amounts. Induction was started from 0.5 mM theophylline addition (dashed arrows). Error bars are shown standard deviation only in the strain s6pdh/fbp (*n*=3).

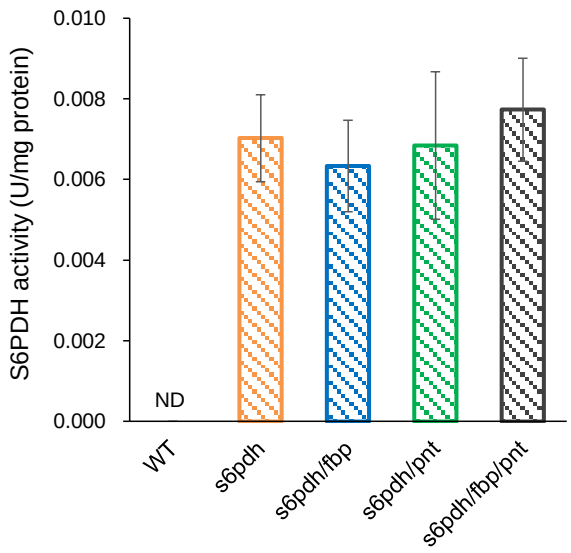

Fig. 1_12. Activity of S6PDH in cells with induction.

S6PDH activity was detected in the cells cultivated for 168 h (0.5 mM theophylline induction for 120 h). Error bars: standard deviation (*n*=3). ND, not detected.

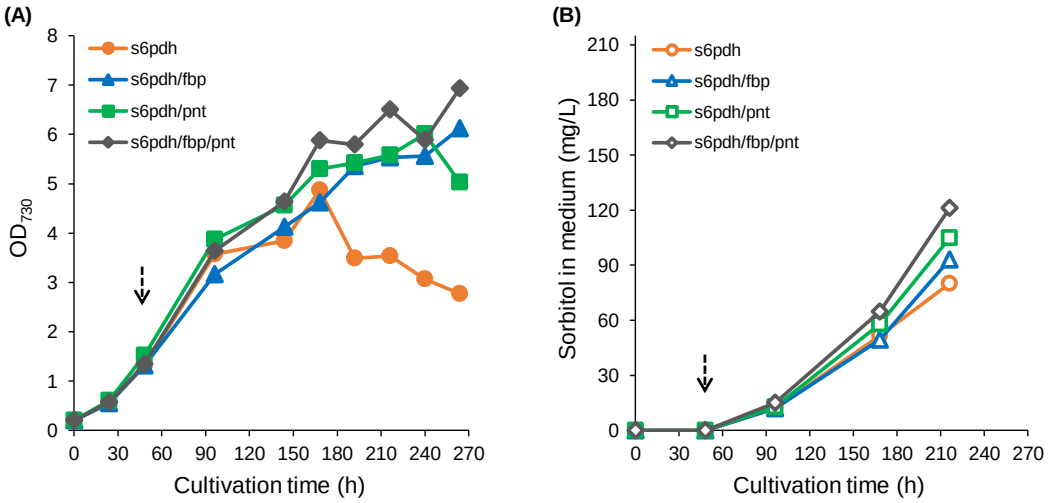

Fig. 1_13. High CO_2 cultivation of sorbitol-producing cyanobacteria.
Growth (A) and sorbitol production (B) were assayed during cultivation with bubbling of 4% (v/v) CO_2. Dashed arrows denote 0.5 mM theophylline addition.

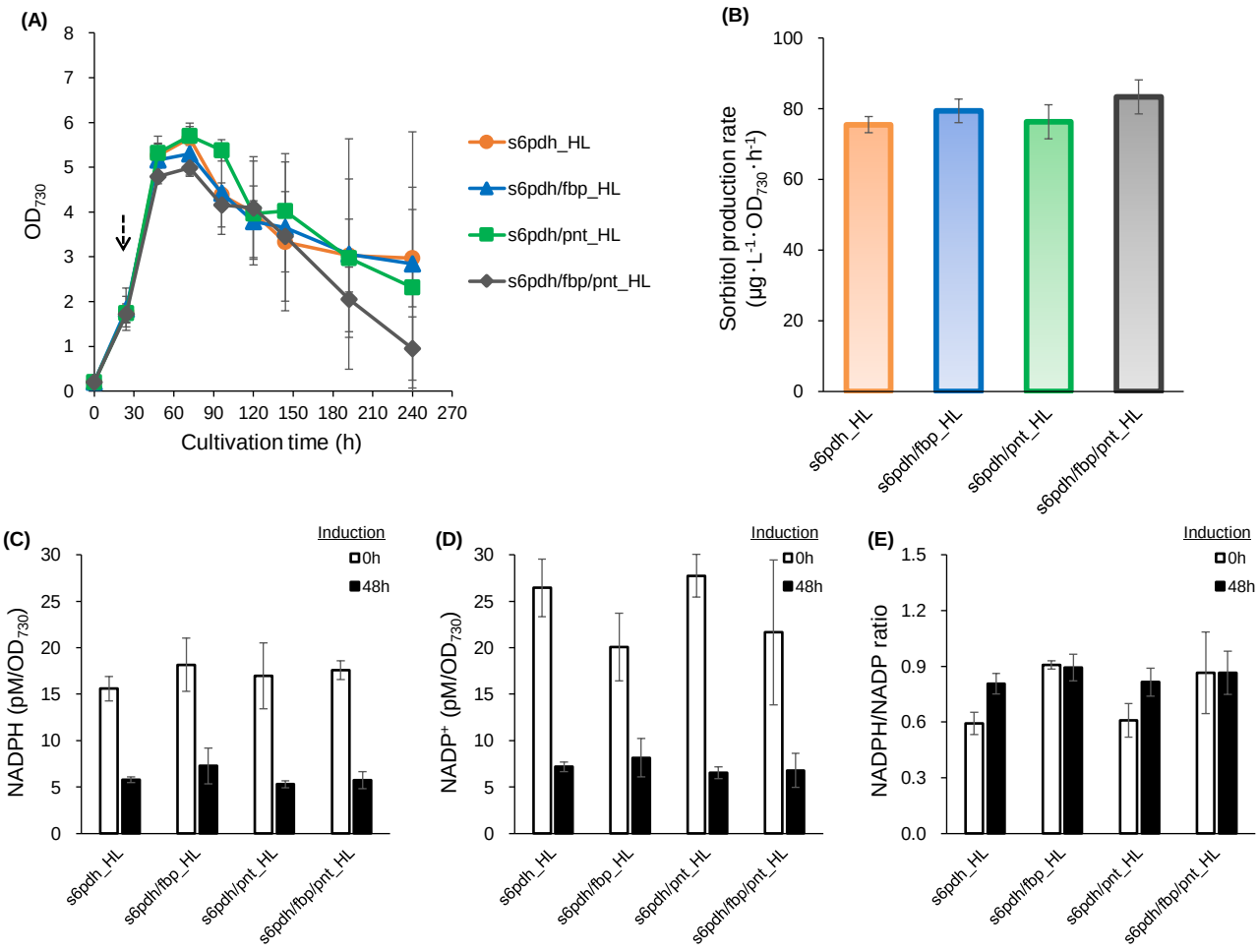

Fig. 1_14. High light cultivation of sorbitol-producing cyanobacteria.

Sorbitol-producing cells were cultivated under continuous illumination at 100 μmol photons · m^{-2} · s^{-1} (HL). Growth (A) was assayed at each cultivation time. Sorbitol production rate (B) was calculated using the values of OD_{730} and sorbitol measured in the medium after 48 h induction. NADPH (C) and $NADP^+$ (D) were quantified using cells before (0 h) and after (48 h) induction. NADPH/$NADP^+$ ratio (E) was calculated from these amounts. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (*n*=3).

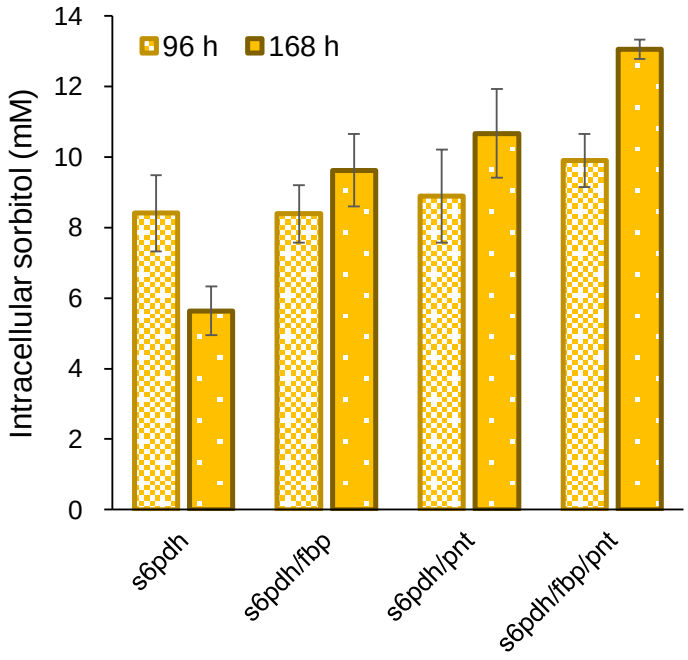

Fig. 1_15. Intracellular sorbitol concentrations in sorbitol-producing cells.
Intracellular sorbitol was quantified using the crude extract of cells harvested at 96 and 168 h of cultivation with 0.5 mM theophylline. Error bars: standard deviation (*n*=3).

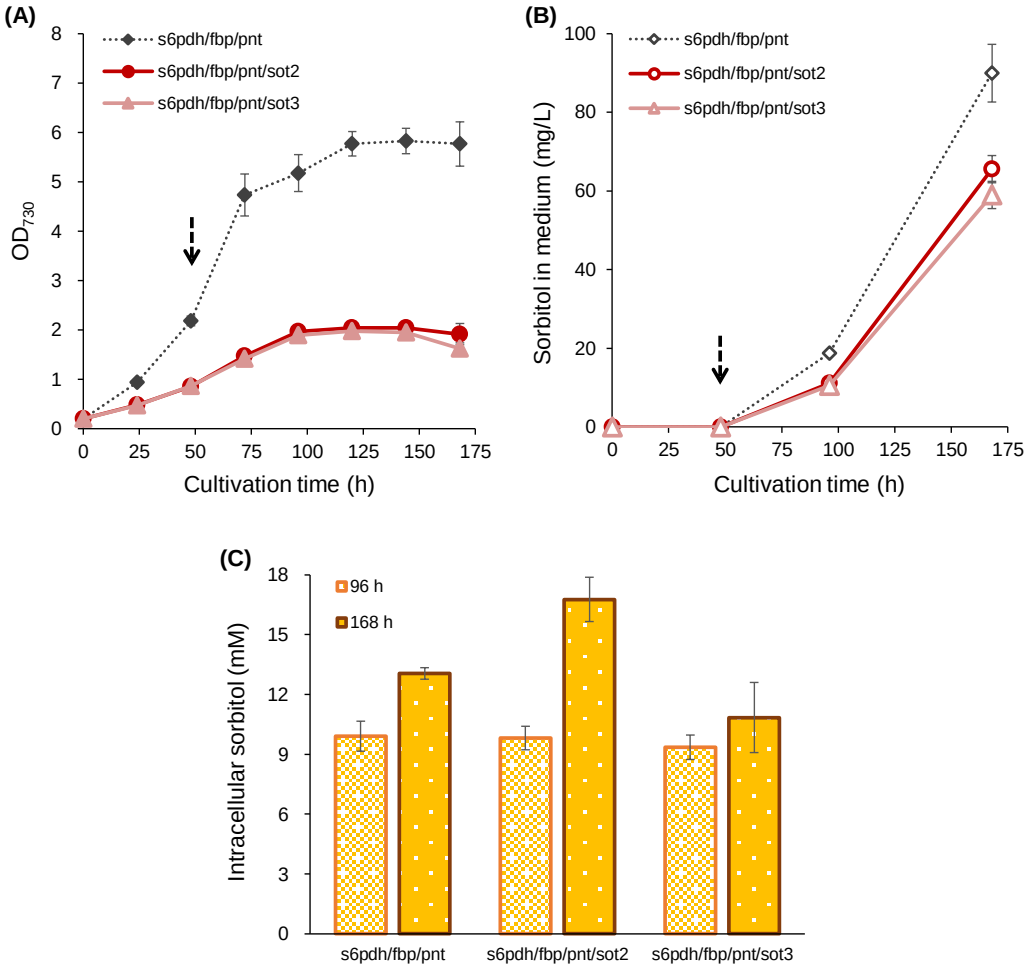

Fig. 1_16. Expression of apple sorbitol transporter in sorbitol-producing cyanobacteria.

Genes of apple sorbitol transporters, *sot2* and *sot3*, were expressed in the strain s6pdh/fbp/pnt. Growth (A) and sorbitol production (B) were assayed at each cultivation time. Intracellular sorbitol (C) was quantified using the crude extract of cells harvested at 96 and 168 h of cultivation with theophylline. Dashed arrows denote 0.5 mM theophylline addition. Error bars: standard deviation (n=3).

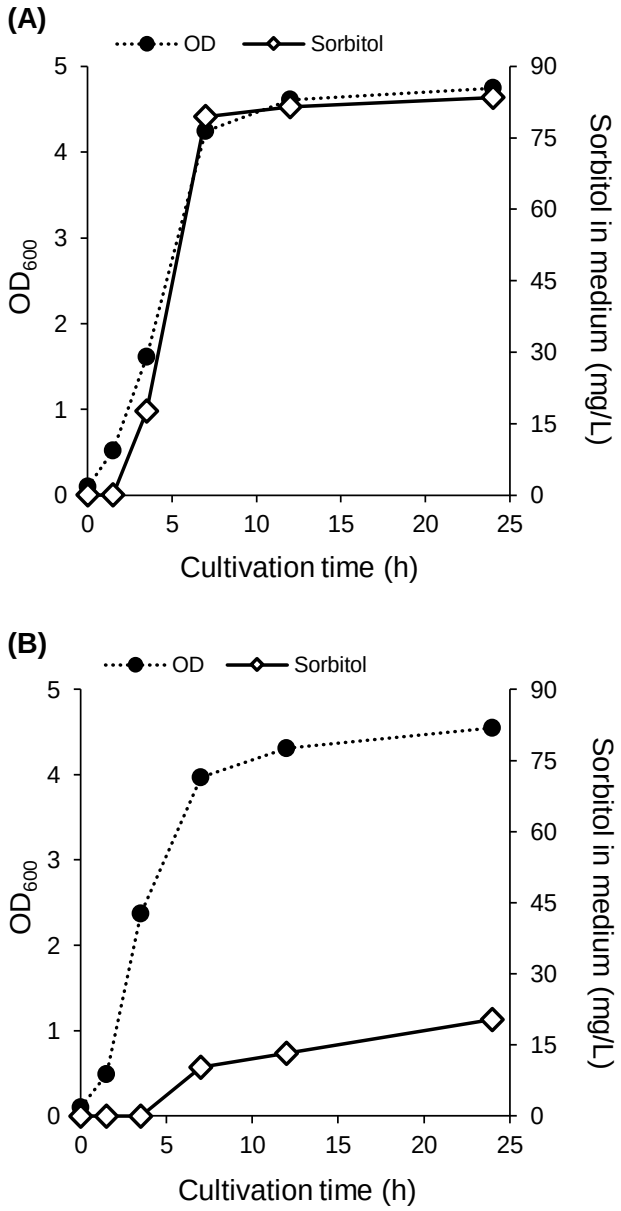

Fig. 1_17. Sorbitol production in *E. coli* expressing sorbitol-6-phosphate dehydrogenase.

S6PDH (A) or SrlD2 (B) was overexpressed by *trc* promoter in the cells of *E. coli* JM109 harboring the plasmid pS46KTs6pdhT or pB46KTsrlD2, respectively. Growth (OD_{600}) and sorbitol were assayed during cultivation in 2 mL of LB medium with 50 mM glucose and 20 μg/mL of kanamycin.

(A) NAD-dependent sorbitol-6-phosphate dehydrogenase (*srlD2*) gene

ATGAATAATAGTTGGATCAACATCTCCGGAAAGGTTTATGTAGTAACTGGAGGTTCCTCTGGTA
TTGGGAAAGCTATTGTCACAGAACTGTTGAATAATGGTGCCATCGTGTATAACGCAGACCTGA
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AGGCCGTAACTCACCAATTTGAGAAACAACAATACGGCGTAATTGTCAACATGAGCAGTGAG
GCTGGGCTGGAGGGCAGCCAAGGACAAAGCGTGTATAGTGCTACCAAGGGCGCGATCAATGG
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GGGTATTATGGAAGCTACCGGATTGCGAACTCGAAGTTATGAGGAGGCTCTGGCATATACGCG
CCACAAAACTGTGGACGATATTCGCGCTGGTTATAAATCCACCTCCACCACGCCCTTAGGGCG
CAGTGGCAAGTTGTCCGAAGTGGCCGACGCCGTCAATTATCTACTCTCCGAGCGAGCCTCCTA
TATCACCGGGGTTACGATTAATGTGGCGGGCGGGAAAAGCCGAGGC

(B) NADP-dependent sorbitol-6-phosphate dehydrogenase (*s6pdh*) gene

ATGAGTACCGTCACCCTATCCTCCGGTTATGAGATGCCGGTGATCGGTCTCGGATTATGGCGTC
TAGAGAAAGACGAGTTAAAAGAGGTGATCCTAAATGCAATCAAAATTGGCTACCGGCACTTC
GACTGCGCTGCACATTACAAATCCGAGGCCGATGTGGGAGAAGCCCTGGCCGAGGCCTTCAA
AACCGGGCTGGTCAAACGAGAGGAGCTATTCATTACCACCAAGATCTGGAACTCCGACCACG
GGCACGTGGTTGAGGCTTGTAAGAACAGTTTGGAGAAGCTGCAAATCGACTATTTGGACCTC
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TCCTGTTTTGAACGATGTGGCCAAGAAGTACGGGAAGTCTGTGGCCCAGATCTGTCTACGATG
GGGTATTCAACGCAAAACGGCCGTTATCCCCAAATCTTCCAAAATCCAACGGTTGAAGGAGA
ATCTAGAAGTTTTAGAGTTTCAATTATCTGATGAAGACATGCAATTAATCTATAGCATTGACCGT
AAATACCGCACTAGCTTACCCTCTAAGACATGGGGCTTGGACGTGTACGCC

(C) Fructose-1,6-bisphosphatase (*fbp*) gene

ATGGCGGCCGTAGGGGAGGCAGCGACGGAGACTAAGGCCCGCACCCGAAGTAAGTACGAAA
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ACTATCGTACTGTCCTCTATTAGCTTAGCTTGTAAACAGATTGCATCTTTAGTTCAACGTGCTGG
GATCTCTAACTTGACCGGAATTCAAGGCGCTGTCAACATTCAGGGCGAAGATCAGAAGAAAC
TCGATGTAGTGAGTAATGAGGTTTTCAGTTCTTGTTTGCGGTCCAGTGGTCGGACCGGTATCAT
TGCTAGCGAGGAAGAAGATGTTCCCGTGGCCGTGGAAGAAAGCTATTCCGGTAATTATATCGT
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ATATATGGACGATCTAAAGGAACCAGGTGAATCTCAGAAACCTTATAGTAGTCGGTACATTGGT
AGTCTAGTGGGAGACTTTCACCGTACCCTGCTCTATGGTGGGATCTATGGGTATCCACGGGAC
GCCAAAAGCAAGAATGGCAAGCTCCGTTTGTTGTATGAATGCGCCCCTATGTCTTTCATTGTA
GAACAAGCTGGCGGAAAAGGTAGCGATGGCCATCAACGAATCTTAGACATTCAGCCCACCGA
AATTCATCAGCGCGTTCCCCTATATATTGGATCTGTCGAAGAAGTAGAAAAGTTAGAGAAATAC
CTAGCATAG

(D) Sorbitol transporter 2 (*sot2*) gene

ATGACAAGTATTCTATTAGGCTACGATATTGGTGTAATGAGTGGCGCATCCCTCTTCATCAAAG
AGAACCTGAAGATTAGTGATGTTCAAGTCGAGATCATGAACGGCACTTTGAATCTATATTCCCT
AATTGGTAGCGCGTTAGCAGGCCGAACCTCTGATTGGATTGGGCGACGCTATACAATTGTACT
GGCTGGAACAATTTTCTTCATTGGCGCCCTATTGATGGGCTTTGCTCCGAATTATGCTTTCCTGA
TGTTCGGACGCTTCGTGGCCGGAGTCGGTGTTGGCTACGCCCTGATGATCGCCCCGGTTTACA
CCGCTGAGATCAGCCCCGCGTCCTTTCGCGGTTTCCTAACTTCCTTCCCCGAGGTGTTTGTGA
ATATCGGCATCTTATTGGGATATGTTTCTAATTATGCGTTCAGCAAACTGCCTATTCACTTAAATT
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TGCCGGAGAGCCCGCGGTGGTTAGTGATGCAGGGACGATTGGGCGACGCCAAGCGAGTCTTA
CAGAAGACCTCCGAAAGTATTGAAGAATGTCAACTCCGTCTGGACGACATCAAAGAGGCAGC
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TTCGAGAAGGCTGGTATTACCAGCTACGATCATAAACTGTTAGCCACCGTTGCAGTTGGCGTT
GTCAAGACGATCTGCATCCTCGTAGCAACCGTCTTCTTGGACAAGTTTGGGCGCCGGCCCCTC
CTATTAACCTCTGTCGCGGGGATGGTGTTCTCCCTGAGTTGTTTGGGCGCATCCCTGACTATCG
TCGACCAACAGCATGGCAAGATTATGTGGGCGATCGTATTGTGCATTACTATGGTCTTACTAAA
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TTACAACTGCGAGCTCAAGGTTGTTCCATGGGAGTGGCCGTTAATCGTGTAACCAGCGGGGT
GATTTCTATGACGTTCATCTCTTTATATAAAGCAATTACTATTGGAGGAGCCTTCTTTCTCTACG
CCGGCATTGCGGCAGTAGGTTGGGTCTTCTTCTACATGCTATACCCGGAGACCCAAGGACGCA
CCCTAGAAGATATGGAGGTTCTCTTCGGTAAATATCATAAATGGCGTGAAGCAAACGCTCTATT
GCAGAAGGCTAAACAAGTGGACGGAGACGACAAAGGACAAGTTCACTAG

(E) Sorbitol transporter 3 (*sot3*) gene

ATGGCTGATCGGCGAGCTGAGGAGAATGCGGTTACAGGTGGGCCACAGAACACATCTATTGA
GGACTTCGATCCTCCAATGAAGCCTAAGACGAGCAAATTCGCTATCGCCTGTGCATTGCTGGC
TTGCACCACTTCCGTATTACTGGGCTATGACATCGGGGTCATGTCTGGCGCGAGTCTGTACATT
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GTGTACACAGCTGAGATTTCCCCTGCGTCTTTTCGCGGCTTCCTGACTAGCTTCCCAGAAGTT
TTCGTGAACGTGGGAATTCTACTGGGATATATCGCAAACTATGCATTCAGCAAATTGCCTCTGC
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TCGCCGCGGTTGGCATTCACTTCTTTGAACAGGCTAGTGGGATTGACACCGTAGTCTTGTATA
GCCCTCGTATCTTTGCGAAAGCTGGGATCACCAGTAGTAACCATAAACTCTTGGCAACTGTCG
CCGTAGGGTTCACGAAGACAGTTTTCATTCTCGTTGCGACGTTCTTCTTAGACAAGTTCGGTC
GTCGGCCACTATTGCTAACGAGTGTCGGTGGAATGGTGTTTAGCTTGATGTTCCTGGGTGTCG
GTCTAACTATTGTCGACCATCACAAGGGATCTGTTCCCTGGGCCATTGGGTTATGCATGGCGAT
GGTTTACTTTAACGTGGCCTTCTTTTCCATTGGCCTGGGACCTATTACATGGGTATATTCTAGTG
AAATCTTCCCACTCAAATTACGGGCCCAAGGTGTTAGTATCGGTGTAGCCTGTAATCGGGTGA
CATCTGGCGTGGTCAGTATGACGTTTATCAGTCTATATAAAGCAATCACCATCGGAGGCGCGTT
CTTCCTATACGCTGGCATCTCCGCTGCGGCCTGGATCTTCTTTTATACTATGTTGCCAGAGACTC
AAGGTCGCACTCTGGAAGACACTGAAGTCCTATTCGGCAAATATCATCGATGGCGTAAGGCGA
ATGCAATGCTCAAGGAGCGAAAGCAAGTAGATGGGGACGACAATAATAACGCACAGGTGTTC
TAG

Fig. 1_18. Codon-optimized nucleotide sequences for *Synechocystis* sp. PCC 6803.

NAD-dependent sorbitol-6-phosphate dehydrogenase (*srlD2*) gene from *Lactobacillus plantarum* WCFS1 (A), NADP-dependent sorbitol-6-phosphate dehydrogenase (*s6pdh*) gene from *Malus domestica* (B), fructose-1,6-bisphosphatase (*fbp*) gene from *Spinacia oleracea* (C), and sorbitol transporter 2 (*sot2*) and 3 (*sot3*) genes from *Malus domestica* (D and E).

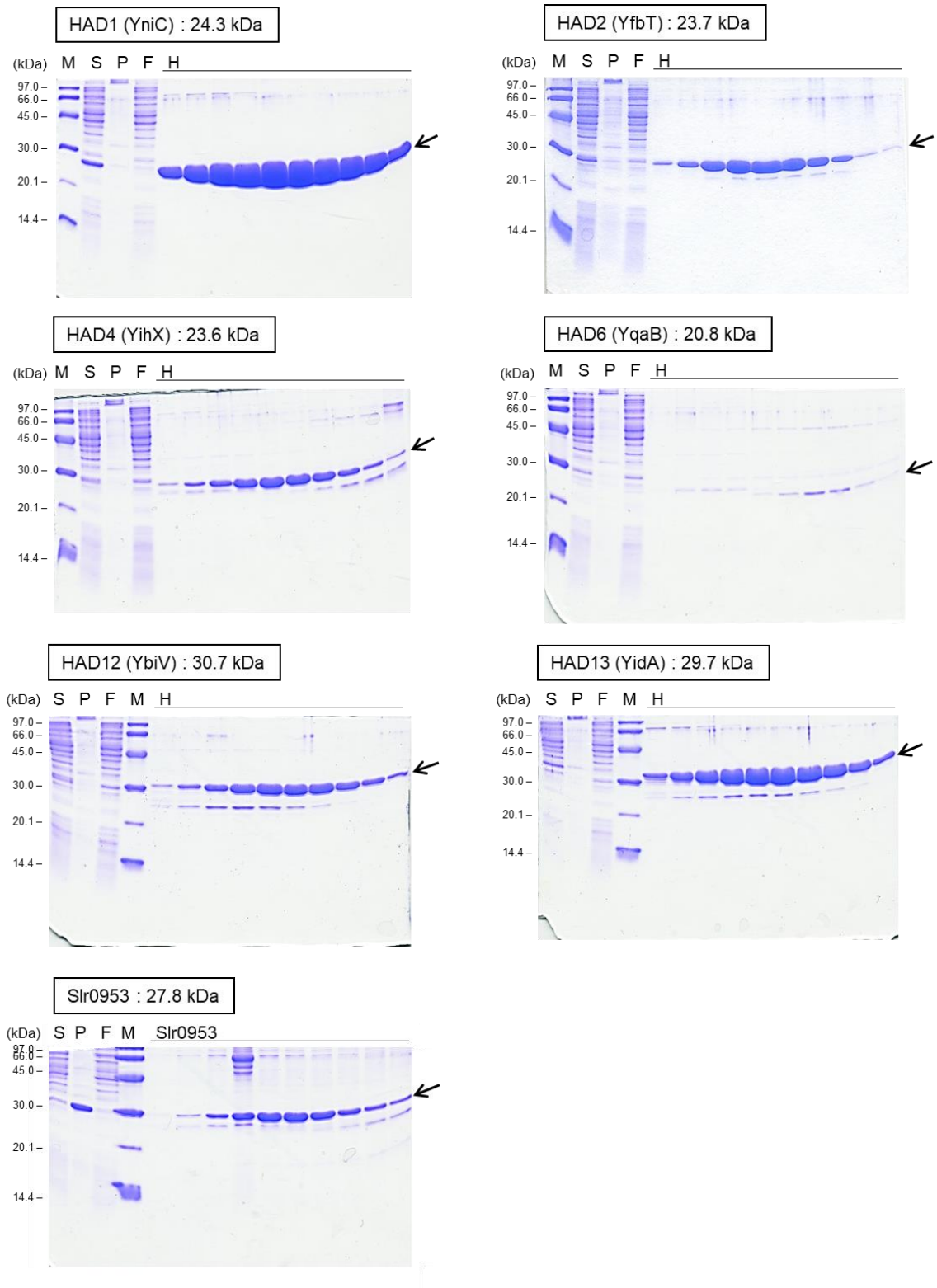

Fig. 2_1. SDS-PAGE analysis of HADs from *E. coli* and *Synechocystis*.

The arrows indicate the His-tagged HADs. M, marker; S, supernatant of cell extracts; P, pellet of cell extracts; F, flow-through fraction of the Ni^{2+} chromatography; H, purified *E. coli* HADs fraction; Slr0953, purified *Synechocystis* HAD (sucrose-phosphatase) fraction.

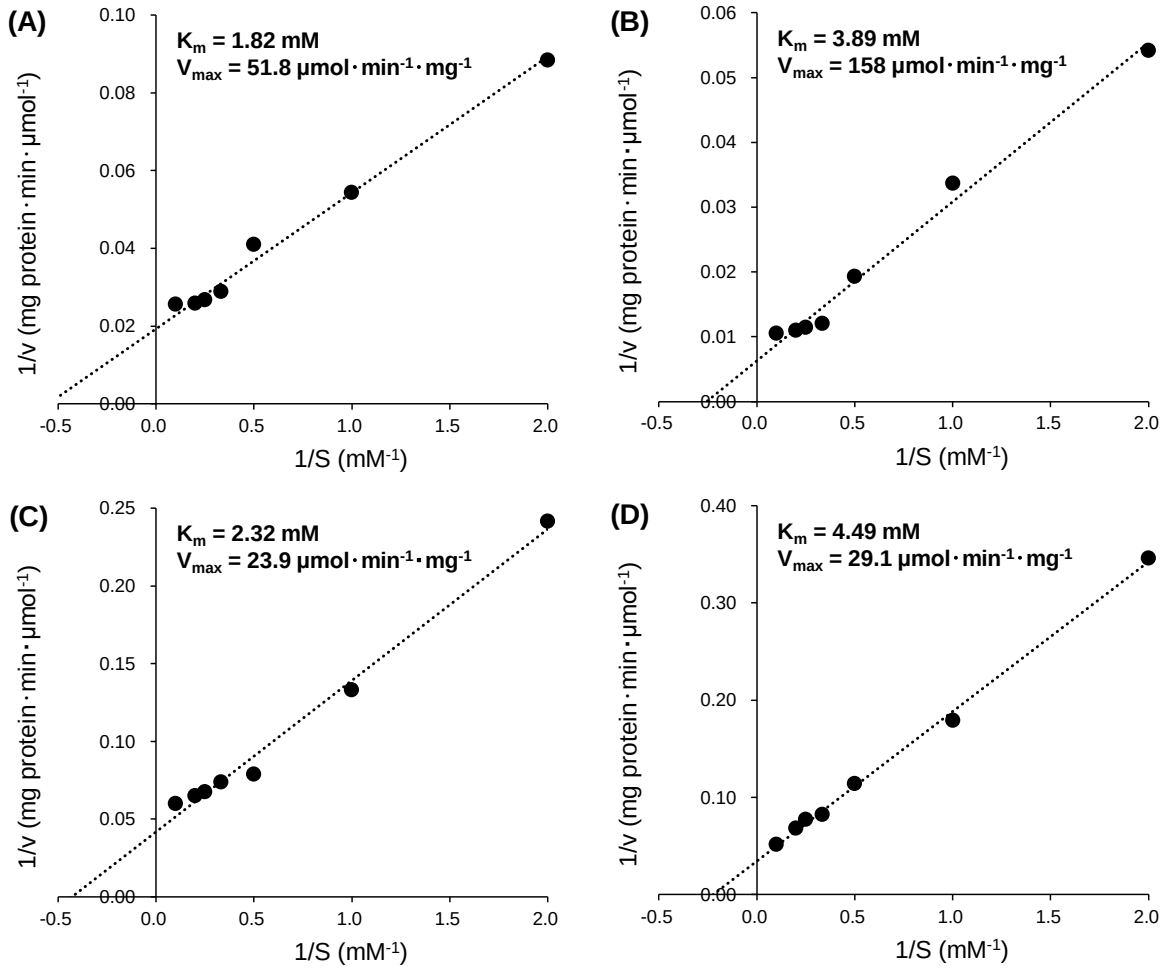

Fig. 2_2. Lineweaver-Burk plots of phosphatase activity of HADs for sorbitol-6-phosphate.

Assays were performed using 1 μg each of purified HAD1 (A), HAD2 (B), HAD12 (C), or HAD13 (D) in the reaction mixture containing 50 mM HEPES-KOH (pH 7.0) and 2 mM $MgCl_2$.

※近い将来において刊行される期待がある内容（5 年以内に出版予定）

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