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Enhanced productions of poly-3-hydroxybutyrate and glycogen in cyanobacterium *Synechocystis* sp. PCC 6803 by disrupting related biosynthetic pathways under phosphorus deprivation

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ABSTRACT: Under normal growth (NORMAL) condition with adequate supply of phosphorus and nitrogen, the well-studied cyanobacterium *Synechocystis* wild type (WT) produced a high biomass level but subtle contents of the two biopolymers: poly-3-hydroxybutyrate (PHB) and glycogen. In contrast, under nitrogen deprivation, growth of WT was terminated, while the accumulations of PHB and glycogen were increased. Thus, a strain and a cultivation strategy that simultaneously allows both biomass production and the increased storages of the two biopolymers are desirable. Here, under phosphorus deprivation (-P), WT grew and still produced biomass at 850 mg/l (accounting for 62% of the biomass level obtained under NORMAL cultivation) with slightly increased contents of PHB and glycogen at 0.5% and 5.5% (w/w DW), respectively. Under -P, the ΔG mutant with the inactivated glycogen synthesis produced biomass at 751 mg/l and enhanced PHB accumulation to 4.9% (w/w DW), corresponding to PHB production of 36 mg/l. The ΔHP mutant with the inactivation of both PHB and hydrogen-gas syntheses under -P produced biomass at 713 mg/l and increased glycogen storage to 14.6% (w/w DW), corresponding to glycogen production of 118 mg/l. The significantly reduced levels of both chlorophyll and phycobilisomes (the main photosynthetic protein complex) found in ΔG and ΔHP mutants under -P suggested that photosynthetic proteins were degraded into amino acids which might subsequently be used for PHB and glycogen syntheses. The results indicated that the metabolic engineering combined with -P cultivation concurrently allowed cell growth and increased the production of two biopolymers in the studied *Synechocystis*.

KEYWORDS: cyanobacteria, biopolymer, bioplastic, phosphorus, photosynthesis

INTRODUCTION

The well-studied cyanobacterium *Synechocystis* sp. PCC 6803 (hereafter, *Synechocystis*) is a promising photoautotrophic microorganism for producing bioplastic poly-3-hydroxybutyrate (PHB) and glycogen (the polymer of glucose used as a substrate for bioethanol production) using the greenhouse gas carbon dioxide as a sole carbon source [1, 2]. Under normal growth (NORMAL) condition with adequate supply of phosphorus and nitrogen, cells grow normally and contain a relatively high protein content [3, 4], but low PHB and glycogen accumulation [1, 5, 6]. In contrast, under nitrogen deprivation (-N), cell growth is terminated, and the cells reduced protein content, including phycobilisomes [4, 7], but increased the accumulations of PHB and glycogen [1, 5, 8, 9]. To obtain high levels of both the biomass production and the high contents of the two polymers, a two-stage cultivation has been conducted [1, 10–12]. The cells were first precultured under NORMAL condition to produce a high biomass level, and the biomass was subsequently cultured under -N to obtain a high accumulation of PHB and glycogen. Although the two-stage cultivation improved the production of PHB and glycogen [1, 10–12], this approach required a long cultivation time and excessive media and multiple cell harvest which restricted production feasibility. Therefore, single-stage cultivation that yields high levels of both the biomass production and the accumulations of the two biopolymers has yet to be developed. It was reported that *Synechocystis* can still grow under phosphorus deprivation (-P), but at a moderately slower rate than those cells cultivated under NORMAL condition [13]. This might be due to the ability that *Synechocystis* can utilize the accumulated polyphosphates for cell growth under -P [14, 15]. Interestingly, under -P, the wild type (WT) *Synechocystis* also slightly increased the accumulations of PHB and glycogen [1, 5, 8, 9]. Thus, -P cultivation is a potential single-stage cultivation approach for producing the two biopolymers because it allows both the cell growth and the increased contents of the two biopolymers. However, the levels of PHB and glycogen accumulation of the WT strains under -P have yet to be improved.

We previously demonstrated that the metabolically engineered *Synechocystis* mutants with the inactivation of biosynthetic pathways: glycogen synthesis, PHB synthesis, and/or hydrogen-gas synthesis improved the accumulation of PHB and/or glycogen under -N cultivation [6]. In this study, we determined

whether the use of such mutants cultured under -P cultivation can improve the accumulations and the production level of the two biopolymers. To examine whether the produced PHB and glycogen of the mutants under -P are related to the conversion of photosynthetic proteins into such two polymers, the levels of chlorophyll and phycobilisomes were determined.

MATERIALS AND METHODS

Cyanobacterial cultivation conditions

The strains used in this study were listed in Table 1. The cells were cultivated in the standard Blue-Green 11 (BG11) medium [16] supplemented with 20 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH (HEPES-NaOH), pH 7.5. The cell cultures were placed at 28 ± 2 °C under continuous illumination of 50 µmol photon/m^2s with culture shaken at 160 rpm. The phosphorus deprivation (-P) was performed by culturing the cells in the BG11 medium that K_2HPO_4 was replaced with KCl on an equimolar basis. Erlenmeyer flasks (500 ml size) were used to cultivate 200 ml of cyanobacterial cultures. For -P cultivation, exponentially pre-grown cells were harvested and washed twice using -P medium, and subsequently cultured under -P condition using the initial cell concentration at $OD_{730} = 0.5$. The cells were harvested by centrifugation (4000*g* for 10 min) and dried at 60 °C immediately until a constant weight was obtained.

Determination of contents, productions, and productivities of PHB and glycogen

The intracellular PHB content was quantified as previously described [1]. Five mg of dry cells was hydrolysed in 1 ml of 18.4 M sulfuric acid at 100 °C to convert the PHB to crotonic acid (a PHB-hydrolysed product). The hydrolysate was diluted with distilled water until reaching the final concentration of 1.84 M sulfuric acid; then, it was filtered and quantified for the crotonic acid content using high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). The HPLC was operated with InertSustain 3-µm carbon-18 column (GL Sciences, Rolling Hills Estates, USA) and UV detector at 210 nm. Commercial PHB (Sigma-Aldrich, Saint Louis, USA) was analysed in parallel with the PHB standard. The conversion of PHB to crotonic acid was 85 ± 5% w/w. Adipic acid and crotonic acid were used as internal standard and crotonic acid standard, respectively.

The glycogen extraction and quantification were conducted using the previously described method [1, 17]. Glycogen was extracted from five mg of dry cells in 30% (w/v) KOH followed by ethanol precipitation. The precipitated glycogen was analysed for glycogen content using the anthrone method. The pellet was first dissolved in 0.2 ml 1.84 M sulfuric acid, and then 0.8 ml of the anthrone reagent (2 g/l in 18.4 M sulfuric acid) was added. The samples were boiled for 10 min at 100 °C to obtain the bluish-green product which was measured spectrophotometrically at 625 nm (A_{625}). Commercial glycogen from oysters was used as standard for the determination of the glycogen content.

The values were calculated using the equations below.

$$\text{PHB content (\% w/w DW)} = \frac{\text{PHB in cells (mg)}}{\text{cell dry weight (mg)}} \times 100$$

$$\text{Glycogen content (\% w/w DW)} = \frac{\text{glycogen in cells (mg)}}{\text{cell dry weight (mg)}} \times 100$$

$$\text{Biomass production (mg/l)} = \frac{\text{dry biomass (mg)}}{\text{cell culture (l)}}$$

$$\text{PHB production (mg/l)} = \frac{\text{Biomass production} \times \text{PHB content}}{100}$$

$$\text{Glycogen production (mg/l)} = \frac{\text{Biomass production} \times \text{Glycogen content}}{100}$$

$$\text{Biomass productivity (mg/l/day)} = \text{Biomass production/day}$$

$$\text{PHB productivity (mg/l/day)} = \text{PHB production/day}$$

$$\text{Glycogen productivity (mg/l/day)} = \text{Glycogen production/day}$$

Determination of maximal light absorption of chlorophyll and phycobilisomes

To measure the whole-cell absorption spectrum, living cells suspended in -P medium at approximately OD_{730} = 1 were measured for absorbance between 400 and 700 nm using synergy H1 microplate reader spectrophotometry (BioTek, Winooski, USA). The levels of phycobilisomes and chlorophyll were estimated by their specific maximal absorption at 625–630 nm and 665–680 nm, respectively [7, 18, 19].

Statistical analysis

The statistical differences between the data obtained from wild type and mutant strains were determined using the two-tailed *t*-test accepting significance at the $p < 0.05$. The analyses were performed using the IBM SPSS Statistics 23 software (IBM, New York, USA).

RESULTS

All mutant strains were constructed previously [6], and their genotypes and phenotypes regarding the bioproduct production were described in Table 1.

Under NORMAL, the wild type (WT) and all mutants (ΔG, ΔP, ΔGH, and ΔHP) grew at comparable rate from day 0 to 7; but after day 7, the ΔGH mutant showed slower growth than the other strains (Fig. 1A). Under -P, the ΔGH mutant showed a significantly lower growth level than the other strains (Fig. 1A).

It was noted that the cell growth of all strains under -P was slightly slower than those under NORMAL (Fig. 1A). Additionally, under -P, the cell cultures of all mutants (ΔG, ΔP, and ΔGH) had a slightly lower level of green color compared with the WT (Fig. 1B). Unexpectedly, the culture of ΔHP exhibited substantial chlorosis (Fig. 1B).

Table 1 List of the *Synechocystis* strains used in this study.

Strain	Genotype[a]	Produced bioproduct[b]			Reference
		PHB	Glycogen	H_2	
WT	*Synechcoystis* sp. PCC 6803	✓	✓	✓	Pasteur culture collection
ΔG	WT, Δ*glgC::cm*^{R}	✓	NP	✓	[6]
ΔP	WT, Δ*phaE-phaC::spc*^{R}	NP	✓	✓	[6]
ΔGH	ΔG, Δ*hoxY-hoxH::km*^{R}	✓	NP	NP	[6]
ΔHP	ΔH, Δ*phaE-phaC::spc*^{R}	NP	✓	NP	[6]

[a] *glgC*, ADP-glucose pyrophosphorylase gene; *phaE-phaC*, the operon containing of PHB synthase subunit-E gene and PHB synthase subunit-C gene; *hoxY-hoxH*, the operon containing hydrogenase subunit-Y gene and hydrogenase subunit-H gene; *cm*^{R}, chloramphenicol resistant gene; *spc*^{R}, spectinomycin resistant gene; *km*^{R}, kanamycin resistant gene.
[b] ✓: produced; NP: not produced.

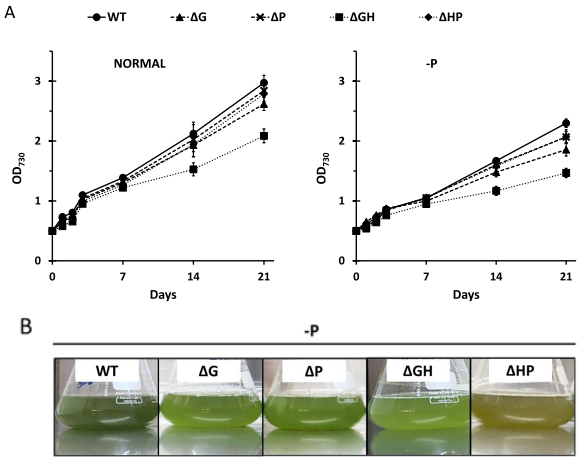

Fig. 1 Cell cultures and growths of the *Synechocystis* wild type (WT) and the gene-deleted mutants. (A), Growths were determined by measuring the optical density of the cell culture at 730 nm (OD_{730}) under normal growth condition (NORMAL) with the adequate supply of phosphorus and under phosphorus deprivation (-P). Each point represents a mean and standard deviation of three independent cultures. (B), Photographs of the cell cultures after 14 days under -P.

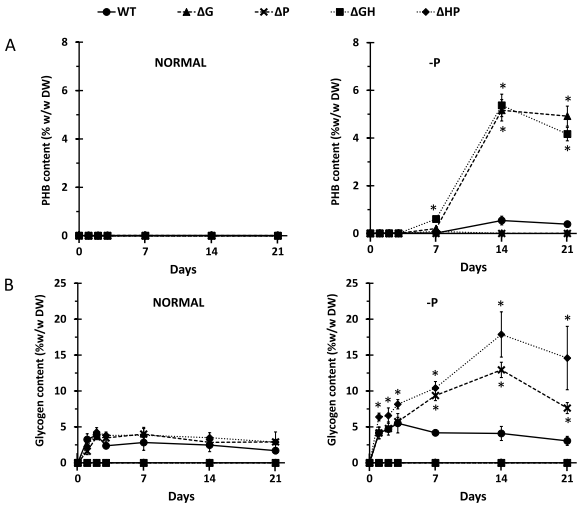

Fig. 2 Cellular accumulation of PHB (A) and glycogen (B). Cells were cultured under a normal growth condition (NORMAL) and phosphorus deprivation (-P). Each point represents a mean of three independent cultures. Asterisks (*) indicate the levels of the mutants that were significantly higher ($p < 0.05$: two-tailed t-test) than those of the WT on the same day of cultivation.

The WT and the two PHB-producing mutants (ΔG and ΔGH) did not produce PHB under NORMAL (Fig. 2A). However, under -P for 14 days, the PHB contents produced by the ΔG and the ΔGH increased up to 5.2% and 5.4% w/w dry weight (DW), respectively, compared with 0.5% (w/w DW) of PHB level in the WT (Fig. 2A). Therefore, disruption of glycogen synthesis increased the PHB accumulation under -P.

The WT and the two glycogen-producing mutants (ΔP and ΔHP) had comparable low glycogen content of less than 5% (w/w DW) under NORMAL (Fig. 2B). Interestingly, under -P, the WT had the maximal glycogen content at only 5.5% (w/w DW), while the ΔP and ΔHP mutants increased glycogen accumulation up to 12.9% and 17.9% (w/w DW), respectively (Fig. 2B). Therefore, the disrupted PHB synthesis significantly increased the glycogen content under -P.

The culture production levels of PHB and glycogen (mg of PHB or glycogen per l of cell culture) under -P were determined. The WT had low PHB production up to 3 mg/l (at day 14, Fig. 3), while the ΔG mutant produced a maximal PHB up to 36 mg/l corresponding to a PHB content of 4.9% (w/w DW) (Fig. 2) and a biomass of 751 mg/l (at day 21, Fig. 3). The ΔGH also produced high PHB level at 32 mg/l (Fig. 3).

For glycogen production, the WT generated a low glycogen level at 28 mg/l (at day 14, Fig. 3), whereas ΔP produced a moderate glycogen level at 87 mg/l (at day 14, Fig. 3). Interestingly, the ΔHP mutant had a maximum glycogen production level at 118 mg/l, which corresponded to a glycogen content of 14.6% (w/w DW) (Fig. 2) and a biomass of 713 mg/l (at

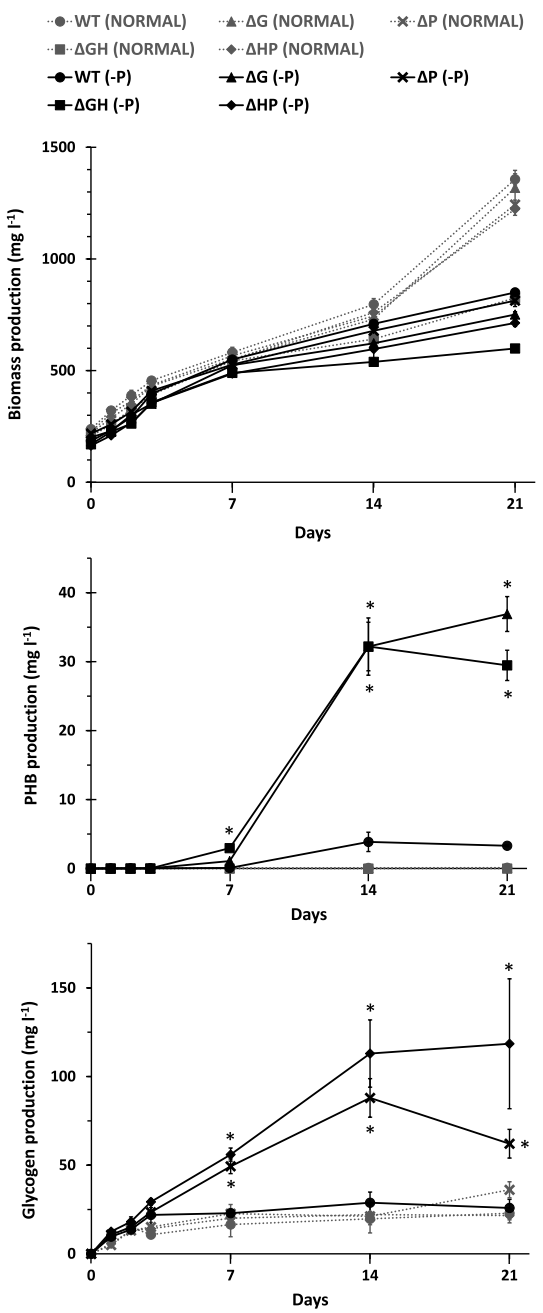

Fig. 3 Culture production levels of PHB and glycogen by the WT and the mutants. Cells were cultured under normal growth condition (NORMAL) and phosphorus deprivation (-P). Each point represents an average of three independent cultures. Asterisks (*) indicate the levels in mutants that were significantly higher than those in the WT on the same cultivation day ($p < 0.05$: two-tailed t-test).

day 21, Fig. 3). Overall, the ΔG and the ΔHP had the highest production level of PHB and glycogen, respectively. Thus, the disrupted glycogen synthesis enhanced PHB production and the disruption of both PHB and hydrogen syntheses significantly increased glycogen production under -P.

The light absorption levels of the phycobilisomes (the main photosynthetic protein complex) and the major photosynthetic pigment chlorophyll were examined under -P which *Synechocystis* accumulated PHB and glycogen. The light absorptions of both phycobilisomes and chlorophyll in all mutants (ΔG, ΔP, ΔGH, and ΔHP) were reduced compared with the WT at days 7, 14, and 21 (Fig. 4). Thus, in all studied mutants, the levels of photosynthetic phycobilisomes and chlorophyll were reduced (Fig. 4), while the cells increased the accumulation of PHB and/or glycogen (Figs. 2 and 3). The results also showed that the disruption of PHB and glycogen syntheses affected the levels of phycobilisomes and chlorophyll under -P.

DISCUSSION

Under NORMAL growth condition with the optimal supply of phosphorus and nitrogen, the *Synechocystis* WT produced a relatively high amount of biomass but low accumulation of PHB and glycogen (Figs. 2 and 3) [1, 5, 6]. Under nitrogen deprivation (-N), the WT terminated cell growth and significantly accumulated PHB up to 0.5–13% (w/w DW) [1, 2, 6, 20, 21] and glycogen up to 8–60% (w/w DW) [1, 6, 9, 20, 21]. The PHB and glycogen accumulations of the mutants used in this study and cultured under -N were reported elsewhere [6]. Unlikely, this study found that under -P, the WT still grew with the biomass up to 850 mg/l (corresponding to 62% of the biomass level produced under NORMAL condition, Fig. 3), and the cells were also able to slightly increase the accumulation of PHB up to 0.5% (w/w DW) and glycogen up to 5.5% (w/w DW) (Fig. 2). Thus, this study showed the novel concept that the -P simultaneously enables WT to increase both the biomass production and the cellular contents of PHB and glycogen, but these subtle increased contents of the two biopolymers have yet to be improved. It is noted that *Synechocystis* can grow under -P due to its ability to utilize accumulated polyphosphates [14, 15]. Here, this study also showed that under -P, the inactivated glycogen synthesis (in the ΔG mutant) increased PHB accumulation up to 5.4% (w/w DW) (Fig. 2), and the inactivated biosynthesis of both PHB and H_2 (the ΔHP mutant) enhanced glycogen accumulation to 17.9% (w/w DW) (Fig. 2). It is also noted that the high PHB contents at 33% and 50% (w/w DW) were obtained by the WT treated with the metabolic inhibitor 2-phenylethanol [22], and the *Synechocystis* with the overexpressing PHB biosynthetic genes and the deleted *pirC* (encoding a protein regulating carbon metabolism) [23], respectively. The high glycogen accumulation at 83–85% (w/w DW) was obtained from the *Synechocystis* WT with the disrupted H_2 synthesis [13]. Thus, it is worth combining

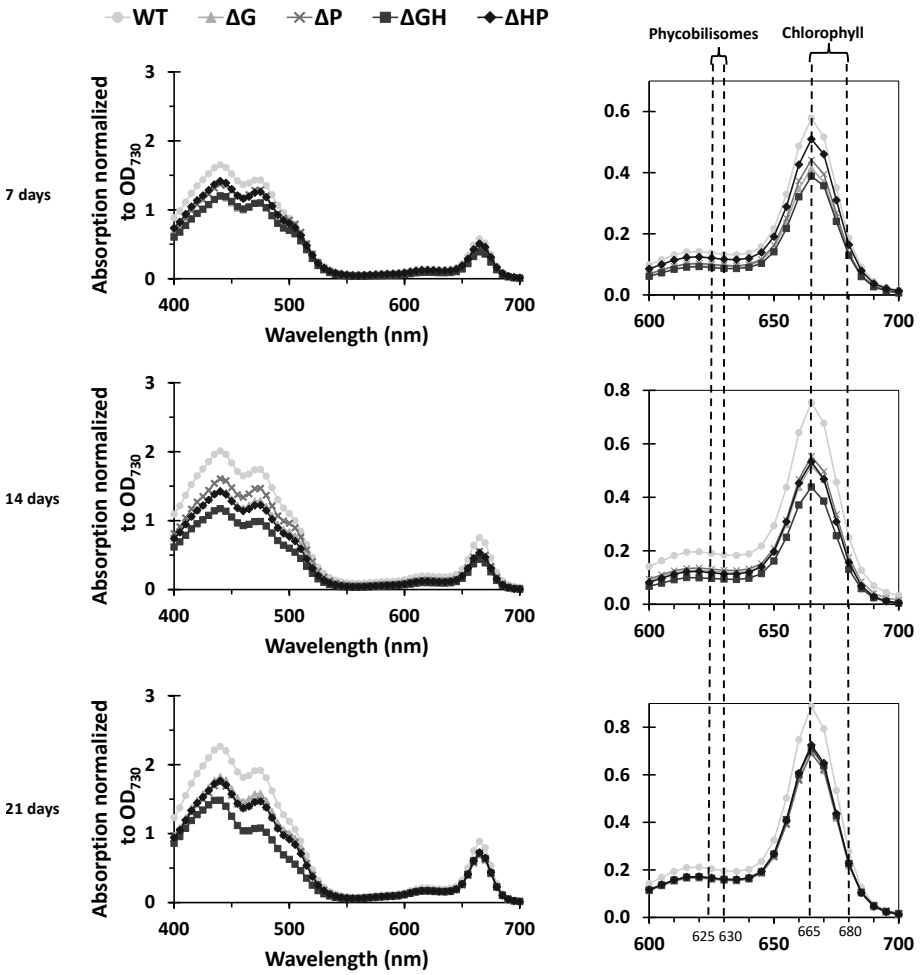

Fig. 4 Light absorption of the WT and the mutants under phosphorus deprivation (-P). Absorption spectra at 400–700 nm (left panel) and 600–700 nm (right panel) of the cell culture were normalized with the optical density of the cell culture at 730 nm (OD_{730}). The WT and the mutants were cultured under -P. The heights of the spectra were normalized to their corresponding cell density at OD_{730}. Spectra were drowned using means of three independent cultures. Broken lines indicate the ranges of the maximal absorption of phycobilisomes at 625–630 nm and Chlorophyll at 665–680 nm [7, 18, 19].

Table 2 PHB and glycogen productivities of *Synechocystis* sp. PCC 6803 obtained from this study and previous reports.

Biopolymer	Productivity (mg/l/day)	*Synechocystis* sp. PCC 6803[a]	% Supply of N and P[b]	Reference
PHB	0.48	Wild type	100% N, 100% P	[1]
	2.16	Wild type	100% N, 20% P	[32]
	2.30	ΔG mutant	100% N, 0% P	This study
	2.30	ΔGH mutant	100% N, 0% P	This study
	6.24	Wild type	50% N, 100% P	[33]
	7.30	The gene-engineered strain with *xfpk* overexpression and with the inactivation of *pta* and *ach*	100% N, 100% P	[34]
Glycogen	4.08	Wild type	100% N, 100% P	[1]
	6.22	The glucose-tolerant strain	100% N, 100% P	[38]
	10.74	ΔP mutant	100% N, 0% P	This study
	12.55	ΔHP mutant	100% N, 0% P	This study
	17.76	Wild type	100% N, 20% P	[32]
	21.30	The glucose-tolerant strain with the inactivation of *apcE*	100% N, 100% P	[38]

[a] *xfpk*, heterologous phosphoketolase gene; *pta*, phosphotransacetylase gene; *ach*, acetyl-CoA hydrolase gene; *apcE*, phycobilisome core-membrane linker polypeptide gene.
[b] % Supply is relative to the nitrogen supply and the phosphorus supply used in the standard BG11 media [16].

such previously reported strategies together with the disrupted biopolymer synthesis and -P as described in this work for further enhancing the accumulations of the two biopolymers.

It has been described that under -N, the inactivated glycogen synthesis (in the ΔG mutant) increased both NAD(P)H and PHB levels [6]. This increased NAD(P)H leads to the enhanced PHB level because NADPH is the cofactor substrate for PHB synthesis [24]. In this study, the inactivated glycogen synthesis (in the ΔG mutant) under -P also increased the PHB level (Fig. 2A). Since NADPH is the essential substrate for PHB biosynthesis [25–27], it might be possible that the inactivated glycogen synthesis under -P also increased cellular NADPH level, and this increased NADPH level promoted PHB synthesis in the ΔG mutant.

It has also been shown that under -N, the disrupted PHB synthesis enhanced glycogen accumulation [6, 9]. This is due to the cause that glycogen can be consumed and metabolically converted to PHB [6, 9]. Thus, the disrupted PHB synthesis terminated such glycogen conversion to PHB; therefore, this led to the increased glycogen level [6]. In this study, glycogen might be converted to PHB under -P as well because the inactivated PHB synthesis also increased glycogen level under -P (Fig. 2).

In the present work, the ΔG and the ΔP mutants increased PHB and glycogen accumulations, respectively; but these two mutants slightly decreased the levels of the major photosynthetic pigments, chlorophyll and phycobilisomes, under -P (Fig. 4). Phycobilisomes are the major component of total cellular proteins responsible for photosynthesis in cyanobacteria [28–30]. Phycobilisomes have been described to be degraded into amino acids which were subsequently metabolized to glycogen and PHB via TCA cycle and gluconeogenesis under -N [22, 31]. This phenomenon of protein conversion to PHB or glycogen has yet to be elucidated under -P. Here, the disruption of either PHB synthesis or glycogen synthesis (ΔG and ΔP) also reduced phycobilisomes levels under -P (Fig. 4), suggesting that such protein conversion to PHB and glycogen also occurs under -P.

Various approaches have been reported on how to increase the culture productivity of PHB in *Synechocystis*. In the WT, NORMAL produced PHB only 0.5 mg/l/day [1], while the cultivation under partial supply of phosphorus at 20% level of the NORMAL cultivation enhanced PHB productivity to 2.2 mg/l/day [32]. The results showed that -P increased the PHB productivity of the ΔG and ΔGH mutants to 2.3 mg/l/day (Table 2). Thus, -P (0% phosphorus) and the 20% phosphorus cultivation yielded comparable PHB productivity. Hence, -P had a lower cost of cultivation than the 20% phosphorus cultivation because no phosphorus addition was required. The high *Synechocystis* PHB productivities at 6.2 and 7.3 mg/l/day were reported in the WT cultivated under the partial supply of nitrogen at 50% level of the NORMAL [33] and in the strain with metabolically engineered an altered cellular central carbon metabolism [34], respectively. It is noted that the drawbacks of utilizing the gene-engineered *Synechocystis* mutants under -P condition were the slower growth rate of the mutants compared with the WT (Fig. 1A) and the lower biomass production compared with the NORMAL cultivation (Fig. 3). It has been reported that phosphorus-limiting cultivation also slightly reduced the levels of photosynthetic proteins [35], ribosomal proteins [35], and NAD(P)H [13] in *Synechocystis* as well as causing partial cell lysis under prolonged cultivation [36]. Furthermore, these gene-engineered mutants must be cultivated in a closed system to avoid the escape of these mutants into the environment causing a horizontal gene transfer from these gene-engineered strains to native organisms [37].

For glycogen productivity of *Synechocystis*, the WT [1] and the glucose tolerant strain [38] produced glycogen at 4.1 and 6.2 mg/l/day, respectively. Under -P in our study, the ΔP and the ΔHP mutants generated glycogen at 10.7 and 12.6 mg/l/day, respectively (Table 2). The high *Synechocystis* glycogen productivity at 17.8 mg/l/day was reported in the WT cultivated under the partial supply of phosphorus at 20% level of NORMAL [32]; while in the strain with the deleted apcE gene to improve photosynthetic efficiency, the productivity was 21.3 mg/l/day [38]. Thus, it is interesting to examine PHB and glycogen productivities in such previously reported metabolic-engineered strains of *Synechocystis* under -P for further improvement of the productivities of these two biopolymers. For *Synechocystis*, a successful 50-l cultivation has been demonstrated both using a bioreactor and outdoor cultivation [39].

The results suggested that such increased PHB and glycogen accumulations were caused by the conversion of photosynthetic proteins to the two biopolymers. Furthermore, enhancing a protein conversion process by metabolic engineering that targets protein degradation or key metabolic steps in PHB and glycogen syntheses [40] might help improving the accumulations of the two biopolymers. This cultivation could also be applied in other cyanobacteria accumulating the two biopolymers and producing high biomass levels such as *Arthrospira platensis* [41] and other novel strains isolated from nature [42], for producing PHB and glycogen.

CONCLUSION

This study showed that -P using the metabolically engineered mutants with the disrupted related biosynthesis simultaneously produced a substantial amount of biomass and significantly enhanced both the cellular

contents and the productivities of PHB or glycogen in *Synechocystis*. Increased PHB and glycogen accumulation using the partially supplied levels of phosphorus and nitrogen might further enhance the productivities of the two biopolymers of the mutants. This single-stage cultivation under -P provides a safe cost and time-saving for PHB and glycogen production rather than the use of a two-stage cultivation.

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