Cyanobacterium *Synechocystis* sp. PCC 6803 lacking *adc1* gene produces higher polyhydroxybutyrate accumulation under modified nutrients of acetate supplementation and nitrogen-phosphorus starvation

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ABSTRACT

Increased polyhydroxybutyrate production in cyanobacterium *Synechocystis* sp. PCC 6803 lacking *adc1* gene (*Δadc1*) is first-timely reported in this study. We constructed the mutant by disrupting *adc1* gene encoding arginine decarboxylase, thereby exhibiting a partial blockade of polyamine synthesis. This *Δadc1* mutant had a proliferative growth and certain contents of intracellular pigments including chlorophyll *a* and carotenoids as similar as those of wild type (WT). Highest PHB production was certainly induced by BG_{11}-N-P+A condition in both WT and *Δadc1* mutant of about 24.9 %w/DCW at day 9 and 36.1 %w/DCW at day 7 of adaptation time, respectively. Abundant PHB granules were also visualized under both BG_{11}-N-P and BG_{11}-N-P+A conditions. All *pha* transcript amounts of *Δadc1* mutant grown at 7 days-adaptation time were clearly upregulated corresponding to its PHB content under BG_{11}-N-P+A condition. Our finding indicated that this *adc1* perturbation is alternatively achieved for PHB production in *Synechocystis sp.* PCC 6803.

1. Introduction

Recently, bio-based and degradable bioplastics, in particular polyhydroxyalkanoates (PHAs) polymers, are better practically produced from prokaryotic organisms, such as well-known bacteria *Alcaligenes eutrophus, Ralstonia eutropha* [4, 21, 24] and cyanobacteria *Spirulina* sp., *Nostoc muscorum, Synechocystis* sp. PCC 6803 [1, 5, 32, 33, 46, 47]. The most common type of PHAs polymers in prokaryotic cells is polyhydroxybutyrate or PHB abundantly found as energy and carbon reserves, especially under starved condition [6]. Nowadays, three main strategies have been implemented to induce PHB production from prokaryotic organisms including nutrient modified medium, environmental stresses, and genetic engineering approaches. First strategy is generalized to accelerate the accumulation of energy reserves in forms of PHB granules inside living cells by generating deprived conditions of essential nutrients. Not only nutrient deficiencies of nitrogen (N) and/or phosphorus (P), but also carbon supplementation, such as acetate and glucose, could induce PHB accumulation in microorganisms via

different patterns upon protein synthesis suppression and expression [10, 13, 32, 37]. The certain increase of PHB accumulation was consequently induced when *Synechocystis* sp. PCC 6803 cells pre-grown in BG_{11} medium containing 0.1% glucose were further adapted in a modified BG_{11} medium lacking phosphorus (P) and adding 0.4 % acetate [32]. Recent reports revealed that carbon pool via glycogen production and catabolism, as well as sugar catabolism, had influenced on PHB production under nitrogen deprivation periods in *Synechocystis* sp. PCC 6803 [22, 27]. Secondly, the environmental stresses could induce PHB production in microorganisms by supporting bacterial viability under adverse environments, such as heat stress, oxidative stress, high salinity [2, 3, 7, 28]. Third strategy relates to genetic and/or metabolic engineering approach. In addition, the integrated strategies are also promising potential for improving PHB quality and productivity. The enhanced PHB accumulation was previously achieved by overexpressing native PHB biosynthetic genes, including *phaA* (encoding beta-ketothiolase), *phaB* (encoding acetoacetyl-CoA reductase), *phaEC* (encoding PHA synthase), in cyanobacterium *Synechocystis* sp.

PCC6803, in particular *phaAB*-overexpressing strain, by 2.6-fold increase of about 26% w/DCW PHB, and up to 35% PHB after adding 0.4% (w/v) acetate [20]. The metabolic engineering approach on competing metabolic pathways for enhancing acetyl-CoA supply to PHB production was employed in *Synechocystis* sp. PCC 6803 via the deletions of *pta* and *ach* genes, encoding phosphotransacetylase and acetyl-CoA hydrolase, respectively, which resulted to block the conversion of acetyl-CoA to acetate, and further combined with a heterologous expression of phosphoketolase encoded by *xfpk* from *Bifidobacterium breve* [8]. On the other hand, the metabolic influence from non-adjacent pathways also has impact on higher PHB production, such as a reduction of phosphate transport, a reduction of proline accumulation via nitrogen metabolism [19, 43]. Recently, the UV-randomly mutated *Synechocystis* sp. PCC 6714, a point mutation of phosphate-specific transport system integral membrane protein A (PstA) proved by genome sequencing, gave 2.5-fold higher PHB productivity than wild type by about 37 % w/DCW PHB under nitrogen and phosphorus starvation [19]. The transposon-mutated *Synechocystis* sp. PCC6803 strains with the disruptions of *sll0461*, encoding gamma-glutamyl phosphate reductase (ProA), and *sll0565* encoding a hypothetical protein, contained higher PHB accumulation [43]. Interestingly, it was noted that after proline reduction via the *sll0461* deletion would gain more glutamate production flowing to main TCA cycle, which not yet clear mechanism how to enhance PHB production. Since the certain knowledge has been known that proline and glutamate, as well as, polyamines tightly involve in stress response of living organisms via arginine utilization [15, 34], the knockout mutation of *adc1* gene encoding arginine decarboxylase in polyamine biosynthesis (*Δadc1* strain) was constructed in this study. Our result was first timely-evident finding that *Δadc1* cells favored highest PHB accumulation after applying nitrogen and phosphorus starvation plus acetate addition for 7 days.

2. Materials and methods

2.1. Construction of Synechocystis sp. PCC 6803 mutant lacking adc gene (Δadc1)

Cyanobacterium *Synechocystis* sp. PCC 6803 mutant lacking *adc1* gene (*Δadc1*) by knockout technique was constructed. To obtain this construct, the gene fragment of *adc1* gene of about 2.4 kb was amplified by PCR from genomic DNA of *Synechocystis* sp. PCC 6803. Primer sequences were designed by adding enzyme restriction sequences shown by underlined letters. Forward primer was designed for about 200-bp upstream of *adc1* gene (5′-GGAATTC<u>CATATG</u>CTCCTGCTGTCAACGGT TAA-3′) with added *Nde*I restriction enzyme sequences whereas reverse primer was about 200-bp downstream of this gene with added *Bam*HI sequences (5′-CG<u>GGATCC</u>GCATCAAGTTACTATCTGAG-3′). Next, this amplified gene fragment was cloned into pGem®-T easy vector (Promega Corporation). Then, a kanamycin resistant cassette (about 1.8 kb) was inserted to interrupt *adc1* gene which cut at one site by *Hin*dIII in that vector by blunt end ligation. After that, the obtained recombinant plasmid was transformed into *Synechocystis* sp. PCC 6803 wild type cells using natural transformation method [20]. *Synechocystis* host cells were freshy grown until their optical density (OD) at 730 nm of about 0.5. Fifty mL of cell culture was harvested and concentrated by centrifugation (2790 × *g*) and resuspended in 0.5 mL of new BG₁₁ medium. About 10 μg of plasmid DNA was added into that concentrated cell suspension, and further incubated under normal growth condition for 6 hours before spreading cell suspension on 0.45 μm sterile mixed cellulose esters (MCE) membrane (MF-Millipore, Merck) placed over a BG₁₁ agar plate. After 24 h incubation, that membrane filter was transferred to place on new BG₁₁ agar containing 20 μg/mL kanamycin. Colonies were grown from that selective antibiotic agar plate under normal growth condition within 3 weeks. To confirm complete segregation of *adc1* knockout strain (*Δadc1*), mutant cells were analyzed by PCR using forward and reverse primers of *adc1*.

2.2. Cell culture and modified nutrient conditions

Synechocystis sp. PCC 6803 cells, both wild type and mutant, were grown in BG₁₁ medium [35] until late-log phase of growth for about 16 days. Growth condition was performed on the rotary shaker under a continuous light (40-50 μE/m²/s) at 28-32 °C. Cell growth was determined spectrophotometrically by measuring OD at 730 nm. Those late-log growing cells (10 days) were applied to various treatments of modified nutrient conditions under the same growth condition for 11 days. There were three modified nutrient media including the deprivation of both nitrogen (N) and phosphorus (P) (BG₁₁-N-P), and the carbon supplementations of 0.4% (w/v) acetate (A) and 0.4% (w/v) glucose (G) into BG₁₁-N-P medium, represented as BG₁₁-N-P+A and BG₁₁-N-P+G, respectively. For BG₁₁-N-P medium, it was BG₁₁ medium lacking NaNO₃ in which ferric ammonium citrate was also replaced by equimolar concentrations of ferrous sulphate heptahydrate whereas phosphorus deprived condition was performed by replacing K₂HPO₄ with equimolar concentration of KCl.

2.3. Determinations of intracellular pigments

Intracellular chlorophyll *a* and carotenoid contents of *Synechocystis* sp. PCC 6803 cells were extracted by N,N-dimethylformamide (DMF) method [9, 16, 25]. Cell culture (1 mL) was harvested by centrifugation at 2790 × *g* at room temperature for 10 minutes. One mL of DMF was used to dissolve cell pellet fraction. After quick spinning, the DMF-extracted supernatant was spectrophotometically measured its absorbances at 461, 625, 664 nm, respectively, and subsequently calculated its intracellular contents according to equations [9, 25].

2.4. Determination of PHB granules by fluorescence microscopy

PHB granules in *Synechocystis* cells were stained by fluorescent dye Nile red, and monitored by fluorescence microscopy. One ml of *Synechocystis* cell culture was harvested by centrifugation at 2790 × *g*. Then, a small loop of cell pellets was resuspended into 3 μl of Nile red staining solution. Then, 0.9 % (w/v) normal saline (100 μL) was subsequently added, mixed and incubated overnight under darkness [20, 44]. To monitor the stained cells, fluorescent microscope equipped with a digital camera (Olympus DP72, Japan) was applied using a filter cup with 535 excitation wavelength, at magnification of 100X.

2.5. PHB extraction and HPLC analysis

In order to extract PHB from *Synechocystis* cells, cultured cells (about 50 mL) were harvested by centrifugation at 6000 × *g* for 10 minutes. Cell pellets were boiled at 100 °C for 60 minutes with 800 μL of concentrated sulfuric acid, and 200 μL of 20 mg/mL adipic acid (an internal standard). This hydrolysis of PHB polymer generated crotonic acid monomers which were detected by high pressure liquid chromatography (HPLC) instrument [44]. Boiled samples were filtered by 0.45 μm polypropylene membrane filter before detecting PHB content by HPLC instrument (Shimadzu HPLC LGE System, Japan) using C18 column, Inert Sustain 3-μm (GL Sciences, Japan). The flow rate was 1.0 ml per minute with UV detector set at 210 nm.. The running buffers were 30% (v/v) acetonitrile and 70% (v/v) of 10 mM KH₂PO₄ (pH 2.3). Authentic commercial PHB (Sigma) was used as standard which prepared as similar as cell sample. The yield of crotonic acid after hydrolysis detected by HPLC was used to generate PHB standard curve and estimated PHB content in cell sample. The unit of PHB content was % PHB weight per dry cell weight (% w/DCW). The dry cell weight was measured after drying harvested cell pellets in 80 °C oven until a constant weight was obtained [20].

2.6. Determination of relative transcript level

Synechocystis cell culture (50 – 100 mL) was harvested by

centrifugation at 12,000 *xg* for 5 minutes and discarded supernatant. Cell pellets were extracted for total RNA by TRIzol Reagent® (Invitrogen). The cDNAs were synthesized from 5 μg of total RNAs later by using SuperScript® III First Strand Synthesis Kit (Invitrogen). After that, these synthesized cDNAs were used as the template for RT-PCR amplification using many specific primers listed in Table 1. The PCR condition for all *pha* genes consisted of 95 °C for 30 seconds, followed by 31 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 35 seconds, and the a final extension at 72 °C for 5 minutes. For *16S* rRNA as a reference, the PCR condition consisted of 95 °C for 30 seconds, followed by 19 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 35 seconds, and the a final extension at 72 °C for 5 minutes. The PCR products were checked by 1.5% (w/v) agarose gel electrophoresis using in 0.5xTAE buffer.

2.7. Results and discussion

The *adc1* gene was inactivated from the *Synechocystis* sp. PCC 6803 wild type, namely Δ*adc1* mutant which contained *adc1*-km^r locus (Fig. 1). Clearly, growth of Δ*adc1* mutant under normal growth condition was similar to that of wild type, except slightly higher during 14 - 16 days of cultivation (Fig. 2A). In general, cell growth at stationary phase was influenced by many substantial factors which directly related to nutrient-depleted medium and changed environment. The changed pH of medium was one of those important factors which impacted on cell growth in longer time period [18]. On the other hand, the similar accumulation of chlorophyll *a* was noted in both WT and mutant strains (Fig. 2B). For carotenoid content, the mutant strain contained significantly higher content than that in WT at 15 days of cultivation (Fig. 2C). These results indicate that the high polyamine accumulation was unnecessary under normal growth condition of *Synechocystis* sp. PCC 6803 as evidently supported by a slight amount of polyamines remained in Δ*adc1* mutant (data not shown). The induced polyamine content was responsive to environmental stresses, especially ionic and osmotic stresses, in *Synechocystis* cells [16]. The regulatory response of *adc2* gene in *Arabidopsis* plant was mainly induced rather than *adc1* by salt stress leading to increase putrescine accumulation [45] whereas the *adc* genes in *Synechocystis* sp. PCC 6803 including *adc1* and *adc2* had differential responses to stresses [17]. Accordingly, we suggested that the *adc1* disruption of *Synechocystis* cells in this study did not inhibit growth and their intracellular pigment accumulations under normal growth condition since their *adc2* gene had certainly functioned.

Recent reports revealed that the maximum PHB production in *Synechocystis* cells was started at the late-log and stationary phases of growth [20, 32]. In this study, we then adapted the harvested

Table 1
PCR primers for RT-PCR.

| Target gene	Name	Oligo sequences	Amplified fragment length (bp)
phaA	*phaAF*	5'-CATGATGGTTTGACGGACAG-3'	310
	phaAR	5'-GACTACAGTTGCCCGCTGTT-3'	
phaB	*phaBF*	5'-ATGCCGGTATCACCAAAGAC-3'	390
	phaBR	5'-CAATTTCCTCCGGTTTACCA- 3'	
phaC	*phaCF*	5'-GGGCACATTTAGCCTGTGTT-3'	346
	phaCR	5'-GTAAGTTTCCCCCGCTTGAT- 3'	
phaE	*phaEF*	5'-GAGCAATATACCGCCACCAC-3'	371
	phaER	5'-TCTTCCATCAAAGCAGCAAA-3'	
16S rRNA	*16F*	5'-AGTTCTGACGGTACCTGATGA-3'	521
	16R	5'-GTCAAGCCTTGGTAAGGTTCT-3'	
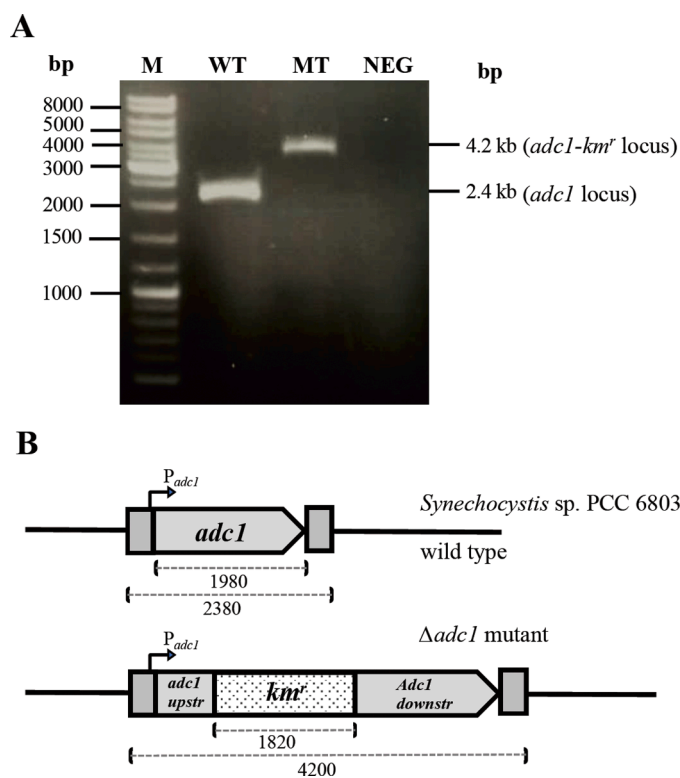
Fig. 1. Inactivation of *adc1* gene. (**A**)PCR analysis of the *adc1* gene in the expected mutant in which a fragment of *adc1* gene was interrupted and replaced with a kanamycin resistant cassette (km^r). Lane M: DNA marker, WT: wild type, MT: Δ*adc1* mutant, NEG: negative control without genomic DNA of *Synechocystis* 6803. (**B**) depiction of the recombinant plasmid used to generate Δ*adc1* mutant strain. The plasmid carries about 4.2 kb fragment of *adc1* gene plus inserted km^r cassette.

Synechocystis cells at late-log phase of growth (10 days of cultivation) into various modified media including BG$_{11}$-N-P, BG$_{11}$-N-P+A, and BG$_{11}$-N-P+G for 11 days (Fig. 3). Unexpectedly, after transferring the mutant cells which were grown till late-log phase of cell growth into new normal BG$_{11}$ medium, their growth recovery was notably less active, as evidently shown in stable level after 7 days of adaptation time when compared with WT (Fig. 3A and B). Our results indicated that growth of both WT and mutant strains was dramatically decreased under BG$_{11}$ medium lacking N and P nutrients (Fig. 3A and B). The supplementation of acetate could slightly increase cell growth of Δ*adc1* mutant when compared to those cells adapted in BG$_{11}$-N-P and BG$_{11}$-N-P+G (Fig. 3B). Glucose addition into BG$_{11}$-N-P medium did not abolish cell growth but severely inhibited chlorophyll *a* and carotenoid accumulations within 7 days of adaptation time (Fig. 3C-F). These results suggested that *Synechocystis* cells would assimilate glucose as carbon source for maintaining cell growth with less photosynthesis, as evident by lower contents of intracellular pigments. On the other hand, we also found that the acetate addition could significantly induce the accumulations of chlorophyll *a* and carotenoids, in particular in Δ*adc1* mutant, when compared with those under BG$_{11}$-N-P and BG$_{11}$-N-P+G (Fig. 3D and F). The supplemented acetate under heterotrophic growth was thoughtfully assimilated by *Synechocystis* cells via acetyl-CoA-derived metabolic intermediate which located in bottleneck flowing to many crucial pathways including Krebs' cycle, PHB, glycogen and fatty acid syntheses [8, 20, 41, 42]. In *Synechocystis*, two mechanism models were considerably proposed for driving the conversion of acetate into acetyl-CoA via either a catalysis of ATP-driven acetyl-CoA synthetase (ACS encoded by *sll0542* gene), or catalyzed reactions of acetate kinase (ACK encoded by *sll1299* gene) and phosphotransacetylase (PTA encoded by *slr2132*) [39, 41]. We demonstrated the PHB production in *Synechocystis* cells adapted
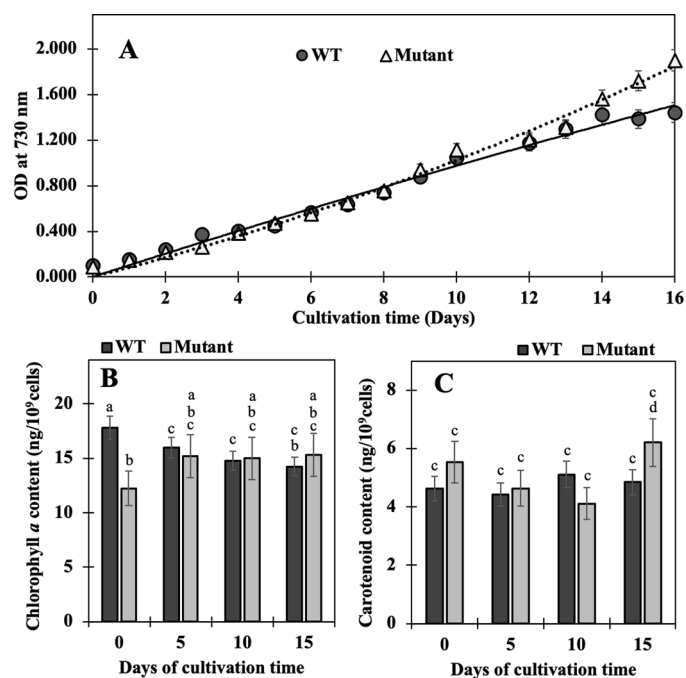
Fig. 2. Growth (**A**) and pigment contents including chlorophyll *a* (**B**) and carotenoids (**C**) of *Synechocystis* sp. PCC 6803 wild-type (WT) and Δ*adc1* mutant (Mutant) cells under normal condition for 16 days. Cells were grown in BG$_{11}$ medium under normal growth condition. The error bars represent standard deviations of means (mean ± S.D., n = 6). In **B** and **C**, means with the same letter have nonsignificant differences at a significance level of *P* < 0.05.

in different modified media (Fig. 4). In *Synechocystis* WT cells, it was found that higher PHB accumulation was significantly induced by all modified media at day 7 of adaptation time (Fig. 4A) whereas the highest increase pointed to PHB contents of about 24.9 %w/DCW at day 9 of adaptation time under BG$_{11}$-N-P+A condition, and stayed constant till day 11 of adaptation time. On the other hand, a sharp increase was shown by about 36.1 %w/DCW in Δ*adc1* mutant at day 7 of adaptation time under BG$_{11}$-N-P+A condition (Fig. 4B) following by that in BG$_{11}$-N-P condition of about 19.8 %w PHB/DCW. These produced PHB inductions occurred faster in Δ*adc1* mutant when compared with WT. It is worth to note that the certain PHB decrease of Δ*adc1* mutant after day 7 of adaptation time occurred in cells which had slight increases of its growth and intracellular pigments at the same time under BG$_{11}$-N-P+A condition. It ruminatively suggested that this cellular condition would result a lack of fatal situation to enhance PHB when compared to WT. Moreover, the substantial decrease of PHB could represent a stored carbon mobilization for survival under scarce conditions, such as carbon limitation, thermal or oxidative stresses [26]. We also showed the Nile-Red stained PHB granules in *Synechocystis* Δ*adc1* mutant (Fig. 5). Abundant PHB granules were apparently found under BG$_{11}$-N-P and BG$_{11}$-N-P+A conditions for 7 days in Δ*adc1* mutant when compared to those in WT (Fig. 5B, C, F and G). According to modified medium, the nutrient deficiency of either nitrogen or phosphorus, herein or both, enabled to efficiently induce higher accumulation of PHB granules in *Synechocystis* sp. PCC 6803, and those granules were further increased by supplying more carbon source of acetate (0.4 %w/v), as recently reported in wild type [32] and engineered strains (Table 2). Recently, large PHB granules were noted by about 81 %w/DCW PHB under nutrient limiting conditions in a metabolically engineered Δ*pirC-REphaAB Synechocystis* sp. PCC6803 strain, with heterologous overexpression of *Cupriavidus necator phaAB* and a disruption of *pirC* gene encoding phosphoglycerate mutase [23]. The *pirC* deletion resulted in certain increase of glycogen degradation and lower glycolysis under nitrogen starvation in which PHB accumulation was augmented [23,
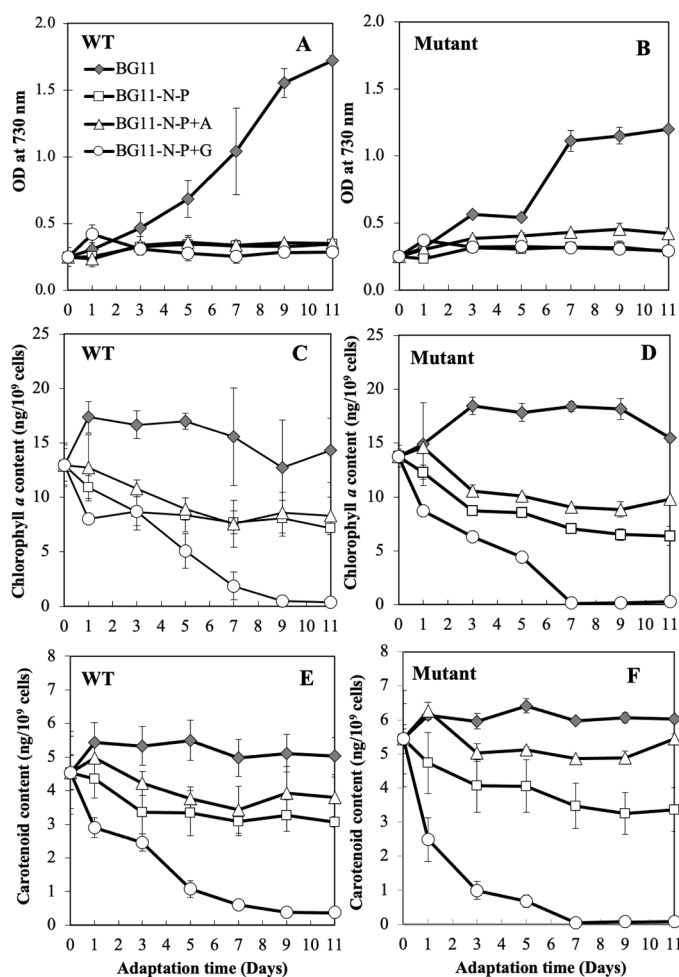
Fig. 3. Growth (**A**) and pigment contents including chlorophyll *a* (**B**) and carotenoids (**C**) of *Synechocystis* sp. PCC 6803 wild-type (WT) and Δ*adc1* mutant (Mutant) cells under adaptation period in various nutrient conditions for 11 days. Cells were grown in various nutrient conditions including normal BG$_{11}$ medium as control, BG$_{11}$ without nitrogen and phosphorus nutrients (or BG$_{11}$-N-P), BG$_{11}$-N-P medium supplemented by 0.4% (w/v) acetate (or BG$_{11}$-N-P+A), BG$_{11}$-N-P medium supplemented by 0.4% (w/v) glucose (or BG$_{11}$-N-P+G). The error bars represent standard deviations of means (mean ± S.D., n = 3).

29]. On the other hand, we considered that the genetic engineering on any transcription factors may give less impact to induce PHB production whereas the metabolic engineering of competing/neighboring pathways would gain more promising for increasing PHB (Table 2), such as *agp* knockout [48], and *Xfpk* expresstion with double *pta_ach* deletion [8]. Importantly, our finding demonstrated that the polyamine synthesis disturbance dramatically induced higher PHB production in *Synechocystis* sp. PCC 6803 up to 36.1% w/DCW under modified nutrient condition. In general, the polyamine pathway in cyanobacteria relates to arginine catabolism, one of amino acids utilized as a source of nitrogen for cell growth [34]. Some arginine amino acids could be converted to polyamines by arginine decarboxylase (encoded by *adc* genes) whereas the main direction of arginine mostly flows to TCA cycle via proline-glutamate pathway [11, 15]. Altogether, we proposed that the *adc1* mutation with trace polyamines (data not shown) might enhance a fast flow of more arginine to TCA cycle, thereby increasing energy metabolism for growth and generating carbon pool, as evident by the higher growth of Δ*adc1* mutant (Fig. 2). Excess acetyl-CoA or carbon pool might then contribute to PHB accumulation, instead of TCA cycle direction, which efficiently induced by our modified nutrient conditions used in this study. On the other hand, as previously noted, Tyo et al. [43] discussed that *Synechocystis* 6803 Δ*sll0461* (or *proA*) strain which
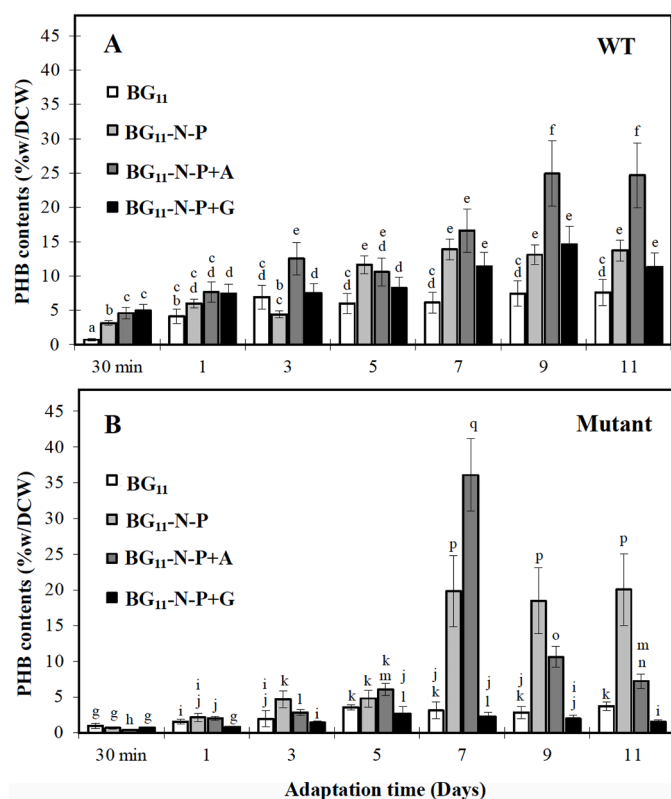
Fig. 4. Polyhydroxybutyrate (PHB) contents of *Synechocystis* sp. PCC 6803 (**A**) wild-type (WT) and (**B**) *Δadc1* mutant (Mutant) cells under adaptation period in various nutrient conditions for 11 days. PHB content (%w/dry cell weight (DCW)) was measured from cells grown in various nutrient conditions including normal BG_{11} medium as control, BG_{11} without nitrogen and phosphorus nutrients (or BG_{11}-N-P), BG_{11}-N-P medium supplemented by 0.4% (w/v) acetate (or BG_{11}-N-P+A), BG_{11}-N-P medium supplemented by 0.4% (w/v) glucose (or BG_{11}-N-P+G). The error bars represent standard deviations of means (mean ± S.D., n = 3). Means with the same letter have nonsignificant differences at a significance level of $P < 0.05$.

represented lower proline was attacked by other stresses which subsequently induced higher PHB accumulation. Rationally, we would then speculate that not only other stresses attacking when cells have less polyamines but also the increase flow of amino acids, herein arginine, to energy metabolism, which favor PHB production in *Synechocystis* sp.

PCC 6803, particularly under stressed condition. For prospective direction, additional experiments on specific gene manipulations related to arginine catabolism and neighboring pathways, as well as up-to-date technology of omics analyses, might verify and gain more understanding whether polyamine pathway disturbance literally influences on PHB production via the increase of the acetyl-CoA pool.

We also demonstrated the changed transcript levels of each gene in PHB biosynthetic pathway under all conditions at day 7 of adaptation time (Fig. 6). Up to date, it is known that there are a cluster of two open reading frames for *phaA* and *phaB* [40], and *phaE* and *phaC* which both responsible to a polyhydroxyalkanoic acid (PHA) synthase [12]. We showed that *Synechocystis* WT had high transcript amounts of *phaA*, *phaB* and *phaC* whereas *phaE* transcript level was lowest under normal growth condition (BG_{11}) (Fig. 6A and B). In previous work, the double overexpression of *phaAB* in *Synechocystis* 6803 had influenced on a certain increase of PHB production [20]. Intriguingly, the *Δadc1* mutant contained higher transcript level of all *pha* genes, except *phaC*, when compared to those of WT (Fig. 6). Modified medium of BG_{11}-N-P+A could dramatically induce all *pha* genes of *Δadc1* mutant among other conditions corresponding to PHB production (Figs. 4 and 5). However, we found that WT cells under BG_{11}-N-P+G condition contained lower transcript levels of *phaA* and *phaB* that did not show a tight connection with PHB content whereas the transcript amounts of both *phaC* and *phaE* did (Fig. 6). Our results indicated that gene expressions of *phaA* and *phaB* mainly occurred in similar tendency whereas the changed transcript proportion of *phaC* and *phaE* was alternately regulated once *phaC* transcript amount was apparently decreased.

3. Conclusion

Synechocystis sp. PCC 6803 lacking *adc1* gene (*Δadc1*) had the highest capacity to synthesize PHB under nutrient modified media. This *Δadc1* mutant could grow as similar as *Synechocystis* wild type. Its highly accumulated PHB occurred up to 36.1 %w/DCW after adaptation in a nitrogen and phosphorus-deprived BG_{11} medium containing 0.4 %(w/v) acetate for 7 days. To understand the actual connection between lower polyamine synthesis and high PHB production in cyanobacteria, further specific gene manipulations on arginine catabolism and neighboring pathways, as well as omics analysis, would be promising in bioenergy and biomaterial fields of Biotechnology. Last but not least, the metabolic engineering approach and product recovery system for preventing intracellular PHB degradation in cyanobacterial cells would draw a spotlight guidance for sustained PHB production from algal resource in practical application.
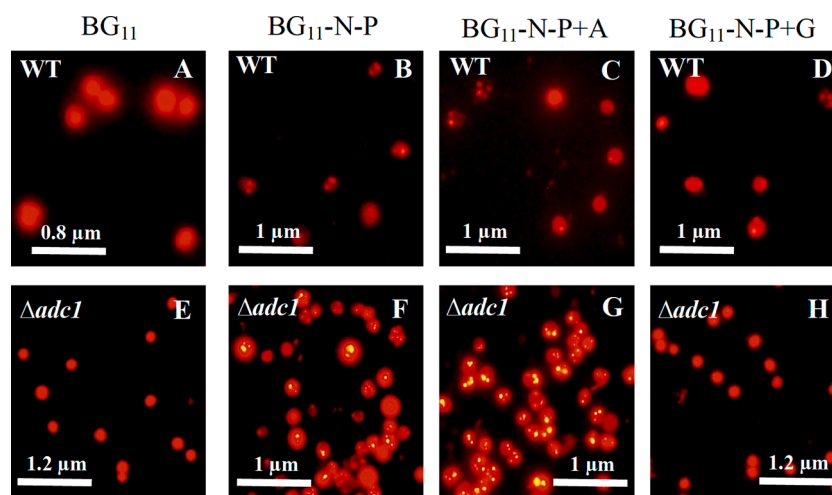
Fig. 5. Images of *Synechocystis* 6803 wild type (**A-D**) and *Δadc1* mutant (**E-H**) adapted for 7 days under various nutrient conditions. Images are from fluorescence microscopy showing PHB granules as bright gold particles with 100x magnification.

Table 2

PHB production in engineered *Synechocystis* sp. PCC6803 (or S6803) strains which directly and indirectly related to its synthetic pathway.

Genetic engineering approach	PHB-producing condition(s)	PHB content	References
Engineered S6803 strain directly relating to PHB synthetic pathway			
Heterologous expression of a gene encoding PHA synthase from *Alcaligenes eutrophus*	BG$_{11}$ containing deprived-N and supplemented acetate, phototrophic growth for 14 days	11 %w/DCW	Sudesh et al. [38]
Heterologous expression of PHA biosynthetic operon from *Microcystis aeruginosa* NIES-843	BG$_{11}$ containing deprived-N, phototrophic growth for 8 days	7 %w/DCW	Hondo et al. [14]
The native overexpression of *phaAB*	BG$_{11}$ containing deprived-N and supplemented acetate, phototrophic growth for 9 days	35 %w/DCW	Khetkorn et al. [20]
The overexpression of *Cupriavidus necator phaAB* combined with *PirC* disruption	BG$_{11}$ containing deprived-N-P and supplemented acetate, phototrophic growth for 20 days	81 %w/DCW	Koch et al. [23]
Engineered S6803 strain indirectly relating to PHB synthetic pathway			
The overexpression of native *SigE* gene encoding RNA polymerase sigma factor	BG$_{11}$ containing deprived-N, phototrophic growth for 9 days	1.4 %w/DCW	Osanai et al. [30]
The overexpression of native *rre37* gene encoding OmpR-type response regulator	BG$_{11}$ containing deprived-N, phototrophic growth	1.2 %w/DCW	Osanai et al. [31]
The knockout of *sll0783* gene encoding transcription factor NtcA	BG$_{11}$ containing deprived-N, phototrophic growth for 120h	> 1 %w/DCW	Schlebusch and Forchhammer [36]
The knockout of *agp* gene encoding ADP-glucose pyrophosphorylase (related to glycogen synthesis)	BG$_{11}$ containing deprived-N, phototrophic growth for 120 hours	18.6 %w/DCW	Wu et al. [48]
Inverse metabolic engineering; transposon random mutation library; mutagenized strains at *sll0461*, and *sll0565* genes	BG$_{11}$ containing 10% phosphate, phototrophic growth for 14 days	9 %w/DCW (Δ*sll0461*), 10 %w/DCW (Δ*sll0565*)	Tyo et al. [43]
the expression of a heterologous phosphoketolase (*XfpK*) from *Bifidobacterium breve* in a double *pta* and *ach* deletion background	Photoautotrophic growth with CO$_2$ bubbling in medium deprived-N-P, for about 32 days	~12 %w/DCW	Carpine et al. [8]
The knockout of *adc1* gene encoding arginine decarboxylase related to polyamine synthesis	BG$_{11}$ containing deprived-N-P and supplemented acetate, phototrophic growth for 7 days	36.1 %w/DCW	This study

Authors' contribution

Suthira Utharn: study conception, main experimenter, data collection, analysis. **Panutda Yodsang:** experimenter (mutant construction). **Aran Incharoensakdi:** study conception, research discussion. **Saowarath Jantaro:** Funding acquisition, project administration, conceptualization, methodology, supervision, validation, writing – review and editing.
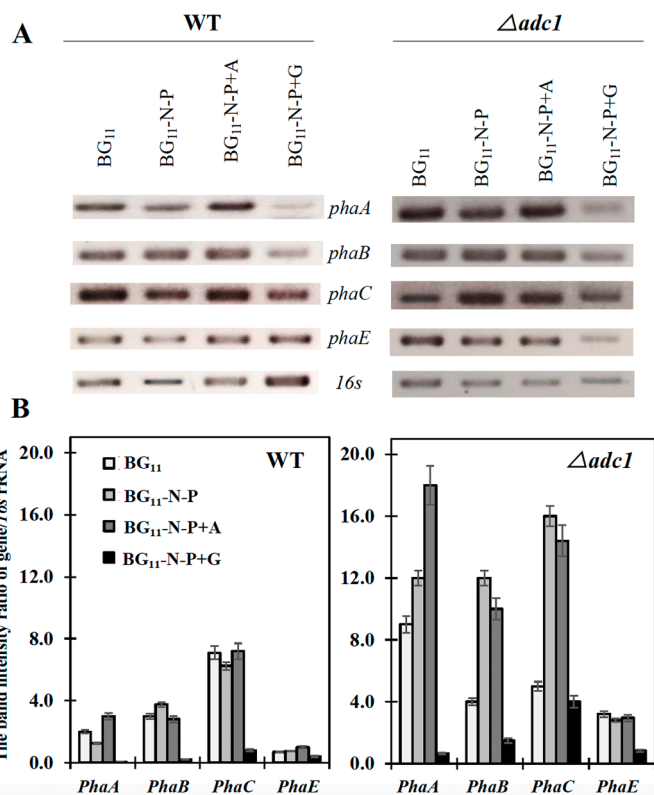
Fig. 6. Relative transcript levels (**A**) of *phaA, phaB, phaC* and *phaE* performed by RT-PCR and the band intensity ratio of each gene/*16s* rRNA (**B**) in WT and Δ*adc1* mutant strains grown under various nutrient conditions for 7 days. The *16S* rRNA was used as reference control.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

References

[1] R.M. Abed, S. Dobretsov, K. Sudesh, Applications of cyanobacteria in biotechnology, J. Appl. Microbiol. 106 (2009) 1–12, https://doi.org/10.1111/j.1365-2672.2008.03918.x.

[2] I.S. Al Rowaihi, A. Paillier, S. Rasul, R. Karan, S.W. Grötzinger, K. Takanabe, J. Eppinger, Poly(3-hydroxybutyrate) production in an integrated electromicrobial setup: investigation under stress-inducing conditions, PLoS ONE 13 (2018), e0196079, https://doi.org/10.1371/journal.pone.0196079.

[3] L.P.S. Alves, A.P. Santana-Filho, G.L. Sassaki, F.O. de Oliveira Pedrosa, E.M. de Souza, L.S. Chubatsu, M. Müller-Santos, 3-Hydroxybutyrate derived from poly-3 hydroxybutyrate (PHB) mobilization alleviates protein aggregation in heat-stressed *Herbaspirillum seropedicae* SmR1, Appl. Environ. Microbiol. 86 (2020), https://doi.org/10.1128/AEM.01265-20 e01265-20.

[4] A.J. Anderson, E.A. Dawes, Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates, Microbiol. Rev. 54 (1990) 450–472. PMID: 2087222.

[5] S. Ansari, T. Fatma, Cyanobacterial polyhydroxybutyrate (PHB): screening, optimization and characterization, PLoS One 11 (2016), e0158168, https://doi.org/10.1371/journal.pone.0158168.

[6] S. Balaji, K. Gopi, B. Muthuvelan, A review on production of poly β hydroxybutyrates from cyanobacteria for the production of bio plastics, Algal Res. 2 (2013) 278–285, https://doi.org/10.1016/j.algal.2013.03.002.

[7] M.B. Batista, C.S. Teixeira, M.Z.T. Sfeir, L.P.S. Alves, G. Valdameri, F. de Oliveira Pedrosa, G.L. Sassaki, M.B.R. Steffens, E.M. de Souza, R. Dixon, M. Müller-Santos, PHB biosynthesis counteracts redox stress in *Herbaspirillum seropedicae*, Front. Microbiol. 9 (2018) 472, https://doi.org/10.3389/fmicb.2018.00472.

[8] R. Carpine, W. Du, G. Olivieri, A. Pollio, K.J. Hellingwerf, A. Marzocchella, F. Branco dos Santos, Genetic engineering of *Synechocystis* sp. PCC6803 for poly-β-hydroxybutyrate overproduction, Agal Res. 25 (2017) 117–127, https://doi.org/10.1016/j.algal.2017.05.013.

[9] D. Chamovitz, G. Sandmann, J. Hirschberg, Molecular and biochemical-characterization of herbicide-resistant mutants of cyanobacteria reveals that phytoene desaturation is a rate-limiting step in carotenoid biosynthesis, J. Biol. Chem. 268 (1993) 17348–17353. PMID: 8349618.

[10] V. Dutt, S. Srivastava, Novel quantitative insights into carbon sources for synthesis of poly hydroxybutyrate in *Synechocystis* PCC 6803, Photosynth Res 136 (2018) 303–314, https://doi.org/10.1007/s11120-017-0464-x.

[11] E Flores, A. Herrero, Assimilatory nitrogen metabolism and its regulation, in: D. A. Bryant (Ed.), The Molecular Biology of Cyanobacteria, Kluwer Academic Publishers, Dordrecht, The Netherlands, 1994, pp. 487–517.

[12] S. Hein, H. Tran, A. Steinbüchel, *Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria, Arch. Microbiol. 170 (1998) 162–170, https://doi.org/10.1007/s002030050629.

[13] K. Hirai, M. Nojo, Y. Sato, M. Tsuzuki, N. Sato, Contribution of protein synthesis depression to poly-β-hydroxybutyrate accumulation in *Synechocystis* Sp. PCC 6803 under nutrient-starved conditions, Sci. Rep. 9 (2019) 19944, https://doi.org/10.1038/s41598-019-56520-w.

[14] S. Hondo, M. Takahashi, T. Osanai, M. Matsuda, T. Hasunuma, A. Tazuke, Y. Nakahira, S. Chohnan, M. Hasegawa, M. Asayama, Genetic engineering and metabolite profiling for overproduction of polyhydroxybutyrate in cyanobacteria, J. Biosci. Bioeng. 120 (2015) 510–517, https://doi.org/10.1016/j.jbiosc.2015.03.004.

[15] S. Jantaro, S. Kanwal, Low molecular weight nitrogenous compounds (GABA and polyamines) in blue green algae, in: R.P. Rastogi, D. Madamwar, A. Pandey (Eds.), Algal green chemistry: recent progress in biotechnology, Elsevier B.V., 2017, pp. 149–169, https://doi.org/10.1016/B978-0-444-63784-0.00008-4.

[16] S. Jantaro, P. Maenpaa, P. Mulo, A. Incharoensakdi, Content and biosynthesis of polyamines in salt and osmotically stressed cells of *Synechocystis* sp. PCC 6803, FEMS Microbiol. Lett. 228 (2003) 129–135, https://doi.org/10.1016/S0378-1097(03)00747-X.

[17] S. Jantaro, A. Pothipongsa, S. Khanthasuwan, A. Incharoensakdi, Short-term UV-B and UV-C radiations preferentially decrease spermidine contents and arginine decarboxylase transcript levels of *Synechocystis* sp. PCC 6803, Curr. Microbiol. 62 (2011) 420–426, https://doi.org/10.1007/s00284-010-9724-0.

[18] H.-B. Jiang, H.-M. Cheng, K. Gao, B.-S. Qiu, Inactivation of Ca^{2+}/H^+ exchanger in *Synechocystis* sp. strain PCC 6803 promotes cyanobacterial calcification by upregulating CO_2-concentrating mechanisms, Appl. Environ. Microbiol. 79 (2013) 4048–4055, https://doi.org/10.1128/AEM.00681-13.

[19] D. Kamravamanesh, T. Kovacs, S. Stefan Pflügl, I. Druzhininab, P. Kroll, M. Maximilian Lackner, C. Herwig, Increased poly-β-hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain *Synechocystis* sp. PCC 6714: Mutant generation and characterization, Bioresour. Technol. 266 (2018) 34–44, https://doi.org/10.1016/j.biortech.2018.06.057.

[20] W. Khetkorn, A. Incharoensakdi, P. Lindblad, S. Jantaro, Enhancement of poly-3-hydroxybutyrate production in *Synechocystis* sp. PCC 6803 by overexpression of its native biosynthetic genes, Bioresour. Technol. 214 (2016) 761–768, https://doi.org/10.1016/j.biortech.2016.05.014.

[21] T. Kichise, T. Fukui, Y. Yoshida, Y. Doi, Biosynthesis of polyhydroxyalkanoates (PHA) by recombinant *Ralstonia eutropha* and effects of PHA synthase activity on in vivo PHA biosynthesis, Int. J. Biol. Macromol. 25 (1999) 69–77, https://doi.org/10.1016/S0141-8130(99)00017-3.

[22] M. Koch, S. Doello, K. Gutekunst, K. Forchhammer, PHB is produced from glycogen turn-over during nitrogen starvation in *Synechocystis* sp. PCC 6803, Int. J. Mol. Sci. 20 (2019) 1942, https://doi.org/10.3390/ijms20081942.

[23] M. Koch, J. Bruckmoser, J. Scholl, W. Hauf, B. Reiger, K. Forchhammer, Maximizing PHB content in *Synechocystis* sp. PCC 6803: a new metabolic engineering strategy based on the regulator PirC, Microb. Cell Fact. 19 (2020) 231, https://doi.org/10.1186/s12934-020-01491-1.

[24] M. Liebergesell, K. Sonomoto, M. Madkour, F. Mayer, A. Steinbuchel, Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules, Eur. J. Biochem. 226 (1994) 71–80, https://doi.org/10.1111/j.1432-1033.1994.tb20027.x.

[25] R. Moran, Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide, Plant Physiol. 69 (1982) 1376–1381, https://doi.org/10.1104/pp.69.6.1376.

[26] M. Müller-Santos, J.J. Koskimäki, L.P.S. Alves, E.M. de Souza, D. Jendrossek, A. M. Pirttilä, The protective role of PHB and its degradation products against stress situations in bacteria, FEMS Microbiol. Rev. 45 (2021) 1–13, https://doi.org/10.1093/femsre/fuaa058.

[27] Y. Nakaya, H. Iijima, J. Takanobu, A. Watanabe, M.Y. Hirai, T. Osanai, One day of nitrogen starvation reveals the effect of *sigE* and *rre37* overexpression on the

[28] expression of genes related to carbon and nitrogen metabolism in *Synechocystis* sp. PCC 6803, J. Biosci. Bioeng. 120 (2015) 128–134, https://doi.org/10.1016/j.jbiosc.2014.12.020.

[28] S. Obruca, P. Sedlacek, M. Koller, D. Kucera, I. Pernicova, Involvement of polyhydroxyalkanoates in stress resistance of microbial cells: Biotechnological consequences and applications, Biotechnol. Adv. 36 (2018) 856–870, https://doi.org/10.1016/j.biotechadv.2017.12.006.

[29] Orthwein, T., Scholl, J., Spät, P., Lucius, S., Koch, M., Macek, B., Hagemann, M., Forchhammer, K., 2020. The novel PII-interacting regulator PirC (Sll0944) identifies 3-phosphoglycerate mutase (PGAM) as central control point of carbon storage metabolism in cyanobacteria. bioRxiv. https://doi.org/10.1101/2020.09.11.292599.

[30] T. Osanai, K. Numata, A. Oikawa, A. Kuwahara, H. Iijima, Y. Doi, K. Tanaka, K. Saito, M.Y. Hirai, Increased bioplastic production with an RNA polymerase sigma factor SigE during nitrogen starvation in *Synechocystis* sp. PCC 6803, DNA Res. 20 (2013) 525–535, https://doi.org/10.1093/dnares/dst028.

[31] T. Osanai, A. Oikawa, K. Numata, A. Kuwahara, H. Iijima, Y. Doi, K. Saito, M. Y. Hirai, Pathway-level acceleration of glycogen catabolism by a response regulator in the cyanobacterium *Synechocystis* species PCC 6803, Plant Physiol. 164 (2014) 1831–1841, https://doi.org/10.1104/pp.113.232025.

[32] B. Panda, P. Jain, L. Sharma, N. Mallick, Optimization of cultural and nutritional conditions for accumulation of poly-beta-hydroxybutyrate in *Synechocystis* sp. PCC 6803, Bioresour. Technol. 97 (2006) 1296–1301, https://doi.org/10.1016/j.biortech.2005.05.013.

[33] B. Panda, N. Mallick, Enhanced poly-beta-hydroxybutyrate accumulation in a unicellular cyanobacterium *Synechocystis* sp. PCC 6803, Lett. Appl. Microbiol. 44 (2007) 194–198, https://doi.org/10.1111/j.1472-765X.2006.02048.x.

[34] M.J. Quintero, A.M. Muro-Pastor, A. Herrero, E. Flores, Arginine catabolism in the cyanobacterium *Synechocystis* sp. strain PCC 6803 involves the urea cycle and arginase pathway, J. Bacteriol. 182 (2000) 1008–1015, https://doi.org/10.1128/jb.182.4.1008-1015.2000.

[35] R. Rippka, J. Deruelles, J.B. Waterbury, M. Herdman, R.Y. Stanier, Generic assignments, strain histories and properties of pure cultures of cyanobacteria, J. Gen. Microbiol. 111 (1979) 1–61, https://doi.org/10.1099/00221287-111-1-1.

[36] M. Schlebusch, K. Forchhammer, Requirement of the nitrogen starvation induced protein *Sll0783* for polyhydroxybutyrate accumulation in *Synechocystis* sp. strain PCC 6803, Appl. Environ. Microbiol. 76 (2010) 6101–6107, https://doi.org/10.1128/AEM.00484-10.

[37] L. Sharma, N. Mallick, Accumulation of poly-beta-hydroxybutyrate in *Nostoc Muscorum*: regulation by pH, light-dark cycles, N and P status and carbon sources, Bioresour. Technol. 96 (2005) 1304–1310, https://doi.org/10.1016/j.biortech.2004.10.009.

[38] K. Sudesh, K. Taguchi, Y. Doi, Effect of increased PHA synthetase activity on polyhydroxyalkanoates biosynthesis in *Synechocystis* sp. PCC 6803, Int. J. Biol. Macromol. 30 (2002) 97–104, https://doi.org/10.1016/s0141-8130(02)00010-7.

[39] M.L. Summers, M.C. Denton, T.R. McDermott, Genes coding for phosphotransacetylase and acetate kinase in *Sinorhizobium meliloti* are in an operon that is inducible by phosphate stress and controlled by PhoB, J. Bacteriol. 181 (1999) 2217–2224, https://doi.org/10.1128/JB.181.7.2217-2224.1999.

[40] G. Taroncher-Oldenburg, K. Nishina, G. Staphanopoulos, Identification and analysis of the polyhydroxyalkanoate-specific β-ketothiolase and acetoacetyl coenzyme A reductase genes in the cyanobacterium *Synechocystis* sp. strain PCC6803, Appl. Environ. Microbiol. 66 (2000) 4440–4448, https://doi.org/10.1128/aem.66.10.4440-4448.2000.

[41] K. Thiel, E. Vuorio, E.-M. Aro, P.T. Kallio, The effect of enhanced acetate influx on *Synechocystis* sp. PCC 6803 metabolism, Microb. Cell Fact. 16 (2017) 21, https://doi.org/10.1186/s12934-017-0640-x.

[42] U. Towijit, N. Songruk, P. Lindblad, A. Incharoensakdi, S. Jantaro, Co-overexpression of native phospholipid-biosynthetic genes *plsX* and *plsC* enhances lipid production in *Synechocystis* sp. PCC 6803, Sci. Rep. 8 (2018) 13510, https://doi.org/10.1038/s41598-018-31789-5.

[43] K.E. Tyo, Y.S. Jin, F.A. Espinoza, G. Stephanopoulos, Identification of gene disruptions for increased poly-3-hydroxybutyrate accumulation in *Synechocystis* PCC 6803, Biotechnol. Prog. 25 (2009) 1236–1243, https://doi.org/10.1002/btpr.228.

[44] K.E. Tyo, H. Zhou, G.N. Stephanopoulos, High-throughput screen for poly-3-hydroxybutyrate in *Escherichia coli* and *Synechocystis* sp. strain PCC6803, Appl. Environ. Microbiol. 72 (2006) 3412–3417, https://doi.org/10.1128/AEM.72.5.3412-3417.2006.

[45] K. Urano, Y. Yoshiba, T. Nanjo, T. Ito, K. Yamaguchi-Shinozaki, K. Shinozaki, *Arabidopsis* stress inducible gene for arginine decarboxylase *AtADC2* is required for accumulation of putrescine in salt tolerance, Biochem. Biophys. Res. Commun. 313 (2004) 369–375, https://doi.org/10.1016/j.bbrc.2003.11.119.

[46] M. Vincenzini, C. Sili, R.D. Philippis, A. Ena, R. Materassi, Occurrence of poly-b-hydroxybutyrate in *Spirulina* species, J. Bacteriol. 172 (1990) 2791–2792, https://doi.org/10.1128/jb.172.5.2791-2792.1990.

[47] B. Wang, S. Pugh, D.R. Nielsen, W. Zhang, D.R. Meldrum, Engineering cyanobacteria for photosynthetic production of 3-hydroxybutyrate directly from CO_2, Metab. Eng. 16 (2013) 68–77, https://doi.org/10.1016/j.ymben.2013.01.001.

[48] G.F. Wu, Z.Y. Shen, Q.Y. Wu, Modification of carbon partitioning to enhance PHB production in *Synechocystis* sp. PCC 6803, Enzyme Microb. Technol. 30 (2002) 710–715, https://doi.org/10.1016/S0141-0229(02)00044-3.