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## Genetic engineering of *Synechocystis* sp. PCC6803 for poly- $\beta$ -hydroxybutyrate overproduction



Roberta Carpine<sup>a</sup>, Wei Du<sup>b</sup>, Giuseppe Olivieri<sup>a</sup>, Antonino Pollio<sup>c</sup>, Klaas J. Hellingwerf<sup>b</sup>, Antonio Marzocchella<sup>a</sup>, Filipe Branco dos Santos<sup>b,\*</sup>

<sup>a</sup> Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, - Università degli Studi di Napoli Federico II, P.le V. Tecchio, 80, 80125 Napoli, Italy

<sup>b</sup> Molecular Microbial Physiology Group, Swammerdam Institute for Life Sciences, Faculty of Science, University of Amsterdam, Science Park 904, 1098 XH Amsterdam, The Netherlands

<sup>c</sup> Biology Department, Università degli Studi di Napoli Federico II, Complesso Universitario Monte S. Angelo, via Cinthia, 4, 80126 Napoli, Italy

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### ABSTRACT

The biosynthesis of poly- $\beta$ -hydroxybutyrate (PHB) directly from carbon dioxide is a sustainable alternative for non-renewable, petroleum-based polymer production. *Synechocystis* sp. PCC6803 can naturally accumulate PHB using CO<sub>2</sub> as the sole carbon source, particularly when major nutrients such as nitrogen become limiting. Many previous studies have tried to genetically engineer PHB overproduction; mostly by increasing the expression of enzymes directly involved in its biosynthesis pathway. Here, we have instead concentrated on engineering the central carbon metabolism of *Synechocystis* such that (i) the PHB synthesis pathway becomes deregulated, and/or (ii) the levels of its substrate, acetyl-CoA, were increased. Seven different mutants were constructed harboring, separately or in combination, three different genetic modifications to *Synechocystis*' metabolic network. These were the deletions of phosphotransacetylase (Pta) and acetyl-CoA hydrolase (Ach), and the expression of a heterologous phosphoketolase (XfpK) from *Bifidobacterium breve*. The wild type *Synechocystis* and the derivative strains were compared in terms of biomass and the PHB production capability during photoautotrophic growth. This was performed in a photobioreactor exposed to a diel light/dark rhythm and using standard BG<sub>11</sub> as the growth medium. We found that the strain that combined all three genetic modifications, i.e. *xfpk* overexpression in a double *pta* and *ach* deletion background, showed the highest levels of PHB production from all the strains tested here. Encouragingly, the production levels obtained: 232 mg L<sup>-1</sup>, ~12% (w/w) of the dry biomass weight, and a productivity of 7.3 mg L<sup>-1</sup> d<sup>-1</sup>; are to the best of our knowledge, the highest ever reported for PHB production directly from CO<sub>2</sub>.

### 1. Introduction

The growing world population and the accompanying increased demand of plastic materials drive our need for more sustainable production of biodegradable polymers [1]. In particular, poly- $\beta$ -hydroxybutyrate (PHB) has received special attention for its interesting features, such as thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility [2]. The global production of PHB was approximately 34 ktonnes in 2014 and is forecasted to grow to 100 ktonnes in 2019 ([www.en.european-bioplastics.org](http://www.en.european-bioplastics.org)).

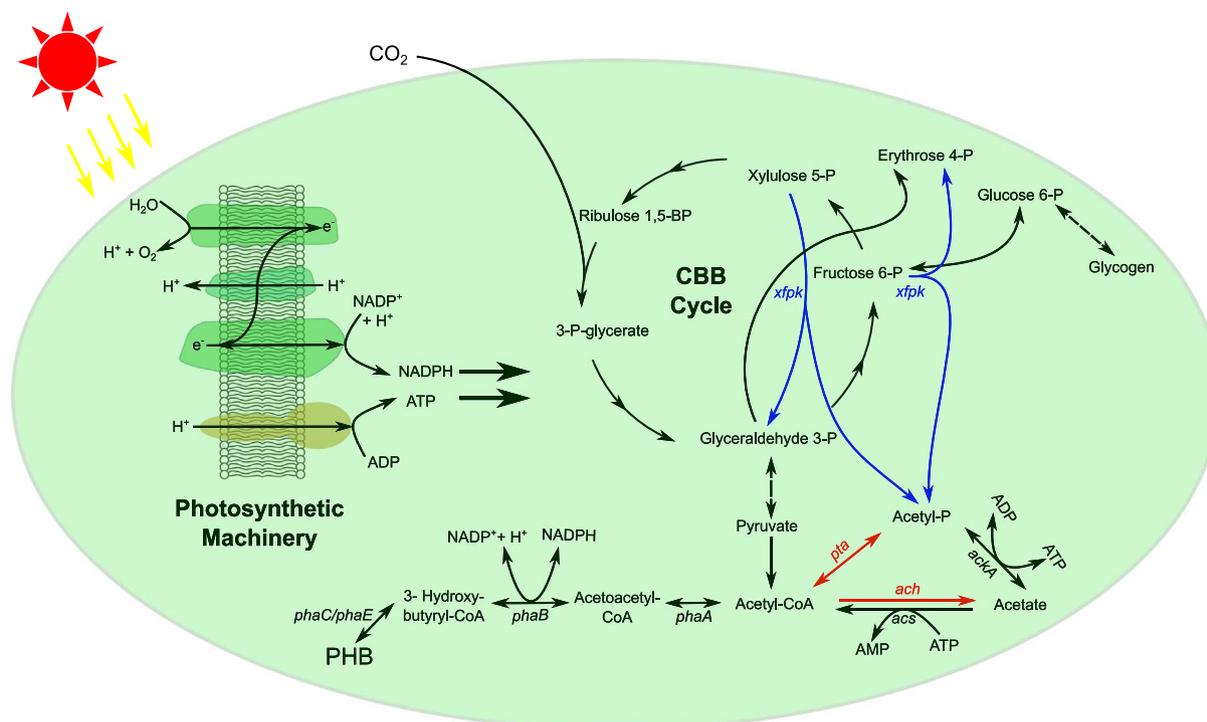
Under optimal conditions, bacteria such as *Cupriavidus necator* (previously known as *Ralstonia eutropha*) H16 can produce PHB up to 80% of their cellular dry weight while relying on fructose as the carbon source [3]. However, costs of raw materials for PHB production by microbial fermentation are still very high, making it of paramount

importance to find other sustainable production routes. This has brought plants into focus as an alternative low cost photosynthetic production system [4,5]. Yet, plant-based expression systems compete directly with subsistence crops for agricultural acreage, and raise further ethical concerns, as the dissemination of transgenic plants is difficult to control. The latter has led to strict regulatory restrictions of transgenic plants in many countries [6]. Cyanobacteria, share all the advantages of photoautotrophic microorganisms when compared to chemotrophs, indeed having the potential to use (sun)light energy to directly convert CO<sub>2</sub> into a product of interest such as PHB, but circumvent the drawbacks of competing with the agro-food market for resources [7–9].

In many cyanobacterial cells, PHB is a native carbon-storing polymer generated via the polyhydroxyalkanoate (PHA) biosynthetic pathway. The latter relies on the activities of three key enzymes: (i)  $\beta$ -

\* Corresponding author.

E-mail address: [f.branco@santosts@uva.nl](mailto:f.branco@santosts@uva.nl) (F. Branco dos Santos).



**Fig. 1.** Overview of the metabolism of *Synechocystis* sp. PCC6803 for PHB production. In this study, genes coloured in red (*pta* and *ach*) were knocked out, while the gene coloured in blue (*xfpk*) was overexpressed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ketothiolase (encoded by *phaA*), catalyzing the conversion of acetyl-CoA to acetoacetyl-CoA; (ii) acetoacetyl-CoA reductase (encoded by *phaB*), which produces the intermediate 3-hydroxybutyryl-CoA (3HB-CoA); and (iii) PHB synthase (encoded by *phaC/phaE*), finally resulting in synthesis of the storage polymer PHB [10]. The main flux of carbon in light-driven CO<sub>2</sub>-based synthesis of PHB by cyanobacterial cells goes from the Calvin–Benson–Bassham (CBB) cycle, through the lower part of glycolysis, to pyruvate and then via acetyl-CoA to PHB (Fig. 1). One of the key intermediates in this route is acetyl-CoA, which acts as the last branching point between PHB synthesis and other competing metabolic pathways. For instance, acetyl-CoA can be metabolized in multiple ways to acetate, such as via phosphotransacetylase (encoded by *pta*) and acetate kinase (encoded by *ackA*), generating ATP in the latter step, or directly catalyzed by an acetyl-CoA hydrolase (encoded by *ach*) [11]. Acetyl-CoA synthase (encoded by *acs*), in contrast, can recover acetate to reconvert it into the much more versatile intermediate acetyl-CoA [12].

A model cyanobacteria, *Synechocystis* sp. PCC6803 (hereafter *Synechocystis*), has been metabolically engineered to synthesize a variety of chemical commodities, such as ethanol, hydrogen, glycerol, ethylene, 2,3-butanediol, isobutanol, lactic acid, and also PHB [13–18]. The introduction of heterologous pathways and/or deletion or overexpression of specific native enzymes can divert the metabolic flux originating from CO<sub>2</sub>, from biomass to a product of interest [19]. The central carbon metabolism of cyanobacteria comprises the CBB cycle, glycolysis, the pentose-phosphate (PP) pathway and the TCA cycle, which collectively form a very complex and interconnected network (Fig. 1). In this study, we concentrated on engineering the native metabolic network of *Synechocystis* to further improve PHB production.

The genetic engineering strategies deployed in this work aimed at increasing acetyl-CoA levels to rewire carbon flux towards the native PHB synthesis pathway. *Synechocystis*, unlike *E. coli* and *Saccharomyces cerevisiae*, is capable of naturally producing PHB as a carbon storage polymer. The deletion of *pta* and *ach* in *Synechocystis* was here used to test the effect of reducing the conversion of acetyl-CoA to acetate. In *E. coli* the effect of the former has been tested, demonstrating that a

reduced phosphotransacetylase activity leads to an increase in acetyl-CoA levels, which ultimately results in an increased PHB accumulation [20]. Moreover, the overexpression of phosphoketolase (encoded by *xfpk*) was also used as a strategy to increase acetyl-CoA levels, and subsequently, PHB production. In yeast, such a strategy has been shown to lead to improved free fatty acid production [21] and PHB accumulation [22]. Phosphoketolases catalyze the cleavage of xylulose 5-phosphate (Xu5P) or fructose 6-phosphate (F6P) to acetyl-P and either glyceraldehyde-3-P or erythrose-4-P, respectively [23], and have only recently been characterized in cyanobacteria [24]. This activity confers flexibility in carbon and energy metabolism, and can be exploited to increase the efficiency of cyanobacterial central metabolism and photosynthetic productivity [24]. The utility of phosphoketolase in redirecting autotrophic metabolism, which can be used in addition to other metabolic engineering strategies, has been used to improve butanol production in *Synechocystis* [25], and acetone in another cyanobacterium, *Synechococcus elongatus* PCC7942 [26].

Here, we report the first study in which *pta* and *ach* deletion and *xfpk* overexpression were evaluated, separately and in combination, as strategies to improve PHB accumulation in cyanobacteria. Ultimately, without genetically engineering the PHB synthesis pathway itself, we have achieved a rise of 6-fold in PHB content and 2.5-fold increase of production rate, while solely engineering the surrounding metabolic network of the production host.

## 2. Materials and method

### 2.1. Genetic engineering

#### 2.1.1. Strains and general cultivation conditions

All the strains used in this study are listed in Table 1. Molecular cloning procedures were carried out in *E. coli* DH5 $\alpha$ , grown either on solidified LB plates containing 1.5% (w/v) agar or in liquid LB medium at 37 °C agitated at 200 rpm. When appropriate, antibiotics were added for propagation of specific plasmids. Concentrations of antibiotics used, alone or in combination, were 100  $\mu$ g mL<sup>-1</sup> for ampicillin and

**Table 1**  
Strains, plasmids and primers used in this study.

Strains, plasmids and primers <sup>a</sup>	Relevant characteristics	Reference
<i>Synechocystis</i>	<i>Synechocystis</i> sp. PCC6803 wild type	D. Bhaya
WD113 (ACH)	<i>Synechocystis ach</i> knock out	This study
WD114 (PTA)	<i>Synechocystis pta</i> knock out	This study
WD115 (DKO)	<i>Synechocystis ach-pta</i> double knock out	This study
WD116 (XFPK)	<i>Synechocystis</i> overexpression of <i>xfpk</i> ; Km <sup>r</sup>	This study
WD117 (ACHX)	<i>Synechocystis ach</i> knock out and <i>xfpk</i> overexpression; Km <sup>r</sup>	This study
WD118 (PTAX)	<i>Synechocystis pta</i> knock out and <i>xfpk</i> overexpression; Km <sup>r</sup>	This study
WD119 (DKOX)	<i>Synechocystis ach-pta</i> knock out and <i>xfpk</i> overexpression; Km <sup>r</sup>	This study
pFL-SA	BioBrick “T” vector with <i>SpeI</i> and <i>AvrII</i> restriction sites	[29]
pWD42	pFL-SA derivative, Amp <sup>r</sup> Km <sup>r</sup> , containing selection cassette originally from pNF1993/4 [28]	This study
pWD026	pFL-SA derivative, Amp <sup>r</sup> , containing <i>ach</i> upstream and downstream homologous regions	This study
pWD027	pFL-SA derivative, Amp <sup>r</sup> Km <sup>r</sup> , containing <i>ach</i> gene upstream homologous region, selection cassette, and downstream homologous region	This study
pWD028	pFL-SA derivative, Amp <sup>r</sup> , containing <i>pta</i> gene upstream and downstream homologous regions	This study
pWD029	pFL-SA derivative, Amp <sup>r</sup> Km <sup>r</sup> , containing <i>pta</i> gene upstream homologous region, selection cassette, and downstream homologous region	This study
pWD030	pFL-SA derivative, Amp <sup>r</sup> , containing <i>ach-pta</i> gene upstream and downstream homologous regions	This study
pWD031	pFL-SA derivative, Amp <sup>r</sup> Km <sup>r</sup> , containing <i>ach-pta</i> gene upstream homologous region, selection cassette, and downstream homologous region	This study
pCyJ02	<i>trc</i> promoter driving overexpression of a phosphoketolase from <i>Bifidobacterium breve</i> ; Km <sup>r</sup>	[25]
ach-upst-Fwd	GGACTATTCACCCAAGAACT	This study
ach-upst-Rev	AGAAAGAAAGACGGCTCAAAtctagaTGTTCTCGCGATCAGAG	This study
ach-downst-Fwd	CTCTGATCGCCAGGAACAtctagaGTTGACCGTCTTCTTCT	This study
ach-downst-Rev	CATCGCCACCACTAAATG	This study
pta-upst-Fwd	CTCAATCGAGGCGATATTC	This study
pta-upst-Rev	ACAACCCCGACTTAGATCAAtctagaATAACCTCAACGTATCCAG	This study
pta-downst-Fwd	CTGGATACGTTGAGGTTATtctagaTGATCTAAGTCGGGGTTGT	This study
pta-downst-Rev	GATGCTTTACTTCATCAATCGG	This study
ach-pta-upst-Fwd	GGACTATTCACCCAAGAACT	This study
ach-pta-upst-Rev	AAACAACCCCGACTTAGATtctagaTTCCTGGCGATCAGAGT	This study
ach-pta-downst-Fwd	ACTCTGATCGCCAGGAAtctagaATCTAAGTCGGGGTTGTTT	This study
ach-pta-downst-Rev	GATGCTTTACTTCATCAATCGG	This study
xfpk-upst-Fwd	GCCTACACTTCGGCTATTAGC	[25]
xfpk-downst-Rev	GCTAAACCCACCTCTTGCC	[25]

<sup>a</sup> primer sequences are given from 5′ → 3′.

50 µg mL<sup>-1</sup> for kanamycin.

A glucose-tolerant *Synechocystis* strain was obtained from D. Bhaya, University of Stanford, Stanford, CA. Unless indicated otherwise, it was cultivated in BG<sub>11</sub> medium [27] at 30 °C in a shaking incubator at 120 rpm (Innova 43, New Brunswick Scientific) under constant white-light illumination (around 30 µE m<sup>-2</sup> s<sup>-1</sup>, measured with a LI-1400, LI-COR, Inc.). For *Synechocystis* mutant construction, kanamycin or nickel sulphate was added to the medium up to a final concentration of 50 µg mL<sup>-1</sup> or 20 µM, respectively. Growth was monitored by recording the optical density at 730 nm (OD<sub>730</sub>; Lightwave II, Biochrom).

### 2.1.2. Plasmid construction

All plasmids used are listed in Table 1. For each markerless gene knockout mutant construction, two plasmids were needed. One contains only the 1 kb homologous regions up- and downstream of the deletion target. While for another plasmid, an extra selection cassette, encoding both a kanamycin resistance fragment and a nickel-induced *mazF* expression fragment, were used. *MazF* encodes an endoribonuclease that cleaves mRNA at the ACA triplet sequence, and thus, acts as a general inhibitor for the synthesis of all cellular proteins [28]. For individual *ach* and *pta* gene knock out plasmid construction, each homologous region was amplified from the genomic DNA of *Synechocystis* by PCR and fused together using Pfu DNA Polymerase (Thermo Scientific). After gel extraction and purification (Zymo Research), an extra adenosine (“A-”) was added to the 3′ overhang of each fused fragment using Taq DNA Polymerase (Thermo Scientific). The fragment with the A-overhang was then ligated to the BioBrick “T-” vector pFL-SA [29], resulting in pWD026 and pWD028, respectively. Since the designed fusion primers possess an *XbaI* restriction site between the homologous regions, and the selection cassette (from pWD42) contains an *XbaI* restriction site on both sides, it can be easily inserted into

pWD026 and pWD028, resulting pWD027 and pWD029, respectively. Since *ach* and *pta* are neighbouring genes in the genome with no obvious other overlapping transcripts [30], the same strategy was applied to delete these two genes simultaneously. The plasmids constructed for this purpose are pWD030 and pWD031. All the fragments amplified in this study were confirmed by Sanger sequencing (Macrogen Europe, The Netherlands), and the primers used are listed in Table 1.

### 2.1.3. *Synechocystis* markerless mutant construction

Using a previously reported method [28], it takes two rounds of transformation to make a clean deletion (i.e. without leaving behind a selection cassette) of one locus in the *Synechocystis* chromosome. The first round of transformation is to isolate a fully segregated mutant with the selection cassette inserted into the chromosome, while the second round of transformation is to completely remove the latter. Briefly, for the first round of transformation, *Synechocystis* wild type cells were collected either directly from the plate or from liquid culture. After washing twice with fresh BG<sub>11</sub> medium by centrifuging at 5000 rpm for 5 min, cells were further concentrated to OD<sub>730</sub> around 2 with a total liquid volume of 200 µL. Plasmid containing a selection cassette was added and mixed with cells to a final concentration of 10 µg mL<sup>-1</sup>. After illumination under moderate light (around 50 µE m<sup>-2</sup> s<sup>-1</sup>) for 4 to 6 h, the mixture was spread on commercial membranes (Pall Corporation, USA) and left resting on BG<sub>11</sub> plates (without antibiotic). After being further illuminated for about 16–24 h, the membrane containing the mixture was transferred to another BG<sub>11</sub> plate with kanamycin. One week later, the colonies that appeared on the plate were picked and seeded sequentially on BG<sub>11</sub> plates supplemented with either kanamycin or nickel. Colonies that grew on the BG<sub>11</sub> plate with kanamycin but not on the BG<sub>11</sub> plate with nickel were candidates for

further PCR confirmation. Further segregation in the liquid culture with higher concentration ( $> 50 \mu\text{g mL}^{-1}$ ) of kanamycin was used when necessary until the desired clone was obtained. For the second round of transformation, which removes the selection cassette, plasmid with only upstream and downstream homologous regions was used for subsequent transformation. Following the protocol just described for the first round of transformation, colonies appeared after about one week on the BG<sub>11</sub> plate with nickel sulphate. Again, colonies were picked and seeded sequentially on new BG<sub>11</sub> plates supplemented with either kanamycin or nickel, but now the colonies that grow on the BG<sub>11</sub> plate with nickel and not on BG<sub>11</sub> plate with kanamycin were chosen for the final PCR confirmation.

For the construction of *xfpk* overexpression mutants, transformation basically followed as described above, but now using kanamycin as the only selection pressure. The cells were transformed using the pCyJ02 plasmid, kindly provided by the Hudson group (KTH, Royal Institute of Technology, Sweden), which harbors a cassette with a strong promoter (Ptrc) upstream of *xfpk* from *Bifidobacterium breve* flanked by regions homologous to the neutral site *slr0168* of the chromosome of *Synechocystis*. The wild type, the strain with *pta* deletion, the strain with *ach* deletion and the strain with the double knock-out (*pta* and *ach*) were transformed with the pCyJ02 plasmid. The integration of the cassette at the *slr0168* site in all the mutants constructed was confirmed by PCR (Fig. 2).

## 2.2. PHB production in the photobioreactor

### 2.2.1. Cultivation conditions and operating procedures

Pre-cultures of 50 mL inoculated from a single isolate picked from a solid plate were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini, Italy) at 28 °C. The chamber was equipped with daylight fluorescent lamps (Philips TLD 30 W/55) set continuously at  $150 \mu\text{E m}^{-2} \text{s}^{-1}$  for 24/24 h [31]. After about two weeks the pre-cultures were used to inoculate the photobioreactors (10% v/v). Inclined bubble column photobioreactors (working volume of 800 mL; internal diameter 5 cm) were adopted for cyanobacterial growth. The photobioreactors were housed in a climate chamber (Solar Neon) at 28 °C. The chamber was also equipped with fluorescent lamps (Philips TLD 30 W/55). A sterilized gas stream using a hydrophobic filter (0.2  $\mu\text{m}$ ) was passed from the bottom of the photobioreactors by means

of multiple-orifices (1 mm ID) in a Teflon tube. The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations as previously described [32]. A gas mixing device (M2 M engineering, Italy) provided the selected concentration of 2% (v/v) of CO<sub>2</sub> in the gas stream fed to the photobioreactors by mixing air and pure carbon dioxide from a pressurized vessel at a final gas flow rate of 4 vessel volumes per minute (vvm). Cultures in inclined column photobioreactors were grown under light/dark cycles using a diel light/dark (L/D) rhythm: 18 h white light sinusoidally varying between 10 and  $260 \mu\text{E m}^{-2} \text{s}^{-1}$ , followed by 6 h darkness. The cultures were sampled every 48 h at the end of dark phase. Samples were characterized in terms of pH (for monitoring purposes of adequate CO<sub>2</sub> inflow to the culture) and concentration of biomass, nitrate, phosphate and PHB content.

Measured data were processed to assess the PHB fraction of biomass ( $\omega_{\text{PHB}}$ ) and PHB productivity ( $P_{\text{PHB}}$ ).  $\omega_{\text{PHB}}$  was calculated according to Eq. (1):

$$\omega_{\text{PHB}} = \frac{[\text{PHB}]}{[X]} \cdot 100 \quad (1)$$

where  $[X]$  is the cyanobacterial biomass concentration, and  $[\text{PHB}]$  the poly- $\beta$ -hydroxybutyrate content. The PHB productivity was calculated according to Eq. (2):

$$P_{\text{PHB}} = \frac{[\text{PHB}]_t}{t} \quad (2)$$

where  $[\text{PHB}]_t$  is the poly- $\beta$ -hydroxybutyrate content measured at the instant  $t$ .

### 2.2.2. Analytical methods

The pH and the total biomass concentration were measured in the culture sampled from the photobioreactors. The sample was then centrifuged at 5000 g for 15 min. The liquid phase was characterized in terms of concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content.

The biomass concentration was measured with a spectrophotometer (Specord 50, Analytic Jena) at 730 nm. Analysis of the dry cell weight (dcw) was carried out using a gravimetric method as previously described [33] by filtering, drying and weighing 10 mL aliquots of culture. The corresponding OD<sub>730</sub> values were paired with the measured dry cell weight, and a correlation factor of 1

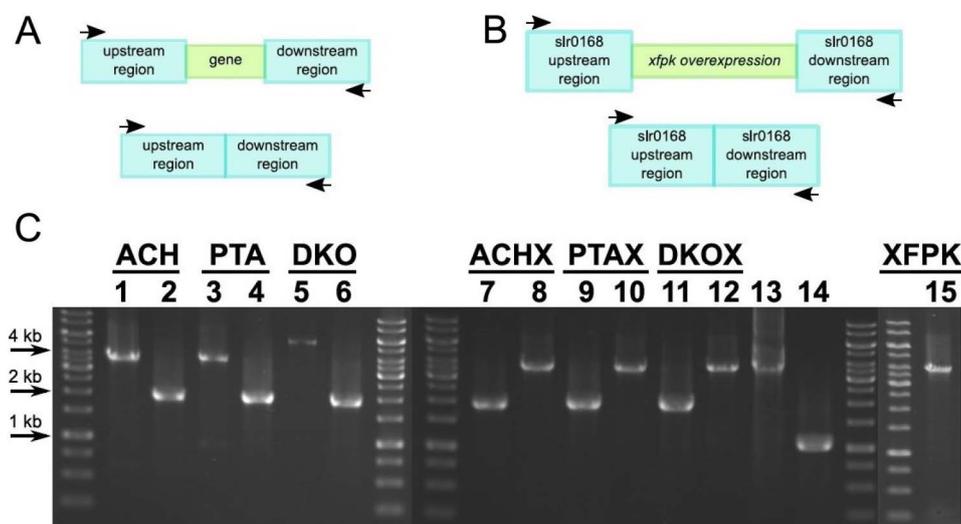


Fig. 2. PCR confirmation of all the mutants constructed in this study. (A) For gene knock out mutants, each homologous region is about 1 kb. Therefore, with the primers indicated as black arrows, a fully segregated markerless knock out mutant gave a single fragment of approximate 2 kb. While for *Synechocystis* wild type, the size of the fragment is larger than its corresponding mutant due to the presented gene. (B) For *xfpk* overexpression mutants, the primers designed would result a fragment of approximate 1 kb for wild type and 4 kb for the mutant. (C) Lane 1, 3, 5 are the results using wild type genomic DNA as controls with each primers specific to each gene, while lane 2, 4, 6, 7, 9, 11 are for their corresponding gene knock out mutants, respectively. Lane 8, 10, 12, 15 are the fragments amplified for *xfpk* overexpression mutants, with lane 13 and 14 as their respective control using the plasmid and wild type genomic DNA as template, respectively.

$OD_{730} = 0.23 \text{ g}_{\text{dcw}} \text{ L}^{-1}$  was found. The pH was measured with a Mettler Toledo pH meter.

The nitrate concentration was determined using a modified method proposed by Collos et al. [34]. The method is based on ultraviolet absorption spectrometry. The absorbance of the liquid phase recovered after the centrifugation was measured at a single wavelength (220 nm).

The phosphate concentration was measured according to the molybdate colorimetric test for ortho-phosphate. The colorimetric test was based on monitoring the redox state of the antimonyl-phosphomolybdate complex produced during the reaction among ammonium molybdate, potassium antimonyl tartrate and ortho-phosphate. This complex is reduced to an intense blue-coloured (molybdenum blue) complex by ascorbic acid [35]. The preparation of the reagents required the mixing of: 250 mL of 4.5 M  $\text{H}_2\text{SO}_4$ ; 45 mL of ammonium molybdate solution,  $95 \text{ g L}^{-1}$ ; 5 mL of potassium antimonyl tartrate solution,  $32.5 \text{ g L}^{-1}$ ; 50 mL of ascorbic acid solution,  $70 \text{ g L}^{-1}$ . 30  $\mu\text{L}$  of reagent and 30  $\mu\text{L}$  of ascorbic acid were added to 940  $\mu\text{L}$  of diluted sample. The absorption time-evolution of each sample was measured using a spectrophotometer at 704 nm and the final endpoint was taken to estimate phosphate concentration.

PHB granules were released by a slightly modified cell rupture protocol [36]. Biomass (10 mL of culture at  $OD_{730} = 1$ ) was harvested by centrifugation (20 min, 5000 g, 4 °C). The cell pellets were dried overnight at 85 °C. The dry pellets were boiled in 1 mL of 18 M  $\text{H}_2\text{SO}_4$  for 40 min, cooled down to room temperature and diluted with 9 mL of 7 mM  $\text{H}_2\text{SO}_4$ , before being filtered through a polyvinylidene difluoride membrane. Quantitative analysis of PHB content in the solution produced from the chemical processing of the cells was carried out by means of HPLC (Agilent 1100 system) equipped with a multi-wave detector set at 210 nm. The separation column used was an Aminex HPX-87H (300 × 7.8 mm). PHB was converted into and measured as crotonic acid, after treatment with sulphuric acid [37], and hence the latter was used as the external standard for HPLC analysis.

### 2.2.3. Statistical analysis

All strains were cultured in three completely independent biological replicates. Each sample collected throughout the cultivations was analyzed in triplicate. We report the mean (data points) along with one standard deviation represented by the error bars, or as otherwise indicated.

## 3. Results and discussion

### 3.1. Genetically engineered *Synechocystis*: Biomass formation and PHB production in photobioreactors

In the present study, the native genes *pta* and *ach* of *Synechocystis* were knocked out, separately and in combination, using a two-step transformation protocol resulting in three markerless derivative mutant strains. All these constructs, along with the *Synechocystis* wild type, were further transformed by integrating a cassette that harbors a heterologous *xfpk* under control of *P*<sub>trc</sub> in the neutral locus *str0168*. In total, seven different mutants were obtained, which were subsequently characterized specifically in terms of biomass and PHB production capability. These strains may also prove to be useful in a chassis for the production of other compounds whose biosynthetic pathways also depend on the availability of acetyl-CoA [e.g. acetone [29], or 1-butanol [38], among others].

*Synechocystis* wild type and mutant strains were grown in BG<sub>11</sub> medium under photoautotrophic conditions exposed to a diel rhythm (18 h light/6 h dark). The biomass concentration and PHB accumulation therein, were periodically monitored in time during the 40 days of cultivation (Fig. 3). The growth rate observed for the wildtype *Synechocystis* under these diel L/D illumination conditions was  $0.036 \text{ h}^{-1}$  (Table 2). Understandably, this value is lower in comparison to previous reports in which it was determined under optimal constant

illumination conditions ( $0.11 \text{ h}^{-1}$ ) [33]. The specific growth rates of all mutant strains were lowered as compared to the wild type *Synechocystis* from which they were derived (Table 2). The deletion of *pta* had the biggest deleterious effect, followed by the *ach* mutant, leading to a 63% and 51% drop in comparison to the wild type, respectively. Somewhat unexpectedly, the double deletion of *pta* and *ach* combined did not lead to a reduction in growth rate as strong as observed for the single deletions, although it was still quite substantial (44% drop in comparison to wild type). Not surprisingly, the overexpression of *xfpk* from *B. brevis* had a negative effect on growth rate relative to the wild type (35% drop). However, it is interesting to note that the opposite effect occurred when comparing the growth rate of the different knock out mutants with that of the corresponding overexpressing strains. Invariably, when combined with *xfpk* overexpression, the single *pta*-, the single *ach*-, and the double *pta* and *ach* deletions show increased growth rate by respectively 69%, 17% and 10%, as compared to the growth rate of the corresponding strain without *xfpk* overexpression.

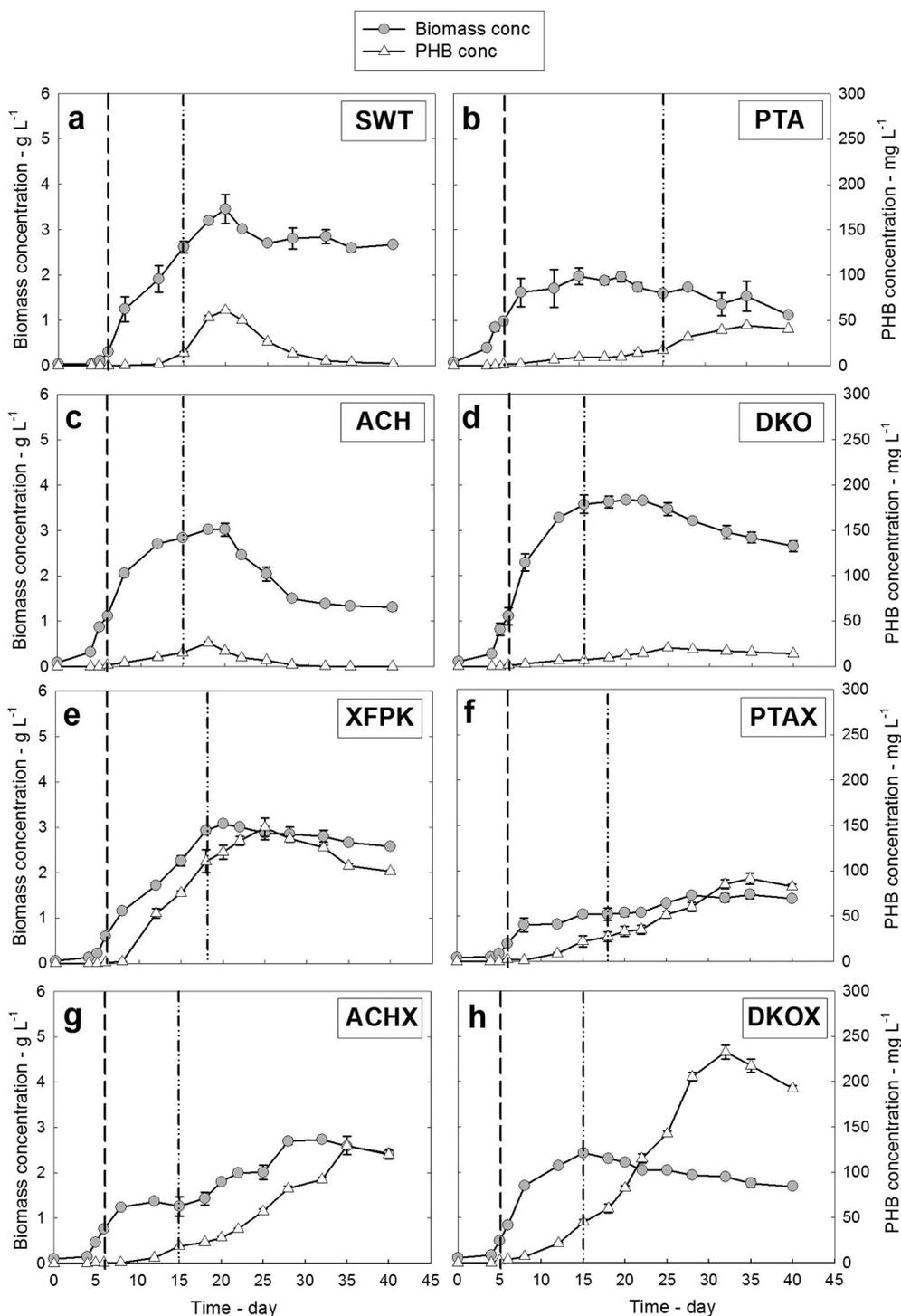
Similar to what was observed for the growth rate, the single *pta* deletion has a great negative effect (43% drop) when it comes to maximum biomass formation, leading to the lowest drop in final biomass concentration. The single *ach* deletion has relatively only a very slight effect on the maximum biomass concentration (13% drop), while the double knockout completely reverts the trend observed for the single *pta* deletion, by not showing any measurable effect on the biomass concentration (Table 2). The *xfpk* overexpression, now in stark contrast to what was observed for the growth rate, was found to consistently have a negative impact on the maximum biomass concentration when compared to the respective background not harboring the heterologous cassette. This apparent trade-off between rate and yield has been noted before in organisms displaying different metabolic strategies [39].

The deletions of *pta* and *ach*, both separately, or in combination, did not lead to a major increase in the maximum PHB concentration reached throughout the diel L/D cultivations. However, unlike what is observed for the wild type and single *ach* mutant, whenever *pta* is knocked out, it seems cells are not able to utilize, during the late stage of cultivation, the PHB storages that they have accumulated earlier (Fig. 3). Regarding the overexpression of *xfpk*, irrespective of the genetic background tested, it had a much more drastic effect on final PHB concentration. This effect can be observed clearly for wildtype and single deletions of *pta* and *ach*, but more importantly, it has a cumulative effect when both of these competing pathways are deleted simultaneously, leading to a PHB accumulation of  $232 \text{ mg L}^{-1}$  in the DKOX strain cultured under photoautotrophic conditions (Table 2).

### 3.2. Effect of phosphate- and nitrogen depletion on biomass and PHB production

During the multiple cultivations under a diel rhythm, the residual phosphate and nitrogen concentration was determined periodically (Fig. 4). Phosphate is rapidly depleted without any measurable phenotypic consequence for biomass formation or PHB accumulation. The rapid depletion of extracellular phosphate pools by *Synechocystis* has been previously described and linked to the rapid build-up of intracellular phosphate storages (e.g. by increasing the number of chromosome copies) that are used in later stages of growth [40]. This phenomenon is thought to be part of the survival strategies deployed by microorganisms that have evolved in aquatic environments, which are at times limited by this nutrient [41].

Nitrogen depletion, under the conditions tested, occurred invariably much later than that of phosphate (Fig. 4). This instant tended to match when the culture was reaching its maximum biomass concentration, and for the wild type, *pta* mutant, *ach* mutant and double *pta* and *ach* mutant strains, it also marked the moment when PHB began to accumulate in the cells. This corroborates previous studies in which it was found that upon N-depletion, cyanobacterial cells initially respond



**Fig. 3.** Biomass and PHB concentration in the diel cultivations of *Synechocystis* and derivative strains: a) depicts *Synechocystis* wild type; b) PTA strain carrying a single *pta* deletion; c) ACH strain carrying a single *ach* deletion; d) DKO strain carrying a double *pta* and *ach* deletion; e) XFPK strain harboring the *xfpk* overexpression in a wildtype background; f) PTAX strain harboring the *xfpk* overexpression in a PTA background; g) ACHX strain harboring the *xfpk* overexpression in an ACH background; h) DKOX strain harboring the *xfpk* overexpression under a DKO background. Grey circle indicates the mean biomass concentration, while white triangles indicate mean PHB concentration of three independent biological replicates analyzed in technical triplicates, with error bars representing standard deviation. Vertical lines mark the depletion of phosphate (long dash line) and nitrogen (dash dot dot line).

by driving the fixed carbon towards storage compounds such as PHB [25]. The genes coding for precursor biosynthesis of PHB in *Synechocystis*, *phaA* (*slr1993*) and *phaB* (*slr1994*), are organized in one operon. PHB synthase, the enzyme catalyzing the polymerization reaction to polyhydroxybutyrate, is encoded in a second operon and is formed by heterodimerization of PhaE (*slr1829*) and PhaC (*slr1830*). Expression of both operons has been reported to be up-regulated upon nitrogen starvation [42]. A remarkable feature of expressing *xfpk* in all the

genetic backgrounds tested was that PHB accumulation occurred long before N-depletion was reached. Mechanistically, this might be a consequence of the increased acetyl-CoA pools, which accompany increased phosphoketolase activity [25], and which would increase the thermodynamic drive to route carbon through the PHB pathway [22]. Alternatively, or in combination with the latter, this could also be a consequence of increased acetyl-P levels, which are known to activate PhaA, the first step of the PHB synthesis pathway [43,44]. Irrespective

**Table 2**

Specific growth rate, maximum biomass concentration, and PHB content, fraction and productivity of *Synechocystis* wild type and derivative strains used in this study (mean value  $\pm$  standard deviation, n = 3).

Strain	$\mu$ (h <sup>-1</sup> ) <sup>a</sup>	Maximum Biomass conc (g L <sup>-1</sup> )	Maximum PHB conc (mg L <sup>-1</sup> )	Maximum PHB fraction (%)	Maximum PHB productivity (mg L <sup>-1</sup> d <sup>-1</sup> )
SWT	0.036 $\pm$ 0.001	3.45 $\pm$ 0.32	50 $\pm$ 0.75	1.8 $\pm$ 0.2	3.05 $\pm$ 0.05
PTA	0.013 $\pm$ 0.002	1.97 $\pm$ 0.18	44 $\pm$ 0.5	3.5 $\pm$ 0.14	1.3 $\pm$ 0.01
ACH	0.017 $\pm$ 0.001	3.01 $\pm$ 0.05	17 $\pm$ 1.25	0.86 $\pm$ 0.04	1.4 $\pm$ 0.08
DKO	0.020 $\pm$ 0.002	3.66 $\pm$ 0.03	20 $\pm$ 0.25	0.58 $\pm$ 0.016	0.8 $\pm$ 0.01
XFPK	0.023 $\pm$ 0.002	3.07 $\pm$ 0.05	150 $\pm$ 10	5.3 $\pm$ 0.6	6.3 $\pm$ 0.57
PTAX	0.022 $\pm$ 0.002	1.47 $\pm$ 0.09	91 $\pm$ 6.25	6.2 $\pm$ 0.03	2.7 $\pm$ 0.16
ACHX	0.020 $\pm$ 0.001	2.73 $\pm$ 0.01	130 $\pm$ 10	5 $\pm$ 0.29	3.7 $\pm$ 0.29
DKOX	0.022 $\pm$ 0.001	2.42 $\pm$ 0.03	232 $\pm$ 7.5	12.4 $\pm$ 0.25	7.3 $\pm$ 0.18

<sup>a</sup> Specific growth rate was calculated based on cell dry weight monitored every 48 h (under diel illumination) during exponential growth phase, when PHB fraction of biomass is still insignificant (< 1%).

of the molecular basis for this phenotype, heterologous *xfpk* expression appears to cause carbon partitioning in cultures in N-replete media to resemble that of cultures in N-limited conditions.

### 3.3. Characterization of the PHB fraction in biomass and overall PHB productivity in the photobioreactors

The PHB fraction in biomass ( $\omega_{\text{PHB}}$ ) and overall productivity ( $P_{\text{PHB}}$ ) throughout the photoautotrophic cultivations under a diel rhythm were calculated based on the biomass and PHB levels (Fig. 5). The  $\omega_{\text{PHB}}$  and  $P_{\text{PHB}}$  are important indicators because the different genetic modifications performed led to changes in both biomass formation and PHB accumulation, which may be misleading if analyzed separately regarding the potential of the different constructs as photoautotrophic cell factories for PHB. The *ach* deletion had a negligible impact on both  $\omega_{\text{PHB}}$  and  $P_{\text{PHB}}$ , regardless of whether it was combined or not. Strain PTA, carrying the single *pta* deletion, did display a sustained increase of  $\omega_{\text{PHB}}$ . However, since this was accompanied as well by a sharp drop in biomass formation, ultimately the overall productivity was not greatly increased. The effect of the *xfpk* overexpression on both  $\omega_{\text{PHB}}$  and  $P_{\text{PHB}}$  was much more pronounced than that of any of the other genetic modifications tested. The mimicking of N-depletion before it actually sets in, which triggers cells to start accumulating PHB at earlier stages of growth, plays a huge role here. Particularly this is the case when the overexpression of *xfpk* was combined with the elimination of both *pta* and *ach* (strain DKOX). This mutant, ultimately reached a PHB content of about 12% (w/w) of the dry cell weight, a value 6-fold higher than that of the wild type strain, and a maximal PHB productivity of  $\sim 7$  mg L<sup>-1</sup> d<sup>-1</sup> (Table 2).

Many different genetic engineering approaches to increase PHB content in cyanobacteria have been described in literature with varying degrees of success [45]. The  $\omega_{\text{PHB}}$  and  $P_{\text{PHB}}$  reported are not always directly comparable, especially because in some instances PHB is not being produced strictly photoautotrophically, i.e. directly from CO<sub>2</sub> alone. We have strived to contextualize the achieved  $\omega_{\text{PHB}}$  and  $P_{\text{PHB}}$  obtained here by surveying the aforementioned reports with respect to: (i) host used; (ii) genetic modifications performed; (iii) carbon source used; (iv) culture condition; and (v) reactor type. Unfortunately, we still found that the overall productivity cannot be compared, because there is not enough data collected in some studies, or the production conditions are simply too distinct. Nonetheless, we found it useful to still compare the different  $\omega_{\text{PHB}}$  obtained (Table 3).

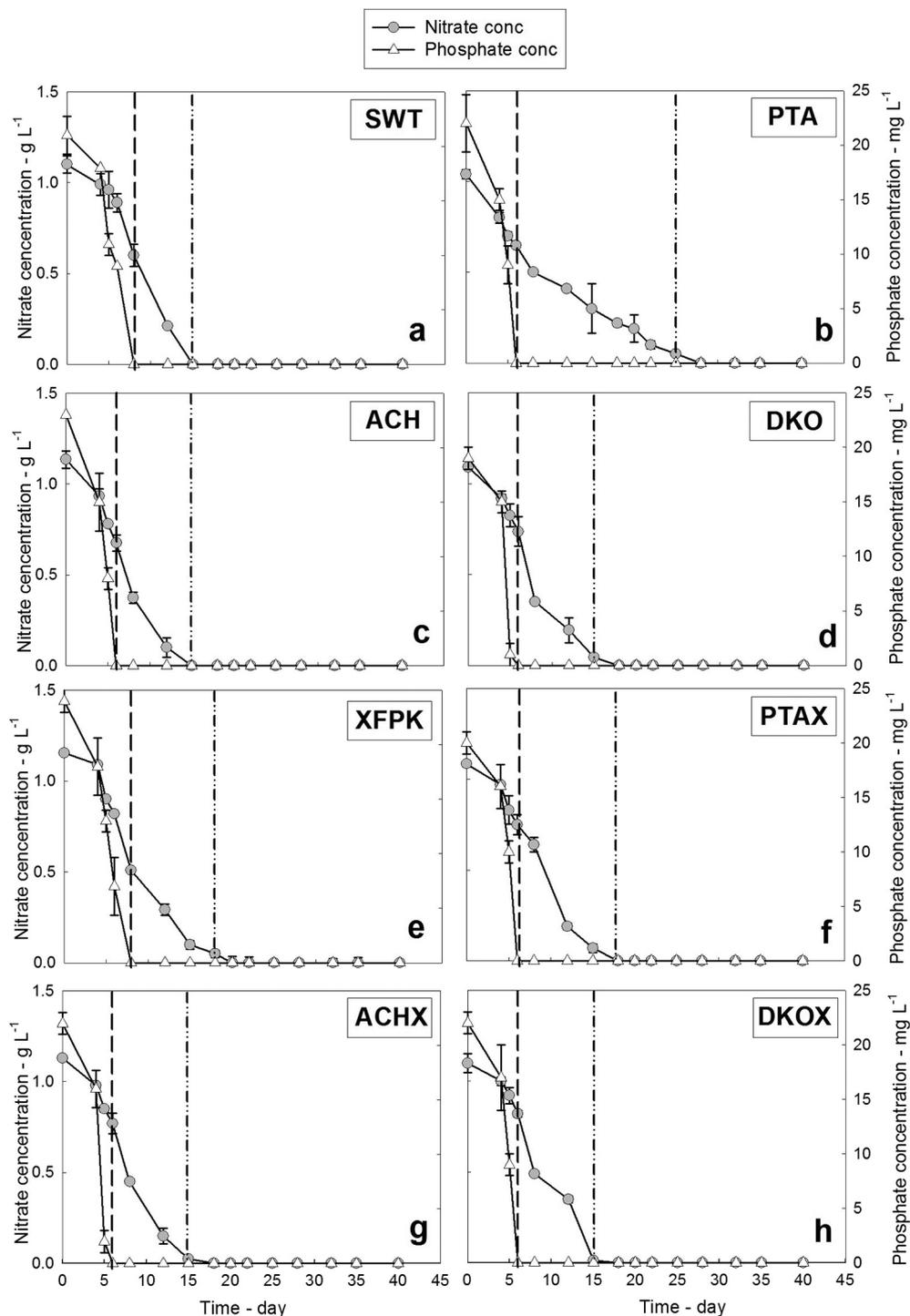
The several studies that have evaluated PHB accumulation using mixotrophic growth conditions [46–49] in mutants of either *Synechocystis* or *Synechococcus* PCC7942, added acetate in the growth medium. Rationale has been that acetate can be converted into acetyl-CoA via mostly the activity of acetyl-CoA synthetase, and thereby support many metabolic pathways such as the TCA cycle for cell growth, glycogen synthesis and fatty acid metabolism (relevant for biofuel production), and also PHB biosynthesis [49]. The heterologous expression of PHA-

synthesizing gene operon from the bacterium *C. necator* in *Synechocystis* sp. PCC6803 increased the PHB content from 7% to 11% of dcw when grown in N-deprived medium with 10 mM acetate [48]. Similar results had already been reported for the cyanobacterium *Synechococcus* sp. PCC7942 heterologously expressing the entire *A. eutrophus* PHB-synthesizing machinery. These recombinant *Synechococcus* cells accumulated 25% PHB of dcw under N- deprivation with 10 mM acetate [47]. The PHB content of the PhaAB strain obtained by Khetkorn et al. [49] in which the native *phaA* and *phaB* genes are overexpressed, is increased to 35% of dcw in the presence of 4 mM acetate under nitrogen deprived condition when compared to about 13% PHB of dcw in the wild type under the same condition.

The numbers reported when  $\omega_{\text{PHB}}$  is evaluated under photoautotrophic growth conditions, using only CO<sub>2</sub> as carbon source, tend to be somewhat more modest [10,50]. The overexpression of the native *sigE* gene integrated in the *Synechocystis* chromosome, increased the PHB content from 0.6% to 1.4% when grown in N-deprived medium [50]. Hondo et al. [10] transformed *Synechocystis* cells with the vector pAM461c harboring a PHA biosynthetic operon from *Microcystis aeruginosa* NIES-843 and reached a PHB content about 7% in N-deprived medium. We have obtained a similar  $\omega_{\text{PHB}}$  by simply overexpressing from a genomic integration *xfpk* under control of a strong promoter (*P*<sub>trc</sub>). Moreover, when the overexpression of *xfpk* was combined with the double deletion of both *pta* and *ach*, we reached a PHB content in biomass > 12%. Such PHB increment in DKOX was mainly due to the rewiring of carbon flux towards PHB synthesis, and not because of potentially lower biomass accumulation (compare Fig. 3a and h). To the best of our knowledge, this value is almost 2-fold higher than ever reported, using CO<sub>2</sub> alone as the direct carbon source.

## 4. Conclusions

The genetic engineering approach adopted in this study concentrated on improving PHB production directly from CO<sub>2</sub> in *Synechocystis*. Focus was placed on trying to engineer the central carbon metabolism of the host such that (i) the PHB synthesis pathway becomes deregulated, and/or (ii) the levels of its substrate, acetyl-CoA, are increased. For this purpose, we constructed seven different mutants, which harbored either separately or in combination, three different genetic modifications to the metabolic network – phosphotransacetylase deletion, acetyl-CoA hydrolase deletion and heterologous phosphoketolase overexpression. These, along with the wildtype *Synechocystis*, were tested for PHB production in photobioreactors under photoautotrophic conditions while exposed to a diel L/D rhythm and using standard BG<sub>11</sub> as the growth medium. Ultimately, all three genetic alterations show a cumulative effect, as the strain that combined them all displayed the highest levels reported of PHB production directly from CO<sub>2</sub>: 232 mg L<sup>-1</sup>,  $\sim 12\%$  (w/w) of the dry biomass weight, and a productivity of 7.3 mg L<sup>-1</sup> d<sup>-1</sup>.



**Fig. 4.** Residual nitrate and phosphate concentration in the photocultivations of *Synechocystis* and derivative strains: a) depicts *Synechocystis* wildtype; b) PTA strain carrying a single *pta* deletion; c) ACH strain carrying a single *ach* deletion; d) DKO strain carrying a double *pta* and *ach* deletion; e) XFPK strain harboring the *xfpk* overexpression in a wildtype background; f) PTAX strain harboring the *xfpk* overexpression in a PTA background; g) ACHX strain harboring the *xfpk* overexpression in an ACH background; h) DKOX strain harboring the *xfpk* overexpression in a DKO background. Data points represent mean values collected from three independent biological replicates analyzed in technical triplicates, with error bars representing standard deviation. Nitrate (grey circles) was found in all cases to be depleted after phosphate (white triangle). Vertical lines mark the sampling point in which depletion of phosphate (long dash line) and nitrogen (dash dot dot line) was considered to occur.

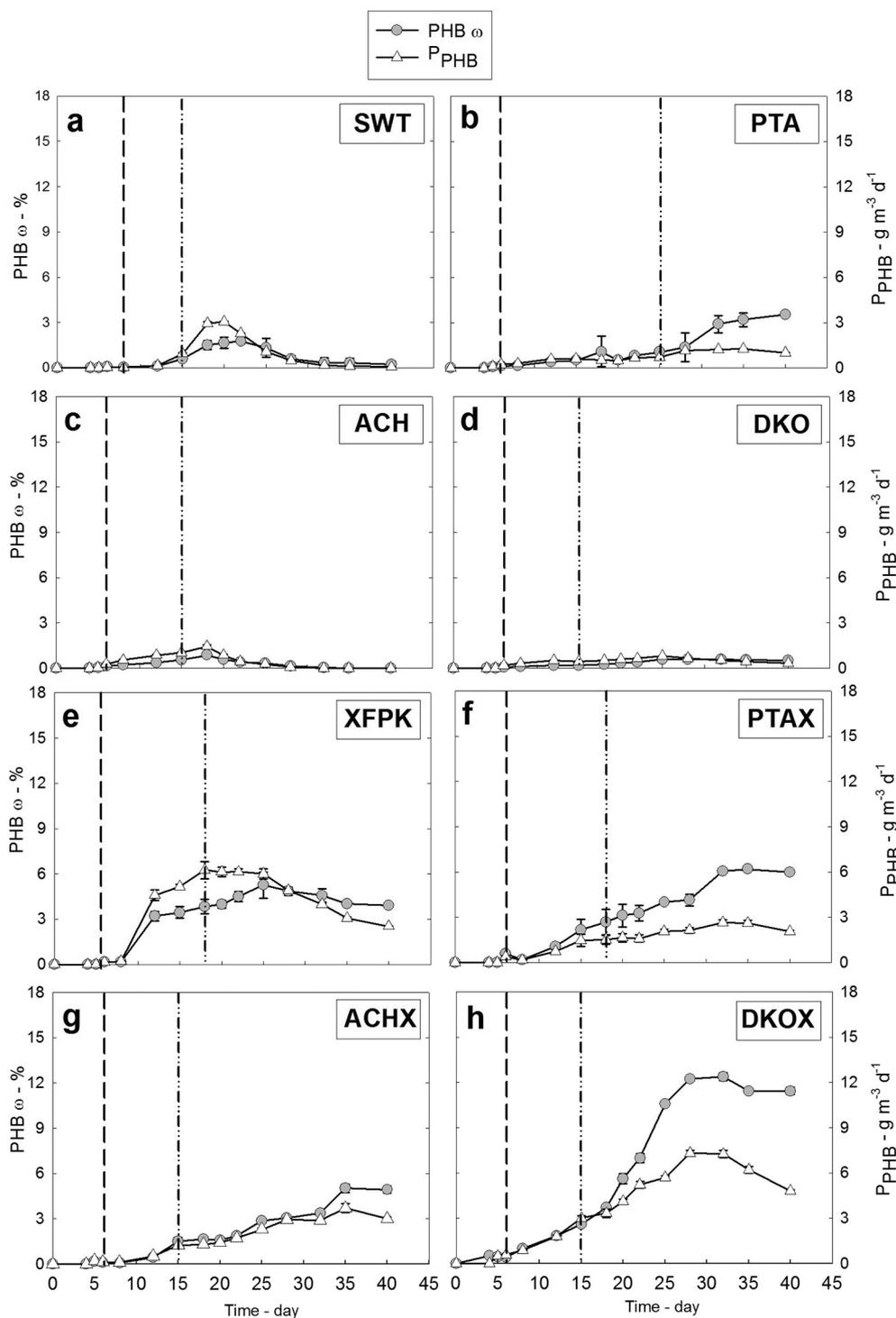
#### Author's Contributions

FBS, KJH, GO and AM conceived the concept of this study based on preliminary results from RC and WD; RC, WD engineered the strains described under the supervision of KJH and FBS; RC, GO, AP and AM were responsible for the photocultivations carried out; RC, WD and FBS analyzed the data; RC, GO, WD and FBS wrote the manuscript with

contributions from all authors.

#### Conflict of interest statement

Klaas J. Hellingwerf is the scientific advisor of Photanol B.V., a University of Amsterdam spin-off company aiming at commercializing sustainable applications with cyanobacteria. He and the other authors



**Fig. 5.** PHB fraction and productivity in the photocultivations of *Synechocystis* and derivative strains: a) depicts *Synechocystis* wildtype; b) PTA strain carrying a single *pta* deletion; c) ACH strain carrying a single *ach* deletion; d) DKO strain carrying a double *pta* and *ach* deletion; e) XFPK strain harboring the *xfpk* overexpression in a wildtype background; f) PTAX strain harboring the *xfpk* overexpression in a PTA background; g) ACHX strain harboring the *xfpk* overexpression in an ACH background; h) DKOX strain harboring the *xfpk* overexpression in a DKO background. Grey circle indicates the mean PHB fraction, while white triangle indicates mean PHB productivity of three independent biological replicates analyzed in triplicate, with error bars representing standard deviation. Vertical lines mark the depletion of phosphate (long dash line) and nitrogen (dash dot dot line).

declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Table 3**  
Comparison of PHB fraction in engineered cyanobacteria cultured under different conditions.

Cyano-bacterial strain	Engineered genes	Carbon source	Culture cond.	Light intensity	Polymer fraction (% dcw)	Ref
<i>Synechocystis</i> derivative	The <i>agg</i> gene was inserted	CO <sub>2</sub> 0.035%, Acetate 5 mM	Nitrogen deprived	~30 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	18.6%	[46]
<i>Synechococcus</i> derivative	A PHB synthesizing enzyme from <i>A. eutrophus</i> was expressed from a plasmid	CO <sub>2</sub> 5%, Acetate 10 mM	Nitrogen deprived	~40 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	25%	[47]
<i>Synechocystis</i> derivative	PHA synthase from <i>C. necator</i> was expressed from a plasmid	CO <sub>2</sub> 1%, Acetate 10 mM	Nitrogen deprived	150 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	11%	[48]
<i>Synechocystis</i> derivative	The native <i>sigE</i> was expressed from the chromosome	CO <sub>2</sub> 1%	Nitrogen deprived	70 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	1.4%	[50]
<i>Synechocystis</i> derivative	A PHA biosynthetic operon from <i>M. aeruginosa</i> was expressed from a plasmid	CO <sub>2</sub> 2%	Nitrogen deprived	100 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	7%	[10]
<i>Synechocystis</i> derivative	The native <i>phaAAB</i> was overexpressed from the chromosome	CO <sub>2</sub> 0.035%, Acetate 4 mM	Nitrogen deprived	40 μE m <sup>-2</sup> s <sup>-1</sup> (continuous light)	35%	[49]
<i>Synechocystis</i> derivative	<i>xfpk</i> from <i>B. breve</i> was expressed in a double <i>pta</i> and <i>ach</i> knock out.	CO <sub>2</sub> 2%	Nitrogen replete	0–260 μE m <sup>-2</sup> s <sup>-1</sup> (18 h light/6 h dark)	12%	This work

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