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Genetic engineering of *Synechocystis* sp. PCC6803 for poly-β-hydroxybutyrate overproduction

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**A R T I C L E   I N F O**

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Cyanobacteria  
Genetic engineering  
Phosphotransacetylase  
Acetyl-CoA hydrolase  
Phosphoketolase

**A B S T R A C T**

The biosynthesis of poly-β-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\textsubscript{2} 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\textsubscript{11} as the growth medium. We found that the strain that combined all three genetic modifications, i.e. xfk 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\textsuperscript{−1}, ~12% (w/w) of the dry biomass weight, and a productivity of 7.3 mg L\textsuperscript{−1} d\textsuperscript{−1}; are to the best of our knowledge, the highest ever reported for PHB production directly from CO\textsubscript{2}.

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-β-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).

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\textsubscript{2} 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) β-
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 CO2-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 ackA), 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 CO2, 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* DH5α, 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 μg mL⁻¹ for ampicillin and
50 μg mL\(^{-1}\) for kanamycin.

A glucose-tolerant *Synechocystis* strain was obtained from D. Bhaya, University of Stanford, Stanford, CA. Unless indicated otherwise, it was cultivated in BG11 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\(^{-2}\) s\(^{-1}\), 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\(^{-1}\) or 20 μM, respectively. Growth was monitored by recording the optical density at 730 nm (OD\(_{730}\); 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, kanamycin or nickel sulphate was added to the medium up to a final concentration of 50 μg mL\(^{-1}\) or 20 μM, respectively. Growth was monitored by recording the optical density at 730 nm (OD\(_{730}\); Lightwave II, Biochrom).

#### Table 1

<table>
<thead>
<tr>
<th>Strains, plasmids and primers</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<tr>
<td><em>Synechocystis</em></td>
<td><em>Synechocystis</em> sp. PC68603 wild type</td>
<td>D. Bhaya</td>
</tr>
<tr>
<td>WD113 (ACH)</td>
<td><em>Synechocystis</em> ach knock out</td>
<td>This study</td>
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<tr>
<td>WD114 (PTA)</td>
<td><em>Synechocystis</em> pta knock out</td>
<td>This study</td>
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<tr>
<td>WD115 (DKO)</td>
<td><em>Synechocystis</em> ach-pta double knock out</td>
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<td>WD116 (XFPK)</td>
<td><em>Synechocystis</em> overexpression of xfpk; Km(^{′})</td>
<td>This study</td>
</tr>
<tr>
<td>WD117 (ACHX)</td>
<td><em>Synechocystis</em> ach knock out and xfpk overexpression; Km(^{′})</td>
<td>This study</td>
</tr>
<tr>
<td>WD118 (PTAX)</td>
<td><em>Synechocystis</em> pta knock out and xfpk overexpression; Km(^{′})</td>
<td>This study</td>
</tr>
<tr>
<td>WD119 (DKOX)</td>
<td><em>Synechocystis</em> ach-pta knock out and xfpk overexpression; Km(^{′})</td>
<td>This study</td>
</tr>
<tr>
<td>pFLSA</td>
<td>BioBrick “T-” vector with SpeI and AvrII restriction sites</td>
<td>[29]</td>
</tr>
<tr>
<td>pWD042</td>
<td>pFL-SA derivate, Amp(^{′})/Kmr(^{′}), containing selection cassette originally from pNF1993/4</td>
<td>This study</td>
</tr>
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<td>pWD026</td>
<td>pFL-SA derivate, Amp(^{′}), containing ach upstream and downstream homologous regions</td>
<td>This study</td>
</tr>
<tr>
<td>pWD027</td>
<td>pFL-SA derivate, Amp(^{′})/Kmr(^{′}), containing ach gene upstream homologous region, selection cassette, and downstream homologous region</td>
<td>This study</td>
</tr>
<tr>
<td>pWD028</td>
<td>pFL-SA derivate, Amp(^{′}), containing pta gene upstream and downstream homologous regions</td>
<td>This study</td>
</tr>
<tr>
<td>pWD029</td>
<td>pFL-SA derivate, Amp(^{′})/Kmr(^{′}), containing pta gene upstream homologous region, selection cassette, and downstream homologous region</td>
<td>This study</td>
</tr>
<tr>
<td>pWD030</td>
<td>pFL-SA derivate, Amp(^{′}), containing ach-pta gene upstream and downstream homologous regions</td>
<td>This study</td>
</tr>
<tr>
<td>pWD031</td>
<td>pFL-SA derivate, Amp(^{′})/Kmr(^{′}), containing ach-pta gene upstream homologous region, selection cassette, and downstream homologous region</td>
<td>This study</td>
</tr>
<tr>
<td>pCyJ02</td>
<td>trc promoter driving overexpression of a phosphoketolase from <em>Bifidobacterium breve</em>; Km(^{′})</td>
<td>[25]</td>
</tr>
<tr>
<td>ach-upst-Fwd</td>
<td>GGACTATTTACCAAGAAGCT</td>
<td>This study</td>
</tr>
<tr>
<td>ach-upst-Rev</td>
<td>AGAAAGAAGACGGGCATTC</td>
<td>This study</td>
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<tr>
<td>ach-downst-Fwd</td>
<td>CTGGATACGTTGAGGTTAT</td>
<td>This study</td>
</tr>
<tr>
<td>ach-downst-Rev</td>
<td>CTCAATCGAGGCGATATTC</td>
<td>This study</td>
</tr>
<tr>
<td>pta-upst-Fwd</td>
<td>CTCAATCGAGGCGATATTC</td>
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<tr>
<td>pta-downst-Fwd</td>
<td>CTGCATCATGTGTTAGTGTTT</td>
<td>This study</td>
</tr>
<tr>
<td>pta-downst-Rev</td>
<td>CTGCATCATGTGTTAGTGTTT</td>
<td>This study</td>
</tr>
<tr>
<td>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.</td>
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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 BG11 medium by centrifuging at 5000 rpm for 5 min, cells were further concentrated to OD\(_{730}\) 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\(^{-1}\). After illumination under moderate light (around 50 μE m\(^{-2}\) s\(^{-1}\) for 4 to 6 h, the mixture was spread on commercial membranes (Pall Corporation, USA) and left resting on BG11 plates (without antibiotic). After being further illuminated for about 16–24 h, the membrane containing the mixture was transferred to another BG11 plate with kanamycin. One week later, the colonies that appeared on the plate were picked and seeded sequentially on BG11 plates supplemented with either kanamycin or nickel. Colonies that grew on the BG11 plate with kanamycin but not on the BG11 plate with nickel were candidates for
Further PCR confirmation. Further segregation in the liquid culture with higher concentration (> 50 μg 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 BG11 plate with nickel sulphate. Again, colonies were picked and seeded sequentially on new BG11 plates supplemented with either kanamycin or nickel, but now the colonies that grow on the BG11 plate with nickel and not on BG11 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 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 μE m^{-2} 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 μm) was sparged at 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 CO2 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 μE m^{-2} 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 CO2 inflow to the culture) and concentration of biomass, nitrate, phosphate and PHB content.

Measured data were processed to assess the PHB fraction of biomass (ωPHB) and PHB productivity (PPHB). ωPHB was calculated according to Eq. (1):

\[ \omega_{\text{PHB}} = \frac{[\text{PHB}]}{[X]} \cdot 100 \]

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

\[ P_{\text{PHB}} = \frac{[\text{PHB}]}{t} \]

where [PHB]t is the poly-β-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 OD730 values were paired with the measured dry cell weight, and a correlation factor of 1.

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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 knockout 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 present 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 dry cell L}^{-1} \text{ 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 antimony tartrate and ortho-phosphate. This complex is reduced to an intense blue-coloured (molybdin blue) complex by ascorbic acid [35]. The preparation of the reagents required the mixing of: 250 mL of 4.5 M H_{2}SO_{4}; 45 mL of ammonium molybdate solution, 95 g L^{-1}; 5 mL of potassium antimony tartrate solution, 32.5 g L^{-1}; 50 mL of ascorbic acid solution, 70 g L^{-1}. 30 mL of reagent and 30 mL of ascorbic acid were added to 940 mL 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 H_{2}SO_{4} for 40 min, cooled down to room temperature and diluted with 9 mL of 7 mM H_{2}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 thatharbors a heterologous xfpk under control of Ptc as the neutral locus stb168. In total, seven different mutants were obtained, which were subsequently analyzed 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 BG11 medium under photautotrophic 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 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 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. Irrespective, 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 strain 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 mg L^{-1} in the DKOX strain cultured under photautotrophic 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
by driving the fixed carbon towards storage compounds such as PHB [25]. The genes coding for precursor biosynthesis of PHB in Synechocystis, \textit{phaA} (\textit{slr1993}) and \textit{phaB} (\textit{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 \textit{PhaE} (\textit{slr1829}) and \textit{PhaC} (\textit{slr1830}). Expression of both operons has been reported to be up-regulated upon nitrogen starvation [42]. A remarkable feature of expressing \textit{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 \textit{PhaA}, the first step of the PHB synthesis pathway [43,44]. Irrespective

Fig. 3. Biomass and PHB concentration in the diel cultivations of \textit{Synechocystis} and derivative strains: a) depicts \textit{Synechocystis} wild type; b) PTA strain carrying a single \textit{pta} deletion; c) ACH strain carrying a single \textit{ach} deletion; d) DKO strain carrying a double \textit{pta} and \textit{ach} deletion; e) XFPK strain harboring the \textit{xfpk} overexpression in a wildtype background; f) PTAX strain harboring the \textit{xfpk} overexpression in a PTA background; g) ACHX strain harboring the \textit{xfpk} overexpression in an ACH background; h) DKOX strain harboring the \textit{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).
of the molecule 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 act 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 ~7 mg L$^{-1}$ d$^{-1}$ (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$_2$ 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 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 Synechocystis 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 nativephaA 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 photautotrophic growth conditions, using only CO$_2$ 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 Micrococcus 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 (Ptec). 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$_2$ alone as the direct carbon source.

### 4. Conclusions

The genetic engineering approach adopted in this study concentrated on improving PHB production directly from CO$_2$ 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$_{11}$ 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$_2$: 232 mg L$^{-1}$, ~12% (w/w) of the dry biomass weight, and a productivity of 7.3 mg L$^{-1}$ d$^{-1}$.

Table 2

<table>
<thead>
<tr>
<th>Strain</th>
<th>$\mu$ (h$^{-1}$)</th>
<th>Maximum Biomass conc (g L$^{-1}$)</th>
<th>Maximum PHB conc (mg L$^{-1}$)</th>
<th>Maximum PHB fraction (%)</th>
<th>Maximum PHB productivity (mg L$^{-1}$ d$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SWT</td>
<td>0.036 ± 0.001</td>
<td>3.45 ± 0.32</td>
<td>50 ± 0.75</td>
<td>1.8 ± 0.2</td>
<td>3.05 ± 0.05</td>
</tr>
<tr>
<td>PTA</td>
<td>0.013 ± 0.002</td>
<td>1.97 ± 0.18</td>
<td>44 ± 0.5</td>
<td>3.5 ± 0.14</td>
<td>1.3 ± 0.01</td>
</tr>
<tr>
<td>ACH</td>
<td>0.017 ± 0.001</td>
<td>3.01 ± 0.05</td>
<td>17 ± 1.25</td>
<td>0.86 ± 0.04</td>
<td>1.4 ± 0.08</td>
</tr>
<tr>
<td>DKO</td>
<td>0.020 ± 0.002</td>
<td>3.66 ± 0.03</td>
<td>50 ± 0.016</td>
<td>0.8 ± 0.01</td>
<td>1.4 ± 0.06</td>
</tr>
<tr>
<td>XFPK</td>
<td>0.023 ± 0.002</td>
<td>3.07 ± 0.05</td>
<td>150 ± 10</td>
<td>5.3 ± 0.6</td>
<td>6.3 ± 0.57</td>
</tr>
<tr>
<td>PTAx</td>
<td>0.022 ± 0.002</td>
<td>1.47 ± 0.09</td>
<td>91 ± 6.25</td>
<td>6.2 ± 0.03</td>
<td>2.7 ± 0.16</td>
</tr>
<tr>
<td>ACHX</td>
<td>0.020 ± 0.001</td>
<td>2.73 ± 0.01</td>
<td>130 ± 10</td>
<td>5 ± 0.29</td>
<td>3.7 ± 0.29</td>
</tr>
<tr>
<td>DKOX</td>
<td>0.022 ± 0.001</td>
<td>2.42 ± 0.03</td>
<td>232 ± 10</td>
<td>12.4 ± 0.25</td>
<td>7.3 ± 0.18</td>
</tr>
</tbody>
</table>

$^*$ 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%).
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...
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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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).
The agp gene was inserted CO₂ 0.035%, Acetate 5 mM Nitrogen deprived ~30 µE m⁻² s⁻¹ (continuous light) 18.6% [46] Synechococcus derivative

A PHB synthesizing enzyme from A. eutrophus was expressed from a plasmid CO₂ 5%, Acetate 10 mM Nitrogen deprived ~40 µE m⁻² s⁻¹ (continuous light) 25% [47] Synechocystis C. necator derivative

The native was expressed from the chromosome CO₂ 1% Nitrogen deprived 70 µE m⁻² s⁻¹ (continuous light) 1.4% [50] Synechocystis M. aeruginosa derivative

Synechocystis was expressed from a plasmid CO₂ 2% Nitrogen deprived 100 µE m⁻² s⁻¹ (continuous light) 7% [10] derivative

Synechocystis B. breve was expressed in a double and ach knock out. CO₂ 2% Nitrogen replete 0 260 µE m⁻² s⁻¹ (18 h light/6 h dark) 12% This work

() CO₂ 2%, Acetate 4 mM Nitrogen deprived 40 µE m⁻² s⁻¹ (continuous light) 35% [49] derivative

Synechocystis was overexpressed from the chromosome CO₂ 0.035%, Acetate 4 mM Nitrogen deprived 40 µE m⁻² s⁻¹ (continuous light) 35% [49] derivative

This work