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Inorganic carbon stimulates the metabolic routes related to the polyhydroxybutyrate production in a *Synechocystis* sp. strain (cyanobacteria) isolated from wastewater

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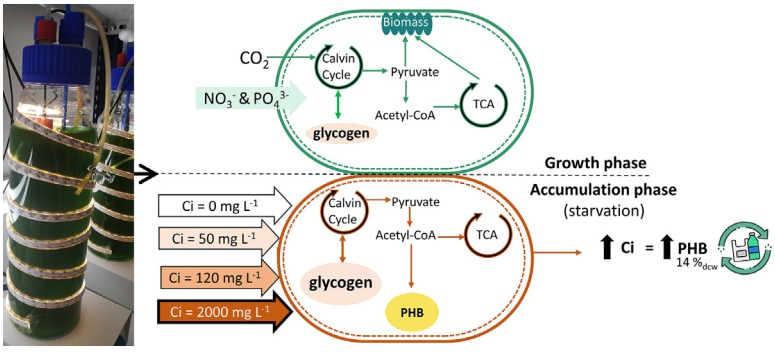
HIGHLIGHTS

- High bicarbonate concentration stimulates cell growth and nutrients consumption.
- High bicarbonate concentration stimulates PHB production.
- A 14%$_{dcw}$ of PHB was reached in *Synechocystis* by using a concentration of 2 gC L^{-1}.
- Glycogen and PHB synthesis genes are overexpressed when there is high DIC content.
- Expression of *glgP2* and *phaC*, *phaB* genes is positively correlated.

GRAPHICAL ABSTRACT



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ABSTRACT

Cyanobacteria are capable of transforming CO_2 into polyhydroxybutyrate (PHB). In this study, different inorganic carbon concentrations (0–2 gC L^{-1}) were evaluated for a *Synechocystis* sp. strain isolated from wastewater. Quantitative RT-qPCR was also performed to decipher the links between inorganic carbon and PHB and glycogen metabolism. 2 gC L^{-1} of bicarbonate stimulated cell growth, nutrients consumption and production of PHB. Using this concentration, a 14%$_{dcw}$ of PHB and an average productivity of 2.45 mgPHB L^{-1} d^{-1} were obtained. Gene expression analysis revealed that these conditions caused the overexpression of genes related to glycogen and PHB synthesis. Moreover, a positive correlation between the genes codifying for the glycogen phosphorylase, the acetyl-CoA reductase and the poly(3-hydroxyalkanoate) polymerase was found, meaning that PHB synthesis and glycogen catabolism are strongly related. These results provide an exhaustive evaluation of the effect of carbon on the PHB production and cyanobacterial metabolism.

Abbreviations: DIC, Dissolved inorganic carbon; PBR, Photobioreactor; gly, Glycogen; CHO, Carbohydrates; PHB, Polyhydroxybutyrate; TCA, Tri-carboxylic acid cycle; μ, Average specific growth rate (d^{-1}); $r_{biomass}$, Average biomass productivity (mgVSS L^{-1} d^{-1}); q_P, Average specific consumption of phosphorus (mgP L^{-1} d^{-1}); $Y_{X/P}$, Biomass yield on phosphorus (mgVSS mgP^{-1}); q_N, Average specific consumption of nitrogen (mgN L^{-1} d^{-1}); $Y_{X/N}$, Biomass yield on nitrogen (mgVSS mgN^{-1}); q_C, Average specific consumption of Carbon (mgC L^{-1} d^{-1}); $Y_{X/C}$, Biomass yield on carbon (mgVSS mgC^{-1}); r_{PHB}, Average PHB productivity (mgPHB L^{-1} d^{-1}); r_{gly}, Average glycogen productivity (mgPHB L^{-1} d^{-1}).

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1. Introduction

Most of the already tested cyanobacteria have the ability to produce polyhydroxybutyrate (PHB), a biodegradable plastic that due to its promising properties, can be a possible alternative to oil-based plastics, avoiding the serious negative environmental impacts related to their use (Gracioso et al., 2021). Cyanobacteria can use both organic carbon or inorganic carbon molecules for the production of PHB. Although it is well known that additions of organic carbon sources in cyanobacterial cultures, such as citrate, acetate, fructose, valerate, glucose, can stimulate the PHB production, their volumetric production is still much lower than that of heterotrophic bacteria (around 10,000 times lower) (Price et al., 2020). Therefore, in theory cyanobacteria can become only economically competitive if instead of using organic carbon sources, which usually accounts for the 25–50% of the heterotrophic PHB production costs, they are cultivated to produce PHB using only inorganic C sources. Moreover, the production of the organic carbon feedstock requires large areas, that should also be taken in consideration. However, to make the autotrophic process viable, the PHB production yield should be improved because it is usually around 5%$_{dcw}$, and only in very exceptional cases over the 20%$_{dcw}$ (Kamravamanesh et al., 2019; Nishioka et al., 2001; Price et al., 2020). Higher PHB contents were only obtained by introducing genetic modifications (up to a 63%$_{dcw}$ autotrophically) (Koch et al., 2020b).

The metabolic function and the conditions that stimulate cyanobacteria PHB metabolic pathways are still unclear (Koch and Forchhammer, 2021). Cyanobacteria produce two carbon storage compounds, PHB and glycogen, being glycogen the primary. Koch et al. (2019) observed, by knocking out some genes related to glycogen catabolism, that PHB was produced from glycogen conversion during prolonged nitrogen starvation, mainly by using the Emden-Meyerhof-Parnas (EMP) pathway. Dutt and Srivastava (2018) observed, by labelling different PHB precursors, that PHB was mainly produced from internal carbon recycling. Considering these evidences, it is believed that during nutrient starvation, PHB serve as an electron sink to rebalance the ATP/NADPH ratio. This process is advantageous with respect to other metabolic processes since the ATP can be recovered while conserving the intracellular carbon storage (Koch and Forchhammer, 2021). Another possible function of PHB (not incompatible with the electron sink function) is as a structural component useful for cell recovery after nitrogen deprivation (Damrow et al., 2016). Jendrossek and Pfeiffer (2014) found that PHB formation is produced attached to the nucleoid, what ensures an almost equal distribution of PHB to the new bacterial cells. Therefore, it is thought, that this substance may be helpful for new cells to recover from chlorosis (Damrow et al., 2016; Jendrossek and Pfeiffer, 2014).

Only a few studies have analysed the effect of inorganic carbon in cultures (either as CO_2 or bicarbonate) in the accumulation of PHB, and the results are varied and somewhat contradictory. For instance, Kamravamanesh et al. (2017) and Urtuvia et al. (2014) found that an increase in CO_2 concentrations had a negative impact on the PHB production. In opposition, Arias et al. (2018) and Kamravamanesh et al. (2018a, 2018b), found a positive relation between PHB production and carbon uptake. In a previous study from the authors (Rueda et al., 2020), the effect of adding carbon in a feast famine regime was evaluated. There, it was observed that PHB and glycogen were both accumulated during the feast phase when 120 mgC L^{-1} were added. While glycogen conversion into PHB was only observed during feast and famine phase (Rueda et al., 2020).

Up to now PHB production with cyanobacteria has been usually tested adding moderate amounts of CO_2 or bicarbonate (usually below 150 mgC L^{-1}) (Kamravamanesh et al., 2017; Troschl et al., 2018). In contrast, in the case of heterotrophic bacteria, the production of PHB usually is attained with concentrations of organic carbon usually ranging between 0.5 gC L^{-1} to 4 gC L^{-1} (Han et al., 2004; Łabuzek and Radecka, 2001; Lasemi et al., 2013; Thirumala et al., 2010), which is certainly much higher than the equivalent inorganic carbon used for cyanobacteria. Just by analogy, it could be hypothesized that much higher inorganic carbon concentrations in the culture could enhance the PHB production. A study already evaluated the gene expression and the proteomics of Synechocystis sp. PCC 6803 when changing the CO_2 content from high (up to a 5%) to low levels (Spät et al., 2021). However, they did not observe significant changes in the proteins directly related with the PHB production, but in the ones related with bicarbonate uptake and carbon and nitrogen assimilation. Another study also tested high CO_2 contents for the production of PHB, lipids and glycogen (Eberly and Ely, 2012). It was observed much higher PHB content when the thermophilic cyanobacteria *Thermosynechococcus elongatus* was grown under atmospheric CO_2 rather than with high CO_2 content (up to 20%). Similarly, they observed a decrease in the PHB content when a high content of $NaHCO_3$ (150 mM) was added. However, the experimental procedure when bicarbonate was used and the evolution of the inorganic carbon was not shown. In the case of glycogen, opposite trends were observed depending on whether CO_2 or $NaHCO_3$ were added (Eberly and Ely, 2012).

In the current study, the effect of inorganic carbon concentrations as $NaHCO_3$ (including very high amounts) on PHB production in *Synechocystis* sp. strain R2020 was tested. Four different concentrations of bicarbonate (0, 30, 120, 2000 mgC L^{-1}) were tested in 3 L lab scale photobioreactors under nutrients starvation. Under carbon limitation (0 mgC L^{-1}) it is expected that internal carbon storage compounds (glycogen and PHB) will be consumed as a carbon source. When moderate concentrations of bicarbonate are added (30 and 120 mgC L^{-1}) it is expected that the accumulated glycogen will be converted into PHB. Eventually, a high concentration of bicarbonate (2000 mgC L^{-1}) is tested with the aim to stimulate both PHB and glycogen accumulation. Furthermore, in order to gain insights in the effect of bicarbonate addition in cyanobacteria metabolism and to decipher which routes are promoted under this condition and how this promotion changes overtime, the expression levels of key genes related to glycogen and PHB metabolisms were analysed. This is the first time that an exhaustive analysis of the effect of bicarbonate addition on cyanobacterial PHB and glycogen production is addressed. These results should contribute to better understand the regulation of PHB production and how these two polymers are related under different abiotic conditions. This new knowledge may also be useful to create new mutants with an enhanced PHB productivity, as well as to optimize the cultivation conditions.

2. Material and methods

2.1. Media and reagents

Modified BG-11 growth medium was used in the reactors in order to obtain the desired concentrations of N and P. Reagents present in this medium, K_2HPO_4, $NaNO_3$, $NaHCO_3$, $CaCl_2{\cdot}2H_2O$, NaOH, Na_2EDTA, $NaHCO_3$ were obtained from Panreac (Barcelona, Spain). $MgSO_4{\cdot}7H_2O$, $C_6H_8FeNO_7$ (ammonium ferric citrate), $C_6H_8O_7$ (citric acid) and HCl were purchased from Scharlau (Barcelona, Spain). The reagents needed for N-NO_3^-, N-NO_2^- and P-PO_4^{3-} analysis were also acquired from Panreac (Barcelona, Spain). HCl, chloroform ($CHCl_3$) and D-glucose needed for glycogen and PHB analysis were purchased from Scharlau (Barcelona, Spain). The *co*-polymer PHB-PHV (86:14% in weight, CAS 80181–31–3) standard was purchased from Sigma-Aldrich (St. Louis, US). Reagents for RT-qPCR were obtained from Thermo fisher Scientific (Waltham, USA).

2.2. Strain and inoculum preparation

The *Synechocystis* sp. strain R2020 (from now on named *Synechocystis* sp.) used in this study was purified from a demonstrative microalgae based wastewater treatment plant as previously described in (Rueda et al., 2020). Its closest relative deposited in the NCBI Genebank database was *Synechocystis* sp. strain YACCYB507 (see the phylogenetic tree in supplementary materials). Note that this is a monoculture of cyanobacteria, but this strain is not axenic and other bacteria has been also identified as shown in (Rueda et al., 2020). Note that wastewater is a relevant alternative for the cultivation of cyanobacteria and bioproducts production.

Therefore, this strain was selected for this study, as it is in theory better adapted to wastewater growing conditions rather than other *Synechocystis* sp. strain (Rueda et al., 2020). *Synechocystis* sp. strain was maintained and grown in BG-11 medium (1500 mg·L^{-1} $NaNO_3$, 31.4 mg·L^{-1} K_2HPO_4, 36 mg·L^{-1} $MgSO_4$, 36.7 mg·L^{-1} $CaCl_2{\cdot}2H_2O$, 20 mg·L^{-1} Na_2CO_3, 1 mg·L^{-1} NaMgEDTA, 5.6 mg·L^{-1} citric acid, 6 mg·L^{-1} Ferric ammonium citrate and 158 mg·L^{-1} $NaHCO_3$) and used as inoculum for the present study. Cultures were continuously mixed by means of sterile air bubbling and kept under 2.1 klx (approximately 37 μmol m^{-2} s^{-1}) and 30 ± 2 °C. Weekly, approximately a third part (330 mL) of the culture was replaced by fresh BG-11 medium, in order to ensure continuous growth.

2.3. Experimental set up

Experiments were carried on in four lab scale (working volume of 3 L) glass photobioreactors (PBR) with a diameter of 11 cm. It is well known that PHB accumulation occur under nutrient limitation (Drosg et al., 2015; Estel Rueda and García, 2021), therefore, two different cultivation phases were used: first, one to grow the cyanobacteria and a second one to stimulate the PHB accumulation. DIC was added only during the accumulation phase, in order to ensure that its effect was not caused by other factors or different biomass states during the growth phase. Experiments were performed in batch in a self-limiting one step culture. To do that, at the beginning of the experiment, during the growth phase, enough nutrients (40 mgN L^{-1} and 7 mgP L^{-1}) to reach nitrogen starvation when light became limiting were added. Nutrients were periodically monitored to detect the point at which the culture had depleted all the nitrogen. Once N was depleted, the content of the four reactors was mixed in a container to ensure an equal state of the biomass before starting the accumulation phase. After mixing, the culture was split into the 4 reactors for the accumulation phase. It was in this phase when different amounts of bicarbonate were added to evaluate the effect of carbon on PHB and glycogen accumulation (Fig. 1).

Reactors were continuously stirred by means of a magnetic stirrer (VELP scientifica, Usmate, Italy) and they were illuminated in day:night cycles of 15 h light and 9 h darkness. Illumination was provided by using 4 external 14 W cool-white LEDs placed at a distance of 20 cm from the reactor and 2.5 m of strip LEDs rolled up around the reactor. All together this provided a homogeneous average illumination of 4 klx (approximately 73 μmol m^{-2} s^{-1}) measured with a luxmeter (HI 97500, HANNA instruments, Italy). pH was recorded with a pH probe (HI1001, HANNA instruments, Italy) inserted into the mix liquor of each PBR and connected to a pH controller (HI 8711, HANNA instruments, Italy) that activated an electrovalve or a peristaltic pump that allowed the injection of CO_2 or HCl (0.1–1 M) in the reactor depending on the culture phase (see below). pH was kept between 7 and 9, which is the optimum reported pH for the genus *Synechocystis* (Touloupakis et al., 2016). Temperature was daily measured off-line and kept at 31.1 ± 0.6 °C.

The four PBRs were initially inoculated with 100 mgVSS L^{-1}, and with enough concentration of bicarbonate N and P (103 mgC L^{-1}, 40 mgN L^{-1} and 7 mgP L^{-1}) in order to reach approximately 1 gVSS L^{-1} when these nutrients were completely depleted (10 days approximately). Nutrients concentration were decided based on previous experiments from the authors (Rueda et al., 2020; Rueda and García, 2021). Since the control reactor was not supposed to have inorganic carbon on it during the accumulation phase, during the growth phase HCl (1–0.1 M) was used to control pH and also to decrease the dissolved inorganic carbon (DIC) content in order to reach 0 mgC L^{-1} before the accumulation phase started.

As mentioned above, once nutrients were depleted, the biomass in the four PBRs was mixed in a 11 L bottle (Nalgene, Waltham, USA) and split in the same 4 reactors to start the accumulation phase. 2 L (instead of the 3 L used for the growth phase) were used for the accumulation phase, because part of the initial culture was evaluated for nutrients and biomass content during the growth phase. During the 26 days that the accumulation phase lasted, different DIC concentrations were maintained by adding the corresponding amount of sodium bicarbonate (Fig. 1). In the first photobioreactor (PBR 1) no additions of DIC were done and pH was controlled by means of HCl additions. In the second photobioreactor (PBR 2) a moderate-low concentration of DIC (30 mgC L^{-1}) was maintained. Note that this low concentration of DIC is extremely difficult to maintain if CO_2 is used to regulate the pH, as the amount of carbon added when CO_2 is injected is difficult to adjust (Rueda et al., 2020). Therefore, in this case, 1 mL of sodium bicarbonate with a concentration of 10 g C L^{-1} was added daily in order to provide approximately 5 mgC L^{-1}, which was the

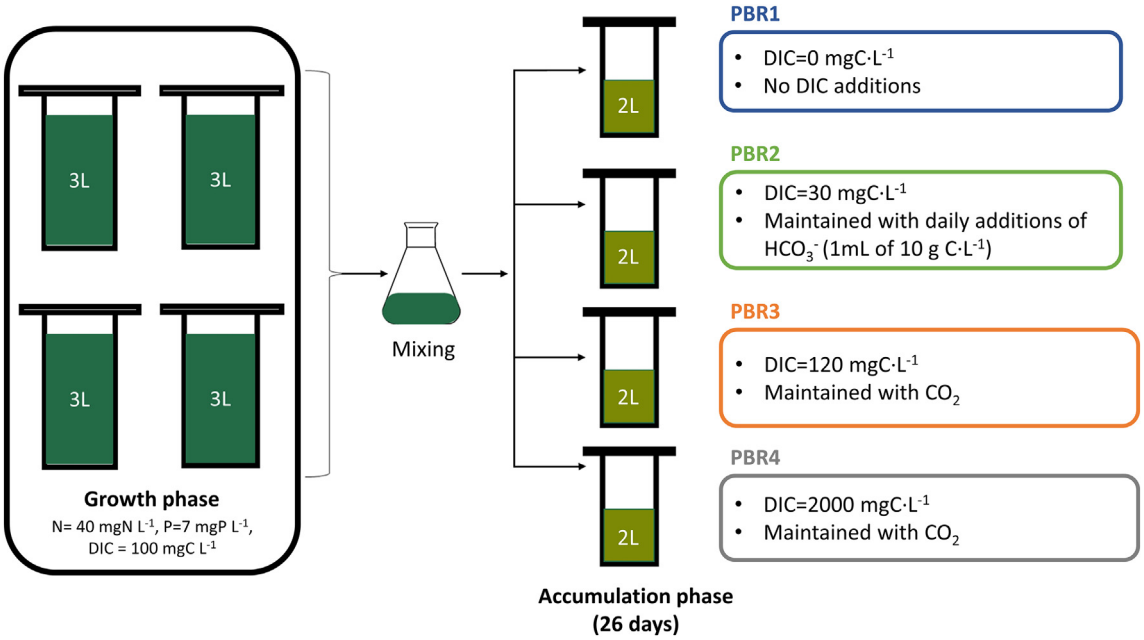


Fig. 1. Graphical representation of the experimental set up. In the growth phase four reactors of 3 L each were inoculated and operated equally. When nutrients were depleted, the biomass from all reactors was mixed to ensure that all experimental conditions in the next phase started from the same biomass. Afterwards, biomass was separated into the same 4 reactors and fed with different amounts of dissolved inorganic carbon (DIC) in each case.

DIC concentration expected to be consumed in 1 day, maintaining therefore the initial 30 mgC L^{-1} concentration. pH in this reactor was also controlled by HCl additions, in order to keep the concentration of carbon at the desired value. In the third photobioreactor (PBR 3) a moderate-high concentration of DIC (120 mgC L^{-1}) was maintained. In this case pH was controlled by injecting CO_2 in to the reactor. These CO_2 injections provided enough carbon to replace the carbon consumed by cyanobacteria. Finally, photobioreactor 4 (PBR 4) had a high content of DIC (2000 mgC L^{-1}). pH and DIC concentrations were maintained as done in PBR 3. DIC content was periodically monitored as described in Section 2.4.

To sum up, four reactors were used to grow cells in the growth phase, after which the reactor contents were mixed together and redistributed back into the four reactors (in order to have the same initial conditions for the accumulation phase). The accumulation phase was studied with four different levels of DIC. In reactors with a moderate-low or without DIC, HCl was used to control the pH. In reactors with a moderate-high or high concentration of DIC pH was controlled by adding CO_2 (Fig. 1).

2.4. Analytical methods

2.4.1. Nutrients analysis

Nutrients (N, P and C), volatile suspended solids (VSS) and turbidity were measured periodically in order to evaluate nutrients uptake rate, cyanobacteria growth rates and to study nutrient evolution and their depletion to allow starting the accumulation phase.

Total nitrogen (TN), total dissolved nitrogen (TDN), nitrite ($N\text{-}NO_2^-$) and ammonia ($N\text{-}NH_4^+$) where measured once per week. Nitrate ($N\text{-}NO_3^-$) was measured daily because is the main form of nitrogen in the BG-11. Dissolved organic nitrogen (DON) and particulate nitrogen (PN) were calculated as described in Eqs. (1) and (2). The colorimetric methods 4500-NO_2^- and 4500-NO_3^- described in Standard methods were used to measure $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ respectively (APHA et al., 2012). $N\text{-}NH_4^+$ was measured using the methodology described in (Solórzano, 1968). TN and TDN were analysed using a C/N analyser (2005, Analytikjena, Germany). Phosphate ($P\text{-}PO_4^{3-}$) was measured daily by the colorimetric method 4500-PE described in the Standard methods (APHA et al., 2012).

$$\text{Dissolved organic nitrogen (DON)} = \text{TDN}-\text{N}-\text{NO}_2-\text{N}-\text{NO}_3-\text{N}-\text{NH}_3 \quad (1)$$

$$\text{Particulate nitrogen (PN)} = \text{TN}-\text{TDN} \quad (2)$$

In order to control that the DIC content was properly maintained, soluble alkalinity M and P were measured using a photometric kit from Lovibond (Tintometer, Amesbury, UK). Alkalinity can be related with DIC as described in Eq. (3). To check that this approach was accurate, DIC was measured once per week together with the TDN by a C/N analyser (2005, Analytikjena, Germany). In average, differences between DIC calculated by using alkalinity or the DIC measured by the C/N analyser were less than 10%. Note that to determine the all dissolved species, samples were previously filtered through 0.7 μm pore glass microfiber filter.

$$\begin{aligned}\text{DIC}\left(\text{mg L}^{-1}\right) &= \text{H}_2\text{CO}_3\left(\text{mg L}^{-1}\right) + \text{HCO}_3^-\left(\text{mg L}^{-1}\right) \\ &\quad + \text{CO}_3^{-2}\left(\text{mg L}^{-1}\right) \\ &= (\text{Alk}-\text{M})-(\text{Alk}-\text{P})\end{aligned} \quad (3)$$

Total suspended solids (TSS) and volatile suspended solids (VSS) were measured every 2 days using the methods 2540C and 2540 D described in Standard Methods (APHA et al., 2012). Turbidity (HI93703, HANNA Instruments) was daily measured as a fast-rough estimation of biomass growth. Turbidity was related to VSS with a calibration curve done by measuring VSS and turbidity at different cultivation times in a culture of *Synechocystis* sp. strain. (see Supplementary material).

Dissolved oxygen (DO) was measured daily with an oxygen meter (HI94142, HANNA Instruments) to evaluate the activity of the culture.

2.4.2. PHB and carbohydrates analysis

PHB and carbohydrates (CHO) concentrations were measured from freeze dried biomass samples (−110 °C and 0.05 hPa, Scanvac, Denmark) once a week during growth phase and twice per week during the accumulation phase. Carbohydrates were measured using the method described by Dubois et al. (1956). In short, 2 mL of 1 N HCl were added to approximately 1–2 mg of freeze-dried biomass and heated to 100 °C for 2 h in a dry-heat thermo-block (Selecta, Spain). Then, 0.5 mL of phenol (5% w/v) and 2.5 mL of concentrated sulfuric acid were added to 1 mL of the digested sample. Samples were mixed and left at room temperature for 10 min. Then, they were introduced in a water bath for 15 min at 35 °C. Finally, the carbohydrates content was determined spectrophotometrically at 492 nm (Spectronic Genesys 8, Spectronic instruments, UK).

PHB was analysed using the method of (Lanham et al., 2013). Around 3–3.5 mg of biomass were weighted and mixed with 1 mL of CH_3OH which contained H_2SO_4 (20% *v*/v) and 1 mL of $CHCl_3$ with 0.05% *w*/w of benzoic acid as internal standard. Then, the samples were incubated for 5 h at 100 °C in a dry-heat thermo-block (Selecta, Spain) Afterwards the reaction was stopped introducing the samples in a cold-water bath for 30 min. Then, 1 mL of deionized water was added to the samples and vortexed. $CHCl_3$ remained in the bottom phase with the PHB dissolved on it and was carefully recovered with a glass pipette. Samples were analysed by gas chromatography (GC) (7820A, Agilent Technologies, USA) using He at 4.5 mL/min as a gas carrier, an injector split ratio of 5:1 and with a temperature of 230 °C and FID temperature of 300 °C. A column DB-WAX 125–7062 was used (Agilent, USA). The concentration of PHB was determined by using a standard curve of the *co*-polymer PHB-HV.

2.5. Primer design and preparation of standards

Genes encoding for key enzymes in the synthesis and catabolism of glycogen (*fbp, glgC, glgA, glgP1, glgP2, glgX*), the synthesis of PHB (*thl, phaB, phaC*) and the introduction of Acetyl-CoA into the tricarboxylic acid cycle (TCA) (*gltA*) were studied to evaluate the effect of incubation conditions on the carbon fluxes inside the cell (Fig. 2). Primers were designed using Primer-BLAST to specifically target the abovementioned genes in members of *Synechocystis*. The designed primers were corroborated by manual alignment against all the sequences available for the corresponding gene within this genus at the National Center for Biotechnology (NCBI; http://www.ncbi.nlm.nih.org/blast/), and experimentally evaluated by PCR using genomic DNA of our *Synechocystis* sp. monoculture obtained using FastDNA™ SPIN Kit (MP biomedicals, Irvine, USA) following the manufacturer instructions. Amplification was achieved using GO Taq® PCR mix (Promega, Madison, WI, USA) in a final volume of 25 μL, containing 1 ul of DNA extract and 25 pmol of the designed primers (Thermo fisher Scientific Waltham, USA) as described elsewhere (Gallego et al., 2014). Amplified fragments were cloned using the pGEM-T Easy Vector system (Promega, Madison, WI, USA), and plasmids purified with the GeneJet Plasmid Miniprep Kit (Thermo fisher Scientific, Waltham, USA). Purified plasmids were sent for sequencing (Macrogen Europe, Amsterdam, The Netherlands) to verify the amplification of the desired genes. Obtained sequences can be found in supplementary material. Eventually, the same clones were used as RT-qPCR standards for quantification and to evaluate amplification efficiency.

2.6. RNA extraction and RT-qPCR

Samples for molecular analyses were collected in triplicates from the reactors at the beginning of the accumulation phase, after 12 days of the starting of the accumulation phase (22 days of experimental time) and at the end of the experiment (36 days of experimental time). 1.5 mL samples of fresh-biomass were collected in triplicate and centrifuged at 11196 ×*g* at 4 °C and stored at −80 °C in an ultra-freezer (Arctiko, Denmark). RNA was isolated using the PureLink RNA Mini Kit (Ambion, Thermo fisher Scientific, Waltham, USA) following the manufacturer's recommendations, adding TRIzol and doing Bead Beating for cell lysis. DNA was removed after

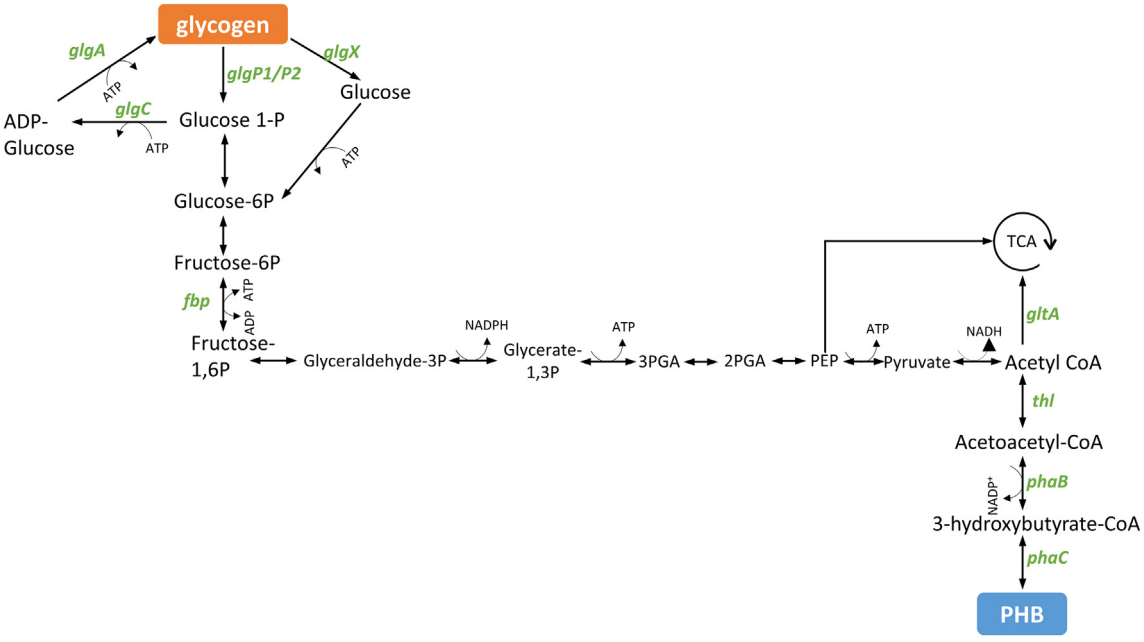


Fig. 2. Simplified representation of the glycogen/PHB metabolism in *Synechocystis* sp. Enzymes codified by the genes studied in this work are highlighted in green. Abbreviations: 3PGA: glycerate-3P; 2PGA: glycerate-2P; PEP: Phenolpyruvate; ATP: Adenosine triphosphate. *glgA*: glycogen synthase, *glgC*: Glucose-1-phosphate adenylyltransferase, *glgP1* and *glgP2*: glycogen phosphorylase, *glgX*: glycogen debranching enzyme, *gltA*: Citrate synthase, *thl*: Acetyl-CoA acetyltransferase, *phaB:* Acetyl-CoA reductase, *phaC:* poly(3-hydroxyalkanoate) synthase.

purification by doing a treatment with DNasa I (Thermo fisher Scientific, Waltham, USA).

Reverse transcription was done using the RevertAid™ Kit (Thermo fisher Scientific, Waltham, USA). 1 μL of the extracted RNA was used for the reverse transcription, together with a combination of random hexamers and oligo (dT) primers following manufactures instructions.

The gene expression was determined using the qPCR thermocycler Quantstudio 3 (Thermo fisher Scientific, Waltham, USA). To do this, the designed primers at 300 nM and the Powerup SYBR master mix (Thermo fisher Scientific, Waltham, USA) were used.

As previously done by Kamravamanesh et al. (2018b), the 16S RNA, the subunit of the ribonuclease P and the Cytochrome b6-f complex subunit genes, which maintain constant expression under normal growth conditions and under N and P starvation were used as normalizers. The selected housekeeping gene was the one with lower variability between the different conditions tested in this case (*petA*). The ΔCt was calculated by subtracting the mean Ct of the control sample (PBR 1-without bicarbonate addition) to the target sample (PBR 2, PBR 3 and PBR 4). Relative expression was calculated as shown in Eq. (4). $\Delta C_{t,tar}$ is the ΔCt calculated for the target gene, and $\Delta C_{t,ref}$ is the ΔCt calculated for the housekeeping gene. Primers efficiencies ($E_{tar,\ ref}$) were calculated by using the slope of a calibration curve with 6 dilutions of the standard per duplicate as shown in Eq. (5).

$$Relative\ expression = \frac{E_{tar}^{\Delta Ct,tar}}{E_{ref}^{\Delta Ct,ref}} \tag{4}$$

$$Etar, ref = 10^{\left(\frac{-1}{slope}\right)} \tag{5}$$

2.7. Calculations

Kinetic coefficients were calculated as follows:

Specific growth rate (d^{-1}) was calculated using the general formula:

$$\mu_X = \frac{\ln(X)_{t2} - \ln(X)_{t1}}{t2 - t1} \tag{6}$$

Where $\ln(X)_{t2}$ and $\ln(X)_{t1}$ are the natural logarithm of the biomass concentration given in mgVSS L^{-1} at the end of each phase (t2) and at the beginning of each phase (t1), respectively. The term t2-t1 is the time span (in days) at which the growth rate is calculated (beginning and end of each phase).

Doubling time (in days) was calculated as:

$$Doubling\ time = \frac{\ln(2)}{\mu_X} \tag{7}$$

Average volumetric growth rate (mg L^{-1} d^{-1}) was calculated as:

$$r_{biomass} = \frac{\sum_{i=1}^{i=n} \frac{VSS_i - VSS_{beginning}}{ti - t1}}{N} \tag{8}$$

Where n is the total number of days in the phase and i the actual day of the experiment. t1 is the day at which the phase started and $VSS_{begining}$ are the VSS at the beginning of the phase. N are the number of points considered in the calculation.

Biomass to nutrients yield were calculated for the growing phase as:

$$Y_{X/N-NO3,P-PO4} = \frac{VSS_{t2} - VSS_{t1}}{N,P_{t1} - N,P_{t2}} \tag{9}$$

Where VSS_{t2} (mg L^{-1}) and VSS_{t1} (mg L^{-1}) are biomass concentrations at the end of each phase (t2) and at the beginning of the phase (t1). N, P_{t2} (mg L^{-1}) and N, P_{t1} (mg L^{-1}) are the nutrient (N-NO_3 or P-PO_4) or concentration at the end and at the beginning of each phase.

Specific consumption rate (mgN, P · mg VSS^{-1} d^{-1}) was calculated as:

$$q_{\frac{X}{N}-NO3,P-PO4} = \frac{\mu_X}{Y_{X/N-NO3,P-PO4}} \tag{10}$$

Carbohydrates and PHB content were calculated as:

$$\%dcw_{CHO,PHB} = \frac{mg\ (CHOPHB)}{mg\ (DCW)} \cdot 100 \quad (11)$$

Average volumetric PHB or carbohydrates productivity (mg L^{-1} d^{-1}) of carbohydrates and PHB in each phase was calculated as:

$$r_{PHB,\mathrm{CHO}} = \frac{\sum_{i=1}^{n} \frac{PHB,\mathrm{CHO}_i - PHB,\mathrm{CHO}_{beginning}}{duration\ of\ the\ phase}}{N} \quad (12)$$

Where PHB,CHO_i, $PHB,CHO_{beginning}$ in (mgPHB,CHO L^{-1}) are the PHB or carbohydrates concentrations in the reactor at the actual time i or at the beginning of the studied phase. n is the total number of days in the phase and i the actual day of the experiment and N are the number of points considered in the calculation.

2.8. Statistical analysis

Statistical analysis was performed using the statistical software GraphPad Prism. A two-way ANOVA with multiple comparison was done to evaluate the differences between samples of the different reactors. Pearson correlation coefficients were calculated to evaluate the possible interaction between genes. *P*-values lower than a 10% were considered statistically significant.

3. Results and discussion

3.1. Effect of DIC on biomass growth and nutrients uptake

During the growth phase the average specific growth rate was 0.14 ± 0.03 d^{-1}. By the end of this phase 0.4 ± 0.06 gVSS L^{-1} were observed. $N\text{-}NO_3^-$ was assimilated at a specific consumption rate of 20.1 mg N $gVSS^{-1}$ d^{-1}, $P\text{-}PO_4^{3-}$ at 1.9 mgP g VSS $^{-1}$ d^{-1} and DIC at 67.7 mgC g VSS $^{-1}$ d^{-1} (Table 1). These results are in agreement with previous findings from the authors using the same strain and reactor design (Rueda et al., 2020). In fact, in these previous work, lower specific growth rates and nutrients consumption (μ = 0.08 d^{-1}, q_N = 5.9 mgN $gVSS^{-1}$ d^{-1}, q_P = 0.4 mgP $gVSS^{-1}$ d^{-1}) were observed there due to lower irradiance in the reactor (36 μmol photon·m^{-2} s^{-1} with respect to 73 μmol photon m^{-2} s^{-1} in the present study). However, in other studies higher values were obtained for other *Synechocystis* strains. Kamravamanesh et al. (2019) found with *Synechocystis* sp. PCC 6714 a specific growth of 1 d^{-1} and a consumption of $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ of 242 mgN $gVSS^{-1}$ d^{-1} and 4.4 mgP $gVSS^{-1}$ d^{-1}, respectively. Gracioso et al. (2021) found with a mangrove-isolated *Synechocystis* sp. and similar irradiance to that of this study, but using a 250 mL Erlenmeyer, a specific growth rate of 0.9 d^{-1}. These differences could be attributed to differences between strains or most probably to different reactor designs (note that reactors used in the aforementioned studies had approximately a third of the reactor diameter used in the present study) which could have enhanced light penetration. Also note that these differences are not relevant for the purposes of the present study.

After the growth phase, when nitrogen was already depleted, the effect of DIC availability on VSS was evaluated (Fig. 3). The highest concentration of VSS (877 mgVSS L^{-1}) was reached in PBR 4, which was the reactor with a higher inorganic C content. Similarly, VSS also increased in the other reactors in this phase reaching a maximum concentration of 625 mgVSS L^{-1}, 583 mgVSS L^{-1} and 653 mgVSS L^{-1} for PBR 1, 2 and 3, respectively. Note that in all PBRs biomass content increased, even after nitrogen depletion. This could be explained by the ability of cyanobacteria to luxury uptake N and P. Thanks to this mechanism, under N depletion, cyanobacteria can continue growing for a short period using the nutrients stored during the previous phase (Zhang et al., 2015). Moreover, it should be noted that here VSS are measured, which accounts for the total biomass weight. Under nitrogen limitation cyanobacteria accumulate carbon storage compounds (glycogen and PHB), which may also increase the weight of the cells. Interestingly, a high concentration of bicarbonate caused an undoubtable increase in the biomass production and nutrients consumption rate in this study. Similar results were obtained by Gracioso et al. (2021), who observed a higher final biomass concentration and a higher growth rate in a culture of *Synechocystis* sp. with 5 g L^{-1} of sodium bicarbonate than in a culture only fed with atmospheric air (0.92 gC L^{-1} with respect to 0.48 gC L^{-1}). Vajravel et al. (2020), also observed a positive impact in ethylene production, cell growth and a prolongation of cell vitality when 1.2–2.4 gC L^{-1} of $NaHCO_3$ were added in a *Synechocystis* sp. genetically modified to produce ethylene. However, they also observed that high amounts of bicarbonate inhibited cell activity (>3.6 gC L^{-1}), probably due to osmotic pressure changes (Vajravel et al., 2020).

3.2. Effect of DIC on PHB and carbohydrates accumulation

Fig. 4 shows the accumulation profiles of PHB and carbohydrates. During the growth phase PHB was very low (<1%$_{dcw}$) while carbohydrates

Table 1
Average component concentrations, kinetic and stoichiometric parameters obtained during the growth phase and for each reactor condition in the accumulation phase. Average consumption and production rates were calculated as the average of the entire experimental phases. Data in brackets is the standard deviation of the calculated average values. Averages in the growth phase include data from the 4 reactors (they were replicates).

	Growth Phase	Accumulation phase			
		PBR 1	PBR 2	PBR 3	PBR 4
DIC (mg L^{-1})	52.2 (38.8)	14.1 (18.8)	78.7 (61.3)	112.5 (43.2)	1748.6 (668,8)
DO (mg L^{-1})	12.5 (4.8)	4.8 (2.2)	4.4 (2.3)	4.5 (2.3)	5.8 (2.2)
max VSS (mg L^{-1})	395	625	583	653	877
μ (d^{-1})	0.14 (0.03)	−0.016	0.023	0.04	0.037
Doubling time (d)	4.9 (1.5)	−43.32	30.14	17.33	18.73
$r_{biomass}$ (mgVSS L^{-1} d^{-1})	47.1 (4.8)	5.1 (24.7)	−9.8 (31.3)	4.9 (28.9)	34.5 (29.2)
q_P (mgP $mgVSS^{-1}$ d^{-1})	1.9 (0.7)	0.02	0.2	0.04	0.1
$Y_{X/P}$	76.7 (17.9)	314.2	106.6	253.0	201.4
q_N (mgN $mgVSS^{-1}$ d^{-1})	20.1 (8.3)	–	–	–	–
$Y_{X/N}$	7.9 (3.2)	–	–	–	–
q_C (mgC $mgVSS^{-1}$ d^{-1})	67.7 (7.6)	–	–	–	–
$Y_{X/C}$	2.1 (0.3)	–	–	–	–
Average PHB (%)	1.32 (0.4)	3.8 (1.9)	2.5 (1.8)	5.3 (3.3)	8.8 (3.9)
max PHB (%)	1.8	5.8	4.8	11.4	14.3
r_{PHB} (mgPHB L^{-1} d^{-1})	0.04 (0.01)	0.48 (0.8)	0.9 (2.0)	0.73 (1.4)	2.45 (1.9)
Average CHO (%)	9.7 (4.5)	15.2 (8.1)	16.1 (5.0)	12.7 (4.7)	12.6 (9.0)
max CHO (%)	14.9	28.7	25.4	18.4	29.8
r_{CHO} (mg gly L^{-1} d^{-1})	0.18 (0.03)	1.7 (3.0)	2.7 (5.3)	0.9 (2.7)	3.2 (2.3)

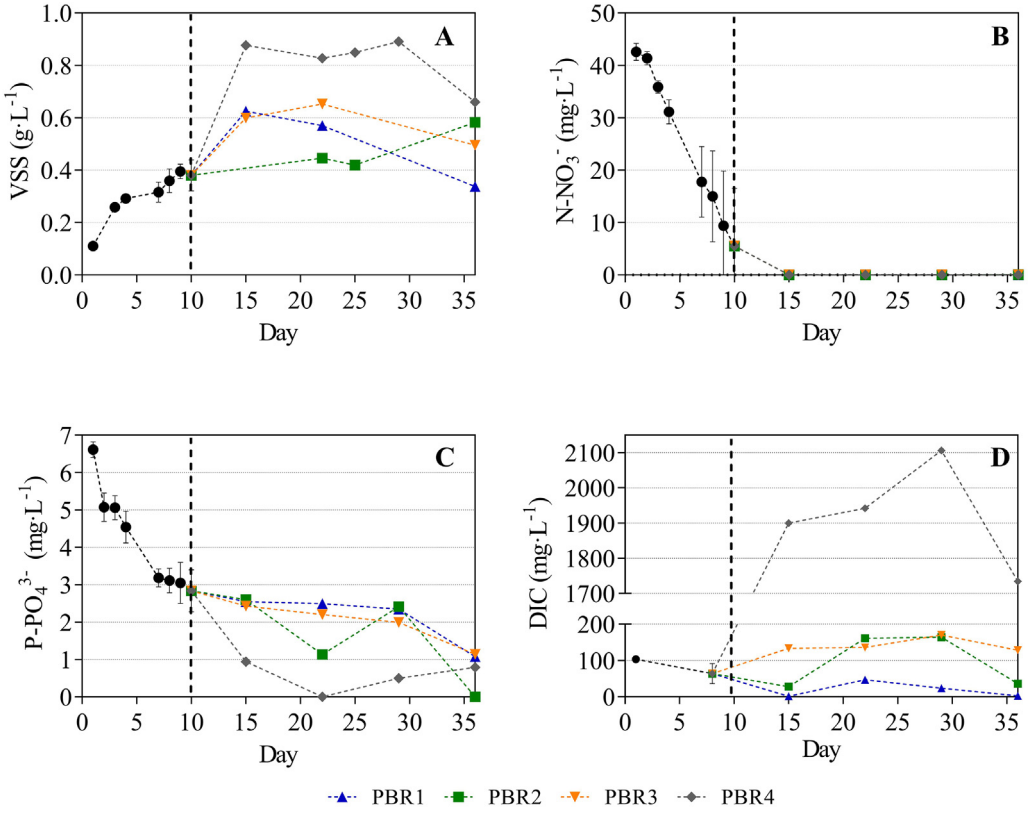


Fig. 3. Concentration changes in volatile suspended solids (A), $N\text{-}NO_3^-$ (B) PO_4^{3-} (C) and DIC (D). Vertical line separates the growth phase (left side) from the accumulation phase (right side). The values in the growth phase are the average and standard deviation (error bars) of the four reactors (replicates).

content was in average 12%$_{dcw}$. Carbohydrates content was in all phases significantly higher than PHB. After N depletion, a fast increase in carbohydrates content was observed in PBR 4 (max 29.8%$_{dcw}$) (Fig. 4). Similarly, but with 5 days of delay, an increase in carbohydrates content was observed in PBR 1 (max 28.7%$_{dcw}$). After reaching this peak concentration, carbohydrates slowly decreased in both reactors. PBR 2 and 3 had a more or less stable concentration during the accumulation phase (maximum content of 25.4%$_{dcw}$ and 18.4%$_{dcw}$ in PBR 2 and PBR 3 respectively). Interestingly, the reactor conditions that reached higher concentrations of carbohydrates were the ones in PBR 4 with a higher DIC content (2 gC L^{-1}) and the ones in PBR 1 with no additions of bicarbonate. These results show that there was no clear effect of inorganic carbon in carbohydrates accumulation, as PBR 2 and 3 had many lower points than PBR 1 (control condition).

PHB content increased in all reactors during the accumulation phase after N depletion. However, only PHB of PBR 3 and PBR 4 exceeded the 5%$_{dcw,}$ being PBR 4 the one with a higher PHB production (2.45 mgPHB L^{-1} d^{-1} and a 14%$_{dcw}$, see Table 1). Considering the time average and deviation of the entire accumulation phase, it can be observed that there are significant differences between reactors with low DIC (PBR 1 and 2) and the reactor with high DIC (PBR 4). However, although higher average PHB was obtained in PBR 4, no statistically significant differences were observed between PBR 3 and PBR 4.

On the other hand, in PBR 4 the increase in carbohydrates was followed by an increase in PHB (Fig. 4). It should be noted that although total carbohydrates are measured here, the glycogen accumulated in the cell is probably one of the major constituents of the total carbohydrates. Having this in

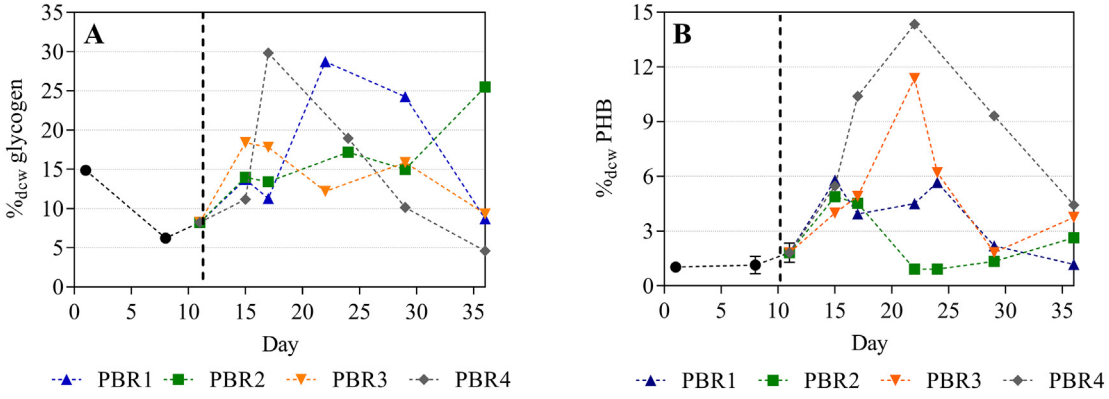


Fig. 4. Changes in the percentage of carbohydrates (A) and PHB (B). Vertical line indicates the beginning of the accumulation phase. The values in the growth phase are the average and standard deviation (error bars) of the four replicates.

mind, the increase in PHB could be related with the conversion from glycogen to PHB, which has been observed previously in other studies (Dutt and Srivastava, 2018; Kamravamanesh et al., 2018a; Koch et al., 2019; Rueda et al., 2020; Troschl et al., 2018). In the last years, there have been many attempts to explain the reason why PHB is produced in cyanobacteria and its relation with the glycogen metabolism. Khetkorn et al. (2016) and Sharma et al. (2007) observed that a wild type *Synechocystis* strain increased its PHB content in either N, P or a combined limitation of both. There, it was suggested that under N deficiency, the synthesis of amino acids is stopped and the NADPH consumption is hindered. PHB is produced to rebalance the NADPH/NADP ratio (Panda and Mallick, 2007). Moreover, due to chlorosis the amino acids released due to phycobiliproteins degradation, can be further used to synthetize glycogen and PHB (Khetkorn et al., 2016). Koch et al. (2019), also suggested that the transformation of glycogen into PHB is turned on during chlorosis to ensure a long-term survival of the cell. In this context, recently, Orthwein et al. (2021) identified a novel regulation mechanisms of the 2,3-phosphoglycerate–independent phosphoglycerate mutase (PGAM). Under N starvation 2-oxoglutarate is accumulated and the interactor PirC is released from P_{II}. PirC is then bound to PGAM what inhibits the conversion of 3-PGA into 2-PGA. The accumulation of 3-PGA promotes the activity of GlgC, which redirects the carbon flux towards glycogen production. During chlorosis this glycogen turnover remains constant and the residual acetyl-CoA molecules arising from the residual glycolytic flux are transformed into PHB during N starvation (Orthwein et al., 2021).

All in all, these results indicate that inorganic carbon stimulate the production of PHB in this strain. Results found here are in contradiction with, Kamravamanesh et al. (2017) and Eberly and Ely (2012), who observed a negative impact of CO_2 in PHB production in the cyanobacteria strain *Synechocystis* sp. PCC6714. This differences may be attributed to the use of a different strain, which may have different isoenzymes related to the glycogen and PHB synthesis (Gründel et al., 2012) and other salinity resistances. Eberly and Ely (2012) also found differences between the glycogen production when using different DIC sources. They reported a positive relation between bicarbonate additions and glycogen production, but a constant glycogen production by increasing the CO_2. All in all, these results suggest that carbon source has a strong influence in the cell metabolism. The reason why this happens is not clear, since considering bicarbonate equilibrium, the added CO_2 should be mainly transformed to bicarbonate at the pH (6–9) in which cyanobacteria cultures are usually maintained (Eberly and Ely, 2012). One factor that should be considered is that addition of sodium bicarbonate increases the culture electrical conductivity (from 1.8 mS cm^{-1} in the normal BG-11 to 12.9 mS cm^{-1} in the culture with 2 g L^{-1} of DIC) increasing the risk of osmotic stress which may promote the production of carbohydrates and PHB. This effect was observed in the halophilic bacteria *Haloferax mediterranei*, which increased the PHB stored along with an increase in electrical conductivity (from about 130 mS cm^{-1} to 320 mS cm^{-1}) (Cui et al., 2017).

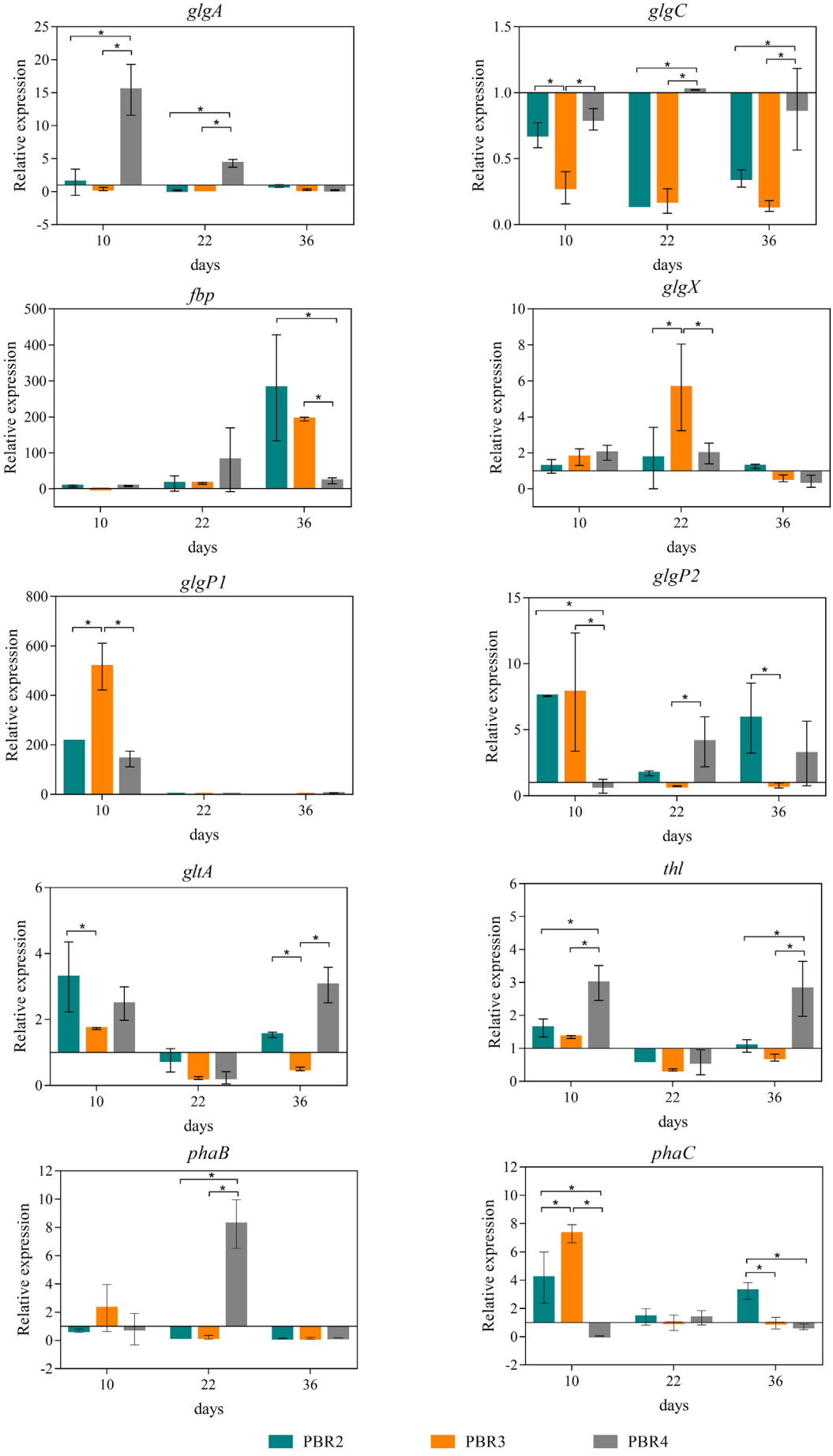
The maximum PHB obtained in this study is in agreement with that found by Troschl et al. (2018) with *Synechocystis* sp. CCALA192 and 129 mg L^{-1} of DIC under N deprivation. However, the productivity in the present study is slightly lower than the one found by Carpine et al. (2018), who obtained a productivity of 6.4 mgPHB L^{-1} d^{-1} or Kamravamanesh et al. (2017, 2018a), who obtained respectively, productivities of 59 mgPHB L^{-1} d^{-1} and 101 mgPHB L^{-1} d^{-1}. Further research is necessary to determine the optimal concentrations of DIC to find the optimal cultivation conditions for *Synechocystis* sp. strain. The optimal point must be chosen considering not only the higher PHB productivity, but also considering the increase of costs due to the use of bicarbonate. As previously mentioned, a possible solution to decrease costs could be the use of residual effluents rich in this component (Markou et al., 2014). Although the PHB production is stimulated by the addition of $NaHCO_3$, the content achieved here is still too low to make this process viable. To these authors knowledge, the highest PHB content reached autotrophically with a wild type *Synechocystis* sp., is the one obtained by Kamravamanesh et al. (2019), who achieved up to a 21%$_{dcw}$ with Synechocystis sp. PCC 6714. Higher PHB contents were obtained autotrophically using other cyanobacteria. For instance, Nishioka et al. (2001), reached a 62%$_{dcw}$ with the thermophilic *Synechococcus* sp. MA19. Or as well as, by generating mutants with a high PHB productivity, such as Kamravamanesh et al. (2018a), who obtained up to a 37%$_{dcw}$ with a randomly muted *Synechocystis* sp. MT_a24. Or Koch et al. (2020b), who obtained introducing genetic modifications up to a 63%$_{dcw}$ autotrophically. High PHB content was also obtained using organic carbon sources. Indeed, Panda and Mallick (2007), obtained a 38%$_{dcw}$ with *Synechocystis* sp. PCC 6803 supplemented with 0.4% of fructose and 0.4% of acetate. Competitive PHB contents can be achieved in cyanobacteria, if a better performing strain is found or by adding organic carbon sources. Nevertheless, the performance of this high production strains should be further evaluated at large scale facilities under non-sterile conditions.

3.3. Analysis of the expression of genes codifying for key enzymes in glycogen and PHB metabolism by quantitative real-time PCR

To unravel the effect of DIC in the regulation of the metabolism of glycogen and PHB by cyanobacteria, the expression of specific genes codifying for key enzymes was analysed in the different reactors (PBR 2-4) and compared with their expression level in the control reactor (the reactor without addition of bicarbonate, PBR 1). Results are shown in Fig. 5. At the beginning of the starvation phase (day 10) the reactor with the highest concentration of DIC, PBR 4, exhibited an overexpression of the genes related to the biosynthesis of glycogen, specially of gene *glgA,* codifying for glycogen synthase. This overexpression is directly related to the increase in glycogen accumulation (see Fig. 4). PBR 2 and 3 had a downregulation in the expression of this gene, what agrees with the lower amount of glycogen accumulated in these reactors. At day 22 the level of expression of the genes related to the glycogen synthesis decreased in comparison to day 10. However, the reactor with the highest content of inorganic carbon (PBR 4) was still the one with the highest expression for genes *glgA* and *fbp* (codifying for fructose-1,6-biphosphatase). Gene *glgC* (glucose-1-phosphate adenylyltransferase) was downregulated in all reactors, meaning that the control reactor had a higher expression of this gene (note that PBR 1, which was the reactor with the second highest glycogen production, was the one used as a control reactor). However, its expression in PBR 4 was still higher compared to the other two reactors. Eventually, by the end of the experiment, the glycogen synthesis was downregulated in all reactors except for gene *fbp*.

PBR 4 also had an overexpression of the genes related with the glycogen catabolism *glgP1* and *glgX* (codifying for glycogen phosphorylase and for a glycogen debranching enzyme, respectively). However, PBR 3 (the reactor with a medium-high concentration of DIC) presented a higher overexpression of this route (genes *glgP1, glgP2* and *glgX* were overexpressed) at the beginning of the starvation. At day 22, PBR 2 and 4 had an overexpression of the gene *glgP2* (glycogen phosphorylase), while an overexpression of gene *glgX* was observed in PBR 3. This trend suggests that glycogen is being catabolized in these two reactors by different metabolic pathways. No differences with respect to the control reactor were observed at day 22 for the *glgP1* expression. This is in agreement with the results by Koch et al. (2019) who observed that a mutant without the gene *glgP1* was equally able to recover from chlorosis and produced the same amount of PHB than the wild type strain, while the mutant without the gene *glgP2* shown a lower capacity to recover from chlorosis and a lower PHB production.

Fig. 5. Relative expression levels compared to PBR 1 of the genes involved in the glycogen synthesis (*glgA, glgC, fbp*), the glycogen catabolism (*glgP1, glgP2, glgX*), the PHB synthesis (*thl, phaB, phaC*) and the transformation of Acetyl-CoA and oxaloacetate to citrate to begin the TCA cycle (*gltA*). Bars represent the mean value and error bars the standard deviation from three independent measures. Statistical analysis was performed using a Two-way ANOVA. * denote statistical significance (p-values < 0.1).


glgA
glgC
fbp
glgX
glgP1
glgP2
gltA
thl
phaB
phaC
Relative expression
days
10
22
36
PBR2
PBR3
PBR4

Doello et al. (2018), also observed that *glgP1* was not regulated, whereas *glgP2* was up-regulated under nitrogen starvation and repressed again when cells are resuscitated from chlorosis. These results show that glycogen granules and glycogen-degrading enzymes are produced simultaneously. This is done to anticipate and prepare the cell for resuscitation from chlorosis, so that glycogen degradation can start as soon as glycogen is available again (Doello et al., 2018). Recently, Selim et al. (2021) observed that the HCO_3^- transporter SbtA, interacts with the second messenger nucleotide *c*-di-AMP to generate the complex SbtB:*c*-di-AMP that regulates the action of the glycogen-branching enzyme GlgB. During the night the complex SbtB:*c*-di-AMP disappears, due to the decrease in the concentration of *c*-di-AMP. Then, the GlgB is deactivated and the glycogen catabolism starts to produce energy for night time survival. GlgP2 may also play a role in this mechanism enhancing the affinity of SbtB for GlgB (Selim et al., 2021).

On the other hand, PBR 4 and PBR 2 presented a higher expression of *gltA,* which codifies for citrate synthase, a pace-making enzyme in the first step of the TCA cycle. Usually PHB is produced when citrate synthase activity is decreased and there are high levels of Acetyl-CoA and NADH (Shrivastav et al., 2010). The higher expression of *gltA* in these reactors, indicates that part of the Acetyl-CoA is derived to the TCA cycle instead of being used to produce PHB. PBR 3 seems to use carbon more efficiently to produce PHB, since this route is in general downregulated. Interestingly, *gltA* was downregulated in the reactors with higher carbon content (PBR 3 and PBR 4) when the maximum concentration of PHB was obtained.

Regarding the metabolism of PHB, gene *phaB* (Acetoacetyl-CoA reductase) was highly overexpressed in PBR 4 but only at day 22. Previous studies suggested that PhaB is the controlling enzyme of the PHB pathway in heterotrophic bacteria (Tyo et al., 2010). This agrees with results in this study, since the reactor with a higher PHB content was the one with a higher expression of this gene. An overexpression of the gene *thl* (Acetyl-CoA acetyltransferase), at the beginning and at the end of the accumulation phase was also detected in PBR 4. Surprisingly, the genes *thl* and *phaB,* follow a complete opposite tendency in PBR 4. The *Synechocystis* sp. used in this study was isolated from a local wastewater treatment plant, and has not been fully sequenced yet and the exact location of genes *thl* and *phaB* is still unknown. Nevertheless, based on previous studies these two genes should be placed in the same operon (Silvestrini and Drosg, 2016). In order to check the configuration of these genes in this strain, the *thl* Forward and *phaB* Reverse primers designed in this study were used to amplify this region. The amplified region had approximately 1200 bp. To further confirm that the amplified sequence corresponded to the *thl-phaB* operon, it was sequenced (see Supplementary Materials). According to the ncbi-BLAST alignment, the segment of the amplified sequence going from base 1 to 799 were identified as gene *thl* (acetyl-CoA C-acyltransferase). Positions 907-1069 were found to correspond to gene *phaB* (beta-ketoacyl-ACP reductase). Surprisingly, there is a well conserved region between the two genes of approximately 100 bp also observed in other *Synechocystis* sp. These results confirm that these two genes are placed in the same operon, which is the most typical configuration for most cyanobacteria producing PHB (Matsumoto et al., 2013) and in this case gene expression found in this study will be contradictory with this fact. Despite that, it cannot be yet discarded that other copies of this genes exist in a separate region of the genome. As happens in *cupriavidus necator,* which have more than one copy of the gene *phaB* and these copies are found in different operons (Silvestrini and Drosg, 2016). Krasaesueb et al. (2021), also observed with a ΔsphU *Synechocystis* sp. PCC 6803 a higher overexpression of *phaB* in contrast to *thl*. These differences could be attributed to the operon organization, as a higher transcription distance decreases the gene expression (Lim et al., 2011). Further research should be done in the PHB regulation mechanisms to decipher how this particular condition could have affected the expression of these genes.

Although PBR 4 was the reactor with a higher PHB content, PBR 2 and 3 had a higher expression of *phaC* (polyhydroxyalkanoate synthase) at the beginning of the accumulation phase and an equal expression in all reactors for the subsequent experimental times. Similarly, Osanai et al. (2013) found comparable or lower expression of the polyhydroxybutyrate synthases (comprising subunits *phaC and phaE*) in a *Synechocystis* sp. PCC 6803 mutant overexpressing the *sigE* gene that produced 2.3 times more PHB than the wild type. Furthermore, the level of the PhaC and PhaB proteins in the insoluble and soluble fractions were similar or decreased with respect to the wild type strain after 3 days of N depletion. Therefore, results obtained in the present study together with those of Osanai et al. (2013) suggest that a higher PHB synthase expression is not a necessary condition to have a higher PHB content. Indeed, Hiroe et al. (2012) found a negative correlation between PHB synthetase activity and molecular weight of the polymer. Another explanation for the similar *phaC* expression in all reactors

A

		PBR2			PBR3			PBR4		
DIC (mgC L^{-1})		30			120			2000		
Time (days)		10	22	36	10	22	36	10	22	36
Synthesis glycogen	*glgA*	0	-	0	0	-	-	+	+	-
	glgC	-	-	-	-	-	-	-	0	-
	fbp	0	0	+	0	0	+	0	+	+
Catabolism glycogen	*glgP1*	+	0	0	+	0	0	+	0	0
	glgP2	+	+	+	+	0	-	0	+	+
	glgX	0	0	-	+	+	-	+	0	-
TCA cycle	*gltA*	+	+	+	0	-	-	+	-	+
PHB metabolism	*thl*	0	0	0	0	0	-	+	0	+
	phaB	-	-	-	0	-	-	-	+	-
	phaC	+	0	+	++	0	0	-	0	-

B

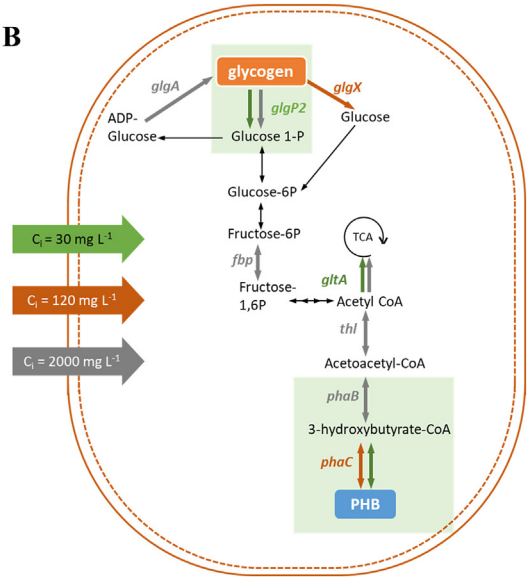


Fig. 6. Summary of the overexpression (" + " or darker colour) or downregulation ("- "or lighter colour) or same expression levels than control reactor ("0" or white colour) for each of the studied genes in each reactor and experimental time (A). And simplified representation of the glycogen and PHB metabolic pathways overexpressed in each condition (B). In A, blue cells represent the genes related to glycogen synthesis, green cells correspond to the genes related with glycogen catabolism, brown cells to the beginning of the TCA cycle and blue-green cells to the PHB synthesis. In B, green arrows represent the metabolic reactions whose genes are overexpressed for PBR 2 (DIC = 30 mgC L^{-1}), orange arrows the ones for PBR 3 (DIC = 120 mgC L^{-1}), grey arrows the ones for PBR 4 (DIC = 20,000 mgC L^{-1}). Green shaded reactions indicate the intercorrelated metabolic routes.

could be that the PhaC protein is post-transcriptionally regulated (Krasaesueb et al., 2021; Nakaya et al., 2015; Osanai et al., 2013). Further investigation in protein concentrations, including the activities of the different enzymes involved in the PHB and glycogen metabolism and the carbon fluxes towards the pools of ADP-glucose and Acetyl-CoA should be done in the future to confirm this hypothesis.

In general terms, PBR 4 had an overexpression of genes related to glycogen and PHB synthesis (except for *phaC*), but as can be seen in Fig. 5 this overexpression is not permanent and stable in time. This made the differences in gene expression not evident because not always the PBRs with the highest DIC content had the higher overexpression. These results, however, agree with the observations made by Kamravamanesh et al. (2018a) and Osanai et al. (2013), who observed no differences in the gene expression of genes related to the PHB metabolism between the wild strain and a mutant, which had a higher PHB productivity. These suggest that these genes are post-transcriptionally regulated and that not always a higher PHB production is related with a higher gene expression. More research efforts should be done in deciphering the post-transcription regulation of these genes and how the environmental conditions affect this regulation. Nevertheless, a positive correlation (Pearson correlation coefficient of 0.7364 and *p*-value 0.0237) between genes *glgP2* and *phaC* was observed. Similar results were obtained for *glgP2* and *phaB* (Pearson correlation coefficient of 0.8608 and p-value 0.0277). This confirms that the glycogen catabolism and PHB synthesis are strongly related (Fig. 6B). This is in agreement with other studies where conversion from glycogen to PHB has been observed (Dutt and Srivastava, 2018; Kamravamanesh et al., 2018a; Koch et al., 2019; Troschl et al., 2018). This degradation of glycogen and transformation into PHB occurs during nitrogen starvation, to allow the cell to produce ATP to maintain the basic cellular functions, while conserving the carbon pool (Koch et al., 2020a).

Overall, results obtained in this study show that the content of DIC necessary to stimulate PHB should be higher than the normally used in cyanobacteria cultures, as well as, provide new insights in the effect of carbon in the metabolism of PHB and glycogen by cyanobacteria, which may help to create new strategies to stimulate the PHB production.

4. Conclusions

The effect of inorganic carbon availability (up to 2 gC L^{-1} in form of $NaHCO_3$) in the PHB and carbohydrates production and metabolism of the wastewater-borne *Synechocystis* sp. strain were evaluated in this study for the first time. High availability of bicarbonate stimulated PHB production (maximum content of 14.3%$_{dcw}$). Gene expression analysis unequivocally demonstrated that the reactor with higher content of bicarbonate overexpressed the genes related to glycogen and PHB synthesis. Moreover, a positive correlation between *glgP2* and *phaC* and *phaB* was observed indicating a clear relationship between the glycogen catabolism and the synthesis of PHB.

CRediT authorship contribution statement

Estel Rueda: Conceptualization, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Ana Álvarez-González:** Investigation, Writing – review & editing. **Joaquim Vila:** Conceptualization, Formal analysis, Writing – review & editing, Supervision. **Rubén Díez-Montero:** Writing – review & editing, Supervision. **Magdalena Grifoll:** Resources, Writing – review & editing. **Joan García:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.154691.

References

APHA, AWWA, WEF, 2012. Standard Methods for the Examination of Water and Wastewater. Stand. Methods. 541.

Arias, D.M., Fradinho, J.C., Uggetti, E., García, J., Oehmen, A., Reis, M.A.M., 2018. Polymer accumulation in mixed cyanobacterial cultures selected under the feast and famine strategy. Algal Res. 33, 99–108.

Carpine, R., Raganati, F., Olivieri, G., Hellingwerf, K., Pollio, A., Salatino, P., Marzocchella, A., 2018. Poly-β-hydroxybutyrate (PHB) production by Synechocystis PCC6803 from Co2. Model development (2018).pdf. Algal Res. 29, 46–60.

Cui, Y.W., Gong, X.Y., Shi, Y.P., Wang, Z., 2017. Salinity effect on production of PHA and EPS by: Haloferax mediterranei. RSC Adv. 7, 53587–53595.

Damrow, R., Maldener, I., Zilliges, Y., 2016. The multiple functions of common microbial carbon polymers, glycogen and PHB, during stress responses in the non-diazotrophic cyanobacterium Synechocystis sp. PCC 6803. Front. Microbiol. 7, 1–10.

Doello, S., Klotz, A., Makowka, A., Gutekunst, K., Forchhammer, K., 2018. A specific glycogen mobilization strategy enables rapid awakening of dormant cyanobacteria from chlorosis. Plant Physiol. 177, 594–603.

Drosg, B., Fritz, I., Gattermayr, F., Silvestrini, L., 2015. Photo-autotrophic production of poly (hydroxyalkanoates) in cyanobacteria. Chem. Biochem. Eng. Q. 29, 145–156.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Div. Biochem. 28, 350–356.

Dutt, V., Srivastava, S., 2018. Novel quantitative insights into carbon sources for synthesis of poly hydroxybutyrate in Synechocystis PCC 6803. Photosynth. Res. 136, 303–314.

Eberly, J.O., Ely, R.L., 2012. Photosynthetic accumulation of carbon storage compounds under CO 2 enrichment by the thermophilic cyanobacterium Thermosynechococcus elongatus. J. Ind. Microbiol. Biotechnol. 39, 843–850.

Gallego, S., Vila, J., Tauler, M., Nieto, J.M., Breugelmans, P., Springael, D., Grifoll, M., 2014. Community structure and PAH ring-hydroxylating dioxygenase genes of a marine pyrene-degrading microbial consortium. Biodegradation 25, 543–556.

Gracioso, L.H., Bellan, A., Karolski, B., Cardoso, L.O.B., Perpetuo, E.A., Nascimento, C.A.O.do, Giudici, R., Pizzocchero, V., Basaglia, M., Morosinotto, T., 2021. Light excess stimulates poly-beta-hydroxybutyrate yield in a mangrove-isolated strain of Synechocystis sp. Bioresour. Technol. 320, 124379.

Gründel, M., Scheunemann, R., Lockau, W., Zilliges, Y., 2012. Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium Synechocystis sp. PCC 6803. Microbiology 158, 3032–3043.

Han, J., Qiu, Y.Z., Liu, D.C., Chen, G.Q., 2004. Engineered Aeromonas hydrophila for enhanced production of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) with alterable monomers composition. FEMS Microbiol. Lett. 239, 195–201.

Hiroe, A., Tsuge, K., Nomura, C.T., Itaya, M., Tsuge, T., 2012. Rearrangement of gene order in the phaCAB operon leads to effective production of ultrahigh-molecular-weight poly[(R)-3-hydroxybutyrate] in genetically engineered Escherichia coli. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.07715-11.

Jendrossek, D., Pfeiffer, D., 2014. New insights in the formation of polyhydroxyalkanoate granules (carbonosomes) and novel functions of poly(3-hydroxybutyrate). Environ. Microbiol. 16, 2357–2373.

Kamravamanesh, D., Pflügl, S., Nischkauer, W., Limbeck, A., Lackner, M., Herwig, C., 2017. Photosynthetic poly-β-hydroxybutyrate accumulation in unicellular cyanobacterium Synechocystis sp. PCC 6714. AMB Express 7.

Kamravamanesh, D., Kovacs, T., Pflügl, S., Druzhinina, I., Kroll, P., Lackner, M., Herwig, C., 2018a. Increased poly-B-hydroxybutyrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain Synechocystis sp. PCC 6714: mutant generation and characterization. Bioresour. Technol. 266, 34–44.

Kamravamanesh, D., Lackner, M., Herwig, C., 2018b. Bioprocess engineering aspects of sustainable polyhydroxyalkanoate production in cyanobacteria. Bioengineering 5, 111.

Kamravamanesh, D., Slouka, C., Limbeck, A., Lackner, M., Herwig, C., 2019. Increased carbohydrate production from carbon dioxide in randomly mutated cells of cyanobacterial strain Synechocystis sp. PCC 6714: bioprocess understanding and evaluation of productivities. Bioresour. Technol. 273, 277–287.

Khetkorn, W., Incharoensakdi, A., Lindblad, P., Jantaro, S., 2016. Enhancement of poly-3-hydroxybutyrate production in Synechocystis sp. PCC 6803 by overexpression of its native biosynthetic genes. Bioresour. Technol. 214, 761–768.

Koch, M., Forchhammer, K., 2021. Polyhydroxybutyrate: a useful product of chlorotic cyanobacteria. Microb. Physiol. https://doi.org/10.1159/000515617.

Koch, M., Doello, S., Gutekunst, K., Forchhammer, K., 2019. PHB is produced from glycogen turn-over during nitrogen starvation in Synechocystis sp. PCC 6803. Int. J. Mol. Sci. 20.

Koch, M., Berendzen, K.W., Forchhammer, K., 2020. On the role and production of polyhydroxybutyrate (Phb) in the cyanobacterium Synechocystis sp. pcc 6803. Life 10.

Koch, M., Bruckmoser, J., Scholl, J., Hauf, W., Rieger, B., Forchhammer, K., 2020b. Maximizing PHB content in Synechocystis sp. PCC 6803: a new metabolic engineering strategy based on the regulator PirC. Microb. Cell Factories 19, 1–12. https://doi.org/10.1186/s12934-020-01491-1.

Krasaesueb, N., Promariya, A., Raksajit, W., Khetkorn, W., 2021. Inactivation of phosphate regulator (SphU) in cyanobacterium Synechocystis sp. 6803 directly induced acetyl phosphate pathway leading to enhanced PHB level under nitrogen-sufficient condition. J. Appl. Phycol. 33, 2135–2144. https://doi.org/10.1007/s10811-021-02460-w.

Łabuzek, S., Radecka, I., 2001. Biosynthesis of PHB tercopolymer by Bacillus cereus UW85. J. Appl. Microbiol. 90, 353–357.

Lanham, A.B., Rita, A., Albuquerque, M.G.E., Pardelha, F., Carvalheira, M., Coma, M., Fradinho, J., Carvalho, G., Oehmen, A., Reis, M.A.M., 2013. Determination of the extraction kinetics for the quantification of polyhydroxyalkanoate monomers in mixed microbial systems. Process Biochem. 48, 1626–1634.

Lasemi, Z., Darzi, G.N., Baei, M.S., 2013. Media optimization for poly(β-hydroxybutyrate) production using Azotobacter beijerinckii. Int. J. Polym. Mater. Polym. Biomater. 62, 265–269.

Lim, H.N., Lee, Y., Hussein, R., 2011. Fundamental relationship between operon organization and gene expression. Proc. Natl. Acad. Sci. U. S. A. https://doi.org/10.1073/pnas.1105692108.

Markou, G., Vandamme, D., Muylaert, K., 2014. Microalgal and cyanobacterial cultivation: the supply of nutrients. Water Res. 65, 186–202.

Matsumoto, K., Tanaka, Y., Watanabe, T., Motohashi, R., Ikeda, K., Tobitani, K., Yao, M., Tanaka, I., Taguchi, S., 2013. Directed evolution and structural analysis of nadph-dependent acetoacetyl coenzyme A(acetoacetyl-CoA) reductase from ralstonia eutropha reveals two mutations responsible for enhanced kinetics. Appl. Environ. Microbiol. https://doi.org/10.1128/AEM.01768-13.

Nakaya, Y., Iijima, H., Takanobu, J., Watanabe, A., Hirai, M.Y., Osanai, T., 2015. One day of nitrogen starvation reveals the effect of sigE and rre37 overexpression on the expression of genes related to carbon and nitrogen metabolism in Synechocystis sp. PCC 6803. J. Biosci. Bioeng. 120, 128–134. https://doi.org/10.1016/j.jbiosc.2014.12.020.

Nishioka, M., Nakai, K., Miyake, M., Asada, Y., Taya, M., 2001. Production of poly-β-hydroxybutyrate by thermophilic cyanobacterium, Synechococcus sp. MA19, under phosphate-limited conditions. Biotechnol. Lett. 23, 1095–1099.

Orthwein, T., Scholl, J., Spät, P., Lucius, S., Koch, M., Macek, B., Hagemann, M., Forchhammer, K., 2021. The novel PII-interactor PirC identifies phosphoglycerate mutase as key control point of carbon storage metabolism in cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. 118, 1–9. https://doi.org/10.1073/pnas.2019988118.

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

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

Price, S., Kuzhiumparambil, U., Pernice, M., Ralph, P.J., 2020. Cyanobacterial polyhydroxybutyrate for sustainable bioplastic production: critical review and perspectives. J. Environ. Chem. Eng. 8, 104007. https://doi.org/10.1016/j.jece.2020.104007.

Rueda, Estel, García, J., 2021. Optimization of the phototrophic Cyanobacteria polyhydroxybutyrate (PHB) production by kinetic model simulation. Sci. Total Environ. 800, 149561. https://doi.org/10.1016/j.scitotenv.2021.149561.

Rueda, E., García-Galán, M.J., Díez-Montero, R., Vila, J., Grifoll, M., García, J., 2020. Polyhydroxybutyrate and glycogen production in photobioreactors inoculated with wastewater borne cyanobacteria monocultures. Bioresour. Technol. 295. https://doi.org/10.1016/j.biortech.2019.122233.

Selim, K.A., Haffner, M., Burkhardt, M., Mantovani, O., Neumann, N., Albrecht, R., Seifert, R., Krüger, L., Stülke, J., Hartmann, M.D., Hagemann, M., Forchhammer, K., 2021. Diurnal metabolic control in cyanobacteria requires perception of second messenger signaling molecule c-di-AMP by the carbon control protein SbtB. Sci. Adv. 7, 1–14. https://doi.org/10.1126/sciadv.abk0568.

Sharma, L., Kumar Singh, A., Panda, B., Mallick, N., 2007. Process optimization for poly-ß-hydroxybutyrate production in a nitrogen fixing cyanobacterium, Nostoc muscorum using response surface methodology. Bioresour. Technol. 98, 987–993. https://doi.org/10.1016/j.biortech.2006.04.016.

Shrivastav, A., Mishra, S.K., Mishra, S., 2010. Polyhydroxyalkanoate (PHA) synthesis by Spirulina subsalsa from Gujarat coast of India. Int. J. Biol. Macromol. 46, 255–260.

Silvestrini, L., Drosg, B., 2016. Identification of four polyhydroxyalkanoate structural genes in Synechocystis cf. salina PCC6909: in silico evidences. J. Proteomics Bioinform. 09. https://doi.org/10.4172/jpb.1000386.

Solórzano, L., 1968. Determination of ammonia in natural waters by the phenolhypochlorite method. Liminology Oceanogr. 799–801.

Spät, P., Barske, T., Macek, B., Hagemann, M., 2021. Alterations in the CO_2 availability induce alterations in the phosphoproteome of the cyanobacterium Synechocystis sp. PCC 6803. New Phytol. 231, 1123–1137. https://doi.org/10.1111/nph.17423.

Thirumala, M., Reddy, S.V., Mahmood, S.K., 2010. Production and characterization of PHB from two novel strains of Bacillus spp. isolated from soil and activated sludge. J. Ind. Microbiol. Biotechnol. 37, 271–278.

Touloupakis, E., Cicchi, B., Margarita, A., Benavides, S., Torzillo, G., 2016. Effect of High pH on Growth of Synechocystis sp . PCC 6803 Cultures and Their Contamination by Golden Algae (Poterioochromonas sp.), pp. 1333–1341.

Troschl, C., Meixner, K., Fritz, I., Leitner, K., Romero, A.P., Kovalcik, A., Sedlacek, P., Drosg, B., 2018. Pilot-scale production of poly-β-hydroxybutyrate with the cyanobacterium Synechocytis sp. CCALA192 in a non-sterile tubular photobioreactor. Algal Res. 34, 116–125.

Tyo, K.E.J., Fischer, C.R., Simeon, F., Stephanopoulos, G., 2010. Analysis of polyhydroxybutyrate flux limitations by systematic genetic and metabolic perturbations. Metab. Eng. https://doi.org/10.1016/j.ymben.2009.10.005.

Urtuvia, V., Villegas, P., González, M., Seeger, M., 2014. Bacterial production of the biodegradable plastics polyhydroxyalkanoates. Int. J. Biol. Macromol. 70, 208–213.

Vajravel, S., Sirin, S., Kosourov, S., Allahverdiyeva, Y., 2020. Towards sustainable ethylene production with cyanobacterial artificial biofilms. Green Chem. 22, 6404–6414.

Zhang, D., Dechatiwongse, P., Del-Rio-Chanona, E.A., Hellgardt, K., Maitland, G.C., Vassiliadis, V.S., 2015. Analysis of the cyanobacterial hydrogen photoproduction process via model identification and process simulation. Chem. Eng. Sci. 128, 130–146.