Polymer accumulation in mixed cyanobacterial cultures selected under the feast and famine strategy

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Abstract

In this study, a sequencing batch reactor (SBR), operated with transient carbon availability (feast and famine) and different nutrients loads, was used to select cyanobacteria accumulating poly (3-hydroxyalkanoate) (PHB) and carbohydrates from a mixed wastewater-borne microbial culture. The SBR was operated with 12h aerobic light and 12h anaerobic dark phases, evaluating the effect of three different operational conditions consisting on; 1) carbon limitation, 2) carbon and phosphorus limitation and 3) phosphorus limitation. Once a steady state was reached in each operational period of the SBR, part of the biomass was collected and submitted to separate batch tests in order to investigate the maximum PHB and carbohydrates accumulation levels. Batch tests were performed during 24h of illuminated aerobic condition and 24h of dark anaerobic condition, while inorganic carbon was constantly present. During the SBR operation, inorganic carbon was mostly used for biomass and carbohydrate production, showing very low PHB accumulation levels (<1%). Notwithstanding, in subsequent batch tests, PHB was accumulated after a complete depletion of nitrogen, reaching almost 4%.

Concerning carbohydrates, it was found that phosphorus limitation (with and without carbon limitation) led to a culture mostly dominated by cyanobacteria and higher levels of carbohydrate content (43%-48%) than the culture with carbon limitation and high loads of nitrogen and phosphorus (29%). Such contents were obtained in only 24h of incubation under aerobic illuminated conditions. Hence, these encouraging results indicate that carbon uptake and the consequent polymers production from cyanobacteria can be enhanced through carbon and nutrient feeding strategies.

Keywords: Algae, wastewater-borne cyanobacteria, bioproducts, wastewater.
1. Introduction

Cyanobacteria are prokaryotes capable to perform oxygenic photosynthesis and they can be found in almost every environment on earth [1]. During the last decades, they have received much attention as a rich source of polymers, being considered as one of the most promising group of organisms to produce them [2]. Cyanobacteria are able to accumulate both carbohydrates in form of glycogen and and polyhydroxyalkanoates (PHA), e.g., poly (3-hydroxyalkanoate) (PHB). Carbohydrates and PHB are attracting increasing interest due to their potential as a biofuel substrate and as a bioplastic, respectively. Although those polymers are also accumulated in other photosynthetic and non-photosynthetic bacteria, the studies that have been done thus far have based their polymers production on the utilization of organic molecules as C source [3]. In the case of cyanobacteria, their mechanism for polymer production is based on carbon storage through oxygenic photosynthesis implying simple requirements for cultivation and the utilization of CO$_2$ as carbon source [4]. This ability for CO$_2$ fixation and conversion into biopolymers is nowadays significantly attractive due to the worldwide concern with the CO$_2$ impact in climate change.

Until now, experiments on carbohydrates and PHB production from cyanobacteria have been performed through pure strains and genetically modified species [5–9], implying strictly controlled processes leading to high production costs, and subsequently expensive products [10]. In this context, a more sustainable alternative for the production of polymers from cyanobacteria could be the use of wastewater-borne cultures. This approach implies the lack of sterilization of substrates or reactors and cheaper equipment that could reduce the production costs compared to pure culture processes. Nevertheless, in spite of being an attractive alternative, the utilization of mixed cyanobacterial cultures to produce biomass and polymers strictly depends on the composition of the culture.
Indeed, a conventional mixed wastewater-borne culture is composed by a mixture of cyanobacteria, other bacteria (which also could accumulate carbohydrates and PHB) and eukaryotic microorganisms, such as green algae, diatoms, metazoa and protozoa, which are unable to produce both polymers. Hence, a certain control of the consortium composition would be necessary in order to achieve favorable yields.

Previous studies, mainly carried out in lakes and reservoirs [11–14], but also in wastewater systems [15,16], highlighted that absolute nutrients concentration and ratio (N : P) are the two most important factors influencing the competition of cyanobacteria with other species (i.e., green algae) [12,17]. More specifically, the dominance of cyanobacteria has been related to their high affinity for N and P and capacity to store them intracellularly [18].

Concerning the polymer accumulation capacity of cyanobacteria, it has been demonstrated that nutrient limitation coupled with carbon excess are determining factors to increase polymer accumulation [19]. Thus, due to their high tolerance to nutrient changes and carbon availability, cyanobacteria polymer production usually requires prolonged periods. The low carbon uptake efficiency turns the polymer production into a slow process compared with processes involving heterotrophic bacteria. Indeed, the maximum accumulation of polymers in cyanobacteria usually takes more than 9 days of incubation for carbohydrates and more than 11 days for PHB accumulation [20–22]. This fact highlights the need for new strategies to improve the efficiency of inorganic carbon (IC) uptake in cyanobacteria and its transformation into polymers.

Considering the example of mixed bacterial cultures, one of the most feasible strategies to select specific accumulating microorganisms and improve PHB and carbohydrate production is the application of unbalanced growth, also called feast and famine [23].
This process consists of a transitory carbon supply, in which the biomass is subjected to a period of carbon availability and a subsequent absence of carbon. With this process, cell growth and storage products are enhanced while the microorganisms able to store carbon and utilize their own reserves are selected.

In this work, feast and famine is proposed as a strategy for the selection of autotrophic cyanobacteria accumulating polymers. To the authors’ knowledge this is the first time that this strategy is employed to select cyanobacteria from a wastewater-borne culture using inorganic carbon as substrate to produce value-added polymers. In the present study, a mixed wastewater consortium was cultivated in a sequencing batch reactor (SBR), evaluating the effect of different nutrients ratios and loads under transient carbon availability on polymer production during the intercalation of aerobic and anaerobic phases. In addition to the effect of those factors on polymer production, the effects of other parameters such as microbial composition, nutrient uptake and oxygen production are also considered and discussed.

2. Material and methods

2.1 Sequencing batch reactor set-up

For the enrichment of cyanobacteria producing PHB and carbohydrates, a double jacket acrylic reactor with a working volume of 2 L was used. The reactor was operated as a non-sterile sequencing batch reactor (SBR). The inoculum utilized consisted of a consortium of green algae, cyanobacteria, bacteria and protozoa, obtained from a pilot photobioreactor described elsewhere [16].

The SBR was operated with a hydraulic retention time (HRT) of 2 days and sludge retention time (SRT) of 10 days. The reactor operation was based on 24h cycles according to the following scheme (Fig. A1):
1. Light aerobic phase (12h): i) 9:30 am, nutrient uptake period, starting with the addition of 1000 mL of growth medium to the reactor (6h), ii) 3:00 pm, carbon uptake period (carbohydrate accumulation), starting with a pulse of 6 mL of 0.442 g L\(^{-1}\) of Na\(_2\)CO\(_3\) (0.050 g C L\(^{-1}\)).

2. Dark anaerobic phase (12h): iii) 9:30 pm, start of the anaerobic phase in which argon was sparged into the culture (250 mL min\(^{-1}\) for 20 min every 2 hours) in order to remove oxygen, iv) 8:50 am, effluent withdrawal phase (2 min) in which 200 mL of the mixed liquor was removed from the culture, v) 9:00 am, settling phase (30 min without stirring), vi.) Supernatant withdrawal phase in which 800 mL were removed from the medium.

During the light phase, the SBR was continuously illuminated with two external LED lamps (23W) placed on the two sides of the SBR giving a light intensity of 91 W/m\(^2\), corresponding to a volumetric light intensity of 2.1 W/L.

Throughout the whole cycle (with the exception of the settling and supernatant withdrawal phases), the medium was stirred using a magnetic stirrer (VELP scientific, USA). The reactor temperature was controlled at 27 °C by means of a water jacket and a thermostat bath (Julabo, Germany). The pH of the reactor was maintained at 8.2 using dosages of 0.01 M NaOH and 0.05 M HCl.

The SBR was operated for 6 months during which three different conditions were tested as shown in Table 1. In the first condition high N and P loads were applied to the culture while it was exposed to a low carbon load. In the second condition a low carbon load was coupled with a low phosphorus load. Lastly, in the third condition, the effect of only low phosphorus load was tested. Those conditions tested the influence of loads on cyanobacteria dominance, growth and production of PHB and carbohydrates. Each condition was tested when the reactor reached steady state. The reactor was considered
to be in steady state when the concentration of total suspended solids (TSS) at the end of
the cycle showed stable results or after the system reached three SRTs.

The growth solution added at the beginning of the light phase in each cycle consisted in
growth medium containing: 0.049 g L$^{-1}$ NH$_4$Cl, 0.072 g L$^{-1}$ CaCl$_2$·2H$_2$O, 0.001 g L$^{-1}$
Na$_2$EDTA, 0.075 g L$^{-1}$ MgSO$_4$·7H$_2$O, 0.6 g L$^{-1}$ C$_6$H$_8$FeNO$_7$ (ammonium ferric citrate),
0.006 g L$^{-1}$ C$_6$H$_8$O$_7$ (citric acid), and 1.0 ml L$^{-1}$ of trace elements: 2.86 g H$_3$BO$_3$, 0.39 g
Na$_2$MoO$_4$·2H$_2$O, 1.8 g MnCl$_2$·4H$_2$O, 0.08 g CuSO$_4$·5H$_2$O, 0.22 g ZnSO$_4$·7H$_2$O and 0.05 g
Co(NO$_3$)$_2$·6H$_2$O. Depending on the operation of the SBR, K$_2$HPO$_4$ concentration varied
from 0.24 g L$^{-1}$ in operation 1 and 0.0056 g L$^{-1}$ for operations 2 and 3. Na$_2$CO$_3$ was added
independently as the inorganic carbon source by the addition of 1 pulse of 6 mL
containing 0.442 g L$^{-1}$ (0.050 g C L$^{-1}$) at the beginning of the carbon phase during
operations 1 and 2, and 2 pulses of 0.442 g L$^{-1}$, one at the beginning and the other in the
middle of the light phase in condition 3.

Table 1. Experimental operating conditions of the SBR.

<table>
<thead>
<tr>
<th>Operation period</th>
<th>C load (mg L$^{-1}$ d$^{-1}$)$^a$</th>
<th>N load (mg L$^{-1}$ d$^{-1}$)$^a$</th>
<th>P load (mg L$^{-1}$ d$^{-1}$)$^a$</th>
<th>C:N:P ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition 1</td>
<td>50</td>
<td>12.5</td>
<td>4.2</td>
<td>12:3:1</td>
</tr>
<tr>
<td>Condition 2</td>
<td>50</td>
<td>12.5</td>
<td>1</td>
<td>50:12.5:1</td>
</tr>
<tr>
<td>Condition 3</td>
<td>100</td>
<td>12.5</td>
<td>1</td>
<td>100:12.5:1</td>
</tr>
</tbody>
</table>

$^a$ Volumetric load per volume of reactor.

2.2 Batch test for polymer accumulation

For each operation period, when steady state was reached, 200 mL of mixed liquor were
collected and placed in a separate batch reactor, filled to 400 mL with deionized water
and used for polymer production batch experiments. In these tests, the operation of the
reactor was based on a 48 h cycle, starting with 24 h of light aerobic condition followed
by 24h of dark anaerobic condition. During the light aerobic phase, the reactor worked as
an open photoreactor while in the anaerobic phase the reactor was hermetically closed.
This strategy was performed in order to accumulate high carbohydrate content during the
light aerobic phase and to enhance the possibility of a further conversion of the
carbohydrates to PHB during the anaerobic dark phase. Similar behavior can be found in
other microorganisms, such as polyphosphate accumulating organisms (PAOs), glycogen
accumulating organisms (GAOs) and other photosynthetic bacteria [24,25].

During the light phase of these batch tests, the reactor was illuminated by two external
halogen lamps (100W) placed on the two sides of the reactor at a light intensity of 343
W/m², corresponding to a volumetric light intensity of 2.2 W/L.

At the beginning of the light phase, a pulse of 10 mL containing 100 mg of carbon in the
form of Na₂CO₃ (C-Na₂CO₃) was fed to the reactor to support polymer accumulation.
Further carbon addition was supplied manually, as pulses of Na₂CO₃, to prevent carbon
depletion. No N and P sources were added, thus, the only amount of nitrogen and phosphorus was the one remaining from the previous SBR cycle, leading to nutrient
limitation in all the experiments.

2.3 Analytical Methods
Each time that the SBR reached steady state, three cycles of the reactor were monitored.
Biomass was harvested from the reactor at different time points (9-11 samples per cycle)
in order to analyze biomass concentration and composition, nutrients concentration, and
PHB/carbohydrate production capacity of the biomass. The samples taken from the mixed
liquor were filtered (~0.2 μm glass microfiber filter, Whatman, UK) and used to analyze
soluble inorganic carbon (IC), soluble organic carbon (OC), orthophosphate (dissolved
reactive phosphorus) (P-PO₄³⁻), nitrite (N-NO₂⁻), nitrate (N-NO₃⁻), and ammonium (N-
Unfiltered samples were used to analyze total nitrogen (TN) and total phosphorus (TP). Particulate Organic Nitrogen (PON) was calculated as the difference between TN and NH$_4^+$, N-NO$_2^-$ and N-NO$_3^-$, whereas particulate phosphorus (PP) was determined as the difference between TP and P-PO$_4^{3-}$, it should be notice that PP is composed of intracellular particulate inorganic P (phosphate, pyrophosphate, and polyphosphate) and organic P. OC and IC were measured by means of the TOC analyzer (Shimadzu, Japan) while P-PO$_4^{3-}$, NH$_4^+$, N-NO$_2^-$, N-NO$_3^-$ concentrations were measured by colorimetry using a segmented flow analyzer (Skalar, The Netherlands). TN was analyzed spectrophotometrically with total nitrogen kits (Hach, Germany), and TP was analyzed following the methodology described in Standard Methods (APHA-AWWA-WPCF, 2001).

Dissolved oxygen (DO), pH and temperature in the reactor were measured in situ with a DO electrode (Mettler Toledo, USA) and a pH electrode (Mettler Toledo, USA). On line measurements (DO, pH, and temperature) were acquired with the software BIOCTR17.

The biomass concentration was measured as total suspended solids (TSS) and volatile suspended solids (VSS) by filtration according to standard methods (APHA-AWWA-WPCF, 2001).

Carbohydrates and PHB content were measured according to the methods described by [26] and [27] with minor modifications. Thus, the biomass collected was centrifuged, ultra-frozen and finally freeze dried. Carbohydrates were determined by mixing the freeze-dried biomass with 2 mL of 0.9 M HCl and digested for 2 h at 100 °C. The supernatant was filtered (0.45 μm membrane) and glucose was analyzed by HPLC using D-glucose as a standard. For PHB determination, freeze-dried biomass was digested for 2h at 100 °C with 1 mL of chloroform and 1 mL of methanol with 20% sulfuric acid. 1
ml of the organic phase was extracted into GC vials and measured by gas chromatography. The gas chromatograph used was coupled to a Flame Ionization Detector (GC-FID Varian CP-3800) and equipped with a Stalbiwax column (Resek, USA). Helium was utilized as the carrier gas at a flow rate of 1 mL/min. The co-polymer Poly (3-hydroxybutyric acid-co-3-hydroxyvaleric acid) (86:14 wt) (Sigma-Aldrich) was used as a standard for hydroxybutyrate (HB) and hydroxyvalerate (HV), and heptadecane was used as the internal standard. Standards with six different concentrations were processed in the same way as the samples.

Biomass composition within the SBR was monitored under microscopy for qualitative observation of microscopic populations. Microbial observation was performed in an epifluorescence microscope (Olympus BX51, Japan). Abundance of the different organisms was determined qualitatively with microscopic observations of the biomass. Cyanobacteria and microalgae species were identified in vivo using conventional taxonomic books (Bourrelly, 1985; Palmer, 1962), as well as a database of cyanobacteria genus (Komárek and Hauer, 2013).

2.4. General calculations and kinetic and stoichiometric parameters

Nutritional parameters applied as C volumetric load (C load) [mg C-Na$_2$CO$_3$ L$^{-1}$ d$^{-1}$], P volumetric load (P load) [mg P-PO$_4^{3-}$ L$^{-1}$ d$^{-1}$] and N volumetric load (N load) [mg N-NH$_4^+$ L$^{-1}$ d$^{-1}$] were calculated following Eq. (1):

$$C\text{ load, } P\text{ load, } N\text{ load} = \frac{C\text{-Na}_2\text{CO}_3 \text{ or } P\text{-PO}_4^{3-} \text{ or } N\text{-NH}_4^+ * Q}{V}$$  \hspace{1cm} (1)

Where Q is the flow [L$^{-1}$ d$^{-1}$], P-PO$_4^{3-}$ or N-NH$_4^+$ is the influent concentration [mg L$^{-1}$] and V [L$^{-1}$] is the volume of the SBR.
The active biomass concentration was calculated by assuming a composition of \( \text{CH}_{1.566}\text{O}_{0.405}\text{N}_{0.192}\text{S}_{0.005}\text{P}_{0.06} \) with a molecular weight of 23.08 g Cmol\(^{-1}\) [31]. Thus, the active biomass \( X \) was calculated as:

\[
X = (\text{VSS} - \text{Carbohydrates} - \text{PHB}) \tag{2}
\]

Carbohydrates and PHB were calculated according to [32], in terms of percentage of VSS:

\[
\%\text{Carbohydrates or PHB} = \frac{\text{gPHB or gCarbohydrates}}{\text{gVSS}} \times 100 \tag{3}
\]

The yields of PHB \( Y_{\text{PHB/S}} \) and carbohydrates \( Y_{\text{carbs/S}} \) per substrate consumed [Cmol PHB or carbohydrates/Cmol IC] were calculated following eq. (4):

\[
Y_{\text{PHB/S or Carbohydrates/S}} = \frac{\text{PHB or Carbohydrates accumulated}}{\text{IC consumed}} \tag{4}
\]

The maximum specific substrate uptake rate \( -q_S \) [Cmol IC/Cmol X d\(^{-1}\)], maximum specific polymer production rate \( (q_{\text{PHB}}, q_{\text{Carbs}}) \) [Cmol PHB or carbohydrates/Cmol X d\(^{-1}\)], were determined by dividing the slope of the linear function of experimental results along the cycle by the average of active biomass.

### 3. Results and discussion

#### 3.1 Sequencing batch reactor (SBR) performance

##### 3.1.1 General conditions

Throughout the SBR operation, the main goal was the selection and enrichment of polymer producing cyanobacteria from the initial mixed culture. Along this selection phase, three different conditions were imposed on the SBR. In Figure 1, pH, oxygen and temperature profiles during the three operating conditions of the PBR are shown. In the first operating condition (high N and P loads and low carbon loads - Table 1), in spite of not having carbon addition in the first 6 hours of light, photosynthetic activity was
registered due to the residual IC present in the bulk culture at the start of the light phase (Fig. 1a). Hence, during the first 2 hours of the light phase and until all IC was consumed, oxygen increased to 12.5 mg L\(^{-1}\). After that time, a gradual decrease of dissolved oxygen was observed until reaching 9.8 mg L\(^{-1}\). Once the carbon was supplied to the culture, an increasing pattern in the oxygen concentration was observed, until reaching a concentration around 40 mg L\(^{-1}\). At the same time, the IC content in the medium declined reaching a specific uptake rate of 0.019±0.004 Cmol IC/Cmol X d\(^{-1}\) (Table 2). As soon as the IC was completely consumed (approximately after 4.5 hours), the oxygen gradually decreased until the dark phase started. Then argon was supplied to the culture, and oxygen quickly reached zero. During the addition of carbon and IC uptake, pH tended to increase due to the photosynthesis performed by microalgae/cyanobacteria and during the dark phase, pH tended to decrease likely because of the organic acids released by the culture [33,34]. This is a plausible assumption since a concentration of 7.8 mg L\(^{-1}\) of OC was measured at the end of the dark phase. In both cases, when pH tended to increase or decrease, there was an addition of acid or base solutions to maintain pH value around 8.2, which can be observed in the oscillations in Figure 1.

Concerning condition 2, where low carbon load was coupled with low phosphorus, a profile similar to condition 1 was observed. Thus, oxygenic activity was observed from the start of the light phase as consequence of the residual IC (around 2.7 mg L\(^{-1}\)) (Fig. 1b), following the same trend on pH, oxygen and IC as condition 1. However, a faster uptake of IC 0.039±0.002 Cmol IC/Cmol X d\(^{-1}\) (Table 2) and higher concentrations of oxygen (up to 45 mg L\(^{-1}\)) were reached. During condition 2, pH oscillations were less frequent than condition 1 during the dark phase. Indeed, OC released during the dark phase was slightly lower than in condition 1, reaching 6.5 mg L\(^{-1}\).
Contrary to the conditions previously mentioned, differences in the oxygen profiles were observed in condition 3. In this case, due to the addition of IC from the start of the light phase, the oxygen started increasing until reaching values of 34.42 mg L$^{-1}$ after 8 hours and maintaining constant values ($\pm 0.29$ mg L$^{-1}$) during the rest of this period. According to these results, pH oscillated along the entire light phase. Like under the previous conditions, oxygen was always present in the culture while most of the total IC consumption occurred in the light phase, and only 4% of IC was consumed during the dark period. In spite of having more IC availability, the IC uptake rate was lower when compared with conditions 1 and 2, having only a rate of $0.016 \pm 0.002$ Cmol IC/Cmol X d$^{-1}$ (Table 2). Also, several oscillations in pH values were observed during the dark phase, recording high concentrations of OC ($\sim 18$ mg L$^{-1}$) at the end of the dark phase.

Interestingly, the patterns observed in pH and oxygen with relation to IC profiles, enabled an non-direct determination of the periods of IC consumption and the moments when the IC was depleted, all without the need of measuring the carbon in the medium.

Comparing the three conditions, it can be observed that condition 2 led to higher oxygen concentrations and faster IC uptake ($0.039 \pm 0.002$ Cmol IC/Cmol X d$^{-1}$). This fact could be attributed to a more efficient photosynthetic performance when the culture was under low P and C loads in comparison with the other conditions. On the contrary, the lowest values were reached under condition 3, when the carbon was present during the entire light phase.

It has to be considered that oxygen reached very high levels in the SBR due to the reduced headspace of the reactor and the poor oxygen transfer between the reactor and the atmosphere. In this study, values up to 560% were reached, considering that 100% of air saturation at the average temperature in the three conditions (8 mg O L$^{-1}$, 26.9 °C). In
closed reactors, microalgae and cyanobacteria deal with high oxygen levels due to the photosynthetic process. In most of the cases, the high concentrations of dissolved oxygen causes damage to cellular metabolism and thus, causes inhibition depending on the species and the time of exposure [35,36]. In general, it is stated that the maximum tolerable dissolved oxygen level is 400% of air saturation [37]. In this work, although the culture was exposed to a maximum of 560% of air saturation (condition 2), the three reactors maintained higher values than 400% and the time of exposure to such values was only 4-6 hours per day, thus no toxic effects were noticed.

Fig. 1. Oxygen, temperature, pH and inorganic carbon (IC) patterns during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). White zone represents the light phase and grey zone represents the dark anaerobic phase.

Table 2. Kinetic and stoichiometric parameters of the SBR performance during the different operational conditions. Average and standard deviations (in parentheses) calculated from the three repeated cycles.

<table>
<thead>
<tr>
<th>Operation</th>
<th>Condition 1</th>
<th>Condition 2</th>
<th>Condition 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-q_{IC}) [Cmol IC/Cmol X d(^{-1})]</td>
<td>0.019 (0.004)</td>
<td>0.039 (0.002)</td>
<td>0.016 (0.002)</td>
</tr>
<tr>
<td>(q_{PHB}) [Cmol PHB/Cmol X d(^{-1})]</td>
<td>-</td>
<td>-</td>
<td>0.00032 (0.0003)</td>
</tr>
<tr>
<td>(q_{carbs}) [Cmol Carbs/Cmol X d(^{-1})]</td>
<td>0.011 (0.002)</td>
<td>0.049 (0.003)</td>
<td>0.0085 (0.003)</td>
</tr>
<tr>
<td>(Y_{PHB/IC}) [Cmol PHB/Cmol IC]</td>
<td>-</td>
<td>-</td>
<td>0.009 (0.004)(^a)</td>
</tr>
<tr>
<td>(Y_{PHB/carbs}) [Cmol PHB/Cmol carbs]</td>
<td>-</td>
<td>-</td>
<td>0.018 (0.015)(^b)</td>
</tr>
<tr>
<td>(Y_{Carbs/IC}) [Cmol Carbs/Cmol IC]</td>
<td>0.4 (0.2)</td>
<td>0.93 (0.3)</td>
<td>0.4 (0.2)</td>
</tr>
</tbody>
</table>

\(^a\) yield calculated within 12 h of illumination.
\(^b\) yield calculated within 12 h of dark.

3.1.2 Nutrient profiles
During steady-state operation in condition 1, the profile of N-NH$_4^+$ showed a higher consumption than the uptake of P-PO$_4^{3-}$, as it can be seen in Fig. 2a. Along the light phase, the initial nitrogen source was reduced by about 60%, while P source was consumed by 16%. On the other hand, a removal lower than 0.5% was found for both nutrients during the night. Specific uptake rates during the light phase were 1.08 mg N-NH$_4^+/g$ X d$^{-1}$ and 0.22 mg P-PO$_4^{3-}/g$ X d$^{-1}$, respectively. Meanwhile, particulate organic nitrogen (PON) and particulate phosphorus (PP) concentrations, during this condition, had an average of 53±22 mg L$^{-1}$ and 3.1±0.8 mg L$^{-1}$ respectively, corresponding to 0.6 and 9.6 % g·g X$^{-1}$ (Table 3). The biomass concentration present in the culture was on average 0.66±0.06 g VSS L$^{-1}$ in the three cycles monitored. The active biomass maintained a constant concentration during the first eight hours of the light phase (0.55±0.04 g X L$^{-1}$), and posteriorly gradually increased to 0.76 g X L$^{-1}$ after the addition of carbon, and even after IC was completely removed from the medium.

In condition 2, the N-NH$_4^+$ profile showed a similar pattern as in condition 1 (60% of removal in the light phase), although the N-NH$_4^+$ specific uptake rate was slightly higher (1.24 mg N-NH$_4^+/g$ X d$^{-1}$). However, the highest uptake of N-NH$_4^+$ was found after the pulse of carbon in the middle of the light phase, where 53% of the N-NH$_4^+$ was achieved.

Table 3. Particulate phosphorus (PP) and organic nitrogen (PON) during the different operational conditions. Average and standard deviations calculated from the three repeated cycles.

<table>
<thead>
<tr>
<th></th>
<th>PP</th>
<th>PON</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[% g·g X$^{-1}$]</td>
<td>[% g·g X$^{-1}$]</td>
</tr>
<tr>
<td>Condition 1</td>
<td>0.6 (0.1)</td>
<td>9.6 (4)</td>
</tr>
<tr>
<td>Condition 2</td>
<td>0.5 (0.2)</td>
<td>8.7 (2.3)</td>
</tr>
<tr>
<td>Condition 3</td>
<td>1.5 (0.2)</td>
<td>8.2 (0.9)</td>
</tr>
</tbody>
</table>
On the other hand, contrarily to condition 1, the phosphate uptake rate was lower (0.103 mg P-PO$_4^{3-}$/g X d$^{-1}$) and phosphate was completely removed after 9 hours (Fig. 2b). In this condition, PON and PP showed similar values and percentages in terms of active biomass than condition 1 (65±17 mg L$^{-1}$ and 3.7±1.8 mg L$^{-1}$, respectively, Table 3). The biomass concentration was slightly higher than condition 1, (0.74±0.05 g VSS L$^{-1}$). The active biomass profile was constant during the first eight hours of the light phase (0.71±0.04 g X L$^{-1}$) and posteriorly increased to 0.78 g X L$^{-1}$ in the last 4 hours of light.

Finally, in condition 3, the simultaneous addition of carbon and nutrients led to the maximum N and P removals observed in the SBR. Thus, after adding the nutrients to the culture, N-NH$_4^+$ and P-PO$_4^{3-}$ were almost completely consumed. With respect to the specific uptake rates, N uptake almost doubled the value of the previous conditions with 2.3 mg N-NH$_4^+/g$ X d$^{-1}$ and P uptake showed similar uptake than condition 1 with 0.22 mg P-PO$_4^{3-}$/g X d$^{-1}$. In this condition, the VSS concentration raised to an average of 1.2±0.1 g VSS L$^{-1}$ during the three cycles, while the active biomass averaged 0.99±0.09 g X L$^{-1}$, approximately 50% higher than the values of condition 1 (0.66 g VSS L$^{-1}$). Considering the removal of N and P, it can be assumed that this condition led to nitrogen and phosphorus limitation during most of the operational time. As shown in Fig.2c, the nutrients supplied at the beginning of the light phase were coupled with the carbon addition. During steady-state operation, as it can be seen in Fig. 2c, 84.7% of N-NH$_4^+$ removal was found during the first hour of the light phase, and was completely removed after 4 hours. Concurrently, P-PO$_4^{3-}$ was completely consumed as soon as it was added. Due to the increment in inorganic nitrogen removal, PON concentration increased to 81±9.06 but maintaining similar percentages than the other conditions (8.2±0.9 % g·g X$^{-1}$), while OP registered a concentration of 15±1.8 mg L$^{-1}$ and a percentage of 1.5 % g·g X$^{-1}$ (Table 3).
It should be noticed that other nitrogen forms, as N-NO₂ and N-NO₃, showed values <0.05 mg L⁻¹ under all the conditions, which implies a lack of nitrification or denitrification processes in the mixed liquor.

The N/P ratio of conditions 1, 2 and 3 were 4.9, 12.03 and 10.45 mg N⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻禳

3.1.3 Polymer accumulation in the sequencing batch reactor operation

Due to the different operating strategies to which the culture was submitted (combinations of nutrients and carbon limitation), differences in active biomass, PHA and carbohydrates between each operational condition were observed. It should be noted that throughout this
study only hydroxybutyrate monomers were detected, indicating the sole production of a PHB homopolymer. During the steady-state of condition 1, a maximum of 10.3±3.0 % of carbohydrate was reached after 4.5 h of the addition of carbon with a maximum specific carbohydrates rate of 0.011 Cmol carbs/Cmol X d⁻¹ (Table 2); while PHB content only 0.3±0.1% at the end of the light phase. As shown in Fig. 3a, carbohydrates were accumulated after the pulse of IC to the medium, and increased until reaching 2.02 C mmol L⁻¹. Afterwards, a decline in carbohydrates was observed for the last 2 hours of the light phase (after IC depletion) and posteriorly in the dark phase. This decrease is associated with the stored carbon consumption by cyanobacteria (and other microorganisms in the culture) when carbon is limited in the medium. In the case of green algae metabolism, they ferment starch during anaerobic dark conditions and transform into reducing equivalents for posterior byproducts formation and release, such products are mainly acetate and ethanol, formate, glycerol, lactate, H₂ and CO₂ [34,39]. Otherwise, in the particular case of cyanobacteria and bacteria metabolism during the dark, energy generation can occur from glycogen respiration in the presence of an electron acceptor [40], then, without the presence of an electron acceptor, as occurred in this case, energy generation occurs during glycogen conversion to PHA [41]. This conversion from glycogen to PHAs is a type of metabolism also occurring during the anaerobic phase in certain types of microorganisms, such as glycogen accumulating organisms (GAOs) and (PAOs) [24,41–43], and also occurring in anoxygenic photosynthetic bacteria [44]. In condition 1, due to the anaerobic conditions in the dark phase, carbohydrates were consumed but only marginally, which can explain why PHB maintained the same low values reached in the light phase (0.3±0.1%).

In condition 2, when the culture was submitted to low carbon and P loads, a more efficient utilization of carbon transformation into carbon storage was observed. A maximum
carbohydrates accumulation of 18.87% was achieved after 3 hours from the carbon pulse reaching 5.2±1.0 Cmmol Carbs L\(^{-1}\) and a maximum specific carbohydrate rate of 0.049 Cmol Carbs/Cmol X d\(^{-1}\), more than four times the rate obtained in condition 1 (Table 2). After finishing the IC available, carbohydrates were quickly consumed before the end of the light phase, but slightly consumed during the dark phase (Fig. 3b). This slight consumption led again to a lack of carbohydrate conversion to PHA during the night; PHA were not observed in any phase of this operational condition.

Unlike the previous operations in the SBR, in the steady-state of condition 3, in spite of achieving a lower carbohydrate content (13.8 %), a higher concentration was reached, 5.7±0.5 Cmmol carbs L\(^{-1}\), in the last hours of the light phase (Fig. 3c). This is likely due to the high biomass concentration reached under this condition (1.2 g VSS L\(^{-1}\)). Additionally, PHB accumulation of 1.03±0.07% was achieved at the end of the dark anaerobic phase. This accumulation of PHB was likely performed by means of carbohydrate conversion since IC was not available.

Fig. 3. Inorganic carbon (IC) consumption profile and transformation of poly (3-hydroxyalkanotes) (PHB) and carbohydrates during one cycle of operation of a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). The white zone represents the light phase and grey zone represents the dark anaerobic phase.
In general, results indicated that the persistent availability of N and P coupled with low loads of carbon in condition 1 did not substantially improve carbohydrate accumulation. Under this condition only a low yield of carbohydrates of 0.04 Cmol Carbs/Cmol IC was obtained. Additionally, under this condition the culture was unable to accumulate PHB. Meanwhile, when the culture was submitted to low carbon loads with P limitation in condition 2, the carbohydrate accumulation efficiency was improved, reaching yields of 0.93 Cmol Carbs/Cmol IC. However, PHB was not accumulated (Table 2). On the other hand, when carbon was always available and the culture had P limitation (condition 3), nitrogen also became limiting and the efficiency of carbohydrate production decreased (Ycarbs 0.093 Cmol Carbs/Cmol IC). However, the culture was able to produce PHB with a higher efficiency by means of carbohydrates conversion during the dark anaerobic phase (0.018 Cmol PHB/Cmol carbs) than by means of IC uptake (0.009 Cmol PHB/Cmol IC). This was likely due to the highest carbohydrate concentration in condition 3, in comparison with other conditions.

3.1.4 Biomass microbial composition

Throughout the operation of the SBR, changes in the culture’s composition were observed in each operation mode. While the culture was originally composed by a consortium mostly formed by cyanobacteria (abundance ~60-70%), their dominance in the culture depended on the limitation of P in the culture. Hence, during the steady state of condition 1, where N and P were not limited in the culture, the mixed culture was composed by ~50% cyanobacteria and ~50% of other Phyla such as Chlorophyta (green algae) and Bacillariophyta (Diatoms). Due to their morphologic characteristics, the cyanobacteria present in the culture were identified to genus level as Aphanocapsa sp., Chroococcus sp., Microcystis sp., Pseudoanabaena sp. and Oscillatoria sp.. Likewise, it was identified species belonging to Chlorophyta were identified, such as Chlorella sp., Ulothrix sp. and
Scenedesmus sp.. Other species of diatoms and animals like Rotifera were frequently observed in the culture during this operation mode (Fig. A2).

When changing to condition 2, the biomass turned to an almost complete cyanobacteria dominated culture (abundance ~90%), mainly by Chroococcus sp., with some Pseudoanabaena sp., and Aphanocapsa sp. (Fig. A3). Green algae Chlorella sp. and diatoms were occasionally present, while rotifers were rarely observed in the culture. Hence, it is assumed that non-cyanobacteria microorganisms cannot grow so effectively in low P volumetric loads (1 mg L\(^{-1}\) d\(^{-1}\)) and P limitation. It should be noticed that under this condition the highest carbohydrate conversion efficiency was achieved, implicating that Chroococcus sp. are able to achieve high amounts of carbohydrates when is submitted to low carbon loads during the aerobic illuminated conditions.

In condition 3, a microbial consortium similar to condition 2 was observed, however, Aphanocapsa sp. was rarely observed, which suggests that appearance and dominance of this cyanobacteria depends not only on the phosphorus limitation, but also on the availability of nitrogen in the culture. This fact was also observed in a previous study by Arias et al., [16], where this organism dominated over green algae and other cyanobacteria when phosphorus was limiting and nitrogen was available. On the contrary, in the present work Chroococcus sp. was dominated the culture (abundance ~80%) while Pseudoanabaena sp. (abundance ~10%) was frequently observed. This fact implies that Chroococcus sp. can tolerate both nutrients limitation, however, the lack of nitrogen restricted carbohydrate accumulation. Species belonging to other Phyla were rarely observed under this condition (Fig. A4).
The activity of other microorganisms, such as nitrifying and denitrifying bacteria, as well as other heterotrophic microorganisms, was discarded due to the low values registered in N-NO₂ and N-NO₃ as well as the lack of external sources of organic carbon.

In summary, general results obtained in the continuous operation of the SBR indicated that the application of different operating strategies can be applied in order to select different cultures. Hence, the application of low carbon loads during the light phase coupled with high load of nitrogen and phosphorus (50/12.5/4.2 mg C-Na₂CO₃·N-NH₄⁺·P-PO₃⁴⁻ d⁻¹), promoted low biomass concentration (0.6 mg VSS L⁻¹), low N and P uptake, non-efficient carbohydrate production, and no PHB production. Moreover, this condition cannot be used for a cyanobacteria dominated culture selection, since the growth of other species, like green algae, diatoms and protozoa, is also promoted. When a culture is operated under both low carbon and phosphorus loads with high nitrogen (50/12.5/1 mg C-Na₂CO₃·N-NH₄⁺·P-PO₃⁴⁻ d⁻¹), a culture dominated by cyanobacteria was selected. Furthermore, although the low load of P was completely consumed, nitrogen uptake was not improved as consequence of carbon limitation, causing the utilization of the carbon available for carbohydrate production instead of biomass production, while no PHB was accumulated. On the other hand, when the culture had high loads of carbon and nitrogen and low loads of phosphorus (100/12.5/1 mg C-Na₂CO₃·N-NH₄⁺·P-PO₃⁴⁻ d⁻¹), a selected cyanobacteria dominated culture could take up more nitrogen until the point of being depleted few hours after its addition. Therefore, the carbohydrates accumulation performed during the light phase was not efficient, due to the utilization of nitrogen and carbon for biomass growth (0.99 g X L⁻¹, ~40% more than in previous conditions). Nevertheless, under this operating condition the total amount of carbohydrates accumulated during the light phase increased and could, therefore, be slightly converted into PHB during the dark phase due to the anaerobic conditions.
3.2 Polymers accumulation in batch test experiments

A batch test was performed after reaching the steady state of each operational condition tested in the SBR in order to assess the polymer (PHB and carbohydrates) storage capacity. These experiments started with biomass concentrations of 0.46, 0.61 and 1.51 g-VSS·L⁻¹, for conditions 1, 2 and 3, respectively. Since no additional nutrient source was supplied to the biomass, the remaining nitrogen (N-NH₄⁺ of 1.8 and 1.1 mgL⁻¹ for condition 1 and 2, respectively) was consumed in the first hours of the tests, while no remaining N was observed in the batch test corresponding to condition 3. In the case of P-PO₄³⁻, this nutrient was only observed in the first hours of condition 1, at 1.15 mg P-PO₄³⁻·L⁻¹ and not observed in any of the other accumulation tests. Furthermore, other parameters, such as dissolved oxygen, did not reach the levels achieved in the continuous operation during the light phase due to a greater oxygen transfer, since it was an open system (9.6 mg O₂·L⁻¹, 28°C).

The accumulation of carbohydrates was achieved during the light aerobic phase in all the conditions. Hence, 6.62, 11.41 and 15.57 Cmmols Carbs·L⁻¹, corresponding to percentages of 29, 48 and 43%, were obtained in the batch test of conditions 1, 2 and 3, respectively, after 24h of incubation (Fig. 4). Although a slight increase on carbohydrate content was observed in the first 4 hours of the dark anaerobic phase in condition 2 (from 48 to 53%), the carbohydrates content declined in all the conditions during the dark anaerobic phase. On the other hand, PHB started increasing in the light phase, as long as the N-NH₄⁺ was depleted in the medium. Contrary to carbohydrate patterns, PHB accumulation also continued in the dark anaerobic phase for batch tests of conditions 1 and 2 likely occurring by means of carbohydrate conversion to PHB. In this case, PHB reached values of 1.63 and 1.08 Cmmol PHB·L⁻¹, corresponding to percentages of 3.9 and 3.5%, respectively, after 48h of incubation. This pattern was not observed in
condition 3, in which 1.89 Cmmol PHB L\(^{-1}\), corresponding to 3.8 %, were obtained after 24h of light aerobic incubation. In this case, the culture already started with 1% of PHB from the SBR operation. After changing the condition to dark anaerobic phase, PHB decreased from 3.8% to less than 1% in the first 4 hours. It should be noticed that during the batch tests for conditions 1 and 2, IC was available during the dark phase, while it was almost depleted in condition 3. Hence, the decrease in PHB after finishing the light phase can be attributed to the lack of IC in the medium. This suggests that the presence of IC must be rigorously controlled during PHB production trials, otherwise this polymer may be quickly consumed by organisms in famine.
Fig. 4. Inorganic carbon (IC) consumption profile, poly (3-hydroxyalkanoates) (PHB) and carbohydrates transformation in batch tests performed with biomass collected from the sequencing batch reactor (SBR) during steady-state operation under a) carbon limitation (Condition 1) b) carbon and phosphorus limitation (condition 2), and c) phosphorus limitation (condition 3). Zone represents the light phase and grey zone represents the dark anaerobic phase.

Reviewing the specific kinetic rates in all the batch tests (Table 4), the highest consumption of IC was obtained with sludge selected under condition 3, with 0.0054 Cmol IC/Cmol X d⁻¹. However, this value and the rates observed in conditions 1 and 2...
were lower than the ones obtained in the SBR (0.016-0.039 Cmol IC/Cmol X d⁻¹).

Likewise, the maximum specific carbohydrate production rate was reached under condition 2, notwithstanding, it was achieved two times lower values than that obtained in the SBR (0.049 Cmol IC/Cmol X d⁻¹). The explanation for such results may be the change of conditions in the batch test, for example, the light type. Since the type of light provided in the SBR corresponded to a wavelength spectrum of 380–780 nm, the photosynthetically active radiation is promoted. On the other hand, the light used in the batch tests was halogen light. Although the same volumetric light intensity was provided as in the SBR (~2.2 W/L), its spectrum spanned from 380 to ~1000. Thus decreasing the energy available in the PAR region, and likely leading to a decrease in the metabolic rates. Nevertheless, despite the fact that these rates decreased, the batch tests enabled the understanding of the most favorable conditions for polymer production. This fact can be seen in the percentages of polymer content and polymer yields reached (Table 4). Thus, although carbohydrate yields reached lower values in biomass taken from conditions 1 and 2, the biomass from condition 3 increased more than twice the values obtained in the SBR (0.5 Cmol Carbs/Cmol IC). Likewise, PHB yields considerably increased as well. Although no PHB was detected for conditions 1 and 2 in the operation of the SBR, the batch test allowed those cultures to accumulate PHB in the light and dark anaerobic phase. This results in the highest yields during the dark anaerobic phase, when PHB accumulation occurred by means of carbohydrate conversion. Although the yield Y_{PHB/carbs} reached in the batch test of condition 1 was the highest of all conditions for this study (0.37 Cmol PHB/Cmol carbs) (Table 4), this value is lower than the yield achieved by other anoxygenic photosynthetic bacteria 0.94 Cmol PHB/Cmol carbs [25]. It is noteworthy that the highest PHB yield during the dark phase was achieved in the biomass from condition 1, which was composed by 50% of other microorganisms (i.e., green
algae, diatoms, protozoa) unable to produce PHB and 50% of cyanobacteria able to accumulate PHB. This fact indicates that the cyanobacteria community from that culture with a diverse species, such as *Aphanocapsa* sp. and *Microcystis* sp., was more efficient accumulating PHB than the other cyanobacteria dominated consortiums, mainly composed by *Chroococcus* sp.

Table 4. Kinetic and stoichiometric parameters of the batch tests performed during a steady state of the SBR for each operational condition.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Light aerobic</th>
<th>Dark anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condition 1</strong></td>
<td><strong>QIC / Cmol IC [Cmol X d⁻¹]</strong></td>
<td><strong>Qcarbohydrates / Cmol Carbs [Cmol X d⁻¹]</strong></td>
</tr>
<tr>
<td>Light aerobic</td>
<td>0.023ᵃ</td>
<td>0.0079</td>
</tr>
<tr>
<td>Dark anaerobic</td>
<td>0.0011</td>
<td>-0.002</td>
</tr>
<tr>
<td><strong>Condition 2</strong></td>
<td>Light aerobic</td>
<td>0.034ᵃ</td>
</tr>
<tr>
<td>Dark anaerobic</td>
<td>0.0034</td>
<td>-0.0081</td>
</tr>
<tr>
<td><strong>Condition 3</strong></td>
<td>Light aerobic</td>
<td>0.0054ᵃ</td>
</tr>
<tr>
<td>Dark anaerobic</td>
<td>0.0002</td>
<td>-0.0083</td>
</tr>
</tbody>
</table>

ᵃ Value calculated considering the two pulses of carbon as one sole dosage of carbon. ᵇ \(Y_{PHB} [\text{Cmol PHB/Cmol Carbs}].\)

The process of transient carbon availability has been widely employed in diverse bacterial cultures and has demonstrated to improve polymer production efficiencies mainly in terms of PHB [23]. However, the use of this process to improve polymer production in cyanobacteria has not been previously performed. Given the experimental design of the batch tests, the maximum polymer accumulation capacity of the culture is uncertain. In the case of carbohydrate content, it is clear that the dark condition stopped the accumulation process because carbohydrate accumulation depends on the CO₂ uptake, which in turn depends on the light availability. Once in the dark phase, and due to the lack of oxygen (electron acceptor), cyanobacteria were forced to perform anaerobic dark energy generation using carbohydrates as endogenous compounds [40]. On the other hand, PHB accumulated under both light and dark conditions during the aerobic light
phase as long as IC was available (and N was limited), and during the anaerobic dark phase from the carbohydrates stored in the previous illuminated phase.

Results obtained in the batch tests indicate, in general, that the best condition to accumulate PHB and carbohydrates is to use a culture previously adapted to carbon availability during the light phase along with high N and P limitation (Condition 3). Under these conditions, microorganisms obtained the highest $Y_{PHB}$ and $Y_{carbs}$. Furthermore, the highest PHB accumulation occurred in a shorter period in comparison to other conditions. From all the batch tests it is clear that high carbohydrate production depends on phosphorus limitation coupled with IC availability and the presence of light, while PHB can be accumulated during N limitation through IC uptake, during the presence of light, and by carbohydrates conversion, during the dark anaerobic phase. In terms of PHB, IC availability during the transition from light to dark conditions, when anaerobic condition is not well established, is necessary to avoid PHB consumption as a source of energy, as it was observed in condition 3. Having said that, values here obtained suggest that PHB and carbohydrates can be produced efficiently in the light phase but also PHB accumulation can continue by means of carbohydrate conversion during the dark. The possibility of employing a light/dark process implies a reduction on the cost related to artificial illumination in a full scale process. In such case, future studies should be conducted to evaluate PHB and carbohydrate accumulation in batch tests during 12h/12h light/dark periods for several days, to simulate real daylight illumination.

Considering that the main goal of his study was to produce both polymers; this research is contributing to the advance in the limited knowledge of feast and famine application to cyanobacteria producing PHB and carbohydrates. Among the possible applications for both polymers produced, carbohydrates can be used as substrate to obtain biodiesel,
bioethanol and biomethane, while PHB can be directed for bioplastics generation. The application of either of the two polymers will depend on the economic feasibility of each process and the commercial interest of the final product.

3.2.1 Comparison of a mixed cyanobacterial culture enhancement in a sequencing batch reactor (SBR) with other studies on polymer accumulation in batch tests

For comparison purposes, the percentages obtained in this work are here compared with batch experiments performed with cyanobacteria cultures submitted to aerobic illuminated autotrophic conditions (Table 5). In general, carbohydrate content obtained in the batch test of condition 1 (29%) was similar to those obtained by most of the previous studies carried out in pure cultures [19,20,45]. However, most of the other studies are surpassed by the values obtained in the batch tests of conditions 2 and 3. Only the study of Aikawa et al., [46] obtained a percentage of 47% in the first 24 h using a specific strain (Arthrospira platensis) that already contained 17% of carbohydrates.

Concerning the PHB content, results obtained with the wastewater-borne cyanobacteria utilized in this study, for all the conditions, achieved a higher PHB content in shorter time in comparison with pure cultures and even with the same type of culture [47]. Generally, most of the studies showed a very low process of PHB accumulation, reaching percentages lower than 1% in the first 24 h. In the particular case of Monshupanee et al., [22], PHB accumulation started after 120 h of incubation achieving a maximum of 13% after 288 h. The results of this study are only similar to those of Nishioka et al., [48], whose specific strain (Synechocystis sp. MA19) was able to start PHB accumulation after 10 h and reached 2% in 24 h. Remarkably, these authors reached 55% PHB content after an incubation of 120 h.
Table 5. Summary of the carbohydrate and PHB contents obtained in accumulation tests performed in this study compared with other aerobic batch studies performed with 24 h illumination.

<table>
<thead>
<tr>
<th>Cyanobacteria cultivated</th>
<th>Carbohydrates percentage obtained in 24h (% VSS)</th>
<th>PHB percentage obtained in 24h (% VSS)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanobacteria dominated mixed culture</td>
<td>29</td>
<td>2.6</td>
<td>This study$^a$</td>
</tr>
<tr>
<td>Cyanobacteria dominated mixed culture</td>
<td>48</td>
<td>2.04</td>
<td>This study$^b$</td>
</tr>
<tr>
<td>Cyanobacteria dominated mixed culture</td>
<td>43</td>
<td>3.8</td>
<td>This study$^c$</td>
</tr>
<tr>
<td><em>Arthrospira platensis</em></td>
<td>47$^d$</td>
<td>-</td>
<td>[46]</td>
</tr>
<tr>
<td>Cyanobacteria dominated mixed culture</td>
<td>16-22</td>
<td>0.7-0.9</td>
<td>[47]</td>
</tr>
<tr>
<td><em>Spirulina maxima</em></td>
<td>34$^e$-35$^f$</td>
<td>0.4$^f$-0.9$^f$</td>
<td>[19]</td>
</tr>
<tr>
<td><em>Anabaena cylindrica</em></td>
<td>-</td>
<td>0.1$^d$</td>
<td>[49]</td>
</tr>
<tr>
<td><em>Spitulina platensis</em></td>
<td>30$^f$</td>
<td>-</td>
<td>[20]</td>
</tr>
<tr>
<td><em>Synechocystis sp. PCC 6803</em></td>
<td>0.5$^d$</td>
<td>0$^d$</td>
<td>[22]</td>
</tr>
<tr>
<td><em>Synechocystis sp. MA19</em></td>
<td>-</td>
<td>2$^d$</td>
<td>[48]</td>
</tr>
<tr>
<td><em>Arthrospira platensis</em></td>
<td>32.5$^f$</td>
<td>-</td>
<td>[45]</td>
</tr>
</tbody>
</table>

$^a$ Results obtained in condition 1
$^b$ Results obtained in condition 2
$^c$ Results obtained in condition 3
$^d$ Values estimated from the figures in the original reference
$^e$ Value obtained in 9 h of incubation
$^f$ Values assumed as the half of the maximum content.

From a general view, this study highlighted that the improvement of PHB and carbohydrate accumulation in wastewater borne cyanobacteria can be achieved. The continuous process of cultivation and selection of specific microorganisms, through transient carbon regimes, enhanced the carbon uptake efficiency and the accumulation capacity of polymers. Thus, when the culture is submitted to a posterior accumulation process, a higher polymer content can be obtained. According to the encouraging results found in this study, further research should be directed to batch/fedbatch experiments testing 12h light/dark cultivation under controlled conditions, and then a posterior
enrichment of the microorganisms with real wastewater streams can be performed. In this last case, a double benefit could be achieved by producing valuable products while wastewater treatment is performed. Moreover, the production of polymers from wastewater-borne cyanobacterial cultures could be a cost-effective alternative to controlled pure cultures.

4. Conclusions

In this study, carbohydrate (biofuel substrates) and PHB (bioplastics) accumulation in wastewater-borne cyanobacteria was enhanced through transient carbon regimes in a sequencing batch reactor. During the continuous operation of the reactor, inorganic carbon was mostly used for biomass and carbohydrate production, showing very low PHB accumulation levels. Notwithstanding, in subsequent batch tests, PHB was accumulated after a complete depletion of nitrogen, reaching almost 4% of PHB. Concerning carbohydrates, it was found that phosphorus limitation (with and without carbon limitation) led to a culture mostly dominated by cyanobacteria, and to higher levels of carbohydrates content (43%-48%) than the culture with carbon limitation and high loads of nitrogen and phosphorus (29%). Such contents were obtained in only 24h of incubation under aerobic illuminated conditions. Hence, these encouraging results indicate that carbon uptake and the consequent polymers production can be enhanced through carbon and nutrient feeding strategies.

Authors' contribution

D.M. Arias performed the experiments, analysis and interpretation of the data, and drafted the manuscript. J.C. Fradinho supervised the experiments, contributed to the interpretation of the data and critically reviewed the manuscript. E. Uggetti, J. García, A.
Oehmen and M. A. M. Reis contributed to the interpretation of the data and critically reviewed the manuscript. All the authors read, edited and approved the manuscript.

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**Conflict of Interest Statement**

- The authors declare no conflicts of interest including any financial or other interests that could be perceived to influence the outcomes of the research.

- No conflicts, informed consent, human or animal rights applicable.

- All authors have agreed to authorship and submission of the manuscript for peer review.

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