Pilot-scale production of poly-β-hydroxybutyrate with the cyanobacterium *Synechocytis sp.* CCALA192 in a non-sterile tubular photobioreactor

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ABSTRACT

Poly-β-hydroxybutyrate (PHB) is considered as one of the most promising bioplastics. It has a broad range of applications and is degraded relatively rapidly by soil organisms. Like many prokaryotes, the cyanobacterium *Synechocystis sp.* CCALA192 produces this biopolymer as a storage compound, especially under nutrient starvation.

In a 200-liter tubular photobioreactor, we cultivated *Synechocystis sp.* CCALA192 semi-continuously over a period of 75 days with CO_2 as sole carbon source. A two-stage cultivation strategy was performed, where after 5-7 days nitrogen was depleted and the culture started to produce PHB and gradually turned from blue-green to yellow. After 16-20 days, 90% of the culture was harvested and the residual 10% was used as inoculum for the following cycle. The harvested culture had an average biomass concentration of 1.0 g/L with an average PHB content of 12.5% of cell dry weight. After restarting with fresh nutrients, the yellow culture turned blue-green again and degraded the PHB within 24-48 hours. When nitrogen of the medium was consumed, PHB was produced again and the cycle continued. In the late stage of each production cycle, a ripening process was observed, where no CO_2 was consumed but the PHB concentration was still rising at the expense of the existing glycogen rich biomass.

Establishing a stable *Synechocystis sp.* CCALA192 culture under non-sterile conditions turned out to be difficult, as this small unicellular organism is very sensitive and easily grazed by protozoa. Therefore, a special cultivation strategy with partially anoxic conditions was necessary.

Introduction

According to the Intergovernmental Panel on Climate Change (IPCC), scientific evidence for anthropogenic carbon dioxide (CO_2) as the main driver for climate change is unequivocal [1]. Therefore, more and more effort has been made to utilize CO_2 as raw material and transform it into different products [2]. Apart from the chemical possibilities, there are also biotechnological ways using CO_2 as a substrate. Cultivating microalgae with CO_2 as carbon source is one promising approach [3,4] and there is a wide variety of microalgal products, ranging from fuels like biodiesel [5–8] and bioethanol [9,10] over platform chemicals like isoprene [11] to high-value products like polyunsaturated fatty acids or astaxanthin [12,13].

Another interesting product synthesized by blue-green algae, better known as cyanobacteria, is the bioplastic poly-β-hydroxybutyrate (PHB). PHB is the most common polymer in the group of polyhydroxyalkanoates (PHAs) and the only PHA produced, when using CO_2 as sole carbon source. It has favorable mechanical properties similar to polypropylene and can be processed by thermoplastic methods, including fiber spinning or injection molding. But in opposite to petroleum-based polypropylene, it is compostable and degraded rapidly by soil organisms [14]. This could avoid serious environmental problems caused by spillage and litter from petroleum-based plastics. For example, in the Austrian Danube river, plastic litter had temporarily outnumbered fish larvae. The small plastic particles are ingested by a wide range of organisms with yet unknown consequences [15].

Cyanobacteria produce PHB as an intracellular energy and carbon storage compound. The most important trigger for PHB production is nutrient deprivation, especially nitrogen limitation. The role of PHB in cyanobacteria is still not clear; besides its storage function, it is supposed that it serves as an electron sink and helps to restore $NADP^+$ and maybe support the cell's stress resistance [16]. Generally, PHB concentrations in nutrient depleted, photoautotrophic cyanobacteria range between 5-20%, which is still quite low, compared to

heterotrophic PHB producers with concentrations above 70%. However, in contrast to heterotrophic bacteria, cyanobacteria do not consume sugars, that are responsible for an estimated 50% of the total production costs. Furthermore, cyanobacteria do no depend on agricultural crops, what makes them even more attractive as sustainable biomass producers [17–20].

Although there a numerous reports about PHB production with cyanobacteria, there are hardly any reports about pilot-scale production under non-sterile conditions. Apart from *Arthrospira sp.,* which can be grown in a highly alkaline environment, cyanobacteria are not grown in an industrial scale. *Arthrospira sp.* produces comparable low amounts of PHB, therefore other strains like *Synechocystis sp.* or *Synechococcus sp.* are more promising. There are next to no reports about full-scale production plants with these cyanobacteria. Indications are strong that the primary reason may be the difficulty of establishing a stable process – avoiding disturbances or overgrowth by other microorganisms. The main difference of large photobioreactors compared to lab-scale systems is the impossibility of sterilization. Therefore, large photobioreactors are susceptible to biological pollutants. Contamination problems due to protozoa are a major drawback when cultivating microalgae in an industrial scale [21].

This work investigates the pilot-scale production of PHB with *Synechoystis sp.* CCALA192 in a non-sterile photobioreactor with a volume of 200 liter. Over a period of 75 days, four production cycles were performed with a special cultivation strategy of partially anoxic conditions. Several parameters such as biomass concentration, PHB concentration, glycogen concentration and CO_2 consumption were measured. The culture was routinely observed in the microscope and occurring contaminants are described. Furthermore, a new operation mode is proposed for a more efficient PHB production with cyanobacteria.

Material and methods

Organism and culture conditions for strain maintenance

Synechocystis sp. CCALA192 was ordered from the Culture Collection of Autotrophic Organisms, Institute of Botany, Trebon, Czech Republic. For strain maintenance in the laboratory, the strain was cultivated in BG11 medium [22] supplemented with 1 g/L sodium hydrogencarbonate ($NaHCO_3$) with a resulting pH of 8.5. Shaking flasks containing 50 mL of medium were used, light intensity was 1000 lx with a light/dark cycle of 16/8 and a temperature of 25°C.

Photobioreactor and online analytics

The photobioreactor used in this study is situated in a small glass house at the coal power plant in Dürnrohr, Austria. It is a tubular reactor build with glass tubes from the Schott AG, Germany, with an inner diameter of 60 mm. The volume of the reactor is 200 liter and the total length is 80 meter. A centrifugal pump was used for circulating the medium with a velocity of 0.7 m/s. A bubble column with a height of two meter served as degasser. Pure nitrogen gas (N_2) with a constant flowrate of 500 mL/min served for oxygen removal. Pure carbon dioxide (CO_2) was injected over a PI (proportional-integral) operated mass flow controller to keep the pH value. This allows online monitoring of CO_2 consumption as well as precise pH control. The pH value and oxygen concentration were continuously measured with probes (CPS11D and COS51D, both Endress+Hauser GmbH, Austria). The photosynthetically active radiation was measured with a PAR sensor (Theodor Friedrichs & Co., Germany). An additional artificial light was supplied with four 250 W gas discharge lamps (2 x Philips Maser HPI-T and 2 x Philips Master SON-T) and 50 meter of LED strips (60 SMD-LED, 14.4 W per meter, 2700-3000 K). The LED strips were mounted directly on the glass tubes. The temperature in the glasshouse was controlled with an air conditioning

system to 25°C +/- 2°C. The data from all probes was captured by a data acquisition board (National Instruments) and connected to a computer for measurement and control.

Culture medium and cultivation strategy in the photobioreactor

Synechocystis sp. CCALA192 was cultivated in the photobioreactor with a modified BG11 medium. This BG11 contained no citric acid and was supplemented with 0.5 g/L $NaHCO_3$ and 0.5 g/L Na_2CO_3. The pH was then adjusted to pH 10 (production cycle 1 and 2) respectively pH 9 (production cycle 3 and 4). 0.4 g/L instead of 1.5 g/L $NaNO_3$ was used, leading to self-limitation of the culture. After 16-20 days of cultivation, 90% of the culture was harvested and the remaining 10% were used as inoculum for the next production cycle. Due to degassing with pure nitrogen gas, the oxygen concentration in the reactor decreased below detection limit during night. This approach was necessary for contamination control and led to stable growth conditions.

Analytical methods

For cell dry weight determination, 50 mL of the culture was centrifuged at 4000g for 10 min. The pellet was washed with deionized water, centrifuged again and dried at 105°C overnight. After weighing, the dried pellet was then used for PHB determination.

The determination of PHB concentration in the biomass was performed with a modified method after Karr et al. [23]. The dried pellet was heated at 95°C for 4 hours in 1 mL of concentrated sulfuric acid. This step converts PHB to crotonic acid. After diluting to 25 mL with deionized water, crotonic acid was measured on a HPLC system (Agilent 1100, column: Transgenomic CARBOSep COREGEL 87H).

The determination of glycogen concentration was performed with a modified method after Maurer at al. [24]. 10 mL of culture was centrifuged and the pellet was resuspended in 2 mL 6 M hydrochloric acid and heated at 95°C for 2 hours for total breakdown of the cells and

digestion of glycogen. The solution was then diluted to 25 mL with deionized water and glucose was measured on a HPLC system (Agilent 1100, column: Transgenomic CARBOSep COREGEL 87H).

The optical density of wavelengths 400-800 nm was measured with a spectrophotometer (Hach-Lange DR-3900).

Nitrate concentration of the cell-free culture supernatant was measured with a commercial test kit (Hach-Lange LCK339).

Harvest and PHB purification

For harvesting, 150 liters of the PHB rich culture of cycle 3 was flocculated using 150 mL of a 40% $FeCl_3$ solution. After sedimentation, the algae sludge was centrifuged to further remove water and finally freeze-dried. The freeze-dried biomass was then treated with 6% sodium hypochlorite solution on ice for 1 hour (10 mL of solution on 1 g of dried biomass). The sample was then centrifuged and washed twice with deionized water and dried at 60°C. The sample was then extracted with hot chloroform in a soxhlet extractor overnight. The PHB rich extract was precipitated with cold ethanol, centrifuged and dried to finally obtain pure PHB.

Analysis and characterization of PHB

The chemical structure of PHB derived from biomass produced by *Synechocystis sp.* CCALA192 was characterized by Fourier transform infrared spectroscopy. FTIR spectra were obtained in Attenuated Total Reflection (ATR) mode with single-reflection diamond crystal using a Nicolet iS50 spectrometer. Spectrum was collected as the average of 64 scans in the frequency range of 4000 – 800 cm^{-1} with the resolution of 4 cm^{-1}.For the measurement was applied the frequency range of 4000 – 800 cm^{-1} and 64 scans with the resolution of 4 cm^{-1}. Elemental composition of PHB was determined using a CHNS analyzer EuroVector EA 3000. The sample was sealed in tin pans and heated in the oven up to 980°C in an oxygen atmosphere. The concentration of CHNS elements has been determined by using thermal conductivity detector (TCD) and calibration with the sulphanilamide. Average molecular weights (M_n and M_w) and polydispersity ($Đ_M$=M_w/M_n) of extracted polymer were analyzed by

gel Size Exclusion Chromatography (Agilent, Infinity 1260 system, PLgel MIXED-C column) with Multiangle Light Scattering (Wyatt Technology, Dawn Heleos II) and Differential Refractive Index (Wyatt Technology, Optilab T-rEX) detection. PHA was dissolved in HPLC-grade chloroform (4 mg mL^{-1}) overnight and after dissolution was analyzed. Chloroform was used as eluent at a flow rate of 0.6 ml min^{-1} and the injection volume was 100 μL. The obtained molecular weights were calculated using the value of refractive index increment of PHB (dn/dc) 0.0336 mL/g, as was determined from the differential refractometer response assuming a 100% sample mass recovery from the column. Thermal stability of the extracted polymer (5 mg of sample sealed in an aluminium crucible) was determined by thermogravimetric analysis (TGA) on a TGA Q50 (TA Instruments) under nitrogen flow of 50 ml min^{-1}, in the temperature range from 25°C to 500°C with a heating rate of 10°C min^{-1}. Thermal behavior of PHB was analyzed with differential scanning calorimetry (DSC), using DSC Q20 (TA Instruments). Sample of approximately 5 mg was sealed in aluminium pan and analyzed with heating and cooling scanning within the temperature range of -50°C to 190°C according to the methodology reported by Kovalcik et al. [25]

Microscopy

The culture was routinely observed in the microscope (Olympus AHBT3 VANOX) for evaluation of contaminations.

Results and discussion

Description of the culture and contamination monitoring

We previously described the necessary contamination control for the non-sterile cultivation of *Synechocystis sp.* [26]. Stable cultivation of this small cyanobacterium was only possible with partially anoxic conditions. Through degassing with pure nitrogen, the oxygen in the culture was removed during the night and inhibited the ciliate *Colpoda steinii*. This cultivation method was performed throughout all cultivations in this study. Furthermore, *Synechocystis sp.* was cultivated at relatively high pH values above 9.

Even with these selective conditions, diverse accompanying microbial flora was still present. The regular microscopic contamination monitoring revealed the presence of several protozoa and some bacteria. Other cyanobacteria or green algae were not seen. Figure 1 shows four representative microscopic pictures of the culture. At higher pH during the first and second cultivation cycle, more amoeba were visible while rotifers were only visible at the third and fourth cultivation cycle with lower pH. These organisms have never caused serious problems such as culture crashes or strong biomass losses.

However, there is a rather high variance in most obtained datasets noticeable. The reason for this could be the complex ecosystem in the photobioreactor, as it is unknown how many different organisms are actually present and how they influence each other. Most important for a production plant is the dominance of the production organism and the establishment of a stable culture. Culture crashes should be avoided, as they cause long disruptions for reactor cleaning and cultivation of the seed culture. Another reason for the variance of the data is that the photobioreactor is located in a glasshouse and subject to light fluctuations.

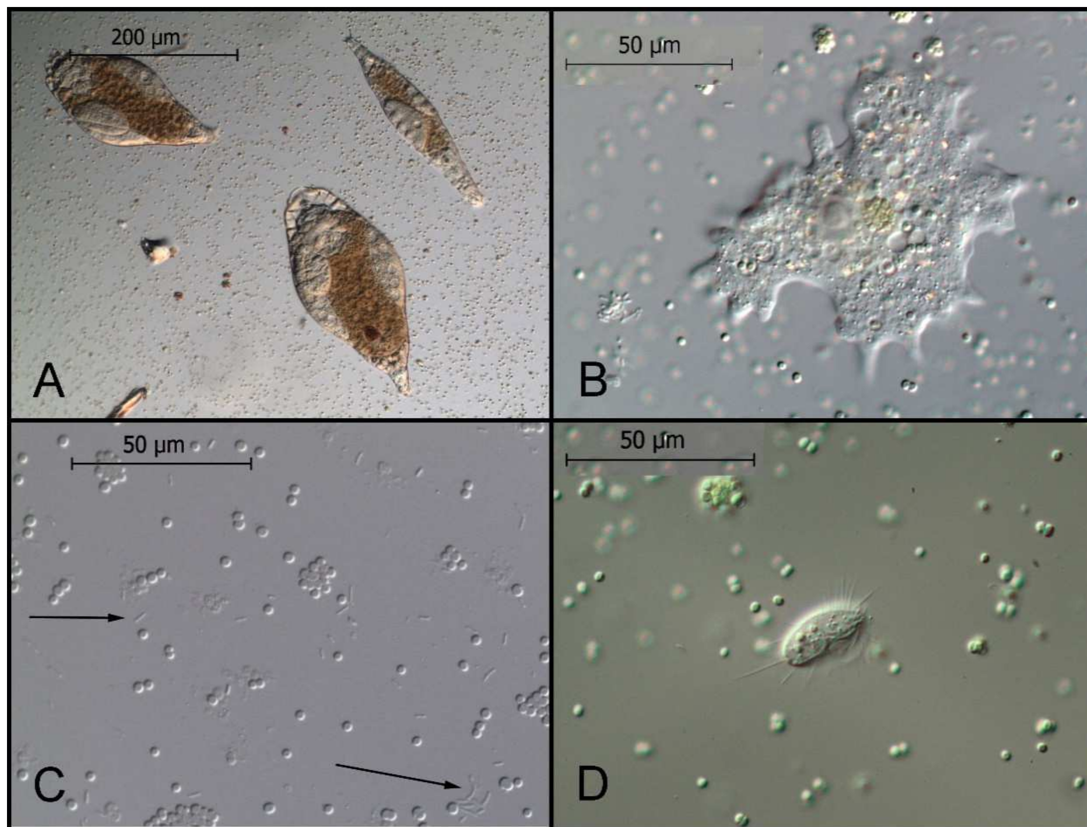

Figure 1: (A) rotifers graze on *Synechocystis*, (B) an amoeba is slowly ingesting *Synechocystis*, (C) bacteria are present in the culture, (D) a small ciliate swims in the culture.

Less contamination problems are one of the main advantages of closed photobioreactors often described in literature [27,28], but this assumption is obsolete. Apart from the reactor itself, there is peripheral equipment like water tanks or nutrient tanks where possible contaminants may enter the culture. Dust is suspected as the main vector for contaminants. Once a contaminant has entered the reactor, it is difficult to remove it. Usually, larger closed tubular photobioreactors have hundreds of joints and several valves, where some biomass remains in the small cavities after cleaning. Furthermore, many protozoa are known to form resilient resting cysts.

The vulnerability of a culture is mainly dependent on the microalgae species. Compared to *Arthrospira sp.* or green algae like *Chlorella sp.,* which are also cultivated at our institute,

Synechocystis sp. appeared to be a rather sensitive organism. The reason for this could be the small size and the prokaryotic cell wall. The cell wall of many eukaryotic green algae is known for their recalcitrance, complicating the extraction of intracellular products. For example, the call wall of *Nannochloropsis gaditana* consists of a bilayer structure consisting of a cellulosic inner wall protected by an outer hydrophobic alganean layer [29]. Such structures are more difficult to digest for predators than the gram-negative cell wall of *Synechocystis sp.* Although there are various interesting products from *Synechocystis sp.,* the vast majority of publications did not exceed the level of shaking flasks or small sterile laboratory reactors, what shows the difficulty of growing *Synechocystis sp.* under non-sterile conditions.

Another aspect of the culture was that *Synechocystis sp.* was capable of forming flocs spontaneously, both at lower and higher pH values. Although no specific trigger was determined, the capability of auto flocculation is especially interesting for an economic harvesting and has been shown and described for a variety of algal species [30–32].

Biomass, PHB and glycogen accumulation

The tubular photobioreactor was running semi-continuously in the time from June to November. Four production cycles in a row over a period of 75 days were monitored. While cycle 1 and 2 were performed at pH 10, cycle 3 and 4 were performed at pH 9. Each production cycle was started with a volume of 20 liters (10%) of the pre-existing, stationary, PHB and glycogen rich culture. Figure 2 shows biomass, glycogen and PHB production of those four production cycles. Biomass concentration reached its maximum value of 1.2 g/L ± 0.2 g/L 12-14 days after starting and was then decreasing to 1.0 g/L ± 0.2 g/L. PHB and glycogen were consumed within the first 2-3 days in all production cycles. Nitrate, as sole nitrogen source, was consumed within 5-6 days, leading to limitation and PHB and glycogen was produced again. Glycogen concentration reached its maximum values concurrently with the maximum biomass concentration and was then decreasing towards the end as well. PHB concentration, on the other hand, was increasing steadily with highest concentrations of 12.5% ± 1.4% of cell dry weight at the end. Mean volumetric PHB productivity was 7.0 ± 1.1 mg/L/d. Production cycles 1 and 2 at higher pH showed lower maximum glycogen concentrations of 26.9% and 21.2% compared to 37.2% and 40.3%. Table 1 summarizes the results of the four production cycles.

Other reports of photoautotrophic cultivation of wild type *Synechocystis sp.* have shown PHB concentrations ranging from 4.1% - 16.5% [33–35]. Therefore, *Synechocystis sp.* CCALA192 with 12.5% PHB of cell dry weight can be considered to be within the most interesting cyanobacteria. The stationary growth phase showed decreasing biomass concentration and increasing PHB concentration. This growth pattern was also shown by Kamravamanesh and colleagues with *Synechocystis sp.* PCC6714 [33].

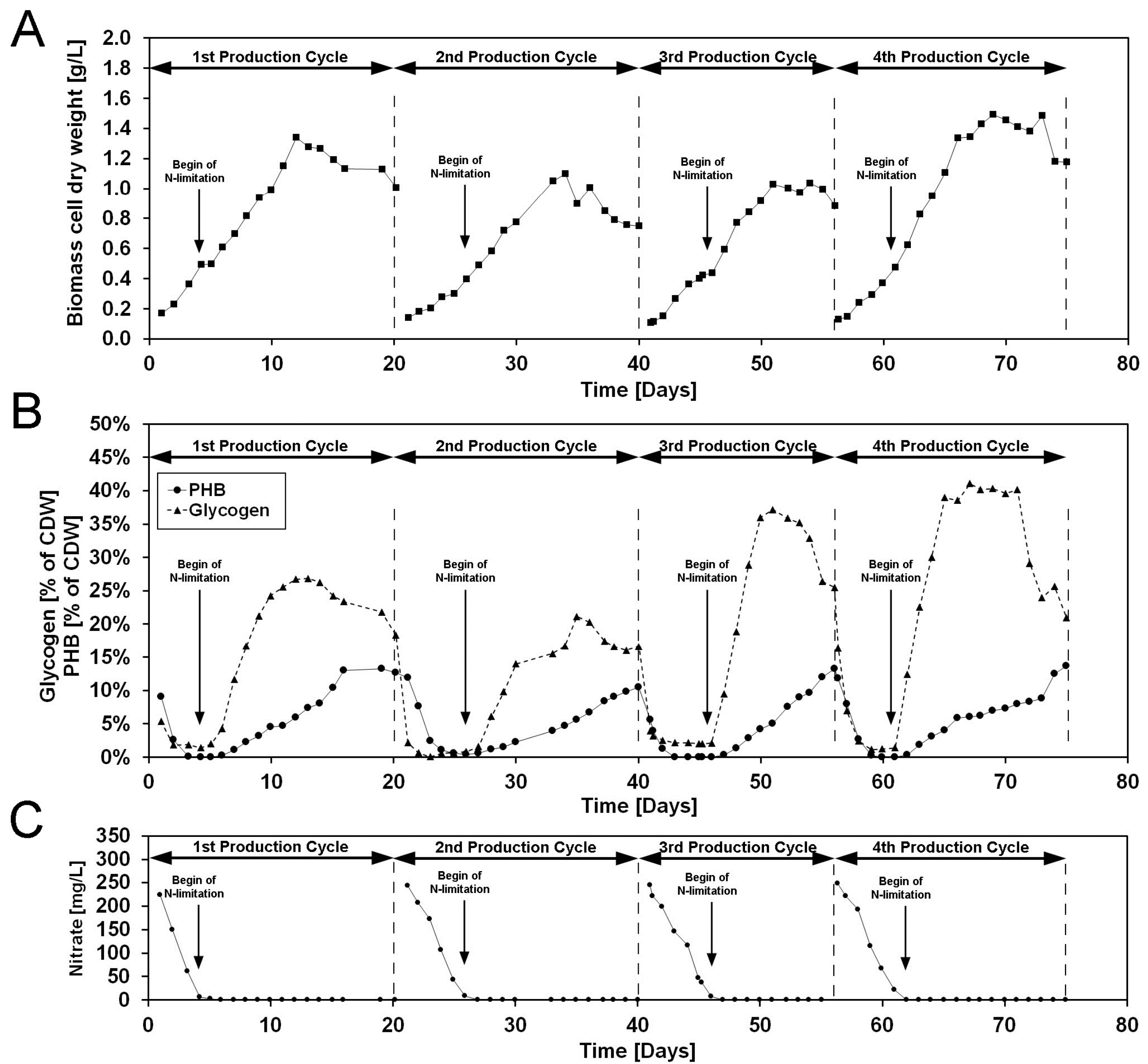

Figure 2: (A) Graph depicts biomass cell dry weight of four production cycles. Each production cycle shows growth for 10-14 days and a decreasing biomass concentration towards the end. (B) Graph depicts PHB and glycogen concentration of four production cycles. Both are consumed within the first 2-3 days and produced again after nitrogen limitation. Glycogen concentration decreases towards end of each cycle. (C) Nitrate is consumed within 4-6 days of each cycle.

Table 1: Results of four production cycles are summarized.

	Duration	pH	Maximum biomass concentration	Final biomass concentration	Final = max. PHB concentration [% of cdw]	Volumetric PHB production rate	Maximum glycogen concentration [% of cdw]	Final Glycogen concentration [% of cdw]	Total CO_2 consumption
1st Production Cycle	20 days	10	1.3 g/L	1.0 g/L	12.7 %	6.3 mg/L/d	27.9 %	18.3 %	2.2 g/L
2nd Production Cycle	20 days	10	1.1 g/L	0.8 g/L	10.5 %	6.0 mg/L/d	21.2 %	16.6 %	2.0 g/L
3rd Production Cycle	16 days	9	1.0 g/L	0.8 g/L	13.3 %	7.3 mg/L/d	37.2 %	25.5 %	2.2 g/L
4th Production Cycle	19 days	9	1.5 g/L	1.1 g/L	13.7 %	8.5 mg/L/d	40.3 %	21.0 %	1.6 g/L

Average			1.2 g/L ± 0.2 g/L	1.0 g/L ± 0.2 g/L	12.5% ± 1.4 %	7.0 ± 1.1 mg/L/d	31.3 % ± 8.9 %	20.4 % ± 3.8 %	2.0 g/L ± 0.3 g/L

CO_2 consumption and pH control

Carbon nutrition in form of CO_2 is substantial for photoautotrophic growth. As CO_2 is one of the main drivers of the production costs, losses should be kept as low as possible. In the present tubular photobioreactor, pure CO_2 was injected before the pump, allowing the CO_2 bubbles to dissolve in the glass tubes during the circulation time of 2.5 minutes. Due to the high pH values of the culture medium, dissolved CO_2 rapidly converts to HCO_3^- and CO_3^{2-}, respectively. These ionic forms cannot leave the reactor over the degasser. According to the carbonate equilibrium, at pH 9, less than 0.2 mol % of the three species is present in form of CO_2. Therefore, there are only negligible losses of CO_2 due to stripping.

Photosynthetic activity of microalgal growth increases the pH value. Alkalinisation of the culture medium has been widely reported as a result of CO_2 uptake [36–39] and was also observed during our experiments. Via injection of pure CO_2 over a proportionally-integrally (PI) operated mass flow controller, the pH was maintained at a certain set point and consumed CO_2 was online monitored. Figure 3 shows the pH of the culture and the CO_2 mass flow over a period of five days. When the lamps turned on, the pH was rising until it reached the setpoint. The CO_2 mass flow controller then started injecting pure CO_2. When the lamps turned off, the pH decreased and no CO_2 was injected during the night. To the best of our knowledge, this photobioreactor is the only one reported with PI control and online CO_2 monitoring. Regarding to the literature, on-off controllers are mostly applied [37,40,41], but they often lack a real time monitoring of the consumed CO_2.

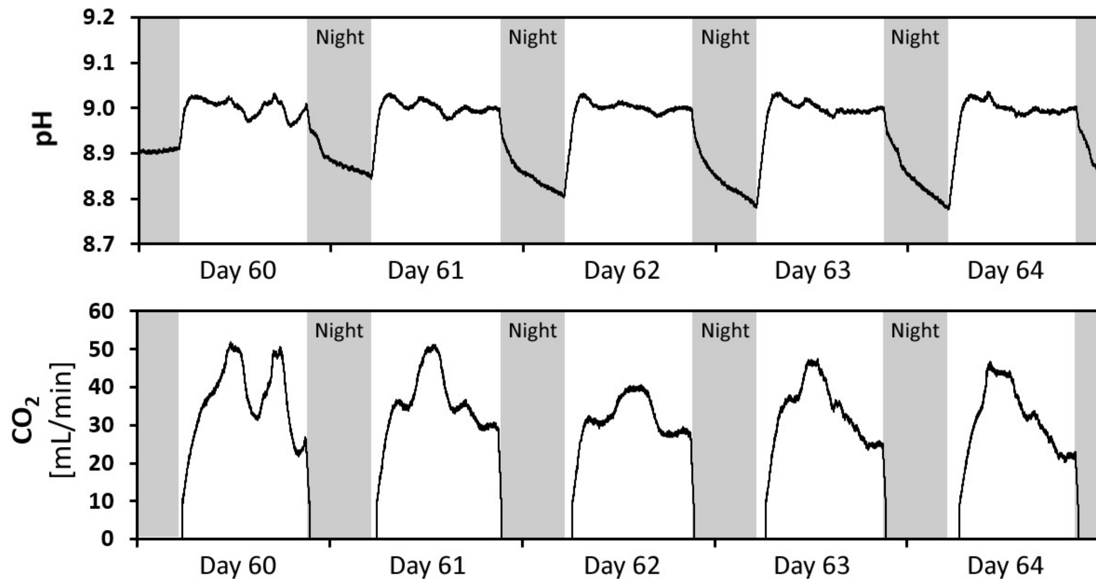

Figure 3: The pH is precisely controlled with a PI-controlled mass flow controller for CO_2. During the day, CO_2 is injected to keep the pH at a set point of 9. During the night, no CO_2 is injected and the pH is slightly decreasing due to respiration of the cells.

Figure 4 shows the daily CO_2 consumption throughout all cultivation cycles. A temporary decrease of CO_2 consumption could be noticed, when nitrogen was depleted. The reason for this could be the extensive metabolic reorganization of the cells during limitation. For producing 1 g of biomass, 2.0 g CO_2 were used. This value is in accordance with other reported microalgae CO_2 yield coefficients such as 2.31 $g_{CO2}/g_{biomass}$ [41] and 1.8 $g_{CO2}/g_{biomass}$ [6].

There was a strong decrease of CO_2 consumption towards the end and almost no CO_2 was consumed in the last five days of each production cycle, although PHB was still produced. In contrast, glycogen concentration was decreasing, indicating a conversion of glycogen to PHB. Conversion of glycogen to PHB was also described by Stal in 1992 [42]. In a very recent study, Dutt and colleagues have shown with ^{13}C labeling studies, that after nitrogen limitation, carbon from CO_2 contributes only 26% of the carbon for PHB synthesis. Intracellular carbon recycling is the most important carbon source for PHB synthesis [43].

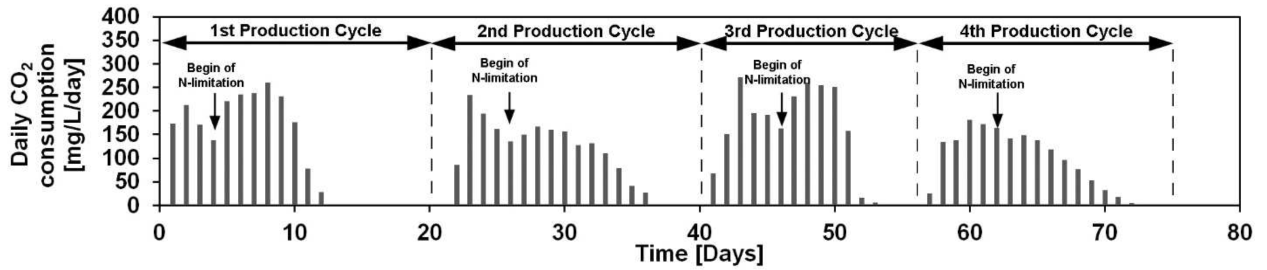

Figure 4: CO_2 consumption of each day is depicted. There is a strong decrease of CO_2 consumption in the late stage of each production cycle.

Oxygen evolution

The nitrogen gas flow in the degasser and the pump speed, responsible for the circulation time, were the most important factors to manipulate the oxygen concentration of the culture. These two parameters were held constant throughout the experiments. Therefore, the oxygen concentration was mainly dependent on the photosynthetic activity of the culture. Figure 5 shows the oxygen concentration and the photosynthetically active radiation (PAR) over a period of five days. During daytime, oxygen concentration reaches peak values of over 20 mg/L, while there are anoxic conditions during nighttime. The overall oxygen evolution was measured in terms of 24-hour mean dissolved oxygen concentrations (Figure 6) and must be regarded as relative values. Similar to the CO_2 consumption, the mean dissolved oxygen concentration decreased temporarily when nitrogen was depleted. In the late cultivation stage, there was hardly any oxygen evolution measured.

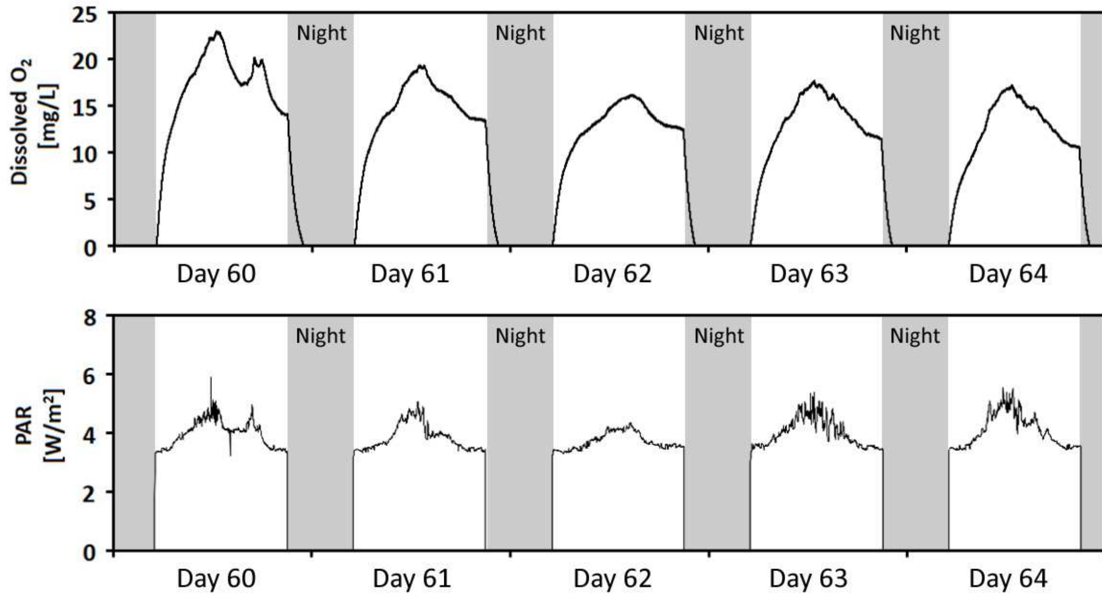

Figure 5: Dissolved oxygen concentration and photosynthetivally active radiation (PAR) are depicted over a period of five days.

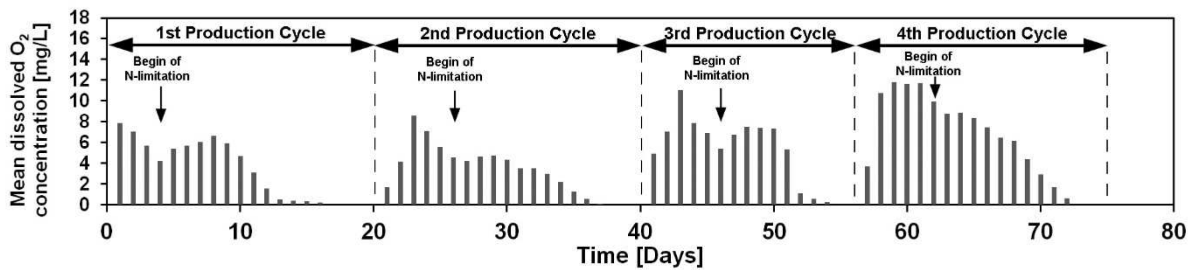

Figure 6: 24-hour mean dissolved oxygen concentration of each day is depicted. There is a strong decrease in the late stage of each production cycle, indicating poor photosynthetic activity.

A proposed operation mode for PHB production

22 out of 75 production days have shown very low CO_2 consumption (less than 10 mg/L/day and little oxygen evolution. All of these 22 days were part of the late stationary phase. During those periods, marginal amounts or no oxygen was produced. These findings indicate the poor photosynthetic activity in the late stationary phase. However, the PHB concentration was still rising due to intracellular conversion. This can be considered as an important ripening process. No illumination is necessary for this process as dark incubation for increasing the PHB concentration has already been described [44–46]. Considering a production plant, 30% occupation of the photobioreactor with little photosynthetic activity due to a ripening process would be costly and should be avoided. The ripening process should take place outside the reactor in stirred tanks. In these tanks PHB synthesis could be further stimulated by addition of substances like acetate, as it is widely reported to increase PHB concentration [33,35,44,47].

Here, a three-stage operation mode for optimal cyanobacterial PHB production is proposed and shown in Figure 7. The first is the green stage when biomass is growing and nitrogen is consumed. The second is the yellow stage, when the culture is nitrogen limited and starts to produce glycogen and PHB. The third is the ripening stage, where intracellular conversion takes place in stirred tanks and PHB concentration is further increased.

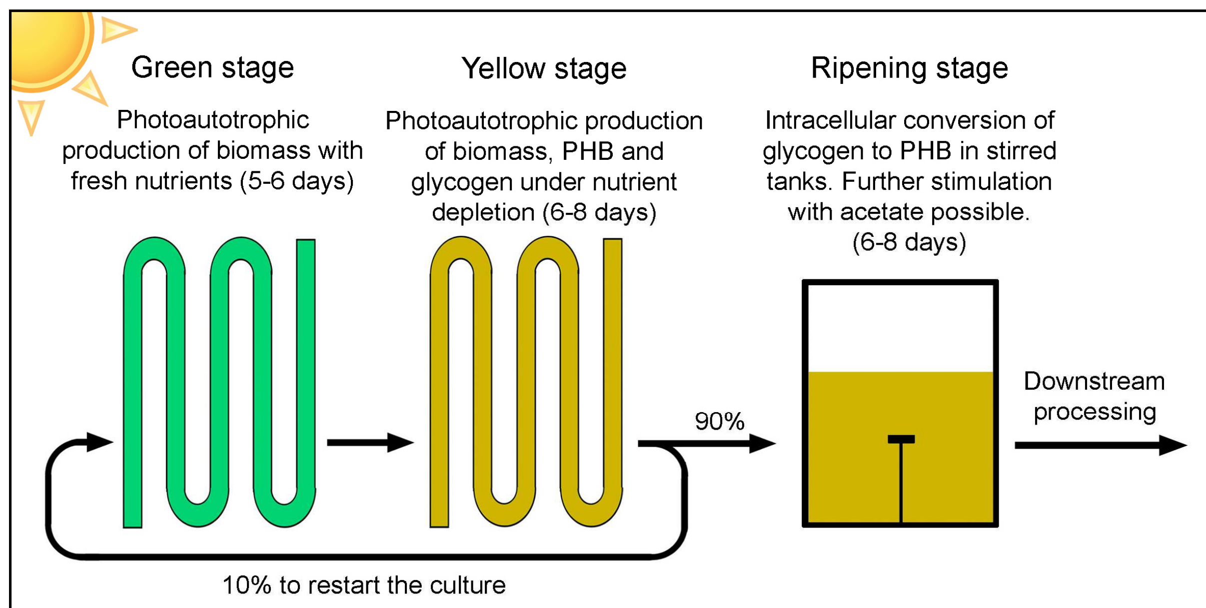

Figure 7: A proposed operation mode for cyanobacterial PHB production is shown. In order to fully use the photosynthetic capacity of the PBR, a ripening tank serves for PHB production in the late stage of the culture, when glycogen is converted to PHB and no CO_2 is consumed.

Downstream, PHB purification and analysis

Flocculation of *Synechocystis sp.* CCALA192 culture with iron chloride led to dense precipitation and a clear supernatant. Approximately 90% of the water could be removed with this step. The biomass was further dewatered via centrifugation and finally freeze-dried. The freeze-dried biomass had to be treated with alkaline sodium hypochlorite prior extraction with hot chloroform, as otherwise the extraction yield was unsatisfactory.

FT-IR spectrum displayed in Figure 8, shows functional groups typical for poly(3-hydroxybutyrate) (PHB). The bands in the range of 3000-2800 cm^{-1} are assigned to methyl and methylene stretching vibrations. The intense band at 1722 cm^{-1} represents carbonyl stretching of an ester group. Asymmetrical shape of this band with a typical shift of its maximum towards lower wavenumbers indicates a significant crystallinity of PHB.The bands at 1453 cm^{-1} and 1379 cm^{-1} correspond with the asymmetric and symmetric deformation vibrations of methyl groups. The bands corresponding with ester group stretching

vibrations in the range of 1300-1100 cm^{-1} indicate the proportion of the crystalline (represented by bands at 1229 and 1279 cm^{-1}) and the amorphous phase in PHB (band at 1180 cm^{-1}). The elemental composition (CHNS) of the extracted polymer was as following: carbon (54.4 %), hydrogen (7.7 %), nitrogen (0 %) and sulphur (0 %). The absence of nitrogen and sulphur is connected with the purification method, giving polymer without protein. The determined weight average molecular weight and polydispersity index of the extracted PHB was 930 kDa and 4.4, respectively. A broad molecular weight distribution of PHB produced by *Synechocystis sp.* CCALA192 corresponds with the applied harvesting and downstream methodology. The thermal properties of the extracted PHB correlate with the determined molecular weight, giving the glass transition (Tg) at -30.5°C, the melting temperature (Tm) at 174.0°C, the crystallinity (Xc) of 56.6% and the start of the thermal degradation (Tonset) at 273.0°C.

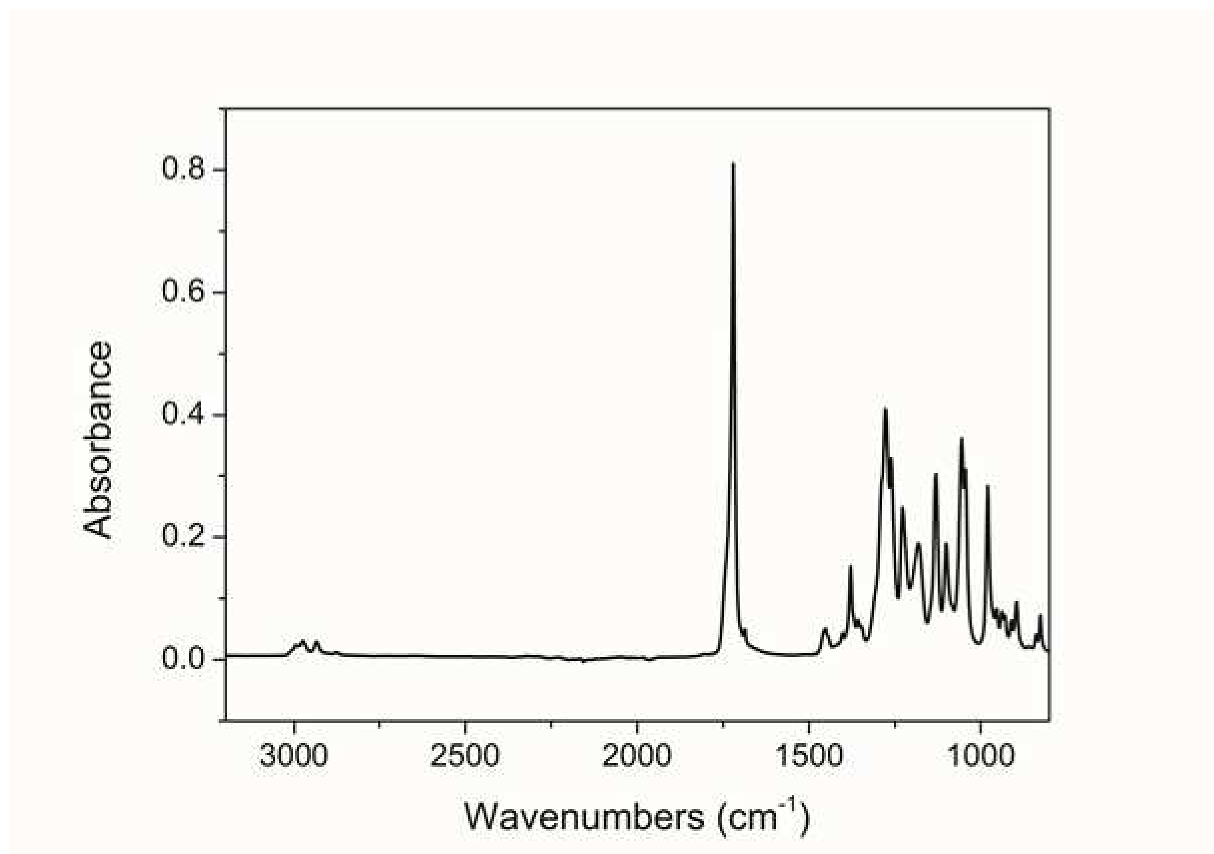

Figure 8: FT-IR spectrum of PHB produced by *Synechocystis sp.*

Deeper insights into downstream processing of *Synechocystis sp.* and possible side products are provided elsewhere [48].

Nitrogen limitation and chlorosis

Nitrogen limitation not only led to production of PHB and glycogen, but also to a strong change of the culture color. The blue-green culture turned gradually to yellow. The wavelength scans (Figure 9) show the relative decrease of phycocyanin and increase of carotenoids. Figure 10 shows photographs of the photobioreactor at the blue-green stage and the yellow stage.

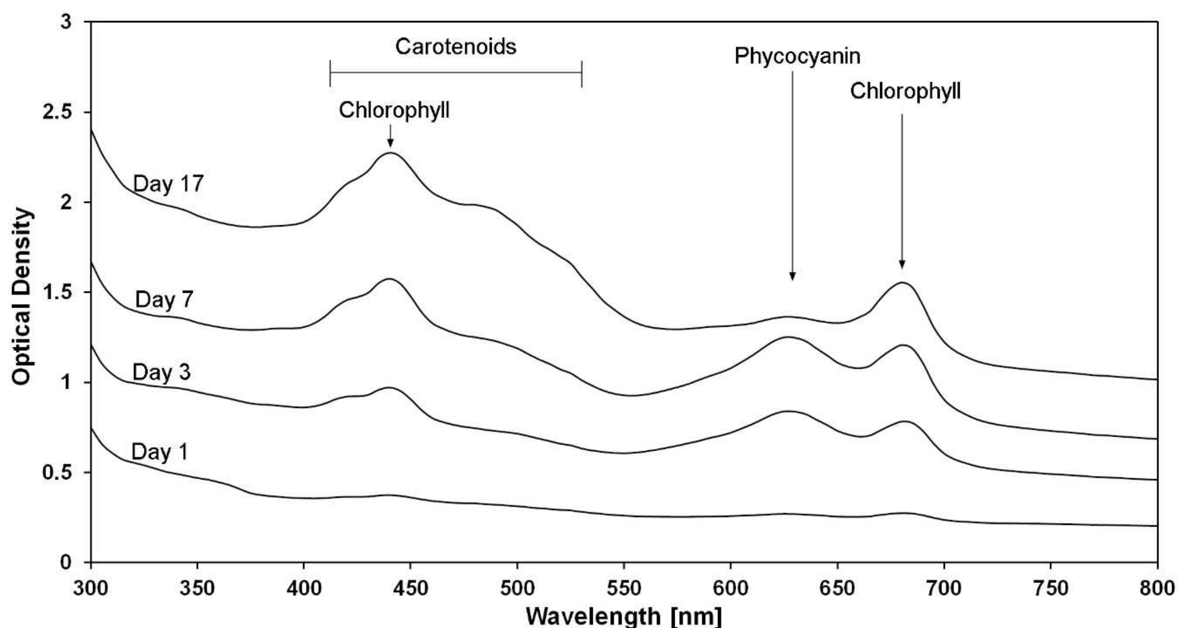

Figure 9: Wavelength scans (production cycle 1) show the relative increase of carotenoids and decrease of phycocyanin in the late stationary stage.

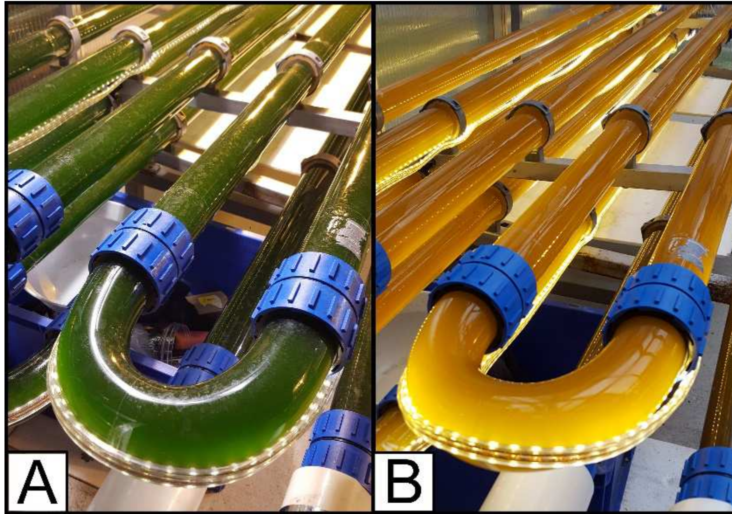

Figure 10: The culture in the tubular photobioreactor undergoes a strong change of color during each production cycle from blue green (A) to yellow (B).

Conclusion

This study demonstrates the long term, non-sterile cultivation of *Synechocystis sp.* CCALA192 in a tubular photobioreactor for PHB production. A special cultivation strategy is necessary to maintain a stable culture, as this small unicellular cyanobacterium is vulnerable to predators. However, even with selective conditions such as high pH values and partially anoxic conditions, the cyanobacterial culture appeared as a complex ecosystem and side organisms such as bacteria, ciliates, amoeba and rotifers were present. We see this work as a first step towards large-scale, non-sterile cultivation of *Synechocystis sp.* and further improvements could be made with variation of different cultivation parameters.

The thermoplastic, semi-crystalline PHB was produced in a two-stage process with nutrient depleting conditions. The nutrient concentration of the culture medium needs to be in a certain range, as too much would not lead to the necessary starvation and too little would cause a poor biomass production. Regarding the cultivation time, there is a necessary compromise between PHB concentration and overall productivity. The longer the cultivation time, the higher the PHB concentration. On the contrary, there is hardly any CO_2 fixed with low photosynthetic activity at the late cultivation stage. We see this as an important ripening process and it should take place separately in stirred tanks to free the reactor for the next cultivation cycle.

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Declaration of author's contribution

Clemens Troschl designed and performed experiments, analyzed data and wrote the article. Katharina Meixner has done substantial work at the photobioreactor and revised the manuscript. Klaus Leitner and Alejandra Palacios Romero performed experiments. Adriana Kovalcik and Petr Sedlacek analyzed the purified PHB sample and revised the manuscript. Ines Fritz and Bernhard Drosg supervised work, analyzed data and revised the manuscript.

Conflict of interest statement

The authors declare no conflict of interest.

Statement of informed consent, human/animal rights

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

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