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Bioplastic production with cyanobacteria

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ABSTRACT

Cyanobacteria, as photoautotrophic organisms, possess the metabolism to transform climate-damaging carbon dioxide (CO_2) to biomass with light as energy source. This ability makes them to promising green cell factories for a more sustainable production of useful molecules. One of the most interesting products synthesized by cyanobacteria is the biopolymer poly-β-hydroxybutyrate (PHB). PHB has similar material properties like polypropylene and can be used as a bioplastic. In contrast to conventional plastics, PHB is biodegradable and serious environmental problems due to plastic waste could be avoided. Cyanobacteria produce PHB as an intracellular energy and carbon storage molecule. Nutrient starvation is the most important trigger for PHB production and leads to the production of the storage molecule glycogen, too.

In this work, photoautotrophic PHB production in pilot-scale was realized and investigated. In a 200-liter tubular photobioreactor, the strain *Synechocystis* sp. CCALA192 was cultivated over several months. A self-limiting medium was used with a two-stage cultivation strategy. The first stage is the green stage, when nutrients are available. The second stage is the yellow stage, when nutrients are consumed and the culture turns yellow and starts to produce PHB. One production cycle took 16-20 days and the final biomass concentration was 1.0 g/L with a PHB content of 12.5%. It could be observed, that no CO_2 was consumed in the late stage of the production cycle, although the PHB concentration was still rising. In this stage, glycogen was metabolized and partly transformed to PHB. Therefore, a totally new three-stage production process with a ripening stage is proposed.

The ciliate *Colpoda steinii,* an ubiquitous soil organism, was experienced as a serious pest for *Synechocystis* cultures. This predator was capable to clear a dense culture within 2-3 days. Several strategies were tested without success and *Colpoda steinii* turned out to be highly resilient. A special new cultivation strategy of anoxic conditions during the night finally inhibited the ciliate and led to stable growth without the use of chemicals like insecticides.

In summary, the pilot-scale production of the bioplastic PHB with cyanobacteria was successfully demonstrated and strategies to prevent the pest and to improve the production process were developed in this thesis.

KURZFASSUNG

Cyanobakterien, als photoautotrophe Organismen, besitzen einen Stoffwechselapparat um klimaschädliches Kohlenstoffdioxid (CO_2) mit Licht als Energiequelle zu Biomasse umzuwandeln. Diese Fähigkeit macht sie zu vielversprechenden grünen Zellfabriken für eine nachhaltigere Produktion verschiedenster Moleküle. Eines der interessantesten Produkte von Cyanobakterien ist das Biopolymer Poly-β-hydroxybutyrat (PHB). PHB hat ähnliche Materialeigenschaften wie Polypropylen und findet Anwendung als Bioplastik. Im Gegensatz zu herkömmlichen Kunststoffen ist PHB biologisch abbaubar. Dadurch könnten die negativen Umweltauswirkungen von Plastikmüll vermieden werden. Cyanobakterien produzieren PHB als intrazellulären Kohlenstoff- und Energiespeicher. Nährstoffmangel ist die wichtigste Voraussetzung für PHB Produktion und führt daneben auch zur Produktion des Speichermoleküls Glykogen.

In dieser Arbeit wurde eine photoautotrophe PHB Produktion im Pilotmaßstab realisiert und untersucht. In einem 200-Liter tubulären Photobioreaktor wurde der Stamm *Synechocystis* sp. CCALA192 über mehrere Monate kultiviert. Ein selbst-limitierendes Medium wurde verwendet, welches zu einer zweiphasigen Kultivierung führt. Die erste Phase ist die grüne Phase, wenn Nährstoffe noch vorhanden sind. Die zweite Phase ist die gelbe Phase, wenn Nährstoffe aufgebraucht sind und die Kultur die Farbe ändert und PHB produziert. Ein Produktionszyklus dauerte 16-20 Tage. Dabei wurde eine Biomassekonzentration von 1.0 g/L erreicht mit einer PHB Konzentration von 12.5%. Am Ende des Produktionszyklus wurde kein CO_2 mehr verbraucht, obwohl die PHB Konzentration noch immer anstieg. In dieser Phase wurde Glykogen teilweise zu PHB verstoffwechselt. Daher wird in dieser Arbeit ein vollkommen neuer, dreiphasiger Produktionsprozess mit einer Reifephase vorgeschlagen.

Der Ciliat *Colpoda steinii,* ein ubiquitär vorkommender Bodenorganismus, trat als ernsthafter Schädling auf und konnte *Synechocystis* Kulturen innerhalb von 2-3 Tagen vernichten. Verschiedene Strategien wurden erfolglos getestet und *Colpoda steinii* erwies sich als sehr resistent. Eine spezielle neue Kultivierungsstrategie mit anoxischen Bedingungen während der Nacht hemmte schließlich *Colpoda steinii* und führte zu stabilem Wachstum.

Zusammenfassend konnten in dieser Dissertation die Produktion des Bioplastiks PHB im Pilotmaßstab erfolgreich demonstriert und Strategien zur Kontaminationsbekämpfung sowie zur Prozessoptimierung entwickelt werden.

OBJECTIVES

The work of this thesis was performed within the FFG (Forschungsförderungsgesellschaft) funded project CO2USE+EPP (Grant number 848783). The overall aim of the project and this thesis is to produce the biodegradable plastic poly-β-hydroxybutyrate (PHB) with cyanobacteria in pilot-scale. Carbon dioxide (CO_2) from the coal power plant station in Dürnrohr should be used with light as energy source. In order to achieve this goal, the small cyanobacterium *Synechocystis* sp. CCALA192 is cultivated in a pilot photobioreactor in Dürnrohr. This photoautotrophic organism is able to convert CO_2 to PHB. The analysis of culture parameters and a deeper understanding of the process are further aims and a final conclusion should be given.

PREAMBLE

As a cumulative thesis, this work includes three publications (Chapter 2-4). The introduction of the thesis itself is supposed to explain the background and give an overview about microalgae, cyanobacteria and the used cultivation system, tubular photobioreactors. Deeper insights about cyanobacterial PHA production are given in Chapter 2, which is a review paper. Chapter 3 and 4 are published works with results gained during the project. Chapter 5 and 6 close this thesis with a general discussion and conclusion about cyanobacterial PHA production.

1. INTRODUCTION

1.1. Anthropogenic microplastic pollution and CO_2 emissions – Markers of the Anthropocene

The Anthropocene is a proposed geological epoch dating from the beginning of significant human impact on planet Earth. The term was popularized by the atmospheric chemist Paul J. Crutzen, who regards the influence of human activities so profound to constitute a new geological epoch (Crutzen 2002). Within the several signals marking the change to the Anthropocene is the CO_2 concentration, which has been stable throughout the current epoch, the Holocene, for the last 12 000 years (Figure 1B). Even when looking at the last 800 000 years, the CO_2 concentration has always been between 150 and 300 ppm, but there has been a drastic increase of the CO_2 concentration since the year 1900. Due to burning of fossil fuels, the atmospheric CO_2 has risen from 300 ppm to over 400 ppm in only 100 years. This anthropogenic CO_2 is one of the main drivers of climate change. The Intergovernmental Panel on Climate Change (IPCC) regularly describes the observed changes. The most important are (1) the warming of the average land and ocean surface temperature of 0.85°C over the period 1880-2012, (2) the rise of the sea level of 0.19 m over the period of 1901-2010, (3) the mass loss of Greenland and Antarctic ice sheets as well as shrinkage of glaciers worldwide. The impacts attributed to climate change are widespread and complex. One example is the very likely increase in extreme weather events such as high sea levels and heavy precipitation in a number of regions (Pachauri et al. 2014).

In recent years, several efforts have been made to finding ways for a reduction of CO_2 emissions. One is the biological CO_2 fixation with microalgae and cyanobacteria. These organisms have been described as promising tools due to their high growth rate and biofixation efficieny, which is 10-50 times higher, compared to terrestrial plants (Cuellar-Bermudez et al. 2015; Cheah et al. 2015).

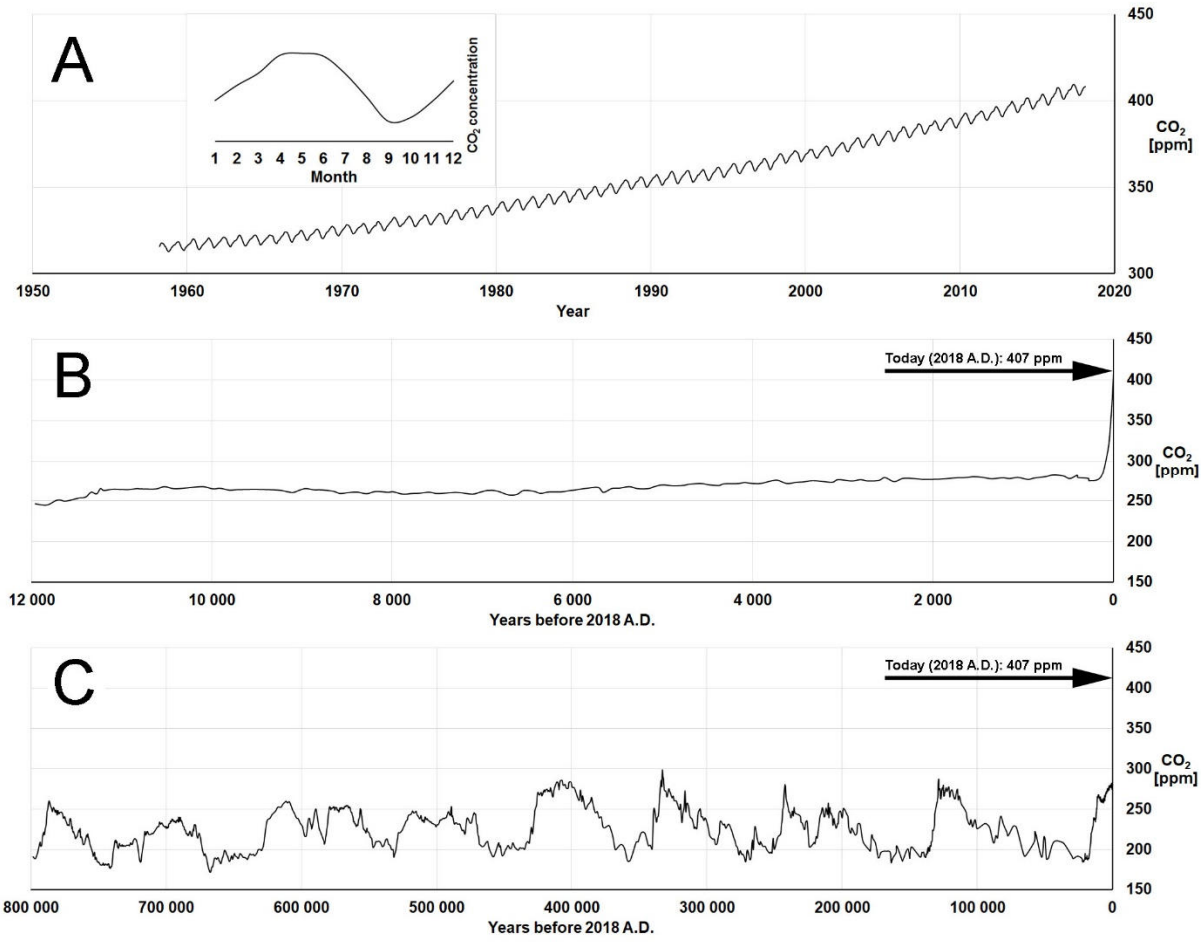

Figure 1: (A) The Keeling curve, named after the chemist Charles David Keeling, shows the CO_2 concentration of the atmosphere since the 1950s. (B) The CO_2 concentration of the last 12 000 years is depicted. (C) The CO_2 concentration of the last 800 000 years is depicted. Data from (Lüthi et al. 2008).

Another proposed marker for the Anthropocene is the occurrence of microplastics in marine sediments, as they will leave identifiable fossils. Microplastics are small particles with a diameter of less than 5 mm found in the environment. They come from a variety of sources and are, in any case, anthropogenic. They form via fragmentation of larger plastic material through photooxidation, mechanical action and biodegradation (Gewert et al. 2015). The growing burden of plastic waste is caused by inadequate waste management and the fact, that around 50% of plastic items are used only once before discarded. Waste management requires technical knowledge and support, which is often absent, especially in developing countries. Solid waste is buried in landfills and plastic particles can be blown away. However, most plastic particles end up in the environment because of litter thrown away carelessly. The plastics spread rapidly via rivers into the sea and settle down in marine sediments. In 2010, an estimated 5-12 tons of plastic was discharged into the oceans (Jambeck et al. 2015). The

long-term environmental impact of microplastics are yet unknown. Various animals ingest the small particles, when its size is within the range of the natural prey and may die (Galloway et al. 2017). Plastic additives increase the harm, as they often show a hormone effects and alter the animals behavior. These problems could be avoided by using degradable bioplastics.

1.2. Bioplastics

Plastic is material consisting of organic compounds that are malleable and so can be formed into solid objects. They are part of our everyday life and are used as packaging material, textile fibres, insulation material, pipes, flooring material, tires and much more. The very resistant and durable materials are processed fast in high amounts with well-established methods like injection molding or extrusion. The versatility and the cheap price led to the global success of plastics. The production rose from 2 million tons in 1950 to 335 million tons in 2016 (PlasticsEurope, 2017). Conventional plastics such as polyethylene, polypropylene or polyvinylchloride derive from fossil feedstocks such as natural gas or oil. About 4-6% of fossil gas and oil used in Europe is converted into plastics (PlasticsEurope, 2017). Plastic materials can be also biobased, when the feedstock come from renewable sources. However, the origin of the feedstock does not indicate its biodegradability, as there are biobased, non-degradable plastics such as biobased polyethylene. Figure 2 shows the classification of bioplastics.

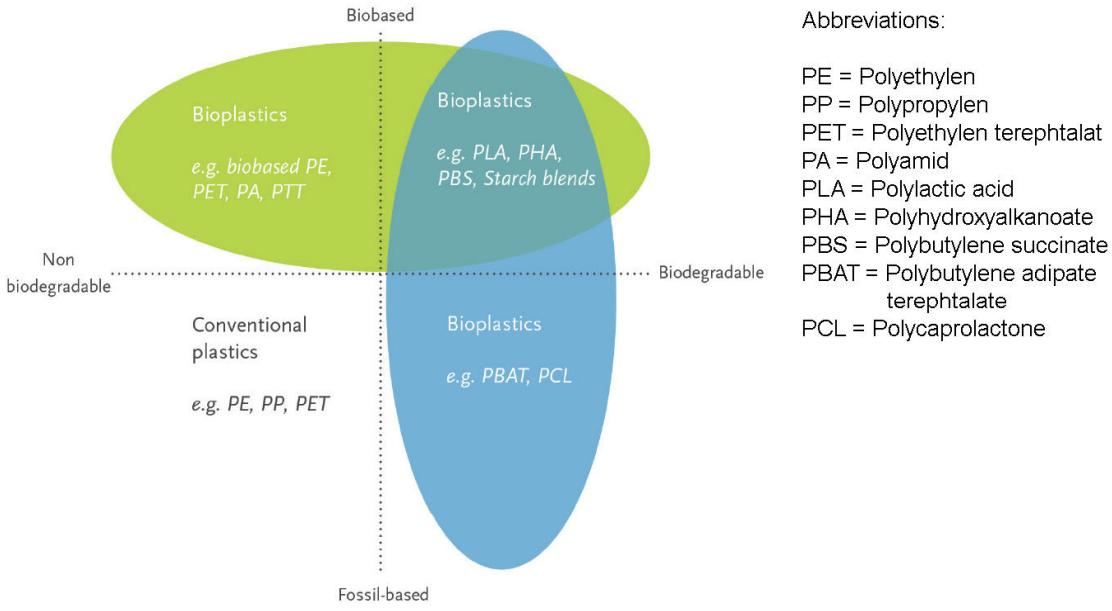

Figure 2: Classification of bioplastics according to their biodegradability and biobased content. (European Bioplastics, 2016 http://docs.european-bioplastics.org/2016/publications/fs/EUBP_fs_what_are_bioplastics.pdf, visited 14.06.2018)

1.3. Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are thermoplastic polyesters synthesized mainly by prokaryotes as intracellular storage granules. Bacteria like *Cupriavidus necator* (formerly known as *Ralstonia eutropha)* are capable to produce more than 80% PHAs of cell dry weight (Lee et al. 1999). PHAs have gained interest, as they are rapidly degraded under aerobic as well as anaerobic conditions (Tokiwa et al. 2009). The material properties of PHAs are highly dependant on the type of PHA. The generic formula of PHAs is depicted in Figure 3.

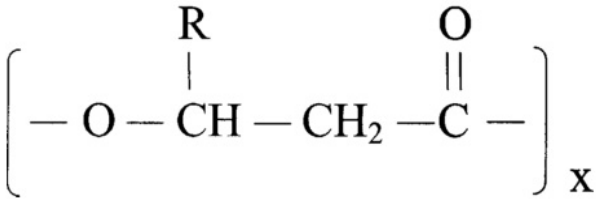

Figure 3: Generic formula of polyhydroxyalkanoates.

The most common and best-described PHA is poly-β-hydroxybutyrate (PHB). PHB has a methyl group as side chain. It is a crystalline material and therefore relatively stiff. Although some material properties as the Young's module and the tensile strength are similar to polypropylene, other properties differ, which makes the commercial use difficult. It shows poor melt stability and decomposes at approximately 200°C, which is close to its melt temperature. Furthermore, it becomes brittle within several days to weeks after production. This aging process can be reduced by blending with other polymers or by incorporating co-monomers to produce co-polymers. When feeding with 3-hydroxyvalerate as co-substrate, the co-polymer [P(3HB-3HV)] can be produced, which is less brittle (Lee 1996; Reddy et al. 2003).

Medium chain length PHAs (mcl-PHAs) are PHAs with longer side chains (C_6-C_{14} carbon atoms). They have different material properties compares to PHB like lower crystallinity and higher elongation to break. These materials might be suitable for biomedical applications such as heart valves. Mcl-PHAs can be produced with *Pseudomonas* sp., as the PHA-synthase of this organism shows low substrate specifity and accepts monomers with longer side chain. For producing mcl-PHAs, carbon sources such as n-hexane or n-octane are added to the medium (Rai et al. 2011).

The quite rare long chain length PHAs have side chains with 15 or more C atoms.

1.4. Cyanobacteria, microalgae and microalgal cultivation

Microalgae are a diverse group of microscopic organisms that are able to perform oxygenic photosynthesis. There is no generally accepted definition of what is actually an alga. The phycologist Robert Edward Lee defines any organisms with chlorophyll and a thallus not differentiated into roots, stem and leaves as an algae. Cyanobacteria are included in this definition, although they are prokaryotic organisms. As algae do not share an immediate common ancestor, it is a polyphyletic group of organisms.

As phytoplankton in the oceans, microalgae have large impact on the global oxygen and carbon cycles and therefore, on the Earth's climate. Algae can form algal blooms with a size of several thousand square kilometres (Figure 4). They are producing an estimated 50-70% of atmospheric oxygen (Sekerci et al. 2015). Fossil oil derives to large quantities from ancient algae that settled to the sea bottom and built up large sediment layers. Over millions of years with intense heat and pressure this biomass ultimately converted to petroleum.

Although vast amounts of microalgae are growing in the oceans, this resource cannot be exploited, as there is no possibility of harvesting the highly diluted algae biomass.

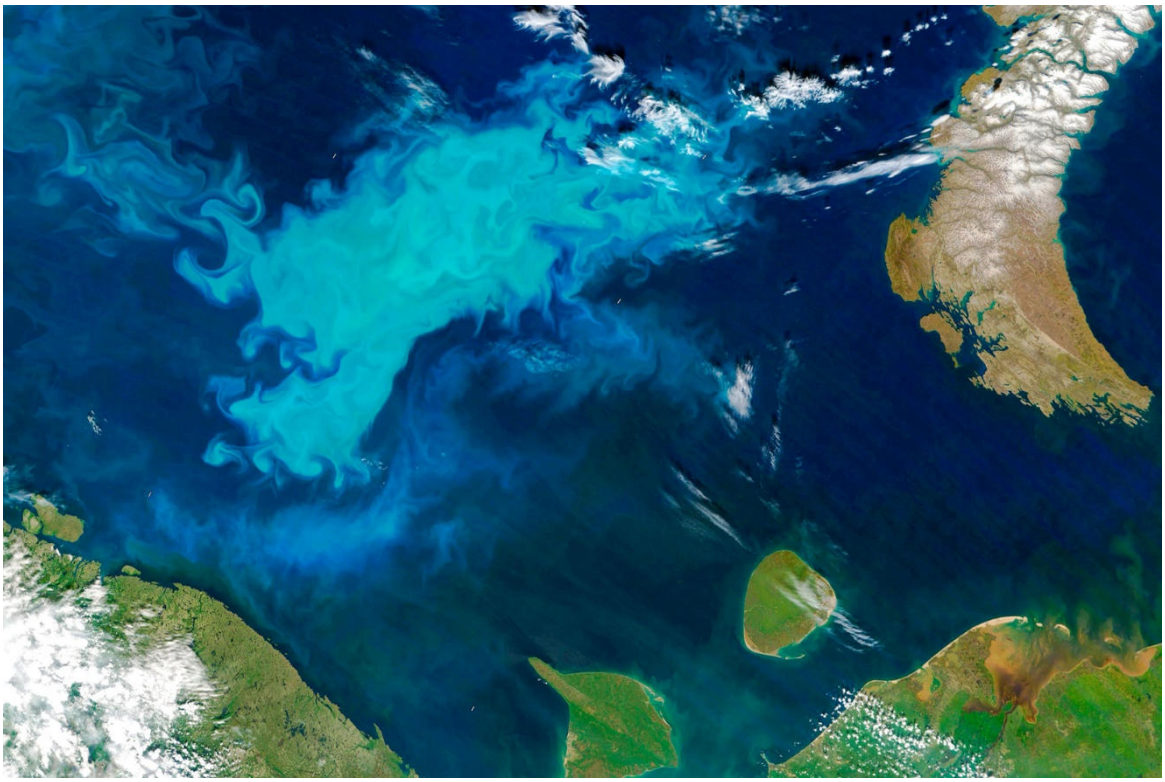

Figure 4: Algal bloom in the Barents Sea. Image acquired on July 6, 2016 from the Terra satellite of NASA. (https://www.nasa.gov/image-feature/the-barents-sea-abloom, visited 14.06.2018)

From estimated 72 500 algal species (Guiry 2012), only about 5-10 are cultivated in an industrial scale. The main application for algae today is the use as nutraceuticals in human nutrition. Most important is the edible cyanobacterium *Spirulina* sp. (also known as *Arthrospira* sp.). It has shown anti-oxidative effects and has a relatively high protein content (Wu et al. 2016). The blue pigment phycocyanin can be extracted from *Spirulina* sp. and used as natural blue food colorant. The main reason why *Spirulina* sp. is the most-produced algae today, is its ease of growth. It is cultivated in a highly alkaline medium with pH values above 10. Due to these selective conditions, it can be cultivated outdoors in open ponds with little risk of contamination. Another advantage of *Spirulina* sp. is its relatively large size of 100 µm, that facilitates the harvesting process, as simple filters are sufficient and no centrifuges are necessary. The annual global production in 2013 was estimated between 5 000 – 10 000 tons (Richmond 2013). Figure 5 shows a typical raceway pond for *Spirulina* sp. production.

Apart from *Spirulina* sp., *Chlorella* sp. and *Dunaliella* sp. are important species that are cultivated in open ponds. *Chlorella* sp. is used in human nutrition as well as in animal feed. Taiwan and Japan are the leading producers with an estimated annual production of 3 500 tons. *Dunaliella* sp. is a producer of natural beta-carotene. It thrives in highly saline waters with salinities between 200 and 300 g/L, which provides selective conditions. Isreal and Australia are the leading *Dunaliella* sp. producers.

Raceway ponds have little investment costs and little operational costs, compared to photobioreactors. The depth is usually between 20-30 cm and the mixing is done with a paddlewheel, which is located on one end of the raceway pond. The main disadvantages are the limitation to selective or highly robust algal species, the low process control, the large land use as well as low cell densities. Compared to closed photobioreactors, the volumetric production rates are 3-10 times lower. However, producing algae biomass with open ponds is still more economic (Kumar et al. 2015; Slade et al. 2013).

Figure 5: Open pond for *Spirulina* production in Hawaii by the company nutrex. (https://www.nutrex-hawaii.com/)

1.5. Tubular photobioreactors

Closed photobioreactors (PBRs) offer the possibility of high process control, which is advisable for the growth of most microalgae. Especially for the cultivation of sensitive strains and the production of high-value compounds, where process stability is required, closed PBRs should be used. From the several closed PBRs, which were proposed in recent years, tubular PBRs are by far the most common and most important closed systems for algae cultivation. An example of a tubular PBR is shown in Figure 6.

Tubular PBRs consist of long transparent tubes made of glass or sometimes PMMA (Polymethylmethacrylate). Although PMMA is cheaper, most production plants use glass due to its durability. The diameter of the tubes is a crucial aspect in the design of a tubular PBR. They usually vary between 40 and 90 mm. The smaller the diameter, the higher is the surface to volume ratio and therefore the illumination of the culture. This leads to higher volumetric productivity as well as higher biomass concentrations. Particularly when cultivating small-size organisms like *Synechocystis* sp., higher biomass concentration is preferable, as it facilitates the downstream process. However, smaller tubes are more expensive in purchase and installation.

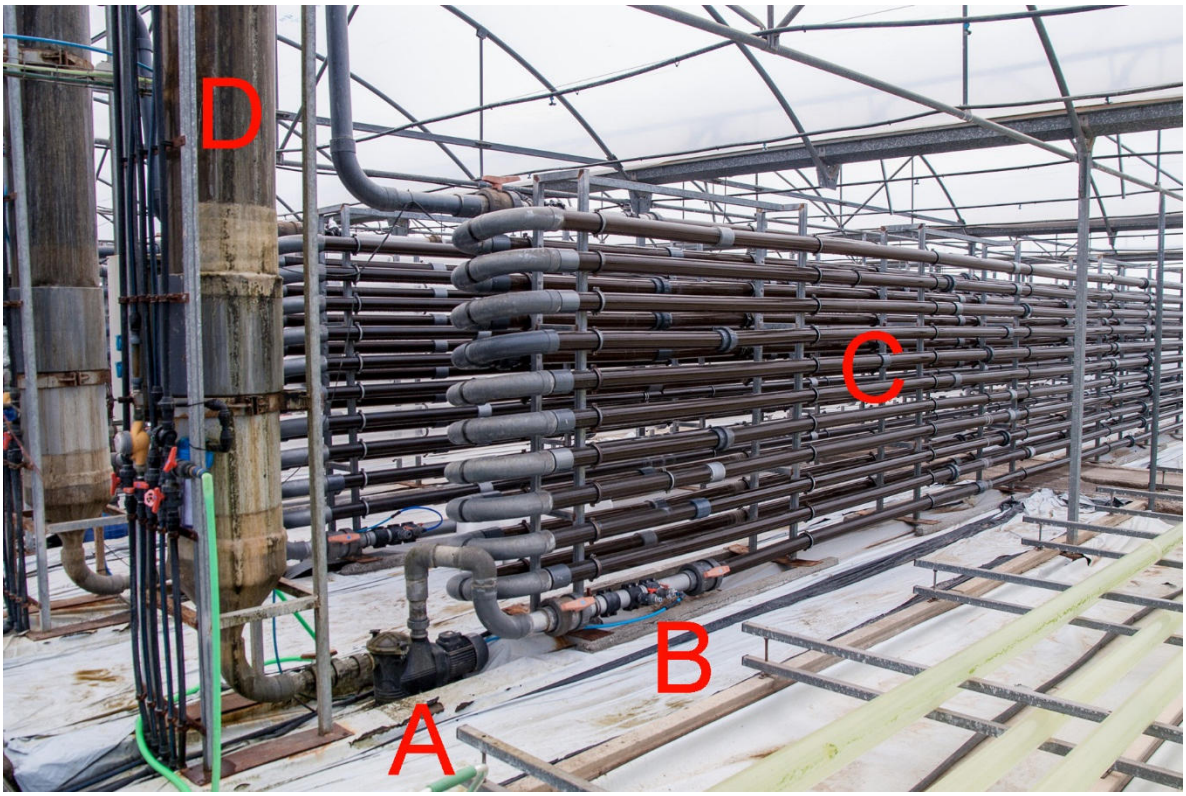

Figure 6: Tubular photobioreactor located at the research facility „Las Palmerillas“ in Almería, Spain. The most important parts are (A) the pump, (B) the CO_2 injection, (C) the glass tubes and (D) the degasser. Photograph taken by the author in April 2016.

Furthermore, the pressure loss of small tubes is higher, leading to higher pumping costs, which are one of the main drivers of the high production costs in tubular PBRs (Acién et al. 2012). The pressure loss of a straight tube can be calculated with Formula 1.

$$\Delta p = \frac{\lambda * L * \rho * v^2}{2 * d}$$

Formula 1: Pressure loss of a straight tube.

p = Pressure loss (Pa)
λ = Friction factor
L = Tube length (m)
ρ = Density (kg/m³)
v = Fluid velocity (m/s)
d = Diameter (m)

Due to photosynthetic activity of the microalgae, oxygen is produced and accumulates in the reactor. High oxygen concentrations should be avoided, as they negatively affect the growth of microalgae. For example, the specific growth rate of the green algae *Neochloris oleoabundans* dropped by 50%, when the partial oxygen pressure was increased from 8 mg/L to 28 mg/L (Sousa et al. 2013). The oxygen concentration increases over the length of the tube and is stripped with pressurized air at the degasser. During day, the oxygen concentration is always oversaturated (100% saturation with air is roughly 8 mg/L at 25°C or 0.24 bar partial pressure).

Therefore, a perfect degasser can only decrease the oxygen concentration to 100% saturation, as long as pressurized air is used. Figure 7 shows the dissolved oxygen concentration in a 400 m tubular PBR. In this case, the oxygen concentration increases from 20 mg/L to 26 mg/L. This degasser could only partly remove the oxygen, as the remaining 20 mg/L is still 250% saturation.

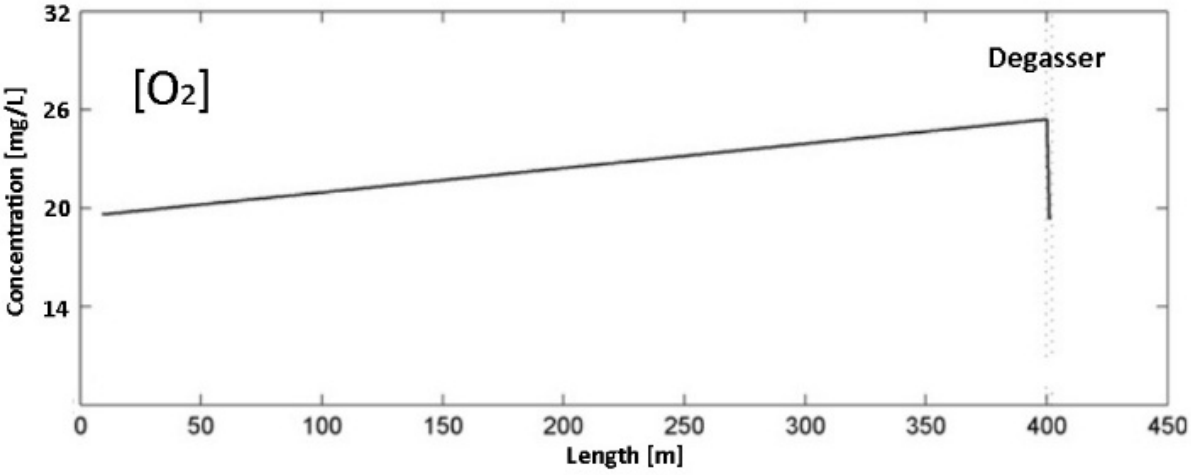

Figure 7: Profile of dissolved oxygen concentration in a 400m tubular photobioreactor with circulation time of 10 min, Adapted image from (Fernández et al. 2012)

In order to further decrease the oxygen concentration, the fluid velocity has to be increased to shorten the circulation time. However, the pressure loss increases with the square of the fluid velocity. Therefore, also the required power of the pump increases with the square of the fluid velocity.

One way to overcome this problem is the use of manifolds. In this type of tubular PBR, manifolds split and merge the tubes. Figure 8 shows a PBR, where the tube after the pump is split into 20 tubes with several manifolds. The white tower on the left side is equipped with devices for degassing and cooling, as well as for exchanging the medium.

Figure 8: Tubular photobioreactor at Necton S.A., Olhao, Portugal. The manifolds to split the tubes can be seen on the right side. The white tower for degassing and cooling can be seen on the left side. Photograph taken by the author in April 2016.

The microalgal suspension in the tubes has to be mixed to ensure that the cells are regularly exposed to light. When the culture has reached higher cell densities, the inner part of the tube is a dark zone. On the other hand, the outer parts of the tube can show high light intensities, which are greater than required by the cells. Due to the turbulent flow in the tubes, the cells steadily change from darker inner zones to brighter outer zones of the tubes. For example, Molina and colleagues estimated the frequency of the cells changing from dark to light zones to be 0.6 Hz (tube diameter=0.6m v=0.3 m/s) (Molina et al., 2000). While the radial mixing is performed relatively fast, the axial mixing is very slow, as tubular PBRs are classical examples for plug flow reactors.

The slow axial mixing is shown in Figure 9. After adding an alkaline solution, it took about 3 hours for a proper mixed culture.

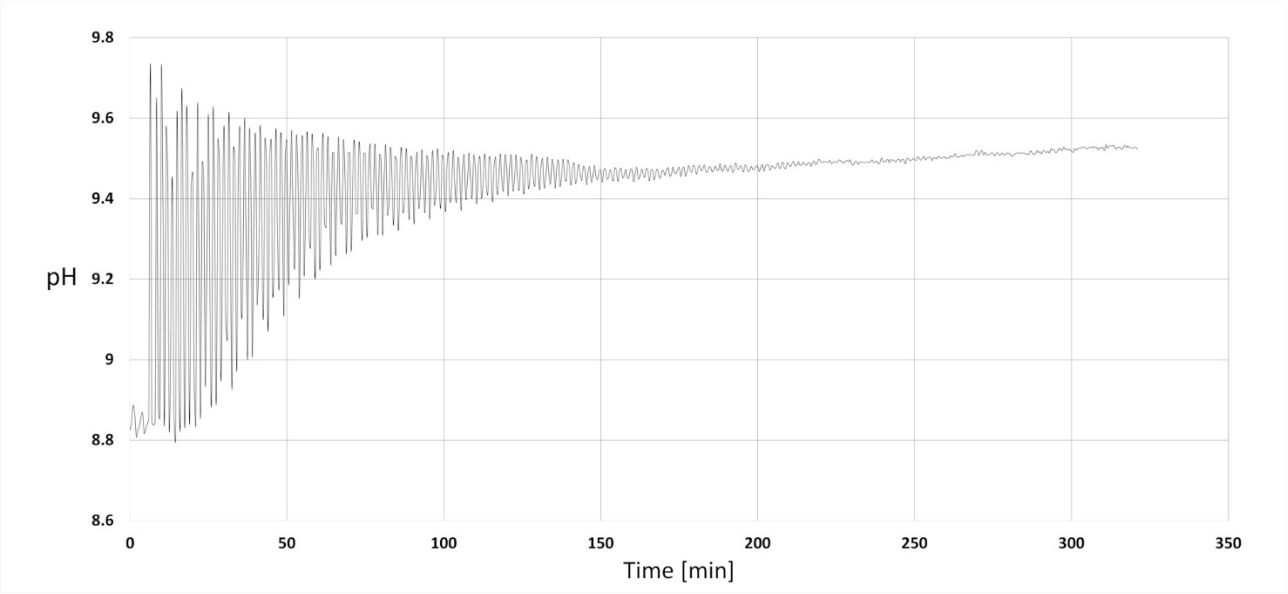

Figure 9: The pH in a tubular PBR (initial pH=8.8) is measured after adding an alkaline solution. The mixing takes 150-200 minutes (Proprietary work).

Like in any closed system, biofouling occurs frequently in tubular PBRs. Microalgae and cyanobacteria can adhere on the inner glass surface of the tubes and form a biofilm. Extracellular polymeric substances (EPS) play a key role in the formation of these biofilms. The EPS form a matrix, which protects the cells inside the biofilm and may lead to conditions for secondary colonization with organisms e.g. bacteria or fungi. The biofilm ultimately shades the algal culture and causes to a strong decrease of productivity. The formation of algal biofilms is a complex process and not only influenced by the tube material and the strain but also strongly by the culture medium (Berner et al. 2015).

Mechanical cleaning of the inner walls can be an annoying problem. Convenient cleaning in practice should be included in the design of a tubular PBR. One way is the use of round sponges, which are pushed into the tubes and pumped through for mechanical cleaning. Another way to reduce the biofilm formation is the use of small cleaning particles, which are pumped together with the algal culture. The particles scrub the inner wall of the tubes and have been successfully applied already (Acién et al. 2012).

1.6. *Synechocystis* and PHB metabolism

Here, *Synechocystis* and its PHB metabolism is shortly explained. Deeper insights are shown in Chapter 2.

Synechocystis is a small (0.5-2µm) unicellular cyanobacterium. It has been used in research as a model organism for studying oxygenic photosynthesis for a long time. Compared to higher plants, it has a relatively simple genome, facilitating genetic manipulation. The genome of the strain *Synechocystis* sp. PCC6803 has been sequenced in 1996 (Kaneko et al. 1996) and was the third fully sequenced organism after *Haemophilus influenzae* and *Mycoplasma genitalium.*

Figure 10 shows the PHB metabolism in *Synechocystis*, which takes place in three steps. Two molecules acetyl-CoA are combined in a Claisen-type condensation reaction to acetoacetyl-CoA with the enzyme β-ketothiolase. Then, acetoacetyl-CoA is reduced to R-3-Hydroxybutyryl-CoA with the enzyme Acetoacetyl-CoA reductase. The enzyme PHA synthase finally produces the biopolymer PHB in a polymerization reaction (Taroncher-Oldenburg et al. 2000).

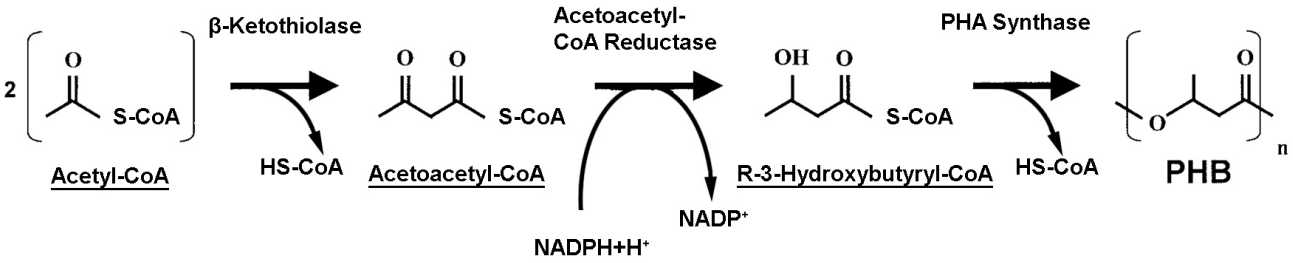

Figure 10: PHB metabolism in *Synechocystis* with Acetyl-CoA as precursor (Taroncher-Oldenburg et al. 2000).

PHB is produced as result of a stress reaction, typically caused by nutrient starvation. After limitation, *Synechocystis* changes from a vegetative cell cycle into a stationary, dormant state. The main features of this process is the degradation of the photosynthesis apparatus, the storage of carbon and energy rich polymers (glycogen and PHB) and the production of carotenoids for UV-protection. In this state, the cells can endure harsh conditions for longer periods. When conditions become better and nutrients are supplied, the cells rapidly change into a vegetative cell cycle again. This was recently shown in an interesting study by (Klotz et al. 2016).

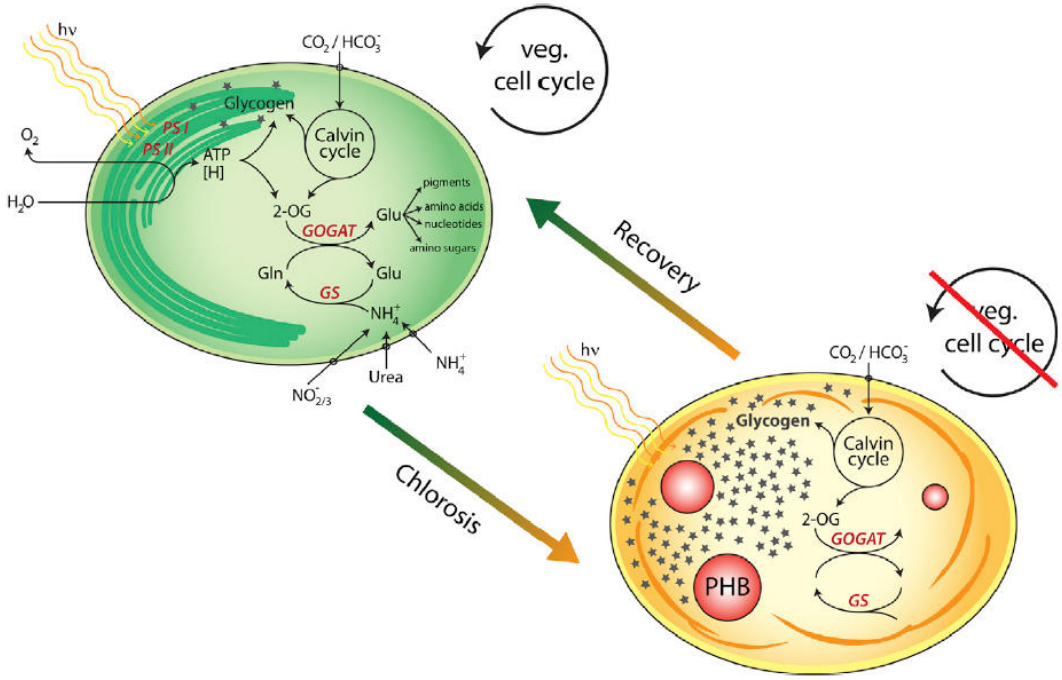

Figure 11: Morphological and metabolic changes during chlorosis caused by nitrogen starvation are shown (Klotz et al. 2016).

While glycogen is produced in many small granules throughout the cell, PHB is produced in 3-8 larger granules. These granules can be visualized with fluorescent dyes such as nile red (Figure 12). Nile red binds to PHB due to its hydrophobic nature.

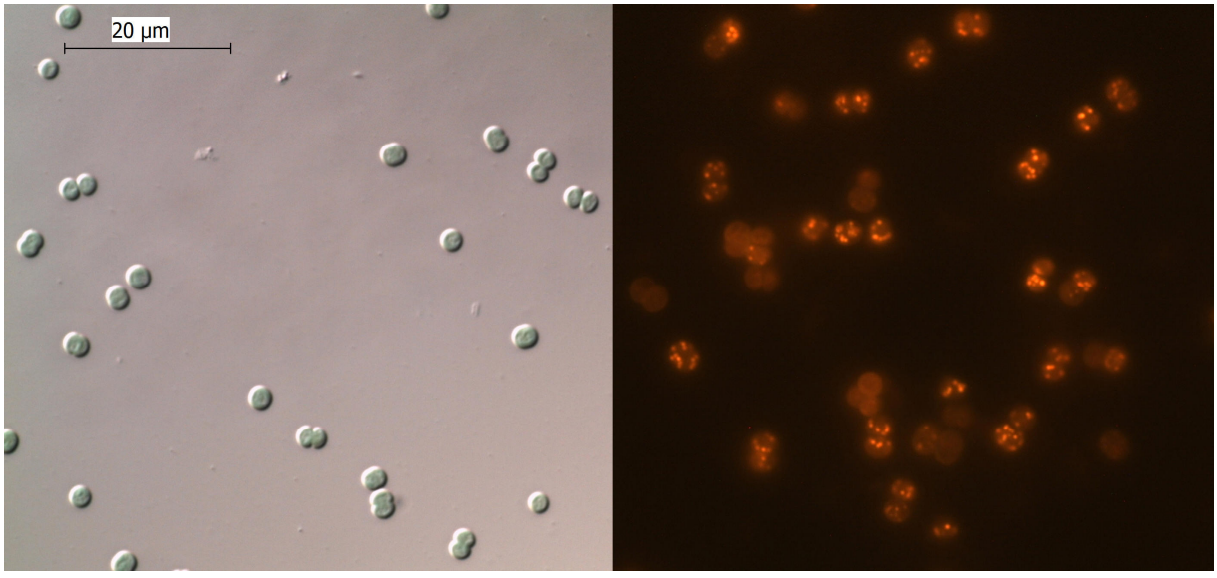

Figure 12: (Left side): Microscopic image of vegetative *Synechocystis* cells; (Right side): Fluorescent microscopic image of dormant *Synechocystis* cells with stained PHB granules (Proprietary work).

2. CYANOBACTERIAL PHA PRODUCTION – REVIEW OF RECENT ADVANCES AND A SUMMARY OF THREE YEARS' WORKING EXPERIENCE RUNNING A PILOT PLANT

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Review

Cyanobacterial PHA Production—Review of Recent Advances and a Summary of Three Years' Working Experience Running a Pilot Plant

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Abstract: Cyanobacteria, as photoautotrophic organisms, provide the opportunity to convert CO_2 to biomass with light as the sole energy source. Like many other prokaryotes, especially under nutrient deprivation, most cyanobacteria are able to produce polyhydroxyalkanoates (PHAs) as intracellular energy and carbon storage compounds. In contrast to heterotrophic PHA producers, photoautotrophic cyanobacteria do not consume sugars and, therefore, do not depend on agricultural crops, which makes them a green alternative production system. This review summarizes the recent advances in cyanobacterial PHA production. Furthermore, this study reports the working experience with different strains and cultivating conditions in a 200 L pilot plant. The tubular photobioreactor was built at the coal power plant in Dürnrohr, Austria in 2013 for direct utilization of flue gases. The main challenges were the selection of robust production strains, process optimization, and automation, as well as the CO_2 availability.

Keywords: cyanobacteria; polyhydroxyalkanoates; CO_2 mitigation; flue gas utilization; photobioreactor

1. Introduction

Polyhydroxyalkanoates (PHAs) are considered as one of the most promising bioplastics. Their mechanical properties are similar to polypropylene and they can be processed in a similar way, including extrusion, injection molding, or fiber spinning [1]. One of the major advantages of PHAs are their biodegradability. They are degraded relatively rapidly by soil organisms, allowing easy composting of PHA waste material [2].

Currently, PHA is produced in large fermenters by heterotrophic bacteria, like *Cupriavidus necator* or recombinant *Escherichia coli* [3]. For these fermentation processes large amounts of organic carbon sources like glucose are necessary, accounting for approximately 50% of the total production costs [4]. An alternative way of producing PHA is the use of prokaryotic algae, better known as cyanobacteria. As part of the phytoplankton, they are global primary biomass producers using light as the sole energy source to bind atmospheric CO_2 [5]. Burning of fossil fuels has increased the atmospheric CO_2 concentration from approximately 300 ppm in 1900 to over 400 ppm today. The latest report of the intergovernmental panel on climate change (IPCC) clearly indicates anthropogenic CO_2 emissions as the main driver for climate change [6]. Given these facts, cultivation of cyanobacteria for PHA production could be a more sustainable way of producing bioplastics.

2. Cyanobacteria and Cyanobacterial Energy and Carbon Storage Compounds

Cyanobacteria are Gram-negative prokaryotes that perform oxygenic photosynthesis. They are abundant in illuminated aquatic ecosystems and contribute significantly to the world carbon and oxygen cycle [7]. According to current evidence, oxygen was nearly absent in the Earth's early atmosphere until 2.4 billion years ago [8]. Due to oxygenic photosynthesis of early cyanobacteria the CO_2-rich atmosphere gradually turned into an oxygen-rich atmosphere, providing the conditions for multicellular life [9,10]. Today there are an estimated 6000 species of cyanobacteria with great diversity, for example ranging in size from the 1 µm small unicellular *Synechocystis* sp. to the several millimeter-long multicellular filaments of *Oscillatoria* sp. [11]. The common feature of cyanobacteria is the presence of the pigment phycocyanin, which gives them their typical blue-green color. Figure 1 shows photographs of four different cyanobacterial species.

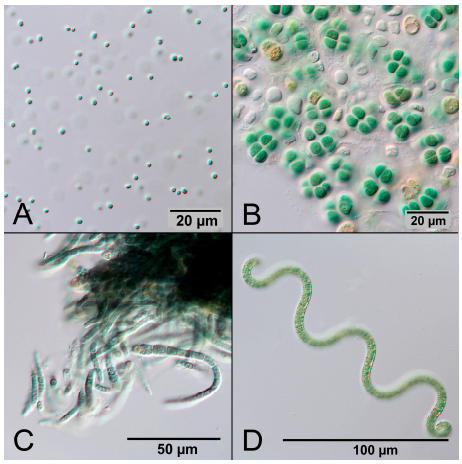

Figure 1. Microscopic photographs of different cyanobacterial species made in DIC (differential interference contrast). (**A**) *Synechocystis* sp.; (**B**) *Cyanosarcina* sp.; (**C**) *Calothrix* sp.; and (**D**) *Arthrospira* sp.

2.1. Cyanobacteria–Microalgae or Not?

For more than a century, cyanobacteria were considered as an algal group under the general name "blue-green algae". They were classified under the International Code of Botanical Nomenclature, nowadays called the International Code of Nomenclature for Algae, Fungi, and Plants (ICN). In 1980 the International Code of Nomenclature of Bacteria, nowadays called the International Code of Nomenclature of Prokaryotes (ICNP), was established. Stanier, one of the leading cyanobacteria researchers at that time, proposed the inclusion of cyanobacteria in the ICNP [12]. Nevertheless, the ICNP was not consistently applied for cyanobacteria and cyanobacteria are still covered by the ICN as well. The latest preamble of the ICN clarifies, that this code applies to all organisms traditionally treated as algae, fungi, or plants, including cyanobacteria [13]. Today cyanobacteria continue to be covered by both the Botanical Code (ICN) and Prokaryotic Code (ICNP). An effort to reconcile the status of this group of bacteria has been underway for several decades. Although some progress has been made, a final decision has not yet been reached [14]. From a phylogenetic point of view, there is a clear distinction between prokaryotic cyanobacteria and eukaryotic green algae. However, phycologists regard any organism with chlorophyll *a* and a thallus not differentiated into roots, stem, and leaves to be an alga. Therefore, in phycology, the term microalgae refers to both eukaryotic green algae and cyanobacteria, microscopic in size [15].

2.2. Cyanobacterial PHA

Polyhydroxyalkanoates (PHAs) can be classified into three groups: short-chain-length-PHA (scl-PHA), medium-chain-length-PHA (mcl-PHA), and long-chain-length-PHA (lcl-PHA). They differ

in mechanical and thermal properties [16]. Among the different PHAs, polyhydroxybutyrate (PHB) is by far the most common and the only PHA produced under photoautotrophic conditions reported so far. Other scl-PHAs, like P[3HB-co-3HV], are only produced when adding organic carbon precursors, like valerate, to the medium. No mcl-PHA or lcl-PHA have been reported in cyanobacteria. Therefore, the term PHB is used in this study, if no other specific PHA is described.

PHB is frequently found in cyanobacteria as an energy and carbon storage compound. In the biosphere they often have to cope with unfavourable environmental conditions. One of the most important growth limiting factors is the absence of nutrients. Nitrogen limitation is the most important and best studied trigger for PHB production in cyanobacteria [17–19]. Non-diazotrophic strains are not able to bind molecular nitrogen and depend on nitrogen in the form of nitrate or ammonium. Nitrogen-depleted cells cannot synthesize the necessary proteins for reproduction and, therefore, start to accumulate storage compounds like PHB. Another important function of PHB synthesis is to compensate imbalanced metabolic situations, as it acts as an electron sink and delivers new reduction equivalents in the form of $NADP^+$ [18–20].

The model organism *Synechocystis* PCC6803 is the best-studied cyanobacterium, and its genome was fully sequenced in 1997 [21]. Most of the understanding of cyanobacterial PHB formation was gained by research done with *Synechocystis* PCC6803. Biosynthesis of PHB from the precursor acetyl-CoA takes place in three steps. Acetoacetyl-CoA is produced from two molecules of acetyl-CoA in a Claisen type condensation by β-ketothiolase. Next step is the reduction of acetoacetyl-CoA by the acetoacetyl-CoA reductase to form D-3-hydroxybutyryl-CoA. Ultimately, PHB is formed in a polymerization reaction by the PHA-synthase. The necessary three enzymes are encoded by the four genes phaA (slr1993), phaB (slr1994), phaC (slr1830), and phaE (slr1829). phaA and phaB are organized in one operon encoding for the β-ketothiolase and acetoacetyl-CoA reductase. phaC and phaE are also organized in one operon encoding the two subunits of the type III PHA synthase [22,23].

2.3. Cyanobacterial Glycogen

Regarding PHB synthesis, it should be kept in mind that cyanobacteria also produce glycogen as a second carbon and energy storage compound under nitrogen depletion. In fact, the glycogen content is most often higher than the PHB content and varies between 20% and 60% [24–28]. While PHB is produced in 3–8 larger granules, glycogen is stored in many small granules [18,29–33]. Glycogen is synthesized instantly after nitrogen depletion while PHB synthesis is slower [34]. Glycogen is also produced in non-depleted cells with lower content, aiding the cell to cope with short term energy deficits like the day-night cycle. Glycogen deficient mutants were shown to be highly sensitive to day-night cycles [35]. Glycogen synthesis is a highly-conserved feature abundant in all cyanobacterial genomes reported so far [36]. PHB synthesis on the other hand is common in many, but not all, cyanobacteria [37,38]. Glycogen shows similarities to starch in green algae, while PHB synthesis shows some similarities to triacylglycerol (TAG) synthesis in green algae, where TAG synthesis also serves as an electron sink and consumes excess NADPH [39,40].

In a recent study Damrow and colleagues compared PHB-deficient mutants to glycogen-deficient mutants of *Synechocystis* PCC6803. Glycogen-deficient mutants could not switch to a dormant metabolic state and could not recover from nitrogen depletion. Excess carbon was mostly secreted into the medium in the form of 2-oxoglutaric acid and pyruvate, although the PHB content also increased from 8% to 13%. PHB-deficient mutants, on the other hand, behaved very much like the wild-type with the same amount of glycogen accumulation and the same recovery capability. Only double-knockout mutants (glycogen and PHB deficient) were most sensitive and showed a reduced growth rate, signs for a very specific role of PHB in cyanobacteria, which is still not totally clear [41]. The reported studies show that inhibiting glycogen synthesis increases the PHB production, although cells suffer as glycogen plays an important role.

2.4. Nitrogen Chlorosis and Photosynthetic Activity

During nitrogen starvation the cells gradually change from a vegetative state to a dormant state. The most obvious feature of this is the change in colour from blue-green to brownish-yellow. This phenomenon is called "nitrogen chlorosis" and was described already at the begin of the 20th century [42]. It is caused by the degradation of the pigments phycocyanin and chlorophyll. When transferring *Synechococcus* PCC7942 to a nitrogen depleted medium, 95% phycocyanin was degraded within 24 h, and after 10 days 95% of the chlorophyll was also degraded [43]. Concomitantly, the activities of the photosystems (PS) I and II decrease strongly and are only about 0.1% compared to vegetative cells [44]. A recent and very interesting study examined the awakening of a dormant *Synechocystis* PCC6803 cell. After the addition of nitrate the yellow culture turned green again within 36 hours. Transmission electron microscopy revealed the rapid degradation of glycogen and PHB. During the first 24 h of this process the cells consumed oxygen. Transcriptome analysis showed the induction of RuBisCO and carboxysom associated RNAs, as well as the photosystem-related RNAs to prepare the cells for vegetative photoautotrophic growth [34]. The results indicate the decrease in photosynthetic activity during nitrogen starvation, which can be considered a significant challenge to photoautotrophic PHB production.

3. Different Cyanobacteria as PHA Producers

3.1. Synechocystis and Synechococcus

Synechocystis and *Synechococcus* are very small (0.5–2 µm) unicellular cyanobacteria abundant in almost all illuminated saltwater and freshwater ecosystems. One of the first detailed descriptions of PHB accumulation in *Synechocystis* PCC6803 was provided by Wu and colleagues. Nitrogen starved cells produced 4.1% PHB of cdw while under-balanced culturing conditions PHB content were under the detection limit [45]. The same strain was examined for PHB production some years later, where 9.5% PHB of cdw were produced under nitrogen limitation. Phosphorous-depleted cells showed 11.2% PHA of cdw. Interestingly, balanced cultivated control cultures already contained 4.5% PHB of cdw. Supplementation of acetate and fructose lead to a PHB content of 38% of cdw [46]. Recently, recombinant *Synechocystis* PCC6803 with overexpression of the native PHA genes were constructed. They showed a PHB content of 26% of cdw under nitrogen-depleted culturing conditions compared to 9.5% of cdw of the wild-type [47]. However, it must be considered that there are legal issues in most countries when cultivating recombinant strains outdoors. In another study the thermophilic strain *Synechococcus* MA19 showed a PHB content of 55% under phosphate-limited culturing conditions. This study was published in 2001 and still reports the highest PHB content under photoautotrophic conditions [48]. Table 1 shows reported PHA values of *Synechocystis* and *Synechococcus*.

Table 1. *Synechocystis* and *Synechococcus* as PHA producers. (cdw = cell dry weight, n.r. = not reported).

Carbon Source	Cyanobacterium	Culture Condition	%PHA of cdw	PHA Composition	Total cdw	Reference
Photoautotrophic	*Synechocystis* PCC6803	Photoautotrophic, nitrogen lim.	4.1%	PHB	0.65 g/L	[45]
	Synechocystis PCC6803	Photoautotrophic, nitrogen lim.	9.5%	PHB	n.r.	[46]
	Synechocystis PCC6803	Photoautotrophic, phosphate lim.	11.2%	PHB	n.r.	[46]
	Synechocystis PCC6803 (recombinant)	Photoautotrophic, nitrogen lim.	26%	PHB	n.r.	[47]
	Synechococcus MA19	Photoautotrophic, phosphate lim., 50 °C	55%	PHB	4.4 g/L	[48]
Heterotrophic	*Synechocystis* PCC6803	Acetate + Fructose supplementation	38%	PHB	n.r.	[46]
	Synechocystis PCC6803 (recombinant)	Acetate supplementation	35%	PHB	n.r.	[47]

3.2. *Arthrospira (Spirulina)*

Arthrospira (formally *Spirulina*) is a species of filamentous cyanobacteria that grows naturally in alkaline salt lakes. It has a high protein and vitamin content and is mainly grown as a food supplement. Recent studies have shown its antioxidant, immunomodulatory, and anti-inflammatory activities [49]. From all cyanobacterial species known, only *Arthrospira* sp. is produced at an industrial scale. The main reason for that is the possibility of cultivation in a highly alkaline environment that prevents contamination and enables the maintenance of a stable culture in open ponds. No exact data are available; however, we estimate the world annual production of around 5000–15,000 tons *Arthrospira* sp. dry weight per year [50–53].

The first description of PHB accumulation in *Arthrospira* was reported by Campbell and colleagues, who described a PHB content of 6% of cdw in a non-optimized mineral medium. Interestingly, the highest PHB content was measured at the end of exponential growth and decreased during stationary phase [54]. In a screening of 23 cyanobacterial strains, *Arthrospira platensis* had the lowest PHB concentration of only 0.5% in a non-optimized medium [37]. In a screening of several *Arthrospira* species the PHB amount never exceeded 1% of cdw in photoautotrophic growth. Addition of sodium acetate led to a PHB amount of 2.5% of cdw [55]. In another experiment *Arthrospira platensis* was grown under phosphate limitation and reached 3.5% PHB of cdw [56]. *Arthrospira subsalsa*, a strain isolated from the Gujarat coast, India, produced 14.7% PHB of cdw under increased salinity [57]. A detailed ultrastructural analysis of *Arthrospira* strain PCC8005 was conducted by Deschoenmaker and colleagues. Under nitrogen depleted conditions PHB granules were more abundant and larger. The nitrogen-starved cells showed an estimated four times higher PHB concentration [27]. Nitrogen starvation was performed in *Arthrospira maxima* and glycogen and PHB content was measured. While the glycogen content increased from around 10% to 60%–70% of cdw, PHB amount remained low at 0.7% of cdw. The addition of sodium acetate increased the PHB amount to 3% of cdw [26].

The performed studies support the idea, that PHB production in *Arthrospira* is highly strain-dependent. Most *Arthrospira* species produce PHB only in amounts of lower than 5%, even with the addition of sodium acetate. *Arthrospira* produces glycogen as storage compound, what has been shown in ultrastructural research, too [27]. Nevertheless, it must be emphasized that *Arthrospira*, at an industrial scale, is still one of the most promising candidates for PHB production with cyanobacteria. Indeed, PHB nanofibers were produced recently from *Arthrospira* PHB and showed highly favourable properties [58,59]. The biggest challenge for further research is to increase the relatively low PHB content of *Arthrospira*. Table 2 shows reported PHA values of *Arthrospira*.

Table 2. *Arthrospira* as a PHA producer. (cdw = cell dry weight, n.r. = not reported).

Carbon Source	Cyanobacterium	Culture Condition	%PHA of cdw	PHA Composition	Total cdw	Reference
Photoautotrophic	*Arthrospira platensis*	Photoautotrophic	6%	PHB	n.r.	[54]
	Arthrospira sp.	Photoautotrophic	<1%	PHB	n.r.	[55]
	Arthrospira platensis	Photoautotrophic, phosphate lim.	3.5%	PHB	0.3 g/L	[56]
	Arthrospira subsalsa	Photoautotrophic, nitrogen lim.	14.7%	PHB	1.97 g/L	[57]
	Arthrospira platensis	n.r.	22%	PHB	n.r.	[59]
Heterotrophic	*Arthrospira maxima*	Acetate + CO_2	5%	PHB	1.4 g/L	[26]
	Arthrospira sp.	Acetate + CO_2	2.5%	PHB	n.r.	[55]

3.3. *Nostoc*

Nostoc is a group of filamentous cyanobacteria very common in terrestrial and aquatic habitats. They are capable of fixing atmospheric nitrogen with specialized heterocysts and are suspected to maintain soil fertility in rice fields due to nitrogen fixation [60]. Ge-Xian-Mi, an edible *Nostoc* species, forms spherical colonies that have been collected in China for centuries [61]. The first reports found for PHB production in *Nosctoc muscorum* are from 2005, when Sharma and Mallick

showed that *Nostoc muscroum* produced 8.6% PHB of cdw under phosphate and nitrogen limitation during the stationary phase. PHB content could be boosted to 35% of cdw with 0.2% acetate and seven days dark incubation [62]. Limited gas exchange and supply with 0.4% acetate increased the PHB content to 40% [63]. *Nostoc muscorum* was grown photoautotrophically without combined nitrogen sources and four days of phosphate deficiency increased PHB content from 4% to 22% [56]. The co-polymer P[3HB-co-3HV] could be produced by *Nostoc* in a propionate- and valerate-supplied medium. The 3HV fraction ranged from 10–40 mol% and showed desirable properties in terms of flexibility and toughness. Nitrogen and phosphate depletion led to a PHA content of 58%–60% of cdw, however, the total cdw did not exceed 1 g/L [64]. Further process optimization led to a PHA productivity of 110 mg/L/d and a P[3HB-co-3HV] content of 78% of cdw, the highest yield in heterotrophic grown cyanobacteria reported so far [65]. Recently, poultry litter was used for cultivation of *Nostoc muscorum agardh*. The poultry litter contained phosphate, ammonium, nitrate, and nitrite as nutrients for cyanobacterial growth. Optimized conditions, which included the addition of acetate, glucose, valerate, and CO_2-enriched air, led to a P[3HB-co-3HV] content of 70% cdw. However, total cdw remained relatively low at 0.68 g/L [66].

The reported studies show that PHB content in *Nostoc* can be significantly increased with organic carbon sources, especially in the form of acetate. However, those organic carbon sources lead to heterotrophic growth and may suppress CO_2 uptake by the cells, which is the most important argument for using cyanobacteria as PHA producers. All of the reported experiments of *Nostoc* were performed in shaking flasks or small reactors under sterile conditions. In mass cultivation *Nostoc* would have to be cultivated under non-sterile conditions and organic carbon sources could cause problems maintaining stable cultures. Although optimized conditions of several experiments lead to PHA contents of more than 50% of cdw, the total cdw remained mostly under 1 g/L and the overall productivity and growth rate of *Nostoc* is relatively low. Table 3 shows reported PHA values of *Nostoc*.

Table 3. *Nostoc* as a PHA producer. (cdw = cell dry weight, n.r. = not reported).

Carbon Source	Cyanobacterium	Culture Condition	%PHA of cdw	PHA Composition	Total cdw	Reference
Photoautotrophic	*Nostoc muscorum*	Photoautotrophic, nitrogen and phosphorous lim.	8.7%	PHB	n.r.	[62]
	Nostoc muscorum agardh	Photoautotrophic, 10% CO_2	22%	PHB	1.1 g/L	[66]
	Nostoc muscorum	Photoautotrophic, nitrogen and phosphorous lim.	22%	PHB	0.13 g/L	[56]
Heterotrophic	*Nostoc muscorum agardh*	Acetate, valerate, nitrogen lim.	58%	P[3HB-co-3HV]	0.29 g/L	[64]
	Nostoc muscorum	Acetate, limited gas exchange	40%	PHB	n.r.	[63]
	Nostoc muscorum agardh	Acetate, glucose, valerate, 10% CO_2	70%	P[3HB-co-3HV]	0.98 g/L	[66]
	Nostoc muscorum agardh	Acetate, glucose, valerate, nitrogen lim.	78%	P[3HB-co-3HV]	0.56 g/L	[65]
	Nostoc muscorum	Acetate, dark incubation, nitrogen and phosphorous lim.	35%	PHB	n.r.	[62]

3.4. Other Cyanobacteria

Recently, the PHB content of 137 cyanobacterial strains representing 88 species in 26 genera was determined under photoautotrophic conditions. High PHB content was highly strain-specific and was not associated with the genera. From the 137 tested strains, 134 produced PHB and the highest content was measured in *Calothrix scytonemicola* TISTR 8095 (Thailand Institute of Scientific and Technological Research). This strain produced 356.6 mg/L PHB in 44 days and reached a PHB content of 25% of cdw and a total biomass of 1.4 g/L. The PHB content of 25% was reached under nitrogen depletion, while cells with nitrogen supply reached a PHB content of only 0.4%. From the 19 tested *Calothrix* strains, only six produced more than 5% PHB of cdw. One of the greatest advantages of *Calothrix* is the relative ease of harvesting the dense flocs of algae, but cultivation of *Calothrix* is still at a very early stage [38].

The filamentous diazotroph cyanobacterium *Aulosira fertilissima* produced 10% PHB of cdw under photoautotrophic conditions and phosphate deficiency. The PHB content was boosted to 77% under phosphate deficiency with 0.5% acetate supplementation. This study also shows the positive effect of other carbon sources like citrate, glucose, fructose and maltose on PHB production [67]. Anabaena cylindrica, a filamentous cyanobacterium, was examined for PHB and P[3HB-co-3HV] production. Under nitrogen depletion with acetate supply, *Anabeana cylindrica* produced 2% PHB of cdw and a total biomass of 0.6 g/L. This organism was also able to produce the co-polymer P[3HB-co-3HV] when supplemented with valerate and propionate [68]. Table 4 shows reported PHA values of different cyanobacterial species.

Table 4. Different cyanobacterial species as PHA producers. (cdw = cell dry weight, n.r. = not reported).

Carbon Source	Cyanobacterium	Culture Condition	%PHA of cdw	PHA Composition	Total cdw	Reference
Photoautotrophic	*Phormidium* sp. TISTR 8462	Photoautotrophic, nitrogen lim.	14.8%	PHB	n.r.	[38]
	Oscillatoria jasorvensis TISTR 8980	Photoautotrophic, nitrogen lim.	15.7%	PHB	n.r.	[38]
	Calothrix scytonemicola TISTR 8095	Photoautotrophic, nitrogen lim.	25.2%	PHB	n.r.	[38]
	Anabaena sp.	Photoautotrophic	2.3%	PHB	n.r.	[69]
	Aulosira fertilissima	Photoautotrophic, phosphorous lim.	10%	PHB	n.r.	[67]
Heterotrophic	*Aulosira fertilissima*	Acetate, phosphorous lim.	77%	PHB	n.r.	[67]
	Aulosira fertilissima	Maltose, balanced	15.9%	PHB	2.3 g/L	[67]

4. CO_2 and Nutrient Supply for Mass Cultivation of Cyanobacteria

4.1. CO_2 Supply

Today, commercial microalgae production is still mainly taking place in open ponds. Here, the C source is normally sodium bicarbonate or atmospheric CO_2. In order to boost productivities in open systems, or if photobioreactor systems are employed, the use of commercial CO_2 from gas cylinders is common [70].

However, current production systems are used for the production of high value products (food, feed additives), where CO_2 price is not critical. If PHA is to be produced, which has a lower economic value, cheap CO_2 sources are of interest. Although there is considerable literature on various CO_2-sources (e.g., flue gases) and microalgae growth, there is very limited literature available on cyanobacteria and alternative CO_2-sources. Table 5 summarizes the literature on cyanobacterial growth on flue gases or fermentation gases.

Table 5. Growing cyanobacteria with alternative CO_2-sources.

Type of Gas	Cyanobacterium	CO_2 Source	Reference
Flue gases	*Phormidium valderianum*	Coal combustion flue gas	[71]
	Atrhrospira platensis	Coal combustion flue gas	[72]
	Arthrospira sp.	Synthetic flue gas	[73]
	Synechocystis sp.	Flue gas from natural gas combustion	[74]
CO_2 rich fermentation gases	*Arthrospira platensis*	CO_2-offgas from ethanol fermentation	[75]
	Arthrospira platensis	Biogas	[76]

4.2. Nutrient Supply

The cultivation of microalgae and cyanobacteria consume high amounts of nutrients, mainly nitrogen and phosphorous [77]. For research, and even cultivation, mainly synthetic nutrient sources are used [78]. By using alternative nutrient sources, like agro-industrial effluents, waste waters, or

anaerobic digestate, questions concerning sustainability of cyanobacteria cultivation, which arise by using fertilizer as a synthetic nutrient source, can be answered [78]. The biomass concentration achieved in open, as well as in closed, cultivation systems are 0.5–1 g/L and 2–9 g/L, respectively [79]. Therefore, large amounts of water are needed. Recycling of process water is another important approach for a more sustainable microalgae cultivation.

In addition to their low costs, the advantages of using alternative nitrogen and phosphorous sources include the production of valuable biomass while removing nutrients from wastewaters, as well as the prevention of competition with food and feed production [78]. On the other hand, new challenges arise, including microbial contaminations, heavy metals and growth inhibitors, suspended solids, or dissolved organic compounds contained in wastewaters, as well as the seasonal composition and fluctuation in amounts [80]. To cope with these challenges recent research focused on cultivating cyanobacteria in anaerobic digestate and agro-industrial effluents or wastewaters for removing nutrients [81–86] (see Table 6) and on integrating cultivation processes into biorefinery systems [83].

Additionally, process water and nutrients after harvesting cyanobacterial biomass [79] and product extraction can be directly recycled. Biomass can also be anaerobically digested [87,88] or hydrothermally liquefied via HTL (mineralization of organic nutrients) [89,90] and then recycled. Recycling process water directly can increase the concentration of inhibitory substances and dissolved organic matter from the previous batch produced by cyanobacteria [91], which decrease the productivity of cyanobacteria. Furthermore, nutrient competition may arise by enhanced bacterial growth [79].

Although many publications deal with alternative nutrient sources for cultivating cyanobacteria, hardly any of them focus on cyanobacterial PHA production [66,92]. Reasons for that may be that PHA production requires nutrient limitation [93] and the balance between nutrient limitation, decreased growth and production rates is difficult. The colouring of the nutrient source must be respected as well [94].

Table 6. Overview of agro-industrial effluents and wastewaters and anaerobic digestates used as nutrient sources for cultivating cyanobacteria.

Nutrient Source		Cyanobacterium	Total cdw/Growth Rate	Product/Purpose	Reference
Agro-industrial effluents and waste waters	Raw cow manure	*Arthrospira maxima*	3.15 g/L	Biomass production	[80]
	Molasses	*Arthrospira platensis*	2.9 g/L	Biomass production	[95]
	Olive-oil mill wastewater	*Arthrospira platensis*	1.69 g/L	Nutrient removal	[84]
	Poultry litter	*Nostoc muscorum agardh*	0.62 g/L	PHA production	[66]
Anaerobic digestate	Waste from pig farm	*Arthrospira platensis*	20 $g/m^2/d$	Nutrient removal	[81]
	Digested sago effluent	*Arthrospira platensis*	0.52–0.61 g/L	Nutrient removal	[96]
	Digestate from municipal solid waste	*Arthrospira platensis*	Growth rate 0.04 d^{-1}	Nutrient removal	[97]
	Digestate from vegetable waste	*Arthrospira platensis*	Growth rate 0.20 d^{-1}	Nutrient removal	[97]
	Waste from pig farm	*Arthrospira* sp.	15 $g/m^2/d$	Nutrient removal	[85]
	Algal digestate	*Chroococcus* sp.	0.79 g/L	Nutrient removal	[86]
	Digestate sludges	*Lyngbya aestuarii*	0.28 g/L	Biomass production	[83]
	Digestates of *Scenedesmus* spp.	*Lyngbya aestuarii*	0.11 g/L	Biomass production	[83]
	Thin stillage digestate	*Synechocystis* cf. *salina Wislouch*	1.6 g/L	PHB production	[92]
	Anaerobic digester effluent	*Synechocystis* sp.	0.15 g/L	Lipid production	[98]

5. Three Years' Working Experience Running a Pilot Plant for Photoautotrophic PHB Production

5.1. Location and Reactor Description

The photobioreactor is situated in a glass house at the coal power station in Dürnrohr, Austria. It is a tubular system built from glass elements of Schott AG with an inner diameter of 60 mm, a total

length of 80 m and a volume of 200 L (Figure 2). The main design of the photobioreactor is described elsewhere [99,100]. A central degassing unit serves to remove the oxygen as well as to compensate filling level. The medium is circulated with a 400 W centrifugal pump. pH value can be controlled through injection of pure CO_2 via a mass flow controller. Additional artificial light is provided by six 250 W gas-discharge lamps. Temperature is controlled with an air conditioner.

Figure 2. Two-hundred litre tubular photobioreactor with *Synechocystis salina* CCALA192. The central tower serves as a degasser. The centrifugal pump is situated at the lowest point of the reactor on the left side.

5.2. CO_2 Supply of the Reactor

The flue gases of the power plant at Dürnrohr usually contain between 11%–13% CO_2. Next to the chimney is a CO_2 separation plant (acronym SEPPL), providing the possibility to concentrate the CO_2 and fill it into gas bottles [101] though, for a more economic approach, the CO_2 should be used directly without prior compression. The SEPPL provides this option, as well as the possibility to wash the flue gases after the flue gas cleaning of the plant itself to remove residual NO_x and SO_x. Unfortunately for our research project, due to the current situation on the energy market, the power station is no longer run in full operation and only runs occasionally for balancing peak demands of the electrical grid. Therefore, a continuous cultivation with direct utilization of flue gas is not possible. Aspects like this must be respected when planning large industrial cultivation plants.

5.3. Automation and pH Control

The pH value is one of the most crucial parameters and needs to be controlled carefully. Due to CO_2 consumption, the pH value rises during photoautotrophic growth. This can be observed when turning on illumination. The tubular photobioreactor is equipped with a PI (Proportional–Integral) controller for pH setting which adjusts the mass-flow controller for CO_2 inlet. This allows an online control of the currently consumed CO_2, which is a suitable parameter for growth monitoring. Figure 3 shows a 24-h course of the pH value and the CO_2 mass flow. Lamps turned on at 02:00 and off at 22:00, causing a rise and decrease of the pH value, respectively. The setpoint of 8.5 is reached after first

overshooting and held during the day. The decrease of CO_2 consumption at noon is caused by the shadow of the power plant's chimney that casts upon the greenhouse at this time.

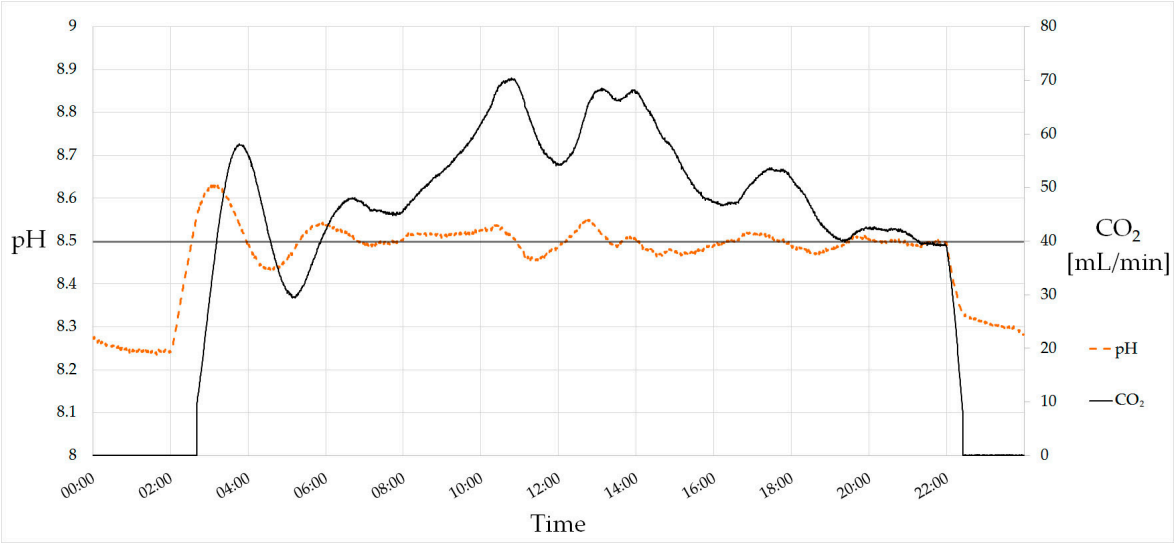

Figure 3. PI-controlled pH value. The setpoint of the pH is 8.5. Lamps turn on at 02:00 and turn off at 22:00, causing a rise and decrease of the pH value due to CO_2 consumption. In total, 59 L (118 g) of CO_2 were consumed on this day.

5.4. Overview of PHB Production Trials

Most of the trials (overview shown in Table 7) were performed using a modified BG 11 medium [102]. Modification in terms of PHB production is necessary, as normal BG 11 medium contains high amounts of nitrogen (1.5 g/L $NaNO_3$) and would not lead to nitrogen limitation. The modified BG 11 contains 0.45 g/L $NaNO_3$ and leads to a self-limitation of the culture. After 8–12 days nitrogen is consumed, PHB production starts and the color of the culture gradually turns yellow. This approach is necessary, as it is not possible to transfer large-scale cultures into a nitrogen-free medium [103].

Synechocystis salina CCALA192 was found to be a very suitable cyanobacterium. It is easy to handle and grows with small inoculation volumes of 1:50. Final biomass and PHB concentrations were in the range of 0.9–2.1 g/L and 4.8% to 9% of cdw, respectively. *Synechocystis salina* CCALA192 also grew with the addition of acetate, but no significant increase of biomass and PHB concentration was observed compared to photoautotrophic growth. When using acetate, contaminations with fungi were likely to occur and trials had to be stopped. Therefore, this approach was finally abandoned.

Digestate from a biogas reactor was successfully tested as an alternative nutrient source. The supernatant was produced by centrifugation with prior addition of precipitating agents. Before usage the supernatant was autoclaved and diluted 1:3 with water [92]. Figure 4 shows biomass and PHB production using digestate as nutrient source.

After one and a half years a new degassing system was installed, as the oxygen concentration was mostly above 250% saturation during the day. For an ideal cultivation of cyanobacteria the oxygen saturation should not exceed 200%. The new degasser led to a rise in biomass production with a maximum production rate of 0.25 g/L/d. Efficient degassing affected the cyanobacteria positively. However, during installation of the degasser dirt from the surrounding soil was brought into the reactor and from that time on culture crashes occurred due to ciliate contaminations (see Section 5.6).

The other tested cyanobacteria *Chlorogloeopsis fritschii* and *Arthrospira* sp. could not be successfully cultivated in the photobioreactor. It is assumed that these strains were sensitive to shear stress caused by the centrifugal pump [104].

Table 7. Overview of selected trials conducted in a tubular photobioreactor at pilot scale.

Trial	Strain	Nutrient Solution	Cultivation Time	Final Biomass Concentration	Final PHB-Concentration of cdw
1. Mineral medium	*Synechocystis salina* CCALA192	Optimized BG11	June 21 days	2.0 ± 0.12 g/L	6.6% ± 0.5%
2. Acetate addition	*Synechocystis salina* CCALA192	Optimized BG11, 20 mM acetate	July 26 days	1.9 ± 0.02 g/L	6.0% ± 0.1%
3. Acetate addition	*Synechocystis salina* CCALA192	Optimized BG11, 60 mM acetate	September 24 days	Trial cancelled, due to contaminations with fungi	
4. 24 h illumination	*Synechocystis salina* CCALA192	Optimized BG11	October 27 days	1.8 ± 0.02 g/L	4.8% ± 0.0%
5. Alternative nutrient source	*Synechocystis salina* CCALA192	Digestate supernatant	November–December 40 days	1.6 ± 0.02 g/L	5.5% ± 0.3%
6. Mineral medium	*Synechocystis salina* CCALA192	Optimized BG11	December–January 30 days	2.1 ± 0.03 g/L	6.0% ± 0.02%
7. Optimal degassing	*Synechocystis salina* CCALA192	Optimized BG11	May 7 days	0.9 ± 0.03 g/L (Trial prematurely cancelled due to ciliates)	9% ± 0.1% (Trial prematurely cancelled due to ciliates)
8. Chlorogloeopsis fritschii CCALA39	*Chlorogloeopsis fritschii* CCALA39	Optimized BG11	February 11 days	Trial cancelled, due to lack of growth	
9. Arthrospira	*Arthrospira* sp.	Spirulina Medium	October 7 days	Trial cancelled, due to lack of growth	

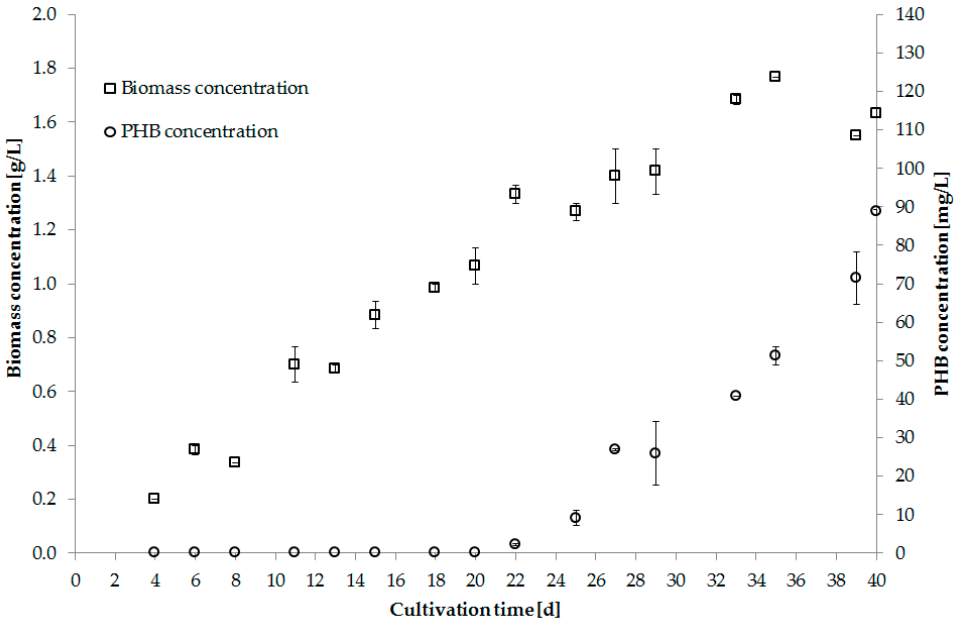

Figure 4. Biomass [g/L] and PHB [g/L] concentration of *Synechocystis salina* using digestate supernatant as nutrient source (Trial 5).

5.5. Downstream Processing of Cyanobacterial Biomass

Downstreaming of cyanobacterial cultures is particularly difficult, as cell densities are much lower compared to heterotrophic bacteria. Typical harvesting methods are sedimentation, filtration, or centrifugation [105]. The cyanobacterial biomass was harvested with a nozzle separator and stored at −20 °C. The biomass was then used to evaluate processing steps necessary to gain clean

PHB-samples for quality analysis. These downstream trials include (i) different cell disruption methods (milling, ultrasound, French press); (ii) different pigment extraction methods (with acetone and ethanol/methanol before or after extracting PHB); and (iii) different PHB extraction methods (soxhlet extraction with chloroform, biomass digestion with sodium hypochlorite) [106].

These trials showed that cell disruption with French press worked quite well but is very time consuming. Milling is assumed to decrease the molecular weight (polymer chain length). Pigment removal turned out to be necessary prior to PHB extraction, as pigments influenced the PHB properties negatively. This process step can be of advantage due to the generation of phycocyanin as a valuable side product [107]. A mixture of acetone and ethanol (70:30) was most suitable for this purpose. PHB extraction was performed with hot chloroform via a soxhlet extractor. Figure 5 compares the necessary processing steps of heterotrophs and cyanobacteria.

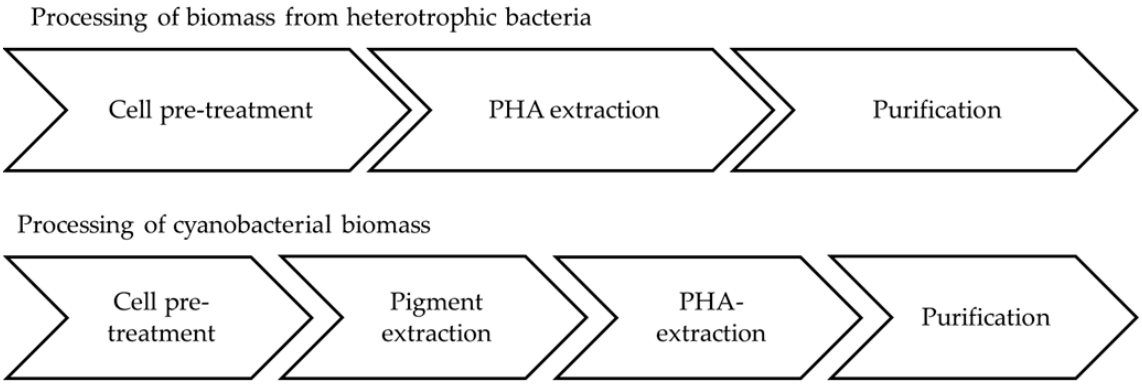

Figure 5. Comparison of processing steps needed to extract PHA from heterotrophic bacteria and cyanobacteria.

The PHB analysis showed that the polymers extracted from cyanobacterial biomass are comparable to commercially available PHB. Furthermore, it was shown that not only did the nutrient source, but also biomass pre-treatment and the method of polymer extraction influence the PHB properties. Pigment extraction and sample pre-treatment increased the average molecular weight (M_w) from 0.3 to 1.4 MD, but decreased degradation temperatures and crystallinity from 282 °C to 275 °C (T_{onset}) and from 296 °C to 287 °C (T_{max}), respectively. The M_w ranged from 5.8 to 8.0 MD, by using mineral medium and digestate, respectively. The thermal properties (T_{onset}: 283–282 °C; T_{max}: 293–292 °C), which are important for processing the polymer, are only slightly influenced by the nutrient source and are lower than, but comparable to, commercially available PHB. The crystallinity, responsible for higher final brittleness of the products, is about 17% lower than commercially available PHA.

5.6. Contaminations

Contaminations in non-sterile mass cultivation of microalgae are inevitable. It is only a matter of time before first contaminations appear, whether cultivation is done in open ponds or closed photobioreactors [108]. We observed certain bacterial and fungal contaminations with minor effects on *Synechocystis salina* CCALA192, when using CO_2 as the sole carbon source. Though, when adding acetate to the medium fungal contaminations were prevalent and difficult to control. After one and a half years the pilot plant was revised and a new degassing system was installed. From this moment on rapid culture crashes occurred. The microscopic image revealed a ciliated protozoa ingesting *Synechocystis* rapidly.

This ciliate forms highly resistant cysts and it is assumed that cysts from the surrounding soil were brought into the system during the revision work. Facts about contaminations in mass cultivation of *Synechocystis* are scarcely reported. Touloupakis and colleagues reported the grazing of *Synechocystis* PCC6803 by golden algae *Poterioochromonas* sp. [109]. High pH values of 10 and above helped to control

the contaminant and maintain a stable culture. Unfortunately, the ciliate in our cultures survives those high pH values. Thoroughly cleaning and sanitizing the photobioreactor brought some success, but the ciliate is still occurring and leading to culture crashes. Due to the ciliate's capability to form cysts, it is very difficult to completely eliminate it from the reactor. Heat sterilization is not possible in tubular photobioreactors. Addressing further research, there is need for special cultivation methods for robustly growing *Synechocystis* in non-sterile environment.

6. Conclusions

Although not economical today, the idea of a sustainable PHB production with cyanobacteria, CO_2, and sunlight is still attractive and, more and more, researchers are working in this field. The main challenges today are similar to biofuel production with green algae: (i) realization of efficient low-cost cultivation systems at large scale; (ii) maintaining stable cultures under non-sterile conditions; (iii) increasing the total productivity and yield; and (iv) economic downstream processing and utilization of the residual biomass.

Looking for suitable production strains it must be considered that PHB production is a very common feature of many, but not all, cyanobacteria. The PHB content of cyanobacteria is highly strain specific, as strains of the same genus were reported with highly varying PHB contents. In addition to the PHB content the growth rate and robustness of a strain is particularly important. The only cyanobacterium cultivated in mass cultivation today is *Arthrospira* sp. and, therefore, one of the most promising candidates for photoautotrophic PHB production, although most *Arthrospira* sp. strains still show little PHB content.

Heterotrophic cultivation with acetate boosts the PHB content remarkably, as most reported values over 30% were achieved this way. However, it needs to be considered that using an organic carbon source impairs the most attractive feature of cyanobacteria, converting CO_2 to PHB. Using organic sources will also complicate non-sterile mass cultivation and could easily lead to contaminations and culture crashes. PHB production with organic carbon sources should be performed with heterotrophic bacteria, as their PHB productivity, as well as their cell density, are 10–100 times higher.

Nitrogen and phosphorous depletion are the most important factors to increase the PHB content and are often necessary to produce any PHB at all. Therefore, a two-stage cultivation with a self-limiting medium is necessary for large-scale photoautotrophic PHB production. With this strategy PHB was successfully produced in our 200 L photobioreactor. In tubular systems small unicellular organisms, like *Synechocystis* sp., are preferred over filamentous organisms, mainly because of the shear stress of the pump. Considering all of the difficulties to overcome, establishing a stable cyanobacterial culture is most important and most difficult to achieve.

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3. CONTAMINATIONS IN MASS CULTIVATION OF CYANOBACTERIA: HIGHLY RESILIENT *COLPODA STEINII* LEADS TO RAPID CRASH OF *SYNECHOCYSTIS* SP. CULTURES AND IS INHIBITED BY PARTIALLY ANOXIC CONDITIONS

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Contaminations in mass cultivation of cyanobacteria: Highly resilient *Colpoda steinii* leads to rapid crash of *Synechocystis* sp. cultures and is inhibited by partially anoxic conditions

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ABSTRACT

Contaminations in microalgae cultures are one of the major drawbacks when cultivating in an industrial scale. What is often experienced as culture crash has been rarely investigated in detail. Here, we show that the ciliated protozoa *Colpoda steinii* is capable to clear a dense culture of the cyanobacterium *Synechocystis* sp. within 2–3 days. In a 200 L tubular photobioreactor, this ciliate frequently appeared and led to great losses. The ciliate was isolated and characterized in the laboratory. Its grazing rate exceeded the growth rate of three tested *Synechocystis* strains (*Synechocystis* PCC6803, *Synechocystis* CCALA192 and *Synechocystis minuscula* SAG 258.80) by far. Due to its devastating effect on *Synechocystis* sp., its ability to form cysts and its resilience, *Colpoda steinii* was experienced as serious threat for large scale cultures of *Synechocystis* sp. The most common means against contaminations are high salinities and high pH values. Though, cultivating with salinities up to 20‰ and pH values up to 10 did not inhibit *Colpoda steinii*. Other strategies like high ammonia concentration, carbon dioxide (CO_2) asphyxiation and anoxic conditions during the dark period were tested against the ciliate as well. While high ammonia concentration and CO_2 asphyxiation did no inhibit *Colpoda steinii*, partially anoxic conditions inhibited this ciliate effectively and appears to be a promising cultivation method for *Synechocystis* sp. under non-sterile conditions.

1. Introduction

Cyanobacteria are phototrophic prokaryotes playing a substantial role in the global carbon and oxygen cycle [1]. They take up carbon dioxide (CO_2) from the atmosphere and are considered as sustainable biomass producers. From the estimated 6000 cyanobacterial species [2] only *Arthrospira* sp. is produced in a larger scale, mainly for food purposes. However, the small unicellular cyanobacterium *Synechocystis* sp. is a promising production organism for a big variety of products and a lot of research has been published in recent years. The genome of *Synechocystis* PCC6803 has been fully sequenced in 1997 and all basic molecular biology techniques are available for metabolic engineering [3]. Wild type strains are capable to produce the degradable bioplastic polyhydroxybutyrate (PHB) [4–7], giving the opportunity to produce bioplastics directly from CO_2. Another highly interesting cell component is the blue fluorescent pigment phycocyanin, which can be used as food colorant [8–10]. *Synechocystis* PCC6803 has also been reported as promising vehicle for sustainable energy supply, as it can produce energy rich molecules like hydrogen or ethanol via photosynthesis [11–14]. Further promising products from *Synechocystis* sp. are platform chemicals like lactic acid [15] or isoprene [16–19].

For an economic production of most of these products, *Synechocystis* sp. needs to be cultivated in ponds or large photobioreactors. Generally, these systems do not provide the possibility of sterilization and also when closed photobioreactors are used, biological pollutants will inevitably enter the culture [20]. *Synechocystis* sp., as small phytoplankton, is situated at the very beginning of the food chain and is grazed by zooplankton, what makes the establishment of a large-scale culture under non-sterile conditions very difficult. Although there are many possible applications for *Synechocystis* sp., large-scale experiments under non-sterile conditions have been scarcely reported. Touloupakis and colleagues produced hydrogen with *Synechocystis* PCC6803 [12] and had to deal with contaminations as well, as they reported culture crashes due to *Poterioochromonas* sp. [21].

In this study, we report the effect of *Colpoda steinii* on a large-scale culture of *Synechocystis* CCALA192 and a cultivation strategy to inhibit this contaminant. Our attempts to produce polyhydroxybutyrate with *Synechocystis* CCALA192 repeatedly failed due to culture crashes. We

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successfully isolated *Colpoda steinii*, a 30 μm large ciliate, and characterized it in the laboratory. Several strategies against *Colpoda steinii* were tested and the predator – prey relations are discussed.

2. Material and methods

2.1. Organisms and standard culture conditions

Three different *Synechocystis* strains were used for this research. *Synechocystis* CCALA192 was ordered from the Culture Collection of Autotrophic Organisms, Institute of Botany, Trebon, Czech Republic. *Synechocystis* PCC6803 was ordered from the Pasteur Culture Collection of Cyanobacteria, Institute Pasteur, Paris, France. *Synechocystis minuscula* SAG 258.80 was ordered from the Culture Collection of Algae at Göttingen University, Göttingen, Germany. *Synechocystis* CCALA192 and *Synechocystis* PCC6803 were grown in BG11 medium [22] supplemented with 1 g/L sodium hydrogen carbonate ($NaHCO_3$) with a resulting pH of 8.5. *S. minuscula* SAG 258.80, originally isolated from a natron lake, was grown in BG11 medium supplemented with 5 g/L $NaHCO_3$ with a resulting pH of 9.6. All strains were grown in 50 mL shaking flasks under a light intensity of 1000 lx at 25 °C. Light-dark cycle was 16 h/8 h.

The ciliate *Colpoda steinii* was isolated from the photobioreactor and cultivated in the lab in 50 mL shaking flasks with *Synechocystis* CCALA192 as prey. For this, 1 mL of *Colpoda steinii* containing culture was transferred into a well-grown *Synechocystis* CCALA192 culture with above described standard culture conditions. Keeping *Colpoda steinii* for a longer period of time turned out to be simple due to the formation of resting cysts, that excyst 24–48 h after transferring into a fresh, well-grown *Synechocystis* CCALA192 culture.

2.2. Photobioreactor

The photobioreactor used in this research is located in a small greenhouse at the coal power plant site in Dürnrohr, Austria. It is a tubular photobioreactor with the system of the Schott AG, Germany, with a volume of 200 L. For additional artificial light, six gas-discharge lamps were used with light-dark cycle of 16 h/8 h. A degasser with pressurized air served for oxygen removal and a centrifugal pump circulated the algal culture. The degasser could be operated with nitrogen gas instead of pressurized air as well. The pH value and oxygen concentration were continuously measured with probes (CPS11D and COS51D, both Endress + Hauser GmbH, Austria) and pH was controlled with injection of pure CO_2. Photosynthetically active radiation (PAR) was measured with a PAR sensor (Theodor Friedrichs & Co., Germany). Temperature in the glasshouse was controlled with an air conditioning system (Daikin Industries) and held at 25 °C ± 2 °C.

2.3. Measurement of biomass dry weight and optical density

For biomass dry weight determination, 30 mL of the algal culture were centrifuged. The pellet was washed with deionized water, centrifuged again and dried at 105 °C overnight. Optical density was measured with a plate reader (Tecan Infinite M200 pro) at 680 nm.

2.4. Strategies against Colpoda steinii in the photobioreactor

The photobioreactor was operated with *Synechocystis* CCALA192. After the first culture crashes appeared, several different strategies were tested to inhibit the ciliate and establish a stable culture. The main focus was to remove or inhibit the ciliate. Possible growth rate reduction of *Synechocystis* CCALA192 was not studied. Following strategies were conducted in the photobioreactor.

Sanitizing the photobioreactor: The photobioreactor was thoroughly cleaned after culture crash and sanitized with a 0.1% sodium hypochlorite (NaClO) solution. In another attempt, the photobioreactor was sanitized with 0.5% ammonia solution.

High salinity: As a brackish water species, *Synechocystis* CCALA192 is able to grow with salt concentration up to about 20 g/L without growth inhibition. BG11 medium was supplemented with 20 g/L NaCl to test if this higher salinity inhibited the ciliate.

High pH value: BG11 medium was supplemented with 0.5 g/L sodium carbonate (Na_2CO_3) and then adjusted to a pH value of 10 with pure CO_2.

High ammonia concentration: Nitrate-free BG11 medium was supplemented with 0.6 g/L NH_4Cl (≙0.2 g/L NH_4^+) as sole nitrogen source and adjusted with $NaHCO_3$ and pure CO_2 to pH 8.5 to test ammonia toxicity.

CO_2 asphyxiation: BG11 medium was supplemented with 1 g/L $NaHCO_3$ and pH value was adjusted to 6.35 with pure CO_2 leading to a calculated dissolved CO_2 concentration of 262 mg/L.

Partially anoxic conditions: BG11 medium was supplemented with 1 g/L $NaHCO_3$. Pure CO_2 was used to keep the pH at a maximum value of pH 9. The photobioreactor was degassed with nitrogen gas at a constant flow rate of 500 mL/min leading to anoxic conditions during the dark period.

2.5. Effect of Colpoda steinii to different Synechocystis strains

The effect of *Colpoda steinii* to other *Synechocystis* strains than CCALA192 was tested in the laboratory. Those were *Synechocystis* PCC6803, the well-studied model organism, and *S. minuscula* SAG 258.80, cultivated with above described standard culture conditions. *Synechocystis* CCALA192 was also tested for comparison. 50 mL shaking flasks were inoculated to a final cell number of 10^7 *Synechocystis* cells per mL and after eight days of growth, 1 mL containing approximately 10^4 ciliates were added to the cultures. Each experiment was performed in triplicate.

2.6. Toxicity test of quinine sulphate on Synechocystis and Colpoda steinii

Moreno-Garrido et al. reported the successful application of 10 mg/L quinine sulphate against a not determined ciliate with a size of around 100 μm in *Dunaliella* cultures [23]. Therefore, we tested this substance against *Colpoda steinii*. Quinine sulphate was ordered from Alfa Aesar. First, toxicity of quinine sulphate was tested on *Synechocystis* CCALA192 in concentrations ranging from 5 to 50 mg/L in 50 mL shaking flasks (standard conditions, pH 8.6) and OD680 was measured daily. The cultures were started with an initial OD680 of 0.4 and every concentration was tested in triplicate. The areas under the growth curves for 120 h cultivation time were calculated. The control group without quinine sulphate represented 100%. For the toxicity test against the ciliate, 100 μL of a *Colpoda steinii* culture (three days after transferring) were placed into 96-well plates. Quinine sulphate was added to final concentrations ranging from 5 to 50 mg/L. After 24 h, mobility of the ciliates was checked in the microscope as criterion of death. Every concentration was tested in triplicate.

2.7. Microscopy

Microscopic images were made with an Olympus AHBT3 VANOX microscope in differential-interference contrast mode. Samsung Galaxy S6 was used with an ocular adapter for imaging.

3. Results and discussion

3.1. Ciliate isolation and identification

After first installation of the photobioreactor, *Synechocystis* CCALA192 was grown succesfully for approximately 18 months, when first culture crashes were experienced. Those were first noticed through (i) brownish foam in the degasser, (ii) decreasing oxygen concentration

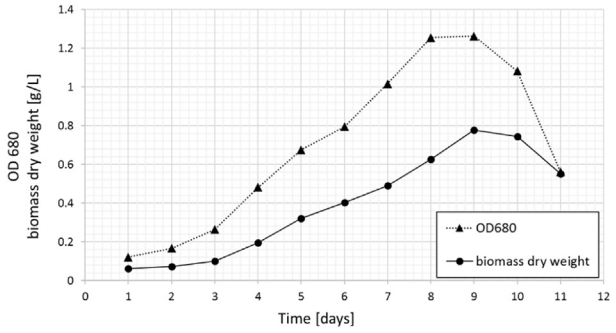

Fig. 1. Growth curve of *Synechocystis* CCALA192 in the tubular photobioreactor. Culture crash due to *Colpoda steinii* occurred between day 8–11. Data represent a single cultivation performed in the photobioreactor.

of the culture, (iii) decreasing CO_2 consumption of the culture and (iv) typical smell. Fig. 1 shows a run in the photobioreactor with culture crash due to *Colpoda steinii*.

The microsopic image revealed ciliated protozoa, that were moving fast and ingesting *Synechocystis* CCALA192 cells quickly (Video supplementary material). Through its morphology, its typical shape of the macronucleus and micronucleus, and its cysts, the ciliate was identified as *Colpoda steinii* [24]. *Colpoda steinii* is globally distributed and has an extremely broad ecological range. It occurs wherever there is adequate bacterial food and not too much competition. It forms resting cysts especially in response to unfavourable conditions like desiccation and can excyst fast when conditions become better [24,25]. Fig. 2 shows the different stages in the life cycle of *Colpoda steinii*.

The grazing capacity of *Colpoda steinii* is remarkable, as we found well-fed examples containing an estimated 300–400 *Synechocystis* cells, which can be seen inside *Colpoda steinii* due to their blue-green color. Sherr and colleagues reported grazing of *Colpoda steinii* on *Synechococcus* cells in Lake Kinneret, Isreal with a doubling time of 4.5 h. It was estimated that they consume the natural daily phytoplankton production [26]. In other reports predator – prey relations of *Colpoda steinii* were studied. Interestingly, the relations were found to be very unstable where episodes of heavy grazing follow episodes of encystment and bacterial population then recover, followed by the next episode of excystment and grazing [27,28]. This is what we observed in some experiments as well, when after days of heavy grazing *Colpoda steinii* was encysting and *Synechocystis* recovered for some time.

3.2. Evaluating strategies against Colpoda steinii in the photobioreactor

Several strategies to achieve a stable cultivation of *Synechocystis* CCALA192 were performed in the photobioreactor (overview shown in table 1). After culture crash, the photobioreactor was thoroughly cleaned and sanitized with 0.1% sodium hypochlorite (NaClO) solution. The photobioreactor was reinoculated with *Synechocystis* CCALA192

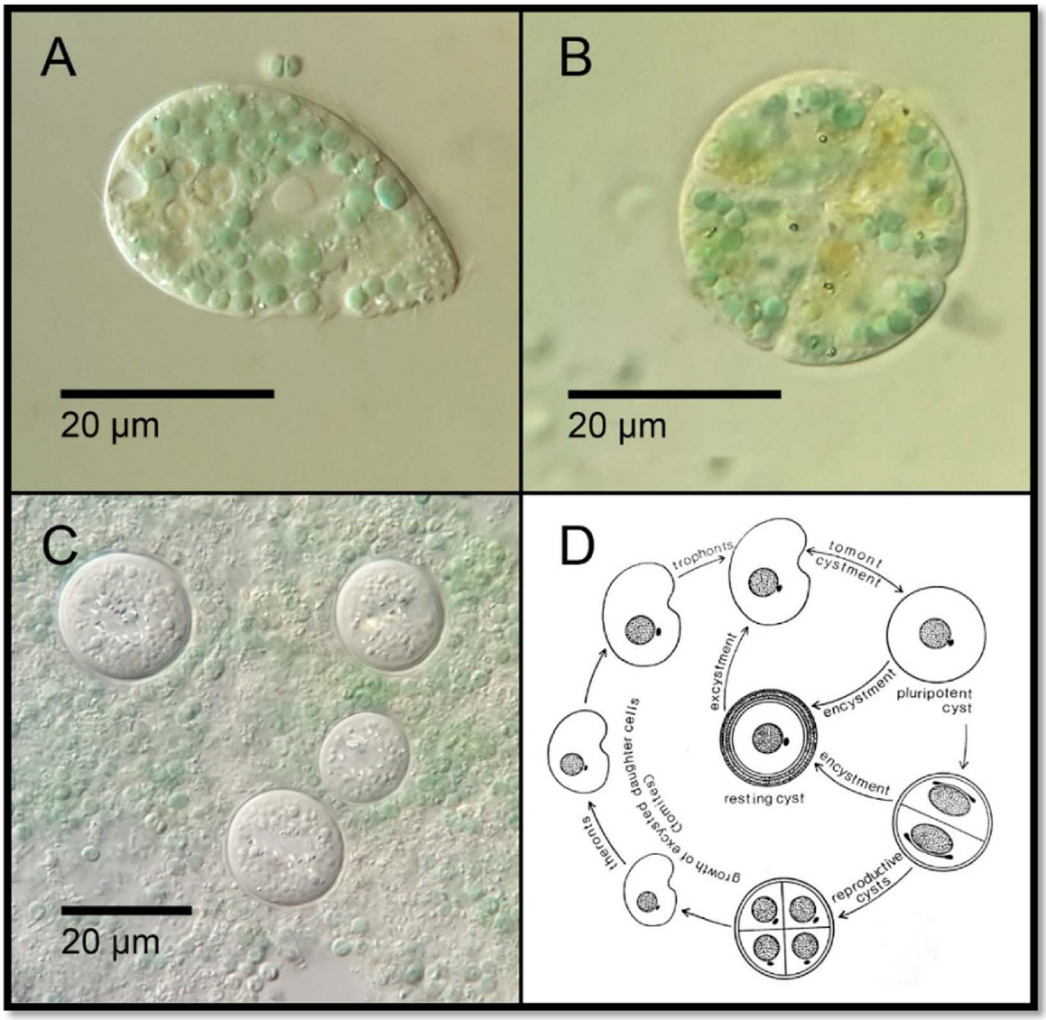

Fig. 2. (A) Grazing *Colpoda steinii* is filled with numerous *Synechocystis* cells. The nucleus can be seen in the center. At the bottom side the oral apparatus can be seen, on the left rear end the contractile vacuole is visible. (B) Reproductive division cyst of *Colpoda steinii.* (C) Resting cysts of *Colpoda steinii*. (D) Life cycle of colpodid ciliates [24].

Table 1
Evaluation of different cultivating strategies against *Colpoda steinii*.

Cultivation strategy in the photobioreactor	Outcome
Sanitizing with 0.1% NaClO solution	Culture crash 4–5 days after inoculation
Sanitizing with 0.5% NH_4 solution	Culture crash 4–5 days after inoculation
High salinity (20 g/L NaCl)	No inhibition of *Colpoda steinii*
Cultivating at pH 10	No inhibition of *Colpoda steinii*
High ammonium (200 mg/L NH_4^+ at pH 8.5)	No inhibition of *Colpoda steinii*
CO_2 asphyxiation (262 mg/L dissolved CO_2 at pH 6.35)	No inhibition of *Coploda steinii*
Partially anoxic conditions	Stable cultivation of *Synechocystis* CCALA192

and after 4–5 days culture crash due to *Colpoda steinii* occured again. As the photobioreactor has several joints and valves there is always some space, where the sanitizing agent cannot work properly and resting cysts survive. Sanitizing with 0.5% ammonia solution led to a similar result. Heat sterilization of the photobioreactor is not possible because of the PVC elements. The next strategies were growing at high salinity or high pH values. Though, neither growing *Synechocystis* CCALA192 with salt concentrations of 20 g/L NaCl nor growing at pH 10 affected *Colpoda steinii*. After inoculation with *Synechocystis* CCALA192, growth was observed for 4–5 days followed by a total clearance of the culture. Higher ammonium concentration was tested, as this was reported to be toxic to ciliates [23,29] and suitable for zooplankton control [30]. Though, *Colpoda steinii* was not affected by ammonium concentrations up to 200 mg/L at pH 8.5. Recently, CO_2 asphyxiation was reported as suitable method for zooplankton control with a tested concentration of 330 mg/L, which was already lethal within 300 s to the ciliate *Paramecium* sp. [31]. For the CO_2 asphyxiation experiment, CO_2 mass flow was gradually increased until pH 6.35 with a dissolved CO_2 concentration of 262 mg/L. After two days, *Colpoda steinii* was still active grazing and the culture showed decreasing oxygen production. We experienced *Colpoda steinii* as serious and completely unexpected problem for our research project, as we spend more than a year and had to cancel numerous trials performed in the photobioreactor.

The last performed strategy was anoxic conditions during the dark period. Oxygen is produced by *Synechocystis* during photosynthesis and therefore, anoxic conditions can only be achieved without light. When degassing with nitrogen gas instead of pressurized air, oxygen concentration was rapidly decreasing when the light turned off, leading to anoxic conditions during the night. Fig. 3 shows the oxygen concentration and photosynthetically active radiation (PAR) over a period of 4 days in the photobioreactor. This strategy finally led to good growth and a stable cultivation. Interestingly, *Colpoda steinii* was sometimes visible in the microscope but never caused culture crash. It was reported, that *Colpoda steinii* is actually able to survive under anoxic conditions [32]. We suppose, that the rapid decrease of oxygen concentration in the culture may harm the active, grazing stages of *Colpoda steinii*. The exact inhibition mechanism is not yet known.

According to current evidence, Earth's early atmosphere was nearly absent of oxygen and ancestors of today's cyanobacteria, responsible for the great oxygenation [33,34], had to grow in an anoxic environment. Anoxic growth of several cyanobacterial strains has been demonstrated by Thomas and colleagues [35]. We observed good growth of *Synechocystis* CCALA192 with partially anoxic conditions in the photobioreactor, a growth curve is shown in Fig. 4.

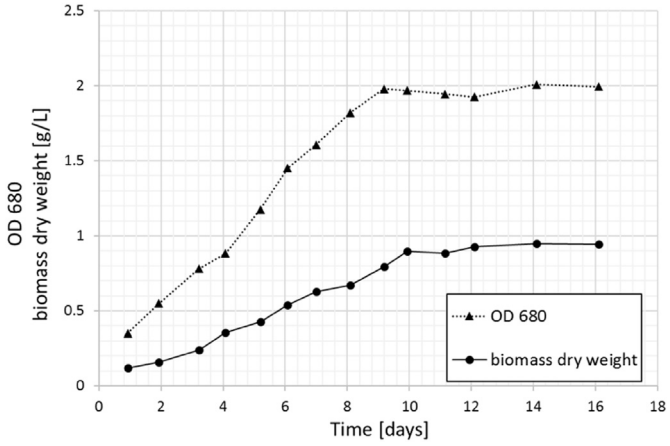

Fig. 4. Growth curve of a stable *Synechocystis* CCALA192 culture with partially anoxic conditions. Data represent a single cultivation performed in the photobioreactor.

As there are no chemicals needed and the implementation of this cultivation strategy is easy, we consider it as highly promising for large scale cultivation of *Synechocystis* sp. This cultivation strategy can be implemented in any closed photobioreactor system by simply changing the pressurized air to nitrogen gas. The costs can be lowered to a minimum, if nitrogen gas will be used during the dark period only.

3.3. *Colpoda steinii grazing on different Synechocystis strains*

After having tested several means for contamination control with *Synechocystis* CCALA192, we compared the grazing behaviour of *Colpoda steinii* on other *Synechocystis* species. The model organism *Synechocystis* PCC6803 is slightly larger than *Synechocystis* CCALA192, while *S. minuscula* SAG 258.80 needs to be cultivated in BG11 medium with increased $NaHCO_3$ concentration (see material and methods). Fig. 5 shows the growth curves of *Synechocystis* CCALA192 and *Synechocystis* PCC6803. Ciliates were added on the ninth day and approximately 40 h later a strong decrease of the optical density was measured in all shaking flasks. After clearance, the cysts of *Colpoda steinii* tend to stick at the liquid – air interface and can be seen as a ring on the glass wall of the shaking flasks (Figure supplementary material).

Fig. 6 shows the growth curves of *S. minuscula* SAG 258.80. In difference to the other *Synechocystis* strains, the measured shaking flasks of *S. minuscula* SAG 258.80 were not cleared simultaneously. We assume, that *Colpoda steinii* had to adapt to the higher $NaHCO_3$ concentrations and therefore, there was longer delay compared to the other *Synechocystis* strains. Once *Colpoda steinii* adapted, cultures were cleared within 2–3 days as well.

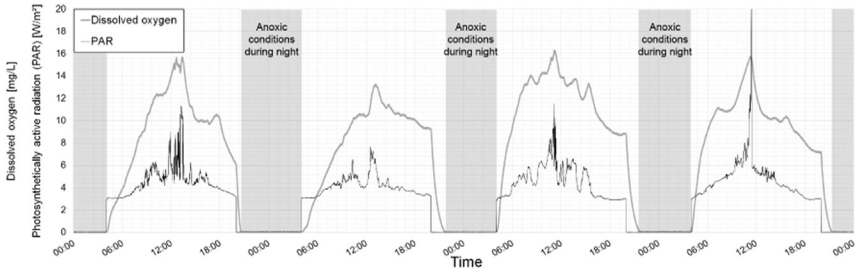

Fig. 3. Partially anoxic culture conditions: Dissolved oxygen concentration and photosynthetically active radiation (PAR) in the tubular photobioreactor are plotted over a period of 4 days. Lamps turn on at 04:00 and turn off at 20:00. During the night, oxygen concentration decreases to zero due to degassing of the culture with nitrogen, leading to anoxic conditions until the lamps turn on and oxygen is produced again.

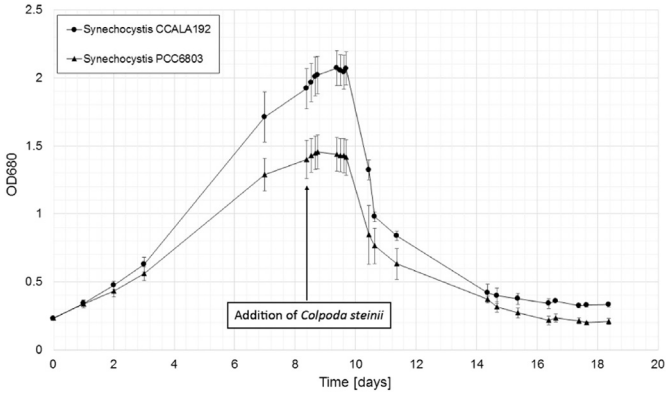

Fig. 5. Growth curve of *Synechocystis* CCALA192 and *Synechocystis* PCC6803. Forty h after addition of *Colpoda steinii*, a rapid decrease of the optical density was measured (n = 3, bars = standard deviation).

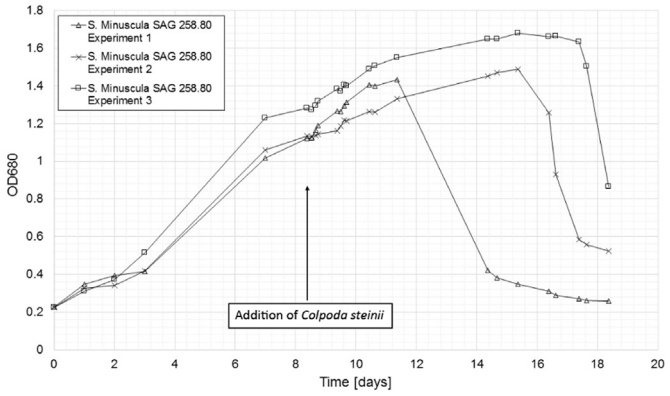

Fig. 6. Growth curves of three independent experiments of *S. minuscula* SAG258.80. Clearance of the cultures occurred approximately 5, 9 and 10 days after addition of *Colpoda steinii*.

Table 2
Results of the toxicity test of quinine sulphate on *Synechocystis* CCALA192 and *Colpoda steinii*. *Colpoda steinii* is affected by 30 mg/L and 50 mg/L is lethal. On the other hand, *Synechocystis* CCALA192 is affected by 5 mg/L and 20 mg/L is lethal. Growth of *Colpoda steinii* could not be quantified.

Concentration of quinine sulphate [mg/L]	0 mg/L	5 mg/L	10 mg/L	20 mg/L	30 mg/L	50 mg/L
Growth of *Synechocystis* CCALA192 in %	100%	78.4%	49.5%	0%	0%	0%
Inhibition of *Colpoda steinii* (−/~/+)	−	−	−	−	~	+

3.4. Toxicity test of quinine sulphate on Synechocystis and Colpoda steinii

Synechocystis CCALA192 was already affected by the lowest tested concentration of 5 mg/L quinine sulphate. Concentrations above 20 mg/L were lethal. *Colpoda steinii*, on the other hand, was more robust to quinine sulphate with lethal concentration between 30 and 50 mg/L. Results of the test are shown in Table 2.

4. Conclusion

While in conventional agriculture most pests of certain crops are well known, there is still little knowledge of pests affecting microalgae cultures. We experienced the 30 µm large ciliate *Colpoda steinii* as a serious threat to cultures of the cyanobacterium *Synechocystis* sp. In a 200 L tubular photobioreactor, this ciliate was the cause of numerous culture crashes. Once it has entered the photobioreactor, it was difficult to remove it, due to its highly resilient cysts. This study leads to the assumption, that small cyanobacteria like *Synechocystis* or *Synchococcus* with a size of 0.5–3 µm are highly vulnerable to *Colpoda steinii*. This ciliate is worldwide distributed and its cysts are resistant to desiccation. Therefore, dust is suspected to be the main vector for entering algae cultures. To be able to unleash the potential of *Synechocystis* sp. as an industrial producer for CO_2-based food ingredients, bioplastics, chemicals or fuels, certain means or special strategies are necessary. Our performed cultivation strategy of partially anoxic conditions led to good growth and inhibited the ciliate. This cultivation strategy is easy to establish and requires only nitrogen gas. As it is applicable to all closed photobioreactors, we see great potential.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2017.11.002.

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Declaration of author's contribution

Clemens Troschl identified the ciliate, designed and performed most experiments, analyzed the data and wrote the article. Katharina Sodnikar performed experiments and counted the cells. Ines Fritz and Bernhard Drosg obtained the funding, 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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4. PILOT-SCALE PRODUCTION OF POLY-β-HYDROXYBUTYRATE WITH THE CYANOBACTERIUM *SYNECHOCYSTIS* SP. CCALA192 IN A NON-STERILE TUBULAR PHOTOBIOREACTOR

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

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Keywords: Cyanobacteria, *Synechocystis* sp. CCALA192, PHB, tubular photobioreactor

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ABSTRACT

The biopolymer poly-β-hydroxybutyrate (PHB) can be used as a promising bioplastic. 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 limitation.

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 were harvested and the residual 10% were 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 cyanobacteria, formerly known as blue-green algae, 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 persistent 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 supports the cell's stress resistance [16]. Generally, PHB concentrations in nutrient-deprived, photoautotrophic cyanobacteria range between 5-20% of cell dry weight (cdw), which is still quite low, compared

to heterotrophic PHB producers with concentrations above 70% of cdw. However, in contrast to heterotrophic bacteria, cyanobacteria do not consume sugars, that are responsible for an estimated 50% of the total production costs [17]. Furthermore, cyanobacteria do not depend on agricultural crops, what makes them even more attractive as sustainable biomass producers. There are numerous reports about PHB production with cyanobacteria: Kamravamanesh et al. produced 16.4% PHB of cdw with *Synechocystis* sp. PCC6714 [18]. Panda et al. produced 11.2% PHB of cdw with *Synechocystis* sp. PCC6803 [19]. Khetkorn et al. produced 26% PHB of cdw with a genetically modified *Synechocystis* sp. PCC6803 [20]. An exceptional high PHB content of 55% of cdw was produced with the thermophilic *Synechococcus* sp. MA19 [21]. Generally higher PHB contents could be produced with adding carbon sources like acetate or glucose. With this strategy, Bhati et al. produced 78% PHB of cdw with *Nostoc Muscorum* Agardh [22]. However, the addition of organic carbon sources will change the metabolism from autotrophic growth to heterotrophic growth. For further examples we recommend the latest reviews about this topic [23–26].

Almost all of the reported experiments were performed under sterile laboratory conditions and there are hardly any reports about pilot-scale production of PHB 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 small 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 [27].

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 examined under 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. This strain was chosen as it proved to be the most promising PHB producer within a previously performed screening of roughly 30 strains. For strain maintenance in the laboratory, the strain was cultivated in BG11 medium [28] 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 built 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. The basic design of the reactor is described elsewhere [29]. 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) with variable flowrate 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 artificial illumination was installed to somehow compensate for the unfavorable situation, that the power plant was partially shading the glasshouse. The light situation in the glass house was therefore very complex and several PAR sensors would have been necessary for acquisition of adequate data for calculations. PAR is therefore only used in Figure 5. 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 adjusted to pH 10 (production cycle 1 and 2) and pH 9 (production cycle 3 and 4), respectively. 0.4 g/L instead of 1.5 g/L $NaNO_3$ were used, leading to self-limitation of the culture. After 16-20 days of cultivation, 90% of the culture were 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 (cdw) determination, 50 mL of the culture were 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. [30]. 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). Commercially available PHB and pure crotonic acid were used as standards.

Volumetric PHB production rate was calculated as a mean value for each production cycle.

$$Volumetric\ PHB\ production\ rate\ = \frac{Final\ volumetric\ PHB\ concentration}{Duration\ of\ production\ cycle}$$

The determination of glycogen concentration was performed with a modified method after Maurer at al. [31]. 10 mL of the culture were 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). Pure glucose was used as a standard.

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 were flocculated using 150 mL $FeCl_3$ (40% w/w) 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, 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. FT-IR 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}. 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) 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. [32]

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. [33]. 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 growth of the ciliate *Colpoda steinii*. This cultivation method was performed throughout all cultivations in this study. Furthermore, *Synechocystis* sp. CCALA192 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.

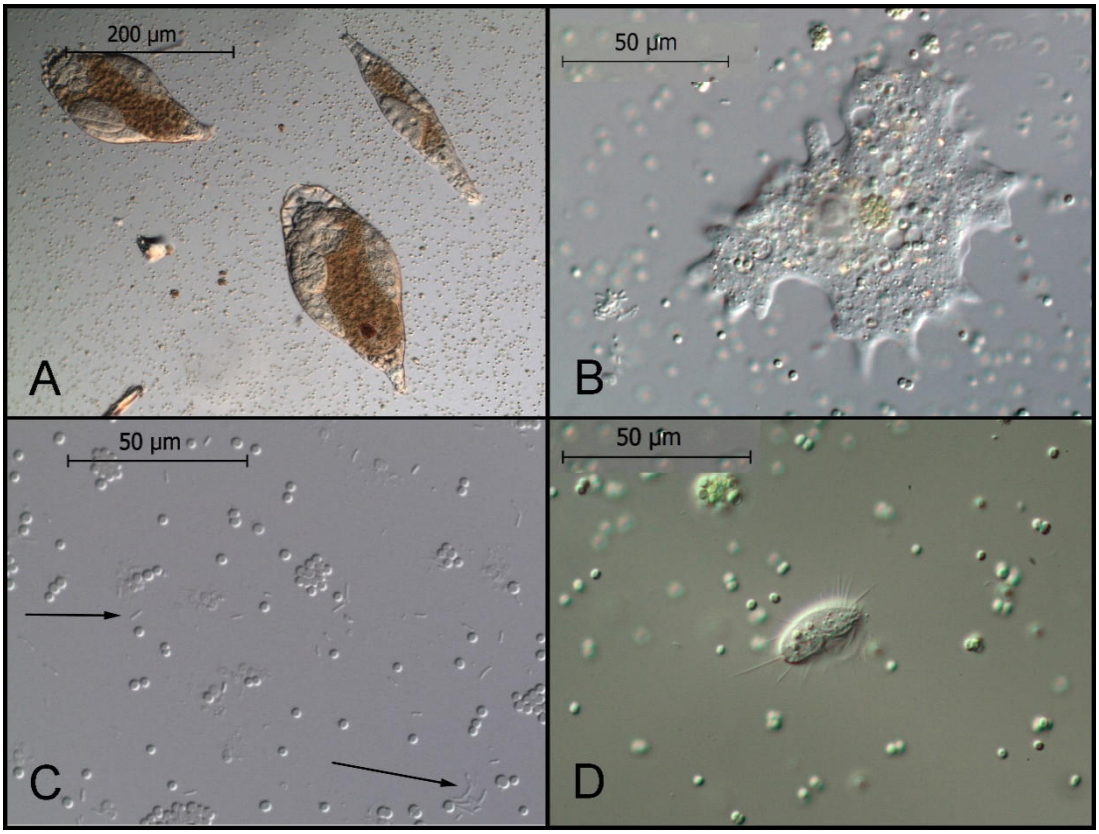

Figure 1: Microscopic images of different organisms, that were observed during the experiments: (A) Rotifers graze on *Synechocystis*, (B) an amoeba is slowly ingesting *Synechocystis*, (C) bacteria are present in the culture, (D) a small ciliate swims in the culture.

Less contamination problems are one of the main advantages of closed photobioreactors often described in literature [34,35], 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 cell wall of *Nannochloropsis gaditana* consists of a bilayer structure consisting of a cellulosic inner wall protected by an outer hydrophobic alganean layer [36]. 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. CCALA192 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 and similar processes [37–39].

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 total volume) 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 ± 0.2 g/L 12-14 days after starting and was then decreasing to 1.0 ± 0.2 g/L. PHB and glycogen, which was accumulated in one production cycle, was consumed in the following cycle during the first 2-3 days. 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 cdw 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% of cdw. 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% of cdw [18,19,25,40]. Therefore, *Synechocystis* sp. CCALA192 with 12.5% PHB of cdw 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 [18].

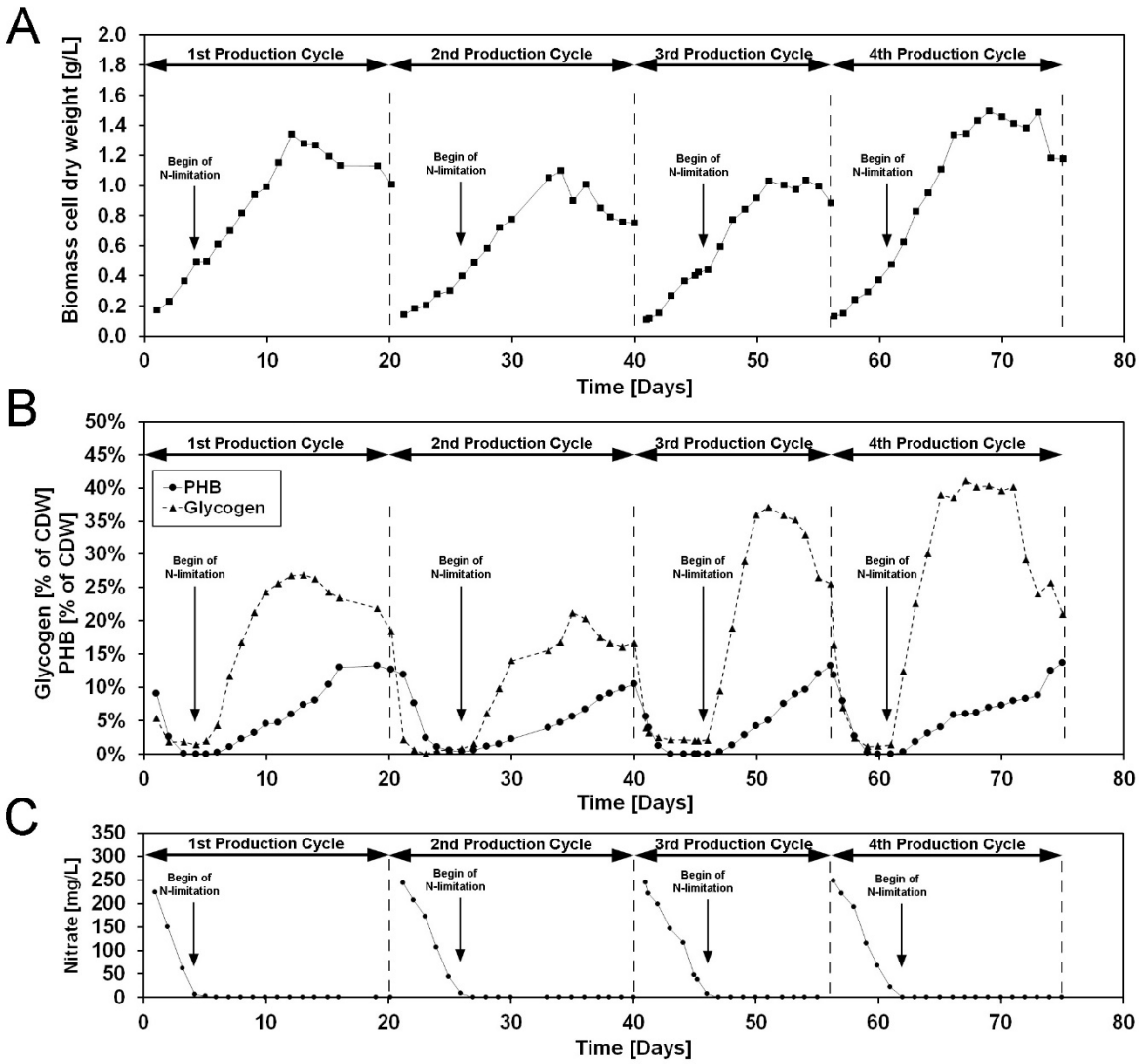

Figure 2: (A) Graph depicts biomass cell dry weight of four production cycles. Each production cycle shows growth for 10-14 days and a decreasing biomass concentration towards the end. (B) Graph depicts PHB and glycogen concentration of four production cycles. Both are consumed within the first 2-3 days 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. Vertical arrows indicate the begin of nitrogen limitation.

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 ± 0.2 g/L	1.0 ± 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 ± 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 are 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 [41–44] 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. Due to respiration of the cells, the pH was slightly decreasing during the dark period. 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 [29,42,45], but they often lack a real time monitoring of the consumed CO_2.

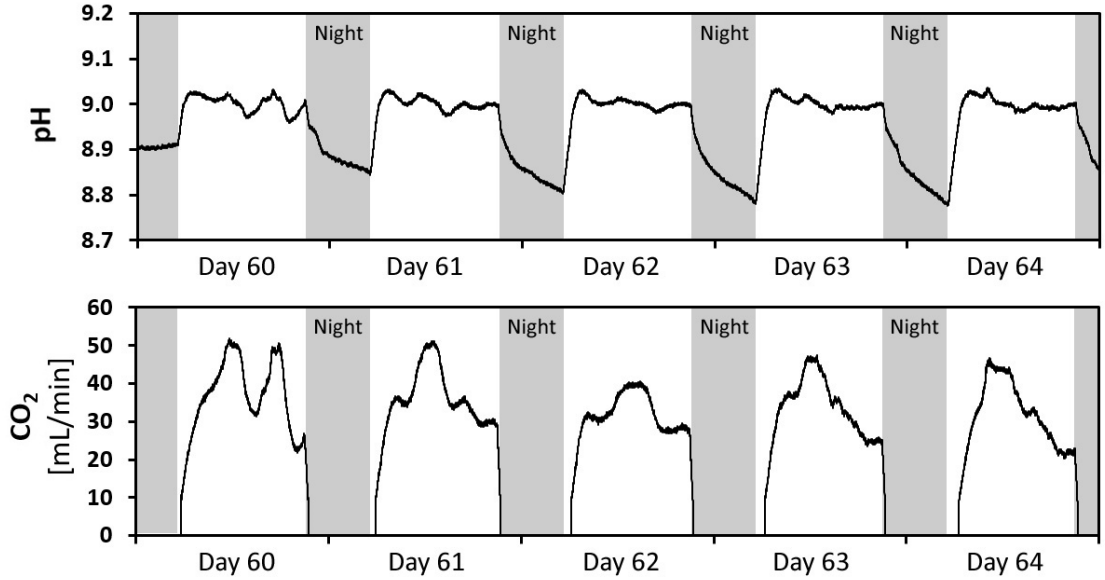

Figure 3: Course of pH-values and CO_2 dosage over five representative production days is depicted. During the day, CO_2 is injected to keep the pH at a set point of 9. During the night, no CO_2 is injected and the pH is slightly decreasing.

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 $g_{CO2}/g_{biomass}$ [45] 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 [46]. 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 [47].

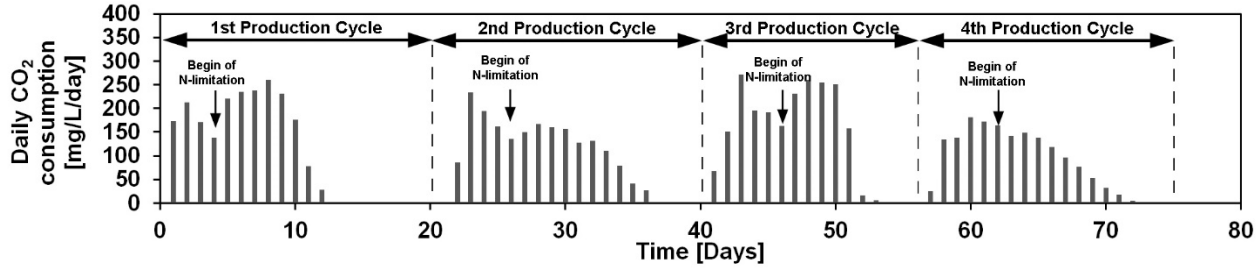

Figure 4: CO_2 consumption of each day is depicted. There is a strong decrease of CO_2 consumption in the late stage of each production cycle. Vertical arrows indicate the begin of nitrogen limitation.

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, indicating the poor photosynthetic activity.

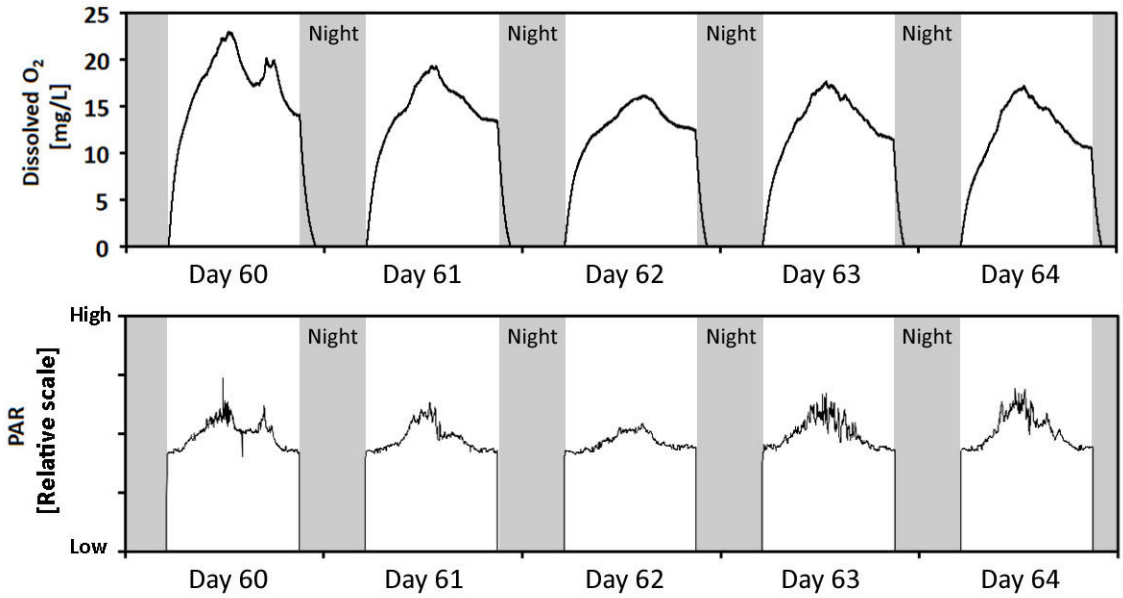

Figure 5: Dissolved oxygen concentration and photosynthetivally active radiation (PAR) are depicted over a period of five days.

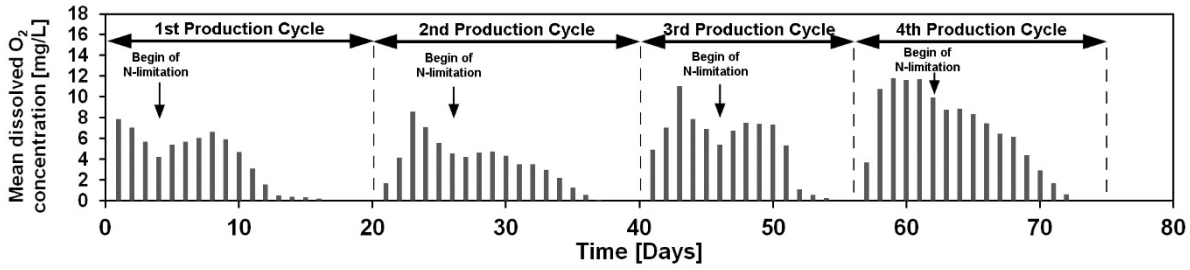

Figure 6: 24-hour mean dissolved oxygen concentration of each day is depicted. There is a strong decrease in the late stage of each production cycle. Vertical arrows indicate the begin of nitrogen limitation.

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 [48–50]. 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 [18–20,48].

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.

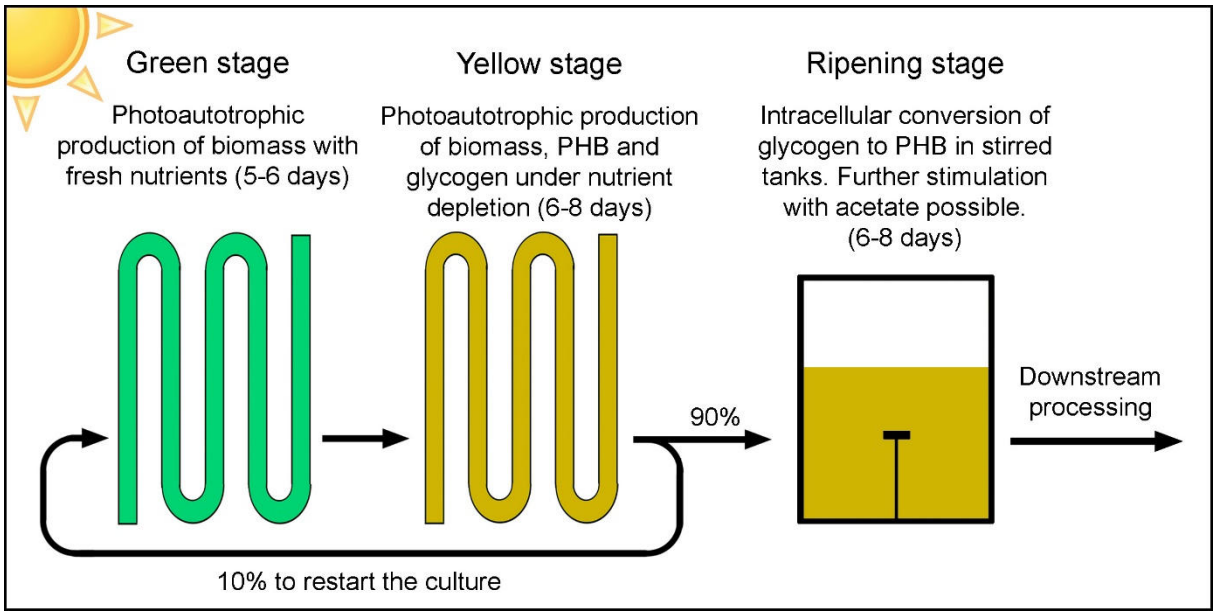

Figure 7: A proposed operation mode for cyanobacterial PHB production is shown. In order to fully use the photosynthetic capacity of the PBR, a ripening tank serves for PHB production in the late stage of the culture, when glycogen is converted to PHB and no CO_2 is consumed.

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 8) show the relative decrease of phycocyanin and increase of carotenoids. Figure 9 shows photographs of the photobioreactor at the blue-green stage and the yellow stage.

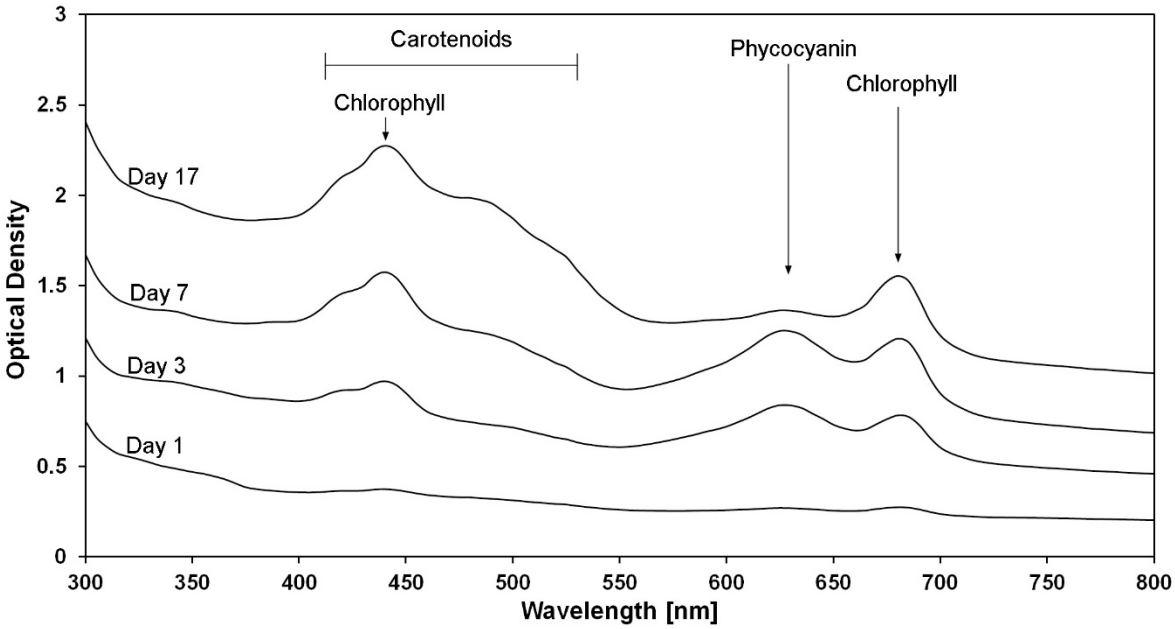

Figure 8: Wavelength scans of four days (day 1, day 3, day 7, day 17) of production cycle 1 are depicted. An increase of carotenoids and a relative decrease of phycocyanin can be seen.

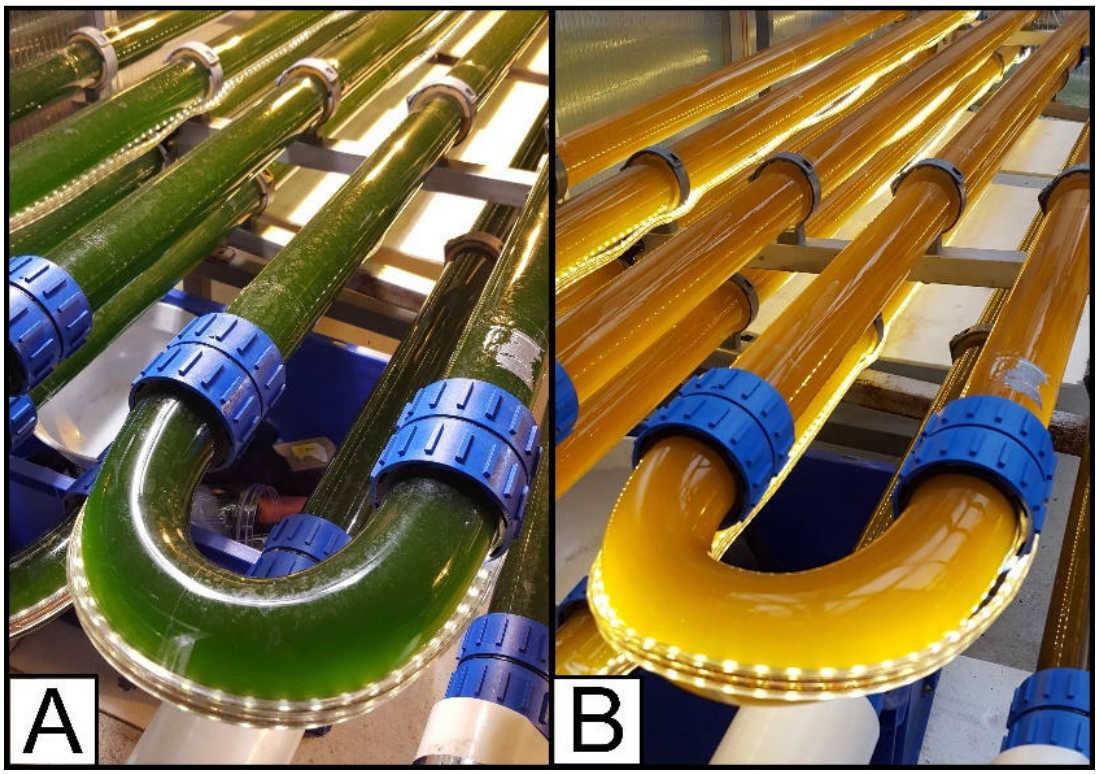

Figure 9: (A) Photograph of the photobioreactor during the green stage of the cultivation process.
(B) Photograph of the photobioreactor during the yellow stage of the cultivation process.

Downstream, PHB purification and analysis

Flocculation of *Synechocystis* sp. CCALA192 culture with iron (III) 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 10, 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 265.0°C.

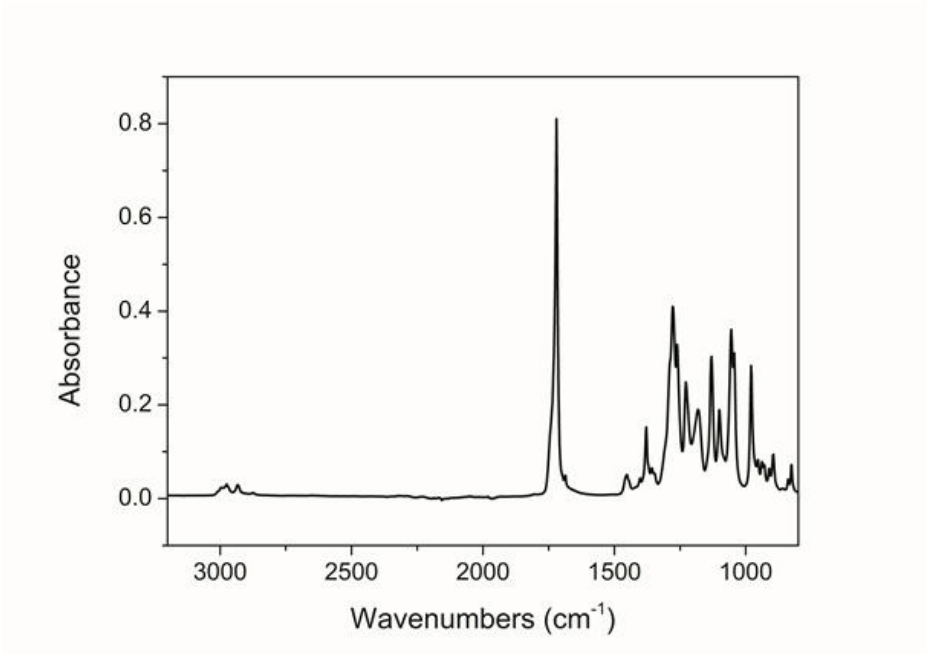

Figure 10: FT-IR spectrum of PHB produced by *Synechocystis* sp.

Deeper insights into downstream processing of *Synechocystis* sp. and possible side products are provided elsewhere [51].

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 as a basis for future work on improving 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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5. GENERAL DISCUSSION

The aim of this work was to produce the bioplastic poly-β-hydroxybutyrate (PHB) photoautotrophically with cyanobacteria and to investigate and describe the process. In this section, all necessary steps for a cyanobacterial PHB production, from the selection of the location to the purification of the final product, are discussed. Special emphasis is placed on bioeconomic aspects. PHB (except for medical applications) is a low value product, which is still not produced in large quantities for serving a mass market, neither with cyanobacteria nor with heterotrophic bacteria. The price should come to 3-5$ per kg to be commercially viable (Reddy et al. 2003). Apart from the economic feasibility, there are other important aspects to promote and justify the complex production process, especially when it comes to cyanobacterial PHB. The whole process must bind more CO_2 than is produced, as the most important argument for using cyanobacteria is their ability of using CO_2 as a substrate. Furthermore, the overall energy balance should be positive.

In recent years, there has been a hype about the production of biofuels from microalgae. Several companies claimed to be able to produce biodiesel or bioethanol at costs competing with fossil fuels. However, to date, biofuel production with microalgae has not been realized and the short-term outlook is rather pessimistic (Wijffels et al. 2010; Chisti 2013; Chen et al. 2011). For the evaluation of the feasibility of this new technology, several universities and institutes performed research projects and a lot of literature is available today. Biodiesel production with microalgae is very much comparable to PHB production with cyanobacteria. Green algae produce lipids as a storage compound under nutrient starvation, there is a complicated downstream process and biodiesel is a low cost product, too. Therefore, interesting results from literature about biodiesel production with green algae are discussed here within.

Requirements for the location

Large quantities of microalgae or cyanobacteria for low-value products should be produced at locations only, which can fulfill some criteria. These are (1) high solar radiation, (2) water supply, (3) CO_2 supply and (4) nutrient supply. As the investment costs of a microalgae production facility are high, satisfying these requirements is necessary for a high yield and an economic production. Regions with relatively low radiation like Central Europe are not suitable for production of low cost products like PHB. Arid places like the Sahara or the Australian desert provide extremely high solar irradiance, but are not suitable either, as there is no water available. The ideal locations might be arid coastal areas with high solar irradiance and

available sea water such as Northern Africa, Mexico or Western Australia. However, the supply of CO_2 and nutrients is still difficult. Between 1.8 - 2.3 kg CO_2 is needed for producing 1 kg of algal biomass (Acién et al. 2012; Chisti 2007), assuming that no CO_2 is lost due to degassing. Atmospheric CO_2 concentrations (0.040% v/v) are generally too low to assure high productivities (Lam et al. 2012). Industrial sites with CO_2 emissions such as thermal power plants or cement factories are good candidates for a CO_2 supply, but they have to be close to the microalgal production facility. The concentrations of other substances in the flue gas such as heavy metals, NOx or SOx have to be respected, too. The production of microalgae and cyanobacteria require furthermore considerable amounts of nutrients. Especially nitrogen and phosphorous are important, but sulfur, potassium, iron and micronutrients like manganese have to be supplied too (Markou et al. 2014). Several successful attempts of using wasterwater and agro-industrial wastes like digestate as nutrient source have been reported in recent years (Markou et al. 2011; Troschl et al. 2017). In a previous research project performed in Dürnrohr, digestate was successfully used as nutrient source for the cultivation of *Synechocystis salina*. Wastes as nutrient sources are promising, but a steady supply have to be ensured, as some waste streams are only seasonal (Meixner et al. 2016).

The powerplant station Dürnrohr is a good place for operating a pilot-plant, as necessary infrastructure is already available. The laboratories of the University of Natural Resources and Life Sciences (BOKU) are only 15 minutes away to measure samples. There is also a CO_2 separation plant in Dürnrohr, which had been erected in 2010 by EVN, ANDRITZ and the Technical University Graz (Rabensteiner et al. 2014). Unfortunately, the powerplant Dürnrohr is operated only occasionally and therefore, no flue gas experiments could be performed in the present thesis. However, even when using the flue gases as a free carbon source, Dürnrohr would not be suitable for an economic cyanobacterial PHB production, as the annual solar radiation is too low. Furthermore, the region around Dürnrohr is intensely agriculturally used. This land had to be used for erecting the photobioreactors, but one of the most important arguments promoting microalgae and cyanobacteria is that they do not compete with arable land (Wijffels et al. 2010; Gouveia et al. 2009).

Requirements for the production organism

The ideal production organism for a cyanobacterial PHB production must fulfill certain criteria as well. First, it needs to be a strain that is capable to produce PHB. Most cyanobacteria fulfill this criteria, as in a recent PHB screening study of 137 cyanobacteria strains, 134 were capable to produce PHB (Kaewbai-ngam et al. 2016). The PHB content of the strain is an important selection criterion, but there are other criteria that should not be overlooked. For example, *Calothrix scytonemicola* was capable to produce 25.4% PHB of cell dry weight, which is comparably high. However, it took 44 days to reach this PHB content at a total biomass of only 1.4 g/L. For comparison, *Spirulina* sp. was reported to reach such cell densities in 3-6 days (Ferreira et al. 2012; Depraetere et al. 2015). The overall growth rate is at least as important as the PHB content.

Screening studies are usually performed with sterile shaking flasks, as many experiments in parallel are necessary for screening. However, shaking flasks are not appropriate for mass cultivation. The performance of a strain will differ significantly from the results of shaking flask experiments, when growing in large non-sterile production facilities. Facilities like open ponds or tubular photobioreactors are always non-sterile and a complex ecosystem is inherent. Especially predators can be a major problem, as shown in Chapter 3. Furthermore, present wild algae or cyanobacteria of the surrounding environment could overgrow the production organism. The robustness of the strain and the adaptation to the production facility are particularly important.

In this thesis, *Synechocystis* sp. CCALA192 was used as production organism. With the necessary selective conditions, it was possible to produce PHB in the tubular PBR. However, there are disadvantages of *Synechocystis* sp. like its small size of only 0.5-2 µm. This makes the organisms vulnerable to predators and complicates the downstream processing significantly. Moreover, the PHB content was still low. Considering the overall production process, a robust and easy-to-harvest strain like *Spirulina* sp. would by favorable, if it also had high PHB concentrations.

The cultivation of cyanobacteria

In this thesis, a tubular PBR was used for cultivation of cyanobacteria. The ensured high process control was the main reason. Moreover, several parameters could be easily changed and adjusted, which is favorable for a research facility. Furthermore, the tubular PBR is relatively compact and fits in the small glass house located in Dürnrohr. However, summarized research performed in the last years has shown, that tubular PBRs do have a negative energy balance, meaning that the production of the biomass requires more energy than the biomass itself contains. The pump for circulating the medium is the major energy consumer (Prokop et al. 2015). In a comprehensive study comparing several life cycle assessments, no closed system achieved a positive energy balance. Considering a sustainable and economic feasible production plant, a positive energy balance is necessary, but were only achieved with raceway ponds (Slade et al. 2013). In a recognized study from Almería in Southern Spain, the costs of producing algae biomass in tubular photobioreactor was estimated with 69 €/kg. The best-case up-scale scenario showed production costs of 12.6 €/kg (Acién et al. 2012). Assuming that cyanobacterial biomass with optimistic 25% PHB of cell dry weight were produced, costs of PHB would be at least 50 €/kg. Open ponds might be more economic to produce PHB with cyanobacteria, but today, only a few strains like *Spirulina* sp. are known to be cultivable in open ponds. As shown in Chapter 4, a ripening process should be integrated in the overall production process. The ripening process can boost the PHB content and makes the reactor free tor the next cultivation cycle. However, further equipment like stirred tanks are also necessary for this approach.

Downstream process

The harvest of cyanobacteria and microalgae is considered as one of the major challenges for an economic production. After cultivation, the cell suspension contains typically around 1 g/L biomass dry weight. Therefore, 99.9% is water that needs to be removed. Additional challenge comes from the small size, as most microalgae are below 30 µm. Sedimentation is the easiest way of harvesting, but due to the negative surface charge of the algae, they tend to stay in dispersed stable algal suspensions and sediment slowly. Centrifugation is the most common way of harvesting algae, but with the disadvantage of high capital and operational costs. Another method of harvesting is filtration, which is best suited for larger cells.

After the first concentration step, the dry matter is between 2-20%, depending on the method. The residual water has to be removed via drying, which is a highly energy consuming process.

The energy input for the evaporation of water at 20°C and atmospheric pressure water is approximately 700 kWh/m³ (Milledge et al. 2013).

As PHB is an intracellular product, the cells have to be disrupted during the drying process in order to receive a pure product. Even when using organic solvents for extracting the PHB out of the dried biomass, cell disruption is favorable as it leads to a higher yield. The use of organic solvents like Chloroform is suited for lab scale, but difficult to realize in an industrial scale. Another common purification method for PHB is the use of sodium hypochlorite, which digests the biomass and leads to a relatively pure PHB. However, PHB may be also partly digested, leading to a smaller molecular weight (Madkour et al. 2013).

6. CONCLUSION

Bioplastic production with cyanobacteria is a highly complex process. Many aspects have to be taken into account, from the selection of the right location over the reactor design and the production strain to the harvest and purification of the final product. In order to serve a larger market with cyanobacterial PHB, the overall process should be economically feasible. To date, it is not possible to accomplish this challenging task. The reached PHB concentration of 12.5% of cell dry weight is within the highest reported, but still quite low. The concentration has to be significantly higher for an economic production. Cyanobacteria produce glycogen as the major storage compound and PHB as a second storage compound and as a supposed electron sink. As CO_2 is not fixed during the late stationary phase, while PHB concentration is still rising at the expense of the glycogen, a purely photoautotrophic PHB production is unrealistic. An external ripening tank to promote PHB production with organic substrates should be installed. Most research concerning cyanobacterial PHB is still performed in lab-scale under sterile conditions, which is far away from a real production plant. This work demonstrates a pilot-scale production of PHB with the cyanobacterium *Synechocystis* sp. CCALA192 and must be seen as another small step towards utilization of cyanobacteria for production of PHB or other molecules. The establishment of a stable culture under non-sterile conditions is difficult and special cultivation strategies have to be used to avoid growth of dangerous pests like *Colpoda steinii,* as demonstrated in this work. The ecosystem in microalgal cultivation is an underestimated, but highly important aspect.

LITERATURE

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I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly marked all material, which has been quoted either literally or by content from the used source.

Date…… Signature ……

Danke meiner lieben Familie!