Photo-autotrophic Production of Poly(hydroxyalkanoates) in Cyanobacteria

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In the last two decades, poly(hydroxyalkanoates) (PHA) were solely produced using heterotrophic bacteria in aerobic cultivation. With respect to the great potential (500 Mt yr^{-1}) of raw industrial CO_2 streams and even greater potential of flue gases, the focus on photo-autotrophic biotechnological processes is increasing steadily. Primarily, PHA-gene transfer from heterotrophic bacteria into algae and plant cells was attempted, with the intention to combine the known biosynthesis pathway with autotrophic cultivation. The natural occurrence of PHA in cyanobacteria is known at least since 1966. However, cyanobacteria were never considered for commercial production because the PHA amount based on cell mass and based on volumetric productivity is generally very low. Therefore, strain improvements were suggested, either by gene amplification or by suppression of biochemical pathways competing for the cell's acetate pool. In the late 1990s, the success of genetic modification was confirmed experimentally, elevating the cyanobacteria cell's PHA content. With additional optimization, PHB amounts up to 50 % w/w of biomass dry matter or up to about 2.4 g L^{-1} bioreactor volume could be produced within 11 days. Considering the land use for agriculture and the competition for plant biomass between food, feed, fuel and energy production, the binding of CO_2 in a biotechnological process using photo-autotrophic microorganisms may become a promising option.

Key words:
poly(hydroxyalkanoates), cyanobacteria, algae, phototrophic, CO_2 binding

Introduction

Replacing conventional by biodegradable plastics is claimed to be beneficial for the environment, especially when the drastic negative consequences of litter are considered. Such plastics are called biobased if the basic chemicals derive from organisms, preferably from autotrophic organisms, such as higher plants, algae or cyanobacteria.[1]

Established biotechnological processes convert carbohydrates by heterotrophic bacteria into the polymer storage substance poly(hydroxyalkanoates) (PHA). These PHAs, especially poly(hydroxybutyrate) (PHB) and poly(hydroxyvalerate) (PHV), belong to the most promising polymers replacing conventional plastic, as they are thermoplastic, hydrophobic and biodegradable at usual ambient conditions. Monomeric sugar is used as carbon and energy source for microbial growth. The sugars derive from plants, such as sugar cane or from corn (starch) or wood (cellulose) after hydrolysis. Process optimization and the use of carbon sources from industrial waste (e.g. food or slaughterhouse waste) have been reported, mainly for reduction of production costs.[2,3]

All those plants grow on agricultural or forestry areas, and the carbohydrates are highly demanded for food, feed, fuels, biobased materials and chemicals as well. Industry claims more and more biomass as their raw material responding to the rising demand for alternative production. However, even small and medium-scale solutions will occupy more agricultural land area as ethically reasonable. Unfortunately, there will not be enough biomass available when plants are harvested only from areas which could not be used for food and feed production.[4,5]

One of the possibilities to avoid ethical conflicts and dodge a disadvantageous development on the resources market is the production of biomass by photo-autotrophic microorganisms, such as algae and cyanobacteria. Closed photobioreactor types may be installed on building fronts and roofs or may cover traffic areas – if the local climate allows their operation.

PHA is a natural energy and carbon storage product of prokaryotes. That includes heterotrophic bacteria, such as *Cupriavidus sp.* and autotrophic bacteria such as cyanobacteria (aerobic) or purple bacteria (anaerobic).[6] For production in eukaryotes the responsible genes have to be transferred from bacteria to plants or algae cells. However, genetically modified plants face some critics, are widely al-

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most not accepted and, finally, are not the best option to ease the competition for agricultural land.

Cyanobacteria, as primary producers, once opened up the way for life as it is today, altering earth's atmosphere deeply due to their photosynthetic consumption of CO_2, leading to the production of oxygen gas.[7] Once again, they could play a role in helping to reduce the impact of CO_2 on earth´s climate. However, from an economic point of view, they have two major drawbacks: (1) their native PHA content is low, almost always below 10 % of cell biomass, in most cases even below 2 % and (2) genetic modification to achieve an essentially increased productivity has rarely been successful.

The following paragraphs give an overview of several aspects of PHA production with photo-autotrophic microorganisms, with special focus on cyanobacteria.

Fundamentals of PHA production in photo-autotrophic microorganisms

As for any other biotechnological process, PHA production with photo-autotrophic microorganisms demands short fermentation times, high volumetric productivity, simple handling, low risk of contaminations and low operating cost.

Cyanobacteria cope with most of these criteria. They became, especially in the period 1980 – 2001, the central research topic of many working groups worldwide (see Table 1). Strains and cultivation conditions were improved for significantly shorter fermentation times and impressive high PHA contents. More specifically, all authors report PHB to be the dominant, if not the only poly(hydroxyalkanoate) in cyanobacteria. Another storage product is the carbohydrate glycogen which is produced growth associated and in competition for the acetate pool with PHB synthesis.[8]

Other than glycogen, PHA in cyanobacteria seems to act as a buffering pathway catching excess Acetyl-CoA and reduction equivalents (NADH). Because of an interrupted tricarboxylic acid cycle, PHA cannot be utilized as good as glycogen.[9] The consequence for the biotechnological process with cyanobacteria is that during night-time and in case of temporally unfavourable fermentation conditions, the glycogen will be consumed first and PHA second for energy production.

Table 1 – *Reports of qualitative and quantitative PHA appearance in cyanobacteria. bal. = balanced (not starved) medium composition; lim. = nutrient limitation; n.spec. = not specified; n.q. = not quantified; bm = based on dry biomass. (Data modified and extended, from Asada[10]).*

Species	C source for PHA*)	Nutrients	Growth time (d)	PHA content (% bm)	Year of publication	Ref.
Chlorogloea fritschii	Acetate	bal.	8	n.q.	1966	[11]
Chlorogloea fritschii	CO_2	bal.	n.spec.	n.q.	1971	[12]
Chlorogloea fritschii	Acetate	bal.	8	10	1982	[13]
Spirulina platensis	CO_2	bal.	8	6	1982	[13]
Spirulina sp. (6 strains)	Acetate	bal.	7	0.3–0.7	1990	[14]
Spirulina maxima	CO_2	bal.	9	< 0.005	1992	[9]
Spirulina maxima	CO_2	N lim.	> 7	0.7	1992	[9]
Spirulina maxima	CO_2	P lim.	7–8	1.2	1992	[9]
Gloeothece sp.	Acetate	bal.		6	1992	[15]
Oscillatoria limosa	Acetate	bal.		6	1992	[15]
Gloeothece PCC6909	CO_2			n.q.	1995	[16]
Anabaena cylindrica	CO_2	bal.	21	< 0.005	1995	[17]
Anabaena cylindrica	Acetate	bal.	21	2	1995	[17]
Synechococcus MA19	CO_2	N lim.	8	21	1996	[18]
Synechococcus PCC7942	CO_2	N lim.	14	3	1998	[19]
Synechococcus PCC7942	Acetate	N lim.	14	25.6	1998	[19]
Synechococcus MA19	CO_2	P & N lim.	11	55–62	2001	[20]
Nostoc muscorum	Acetate	P & N lim.	4	up to 47	2007	[21]

*) Acetate was added as an inducer for the PHA-synthesis pathway, not as a carbon or energy source for biomass growth.

Table 2 – *Comparison of different autotrophic growth systems to a reference of heterotrophic growth, with regard to growth time and PHA content (bm = based on dry biomass)*

Organism	Species	C source	Growth time (d)	PHA content (% bm)	Ref.
		Heterotrophic growth			
Bacteria	*Cupriavidus necator*	Glucose	3	80	[22]
		Autotrophic growth			
Cyanobacteria	*Spirulina platensis*	CO_2	8	6	[13]
Cyanobacteria (GMO strain)	*Synechococcus* MA19	CO_2	11	62	[20]
Microalgae (eukaryotic, GMO strain)	*Phaeodactylum tricornutum*	CO_2	7	11	[23]
Higher plant	*Arabidopsis thaliana*	CO_2	-	14	[24]

For a comparison of reported PHA cell contents of cyanobacteria compared to other organisms see Table 2.

Key enzymes in PHB biosynthesis

On the example of the strain *Synechocystis* PCC 6803[25] the pathway can be drawn as given in Figure 1. Four enzymes are necessary to synthesize polymeric PHB from Acetyl-CoA. This general synthesis pathway does not differ much between hetero- and photo-autotrophic prokaryotes. However, in cyanobacteria, the four genes are located on two different positions in the genome (see Fig. 4) and are regulated by two different operons. Wang *et al.* identified *phaEC* to be the bottleneck in PHB synthesis and replaced the native genes with the more efficient thioesterase II (TesB) deriving from *E. coli.*[25]

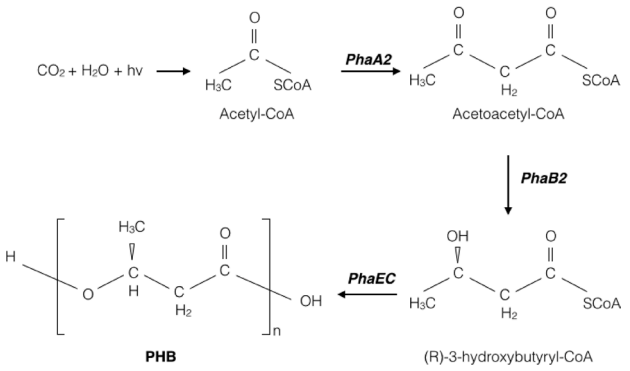

Fig. 1 – *PHB biosynthetic pathway from Acetyl-CoenzymeA, described for Synechocystis PCC 6803 by Wang (2013)*[25]

Potential of CO_2 as a substrate in biotechnological processes

For biotechnological growth of photo-autotrophic organisms, different carbon dioxide sources can be used. In nature, these organisms use the CO_2 present in ambient air. However, CO_2 concentrations are very low, which makes air less interesting for industrial processes. More interesting are higher concentrated CO_2 sources. In the following text, two different types of gases are focussed on: flue gases, which are exhaust gases from combustion processes (e.g. power plants), and "raw industrial CO_2 off-gases", which are gaseous by-products from industrial processes without direct combustion (e.g. fermentation).

Potential of utilising flue gases

Currently, the main drivers for utilising flue gases are reducing emissions by biological carbon capture and utilisation (bio CCU). In flue gases, CO_2 concentrations typically range from 3 to 15 %.[26] Cement plants show even higher CO_2 concentrations of 20 %.[26] The advantage of utilising flue gas is that it is very cheaply available and sources are located all over the world. In addition, the amounts available are endless. To give an example, 13,000 Mt yr^{-1} of CO_2 are emitted as flue gases from stationary sources (> 0.1 Mt yr^{-1} of CO_2) according to IPCC.[26] However, depending on the process, direct flue gas utilisation will need some conditioning like cooling, dust removal or removal of other incineration residues. In addition, legal issues can become challenging, as products derived from flue gases might be considered waste under current legislation.

Potential of utilising raw industrial CO_2 off-gases

From a mere biotechnological point of view, the utilisation of industrial off-gases with high CO_2 concentrations sounds as a very promising alternative. As carbon dioxide is considered waste in several industrial processes, it is attractive to reuse it as a raw material in industry. In addition, there seems to be a very high potential, about 507 Mt yr^{-1} of such off-gases are available worldwide (see Table 3), whereas only about 110 Mt yr^{-1} are already used as raw material in the chemical industry.[27] Thus,

Table 3 – *Worldwide overview of pure or high concentration CO_2 off-gases from industrial processes (adapted after Markl, 2014)*[28]

	Process	CO_2 (10^6 t yr^{-1})	Off-gas CO_2 concentration (%)	Source
Fermentative processes	Bioethanol	71.7	only minor impurities	Estimations based on Renewable Fuels Association[29]
	Breweries	6.9	only minor impurities	Estimations based on Barth Report[30]
	Biogas (off-gas from upgrading plants)	2.6	only minor impurities	Estimations based on list of biogas upgrading plants from IEA Bioenergy[31]
Technical processes	Natural gas processing	160.0	95–100 %	UNIDO[32]
	Ammonia production	239.4	30–100 %	UNIDO[32]
	Ethylene oxide production	6.3	95–100 %	UNIDO[32]
	Coal-to-liquids	20.0	30–100 %	UNIDO[32]
	Total	**506.9**		

78 % of these off-gases currently remain untapped. Table 3 gives a detailed overview of worldwide accumulation of such high concentration CO_2 off-gases.

In large-scale photo-autotrophic PHA production facilities, the transportation of such off-gases can become an issue, as they are less abundant than flue gases. This will lead, however, to increased overall costs. For this reason, while these CO_2-sources are still mostly untapped, first facilities should be built in the vicinity of the CO_2-sources, so that short pipelines can be used for gas transport.

Photo-autotrophic cultivation conditions

Chlorophyll is the most common pigment used by microorganisms for light absorption. Smaller variations in the molecule lead to several types of chlorophyll and bacteriochlorophyll, each with a small deviation in the absorbance spectrum as well. Secondary or accessorial pigments, such as carotenoids, phycobilins and rhodopsins complement the energy absorbance by utilizing the centre of the light spectrum.[33,34] What nature developed by evolution, namely specific light demands, favourable pH-values and salinity as well as low concentration of nutrients can be set for almost selective conditions in a biotechnological production with cyanobacteria.

Water usually acts as the electron donor in the aerobic photo-autotrophic metabolism, releasing oxygen during the illuminated growth phases. Instead of water, H_2S acts as electron donor in the anaerobic or anoxygenic photosynthesis, releasing sulphur. In dark periods, all photo-autotrophic microorganisms switch to a heterotrophic metabolism, aerobically consuming oxygen and always releasing carbon dioxide. Any biotechnological production must consider these general differences between light and dark phases. Practically speaking, an oxygenic photo-autotrophic fermentation needs constant flow of carbon dioxide and a constant dissipation of oxygen during the day (illumination) and the reverse must be provided during night (darkness). Production of high value biochemicals, such as carotenoids or vitamins, may justify the high energy demand for a 24 hour artificial illumination. However, low price mass chemicals, such as carbohydrates, lipids or PHA, should be produced by utilization of natural sunlight.

Light quantity and quality

Cyanobacteria generally need light within the photosynthetically active radiation (PAR) in order to operate photosynthesis, which converts light energy to chemical energy. PAR is considered as the wavelength range between 400 nm and 700 nm and is commonly quantified as photosynthetic photon flux (μmol photon m^{-2} s^{-1}) or expressed as energy unit irradiance (PAR)/(W m^{-2}). By extending the PAR band, *Acaryochloris marina* can even exploit the near-infrared light.[35] Given solar radiation, not more than about 9 % of the full spectrum energy can be converted into biomass.[36] However, considering only the PAR region, the maximal efficiency of photosynthesis is estimated to be 11.3 %.[37]

For satisfying biomass production, the light intensity has to be in a certain range. If the intensity is too low, light becomes a limiting factor, which is of course undesirable. A light intensity too high, on the other hand, can lead to photoinhibition. This generally means the cyanobacteria are no longer able to repair the photosystem II (PSII), which further leads to a loss in the activity of the oxygen-evolving complex.[38,39,40]

As the cells should not constantly remain in the dark, sufficient mixing is crucial. In a photobioreactor, this means a turbulent flow has to be maintained to allow the cells a continuous change between light and dark zone. The light/dark cycle time is defined as the sum of the time in the bright vs. the time in

the dark zone. This cycling not only prevents the organism from light starvation, but also allows the dark catalytic reactions of photosynthesis to complete, in order to restore the full capacity of the photosynthetic apparatus.[41,42] Kroon reports that photosynthetic efficiency is highest when the turnover rate of electrons in PSII is equal to the frequency of the change between light and dark.[43]

According to variations in the pigment composition, different wavelengths are absorbed with different efficiencies. Next to other factors, this composition is also dependent on nutrient status.[44] This has to be considered if PHA production is carried out under certain nutrient limitations. Mohsenpour *et al.* recently reported that, provided a well-mixed PBR, red light can promote the synthesis of phycobiliproteins as well as biomass production in *Gloeothece membranacea*.[45] However, research by Wyman *et al*. shows that the spectral influence on growth and biomass compositions depends much more on the organism used.[46]

From the aspect of an economically beneficial fermentation, sufficient biomass concentration has to be produced. However, there is a theoretical limit to the productivity of a mass culture, which is heavily determined by the average irradiance per cell, the mixing, the gas exchange, and the temperature. Eventually, the theoretical maximum growth rate is limited by the rate of photosynthesis. This rate has to be maximized by making sure that the culture is able to use all the light it is delivered. This is achieved either by intense mixing or by using special photobioreactor designs.[47]

Carbon source

All photo-autotrophic organisms use inorganic carbon as source, mostly in the form of one of the dissociated dissolved ions of CO_2.[9,10,11,12,13,15,16,17,18,19,20,21,48,49,50] The active membrane transport species is almost always hydrogen-carbonate (HCO_3^-).

Cyanobacteria fix carbon via the Calvin Cycle (also known as reductive pentose phosphate cycle or Calvin-Benson-Bassham cycle) driven by the energy gained from photosynthesis.[33,51]

As the enzyme ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO) happens to have a low affinity for CO_2 (50 % saturation at the normal atmospheric level of currently 390 ppm CO_2), cyanobacteria have mechanisms to increase the concentration of this enzyme, so-called CCMs (carbon-concentrating mechanisms).[38] They can actively take up and accumulate inorganic carbon from external sources, which gives them the ability to maintain photosynthesis under different carbon concentrations.[52] The use of carbon dioxide as a sole carbon source for microalgal production is not only cheap, but offers a way of utilizing the gas as a raw material instead of emitting it straight into the atmosphere.

Besides a carbon supply, which allows for unlimited photosynthesis, an economic use of CO_2 is important for large-scale production. The dosage has to be maintained in a way that, first, it does not affect the pH in an unfavourable way and, second, the carbon loss is kept to a minimum. Small bubbles and a long enough retention time are beneficial in order to dissolve the gaseous CO_2 completely. Measuring the pH and maintaining it at a defined level through CO_2 injection would be a probate strategy to control carbon supply.[53,54] It is pointed out by Behrens that this system only works well as long as there is nitrate uptake, thus, an alkalisation.[54] This can easily become a problem in N-limited cultures, as for example, during PHA production. It is suggested to use a CO_2 probe or a gaseous infrared carbon dioxide analyser instead. Rubio[53] and Sánchez[55] report about methods for the prediction of dissolved CO_2 in photobioreactors and the minimization of losses thereof.

It should be mentioned that many cyanobacteria could be cultivated under organo-heterotrophic conditions in the dark with similar growth rates compared to autotrophic conditions.[57] As it is mentioned by many authors (see Table 1), acetate can act as transcription inducer and as a carbon source for the metabolic chain of PHA biosynthesis. However, organo-heterotrophic cyanobacteria growth will not be described in this manuscript.

Nutrients

In many freshwater environments, phosphorus is often the limiting nutrient and therefore controls the abundance of natural cyanobacterial populations.[56,58] For mass development of cyanobacteria, typically less than 0.03 mg L^{-1} of P are required.[56] The fact is that, almost all media in the overview of Andersen[59], show higher P concentrations than necessary and much higher than common in nature. The BG-11 medium[60] for instance has 7 mg L^{-1} P. Cyanobacterial blooms, which regularly occur in eutrophic waters, lead to the assumption that they need high phosphorus and nitrogen (N) concentrations. In reality, cyanobacterial blooms often appear when concentrations of dissolved phosphate are low. In fact, many cyanobacteria show a higher affinity to both nitrogen and phosphorus, compared to other photosynthetic organisms and therefore can excel competitors under P and N limitation. Moreover, they can, like most other phytoplankton, store sufficient phosphorus (mostly as polyphosphate) which is enough for several cell divisions.[61] One possibility to increase PHA production is to limit the culture in phosphorus content, if at the same time acetate as carbon source is provided in excess.[62,63]

Nitrogen content in cyanobacteria can make up to 10 % of dry matter.[64] It is crucial for growth because a lack of N will cause less efficient light harvesting due to decreasing phycobiliprotein content.[65] Many species have the ability to fix atmospheric N_2 when dissolved nitrogen concentrations are low.[54] However, they can also lose this ability if maintained for a long time in media with a combined nitrogen source.[66] In any case, they preferably assimilate ammonium and favour utilizing combined nitrogen (e.g., nitrate) instead of fixing N_2. In fact, cyanobacteria do not assimilate other forms of nitrogen if ammonium (NH_4^+) is present.[67] The whole process of nitrogen uptake is strongly connected with photosynthesis and therefore also closely related to CO_2 fixation. These two processes compete for electron donors like ferredoxin and energy provided by photosynthesis.[68,61] If nitrogen is limited, carbohydrate or lipid reserves are built[64]. This is actually one of the main requirements for PHA production.[10]

Like with phosphorus, cyanobacteria are capable of storing nitrogen too. This is possible in the form of the accessory pigment phycocyanin and cyanophycin, a copolymer of aspartate and arginine.[61] The comprehensive list by Andersen[59] reveals that most of the common media are high in nitrogen. Nitrogen starvation can also lead to increased PHA production as reported by Wu[69] and Stal[70].

Cyanobacterial growth, according to Paerl[71], is also dependent on iron (Fe) and many trace metal micronutrients, such as Zn, Cu and Ni.

Because of N and P starvation combined with energy and carbon excess, the cells synthesize preferably storage products, such as carbohydrates and hydrocarbons. However, minimum intracellular nutrient levels have to be kept, sufficient for overall metabolic activity, which is required for PHA synthesis.[72] It can be seen from Fig. 2 that the biomass growth was dependent on the amount of nitrogen to a higher extent than the PHA production.

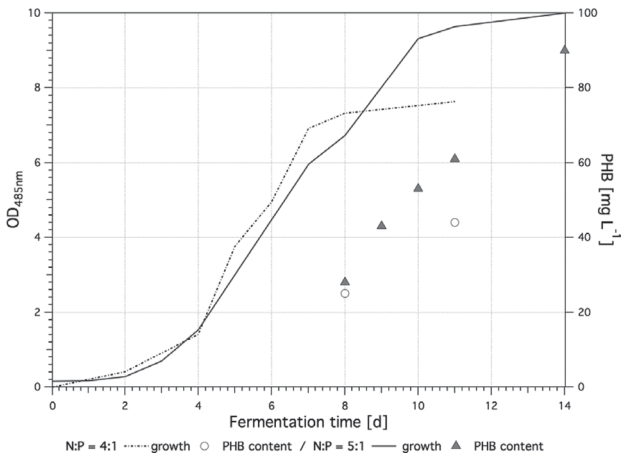

Fig. 2 – *Growth and dynamics of PHB production of Synechocystis cf. salina in a self-limiting medium in a one-stage batch process (physiologically a two-stage process, as PHB production does not start before depletion of nutrients)*

pH-Value

According to Brock,[73] cyanobacteria generally seem to be unable to grow at a pH lower than 4 to 5. In fact, most are alkalophiles having their growth optima between pH 7.5 to 10.[74] Although pH next to alkalinity and temperature influences the interspeciation of dissolved inorganic carbon, it has an effect on growth independently.[75] Overall, the optimal pH for maximal growth rate cannot be generalized as it varies from strain to strain and depends on their natural environment.

Temperature

As for pH, also for temperature no general optimum can be mentioned. Cyanobacteria growth is reported from cryophilic (+4 °C) up to thermophilic conditions (e.g. *Synechococcus lividus*, 75 °C).[76] Photosynthetic activity, without observable growth, was reported by De Vera[77] even at –30 °C. Miyake[18] and Nishioka[20] reported PHA production with *Synechococcus* MA19 at 50 °C, while almost all the other authors had done their cultivation experiments in the range between 20 and 30 °C.

Thermophilic conditions are beneficial because of increased metabolic turnover and because of a significantly reduced contamination risk. However, thermophilic cyanobacteria able to produce PHA are rare, and thermophilic production in a large-scale photobioreactor will cause very high effort for thermal insulation.

Two-stage cultivation

All the authors who reported increased PHA production under nitrogen or phosphorous starvation (see Table 1) performed a two-stage cultivation. At first, the cyanobacteria were grown for biomass production in a nutrient-rich medium, and were then transferred into a deficient medium to initiate the synthesis of PHA and other storage products.

Strictly following this principle in large-scale production will result in enormous efforts for the separation of cells from residual medium solution. In addition, this will apply some stress to the cells, such as shear forces and oxygen deficiency, and thus cause a new lag phase.

We tried to optimize a medium composition, providing enough nutrients in balanced composition for cyanobacteria growth up to a cell density of about 2 g L^{-1} based on dry matter (see Fig. 2). Between cultivation day 7 and 8, the optical density

reached a value of around 6–7, appearing almost black. At the same time, all dissolved nitrogen and phosphorous was consumed by the microorganisms initiating starvation and PHA production without medium transfer. Until cultivation day 14 about 90 mg L^{-1} PHA (accordingly 4.5 % w/w on cell dry mass) was produced by the genetically nonmodified strain *Synechocystis cf. salina* PCC6909 (CCALA #192).

Applying a physiologically two-stage process operationally in one-stage will improve biomass growth, accelerate the PHA synthesis, and reduce the overall production cost (Fig. 2).

Auto-selectivity and contaminations

Auto-selectivity, a combination of cultivation conditions favourable for the intended strain and unfavourable for all potential contaminants, is a serious goal for all biotechnological processes. For cyanobacteria, this selectivity can be achieved by setting several parameters simultaneously: the lack of dissolved organic carbon, limiting concentrations of nitrogen and phosphorous, and a pH-value at or above 8.5. In our own experiments we, nevertheless, observed repeatedly some growth of green algae (*Chlorella* sp.).

As the culture reaches its stationary phase, cells will die and release their content. This may be a carbon and energy source for heterotrophic contaminants, making long time running batch processes critical.

Photo-autotrophic cultivation systems

Generally, there are two types of systems to cultivate photo-autotrophic microorganisms at larger scales: open and closed systems (see Fig. 3). Advantages and disadvantages of the different systems are illustrated in Table 4.

Table 4 – *Advantages and disadvantages of open and closed systems for photo-autotrophic cultivation (for the evaluation "+" indicates a favourable, and "–" an unfavourable parameter)*

	Open systems	Closed vessels
Investment costs	+ +	– –
Cell concentration	–	+
Maintenance/Handling	+	–
Problems with contaminations	– –	+
Control of conditions	–	+ +
Accuracy of CO_2-dosing	–	+ +

Fig. 3 – *Cultivation systems for photo-autotrophic microorganisms: open cascade system (top – © Jiri Kopecky, Institute of Microbiology), closed tubular photobioreactor at pilot-scale (bottom – © Katharina Meixner, IFA Tulln/University of Natural Resources and Life Sciences)*

Cultivation in open cultivation systems

According to Pulz,[78] open cultivation systems can be divided into open vessels, natural water, cascade systems (see Fig. 3), and raceway ponds. The latter is the most applied open cultivation system. These open systems have some serious drawbacks, such as the lack of good monitoring and control possibilities for parameters like pH, temperature, mixing, and light availability. Sparged CO_2 has a very short residence time, resulting in both high losses and bad solubility. Seasonal variations as well make it nearly impossible to produce replicable data. Other disadvantages are high water losses due to evaporation and a major risk of contamination by predators and other fast growing autotrophs, which can lead to either poor productivity or a total loss of the desired production strain.[37,38,78,79]

Cultivation in closed vessels

Among the closed systems, there exist several types of photobioreactors. These are tubular reactors (Fig. 3), laminar (or flat panel) reactors, hanging plastic sleeves or fermenter-like tank reactors.[78]

The latter have to be artificially illuminated, while the others are, mostly, operated without artificial illumination. Although closed systems are more complex, they offer a better control of crucial parameters, and at the same time contamination becomes less likely.[40] The main challenges in any photobioreactor system are: light availability, CO_2 introduction, O_2 removal and sufficient mixing. Despite all the advantages, it has to be stated clearly that closed systems are much more expensive than open pond systems, provided that the ground area is cheaply available.

Cultivating and processing PHA-producing cyanobacteria

As photo-autotrophic PHA-production is still in the research phase, no experiences of optimal cultivation systems at industrial-scale have been made. In general, closed systems are definitely to be preferred. However, in order to save considerable investment costs, it might also be possible to use open cultivation systems in an auto-selective process like, for example, with halophilic cyanobacteria. With regard to productivity, it should be considered that biomass concentrations in photo-autotrophic systems are generally low. Typical concentrations are between 0.3 and 8.0 g L^{-1} according to Pulz,[78] which is much lower than in heterotrophic systems where biomass concentrations of 30–221 g L^{-1} can be achieved in fed-batch systems.[80] Such low cell densities in photo-autotrophic processes have two main drawbacks: high investment costs for cultivation systems, and high effort in downstream processing.

Downstream processing and product quality

PHA production by cyanobacteria has yet to reach the commercial production scale. No specific data for the efforts and cost of downstream processing is available. As cyanobacteria are Gram negative bacteria, it can be assumed that their isolation and purification will not be much different from the isolation and purification of PHA from other Gram negative heterotrophic bacteria when considering the generally lower content in the cell. Some experiments have been done in our institute in laboratory scale, demonstrating the general possibility of PHA isolation and purification by solvent extraction from dry or wet biomass. No new techniques were needed.

Impurities not found in other microbial PHA are colour, most probably residues and breakdown products from chlorophyll. It is too early to specify qualities of the isolated PHA and quantities of impurities or to predict the influence of pigment residues on injection molding or on the usability of products.

Recombinant cyanobacteria for PHA production

Currently, intensive research is aimed at producing high amounts of polyesters through recombinant microbial cell factories able to accumulate polymers intracellularly in the form of storage granules. The utilization of recombinant bacteria harbouring heterologous PHA biosynthetic genes permits achieving a high level of sub-cellular PHA accumulation, up to 80 % of bacterial dry cell weight.[81] Despite the advantage of high productivity, the process has elevated costs, which represent a commercial problem. On the basis of this evidence, many studies have focused on the utilization of photosynthetic and heterotrophic organisms.

Microorganisms with modified promoters or modified genes are GMOs of safety classes L1 or L2. Cultivation and manipulation has to follow national rules, which includes safety precautions, such as tight reactor, security gates, sanitation of waste, sanitation of biomass. All these precautions and installations increase investment or operating costs.

In comparison to algae and plants, cyanobacteria are easier to genetically manipulate[82] either *in cis*, through chromosome modification, or *in trans*, through plasmid introduction[82]. Some cyanobacteria, e.g. *Gloeocapsa* sp., *Spirulina platensis*, *Aphanotece* sp., *Oscillatoria limosa*, *Anabaena cylindrica*, *Synechococcus* sp., *Synechocystis* sp., *Nostoc muscorum* (Table 1), naturally possess genes for PHA biosynthesis. In the model cyanobacterium *Synechocystis* sp. PCC6803, PHA production is modulated by the choice of carbon and nitrogen sources, and PHA content of 10 % of the dry cell weight can be achieved.[83] The natural capability of polymer production in an unmodified strain inspires the construction of an optimal recombinant PHA producer. In the 1970s, the identification of *Cupriavidus necator* as bacterium able to produce high-molecular weight of P(3HB) allowed the identification of PHA biosynthetic operon.

Synechococcus PCC7942 (Table 1) represents the first example of recombinant cyanobacteria for PHA production. The introduction of *C. necator* PHA operon in *Synechococcus* PCC7942 conferred to the cyanobacterium the capability to improve PHA accumulation within the cell, from 3 up to 25 % of the dry cell weight.[84] In contrast, PHAs are naturally produced in *Synechocystis* PCC6803, whose entire genome was sequenced in 1996[85] allowing the recognition of four PHA_{Syn} genes in two distinct loci. Such genes are clusterized two by two, respectively forming the *phaA*-B_{Syn} cluster and the *phaE*-C_{Syn} cluster (Fig. 4). The *phaA* (slr1993) and the *phaB* (slr1994) genes are co-linear, putatively co-expressed and encode for the PHA-specific β-ketothiolase and the acetoacetyl-coenzyme A reductase, respectively. The

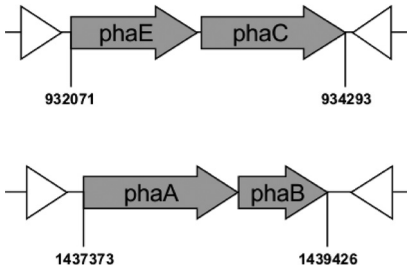

Fig. 4 – *PHA genes location in Synechocystis sp. PCC6803 genome*

phaE-C$_{Syn}$ operon, harbouring the *phaE* (slr1829) and the *phaC* (slr1830) genes, encodes for the putative poly(3-hydroxyalkanoate) synthase component and the poly(3-hydroxyalkanoate) synthase.[83] Plausibly, the latter form a protein complex involved in the modulation of PHA polymers.

The consistent amount of available genome data described the first complete PHA biosynthesis pathway known in cyanobacteria and opened the possibility to genetically manipulate *Synechocystis* sp. PCC6803 in order to improve the PHA intracellular accumulation. The potential of *Synechocystis* as a model for PHA genetic engineering was demonstrated by the inactivation of PHA synthase through the disruption of *phaE-C*$_{Syn}$ cluster using a PCR-based gene disruption method.[23] This study allowed the identification and characterization of other genes involved in the PHA synthesis. Considering that *Synechocystis* harbours from six to ten copies of its genome, the latter method permits the replacement of all the genome target genes, increasing the possibility to genetically manipulate this organism, also if the mechanism of total replacement is still unknown.[87]

One of the most difficult aspects for the generation of PHA recombinant cyanobacteria *in trans* was the plasmid stability, often replaced by a chromosome integration strategy. An antibiotic-free method developed in 2011 implies a complementation strategy based on the presence of *Escherichia coli recA* gene able to complement the *Synechocystis recA* null mutant on the plasmid carrying the PHA operon. This transgenic strain accumulated PHA up to 52 % of its dry weight under nitrogen limitation conditions, which is among the highest amounts reported for cyanobacteria.[88]

The high-CO_2 response mechanisms described in microalgae like *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp. and *Chlorococcum* sp. initiated an increasing interest in the CO_2-fixing conserved enzyme RuBisCO (EC 4.1.1.39) involved in the Calvin-Benson cycle.[89] In the cyanobacterium *Synechococcus* PCC7002 RuBisCO gene (*rbc*) possesses a strong promoter inducible by the CO_2 concentration. This promoter has been recently used successfully as a regulating sequence for the PHA genes´ expression and production in *Synechocystis* sp. PCC6803.[90] In this sense, the PHA production in cyanobacteria assumes a promising meaning for the industrial conversion of CO_2 in useful biomaterial without the high costs applied for PHA production in bacteria strains. In general, the continuous development of genetic strategies applicable to cyanobacteria and the increasing number of available genome data, e.g. genome sequencing and RNA-seq transcriptome analysis in *Synechocystis* sp., makes these organisms suitable as efficient industrial cell factories in the conversion of waste materials to useful industrial biomaterials. Future work will be aimed at optimizing as much as possible the polymers production potential of cyanobacteria factories by metabolic engineering and genetic modifications.

Recombinant eukaryotic microalgae for PHA production

Microalgae represent a great potential, especially as new expression systems applied for industrial and therapeutic purposes, as well as for the synthesis of biotechnologically relevant polymers such as PHA and PHB. Similar to cyanobacteria, algae possess a high-growth rate and are easy to handle and cultivate. Recently, Hempel and coworkers described the first process of PHA biosynthesis in the diatom *Phaeodactylum tricornutum*, where the *C. necator* PHA operon was introduced and expressed in the cytosol, achieving a PHA production of 10.6 % of algal dry weight only after 7 days.[23] This example encourages the idea that PHA/PHB production is possible in microalgae, with an expression level 100-fold higher than in plants, due to their capability of accumulating huge amounts of omega-3-fatty acids. In comparison to cyanobacteria, the transformation method for algae is less developed, making it difficult to carry out genetic manipulation and create a recombinant strain. Until now, some methods for chloroplasts and nucleus stable transformation have been developed, e.g. particle bombardment, electroporation, *A. tumefaciens*-mediated transformation. DNA-coated metal particle bombardment has proven to be the most efficient method for algae transformation.[91] Same as with cyanobacteria, microalgae also requires genetic modifications by classical mutagenesis together with metabolic engineering in order to achieve significant PHA amounts.

Concerning the law, the practice of GM-algae production systems is limited, especially in open environments (e.g. ponds) where the introduction can be considered intentional. What should always be considered are the adverse effects that GM-algae can have on the environment, especially regarding the competition of recombinant algae and wild

types, their spreading and the time of their stability. In this sense, the transgene inserted achieves the main importance because it can confer an environmental advantage for the survival, e.g. GM-algae producing antimicrobial peptides. Interesting is the increasing use of marine GM-microalgae due to their inability to contaminate lands and fresh water. The main problem is that they should be domesticated by developing useful genetic tools. In this sense, the application of recombinant algae to produce bio-chemicals, bio-products and bio-fuel is an expanding field with high expectations. It is always important to establish the strain identity because some algal and cyanobacteria species produce toxins impacting humans and environment. [92,93,94]

Conclusions and perspectives

Industry is starting to show more and more interest in the use of CO_2 as a raw material for their processes. Societal and legal drivers are also going in this direction, as direct competition with food crops can be avoided. Nevertheless, industrial photo-autotrophic production of PHA has yet far to go, as the process optimisation is still going on. There is an immense potential for the use of waste carbon dioxide either from flue gases or raw industrial CO_2 off-gases. Several challenges remain to establish an economically viable process:

– Elevated PHA content in the photo-autotrophic cells

– Increased cell density in the photobioreactor

– Scale-up of photobioreactors by innovative design

– Closed loop of nutrients and water (optimized downstream processing)

– Definition of quality criteria for CO_2 containing off-gases

– Improved CO_2-uptake dynamics

Currently achieved PHA concentrations are very low, so that strain improvement of cyanobacteria is intensively elaborated on, where up to a ten-fold increase of PHA yields is in reach. Gene amplification and gene transfer are the most promising techniques to achieve this goal. Unfortunately, cell densities in photo-autotrophic cultivation are at least by a factor of 10 (more probably by a factor of 20) lower than in heterotrophic cultivation. With the same factor, the volumetric productivity is lower and the investment costs higher. The higher the volume, the higher is the effort for downstream processing. Volumes of open ponds cannot be increased infinitely, the same as tube length or tube diameters in closed photobioreactors. CO_2 saturation and degassing is the limiting factor for all closed reactors. Reactor design needs to be optimised for energy efficiency, light utilisation, flow management, mixing, and distribution of cells and nutrients. The whole production plant needs to be designed for water and nutrient recycling, avoiding the need of finite resources. For example, coupling the photo-autotrophic growth with anaerobic digestion is a viable option. It seems to be obvious that acidic flue gas contents, such as SO_2 or NO_x and toxic heavy metals are disadvantageous for photo-autotrophic microorganisms and need to be removed. Not so clear is the influence of solid particles (dust) or microbial contaminants (from biotechnological sources) on the performance and stability of a photo-autotrophic process. Depending on the CO_2-absorption kinetics, the growth of many photo-autotrophic bacteria will be dependent on the CO_2 content in the source gas.

Now, PHA is successfully produced by heterotrophic fermentation and this will continue for the next decade. We believe in the success of PHA production by photo-autotrophic biotechnological processes in the long run.

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