Production of polymers by cyanobacteria grown in wastewater: current status, challenges and future perspectives

Dulce María Arias[1*], Joan García[1] and Enrica Uggetti[1]

[1] GEMMA - Environmental Engineering and Microbiology Research Group, Department of Hydraulic, Maritime and Environmental Engineering, Polytechnic University of Catalonia - BarcelonaTech, c/ Jordi Girona 1-3, Building D1, E-08034, Barcelona, Spain.

* Corresponding author:

Tel.: +34 934015952

Fax: +34 934017357

E-mail address: dulce.maria.arias@upc.edu

Abstract

Cyanobacteria are prokaryotic oxygenic phototrophs receiving attention in a wide variety of technological applications such as food and feed supplements, and production of valuable polymers. Among these, carbohydrates (e.g. glycogen) and polyhydroxyalkanoates (PHAs) are of increasing interest due to their potential as a biofuel substrate and bioplastics, respectively. However, biofuels and bioplastics from cyanobacteria have seen many years of effort towards commercialization with only limited success. Their main limitation for polymer production is the high cost of the nutrient source; wastewater, as an inexpensive and widely available alternative, may overcome this bottleneck. Though cyanobacteria have demonstrated a capacity to treat wastewater effluents, their cultivation in such a variable environment involves certain challenges of which the chief one is linked to contamination by other species, especially green algae. This would represent a serious drawback during cyanobacterial biomass production and affects further PHA and carbohydrate production. The present study reviews the potential of cyanobacteria to grow in wastewater effluents from different sources. Conditions favoring them in mixed-culture reactors are described, focusing on nutritional and operational aspects. Current advances and future prospects in PHA and carbohydrate production are explored and discussed.

Keywords: Biofuels, Bioplastics, Mixed cultures, Microalgae, Wastewater treatment.

Introduction

Cyanobacteria, also known as blue-green algae, have existed for more than 3.5 billion years. They were the first organisms to produce molecular oxygen, and thus contributed to changing the biosphere from anaerobic to largely aerobic conditions [1]. They are by far the largest group of photosynthetic prokaryotic organisms as judged by their widespread occurrence, frequency, abundance and morphological diversity [2,3]. Moreover, when growth conditions are not suitable, some species can survive adverse conditions for long periods [4]. In general, efficient adaptation of cyanobacteria to almost any environment implies that their cultivation does not require energy-rich compounds as do other non-photosynthetic microorganisms [5]. Simple nutrient requirements give them potential to create low cost, eco-friendly technologies for wastewater treatment as well as generation of bioproducts [5]. The capacity of cyanobacteria to grow in different effluents such as municipal and industrial wastewater has been already documented [6,7]. The strategy of growing cyanobacteria in wastewaters offers a second advantage as wastewater is treated to produce clean - or at least cleaner - water, in addition to economically valuable byproducts [6,8]. Indeed, they are known to store polymeric compounds such as polyhydroxyalkanoates (PHAs) and carbohydrates under certain nutrient deprived conditions [9]. PHA and carbohydrate production from cyanobacteria has received considerable attention because of their potential uses as substrates for bioplastics and biofuels production, respectively.

Commercialization of cyanobacteria bioplastics and biofuels has met with limited success [10,11], the main bottleneck being the high cost of nutrients. The use of wastewater as feedstock may overcome this bottleneck by offering an inexpensive and readily available substrate. Although cyanobacteria have demonstrated their capacity to treat a range of wastewater effluents, cultivation in variable environments such as many wastewater streams involves certain challenges of which the main one is contamination by other species, especially by green algae [12]. This could be seriously detrimental to cyanobacterial biomass production and affect further PHA and carbohydrate production.

This review considers the characteristics of cyanobacteria, their potential to grow in wastewater effluents, and their use for polymer production. A critical analysis of the conditions favoring cyanobacteria in mixed-culture reactors is provided, as well as a description of current advances and future prospects in polymer production from mixed cyanobacterial cultures.

Overview of cyanobacteria

Cyanobacteria are prokaryotic oxygenic phototrophs found in almost every habitat [13]. They constitute one of the largest groups of Gram-negative prokaryotes with a wide diversity in morphology, physiology, cell division patterns, cell differentiation and habitats (**Figure 1**) [14]. Due to their high protein and vitamin content, and good digestibility, cyanobacteria are used commercially for a variety of purposes including food and feed supplements as well as many other applications [13,15–17]. Since cyanobacteria are naturally transformable, they can be genetically manipulated relatively easily, leading to successful applications in genetic engineering [18,19], including production of a number of value-added compounds at high rates at laboratory scale (*e.g.* ethanol, isobutyraldehyde, isobutanol, 1-butanol, isoprene, ethylene, hexoses, cellulose, mannitol, lactic acid, fatty acids) [15,20]. Recent studies have demonstrated that cyanobacteria form ideal consortia with chemotrophic bacteria and can effectively be used to clean oil-contaminated sediments and wastewaters [13]. In these processes, wastewater streams have been frequently used as a readily available and low-cost substrate for growth, biomass production and nutrient removal and have been recognized as the only economic and sustainable source of nutrients for cyanobacteria biomass cultivation [21].

Cyanobacteria cultivation in wastewater treatments systems

The use of photosynthetic microorganisms (eukaryotic microalgae as well as cyanobacteria) for wastewater treatment was first proposed by Oswald and Golueke [22]. Through the use of open raceway ponds in California for municipal wastewater treatment, cyanobacteria and eukaryotic microalgae were grown in association with aerobic heterotrophic bacteria [13,23]. In this process, photosynthetic microorganisms produce molecular oxygen to be used as an electron acceptor by bacteria degrading organic matter. In return, bacteria release CO_2 during the mineralization process, providing substrate for photosynthesis [13]. Cyanobacteria can be used in wastewater treatment for a range of purposes such as the removal of nutrients, the reduction of both Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD) and also for the removal of heavy metals [24–26]. This kind of wastewater treatment is regarded as an economical and environmentally friendly process with no secondary pollution as long as the biomass produced can be reused and allows efficient nutrient recycling [21,26]. Technologies based on cyanobacteria have been successfully applied to the treatment of municipal, livestock and industrial wastewaters [27]. Efficiencies found are often superior to those achieved in conventional aerobic activated sludge processes in terms of nutrient assimilation and pathogen removal, with much lower energy demands [28]. However, most of the studies have been performed at laboratory scale and with monocultures. **Table 1** shows how cyanobacteria cultures can be successfully applied for wastewater treatment purposes. The comparison of all these results is only indicative, since they have been carried out under different operating conditions.

Municipal wastewater

Removal of N and P has been the main objective of cyanobacteria cultures for municipal wastewater systems, since nutrient enrichment and consequent eutrophication can alter the structure and function of aquatic ecosystems, potentially endangering health, biodiversity and ecosystem sustainability [29]. Cyanobacteria are used for this purpose since they can assimilate different forms of N and P for growth (NH_4^+, NO_2^-, NO_3^- and PO_4^{3-}) [24]. Different species of cyanobacteria, mostly filamentous such as *Phormidium sp*. [30] or *Spirulina platensis* [31] have been successfully cultivated using municipal wastewater, not only for the removal of N and P (>60%), but also for the reduction of COD and BOD (>80%) indicating the presence of heterotrophic bacteria in the culture (Table 1).

Industrial wastewater

The diversity and dominance of cyanobacterial species in different aquatic polluted bodies demonstrate the tolerance of this group to a wide variety of contaminants [32]. Through several studies, cyanobacteria have demonstrated their ability to biodegrade and biosorb persistent chlorinated hydrocarbons and heavy metals, respectively [33,34]. Filamentous *Oscillatoria brevis*, *Westiellopsis prolifica, Anabaena sp.* and *Nostoc muscorum* have been shown encouraging results in treatment of several industrial effluents, including from cheese factory [35], oil, soap and fodder industry [36], dye industry [37], distillery [38], and aquaculture [39], reducing nutrients and organic matter by 50-100% (Table 1). Other studies also include the biodegradation of phenol and dichloroacetate from pulp-and-paper wastewater (up to c. 100%) [40] and the removal of copper (75%), cobalt (11.8%), lead (100%) and manganese (61.5%) from mixed domestic-industrial effluent [34]. These studies have been only performed at laboratory scale.

Secondary effluents

Tertiary wastewater processes aim at removing NH_4^+, NO_3^- and PO_4^{3-} from secondary effluents, and generally are more expensive than secondary treatments. Cyanobacteria cultures could offer an efficient solution for tertiary treatment due to their ability to use remaining nutrients from previous wastewater treatments due to their low nutrient requirements. The utilization of cyanobacteria has been documented for tertiary treatment of different secondary effluents by filamentous species as *Oscillatoria* sp. [12], *Phormidium* sp. [35] and *Spirulina maxima* [41] and mixed cultures dominated by cyanobacteria *Aphanocapsa* sp. and *Phormidium* sp. [42] (Table 1).

Digestates

Recent research has been focused on the use of the liquid part of anaerobic digestion effluents for microalgal and cyanobacterial growth. Due to the characteristics of digestate, which is rich in NH_3, PO_4^{3-} and acids, it is a potential substrate for cyanobacteria requirements [43,44]. Several species of microalgae and cyanobacteria can grow on diluted and undiluted digestates from various sources, including swine slurry [43], municipal organic waste [45], abattoir digestate [46], swine manure [41,47], poultry manure [48] and microalgal anaerobic digestate [49,50]. Thus, recycling nutrients from digestates could also be an economic and environmentally friendly option to growing cyanobacterial biomass for nonfood byproducts. These studies demonstrated the successful application of cyanobacteria to treat wastewater effluents; however, the critical challenge concerning the maintenance of a culture dominated by the same species of cyanobacteria during the entire cultivation period was not described.

Selection and maintenance of cyanobacteria-dominated cultures in wastewater treatment systems

Cyanobacteria cultivation in such a variable medium implies competition with other microorganisms, especially green microalgae. Thus, a challenge facing cyanobacteria cultivation in laboratory and large-scale open (e.g., high rate algal ponds (HRAP)) or closed (e.g., photobioreactors (PBR)) systems is maintaining the dominance of these microorganisms throughout long periods. Dominance of a single species typically occurs when it is able to adapt to certain environmental conditions better than other species [51]. Most of the studies on cyanobacteria interactions and dominance have occurred in natural ecosystems such as lakes and rivers and growth has been related to complex interactions among biotic (competition for resources and selective predation by zooplankton grazers) [52] and abiotic factors (e.g. light intensity, temperature, turbulence, pH and nutrients [52–56]). Some cyanobacteria are able to survive and compete across a wide range of water temperature, light, oxygen, pH and nutritional conditions [52,56–59]. Most studies agree that the nitrogen and phosphorus ratio (N:P), and absolute concentration levels are the two key factors determining competition capacity of cyanobacteria [53,56,60–65]. It is reasonable to assume that cyanobacteria behave in a similar way in open and closed wastewater treatment systems carried out in HRAP and PBR. Feasibility of controlling dominant cyanobacteria or microalgae in either system is limited to a few studies focused on the relationship between nutrient conditions in wastewater [42,50] and the factors controlling the competitive relationships in these systems are poorly understood [66,67].

From the first studies in open pond systems treating wastewater and in contaminated aquatic ecosystems, an enormous diversity of microalgae, cyanobacteria, protozoa and small metazoan has been identified [68]. Cyanobacteria *Oscillatoria* sp. and *Phormidium* sp., together with some species of green algae (*Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp., *Stigeoclonium* sp., the diatom *Nitzschia* sp., and the protist *Euglena* sp.) were found to be the photosynthetic taxa most tolerant to polluted environments [68]. Starting from these first studies, small and large-scale wastewater treatment systems were inoculated with pure or mixed cultures of these photosynthetic microorganisms. For cyanobacteria monocultures, many species, especially filamentous ones, were cultivated on batch laboratory scale [30,64,69]. Despite being a relevant issue, the contamination by other microorganisms in the culture was not mentioned.

Several studies using wastewater-borne mixed cultures in open and closed systems have been performed in order to treating wastewater from different sources. The type of influent, total nutrient concentration and operation of the system have defined the dominance of green algae or cyanobacteria in the culture, as seen in **Table 2**. Important environmental conditions that may affect cyanobacteria dominance can be found below.

Role of nitrogen and phosphorus

Filamentous cyanobacteria *Pseudanabaena* sp. and colonial *Aphanocapsa* sp. are the genera most frequently dominant in waste streams, out-competing green algae genera such as *Chlorella* sp. and *Stigeoclonium* sp. These cyanobacteria genera grow in different types of wastewater, and tolerate a wide range of nutrient concentrations. A 50% dominance of *Pseudanabaena* sp. was achieved in open systems with high N and P concentrations in the mixed liquor (Total Inorganic Nitrogen (TIN) 100 mg L^{-1}, Total Inorganic Phosphorus (TIP) 14.5 mg L^{-1}) [73], while the same organisms were observed at lower N and P concentrations (TIN 12.88 mg L^{-1}, TIP 0.90 mg L^{-1}) [52]. Similarly, in the case of *Aphanocapsa* sp., studies found this organism to be dominant in wastewaters exhibiting TIN and TIP concentrations of 4.12-13.82 and 0.20-0.89 mg L^{-1}, respectively [52,74], while *Aphanocapsa* was reported dominant in systems exhibiting TIN and TIP values of 2.99 and 2.17 mg L^{-1}[44]. One remarkable characteristic in these studies was that the N:P ratios in selected wastewaters were all higher than 32:1, despite the wide range in concentrations. The only exception to this was [42]. In general, the N:P ratio favoring dominance of *Pseudanabaena* and *Aphanocapsa* was more than twice the Redfield ratio (inorganic N:P molar ratio of 16:1), considered the standard for microalgal growth [42]. These studies suggest that a very low phosphorus concentration or phosphorus limitation with respect to nitrogen is an important factor determining the dominance of cyanobacteria in wastewater systems. This ability to out-compete other microalgae under P limitation may be related to their higher affinity for phosphorus than many other photosynthetic organisms [70,71], attributed to their capacity for luxury uptake by storage as polyphosphate [61,72].

Role of operational conditions

While nutrients play a crucial role in species dominance, this factor cannot be considered the only aspect favoring the competition of cyanobacteria. Some systems studied have noted absence or low abundance of cyanobacteria in environments of high N:P ratios (Table 2) [52,80-82]. This indicates that other factors influence the proportion of cyanobacteria to green algae. Many types of operating conditions, such as hydraulic regimes (continuous, sequencing batch, semi-continuous), solids retention time (SRT), hydraulic retention time (HRT), organic loadings, incorporation of a settling period and recirculation of biomass all may have substantial impacts on microbial dynamics.

Hydraulic regimes (continuous, sequencing batch, semi-continuous)

In general, closed photobioreactors are considered the most appropriate systems for effective species control because open systems are more susceptible to changes of environmental conditions (pH, temperature). Dominance of cyanobacteria has been mainly obtained in closed systems (Table 2). *Pseudanabaena* sp. dominance was achieved in HRAP only in the reports of [73] and [74], probably as a result of operations being based on recirculation and in sequencing batch modes, respectively.

Continuous and semi-continuous feeding are the most conventional hydraulic regimes in closed and open systems. Continuous feeding is used in HRAP at full scale for practical reasons [75] and consists of constant feeding of the reactor over time (usually with variable flows and concentrations) (**Figure 2**a). This usually promotes the growth and dominance of green algae (*Chlorella* sp., *Stigeoclonium* sp.), and usually results in strongly seasonal microbial variability [76,77]. Such changing conditions make it difficult to select preferred species (e.g. for harvest). In contrast, photobioreactors operated in a semi-continuous feeding mode, with a single feeding per day (Figure 2b), result in an environment which is more favorable for microorganisms such as cyanobacteria which possess a higher affinity for nutrients compared to many other photosynthetic organisms [70,71]. Dominance of cyanobacteria under this type of operation has been achieved in [50].

Another less conventional regime in green algae/cyanobacteria-based wastewater treatment systems is the sequencing batch operation, successfully used for activated sludge systems [78,22,79] and implemented in both HRAP and PBR [42,74] with green algae/cyanobacteria cultures. It consists of a single feeding per day in conjunction with uncoupled SRT and HRT. The process begins as a volume of mixed liquor is discharged from the reactor, followed by a settling period and removal of supernatant (Figure 2c). Such operation provides several advantages over semi-continuous operation since nutrient loads can be easily controlled while maintaining SRT adequate for specific species. The settling period encourages the formation of flocs with faster settleability, while unsettleable cells are removed with the supernatant [80]. In contrast to continuous and semi-continuous hydraulic regimes, which do not promote extensive spontaneous flocculation, this approach can facilitate subsequent harvesting. Thus, it presents a logistical advantage by favoring microorganisms such as cyanobacteria that form spontaneous aggregates [81,82], while removing non-settling green algae (*Chlorella* sp., *Scenedesmus* sp., *Chlamydomonas* sp. *Desmodesmus* sp., *Micractinium* sp.)with poor settling characteristics [74] (**Figure 3**).

- *Other operational factors*

An important factor also affecting cyanobacteria selection is SRT. In general, SRT greater than 10 days are adequate for cyanobacterial cultures mainly composed of filamentous species such as *Phormidium* sp. and *Pseudanabaena* sp. [50,67,81,83–85]. SRTs of less than 8 days promote species of green algae such as *Scenedesmus* sp. *Chlorella* sp. and *Stigeoclonium* sp**.** [86–88]. Another operational process influencing microbial dominance is the recirculation of material from settler tanks (e.g. used for harvesting) to reactors to maintain biomass production and species composition in the reactors (Figure 2d). Studies of this type of operation report only improvement in selection and maintenance of green algae [75,86,89,90]; it fails when applied to cyanobacteria maintenance in HRAP [91]. The dominance of the cyanobacteria *Oscillatoria* sp. (90%) was maintained in a closed PBR for 90 days under a recirculation flow rate of 4.2 L min^{-2} min^{-1} and 10 days SRT [50]; *Oscillatoria* dominance declined when the recirculation flow increased and the SRT decreased.

Polymer accumulation in cyanobacteria

Cyanobacteria have received attention as a rich source of bioactive compounds and have been considered as one of the most promising groups of organisms producing them [13]. The use of waste streams as sources of nutrients reduces the range of biomass production applications due to possible contamination by various pollutants present in wastewater [8]. Thus, cyanobacteria grown in wastewater should be used mainly for production of non-food applications, e.g. bioenergy, biofuels and bioplastics [64]. Interest in cyanobacteria cultivation has focused on the production of PHAs and carbohydrates, used as a bioplastic and a biofuel substrate respectively. Cyanobacteria producing both polymers have been mainly studied in pure cultures, but recent investigations have focused on the utilization of mixed non-sterile cultures.

Production of PHAs in pure cyanobacteria cultures

PHAs are polyesters of 2-, 3-, 4-, 5- or 6-hydroxyacids with potential application as renewable and biodegradable plastics [92–94]. They are produced and stored as carbon reserve polymer by a variety of prokaryotes [111] acting on a wide range of substrates from renewable sources (sucrose, starch, cellulose, triacylglycerols), fossil resources (methane, mineral oil, lignite, hard coal), byproducts from food processing (molasses, whey, glycerol), chemicals (propionic acid, 4-hydroxybutyric acid) and carbon dioxide [95].

Cyanobacteria can accumulate PHAs as carbon and energy storage material [111]. They hold special interest as low-cost PHA producers due to their low nutrient requirements and ability to accumulate PHAs by oxygenic photosynthesis [13,96,97]. Several studies have demonstrated their capacity to accumulate bioplastics in both autotrophic and heterotrophic conditions, mainly in the form of polyhydroxybutyrate (PHB), but also as co-polymers of PHA as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) [98]. Similar to other prokaryotes, cyanobacteria are capable of storing nutrients for future consumption in conditions of essential nutrient excess [98]. Table 3 includes species that have been assessed for the presence of PHAs, mostly in form of PHB. In most studies, PHA accumulation was species-dependent and a function of operational and nutritional conditions affecting growth (light-dark cycles, temperature, pH, nutrient limitation), as well as carbon source (organic or inorganic carbon). Among these conditions, nutrient limitation is the most common approach to increase PHA accumulation. As shown in Table 3, nutrient limitation, along with organic carbon sources (as glucose or acetate) have increased PHB content to from 10 to 46% in terms of cell dry weight (cdw) *Synechococcus* sp., *Spirulina* sp. and *Nostoc* sp., reaching the maximum of 85% cdw in *Aulosira fertilissima*, a similar percentage to the obtained by *Ralstonia eutropha* (90% of PHB) [99], the most promising heterotrophic bacteria for PHA production. The use of inorganic carbon sources with N or P limitation has increased PHB concentration up to 20% cdw. The specific strain used in [107], *Synechocystis sp. MA19*, reached the highest accumulation of 60% of PHB.

Production of carbohydrates in pure cyanobacterial cultures

Carbohydrates, including monosaccharides and glucose-based polymers (di-, oligo-, and poly-saccharides), are energy-storage and cell wall components in green algae/cyanobacteria cells. Cyanobacteria synthesize glycogen (α- 1,4 linked glucan) during photosynthesis and deposit it as granules within the thylakoid membrane [100], with the physiological function of providing maintenance energy for cell integrity and viability in dark periods [101]. Accumulation of carbohydrates by cyanobacteria has attracted attention as an alternative to first generation biodiesel feedstocks (sugar, grain and oleaginous crops) that have significant drawbacks related to the "food versus fuel" tradeoff [102–105]. The use of cyanobacterial or microalgal carbohydrates has several advantages, including their high growth rate, high areal (or volumetric) productivity, and capacity to grow in wastewater effluents. The harvesting cycles of cyanobacteria are shorter (~1–10 days), compared to other feedstock (harvest once or twice a year). These advantages improve suitability for ethanol production [106]. Furthermore, glycogen from cyanobacteria compared with carbohydrates from other higher plants or green algae is also advantageous [20] due to a lack of a cellulose cell wall, which typically requires additional pretreatments and expensive conversion processes [16,20,107]. Although cyanobacterial cultivation offers several advantages for bioethanol production, their potential to become a competitive raw material in this area is still limited by several factors, including the cultivation of algae and the production of carbohydrate-enriched biomass, dehydration and collection, pre-treatment of the biomass and ensuring the maximum fermentation [103]. These factors need to be optimized in order to achieve cost-effective and feasible bioethanol production.

Similar to PHAs, intracellular accumulation of carbohydrates in cyanobacteria has been facilitated by manipulation of environmental and cultivation factors, mainly N and P deficiency using inorganic carbon (**Table 4**). In general, the highest carbohydrate content is obtained under N limitation (65-70%), although maximum carbohydrate content and periods of accumulation are species-dependent. *Spirulina maxima* exhibited higher accumulation (70%) under N-deficiency, but lower content under P-deficiency for a period of 2.7 days [108].

Polyhydroxybutyrate and carbohydrate competition

Table 3 shows that PHB accumulation from inorganic carbon obtained generally lower values than with organic carbon sources. There are few reports of the interaction between glycogen and PHB. The studies [108–110] confirmed that glycogen in cyanobacteria plays a greater role (in terms of energy storage) than PHB in survival under adverse conditions such as nutrient starvation, as shown in **Figure 4**. However, the advantage of cyanobacteria is that PHB or other PHA compounds can also accumulate in heterotrophic conditions. Co-polymer P(3HB-co-3HV) and polyhydroxyvaletare (PHV) can be obtained using glucose, acetate, propionic and valeric acids as carbon source. In this case, the process involves as intermediate either acetyl-CoA (Ac-CoA) or acyl-CoA and ends with monomer polymerization by PHA synthases [98,111,112].

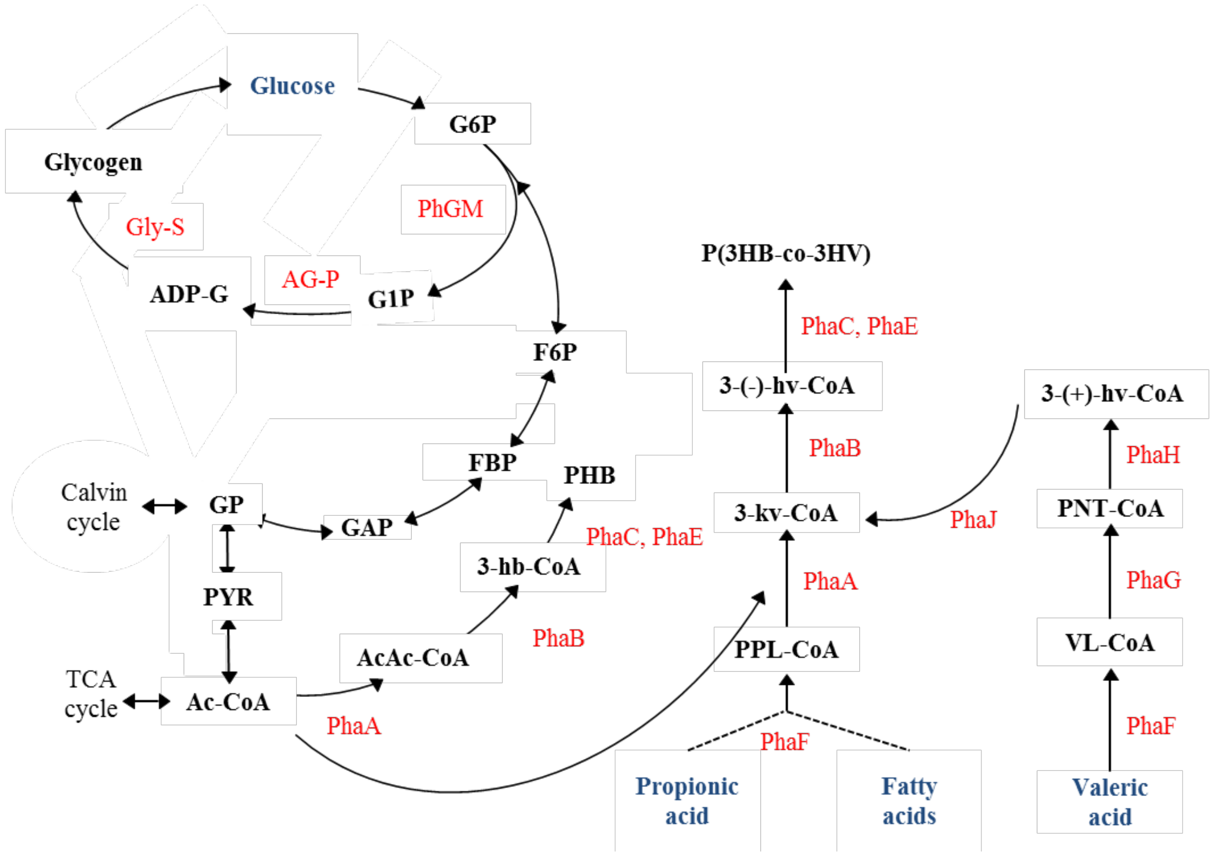

Figure 4. Cyanobacteria metabolic pathways for polyhydroxybutyrate (PHB), co-polymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (P(3HB-co-3HV)) and glycogen from different

carbon sources (in blue color). Abbreviations: acetyl-CoA (Ac-CoA), pyruvate (PYR), glycerate-3-P (GP), glyceraldehyde-3-P (GAP), fructose-1,6-bis-P (FBP), fructose-6-P (F6P), glucose-6-P (G6P); glucose-1-P (G1P), ADP-glucose (ADP-G), acetoacetyl-CoA (AcAc-CoA), 3-hydroxybutyryl-CoA (3-hb-CoA), propionyl-CoA (PPL-CoA), 3-ketovaleryl-CoA (3-kv-CoA), 3-hydrohyvaleryl-CoA (3-hv-CoA), pentenoyl-CoA (PNT-CoA), valeryl-CoA (VL-CoA). Enzymes involved in this pathway: phosphoglucomutase (PhGM), ADP-glucose pyrophosphorylase (AG-P), glycogen synthase (Gly-S), PhaA, β-ketothiolase; PhaB, acetoacetyl-CoA reductase; P(3HB) polymerase (PhaC), PHA synthase (PhaE), Acyl-CoA synthase (PhaF), Acyl-CoA-deshidrogenase (PhaG), 3-hidroxyacyl-coA-hydrolase (PhaH), 3-hidroxyacyl-CoA-dehidrogenase (PhaJ). Adapted from [98,109,111,112].

Polyhydroxybutyrate and carbohydrate production in cyanobacterial cultures using wastewater substrates

A few studies have focused on processes of wastewater treatment and simultaneous polymer production. *Aulossira fertilissima* has been grown using wastewater from an aquaculture system operating in batch conditions [9] with promising results of 200 mg L^{-1} of PHB after 15 days incubation, while P-PO_4, N-NO_3 and N-NH_4 were completely depleted. Cultivation of *Synechocystis salina* in a pilot scale PBR (200 L) has been described using diluted digestate from municipal sludge (1:3 in distilled water) [113]. After 40 days of incubation in batch conditions, 88 mg L^{-1} of PHB accumulated while 86%, 50% and 60% of COD, TN and TP, respectively, were removed.

As noted, the potential production of PHB and carbohydrates from cyanobacteria has been based on pure strains fed with sterile growth media [114,115]. This practice is associated with high operational cost, increased polymer production costs, may prevent further scale-up of the technology and limits its marketability. A more sustainable alternative approach could be the use of mixed wastewater-borne cultures dominated by cyanobacteria. Although previous reports of PHB production by pure cultures have used organic carbon, in mixed cultures this substrate would decrease cyanobacterial dominance and increase heterotrophic bacteria activity. Thus, the use of inorganic carbon is clearly the most favored option in the case of mixed cultures and is made more attractive due to the role of CO_2 in climate change. The availability of cyanobacteria-dominated cultures, instead of pure cultures, for producing PHBs and carbohydrates using inorganic carbon as substrate has been reported. Mixed wastewater-borne cyanobacteria were subjected to N and deficiency under different photoperiods (24 h light, 12h light dark) during 15 days of incubation [123]. Under conditions of N-deficiency and 12h light/dark, the maximum PHB content obtained was 6.5%, derived from 75% cdw of carbohydrates accumulated in 12 days. In another study [116], the time of incubation was reduced by employing a carbon "feast and famine" strategy, mostly used in bacterial fermentation systems [117–119]. This strategy consisted of brief periods of carbon availability during the cultivation period, in conjunction with nutrient deficiency. The use of this strategy during the cultivation period led to 3.8% cdw of PHB and 53% cdw of carbohydrates in less than 1.2 days of incubation.

PHB content of wastewater-borne cyanobacteria reported in [123, 124] are within the range from pure cultures using inorganic carbon as substrate (0.2-8.5%) [96,108,109,113,115,120]. Exceptionally, a slightly higher accumulation percentage was observed, but only after longer periods of time (21 days) [121]. Only the studies in [114] reported higher PHB content (55%) in only 5 days with specific strains of cyanobacteria *Synechococcus* sp. MA19 submitted to nutrient limited conditions with inorganic carbon as the carbon source (Table 3). The percentages and contents of carbohydrate obtained with wastewater-borne cyanobacteria under N-limitation in [122] were similar to those obtained in most of the previous studies carried out in pure cultures of *Spirulina/Arthrospira* sp. (Table 4) [103,108,123]. Although percentages of both PHB and carbohydrates reported [116] are lower than those in most of the other studies, they correspond to only 1.2 days of incubation, while most other studies required more than 10 days. This means that the “feast and famine” strategy during the cultivation phase of cyanobacteria can reduce the prolonged periods necessary to accumulate both polymers, resulting in incubation periods even shorter than those required by pure cultures (Table 4).

Promising results for wastewater treatment and PHB and/or carbohydrate accumulation lead one to conclude that waste streams could be used as substrate to obtain valuable products from cyanobacteria without the additional costs related to consumption of freshwater and nutrients used as growth medium in pure cultures.

Aspects to consider in production of polymers from cyanobacteria cultivated in wastewater and future approach

Through several studies, it is clear that the production of polymers from cyanobacteria cultivated in wastewater is promising, but still has challenges to be overcome prior to scale-up. The first is the requirement to maintain cyanobacteria-dominated cultures over prolonged periods. The keys for the control and maintenance of the culture are to establish the most appropriate medium for growing cyanobacteria, while hindering other microorganisms. The control of nutrient concentrations and (molar) ratios, as well as optimal operational conditions, are crucial in order to maintaining cyanobacteria dominance in mixed culture, and preventing contamination. For nutrients, the best approach favoring cyanobacteria seems to be the maintenance of high N:P ratios (>32:1). The high affinity for P exhibited by cyanobacteria and their capacity through luxury uptake to store it as polyphosphate could increase the likelihood of dominance over green algae species *Chlorella* sp. and *Stigeoclonium* sp. In terms of influent sources, it seems that the utilization of waste streams with stable characteristics (e.g. diluted digestate) or low nutrient sources (e.g. secondary effluents) can be the most appropriate for cyanobacterial selection. Nutrient loading from these waste streams can be more easily controlled than in others with more variable nutrient concentrations (e.g. municipal wastewaters). Other factors to consider are operating conditions; literature suggests that the sequencing batch operation is most appropriate for the selection of easy settleable cyanobacteria. However, nutrient loads and adequate SRT should be carefully tested, mostly due to the appearance of other easy settleable microorganisms like *Stigeoclonium* sp. or *Pediastrum* sp.

Another challenge concerns carbohydrate and PHB production from mixed wastewater-borne cyanobacteria. Results suggest that mixed cyanobacterial cultures can compete with pure cultures. The occurrence of both polymers has been confirmed in mixed cultures dominated by several cyanobacteria species. Using mixed cultures can avoid the high production costs required for controlled sterile conditions which are necessary for maintenance of pure cultures, where high production costs limit large-scale production. Overall, mixed cyanobacteria-bacteria cultures grown in wastewater treatment systems offer an alternative for polymer production and enhance the environmental and economic benefits of bioplastics and biofuels. The efficient production of PHAs (bioplastics) and carbohydrates (biofuel substrate) using cyanobacteria cultivated in wastewater is still limited to a few studies carried out at the laboratory and pilot scales, under controlled conditions. Further research needs to focus on scale-up of the technology and careful analyisi of the effects of outdoor conditions (e.g. direct sunlight and temperature).

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