1. Introduction

Plastic is an essential material with numerous applications across various industries and activities, including packaging, construction, automotive, electronics, household goods, medicine, and agriculture. However, the adverse effects of petroleum-based plastics and chemicals on the environment cannot be denied. In fact, it is estimated that 60 % of all produced plastics have been discarded into landfills or natural ecosystems (Geyer et al., 2017). In response, strategies and legal acts like the Directive (EU) 2019/904 of the European Parliament and of the Council on the reduction of the impact of certain plastic products on the environment, adopted in June 2021, have been implemented. This directive is part of a broader movement towards developing sustainable production processes to replace petroleum-based materials. Despite significant advancements in green chemistry and exploring biomass feedstocks as alternatives to petroleum, the transition towards a bio-based chemical industry remains relatively slow. This is largely due to scientific and technological challenges in developing highly efficient cell factories, as well as economic challenges in competing with the low costs of established petroleum-based processes (Nielsen et al., 2022).

Despite these difficulties, research in this field has demonstrated promising alternatives to fossil-based commodities. Several innovations have already entered the market (Nielsen et al., 2022). An illustrative example is bioplastic. According to the European Bioplastics association, bioplastics are defined as a group of polymers produced from natural or renewable sources (biobased), biodegradable, or both ("European Bioplastics e.V.," 2023). Among the most interesting bioplastics are polyhydroxyalkanoates (PHAs) as they are biobased and biodegradable. Compared to other biodegradable bioplastics, such as polylactic acid (PLA) and starch-based bioplastics, PHAs exhibit superior biodegradability ("Nova Institut," 2020). Their complete degradation in diverse

environments, including soil, marine, and composting facilities, highlights their potential to reduce plastic waste accumulation and minimize environmental damage. PHAs offer a promising alternative to conventional plastics across various sectors, including packaging, biomedical, and agriculture, due to their thermal processability, biodegradability, and biocompatibility properties (Rueda et al., 2024).

Industrial-scale PHA production is achieved by various companies, primarily located in Asia, using defined heterotrophic bacteria strains such as *Cupriavidus necator*, *Halomonas* sp., or recombinant *Escherichia coli*. The process typically relies on refined feedstocks like glucose or sucrose, increasing the production costs (Koller and Mukherjee, 2022). These high production costs, which are approximately five to ten times higher than those of petroleum-based polymers like polypropylene (Ali Raza et al., 2018), represent a significant barrier to the adoption of PHAs in the market. To address this challenge and scale up production to meet market demands, research efforts must focus on discovering new productive strains, enhancing production and downstream processes, and expanding applications.

Exploring alternatives to heterotrophic bacteria for PHA production includes investigating other microorganisms, such as cyanobacteria. Unlike heterotrophic bacteria, cyanobacteria are photoautotrophic, relying primarily on CO_2 and sunlight for growth rather than organic carbon sources. They have garnered significant interest as platforms for the sustainable production of high-value products (such as pigments, extracellular polymeric substances, and lipids), or biomass (Chentir et al., 2017; Senatore et al., 2023). However, research on PHA production using cyanobacteria remains limited compared to heterotrophs, with less than 5 % of scientific publications on PHAs production focusing on cyanobacteria (Rueda et al., 2024). Furthermore, in pursuing sustainable and economically feasible alternatives, researchers have also

explored the use of mixed microbial consortia (or microbiomes - a diverse microbial culture comprising various microorganisms).

One of the key benefits of microbiomes is their functional robustness and flexibility in responding to environmental changes (Santinello et al., 2024). Their self-stabilizing capacity is particularly significant, as it eliminates the need for sterile conditions typically required in monoculture systems. Moreover, using microbiomes opens opportunities for using low-cost waste streams as feedstock, thereby reducing production costs and supporting waste management and circular economy principles. Nevertheless, while research on cyanobacteria monocultures for PHB production is comparatively scarce, studies involving cyanobacteria microbiomes - a diverse microbial culture comprising various cyanobacterial strains and other microorganisms - are even more uncommon. Despite this, the combined advantages of cyanobacteria and mixed cultures present a promising area for further exploration.

This review focuses on the production of PHB by cyanobacteria, with particular emphasis on research involving cyanobacteria microbiomes collected from natural environments. The analysis covers the results obtained from studies using natural cyanobacteria microbiomes for PHB production. It evaluates current methodologies and their efficiencies, highlighting the potential of these cultures for sustainable biopolymer production. It is important to clarify that within the scope of this review, the term "cyanobacteria microbiome" refers explicitly to scenarios involving mixed cultures derived from environmental samples (water bodies) where cyanobacteria are the main PHB producers.

2. PHB production by cyanobacteria

Cyanobacteria have emerged as a promising source of PHB production. Beyond PHB, they can also produce other PHA co-polymers, such as poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) (PHBV), by incorporating organic carbon supplements like propionate, valerate or fructose into the cultivation medium (Tanweer and Panda, 2020; Tarawat et al., 2020). This versatility expands the range of biopolymers that cyanobacteria can synthesize, underscoring their potential in producing various PHAs. Nevertheless, research predominantly focuses on identifying PHB-producing cyanobacteria species and optimizing culture conditions to enhance biopolymer synthesis. There are very few studies on copolymer synthesis by cyanobacteria and the knowledge is undoubtedly very insufficient.

3. Cyanobacteria microbiomes for PHB production

Numerous cyanobacterial species capable of producing PHB have been identified, with significant attention in the genera *Arthrospira*, *Nostoc*, *Synechocystis*, and *Synechococcus* (Rueda et al., 2024). The production of PHB by cyanobacteria can be achieved through either pure (mono-) cultures or microbiomes. Pure cultures require a sterile environment, necessitating refined substrates and sterilized equipment, contributing to high operational costs. On the other hand, the utilization of microbiomes offers a pathway to reduce production expenses by eliminating the need for sterilization of the reactor, enabling for greater flexibility in adapting to a wide range of inexpensive and complex media, including wastewater (Rueda et al., 2024).

Although research on cyanobacteria microbiomes for PHB production remains limited (Table 1), the primary advantage of employing microbiomes from field samples lies in: i) the preservation of natural diversity, including rare and uncultured species that might not be represented in laboratory collections, ii) retaining ecological relationships and interactions inherent to natural environments, and iii) adaptive mechanisms and evolutionary strategies shaped by local environmental factors (Louca et al., 2018). All these aspects may not be discernible in microorganisms from culture collections.

The exploration of cyanobacteria microbiomes from field samples for PHA (and other bioproducts) synthesis comprises four stages: (i) sample procurement; (ii) testing for bioproduct production; (iii) optimization of bioproduct synthesis; and (iv) production of the bioproduct (Figure 1).

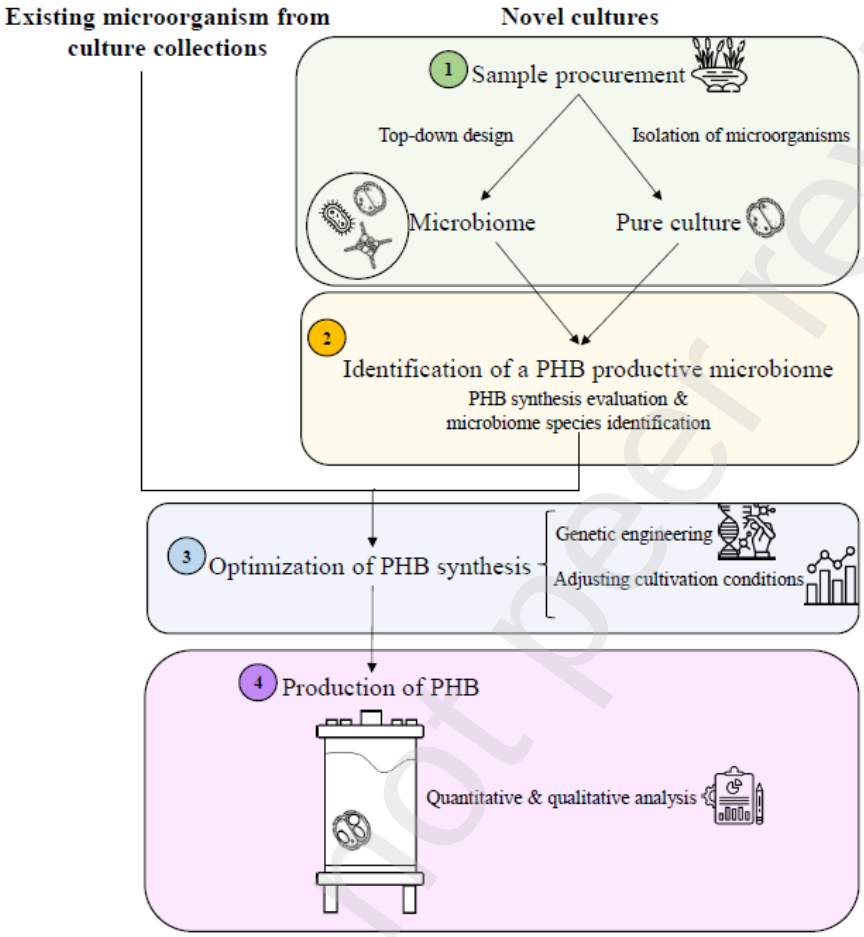

Figure 1. Schematic workflow processes for PHA research, illustrating two approaches: the use of existing microorganisms and the exploration of novel microbial cultures. This review is focused on the latter, particularly cyanobacteria-dominated microbiomes from environmental samples. The step-by-step process for exploring these microbiomes for PHB synthesis is outlined, including: (1) sample procurement, (2) identification, (3) process optimization, and (4) PHB production.

112 **Table 1.** Summary of PHB production studies conducted with cyanobacteria microbiomes.

Origin	Culture conditions during PHB accumulation				PHB [%dcw]	Ref.
	Nutrient limitation	Light:dark photoperiod [h:h]	C supplement	Working V [L]		
Wastewater-borne cyanobacteria	N and P	12:12	IC (Na_2CO_3)	0.4	4	(Arias et al., 2018a)
Wastewater-borne cyanobacteria	N	24:0	IC ($NaHCO_3$)	1	5	(Arias et al., 2018b)
	P	24:0	IC ($NaHCO_3$)	1	6	
	N	12:12	IC ($NaHCO_3$)	1	7	
	P	12:12	IC ($NaHCO_3$)	1	6	
Wastewater-borne cyanobacteria	None*	12:12	None	2.5	< 1	(Arias et al., 2018c)
Wastewater-borne cyanobacteria	N and P	15:09	IC (CO_2 + $NaHCO_3$)	11,700	5	(Rueda et al., 2020b)
River sample enriched in cyanobacteria	N and P	0:24	OC (Ac)	2.5	27	(Altamira-Algarra et al., 2024b)
Urban pond sample enriched in cyanobacteria and microalgae	N and P	0:24	OC (Ac)	2.5	8	
Canal sample enriched in cyanobacteria	N and P	0:24	OC (Ac)	2.5	22	(Altamira-Algarra et al., 2024a)
Canal sample enriched in cyanobacteria and microalgae	N and P	0:24	OC (Ac)	2.5	5	

Table 1 (Continued)

Origin	Culture conditions during PHB accumulation				PHB [%dcw]	Ref.
	Nutrient limitation	Light:dark photoperiod [h:h]	C supplement	Working V [L]		
Urban pond sample enriched in cyanobacteria	N and P	24:0	IC ($NaHCO_3$)	0.05	0	(Altamira-Algarra et al., 2023)
	N and P	0:24	IC ($NaHCO_3$)	0.05	1	
	N and P	24:0	OC (Ac)	0.05	6	
	N and P	0:24	OC (Ac)	0.05	4	
	N and P	24:0	OC (Ac) + IC ($NaHCO_3$)	0.05	3	
	N and P	0:24	OC (Ac) + IC ($NaHCO_3$)	0.05	6	
River sample enriched in cyanobacteria	N and P	24:0	IC (NaHCO3)	0.05	1	
	N and P	0:24	IC (NaHCO3)	0.05	3	
	N and P	24:0	OC (Ac)	0.05	4	
	N and P	0:24	OC (Ac)	0.05	6	
	N and P	24:0	OC (Ac) + IC ($NaHCO_3$)	0.05	4	
	N and P	0:24	OC (Ac) + IC ($NaHCO_3$)	0.05	4	

Table 1. (Continued)

Origin	Culture conditions during PHB accumulation				PHB [%dcw]	Ref.
	Nutrient limitation	Light:dark photoperiod [h:h]	C supplement	Working V [L]		
Canal sample enriched in cyanobacteria	N and P	24:0	IC ($NaHCO_3$)	0.05	0	(Altamira-Algarra et al., 2023)
	N and P	0:24	IC ($NaHCO_3$)	0.05	0	
	N and P	24:0	OC (Ac)	0.05	11	
	N and P	0:24	OC (Ac)	0.05	14	
	N and P	24:0	OC (Ac) + IC ($NaHCO_3$)	0.05	11	
	N and P	0:24	OC (Ac) + IC ($NaHCO_3$)	0.05	8	
Constructed wetland sample enriched in cyanobacteria	N and P	24:0	IC ($NaHCO_3$)	0.05	1	
	N and P	0:24	IC ($NaHCO_3$)	0.05	0	
	N and P	24:0	OC (Ac)	0.05	7	
	N and P	0:24	OC (Ac)	0.05	5	
	N and P	24:0	OC (Ac) + IC ($NaHCO_3$)	0.05	7	
	N and P	0:24	OC (Ac) + IC ($NaHCO_3$)	0.05	8	

1. Sample procurement

The process begins by collecting natural microbiomes from diverse environments to ensure broad microbial diversity. These environmental samples contain a wide range of microorganisms, including bacteria, diatoms, green algae, protozoa, and rotifers. Some studies have focused on isolating cyanobacteria strains from water bodies , such as wastewater streams, ponds, or lakes, through prolonged sub-culturing, where colonies are scaled up from plates to lab-scale flasks (up to 1 L) (Djebaili et al., 2022; Meixner et al., 2022; Rueda et al., 2020a). However, these efforts culminate in using axenic cultures under sterile conditions, which does not leverage the primary benefits of cyanobacteria microbiomes.

In contrast, to develop a microbiome capable of producing specific bioproducts—such as a photosynthetic microbiome for PHB production—it is necessary to apply conditions that selectively promote the growth of target microorganisms within competitive environments. This method is known as “top-down” strategy, which involves optimizing natural microbial communities by optimizing physical and chemical parameters to maximize the community function (Liang et al., 2022). For example, (Arias et al., 2017) highlighted the key role of nutrient (nitrogen (N) and phosphorus (P)) fluctuations in the medium and how their ratio (N:P) influenced the culture composition and biomass concentration. They employed a closed 30 L photobioreactor (PBR) fed with urban secondary treated effluent and digestate, inoculated with a mixed consortium of green algae and cyanobacteria, and operated for one year to select cyanobacteria. The findings revealed that cyanobacteria species dominated over green algae under non-limited carbon conditions and low P content. Nonetheless, biomass production was negatively impacted due to P limitation. Specifically, a volumetric phosphorus loading rate (LvP) around 0.23 mg P-$PO_4^{3-}\cdot L^{-1}\cdot d^{-1}$ significantly enhanced biomass production, averaging at 0.08 g$\cdot L^{-1}\cdot d^{-1}$,

with a maximum production of 0.23 $mg \cdot L^{-1} \cdot d^{-1}$. This LvP level ensured adequate P supply for supporting cellular growth. On the contrary, when the LvP fell below 0.16 mg $P\text{-}PO_4^{3-} \cdot L^{-1} \cdot d^{-1}$, a significant drop in biomass production was noted, decreasing to 0.04 $mg \cdot L^{-1} \cdot d^{-1}$. This decline was attributed to the phosphorus limitation, which reduced the growth and metabolic activities of the microorganisms. These outcomes emphasize the importance of carefully managing nutrient loads to favour cyanobacteria dominance while meeting certain minimal nutrient requirements to achieve high biomass concentrations, aiming for a highly productive culture. In another study (Arias et al., 2019), cultivation under phosphorus limitation increased the proportion of cyanobacteria from an initial 2 % to 70 % of the total population after one month. The sample of this study originated from a pilot-scale tertiary wastewater treatment system, initially predominantly composed of the green microalgae *Scenedesmus* sp. (93 %). In a more recent study (Altamira-Algarra et al., 2023), samples were collected from seven locations, including an urban pond, a river, and a constructed wetland. The water in these locations had different salinity and nutrient content properties. The initial samples contained a mixture of green algae, filamentous cyanobacteria, and diatoms. The study aimed to assess these samples for PHB production by enriching the cultures in cyanobacteria. Cultivation under phosphorus limitation was employed to favour cyanobacteria growth over other phototrophic organisms (such as green algae), given cyanobacteria's greater ability to store phosphorus intracellularly. Over six months, this strategy successfully reduced the diversity of the microbiome, increasing cyanobacterial dominance, which was confirmed through 16S rRNA gene amplicon sequencing. In another related study, (Meixner et al., 2022) used the antibiotic cycloheximide to eliminate eukaryotic microalgae. While this method proved effective in obtaining

eukaryotic algae-free cultures, it also resulted in the loss of cyanobacteria in some cultures.

The species composition of the microbiome is crucial for sustaining consistent PHB production. In (Altamira-Algarra et al., 2024a) a decline in biopolymer yields from 13 to <5 %dcw was observed, attributed to the prevalence of non-PHB-producing microorganisms (green microalgae) within the microbiome. Furthermore, another study (Altamira-Algarra et al., 2024b) emphasized the key role of the initial inoculum composition, suggesting that mitigating the growth of green microalgae competitors becomes progressively challenging once established. These insights might, in our opinion, have a role in explaining the lower productivity reported in studies involving wastewater-borne cyanobacteria (Table 1), where, despite cyanobacteria initially dominating the culture (abundance 60–70 %), shifts in nutrient dynamics, particularly the availability of phosphorus, promoted the proliferation of other microorganisms, which were non-PHB producers, including green microalgae and diatom species (Arias et al., 2018a, 2018b, 2018c). Additionally, in (Rueda et al., 2020b), the utilization of wastewater-born cyanobacteria in an open system supplied with agricultural runoff, where contamination risks by other microorganisms were elevated, likely contributed to the low PHB levels (5 %dcw).

These findings demonstrate that phosphorus limitation can be a powerful tool for shaping the microbiome composition, particularly promoting the development of cyanobacteria-enriched communities (Altamira-Algarra et al., 2024b). However, achieving consistent results requires careful consideration of multiple factors beyond just phosphorus limitation (Altamira-Algarra et al., 2024a). Other influential factors, including inorganic carbon concentrations, light wavelength, and temperature, also enhance the prevalence of desired species within the microbiome (Arias et al., 2017;

Lürling et al., 2018; Tan et al., 2020). Notably, light wavelength significantly influences interspecific competition between green algae and cyanobacteria in co-cultures. Under blue light, *Chlorella pyrenoidosa* (green algae) dominated, whereas *Microcystis aeruginosa* (cyanobacteria) prevailed in red and white light (Tan et al., 2020).

2. Identification of a PHB productive microbiome

2.1. PHB synthesis evaluation

Biochemical composition of cyanobacteria, including yield and production of PHB, can vary significantly among different strains. Therefore, optimizing the yield and production of bioproducts for each culture is crucial.

Small volume tests, such as those conducted in test tubes holding up to 100 mL of culture medium, are ideal for screening cultures for bioproduct synthesis (Altamira-Algarra et al., 2023; Ansari and Fatma, 2016; Kamravamanesh et al., 2017; Rueda et al., 2022a; Thi et al., 2024). These tests allow for the efficient analysis of many samples under controlled conditions, providing valuable insights into the potential of each culture for bioproduct synthesis without the need for extensive resources or infrastructure. (Ansari and Fatma, 2016) analysed up to 23 cyanobacteria strains for PHB production in 50 mL test tubes, showcasing the method's applicability. Moreover, the capacity for analysing even more samples can be enhanced by employing multiwell plates, which feature up to 96 wells functioning as small test tubes. This setup allows rapid and parallel assays across multiple cultures and conditions. Nevertheless, such small-scale studies face limitations in controlling specific culture parameters, such as pH, temperature, nutrient concentration, and mixing, due to practical challenges with sampling, sample volume, and analysis procedures. As a result, the outcomes of these experiments may serve as indicators for further evaluating the process at larger scales, where a strict control of the parameters is feasible.

2.2. Microbiome species identification

Efficient screening methods for PHA producers are crucial when working with natural microbiomes or large sample sets. Common detection techniques include biopolymer staining, using lipophilic dyes like Sudan Black or fluorescent dyes such as Nile Blue (or Red) for optical and fluorescence microscopy, respectively (Ansari and Fatma, 2016; Meixner et al., 2022; Simona et al., 2019; Thi et al., 2024). This approach enables the rapid identification of biopolymer producers within just 10 minutes (Ansari and Fatma, 2016; Meixner et al., 2022). For example, (Thi et al., 2024) screened 47 microalgae and cyanobacteria strains from various locations and environmental conditions. Among these, 15 strains were found to accumulate biopolymers through Nile Red staining, with the cyanobacteria *Arthrospira* sp. achieving the highest yield (4 %dcw) under the conditions tested (standard Zarrouk medium and 14:10 h light:dark photoperiod).

Moreover, microbial characterization is essential to identify the biopolymer producers. Molecular techniques, such as 16S rRNA gene amplicon sequencing and fluorescence in situ hybridization (FISH), are widely used for this purpose (Altamira-Algarra et al., 2023; Crognale et al., 2022; Djebaili et al., 2022; Meixner et al., 2022; Pei et al., 2022). When combined with staining protocols for PHB detection, these methods are powerful tools for identifying PHB-producing microorganisms within complex communities or isolates. However, it is important to recognize that these techniques involve labour-intensive protocols and subsequent bioinformatic analysis, requiring specialized expertise.

3. Optimization of PHB synthesis

After identifying a potentially productive microbiome, optimizing bioproduct synthesis becomes a critical next step. Several strategies and considerations have been explored to

enhance PHB production in cyanobacteria, involving (i) genetic engineering and (ii) adjusting cultivation conditions.

3.1. Genetic engineering

Genetic engineering of cyanobacteria has emerged as a powerful approach to boost PHB biosynthesis, especially on strains from culture collections (Figure 1). By modifying cyanobacterial genomes, researchers aim to enhance existing PHB synthesis pathways to improve biopolymer production. This requires transforming cyanobacteria strains with foreign key genes involved in biopolymer synthesis. A widely employed method involves introducing genes from *C. necator*, such as acetoacetyl-CoA reductase, β-ketothiolase, and PHB synthase (Carpine et al., 2017; Koch et al., 2020; Lee et al., 2024). Notably, up to 12 %dcw PHB was produced directly from CO_2 by a genetically modified *Synechocystis* sp. PCC6803 under nitrogen and phosphorus depleted medium (Carpine et al., 2017). Another approach involves genetic engineering techniques to modify existing metabolic pathways and introduce new enzymes to optimize carbon flux towards PHB synthesis. This includes two strategies: (i) improving the efficiency of carbon fixation to increase the amount of carbon available for biopolymer synthesis; and (ii) redirecting carbon flux away from non-essential metabolic pathways and towards PHB synthesis (Koch et al., 2020; Krasaesueb et al., 2021; Orthwein et al., 2021). An illustrative example is the work from (Koch et al., 2020), where *Synechocystis* sp. PCC 6803 deficient in the regulatory protein PirC, exhibited a higher phosphoglycerate mutase activity, resulting in increased PHB pools under nutrient-limiting conditions. Further enhancements were achieved by introducing *phaA* and *phaB* genes from *C. necator*, leading to up to 63 %dcw PHB production, and up to 81 %dcw upon the addition of acetate.

It is important to mention that while recombinant strains have shown promise results in small-scale flask experiments (typically <100 mL volume), these studies often fail to assess the feasibility of scaling up the process. Notable exceptions include the work by (Krasaesueb et al., 2019), who evaluated the reuse of shrimp wastewater for low-cost cyanobacteria cultivation to produce PHB. Their study involved culturing *Synechocystis* sp. PCC 6803 lacking the phosphate regulator SphU in a batch PBR using shrimp wastewater as growth medium. During the initial growth phase (0–4 days), low levels of PHB were detected due to the presence of nutrients. However, PHB accumulation rapidly increased from 0.3 %dcw to 30 %dcw when nitrogen and phosphorus were depleted (4–14 days). Despite such example, the economic viability of relying solely on engineered strains for industrial applications appears limited. The high costs of strain development, maintenance, and scale-up, pose substantial barriers. Furthermore, yield and productivity often fail to meet the requirements for industrial-scale expansion, mainly due to metabolic stress these manipulations impose, which slows microbial growth (Heieck et al., 2023). Consequently, the preference lies in using wild-type strains and microbiomes, exploring strategies for process improvement through adjustments to cultivation parameters.

3.2. Adjusting cultivation conditions

This approach aims to optimize physical and chemical factors influencing PHB accumulation, as the environment in which microorganisms grow is a key element controlling their traits and properties and can be exploited to optimize their functions. Abiotic environmental factors such as nutrient deficiency, presence of inorganic or organic carbon sources, pH, salinity, temperature, and light intensity have been evaluated in cyanobacteria cultures to increase PHB synthesis (Rueda et al., 2024). These studies are usually performed in small-scale flasks, up to 150 mL, to evaluate

PHB production under different conditions to optimize critical factors by multivariable experimental designs (Altamira-Algarra et al., 2023; Ansari and Fatma, 2016; Kamravamanesh et al., 2017; Rueda et al., 2022a; Samantaray and Mallick, 2012; Yashavanth and Maiti, 2024). For example, in the study by (Rueda et al., 2022a), the authors investigated the effects of acetate (as an organic carbon supplement), sodium bicarbonate (as a source of inorganic carbon), salinity, and days in darkness on biopolymer synthesis by response surface methodology (RSM). The optimized conditions for PHB accumulation in *Synechocystis* sp. were identified as 1.2 $g \cdot L^{-1}$ Ac, 4 $g \cdot L^{-1}$ inorganic carbon (as sodium bicarbonate), 18 $g \cdot L^{-1}$ NaCl and lack of darkness. For *Synechococcus* sp., the same acetate concentration and lack of darkness, and 0.05 $g \cdot L^{-1}$ inorganic carbon and 9 $g \cdot L^{-1}$ NaCl were required. Under these conditions, *Synechocystis* sp. and *Synechococcus* sp. accumulated 6 %dcw and 26 %dcw PHB, respectively. Similar PHB yield was achieved by *Nostoc muscorum* (26 %dcw PHB) under optimized conditions for PHB accumulation, including pH 7.5, 30 °C, 10:14h light:dark cycles, P-deficiency, and 6 $g \cdot L^{-1}$ glucose supplementation (Ansari and Fatma, 2016).

Based on the findings from these studies, it appears that key nutrients deficiency, especially nitrogen and phosphorus, is essential to promote PHB synthesis in cyanobacteria. In addition, supplementing the culture with an organic carbon source has proven successful results in enhancing PHB synthesis in pure cyanobacteria cultures (Duangsri et al., 2020; Panda et al., 2006; Rueda et al., 2022a), as well as, in photosynthetic microbiomes (Table 1). For example, adding acetate significantly enhanced PHB accumulation in *Synechocystis* sp. reaching up to 29 %dcw, which was almost six times higher compared to conditions without acetate (Panda et al., 2006). Other organic carbon supplements, like butyrate and glucose, have been evaluated but the PHB synthesis achieved was lower than that obtained with acetate (Duangsri et al.,

2020; Simonazzi et al., 2021; Wu et al., 2002). For example, when using glucose as the carbon supplement, only 11 %dcw PHB was achieved by *Anabaena* sp., compared to 40 %dcw with acetate addition (Simonazzi et al., 2021). This variation could be due to differences in metabolic pathways or energy conversion efficiencies between glucose and acetate metabolism within the cyanobacterial cells (Wu et al., 2002). The positive effect of acetate on biopolymer synthesis is attributed to the conversion of the up-taken acetate into acetyl-CoA, the primary precursor of PHB, thereby increasing the acetyl-CoA pool (Figure 2).

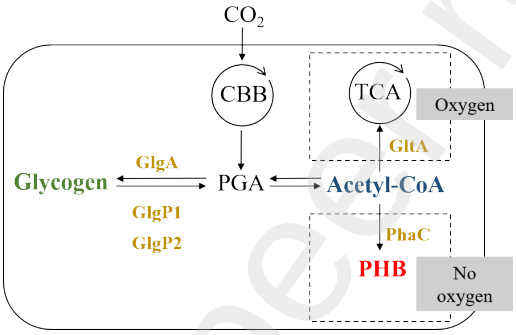

Figure 2. Biosynthetic pathways of PHB and glycogen in cyanobacteria, highlighting the fate of Acetyl-CoA under aerobic and anaerobic conditions. Key enzymes are represented in yellow. For synthesis of PHB, PhaC: poly(3-hydroxyalkanoate) synthase; for glycogen synthesis, GlgA, glycogen synthase; and catabolism, GlgP1 and GlgP2: glycogen phosphorylase; and for the tricarboxylic acid (TCA) cycle, GltA: citrate synthase. Other abbreviations: CBB: Calvin- Benson–Bassham cycle; PGA: 3-phosphoglycerate.

The decrease in dissolved oxygen (or even depletion) within the PBR is indeed another factor enhancing PHB synthesis in cyanobacteria (Table 1). The creation of low oxygen or anoxic environments is possible by dark incubation, which stimulates glycogen degradation and the subsequent production of PHB (Koch et al., 2019) (Figure 2). Glycogen is the primary reservoir for carbon and energy in cyanobacteria, to acclimate and cope with starvation conditions, particularly nitrogen starvation (Damrow et al., 2016). Under extended stress periods, the stored glycogen is progressively metabolized and transformed into PHB (Altamira-Algarra et al., 2024b; Koch et al., 2019; Rueda et al., 2022b). Indeed, the overexpression of the *glgA* gene, which encodes for glycogen synthase, occurs early in the starvation phase, coinciding with elevated glycogen levels.

As starvation phase progresses, glycogen concentrations decrease, leading to sustained or increased PHB concentrations (Altamira-Algarra et al., 2024b). Concurrently, *glgA* overexpression ceases (Altamira-Algarra et al., 2024b; Rueda et al., 2022b). Notably, a consistent PHB content of 23 %dcw was maintained during the final three days of the accumulation phase in a cyanobacteria-rich microbiome (Altamira-Algarra et al., 2024b). However, contradictory findings were reported by (Rueda et al., 2022b), who did not observe similar maintenance of PHB levels. Following 20 days of starvation, where PHB concentration reached 14 %dcw, it subsequently decreased to 5% dcw in two weeks. Key differences in experimental conditions may explain these discrepancies: (i) external carbon supplementation, and (ii) oxygen levels. In (Altamira-Algarra et al., 2024b), 600 $mg \cdot L^{-1}$ of acetate was added at the beginning of the accumulation phase, likely playing a critical role in boosting PHB synthesis. Additionally, the absence of oxygen favoured the conversion of glycogen into PHB. Conversely, (Rueda et al., 2022b) maintained photoperiods during the starvation phase, allowing oxygen to persist. As illustrated in Figure 2, acetyl-CoA plays a central role in the citric acid cycle (also known as the Krebs cycle or TCA cycle) and PHB synthesis. In the presence of oxygen, acetyl-CoA preferentially enters the TCA cycle (Ciebiada et al., 2020), reducing PHB yield. These findings suggest that while initial PHB synthesis depends on glycogen breakdown, sustained production requires environmental factors like acetate supplementation or anoxic conditions, or both. Without these conditions, PHB synthesis eventually ceases. Future studies should consider these factors when designing experiments to optimize PHB yield in cyanobacteria, as the addition of external carbon sources and low-oxygen or anoxic environments may be critical for achieving consistent, high-yield PHB production in these microorganisms.

Research by (Ansari and Fatma, 2016) found that *Nostoc* sp. cells grown in a 14:10 h light-dark photoperiod exhibited a significant increase in PHB accumulation, reaching 8 %dcw, substantially higher than those grown under constant light (3 %dcw) or continuous darkness (2 %dcw). This observation underscores the significance of alternating light and dark periods for promoting optimal growth and efficient glycogen storage, which is later catabolized and converted into PHB. Furthermore, transitioning stationary phase cultures of cyanobacterial cells to a medium optimized for biopolymer synthesis—characterized by acetate supplementation, darkness, and nutrient limitation—resulted in substantial increases in PHB levels (Monshupanee et al., 2016; Samantaray and Mallick, 2012). These findings led to developing a cycle approach based on alternating biomass growth and PHB accumulation phases. This two-stage methodology was explicitly developed for cyanobacteria-rich microbiomes in (Altamira-Algarra et al., 2024a) and further validated in (Altamira-Algarra et al., 2024b). The first phase aims at maximizing biomass growth, followed by a subsequent phase designed for PHB synthesis under nutrient limitation, acetate supplementation and dark conditions, promoting an oxygen-limited environment. Up to 27 %dcw PHB was achieved following this approach (Altamira-Algarra et al., 2024b).

According to (Altamira-Algarra et al., 2024b) the cells reach a PHB synthesis peak in three days. Beyond this period, the rate of PHB synthesis tends to maintain a plateau or decrease, indicating that the cells have reached their capacity for PHB production under the specific culture conditions. Interestingly, pulse-wise feeding, where brief bursts of acetate are administered to the culture, could be tested to determine whether additional PHB production is possible, or if the cells have indeed reached their maximum capacity. In activated sludge, pulse feeding resulted in higher PHA content compared to excess substrate supply, achieving 32 %dcw versus 20 %dcw PHB (Estévez-Alonso et al.,

2022), or 78 %dcw versus 57 %dcw PHB (Serafim et al., 2004), respectively. Interestingly, microbial characterization studies showed that the feeding regime did not change the microbial composition (Ciggin et al., 2012).

Quantitative real-time PCR (RT-qPCR) analysis of genes related to PHB metabolism offers valuable insights into how environmental factors influence PHB synthesis (Altamira-Algarra et al., 2024b; Duangsri et al., 2020; Rueda et al., 2022b). This technique effectively compares gene expression levels under different conditions, revealing key influences on PHB production. For instance, in *Anabaena platensis* grown in nitrogen-deprived medium with acetate supplementation, the expression of *phaB* and *phaC*, encoding acetoacetyl-CoA reductase and PHA synthase, respectively, increased approximately 5- and 4-fold compared to cells without carbon supplementation (Duangsri et al., 2020). These results suggest that acetate supplementation enhances *phaB* and *phaC* expression, thereby boosting PHB production through the induction of PHB synthesis-related enzymes. Moreover, the expression levels of both genes were evaluated in cultures supplemented with alternative carbon substrates (butyrate and glucose), which showed significantly lower expression than acetate-supplemented cultures. This finding suggests that acetate is a more effective inducer for PHB production.

Furthermore, gene analysis reveals important connections between glycogen metabolism, the TCA cycle and PHB synthesis (Figure 2). In the study by (Rueda et al., 2022b), bicarbonate supplementation in *Synechocystis* sp. cultures significantly increased PHB production, reaching a maximum of 14 %dcw PHB. Interestingly, gene expression analysis showed upregulation of the genes *glgA* and *phaC*, which are related to glycogen and PHB synthesis, respectively, in the cultures with the highest bicarbonate levels. Additionally, a positive correlation between *glgP2* (encoding

glycogen phosphorylase), *phaB* and *phaC* further indicated that glycogen catabolism supports PHB synthesis (Rueda et al., 2022b). This observation indicates that glycogen is a primary precursor for PHB production during prolonged nitrogen starvation in cyanobacteria. This conclusion is supported by observations in mutant cells of *Synechocystis* sp., where the absence of glycogen synthase (GlgA1) reduced PHB production (Koch et al., 2019). The findings highlight the potential for optimizing PHB yield by modulating glycogen metabolism, achievable by adjusting culture parameters to promote glycogen synthesis.

4. Production of PHB

Performance of the cultures with the optimized conditions found in the previous stage is essential for evaluating PHB production in more significant volumes, ideally in 1 - 5 L PBRs. Here a major drawback of studies related to cyanobacteria PHB production appears. Current studies predominantly focus on batch experiments conducted under sterile conditions, involving a short-term biopolymer accumulation phase spanning just a few weeks in flasks with working volumes lower than 1 L (Gracioso et al., 2021; Monshupanee and Incharoensakdi, 2014). This approach limits the applicability of findings to real-world scenarios, where scalability and non-sterile conditions are essential for practical implementation. Departing from these small volume experiments, some studies have extended their focus to assess PHB synthesis in lab-scale PBRs of up to 5 L, using pure cyanobacteria cultures and cyanobacteria dominated microbiomes, yielding promising results (Table 2).

434 **Table 2.** Summary of PHB production studies conducted with cyanobacteria cultures in working volumes > 1 L.
435

Strain	Sterility	Working V [L]	Period [d]		Culture conditions for PHB accumulation	PHB [%dcw]	Ref.
			Biomass growth	PHB accumulation			
Synechocystis sp. PCC 6714	Yes	1.5	5	15	N and P limitation 2 % CO_2	16	(Kamravamanesh et al., 2017)
Chlorogloea fritschii	Yes	2	9	4	N and P limitation 0.4 g·L^{-1} Ac Darkness	32	(Yashavanth and Maiti, 2024)
Synechocystis sp.	Yes	2.5	30	14	N and P limitation IC feeding feast-famine	5	(Rueda et al., 2020a)
Synechocystis sp.	Yes	2.5	25	15	N and P limitation 2 g·L^{-1} IC 12 g·L^{-1} NaCl	8	(Rueda et al., 2022c)
Synechocystis sp.	Yes	2.5	24	18	N and P limitation 1.2 g·L^{-1} Ac 4 g·L^{-1} IC 18 g·L^{-1} NaCl	6	(Rueda et al., 2022a)
Synechococcus sp	Yes	2.5	24	18	N and P limitation 1.2 g·L^{-1} Ac 0.05 g·L^{-1} IC 9 g·L^{-1} NaCl	26	
Synechocystis sp.	Yes	2.5	10	15	N and P limitation 2 g·L^{-1} IC	14	(Rueda et al., 2022b)

Table 2. (Continued)

Strain	Sterility	Working V [L]	Period [d]		Culture conditions for PHB accumulation	PHB [%dcw]	Ref.
			Biomass growth	PHB accumulation			
Nostoc muscorum	Yes	4	20	12	15 % CO_2	22	(Bhati and Mallick, 2016)
Chlorogloea fritschii	No	5	21*		N and P limitation	5	(Meixner et al., 2022)
Cyanobacteria dominated mixed culture	No	1	-	15	N limitation	7	(Arias et al., 2018b)
Cyanobacteria dominated mixed culture	No	2.5	7	14**	N and P limitation 0.6 g·L^{-1} Ac Darkness	22	(Altamira-Algarra et al., 2024a)
Cyanobacteria dominated mixed culture	No	2.5	7	14**	N and P limitation 0.6 g·L^{-1} Ac Darkness	27	(Altamira-Algarra et al., 2024b)
Microalgae mixed microbiome	No	2.5	30*		N and P availability	< 1	(Arias et al., 2018c)

Note. *Growth and accumulation phase were not distinguished ** PHB accumulation cycles lasting 14 days. Abbreviations: N: nitrogen; P: phosphorus; Ac: acetate; IC: inorganic carbon; Glu: glucose

4.1. Quantitative and qualitative analysis

During the PHB synthesis step, precise PHB quantification is essential to measure the amount of polymer produced accurately. Gas chromatography (GC), coupled with either flame ionization detection (FID) or mass spectrometry (MS), is the most widely used method for reliable PHB content quantification. Biopolymer quantification by GC requires PHB extraction from freeze-dried biomass, which involves a laborious and time-consuming protocol that takes more than one day to complete (Lanham et al., 2013). Moreover, hazardous chemicals, like chloroform, are used during sample preparation. Considering effectiveness, especially at the laboratory level, there is a need for fast, cost-effective, and reliable analytical methods that can be easily integrated into routine workflows, allowing fast decision-making, such as cell harvesting when the maximum biopolymer content is achieved or addition of more feedstock.

Alternatives to GC for PHB quantification include fluorescence spectroscopy and flow cytometry (Table 3). Both methods use lipophilic fluorescent dyes, such as Nile Blue or its oxidized form, Nile Red.

Fluorescence spectroscopy operates on the principle that the emitted fluorescence intensity is directly proportional to the PHB concentration in the sample, making it a simple and reliable method for quantification (Rajankar et al., 2018; Zuriani et al., 2013). The method is promising for rapid PHB quantification because of its sensitive signals, high accuracy, time- saving and safe procedure. Furthermore, fluorescence spectroscopy facilitates the concurrent analysis of up to 96 samples via standard microplate readers, a marked contrast to the individual analysis of samples by GC, making it a valuable tool for researchers.

Recent advancements in the field have introduced two-dimensional (2D) fluorescence spectroscopy for real-time PHA content monitoring. Proposed by (Guarda et al., 2024),

this non-destructive, solvent-free, and non-invasive technique demonstrated outstanding potential for assessing intracellular PHA content in activated sludge. Notably, this method could predict new PHA content with an average error of 4 %dcw, showcasing its reliability and precision.

Flow cytometry is a highly sensitive technique used to analyze and separate cells based on their physical and chemical characteristics. For PHB quantification, Nile Blue or Red is sometimes avoided as they can bind to phospholipids, leading to false positives (Saranya et al., 2012). To address this, (Vidal-Mas et al., 2001) combined Nile Red with SYTO-13, a specific bacterial dye, ensuring accurate PHA quantification by excluding interference from extracellular lipids and free PHA granules. Alternative fluorochromes, such as LipidGreen and BODIPY, have also proven effective for rapid PHB quantification in *C. necator* and recombinant *E. coli* (Choi et al., 2015; Kettner et al., 2022).

Flow cytometry is also a powerful tool for rapid microbiome characterization, enabling the detection of population changes and serving as a sensitive indicator of potential contamination. Methods like 16S rRNA or 18S rRNA gene amplicon sequencing and FISH have been used to evaluate population characteristics within cultures, as discussed in section *2.2. Microbiome species identification*. However, flow cytometry presents a compelling alternative that potentially streamlines the process since it enables rapid, high-throughput, and real-time monitoring. While fluorescence spectroscopy and flow cytometry have shown promising results as tools for real-time process monitoring, the research in this area, particularly for complex systems, remains limited. While these methods offer fast, cost-effective protocols, most studies focus on pure cultures, with few addressing cyanobacteria or mixed microbial communities (heterotrophic and autotrophic).

In addition to quantitative analysis, qualitative analysis is crucial for characterizing the structure of the biopolymer produced, which is vital for understanding its physical and thermal properties (Mai et al., 2024). The importance of structural characterization becomes evident when considering the diverse properties exhibited by different PHAs. Spectroscopic methods such as infrared spectroscopy (IR) and Nuclear Magnetic Resonance (NMR) are commonly employed for the structural characterization of PHA (Table 3). IR spectroscopy provides insights into the functional groups present in the polyester and their interactions, while NMR helps identify different monomeric building blocks (Koller and Rodríguez-Contreras, 2015). Due to their time-consuming nature, these techniques are typically conducted after successful production processes to analyse further the produced PHA (Altamira-Algarra et al., 2024b; Ansari and Fatma, 2016; Koch et al., 2020). Fourier transform infrared spectroscopy (FTIR) has also been evaluated as a quantification method for PHA, correlating infrared spectra with reference PHB contents (Arcos-Hernandez et al., 2010; Kansiz et al., 2000). FTIR can even be used for biopolymer quantification in intact cells taken from both pure (Jarute et al., 2004; Kansiz et al., 2000) and mixed cultures (Arcos-Hernandez et al., 2010), further streamlining the process by eliminating the necessity for biomass freeze-drying. Although a step of sample drying is necessary (Arcos-Hernandez et al., 2010), (Jarute et al., 2004) developed an experimental setup designed for the online and automated monitoring of intracellular PHB in recombinant *E. coli*, making it feasible to use this approach as a routine protocol.

509 **Table 3.** Overview of techniques employed in microbial identification, PHA detection and determination.
510

Aim	Technique	Principle	Labor	Suitable for routine analysis	Advantages	Drawbacks	Ref.
Identification of PHB producing microorganisms	Microscopy	Staining	Low	Yes	Easy, fast, inexpensive, small sample required	Susceptible to errors by staining other lipophilic inclusions	(Altamira-Algarra et al., 2024b; Ansari and Fatma, 2016; Meixner et al., 2022; Thi et al., 2024)
	PCR	Gene amplification	High	No	Highly sensitive, highly selective, high throughput, small sample required	Limited taxonomic resolution, time-consuming	(Altamira-Algarra et al., 2023; Crognale et al., 2019; Meixner et al., 2022)
	FISH	Gene localization	High	No	Direct visualization	Complex probe design, time-consuming	(Crognale et al., 2022; Pei et al., 2022)
Quantification analysis	GC-FID	Separation of building blocks on nonpolar column and detection by FID	High	No	Highly sensitive, quantitative, and qualitative results	Time consuming, use of chloroform	(Lanham et al., 2013)
	Spectrofluorometry	Fluorescence emission	Low	Yes	Easy, fast, and inexpensive	Interference and background	(Guarda et al., 2024; Rajankar et al., 2018; Zuriani et al., 2013)
	Flow cytometry	Fluorescence emission	Low	Yes	Fast and high-throughput, single cell resolution	Sample preparation, expensive	(García et al., 2020; Saranya et al., 2012; Vidal-Mas et al., 2001)

Table 3. (Continued)

Aim	Technique	Principle	Labor	Suitable for routine analysis	Advantages	Drawbacks	Ref.
Qualitative analysis	IR spectroscopy	Absorption and emission of infrared radiation of PHA	Low	Yes	Short analysis time, non-invasive technique for PHB quantification	Cannot distinguish between heteropolyesters and blends of homopolyesters.	(Arcos-Hernandez et al., 2010; Kansiz et al., 2000)
	NMR	Interaction between atomic nuclei and magnetic fields	High	No	High resolution, distinguish between heteropolyesters and blends of homopolyesters,	Time consuming, requires polymer extraction	(Altamira-Algarra et al., 2024b; Ansari and Fatma, 2016; Koch et al., 2020)

511

4. Approach for large-scale PHB production

Scaling up photosynthetic cultures for large-scale PHB production encounters numerous challenges, complicating the shift from lab-scale to industrial-scale operations. Several pilot-scale studies (> 30 L PBRs) have explored PHB production using cyanobacteria cultures (Table 4). Notably, the most extended cultivation system operated for over eight months, using wastewater-borne cyanobacteria in demonstration-scale PBRs (11.7 m^3), with agricultural runoff as feedstock (Rueda et al., 2020b). The highest biopolymer content achieved at pilot scale was 23 % dcw, produced by a randomly mutated *Synechocystis* sp. in a 100 L thin layer raceway pond after 31 days of operation (Grivalský et al., 2024). However, engineered strains have limited application in open systems. Trials with wild-type strains have resulted in lower PHB accumulation (Table 4). Nonetheless, these studies demonstrate that scaling up cyanobacteria cultivation in closed or semi-closed systems over extended periods is feasible and pave the way for further exploration of scaling up PHB production.

Consistent with the reviewed trends, a notable approach for sustainable PHB production by cyanobacteria microbiomes involves a two-stage cultivation process: (i) an initial PBR dedicated to biomass growth, followed by (ii) a tank for PHB synthesis (Figure 3). The cells are first grown under photoautotroph metabolism (CO_2 and sunlight) to obtain the desired biomass concentration, followed by cultivation under dark conditions to achieve absence of oxygen, with organic carbon supplementation to produce PHB.

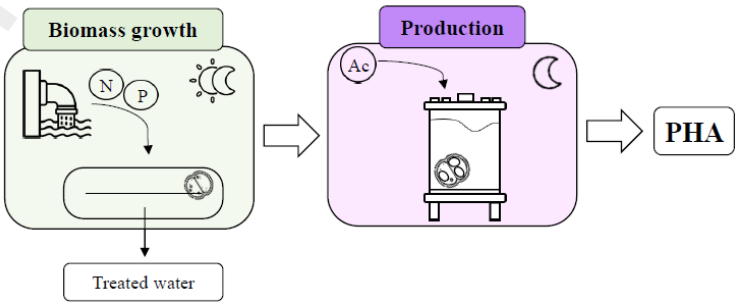

Figure 3. Schematic representation of a two-stage approach for large-scale PHB production: (i) biomass growth, followed by (ii) PHB synthesis.

First stage: biomass growth

During the initial stage, biomass grows and nutrients are consumed. While pure synthetic medium has been commonly employed in laboratory-scale studies, considering cheaper alternatives becomes essential for developing economically viable processes. Indeed, employing treated wastewater for cultivation can decrease the expenses related to PHB production by nearly half (Rueda et al., 2023). This approach not only lowers operational costs but also supports sustainability by reusing wastewater and its nutrient content, mitigating eutrophication by minimizing nutrient releases into aquatic environments.

Raw or partially treated wastewater, including urban wastewater and agro-industrial effluents, and anaerobic digestate, can potentially serve as a resource for cultivating cyanobacteria (Bhati and Mallick, 2016; Meixner et al., 2016). When properly managed, these cultures show remarkable nutrient removal efficiency, eliminating up to 99 % of nutrients from effluents (Arias et al., 2018c; Rueda et al., 2020b; Senatore et al., 2023). Nevertheless, using untreated waste effluents without any pre-treatment may not be optimal for cultures that rely on the specific microbiome composition to produce targeted bioproducts like PHAs. The presence of diverse (non-producers) microorganisms in these streams can lead to competition for resources and potential contamination from the overgrowth of other microorganisms, thereby reducing bioproduct yields (Arias et al., 2018a, 2018c; Troschl et al., 2018, 2017). For instance, in an open system culturing *Synechocystis* sp., contamination by grazers (*Poterioochromonas malhamensis*) was effectively controlled by maintaining a highly alkaline environment (pH ~10.5), without significantly affecting the culture's growth (Grivalský et al., 2024). Nevertheless, this approach did not successfully prevent

559 ciliated protozoa in another study working in a 200 L tubular PBR with *Synechocystis*
560 *salina* (Troschl et al., 2017).

561 Pretreated wastewater, such as secondary effluent from a municipal wastewater
562 treatment plant or certain industrial effluents with reduced organic matter and nutrients,
563 is more suitable in such cases. Additionally, incorporating pre-treatment steps, like UV
564 sterilization or autoclaving, may allow using urban wastewater or digestate (Grivalský
565 et al., 2024; Meixner et al., 2016). Therefore, further research should focus on
566 microbiome dynamics when using these effluents to ensure consistent bioproduct
567 synthesis and develop cultivation methods supporting robust cyanobacterial growth in
568 non-sterile environments.

569 The biomass growth stage can be carried out in a raceway pond using treated effluents
570 or specific industrial wastewater, allowing continuous microbiome growth (Figure 3).
571 This system consists of a shallow, open-channel pond with a continuous water
572 circulation to promote biomass growth. Raceway ponds are considered the most cost-
573 effective method for microalgal production, both in construction and operation
574 (Novoveská et al., 2023). As the culture moves through the system, cells remove
575 nutrients (such as nitrogen and phosphorus) through assimilation into biomass, thereby
576 enhancing water quality.

577 *Second stage: PHB synthesis*

578 Biomass from these raceway ponds is used to produce PHB in specific reactors.
579 Periodically, a certain volume of the raceway pond is pumped to the second stage
580 system. Prior, a first step includes biomass harvesting to obtain a concentrated slurry.
581 Here different procedures could be combined in sequential steps, such as centrifugation
582 or membrane filtration, being the latter a more promising technique (Singh and Patidar,
583 2018). This step is essential to separate the biomass from the medium, and ensure an

environment devoid of nutrients, which is a prerequisite for the adequate accumulation of PHB.

After, the biomass is inoculated in the reactor designed for biopolymer synthesis. This reactor is configured as a tank to minimize exposure to light and enable depleted dissolved oxygen concentrations. In addition, acetate is added to the reactor to stimulate biopolymer synthesis. According to (Altamira-Algarra et al., 2024b) this production stage could be operating for three days. Afterwards, a PHB recovery system will be implemented, following biomass harvesting, PHB extraction and PHB recovery.

All in all, this approach possesses transformative potential across three critical sectors: cyanobacteria cultivation, bioplastics innovation, and wastewater treatment advancements.

Table 4. Summary of PHB production studies conducted in large-scale photobioreactors (PBRs > 30 L) with cyanobacteria cultures.

Strain	PBR		Time	Culture conditions	PHB [%dcw]	Ref.
	Type	V [L]				
Synechococcus leopoliensis	Open thin-layer	200	16 d	Growth in mineral medium and two waste streams. PHB accumulation in 250 mL flasks for 10 d	1	(Mariotto et al., 2023)
Synechocystis sp. CCALA192	Tubular horizontal	200	21 d	Optimized BG-11 medium	7	(Troschl et al., 2017)
			26 d	Optimized BG-11 medium. Acetate supplementation.	6	
			40 d	Digestate supernatant	6	
Synechocystis sp. CCALA192	Tubular horizontal	200	75 d	Optimized BG-11 medium. Two-stage cultivation	13	(Troschl et al., 2018)
Synechocystis cf. *salina* Wislouch	Tubular horizontal	200	40 d	Diluted and autoclaved digestate	6	(Meixner et al., 2016)
Synechocystis sp. PCC6803	Serpentine	400	23 d	Sterilised BG-11 medium. Acetate supplementation.	4	(Elghazy et al., 2024)
Randomly mutated *Synechocystis* sp. PCC6714	Tubular vertical air-lift	40	10 d	Modified BG-11 medium	11	(Kamravamanesh et al., 2019)

Randomly mutated *Synechocystis* sp. PCC6714	Thin-layer raceway pond	100	31 d	Urban WW pre-treated by UV sterilization	23	(Grivalský et al., 2024)
Waswater-borne *Synechocystis* sp.	Tubular vertical	30	3 m	Growth with secondary effluent of urban-treated WW. PHB accumulation in 5 L reactor for 7 d	5	(Senatore et al., 2023)
Waswater-borne cyanonacteria microbiome	Tubular horizontal semi-closed	11,700	8 m	Agricultural runoff	5	(Rueda et al., 2020b)

Note. d: days; m: months; WW: wastewater

5. Directions for future research

Despite progress in PHB production with cyanobacteria microbiomes, the production yield remains the primary barrier to scaling up PHB production using photoautotrophic microbiomes. However, recent findings in implementing an iterative two-stage strategy offer promising avenues for improvement. This approach involves an initial biomass growth phase, followed by a PHB accumulation phase, with the process repeated multiple times. Building on this success, future research efforts should prioritize optimizing both stages to maximize overall yield. Here are key areas to focus on:

Optimization of biomass growth: Future research should focus on refining cultivation parameters to address the challenges related to microorganisms' growth. This involves adjusting variables such as temperature, pH, light intensity, nutrient availability, and oxygen levels to create an environment conducive to the desired microorganism's proliferation. This is especially crucial for open systems, where controlling these parameters is more challenging than in laboratory setups (Mariotto et al., 2023; Rueda et al., 2020b; Troschl et al., 2018).

PHB synthesis conditions: Understanding the optimal conditions for PHB synthesis in cyanobacteria is vital for achieving high production yields. Recognizing the importance of dark conditions and reduced/depleted dissolved oxygen levels in boosting PHB

synthesis, alongside the induction with organic carbon supplements such as acetate, future research should aim to refine these conditions further. Specifically, attention should be directed towards optimizing organic carbon supplement concentration, studying the impact of different regime types to identify the most beneficial approach, such as pulse-wise or continuous acetate supply, and exploring the potential of combining different organic carbon sources to synthesize various types of PHAs, aiming to the production of co-polymers. Future research should also prioritize comprehensive assessments of the intricate relationship between glycogen and PHB metabolisms focusing on optimizing glycogen metabolism to enhance PHB production.

Microbiome stability and composition: Ensuring the dominance of PHB-producing cyanobacteria within the microbiome is essential for stable and efficient PHB production (Altamira-Algarra et al., 2024a, 2024b). Strategies to selectively promote cyanobacteria growth over non-PHB producers like green microalgae have been developed, but further research is needed to effectively control non-PHB-producing microorganisms for optimal PHB production environments. Microscopic techniques can visualize PHB-producing cells, but may not be suitable for complex microbiomes. 16S rRNA amplicon sequencing or FISH provide insights into community composition, helping track microbiome changes. Additionally, flow cytometry offers a high-throughput, efficient approach for studying microbial dynamics. Hence, developing protocols for analyzing photosynthetic microbiomes has become a promising tool. Developing rapid and effective strategies to mitigate their growth should they unexpectedly emerge is crucial.

6. Cyanobacteria versus heterotrophic microbiomes

Cyanobacteria microbiomes combine the benefits of mixed cultures (such as non-sterility) alongside the inherent capabilities of cyanobacteria (e.g., CO_2 fixation,

641 sunlight utilization) while eliminating the need for aeration and decreasing the use of
642 organic carbon sources, which can account for up to 50% of the total cost (Troschl et
643 al., 2017). Photoautotrophic PHB production offers significant environmental benefits
644 over heterotrophic methods, which typically rely on sugars from cultivated plant
645 feedstocks. Recent life cycle assessments highlight the advantages of cyanobacteria-
646 based PHA in various impact categories, including global warming potential, freshwater
647 eutrophication, and land and water use (Koch et al., 2023). Nevertheless, it is important
648 to mention that research on heterotrophic microbiomes has explored the use of waste
649 streams enriched in volatile fatty acids to produce PHAs (Estévez-Alonso et al., 2021).
650 While supplementing with organic carbon holds promise for achieving high PHB
651 contents in cyanobacteria microbiomes (Table 1), their application could increase
652 production costs, potentially undermining the competitiveness of cyanobacteria-based
653 biopolymer production relative to heterotrophic bacteria. However, supplementation in
654 cyanobacteria cultures is much less demanding than the high substrate demands of
655 heterotrophs (about 10 times lower). Indeed, the addition of acetate to cyanobacteria
656 cultures typically ranges from 0.2 to 5 $g \cdot L^{-1}$, contrasting with the broader range of 1 to
657 50 $g \cdot L^{-1}$ commonly employed in activated sludge cultures (Altamira-Algarra et al.,
658 2024a; Estévez-Alonso et al., 2022; Monshupanee et al., 2016; Oliveira et al., 2017;
659 Rueda et al., 2022a). This difference in concentration arises because, in cyanobacteria,
660 acetate is a supplement to boost PHB synthesis, whereas in heterotrophic cultures, it is
661 utilized to support biomass growth.
662 Activated sludge from wastewater treatment plants is the most studied mixed culture for
663 PHB production under heterotrophic conditions. Lab-scale to pilot-scale studies (up to
664 500 L) have been conducted, showing PHB production varying from 20 to 80 %dcw
665 PHB (Estévez-Alonso et al., 2022; Morgan-Sagastume et al., 2020; Oliveira et al.,

2017). However, industrialization faces several challenges, including enriching PHA-accumulating bacteria, achieving high productivity with waste streams, and developing efficient downstream processes for bioproduct recovery (Estévez-Alonso et al., 2021).

7. Conclusions

Recent studies highlight the potential of cyanobacteria-rich microbiomes for PHB production, achieving high yields under non-sterile conditions over extended periods, offering new possibilities for the bioplastics industry.

Optimizing microbial community and culture conditions is key to achieving high PHB yields. On one hand, non-PHB-producing microorganisms negatively impact production, so strategies to control their growth are essential. On the other hand, cultivation factors such as acetate supplementation and dissolved oxygen levels within photobioreactors significantly enhance PHB synthesis.

Moreover, analytical techniques for identifying PHB producers and quantifying biopolymer offer fast and cost-effective solutions for real-time monitoring.

Future studies should explore scaling these findings to larger setups. While upscaling poses challenges, large-scale cyanobacteria cultivation in open systems appears feasible. A promising two-stage strategy integrates biomass growth in the first stage, followed by PHB synthesis in the second. These advancements pave the way for sustainable and scalable PHB production using cyanobacteria-rich microbiomes.

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Table 3. Overview of techniques employed in microbial identification, PHA detection and determination.

Aim	Technique	Principle	Labor	Suitable for routine analysis	Advantages	Drawbacks	Ref.
Identification of PHB producing microorganisms	Microscopy	Staining	Low	Yes	Easy, fast, inexpensive, small sample required	Susceptible to errors by staining other lipophilic inclusions	(Altamira-Algarra et al., 2024b; Ansari and Fatma, 2016; Meixner et al., 2022; Thi et al., 2024)
	PCR	Gene amplification	High	No	Highly sensitive, highly selective, high throughput, small sample required	Limited taxonomic resolution, time-consuming	(Altamira-Algarra et al., 2023; Crognale et al., 2019; Meixner et al., 2022)
	FISH	Gene localization	High	No	Direct visualization	Complex probe design, time-consuming	(Crognale et al., 2022; Pei et al., 2022b)
Quantification analysis	GC-FID	Separation of building blocks on nonpolar column and detection by FID	High	No	Highly sensitive, quantitative, and qualitative results	Time consuming, use of chloroform	(Lanham et al., 2013)
	Spectrofluorometry	Fluorescence emission	Low	Yes	Easy, fast, and inexpensive	Interference and background	(Guarda et al., 2024; Rajankar et al., 2018; Zuriani et al., 2013)
	Flow cytometry	Fluorescence emission	Low	Yes	Fast and high-throughput, single cell resolution	Sample preparation, expensive	(Degelau et al., 1995; Li and Wilkins, 2020a; Saranya et al., 2012; Vidal-Mas et al., 2001)

Table 3. (Continued)

Aim	Technique	Principle	Labor	Suitable for routine analysis	Advantages	Drawbacks	Ref.
Qualitative analysis	IR spectroscopy	Absorption and emission of infrared radiation of PHA	Low	Yes	Short analysis time, non-invasive technique for PHB quantification	Cannot distinguish between heteropolyesters and blends of homopolyesters.	(Arcos-Hernandez et al., 2010; Kansiz et al., 2000)
	NMR	Interaction between atomic nuclei and magnetic fields	High	No	High resolution, distinguish between heteropolyesters and blends of homopolyesters,	Time consuming, requires polymer extraction	(Altamira-Algarra et al., 2024b; Ansari and Fatma, 2016; Koch et al., 2020b)

Figure 1. Schematic workflow processes for PHA research, illustrating two approaches: the use of existing microorganisms and the exploration of novel microbial cultures. This review is focused on the latter, particularly cyanobacteria-dominated microbiomes from environmental samples. The step-by-step process for exploring these microbiomes for PHB synthesis is outlined, including: (1) sample procurement, (2) identification, (3) process optimization, and (4) PHB production.

Figure 2. Biosynthetic pathways of PHB and glycogen in cyanobacteria, highlighting the fate of Acetyl-CoA under aerobic and anaerobic conditions. Key enzymes are represented in yellow. For synthesis of PHB, PhaC: poly(3-hydroxyalkanoate) synthase; for glycogen synthesis, GlgA, glycogen synthase; and catabolism, GlgP1 and GlgP2: glycogen phosphorylase; and for the tricarboxylic acid (TCA) cycle, GltA: citrate synthase. Other abbreviations: CBB: Calvin-Benson–Bassham cycle; PGA: 3-phosphoglycerate.

Figure 3. Schematic representation of a two-stage approach for large-scale PHB production: (i) biomass growth, followed by (ii) PHB synthesis.