Production of polyhydroxybutyrate by the cyanobacterium cf. *Anabaena* sp.

Mara Simonazzi,[a] Laura Pezzolesi,[a,b] Paola Galletti,[b,c] Chiara Gualandi,[b,d] Rossella Pistocchi,[a,b] Nicole De Marco,[a] Zoe Paganelli,[a] Chiara Samorì[*,b,c]

[a] Dipartimento di Scienze Biologiche, Geologiche ed Ambientali (BiGeA), Università di Bologna, via S. Alberto 163, Ravenna (Italy)

[b] Centro Interdipartimentale di Ricerca Industriale Fonti Rinnovabili, Ambiente, Mare ed Energia (CIRI-FRAME), Università di Bologna, via S. Alberto 163, Ravenna (Italy)

[c] Dipartimento di Chimica "Giacomo Ciamician", Università di Bologna, via S. Alberto 163, Ravenna (Italy)

[d] Centro Interdipartimentale di Ricerca Industriale (CIRI) Meccanica Avanzata e Materiali (CIRI-MAM) Università di Bologna, Viale Risorgimento 2, Bologna (Italy)

[*]corresponding author: chiara.samori3@unibo.it

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Abstract

Polyhydroxybutyrate (PHB) production by the cyanobacterium cf. *Anabaena* sp. was here studied by varying the medium composition and the carbon source used to induce mixotrophic growth conditions. The highest PHB productivity (0.06 g_{PHB} $g_{biomass}^{-1}$ d^{-1}) was observed when cultivating cf. *Anabaena* sp. in phosphorous-free medium and in the presence of sodium acetate (5 g L^{-1} concentration), after an incubation period of 7 days. A content of 40% of PHB, a dry weight of 0.1 g L^{-1}, and a photosynthetic efficiency equal to the control were obtained. Cf. *Anabaena* sp. was then grown on a larger scale (10 L) to evaluate the characteristic of the produced PHB in relationship to the main composition of the biomass (the content of proteins, polysaccharides, and lipids): after an

incubation period of 7 days, a content of 6% of lipids (52% of which as unsaturated fatty acids with 18 carbon atoms), 12% of polysaccharides, 28% of proteins, and 46% of PHB was reached. The extracted PHB had a molecular weight of 3 MDa and a PDI of 1.7. These promising results demonstrated that cf. *Anabaena* sp. can be included among the Cyanobacteria species capable to produce polyhydroxyalkanoates (PHA) either in photoautotrophic or mixotrophic conditions, especially when it is grown under phosphorous-free conditions.

1. Introduction

Polyhydroxyalkanoates (PHAs) are a family of bio-based polyesters produced as intracellular inclusions in a wide variety of photosynthetic and heterotrophic organisms (e.g. the gram-negative bacterium *Cupriavidus necator*). The interest towards these biopolymers comes from their bio-based, biodegradable, compostable, and biocompatible nature, as well as on the possibility of tuning their chemo-physical characteristics by changing the carbon source used to feed the PHA-producers (the thermoplastic properties of PHAs can be tuned according to their co-monomer combination, that, in turns, is determined by the carbon source). These unique features make PHAs promising candidates for the replacement of petroleum-based non-biodegradable plastics. However, the current share of PHAs in the biopolymer market is limited to around 2% of all available biopolymers [1], and almost the entire global production is presently achieved by exploiting cultures of the gram-negative bacterium *C. necator*. Among the gram-negative bacteria known to produce PHAs, some species of Cyanobacteria have been described as capable of accumulating PHAs as secondary metabolites. Cyanobacteria are unicellular organisms constituting a phylogenetically coherent group of long evolutionary history prokaryotes, able to carry out oxygenic photosynthesis through chlorophyll *a*, as the major photosynthetic pigment, and phycobiliproteins that serve as light-harvesting pigments [2]. They are widely distributed throughout different aquatic and terrestrial environments, in which they play a key role in carbon and nitrogen fixation, thus impacting biogeochemical cycles in aquatic ecosystems as well as improving soil fertility [3]. Cyanobacteria are photoautotrophic and grow using

CO_2 as the sole source of carbon, although some strains have displayed an heterotrophic behavior especially when adapting to dark or semi-dark conditions, thus using other C-feedstocks like sugars under mixotrophic or heterotrophic regimes [4]. Cyanobacteria play significant roles both in the global primary production of the oceans (with a net primary production exceeding 1 Gt of wet biomass [2]), and in the biotechnological market [5], producing a variety of primary and secondary metabolites like proteins, phycobiliproteins (i.e. phycoerythrin and phycocyanin), and PHAs. PHAs can be produced by some Cyanobacteria strains via photosynthetic CO_2-fixation (photoautotrophic conditions) [6], and their PHA content is boosted when a mixotrophic metabolism is induced by the addition of carbon compounds (e.g. acetate) under specific growth conditions (e.g. lack of nitrate) [7]. The accumulation of PHAs by Cyanobacteria seems to be species-specific, even if a general low PHA-productivity is usually observed: the best PHA-cyanobacterial producers (e.g. *Nostoc muscorum*, *Synechococcus* sp., or *Synechocystis* sp.) are capable of accumulating comparable amounts of PHAs than heterotrophic bacteria (e.g. *Cupriavidus necator*) (40-50 *vs* 60-70%) but their PHA-productivity is 150-200-folds lower (in the order of g L^{-1} d^{-1} for Cyanobacteria *vs* g L^{-1} h^{-1} for heterotrophic bacteria, respectively) [7]. The relatively lower PHB amount and productivity observed for Cyanobacteria have been ascribed to their larger cellular size and mass with thicker cell walls than heterotrophic bacterial cells [8], confirming similar PHA-synthesizing ability [9]. Moreover, it has been estimated that the costs related to the carbon source (the amount of exogenous carbon is more than one order of magnitude lower if compared to that needed by heterotrophic bacteria [6]), the nutrient requirements (Cyanobacteria can be cultivated in wastewaters, using inorganic nitrogen and phosphorous for their metabolism), and the oxygen supply necessary in PHAs production processes could be lower when Cyanobacteria are used than when heterotrophic bacteria like *C. necator* are used, *de facto* lowering the final PHAs cost [8–11]. The capability of fixing nitrogen seems not to be directly correlated to the capability of producing PHAs, since among the best PHA-cyanobacterial producers, there are both nitrogen-fixing (e.g. *Nostoc muscorum*) and non-fixing Cyanobacteria (e.g. *Synechocystis* sp. and *Synechococcus* sp.) [12]. Among the diazotrophic Cyanobacteria, *Anabaena*

cylindrica has been reported to accumulate PHAs; however, this characteristic is not common to the other species of the *Anabaena* genus [13]. Although *A. cylindrica* accumulates polyhydroxybutyrate (PHB, the homopolymer constituted by 3-hydroxybutyrate units) when grown with acetate as carbon source and under nitrogen starvation, nor *A. variabilis* neither *A. torulosa* were able to accumulate the polymer under similar conditions [13–15]; the maximum PHB content accumulated by *Anabaena* spp. Was 2% when cultivated with acetate and without nitrogen, 10-20 times lower than what was observed for other nitrogen-fixing Cyanobacteria such as *Nostoc muscorum* [12,13].

The present paper aims at deepening the knowledge on PHB production by a newly isolated strain of the genus *Anabaena* to better understand its potential as a PHB producer. The growth conditions were initially optimized to maximize PHB accumulation, and then the cultivation was scaled up (10 L) to study the whole biomass composition (in terms of PHB, lipids, fatty acids, polysaccharides, and proteins) and the PHB recovery in terms of extraction yields and PHB characteristics.

2. Materials and methods

2.1. Chemicals. All reagents, solvents and chemicals used in this work were of analytical grade and purchased from various commercial suppliers. Commercial polyhydroxybutyrate (PHB) was bought from Biomer (Germany).

2.2. Cf. *Anabaena* sp. isolation. The cyanobacterial strain was isolated from surface water collected in Reno river (Emilia-Romagna, Italy) in July 2018, and morphologically identified as cf. *Anabaena* sp. (hereafter referred to as *Anabaena* sp.) using an inverted light microscope (ZEISS Axiovert 100) at 320x magnification according to Komárek [16]. Single trichomes were manually isolate through capillary pipetting and carefully washed 2-3 times with sterile distilled water before inoculation in 24-well plates.

2.3. *Anabaena sp.* growth under balanced and mixotrophic conditions. Mono-specific cultures (0.2 L) of *Anabaena* sp. were grown in Erlenmeyer flasks under balanced nutritional conditions, using Jaworski's medium (hereafter JM) [17], at a temperature of 27±2 °C, and light intensity of 90-120 µmol m^{-2} s^{-1} (16:8 h light:dark photoperiod). Cyanobacterial biomass at the beginning of stationary phase was collected by centrifugation and subsequently transferred in a low-light environment (30-50 µmol m^{-2} s^{-1}) using modified JM media lacking nitrogen and/or phosphorous (ØN medium, ØP medium or ØNP medium). To stimulate mixotrophy, cultures were subjected to the addition of different carbon sources at various concentrations. The tested conditions were:

i) growth in nitrogen-free JM medium (ØN), with the addition of 5 g L^{-1} sodium acetate (hereafter NaOAc);

ii) growth in phosphorous-free (ØP) or phosphorous- and nitrogen-free (ØNP) JM media, with the addition of 5 g L^{-1} NaOAc;

iii) growth in ØP medium, varying the concentration of NaOAc at 0.5, 1.0, 2.5 g L^{-1};

iv) growth in ØP medium, varying the carbon source (glucose at 0.5, 1.0, 2.5, 5 g L^{-1}).

All tested conditions were compared to control cultures grown in balanced JM medium but without exogenous C sources and exposed to the same conditions of low-light and temperature as for the mixotrophic ones.

Photosynthetic activity (%) of *Anabaena* sp. during the growth under balanced or mixotrophic conditions was evaluated through PAM fluorometry measurements, according to Samorì et al. [18]. Kinetics and parameters of Photosystem II (PSII) were measured by pulse-amplitude modulated fluorometry. The model used was: 101-PAM (H. Walz, Effeltrich, Germany) connected to a PDA-100 data acquisition system, red high-power LED Lamp Control unit HPL-C and LED-Array-Cone HPL-L655 to supply saturated pulses, US-L655 and 102-FR to provide far red light and measuring light, respectively. Before and after the tests, subsamples of *Anabaena* sp. cultures (3 mL) were analyzed in 4-clear sided quartz cuvettes (10x10 mm) mounted on an optical unit ED-101US/M.

Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (ΦPSII) and calculated as:

$$\Phi_{PSII} = \frac{F_m - F_0}{F_m} = \frac{F_v}{F_m}$$

The samples were dark-adapted for 20 min, and minimal fluorescence (F_0) was measured using modulated light of low intensity (2 µmol m^{-2} s^{-1}). Then, a short saturating pulse of 3000 µmol m^{-2} s^{-1} for 0.8 s induced the maximal fluorescence yield (F_m). Photosynthetic activity (%) was calculated by dividing the maximum quantum yield of PSII (ΦPSII) of the cultures grown under mixotrophic conditions with the maximum quantum yield of PSII (ΦPSII) of the control sample expressed as percentage.

2.4. Cultivation scale-up. A scale-up cultivation of *Anabaena* sp. was performed using glass bottles of 10 L, monitoring the growth every two days per 7 days in a ØP medium with the addition of 5 g L^{-1} NaOAc. The growth was monitored as dry weight (g L^{-1}), filtering an aliquot of the culture (20 mL) with pre-weighted glass microfiber filters (Whatman GF/F, 47 mm, nominal pore size 0.7 µm). The filter was then washed with a double volume of distilled water (40 mL), and subsequently dried at 105°C for 2 h before gross weight determination.

2.5. PHB content determination. Freeze-dried samples of *Anabaena* sp. (10 mg) or standard PHB (1-2 mg) were charged in screw-cap vials (4 mL volume, 50 mm high) and then placed on a heating plate at 350°C. After 20 min, the vials were removed from the heating plate and let cooling down to RT before adding the internal standard (2-ethylbutanoic acid, 0.1 mL of a solution 5000 ppm in acetonitrile). The sample was then diluted with acetonitrile (4 mL) and analyzed by GC-MS as described below (section 2.10) [19]. PHB content was expressed on biomass weight basis ($g_{PHB}/g_{biomass}$ %). Specific PHB productivity ($g_{PHB}\ g_{biomass}^{-1}\ d^{-1}$) has been calculated based on the cultivation period (3, 5, 7, 11, or 15 d) as follows:

$$PHB\ productivity\ \left(\frac{g\ PHB}{g\ biomass * d}\right) = \frac{PHB\ content\ \left(\frac{g\ PHB}{g\ biomass}\%\right)}{cultivation\ period\ (d)}$$

2.6. PHB extraction and characterization. Freeze-dried samples of *Anabaena* sp. (100 mg) were extracted with chloroform (50 mL) for 2 h under reflux and under magnetic stirring. The extraction was repeated twice, then the solvent phases were collected, centrifuged at 4000 rpm for 2 min, filtered with polypropylene membrane filters of 0.45 μm porosity, and dried under vacuum. PHB films precipitated after the addition of acetone (10 mL); the films were washed several times with acetone and then dried overnight at 40°C. PHB yield was calculated gravimetrically on *Anabaena* sp. biomass weight basis ($g_{PHB}/g_{biomass}$%). PHB recovery (%) was calculated as follows:

$$PHB\ recovery\ (\%) = \frac{PHB\ yield\ (g\ PHB/g\ biomass\%)}{PHB\ content\ in\ Anabaena\ sp.\ (g\ PHB/g\ biomass\%)}$$

where the determination of PHB content in *Anabaena* sp. biomass has been described in section 2.5. Molecular weight and polydispersity index of the recovered PHB were determined in a chloroform solution by size exclusion chromatography (SEC) using an HPLC Lab Flow 2000 apparatus working

with a 1 mL min^{-1} flow, equipped with an injector Rheodyne 7725i, a Phenomenex Phenogel 5u 10E6A column and a RI detector Knauer RI K-2301. Calibration curves were obtained using several monodisperse polystyrene standards.

Thermogravimetric analyses (TGA) were carried out using a TGA Q500 thermogravimetric analyzer (TA Instruments). Analyses were performed from RT to 600 °C, at a heating rate of 10 °C min^{-1}, under nitrogen flow. The temperature of degradation (T_{deg}) was taken from the peak maximum in the corresponding derivative curve.

Thermal transitions were measured by means of a differential scanning calorimeter (DSC Q100; TA Instruments), equipped with a refrigerated cooling system (RCS). Samples, under nitrogen flow, were subjected to a first heating scan at 20 °C min^{-1}, from -90 °C to 195 °C, to erase polymer thermal history. The samples were then cooled at 10 °C min^{-1} and a second heating scan was applied where the glass transition temperature (T_g) was taken at half-height of the glass transition heat capacity step, while the melting temperature (T_m) was taken at the peak maximum of the melting endotherm. The degree of crystallinity (χ_c) was calculated as follows:

$$\chi_c = \frac{\Delta H_m}{\Delta H_m^0} \cdot 100$$

Where ΔH_m is the melting enthalpy associated to the heating scan and ΔH_m^0 0 is the theoretical melting enthalpy of the 100% crystalline PHB, equal to 146 J g^{-1} [20].

The elemental composition of the extracted polymer was determined using an elemental analyzer (Thermo Scientific, Flash2000, Organic Elemental Analyzer) by means of the flash combustion technique.

2.7. Protein content determination. Protein determination was performed on freeze-dried *Anabaena* sp. biomass (10-15 mg). The samples were extracted with 3.0 mL of NaOH (0.5 M) and incubated at 90°C for 8 min under magnetic stirring, then transferred in ice for 2 min, and subsequently centrifuged (2550×*g*, 10 min). The resulting supernatant was collected, and the extraction procedure was repeated three-times. Protein content was determined on the collected supernatant with the Folin phenol reagent according to Lowry et al. [21].

2.8. Polysaccharide content determination. Intracellular polysaccharides were extracted from freeze-dried samples (5-10 mg) according to Myklestad and Haug [22], and quantified spectrophotometrically through the phenol-sulfuric acid colorimetric reaction [23].

2.9. Lipid content determination and total fatty acids (TFA) analysis. Freeze-dried samples of *Anabaena* sp. (100 mg) were extracted with a mixture of methanol (1 mL) and chloroform (2 mL) for 2 h at 50°C under magnetic stirring. The extraction was repeated three times, then the solvent phases were collected, centrifuged at 4000 rpm for 10 min, and dried under nitrogen. Lipid samples (about 2 mg) were dissolved in DMC (0.4 mL). 2,2-dimethoxypropane (0.1 mL) and 0.5 M NaOH in MeOH (0.1 mL) were then added; the samples were placed at 80°C for 30 min. After cooling for 5 min to RT, 1.3 M BF_3-methanol 10% (w/w) reagent (0.7 mL) was added before repeating the incubation at 70°C for 30 min. After cooling for 5 min to RT, saturated NaCl aqueous solution (2 mL) and hexane (1 mL) containing methyl nonadecanoate (0.02 mL of a solution 1000 ppm in hexane) were added and the samples were centrifuged at 4000 rpm for 1 min. The upper hexane-DMC layer, containing TFA, was then analyzed by GC-MS as described below (section 2.10).

2.10. GC-MS analysis. GC-MS analysis to determine the PHB content of *Anabaena* sp. biomass was performed using an Agilent 7820A gas chromatograph connected to an Agilent 5977E quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated on a DB-FFAP polar column (30 m length, 0.25 mm i.d., 0.25 μm film thickness), with helium flow of 1 mL min^{-1}. Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 29-450 m/z range. The temperature of the column was set to 50°C (5 min) and increased to 250°C (10°C min^{-1}). GC-MS analysis to determine the total fatty acids (TFA) content of the lipid fraction extracted from *Anabaena* sp. biomass was performed using a 6850 Agilent HP gas chromatograph connected to a 5975 Agilent HP quadrupole mass spectrometer. The injection port temperature was 280°C. Analytes were separated by a HP-5 fused-silica capillary column (stationary phase poly[5% diphenyl/95% dimethyl]siloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness), with helium as carrier gas (at constant pressure, 33 cm s^{-1} linear velocity at 200°C). Mass spectra were recorded under electron ionization (70 eV) at a frequency of 1 scan s^{-1} within the 12-600 m/z range. The temperature of the column was increased from 50°C up to 180°C at 50°C min^{-1}, then from 180°C up to 300°C at 5°C min^{-1}.

3. Results and Discussion

3.1. *Anabaena* sp. growth under balanced and mixotrophic conditions

Anabaena sp. was initially cultivated in a nitrogen-free (ØN) growth medium with sodium acetate (NaOAc, 5 g L^{-1}); the culture was placed in a low-light environment for 7 and 11 d and compared with the control (balanced growth medium in a low-light environment) in terms of PHB content, dry weight, and photosynthetic efficiency (Table 1, entries 1-3). As expected, *Anabaena* sp. grown in a balanced medium with no additional source of NaOAc did not accumulate PHB (entry 1), in line with what was reported in the literature [13]. The addition of exogenous carbon sources like NaOAc was mandatory for achieving stimulation of PHB accumulation, reaching a value of 22.7 ± 2.0% (entry

2). A decrease in PHB content (14%) was observed when the cultivation period was increased up to 11 d (entry 3), presumably due to the consumption of PHB operated by *Anabaena* sp. for its metabolism. The photosynthetic efficiency of *Anabaena* sp. grown under balanced or mixotrophic conditions did not substantially change over the test period, indicating a comparable and healthy status of the cells. Therefore, giving these promising results, optimization of the conditions inducing a higher PHB accumulation was performed by varying the medium composition, the concentration of NaOAc, and the carbon source.

Effect of medium composition

Growth media without phosphorous (ØP, Table 1, entries 4-6) or without both phosphorous and nitrogen (ØNP) (Table 1, entries 7-9) were tested in combination with a concentration of 5 g L^{-1} of NaOAc. The PHB production was largely boosted when *Anabaena* sp. was grown in a ØP medium (entry 4): PHB content doubled from 22.7 % (entry 2) to almost 40%, then a progressive decrease to a value of 10% was observed after 15 d (entries 5 and 6); this doubling was already observed for *Synechococcus* sp. fed with CO_2 in nitrate- or phosphate-free media [24]. The same PHB content and progressive decrease by time were found when *Anabaena* sp. was cultivated in a ØNP medium (entries 7, 8, and 9). The dry weight achieved in a ØP medium (entry 4) was 2-times higher (0.1 g L^{-1}) than the control (entry 1), the ØN condition (entry 2), and the ØNP condition (entry 7). The photosynthetic efficiency of cells grown in the ØP medium did not substantially change over the test period in comparison to the control, whereas a progressive decrease was observed for the cells grown with NaOAc in a ØNP medium (from 80% of the photosynthetic efficiency of the control after 7 days to 21% after 15 days). These findings suggested that:

- the absence of phosphate from the cultivation medium is a key factor for boosting PHB accumulation, confirming the fact that PHB biosynthesis is promoted by a nutritional deficiency of key nutrients (e.g. nitrogen or phosphorous) and that this regulation could be strain specific.

Specifically, lack of phosphate could affect the balanced intracellular formation of ATP and NADPH through photosynthesis, thus leading to a reducing potential responsible for the metabolic shift to PHB synthesis [25]; this deficiency was effective for species like *Synechococcus* sp., *Arthrospira platensis* and *Nostoc muscorum* [11,24,26], indicating that 2-4-folds rise in PHB accumulation can be achieved just by manipulating nutrient supply and cultivation conditions [11];

- even though capable of nitrogen-fixation, *Anabaena* sp. needed nitrogen supply to sustain its growth and reaching a higher dry weight, and higher PHB productivity;
- *Anabaena* sp. reached its maximum potential of PHB accumulation in 7 d, in line with other cyanobacterial species (e.g. *Synechococcus* sp.) [24];
- *Anabaena* sp. partially consumed its intracellular PHB after longer cultivation periods for its cellular metabolism, as already observed for both heterotrophic and other phototrophic microorganisms [12].

Effect of NaOAc concentration

Since a wide range of NaOAc concentrations have been tested for inducing PHB accumulation in various Cyanobacteria demonstrating that the optimal dose of exogenous carbon could be strain-specific, herein concentrations of 0.5, 1 and 2.5 g L^{-1} were also tested (Table 1, entries 10-12). PHB content increased by increasing NaOAc concentration, varying between 34% (0.5 g L^{-1}, entry 10) to 46% (2.5 g L^{-1}, entry 12). However, as NaOAc concentration increased, the dry weight decreased from 0.06 to 0.04 g L^{-1}, as well as the photosynthetic efficiency (from 100% when the cells were treated with 0.5 g L^{-1}, to 74% at the dose of 2.5 g L^{-1}).

Effect of different carbon sources

Since the use of glucose increased by 3-times the accumulation of intracellular PHB in a strain of *A. cylindrica* than under phototrophic growth conditions [13], this carbon source was also tested at

concentrations of 0.5, 1, 2.5 and 5 g L^{-1} (Table 1, entries 13-16) in a ØP medium. No one of the doses tested was capable to induce an accumulation of PHB higher than 10%, and the dry weight of the cultures was lower than the control (even if the photosynthetic efficiency was not different from the control). Thus, NaOAc was confirmed as a better carbon source than glucose, presumably for its direct utilization in the synthesis of PHB (glucose is claimed to have a cascade effect boosting the synthesis of reduced cofactor fundamental for the enzyme acetoacetyl-CoA reductase involved in the synthesis of PHB) [11].

This initial screening phase allowed the definition of the best cultivation protocol oriented to the maximum PHB accumulation in *Anabaena* sp.: the combination of 7 days of growth in a medium lacking phosphate with 5 g L^{-1} of NaOAc as carbon source (Table 1, entry 4) provided 40%, the highest dry weight (0.1 g L^{-1}), and a specific PHB productivity of 0.06 g_{PHB} $g_{biomass}^{-1}$ d^{-1}, in line with what was reported for *Synechococcus* sp. or *Nostoc muscorum* grown in a phosphate-free medium [24,26]. Moreover, the dry weight reached a maximum value of 0.1 g L^{-1}, and the photosynthetic efficiency was not different from the control.

3.2. Scaled-up production of PHB by *Anabaena* sp.

When scaled-up in a 10-L cultivation system, *Anabaena* sp. accumulated PHB analogously to what occurred on a lower scale (0.2 L), with similar specific PHB productivity after 7 days of cultivation (0.06 g_{PHB} $g_{biomass}^{-1}$ d^{-1} *vs* 0.07 g_{PHB} $g_{biomass}^{-1}$ d^{-1}, respectively). PHB content progressively increased from 21.4±1.6% (d 3, Table 2, entry 2) to 32.1±2.4% (d 5, entry 3), reaching 46.4±5.9% at the end of the cultivation period (d 7, entry 4). The dry weight was 0.2±0.02 and 0.1±0.02 g L^{-1} after 3 and 5 d, respectively, whereas it drastically decreased the last day, reaching a value of 0.02±0.001 g L^{-1}. However, also the dry weight of the control sample followed a similar trend, suggesting that the scaling-up performed in a low-light environment with a less efficient mixing or aeration of the culture could somehow hamper the growth on prolonged cultivation periods. The photosynthetic efficiency

decrease seemed to confirm this hypothesis (Table 2), when compared to the higher values observed on a lower scale (Table 1, entries 4-6).

The lipid content of the control was stable over the cultivation period and 2-times higher (about 20%) than that achieved under mixotrophic conditions (about 8%) (Fig. 1); also the polysaccharide and protein content remained stable over time in the control (22 and 36%, respectively), but when mixotrophic conditions were applied the composition changed: proteins slightly decreased (30%) and remained constant over the time, whereas polysaccharides drastically decreased the last day of the experiment (from 26-29% to 12%) (Fig. 1). Similarly, the lack of phosphorous in the growth medium (eventually coupled to the addition of an external carbon source) led to a marked decrease in exopolysaccharide (EPS) production by different *Anabaena* strains (i.e. *A. cylindrica*, *Anabaena* sp., and *A. torulosa*) [13,27]. It is known that specific nutrient limitation can finely tune the biochemical composition and growth of Cyanobacteria and microalgae, and here the absence of phosphate seemed to have a greater impact on lipids and polysaccharides than on proteins. However, the decrease in lipids could also occur as a direct consequence of PHB accumulation with time, since the carbon normally used for biosynthesizing storage-lipids can be diverted to PHB synthesis (as occurred for *Synechococcus subsalsus* and *Arthrospira* sp.) [28].

The amount of the total fatty acids (TFA, bounded as esters in triglycerides, phospholipids, or glycolipids, or in free form) in the lipid extract was higher in *Anabaena* sp. cultivated under mixotrophic conditions (18-25 $g_{TFA}\ g_{lipids}^{-1}$ %) than in the control (14-18 $g_{TFA}\ g_{lipids}^{-1}$ %). The TFA profile was also different among the two conditions, confirming it as a "fingerprint" for a certain strain when grown under a specific cultivation mode (Table 3): the percentage of unsaturated fatty acids, as well as the percentage of C18-compounds, was higher under mixotrophic conditions than balanced ones, and polyunsaturated fatty acids (e.g. arachidonic acid and eicosapentenoic acid, C20:4 and C20:5, respectively) were present only when NaOAc was added in a ØP medium. Therefore, the ratio "$saturated_{TFA}$/$unsaturated_{TFA}$" was higher under balanced conditions than mixotrophic ones.

3.3. PHB extraction and characterization

The extraction of PHB from heterotrophic bacteria is a challenging issue strictly connected to the characteristics of the polymer itself and of the surrounding bacterial biomass [29]. If the downstream phase is gaining attention in the field of heterotrophic PHAs productions for the associated environmental and economic impacts, this aspect is almost neglected in the case of Cyanobacteria [7]: a scarcity of data is found in terms of recovery strategies, recovery percentages, and polymer molecular weight. Just a few authors have clearly underlined that the extraction of PHAs from Cyanobacteria could be as challenging as for heterotrophic bacteria, since cyanobacterial PHAs are accumulated as spherical granules (100-800 nm diameter) [15], closely associated with thylakoid membranes (evidences reported for *Synechocystis* sp., *Synechococcus*, and *A. platensis*) [30–32]. However, the association "thylakoids-PHAs" seems to be strain-specific, even within the same cyanobacterial species (in *Synechococcus* sp. MA19 PHB granules are surrounded with the thylakoid membranes while this holds not true for *Synechococcus* sp. PCC7942) [33]. Herein, PHB from *Anabaena* sp. was extracted with chloroform (the solvent of choice for PHAs extraction from heterotrophic bacteria) after 3, 5, and 7 d of mixotrophic growth, and characterized in terms of elemental composition (Table 4), molecular weight, polydispersity index, and thermal properties (Table 5). The yield of the extracted PHB was constant, independently by the time and the initial PHB content, therefore the recovery decreased by the time from almost 60% to 26% (Table 4). A low polymer recovery was also observed in *Synechocystis* sp. by Sudesh et al., who reported the presence of intact intracellular PHAs granules even after long extraction in hot chloroform [30]. These data highlight the need for further investigations specifically addressed to downstream processing and strategies for recovery of PHB from Cyanobacteria, and that there is an analogy with the extraction issue observed for heterotrophic bacteria, confirming the downstream phase as one of the most critical parts of the overall PHAs production [29]. The PHB that was here recovered from *Anabaena* sp. after 3, 5, and 7 d of mixotrophic growth had two peculiar characteristics:

- very high molecular weight (above 3 MDa) and low PDI (1.6-1.7) (Table 5); the M_w values here obtained were higher than what previously obtained with other Cyanobacteria like *Synechocystis* sp. (0.5 MDa) [30], *Synechococcus subsalsus* or *Spirulina* sp. (0.2 MDa) [28], confirming that the polymer length is strain-specific. The PDI value was largely lower than what was previously obtained with *Synechocystis* sp. (4.3), suggesting a more homogeneous polymer; the thermal properties of the PHB films produced by *Anabaena* sp. after 5 and 7 d were comparable with those of PHB obtained from other Cyanobacteria and bacteria (*C. necator*) [34].
- a time-dependent pigmentation, from deep green (d 3) to light green (d 5), and light pink (d 7) (Fig. 2). The association between PHB granules and thylakoid membranes found for other Cyanobacteria was suggested to be responsible for the great difficulty in separating PHB from photosynthetic pigments, especially chlorophyll *a* [7,12,32]. Even if an *ad-hoc* investigation of the location of PHB granules in *Anabaena* sp. was not done, the color of the extracted PHB films seemed to suggest a similar association with thylakoid membranes, and consequent contamination of the extracted PHB with photosynthetic pigments. The elemental analysis of the extracted PHB (Table 4) revealed that the composition of the PHB recovered after 7 d was almost identical to the one of commercial PHB (C 55.6±0.8%, H 7.0±0.1%) with just a minimal trace of nitrogen, a possible indication of the presence of N-containing compounds like photosynthetic pigments (e.g. chlorophyll *a*, phycobiliproteins); on the other hand, the PHB recovered after 3 d was characterized by a relevant amount of N (more than 6%), and the carbon content was largely lower than the one of commercial PHB, suggesting that this polymer was the most "contaminated" by pigments among the three ones.

4. Conclusion

The mixotrophic production of PHB by Cyanobacteria is rarely studied in comparison to the heterotrophic one operated by non-photosynthetic microorganisms. However, some cyanobacterial

strains have been reported to accumulate high PHB amounts, comparable to what occurs for heterotrophic bacteria. The unique potential of Cyanobacteria to produce PHB exploiting CO_2 as the sole carbon source can be boosted when an exogenous carbon source is provided, nutrients are limited, or stress conditions are applied. Moreover, Cyanobacteria can grow even under extreme conditions and environments, coupling the production of secondary metabolites like PHB or phycobiliproteins with other activities like CO_2 capture or wastewater remediation. The cyanobacterium cf. *Anabaena* sp. here studied behaved like this, demonstrating its ability to produce PHB under mixotrophic conditions. A 40% content of PHB was observed in 7 days under phosphorous-deficiency conditions and with the addition of 5 g L^{-1} of NaOAc, both at 0.2 L and 10 L-scale. This value is among the highest ones reported for Cyanobacteria. Moreover, the benthic and filamentous nature of cf. *Anabaena* sp. could facilitate an easier and more cost-effective harvesting of biomass than other unicellular and/or planktonic Cyanobacteria (e.g. *Synechococcus* sp.). Moreover, the characteristics of the isolated PHB (molecular weight, polydispersity index, and thermogravimetric properties) were similar to those observed for chemoheterotrophic bacterial PHB, suggesting their equivalency in the biopolymer scenario. Lastly, the biochemical composition (proteins, polysaccharides, and lipids) of cf. *Anabaena* sp substantially changed under mixotrophic conditions from 3 to 7 d, especially in terms of lipids and polysaccharides that decreased as PHB was accumulated, suggesting strong metabolic plasticity of cf. *Anabaena* sp. that can be exploited to optimize PHB productivity.

Author contributions

C.S. and M.S. have made substantial contributions to the conception and design of the study; C.G., M.S., N.D., and Z.P. have made substantial contributions to the acquisition, collection and assembly of data; C.S., M.S., L.P., and C.G. have made substantial contributions to the analysis and interpretation of data; C.S. has made substantial contributions to the drafting of the article; P.G., R.P. and L.P. have made substantial contributions to the critical revision of the article for important

intellectual content; all authors have made substantial contributions to the final approval of the version to be submitted.

Declaration of Competing Interest

The Authors declare no conflict of interest.

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Tables

Table 1. PHB accumulation by *Anabaena* sp. under different growth conditions, related dry weight (g L^{-1}) and photosynthetic efficiency (%) determined after 7, 11 or 15 d of cultivation.

Entry	Growth conditions	PHB content (%)	Dry weight (g L^{-1})	Specific PHB productivity (g_{PHB} $g_{biomass}^{-1}$ d^{-1})	Photosynthetic efficiency (%)
1	Balanced medium 7 d	-	0.04	-	-
2	ØN NaOAc 5 g L^{-1} 7 d	22.7±2.0	0.05	0.03	100
3	ØN NaOAc 5 g L^{-1} 11 d	14±1.6	0.07	0.01	100
4	ØP NaOAc 5 g L^{-1} 7 d	39.7±1.8	0.10	0.06	100
5	ØP NaOAc 5 g L^{-1} 11 d	25.5±2.1	0.03	0.02	100
6	ØP NaOAc 5 g L^{-1} 15 d	10.4±1.7	0.07	0.007	100
7	ØNP NaOAc 5 g L^{-1} 7 d	39.6±2.3	0.04	0.06	80
8	ØNP NaOAc 5 g L^{-1} 11 d	22.0±1.8	0.03	0.02	38
9	ØNP NaOAc 5 g L^{-1}	4.1±0.9	0.02	0.003	21

	15 d ØP				
10	NaOAc 0.5 g L^{-1} 7 d ØP	33.9±2.8	0.06	0.05	100
11	NaOAc 1 g L^{-1} 7 d ØP	37.4±3.0	0.07	0.05	96
12	NaOAc 2.5 g L^{-1} 7 d ØP	45.6±3.2	0.04	0.06	74
13	glucose 0.5 g L^{-1} 7 d ØP	9.5±1.2	0.03	0.01	100
14	glucose 1 g L^{-1} 7 d ØP	5.9±1.1	0.03	0.008	100
15	glucose 2.5 g L^{-1} 7 d ØP	9.7±1.5	0.03	0.01	91
16	glucose 5 g L^{-1} 7 d	10.6±2.2	0.03	0.01	89

Table 2. PHB accumulation by *Anabaena* sp. after 3, 5 and 7 d of balanced or mixotrophic growth (ØP medium and 5 g L^{-1} of NaOAc) at 10-L scale, and related dry weight (g L^{-1}) and photosynthetic efficiency (%).

Entry	Cultivation period (d)	PHB content (%)	Dry weight (g L^{-1})	Specific PHB productivity (g_{PHB} $g_{biomass}^{-1}$ d^{-1})	Photosynthetic efficiency (%)
1	3	21.4±1.6	0.17±0.02	0.07	71±3
2	5	32.1±2.4	0.10±0.02	0.06	51±2
3	7	46.4±5.9	0.02±0.001	0.07	78±5

Table 3. Fatty acid composition (%) of *Anabaena* sp. after 3, 5 and 7 d of balanced or mixotrophic (ØP medium and 5 g L^{-1} of NaOAc) growth at 10-L scale.

Fatty acid	**Relative abundance (%)**					
	Balanced medium			*Phosphate-free medium*		
	d3	*d5*	*d7*	*d3*	*d5*	*d7*
C14:0	1.1	1.2	1.2	0.9	1.8	0.9
C15:0	0.6	0.7	0.8	0.2	0.3	0.3
C16:1	22.3	21.3	20.3	18.4	15.5	15.1
C16:0	31.9	28.7	25.4	17.0	18.2	18.5
C17:0	0.5	0.5	0.5	0.3	0.4	0.3
C18:3/C18:2/C18:1	37.3	40.5	43.8	55.0	51.2	51.9
C18:0	3.1	3.3	3.4	3.7	6.9	4.5
C20:3	-	0.3	0.6	-	-	-
C20:4	-	-	-	0.3	0.4	0.7
C20:5	-	-	-	0.8	1.0	1.2
Unidentified	3.2	3.6	4.0	3.4	4.2	6.7
Σ saturated/ Σ unsaturated	0.6	0.6	0.5	0.3	0.4	0.3

Table 4. Extraction yield, recovery, and elemental analysis of PHB obtained from *Anabaena* sp. after 3, 5 and 7 d of mixotrophic growth at 10-L cultivation scale.

Cultivation period (d)	**PHB extraction yield (%)**	**PHB recovery (%)**	**N (%)**	**C (%)**	**H (%)**
3	12.3	58.7	6.2±0.2	46.6±1.8	6.1±0.4
5	12.0	38.8	0.9±0.1	57.9±2.2	6.8±0.3
7	12.1	26.3	0.4±0.01	55.5±0.5	7.2±0.03

Table 5. Physical characteristics and thermal properties of PHB obtained from *Anabaena* sp. extracted after 5 and 7 d of mixotrophic growth at 10-L cultivation scale (the analysis for the PHB extracted after 3 days were not carried out because of the highest level of impurities of the recovered material; M_w: average molecular weight; PDI: polydispersity index; T_{deg}: maximum decomposition temperature; T_g: glass transition temperature; T_m: melting temperature; ΔH_m: melting enthalpy; χ_c: degree of crystallinity).

Cultivation period (d)	**M_w (MDa)**	**PDI**	**T_{deg} (°C)**	**T_g (°C)**	**T_m (°C)**	**ΔH_m (J g^{-1})**	**χ_c (%)**
5	3.15	1.63	291	3	170	81	55
7	3.02	1.71	285	1	171	82	56

Figure captions

Figure 1. Lipids, polysaccharides, proteins, and PHB content in *Anabaena* sp. after 3, 5 and 7 d of balanced or mixotrophic (ØP medium and 5 g L^{-1} of NaOAc) growth at 10-L scale.

Figure 2. PHB extracted from *Anabaena* sp. scale-up cultivation (10 L) after 3, 5 and 7 d of mixotrophic growth.

Figures

Fig. 1

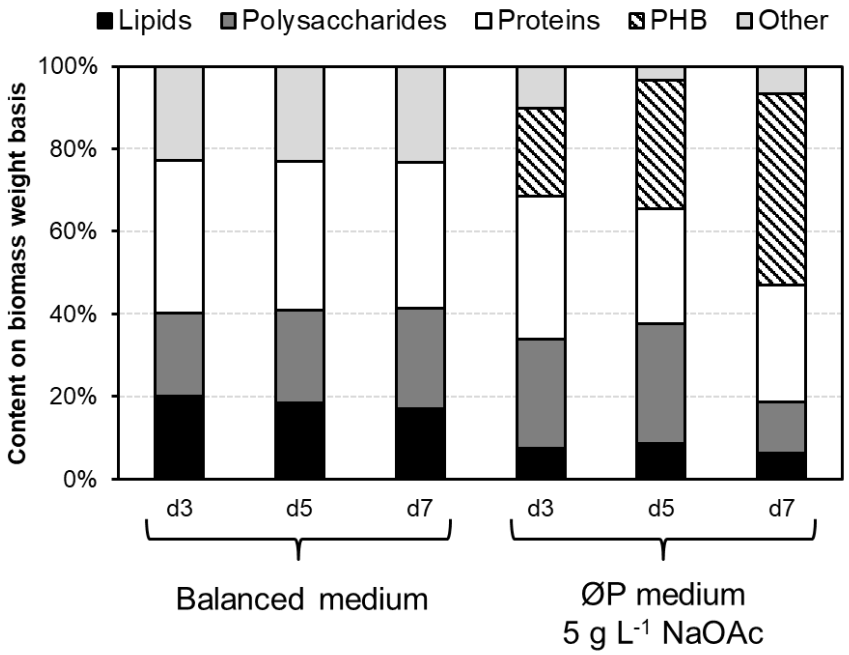

Fig. 2

PHB d3 PHB d5 PHB d7