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PHOTOAUTOTROPHIC PRODUCTION OF POLY-B-HYDROXYBUTYRATE (PHB) FROM CYANOBACTERIA: NITRATE EFFECTS AND SCREENING OF STRAINS

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Abstract

The polyhydroxybutyrate (PHB) production by photoautotrophic cultures is a potential eco-sustainable process to produce bioplastics. The biosynthesis of PHB by cyanobacteria meets a twofold target: the use of CO_2 as feedstock alternative to the fossil resource used in the conventional processes; the contribution to the reduction of the CO_2 concentration in the atmosphere. This contribution reports results of a study regarding the optimal growth conditions for cyanobacteria to produce PHB under photoautotrophic conditions using 16h/8h light/dark irradiation strategy.
Synechocystis PCC6803 was selected as the model organism for optimization of the medium composition with respect to PHB production. Four culture media, characterized by different nitrate concentrations, were investigated: BG_{11} (optimal nitrate concentration), $BG_{1/2}$ (half of optimal nitrate concentration), $BG_{1/4}$ (one fourth of the optimal nitrate concentration) and BG_0 (nitrogen-starved conditions). $BG_{1/2}$ proofed to be the best medium to optimize the PHB production in terms of PHB fraction of cell (8% $_{DCW}$) and PHB productivity (7g/Ld).
Five strains of cyanobacteria were then compared to select the best strain to produce PHB. *Synechocystis* PCC6803 and *Synechocystis aquatilis* were the best strains for PHB production. A PHB fraction and productivity of about 8% $_{DCW}$ and 7g/Ld were obtained for both strains. The production performance was promising when considering the free substrate (CO_2) used.

Keywords: cyanobacteria, nitrogen-starvation, photobioreactor, polyhydroxybutyrate (PHB), screening, strain

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1. Introduction

Modern society continuously asks for plastic items inexpensive, versatile, and eco sustainable. Indeed, the plastic worldwide demand - more than 300 million tons of plastic per year –has included the plastics among the key player of the today's economy (http://www.european-bioplastics.org/). Although the central role of the plastics in the modern society, two main issues penalize their future: i) petroleum-based plastics are a major constituent of world plastic consumption; ii) plastics are environmentally unfriendly. In particular, the fossil-based plastics have several environmental disadvantages: a large amount of CO_2 is released in the environment during the production and the conventional waste disposal processes; they accumulate in the environment because they are typically non-degradable. Alternative process of producing plastics in large quantities that are both economically and environmental friendly

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have recently gained considerable attention (Bhati et al., 2010). Roughly 85% of plastics could technically be substituted with biobased plastics (http://www.european-bioplastics.org/): plastics produced by renewable resources. The use of renewable resources contributes to the reduction of the CO_2 concentration in the atmosphere. The reduction is still more marked if the CO_2 is captured from gas streams produced by anthropic activities.

The shift from traditional plastics to biodegradable plastics can potentially reduce the total plastic waste by up to 20% (Dias et al., 2006). A large fraction of plastic products may be made by biopolymers belonging to the polyhydroxyalkanoates (PHAs) class. Indeed, PHA based products have a wide spectrum of potential applications: packaging, medical uses, agricultural uses, and in carbon nano tubes (Akaraonye et al., 2010). In particular, Poly(3-hydroxybutyrate) (PHB) received a special attention for its interesting features: thermoplastic processability, hydrophobicity, complete biodegradability and biocompatibility (Braunegg et al., 1998). According to european-bioplastics, the global production of poly-β-hydroxybutyrate (PHB), was approximately 34 ktonnes in 2014 and is forecasted to grow at a 100 ktonnes in 2019.

PHB is a biopolymer that exhibits thermal properties and a tensile strength equivalent to those of petroleum-based plastics (Verlinden et al., 2007). Moreover, PHB is also biocompatible to various human tissues and it has been used as a biomaterial for tissue engineering. Currently, PHB is commercially synthesized by heterotrophic bacteria by fermentation of expensive feedstocks. Indeed, bacteria such as Ralstonia eutropha H16 can produce up to 80% of PHB of cellular dry weight under optimal conditions (Hempel et al., 2011; Pötter et al., 2004). However, the high cost of the feedstock (about 30–40% of the total production cost) for PHB production by bacterial fermentation makes this process not economically competitive (about 2.6 $/kg as reported by Lopez-Arenas et al., 2017). A potential alternative to the heterotrophic bacteria fermentation is the exploitation of photosynthesis production system (Mittendorf et al., 1998; Poirier et al., 1992). Indeed, autotrophic cultures use CO_2 as carbon source and light as energy source. Therefore, autotrophic cultures could be a promising system using solar energy to generate reducing equivalents and incorporate atmospheric CO_2 into organic molecules (Atsumi et al., 2013). As a matter of fact, the PHB can be produced by some photoautotrophic cyanobacteria using abundant solar energy and CO_2 - in particular that presents in gas streams produced by anthropic activities - as the sole carbon substrate (De Philippis et al., 1992; Gopi et al., 2014; Hai et al., 2001; Wijffels et al., 2013).

The tuning of substrate concentration in the cyanobacteria culture media can induce drastic shifts of the central carbon metabolism in non diazotrophic cyanobacteria such as Synechocystis. In particular, under nitrogen depletion conditions, Anfelt et al. (2015) proved that the carbon flux is directed to storage compounds such as PHBs. Indeed, PHB synthase enzyme is activated by acetyl phosphate synthesized by phosphotransacetylase. The expression of the enzyme may occur under nitrogen starved conditions according a sequence of phenomena including: i) the increase of acetyl-CoA flux because of reduced amino acid synthesis due to nitrogen starvation; ii) the increase phosphoacetyltransferase activity; iii) the increase of the acetyl phosphate concentration; iv) the activation of the PHB synthase (Asada et al, 1999). Although the effect of the nitrogen concentration on PHB has been addressed from the pathway point of view, the role of the concentration of the nitrogen source on the PHB production is still lacking.This paper reports the results of a systematic study aimed at the optimization of the cyanobacterial growth media to increase the PHB production in photobioreactor by tuning the nitrate concentration. Synechocystis PCC6803 was selected as the model organism for the optimization of the media culture tests. The investigation was extended to five cyanobacteria strains to select a high PHB producer

2. Material and methods

2.1. Microorganism and medium

Synechocystis PCC6803 (used for culture medium optimization) was provided by the Laboratory of Microbiology of University of Amsterdam. *Synechocystis aquatilis*, *Synechocystis fuscopigmentosa*, *Synecoccoccus nidulans* and *Chlorogloeopsis fritshii* were investigated during the screening investigation and were provided by ACUF collection (http://www.acuf.net).

S. aquatilis cells are characterized by rounded shape with a very thin mucilaginous sheath around the cells. The thylakoid membranes are located in a peripheral position. Cell division occurs by two perpendicular planes of division in successive generations (Korelusova et al., 2009).

The strain *S*. PPC6803 is frequently used as a model organism in a wide spectrum of biochemical studies. The *S*. PPC6803 cells are characterized by morphological features like to those of *S. aquatilis* cells. Indeed, *S*. PPC6803 is often considered as the reference strains of *S. aquatilis* (Komárek et al., 2014). *S. fuscopigmentosa* Kováčik is characterized by morphological features like those of *S. aquatilis* too. However, *S. fuscopigmentosa* Kováčik is characterized by the extracellular excretion of a brown pigment (Kováčik, 1988).

The *S. nidulans* cells are cylindrical rod-shaped, often bent at one or both ends, without mucilage and are characterized by homogeneous cell content. Thylakoids are generally in a peripheral position, located around the cell wall. Cells divide symmetrically by binary fission in a plane producing two equal cells (McGregor et al., 2007).

C. fritshii cells are cylindrical and forming regular clusters of cells and small filaments, about 3 μm in diameter, rarely formed by more than 10 cells

(Komarek, 2013). Thylakoids are generally oriented parallel to the cell wall. Cells reproduce by endospores that originate clumps or filaments. Heterocyst-like cells form about 5% total cell population in heterotrophic cultures under nitrogen starvation (Whitton and Peat, 1967).

For culture medium optimization tests, *S.* PCC6803 was grown under photoautotrophic conditions in four growth media: BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11}. The composition of the four media as regards the common species is: 0.04 g/L K_2HPO_4; 0.075 g/L $MgSO_4$; 0.036 g/L $CaCl_2*2H_2O$; 0.006 g/L Citric acid; 0.006 g/L ammonium ferric citrate; 0.001 g/L $Na_2EDTA*2H2O$; 0.05 g/L $NaCO_3$; 2.86 g/L HBO_3; 1.81 g/L Mn Cl_2 * $4H_2O$; 0.22 g/L $ZnSO_4$ * $7H_2O$; 0.39 g/L Na_2MoO_4 *2 H_2O; 0.05 g/L $Co(NO_3)_2$. The four media differ between each other with respect to the nitrate content: BG_{11} contained the optimal concentration of $NaNO_3$ (1.5 g/L) for cyanobacterial growth according to Waterbury et al. (1981); BG_0 did not contain nitrate; $BG_{1/2}$ contained half the optimal nitrate concentration (0.75 g/L); $BG_{1/4}$ contained one fourth of the optimal nitrate concentration (0.375 g/L).

The screening tests were carried out under photoautotrophic conditions using the medium selected from the optimization tests ($BG_{1/2}$).

2.2. Analytical

The pH and the cell concentration (X) were measured in the culture sampled from the photobioreactors. The sample was centrifuged at 4,890 g for 15 min. The liquid phase was characterized in terms of concentration of nitrate and phosphate. The solid phase was processed to assess the PHB content.

The concentration of cyanobacteria was measured with a spectrophotometer Specord 50 - Analytic Jena at wavelength of 730 nm. The dry cell weight was calculated from the optical density measured by the spectrometer according to a calibration procedure pairing OD and dry cell weight. The dry cell weight was carried out according to the following procedure: i) 10 mL samples of cultures were filtered by means of a Whatman filter; ii) filters were dried at 85°C until a constant weight was approached; iii) the dry cell weight of the cyanobacteria was calculated as cyanobacteria-filter weight minus the dry weight of the filter. The conversion factor from OD to cell concentration was: 1OD=0.23 g_{DM}/L. pH was measured with a Mettler Toledo pH meter.

The nitrate concentration was determined using a modified method proposed by Collos et al. (1999). The method was based on ultraviolet absorption spectrometry. The absorbance of the liquid phase recovered after the centrifugation was measured at a single wavelength (220 nm) supplementing 20 µL of HCl 1M to 1mL of sample.

The phosphate concentration was measured according to the molybdate colorimetric test for ortho-phosphate. The colorimetric test was based on the monitoring the redox state the antimonyl-phosphomolybdate complex produced during the reaction among ammonium molybdate, potassium antimonyl tartrate and ortho-Phosphate. This complex is reduced to an intense blue-colored (molybdenum blue) complex by ascorbic acid (Pierzynski et al., 2000). The absorption time-evolution of each sample was measured by a spectrophotometer at 704 nm.

Cell rupture was required to release PHB granules. A procedure based on that proposed by Schlebusch and Forchhammer, (2010) was used for the cell rupture. Biomass (10 mL of culture at 1 OD) was harvested by centrifugation (20 min, 4,890g, 4°C). The cell pellets were dried overnight at 85°C. The dry pellets were boiled in 1 mL of H_2SO_4 96% for 40 min, diluted with 9 mL of 0.007 M H_2SO_4, and filtered by means of a polyvinylidene difluoride membrane. Quantitative analysis of PHB content in the solution produced from the chemical processing of the cells was carried out by means of HPLC (Agilent 1100 system) equipped with a multi-wave detector set at 210 nm. The separation column was Aminex HPX-87H (300x7.8 mm). Crotonic acid was used as the external standard for HPLC analysis. Indeed, PHB was converted in crotonic acid after treatment with sulphuric acid (Karr et al., 1983).

The presence of PHB in cells was visualized by means of the Nile red staining method. 200 µL of samples with cyanobacteria were supplemented with 50 µL of Nile red dye (from a stock with 1mg/mL in DMSO) and incubated for 10 minutes at room temperature. The suspension was rinsed with double distilled water. Slides with stained cyanobacterial cells were prepared according to the wet mount method and observed at fluorescent microscope settings with the excitation at 465 nm. The PHB granules were visible as red dots in the cells.

2.3. Apparatus

Pre-cultures were grown in 250 mL Erlenmeyer flasks housed in a climatic chamber (Gibertini) at 25 °C. The chamber was equipped with daylight fluorescent Philips lamps (TLD 30W/55) set at 150 $\mu Em^{-2}s^{-1}$ for 24/24 h (Carpine et al., 2017).

The cyanobacterial cultures were carried out in inclined bubble column photobioreactors (IBCPs) (volume: 800 mL) in a climate chamber (Solar Neon) at 25 °C. Light was provided by fluorescent lamps (Philips TLD 30 W/55). Each IBCP was equipped with a multiple-orifice (1 mm ID) Teflon tube at the bottom to sparge the gas stream. The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations (Olivieri et al. 2013). Air and pure carbon dioxide from a pressurized vessel were mixed by means of a gas mixing device (M2M engineering) to provide the selected concentration of CO_2 in the gas stream fed to the IBCPs. The CO_2 concentration in the air stream was set at 2% (v/v). Gas flow rate was set at 4 vvm.

The IBCPs and the medium were sterilized in autoclave. A hydrophobic filter (0.2 µm) sterilized the gas stream fed to the IBCPs.

2.4. Procedures

The cyanobacteria were transferred from a Petri plate into 250 mL Erlenmeyer flasks containing 50 mL medium. After about two weeks the pre-cultures were inoculated into IBCPs: 10% (v/v) of actively growing pre-cultures. Cultures in IBCPs were carried out under light/dark cycle conditions: 16 h light at 150 $\mu Em^{-2}s^{-1}$ and 8 h dark. The cultures were sampled every 48 h. Samples were characterized in terms of pH (for CO_2 inflow maintenance) and concentration of biomass, nitrate, phosphate and PHB concentration. Each test was carried out in triplicate and the mean values are reported as results. The error was typically lower than 5%.

Measured data were processed to assess the PHB fraction (%) (ω_{PHB}) and PHB productivity. ω_{PHB} was calculated according to (Eq. 1):

$$\omega_{PHB} = \frac{PHB}{X} \cdot 100 \quad (1)$$

where: X is the cyanobacterial cell concentration, and PHB concentration? The PHB productivity (mg/Ld) (P_{PHB}) was calculated according to Eq. (2):

$$P_{PHB} = \frac{PHB/_t}{t} \quad (2)$$

where: $PHB|_t$ is the poly-β-hydroxybutyrate concentration measured at the instant t.

3. Results and discussion

3.1. Nitrogen optimal composition

Fig. 1 reports the time resolved data regarding autotrophic cultures of *S.* PCC6803 using the four culture media. In particular, Figs. 1a-e reports biomass concentration, PHB fraction, PHB productivity, nitrate concentration and phosphate concentration measured during the growth using the four tested media, respectively. The pH ranged between 6.5-7.5 for all the tests: the CO_2 concentration was always close to the saturation value. The typical four phases of the cell growth may be observed in Fig. 1a:

- a lag phase: cell concentration was almost constant and PHB was not produced;
- an exponential phase: cell concentration increased sharply and the PHB concentration increased remarkably;
- a steady phase: the cell concentration was almost constant and the PHB production was active.

The analysis of the nitrogen and phosphate concentration (Figs. 1d and e) and of the PHB fraction points out that: i) the phosphate starvation occurred always late than nitrogen starvation; ii) the PHB accumulation started at the onset of the nitrogen starvation; iii) the PHB accumulated even though the phosphate source was present in the medium.

The vertical dashed lines in Fig. 1 mark the nitrogen starvation for each medium (reported in the label on the top of the Figures). The PHB accumulation started at the vertical dashed line.

As regards phosphate (Fig 1e) it can be noted that it is depleted without any measurable phenotypic consequence for biomass formation and PHB accumulation. The extracellular phosphate pool was depleted very quickly by *Synechocystis*. Indeed, the phosphate was used by the cells for the rapid build-up of intracellular phosphate supply to be used in later stages of growth (Zerulla et al., 2016). This phenomenon was used by microorganism as strategy to survive under depleted conditions of this nutrient in aquatic environments (Oliver and Ganf, 2000).

The analysis of Fig. 1a suggests that cyanobacteria continued to grow even though nitrogen starvation occurred. This observation may be explained by taking into account the spectrum of sources available for growth of the microorganism: extracellular substrates are consumed under nutrient replete condition; stored intracellular nutrients are consumed under nutrient depletion conditions. In particular, Palabhanvi et al. (2014) reported that the stored intracellular nutrients are present in three forms: (i) structural form of nutrient, SFN (structural protein, DNA, rRNA, etc.); (ii) readily utilizable nutrient, RUN (inorganic phosphate, nitrate, ammonia etc.); and (iii) non-readily utilizable nutrient, Non-RUN (some protein, mRNA, polyphosphate bodies etc.). Therefore, it is expected that investigated cyanobacteria used intracellular nitrogen during the large part of the exponential growth.

The comparison of the PHB fraction of cells measured during the culture carried out in the four media may be done by analysing data reported in Fig. 1b. This data analysis points out that $BG_{1/2}$ was the best compromise between cell growth and PHB accumulation. Indeed, the maximum cell concentration does not change markedly for the cultures carried out in $BG_{1/2}$ and BG_{11} media but the cellular PHB accumulation was high and early in $BG_{1/2}$ growth medium. Fig. 1c reports a comparison of the PHB productivity measured during the culture carried out in the four media. This data analysis reveals that the PHB productivity was characterized by a maximum for all the tested media. In particular, the maximum value of PHB productivity was found using $BG_{1/2}$ media as growth medium. The analysis of Fig. 1b and 1€ pointed out that the instant at which the maximum PHB fraction was measured was different from that at which PHB productivity was measured. A choice between the two targets must be made based on post-processing operations: economic and technical analysis is required.

The best results were measured for the culture carried out in the $BG_{1/2}$ medium: PHB content of about 8% and PHB productivity of about 7 mg/Ld.

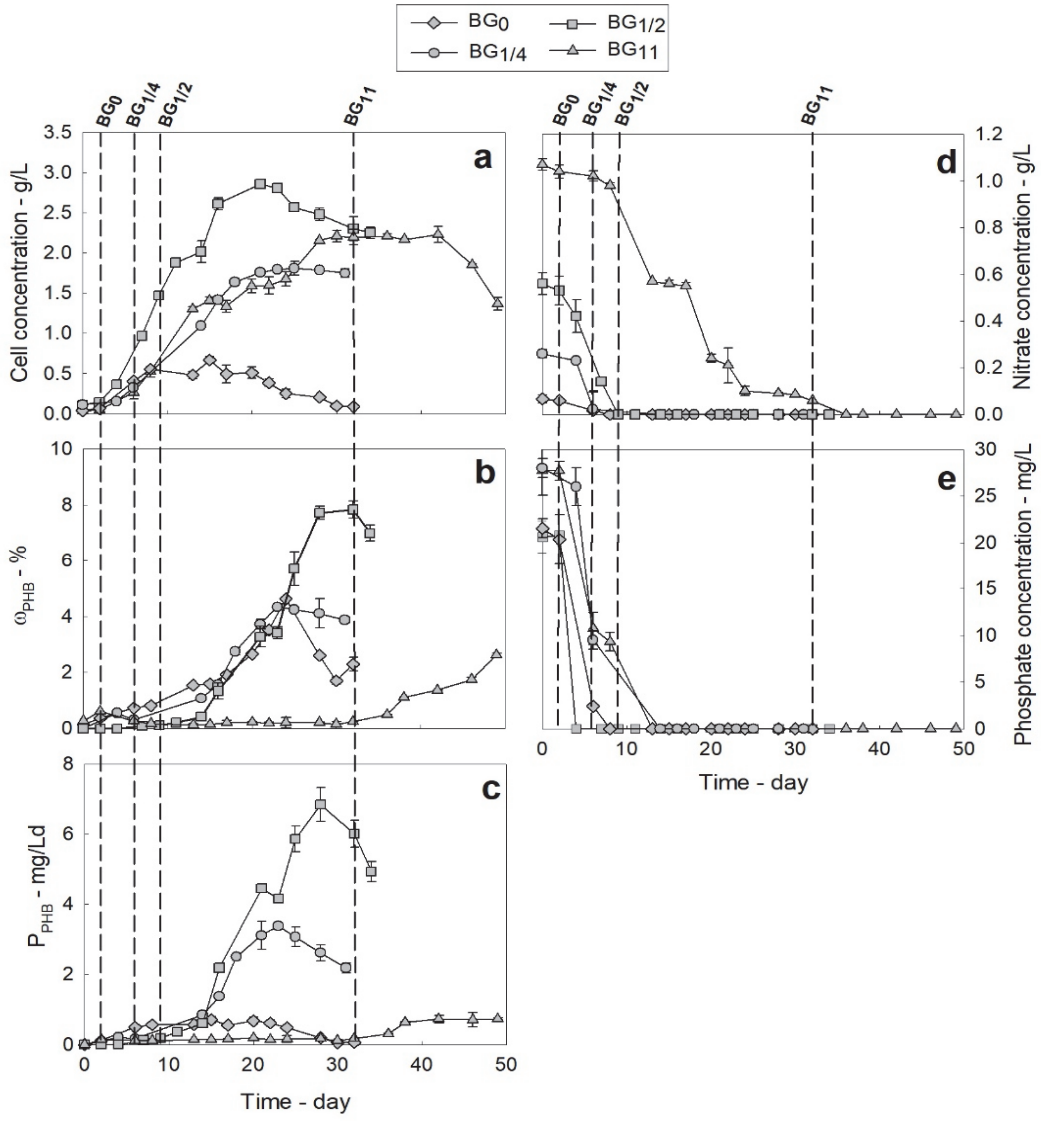

Fig. 1. Data measured during the culture of SynechocystisPCC6803in the four investigated media (BG_0, $BG_{1/4}$, $BG_{1/2}$ and BG_{11}): (a) cell concentration; (b) PHB-fraction; (c) PHB productivity; (d) nitrate concentration; € phosphate concentration. Reported data are the average of triplicate test and the error was typically lower than 5%. Vertical lines mark the nitrogen starvation

A potential interpretation of the high accumulation of PHB under nitrogen-starvation conditions is based on the observation that the nitrogen was absent in the medium and the photosynthesis was active, and fixed CO_2. As a matter of fact, cells did not grow because they cannot synthesize proteins and the fixed CO_2 was accumulated as carbon/energy reservoir as PHB (Smith, 1982). As a result of the flux re-direction under nitrogen-starved conditions, it is interesting to note that $BG_{1/2}$ medium was better than BG_{11} because in this medium nitrogen starvation was reached in a shorter period of time.

Moreover, $BG_{1/2}$ medium was better than BG_0 medium because in BG_0 medium PHB was produced from the beginning of the test but, the cells concentration remained very low. With $BG_{1/4}$ medium the PHB fraction reached during cyanobacteria growth, was higher than PHB fraction reached with BG_0 and BG_{11}, but lower than PHB fraction reached using $BG_{1/2}$ medium.

Table 1 reports a comparison of data regarding the PHB production by cyanobacteria reported in the literature. The main observations are reported hereinafter:

- the maximum PHB productivity and/or concentration are obtained under heterotrophic growth conditions (Bhati and Mallick, 2015; Panda and Mallick, 2007; Samantaray and Mallick, 2014). It should be noted that the high PHB production requires the feeding of an organic source of carbon that it is expected to be expensive.

Coelho et al. (2015), Haase et al. (2012) and Kaewbai-ngam et al. (2016), reported interesting results for autotrophic growth cultures carried out in flasks. They cultivated *Nostoc muscorum*, *Calothrix scytonemicola* and *Spirulina* sp. LEB 18, respectively. However, these strains are biofilm producing strains.

Table 1. Data of PHB production reported in the literature

Polymer concentration (mg/L)	438.9	nr	537.5	nr	nr	356	27	nr	**180**
Polymer fraction (% dcw)	78	31	77	38	22	25	4	55	**8**
Polymer productivity (mg/L*day)	109.7	nr	38	nr	nr	nr	nr	220,8	7
Type of polymer	P(3HB-co-3HV)	PHB	P(3HB-co-3HV)	PHB	PHB	PHB	PHB	PHB	PHB
Light intensity (µmol photon m^{-2} s^{-1})	75 (14h light/10h dark)	59.2, (12h light/ 12 h dark)	nr	75 (14h light/10h dark	85 (continuous light)	50 (continuous light)	4000 lux (continuous light)	50 (continuous light)	180 (18h light/6h dark)
Reactor	flask	flask	flask	flask	flask	flask	flask	flask	photobioreactor
Culture condition	Nitrogen and phosphorous depletion	0.05 g/L sodium nitrate; 0.5 g/L potassium phosphate	Phosphorous depletion	Nitrogen depletion	Phosphorous depletion	Nitrogen depletion	Nitrogen depletion	phosphate-free medium with $Ca_3(PO_4)_2$	Nitrate concentration is the half of the optimal concentration
Carbon source	Glucose	Sodium bicarbonate	Fructose + Valerate	Fructose + Acetate	CO_2	atmospheric CO_2	CO_2	CO_2	CO_2 (2%)
Cyanobacteria strain	*Nostoc muscorum* Agardh	*Spirulina sp.* LEB 18	*Aulosira fertilissima* CCC 444	*Synechocystis* sp. PCC 6803	*Nostoc muscorum*	*Calothrix scytonemicola* TISTR 8095	*Synechocystis* sp. PCC 6803	*Synechococcus* sp. MA19	*Synechocystis* sp. PCC 6803
References	Bhati and Mallick (2015)	Coelho et al., (2015)	Samantaray and Mallick (2014)	Panda and Mallick (2007)	Haase et al.,(2012)	Kaewbai-Ngam et al., (2016)	Wu et al. (2000)	Nishioka et al., (2001)	This work

• *Synechococcus* sp. MA19 was characterized by high PHB productivity under autotrophic condition (Nishioka et al., 2001). In contrast, the thermophilic nature of *Synechococcus* sp. MA19 made it unsuitable for large-scale exploitation because of the high-energy requirement to keep the growth temperature at 50 °C (Panda et al, 2007).

• *Synechocystis* PCC6803 was characterized by high PHB productivity under heterotrophic conditions (Panda et al., 2007). However, the performance drastically reduced when growth under autotrophic conditions (Wu et al., 2001). Indeed, the PHB fraction was half the value reported in this work.

The reported results and comparison with the literature proved that the selected medium composition was optimal for PHB production under autotrophic conditions and it was used in the screening of the strains.

3.2. Strain screening

Pure cultures of *S.* PCC6803, *S. aquatilis*, *S. fuscopigmentosa*, *S. nidulans*, and *C. fritshii* were investigated for the PHB production screening. In particular, PHB accumulation was assessed by microscopic observations of Nile red stained cell method. The five investigated strains accumulated a detectable amount of PHB: PHB inclusions were highlighted as bright orange intracellular granules (Fig. 2). The colorimetric assay did not allow to assess the PHB fraction but it was used just to point out the presence of PHB in the cells. Fig. 3 reports data measured during cultures the screened strains of cyanobacteria using the $BG_{1/2}$ medium: *S.* PCC6803, *S. aquatilis*, *S. nidulans* and *C. fritshii*. The pH ranged between 6.5-7.5 for all the tests: the CO_2 concentration was always close to the saturation value. Data regarding the culture of *S. fuscopigmentosa* were not reported because the PHB concentration was very low. This result confirms that the colorimetric assay did not allow to assess the PHB fraction but it was just addressed to highlight its presence even in trace.

Cyanobacteria cell concentration was reported in Fig. 3a, PHB content, PHB productivity, nitrate concentration and phosphate concentration were reported in Figs. 4 b-e, respectively. The beginning of the nitrogen starvation was marked by vertical dashed lines (the vertical label above the vertical lines indicate the strain).

Except for *C. fritshii*, all the screened cyanobacteria strains continued to grow even though nitrogen starvation occurred (Fig. 3a), according to the results pointed out during the medium optimization tests. The time resolved plot of the cell-concentration was quite similar for *S.* PCC6803, *S. aquatilis*, and *S. nidulans*. The maximum cell concentration of *S.* PCC6803 was about 30% larger than that of *S. aquatilis*, and *S. nidulans*. *C. fritshii* was characterized by a longer lag phase and by lysis as nitrogen starvation occurred. *S.* PCC6803 and *S. aquatilis* were the best strains for PHB production. PHB content and productivity were more or less constant for the two strains: about 7.7 % and 6.8 g/m^3d for *S. PCC6803* and about 7.75% and 6.4 g/m^3d for *S. aquatilis*.

The analysis of Figs. 3b and 3c suggests that *S.* PCC6803 and *S. aquatilis* were characterized by the highest PHB content and productivity among the investigated strains. The main differences between these strains were:

i) *S. aquatilis* produced PHB since the beginning of the growth. PHB production continued even after nitrogen starvation.

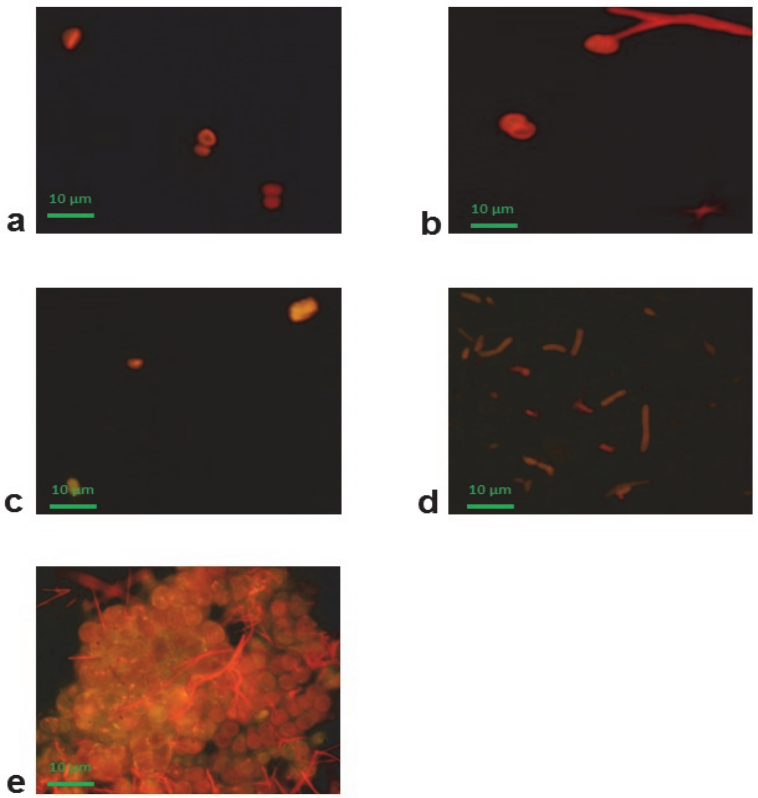

Fig. 2. Microscope observation of Nile red stained cells of cyanobacteria: A) *Synechocystis* sp. PCC6803; B) *S. fuscopigmentosa*; C) *S. aquatilis*; D) *S. nidulans*; E) *C. fritshii*. Bright orange intracellular granules highlighted PHB inclusions

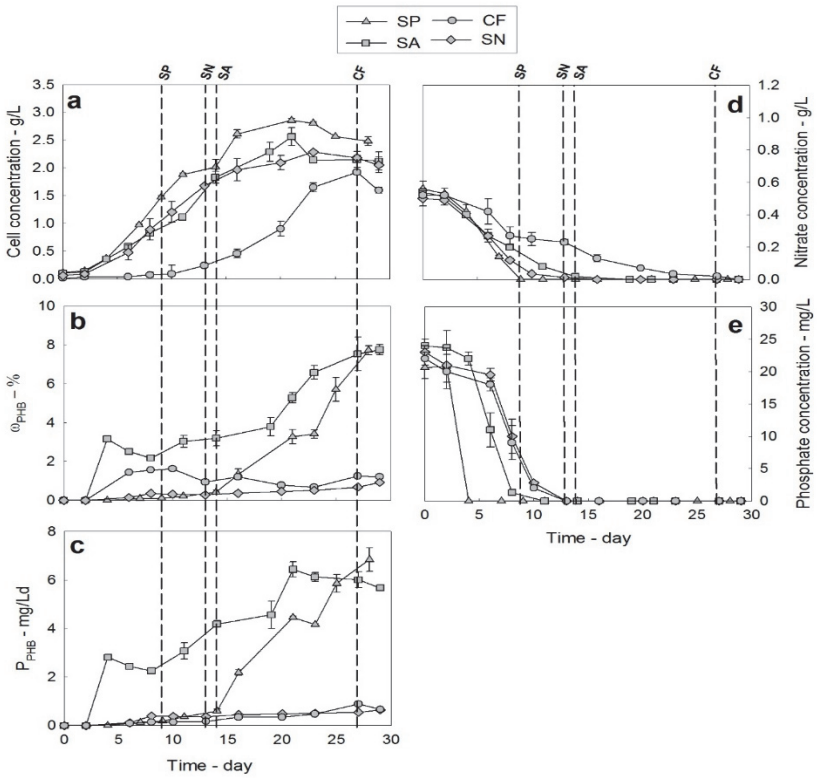

Fig. 3. Data measured during the growth of the four screened cyanobacteria strains (*S. PCC6803, S. aquatilis, C. Fritshii, S. nidulans,*) using $BG_{1/2}$ medium: a) cell concentration; b) PHB-fraction; c) PHB productivity; d) nitrate concentration; e) phosphate concentration. Reported data are the average of triplicate test and the error was typically lower than 5%.
Vertical lines mark the nitrogen starvation

The observed behavior is very interesting from the point of view of continuous PHB production. Indeed, the continuous PHB production coupled with the cell growth may be exploited by using a continuous photobioreactor: continuous harvesting of PHB-rich cells. Indeed, the batch-wise related bottlenecks that reduce the PHB productivity (e.g. lag phase and dead time between successive batches) are absents in continuous productions;

ii) *S.*PCC6803 began to produce PHB at the nitrogen starvation onset.

iii) *S. aquatilis* reached the maximum value of the PHB productivity in a period shorter than that required by *S*. PCC6803.

Further investigation on nitrate concentration effects should be carried out with S. *nidulans* and C. f*ritshii* to verify that $BG_{1/2}$ is the optimal medium for these cyanobacterium strains too.

4. Conclusions

The biosynthesis of PHB in autotrophic cultures of cyanobacteria was successfully carried out: it was enhanced under nitrogen-starvation conditions.

The medium optimization for PHB production was investigated with reference to *S.* PCC6803 (model strain). The optimal nitrogen concentration was half the optimal concentration assessed for cell growth. Under the optimized conditions the maximum PHB-cell fraction and the maximum PHB productivity were: 8 % and 7 g/m^{3}d, respectively.

The screening tests pointed out that *S.* PCC6803 and *S. aquatilis* were the best strains for PHB production. PHB fraction and productivity of about 8% and 7 g/m^{3}d were obtained for both strains. The results are very promising to support the autotrophic cyanobacterium cultures as a production route for PHB from an abundant and free substrate, the carbon dioxide. The economic potential of PHB production by means of autotrophic cyanobacterium cultures with respect to heterotrophic cultures based on expensive feedstocks should be proved by a techno-economic investigation.

The production of PHB by means of autotrophic cultures may be proposed as an eco-sustainable production process because it couples the production of bio-polymers with the capture of the CO_2 from gas streams produced by anthropic activities.

Abbreviations:

BG_{11}, medium with the optimal nitrate concentration; **BG_0**, medium without nitrate; **$BG_{1/2}$**, medium in which nitrate concentration was the half of optimal concentration; **$BG_{1/4}$**, medium in which nitrate concentration was one fourth of the optimal concentration; **CF**, *Chlorogloeopsis fritshii;* **PHB**, poly-β-hydroxybutyrate; **SP**, *Synechocystis* PCC6803; **SA**, *Synechocystis aquatilis*; **SF**, *Synechocystis fuscopgmentosa*; **SN**, *Synechococcus nidulans*.

Nomenclature

P_{PHB}	[g/m^{3}d]	PHB productivity
t	[day]	time
ω_{PHB}	[% w/w]	PHB fraction
X	[g_{DM}/L]	cyanobacteria cell concentration

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