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The Cyanobacterial Route to Produce Poly-β-hydroxybutyrate

Roberta Carpine[a], Giuseppe Olivieri[a], Klaas Hellingwerf[b], Antonino Pollio[c], Antonio Marzocchella[*a]

[a] Dipartimento di Ingegneria Chimica, dei Materali e della Produzione Industriale - Università degli Studi di Napoli "Federico II" – P.le V. Tecchio, 80 - 80125 Napoli, Italy
[b] Laboratory for Microbiology, Swammerdam Institute for Life Sciences - Science Park 904 1098 XH Amsterdam, The Netherlands
[c] Biology Department - Università degli Studi di Napoli "Federico II" - Via Foria, 223 - 80139 Napoli, Italy
antonio.marzocchella@unina.it

The amount of plastics produced in the world and released in the environment has dramatically increased putting societies and the environment to hard test. The biodegradable plastics are a potential solutions to reduce the environmental impact and the fossil resource exploitation.
Polyhydroxyalkanoates (PHAs) are widely adopted as building blocks for biodegradable plastic production and they may be produced by microorganisms. The poly-β-hydroxybutyrate (PHB) is the most widespread and thoroughly characterized PHA found in bacteria. In particular, cyanobacteria are potential host systems for the PHB production, as they may synthesize this polymer from CO_2.
This contribution reports a joint research between the University of Napoli and the University of Amsterdam on the feasibility of PHB production by Synechocystis PCC6803. Both BG_{11} (balanced conditions) and BG_0 (nitrogen-starved conditions) media were investigated to find the best growth conditions and optimal PHB production. Batch autotrophic cultures were carried out in inclined bubble column photobioreactors under 12h/12h light/dark cycles.
Cultures were characterized in terms of biomass, PHB content, pH and nitrate consumption. After cell-rupture with sonication the PHB concentration was assessed by means of GC, adopting a modified propanolysis method. The maximum PHB concentration was 4 mg/L and the maximum PHB productivity was 0.19 mg/(L day). The PHB content of the cells is definitively higher in nitrogen-starved medium (1 %) than in balanced medium (0.25 %).

1. Introduction

Plastics are among the materials widely used all over the world. Nowadays, plastics are indispensable as components of cars, home appliances, and computer equipment, in packaging and as medical tool/devices. Although mechanical-technological features of plastics have worldwide been recognized, plastics have been vilified because they are environmentally unfriendly. Indeed, plastics are not biologically degradable and accumulate in the environment producing mountains of pollutants (Sharma et al., 2007).
Bioplastics have a history of approximately 150 years. The first artificial thermoplastic "celluloid" was invented in the 1860s. Since then, numerous new compounds were produced from renewable resources. The advent of the fossil-derived plastics caused the industrial failure of plastics derived from renewable resources: synthetic polymers have been successfully produced at industrial scale since the 1950s. The oil price shocks of the last decades of the last millennium, in combination with the environmental consciousness that arose during the last decades, have refueled the interest in bio-based chemistry and in particular in bio-based plastics (European Bioplastics, 2009). Biodegradable plastics are a class of bioplastics that are both produced from biomasses (biobased materials) and are biodegradable: microorganisms are able to convert them into simple, elemental, substances (e.g. water, carbon dioxide).
Polyhydroxyalkanoates (PHAs) are fundamental building blocks for biodegradable plastics. PHAs are microbial polyesters that may be produced by various microorganisms, cyanobacteria included. PHAs are

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among the most investigated biodegradable polymers in recent years, due to their chemical and physical features that include: non-toxicity, biocompatibility, and biodegradability. Particular attention has been paid to poly-β-hydroxybutyrates (PHBs) because of their interesting features: thermoplastic processing, hydrophobicity, complete biodegradability, and biocompatibility with optical purity (Braunegg et al., 1998).
Cyanobacteria – or blue-green algae - are prokaryotic organisms. They may carry out oxygenic photosynthesis under photoautotrophic conditions and are characterized by a short duplication time (Gopi et al. 2014). Cyanobacteria can be considered as an alternative host system for the production of PHBs because they are photoautotrophic and require minimal nutrients. They need some simple inorganic salts such as phosphate, nitrate, magnesium and calcium, as macro-, and ferrous, manganese, zinc, cobalt, and copper as micronutrients for their growth and multiplication (Balaji et al., 2013). Cyanobacterial species may accumulate the homopolymer of PHB under photoautotrophic conditions. Indeed, PHB functions as a specialized reserve material for carbon- and energy supply: storage accumulated in a variety of microorganisms under conditions of nutrient imbalance (Wu et al., 2000).
Synechocystis PCC6803 is an extensively investigated cyanobacterium. It is a naturally transformable organism, genetically and physiologically well characterized. Its growth is quite fast (minimal doubling time less than seven hours) and it does not have specific nutritional demands (Angermayr et al., 2009). Figure 1 shows its most important metabolic pathways.

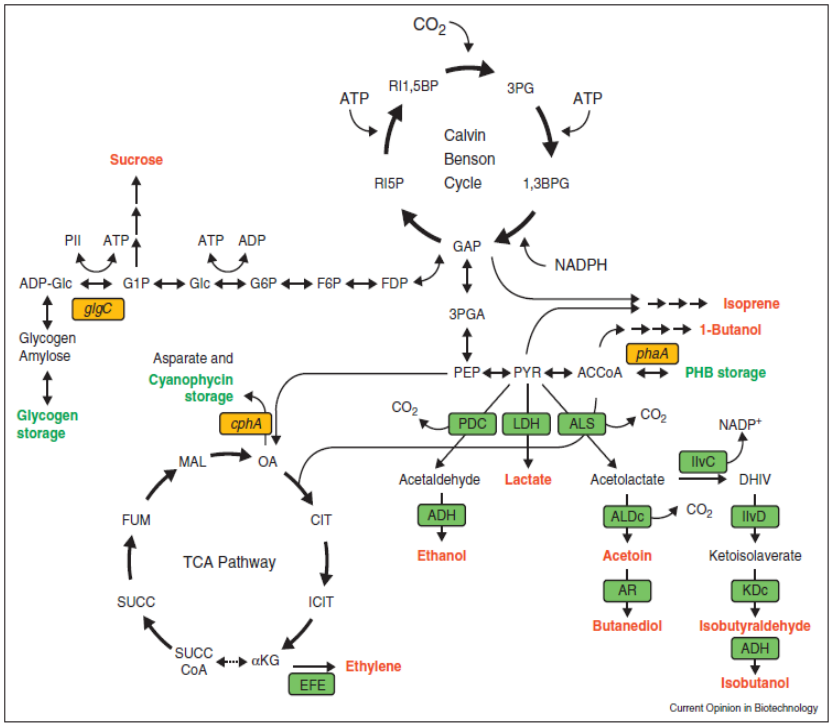

Figure 1: Synechocystis PCC6803 intermediary metabolism (Wijffels et al., 2013)

Present contribution reports some results of a joint study between the University of Study of Napoli Federico II and the University of Amsterdam on PHBs production by means of autotrophic cultures of cyanobacteria. The strain *Synechocystis sp.* PCC6803 was selected as the model organism for these studies. The production of PHBs was investigated in two media (i.e. with and without added nitrate). Cultures were grown in inclined bubble column photobioreactors with alternating dark/light cycles.

2. Materials and method

2.1 Microorganism and medium

Synechocystis sp. PCC6803 was provided by the University of Amsterdam. Cyanobacteria were grown under photoautotrophic conditions in two growth media: BG_{11} and BG_0 medium (Table 1). The difference between the two media is in the nitrate content: BG_0 does not contain added nitrate.

Table 1: BG_{11} and BG_0 composition (g/L)

	BG_{11}	BG_0
$NaNO_3$	1.5	0
K_2HPO_4	0.04	0.04
$MgSO_4$	0.075	0.075
$CaCl_2*2H_2O$	0.036	0.036
Citric acid	0.006	0.006
Ammonium ferric citrate	0.006	0.006
$Na_2EDTA*2H_2O$	0.001	0.001
$NaCO_3$	0.02	0.02
HBO_3	2.86	2.86
$Mn\ Cl_2 * 4H_2O$	1.81	1.81
$ZnSO_4 * 7H_2O$	0.22	0.22
$Na_2MoO_4 *2\ H_2O$	0.39	0.39
$Co(NO_3)_2$	0.05	0.05

2.2 Physico-chemical methods

A fraction of the sampled cell broth was centrifuged at 5,000 rpm for 15 min. Nitrate concentration and pH were measured in the supernatant with ion selective electrodes (Hanna Instruments) and a Mettler Toledo pH meter, respectively.
The biomass (i.e. cyanobacteria) concentration was measured with a spectrophotometer Specord 50 – Analytic Jena at wavelength of 730 nm. Analysis of the dry cell weight was carried out by filtering 10 mL aliquots of culture on a Whatman filter. Each filter was dried at 60°C until the weight approached a constant value. The dry weight of each filter was subtracted from that of the cyanobacteria-containing filter, to obtain the dry cell weight of the cyanobacteria. The OD values were converted to biomass concentration via an appropriate calibration line between OD and dry cell weight, showing the conversion factor.

2.3 Assay of PHBs

The PHB assays required cell rupture, which was carried out by means of sonication. A 50 mL sample was sonicated with a Sonics Vibracell VCX 500 for 30 min (10 s on/ 10 s off), amplitude 21%, and energy 90 kJ. The sample is cooled in ice during sonication to prevent overheating.
Sonicated samples were centrifuged in glass tubes at 3,000 rpm for 1 h and the resulting pellets were dried overnight at 40 °C.
PHB was quantified using a slight modification of the gas chromatographic method of Riis and Mai (1988). Two mL of 1,2-Dichloroethane (DCE), 2 mL n-Propanol, containing hydrochloric acid (HCl) (1 volume concentrated HCl + 4 volume n-Propanol), and 200 µL internal standard (2.0 g benzoic acid in 50 ml n-Propanol) were added to the dried pellet. The mix was incubated for 4 h in a water bath at 85 °C with intermittent shaking. After cooling to room temperature, 4 mL water were added and the mixture shaken for an additional 20-30 s. The heavier DCE-Propanol phase was collected and injected directly into a gas chromatograph. Quantitative evaluation was based on peak areas of hydroxy-butyric acid and benzoic acid (Nagamani et al., 2011).
An Agilent 6890 gas chromatograph was fitted with an automatic injector and a flame ionization detector. The injection split ratio was 100:1 with a helium flow of 0.9 mL/min through the 25 m Poraplot Q capillary column. The injector port temperature was 120 °C and increased by 8 °C per min to a final temperature of 210 °C. Injections of 5 µL were made and the retention times for the PHB and benzoic acid were 4.1 and 7.0 min respectively (Aremu et al., 2010).
PHB calibration was carried out by dissolving 200 mg of PHB standard (Sigma Aldrich) in warm DCE. After cooling at room temperature, the mixture was made up to 10 mL. Volumes of 200, 400, 600 and 800 µL of this solution were treated as outlined above. The relationship between the peak-area quotient and the quantity of PHB was observed to be linear.
The concentration of PHB measured during the tests was referred to the unit of volume of the culture.

2.4 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.
Inclined bubble column photobioreactors (volume: 800 mL) were adopted for cyanobacterial growth. The photobioreactors where housed in climate chamber (Solar Neon) at 25 °C, equipped with lamp. Gas stream

was sparged through the bottom of the photobioreactors by means of multiple-orifice (1 mm ID) Teflon tube. A hydrophobic filter (0.2 μm) sterilized the gas flow inlet. A gas mixing device (M2M engineering) provided the selected concentration of CO_2 in the gas stream fed to the photobioreactors, by mixing air and pure carbon dioxide from a pressurized vessel. Gas flow rate was set at 4 vvm. The CO_2 concentration in the air stream was set at 2% (v/v). The head of the photobioreactors was equipped with three ports for gas inlet/outlet and sampling operations (Gargano et al., 2013).

2.5 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 photobioreactors: 10% (v/v) of actively growing pre-cultures.
Cultures in inclined column photobioreactors were grown in light/dark cycles: 12 h light at 150 $\mu Em^{-2}s^{-1}$, 12 h dark. The cultures were sampled every 48 h. The samples were characterized with respect to biomass content, pH and PHB concentration.
Measured data were processed to assess the PHB concentration in the biomass according to the relationship:

$$\omega_{PHB} = \frac{PHB}{X} \tag{1}$$

where X is the cyanobacterial concentration, and PHB the poly-β-hydroxybutyrate concentration.

3. Results

Figure 2 reports the time resolved data regarding two autotrophic cultures of *Synechocystis sp.* PCC6803. Data reported in the figures are: the cell concentration, the PHB-cell concentration assessed according to eq (1), and the PHB concentration. The runs refer to a culture carried out with a balanced-medium (BG_{11}) (Figure 2 A and C) and to a culture carried out with a nitrogen-deplete medium (BG_0) (Figure 2 B and D). The growth in the balanced medium was quite exponential during the investigated growth period. The typical S-shaped curve cell-concentration vs. time was observed for the culture in nitrogen-less medium to highlight the gradual decrease of key substrate.

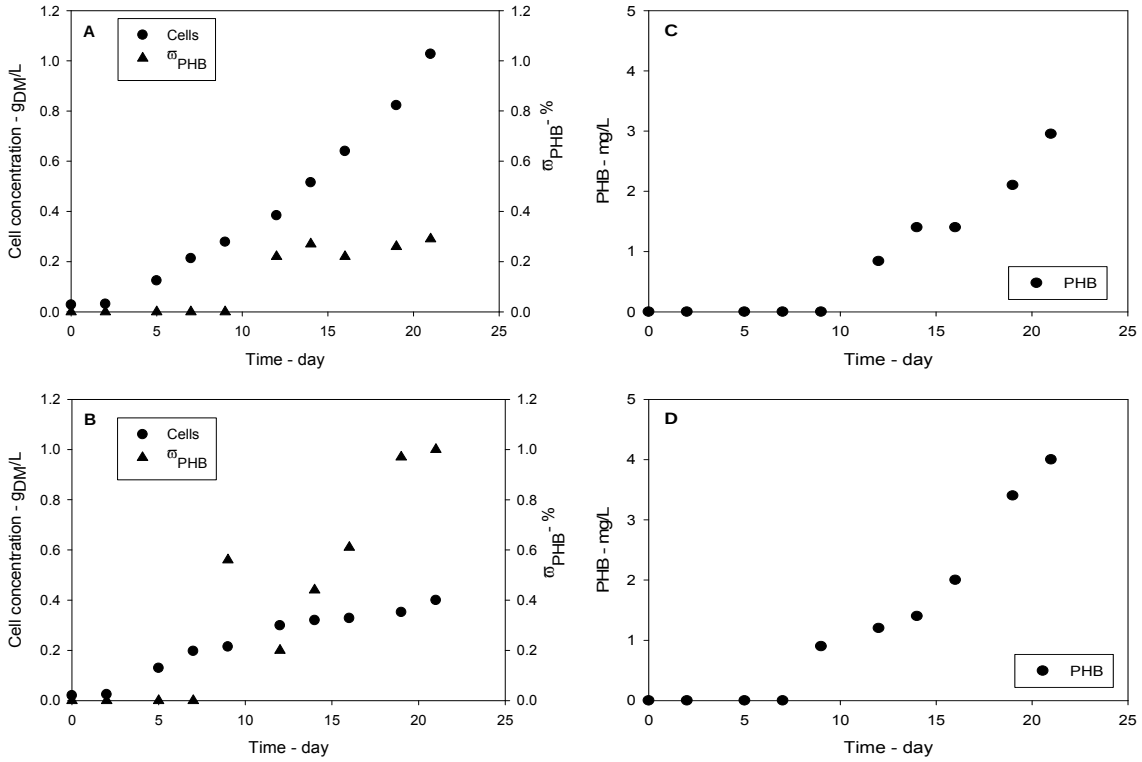

Figure 2: Data measured during the growth of Synechocystis PCC6803: cell concentration, PHB concentration, PHB-cell composition (ω_{PHB}). A & C): balanced medium; B & D) nitrogen-deplete medium.

Figure 3 shows the pictures of the cultures at day 21. The blue-green cultures typically of the balanced cultures turned into yellow-green for nitrogen-depleted culture. The cell color changed from blue-green to yellow-green because under nitrogen-starvation conditions the phycobiliproteins are degraded. Indeed, phycobiliproteins are nitrogen storage compounds with deep blue color in cyanobacteria (Allen, 1984). The amino acids released by the phycobiliprotein breakdown can be further used to synthesize new cellular materials to sustain cell growth.

Figure 3: Pictures of Synechocystis sp. PCC6803 cultures in balanced medium (BG_{11}) and in medium lacking added nitrate (BG_0). Cultures were grown for 21 days.

Figure 4 shows the PHB accumulated at the bottom of vials (white region). The samples were from nitrate free cultures.
The analysis of the data of PHB concentration vs. time assessed for both cultures (Figure 2 B and D) highlights that nitrogen deficiency was favorable to PHB synthesis. Indeed, the PHB concentration at the end of the 21 day culture was about 4 mg/L for the nitrogen-starved conditions and just 2.95 mg/L for the balanced cultures. Moreover, the PHB concentration is almost constant at about 0.25 % during the balanced-medium cultures and it gradually increases up to 1 % in the nitrogen-starved culture.
The different PHB final concentrations are in agreement with the results reported in the literature. In particular, the higher accumulation of PHB under nitrogen-starvation conditions may be interpreted taking into account the absence of nitrogen into the medium while the photosynthetic activity fixes CO_2. Since the cells cannot synthesize protein, the carbon/energy is accumulated by cells also as PHB (Smith, 1982). As a result of the flux re-direct under nitrogen-starved conditions, it is interesting to note that the PHB yield is higher for the nitrogen-starved culture than that for the balanced medium culture.
The PHB productivity – assessed as the final concentration divided for the culture time – is 0.19 mg/(L d).

Figure 4: PHB pellet after sonication: white region (cells were grown in BG_0).

The comparison of the final concentration of PHB in the cell and of the PHB productivity with data reported in the literature for autotrophic cultures points out that present results are promising. Although the final concentration of PHB in the cell and the PHB productivity measured for heterotrophic-mixed cultures (e.g. Jiang et al., 2011) are larger than those found in the present investigation, it should be noted that the feedstock used for the present investigation (CO_2) does not compete with any food/feed resource.

4. Final remarks

The production of PHB by means of autotrophically-grown *Synechocystis sp.* PCC6803 was successfully carried out. The cyanobacteria were able to produce PHB just from CO_2. The level of PHB production is

enhanced under nitrogen-starvation conditions. In nitrogen-starved cultures about 4 mg/L of PHB was produced, as compared to about 25 % of this amount in nitrogen replete cultures.
Although the PHB production was quite low with respect to data reported in the literature, the results are promising, be it that it is necessary to adopt a strategy to improve the PHB final concentration and the PHB productivity.

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