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PHB-Rich Biomass and BioH_2 Production by Means of Photosynthetic Microorganisms

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Polyhydroxyalkanoates (PHAs) are a family of biopolyesters produced by many bacteria as intracellular storage carbon and energy source. Poly-β-hydroxybutyrate (PHB) is probably the most common type of PHA. It is biodegradable and renewable, with relevant thermoplastic properties along with adjustable thermal and mechanical properties. The thermoplastic properties of PHB and its biodegradability make it a potential alternative to petroleum-based plastics. Several microorganisms growing in the dark and/or in the light produce PHB. The polymer is mainly accumulated in the cytoplasm of cells when microorganisms are growing under conditions of stress. If purple non-sulfur photosynthetic bacteria (PNSB) are grown under nitrogen starvation conditions, a photoevolution of molecular hydrogen occurs as well. The PHB amount increases when carbon and energy sources are in excess, but the growth is limited, for example, by the lack of a nitrogen, phosphorous or sulfur source. This work deals the possibility of producing PHAs by photosynthetic microorganisms belonging to cyanobacteria and PNSB. Different culture broths, with and without organic carbon sources, were investigated to maximize PHA production by photosynthetic microorganisms. An unbalanced agro-industrial wastewater has been also investigated in the present study. It concerns the olive mill wastewater (OMW) containing significant reusable carbon fractions suitable for an eco-efficient valorization by feeding photosynthetic processes. The maximum PHA concentration in a cyanobacterium dry-biomass was 317 mg/L, when growing cells in a medium with a low content of acetic acid (LAC). In PNSB dry-biomass the maximum PHB content was 215 mg/L, when growing PNSB in a synthetic medium. A simultaneous H2 co-production (1,295 mL/L of culture) was cumulated as well, at the end of the process.

1. Introduction

PHB is a storage compound typically produced by prokaryotic organisms; it was discovered for the first time in 1920 by Maurice Lemoigne. The properties of this compound (e.g. thermoplastic process ability and complete biodegradability even in a marine environment) suggest that it can be an attractive alternative to common plastics. Bioplastics are still prohibitively expensive for many of the current applications and their production is often based on the use of sources (starch and glucose) in competition with the food sector. On the other hand, the non-biodegradable plastics accumulate in the environment at a rate of 25 million tons per year (Balaji et al., 2013) and today they are one of the most widely used materials. Thus, over the next few years, the use of biodegradable plastics will surely increase in order to substitute conventional plastics and thus reduce the environmental impact of these materials. Research correlated with the production of PHAs is now focusing on the improvement and consistency of properties of these biopolymers in order to make their exploitation and industrialization easier with the goal of producing PHAs at competitive costs. PHA has not to be compared only to biodegradable polymers on the market, but also to non-biodegradable plastics. Cyanobacteria, together with bacteria, can be considered host polymer cell-factories for the production of PHA. Some cyanobacterial strains are able to accumulate this polymer even in photoautotrophic conditions (Balaji et al., 2013; Carpine et al., 2015), without organic carbon-sources. Cyanobacteria can also grow in different kind of wastes: textile effluent, dairy wastes, paper mill wastes, etc. Saharan et al. (2014) reported a PHB content up

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to 55% of cellular dry weight for a cyanobacterium as Synechococcus sp. MA19. Many PNSB, like *Rhodobacter sphaeroides*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum*, are able to co-produce PHA and H2 under nutrient-limited conditions. These two metabolic pathways are in competition, but the combined production of H2 and PHB would be a major advantage for the environment since it would be possible to use solar energy bioconversion to obtain alternative sources of energy and recyclable plastics (Khatipov et al., 1998).
The present study represents a joint preliminary investigation between the National Research Council and the University of Pisa about the possibility to obtain bioplastics from cyanobacteria or PNSB, by setting up a suitable and eco-sustainable process.

2. Materials and Methods

2.1. Microorganisms and culture conditions

In the present work, cyanobacteria and PNSB were investigated, both belonging to the culture collection of the Institute of Ecosystem Study of National Research Council (ISE-CNR), Florence, Italy. Cyanobacteria were grown in a modified BG11: the concentration of K, Ca and Mg salts was doubled while the other salts were kept at standard concentrations. Increasing quantities of acetic acid from 0 to 8 g/L were added, when needed, to induce PHB accumulation in the cultures. These experiments were carried out under either a continuous light intensity of 150 $\mu E/m^2/s$ on both sides of the reactor or under a light:dark cycle - 15:9 h irradiating only one side of the reactor. The cultures were mixed by means of an air flow mixture (98% air and 2% of CO_2) that made it possible to maintain the pH value within a range of 7.0 to 7.8. PNSB were grown using two different synthetic media: (i) a modified van Niel medium (synthetic growth medium, SGM) with the following composition (1.0 L): 4 g butyric acid; 0.5 g NH_4Cl; 1 g KH_2PO_4; 0.4 g NaCl; 0.05 g $CaCl_2 \cdot 2H_2O$; 0.4 g $MgSO_4 \cdot 7H_2O$; 0.1 g para-aminobenzoic acid; 10 mL of trace element solution (per 1 L of deionized water: 30 mg H_3BO_3; 3 mg $MnCl_2 \cdot 4H_2O$; 500 mg $Na_2MoO_4 \cdot 2H_2O$; 10 mg $ZnSO_4 \cdot 7H_2O$; 1 mg $CuCl_2 \cdot 2H_2O$; 2 mg $NiCl_2 \cdot 6H_2O$; 24.5 mg $CoNO_3 \cdot 6H_2O$); pH adjusted to 6.8; (ii) a modified van Niel medium for hydrogen production (synthetic medium for hydrogen, SHM) that has the same composition as the previous medium except for NH_4Cl that is substituted by Na-glutamate 1.0 g/L. An effluent originated from a pretreatment of olive mill wastewater (OMW_{eff}) was also tested according to Carlozzi et al., 2015. This effluent was diluted with sterile deionized water and used for producing both H_2 and PHB-rich biomass. A 150-W OSRAM power-star HQI-TS lamp was used to expose the culture to a continuous irradiance of 74 W/m^2 on one side of the PBR. All experiments were carried out at 30 ± 0.2 °C.

2.2. Photobioreactors

Cyanobacteria were grown in a flat glass photobioreactor (FGPBR) previously described in Vaičiulytė et al., 2014. The FGPBR had an internal glass tube ($Ø_{int}$ = 10 mm) equipped with an air stone sparger. Compressed air and CO_2 flowed inside the inner tube to mix the cyanobacterial culture and to control the culture pH. The FGPBR was equipped with three probes for the continuous control of culture parameters such as temperature, pH and dissolved oxygen concentration. The probes were connected to a control unit (Chemitec srl, Florence, Italy). PNSB were grown in a cylindrical glass photobioreactor ($Ø_{in}$= 4.0 cm). The culture was mixed by using a magnetic stirrer. The growth parameters (temperature and pH) were monitored using probes connected to a control unit (Chemitec srl, Florence, Italy). The details about the schematic diagram of the cylindrical photobioreactor used for the investigation can be found elsewhere: Carlozzi, 2012. The hydrogen generated by bacterial cells flowed into a CO_2-absorber solution (saline solution of NaOH), and it was then trapped in a calibrated column, where it was collected and the volume was measured to calculate the hydrogen production. The calibrated column was refilled with the saline solution of NaOH every 24 h.

2.3. Analytical Methods

The optical density of the cyanobacterial culture was determined at 730 nm using a spectrophotometer Cary 50 (Agilent Technologies Inc., Santa Clara, CA, USA). With the same spectrophotometer, the bacteriochlorophyll (Bchl) was also measured, according to Carlozzi et al., 2006; as well as the amount of volatile fatty acids (VFAs) of the OMW_{eff} at 530 nm (Mato et al., 2005). The COD amount was determined according to Ena et al., 2007. The acetic acid concentration was evaluated according to Carlozzi and Lambardi, 2009. Approximately 6 mg of lyophilized dry biomass was analyzed for PHB content by HPLC following acid digestion to crotonic acid by boiling cells in 1 mL of pure sulfuric acid in screw-cap glass test tubes for 30 min. Extracts were appropriately diluted with water, filtered and injected into the HPLC for analysis (Thermo Finnigan-Spectra System 6000 LP). Crotonic acid was eluted from a Synergy-Hydro-RP C-18 column (250 x 4.6 mm id.). A mobile phase comprising 15% (v/v) acetonitrile in 0.1 % (v/v) H_3PO_4 in aqueous solution was employed at a flow rate of 1 mL/min. The compound was detected at 214 nm. Peak areas were compared to a calibration curve realized with commercial PHB (Biomer). All analyses were carried

out in triplicate. The light intensity was measured with the use of a Quantum/Radiometer/Photometer (model LI-185B, Li-COR, Lincoln, NE, USA).

3. Results

3.1 PHB accumulation in cyanobacterial cells

A cyanobacterium belonging to the order of *Chroococcales* owned by the ISE-CNR was cultivated first in basic BG11 medium (Rippka et al., 1979) to test its capacity to accumulate polyhydroxyalcanoates. The investigation was carried out applying a light/dark cycle of 15/9 hours. The growth, determined as optical density at 730 nm (OD_{730nm}), is shown in Figure 1-a. The cumulative amount of PHB is reported in Figure 1-b. The addition of acetic acid, represented in the figure by arrows, started at t_{210}. As can be noted, no PHB accumulation occurs when cultivating the cyanobacteria in basic BG11 medium (autotrophically) with a light:dark cycle. In fact, from the start of the experiment to t_{210} the concentration of PHB in the culture swung from 0.10 to 0.20 mg/L. When the culture started to grow mixotrophycally, i.e. in the presence of organic and inorganic carbon sources, PHB rapidly increased, reaching the concentration of 82.14 mg/L and an OD_{730nm} of almost 5 (Fig. 1a and b). Further studies on polymer accumulation were carried out by growing cyanobacteria at both high acetate concentration (HAC) and at low acetate concentration (LAC).

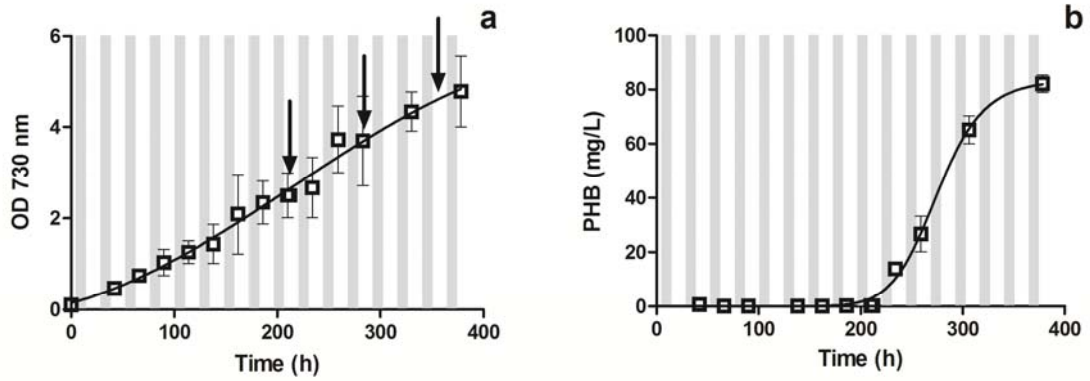

Figure 1: OD at 730 nm (a) and PHB accumulation (b) in cyanobacterial species applying a light/dark cycle of 15/9 hours; dark (grey bars). Arrows indicate the acetic acid additions. Symbols represent the mean values of three replications ± SD.

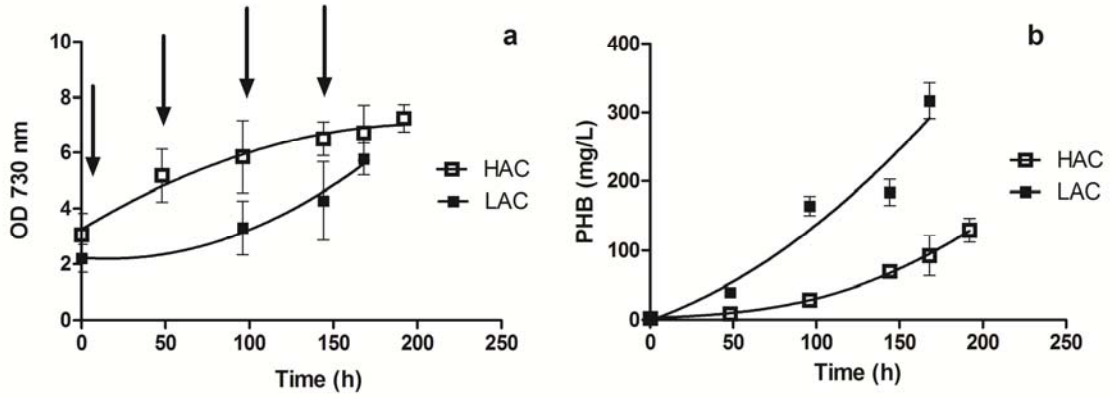

Figure 2: OD at 730 nm (a) and PHB accumulation (b) in cyanobacterial species in HAC and LAC conditions. Arrows indicate the acetic acid additions. Symbols represent the mean values of three replications ± SD.

The results are shown in Figure 2. The cyanobacterial growth was evaluated by measuring the optical density (OD730nm): the maximum OD was 7.2 at HAC and 5.8 at LAC (Figure 2-a). This behaviour was completely inverted when looking at the cumulative PHB in the cultures. In fact, at HAC about 130 mg/L of PHB were reached, whereas at LAC the accumulation of PHB boosted as high as 317 mg/L (Figure 2-b).

3.2. PHB accumulation in purple-non sulfur bacteria

A selected PNSB belonging to the genus *Rhodopseudomonas* was chosen to study cumulative PHB. When growing under photoautotrophic conditions, *R. palustris* fixes CO_2, provided an inorganic electron donor is present (H_2, $S_2O_3^{2-}$, weak H_2S). *R. palustris* can also grow richly in a synthetic medium that contains organic compounds such as organic acids, alcohols or aromatic substances (Carlozzi and Sacchi, 2001). Thus, several organic sources are adequate as carbon source and e- donor. Three different media were tested: a synthetic growth medium (SGM) containing 4.0 g/L of butyric acid and 0.5 g/L of NH_4Cl; a synthetic medium for hydrogen production (SHM) containing 4.0 g/L of butyric acid and 1.0 g/L of Na-glutamate and an effluent coming from an OMW pretreatment (OMWeff) containing 4.2 g/L of volatile fatty acids (VFAs).

Figure 3a shows the BChl growth versus time. Bchl grew very quickly in SGM, reaching the concentration of 33 mg/L after only 168 hours. Almost the same Bchl concentration (35 mg/L) was reached in SHM, in only 336 hours. The culture grown with the effluent derived from OMW pretreatment (OMW_{eff}) was not able to reach such high values of Bchl concentration as those obtained by using synthetic broths: even if the experiment lasted more than 400 h the maximum Bchl concentration was only 25 mg/L. The highest PHB concentration (215 mg/L) was reached in SHM culture, after 240 hours of growth, then it slightly decreased (Figure 3b). In the culture broth containing OMW_{eff}, a PHB concentration of 175 mg/L at t_{408} was found. The lowest PHB concentration (156 mg/L) was found in the culture grown in SGM at t_{168}. Besides the accumulation of bioplastics, *R. palustris* was able to produce $BioH_2$. Cumulative hydrogen volumes are reported in Figure 3c. $BioH_2$ production was observed in two culture broths only: SHM and OMW_{eff}, but no gas was produced in SGM. The best results were obtained for the culture broth containing OMW_{eff}. Under this condition, a cumulative H_2 volume of 1,825 mL/L of culture was attained. In SHM 30% less H_2 was produced, in comparison with the previous result. During the experiment, the consumption of VFAs was checked. The results are shown in Figure 3d. The reduction in the VFAs content was very fast in the two synthetic culture broths; while it was slower in the culture containing OMW_{eff}. In this last culture, the COD consumption was also monitored. The COD concentration decreased slightly (about 23%).

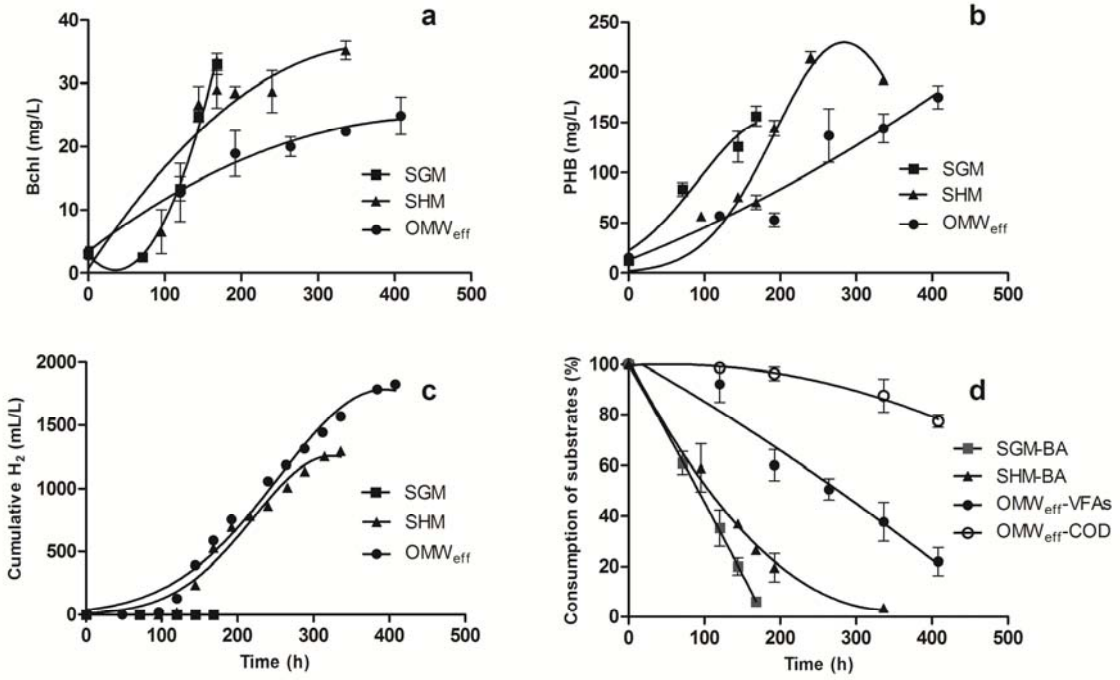

Figure 3: Growth trends of R. palustris versus time: Bchl concentration (a), cumulative PHB (b), cumulative hydrogen (c) and consumption of substrates (d). The experiments were carried out feeding the photobioreactor with two different synthetic media (SGM and SHM), and a third containing pre-treated olive mill wastewater (OMW_{eff}). The symbols represent the mean values of three replications ± SD. BA: butyric acid; VFAs: volatile fatty acids; COD: chemical oxygen demand.

4. Discussion

PHB accumulation can represent an alternative pathway for discharging excess reducing power. Usually, a reserve material such as PHB increases under unbalanced growth conditions, for example when nutrients are limited. In the experiments carried out with the cyanobacterial species, the need of an additional organic carbon source to open up the PHB-accumulation pathway has been underlined, as already observed by Mallick (2007). In fact, no increase in PHB concentration was observed before adding acetate (Figure 1). Recently, Carpine et al. (2015) investigated the growth of a selected cyanobacterial strain in a mineral medium, even with macronutrient depletion; they obtained a limited PHB accumulation of 4 mg/L only. On the contrary, as can be observed in Figure 2, an excess of organic substrate did not push the accumulation of the PHB further, so it is fundamental to find out the optimum concentration and the growth strategy in order to allow cyanobacteria to accumulate as much polymer as possible. Conversely, when a microorganism has the genetic arrangement that supports PHB production, this polymer could be produced even without stresses or nutrient starvation. The capability to accumulate this polymer can vary greatly among microorganism species as reported in Table 1. Furthermore, on changing the growth conditions, there can be important differences in PHB accumulation, even for the same bacterial strain. Belonging to PNSB, the bacterial genus that seems more able to accumulate a substantial quantity of PHB are *Rhodobacter* and *Rhodospirillum*, thus suggesting that the efficiency of this biochemical pathway depends on the particular species. In general, macronutrient depletion (N and/or P) could help to create a stressful growth condition and lead to an extra accumulation of the polymer. By culturing R. palustris in a standard medium, without stresses, it was possible to reach a PHB concentration of 150 mg/L (Figure 3b). When a lack of nitrogen occurred, the bacteria started to produce both hydrogen and PHB. Nevertheless, the two metabolic pathways are in competition (Khatipov et al., 1998); this means that the higher the cumulative hydrogen volume, the lower the amount of PHB. Depending on the goals, the coproduction of hydrogen could be an obstacle to obtaining the maximum amount of PHB in the microbial dry-biomass; or an interesting co-product that makes the entire biotechnological process more ecological (Khatipov et al., 1998; Oliveira et al., 2015). In this work, it was possible to reach a good level of PHB accumulation (175 mg/L) together with the hydrogen production of 1,825 mL/L of culture using the broth containing pre-treated olive mill wastewater (OMW_{eff}).

Table 1: Comparison of data on PHB content obtained in this work with those reported in literature, growing PNSB under different regimens

PNSB	Growth regimen	C source	Depletion	H_2 production	PHB content (%)	Ref.
Rhodobacter capsulatus Kb1	Batch	Acetic Acid	N	--	34	Liebergesell et al., 1991
Rhodobacter sphaeroides Y	Batch	Acetic Acid	N	--	63	
Rhodobacter sphaeroides RV	Batch	Acetic Acid		no	38	Khatipov et al.,1998
	Batch	Glucose		no	8	
Rhodopseudomonas palustris 42OL	Batch	Acetic Acid		no	1-4	Carlozzi and Sacchi, 2001
	Batch	Butyric Acid		yes	18.1	
Rhodopseudomonas palustris 42OL	Batch	Malic Acid		yes	7	Vincenzini et al., 1997
	Batch	Malic Acid	P	no	18	
Rhodopseudomonas palustris sp.	Batch	Butyric Acid		yes	7	This work
	Batch	OMW_{eff}		yes	9	
Rhodopseudomonas palustris 1850	Batch	Acetic Acid	N	--	15	Liebergesell et al., 1991
Rhodospirillum rubrum UR2	Batch	Malic Acid		yes	40	Melnicki et al., 2009
	Batch	Malic Acid	S	--	59	

With the prospect of a future industrial application, the whole process can be very desirable and environmentally friendly, since we can attain: i) the biological treatment of a highly polluting waste; ii) the production of bioH_2 from renewable sources and iii) the production of an extremely biodegradable bioplastic.

3. Conclusions

The present study investigated the possibility to produce bioplastics by means of photosynthetic pathways using either blu-green or purple photosynthetic microorganisms (cyanobacteria and PNSB). No production of PHB was observed when cultivating cyanobacteria in autotrophic conditions. On the contrary, when adding acetate (mixotrophic condition), it was possible to reach a PHB concentration of 317 mg/L. A co-production of PHB and hydrogen was also possible when growing PNSB, such as *R. palustris*. Biological processes used to produce bioplastics are very attractive because of the possibility to use renewable resources by feeding also microorganisms with pre-treated wastewater. If this technology was properly set up to maximize the green products, it could be an environmentally friendly alternative to the production of plastics from fossil sources.

Acknowledgments

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