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Poly-β-Hydroxybutyrate Production by *Rhodopseudomonas* sp. Grown in Semi-Continuous Mode in a 4 L Photobioreactor

Eleftherios Touloupakis [1,*], Eleni G. Poloniataki [2], Martina Casciana [3], Demetrios F. Ghanotakis [2] and Pietro Carlozzi [1]

[1] Research Institute on Terrestrial Ecosystems, National Research Council, Madonna del Piano 10, 50019 Sesto Fiorentino, Italy; pietromario.carlozzi@cnr.it
[2] Department of Chemistry, University of Crete, Voutes Campus, 70013 Heraklion, Greece; helenpol95@unist.ac.kr (E.G.P.); ghanotakis@uoc.gr (D.F.G.)
[3] Department of Biology, University of Florence, Via Madonna del Piano 6, 50019 Sesto Fiorentino, Italy; martina.casciana@stud.unifi.it
* Correspondence: eleftherios.touloupakis@cnr.it; Tel.: +39-055-5225-936

Abstract: The synthesis of polyhydroxybutyrate (PHB) by photosynthetic non-sulfur bacteria is a potential approach for producing biodegradable plastics. In this work, acetate was used as a single carbon source to study the effect on PHB formation in *Rhodopseudomonas* sp. cultured in a cylindrical four-liter photobioreactor under semi-continuous mode. The cultivation process is divided into a symmetrical growth phase and a PHB accumulation phase separated temporally. The symmetrical growth phase (nutrient sufficient conditions) was followed by a sulfur-limited phase to promote PHB accumulation. The main novelty is the progressive lowering of the sulfur concentration into *Rhodopseudomonas* culture, which was obtained by two concomitant conditions: (1) sulfur consumption during the bacterial growth and (2) semi-continuous growth strategy. This caused a progressive lowering of the sulfur concentration into *Rhodopseudomonas* culturedue to the sulfur-free medium used to replace 2 L of culture (50% of the total) that was withdrawn from the photobioreactor at each dilution. The PHB content ranged from 9.26% to 15.24% of cell dry weight. At the steady state phase, the average cumulative PHB was >210 mg/L. Sulfur deficiency proved to be one of the most suitable conditions to obtain high cumulative PHB in *Rhodopseudomonas* culture.

Keywords: purple non-sulfur bacteria; polyhydroxyalkanoate; *Rhodopseudomonas* sp.; polyhydroxybutyrate; photobioreactor

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1. Introduction

Environmental pollution, in addition to recent pressure on fossil energy resources, is forcing the research society to find a green, circular economy solution that uses mainly renewable resources [1]. Application of photosynthesis could be a possible solution to address environmental problems and to overcome the fossil-fuel based economy. Photosynthetic microorganisms, such as microalgae and photosynthetic non-sulfur bacteria (PNSB), are promising alternative candidates for providing value-added compounds such as biofuels and biomaterials [2–5]. Compared to chemical synthesis, these microorganisms have many advantages because they can grow by using natural light and have minimal nutritional requirements.

Plastic pollution is one of the main environmental problems worldwide. Replacement of petroleum-based plastics with biodegradable bioplastics could be a realistic solution to this environmental problem [6]. Therefore, research efforts are rising in the bioplastics sector to reduce the problem of recycling of petroleum-based plastics and minimize their environmental impact. In many countries, traditional plastic objects are already replaced with biodegradable ones, e.g., in applications for packaging and bio-shoppers. Despite the environmental benefits of bioplastics, their actual worldwide production is only ca. 1 Mt/y [7].

Today, applications of naturally obtained bioplastics such as polyhydroxyalkanoate (PHA) are enormously increased. PHAs are bio-based polyesters of hydroxyalkanoic acids produced by a variety of microorganisms such as heterotrophic bacteria, cyanobacteria, and PNSB [2,8–11]. More than 300 heterotrophic bacterial species are reported to produce PHA. Recently, many bacterial strains that belong to the genera of *Arenibacter*, *Vibrio*, *Ralstonia*, *Bacillus*, *Halomonas*, *Alcaligenes*, *Shewanella*, and *Pseudomonas* have been studied regarding their PHA production potential [1,12–17]. They are usually produced through a two-stage process and their production depends on bacterial strain and growth conditions [18,19]. The first stage (excess of nutrients) promotes growth, while the second (nutrient limitation) drives PHA accumulation [20]. PHA is produced from acetyl-coenzyme through three enzyme reactions [21]. Initially acetoacetyl-CoA is formed from two acetyl-CoA by ketothiolase. Acetoacetyl reductase reduces acetoacetyl-CoA to 3-hydroxyacyl-CoA. Finally, (R)-3-hydroxyacyl-CoA is catalyzed to PHA by the PHA synthase enzyme. A few bacteria, such as *Alcaligenes latus* and *Azotobacter vinelandii*, produce PHA during growth phase without nutrient limitation, while others require nutrient limitation for PHA production [22–24]. Poly(3-hydroxybutyrate) (PHB) represents the most basic PHA form commercially available. PHB, a semi-crystalline isotactic polyester, is obtained by the PHA monomer called 3-hydroxybutyrate. The PHA production cost on a large scale is high due to cost of the carbon source used in the growth media [25,26]. A strategy to reduce PHA production costs is the use of food wastes, such as whey and plant oils [27–30]. PHA copolymers formation can significantly improve chemical and physical performances. Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) obtained by introducing3-hydroxypentyl ester into PHB. P(3HB-co-4HB) is obtained by introducing a 4-hydroxybutyrate unit into PHB and PHBHHx is obtained by introducing a 3-hydroxyhexanoic acid unit into PHB [31]. PHBV is produced by culturing Gram-negative bacteria such as *Cupriavidus necator* and *Pseudomonas oleovorans* in the presence of glucose and propionic acid [32]. PHA monomers can be combined in a variety of configurations to produce a wide range of different physical properties. They are insoluble in water and possess similar physical and chemical properties to conventional synthetic plastics. They are environmentally friendly, biocompatible, and biodegradable materials [33]. PHA is degraded by various microorganisms that produce and secrete extracellular enzymes such as PHA hydrolases and PHA depolymerase [34–36]. Depolymerases catalyze hydrolysis, thus producing free D(−)-3-hydroxybutyrate, which is then oxidized to acetoacetate by a NAD-specific dehydrogenase. Degradation of PHA can be realized in various environments such as soil, fresh water, and marine environments [37,38]. Some of the most dominant PHA-degrading microorganisms belong to the bacterial genera of *Bacillus*, *Ralstonia*, *Pseudomonas*, *Alcaligenes*, *Mycobacterium*, *Comamonas*, *Acinetobacter*, *Azospirillum*, and *Streptomyces* [37,39,40]. PHA is a suitable material for applications such as the slow release of chemicals [39]. PHAs are also applied in the biomedical field mainly for tissue engineering, as drug delivery carriers, and in many single use applications such as disposable tableware, food packaging, plant pots, etc. [41–43].

PNSB are metabolically versatile organisms that are capable of phototrophic growth using light as an energy source and organic carbon compounds as electron donors for CO_2 fixation [44]. Moreover, they have been selected for applications in the environmental protection and agriculture research areas [45–47]. PNSB accumulate PHB as a carbon reserve material when they are grown under nutrient (such as nitrogen or phosphorus)-limiting conditions [48]. *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* represent the most suitable genera for PHB accumulation [49]. They can metabolize organic compounds such as volatile fatty acids [27,50,51] to produce PHA via the acetyl-CoA or propionyl-CoA metabolic pathway [52].

The possibility of marketing PNSB as a reservoir of bio-products, such as PHA, requires the use of controlled growth in efficient photobioreactors (PBRs). Efficient PBRs should be developed in order to have an efficient use of light and good mixing to ensure symmetrical and homogeneous illumination, and nutrient distribution to obtain high

growthrate and productivity. Optimization of the starting culture biomass density is an important issue.

In this investigation, acetate was used as a single carbon source to study the effect on *Rhodopseudomonas* sp. growth and PHB formation in a cylindrical four-liter PBR. The use of PBR provided optimal growth under fully controlled culture conditions and minimized the risk of culture contamination. Moreover, the present investigation focused on PNSB cultivation initially under sulfur-sufficient and subsequently under sulfur-deficient conditions. This last growth condition should lead to an increased PHB content in bacterial cells.

2. Materials and Methods

2.1. Organism and Culture Conditions

Rhodopseudomonas sp. was grown using a modified Van Niel growth medium (in 1.0 L): 6.0 g acetate, 0.5 g NH_4Cl, 1.0 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.05 g $CaCl_2 \cdot 2H_2O$, 0.4 g NaCl, 0.1 mg para-aminobenzoic acid, and 10 mL of micronutrients solution; and a micronutrient solution (in 1.0 L): 2.0 mg $NiCl_2 \cdot 6H_2O$, 1.0 mg $CuCl_2 \cdot 2H_2O$, 20 mg $CoCl_2 \cdot 6H_2O$, 3.0 mg $MnCl_2 \cdot 4H_2O$, 30 mg H_3BO_3, 10 mg $ZnSO_4 \cdot 7H_2O$, 500 mg $Na_2MoO_4 \cdot 7H_2O$, and 200 mg $FeSO_4 \cdot 7H_2O$. *Rhodopseudomonas* sp. cells were previously acclimated for seven days in the media containing acetate and ammonium chloride. Prior to each experiment, the culture was dark-incubated for 18 h in order to obtain anaerobiosis.

The culture was operated in semi-continuous mode. After an initial start-up phase, 50% of the culture volume (2.0 L) was withdrawn from the reactor and replaced by an equal volume of fresh medium presenting 0.25 g/L NH_4Cl and 0.02 g/L $MgSO_4 \cdot 7H_2O$. At each dilution, acetate concentration in the culture was fixed to 6.0 g/L. This feeding strategy was used for long-term investigation (30 days). Growth media were sterilized by autoclaving for 20 min at 121 °C and 2.0 atm pressure in a Fedegari FVA-2 autoclave (Fedegari Autoclavi SpA, Albuzzano (PV), Italy).

2.2. Culture System

A 4 L PBR was used for *Rhodopseudomonas* sp. cultivation (Figure 1) [50]. The PBR consisted of a cylindrical chamber with an internal rotor (9.1 cm Ø, 20 cm high) equipped with four symmetrical paddles to mix the culture (170 rpm). Culture illumination (80.8 ± 4.5 W/m^2) was provided by two OSRAM power-star 150 Watt HQI-TS lamps. Culture temperature (30.0 ± 0.1 °C) was controlled with the use of a refrigerated-heating circulator (Julabo, Seelbach, Germany) and pH = 7.1 was maintained constant by the addition of a sterile HCl solution (10 mM). The pH values were monitored by using a probe connected to a control unit (Chemitec srl, Florence, Italy).

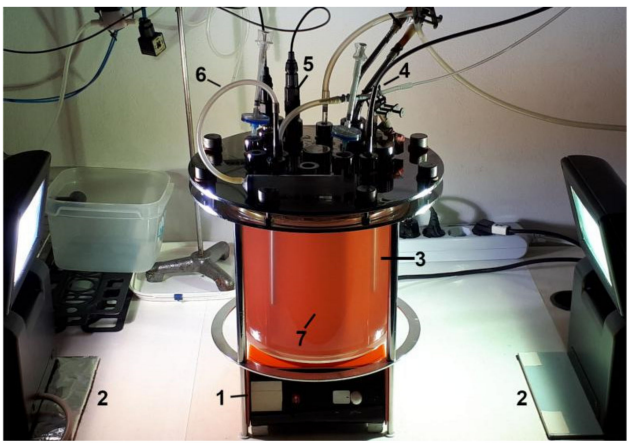


Figure 1. The cylindrical 4.0 L photobioreactor utilized for culturing *Rhodopseudomonas* sp. Magnetic stirrer (1); lamps (2); temperature probe (3); heating/cold finger (4); pH probe (5); sample port (6); 4-paddle rotor (7).

2.3. Analytical Methods

Cell dry weight (CDW) was determined in triplicate in accordance with Carlozzi and Sacchi [53]. A total of 5.0 mL of the culture sample was diluted to 50 mL with distilled water and filtered through a pre-weighed cellulose nitrate membrane with a 0.45 µm pore size (Sartorius Stedim Biotech GmbH, Göttingen, Germany) and dried at 105 °C until a constant weight was reached [53]. Bacteriochlorophyll concentration was estimated spectrophotometrically in accordance with Carlozzi and Sacchi [53]. The acetate concentration was determined in accordance with Carlozzi et al. [50].

PHB was determined in the form of crotonic acid by HPLC. We used 5.0 mL of culture for acid digestion to crotonic acid by boiling them in 1.0 mL of pure sulfuric acid for 30 min. This treatment converts PHB into crotonic acid, which was assayed by HPLC. The latter was performed by using an HPLC-Thermo Finnigan-Spectra System 6000LP (Thermo Finnigan, San Jose (CA), USA), equipped with a Synergi-Hydro-RP C-18 column (250 × 4.6 mm i.d.) (Phenomenex International, Torrance (CA), USA) and an ultraviolet detector (214 nm). A mobile phase comprising 15% (*v*/*v*) acetonitrile and 0.1% (*v*/*v*) H_3PO_4 in aqueous solution was employed using a flow rate of 1.0mL/min [54]. Pure PHB (Biomer, Krailling, Germany) converted to crotonic acid was used for the calibration curve. All analyses were carried out in triplicate.

3. Results and Discussion

Very often, PHAs are synthesized under stressful conditions by several heterotrophic bacteria and many photosynthetic microorganisms such as cyanobacteria and PNSB [11]. PHA production using microalgae is also being explored [8]. When microalgae grow in nitrogen- and/or phosphorus-depleted medium, they accumulate intracellularly carbon-rich compounds, such as PHAs, as an energy source. However, the low PHB productivity reported for cyanobacteria presents the biggest obstacle preventing economic PHB production [55]. Among photosynthetic microorganisms, PNSBs, such as *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Rhodopseudomonas* sp., and *Rhodovulum sulfidophilum*, are known to have a better ability to produce PHA [21].

Mainly the type of carbon source, C/N ratio, pH, and the used strain influence PHB accumulation in PNSB cells [49]. A low C/N ratio is beneficial for cell growth, while a high C/N ratio boosts PHB production. Generally, PHB accumulation in PNSB cells increases symmetrically in the presence of excess carbon and under nutrient-limited conditions [21]. For instance, the PHB content in *Rhodobacter sphaeroides* was high (51.57% of CDW) when the bacterium was cultured at a C/N ratio of 15:1 [56]. These nutrient-limiting conditions cause a decrease in cell growth and division, and a redirection of metabolism toward PHB biosynthesis.

During the photoheterotrophic growth of a *Rhodopseudomonas* cell, anaerobiosis and light conditions are required [53]. The types of culture and cultivation system are also important for the efficient production of PHB. Fed-batch cultivation combines a high cell density cultures with a high percentage of PHB. This type of culture is started in batch mode, and the culture is fed with nutritional components for growth and/or PHB synthesis in order to prolong the exponential-phase growth at a high rate, thereby maximizing cell density [57].

The possibility of using PNSB for PHB large-scale production requires the use of efficient cultivation systems such as closed PBRs and controlled culture conditions. This is necessary to reach a better understanding of how PNSB cells acclimate to certain culture conditions. Thus, optimization of culture conditions, growth media, process parameters, and bioreactor design should be managed. By using closed PBRs culture parameters such as nutrients, pH, temperature, and light intensity, mixing can be fully controlled. Moreover, they are suitable to prevent culture contamination by unwanted microorganisms. Many PBR designs with symmetrical shapes have been proposed and optimized for the cultivation of PNSB [2,10,58–60]. To date, many studies on PHB production by PNSB have been carried out using small, closed-type PBRs [27,50]. PBR efficiency is determined by the

combination of light capture, transmission, and symmetrical distribution. Mixing is also important for homogenous nutrient distribution, gas exchange, temperature, and culture pH maintenance.

A novel four-liter cylindrical PBR was used to grow *Rhodopseudomonas* sp. cells under semi-continuous mode (Figure 1). PBR shape and the presence of the four-paddle rotor offered an optimal use of light (4 cm light path) and suitable mixing to ensure homogeneous illumination of the culture. PBR provided optimal growth under fully controlled culture conditions and minimized the risk of culture contamination. The cultivation process is divided into a growth phase and a PHB accumulation phase, which are separated temporally. The growth phase (nutrient sufficient conditions) was followed by a nutrient-limited phase (absence of S) to promote high PHB at the time of harvest. After an initial start-up phase, 2.0 L of the culture was replaced by an equal volume of fresh medium that was sulfur-free. The following dilutions were produced by using fresh culture medium containing 0.25 g/L NH_4Cl and 0.02 g/L $MgSO_4$. The NH_4Cl was added, as the Nsource (C/N ratio = 35), in order to inhibit nitrogenase activity and consecutively hydrogen production. It is known that hydrogen production is incompatible with PHB accumulation because of the result of competition for assimilation of reducing equivalents [61]. In our previous study, we tested different carbon sources with the same microorganism grown in a smaller PBR (0.22 L) and we concluded that acetate was the best carbon source in terms of the PHB content [10]. Sulfur concentration is maintained sufficiently low in order to maintain high cell viability and to gain sulfur limitation conditions, slowly, in *Rhodopseudomonas* culture. Sulfur deficiency prevents de novo biosynthesis of sulfur-containing amino acids, thereby blocking protein biosynthesis and cell growth. PNSB cells maintain their metabolic activity in a non-growing state for a long period of time [62] and produce PHB as an energy storage polymer [63]. PHB accumulation is associated with functional pre-existing PHB synthesis enzymes and the nitrogen availability in the growth medium [64].

Rhodopseudomonas sp. grew well on acetate as a carbon source, showing a fast CDW increase after each culture dilution (Figure 2). The acetate concentration decreased progressively during cell growth after each culture dilution (Figure 3). Bacteriochlorophyll concentration during the experiment was from 2% to 3% of CDW (data not shown). In the presence of acetate and NH_4Cl, the nitrogenase enzyme was inactive and only PHB production was achieved. PHB production has been previously reported by several *Rhodopseudomonas* strains [61,65,66].

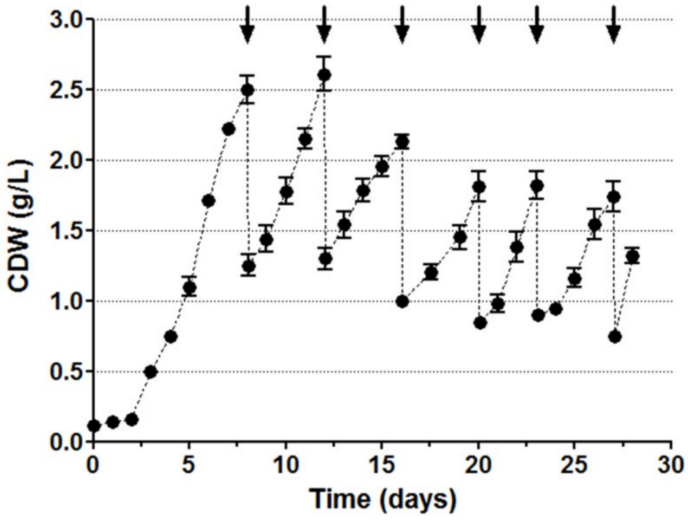


Figure 2. Changes in CDW over time during *Rhodopseudomonas* sp. growth in the 4.0 L photobioreactor (arrows indicate culture dilution times).

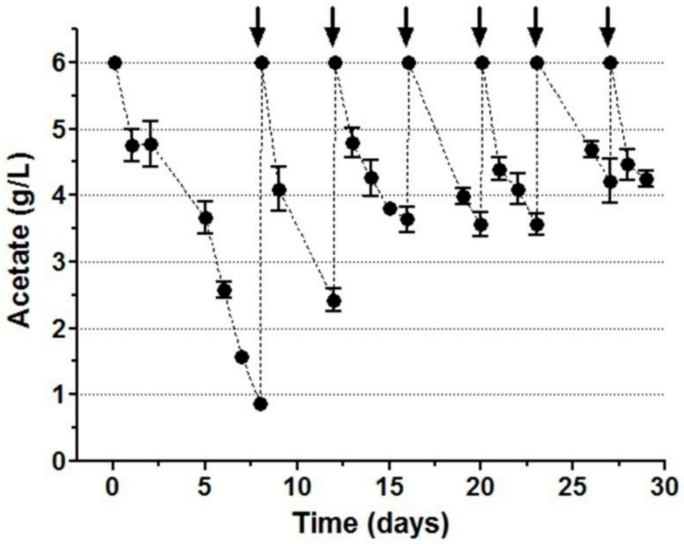
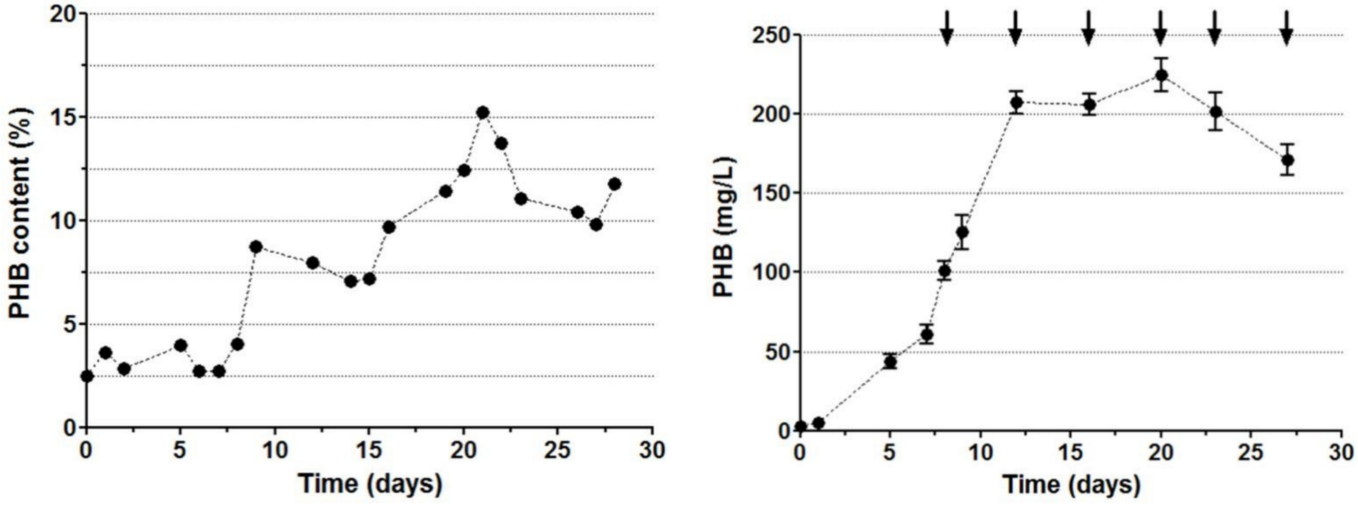


Figure 3. Changes in acetate concentration over time during *Rhodopseudomonas* sp. growth (arrows indicate culture dilution times).

PHB accumulation in *Rhodopseudomonas* is triggered in the absence of essential nutrients (such as N, P, or S) and with an excess of a carbon source [67]. Under such nutrient-limited conditions, the culture enters a steady-state phase directing carbon flux from biomass production toward the PHB biosynthesis pathway [67,68]. The use of fresh S-free medium (at day 8) and later (from day 12) with a medium containing a low sulfur concentration caused a continuous decrease in sulfur concentration in the culture. This new condition caused a progressive increase in total PHB in the *Rhodopseudomonas* culture and PHB amount in CDW (Figure 4). After 16 days, the culture entered a steady-state phase (Figure 2). The PHB content in the culture, during the steady-state phase, was from 9.26% to 15.24% of CDW (Figure 4a). The maximum intracellular PHB was 224.6 mg/L (Figure 4b). PHB content (~15% of CDW) was near the value (~18% of CDW) obtained recently from our group [10] by using *Rhodopseudomonas* cells cultured in a 0.22 L cylindrical PBR by using acetate as the carbon source (under sulfur deficiency conditions).



(**a**) (**b**)

Figure 4. (**a**) PHB content (% of CDW) inthe *Rhodopseudomonas* cells; (**b**) intracellular PHB concentration (mg/L) versus time (arrows indicate culture dilution times).

4. Conclusions

This study investigated the influence of cultural conditions, such as sulfur deficiency, obtained by sulfur consumption together with the semi-continuous growth strategy. This caused a progressive lowering of the sulfur concentration in *Rhodopseudomonas* culturedue to the sulfur-free medium used to replace 2 L of the culture (50% of the total) that was withdrawn from the PBR at each dilution. Sulfur deficiency provedto be one of the most suitable conditions to obtain high cumulative PHB in *Rhodopseudomonas* culture.

Author Contributions: Conceptualization, P.C. and E.T.; supervision, P.C., E.T. and D.F.G.; methodology, E.G.P., M.C. and E.T.; validation, E.G.P. and M.C.; investigation, E.T., P.C., E.G.P. and M.C.; resources, E.T. and P.C.; writing—original draft preparation, E.T.; writing—review and editing, E.T., P.C. and D.F.G.; visualization, E.G.P. and M.C.; funding acquisition, P.C. All authors have read and agreed to the published version of the manuscript.

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