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Poly(3-hydroxybutyrate) production by *Rhodopseudomonas* sp. S16-VOGS3 cells grown in digested sludge

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ABSTRACT

The biomass produced by cultivating photosynthetic bacteria could be used for a variety of applications, such as feedstock for laying hens and/or bioplastic production. To achieve high productivity, it is important to optimize certain criteria such as the availability of water, nutrients, and light. Due to the high price of nutrients, alternatives such as the use of digested sludge as a nutrient source have been explored. In this study, digested sludge was tested as cultivation medium in order to promote *Rhodopseudomonas* sp. S16-VOGS3 growth and poly(3-hydroxybutyrate) (PHB) production. A cylindrical photobioreactor (0.22-L working volume) was sufficient to ensure optimal mixing of the culture and adequate exposure of the bacterial cells to the light. By using 8% of the digested sludge diluted in sterile deionized water, *Rhodopseudomonas* cells grew fast and stabilized at a value of 0.37 $\pm$ 0.01 g L^{-1} of cell dry weight (CDW). Amounts higher than 8%–12% of digested sludge should be investigated to improve the process efficiency. PHB production started as soon as phosphate was limited in the culture and continued over time to a maximum concentration of 18.5 mg L^{-1} and 5.2 $\pm$ 0.2% of CDW. This study demonstrated that digested sludge can be effectively used as a suitable feedstock for the cultivation of *Rhodopseudomonas* sp. S16-VOGS3.

1. Introduction

One of the largest environmental problems in the world today is plastic waste. Today, plastics are widely used and have a significant economic impact in many industries. According to a recent study (Plastics Europe, 2022), 44% of plastics demand is for packaging, 18% for building and construction, and 8% for the automotive industry.

Petroleum-based plastics are not significantly biodegradable in nature; moreover, they can break down when exposed to UV light, high temperatures, or other natural stresses, creating tiny plastic particles (Sarijan et al., 2021). The presence of microplastics poses a threat to soil ecology and ecosystems because they have long life cycles, potentially harmful effects, and long lifespans (Sajjad et al., 2022). Microplastics can alter soil properties, affect soil microbial activity, harm soil fauna and affect plant growth. Living organisms can consume particles smaller than 150 nm and microparticles can contaminate drinking water, accumulate in the food chain, and release harmful compounds (Yuan et al., 2022). Since acrylonitrile-based plastics are not biodegradable, their combustion produces hydrocyanic acid, which poses a major risk to both

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health and the environment. Harmful substances in plastics that can leach out and affect the environment and people's health include phthalates, polyfluorinated compounds and bisphenol A. Exposure to these hazardous chemicals can lead to serious negative health effects, including cancer, weakened immune system, endocrine disruption, developmental and reproductive harm (Rustagi et al., 2011). A viable solution to this environmental problem would be to switch to biodegradable bioplastics instead of petroleum-based plastics. Therefore, research activities in the field of bioplastics are increasing worldwide in order to reduce the difficulties of recycling petroleum-based plastics (Nanda et al., 2022; Moshood et al., 2022).

Polyhydroxyalkanoates (PHAs) are inert, water-insoluble, nontoxic polyesters (Samui and Kanai, 2019). They have excellent thermoplastic and mechanical properties and are biodegradable in various ecosystems (Meereboer et al., 2020). PHAs are used as biomaterials in medical and therapeutic applications, as well as in the production of biodegradable products for a variety of industrial applications (Alves et al., 2022). The synthesis of a PHA, such as poly (3-hydroxybutyrate) (PHB), depends on the bacterial strain and growth conditions (Carlozzi and Touloupakis, 2021). The fundamental pathways and core metabolism for PHA synthesis as a function of various substrates from major global waste streams were documented by Koller et al. (2010). Volatile fatty acids (VFAs), byproducts of anaerobic digestion, are useful substrates for several bioprocesses, including PHA production (Valentino et al., 2018). Initially, cell growth is favored by excess of nutrients, but PHA production occurs due to a nutrient limitation (Fernández-Dacosta et al., 2015).

PHA-accumulating bacteria (Eubacteria and Archaea) have been used to produce bioplastic, which gained high interest in recent years as an ecological solution to the problem of plastic pollution (Raza et al., 2018). Recently, a study on PHA production, via a fermentative process, has been reported by using the heterotrophic bacterium *Thauera* sp. fed with digestate, obtained from the anaerobic digestion plant (Critelli et al., 2022). In 2021, Tyagi and Sharma focused their studies on the utilization of untreated crude paper industry effluent (PIE) as a substrate for PHA production in order to utilize the unused carbon and thus develop a feasible production process (Tyagi and Sharma, 2021). They concluded that crude PIE could be used as a substrate if a correct bacterium (i.e., *Ancylobacter* sp.) is employed to obtain sustainable PHA production. In addition, microalgae, cyanobacteria, and purple non-sulfur bacteria (PNSB) are examples of photosynthetic microorganisms that could be used as feedstock for bioplastics (Carlozzi et al., 2019c). They can be used to produce high-value products, as well as biomass and bioenergy (Tiwari et al., 2018; Touloupakis et al., 2021a).

PNSB have been proven to be an alternative biological platform for wastewater treatment due to their high carbon uptake capacity (Sepúlveda-Muñoz et al., 2020, 2022). In addition, they can grow at low temperatures, are tolerant of high salinity and pollutants present in most wastewaters, can assimilate all forms of nitrogen, and exhibit high growth rates under photoheterotrophic conditions. The great metabolic adaptability of PNSB, which can thrive in both aerobic and anaerobic environments, makes them a unique candidate for developing appropriate approaches for wastewater treatment. PNSB, such as *Rhodospirillum rubrum*, *Rhodopseudomonas* sp., and *Rhodobacter sphaeroides*, are metabolically versatile and thus can grow as photoheterotrophic, photoautotrophic, or chemoheterotrophic, switching from one mode to the other depending on available conditions. They can use light energy, which increases its efficiency in recycling organic matter thus reducing energy costs.

PNSB can accumulate a significant amount of polyesters as a carbon storage material (Ye et al., 2013; Cabecas Segura et al., 2022). Under growth-limiting conditions, PNSB produce considerable amounts of polyesters such as PHAs (Carlozzi et al., 2019c; Touloupakis et al., 2021b).

The use of current renewable biomass sources to produce biodegradable polymers such as PHA may increasingly compete with their conventional food applications. Organic wastes, such as digested sludge, lignocellulosic waste, paper industry effluent, and protein hydrolysates generated from agricultural residues could be successfully used as feedstocks to produce biologically derived growth media containing volatile fatty acids (Allegue et al., 2021; Tamang et al., 2021; Saravanan et al., 2022). These can be used as precursors of PHA, which lowers the production cost of PHA and makes PHA-based bioplastics competitive with standard fossil fuel-based polymers (Rossi et al., 2022a).

Digested sludge is currently underutilized and often disposed of in landfills or incinerated in waste-to-energy plants, and only a portion is being transferred to co-composting facilities (Albini et al., 2019). The digested sludge obtained from the processes of dark fermentation of organic wastes can also be an interesting substrate for the increasing development of new technologies for their treatment. The concept of anaerobic biorefinery, from the point of view of the circular economy, allows the recovery of energy and matter as a bioproduct (Pecorini et al., 2012; Baccioli et al., 2018). Depending on the technical process and the input materials, digested sludge can have a wide range of chemical and biological compositions (Rossi et al., 2022a). The digestate produced during the anaerobic digestion of sludge has a high content of organic matter and nutrients and is therefore highly suitable for the cultivation of photosynthetic bacteria (Pecorini et al., 2020).

In the present study, the effect of diluted digested sludge on *Rhodopseudomonas* sp. S16-VOGS3 (hereafter *Rhodopseudomonas*) growth and PHB production was investigated. The abovementioned bacterium was cultivated in a 0.22-L cylindrical photobioreactor (PBR). The *Rhodopseudomonas* cell growth potential and PHB production were evaluated. The use of the PBR reduced the possibility of contamination of the culture while allowing excellent development under well-controlled culture conditions.

Table 1
Composition of the digested sludge.

Compound	Concentration (mg Kg^{-1})
Acetic Acid	1740 ± 260
Propionic Acid	4640 ± 700
Ammonium	3130 ± 470
Phosphate	589 ± 71
Al	640 ± 180
Ba	254 ± 53
Ca	14400 ± 53
Fe	1460 ± 130
Mg	890 ± 270
Mn	68 ± 22
Na	1560 ± 500
K	2920 ± 440
S	< 50
SiO_2	61600 ± 9200
Zn	29.0 ± 4.4

2. Materials and methods

2.1. Materials

The digested sludge used in the present study was from a pilot Plug-flow reactor fed with the organic fraction of municipal solid waste (OFMSW) (managed by DESTEC UNIPI) (Rossi et al., 2022b). The feeding slurry had a dry matter content of 33%, and the trial lasted about 330 days (Rossi et al., 2022c). A sample of the digestate was collected every month in order to obtain a representative sample of sludge from the trial. Each sample was centrifuged to separate the solid fraction from the liquid to simulate an industrial pretreatment process and obtain a digested liquid sludge (1%–2% Total Solid). After centrifugation at 13,500 rpm for 20 min and filtration with a 0.45 μm membrane the digested sludge was frozen to transfer it to the next trial. The composition of the above digested sludge is shown in Table 1. The digested sludge had a total organic carbon (TOC) = 8.8 ± 1.3%, a C/N = 21.0 ± 2.1 and a pH of 7.9 ± 0.4.

2.2. Microorganism

Investigations were performed by culturing the bacterium *Rhodopseudomonas* sp. S16-VOGS3 from the culture collection of the Research Institute on Terrestrial Ecosystems (IRET), National Research Council (CNR), Florence – Italy. The 16S sequence of the bacterium was deposited in the GenBank database (accession numbers: KU899101–KU899105).

2.3. Culture conditions

A modified van Niel medium was used as control, whose composition was 2.0 g L^{-1} of acetate, 0.5 g L^{-1} NH_4Cl, 1.0 g L^{-1} KH_2PO_4, 0.4 g L^{-1} NaCl, 0.4 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.05 g L^{-1} $CaCl_2{\cdot}2H_2O$, 0.1 mg L^{-1} p-aminobenzoic acid, 0.005 g L^{-1} ferric citrate and 10 mL L^{-1} of mineral solution for micronutrients. Mineral solution (1 L) contained 1.0 mg $CuCl_2{\cdot}2H_2O$, 2.0 mg $NiCl_2{\cdot}6H_2O$, 3.0 mg $MnCl_2{\cdot}4H_2O$, 10 mg $ZnSO_4{\cdot}7H_2O$, 20 mg $CoCl_2{\cdot}6H_2O$, 30 mg H_3BO_3, 200 mg $FeSO_4{\cdot}7H_2O$, and 500 mg $Na_2MoO_4{\cdot}7H_2O$. The pH of the medium was adjusted to 6.8 by using HCl 10 mM or NaOH 5% w/v. *Rhodopseudomonas* cells were previously acclimated for 7 days in the media containing acetate (2.0 g L^{-1}) and ammonium chloride (0.5 g L^{-1}).

2.4. Tube experiment

The solid fraction of the digested sludge was removed by centrifugation, and the liquid fraction was filtered through 0.45 μm filters and diluted with distilled water (2, 4, 8, 12%, v/v). *Rhodopseudomonas* growth experiments were performed in 20 mL glass tubes at a constant temperature of 30.0 ± 0.1°C and a continuous irradiance of 80 W m^{-2} (Fig. 1). A halogen lamp (150-W OSRAM power-star HQI-TS) was used to illuminate the cultures. A radiometer (model LI-185B, LICOR, Lincoln, Nebraska, USA) was used to measure irradiance. The amount of precultured *Rhodopseudomonas* inoculated into the tubes was 10% of the volume of the synthetic medium. To achieve anaerobiosis, each experiment was preceded by a 24 hour dark incubation. Every day, each culture tube was vortexed for 1 min. Experiments were performed in triplicate.

2.5. Photobioreactor experiment

A sterilized cylindrical glass PBR with a working volume of 220 mL and an internal diameter of 4 cm was used. An appropriate amount of precultured *Rhodopseudomonas* was taken, and the cells were centrifuged and washed with physiological solution to remove traces of salts. To study the effect of the medium on cell growth and PHB formation, the cells were suspended in a culture broth containing the diluted (8% v/v) digested sludge. The initial concentrations

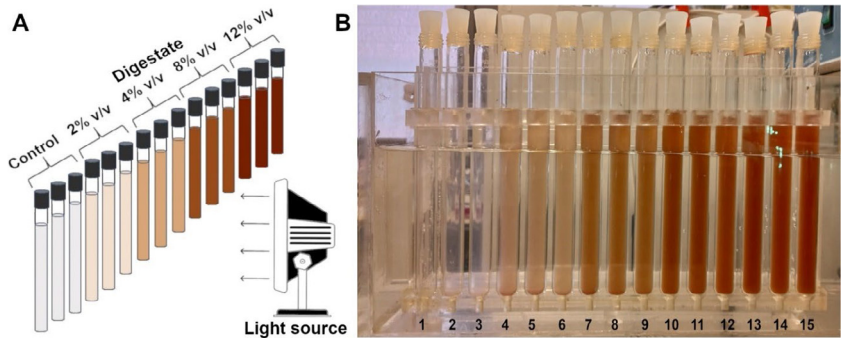

Fig. 1. *Rhodopseudomonas* sp. S16-VOGS3 cultural growth system using digested sludge diluted with deionized sterile water. (A) Schematic representation of the cultural system; (B) A picture of the cultural system. Tubes 1–3: control; tubes 4–6: digestate 2%; tubes 7–9: digestate 4%; tubes 10-12: digestate 8%; tubes 13-15: digestate 12%.

of bacteriochlorophyll (Bchl) and cell dry weight (CDW) were 0.75 mg L^{-1} and 0.06 g L^{-1}, respectively. The culture was stirred with a magnetic stirrer and maintained in a constant temperature plexiglass water bath with a heat exchanger. Two needles were inserted into the silicone plug at the top of the reactor, one for the addition of sterile distilled water and the second for the addition of a HCl solution. The culture was maintained in batch-growth regimen. Each day, a small volume (10 mL) was removed from the reactor to collect a culture sample and then an appropriate volume of sterile distilled water was added. The pH of the culture was initially adjusted to 7.2 and then was adjusted every 24 hours. Two probes connected to a control unit (Chemitec srl, Florence, Italy) were used to monitor pH and the oxidation-reduction potential.

2.6. Analytical procedures

CDW was determined by filtering five milliliters of culture through a pre-weighed cellulose nitrate membrane (Sartorius Stedim Biotech GmbH, 37070 Goettingen, Germany) with a pore size of 0.45 μm. The membrane was then dried at 80 °C to a constant weight. Bchl was calculated according to the method of Carlozzi and Touloupakis (2021). PHB was determined in the form of crotonic acid by HPLC using the following procedure. Five milliliters of the culture were centrifuged in a Sorvall Super T21 centrifuge at 5000 g for 10 min, and the pellet was used for acid digestion with 1 mL of pure sulfuric acid at 105 °C to convert PHB to crotonic acid. Crotonic acid was eluted from a Synergy-Hydro-RP C-18 column (205 × 4.6 mm i.d.) and measured by ultraviolet detection at 214 nm. The mobile phase was 15% (v/v) acetonitrile in 0.1% (v/v) H_3PO_4 in aqueous solution at a flow rate of 1 mL min^{-1}. Every analysis was performed in triplicate. Total volatile organic acids were quantified by using the Spectroquant® Volatile organic Acids Photometric Test (Merck, Darmstadt, Germany). Ammonia was tested by using the Hanna ammonia reagent kit (HI93715-01) and the C99 Multiparameter Bench Photometer (HANNA Instruments, Lucca, Italy).

3. Results

The effect of diluted digested sludge on *Rhodopseudomonas* was studied by feeding various concentrations of digested sludge (2, 4, 8, and 12% v/v) diluted with deionized sterile water in 20 mL glass tubes (Fig. 1). The main components of the digestate after centrifugation and filtration were: Acetic acid = 1.74 ± 0.26 g kg^{-1}; Propionic acid = 4.64 ± 0.70 g kg^{-1}; Ammonium = 3.13 ± 0.47 g kg^{-1}; Phosphate = 0.59 ± 0.07 g kg^{-1}, pH = 7.9.

Rhodopseudomonas cells grew well in all digestate dilutions. Fig. 2 shows the changes in biomass (CDW), VFAs and PHB after a growth period of 192 hours. PHB formation and biomass growth were stimulated in the cultures in the presence of diluted digestate.

Optimal conditions for bacterial growth and PHB production were achieved by using 8% and 12% dilutions of the digested sludge. A CDW of 0.65 g L^{-1}, a Bchl concentration of 4.77 mg L^{-1}, and a PHB concentration of 7.99 ± 0.05 mg L^{-1} were achieved with the 8% dilution. The cell biomass contained 1.21% PHB. With the 12% dilution of digested sludge, a final CDW of 0.84 g L^{-1}, a Bchl concentration of 6.00 mg L^{-1} and a PHB concentration of 17.53 ± 0.05 mg L^{-1} (2.09% of CDW) were achieved.

Rhodopseudomonas cells were subsequently cultured in the presence of the diluted digestate, for 8 days, in a cylindrical PBR (working volume of 220 mL). To maintain a balance of VFAs and phosphate contents, the 8% of the diluted digestate was used to feed *Rhodopseudomonas*. The low phosphate content in the digestate could facilitate the PHB production process. The use of 8% diluted digestate would reduce the effects of dark color and ammonium concentration in the culture. The bacterial strain was cultured in the culture system shown in Fig. 3. Both pH and oxidation–reduction parameters of the culture were measured regularly. The pH (7.2) was kept reasonably constant over time by the addition of 0.1 mM HCl.

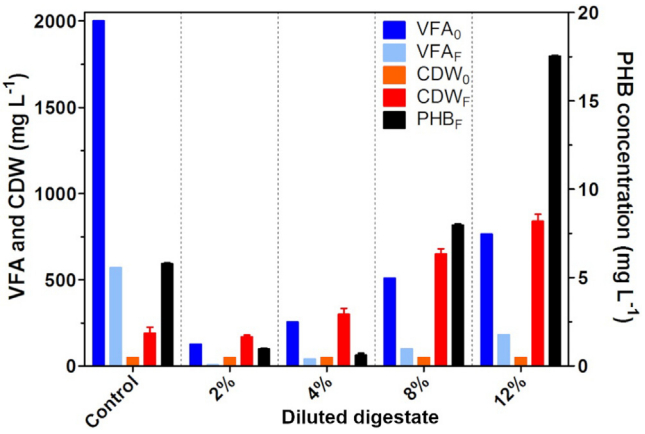

Fig. 2. Volatile fatty acids (VFA), cell dry weight (CDW) and poly (3-hydroxybutyrate) (PHB) at the beginning and end of cultivation of *Rhodopseudomonas* sp. S16-VOGS3 in glass tubes containing diluted digested sludge. Synthetic growth medium containing acetate 2 g L^{-1} was used as a control. CDW_0 = initial CDW; CDW_f = final CDW; VFA_0 = initial VFA; VFA_f = final VFA; PHB_f = final PHB.

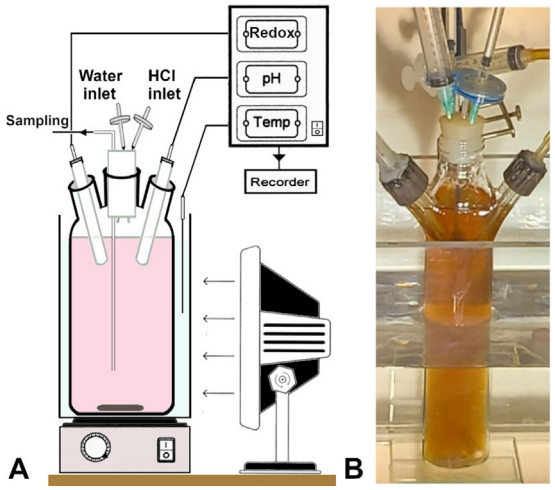

Fig. 3. The 0.22 L PBR for testing *Rhodopseudomonas* sp. S16-VOGS3 fed with digested sludge diluted with distilled water (8% v/v). (A) schematic representation of the photobioreactor; (B) image of the photobioreactor.

Rhodopseudomonas grew well in the presence of the diluted digestate, indicating that it can assimilate the organic fatty acids it contains for cell division. This is in accordance with Touloupakis et al. (2021c). The CDW increased rapidly and stabilized at a stationary phase at a value of 0.37 ± 0.01 g L^{-1} (Fig. 4). The Bchl concentration of the culture increased rapidly and then stabilized at about 2.5 mg L^{-1} (Fig. 4). The highest Bchl concentration was 2.67 ± 0.01 mg L^{-1}.

VFAs in the digestate (mainly acetic acid and propionic acid) were rapidly consumed by *Rhodopseudomonas* cells (Fig. 5). Similarly, ammonium consumption was observed until the final concentration of 36.5 mg L^{-1}. No H_2 production was observed during the experiment. This is due to the presence of ammonium, which has an inhibitory effect on H_2 production.

As shown, cell growth and PHB production led to a significant decrease in the VFA content in the culture broth. The cumulative production of PHB by *Rhodopseudomonas* is shown in Fig. 6. PHB production began as soon as phosphate was limited in the growth medium and continued over time to a maximum concentration of 18.5 mg L^{-1} (5.2 ± 0.2% of CDW).

4. Discussion

PHAs have attracted the attention of academia and industry, but their major drawback is the high cost of production. The cost of PHAs has decreased over time, from 15–17$ kg^{-1} in the 1990s to 3–5$ kg^{-1} currently, because of main advances in research (Gahlawat et al., 2020). This market price is still not comparable to petroleum-based polymers. The carbon source accounts for 30%–50% of the cost of producing PHAs (Gahlawat et al., 2020); therefore, it is critical to use affordable carbon sources (Saratale et al., 2019; Policastro et al., 2020). The European Commission recommendation

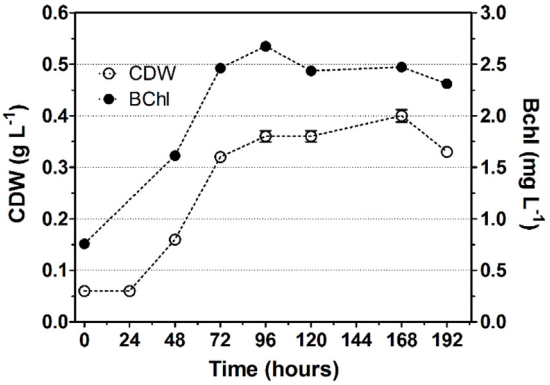

Fig. 4. Changes of cell dry weight (CDW) and bacteriochlorophyll (Bchl) over time during *Rhodopseudomonas* sp. S16-VOGS3 growth using diluted digestate (8% v/v) in the 0.22 L photobioreactor.

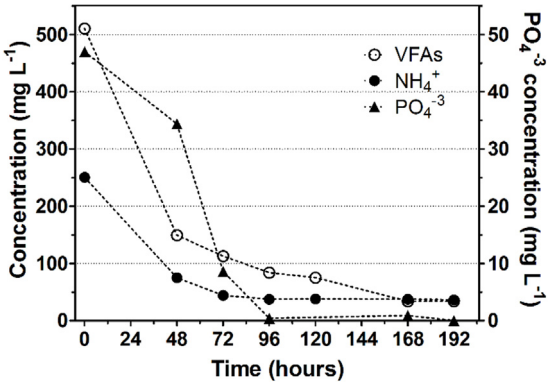

Fig. 5. Changes of total volatile fatty acids (VFAs), ammonium (NH_4^+) and phosphate (PO_4^{-3}) concentration over time during *Rhodopseudomonas* sp. S16-VOGS3 growth using diluted digestate (8% v/v) in the 0.22 L photobioreactor.

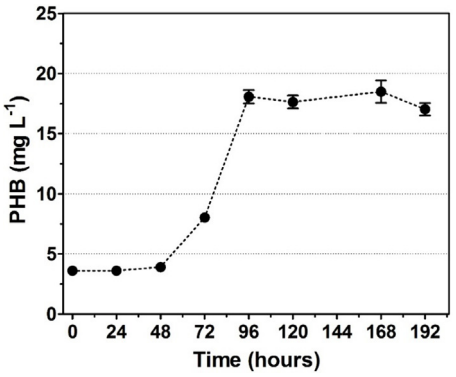

Fig. 6. Changes of poly(3-hydroxybutyrate) (PHB) content (mg L^{-1}) over time during *Rhodopseudomonas* sp. S16-VOGS3 growth using diluted digestate (8% v/v) in the 0.22 L photobioreactor.

(Directive 2018/0172) to use biodegradable plastics, and more recently the adoption of the European Green Deal, pushes for new investment and research in this area. Currently, sugars or other comparable substrates are used as the sole carbon source for aerobic pure cultures producing PHA for industrial use. Compared to aerobic fermentation of microorganisms currently used to produce commercial PHA, photosynthetic bacteria have several advantages. Their use reduces the need for aeration, allows the use of a wide range of wastes as substrates, and allows the accumulation and production of PHA in the same bioreactor, which reduces production costs. The use of low-cost carbon sources, such as agro-industrial waste, which accounts for 30%–50% of the cost of PHA, could further reduce the production cost.

PNSB can grow under a wide range of conditions thanks to their high metabolic flexibility (Dinesh et al., 2020; Brown et al., 2020). They can utilize solar energy under anaerobic conditions without losing CO_2, enabling the development of a high substrate conversion efficiency (Fradinho et al., 2019). PNSB are drawing attention because of their ability to produce PHA under various growth conditions (Ghimire et al., 2016; Luongo et al., 2017). PNSB accumulate PHB as a carbon reserve when growing under nutrient limiting conditions. A lack of nutrients, especially nitrogen and phosphorus, which limits the growth of bacteria and forces them to accumulate excess carbon in the form of PHB, is the main cause of PHB production (Monroy and Buitron, 2020).

The proposed nutritional and working conditions for PHA production by photosynthetic bacterial cultures are described below (Monroy and Buitron, 2020): (i) bacterial cultures should be supplied with high carbon sources (Demiriz et al., 2019); (ii) PHB production is favored by VFAs (Chen et al., 2017); (iii) deficiency or limitation of nutrients such as N, P, S and Mg is required (Carlozzi et al., 2019a; Demiriz et al., 2019); (iv) pH values between 6.5 and 7.0 and temperature between 24 °C and 30 °C are recommended (Ghimire et al., 2016; Kumar et al., 2019).

The cultivation of photosynthetic bacteria using anaerobic digested sludge has several advantages, as it can replace the supply of carbon and nitrogen sources in the form of VFAs and ammonium, as well as phosphorous. The composition of the digested sludge depends on the input material, the digestion process, and the methods used to treat the digestate (Marcilhac et al., 2015). OFMSW is a readily available and cost-effective substrate for the anaerobic digestion process. Anaerobic digestion of OFMSW is an example of a biorefinery catalyzed by a bacterial community that can convert biodegradable materials into byproducts. The digested sludge is easy to handle because it has low dewatering costs and is rich in nutrients (Fagbohungbe et al., 2015). The dark color and high ammonium–nitrogen concentration of the digestate are the main drawbacks when used as a medium for cultivating photosynthetic microorganisms. Since PHB and H_2 metabolic pathways are in competition the presence of ammonium in the digestate is an advantage. The presence of ammonium inhibits the photofermentation process in *Rhodopseudomonas* by suppressing the nitrogenase enzyme responsible for H_2 production (Androga et al., 2012). Since nitrogen fixation requires a lot of energy, nitrogenase synthesis is inhibited when ammonium is present (Masepohl and Hallenbeck, 2010). In order to determine which digestate concentration is most suitable for *Rhodopseudomonas* growth and PHB production, a preliminary experiment was performed by using cylindrical glass tubes. PHB is produced with various substrates in different amounts (Li et al., 2022). For example, PHB synthesis in *Rhodopseudomonas palustris* is triggered exclusively by acetate and propionate (Wu et al., 2012). PHB synthesis with combinations of acetate, butyrate, and propionate as substrates was demonstrated by Cardeña et al. (2017). When acetate is used as a substrate, it is utilized for PHB production via the glyoxylate pathway under conditions with limited ammonium concentrations; however, when nitrogen is scarce, acetate is consumed via the TCA cycle (McKinlay et al., 2014).

Rhodopseudomonas was recently studied by our research group under single or combined nutrient deficiency; acetate, butyrate, lactate, and propionate were used as carbon sources and either NH_4Cl or glutamate as nitrogen sources (Carlozzi et al., 2019a). The carbon sources present in the digested sludge, such as acetate and propionate, which were used to feed the bacterium, together with the nitrogen source (NH_4Cl) and phosphate promoted the metabolism of *Rhodopseudomonas*. Our results show that *Rhodopseudomonas* grew well in all digestate dilutions. In the presence of 4, 8 and 12% diluted digestate, cell growth was even higher compared to the control. Carlozzi et al. (2019b) reported a similar result after giving *Rhodopseudomonas* sp. S16-FVPT5 raw olive mill wastewater diluted with distilled water.

The main objective of the present study is to promote the production of bioplastics, such as poly (3-hydroxybutyrate), by recycling digested sludge; essentially, this objective can be achieved by a photofermentative process carried out by culturing *Rhodopseudomonas sp*. S16-VOGS3. The novelty of this work is the use of digested sludge (an environmentally harmful by-product) as a no cost nutrient for feeding PNSB.

The amount of diluted digestate added to *Rhodopseudomonas* in the PBR was 8% in order to achieve suitable VFA, and ammonium levels and reduce the effects of dark coloration. *Rhodopseudomonas* growth was induced and a CDW of 0.33 ± 0.01 g L^{-1} was observed. On the contrary, the low phosphate concentration (47.1 mg L^{-1}) in the culture broth containing 8% diluted digested sludge was an optimal condition to favor PHA accumulation in *Rhodopseudomonas* cells. PNSB cultures grown under phosphorus deficiency were shown to accumulate more PHA than those grown with sufficient phosphate (Carlozzi et al., 2019a). It was also demonstrated that culturing *Rhodopseudomonas* under low phosphorus conditions is a good strategy to achieve a high PHB production rate.

At the end of the experiment, the cell culture contained 17.03 mg L^{-1} PHB (5.2% of CDW). The VFAs measured at the end of the experiment (in the bacterial culture) showed that part (about 6%) of the initial content (510.4 mg L^{-1}) was not consumed by the bacteria. This remaining VFA content (30.0 mg L^{-1}) was lower than the percentage found when the bacterium was tested in the glass tubes (99.6 mg L^{-1}) (Fig. 2). This was due to the better conditions that the bacterium found during growth in the PBR equipped with continuous mixing.

Table 2 compares the PHB content from many studies using wastewater as a carbon source for photosynthetic bacteria.

It should be mentioned that the amount of PHB observed in the present study (18.5 mg L^{-1} PHB) is much lower compared to that obtained in our previous work (1066.4 mg L^{-1} of PHB) with the same strain when cultivated in 25% dark fermented cheese whey effluent or in the presence of glycerol (Carlozzi et al., 2019c). The difference in these values is most probably due to the conditions used for cultivation (semi-continuous) and the higher VFA content in the dark fermented effluent used to feed the bacterium. In the same microorganism, PHA content varies and is influenced by nutrient supply,

Table 2
Comparison of PHB content from several studies carried out with different photosynthetic bacteria strains using different carbon sources and photobioreactor (PBR) types.

Purple bacterial strain	Carbon source	PHB (mg L^{-1})	PHB (% CDW)	PBR (volume)	Culture time (h)	References
Rhodopseudomonas sp. S16-FVPT5	Dephenolized Olive mill wastewater (25%)	42.0	3.2	Cylindrical 0.22 L	384	Carlozzi et al. (2019b)
Rhodobacter sphaeroides O.U.001	Olive mill wastewater (2%)	39.0	–	Column 0.4 L	124	Eroğlu et al. (2010)
Rhodobacter sphaeroides AV1b	Dark fermentation effluent	1864	39.2	1.5 L	960	Ghimire et al. (2016)
Enriched photoheterotrophic culture	Dark fermentation effluent	–	6.3	1.5 L	960	Ghimire et al. (2016)
Rhodobacter capsulatus ATCC 17015	Dark fermentation effluent of fruit vegetable wastes	–	22.0	0.12 L	720	Corona et al. (2017)
Rhodopseudomonas palustris	Olive mill wastewater	175	9.0	Cylindrical 0.25 L	400	Padovani et al. (2016)
Rhodopseudomonas sp. S16-VOGS3	Dark fermentation effluent of molasses (20%)	1066	41.7	Cylindrical 0.22 L	600	Carlozzi et al. (2019c)
Rhodopseudomonas sp. S16-VOGS3	Digested sludge (8%)	18.5	5.2	Cylindrical 0.22 L	192	This work

absorption pathways, and culture conditions. According to a recent study by Higuchi-Takeuchi and Numata (2019), *Rhodovulum sulfidophilum* produced more PHB under aerobic conditions in a nitrogen- and phosphorus-rich growth medium.

The high efficiency of PNSB in consuming complex organic substrates has been demonstrated in several studies (Dinesh et al., 2020; Montiel-Corona and Buitrón, 2021). Fradinho et al. (2019) were able to produce large amounts of PHA by using fermented cheese whey. Carlozzi et al. (2019c), obtained 1.07 g L^{-1} PHB by culturing the same *Rhodopseudomonas* strain in semi-continuous mode using 20% dark fermented effluent of molasses. Municipal organic waste and food waste were used in several studies to obtain VFA-rich effluents with a high potential for PHA production (Kumar et al., 2020; Yukesh Kannah et al., 2022).

High cell density and high PHA production rates are required for industrial production. Most studies show low PNSB biomass concentrations, which in turn leads to low PHA concentrations. The low PHA production rates of PNSB, which should be increased by using novel approaches, represent a niche to focus on. Low PHA production rates directly impact the ability to remain financially viable. Light distribution in the culture broth, mixing of the culture, and the amount of cell biomass produced are among the elements directly responsible for low PHA production rates. Further studies are needed to evaluate and compare different operating conditions and to implement methods that allow simultaneous production of PHA and other value-added products to support the economic feasibility of the process.

The current work has shown that diluted digestate, with sterile freshwater, can be fed to *Rhodopseudomonas* to produce PHB. Photosynthetic bacteria could provide a very simple solution with relatively low investment and resource requirements, since most of the energy conversion occurs within the cell, reducing the need for complex plants. This result also provides an important direction for future research as a treatment alternative for nutrient consumption in wastewater treatment and liquid digestate in general. In both wastewater treatment plants and solid waste anaerobic digestion plants where a liquid digestate is produced, conversion by biological means to PHB could be a technique that closes the anaerobic biorefinery loop in terms of a circular bioeconomy (Rossi et al., 2022a; Critelli et al., 2022).

Since production methods are difficult to scale up, PHB synthesis by photosynthetic bacteria has not yet found an application. One of the major drawbacks in culturing photosynthetic bacteria could be the lighting requirements. The light distribution in the culture, which is influenced by the area/volume ratio of the reactors used, the color of the substrate, and the amount of cell biomass, is directly responsible for poor PHA production rates. According to recently analyzed data, the lighting cost for the production of 1 kg of biomass is 1.68€, which would make the process economically unviable (Capson-Tojo et al., 2020). In scaling up the proposed process, several strategies could be used to reduce lighting costs (Allegue et al., 2021). The use of sunlight for the process is essential from both an economic and environmental perspective. For a successful integrated scale-up, proper technology transfer from laboratory to industrial scale is of utmost importance.

5. Conclusions

This study has demonstrated that digested sludge can be effectively used as a suitable feedstock for the growth of *Rhodopseudomonas* sp. S16-VOGS3. The results obtained by using the small-scale cylindrical PBR showed that the abovementioned bacterial strain can support the production of PHB when grown in digested sludge. This photo-fermentative process is an attractive alternative to the traditional production of petrol-based plastics. Moreover, the

described bioprocess could become very attractive and environmentally feasible, by performing a semi-continuous cultivation strategy, as shown by culturing the same bacterial strain in either dark fermented effluent of whey or molasses (Carlozzi et al., 2019c).

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CRediT authorship contribution statement

Eleftherios Touloupakis: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft, Writing – review and editing, Supervision. **Angeliki Chatziathanasiou:** Methodology, Validation, Investigation, Data curation, Writing – review and editing. **Demetrios F. Ghanotakis:** Resources, Writing – review and editing. **Pietro Carlozzi:** Resources, Writing – review and editing. **Isabella Pecorini:** Methodology, Data curation, Writing – original draft, Writing – review and editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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