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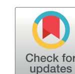
Hydroxytyrosol rich-mixture from olive mill wastewater and production of green products by feeding *Rhodopseudomonas* sp. S16-FVPT5 with the residual effluent



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ABSTRACT

This study disserts on the exploitation of olive mill wastewater (OMW) for the production of both bio-based poly-β-hydroxybutyrate (PHB) and hydrogen (H_2) by using the residual effluent as feedstock for growing purple bacteria after the recovery of hydroxytyrosol-rich mixtures. In particular, *Rhodopseudomonas* sp. S16-FVPT5 was fed with either the virgin OMW or dephenolized-OMW (d-OMW). For polyphenols removal, the OMW was treated with activated carbon; subsequently, acidified ethanol (pH = 3.1) at 50 °C was used as extractor solvent for obtaining hydroxytyrosol-rich mixtures. The maximum hydroxytyrosol content in the resultant polyphenolic mixture was 2.02 g/L. The highest co-production of PHB (315 mg PHB/L) and H_2 (2236 mL H_2/L) were achieved feeding *Rhodopseudomonas* sp. S16-FVPT5 with pure d-OMW. The highest hydrogen yield (4.55 L(H_2)/$L_{d\text{-}OMW}$) was obtained feeding the bacterium with d-OMW, diluted at 25%; by increasing the content of d-OMW into the culture broth the hydrogen yield progressively decreased. Lower results were obtained by feeding the bacterium with a synthetic medium, the cumulative hydrogen was 1855 mL H_2/L); the PHB was 101 mg PHB/L. The highest theoretical light conversion efficiency was 2.36% with the synthetic medium and 1.99% when feeding *Rhodopseudomonas* sp. S16-FVPT5 with d-OMW diluted with water 50%, v/v.

1. Introduction

The effluent coming from olive-oil industry is considered one of the most pollutant wastes in the Mediterranean basin due their high content of phenolic compounds. However, these compounds possess beneficial properties for health; for example, they are strong antioxidants and, consequently, the olive oil residues can be represented as an inexpensive source of these natural antioxidants (Ena et al., 2012). The adsorption by activated carbon (AC) is the most frequently used treatment method to remove toxic pollutants such as phenols from aqueous effluents. The recovery of polyphenols from olive mill wastewater (OMW) by AC and organic solvent, such as methanol, have been widely used for the adsorption/desorption processes (Gamel and Kiritsakis, 1999; Cardoso et al., 2005). The OMW, deprived of polyphenols, can represent a suitable feedstock for feeding purple bacteria because it is rich of nutrients; hence, it can be used in industrial biotechnological processes for the combined production of green products.

Nowadays, global energy requirements are mostly dependent on fossil fuels, which eventually lead to foreseeable depletion due to limited fossil energy resources. In addition, the fast climate changes force the researchers to find a solution to overcome the actual fossil-based economy developing a more suitable green economy based on renewable energy sources. In recent times a great deal of attention is being paid to the usage of hydrogen as alternative and eco-friendly fuel throughout the world (Mohan et al., 2007), because from its combustion to generate electricity only water is formed hence it does not contribute to environmental pollution (Miyake, 2014). Biological production of hydrogen (H_2) from biomass and wastewaters is one of the alternative methods less energy intensive and more environmental friendly where processes can be operated at ambient temperatures and pressures (Mohan et al., 2007; Seifert et al., 2010). The share of the vector hydrogen in the automotive fuel market could grow very fast in the near future, due to both H_2 environmental advantage and the use of low-cost feedstock as industrial agro-food residues and/or wastewaters

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suitable for feeding the photo-fermentative process. Disregarding the artificial photo-assisted hydrogen generation, there are two different light-dependent processes for the H_2 production: water biophotolysis, using cyanobacteria and green algae, and photo-fermentation of organic compounds using photosynthetic bacteria (Hallenbeck et al., 2012). Photosynthetic microorganisms could provide a very simple method with relatively minimal investment and resource requirements because the major part of the energy conversion is carried out within the cellule without the need to use complex plants (Miyake, 2014). Since H_2 production by nitrogenase (N_2ase) is induced by nitrogen deficiency or low nitrogen content in the culture broth, the carbon to nitrogen (C:N) ratio in the medium is a very important parameter. It has been demonstrated that in the absence of nitrogen all the e^{-s} are allocated to H_2 production in the N_2ase reaction (Sakurai et al., 2013).

Among the photosynthetic microorganisms, the purple bacteria (PB), a group of Gram-negative pink to purplish-brown bacteria, contain type II reaction center (RC), which cannot use H_2O as the e^- donor in contrast with organisms like cyanobacteria, algae and land plants that contain the photosystem II (PS II). This is because the oxidant generated by the photochemical reaction center (RC) of type II in purple bacteria is not strong enough to extract e from H_2O (Sakurai et al., 2013). The major groups of bacteria that contain this type of RC are the purple bacteria genera as *Rhodobacter*, *Rhodospirillum*, *Rhodopseudomonas*, etc.; in the purple bacteria, N_2ase activity is induced by nitrogen deficiency. Since nitrogen fixation is a highly energy-demanding process, synthesis of N_2ase is inhibited as long as ammonium is available (Masepohl and Hallenbeck, 2010).

Bioenergy from microorganisms does not compete with food crops, and the photobioreactors for production of green energy can be positioned in marginal areas without competition with agricultural lands (Carlozzi et al., 2010; Carlozzi and Sacchi, 2001).

In conjunction with the production of H_2, photosynthetic processes can be explored to produce biopolymers such as polyhydroxyalkanoates (PHAs). PHAs are polyesters synthesized by numerous bacteria that are attracting a great attention due to their thermoplastic properties, similar to that of polypropylene, good mechanical properties and excellent biodegradability in various ecosystems such as fresh water, soil, industrial/domestic compost and seawater. PHAs are among the most promising candidates for the production of biodegradable items for different industrial applications. But, their relatively high cost (7–12 €/kg), compared to other biodegradable polymers such as poly-lactic acid (PLA), has somehow refrained research activity on their use in commodity applications such as packaging and service items, restricting their use to high-value applications, such as those in medical and pharmaceutical sectors. These high market costs are also partly due to the high costs of used carbon sources (e.g. glucose and sucrose) (Bugnicourt et al., 2014).

PHA production using no-cost agro-industrial wastes such as fermented sugar cane molasses, paper mill effluent, cheese whey, palm oil, municipal wastewater, and olive oil mill wastewater is currently attracting considerable attention from researchers, especially for use in mixed microbial culture technologies in order to reduce the actual PHA production costs (Campanari et al., 2017).

In this study, a multi-step process was developed for the valorization of OMW through a first selective recovery of polyphenols (PPs) and the subsequent combined production of H_2 and poly-β-hydroxybutyrate (PHB) by using *Rhodopseudomonas* sp. S16-FVPT5. The removal of PPs from OMW was carried out by AC. The desorption of the adsorbed PPs was obtained using ethanol; this solvent has several advantages being cheap, recoverable and nontoxic (Mylonaki et al., 2008; Galanakis et al., 2010; Scoma et al., 2012). Multiple adsorption/desorption cycles were performed in order to individuate the optimal operating conditions in terms of adsorption/desorption efficiencies of PPs, chemical oxygen demand (COD) and total volatile fatty acids (TVFAs). The resultant de-phenolized OMW (d-OMW) was used pure or diluted with water as source of nutrients for the combined photo-fermentative production of H_2 and PHB using *Rhodopseudomonas* sp. S16-FVPT5. The performance of the photofermentative process was evaluated varying the dilution of d-OMW in terms of specific cumulative production and yields of H_2 and PHB, and light conversion efficiency.

2. Material and methods

2.1. Materials

The virgin OMW used in the present study came from a three-phase olive oil extraction process in a continuous olive-processing plant located in Tuscany, Italy. The main characteristics of the abovementioned OMW, after centrifugation, were the following: pH = 3.7; COD = 53.2 g/L; PPs = 5.55 g/L and TVFAs = 12.85 g/L. OMW sample (200 L) used for the investigation on the recovering of Hydroxytyrosol rich-mixture and co-production of H_2 and PHB was stored in an underground reservoir for a period of 80 days. The AC used for the PP removal was supplied by Carboplant S.r.l., Vigevano (PV) - Italy. All properties of the AC have been reported elsewhere (Ena et al., 2012). Acetonitrile, methanol, acetic acid and water (for HPLC), were supplied by Carlo Erba Reagents S.r.l., Cornaredo (Mi) – Italy. Ethanol used for regeneration of AC and desorption of PPs were purchased from Sigma–Aldrich (St. Louis, MO, USA). Hydroxytyrosol was supplied by Phytolab (Vestenbergsgreuth, Germany). Folin-Ciocalteu reagent was purchased from Merck (Darmstadt, Germany).

2.2. Analytical methods

Polyphenolic content of the OMW was determined in accordance with the method reported by Folin and Ciocalteu (1927). The total content of PPs was determined at 730 nm by spectrophotometer (Cary 50, Agilent Technologies Inc., Santa Clara, CA, USA). Determination of hydroxytyrosol in the PPs mixture was carried out by high performance liquid chromatography (HPLC) (ProStar/Dynamax System Liquid Chromatograph, Agilent Technologies Inc., Santa Clara, CA, USA) coupled with a ProStar 335 PDA DAD detector and with the use of a binary gradient elution (Ena et al., 2012). A 4.6 × 250-mm, Sinergy Fusion RP18, 4 μm (Phenomenex, Torrance, CA, USA) analytical column was used with the same precolumn (10 × 4 mm). All analyses were carried out in triplicate.

Chemical oxygen demand (COD) measurements were performed using a C99 Multiparameter Bench Photometer (HANNA Instruments, Lucca, Italy). Samples (2 mL) were added to an oxidizing chromatic acid solution and digested for 2 h at 150 °C and the measurements were carried out after cooling.

The cell dry weight (CDW) concentration, carbohydrate and protein content of the biomass, elemental analysis of the biomass (C, H, N, O) and the heat of combustion of ash-free biomass (H_B) were determined in accordance with Carlozzi and Sacchi (2001). The content of PHB in the biomass was determined in accordance with Padovani et al. (2018). The bacteriochlorophyll (Bchl) was measured according to Carlozzi et al. (2006) and the amount of TVFAs was determined at 530 nm, in accordance with Mato et al. (2005).

The gas mixture (H_2 plus CO_2) from the headspace of the photobioreactor (PBR) was first made to flow into a basin containing a saline NaOH solution to adsorb CO_2; successively H_2 was trapped in a calibrated column, where it was collected and the volume measured to determine its production. No CO_2 was found inside the calibrated column. This was checked by sampling (in triplicate) 0.1 mL of the gas from the calibrated column and it was injected into a gas chromatograph (Perkin–Elmer Autosystem) equipped with TCD detector and a Silica Gel 60/80 Grade 12 column (Alltech, Derfield). The calibrated column was refilled with a saline solution of NaOH every morning. The consumption of acetate was checked with a HPLC (Thermo Finnigan - Spectra System 6000 L P). The HPLC was equipped with a C18 analytical column (250 x 4.6 mm) and the column temperature was 25 °C.

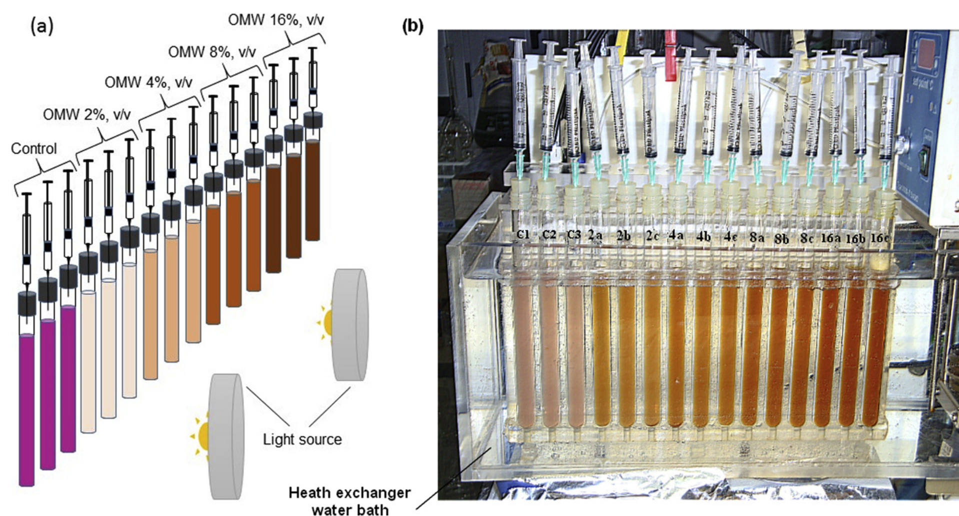

Fig. 1. Cultural system used for testing the growth of *Rhodopseudomonas* sp. S16-FVPT5 using the culture broths containing untreated OMW diluted with distilled water (2, 4, 8 and 16%; v/v). Schematic representation of culture system (a); picture of the cultural system (b).

After the removal of the cells by disposable syringe filter units (MFS-13 mm, 0.45 µm pore size), the supernatant was tested in order to measure the acetate content. The mobile phase consisted of an aqueous 0.1 wt. % H_3PO_4 solution at a flow rate of 1.0 mL/min (Carlozzi et al., 2010).

2.3. Statistical methods

One-way permutation analyses of variance (PERMANOVA; Anderson et al., 2008) were used to test for differences in terms of production of H_2 and biomass among the different concentrations of OMW diluted with water (2, 4, 8 and 16%, v/v) and the control. Levene's tests were performed to check for homogeneity of dispersion. Four factor levels only, respectively control, 2, 4 and 8%, were investigated for BChl. Unrestricted permutation of raw data, Type I of sum of squares and 9999 permutations were chosen according to Anderson (2001). PERMANOVAs were run on the basis of a Euclidean distance matrix. Pairwise permutational post hoc t-tests were applied when appropriate using a Monte-Carlo p-values. Significance was set at p-value = 0.05. No p-values correction was applied to permutational tests as suggested by Anderson (2001).

2.4. Adsorption and desorption process

The phenolic compounds present in OMW could be counted among those with hydro-soluble characteristics. Among these compounds, there are gallic acid and derivatives, hydroxytyrosol and derivatives, tyrosol and derivatives, syringic acid, coumaric acid and oleuropein. This last is a glucoside that quickly degrades in elenolic acid, hydroxytyrosol and aglycone.

For polyphenols (PPs) removal, the OMW was treated with AC by using the device built by Tecnolab srl, Spello, PG, Italy. Then, PP-rich AC was dried at 38 °C and divided into several portions and packed into nylon bags for the subsequent desorptions. PPs were extracted using a TIMATIC extractor (Tecnolab srl, Spello, PG, Italy) that permitted to recover PPs using acidified ethanol (pH = 3.1) at 50 °C as extractor solvent. The time of the extraction varied from 15 to a maximum of 720 min. The process consists of two phases: the first is a dynamic phase comprising a forced filtration; the second is a static phase, during which the adsorbed compounds are transferred from the solid to the liquid phase. In view of the industrial process, either single or double or triple desorption cycles was investigated by using the same extractive solvent.

The efficiency of the adsorption process for PPs, COD and VFAs was evaluated as follows:

$$CRE\ (\%) = (C_0 - C_f)/C_0 \times 100 \quad (1)$$

where CRE is the compound removal efficiency, C_0 and C_f indicate the amount of PPs, COD and VFAs, in the OMW, before and after adsorption, respectively.

2.5. Microorganism and culture conditions

Rhodopseudomonas sp. S16-FVPT5 coming from the collection of Research Institute on Terrestrial Ecosystems, National Research Council (IRET, CNR) has been used in the present work. The 16S sequences were deposited in GenBank under the following accession numbers: **KU899101-KU899105**. *Rhodopseudomonas* sp. S16-FVPT5 was pre-cultured in the synthetic medium (with a composition reported below) at a constant temperature of 30 ± 0.2 °C in anaerobic conditions (Sovirel bottles, 100 mL) under continuous irradiance of 74 W/m^2 using a 150-W OSRAM power-star HQI-TS lamp. The irradiance was measured using a Quantum/Radiometer/Photometer (model LI-185B, LI-COR, Lincoln, Nebraska, USA). The investigation was carried out by feeding the bacterium with virgin OMW and d-OMW. A synthetic medium was used as control, whose composition was 2.0 g/L of acetate, 0.5 g/L NH_4Cl, 1.0 g/L KH_2PO_4, 0.4 g/L NaCl, 0.4 g/L $MgSO_4{\cdot}7H_2O$, 0.05 g/L $CaCl_2{\cdot}2H_2O$, 0.1 mg/L *p*-aminobenzoic acid and 10 mL/L 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 synthetic medium was adjusted to 6.8 by using HCl 0.1 mM or NaOH 5 wt.%. For the combined production of hydrogen and PHB, the synthetic growth medium was modified, the carbon source (acetate) concentration was increased to the optimal value of 4.0 g/L and the nitrogen source (NH_4Cl) was replaced with 1.0 g/L of Na-glutamate.

The virgin OMW was first centrifuged to remove the solid fraction and then the liquid fraction was diluted with distilled water (2, 4, 8 and 16%, v/v); the initial pH of the culture broth was adjusted at 6.8 and sterilized by autoclaving (temperature 121 °C; pressure 2.0 atm.; time 20 min.). The photofermentative growth experiments with *Rhodopseudomonas* sp. S16-FVPT5 were carried out in 20 ml (working volume) glass tubes by using the system shown in Fig. 1(a, b), at a constant temperature of 30 ± 0.2 °C under continuous irradiance of 74 W/m^2. The amount of inoculation to the photobioreactors (tubes) was 10% by volume of pre-cultured *Rhodopseudomonas* sp. S16-FVPT5 in the synthetic medium. Each culture tube was mixed every day with a vortex for 1 min. The experiments were carried out in triplicate. At the

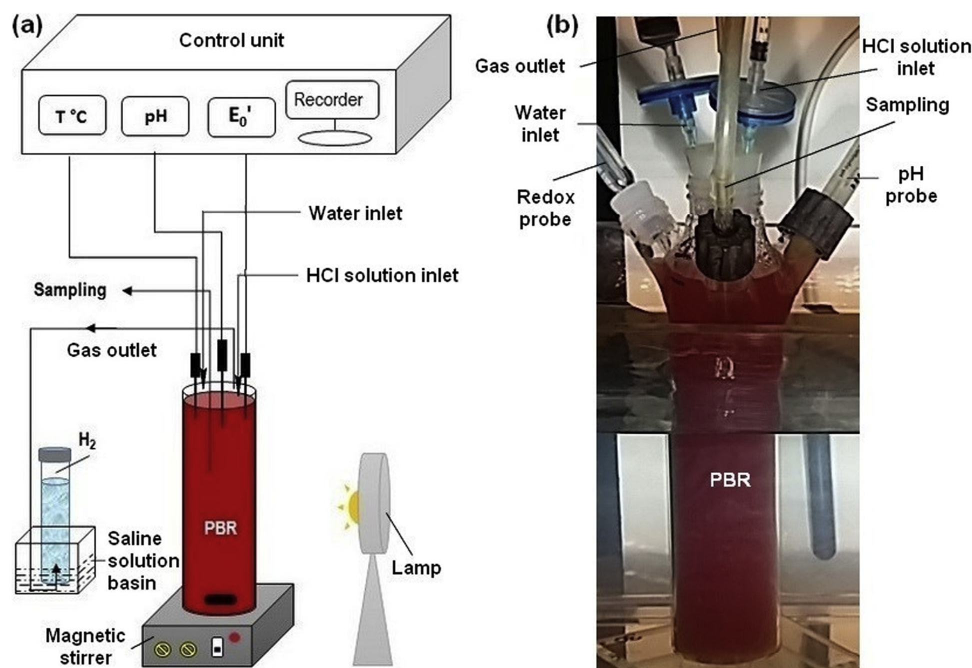

Fig. 2. Photobioreactor used for testing *Rhodopseudomonas* sp. S16-FVPT5 fed with pure d-OMW (100%) or diluted with distilled water (25% v/v and 50% v/v respectively). Schematic representation of the photobioreactor (a); picture of the photobioreactor (b).

end of the experiment, cultures were sampled for analyses.

The d-OMW was used pure (100%) or diluted with distilled water (25% v/v and 50% v/v respectively). A cylindrical-glass photobioreactor with an internal diameter of 4.0 cm and a working volume of 220 mL was used (Fig. 2a, b). The initial pH of the medium was adjusted at 6.8; subsequently, the pH was adjusted to 7.0 every 24 h. The pH and the oxidation-reduction potential (E_0') were monitored continuously. Culture samples were replaced with distilled water.

An appropriate volume of pre-cultured *Rhodopseudomonas* sp. S16-FVPT5 was sampled and cells were collected by means of centrifugation and were washed with physiological solution to remove traces of synthetic salts. The cells were then suspended in the culture broths containing either the synthetic medium or d-OMW (pure o diluted), in order to investigate the medium effect on the PHB accumulation and the hydrogen evolution. The initial BChl and CDW concentrations were 2.0 mg/L and 0.193 g/L respectively. Cultures grown in the photobioreactor shown in Fig. 2(a, b) were mixed continuously by using a magnetic stirrer. The PBR (220 mL working volume) was sterilized through the aforementioned autoclaving process; successively, it was operated under sterile conditions. The growth parameters (temperature, pH and E_0') were monitored using probes connected to a control unit (Chemitec srl, Florence, Italy). All experiments were carried out in a thermostatic room of 22 °C, under atmospheric pressure. In order to deaerate the headspace and culture broths, Argon was flowed for 5 min and subsequently the cultural systems were placed under the above-mentioned continuous light conditions.

2.6. Light conversion efficiency

The theoretical light conversion efficiency (LCE_T) is the key factor in the development of a photosynthetic process aimed at hydrogen production. This factor is calculated on the hypothesis that the substrate is fully converted in bioH_2 in accordance as follows:

$$LCE_T = H_{2,output}/Energy_{,input} \times 100 \tag{2}$$

LCE_T is based on the complete conversion of organic compounds to carbon dioxide and molecular hydrogen (Gest et al., 1962). However, a significant quantitative of biomass is produced together with bioH_2, which is due to the bacterial growth (Basak and Das, 2007). Consequently, a real light conversion efficiency (LCE_R) has to be introduced and assessed as follows:

$$LCE_R = Total\ Energy_{,output}/Energy_{,input} \times 100 \tag{3}$$

where the total energy output is the energy of the H_2 and of the biomass produced, while energy input is the irradiance impinged on the reactor surface and the energy of the organic compounds consumed. Details regarding the Eqs. (2) and (3) are reported elsewhere (Carlozzi, 2009).

3. Results and discussion

3.1. OMW as natural feedstock for feeding purple bacteria

The main characteristics of stored-OMW after centrifugation were the following: pH = 4.3, COD = 51.5 g/L, PPs = 5.4 g/L; TVFAs = 19.5 g/L. The pure effluent is an unsuitable feedstock due to both the high PPs content, which causes an inhibitory effect on the growth of many bacteria, and its black-brownish color (Keskin et al., 2011; Daglia, 2012). Despite the toxic potential of PPs, the possibility of using non-treated diluted OMW was investigated feeding *Rhodopseudomonas* sp. S16-FVPT5 with different concentrations of OMW diluted with water (2, 4, 8 and 16%, v/v). The results are shown in Table 1 in terms of production of H_2 and biomass after a growth time of 192 h. All PERMANOVAs were significant (p-values < < 0.0001) indicating clear differences in terms of production of H_2 and biomass among the different concentrations of OMW diluted with water (2, 4, 8 and 16%, v/v) and the control. Results of pairwise permutational post hoc t-tests are shown in the Supplementary File. Broth cultures containing OMW concentration ≥2%, v/v had a net inhibitory effect on H_2 production, CDW and BChl growth. Conversely, a significant growth stimulation was observed for VFA at OMW concentration ≥2%, v/v. A similar observation was reported by Eroğlu et al., 2006 by feeding *Rhodobacter sphaeroides* O.U.001 with raw OMR diluted with water. Consequently, for the subsequent experiments, OMW was de-phenolized before feeding *Rhodopseudomonas* sp. S16-FVPT5 that was cultured in the controlled photobioreactor with a working volume of 220 mL.

Table 1
Culture broths containing non-pretreated OMW diluted with distilled water were used for feeding *Rhodopseudomonas* sp. S16-FVPT5. A synthetic medium containing acetate was used as control.

Runs	CDW (mg/L)		VFA (g/L)		PP (mg/L)		BChl (mg/L)		Cumulative H_2 (mL H_2/L)
	t_0	t_f	t_0	t_f	t_0	t_f	t_0	t_f	
OMW 2%	74	57 ± 06	0.43	0.257 ± 0.021	111	nd	0.48	0.60 ± 0.08	139 ± 28
OMW 4%	74	130 ± 50	0.86	0.447 ± 0.058	222	nd	0.48	0.87 ± 0.10	178 ± 17
OMW 8%	74	50 ± 26	1.72	1.280 ± 0.056	444	nd	0.48	0.37 ± 0.05	2.2 ± 0.5
OMW 16%	74	20 ± 09	3.44	3.422 ± 0.088	888	nd	0.48	nd	0.0
Control	74	770 ± 26	2.00	0.00 ± 0.00	0	nd	0.48	2.74 ± 0.20	239 ± 22

t_0, Initial time; t_f, final time; nd, not determined.

3.2. Removal of PPs and recovery of hydroxytyrosol-rich mixtures

The main characteristics of OMW after the adsorption process with 100 g(AC)/L were the following: pH = 5.4, COD = 19.9 g/L, PPs ≤0.1 g/L, TVFAs = 8.4 g/L corresponding to average removal efficiency > 98% for PPs, 61.4% for COD and 59.6% for TVFAs. These results demonstrated the high removal efficiency of the used adsorption device, specially, for PP removal from OMW. To desorb PPs and regenerate AC, the acidified ethanol solution was successfully used as extraction solvent showing the feasibility of the adsorption/desorption process investigated. The procedure was optimized in order to fulfill the conditions, which would significantly advantage the feasibility of polyphenols recovery. The temperature at which the operations (desorption) were performed was fixed at 50 °C; lower results were obtained at 30 °C (data not shown). In order to optimize the extraction time, several tests were carried out increasing the time from 15 to 720 min. Table 2 shows the results concerning the desorption of phenolic compounds carried out with the TIMATIC extractor in terms of amount of hydroxytyrosol desorbed by the AC. As shown, the hydroxytyrosol content into the PP mixture raised increasing the extraction cycles and a maximum hydroxytyrosol content of 2.02 g/L was obtained after 3-cycles and after 180 min. However, small differences were observed between the extraction times of 120 and 180, so the time of 120 min could be taken into consideration as an appropriate period for reaching enough content of hydroxytyrosol. The desorption interval times of 15, 120, 180 and 720 min were chosen because they were significantly lower than 24 h, which was a very long time used on previous desorption experiments, carried out by our group (Ena et al., 2012).

The obtained PP mixture is of high interest for both pharmaceutical and cosmetic industries due to the relevant benefits of hydroxytyrosol on human health (Manna et al., 1999; O'Dowd et al., 2004).

3.3. Combined production of PHB and H_2

The resultant d-OMW was used as nutritive solution for feeding the photosynthetic process based on *Rhodopseudomonas* sp. S16-FVPT5. This bacterial strain was grown in the cultural system shown in Fig. 2(a, b) using different culture broths containing pure d-OMW or diluted one with water at 25% and 50% (v/v). Fig. 3(a, b) shows the relative cumulative productions of PHB and H_2 by *Rhodopseudomonas* sp. S16-FVPT5, respectively. As shown, after about 400 h the cumulative PHB and H_2 resulted insignificant when the diluted d-OMW at 50% and 25% (v/v) were used, reaching PHB concentrations of 175 and 43 mg PHB/L and cumulative H_2 productions of 1825 and 1120 mL H_2/L, respectively. On the other hand, using pure d-OMW, the productions continued over time reaching cumulative PHB and H_2 of 315 mg PHB/L and 2236 mL H_2/L, respectively, after about 800 h. These results are attributable to the fact that the concentrations of the nutritive components increased by increasing the percentage of d-OMW in the culture broth with consequent increase of the metabolic activity of the bacterial biomass as evidenced by higher productions of PHB and H_2 obtained

Table 2
Hydroxytyrosol content in the polyphenolic mixture obtained by single, double and triple desorption from AC using the same ethanol solvent.

Hydroxytyrosol amount in the polyphenolic mixture					
Number of desorptions	Unit	Desorption time (min)			
		15	120	180	720
Single	mg/L	622	nd	733	800
Duple	mg/L	1028	1370	1394	1243
Triple	mg/L	nd	1922	2018	1858

nd; not determined.

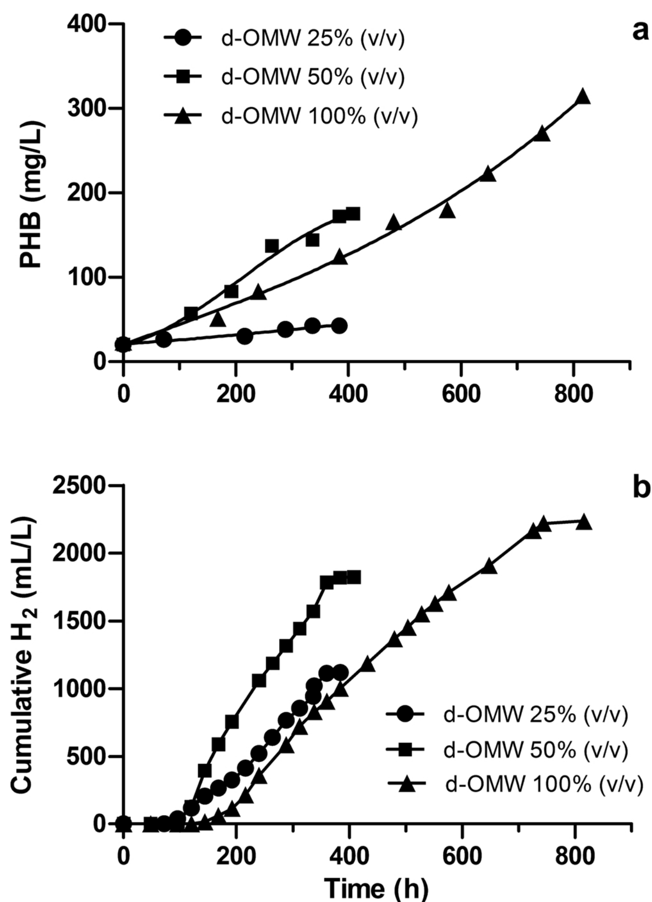

Fig. 3. Combined accumulation of poly-β-hydroxybutyrate (PHB) (a) and H_2 (b) versus time by means of *Rhodopseudomonas* sp. S16-FVPT5 grown in the culture broths containing pure d-OMW or diluted with water (50%, v/v or 25%, v/v).

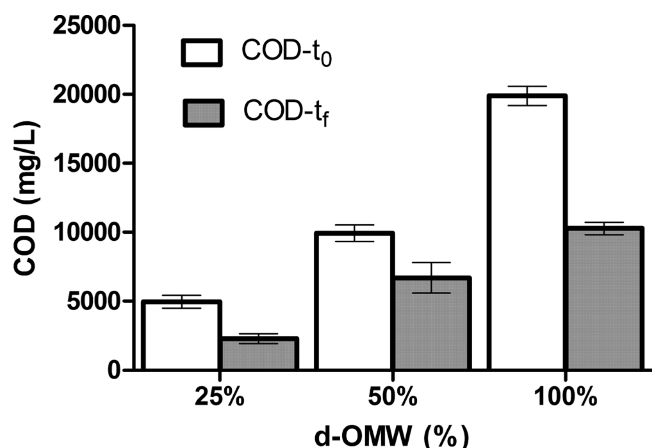

Fig. 4. Chemical oxygen demand (COD) changes in the culture broths containing growing amounts of d-OMW diluted with water (25%, v/v or 50%, v/v) or pure (100%) by means of *Rhodopseudomonas* sp. S16-FVPT5. COD values were evaluated at the initial time (t_0) and at the final time (t_f).

using 100% d-OMW. A combined production of H_2 and PHB is a major advantage for environmentally friendly technology in utilizing solar energy bioconversion and in turning industrial and sewage wastes into alternative sources of energy and recyclable plastics (Khatipov et al., 1998). In the biorefinery process, the production of biological hydrogen is an important challenge, in light of a 100% renewability criterion (Deneyer et al., 2016).

In Fig. 4, the initial and final COD values of the different culture broths containing d-OMW are reported. The final time (t_f), to which the COD values refer are around 400 h for 25 and 50 vol.% d-OMW and 816 h for 100% d-OMW. As shown, a significant reduction of the COD values were observed in the culture broths as a result of the combined productions of PHB and H_2. The highest COD removal (54%) was obtained with the culture broth containing 25% of d-OMW.

Eroğlu et al. (2011) achieved 30% of COD removal when *Rhodobacter sphaeroides* O.U.001 was fed with OMW diluted with water (2%, v/v). They increased COD removal to 48%, by adding in the culture broth both Fe and Mo. Higher COD removal (73%) was reported by Ghimire et al. (2017) feeding enriched photoheterotrophic culture with dark fermentation effluent of food wastes. The pH and E_0' of the cultures were continuously monitored and the results are shown in Fig. 5. The pH was kept quite stable, over time, by adding HCl 0.1 mM; it ranged from 6.8 to 7.2 in all cultures tested. On the contrary, regarding the oxidation potential, some important differences emerged during bacterial growth. The E_0' decreased quickly during the first growth period (24 h) reaching the value of −180 mV; this behavior was observed in all cultures investigated. In the following period, E_0' decreased slowly up to the lowest value (−512 mV). This value stayed stable in the culture with pure d-OMW, but got up to about −400 mV in *Rhodopseudomonas* cultures grown in the broths containing diluted d-OMW. A scheme involving redox potential changes of clusters in nitrogenase proteins were reported by Sakurai et al. (2013). Investigating about Fe-protein-ATP_2 complex and MoFe-protein, the authors showed that the Fe-protein still decreases further up to about −620 mV.

The present study demonstrated that d-OMW could be used pure, without dilution with fresh water, for feeding *Rhodopseudomonas* in order to produce both bioplastic as PHB and bioH_2, so that a dual objective can be reached. This solution avoids the use of clean fresh-water for diluting d-OMW preventing an environmental paradox: "instead of cleaning up the environment, clean fresh water would be contaminated" and satisfy a more general concept "converting waste into resources is better than shredding them".

As comparison, *Rhodopseudomonas* sp. S16-FVPT5 was also investigated using a synthetic medium, as where acetate and glutamate

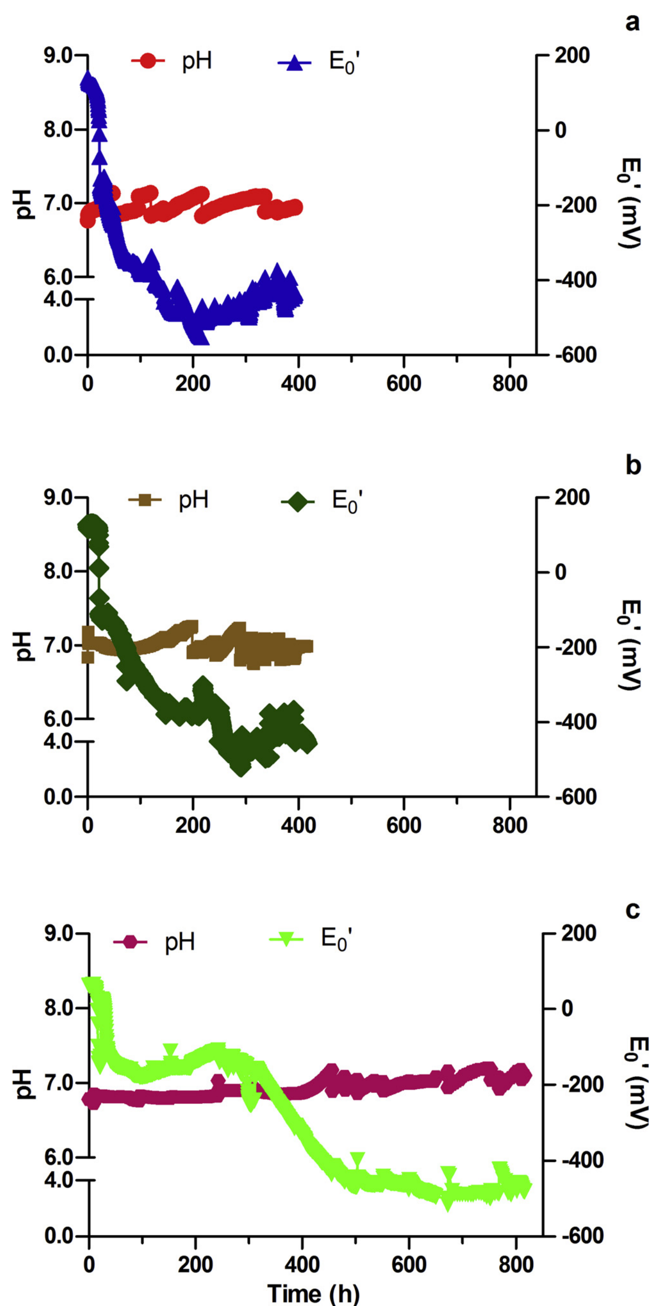

Fig. 5. Changes of pH and oxidation-reduction potential (E_0') vs. time when *Rhodopseudomonas* sp. S16-FVPT5 was fed with three different culture broths containing increasing amounts of d-OMW diluted with distilled water: 25%, v/v (a); 50%, v/v (b) and 100%, v/v (c).

were used as carbon and nitrogen sources, respectively. The investigation was carried out operating the photobioreactor in batch mode; results are shown in Fig. 6(a–c). A combined production of PHB and H_2 were achieved (100 mg PHB/L and 1855 mL H_2/L, respectively) in 336 h (Fig. 6a). The BChl concentration reached about 11.0 mg/L at the end of the growth and the initial amount of acetate (4.0 g/L) was totally consumed (Fig. 6b). The generation of hydrogen began after 72 h and continued until acetate was totally consumed. During the hydrogen photoproduction, the pH was maintained around 7.0 and E_0' was about −550 mV and, only in the last period of growth, it increased to −475 mV (Fig. 6c). An increase of bacterial biomass was also observed, reaching the concentration of 1.46 g (CDW)/L, at t = 336 h (data not shown).

The composition of all biomasses achieved growing

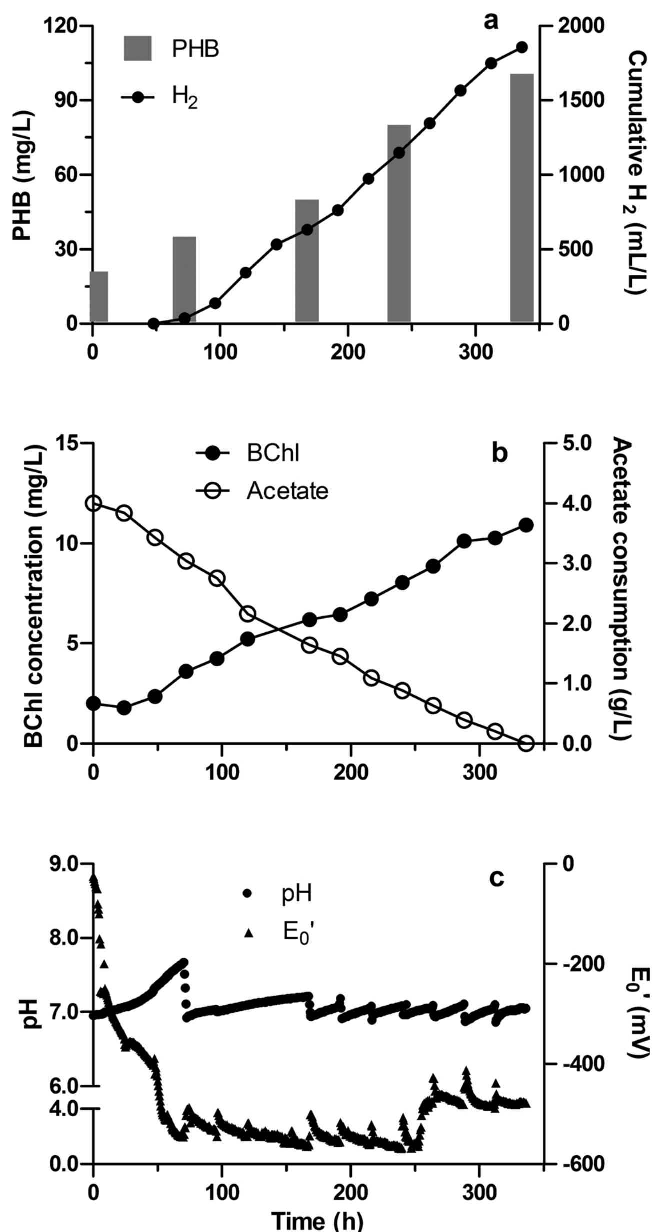

Fig. 6. *Rhodopseudomonas* sp. S16-FVPT5 grown in the synthetic medium containing acetate and glutamate: combined production of poly-β-hydroxybutyrate (PHB) and H_2 (a); bacteriochlorophyll (BChl) enhancement and acetate consumption vs. time (b); trends of pH and oxidation-reduction potential (E_0') vs. time (c).

Rhodopseudomonas sp. S16-FVPT5 with the synthetic medium and with the broths containing different amounts of d-OMW are reported in Table 3. The hydrogen and PHB yields (Y_H, Y_{PHB}) of *Rhodopseudomonas* sp. S16-FVPT5 fed with synthetic medium or d-OMW effluents are shown in Table 3. Data are comparable with those reported by Eroğlu et al. (2006), when *Rhodobacter sphaeroides* O.U.001 was fed with clay-pretreated OMW diluted with water (50%, v/v).

Table 4 compares the PHB content and cumulative H_2 from several studies using different carbon sources as either wastewater or synthetic substrates. The content of PHB in the biomass harvested at the end of the experimental sets raised when increasing the content of d-OMW into culture broths. By culturing *Rhodopseudomonas* sp. S16-FVPT5 into a culture broth containing pure d-OMW, the dry-biomass collected at the end of the process showed the highest PHB content of 15.7%. This value is very high considering that in this case the PHB is a secondary product during the hydrogen generation. A so high PHB content is comparable with that obtained with *Rhodobacter capsulatus* fed with dark fermentation effluent of fruit-vegetable wastes, ranging from 11% to 22% of dry-biomass (Corona et al., 2017). Higher PHB content (32.5% of CDW) have been recently reported by Ghimire et al. (2017) when growing an adapted culture of *Rhodobacter sphaeroides* AV1b. Lower PHB content (6.3% of CDW) was reported by the same researcher team feeding mixed photofermentative cultures with diluted dark fermentation effluents achieving a concomitant production of 169 mL H_2/L of culture.

A comparison of the specific production activities of PHB and H_2 using volatile fatty acids was reported by Cardeña et al. (2017); H_2 and PHB formations compete for the reducing equivalents released from bacteria growing under certain substrates, showing that the formation of H_2 or PHB is detrimental to the formation of each other. The accumulation of PHB in the biomass is undesired for hydrogen photoproduction reducing its yield.

OMW valorization towards PHAs production could be relevant because the procedure did not require any pretreatment of OMW (Campanari et al., 2017). The process investigated in the present study exalts the recovery of the hydroxytyrosol-rich mixture that is a bioactive and profitable product.

3.4. Light conversion efficiency

Several researchers have used the light conversion efficiency as parameter for evaluating the efficiency of photofermentative processes (Carlozzi and Sacchi, 2001; Akkerman et al., 2002; Boran et al., 2012). Sakurai et al. (2013) calculated maximum solar energy conversion efficiencies varying from 11% to 25.6%. A much lower value (7.6%) was reported by Carlozzi and Sacchi (2001), when *Rhodopseudomons palustris* 42OL was cultured, outdoors, using an underwater tubular photobioreactor and taking into account, for the purple bacteria, a photosynthetic available radiation from 400 to 900 nm (64% of the spectrum of solar radiation). Our results regarding the light conversion efficiency are shown in Table 3. The highest LCE_T was obtained by using the

Table 3
Biomass composition, combustion heat (H_B), light conversion efficiencies and both hydrogen and PHB yields (Y_H, Y_{PHB}) of *Rhodopseudomonas* sp. S16-FVPT5 fed with synthetic medium or d-OMW effluents.

Culture broths	Growth mode	Biomass composition		H_B (kcal/g)	LCE_T (%)	LCE_R (%)	Y_H	Y_{PHB}
		Protein (%)	Carbohydrate (%)				L H_2/$L_{d\text{-}OMW}$	mg PHB/$L_{d\text{-}OMW}$
Synthetic	Batch	46.52	19.83	6.2116	2.36	5.03	–	–
d-OMW Diluted (25%)	Batch	45.44	20.94	6.1043	1.31	2.13	4.55	89.6
d-OMW Diluted (50%)	Batch	37.99	10.03	6.6330	1.99	4.85	3.65	350.0
d-OMW Pure (100%)	Batch	39.65	11.27	6.5601	1.18	2.83	2.24	315.0

Table 4
Comparison of PHB content and cumulative H_2 from several studies carried out with different PB strains using different carbon sources.

Purple bacterial strain	Carbon source	Nitrogen source	PHB concentration (mg PHB/L)	PHB (% CDW)	Cumulative H_2 (mL H_2/L)	Culture time (h)	References
Rhodopseudomonas sp. S16-FVPT5	d-OMW (100%)	–	315	15.7	2236	816	This study
	d-OMW diluted (50%)	–	175	11.9	1825	408	
	d-OMW diluted (25%)	–	42	3.2	1118	384	
	Acetate	Glutamate	101	7.5	1855	336	
Rhodobacter sphaeroides O.U. 001	Lactate	Glutamate	200	19.8	–	96	Yiğit et al. (1999)
	30% sugar refinery	Glutamate	500	70.4	35	96	
Rhodobacter sphaeroides O.U.001	OMW diluted with water (2%, v/v)	–	39*	–	250*	124	Eroğlu et al. (2010)
Rhodobacter sphaeroides AV1b *Mixed photofermentative culture*	VFAs (Lactate, propionate, butyrate, acetate) Dark fermentation effluent (DFE)	Ammonium Ammonium	1865 –	32.5 (Max 39.2) 6.3	914 169	960 (792) –	Ghimire et al. (2017)
Rhodobacter sphaeroides RV	Acetate	Ammonium	–	˜39.0	–	13	Khatipov et al. (1998)
	Acetate	N-deficiency	–	˜37.0	–	26	
	Acetate	Glutamate	–	˜27.0	–	26	
	Lactate	N-deficiency	–	˜28.0	yes	22	
	Lactate	Glutamate	–	˜7.0	yes	24	
	Glucose	Ammonium	–	˜8.0	–	13	
Rhodobacter capsulatus ATCC 17015	Dark fermentation effluent (DFE)	–	–	22.0[a]	866[a]	720[a]	Corona et al. (2017)
Enric. photoheterotrophic culture (IZT)			–	11.0[a]	1340[a]	720[a]	
Rhodobacter capsulatus ATCC 17015			–	30.8[b]	590[b]	720[b]	
Rhodopseudomonas sp. S16-VOGS3	Glycerol plus glucose	–	424	18.0	–	700	Padovani et al. (2018)

* mg PHB/L_{OMW}.
[a] Continue illumination.
[b] Light/dark cycle 30/30 min.

synthetic medium (5.03%); a very similar value (4.85%) was also obtained when using d-OMW diluted with water (50%, v/v). LCE_T values were about 2.3 times lower than LCE_R. Hence, LCE_R resulted more adequate than LCE_T for overcoming the gap of the theoretical photo-dissimilation of organic carbon-sources into H_2 and CO_2 against the real photo-conversion into H_2 plus CO_2 plus PHB-rich biomass (Ghimire et al., 2017).

Feeding *Rhodopseudomonas* sp. S16-FVPT5 with d-OMW, a PHB-rich biomass was significantly co-produced together with H_2. An inverse relationship was observed regarding the hydrogen yield (Y_H) and d-OMW content in the culture broth: "The lower the d-OMW amount in the culture broth, the higher the Y_H". On the contrary, the PHB yield (Y_{PHB}) grew when the d-OMW was used pure or diluted with water (50%, v/v). In this last case, the highest Y_{PHB} (350 mg PHB/$L_{d\text{-}OMW}$) was obtained. On the other hand, it is known that the PHB accumulation represents an alternative pathway for discharging excess reducing power and a reserve material such as PHB increases under unbalanced growth conditions (Melnicki et al., 2009).

4. Conclusions

This study demonstrated the efficient use of the OMW in the recovery of valuable polyphenolic mixtures with high concentration of hydroxytyrosol (> 2.0 g/L). The resultant polyphenol-free OMW was used as suitable feedstock for *Rhodopseudomonas* sp. S16-FVPT5 used to co-produce PHB and H_2, which represents both attractive alternatives to common petrol plastics (not biodegradable) and fossil fuels, respectively. The highest Y_H of 4.55 L(H_2)/$L_{d\text{-}OMW}$ was obtained when using the culture broth containing d-OMW, diluted at 25%, while the higher PHB yield of 350 mg PHB/$L_{d\text{-}OMW}$ was achieved when feeding the photobioreactor with culture broth containing 50 vol.% of d-OMW. Nonetheless, the highest content of PHB (15.7%) in dry-biomass was achieved with pure d-OMW. The results, obtained on small scale, showed that the production of PHB by purple bacteria, using d-OMW as feedstock, is feasible. Combining the recovery of hydroxytyrosol rich-mixture with the wastewater treatment and the production of valuable products (PHB and H_2) could render the process very attractive and economically feasible.

Declaration of interests

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.

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"*This paper is dedicated to the memory of our colleague and friend Professor Alessandro Degl'Innocenti*".

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2019.02.006.

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