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Photofermentative production of hydrogen and poly-β-hydroxybutyrate from dark fermentation products

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ABSTRACT

The aim of this work is to investigate the hydrogen and poly-β-hydroxybutyrate (PHB) production during the photofermentative treatment of the effluent from a dark fermentation reactor fed with the organic fraction of municipal solid waste. Two different inocula, an adapted culture of *Rhodobacter sphaeroides* AV1b and a mixed consortium of purple non sulphur bacteria have been investigated under the same operational conditions. Different hydrogen productivities of 364 and 559 N mL H_2 L^{-1} were observed for the *Rhodobacter sphaeroides* and the mixed culture consortium tests, respectively: the consortium of PNSB resulted 1.5-fold more productive than the pure culture. On the other hand, *Rhodobacter sphaeroides* culture showed a higher PHB productivity (155 mg PHB g COD^{-1}) than the mixed culture (55 mg PHB g COD^{-1}). In all the tests, the concomitant H_2 and PHB production was associated to a dissolved COD removal higher than 80%.

1. Introduction

In energy and environmental field, hydrogen (H_2) has gained considerable interests due to its higher specific energy content (122 MJ kg^{-1}), clean combustion (Balat and Kırtay, 2010) and environmental friendliness in production and use (Lin et al., 2014; Andreottola et al., 2012; Ghimire et al., 2015).

At present, the production of H_2 for industrial applications comes mainly from thermo-catalytic and gasification processes, which in turn are fossil fuels dependent. In comparison to these energy intensive physico-chemical routes for H_2 production, biological processes represent a valid alternative as they can utilize renewable biomasses (Ghimire et al., 2015). However, one of the main challenges arising from the use of low value organic biomass for hydrogen production lies in the maximization of hydrogen yields. The dark fermentation (DF) of waste biomass represents the most explored biological route for the biohydrogen production. However, dark fermentative degradation of carbohydrate rich organic biomass normally leads to incomplete substrate conversion and low H_2 yields due to thermodynamic constrains and accumulation of organic acids and alcohols as by-products (De Gioannis et al., 2013; Urbaniec and Bakker, 2015). These different types of carbon can be used as a reducing energy source by other microbial species to perform diverse biochemical reactions (Mattei et al., 2015a). Therefore, combining the DF with other processes such as photo fermentation (PF) or bioelectrochemical systems could lead to higher H_2 yields and enhance the waste biomass valorization (Bastidas-Oyanedel et al., 2015).

Under anaerobic conditions, Purple Non-Sulphur Bacteria (PNSB) carry out an anaerobic photosynthesis using light and reduced carbon sources, such as organic acids and alcohols, to produce H_2. This ability could be exploited for treating dark fermentation effluents (DFE) (Cheng et al., 2015; Rai et al., 2014). Indeed, the combined DF–PF process not only results in a higher hydrogen production (e. g. 4 extra H_2 moles for each mole of acetic acid), but also in the possibility of synthetizing poly-β-hydroxybutyrate, which is a biopolymer precursor of economic interest (Montiel-Corona et al., 2015).

In photofermentative bacteria, PHB is often produced under nutrient starvation and accumulated in the cytoplasm as intracellular carbon and energy storage compounds. Several studies have been conducted on PHB or, generally, on poly-hydroxyalkanoates (PHA) bio-accumulation (Kumar et al., 2016; Korkakaki et al., 2016), as the optimization of the biological production of plastic material may be seen as the way to overpass the environmental and recycling issues deriving from the wide utilization of petrochemical-derived plastic materials. However, their extraction and production procedures do not allow the commercial application due to the high costs required.

H_2 and PHB production strongly depend on the Volatile Fatty Acids (VFAs) present in the DFE used as feedstock for the PF. Based

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on the type and concentration of VFAs in the culture media, PNSB can differently convert organic sources in biological H_2 by several pathways (Ghimire et al., 2016; Kemavongse et al., 2007). Moreover, the structures of these copolymers directly affect their mechanical properties and thus their feasible applications (Reddy et al., 2003).

Several studies report that the synthesis of PHB competes with the H_2 production, as both functions constitute the way to dissipate the excess reducing power (Wu et al., 2012). Nonetheless, a concomitant production of H_2 and PHB is possible, as shown by Montiel-Corona et al. (2015) and Ghimire et al. (2016), but it depends on several operating conditions, such as nutrients availability (carbon to nitrogen (C/N) ratio), PNSB strains (mixed and pure culture), pH, light intensity and presence of physico-chemical stress, e.g. major H_2 inhibitor, ammonium in the culture medium, sulphur deprived conditions (Eroglu and Melis, 2011; Adessi and De Philippis, 2014; Chen et al., 2011; Fermoso et al., 2015). Depending on the aim of the process, PF can be directed towards H_2 production, suppressing the PHB synthesis by genetic modifications of the PNSB (Kim et al., 2011) or towards PHB accumulation in photosynthetic bacteria by controlling acetate and nitrogen availability in the growth medium.

The majority of the studies on both photofermentative H_2 production and PHB accumulation involved the use of pure cultures and simple organic substrates. While the use of pure strains usually results metabolically advantageous, one of the main drawbacks in the scale-up of the PF process relies on the presence of inhibitory compounds or competitive species that can affect the purity of the cultures, reducing the efficiency of the system (Ghosh et al., 2016). These problems could be addressed by the use of mixed cultures, as the synergic interactions of the H_2 producing PNSB in the consortium might enhance the efficiency and the effectiveness of PF in terms of H_2 production.

In this work, the ability of PNSB to produce H_2 and PHB from DFE obtained from the thermophilic DF of the organic fraction of municipal solid wastes (OFMSW) has been investigated. In particular, two different inocula, i.e. *Rhodobacter sphaeroides* AV1b and an enriched mixed culture of PNSB obtained from an anaerobic digestate, were tested under different operating conditions in order to examine the parameters affecting H_2 and PHB productivities. The performances of the different inocula were evaluated in terms of H_2 and PHB production and removal of soluble organic compounds.

2. Materials and methods

2.1. Dark fermentation effluent

The DFE utilized in this study was collected after 110 working days from a thermophilic semi-batch continuous stirred tank reactor with a 0.7 L working volume, a 300 mL headspace and an operating pH of 5.0 (±0.3). The H_2 yields and production rates were 105 (±28) N mL H_2 g VS^{-1} and 205 (±40) N mL H_2 L^{-1} d^{-1} at organic loading rate of 2 g VS L^{-1} d^{-1} and hydraulic retention time of 4 days. The DFE was characterized in terms of total Kjeldhal nitrogen (211 ± 4.0 mg L^{-1}), nitrogen ammonium concentration (1.89 ± 0.3), COD (4672 ± 136 mg L^{-1}) and organic acids concentration (acetic acid 575.90 mg L^{-1}, butyric acid 1117.32 mg L^{-1}, propionic acid 477.90 mg L^{-1} and lactic acid 36.11 mg L^{-1}).

In order to separate the liquid fraction, rich in organic acids, DFE was settled for 30 min, centrifuged at 4500 rpm for 20 min and finally diluted 1:2 with distilled water to obtain a clear medium for PF tests. This enhances the light penetration and reduces the potential hydrolysis of particulate organic materials which might occur otherwise during PF tests.

2.2. PF tests

Two different cultures were compared in this study: an adapted culture of *Rhodobacter sphaeroides* AV1b (RS) isolated from the Averno Lake (Naples, Italy) and a mixed consortium (MC) of PNSB enriched in a lab-scale reactor under continuous illumination. In particular, the mixed culture (MC) was obtained from the digestate of an anaerobic digestion full-scale plant treating buffalo manure as main substrate for methane production. After the clarification procedure, the digestate was inoculated in synthetic VFAs medium under continuous illumination to stimulate the selection of the PNSB species.

The experiments were carried out in triplicate by using 500 mL reactors with a 400 mL working volume, operated in batch conditions. The reactors were equipped with thin tubing on the top for sampling and gas extraction. The light was continuously provided through fluorescent lamps with constant illumination of 4000 lx according to other studies investigating the light effects on growth and H_2 production of photofermentative bacteria (Koku et al., 2002; Sevinç et al., 2012; Androga et al., 2014; Akman et al., 2015). The stirring conditions were fixed to 300 rpm through IKA RT 5 stirrer stations (Sevinç et al., 2012; Androga et al., 2014). The experiments were executed at fixed room temperature (25 °C), flushing the headspace of the reactors with argon gas for different times (0, 10 and 20 min). The PF reactors were fed with the real DFE previously defined or with a synthetic culture medium (preliminary tests only) reproducing the same characteristics of the real DFE. The pH of the medium culture for all the PF tests was initially adjusted to 6.0 with 1 M NaOH to prevent any low pH inhibition due to the presence of organic acids as substrates (Chen et al., 2011; Akroum-Amrouche et al., 2011). Total dissolved nitrogen concentration was kept low by removing the particulate organic components from the DFE. In this way, the protein hydrolysis and further release of ammonium, which usually occurs at high pH values, was limited to avoid nitrogen inhibition on PNSB activity (Keskin et al., 2011). Moreover, high C/N ratios have been found to enhance the production of PHB (Koku et al., 2003; Argun et al., 2008). In addition, the initial VFA concentrations from the DFE were not in the inhibiting range as reported by Han et al. (2012).

The samples were collected every 2–5 days and H_2 production was quantified through water displacement. The measurement system consisted in an acidic water (1.5% HCl) column where the biogas was forced to pass through; specifically, the volume of gas produced was quantified as the volume of water displaced by the overpressure of the reactor headspace. The H_2 production was calculated by considering the total biogas composition under normal conditions.

2.3. Analytical methods

Hydrogen was quantified by a Varian Star 3400 gas chromatograph equipped with ShinCarbon ST 80/100 column and a thermal conductivity detector. Argon was utilized as carrier gas with 20 psi front and rear end pressure. The duration of analysis was 15 min. The VFAs were quantified by high pressure liquid chromatography (HPLC) (Dionex LC 25 Chromatography Oven) equipped with a Synergi 4u Hydro RP 80A (size 250 × 4.60 mm) column and UV detector (Dionex AD25 Absorbance Detector). The isocratic elution consisted of 20% methanol and 10% acetonitrile in 5 mM H_2SO_4, pumped at a rate of 0.9 mL min^{-1} by using a Dionex GP 50 Gradient Pump. The elution time was 18.5 min. For PHB analysis, the samples were lyophilized and the polymers were extracted according to Oehmen et al. (2005). PHB concentration was quantified by a gas chromatograph (GC) equipped with a mass spectrometer (MS) and ZB Semi Volatiles (Zebron) column using helium as carrier gas. The

light intensity was measured with a lux meter (Lutron- LX-107). The COD was determined by the Closed Reflux method and total Kjeldahl nitrogen by macro-Kjeldahl in accordance to Standard Methods (APHA, 2005). Biomass growth was quantified by spectrophotometric measurements of the Optical Density at 660 nm (OD660) (Photolab Spektral, WTW, Germany). Total Suspended Solids (TSS) were quantified after filtering 20 mL of PNSB culture samples on 0.45 μm filters dried at 105 °C for 24 h. Total suspended solids (TSS) were correlated to the OD660 measurements using a specific calibration curve for each culture (OD660 = 3.4534*TSS (R^2 = 0.99845) and OD660 = 3.2413*TSS (R^2 = 0.99837), respectively, for *R. sphaeroides* AV1b and mixed PNSB cultures.).

3. Results and discussion

In all the experiments, the initial TSS content was kept low (<0.05 g L^{-1}) in order to favour light penetration and diffusion in the bulk liquid.

A preliminary set of experiments was conducted with the MC in order to evaluate the effect of argon flushing on the reactor performance. To this aim, a synthetic culture medium reproducing the features of the real DFE in terms of VFAs (acetic acid 563.70 mg L^{-1}, butyric acid 1088.90 mg $L^{-1}$and propionic acid 448.20 mg L^{-1}) was used as feeding solution to the photofermentative batch reactors. The serum bottles were flushed with argon for different times (0 and 10 min) which correspond to the following residual nitrogen percentages (79 and 60) in the reactor headspace. The experimental results showed that the high nitrogen concentration in the headspace observed for a 10 min flushing exerts a negative effect on the cumulative H_2 production and PHB accumulation as the mixed culture was affected by a long lag phase (Fig. 1). Moreover, neither H_2 production (Koku et al., 2002; Sasikala et al., 1990) nor PHB accumulation was detected in the PF tests without argon flushing (data not shown). This may be related to the functioning of nitrogenase and hydrogenase enzymes, which can induce the conversion of dinitrogen gas and protons to ammonia and the H_2 re-oxidization into protons and electron (Ghimire et al., 2016; Wu et al., 2012; Liu et al., 2008; Varley et al., 2015). Indeed, the presence of N_2 promotes nitrogen fixation rather than H_2 production and inhibits the structural genes for the three key enzymes of PHB synthesis from acetyl coenzyme A (Brown et al., 2016; Lee, 1995). Moreover, it can be noted that biomass growth trends were not affected by the initial nitrogen content in the headspace (Fig. 2). Indeed, the TSS concentration in tests with 0 and 10 min argon flushing was comparable to the other tests with 20 min argon flushed reactors fed with synthetic and / or real DFE (Fig. 2). Increasing flushing time (from 10 to 20 min) and progressing from a synthetic to a real DFE, which might be rich in other micronutrients such as iron or molybdenum (Özgür et al., 2010), led to higher H_2 and PHB productivity (Fig. 3).

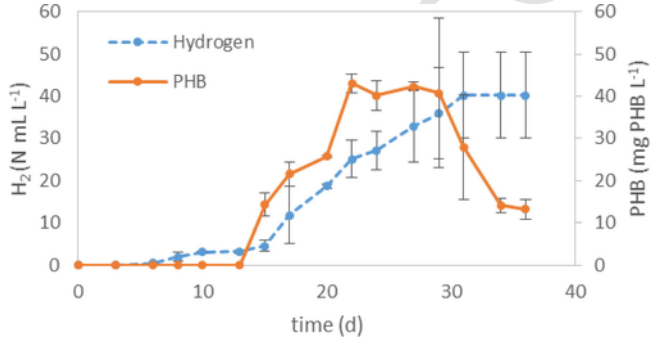

Fig. 1. Cumulative H_2 production and PHB trend from synthetic DFE with 10 min argon flushed test.

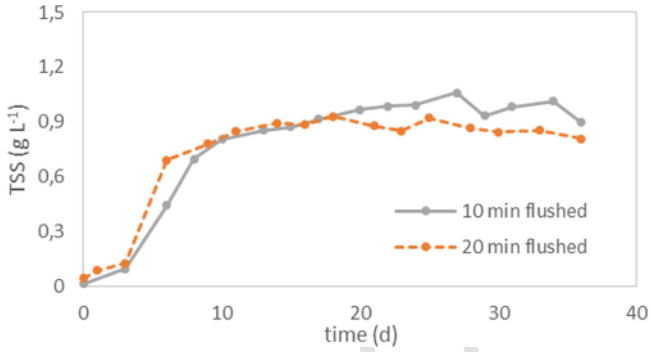

Fig. 2. Biomass growth trends of MC under different operational conditions (10 and 20 min argon flushed reactors).

Based on the results achieved in these preliminary tests, two sets of experiments were conducted by using RS and MC photofermentative reactors flushed for 20 min with argon and fed with the real DFE. The H_2 production (Fig. 3A and D), the concomitant biomass growth in terms of TSS and PHB accumulation (Fig. 3B and E), and the depletion of organic acids (Fig. 3C and F) have been reported. The maximum pH value of 7.3 was reached during the MC tests. For each reactor, similar pH trends were observed with a slight increase during the exponential growth phase and a further stabilization to the not inhibiting value of 7 (Tao et al., 2008; Tawfik et al., 2014; Boran et al., 2012).

After 36 days of incubation, the cumulative volumetric yields of 364 (±9) N mL H_2 L^{-1} and 559 (±58) N mL H_2 L^{-1} were obtained for the RS and MC reactors, respectively. The cumulative H_2 production from RS and MC tests was comparable to the maximum H_2 production of around 1000 N mL H_2 L^{-1} from DFE obtained in Uyar et al. (2009). During the first days, VFA concentrations decreased faster in MC than in RS and the final concentrations observed at the end of the experiments were lower in MC; in particular, the residual butyrate concentration in RS resulted higher than 50 mg L^{-1} at day 36. The concomitant PHB accumulation was observed in both the experiments (Fig. 3B and E). RS test led to the maximum PHB concentration of 882 (±99) mg PHB L^{-1} after 16 days whereas the lower value of 185 (±25) mg PHB L^{-1} was obtained at day 28 in the MC test. According to the past studies by Johnson et al. (2009) and James et al. (1999), a characteristic decrease in PHB concentration during the last days of incubation was observed. PHB represents an intracellular storage of carbon and energy that bacteria are able to use when VFAs start to be depleted or almost completely used (Fig. 3B and E). During the RS test, the PHB consumption was associated to a concomitant enhancement of H_2 cumulative production (Fig. 3A). On the contrary, during the MC experiments, the maximum value for hydrogen production was reached at day 25 and remained constant even after the decrease in PHB concentration (Fig. 3D).

The maximum biomass concentration of 1.06 (±0.02) g TSS L^{-1} and 0.93 (±0.01) g TSS L^{-1} were observed during the RS and MC tests, respectively (Fig. 3B and E). The characteristic exponential phase in bacterial growth was probably limited by the self-shading from light irradiance (Ghimire et al., 2016; Sevinç et al., 2012).

The mixed PNSB culture led to higher H_2 yields in comparison to the pure *R. sphaeroides* AV1b culture. This can be attributed to the adaptation of the mixed PNSB inoculum to H_2 production, confirmed in a study by Montiel-Corona et al. (2015) who obtained higher H_2 production from mixed PNSB consortia compared to a pure culture. On the contrary, PHB productivity in MC, that might not be rich in

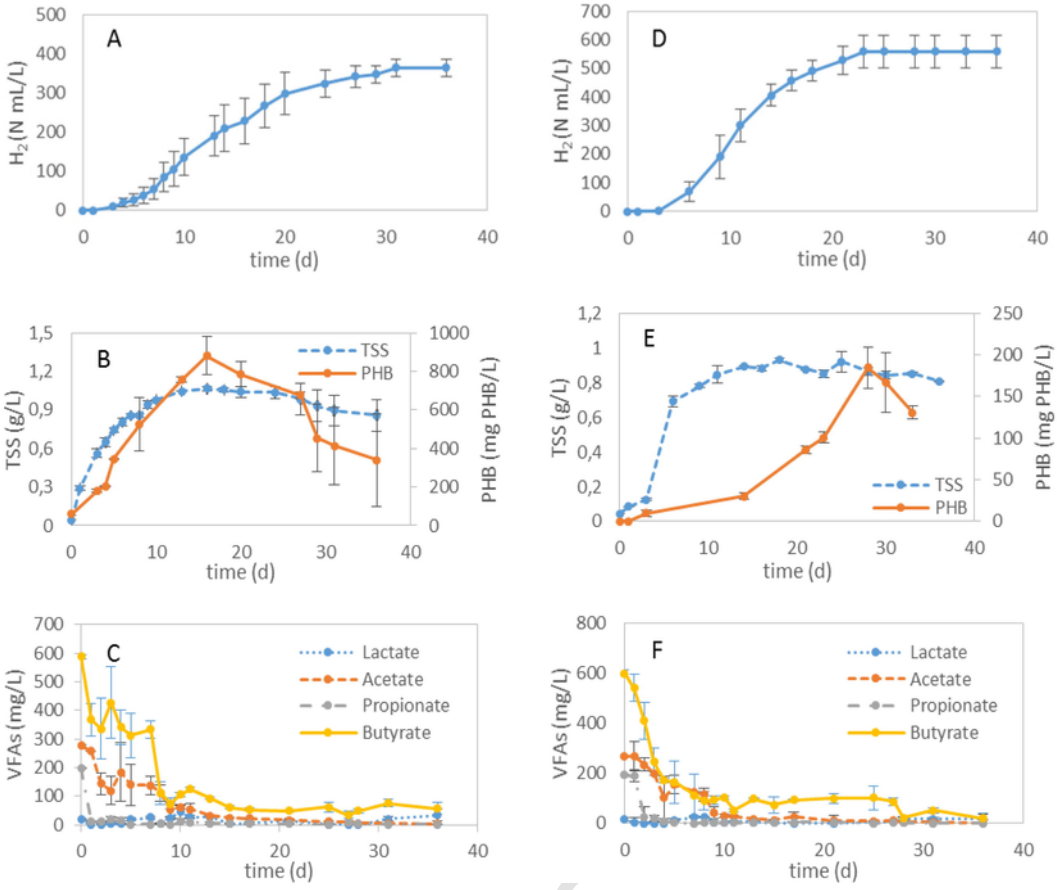

Fig. 3. Cumulative H_2 production (A, D), biomass and PHB trends (B, E) and organic acids depletion in RS (A, B, C) and MC (D, E, F) tests.

PHB producing species, was very low comparing with the RS tests, remarking the importance of pure cultures in PHB production.

A slight difference in COD removal was observed: RS tests reached 82% (±1.5%) of conversion while MC degraded 90% (±1.1%) of the initial soluble COD, indicating that the type of PNSB strain can affect the COD removal. This can be due to the presence of several microbial species in the mixed PNSB culture, which could utilize the different carbon sources present in DFE leading to a higher process robustness. Indeed, the synergies established among the different H_2 producing species might enhance the conversion of the organic substrates to H_2 and play a crucial role in the establishment of a less sensitive system to the operational conditions (e.g. pH and temperature).

In this context, the use of mathematical modelling might be crucially helpful in testing a large variation of environmental and operational conditions affecting the process (Mattei et al., 2015b; D'Acunto et al., 2016).

4. Conclusions

The results demonstrate the possibility of adapting a mixed PNSB culture for higher hydrogen production compared to the pure cultures. However, higher PHB yields was obtained with pure cultures of *R. sphaeroid*[redacted]V1b than the mixed culture. Nonetheless, the use of mixed cu[redacted] could be promising in the scale-up application of the PF systems for the treatment of DFE, as it provides a higher COD removal efficiency and saves the asepsis costs increasing process robustness. Conversely, pure *R. sphaeroides* cultures could be specifically applied for PHB production as a value added products from PF process.

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