



Biological and Bioelectrochemical Systems for Hydrogen Production and Carbon Fixation Using Purple Phototrophic Bacteria

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Domestic and industrial wastewaters contain organic substrates and nutrients that can be recovered instead of being dissipated by emerging efficient technologies. The aim of this study was to promote bio-hydrogen production and carbon fixation using a mixed culture of purple phototrophic bacteria (PPB) that use infrared radiation in presence or absence of an electrode as electron donor. In order to evaluate the hydrogen production under electrode-free conditions, batch experiments were conducted using different nitrogen (NH₄Cl, Na-glutamate, N₂ gas) and carbon sources (malic-, butyric-, acetic- acids) under various COD:N ratios. Results suggested that the efficiency of PPB to produce biogenic H₂ was highly dependent on the substrates used. The maximum hydrogen production (H_{2_max}, 423 mLH₂/L) and production rate (H_{2_rate}, 2.71 mLH₂/Lh) were achieved using malic acid and Na-glutamate at a COD:N ratio of 100:15. Under these optimum conditions, a significant fixation of nitrogen in form of single-cell proteins (874.4 mg/L) was also detected. Under bio-electrochemical conditions using a H-cell bio-electrochemical device, the PPB were grown planktonic in the bio-cathode chamber with the optimum substrate ratio of malic acid and Na-glutamate. A redox potential of -0.5 V (vs. Ag/AgCl) under bio-electrochemical conditions produced comparable amounts of bio-hydrogen but significantly negligible traces of CO₂ as compared to the biological system (11.8 mLCO₂/L). This suggests that PPB can interact with the cathode to extract electrons for further CO₂ re-fixation (coming from the Krebs cycle) into the Calvin cycle, thereby improving the C usage. It has also been observed during cyclic voltammograms that a redox potential of -0.8 V favors considerably the electrons consumption by the PPB culture, suggesting that the PPB can use these electrons to increase the biohydrogen production. These results are expected to prove the feasibility of stimulating PPB through bio-electrochemical processes in the production of H₂ from wastewater resources, which is a field of special novelty and still unexplored.

Keywords: purple phototrophic bacteria, bioelectrochemical, high value-added products, bio-hydrogen, carbon fixation, proteins

INTRODUCTION

Typical wastewater systems entail the dissipation of the contamination. However, the high content of organics and nutrients in industrial and domestic wastewaters is a valuable resource for energy and products recovery (Puyol et al., 2017a). Hence, upgrading of existing WWTP as resource recovery systems by implementing novel technologies, are mandatory steps considering economic and environmental benefits and recent policies within the circular economy.

Among the competing technologies, the biological accumulation of nutrients and their subsequent recovery, has received great attention as an environmental friendly and certainly cost-effective process (Batstone et al., 2015). Purple phototrophic bacteria (PPB) have shown significant accumulation of organics and nutrients from wastewater through assimilative processes (Batstone et al., 2015). PPB is a group of anaerobic facultative microorganisms, which can utilize infrared light (IR) as the main energy source. The use of PPB in the Partition-Release-Recovery concept proved to be far superior to other phototrophic organisms (as algae or cyanobacteria), since they achieve high growth rates and are not inhibited by O₂ (Muñoz and Guieysse, 2006).

PPB are extremely versatile organisms due to their complex metabolic system, involving major C, N, S, P, and Fe pathways, which absorbs the IR energy through their photosystem, composed by carotenoids and bacteriochlorophylls (Hunter, 2008). Anoxygenic photosynthesis generates practically all the energy required for growth via the so-called cyclic electron flow (Klamt et al., 2008). In domestic wastewater treatment, the main metabolism follows photoheterotrophic growth on volatile fatty acids and sugars, although chemoheterotrophy (e.g., fermentation and anaerobic oxidation) can provide the necessary electrons for photoautotrophic growth (via hydrogen; Hülsen et al., 2014, 2016; Puyol et al., 2017a,b). The internal electron recycle, however, can be used for obtaining ammonium through dinitrogen gas fixation or directly dissipating electrons in the nitrogenase complex, which generates bio-hydrogen as the electron acceptor (Koku et al., 2002), or for direct internal accumulation of organic acids as poly-hydroxy-alkanoates (PHA) (Fulop et al., 2012). Moreover, the assimilative partitioning of wastewater macronutrients and organics through PPB leads to the production of one solid bacterial stream rich in proteins.

In this sense, PPB can be used for the extraction of high value-added products from waste sources, such as biofuels like bio-hydrogen, bioplastics as PHA and single-cell proteins. The metabolic pathways to obtain the valuable bioproducts are catalyzed by variant enzymes (McKinlay and Harwood, 2010). Monitoring the functionality of the involved bacteria and following-up their activity, can be of added value toward maximizing the bioproducts' formation. To add to the complexity of the above system, the end product depends greatly on the environmental conditions (IR light intensity, temperature, nutrients concentration, etc.). Thus, wastes rich in nitrogen are good sources for PPB growth producing biomass with high protein content (Verstraete et al., 2009), which can subsequently be used as additive animal food. In organic media lacking

nutrients, PPB can accumulate high quantities of PHA, achieving up to 70–90% w/w (Mas and Van Gernerden, 1995). They are therefore an interesting alternative to fossil-fuels for plastics production. When the organic matter composition is quite high and is more reduced than biomass (i.e., butyrate), the excess of electrons (in absence of ammonium) are driven toward hydrogen production that can be used as a clean and renewable biofuel. Understanding the factors of importance and unraveling their relationship with the desired end bioproduct, remains one of the most important challenges in the ongoing research.

Finally, the internal electron recycling of PPB is a key issue, and an active modification of the electronic fluxes by means of artificial addition of electrons could drive toward different targeted bioproducts (Varfolomeyev, 1992). In this way, the concepts supporting microbial electrochemical technologies (METs) could be used to enhance the biochemical reactions of PPB by supplying electric current to microorganisms using electrodes as electron donors. In this context, METs have received great attention due to their potential applications in nitrate reduction (Pous et al., 2013; Tejedor et al., 2016), methanogenesis (Cheng et al., 2009) and microbial electrosynthesis (Logan and Rabaey, 2012). Likewise, the wise use of electricity to enhance PPB activity toward high value-added compounds (i.e., biohydrogen) through a bio-electrochemical system is undoubtedly an attractive challenge. PPB are highly electroactive organisms with high ability to generate bioelectricity through MFCs (Xing et al., 2008; Park et al., 2014). However, the use of electricity to enhance the PPBs metabolic activity aiming to produce high value-added bioproducts is an unexplored field with high growth potential in the short-term.

Based on the above-mentioned grounds, the aim of the present work was the assessment of PPB to enhance the formation of valuable bioproducts, such as biohydrogen, using electric and light energy as the driving forces. This was accomplished by identifying the biological and electrochemical conditions that influence the process of bio-hydrogen production from PPB. The wise use of electric energy to decontaminate wastewater and to produce bio-hydrogen is undoubtedly an attractive and novel challenge, yielding substantial ecological and economic benefits.

MATERIALS AND METHODS

Chemical Compounds and Growth Media

All the chemicals compounds used were purchased from Sigma-Aldrich. The organic compounds that were used were: L-malic acid (C₄H₆O₅), butyric acid (C₄H₈O₂), acetic acid (C₂H₄O₂), propionic acid (C₃H₆O₂) and ethanol (C₂H₆O). Stock solutions of individual organic compounds (20 gCOD/L) were prepared in ultra-pure water and stored at 4°C. The nitrogen sources used were: ammonium chloride (NH₄Cl) as inorganic N-source, L-glutamic acid monosodium salt monohydrate (Na-glutamate, C₅H₈NNaO₄·H₂O) as organic N-source and nitrogen gas (N₂) as external gaseous source. Stock solutions of both organic and inorganic nitrogen sources (5 gN/L) were prepared in ultra-pure water and stored at 4°C.

Finally, macro- and micro-nutrient solutions were prepared following the recipe proposed by Ormerod et al. (1961). The

macro-nutrient solution contained 10.86 g $K_2HPO_4 \cdot 3H_2O$; 6.66 g KH_2PO_4 ; 2 g $MgSO_4 \cdot 7H_2O$, 0.75 g $CaCl_2 \cdot 2H_2O$; 69 mg $FeCl_2 \cdot 4H_2O$ and 0.2 g EDTA in 1 L ultra-pure water. The micro-nutrient solution contained 1.4 g H_3BO_3 ; 1.013 g $MnCl_2 \cdot 4H_2O$; 274 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 57 mg $ZnCl_2$; 14 mg $CuCl_2 \cdot 2H_2O$; 7.5 mg biotin and 1 g EDTA in 0.5 L ultra-pure water. The pH in all solutions was adjusted to 7.

Purple Phototrophic Bacteria (PPB) Enrichment

All experimental tests were inoculated with a mixed culture of PPB. These bacteria were enriched from a wastewater influent taken from the pilot-scale WWTP located at the Rey Juan Carlos University (Mostoles, Madrid, Spain). Enrichment was performed by inoculating a 1 L suspended growth reactor (SGR) with sludge liquor, and subsequent incubation under near infrared (NIR) light illumination and anaerobic conditions using a synthetic wastewater (SW) as growth medium. The SW (prepared with tap water) contained the 5 different organic carbon sources (acetic acid, malic acid, propionic acid, butyric acid and ethanol) with a total COD concentration of 2 gCOD/L, 0.26 gN/L as NH_4Cl and 1 and 100 mL/L of micro- and macro-nutrient solutions, respectively. After the addition of SW the bioreactor liquor was flushed with argon gas in order to remove any presence of oxygen. The bioreactor was illuminated with LED lamps (850 nm) as IR light source. The reactor's surface was covered with UV-VIS absorbing foil (ND 1.2 299, Transformation Tubes, Banstead, UK). The foil absorbed around 90% of the wavelength below 750 nm. The average light intensity measured on the outside reactor's surface was 13 W/m². The PPB mixed culture was continuously stirred and incubated at room temperature ($25 \pm 1^\circ C$). The liquor of the reactor was refreshed every week with fresh SW (99% volume exchange) to achieve final concentrations of 2 gCOD/L and 0.26 gNH₄-N/L. The pH was weekly adjusted to 6.8 ± 0.1 . The enrichment of PPB was evaluated by the detection of Bacteriochlorophylls (*Bchl*) and carotenoids accumulation by performing VIS-NIR spectra analyses of the culture.

Biological Experiments

The ability of the PPB enriched culture to produce bio-hydrogen using different carbon and nitrogen sources was evaluated in batch assays. Initially, the capacity of the PPB culture to produce hydrogen using different organic and inorganic nitrogen sources was examined. The first set of experiments were conducted by using 2 gCOD/L of L-malic acid as the carbon source. Malic acid was chosen as a suitable carbon source that could favor hydrogen production by PPB (Assawamongkholisiri and Reungsang, 2015). Batch experiments were performed using: inorganic (NH_4Cl) and organic (Na-glutamate) nitrogen, both with concentrations of 75, 150, and 300 mgN/L, and finally dinitrogen gas (60 mL of N_2 in the headspace). Thereafter, two additional experiments were conducted using different carbon sources (butyric- and acetic-acid) at a concentration of 2 gCOD/L each, with 300 mgN/L

of Na-glutamate as organic nitrogen source. A summary of the experimental conditions of the batch assays is shown in **Table 1**.

All the experiments were conducted in 160 mL serum bottles with a working volume of 100 mL. The reactors contained 99 mL of SW medium (prepared as described above) with the corresponding COD and N contents and were inoculated with PPB enriched culture (1% v/v inoculum). The initial pH of the medium was adjusted to 6.8 ± 0.1 using NaOH or H_2SO_4 . The liquid medium of each reactor was flushed with argon for 10 min. Thereafter, the bottles were closed with rubber stoppers and capped with aluminum seals. Subsequently, the headspace of the reactors was flushed again with argon for 2 min except from the reactors where nitrogen gas was used as nitrogen source that were flushed with N_2 gas. The bottles were continuously shaken horizontally at 120 rpm at $25 \pm 1^\circ C$ (Orbital shaker, optic ivymen system) and illuminated at an average light intensity of 20 W/m² using LED lamps for 7 days. The performance for H_2 production using identical conditions but without PPB enriched culture was studied by conducting control experiments under sterilized conditions (all the glassware and media used were autoclaved). During these control experiments, no biomass growth as well as no H_2 production or acid assimilation were detected. Both the liquid and the gas media were sampled periodically to evaluate, the carbon and nitrogen assimilation, the PPB growth and the hydrogen production. All the experiments were conducted in duplicate.

Bio-Electrochemical Experiments

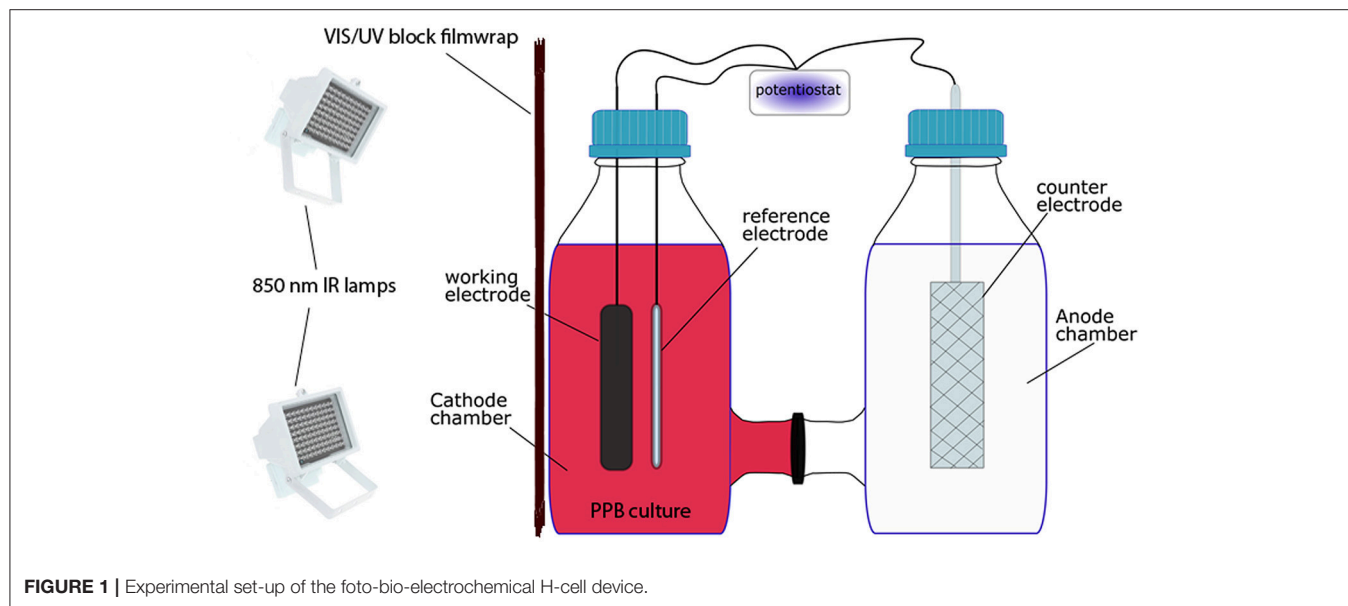
Bio-electrochemical experiments were performed in an H-cell device as shown in **Figure 1**. The device was consisted of two Duran glass bottles (8.6 cm diameter \times 18.1 cm height) serving as two chambers. Each cell (cathode and anode) had a working volume of 500 mL. The cathode chamber was equipped with a working electrode of graphite of 10 \times 100 mm and a reference electrode RE-5B Ag/AgCl. All potentials are quoted vs. Ag/AgCl. The anode chamber was equipped with a counter electrode of Ti/Pt (2.5 micro-m) 100 \times 20 mm in a 10 \times 5 mesh. The cathode and anode chambers were separated with a cationic membrane (RALEX, MEGA a.s., Straz pod Ralskem, Czechia). The working, counter and reference electrodes were connected to a potentiostat NEV4-V2 (Nanoelectra S.L., Alcala de Henares, Spain) with maximum current of ± 100 mA and compliance voltage of ± 10 V. A computer processed by specialized software (Potentiostat NEV4 software, Alcala de Henares, Spain) was used for the automatic recording of data.

As shown in **Figure 1**, the cathode chamber from the H-cell was employed as bio-cathode containing SW (495 ml). The bio-cathode was inoculated with PPB enriched culture (5 mL, 1% v/v inoculum). Malic acid (2 gCOD/L) and Na-glutamate at COD:N ratio of 100:15 were respectively used as carbon and nitrogen sources. The anode chamber was filled with 495 mL of tap water and 5 mL of the macro-nutrients solution. The initial pH in both chambers was adjusted to 6.8 ± 0.1 . The bio-cathode was illuminated with LED lamps as NIR light source with an average light intensity of 20 W/m². Bio-electrochemical experiments were performed at $25 \pm 1^\circ C$ and the cell of bio-cathode was continuously stirred at a speed of 200 rpm. The media in both

TABLE 1 | Experimental runs of PPB biological experiments under different nitrogen and carbon sources.

ID	Carbon source	Organic acid concentration (mgCOD/L)	Nitrogen source	N concentration (mgN/L)	COD:N ratio
1	Malic acid	2,000	NH ₄ Cl	75	100:3.75
2	Malic acid		NH ₄ Cl	150	100:7.5
3	Malic acid		NH ₄ Cl	300	100:15
4	Malic acid	2,000	Na-glutamate	75	100:3.75
5	Malic acid		Na-glutamate	150	100:7.5
6	Malic acid		Na-glutamate	300	100:15
7	Malic acid	2,000	N ₂ gas	8.8*	–
8	Butyric acid	2,000	Na-glutamate	300	100:15
9	Acetic acid	2,000	Na-glutamate	300	100:15

*Based on Henry's Law and the solubility of gases.

**FIGURE 1** | Experimental set-up of the foto-bio-electrochemical H-cell device.

cells were flushed with argon for 20 min. Subsequently, the cells were closed with butyl septa and capped with GL45 Duran caps. The headspaces of the cells were flushed again with argon for 3 min.

Experiments were conducted by setting the potential of bio-cathode at -0.5 V in order to force the PPB culture to be adapted to the electrochemical conditions. The reaction period among the PPB culture and the bio-cathode was chosen to be 1 week, similar to the biological experiments. Control electrochemical (abiotic) experiments were conducted using the same experimental conditions without PPB biomass. In order to determine whether the PPB culture interacted with the cathode or not by means of electron acceptance from PPB, cyclic voltammetry (CV) in the range of -0.8 to 0.8 V was performed during the weekly reaction process.

Analytical Methods

All parameters except total chemical oxygen demand (COD) and total Kjeldahl Nitrogen (TKN) were determined after filtering with a $0.45 \mu\text{m}$ nylon filter (Chrodisc filter/syringe, CHMLab,

Barcelona, Spain). Total and soluble COD were determined using a dichromate-reflux colorimetric method (APHA, 2005). The nitrogen contents of filtered and non-filtered samples were determined by the standard Kjeldahl procedure (Gerhardt TNK, Vapodest 450, Königswinter, Germany) using 20 mL of concentrated H₂SO₄ and K₂SO₄-CuSO₄ as catalyst. Organic nitrogen content of PPB culture was determined as the difference between Kjeldahl nitrogen of filtered and non-filtered sample. The single cell protein (SCP) content of cell dry weight (CDW) was obtained by multiplying the obtained nitrogen value with a conversion factor of 5.33 (Salo-Vaananen and Koivistoinen, 1996). The inorganic nitrogen was analyzed as NH₄Cl using Spectroquant Ammonium Test (Merck, Darmstadt, Germany). The optical density of PPB biomass was measured at 590 nm by UV-VIS spectrophotometer (V-630, Jasco, Madrid, Spain) and the concentration of biomass was calibrated using a standard curve of PPB optical density on the basis of volatile suspended solids (gVSS/L) concentration (Vasiliadou et al., 2008). The VSS concentration (gVSS/L) was measured according to standard methods (APHA, 2005). The detection of *BChl* and carotenoids

of PPB was performed by determining the VIS-NIR spectra (400–950 nm) using a UV-vis spectrophotometer (V-630, Jasco, Madrid, Spain). The pH was measured using a pH meter (Crison GLP22, Hach Lange, Loveland, CO, USA). Illuminance was measured with a VIS-NIR spectroradiometer (STN-Bluewave-V, MTB, Madrid, Spain). The concentrations of VFAs (malic, acetic and butyric acids) in the liquid samples were analyzed using high performance liquid chromatography (HPLC) (Varian 356-LC, Agilent Technologies, Santa Clara, CA, USA), employing refractive index (RI) detector with a MetaCarb 67H 300 × 6.5 mm column (Agilent Technologies, Santa Clara, CA, USA). The oven temperature was 65°C. The mobile phase was 0.25 mM H₂SO₄ at a flow rate of 0.8 mL/min. The volume of the gas was determined by releasing pressure from the reactors headspace using a Boyle-Mariotte Apparatus (3B Scientific S.L., Hamburg, Germany). The composition of each reactors head-space was analyzed using a 7820A GC system equipped with a 3Ft 1/8 2 mm Poropak Q 80/100 SS column, a 6Ft 1/8 2 mm Poropak Q 80/100 SS column and a 6Ft 1/8 2 mm MolSieve 5A 60/80 SS column, a fitting external Luer lock and a thermal conductivity detector (TCD) (Agilent Technologies, Santa Clara, CA, USA). The mobile phase was Argon at a flow rate of 5 mL/min. The temperature of the oven and the detector were 45 and 220°C, respectively.

RESULTS AND DISCUSSION

The following section include all results generated after exploring the physiology of PPB for selecting those culture conditions, including nitrogen and carbon sources, for achieving an optimal conversion of an extracellular source of electrons into hydrogen production and CO₂ fixation.

Enrichment of a PPB Mixed Culture From Domestic Wastewater

The enrichment process was performed aiming to enhance the growth and acclimation of a mixed PPB culture from domestic wastewater, using a specific environment of NIR radiation. The organic mixture used for the enrichment was chosen on the basis that PPB can efficiently produce hydrogen from wastes that contain mixed VFAs (Wu et al., 2012). It should be noted that the optimum COD:N ratio for efficient C and N assimilation from domestic wastewater by PPB was reported to be 100:7.1 (Puyol et al., 2017b). However, during conventional DWW treatment operation, nutrients, such as N and P are usually in excess (Puyol et al., 2017b). Therefore, a COD:N ratio of 100:13.1 was chosen for the enrichment and acclimation process. Following a 2-weeks enrichment period, PPB biomass growth was evidenced through the BChl *a* accumulation as detected from the peaks with maximum absorbance at 590, 805, and 860 nm. This clearly indicated that the enrichment under anaerobic conditions and NIR light source could selectively enrich PPB from wastewater and express their photosynthetic apparatus via bacteriochlorophylls (Melnicki et al., 2008).

Figure 2 shows an example of PPB culture performance during a weekly operating cycle, after a 2-months acclimation

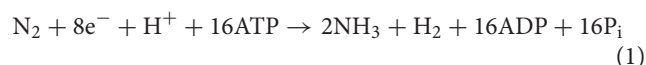
period. **Figure 2A** shows the absorbance spectra of PPB culture aliquots that were taken at different time intervals during the weekly cycle (day 0–7). The PPB culture produced and accumulated with time BChl *a* as well as carotenoids that are naturally synthesized by photosynthetic organisms. The absorption spectrum of BChl *a* appeared in the spectral range between 560 and 930 nm, with maximum peaks at 590, 805, and 860 nm, respectively, while the spectrum of carotenoids appeared in the range between 400 and 550 nm. It has been previously reported that PPB produce molecules referred to as open-chain carotenoids and incorporate them into their photosynthetic system, such as light-harvesting complexes and the bacteriopheophytin-quinone type reaction center (Niedzwiadzki et al., 2017).

As shown in **Figure 2B**, the PPB concentration reached 750 mg/L at the end of the weekly cycle, giving a growth yield of 0.75 ± 0.05 gCOD_{PPB}/gCOD_{VFA}. Moreover, the removal of COD and N by PPB culture over the whole acclimation period resulted to an average COD:N of 100:10, which was higher than the optimum ratio (100:7.1) previously reported for domestic wastewater. This high ratio suggested that the PPB enriched culture may have potential for enhancing nitrogen removal in order to achieve the discharge limits for total nitrogen (Hülßen et al., 2014).

Enriched PPB biomass was used for inoculum purposes in order to study the hydrogen formation from wastewater in presence and absence of an electrode as electron donor.

Effect of Nitrogen Source on Biological Hydrogen Production by PPB

Hydrogen production under nitrogen fixation conditions is described by Equation (1) where molecular nitrogen (N₂) is converted to ammonia (NH₃) and protons (H⁺) to hydrogen (H₂) (Rey et al., 2007).



Biological experiments were conducted in order to extract the optimum biological conditions to maximize hydrogen production while minimizing CO₂ emission. Our first approach was to analysis how biohydrogen production depended on nitrogen substrate at different concentrations by using three different N sources (ammonium, glutamate and nitrogen gas) and malic acid as a model substrate of organic carbon. Interestingly, glutamate increased PPB growth rate by 2-fold in comparison with ammonium or nitrogen gas (see **Figure S1** in Supplementary Information). This also is shown in **Table 2**, where the kinetic parameters of PPB metabolism are included.

Biohydrogen analysis revealed an interesting correlation of hydrogen with the ratio COD:N. So, hydrogen production was enhanced (451 ± 2.1 mLH₂/L) when NH₄Cl was used as inorganic nitrogen at a COD:N ratio of 100:3.75. In contrast, very low amount of hydrogen was produced when higher concentrations of NH₄Cl (COD:N of 100:7.5 and 100:15) were tested (**Figure 3A**; **Table 2**), with 95% confidence values concurring with the zero value. This, in fact, indicates that zero hydrogen production cannot be statistically discarded under these conditions. This is in agreement with the results previously

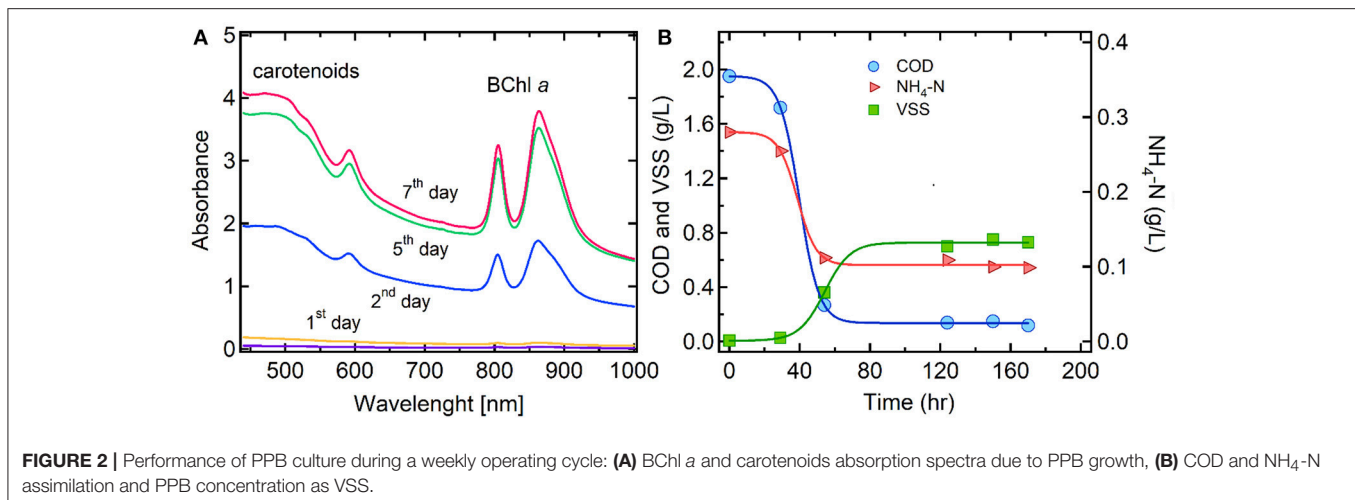


TABLE 2 | Comparison of H₂ production under different nitrogen and carbon sources.

No	Sources	COD:N	R _{PPB} ^a (mgVSS/Lh)	R _{acid} ^b (mMacid/Lh)	H ₂ ^c _{max} (mLH ₂ /L)	H ₂ ^d _{rate} (mLH ₂ /Lh)	Y _{H₂} ^e (LH ₂ /g _{acid})	Y _{molH₂} ^f (molH ₂ /mol _{acid})
1	Malic/NH ₄ Cl	100:3.75	3.67 ± 0.82	0.21 ± 0.05	451.0 ± 2.1	2.63 ± 0.13	0.13 ± 0.01	0.70 ± 0.04
2	Malic/NH ₄ Cl	100:7.5	4.74 ± 0.84	0.19 ± 0.04	2.2 ± 2.3	(1.35 ± 1.1) × 10 ⁻²	(0.59 ± 0.58) × 10 ⁻³	(3.15 ± 3.18) × 10 ⁻³
3	Malic/NH ₄ Cl	100:15	5.47 ± 1.01	0.20 ± 0.04	13.7 ± 14.5	(1.30 ± 0.7) × 10 ⁻²	(0.50 ± 0.63) × 10 ⁻³	(2.55 ± 2.76) × 10 ⁻³
4	Malic/Na-glutamate	100:3.75	3.39 ± 0.97	0.21 ± 0.06	300.2 ± 85.0	2.06 ± 0.90	(8.75 ± 2.89) × 10 ⁻²	0.47 ± 0.16
5	Malic/Na-glutamate	100:7.5	6.26 ± 2.07	0.20 ± 0.06	416.1 ± 148.2	2.57 ± 1.03	0.12 ± 0.05	0.66 ± 0.27
6	Malic/Na-glutamate	100:15	7.56 ± 1.89	0.19 ± 0.04	423.0 ± 40.9	2.71 ± 0.27	0.12 ± 0.01	0.67 ± 0.05
7	Malic/N ₂ gas	–	5.57 ± 1.68	0.21 ± 0.04	12.2 ± 11.3	0.12 ± 0.05	(0.36 ± 0.39) × 10 ⁻²	(1.97 ± 2.12) × 10 ⁻²
8	Butyric/Na-glutamate	100:15	3.23 ± 0.18	0.06 ± 0.01	214.2 ± 7.2	1.21 ± 0.12	0.22 ± 0.02	0.79 ± 0.08
9	Acetic/Na-glutamate	100:15	3.96 ± 0.09	0.16 ± 0.0	320.4 ± 82.5	2.49 ± 0.36	0.21 ± 0.05	0.50 ± 0.13
10	Bio-electrochemical Malic/Na-glutamate*	100:15	5.91	0.17	390	2.32	0.11	0.60

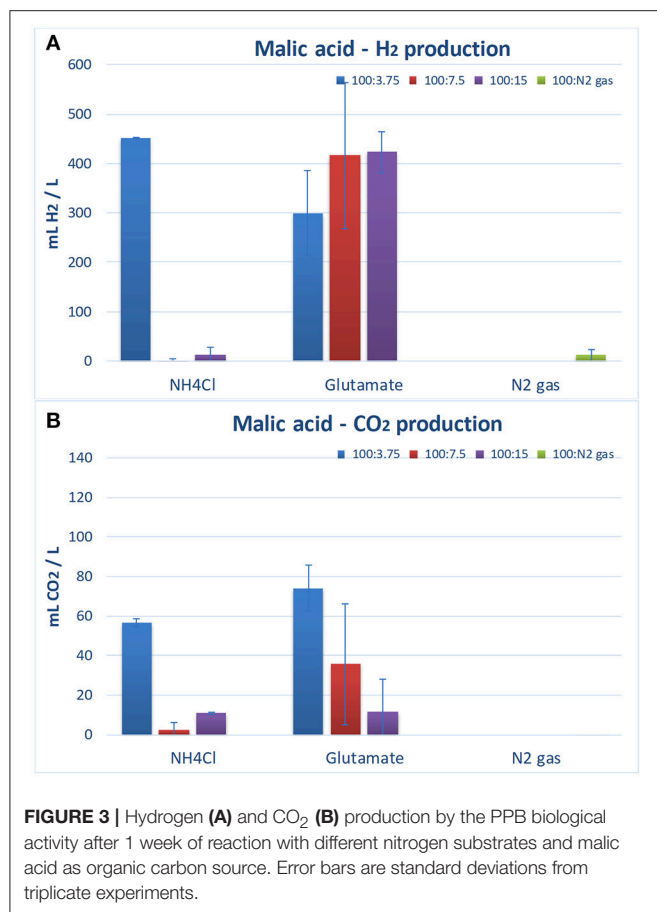
^aPPB growth rate, ^b organic substrate assimilation rate, ^c maximum H₂ production, ^d H₂ production rate, ^e H₂ yield, ^f In the Bio-electrochemical experiments there were no replicates, so no error analysis was able to be conducted.

reported, stating that high NH₄Cl concentration inhibits the function of the enzyme nitrogenase of PPB resulting in lower hydrogen production (Kim et al., 2012a). Alternatively, N₂ gas as a nitrogen source was used to enhance hydrogen production under nitrogen fixation conditions without repressing the expression of nitrogenase genes.

However, it was observed that the use of N₂ gas as nitrogen source did not efficiently produce H₂ (Figure 3A; Table 2). The low H₂ production rate (0.12 ± 0.05 mLH₂/Lh) observed in this experiment was probably attributed to a higher energy requirement for the process (16 ATP per mol of hydrogen produced in the nitrogen fixation case vs. 4 ATP per mol of hydrogen produced in the case of the hydrogen production with no nitrogen fixation in the nitrogenase; McKinlay and Harwood, 2010). Also, the extra consumption of reductants to conduct nitrogen fixation for the heterotrophic growth

may be counteracted by an increase of the consumption of the produced H₂ in autotrophic growth mode. The absence of CO₂ evolution in the N₂ experiments confirmed such a hypothesis. The PPB culture may use the H₂ produced during the N₂ fixation to re-fixate, in the Calvin-Benson-Bassham cycle (Calvin cycle, CBB), the CO₂ produced during the malic acid assimilation. The analysis of the effect of an external electron source (e.g., from the cathode) would give light to this unsolved question and open possibilities for further research.

Finally, the results indicated that PPB culture produced large amounts of hydrogen when Na-glutamate was used as an organic nitrogen source (300–423 mLH₂/L, Table 2). Na-glutamate enhanced the PPB growth and hydrogen production rate (2.1–2.7 mLH₂/Lh). The results of this study are in agreement with those reported by other



researchers, who have shown that Na-glutamate enhances hydrogen production without inhibiting the nitrogenase enzyme (Melnicki et al., 2008; Assawamongkholsiri and Reungsang, 2015). This is due to the fact that organic nitrogen can be directly assimilated into proteins and a less complex metabolic activity is required for the production of amino acids compared to inorganic sources (Merugu et al., 2010).

Considering Na-glutamate concentration, results showed that H₂ production (mLH₂/L) as well as its production rate was increased as the organic nitrogen concentration increased, achieving a maximum H₂ production at a COD:N ratio of 100:15 (Figure 3A; Table 2). Moreover, it is noteworthy to mention that the CO₂ production was reduced as the Na-glutamate concentration increased (Figure 3B). Therefore, the use of Na-glutamate at COD:N ratios of 100:3.75, 100:7.5, and 100:15 resulted to the production of 74.2 ± 11.6, 35.7 ± 30.7, and 11.8 ± 16.0 mLCO₂/L, respectively. PPB that grow on oxidized organic substrates (as malic acid) produce CO₂ due to the oxidation of these substrates. The released CO₂ can then be fixed through the Calvin-Benson-Bassham cycle (Calvin cycle) into cell material as an electron accepting process (McKinlay and Harwood, 2010). This CO₂ fixation via the Calvin cycle enabled PPB to accept excess of electrons and to maintain redox balance and to dispose extra electrons that are generated during use of extra carbon

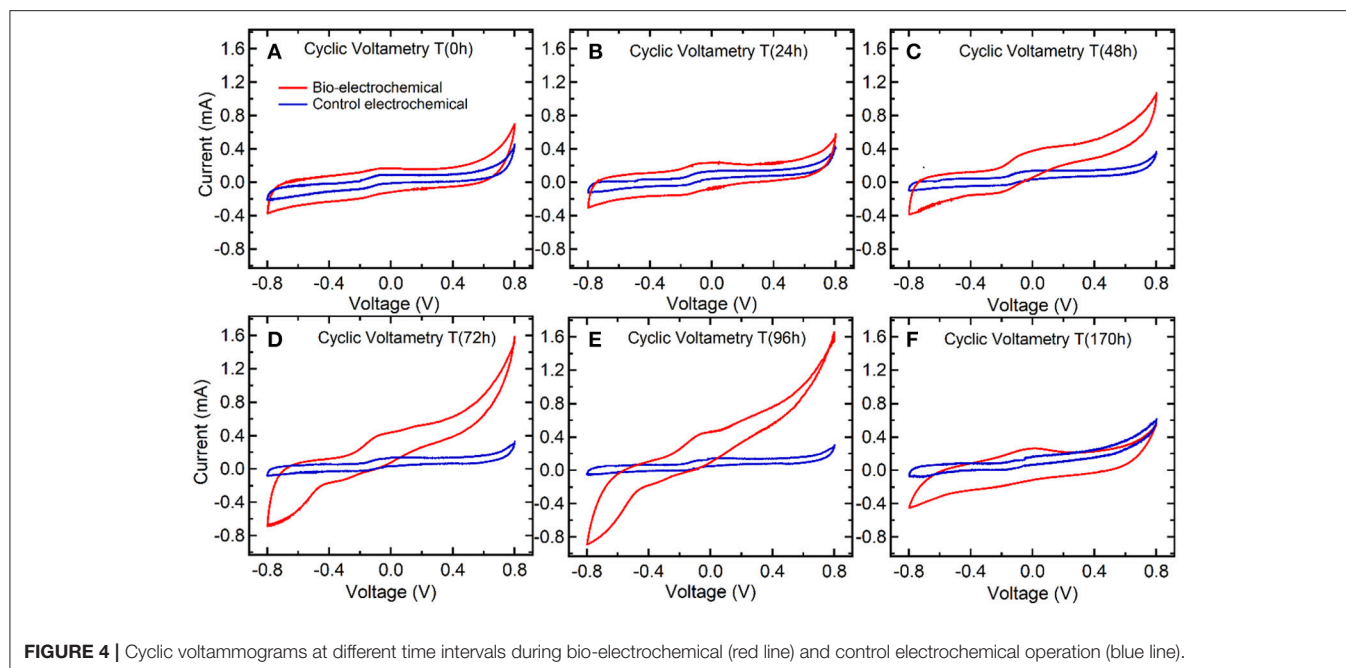
included in Na-glutamate. Therefore, the higher Na-glutamate concentration in the medium could result in a greater CO₂ fixation and lower emission.

It should be highlighted that reducing the NH₄Cl levels (100:3.75 ratio) resulted in a high hydrogen production (Figure 3A) by minimizing the inhibitory effect of this compound on the activity of nitrogenase. In contrast, same conditions resulted in a significant emission of CO₂ (56.7 ± 1.9 mLCO₂/L). In addition, Na-glutamate in the ratio 100:15 could favor the PPB activity toward nitrogen assimilation. In conclusion, based on the above, Na-glutamate at a COD:N ratio of 100:15 was selected as the optimum culturing conditions for maximized H₂ production with minimized CO₂ emission.

PPB Can be Cultured Under Bio-Electrochemical Conditions

Considering the importance of the internal electron recycling, an active modification of the electron fluxes through artificial addition of electrons by applying electrochemical technology may potentially enhance the PPB activity and drive toward an optimum H₂ production process. In this sense, the bio-electrochemical capability of interaction between PPB and graphite-electrode has been explored, specially emphasizing the situation when graphite electrode behaves as an electron donor to PPB (setting graphite-electrode potential at -0.5 V) aiming to increase PPB metabolic paths activity by supplying electric current. Therefore, this study focused on the analysis of a bio-electrochemical device based on PPB, using malic acid as organic source and Na-glutamate as nitrogen source, compared to electrode-free biological systems.

Figure 4 presents the cyclic voltammograms (CV) of bio-electrochemical system as well as the bare graphite-electrode as a control electrochemical process, at different time intervals during a weekly operation. The electrochemical behavior for the bare graphite-electrode in the culture media do not exhibit any electrocatalytic behavior in the whole potential range of study (-0.8 to 0.8 V). As it can be seen in Figure 4, the cyclic voltammograms for bare graphite-electrode (electrochemical abiotic control) has no reductive currents that can be assigned to tentative hydrogen evolution or the malic acid reduction. As expected, only capacitive currents typical from bare graphite-electrode (ideally polarizable electrode, Bard and Faulkner, 2001) were detected in absence of PPB. Only small positive currents were observed at the more positive potentials explored, 0.8 V (vs. Ag/AgCl), probably due to slight water oxidation and graphite surface oxidation. Figure 4 shows the cyclic voltammograms for the bio-electrochemical system during the first 24 h, which are very similar to the bare graphite electrode. Only changes in the capacitive currents were observed, exhibiting a higher value for the interfacial pseudocapacitance under bio-electrochemical conditions, so suggesting an electrode surface modification by bacteria attachment. Just after inoculation no significant electroactive biofilm formed but the presence of bacteria in the interface increases the interfacial pseudocapacitance. This was



probably due to the very low amount of PPB biomass existed at the beginning (Time 0 h) of the experiment (0.01 gVSS/L).

After 48 h of polarizing the electrode at -0.5 V (vs. Ag/AgCl), the cyclic voltammograms revealed the electroactivity of the PPB biofilm interacting with the graphite-electrode surface. These results suggested that PPB started to interact with the working electrode when sufficient amount of biomass (0.1 gVSS/L) and malic acid as carbon source were present in the cathode chamber (**Figure 5A**). It can be observed in **Figure 4C**, how the current was increased in correlation with a potential increase above 0.4 V. This result indicates that PPB biofilm used the electrode as an electron acceptor, probably for malic acid oxidation. This remarkably result indicates the use of PPB for anodic-based oxidations in MET applications. A less noticeable change in current in the negative potential region (between -0.2 and -0.8 V) is starting to develop after 48 h. In **Figure 4D** the negative currents in the potential region between -0.2 and -0.8 V results in a clear negative feature indicating processes related to the interaction of PPB with the graphite electrode as an electron donor. A detailed analysis of the cyclic voltammograms at 72 and 96 h suggests the presence of two processes responsible for the negative feature between -0.2 and -0.8 V. Two processes may be identified: (a) between -0.2 and -0.4 V, there was a steady increase in the negative current (in absolute value), and (b) around -0.6 V there is a steep change in the slope of the negative current indicating the occurrence of a second process. In our experiments, the electrode was polarized at -0.5 V, a potential able to explore the first reductive process from electroactive PPB. Finally, it is noteworthy to mention that after the depletion of malic acid in the medium at 170 h (**Figure 5A**) the magnitude of redox reactions was changed (**Figure 4F**), showing an electrochemical behavior similar to

this of Time 0 h, and suggesting low electroactivity of the PPB culture.

Effect of Carbon Source on Biological Hydrogen Production by PPB

The biological production of hydrogen by PPB was studied by testing different organic carbon sources, as malic acid, butyric acid and acetic acid, using Na-glutamate as optimum nitrogen source at a COD:N ratio of 100:15. Results indicated that the PPB culture was able to assimilate all the organic acids tested toward biomass growth as well as hydrogen production. Maximum PPB growth rate (7.56 mgVSS/Lh) was obtained when malic acid was used as compared to butyric (3.23 mgVSS/Lh) and acetic acid (3.96 mgVSS/Lh; **Table 2**). As a consequence of the higher C assimilation, the N assimilation into bacteria (as proteins) was also enhanced by using malic acid, giving a production of SCP of 874 mg/L as compared to 621 and 346 mg/L obtained with acetic and butyric acids, respectively. It was observed that the use of malic acid as carbon source achieved the highest hydrogen production (H_{2_max} , mLH₂/L) and the highest H₂ production rate (H_{2_rate} , mLH₂/Lh; **Table 2**). Malic acid has widely used as optimum carbon source for H₂ production, probably due to its capacity to directly enter the tricarboxylic acid cycle (Melnicki et al., 2008; Kim et al., 2012b; Assawamongkholisiri and Reungsang, 2015). Other evidences supporting malic acid as the optimum organic to conduct hydrogen production is shown in Supporting Information.

The experimental results obtained from the biological study (electrode-free) of hydrogen production indicated that the combination of malic acid and Na-glutamate was the optimum for maximizing the hydrogen production by PPB. The efficiency of H₂ production from the PPB mixed culture enriched in this

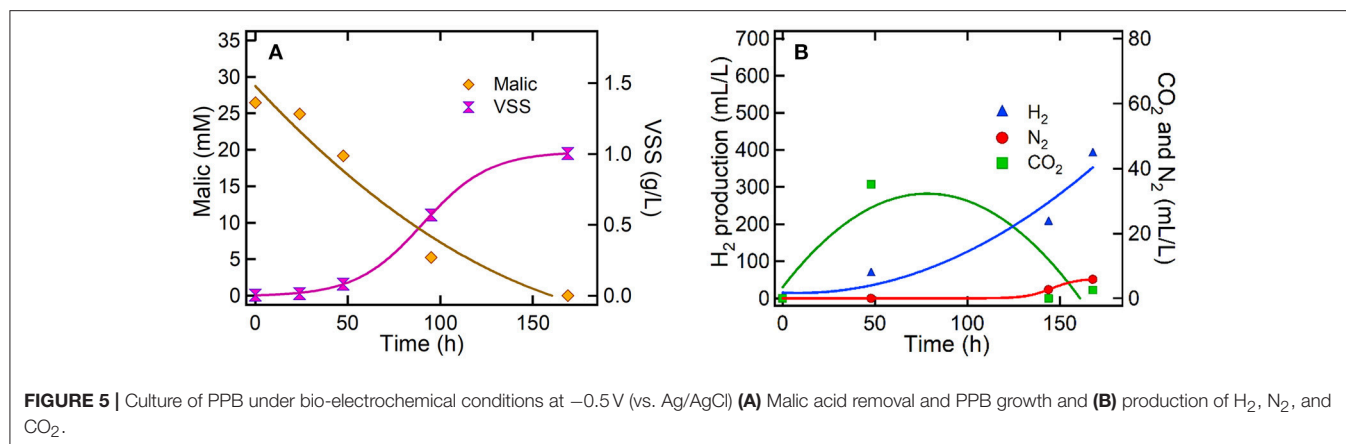


FIGURE 5 | Culture of PPB under bio-electrochemical conditions at -0.5 V (vs. Ag/AgCl) **(A)** Malic acid removal and PPB growth and **(B)** production of H₂, N₂, and CO₂.

study is comparable to those of previous studies where pure or mixed PPB cultures were used (Table 3). In conclusion, the high H₂ production rates achieved in this study showed that the PPB mixed culture could potentially be used for a feasible H₂ production application during wastewater treatment processes. This supports the use of malic acid and Na-glutamate as the C and N sources for the bio-electrochemical production of H₂.

Effect of Bio-Electrochemical Electron Donor on PPB for Producing Hydrogen and Fixing CO₂

Results suggested that bio-electrochemical process of PPB resulted to similar H₂ production rate and hydrogen yields (Table 2) compared to the PPB biological process under the same conditions. However, it was observed that after 1 week of bio-electrochemical reaction PPB fixed all the amount of CO₂ that was produced during the first 50 h (Figure 5B). This resulted to zero CO₂ emission as compare to the PPB biological process (see Supporting Information, Figure S2) that produced an average of 11.8 ± 16.0 mLCO₂/L after 1 week of biological process (Figure 3B). Subsequently, results suggested that CO₂ fixation was the main mechanism of PPB metabolism that was accepting electrons from the bio-cathode. This is in agreement with the negative current values observed during the bio-electrochemical process suggesting that there might be a consumption of electrons due to the PPB activity (Figure 6).

Figure 5 shows the malic acid assimilation, the PPB growth and the evolution of gas production during bio-electrochemical process. The bio-electrochemical setup revealed that PPB can effectively use the graphite-electrode as electron donor (Figures 4C-48 h, D-72 h, and E-96 h) and, subsequently, reduce the levels of CO₂. Carbon dioxide fixation is not detected in such a high extension when system was run in absence of electrode (see Supporting Information, Figure S2). Carbon dioxide fixation seems to occur at the origin of the first reductive process detected between -0.2 and -0.6 V. The extra electron source provided by the electrode promoted carbon dioxide fixation by PPB beyond the standard activity of this bacterial genus in absence of electrodes under limited electrons availability.

It is well-reported that graphite electrodes, and generally carbon electrodes, exhibit a high overpotential for hydrogen evolution and carbon dioxide reduction (Sullivan et al., 1993; DuBois, 2006; Yang et al., 2016) and therefore poor electrocatalytic properties. The standard electrode potential for carbon dioxide reduction to formic acid and oxalic acid are -0.199 and -0.590 V (Sullivan et al., 1993; Eggins et al., 1998; DuBois, 2006; Yang et al., 2016), respectively. These values are reported in the SHE scale, and taking into account that we are working in pH = 7 solutions and Ag/AgCl reference scale (ca. 0.2 V vs. SHE), the standard electrode potential has to be recalculated according to Nernst equation. Nernst equation gives an equilibrium potential of -0.716 V for formate and -0.790 V in the case of oxalate vs. Ag/AgCl. In any case, these electrode potentials are more negative than -0.5 V vs. Ag/AgCl, potential value used in this study. Furthermore, these are the thermodynamic potential values, carbon dioxide reduction has been reported on graphite electrodes taking place at potentials more negative than -0.9 V vs. Ag/AgCl (Eggins et al., 1998). Actually, this fact can be clearly observed in the electrochemical control voltammograms reported in Figure 4. The cyclic voltammogram corresponding to the bare graphite electrode in the same solution but in absence of PPB, displays the classical voltammogram of an ideally polarized electrode, where there are no significant faradaic currents in the whole potential range explored (0.8 to -0.8 V), as it would be expected. Only pseudocapacitive electrochemical behavior is detected in the electrode interface in absence of PPB. Regarding the previous arguments, electrochemical carbon dioxide reduction on bare graphite electrode can be discarded, and the only contribution of PPB metabolism can explain the consumption of carbon dioxide.

In contrast, at -0.5 V, the capability of hydrogen production of the bio-electrochemical system was comparable to the exhibited by PPB in absence of electrode, and no electrode potential was observed in this process. The origin of the second bio-electrochemical process developed below -0.6 V, that can be tentatively assigned to hydrogen production, will require further investigation beyond the scope of this work. The capability of PPB for using graphite-electrode as electron donor was demonstrated. The extra electron donor source can be used in

TABLE 3 | Comparison of hydrogen production rates by different cultures and systems with the one studied in the present work.

PPB culture	IR	Process mode	Carbon/Nitrogen sources	Y_{H_2} (LH ₂ /gacid)	H ₂ rate (mLH ₂ /Lh)	Y_{molH_2} (molH ₂ /mol acid)	References
<i>Rhodospirillum rubrum</i>	60 W/m ²	Batch	Succinate/glutamate	–	21	–	Melnicki et al., 2008
<i>Rhodopseudomonas palustris</i>					4.3		
<i>Rhodobacter sphaeroides</i>	100 W	Batch	Malate/glutamate	0.541	5.1	–	Akkose et al., 2009
			Malate/NH ₄ Cl	0.224–0	4.6–0		
			Acetate/NH ₄ Cl	0.467–0.135	5.8–3.3		
<i>Rhodobacter sphaeroides</i>	5 Klux*	Continuous	Mixture of VFAs**/(NH ₄) ₂ SO ₄	0.185	1.125	–	Ozmihci and Kargi, 2010
<i>Rhodopseudomonas acidophila</i>	2,400 lux*	Batch	Acetate/nitrate	–	2.7	–	Merugu et al., 2012
			Malate/nitrate		2.5		
			Succinate/nitrate		3.3		
			Succinate/N ₂		0.5		
			Succinate/NH ₄ C		1.25		
Mixed culture–dominant <i>Rhodopseudomonas palustris</i>	190 W/m ²	Continuous	Mixture** of acetate, lactate, butyrate, propionate/NH ₄ -N	0.97	121	–	Tawfik et al., 2014
<i>Rhodobacter sphaeroides</i>	10 W/m ²	Batch	Succinate/(NH ₄) ₂ SO ₄	–	31	–	Ryu et al., 2014
<i>Rhodobacter sp. KKU-PS1</i>	2,500 lux*	Batch	Malate/glutamate	–	6.8	–	Assawamongkholisiri and Reungsang, 2015
<i>Rhodopseudomonas palustris</i>	2,000 lux*	Batch	Lactate/glutamate	–	8.4	2.57	Hu et al., 2017
			Butyrate/glutamate		19.9	4.92	
Mixed culture	20 W/m ²	Batch	Malic acid/glutamate	0.12	2.71	0.67	This study

*Illumination intensity was calculated (1 lx = 0.0161028 W/m²), **Dark fermentation effluent.

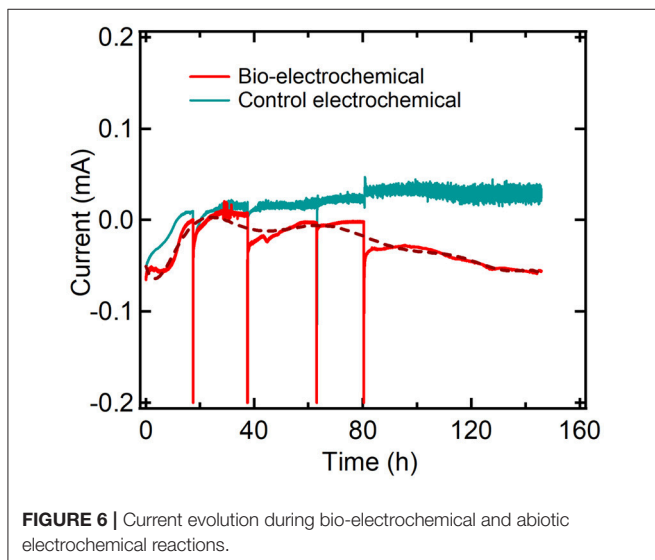


FIGURE 6 | Current evolution during bio-electrochemical and abiotic electrochemical reactions.

more than one metabolic pathway. Polarizing the electrode at –0.5 V, allows PPB to use electrode for carbon fixation reaching almost no carbon dioxide accumulation in contrast to the electrode-free biological system. This is the first study indicating that electroactive capture of CO₂ by PPB is feasible.

Finally, the SCP production achieved by PPB during the bio-electrochemical process (81% mgSCP/mgVSS) was similar

to this observed by PPB growing in absence of electrodes. Therefore, the bio-electrochemical process did not seem to affect proteins yields under the experimental conditions tested.

CONCLUSIONS

This work analyzed the optimum culturing conditions for maximizing the hydrogen production by a mixed culture of purple phototrophic bacteria. In addition, the effect of a negatively polarized bio-electrochemical device on the modification of the behavior of the culture in terms of metabolic shifts and current consumption was explored. The main conclusions extracted from this work are shown below:

- Among all the conditions tested in absence of electrodes, best results on the hydrogen production have been achieved by using malic acid as a carbon source (instead of acetic and butyric) and Na-glutamate as a N source (instead of ammonium and dinitrogen gas), in a COD/N relationship of 100/15. Under these conditions, the production of CO₂ was also minimized.
- Cyclic voltammograms of the bio-electrochemical system shown the appearing of at least three potentials (two negative and one positive) with clear interaction between the PPB culture and the electrode. This makes evident the high electroactivity of PPB cultures and their potential as a MET microbial candidate.

- Negative polarization of the electrode at -0.5 V caused a detectable consumption of electrons associated with a depletion of the produced carbon dioxide, which indicates that the PPB culture was capable of using electrons from the cathode to capture the excess of C released as CO_2 during the CBB cycle. This behavior was not observed before in an indigenous (non-genetically-modified) PPB culture.
- Results presented herein have shown that further in-depth research using different conditions (other polarization of the cathode) will be of extreme benefit and may enhance the H_2 production rate.

AUTHOR CONTRIBUTIONS

IV designed and performed the experiments and wrote the manuscript, AB critically reviewed the manuscript, CM helped in the experimental stage, JM critically reviewed the manuscript, FM critically reviewed the manuscript

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and supervised the work, AE-N and DP designed the experiments, supervised the work, and corrected the manuscript. Both DP and AE-N are corresponding authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenrg.2018.00107/full#supplementary-material>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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