Hydrogen production in single chamber microbial electrolysis cells with different complex substrates

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The use of synthetic wastewater containing carbon sources of different complexity (glycerol, milk and starch) was evaluated in single chamber microbial electrolysis cell (MEC) for hydrogen production. The growth of an anodic syntrophic consortium between fermentative and anode respiring bacteria was operationally enhanced and increased the opportunities of these complex substrates to be treated with this technology. During inoculation, current intensities achieved in single chamber microbial fuel cells were 50, 62.5, and 9 A m⁻³ for glycerol, milk and starch respectively. Both current intensities and coulombic efficiencies were higher than other values reported in previous works. The simultaneous degradation of the three complex substrates favored power production and COD removal. After three months in MEC operation, hydrogen production was only sustained with milk as a single substrate and with the simultaneous degradation of the three substrates. The later had the best results in terms of current intensity (150 A m⁻³), hydrogen production (0.94 m³ m⁻³ d⁻¹) and cathodic gas recovery (91%) at an applied voltage of 0.8 V. Glycerol and starch as substrates in MEC could not avoid the complete proliferation of hydrogen scavengers, even under low hydrogen retention time conditions induced by continuous nitrogen sparging.

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1. Introduction

Bioelectrochemical systems (BES) are a recently developed technology that allows current generation or production of value added compounds, such as hydrogen, from wastewater (Liu et al., 2005). These systems are known as microbial fuel cells (MFC) and microbial electrolysis cells (MEC), respectively. MEC are interesting in view of hydrogen production when compared to alternative technologies such as dark fermentation and photosynthesis because i) they require much lower energy input when compared to hydrogen obtainment from water electrolysis and ii) they have higher hydrogen yield (Lee and Rittmann, 2010).

BES operation relies on the presence of a group of microorganisms that have the ability to use an external insoluble electrode as electron acceptor and are therefore known as exoelectrogens or anode respiring bacteria (ARB). ARB consume organic matter anaerobically, donating the last electron involved in their metabolic pathway to the electrode. The flow of electrons generated because of the organic matter consumption can be used as electricity or to drive specific reduction reactions, such as the reduction of protons to hydrogen, on the cathode. Whereas electricity production in
MFC is a thermodynamically favored process, hydrogen production in MEC requires some energy input. As a consequence, to consider the process economically interesting (i) the energy recovered as hydrogen gas should be higher than the energy input or (ii) the energy input should be lower than energy requirements for treating wastewater in conventional wastewater treatment systems.

Lab scale studies have broadly investigated BES fed with synthetic wastewater containing easily biodegradable substrates, mainly acetic acid. However, real wastewaters contain a wide range of substrates with different biodegradability. In order to reach a real application of this technology, it is fundamental to study the potential of current intensity and hydrogen production simultaneously to the degradation of the organic matter present in real wastewater.

In systems where a complex substrate is used, an initial hydrolysis and fermentation step is necessary to break macromolecules to simpler ones and to convert them to acetate and other readily biodegradable substrates, which will be further degraded by ARB. Hence, a syntrophic consortium between fermentative bacteria and ARB needs to be developed. Syntrophic interactions between fermenters and ARB have been reported to allow the utilization of complex organic matter in BES, where different substrates entail the development of different microbial communities (Kiely et al., 2011; Lu et al., 2012).

BES studies fed with complex substrates as sole carbon source show the necessity to develop such syntrophy in the system (Cheng et al., 2011; Gómez et al., 2011; Lalaurette et al., 2009; Sun et al., 2012; Velasquez-Orta et al., 2011) and present the hydrolysis and fermentation step as the limiting one (Velasquez-Orta et al., 2011). They also show that a pre-acclimation to single products improves later degradation in a complex mixture and increases hydrogen yield (Lalaurette et al., 2009). In general, the substrates that have been tested so far in BES include synthetic wastewater containing starch (Chosh et al., 2012; Herrero-Hernandez et al., 2013; Lu et al., 2009; Velasquez-Orta et al., 2011), cellulose (Cheng et al., 2011; Lalaurette et al., 2009), glycerol (Chignell and Liu, 2011; Escapa et al., 2009; Nimje et al., 2011; Reiche and Kirkwood, 2012), methanol (Montpart et al., 2014), phenol (Song et al., 2014), landfill leachates (Mahmoud et al., 2014), municipal wastewater (Escapa et al., 2012; Heidrich et al., 2013) and industrial wastewater like dairy (Elakkiya and Matheswaran, 2013; Mardanpour et al., 2012), brewery (Cusick et al., 2011) and biodiesel wastewater (Feng et al., 2011).

Practical implementation of MEC will mainly require operation at low cost. From all the various configurations that have been discussed in the literature, a single chamber membrane-less MEC would offer the lowest installation and operation costs because of being a single unit without membrane. In addition, avoiding the use of an ionic membrane decreases the internal resistance of the system, which represents one of the voltage losses appearing in MECs. In addition, the possibility to immobilize the syntrophic consortia in the anode introduces an improvement in the system, since a pre-treatment tank would not be required. Nevertheless, MEC operation in membrane-less single chamber is not straightforward, since it faces some bottlenecks.

One of the major problems that single chamber MEC still deals with is the growth of methanogens (Zhang and Angelidaki, 2014), as the anaerobic environment of a MEC favors their growth. Methanogens compete with exoelectrogens for both substrate and product. Acetoclastic methanogens convert acetate to methane and hydrogenotrophic methanogens consume hydrogen to produce methane. Because of their growth and activity, hydrogen production decreases and the gas obtained is less rich in hydrogen. This represents a loss in terms of energy obtained from MEC, since hydrogen possesses higher combustion energy than methane. It also introduces extra costs when the goal is using hydrogen as a feedstock, since a previous separation process would be necessary. Some long-term and pilot scale studies have shown that once methanogenic archaea takes over it is very complicated to get rid of them (Cusick et al., 2011; Rader and Logan, 2010).

Research on how to limit methanogenic growth and activity includes operational strategies such as temperature, pH, oxygen exposure and periodic aeration (Ajayi et al., 2010; Chae et al., 2010; Wang et al., 2009), loading rate (Lalaurette et al., 2009), applied voltage (Hu et al., 2008; Torres et al., 2009) and dosage of chemical inhibitors like 2-bromoethanesulphonate (Chae et al., 2010; Wang et al., 2009; Zhuang et al., 2010). The latter is an efficient chemical inhibitor of methanogenesis, but its cost limits its usage in MEC. Other strategies like working at low retention time to reduce the interval that hydrogen is available in the system have been suggested (Lalaurette et al., 2009).

The aim of this work was studying the long-term opportunities of various complex substrates for net hydrogen production in single chamber MEC without addition of chemical inhibitors of methanogenesis. Milk, starch and glycerol were chosen as carbon sources in view of their differences in composition and therefore biodegradability. In this sense, glycerol was chosen for being a short chain fermentable substrate, starch a large polysaccharide and milk a mixture of sugars, fats and proteins. These substrates were representative of different industrial wastewater (biomass industry wastewater, potato industry and dairy industry), with wastewater treatment systems that could be upgraded by producing hydrogen with MEC. In addition, the advantages of codigesting the three substrates at the same time were explored, a situation that could be extrapolated to an urban wastewater, where a variety of compounds is typically available.

2. Materials and methods

2.1. Experimental setup

A consortium able to degrade a specific complex substrate was obtained by separately growing fermentative and ARB microbial communities in culture flasks and in MFC respectively. Next, both communities were joined in MFC. Once it was ensured that a syntrophic consortium had developed in MFC, the biologically enriched anodes were moved to MEC.
2.2. Culture flasks

Culture flasks were 100 mL glass bottles tightly capped with PTFE rubber septa and an aluminum crimp top. Bottles were filled up to 70 mL, they were magnetically stirred and kept in a 37 °C room. 45 mL of anaerobic digester sludge (Municipal wastewater treatment plant of Manresa, Catalonia) was used as inoculum in each culture flask. The culture flasks were operated under fed-batch mode with cycles of five days duration. Every time the system was fed, the mixed liquor was centrifuged (4 min at 5000 rpm) to enhance biomass retention, the supernatant medium was discarded, and the sludge was then resuspended in fresh medium. Before closing the bottles, nitrogen was sparged to ensure anaerobic conditions.

Each culture flask treated a different substrate independently. 2-bromoethanesulfonate (50 mM) was used to inhibit the methanogenic activity according to the work of Parameswaran et al. (2011), where it was stated that such concentration would selectively inhibit methanogenic archaea. 2-bromoethanesulfonate had been previously stated to inhibit methanogenic activity (Nollet et al., 1997; Sparling et al., 1997) and to be more effective than other chemical inhibitors or changes in system conditions such as pH and temperature (Chae et al., 2010). Volatile fatty acids (VFAs) and chemical oxygen demand (COD) were measured to assess the development of the fermenting community and gas analyses from the headspace allowed to ensure that no methane was being produced. pH was also measured. Additionally also lactate and glucose were measured for those systems working with starch or milk.

2.3. Microbial fuel cells

MFC were 28 mL methacrylate cylindrical vessels provided with a lateral aperture (3.8 cm diameter), where a PTFE diffusion layer stuck to the cathode permitted oxygen diffusion into the cell while preventing water leakage (Cheng et al., 2006). The cathode consisted of graphite fiber cloth (3.8 cm diameter, 7 cm² total exposed area) coated with platinum (5 mg Pt/cm², ElectroChem Inc.). The anode was a graphite fiber brush (20 mm diameter × 30 mm length; 0.21 m²) made with fibers of diameter 7.2 μm (type PANEX33 160K, ZOLTEK) connected with a titanium wire core. The brush was thermally treated at 450 °C for 30 min to enhance biomass adhesion (Wang et al., 2009). The two electrodes (spaced 2.5 cm apart) were initially connected through a 1000 Ω external resistance. The MFC was inoculated with ARB using the effluent from an already working MFC, accounting for 25% of the total reactor volume. Acetate and propionate were used as carbon source during this ARB inoculation period. Only 50% of the media was replaced at the end of each batch cycle until a steady response in current generation was achieved.

After the ARB inoculation period, sludge from each culture flask was used to inoculate fermentative bacteria in each MFC (25% of the total MFC volume). The development on the anode of the syntrophic consortium was enhanced by a step-wise replacement of the media, beginning with the replacement of 50% of the reactor volume, then 75% and finally replacing the totality of its content. The carbon source used during this period was the complex substrate, without adding any acetate or propionate.

2.4. Microbial electrolysis cells

MEC were analogous to MFC, but the cathode was not exposed to air and the cell had a glass cylinder at the top (16 mL), tightly sealed with a PTFE rubber cap and an aluminum crimp, which enabled gas collection. The system was filled up to 40 mL to guarantee water sealing on joints and to avoid gas leakage. The gas produced was further collected in a gas-tight bag (Ritter, Cali-5-bond) connected to the glass cylinder by means of a PVC hosepipe. Both electrodes were connected to a power source (HQ Power, PS-23023) applying a potential of 0.8 V. Current production was measured quantifying the voltage drop across a 12 Ω external resistance serially connected to the circuit.

2.5. Experimental conditions

Only during MFC inoculation by ARB both acetate and propionate were used as carbon sources (0.5 g L⁻¹ initial concentration of each). After the fermentative bacteria inoculation, the MFC were fed independently with each complex substrate (glycerol, milk and starch) in fed-batch mode, with an initial concentration per compound of 1 g L⁻¹. Glycerol and starch were analytical grade reactants (1.216 g COD g glycerol⁻¹ and 1.185 g COD g starch⁻¹ respectively). Milk was commercial powder milk (1 g COD g milk⁻¹). The codegradation of the three substrates was also explored, having as initial concentration 1 g L⁻¹ of each compound.

The medium used contained per liter: 0.2 g NH₄Cl, 4 mg FeCl₃, 6 mg Na₂S, 5 mL of mineral media solution and 172 mL phosphate buffer solution (PBS). Mineral media solution contained (g L⁻¹): 1 EDTA, 0.164 CoCl₂·6H₂O, 0.228 CaCl₂·2H₂O, 0.02H₂BO₃, 0.04 Na₂MoO₄·2H₂O, 0.002 Na₂SeO₃, 0.02 Na₂WO₄·2H₂O, 0.04 NiCl₂·6H₂O, 2.32 MgCl₂, 1.18 MnCl₂·4H₂O, 0.1 ZnCl₂, 0.02 CuSO₄·5H₂O and 0.02 AlK(SO₄)₂·12H₂O. The PBS stock solution contained per liter 70 g Na₂HPO₄ and 12 g KH₂PO₄. During the first four weeks of the work, in MFC operation mode, 2-bromoethanesulfonate was used as methanogenic activity inhibitor at a concentration of 50 mM. Under regular operation, the cell content was completely replaced with fresh medium when voltage response decreased below half the maximum signal (both in MFC and MEC). MEC were sparged with nitrogen for 10 min (50 mL N min⁻¹) after feeding to guarantee anaerobic conditions. They were kept at room temperature (around 23 °C) during all the operational period. Throughout the document, the reactors are identified as MXC-glycerol, MXC-milk, MXC-starch and MXC-mixed, where MXC stands for either MFC or MEC accordingly.

Voltage evolution was monitored by means of a 16-bit data acquisition card (Advantech PCI-1716) connected to a personal computer with a software developed in LabWindows CVI 2014 for data acquisition.

Samples for chemical analyses were taken at the beginning, at the middle and at the end of each cycle, accounting for a maximum of 10% of the total reactor volume.
2.6. Analytical methods

VFAs concentration (acetate, propionate, butyrate and valerate) was analyzed from 0.22 μm filtered samples with gas chromatography (Agilent Technologies, 7820-A) using a flame ionization detector and helium as carrier gas (oven temperature 85–130 °C, ramp of 3 °C min⁻¹, 130–220 °C, ramp of 35 °C min⁻¹, nitrotetraphthalic acid modified FEG capillary column, detector temperature 275 °C). Hydrogen and methane were also assessed with gas chromatography using a thermal conductivity detector and argon as carrier gas (oven temperature 40 °C, HP-mole sieve column, detector temperature 220 °C). Gas production was evaluated as in Ambler and Logan (Ambler and Logan, 2010).

Glycerol concentration was quantified in 0.22 μm filtered samples with high performance liquid chromatography (HPLC, Dionex Ultimate 3000) provided with a refractive index detector. COD was measured by a colorimetric method after oxidation of the sample with potassium dichromate, which was unfiltered for the systems fed with milk and starch. Commercial test tubes for COD analysis were used (LCK 714 100–600 mg O₂ L⁻¹, Hach Lange). Glucose and lactate were measured with YSI biochemistry analyzer (2700 SELECT Biochemistry Analyzer).

MFC internal resistance (Rint) and maximum power output (Pmax) were assessed from polarization curves. The polarization curve was performed allowing the cell to reach the open circuit voltage for a period of one hour and then progressively changing the external resistance (from high to low resistance) and measuring the cell voltage after 10 min. The set of external resistances used for the polarization curves were 470 kΩ, 218 kΩ, 44.2 kΩ, 24.1 kΩ, 12.1 kΩ, 6.6 kΩ, 3.3 kΩ, 2.0 kΩ, 1.65 kΩ, 1.0 kΩ, 825 Ω, 470 Ω, 250 Ω, 218 Ω, 100 Ω, 50 Ω and 25 Ω. Rext was calculated as the slope in the section where polarization curves follow a linear trend (voltage limitations by ohmic losses).

2.7. DNA extraction

Samples from the anodes were taken after 50 days of operation in MFC mode fed with complex substrates (MFC-glycerol, MFC-milk and MFC-starch). Graphite fibers from the anode were cut and combined for DNA extraction. Previously, the fibers were rinsed with 1 mL of sterile MilliQ water to remove residues from the growth medium or residues from biofilm. Total DNA was extracted from approximately 0.2 g of samples using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instructions. Quality and quantity of the DNA was measured using a NanoDrop® spectrophotometer (ThermoScientific). DNA was visualized under UV in a 0.7% gel electrophoresis with TBE (Tris-Borate 50 mM; EDTA 0.1 mM; pH 7.5–8). No samples from the anodes during MEC operation were taken for microbial community analyses.

2.8. High-throughput 16S rRNA gene pyrosequencing

High-throughput 16S rRNA gene pyrosequencing was performed in a 454 Titanium FLX system by the Research and Testing Laboratory (Lubbock, TX) based upon RTL protocols from anode DNA samples (17–26 ng/μL, quality ratio of 1.6–1.8). DNA samples were analyzed with an average of 3000 reads/assay with the primers couples 357F/926R, comprising the V3–V5 regions of the bacterial 16S rRNA gene (Segata et al., 2011).

Sequences were checked using Uchime (Edgar et al., 2011), sorting and trimming them by using the Pipeline Initial Process at the Ribosomal Database Project (RDP) Pyrosequencing Pipeline (http://rdp.cme.msu.edu/index.jsp; Cole et al., 2009) with the default settings. The RDP Classifier was used to assign 16S rRNA gene sequences to a taxonomical hierarchy with a confidence threshold of 95%, since the DNA sequences were <250 bp (Claesson et al., 2009). The relative abundance of a given phylogenetic group was calculated as the number of sequences associated with that group divided by the total number of sequences per sample.

2.9. Calculations

Cell current intensity and power were calculated according to Ohm’s law (Equations (1) and (2)):

\[
I = \frac{V}{R_{ext}} \\
(1)
\]

\[
P = V \cdot I \\
(2)
\]

where \(V\) is the voltage drop in the resistance (\(V\)), \(R_{ext}\) is the external resistance (\(Ω\)), \(I\) is the current intensity (\(A\)) and \(P\) is the power output (\(W\)). Current intensity was normalized by reactor hold up volume.

Coulombic efficiency (CE) was calculated as stated in Equation (3):

\[
CE = \int_{t_0}^{t} \frac{I(t) \, dt}{\Delta S \cdot b \cdot F \cdot V_L} \\
(3)
\]

where \(t\) is time (\(s\)), \(\Delta S\) is the substrate consumption in terms of COD (mol O₂/L), \(b\) is the stoichiometric number of electrons produced per mol of oxygen (4 mol-e⁻), \(F\) is Faraday’s constant (96,485C/mol-e⁻) and \(V_L\) the liquid volume (L).

Cathodic gas recovery \(r_{CAT}\) was calculated as the ratio of moles of hydrogen measured and moles of hydrogen theoretically produced based on current intensity measured, as presented in Equation (4):

\[
r_{CAT} = \frac{n_{H_2}}{\int_{t_0}^{t} \frac{I(t) \, dt}{2F}} \\
(4)
\]

where \(n_{H_2}\) is the number of moles of hydrogen measured, calculated according to the ideal gases law knowing the hydrogen volume measured and 2 is the number of moles of electrons per mole of hydrogen.

Hydrogen relative composition was calculated as the ratio of hydrogen to the total amount of hydrogen and methane and was calculated as presented in Equation (5):

\[
\text{Relative composition}_{H_2} = \frac{v_{H_2}}{v_{H_2} + v_{CH_4}} \\
(5)
\]

where \(v_{H_2}\) and \(v_{CH_4}\) represent the volume fraction for H₂ and CH₄ respectively.
Carbon dioxide was not included in the relative hydrogen composition calculation because analyses of the headspace showed a rather negligible amount in the gas.

For the culture flasks, the $j$ metabolite concentration was calculated in COD units relative to the initial COD, obtaining in total the percentage of complex substrate remaining, as presented in Equations (6) and (7).

\[
\text{Normalized metabolite concentration} \left( t \right) = \frac{\text{COD}_{t:j}}{\text{COD}_{t:0}} \times 100 \quad (6)
\]

\[
\text{Complex substrate remaining} \left( t \right) = \left( 1 - \sum_{j=1}^{n} \frac{\text{COD}_{t:j}}{\text{COD}_{t:0}} \right) \times 100 \quad (7)
\]

3. Results and discussion

3.1. Development of syntrophic consortia on the anodic biofilm

Obtaining a syntrophic consortium that would allow exoelectrogenesis from complex substrates was a key step in this work. With this aim, both communities were initially grown separately. The growth of a fermenting population able to degrade each complex substrate was assessed by periodically monitoring the metabolites concentration in the culture flasks. Fig. 1 presents the metabolites profile in a conventional batch cycle for each substrate studied. A progressive accumulation of VFAs was observed, especially acetic acid and propionic acid. Lactate, glucose, valeric acid and butyric acid were present in low concentrations (lower than 15 mg L$^{-1}$ for all the period) and hence, were plotted together for glycerol and starch culture flasks. On the contrary, milk-culture flask showed an increasing profile for almost all the intermediates measured.

The glycerol-culture flask presented the lowest fermentation ability, reaching a maximum of 15% degradation of the initial COD, whereas milk and starch-culture flasks could degrade 60% and 70% respectively. pH did not experience a decrease of more than 0.2 units regardless of the VFAs accumulation and no methane was detected.

In parallel, the enrichment of the anodic biofilm on ARB was assessed by monitoring the current intensity generation in MFC (Figure S1). Propionic and acetic acids were used as carbon source in view of a future syntrophy with a fermenting population, which would degrade the more complex substrates to VFAs. The analyses performed at the end of each batch cycle showed that both acetate and propionate were being consumed in the system. Maximum current intensity achieved increased with time until a steady state was reached. At this point it was considered that the MFC were ready to be inoculated with the fermenting community and to be fed with the complex substrate as a sole carbon source.

Each MFC was inoculated with sludge from the corresponding culture flask, accounting for the 25% of the total reactor MFC volume. To inoculate MFC-mixed with a fermenting community, sludge from the three culture flasks was used also accounting in total for a 25% of the reactor volume. From this moment on, the systems were fed exclusively with glycerol, milk or starch as sole carbon source and, in the case of MFC-mixed, the reactor was fed with the mixture of the three complex substrates. Only from day 0 to day 30 of operation in MFC, 2-bromoethanesulfonate was added to inhibit methanogenesis.

Fig. 2 presents the current intensity response and the organic loading rate for the three MFC operating with different single substrate and MFC-mixed since the inoculation. The fermenting community was initially in suspension in the MFCs, which would not be interesting in view of a fed batch or continuous operation, since fermenting microorganisms could be washed out from the system. The growth of fermenting bacteria on the anode surface would be an ideal situation in view of the syntrophy required with ARB. A step-wise replacement of the media was designed to enhance the
A high external resistance of 1000 Ω was only used during the inoculation period to ensure working at a low anode potential, which a priori could enhance the selection of biomass able to grow with a low energy gain (Aelterman et al., 2008; Logan et al., 2006; Schröder, 2007). The external resistances were decreased from 1000 Ω to 100 Ω on day 40 (Fig. 2) to enhance biomass electroactivity, resulting in a significant increase in current intensity for MFC-glycerol, MFC-milk and MFC-mixed.

Table 1 shows the CE for each system evaluated before and after the change in external resistance. Initially, CE values lower than 50% indicated that less than a half of the substrate available was being recovered as current intensity and therefore consumed by ARB. Changing over to a lower external resistance generally produced an increase in CE. Only MFC-starch was negatively affected by the change.

Polarization and power curves were performed before and after the change in external resistance (on days 33 and 49 respectively, see Fig. 2). They did not only aim at estimating the internal resistance of the system but also identifying the limiting step in the power production from a complex substrate. Then, the curves were firstly obtained for the three MFC running with the actual complex substrate and they were repeated for acetate as sole carbon source. Fig. 3 shows the results obtained on day 33 (1000 Ω). Power production was limited by the fermenting community only for MFC-starch, obtaining up to 1.3 mA when acetate was fed and barely reaching 0.4 mA when starch was fed. On the contrary, glycerol and milk-fed MFC did not show a significant difference neither in terms of maximum power output nor in current intensity achieved when they were operating with the complex substrate or acetate. In their case, power production would be limited by ARB community.

Similar power curve profiles (with acetate and with complex substrate) were obtained for the operation with 100 Ω on day 49 (data not shown). According to $R_{int}$ which should coincide with the external resistance to maximize power output, the change from 1000 Ω to 100 Ω was adequate for MFC-glycerol and MFC-milk, although it was not positive for MFC-starch probably because of the fermentative activity limitation. Data related to the comparison among these power curves is further detailed in the supporting information (Table S1).

Samples from the anodes in MFCs treating a single substrate were taken for pyrosequencing on day 50. Fig. 4 presents the results obtained, revealing the coexistence of fermentative and exoelectrogenic bacteria in each of the anodes. The presence of Geobacter sp was common in all the MFCs (15–20% of the total). The growth of Geobacter sp was expected, since acetate was used as carbon source to grow the exoelectrogenic biofilm on the anode (Yates et al., 2012).

<table>
<thead>
<tr>
<th>Table 1 – Average coulombic efficiency for MFC.</th>
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<td>Substrate</td>
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<td>Glycerol</td>
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<td>Milk</td>
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<td>Starch</td>
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<td>Mixed</td>
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Fig. 2 – Current intensity (solid line) and organic loading rate (diamonds) for MFC fed with different complex substrates. Two periods with 1000 Ω and 100 Ω external resistance were evaluated.
Unclassified Bacteria accounted for 10–13% of the total reads. An important range of unclassified genus was obtained for all samples (up to 67% in MFC-starch), indicating the diversity of microorganisms participating in the degradation of each complex substrate.

In MFC-glycerol, a large percentage of Actinomycyes sp was detected (29%), whose growth is favored in presence of glucose as carbon source (El-Nakeeb and Lechevalier, 1963; Vandzuurová et al., 2013). Proteiniphilum and Dethiosulfatibacter sp were detected in much minor proportion (1 and 2% respectively), attributing their presence to the fermentation of organic acids to acetic acid and propionic acid. Pyrosequencing also revealed a wide microbial community development in this MFC, as 53% of the total accounted for unclassified species with an unidentified but probably important role in the cell performance. Among the unclassified species, microorganisms of the phylum Bacteroidetes were found (13%). Their presence is frequent in natural environments such as soils, oceans and fresh water and has also been reported in anaerobic digestion sludge (Zhao et al., 2014), which explains their presence in this system, since anaerobic digestion sludge was used as initial inoculum to grow each fermentative community.

In MFC-milk, a large contribution of Actinomycyes sp was again present in the anode (13%), which are usually responsible of metabolizing lactose to lactic acid (Gillespie and Hawkey, 2006). Lactose is also consumed by Lactococcus sp, with a presence in the sample of 3% of the total. The differences in abundance between both lactic acid bacteria were attributed to the origin of the initial inoculum (anaerobic digestion sludge) used to grow the fermentative community able to degrade milk. In fact, Actinomycyes sp are commonly found in soil and water environments, and therefore their presence in the anaerobic digestion sludge is also favored unlike Lactococcus sp. Petrimonas sp were detected (7%), being responsible of the fermentation of sugars and organic acids to hydrogen and acetate. Other fermentative bacteria were detected in the anode (Proteiniphilum sp, 2% and Dysgonomonas sp 1%), producing volatile fatty acids. Unclassified Bacteroidetes accounted in this sample for 29% of the total. Besides their presence in the original inoculum, microorganisms belonging to this phylum are known to be well degraders of high molecular weight organic matter like proteins and carbohydrates, which explains the higher content in this sample when compared to the sample from MFC-glycerol.

In MFC-starch, besides from Geobacter sp, only Dysgonomonas sp and Proteiniphilum sp were clearly identified in the sample (11% and 7% respectively), as they were able to ferment glucose contained in starch to fatty acids. The presence of Proteiniphilum sp was also reported from anaerobic digestion sludge samples (Zhao et al., 2014). Similarly to the sample from MFC-milk, an important percentage of unclassified Bacteroidetes was detected (28% of the total), participating in the polysaccharide degradation.

Table 2 compares the common performance MFC parameters obtained in this work with similar works from the literature. A direct comparison can be stated with single chamber systems inoculated with a source already containing the specific substrate, e.g. industrial wastewater, since they will most probably already contain fermentative population able to degrade it. Nevertheless, other cases have also been included to have a broader view. The results obtained in terms of current intensity generation per reactor volume and CE were better than those reported in other studies. Thus, developing a syntrophic consortium in the anode was advantageous in such systems. Indeed comparable results were obtained when the system was inoculated with the very same wastewater (Heilmann and Logan, 2006), or with activated sludge treating the same kind of substrate (Mardanpour et al., 2012), as the inoculum was probably already containing the proper fermentative population. In the case of MFC-starch, results were one order of magnitude lower, but they were also comparable with those found by Lu et al. (2009) in a system fed with starch wastewater and inoculated with wastewater from a starch processing plant.

Concerning substrates complexity, it was observed that starch was the most difficult substrate to degrade and to effectively be converted to electricity, which was probably related to its low solubility, introducing an accessibility...
limitation to the substrate, and to the necessity to hydrolyze such a big macromolecule. This implied an important role of fermentative populations, as determined by the pyrosequencing results. In contrast, milk seemed to allow the highest power production regardless of the variety of its content of sugars, fats and proteins. Regarding glycerol, results were comparable to other works, where conditions were stricter and even crude glycerol (biodiesel waste) was used as carbon source (Feng et al., 2011).

In terms of COD removal, milk was the substrate with the lowest efficiency. Nevertheless, when all the substrates were fed together in MFC-mixed, the organic matter was almost totally degraded. In fact, in MFC-mixed, not only COD removal was better than for MFC with single substrates, but also current intensity was boosted.

The evaluation of the performance in each MFC was important in view of the further operation of the inoculated anodes in MEC for hydrogen production, being able to discern whether hydrogen-producing opportunities were limited by the nature of the complex substrate treated or by the metabolism of hydrogen scavengers.

3.2. Complex substrates in MEC

After assessing the development of syntrophic consortia on the anodic biofilm of each system, the anodes were transferred to MEC, which operated for a period of over three months. For MEC-glycerol, MEC-milk, MEC-starch and MEC-mixed, the performance obtained for the four systems in terms of current intensity, CE and relative hydrogen composition is presented in Fig. 5. Relative gas composition was measured at the end of each batch cycle.

Current intensity in MEC increased with respect to MFC operation, indicating higher exoelectrogenic activity. The highest current intensity in MEC fed with a single complex substrate was observed for MEC-glycerol, reaching about 4 mA. 2.6 mA were measured on average for MEC-milk. The lowest current intensity was observed for MEC-starch, barely reaching a maximum of 2 mA. Regarding MEC-mixed, current intensity reached 5 mA in the first two weeks of operation and rose up to 6 mA at the end of the operating period. The trend observed in terms of current intensity for the four systems correlated well with the behavior observed in MFC operation,
i.e. higher exoelectrogenic activity was measured for MEC-glycerol and when the three substrates were being fed simultaneously in MEC-mixed some sort of advantage in the codigestion of the three substrates was experienced.

Although current intensities in MEC are correlated to exoelectrogenic activity, they are not a direct measurement of the hydrogen production rate, as phenomena as hydrogen recycling can increase current intensity without net hydrogen production. Regarding the measured gas production, it was quickly observed for the three complex substrates, with a hydrogen relative composition of 100% even though 2-bromoethanesulfonate was not used. Nevertheless, the long-term stability of hydrogen production was only demonstrated for MEC-milk and MEC-mixed, producing a gas with about 80% of hydrogen volumetric content.

CE followed a similar trend in all the systems: it was initially much higher than 100%, indicating electron recycling, and it decreased up to values ranging from 50 to 70%. Such a decrease in CE coincided with the loss of biogas purity, which, initially much higher than 100%, indicating electron recycling, would not be favored (Montpart et al., 2012). Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling can increase current intensity without substrate. Therefore, methane production and electron recycling would not be favored.

The abovementioned CE trend implied that a shift in hydrogen scavengers occurred. The high initial CE indicated a significant activity of homoacetogenic bacteria and/or hydrogen oxidizing anode respiring bacteria, which was slowly replaced by a methanogenic population. Acetate was detected at the end of the cycle in this first hydrogen recycling period, possibly indicating a steady concentration reached due to homoacetogenic metabolism in MEC. Nevertheless, no microbial analyses were performed, not being able to specify quantitatively the microbial distribution in the system. The behavior between both homoacetogenic bacteria and methanogenic archaea was already discussed by Parameswaran et al. (2010) and Ruiz et al. (2013), proving that homoacetogenesis was only significant when methanogenesis was not active. Table S2 presents the most probable reactions taking place in the system.

The fact that hydrogen was consumed by hydrogen scavengers throughout implies that very low cathodic recoveries were obtained (less than 13%). Although cathodic recovery is indicating the actual performance of the system in terms of net hydrogen production, other parameters to differentiate the fraction of current intensity generated from the substrate added or the one from hydrogen recycling could be calculated to give a fairer view of the activity of ARB (Ruiz et al., 2013). Analogously to cathodic recoveries, net hydrogen production remained at low values, reaching a maximum of 0.08 m³ H₂ m⁻³ reactor d⁻¹ for MEC-milk.

#### 3.3. Methane versus hydrogen production in MEC

To avoid the uptake of hydrogen by other microorganisms or ARB themselves, a strategy to decrease the retention time of hydrogen in the system was followed. With this aim, a constant feeding of nitrogen was supplied to the MEC (indicated as horizontal arrows in Fig. 5) so that hydrogen could be stripped out of the system, leaving hydrogen scavengers without substrate. Therefore, methane production and electron recycling would not be favored (Montpart et al., 2012).

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**Table 2** — Comparison of this work with others studies dealing with complex substrates in MFC.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Reactor configuration</th>
<th>Inoculation</th>
<th>Current intensity [A/m²]</th>
<th>Coulombic efficiency (%)</th>
<th>COD removal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude glycerol</td>
<td>Single chamber</td>
<td>Domestic WW</td>
<td>43.71</td>
<td>18</td>
<td>90</td>
<td>Feng et al., 2011</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Single chamber</td>
<td>Bacillus subtilis</td>
<td>1</td>
<td>23</td>
<td>–</td>
<td>Nimje et al., 2011</td>
</tr>
<tr>
<td>Dairy WW</td>
<td>Annular single chamber</td>
<td>Dairy WWTP activated sludge</td>
<td>42.2</td>
<td>27</td>
<td>91</td>
<td>Mardanpour et al., 2012</td>
</tr>
<tr>
<td>Dairy WW</td>
<td>Double chamber</td>
<td>Dairy WW</td>
<td>–</td>
<td>17</td>
<td>91</td>
<td>Elakkiya and Matheswaran, 2013</td>
</tr>
<tr>
<td>Protein rich</td>
<td>Double chamber</td>
<td>Anaerobic sludge</td>
<td>1.5</td>
<td>12</td>
<td>50</td>
<td>Liu et al., 2009</td>
</tr>
<tr>
<td>Proteins (bovine serum albumin)</td>
<td>Single chamber</td>
<td>Meat processing</td>
<td>27.5</td>
<td>20</td>
<td>90</td>
<td>Heilmann and Logan, 2006</td>
</tr>
<tr>
<td>Starch WW</td>
<td>Single chamber</td>
<td>Starch processing WW</td>
<td>5.25</td>
<td>8</td>
<td>98</td>
<td>Lu et al., 2009, Velasquez-Orta et al., 2011</td>
</tr>
<tr>
<td>Starch</td>
<td>Single chamber</td>
<td>Primary clarifier effluent</td>
<td>3.5</td>
<td>19</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>Single chamber</td>
<td>Fermentative population</td>
<td>50</td>
<td>35</td>
<td>100</td>
<td>This work</td>
</tr>
<tr>
<td>Milk</td>
<td>Single chamber</td>
<td>working MFC</td>
<td>62.5</td>
<td>52</td>
<td>73.5</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td></td>
<td>71.4</td>
<td>28.8</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

a Parameter calculated from the data presented in the specific publication.
b Parameter calculated from the data presented in the specific publication.
The direct effect of sparging nitrogen was the observation of shorter cycles and higher current intensities, which was consequence of reducing the internal resistance of the system due to the stirring created with the nitrogen bubbling. No substrate loses due to stripping were detected in a test performed to evaluate this effect (data not shown). Cycles without stripping were periodically performed to assess the evolution of the gas composition in each MEC.

MEC-glycerol experienced a decrease in CE once methanogenic activity appeared (day 70). Initially CE had values much above 100% indicating the existence of hydrogen recycling phenomena. A continuous nitrogen sparging period began when no hydrogen production was detected at the end of the cycle (day 87, CE 35%). Throughout this period, CE slowly rose up to 100% presumably because of both the stirring effect created by nitrogen sparging and the washout of acetate consumers. The later could have been favored by the operation at lower hydraulic retention time in this period, given that at higher current intensity the substrate was degraded faster and therefore the fedbatch cycle was shortened. After the nitrogen sparging period, CE decreased to values around 70% and the gas collected at the end of each batch cycle still contained exclusively methane (day 127). This showed that methanogens had not been washed out, as before suggested.

MEC-milk also showed a decrease in CE when methane was initially detected, decreasing from about 300 to 170%, which indicated that still the hydrogen recycling phenomena were far more active than methanogenesis. In this point, nitrogen sparging was supplied continuously to avoid hydrogen consuming metabolisms (day 113). CE values of about 100% were directly favored by the stirring effects, lowering up to 50% afterwards. After one week with continuous nitrogen

Fig. 5 — Evolution throughout the operational period of current intensity (solid), coulombic efficiency (●) and relative hydrogen composition (▫) in each MEC. Arrows indicate nitrogen sparging periods.
sparging, hydrogen relative composition kept decreasing. As opposed to MEC-glycerol, hydrogen relative composition did not differ after 24 h from the beginning of the batch cycle and at the end. This could be indicating that methane origin was not related to hydrogen but acetate. A very similar trend to MEC-milk was observed for MEC-mixed.

It is important bearing in mind that nitrogen stripping is only effective against those hydrogen consuming metabolisms, like hydrogenotrophic methanogenesis, homoacetogenesis or hydrogen oxidation by ARB. Methanogenesis could still be possible through acetoclastic methanogenesis. Nevertheless, in MEC, low contribution on methane production was expected from acetoclastic methanogens given the fact that their affinity for acetate is lower than for ARB (Parameswaran et al., 2010), and therefore their growth is less favored.

As mentioned before, methane was detected both in MEC-milk and in MEC-glycerol even after a nitrogen sparging period. Some tests in open circuit configuration were performed with the aim of assessing the origin of methane (day 135–140). Methane formation from electrochemically produced hydrogen was not possible, but still hydrogen could be produced during fermentation. In order to rule out this hydrogen input, only acetate and propionate were used as carbon source in these tests (1 g L\(^{-1}\) of each). A second set of tests was performed aiming at assessing hydrogenotrophic methanogenesis. Hydrogen was initially bubbled in the open circuit MEC configuration and sodium bicarbonate (2.45 g NaHCO\(_3\) L\(^{-1}\)) was added in the medium (no acetate or propionate were added). With this last test, methane could only be produced by hydrogenotrophic methanogens. Excess initial substrate concentration was assumed in all cases, although hydrogen concentration in the liquid was not measured.

Fig. 6 shows the methane production rate for these tests and compares them to the methane production rate in the ordinary MEC configuration, when the complex substrate was fed. It was clearly seen that methane production rate from hydrogen was much lower than from acetate or propionate. Nevertheless, the methane production rate in a regular MEC with the usual carbon source was higher than the total methane from both hydrogen and acetate or propionate, which only accounted for 60% of the methane detected in MEC. When comparing these production rate values, it must be noted that maximum initial reaction rates were assumed.

Differences in the open circuit tests and the usual MEC operation could be attributed to the fermentation step, which also gives some hydrogen that can be converted to methane. This fact would imply that excess hydrogen concentration was not achieved in the open circuit tests and, therefore, they were not working at maximum rate. However, the contribution of hydrogen obtained in the fermentation step was not experimentally assessed. Another possible explanation was that in normal operation methane was being produced by methanogens directly using the cathode as electron donor, which would explain the lower production achieved in open circuit conditions (Cheng et al., 2009; Clauwaert and Verstraete, 2009; Villano et al., 2011).

Far from expecting a quantitative result, these tests qualitatively confirmed that hydrogen stripping with nitrogen alone could not be effective in terms of methane control, because, whereas hydrogenotrophic methanogens and other hydrogen scavengers would be affected, acetoclastic methanogens could proliferate. A rough contribution of hydrogen to methane formation could be calculated, being around 25% for MEC-glycerol and around 20% for MEC-milk.

This study shows the possibility of effectively producing hydrogen in a single chamber MEC for given wastewaters containing dairy industry substrates, which in the long-term operation do not enhance methanogenic archaea proliferation. Even though this is already an important achievement, tests with real dairy wastewater should be done to confirm this potential.

Finally, Table 3 compares the results obtained in this work with results reported on complex substrates for hydrogen production in MEC. Single chamber MEC studies are directly comparable to this work, but also other configurations have been included in the list since their comparison is also interesting. It can be stated that similar results in terms of current intensities were obtained in this work. However very low
hydrogen production values are evidenced in here, mainly due to methanogenic archaea proliferation in the system or to gas leakages. Only MEC-mixed showed a different behavior, recovering up to 90% of the hydrogen produced, and obtaining a biogas mainly composed by hydrogen (80% hydrogen content), even after 100 days operation. Again, MEC-mixed seemed to stand out against MEC-single substrates, confirming the advantages of codigesting substrates of very different nature.

In terms of gas composition, i.e. hydrogen concentration, also MEC-milk presented an interesting behavior, reaching about 75% of hydrogen purity after 100 days operation, unlike MEC-glycerol or MEC-starch, which could not avoid the proliferation of hydrogen scavengers.

Although other works managed net hydrogen production using glycerol, long-term stability of these systems has not been reported. Selembo et al. (2009) produced hydrogen from glycerol and crude glycerol reaching cathodic recoveries higher than 70% and gas purities higher than 85%. A periodic exposure to air was applied to maintain under control methanogenic archaea. However, the period of time that the system was working was not stated. Chignell and Liu (2011) and Escapa et al. (2009) also reached net hydrogen production from glycerol, although Escapa used a system with a gas phase cathode. Chignell and Liu (2011) did not mention the length of the study.

Table 3 – Comparison of this work with others studies dealing with complex substrates in MEC.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Reactor configuration and operation</th>
<th>Current intensity (A/m$^2$)</th>
<th>Hydrogen production (m$^3$/m$^3$·d)</th>
<th>Cathodic gas recovery (%)</th>
<th>Biogas composition (% H$_2$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>Single chamber, $V_{app}$ 0.9 V</td>
<td>221</td>
<td>2</td>
<td>79</td>
<td>88</td>
<td>Selembo et al., 2009</td>
</tr>
<tr>
<td>Crude glycerol</td>
<td></td>
<td>87</td>
<td>0.41</td>
<td>65</td>
<td>87</td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td>Single chamber, $V_{app}$ 0.6 V</td>
<td>238</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
<td>Chignell and Liu, 2011</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Single chamber, gas phase cathode $V_{app}$ 1V 57 days</td>
<td>10.7</td>
<td>0.6</td>
<td>100</td>
<td>98</td>
<td>Escapa et al., 2009</td>
</tr>
<tr>
<td>Cellulose</td>
<td>Single chamber, 2 stage process, &lt;20 days$^*$</td>
<td>1.15</td>
<td>1.11</td>
<td>86</td>
<td>84</td>
<td>Lalauvette et al., 2009</td>
</tr>
<tr>
<td>Proteins</td>
<td>Single chamber</td>
<td>236</td>
<td>0.42</td>
<td>35</td>
<td>100</td>
<td>Lu et al., 2010</td>
</tr>
<tr>
<td>Domestic WW</td>
<td>Double chamber</td>
<td>308$^*$</td>
<td>0.015</td>
<td>60</td>
<td>100</td>
<td>Ditzig et al., 2007</td>
</tr>
<tr>
<td>Domestic WW</td>
<td>Multi cassette, double chamber, 3 months</td>
<td>18.6$^*$</td>
<td>0.05</td>
<td>—</td>
<td>100</td>
<td>Heidrich et al., 2013</td>
</tr>
<tr>
<td>Winery WW</td>
<td>Single chamber, gas phase cathode</td>
<td>7.4</td>
<td>0.19</td>
<td>—</td>
<td>14</td>
<td>Cusick et al., 2011</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Single chamber</td>
<td>100</td>
<td>0.021</td>
<td>4</td>
<td>5</td>
<td>This work</td>
</tr>
<tr>
<td>Starch</td>
<td>$V_{app}$ 0.8 V 100 days</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td>75</td>
<td>0.086</td>
<td>13</td>
<td>76</td>
<td></td>
</tr>
<tr>
<td>Mixed</td>
<td></td>
<td>150</td>
<td>0.94</td>
<td>91</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

Index $^*$ indicates that the parameter has been calculated from the data presented in the specific publication. Biogas composition measured at the end of the batch.

When it comes to the length of the study and the proliferation of methanogenic archaea, it is interesting to have a look to the works of Cusick et al. (2011) and Heidrich et al. (2013). Also the work from Escapa et al. (2009) is comparable to the previous ones in terms of the length of the study. In these works, there is a clear difference in gas composition in the long-term operation depending on the system configuration. In this sense, a single chamber MEC would seem to offer low chance of hydrogen production. Nevertheless, in this work it was managed to produce a gas richer in hydrogen than methane for MEC-milk and MEC-mixed in a long-term operation.

4. Conclusions

Glycerol, starch and milk were explored as complex substrates to treat in single chamber MECs for long-term hydrogen production without addition of any chemical inhibitor of methanogenesis. A consortium development on the anode surface enhanced the bioelectroactivity of the system, avoiding any prefermentation treatment.
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Appendix A. Supplementary data

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References


