

Article

Strategies for Reducing the Start-up Operation of Microbial Electrochemical Treatments of Urban Wastewater

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Abstract: Microbial electrochemical technologies (METs) constitute the core of a number of emerging technologies with a high potential for treating urban wastewater due to a fascinating reaction mechanism—the electron transfer between bacteria and electrodes to transform metabolism into electrical current. In the current work, we focus on the model electroactive microorganism *Geobacter sulfurreducens* to explore both the design of new start-up procedures and electrochemical operations. Our chemostat-grown *plug and play cells*, were able to reduce the start-up period by 20-fold while enhancing chemical oxygen demand (COD) removal by more than 6-fold during this period. Moreover, a filter-press based bioreactor was successfully tested for both acetate-supplemented synthetic wastewater and real urban wastewater. This proof-of-concept pre-pilot treatment included a microbial electrolysis cell (MEC) followed in time by a microbial fuel cell (MFC) to finally generate electrical current of *ca.* 20 A · m^{−2} with a power of 10 W · m^{−2} while removing 42 g COD day^{−1} · m^{−2}. The effective removal of acetate suggests a potential use of this modular technology for treating acetogenic wastewater where *Geobacter sulfurreducens* outcompetes other organisms.

Keywords: bioelectrochemical systems (BES); microbial electrochemical technologies (METs); *Geobacter*; microbial fuel cell (MFC); microbial electrolysis cell (MEC); acetate; acetogenic wastewater; wastewater treatment

1. Introduction

Urban wastewater treatment is a biological process typically associated with energy consumption due to the air supply required for promoting microbial growth [1]. It may be feasible however to turn wastewater treatment into a self-sustaining process by using the energy in the wastewater. The chemical energy contained in the organic matter of wastewater constitutes up to 9-fold more energy than required to treat the wastewater [2,3]. Biogas production through anaerobic treatments is the most common technology so far to achieve the goal of self-sufficiency [4]. However, the use of wastewater as an energy source by using microbial electrochemical technology (MET) based on the electrochemical interaction between microbes and electrodes is also feasible [5]. These biological redox reactions are at the core of METs [6–9]. From the very beginning of this technology's discovery [10] it was proposed to have a promising role in wastewater treatment by allowing for a good effluent quality while converting the biodegradable materials into electric energy.

A large variety of applications have been developed on this basis: direct power generation (microbial fuel cells, MFCs) [11–13], chemical production of H_2 (microbial electrolysis cells, MECs) [14,15], microbial electrosynthesis [16,17], water desalination (microbial desalination cells, MDCs) [18,19], or even microbial electroremediating cells (MERCs) for restoring polluted environments [20].

MFCs and MECs share a common and similar structure, a community of electroactive microorganisms transferring electrons from organic matter to an electrode (anode) [21]. These electrons are then transferred through an external resistor to a cathode for harvesting electricity (MFC), or to a counter electrode under potentiostatic control (MEC). The presence of these electroactive microorganisms catalyse the oxidation of organic matter on the anode, and donate electrons to the anode that can be harvested as electric current [22].

Although urban wastewater has been the most common biodegradable fuel tested in METs, alternative organic matter sources such as cellulose [23], food industry residues [24], brewery wastewater [25], cheese wastewater [26], or root exudates [27] have been extensively tested in the last decade.

The core of a MET-based process mainly lies in: (i) electrochemically active microorganisms [28–31]; (ii) materials for membrane and electrodes [32,33]; and (iii) the operational mode of the system [34,35]. All those factors play an important role in achieving good treatment efficiency and have been greatly studied for increases in operational optimization [36–38].

Since Bond *et al.* [28] discovered bacteria from the *Geobacter* genus in an electrode-colonizing biofilm, this microbial genus has been the model microorganism for exploring MET. They are not the only microorganism able to colonize an electrode, but they outcompete bacteria from other environments like wastewater [25,39,40]. The reason for that is related to the unique physiology of *Geobacter* to couple its oxidative metabolism with the direct electron transfer to extracellular electron acceptors [41]. *Geobacter*'s ability to produce membrane proteins called cytochromes C in large quantities is key for that [42]. Indeed, those electron carriers are directly involved in the electrochemical activity's mechanism [43–45], a process that fails if a severe reduction of cytochrome C is achieved [46]. Moreover, other novel mechanism based on microbial nanowires [47,48] also participate in the inner biofilm conductivity.

The physiological state of electroactive bacteria is key during two different MET operational stages: (i) the start-up period, which is related to the primary colonization and growth of the electroactive biofilm on the electrode surface; and (ii) steady-state period, in which the biofilm is mature and the current harvested by the MET is stable. In this work, we use the model electroactive microorganism *Geobacter sulfurreducens* to explore both, design of new start-up procedures and electrochemical operation where MEC is followed by MFC in order to treat acetogenic wastewater and harvest electric energy.

2. Results and Discussion

The bioelectrochemical system (BES) explored in this study aims to oxidize acetate to CO_2 on the anode (*i.e.*, $C_2H_4O_2 + 2H_2O \rightarrow 2CO_2 + 8e^- + 8H^+$, $E^\circ = -290$ mV). Such a reaction will be tested under two different operational conditions based on the reduction of:

- Water on the cathode to produce hydrogen gas (*i.e.*, $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$, $E^\circ = -830$ mV *vs.* standard hydrogen electrode (SHE) by using a MEC since the reaction is not spontaneous.
- Fe^{3+} on the cathode to produce Fe^{2+} through a spontaneous reaction (Fe^{3+}/Fe^{2+} , $E^\circ = 0.77$ V *vs.* SHE by using an MFC since the reaction is spontaneous.

2.1. Microbial Conversion of Acetate into Electrical Current

Among the three main operational modes of MET: MFC, microbial short circuited (MSC) and MEC, the last one is the most feasible operational mode for advanced wastewater treatment because

of its superior capability for microbial current generation [34]. MEC will be indeed the first operation mode used to test our bioelectrochemical cell.

Our first approach was to use batch-grown cells of *Geobacter sulfurreducens* for inoculating a commercial pre-pilot microbial electrochemical cell reactor (anode set at 0.0 V *vs.* an Ag/AgCl reference electrode).

Under the starting-up operation conditions, batch grown cells showed a long lag period of 10 days before we could notice a current of only 10% of the final steady-state value. In the following week the current density rose continuously until the steady-state value of $1.8 \text{ mA} \cdot \text{cm}^{-2}$ was reached (Figure 1). During the starting-up period acetate was removed at a rate of $1 \text{ mmol} \cdot \text{day}^{-1}$, a biodegradation rate that correspond to a chemical organic demand (COD) removal of *ca.* $6.4 \text{ g COD day}^{-1} \cdot \text{m}^{-2}$. It is important to point out that tested conditions were equivalent to a wastewater with high COD soluble fraction and easily degradable organic matter.

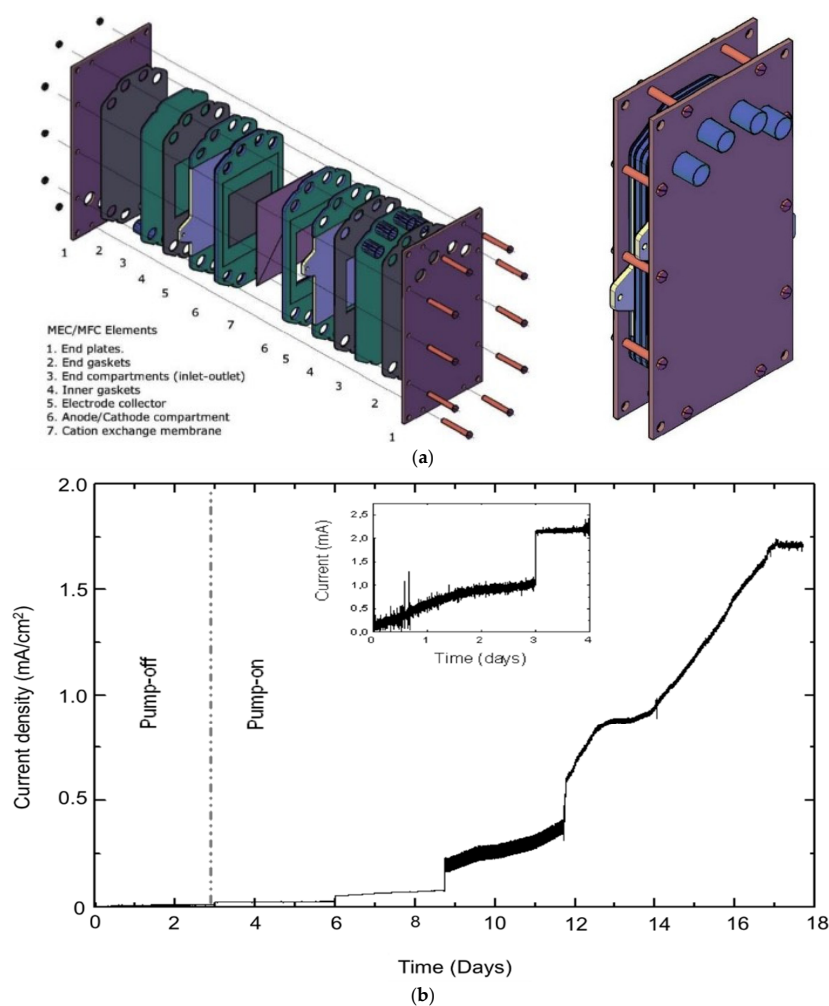


Figure 1. (a) Schematic of the filter press-based bioelectrochemical reactor used for microbial electrolysis cell (MEC) and microbial fuel cell (MFC) operations; (b) electric current production for starting-up the MEC after inoculation with batch-grown cells. Monitoring of current density revealed sudden increases at days 6, 9 and 12. They were due to the replacement the media. Pump-off: peristaltic pump off; Pump-on: peristaltic pump on. Inset: zoom of the electric current production during the first 4 days after inoculation.

Our results (Figure 1) revealed that it was technically possible to operate the MEC at a nominal electric current density of $18 \text{ A} \cdot \text{m}^{-2}$ (180 mA , 100 cm^2 of cross section). The electric consumption for the system (operating at steady-state) was $61.2 \text{ W} \cdot \text{m}^{-2}$ (*i.e.*, electric current = $1.8 \text{ mA} \cdot \text{cm}^{-2}$,

cell potential = 3.4 V). Interestingly, part of this energy could be recovered as hydrogen gas (ca. $58 \text{ L} \cdot \text{day}^{-1} \cdot \text{m}^{-2}$, 298 K and 1 atm) in the cathode chamber although it was not collected in this experimental setup. Furthermore, no biomass presence was found in the anolyte feed tank suggesting that cell growth was restricted the anodic granulated bed.

2.2. Minimizing the Start-up Operation: The Use of Plug and Play Geobacter Cells

The MEC start-up procedure is key to form an electroactive biofilm on the granulated anode suitable for the oxidation of acetate. Typically the experiments based on *Geobacter* have used batch grown cells at the exponential phase as inoculum [28,49,50]. In contrast, we used an alternative approach based on cells cultured in a chemostat [51]. Interestingly, it has been previously reported that electron acceptor-limiting conditions in a chemostat enhances extracellular electron-transfer rates [51] together with overproduction of cytochromes C [44] making *Geobacter* highly electroactive even under planktonic conditions [44], so we used electroactive planktonic cells for inoculating our MEC system and to analyse the early response as part of the start-up system.

Interestingly, the inoculation of those chemostat-cultured cells drastically reduced the lag-phase of the system so current was harvested from the very beginning (Figure 2). In 12 h the MEC reached 10% of the maximum current, 20-fold faster than using standard batch cells. During this period acetate was consumed at $6.6 \text{ mmol} \cdot \text{day}^{-1}$ what correspond to a removal of $42 \text{ g COD day}^{-1} \cdot \text{m}^{-2}$. Moreover, a maximal current of $2 \text{ mA} \cdot \text{cm}^{-2}$ was reached in just 2 days, in contrast with the 17 days required with standard batch-grown cells inoculation performance.

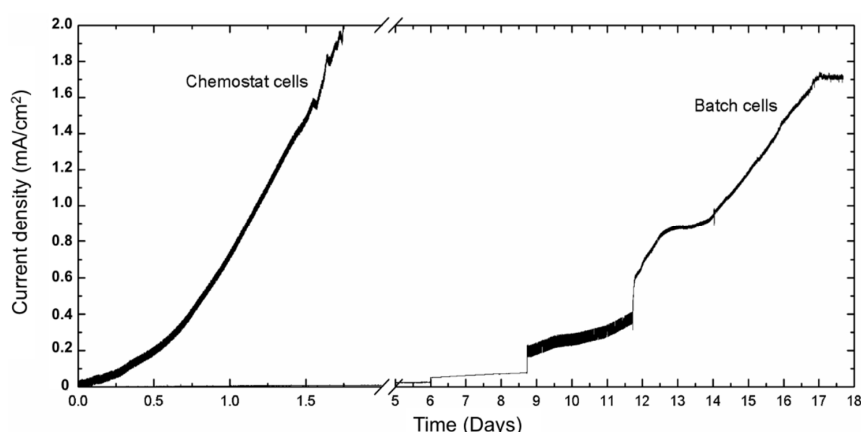


Figure 2. MEC start-up operation using inoculum of *steady-state* cells harvested from a chemostat (left); or exponential phase cells from a batch culture (right).

This result demonstrates that it is possible to start-up a BES in a short period of time when a pre-active inoculum is used to form the biofilm on the anode surface. We have named these cells *plug and play* since they may have an impact on the time required to start-up large microbial electrochemical reactors, one the barriers for the industrial implementations of biofilm-based systems.

2.3. Potential Electrode Competitors and Inhibitors of *Geobacter sulfurreducens*

When steady state is reached, the electroactive biofilms oxidizes soluble organic matter and use the anode (electrode) as terminal electron acceptor, so it is reasonable to consider that other soluble terminal electron acceptors (TEAs) existing in the aqueous media could affect the MEC operation. They could be an alternative electron acceptor for *Geobacter sulfurreducens* or even they could negatively affect the electroactive biofilm. Nitrate, sulphate, Fe(III) and oxygen are among most common soluble electron scavengers as TEA present in real wastewater. Figure 3 shows the influence on the microbial current production when the anolyte was spiked with different electron acceptor.

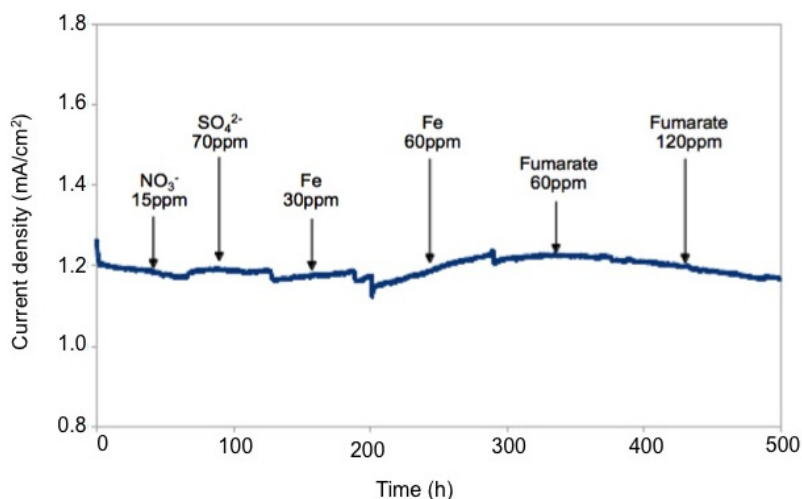


Figure 3. Soluble electron acceptor test on MEC operation behaviour.

Ammonium-oxidizing bacteria present in wastewater are able to generate nitrate [52]. Nitrate concentration in municipal wastewater has been reported to be up to 5 ppm [52], so the anolyte medium was pulsed with nitrate (15 ppm) although no disturbance was observed on the current production.

In relation to sulphate, moderate concentrations between 20 ppm and 150 ppm can be present in wastewater. Interestingly, the addition of 70 ppm of Na_2SO_4 to our inlet media did not show any effect in the current production.

Iron is normally present in relatively low concentrations (less than a few ppm) due to its low solubility at neutral pH and it is typically associated to industrial wastewater discharges. In our case, iron was added to the system in the form of Fe (III)-citrate salt at two different concentrations, 30 ppm and 60 ppm. This concentration was quite high compared with the typical natural level of Fe (III) in domestic wastewaters (less than 3 ppm), but taking the high affinity between iron and *Geobacter* into account [53] we considered testing its competitive role under high doses.

On the other hand, some other soluble compounds produced in the degradation of organic matter could be used as electron acceptors. Thus, we tested fumarate, a known TEA that is reduced to succinate ($\text{fumarate} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{succinate}$, $E^\circ = +0.031\text{ V}$) by *Geobacter sulfurreducens* as part of its respiratory system. As in the previous assays, no current alteration was detected after the TEA addition.

Finally, the competitive effect of oxygen was also tested. Although *Geobacter sulfurreducens* is often wrongly classified as a strict anaerobe, it has been reported to respire oxygen when supplied at low concentrations (10%) [54], so oxygen could act as a true electrode competitor for accepting electrons from microbial metabolism. In addition to this physiological role, oxygen may also be toxic over certain levels by oxidizing the cytochrome network and blocking the electron transfer. Furthermore the presence of oxygen can generate reactive oxygen species (hydrogen peroxide, superoxide radicals) that can damage cell structure, including membrane, DNA and proteins, in a process referred as oxidative stress [55]. Indeed a current drop was measured in our MEC device when oxygen level was increased to 2 ppm (Figure S1). In spite of this negative response, the electroactive biofilm was robust enough to recover the value of steady-state current after restoring the anoxic conditions of the inlet medium.

2.4. Microbial Electrolysis Cells Performance with Real Wastewater

On top of the TEAs, real urban wastewater contains undetermined amounts of diverse recalcitrant compounds like drugs and personal-care chemicals [56] together with trace pollutants like heavy metals (Cr^{4+} , Ni^{2+} , Cu^{2+} , Zn^{2+}) [57] that could be potentially harmful to the biofilm cells, so a

real urban wastewater sample was used as matrix for the preparation of the inlet medium in order to monitor the MEC performance. In addition to the COD content of the wastewater, additional acetate was supplied in order to assure a suitable electron donor for *Geobacter sulfurreducens*. Interestingly, the stationary state current was increased from 20 mA to 37 mA when the acetate-depleted synthetic inlet media was shifted to an acetate-supplemented urban wastewater media. In spite of the positive response, the harvested current was significantly lower than the current harvested using a synthetic freshwater medium (150–175 mA). Such a difference could be due to the non pH-buffered nature of the wastewater if we consider the critical role of biofilm acidification due to acetate oxidation in electroactive *Geobacter sulfurreducens* biofilms [58].

To counterbalance the acidification of the biofilm we proceeded to add bicarbonate, the standard buffer salt used as growth media in *Geobacter* [50]. Interestingly, sequential additions of bicarbonate generate a steady-state current of 140 mA (Figure 4). This suggests that the dynamic balance between proton formation inside the biofilm and the buffer capacity of the bicarbonate in the bulk solution have an impact on the electroactive biofilm's performance [58]. Apparently, acetate oxidation coupled to electrode reduction produces more protons than those neutralised by bicarbonate diffusing from bulk solution to the biofilm. Then, acetate oxidation and subsequently current production (*i.e.*, extracellular respiration rate) will be limited to avoid further damage to the biofilm. Furthermore, the electric conductivity shift provided by the addition of HCO_3^- ions to wastewater was not considered responsible of the current enhancement, since the electrical conductivity ($12 \text{ mS} \cdot \text{cm}^{-1}$ at 25°C) was similar to the one present in the synthetic medium.

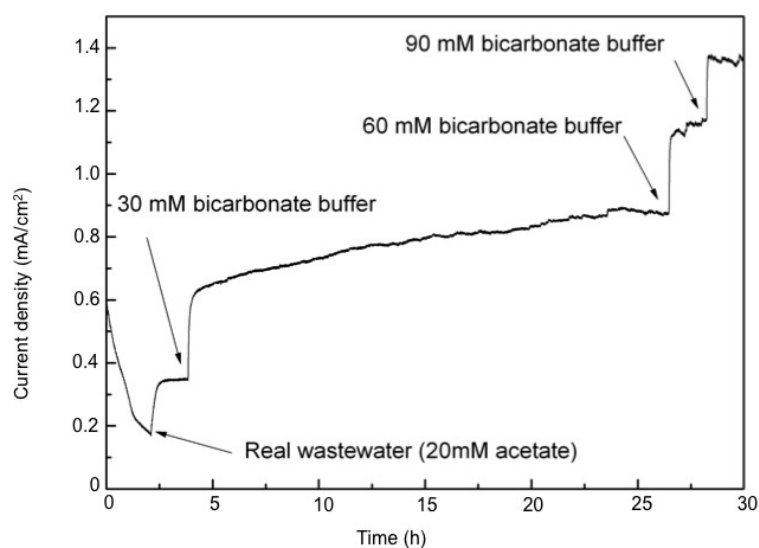


Figure 4. Electric current measured in MEC system when real water matrix is used as fuel (anode at 0.0 V *vs.* a 3.5 M Ag/AgCl reference electrode). Water matrix is supplemented with 20 mM acetate (see Section 2.5).

2.5. Microbial Fuel Cell: Steady State Operation and Power Curve

As described previously, once the steady state has been reached in a MEC (*i.e.*, the electric current is stable), the reactor was converted into a MFC by shifting the catholyte solution to FeCl_3 0.2 M, pH = 1 HCl, then connecting anode and cathode through an external load (resistor 2.1Ω) (Figure S2).

The anode potential value was in the range of -150 mV and -350 mV (*vs.* an Ag/AgCl reference electrode), indicating that the oxidation of acetate to produce CO_2 was occurring on the anode. Consequently, the reduction of Fe^{3+} produced on the cathode was at a potential of between 350 mV and 450 mV . The slight change of the cathode potential during the experimental period was mainly due to the change of the formal potential ($E = E^\circ + RT/nF \ln[\text{Fe}^{2+}]/[\text{Fe}^{3+}]$) as Fe^{3+} concentration decreased and Fe^{2+} concentration increased during the experiment. The membrane potential drop

is considered as an indirect value of the internal resistance of the MFC. In our system it was in the range of 250–400 mV, indicating that an important amount of available energy produced by the oxidation/reduction reaction (*i.e.*, $E_{\text{cell}}^{\circ} = E_{\text{cathode}}^{\circ} - E_{\text{anode}}^{\circ} = 0.77 \text{ V} - (-0.29 \text{ V}) = 1.06 \text{ V}$ in standard conditions) was consumed inside the cell as ohmic drop, lowering the energy efficiency of the system. On the other hand, the measured variations of the anode potential and the membrane potential drop could be attributed to the variations in the electric field inside the cell due to liquid/particles movements and turbulences created inside the anode compartment in the cell.

Figure S3 shows a current density for the MFC in the range of 1.75–2.25 $\text{mA} \cdot \text{cm}^{-2}$ (current intensity 175–225 mA). As previously described, the slight decrease is due to the change in the cathode equilibrium potential that affects current density production.

As the cell's potential depends on the anode's and cathode's potential, as well as the membrane potential drop (*i.e.*, $E_{\text{cell}} = E_{\text{cathode}} - E_{\text{anode}} - E_{\text{mem_drop}}$, being $E_{\text{mem_drop}} = I (R_{\text{membrane}} + R_{\text{anode_compartment}} + R_{\text{cathode_compartment}})$), it is important to study each parameter separately in order to understand the MFC operation. Figure 5 shows the anode, cathode and membrane potential drop *vs.* electric current density for the experiment studied in this section (performed as described in the Materials and Methods section). It is important to note that the slopes for both the anodic and cathodic processes (overvoltage) were higher (absolute value) in the case of bioanode potential and closely related to kinetic hindrance considerations. It can be assumed that the cathode reaction was faster than the anode reaction (catalyzed by the microorganism biofilm), and that explains the different value for the slopes in both processes. Another important point is the membrane potential drop (green line in Figure 5), it represents an important energy loss inside the system. The high value of the membrane potential (300 mV at 1.75 $\text{mA} \cdot \text{cm}^{-2}$) could be related to the low electrical conductivity of the anode solution. Indeed, in this case the main limitation of the system was the high value (*i.e.*, slope) of the membrane potential drop. In a real application the electrical conductivity of the anode stream is determined by the dissolved salts, which is a difficult parameter to change. Reducing the thickness of the anode compartment may be a strategy to optimize the system performance in order to reduce the ohmic drop. Moreover, the use of carbon particles with different size or diameter in order to increase the bed's porosity (*i.e.*, surface area available for the biofilm) could be a suitable strategy for optimization.

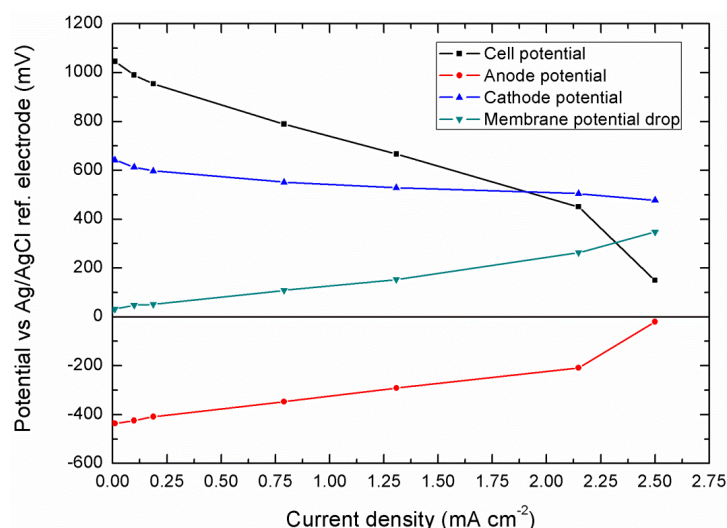


Figure 5. Potential diagram for the MFC device at steady state.

When the MFC was operated at short circuit (external load = 0Ω) the electric power provided by the device was zero, but the consumption of acetate (*i.e.*, wastewater treatment rate in a real application) was maximal. Alternatively, if the MFC is operated at (or near) open voltage circuit

conditions (for example, external load = 10 k Ω), the MFC provides the maximum cell voltage, but the wastewater biodegradation rate would become almost zero. Figure 6 shows the cell potential and the electric power provided by the cell *vs.* current density. The maximum power was reached at 2 mA·cm^{−2} when the cell voltage was 500 mV and the electric power density provided by the MFC was 10.0 W·m^{−2}. This operational condition was reached when an external resistor of 2.1 Ω was connected as external load.

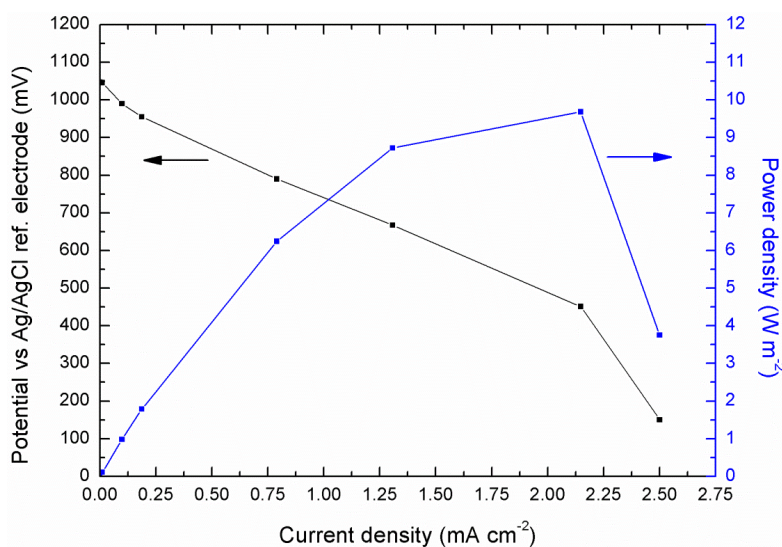


Figure 6. Cell potential and electric power density provided by the MFC at steady state (batch operation mode).

3. Materials and Methods

3.1. Bacterial Strain and Culture Conditions

The Bacterial strain used was *Geobacter sulfurreducens* DL1. This strain was routinely grown at 30 °C in septum-sealed serum bottles containing freshwater medium (FWM, pH = 6.9, EC = 12.4 mS·cm^{−1}) with the following mineral salts: NaHCO₃ 2.5 g·L^{−1}; NH₄Cl 0.25 g·L^{−1}; NaH₂PO₄H₂O 0.06 g·L^{−1}; KCl 0.1 g·L^{−1}; Fe (NH₄)₂(SO₄)₂6H₂O 0.04 g·L^{−1}. The medium was supplemented with a trace mineral and vitamin solution [50]. Sodium acetate (NaC₂H₃O₂ 20 mM, Sigma-Aldrich, Madrid, Spain) was used as electron donor and fumarate (C₄H₄O₄, 40 mM, Sigma-Aldrich, Madrid, Spain) as the sole electron acceptor. The culture media was degassed by using a mixture of N₂/CO₂ (80:20, industrial ALIGAL-12, Air-Liquide, Madrid, Spain). Traces of oxygen were removed from the gas phase by passing the gas mixture through heated copper fillings pre-reduced with H₂.

For continuous culture in chemostat, the microorganism was grown in a 2 L bioreactor (Braun Biostat Bioreactor, Melsungen, Germany). A temperature of 30 °C was kept constant by using a water-jacket device connected to the control unit. Stirring was set at 250 rpm and a level probe was used to maintain the working volume at 2 L. Fresh media water (FMW) for continuous culture experiments was set under fumarate (electron acceptor) limited conditions by using a FWM supplemented with 10 mM acetate and 10 mM fumarate under a growth rate of 0.05 h^{−1}. Both the inlet medium and the bioreactor headspace were made anoxic by using a mixture of N₂/CO₂ (80:20, industrial ALIGAL-12) gas flow. The vessel bioreactor and all associated tubing were sterilized by autoclaving (15 min, 121 °C). Steady-state electroactive cells were obtained after five volume refills.

3.2. Microbial Electrolysis Cell Device

A commercial multipurpose Electro MP-1 electrochemical reactor manufactured by ElectroCell (Tarm, Denmark, projected electrode area 100 cm^2) was used as MEC in this work. The cell design (Figure S4) comprised a compact stack design of several polypropylene compartments and neoprene gaskets for an optimal hermetically seal. The arrangement of these compartments allows to obtaining different configurations from the same cell. In this case, two chambers were used (anodic and cathodic compartments) each with a thickness of 17 mm and a compartment volume 170 cm^3 . The anodic compartment was filled with graphite particles (mean diameter 2.3–4.0 mm, porosity 36%). The surface area of the particles was estimated to be $1.173\text{ m}^2\cdot\text{g}^{-1}$. As the total mass of carbon particles in the anode bed was 178 g, the total surface area of the anode bed electrode could be estimated in 208 m^2 . However, this anode bed electrode area should be only considered indicative for comparison purpose (for example, with other analogous microbial electrochemical devices using filter press configuration) due to the fact that most of this area is not likely available for biofilm attachment/growth. The cathodic compartment was filled with carbon felt RVG4000 (MERSEN Ltd., Barcelona, Spain). Both compartments contained graphite plates as electrical collectors, while a cationic membrane Nafion 324 (DuPont, DE, USA) separated the compartments. The device was closed with stainless steel screws in order to avoid any leakage of the system. The whole system was completed with a 5 L substrate feed tank and 2 L one for catholyte solution.

As it is shown in Figure S5, the MEC reactor was connected to the anolyte and catholyte tanks by using a Pharmed Tubing 1/4" internal diameter (Saint-Gobain, Courbevoie, France). Two-channel peristaltic pump (205 CA, Watson Marlow, Wilmington, MA, USA) were used to recirculate both streams through the batch mode system with a flow rate of $6\text{ L}\cdot\text{h}^{-1}$. Both, the MEC reactor and the anolyte/catholyte tanks were placed in a temperature controlled room at $30\text{ }^\circ\text{C}$. The anolyte tank was kept under anaerobic conditions by fluxing a mixture of N_2/CO_2 (80:20, industrial ALIGAL-12). A reference electrode (Ag/AgCl KCl 3.5 M) was placed in the geometric centre of the anodic compartment, in order to measure anode potential. A Voltalab PGZ100 potentiostat (Radiometer Analytical, Villeurbanne, France) was connected to the MEC system, allowing recording experimental data while performing to control the electrochemical setup.

3.3. Start-up and Operation Procedure

Prior to inoculation, the MEC system was sterilized by recirculation of 70% w/w ethanol/water solution through the whole batch mode system. A filtered gas mixture of N_2/CO_2 was gassed for 2 h in order guarantee both the ethanol evaporation and an anoxic environment inside the MEC.

The anolyte was made of FWM supplemented with 20 mM acetate solution, and the catholyte was made of Na_2SO_4 0.25 M solution. Both solutions were recirculated through the system, and the anode potential (working electrode) was poised at 0.0 V *vs.* reference electrode located in the geometrical centre of the anodic compartment. After overnight operation, the anodic chamber was inoculated by recirculating 200 mL of a batch culture of *Geobacter sulfurreducens* (OD_{600} 0.4) for 2 h in order to ensure the adhesion of cells to the anode particles. In the case of chemostat cells, the anodic chamber was inoculated by recirculating 200 mL of a steady-state culture (OD_{600} 0.11, fumarate concentration $<1\text{ mM}$) for 2 h. Then, the cell culture solution was replaced by FMW supplemented with 20 mM acetate in absence of other electron acceptor. The MEC was operated without recirculation (pump off) until a positive and measurable current was obtained (3 days for batch cells assay and 12 h for chemostat cells assay). After that, acetate-supplemented FWM was recirculated at a flow rate of $6\text{ L}\cdot\text{h}^{-1}$.

3.4. Competitive Assays with Soluble Terminal Electron Acceptors

A number of TEAs were tested to evaluate their influence during electric current production in FWM medium. The anolyte tank was pulsed with 1 mL anoxic solutions of the following chemicals:

nitrate (1.5 ppm), sulphate (70 ppm), iron (30 ppm and 60 ppm), fumarate (60 ppm and 120 ppm). In addition, oxygen influence was evaluated after air-sparging the anolyte tank until making the solution $2 \text{ mg} \cdot \text{L}^{-1}$ in oxygen (Oxi 320 Oximeter, Crison, Barcelona, Spain).

3.5. Operation as a Microbial Fuel Cell

The main objective of this assay was to demonstrate that the anode biofilm was stable during operation and could achieve high current density. Once the electric current provided by the bioanode reached stable values, it could be assumed that the biofilm had achieved a dynamic balance (equilibrium) between cell division and cell death (or detachment), and thus the system could be considered at steady state, at least from the electrochemical point of view. When the bioanode is stable, the system could be operated as MFC (*i.e.*, device that spontaneously produces electric energy from the oxidation of organic matter in the anodic compartment and reduction of a suitable chemical species in the cathodic compartment, as described before) by disconnecting the potentiostat used for biofilm growth, substituting the catholyte solution with a solution with a chemical substance able to act as electron acceptor, and electrically connecting the anode and cathode collector through and external load (resistor). In this study, a FeCl_3 0.20 M pH = 1 (HCl) was used as catholyte solution ($\text{Fe}^{3+}/\text{Fe}^{2+}$, $E^\circ = 0.77 \text{ V vs. HSE}$), a 20 mM acetate + FWM (as described in the previous section) was used as anolyte, and a 2.1Ω resistance was used as external load. The volume of anolyte and catholyte was 10 L and 2 L respectively, and the flow rate was $6 \text{ L} \cdot \text{h}^{-1}$ for both streams (batch operation). Whole configuration represented in Table 1.

Table 1. Bioelectrochemical system (BES) configuration. Freshwater medium: FWM.

	Materials/conditions	Details
BES configuration	Compartments	1 anode compartment; 1 cathode compartment
	Projected area	17 mm
	Compartment thickness	100 cm ²
	Anode electrode	Particles 2.3–4.0 mm diameter mass: 178 g; porosity 36%
	Cathode electrode	Carbon felt
	Electric collectors	Graphite plate
	Membrane	Nafion 324 (DuPont)
	Reference electrodes	Ag/AgCl 3.5 M KCl reference electrodes units located in the geometrical center of each compartment (2 units)
	Flow rate	$6.4 \text{ L} \cdot \text{h}^{-1}$ (both streams)
	Anolyte solution	Acetate 20 mM + FWM (pH = 6.9, EC = $12.4 \text{ mS} \cdot \text{cm}^{-1}$)
	Catholyte solution	MEC operation: Na_2SO_4 0.25 M ($16.0 \text{ mS} \cdot \text{cm}^{-1}$) MFC operation: FeCl_3 0.20 M pH = 1 HCl ($15.6 \text{ mS} \cdot \text{cm}^{-1}$)
	Anolyte tank	5 L
	Catholyte tank	2 L

The polarization curve was obtained by shifting the value of the connected external load (in the range of $0.25\text{--}1000 \Omega$ and then waiting 50–60 min until steady state was reached under a stable electric current generation.

3.6. Assays with Real Urban Wastewater

Urban wastewater was harvested from an anaerobic lagoon effluent at the wastewater treatment plant from the municipality of Carrión de los Cespedes (Sevilla, Spain). Physical chemical characteristics are shown in Table 2. The wastewater was filtered under vacuum with a Kitasato flask (Labbox, Barcelona, Spain) through a Whatman filter paper (Sigma-Aldrich, Madrid, Spain) of $20\text{--}25 \mu\text{m}$ and gassed with a N_2 . The urban wastewater was supplemented with acetate to mimic the FWM acetate composition (20 mM) so microbial current production could be properly compared. The MEC reactor was run under the same operation conditions used for synthetic FWM assays. The pH buffering assays were performed after bicarbonate addition to the anolyte tank and under N_2/CO_2 (80:20).

Table 2. Wastewater characteristics from the anaerobic lagoon of CENTA treatment plant *. Chemical oxygen demand: COD, biological oxygen demand: BOD.

Real wastewater					
Parameter	pH	Conductivity (mS·cm ^{−1})	BOD (mg·L ^{−1})	Total nitrogen (mg·L ^{−1})	Acetate (mM)
Value	7.0	1.5	280	0.5	1.0

* Provided by CENTA (Center for New Water Technologies Foundation, Carrión de los Céspedes, Seville, Spain).

3.7. Analytical Methods

The content of acetate in the cultures was measured with HPLC with a ZORBAX PL Hi-Plex H Guard Column (50 mm × 7.7 mm, Agilent Technologies, Madrid, Spain) and mobile phase of 0.1% H₃PO₄. The sample volume was 50 µL, mobilized at a flow rate of 0.5 mL·min^{−1}. Acetate was detected by using UV at 210 nm. Electric conductivity measurements were carried out using a GLP 31 conductivity meter (Crison, Barcelona, Spain). pH was measured using a GLP 21 pH-meter (Crison, Barcelona, Spain). Both analyses were performed at 25 °C.

3.8. Electrochemical Assays

All experiments were performed using a Voltalab PGZ100 potentiostat (Radiometer Analytical, Villeurbanne, France) by the Voltamaster 4 software. Chronoamperometry was performed at 0.0 V potential *vs.* Ag/AgCl-KCl sat. Reference electrode was located at in the geometric centre of the anodic chamber. Each point was acquired every 10 s. The predictive conversion of acetate into electric current was calculated using the following equation:

$$I = mFn \frac{1}{24 \times 3600 \times 1000} \quad (1)$$

where m is the acetate consumption rate (mmol·d^{−1}), F is Faraday's constant (96,485 C·mol^{−1}), and n is the number of electrons released in the acetate oxidation ($n = 8$, or eight moles of electrons per mol of acetate oxidized to CO₂).

4. Conclusions

BESs are suitable technologies for treating urban wastewater, however a number of factors should be explored in order to optimize the methodology and make them profitable. Although materials like ion interchange membranes and electrodes are typically the key issues under investigation, we have tried a different strategy based on exploring the physiology of the inoculum under a fine-tuning method to optimize the start-up operation. Our chemostat-grown cells were able to reduce the start-up period by 20-fold while enhancing the COD removal by more than 6-fold during the start-up period. The resulting electroactive biofilm was robust to the inhibitory action of additional electron acceptors present in the wastewater. However, the pH of the medium was key for harvesting maximal current. The methodology described in this paper has been successfully tested for both acetate-supplemented synthetic wastewater and real wastewater as a proof-of-concept for a pre-pilot treatment where MEC was followed by a MFC. The effective removal of acetate suggests a potential use of the technology for treating acetogenic wastewater from an anaerobic digester reducing the start-up operation by using plug and play *Geobacter* cells. The modular nature of our system allows a feasible scale-up of the technology.

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