



Monitoreo electroquímico y caracterización microbiana de una celda de combustible microbiana alimentada con aguas residuales domésticas inoculada con lodo anaeróbico

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Resumen

Una celda de combustible microbiana de doble cámara con cátodo inmerso en el agua fue operada con aguas residuales domésticas para investigar la capacidad electrogénica de las bacterias anaeróbicas de una planta de tratamiento de aguas residuales municipales. Se obtuvieron diariamente curvas de potencial de celda vs. densidad de corriente, densidad de potencia vs. densidad de corriente y corriente a una resistencia fija de 100Ω para monitorear la evolución electroquímica del sistema en función del uso del sustrato en varios ciclos de lotes. Se obtuvo una densidad de potencia máxima de $1.11 \mu\text{Wcm}^{-2}$ después de 65 días de operación continua, una eficiencia coulombic del 7% y una disminución de la Demanda de Oxígeno Químico del 76% en el último ciclo del lote. El cultivo anaeróbico de las bacterias de la biopelícula anódica dio como resultado el aislamiento de dos bacterias Gram-positivas y dos Gram-negativas con capacidades de fermentación de azúcar divergentes, mientras que el análisis de los fragmentos del gen 16S rRNA mostró tres clones de los filos Firmicutes, Prote-Proteobacteria y α -Proteobacterias. El análisis de las imágenes de microscopia electronica de barrido reveló un aumento en la diversidad celular y la proliferación de arqueas metanogénicas al cambiar de aguas residuales sintéticas a reales. Estos resultados revelan la influencia de la concentración de sustrato y la presencia de microorganismos metanogénicos en la producción de energía en la celda de combustible microbiana, lo que sugiere que los desarrollos futuros podrían ser una contribución para el uso de esta tecnología para descontaminar las aguas residuales domésticas en las comunidades rurales.

Palabras clave: diversidad bacteriana, pruebas bioquímicas, biofilmes, celda de combustible microbiana, tratamiento de aguas residuales.

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Electrochemical Monitoring and Microbial Characterization of a Domestic Wastewater-Fed Microbial Fuel Cell Inoculated with Anaerobic Sludge

Abstract

A dual-chambered microbial fuel cell with aqueous cathode was operated with domestic wastewater to investigate the electrogenic ability of anaerobic bacteria from a municipal wastewater treatment plant. Curves of cell potential vs. current density, power density vs. current density and current at a fixed load of 100 Ω , were obtained daily to monitor the electrochemical evolution of the system as a function of substrate use in several batch cycles. A maximum power density of 1.11 μWcm^{-2} was obtained after 65 days of continuous operation and a coulombic efficiency of 7% and a chemical oxygen demand removal of 76% were found in the last batch cycle. Anaerobic culture of the bacteria from the anode biofilm resulted in the isolation of two Gram-positive and two Gram-negative bacteria with divergent sugar fermentation capabilities, while analysis of 16S rRNA gene fragments showed three clones from the phyla *Firmicutes*, δ -Proteobacteria and α -Proteobacteria. Scanning electron imaging analysis revealed an increase in cell diversity and proliferation of methanogenic archaea when changing from synthetic to real wastewater. These results reveal the influence of substrate concentration and presence of methanogenic microorganisms on the production of power in microbial fuel cells, suggesting that future developments could be a contribution for the use of this technology to decontaminate domestic wastewater in small communities.

Keywords: Bacterial diversity, biochemical tests, biofilm, microbial fuel cell, wastewater treatment.

1 Introduction

Microbial fuel cells (MFCs) form part of an emerging technology that makes it possible to deal with two of the major problems facing society today: water availability and energy⁽¹⁾. MFCs are electrochemical devices that convert the energy stored in organic molecules into electricity by means of the metabolism of microorganisms grown in an anaerobic environment⁽²⁻⁴⁾. These devices provide a sustainable way of using biomass as a source of electricity for several applications most of which are being tested under laboratory conditions. One of such applications is the possibility of making wastewater treatment process sustainable, by using part of the energy stored in wastewater for the operation requirements of wastewater treatment plants^(1,5,6). Several authors⁽⁷⁻¹⁰⁾ have implemented laboratory scale MFCs for the treatment of wastewater of several origins, while evaluating the amount of power produced by them. Efforts have been mainly concentrated in: i) the design of the reactor⁽¹¹⁻¹³⁾; ii) the modification of electrodes or modification of new materials used as electrodes⁽¹⁴⁻¹⁶⁾; and iii) the characterization of the diversity of the microbial community within these devices⁽¹⁷⁻¹⁹⁾.

Different substrates have been tested so far to analyze their effect in MFC performance in terms of the amount of electricity generated and chemical oxygen demand (COD) removal. These can be divided into synthetic substrates, such as solutions with a single compound as a source of carbon, and real substrates, such as hospital wastewater⁽²⁰⁾, slaughterhouse wastewater⁽²¹⁾, brewery wastewater⁽²²⁾ and municipal wastewater^(20,23). In terms of diversity studies performed on MFC, Phung, *et al.*⁽²⁴⁾ assessed the bacterial diversity in two oligotrophic MFC, one fed with river water and the other with artificial wastewater with acetate. Authors found a shift in microbial communities of the MFC when compared with the river sediment communities used as inoculum. Sequencing of

16S rDNA showed that 80% of bacteria found in the anode belonged to the Proteobacteria (α -Proteobacteria outnumbering other phyla in the acetate fed MFC and β -Proteobacteria outnumbering in the river water fed MFC). Other publications reinforce the predominance of the Proteobacteria in electricity generating bacterial communities^(25,26) and the variation in microbial community composition related with variation in carbon sources used to feed the bacteria.

Works on MFC fed with domestic wastewater have evaluated the performance of these devices on the basis of the current produced using a fixed resistance or by means of the elaboration of polarization curves to find the maximum power density at a given time of operation, while a monitoring of this parameter in time following changes in substrate concentration or substrate origin has not been evaluated^(22, 27, 28). Furthermore, examination on the structure of the anode biofilm have been mainly elaborated using molecular approaches while direct observation of the anode surface under changing conditions of operation has been scarce⁽²⁹⁾. This work aims to evaluate the electrogenic activity of microorganisms from a septic tank of a domestic wastewater treatment plant when fed with domestic wastewater in a MFC by monitoring the evolution of cell voltage and current density. Additionally, this work seeks to characterize the microbial diversity in MFC using molecular and traditional microbiological methods.

2 Material and Methods

2.1 Microbial Fuel Cell Design and Operating Conditions

A two chambered MFC was constructed with two cylindrical containers of 250 mL of working volume each. The containers were opened at the sides and connected with glass flanges of 38 mm of length and 32 mm of internal diameter. In the middle of the flanges a Nafion® 117 membrane was located using a Teflon® ring for adjustment. The membrane was fixed using a stainless steel clamp. Two pieces of carbon cloth were treated with heat and nitric acid for organic contamination removal and then used as anode and cathode. The anode had a geometric area of 51.9 cm² on which the biofilm was later formed. The cathode had 40.8 cm² of geometric area and it was covered with Pt/C catalyst (0.2 mg cm⁻²) by painting. The electrodes were connected to an external circuit with a fixed load of 100 Ω in order to set the MFC in operation. The anodic chamber was fed in batch mode using filtrated sterilized synthetic wastewater with the composition suggested by Mathuriya and Sharma⁽³⁰⁾ with D-glucose as carbon source for the enrichment and acclimation of the microbial community for approximately 2 weeks before feeding with real wastewater for the rest of the experiment. The anodic chamber was maintained with permanent N₂ bubbling in order to guarantee anaerobic conditions. The cathode was maintained in a 250 mL volume of milli Q water saturated with air provided by a peristaltic pump. The MFC was kept in a thermostat at a constant temperature of 27 °C. Figure 1 shows the overall arrangement of the MFC with all the components.

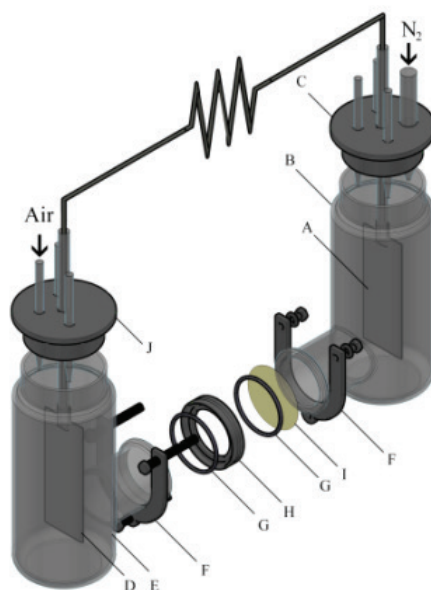


Figure 1. *Microbial Fuel Cell design: A) Anode; B) 250 mL anodic chamber; C) PTFE lid; D) Cathode; E) 250 mL cathodic chamber; F) Stainless steel clamp elements; G) Rubber O-rings; H) PTFE packaging; I) Nafion® 117 membrane; J) Rubber lid.*

2.2 Substrate and Inoculum Source

Domestic wastewater was obtained at the entrance of the septic tanks of a domestic wastewater treatment plant in La Vorágine, at the outskirts of Cali, Colombia. Wastewater was obtained every 3 days when it was required to feed the MFC, once organic matter was depleted in each batch cycle. A sample of 2.5 grams of bottom sludge of the septic tank was used as a source of microorganisms to inoculate the anode chamber of the MFC at the beginning of the experiment.

2.3 Electrochemical Monitoring and Chemical Analyses

Cell potential vs current density curves were obtained daily using the galvanostatic discharge method ⁽³¹⁾ using resistances from 1 Ω to 800 k Ω , after voltage stabilization. This information was also used to obtain daily curves of power density vs. current density (normalized to the anode surface area) in order to monitor the evolution of maximum power density as function of time. Electrochemical performance was also evaluated by measuring the current every 12 hours using an external resistance of 100 Ω . During the last batch, anolyte's COD and dissolved organic carbon (DOC) were determined to quantify the amount of organic matter removed by the system and the coulombic efficiency (C_E) was determined for this batch cycle using the resistance producing the maximum power density.

2.4 Secondary Electron Imaging

Pieces of the anode of about 0.25 cm² were removed at the end of the synthetic wastewater (SW) and real wastewater (RW) feeding stages and they were fixed in a 3% glutaraldehyde solution and dehydrated with increasing concentrations of ethanol solutions and HDMS according to Araujo *et al.* ⁽³²⁾ in order to observe the biofilm on the electrode surface. Samples were sputter coated with gold for 5 minutes using a Denton Vacuum Desk IV coater and secondary electron imaging (SEI) micrographs were obtained in a Jeol JSM 6460 scanning electron microscope at 15kV.

2.5 DNA Analysis

Three sections of approximately 0.5 cm² of the anode were removed and processed using the DNA extraction kit FastDNA™ SPIN KIT of MP Biomedicals following manufacturer instructions for total genomic DNA extraction. For amplification of the 16S rDNA gene, primers fD1 (5'- ccgaattc gtcgacaacAGAGTTTGATCCTGGCTCAG-3') and rD1 (5'- cccgggatccaagcttAAGGAGGTG ATCCAGCC-3') for eubacteria were used ⁽³³⁾. Amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega) and vector ligation was performed using the pGEM®-T Easy Vector System (Promega). Recombinant plasmids were used to transform cells of *Escherichia coli* DH5α and a clone library was elaborated. To identify different 16S rDNA fragments from the colonies, digestion with the restriction enzymes KpnI, NdeI, PstI y DraI was carried out. Extraction and purification of the vectors containing the selected fragments were done using the Wizard® Plus SV Minipreps DNA Purification System (Promega) and the purified products were sent for sequencing (Macrogen, Korea). Sequences were edited using the software Geneious 5.6.4 and were compared for homology with 16S rDNA sequences of the GeneBank using the Basic Local Alignment Search Tool (BLAST, National Center for Biotechnology Information) with the Blast-n algorithm to obtain similar sequences of phylogenetically related species.

2.6 Microbiological Characterization

At the end of the experiment, a piece of 0.3 cm x 7.0 cm of the anode was cut off and introduced in a test tube with sodium thioglycolate. The test tube was vortexed to detach the biofilm from the piece of electrode and then, it was incubated for 48 hours in an anaerobic jar (OXOID). After bacterial growth was evident, plating was performed to isolate individual bacterial colonies in sodium thioglycolate - agar medium and anaerobically incubated. Isolated colonies were Gram stained and morphologically characterized. Biochemical characterization of the isolated bacteria was performed using BBL Crystal™ Gram-Positive ID Kit and Enteric/Non-fermenter ID Kit for Gram-positive and Gram-negative bacteria respectively.

3 Results and Discussion

3.1 Microbial Fuel Cell Operation

The MFC was operated for 70 days and electrochemically monitored for 65 days from day five. During the first days of operation the MFC was fed with SW through five batches followed by a cycle of 1:1 SW:RW and ten cycles of RW, with a hydraulic retention time (HRT) of four days. The cycle containing the 1:1 mixture served to diminish the impact of a sudden change in substrate that could negatively affect the biofilm deposition on the anode because of the presence of probable inhibitors of microbial growth in the RW. Figure 2(a) shows the consecutive power density curves taken daily derived from their respective polarization curves, the black ribbons showing curves taken when the cell was fed with SW and the red ribbons when RW was used. Figure 2(b) illustrates the evolution of the maximum power densities obtained in function of time. It can be seen that there is an overall increase in maximum power density in each anolyte replacement (red triangles) when using SW, which was consistent with the concomitant maturation of the biofilm on the anode. It should be pointed out that the decrease in the peak values reached by polarization curves in the measurements immediate to the anolyte replacement are due to substrate depletion by action of microbial metabolism, which was the indicator to make a new batch cycle. During this enrichment phase, SW allowed for a maximum power density of $1.10 \mu\text{Wcm}^{-2}$ at day 27, while the mixture of SW and RW used prior the transition to RW generated a slightly greater value of $1.14 \mu\text{Wcm}^{-2}$ at day 29. Figure 3(a) shows the behavior of the current density over time when using a 100Ω resistance from the beginning of the experiment to day 56, and from day 57 to 65 when the resistance was changed to $2.74 \text{ k}\Omega$ (hence the sudden drop in current levels) to establish the conditions for maximum power and calculate C_E . Current monitoring was essential for the experiment because it allowed the determination of the HRT, which was used to decide when to replace the volume of anolyte and start a new batch. Current density behavior over time, as can be inferred from the graphic, followed a similar pattern as that of maximum power density, reaching a maximum value of $6.30 \mu\text{A cm}^{-2}$ with RW at day 42. Figure 3(b) shows three representative polarization curves used to elaborate the ribbon diagram in figure 2. Data represented in this figure correspond to days 4, 26 and 46 of the experiment, and allow the observation of the increase in the electrochemical performance of the system over time when there are not considerable changes in substrate concentration. Maximum power incremented from $0.13 \mu\text{Wcm}^{-2}$ (day 4), going through $0.79 \mu\text{W cm}^{-2}$ (day 26) up to $1.11 \mu\text{W cm}^{-2}$ (day 46), which represents an increase of 850%. This tendency would obey the uniform biofilm structure formation to the conditions offered by the system to the bacteria and the increase in active biomass and decrease of void spaces in the biofilm matrix as it has been suggested by Read *et al.* ⁽²⁹⁾, and Zhang *et al.* ⁽³⁴⁾ which, at the same time, optimizes the transport of electron donors from the external surface of the biofilm to the interface biofilm-anode resulting in an increment of the electrogenic capacity.

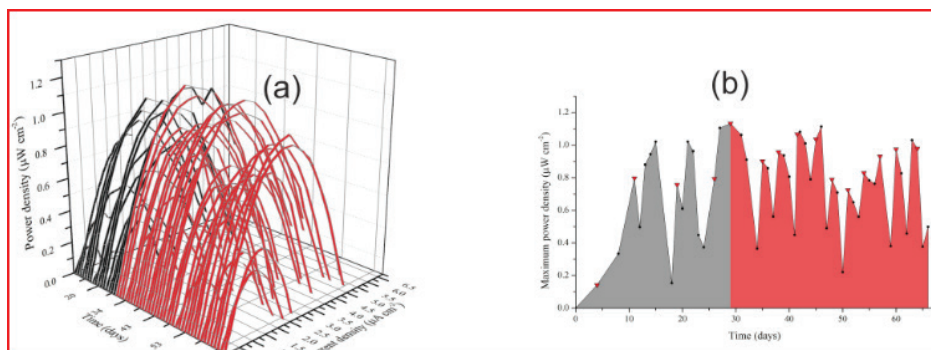


Figure 2. (a) Power density curves over time. Black and red ribbons correspond to curves made when using synthetic and real waste water respectively. (b) Maximum power density over time. The gray shaded area under the line indicates the use of SW as anolyte and the red shaded area the use of RW. The red triangles indicate anolyte replacement.

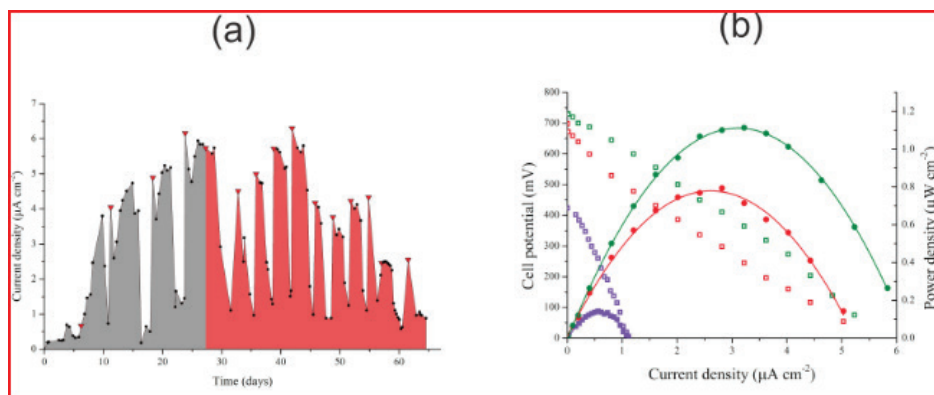


Figure 3. (a) Current density over time. The gray shaded area under the line indicates the use of SW as anolyte and the red shaded area the use of RW. The red triangles indicate anolyte replacement. (b) Cell potential vs. current density and power density vs current density curves taken at days 4 (purple), 26 (red) and 46 (green).

Operation with RW batches was marked at the beginning by a drop in the maximum power density in relation with the mixture of SW and RW, followed by a stepwise increase in successive substrate replacement until a considerable drop was observed at day 48. This drop in power density (as well as in current density, Figure 4) can be attributed to the dilution of the anolyte due to persistent rain in the sampling area diminishing the concentration of substrate (amino acids, sugars, volatile organic acids) and the available carbon sources. As power production in MFC follows saturation kinetics⁽³⁵⁾, a drop in substrate concentration reduces the magnitude of electron recovery by the system. The wastewater treatment plant from where samples of RW were collected serve 70 rural properties, two of which correspond to watering places that during rainy seasons discharge, irregularly, rainwater to the wastewater channels, causing a sudden increase in diluted water inflow to the wastewater treatment plant. The differences in the lower values of maximum power density of the MFC when using RW in comparison with the value obtained when using the mixture can be attributed to factors inherent to the complex nature of the anolyte, presence of growth inhibitors common in wastewaters (ammonia, sulfide, light metal ions) and introduction

of competing bacteria as it has been mentioned by Chen *et al.* ⁽³⁶⁾, and Lee *et al.* ⁽³⁷⁾. The maximum power density value reached when the MFC was fed with RW was $1.11 \mu\text{W cm}^{-2}$, similar to the value obtained with SW.

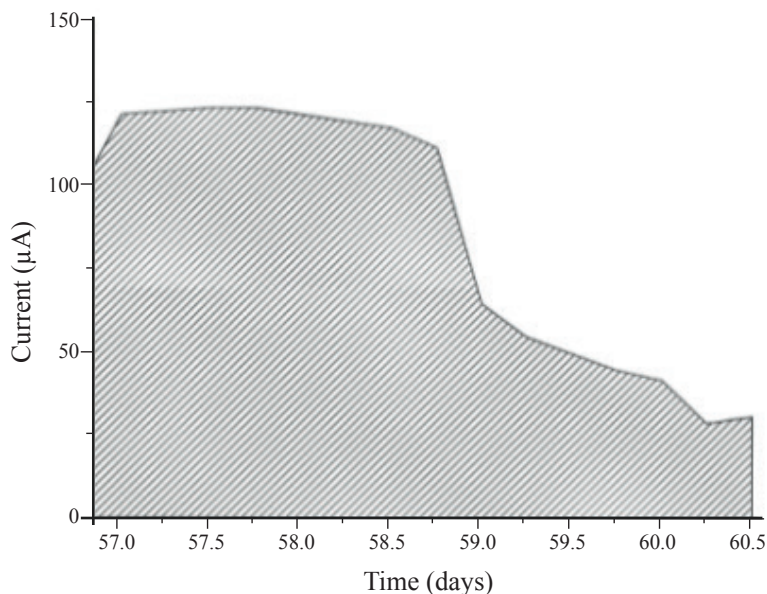


Figure 4. Current variation with time during the last batch at maximum power using a $2.74 \text{ k}\Omega$ resistance used to quantify.

As indicated by the proximity of the values of maximum power density when using SW and RW as substrate, the cell configuration could limit power generation and its effect may overlap with those produced by the chemical differences between the substrates. The electrode spacing was probably the factor with the greatest influence on the limitation of power, as it would be expected from a distance of 10 cm between anode and cathode including the ohmic drop due to the Nafion[®] membrane. In spite of reasonable ohmic drop, the electrogenic activity was considerable. Previous studies have shown that decreasing electrode spacing from 4 to 2 cm can increase by up to a 60% the power density of the system because of a lowering in its ohmic resistance ^(38,39).

Maximum power densities reported in this work are lower than those reported by Liu *et al.* ⁽⁸⁾ in a domestic wastewater feed MFC that produced $2.6 \mu\text{W cm}^{-2}$. This difference can be explained considering that, the MFC used by them was single-chambered and with a continuous flow of anolyte, which tend to increase power outputs. Our microbial fuel cell, with a significant ohmic drop, also presented a lesser cathodic area, and the PEM was physically separated from the cathode, not hot-pressed against it, which contributed to an increase in mass transport losses (proton exchange is hindered from the anode chamber to the cathode chamber). Higher values in maximum power density of $8.23 \mu\text{W cm}^{-2}$ with O_2 -saturated catholyte and $11.93 \mu\text{W cm}^{-2}$ using ferricyanide were obtained by Venkata *et al.* ⁽¹⁰⁾ in a dual-chamber MFC fed with domestic wastewater. They also performed this

experiment in continuous feeding mode, which together with the use of catholytes with higher reducing potentials than ours, explain the higher power outputs. In the same way, Min and Logan⁽²³⁾ working with a lab scale MFC reported a maximum power density of $7.2 \mu\text{W cm}^{-2}$, value that can be attributed to the reactor design, a flat plate MFC with aerated cathode.

3.2 Coulombic Efficiency and COD Removal Efficiency

Using the last batch to calculate the C_E , current was monitored for approximately three days at the fixed load under which power was maximum (2.74 k Ω). A coulombic efficiency of 7% was obtained, and although low, was comparable to those obtained in other studies in which complex mixtures of organic compounds were used: Min and Logan⁽²³⁾ reported a C_E of 6%, Antonopoulou *et al.*⁽⁴⁰⁾ using cheese whey as substrate obtained 1.9%, whereas Li *et al.*⁽⁴¹⁾ reported a value of 2.7%. These results are attributable to substrate use by competing methanogens or suspended bacteria in the anolyte. Studies where higher C_E were obtained, between 60% and 80%,⁽⁴²⁻⁴⁵⁾ used acetate as the sole carbon source from AW, in some instances coupled to ferricyanide in the catholyte. These factors favor higher C_E because prevent the entrance of alternative electron acceptors to the anodic chamber and control competing metabolic routes by using a simpler electron donor in the anolyte that is not fermentable. When using RW as anolyte, studies such as those done by Liu *et al.*⁽⁸⁾ (12%) and Rabaey *et al.*⁽²⁰⁾ (20%) report the highest C_E obtained in a domestic wastewater fed MFC. It should be remarked that in our work we evaluated performance in which voltage and current were maximum which at the same time resulted in greater resistances limiting the flow of current; Moreover, when compared with previous studies which report a higher C_E , our measurements differ from those made by other authors in that they made use of lower external resistances allowing larger currents regardless of power at such loads.

Physicochemical analyses of the MFC effluent showed a decrease of 76.15% in COD and 57.7% in SOC (Table 1) after 3 days. This is consistent with previous researches where decontamination capabilities in terms of COD removal fluctuated from 40% to 80% in a single chamber MFC Liu *et al.*⁽⁸⁾ suggesting a high potential for bioremediation of water contaminated with organic compounds using this technology. While the removal of organic matter is notorious, higher values could not be reached probably because of lack of flow of the anolyte inside the anodic chamber that could have contributed for the proper circulation of the nutrients in the anode.

This is obviously a limitation posed by the design of the cell used, and that can be easily overcome with stirring or other circulation methods provided by more sophisticated designs. Nevertheless, it is remarkable the percentage of COD removal in such a HRT when compared with a single chamber MFC with an air cathode that achieved the same removal of COD but at a longer solid retention time of 10 days⁽²⁸⁾.

Table 1. Physicochemical analysis performed on the real wastewater used as anolyte.

Sample origin	Analyses			
	COD (mgO ₂ L ⁻¹)	SOC (mgO ₂ L ⁻¹)	% COD removal	% SOC removal
Treatment plant influent	173.60	18.79	-	-
MFC effluent	41.40	7.95	76.15	57.70
Treatment plant effluent	41.80	11.31	75.92	39.81

It should be noted that electrogenic activity cannot be pointed as the sole responsible for the removal of organic matter, since it has been shown that near 30% of COD removal is not due to electrogenic phenomena but to parallel metabolic processes that use nitrate and sulphate as electron acceptors and that are very common in domestic wastewater⁽⁴⁶⁾. Furthermore, direct oxidation of organic matter by oxygen diffusion from the cathode chamber could also contribute to the direct oxidation of carbon sources in the anode chamber, as it could happen in our case where a Nafion[®] membrane is used. To avoid this O₂ diffusion, Choi and Chae⁽⁴⁷⁾ used membranes of polydimethylsiloxane (PDMS).

3.3 SEI Analyses of the Biofilm

SEI micrographs from biofilms on the anodic carbon cloth taken after 15 days of operation with AW predominantly showed bacilli of different sizes and some coccobacilli immersed in the gelatinous biofilm matrix (Figure 5). SEI micrographs show individual bacteria making direct contact with the electrode and bacterial clusters of varying thickness. Pili extending from the surface of the cells can also be observed and could be serving as nanocables to transport electrons to the electrode surface. It can be suggested that the bacteria located in the upper layer of the clusters might be fermenting complex sugars to produce simpler volatile organic acids used by bacteria in the lower layer of such clusters (or by those directly attached to the electrode) with direct electrode reducing capability. Nevertheless, bacterial species stratification in the biofilm following electron donor transportation of fermentation products requires further analysis.

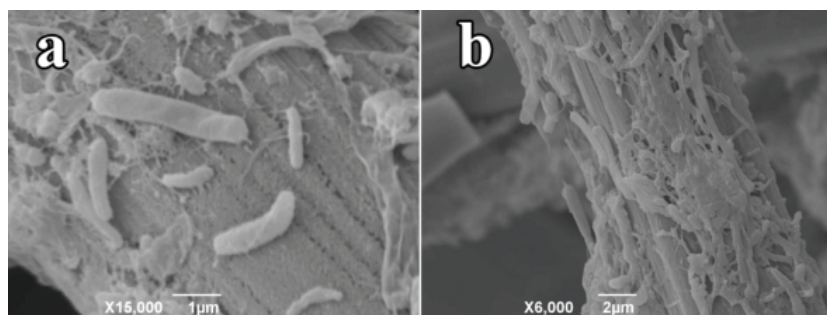


Figure 5. SEI micrographs of bacilli and coccobacilli adhering to anodic carbon cloth surface after 15 days of operation of the MFC. (a) Some of them are shown individually attached to the carbon cloth with visible pili extending out of them (b) others are shown in clusters.

After 60 days of operation, the change in cell diversity was notorious. Operation with RW favored the proliferation of spirilla, cocci and some club-shaped cells as shown in Figure 6. Bacilli and coccobacilli were also observed, but their abundance was much lower than the observed when the cell was operated with AW. The increase in cell diversity is correlated with the increase in substrate types and contributes to explain the decrease in power density levels as pointed out by Chae *et al.* ⁽⁴⁸⁾. Highly diverse MFC tend to produce lower power outputs because of the presence of prokaryotic species capable of competing for organic sources against exoelectrogenic bacteria, which are selected with time as the system becomes more efficient with increases in abundance of electrochemically active bacteria.

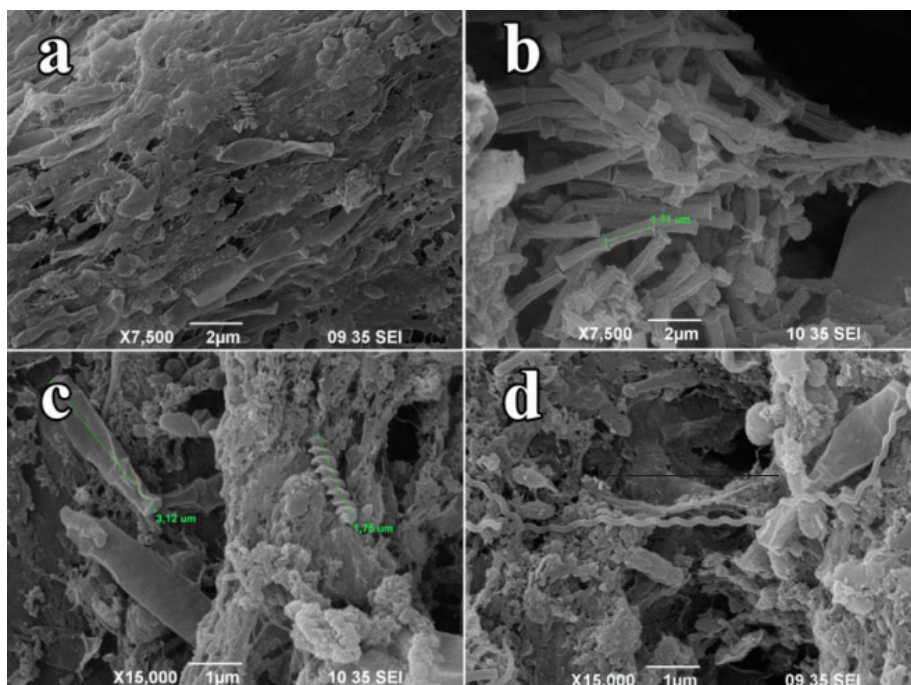


Figure 6. SEI micrographs of prokaryotic cells after feeding the MFC with real wastewater: (a) Groups of club-shaped bacteria), (b) Archaeobacteria of genus *Methanosaeta*, (c and d) spirilli introduced with the change of anolyte.

The presence of at least two morphologically distinct species of spirilli as observed in figure 6 (c and d), belonging to the filum Proteobacteria provides direct observation of electro active bacteria feeding on formic acid as reported by Kiely *et al.* ⁽⁴⁹⁾. This acid is usually present in relatively high concentrations in wastewater of domestic origin. Furthermore, large clusters of cylinder-shaped cells joined at the ends forming thread-like structures correspond to the morphologically characteristic methanogenic archaeobacteria belonging to the genus *Methanosaeta* as described by Araujo *et al.* ⁽³²⁾, which reduces the available lactic acid to produce methane. This can be correlated with the low C_E , due to the decrease in organic compounds available for electrogenic metabolism and with the relatively high efficiency in organic matter removal as evidenced by the decrease in COD ⁽⁷⁾.

3.4 Isolation and Biochemical Characterization of Anodic Bacteria

Four bacterial strains were isolated in a thioglycolate medium, two Gram-positive and two Gram-negative. Growth pattern in test tubes (uniform turbidity from the bottom to the top of the liquid medium in the test tubes) prior plating suggested presence of aerotolerant anaerobic bacteria. Kim *et al.* ⁽⁵⁰⁾ isolated anaerobic bacteria from their MFC, and also obtained a low number of isolated bacteria and suggested that uncultivable bacteria might be depending on syntrophic relationships to grow on the anode as seen in SEI analyses. Such relationships are not maintained when growth on nutrient medium is attempted. Table 2 shows the results of sugar fermentation biochemical tests done in three of the four isolated bacteria. It can be seen that the Gram-negative bacteria and one of the two Gram-positive bacteria showed sugar fermentation capabilities, suggesting that these isolates are not directly involved in the electrode reducing pathway and they are rather decomposing complex carbon sources providing non-fermenting bacteria with simpler substrates. The other isolated Gram negative bacteria was not capable of reducing any of the sugars tested, suggesting that this particular isolate may be reducing the electrode directly while oxidizing simpler organic molecules. This is also an indicator of a syntrophic relationship among bacteria in the MFC that contributes to the overall generation of electricity of the system. Kim *et al.* ⁽⁵⁰⁾ pointed out that electrochemically active bacteria are generally limited to simple organic acids as electron donors such as simple organic acids and are dependent on the fermentation products by fermentative bacteria in the anode biofilm.

Table 2. Biochemical tests on carbohydrate fermentation capabilities on isolated bacteria.

Substrate	Gram Positive		Gram Negative	
	Isolate 1	Substrate	Isolate 2	Isolate 3
Arabinose	+	Trehalose	+	-
Manose	+	lactose	+	-
Sucrose	+	Sucrose	+	-
Melobiose	+	Mannitol	+	-
Sorbitol	+	Maltotriose	+	-
Galactose	+	Arabinose	+	-
Ramnose	-	Glycerol	+	-
Mannitol	-	Fructose	+	-
Adonitol	-			

3.5 16S rDNA Analysis

A total of three OTUs were obtained after cloning and sequencing the extracted 16S rDNA from the anodic bacteria. Table 3 shows the list of the clones with the identification of the accessions in GenBank which have the highest identity. Sequences of the bacteria present in the anode showed the greatest identity with sequences of uncultured species only identified by means of molecular biology tools. Identity percentages of clones 2

and 3 (97, 96 and 95%) might be suggesting the microbial enrichment of novel bacteria in our MFC. As it has been reported in most works analyzing the microbial composition of the anodic community of MFC, phyla Firmicutes (Clone 1), δ -Proteobacteria (Clone 2) and α -Proteobacteria (Clone 3) were detected in our MFC.

Table 3. Recombinant clones comparison for identity percentage with NCBI accessions.

Clone	Most closely related species	Source	Identity %
1	Uncultured bacterium clone E-38 (KC835957)	Bacterial community composition shifts in response to microcystis bloom - an enclosure experiment (Unpublished data)	99
	Unculturable Firmicutes QEDN3BD0 Clone (CU925891)	Mesophilic anaerobic digester (Riviere <i>et al.</i> 2009)	
2	Unculturable bacteria clone N1_3_694 (FJ534955)	Anaerobic sludge digester (Lee <i>et al.</i> 2012)	97
	Unculturable <i>Smithella</i> sp. Clone wn95 (JQ012317)	Activated sludge (Unpublished data)	
3	Unculturable bacteria Clone Ac-2 (HQ602890)	Laboratory scale wastewater treatment reactor (Li <i>et al.</i> 2011)	96
	α -Proteobacteria Clone AS-45-1 (GQ406147)	Sediment sample from benthic ecosystem (Nold <i>et al.</i> 2010)	

Predominance of Firmicutes in MFC has been reported when using propionate as substrate and this phylum has been reported as one of the integral members of the anodic community⁽⁵¹⁻⁵³⁾. On the other hand, identity percentage of the sequence of the clone 2 with *Smithella* sp. offers support to the observed presence of methanogenic archaea with SEI and their syntrophic association, given the fact that the abovementioned bacteria can only grow syntrophically with methanogenic archaea of the class Methanomicrobia, to which *Methanosaeta* sp., belongs⁽⁵⁴⁾. Proteobacteria has been usually identified as the predominant phylum in anodic communities^(17,24,55-57) containing well-characterized electroactive species such as *Geobacter* and *Shewanella*^(45,56-58). Since none of the sequenced OTUs showed identity with these electrochemically active species, it is expected that novel, yet to be characterized bacteria of the same phylum are contributing to the energy generation.

4 Conclusions

The present study showed the ability of bacteria from anaerobic sludge in a domestic wastewater treatment plant, serving a small rural community in Colombia, to produce a maximum power density of 1.11 $\mu\text{W cm}^{-2}$ and a coulombic efficiency of 7%. These results suggest feasibility of applying MFC technology to the treatment of domestic wastewater in small communities located in temperate climates as suggested by the decrease in COD content. It must be remarked that systems with continuous feeding mode must also

be evaluated in order to assess the full potential of the technology for these particular conditions. Biochemical tests on isolated bacteria showed metabolic heterogeneity on the capability to ferment different sugars, which suggests syntrophic relations among the members of the microbial community of the anode. SEI observation showed an increase in cell diversity, which is expected given the fact that substrate diversity also increases when artificial wastewater operation shifts to real wastewater operation. In addition, the presence of methanogenic archaea hinders the overall performance of the MFC, as suggested by SEI and supported by 16S DNA analyses. This factor, along with the decrease in substrate concentration due to a rainy season, contributes to explain the relatively low power outputs and coulombic efficiency. Finally, 16S DNA gene analysis suggests the presence of new species of unidentified bacteria that could be potential electrode reducers. Tests with pure culture inoculated MFCs are being performed with isolated bacteria to assess their individual contribution to electricity generation.

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Abbreviations

AW: Artificial wastewater.
 CE: Coulombic efficiency.
 COD: Chemical Oxygen Demand.
 DOC: Dissolved Organic Carbon.
 HDMS: Hexamethyldisilazane
 HRT: Hydraulic Retention Time.
 MFC: Microbial Fuel Cell.
 OTUs: Operational Taxonomic Unit
 SEI: Secondary Electron Imaging.
 SRT: Solid Retention Time.
 RW: Real Wastewater
 SW: Synthetic Wastewater.

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