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Bioelectricity generation using long-term operated biocathode: RFLP based microbial diversity analysis

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ABSTRACT

In the present work, power generation and substrate removal efficiencies of long-term operated microbial fuel cells, containing abiotic cathodes and biocathodes, were evaluated for 220 days. Among the two microbial fuel cell (MFC) types, the one containing biocathode showed higher power density (54 mW/m^2), current density (122 mA/m^2) coulombic efficiency (33%), and substrate removal efficiency (94%) than the abiotic cathode containing MFC. Voltammetric analysis also witnessed higher and sustainable electron discharge for the MFC with biocathode, when compared with the abiotic cathode MFC. Over the tested period, both MFC have shown a cell voltage drop, after 150 and 165, days, for the MFC with biocathode and abiotic cathodes, respectively. Polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) analysis identified 281 clones. Bacteria belonging to Acinetobacter, *Acidovorax, Pseudomonas* and *Burkholderia* were observed in the abiotic cathode MFC. Bacteria belonging to *Geobacter, Cupriavidus* and *Acidobacteria* were observed in the biocathode MFC. Almost similar types of archaea (*Methanosarcinales, Methanolinea, Nitrososphaera* and *Methanomicrobiales*) were observed in both MFCs.

1. Introduction

Bioelectrochemical system (BES) is an electrochemical system in which biocatalysts perform oxidation and/or reduction of substrates at electrodes [1–3]. Electrons produced by microorganisms through the substrate degradation are moved to the anode and then to the cathode [4,5]. Microbial fuel cell (MFC) are one of the prominent types of BESs, where microorganisms oxidize organic and inorganic materials to produce energy. The anode of MFC works as electron acceptor and moves the electrons to the cathode through a resistor and produce electricity [6–8]. Abiotic cathode microbial fuel cell (in this work named MFC-1) system has been half biological, because only the anode side consists of electrochemically-active microorganisms. The biocathode system in microbial fuel cell (here named MFC-2) contains microorganisms functioning as biocatalysts in the anode, motivating the degradation of organic substances to produce electrons, which travel to the cathode (also contaianing microorganisms) side through an electric circuit. The presence of free electrons on the cathode of MFC initiates a reduction response of oxygen to produce water. In this case, microorganisms, namely bacteria, will take electrons at the cathode by the reduction of electron acceptors such as nitrate, sulfate, perchlorates and metals [9–12]. Biodegradable organic matter is, thus, converted into electricity, hydrogen or other value added materials through MFCs [13–15]. In several studies, researchers used human waste [16], agricultural waste [17] and food waste [18] in MFCs to produce energy or chemicals.

Lately, biocathodes in microbial cells gained focus for the removal of different pollutants due to their low cost, use of self-regenerate catalysts, and maintainable power supply [19,20] and developments were made by using diverse microorganisms as biocatalysts also on biocathodes [19–22]. Studies have reported that MFCs performance can be enhanced

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by the action of microorganisms present at the cathode [21,22,25–27]. Biocathodes which contains biofilms are not only able to catalyze the oxygen reduction at the cathode, but also show comparable or higher performances than abiotic cathode, such as the case of some metal reducing bacteria when in the cathodic chamber [28,29]. Eventhough biocathodes have great potential in MFCs technology, issues such as performance of biocatalysts at terminal acceptor conditions, cathodic activation overpotentials [30,31], biofouling, accumulation and competition of metabolites [32,33] need to be solve aiming its future widely application. For that goal, long-term operation and the scaling-up of biocathodes need to be further discussed to make the biocathode MFCs technology more practicable.

In order to understand the structure and function of the ecosystem in an MFCs, the microorganisms should be identified (and quantified). Advanced molecular methods provided new insights, so isolation and cultivation of microorganisms are no longer required to get information about microbial communities [34]. 16S rRNA based sequencing is the most popular method used for the identification of microbial communities present in soil, sea, river, lake and wastewaters [35]. Currently, researchers are using the on-line tools available through the "Entrez Programming Utilities (E-utilities)" https://eutils.ncbi.nlm.nih.gov/entrez/eutils/) to compare the sequence from various microorganisms throughout the world. However, only a limited number of researchers worked on the electricity generation using long-term operated biotic MFC together with analysis of microbial diversity using polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) technique. Hence, the objectives of this study is the evaluation of the long-term operation of MFC-2 (containing an aerobic biocathode), regarding its overall performance, including power generation and treatment efficiencies which were analyzed and compared with the conventional MFC-1 (which contains an abiotic cathode). The MFCs performance was evaluated based on the parameters like power density (PD), columbic efficiency (CE), open circuit voltage (OCV), and current density (CD); the substrate degradation was evaluated through chemical oxygen demand (COD) and the organic compounds removal efficiency. The microbial populations present in the long-term operation MFCs reactors were evaluated using PCR based RFLP technique.

2. Material and methodology

2.1. Biocatalyst

Mixed aerobic consortium was collected from the wastewater treatment plant (Chelas, Lisbon). It was inoculated in both microbial fuel cells (MFC-1 and MFC-2) anode compartments and in the cathode compartment of MFC-2 (biocathode). MFC-1 cathode was not inoculated with aerobic consortium, so it is considered as an abiotic cathode.

2.2. MFCs operation

Two chambered MFCs (abiotic cathode MFC-1, biocathode MFC-2) with equal working volume (0.36 L) were assembled in the laboratory using acrylic glass material for the anodic and cathodic compartments. Compartments were separated by a Nafion-cation exchange membrane; graphite felts (6 × 6 cm; 5 mm thick; surface area 36 cm^2) were used as electrodes. Oxygen (from air) was provided through an air-pump to the cathodes to maintain the dissolved oxygen concentration. For sampling ports, provisions were made at appropriate places.

MFC-1 and MFC-2 scheme and image are shown in **Fig. 1** and **SFig. 1**, respectively. Both MFCs anodic chambers were fed with simulated wastewater comprising of 0.82 g/L sodium acetate, and 0.31 g/L of sodium carbonate in the biocathode chamber (MFC-2) as the sole carbon sources. MFC-1 cathodic chamber contains 50 mM phosphate buffer. The composition of nutrient solution used in the anodic and biocathode chambers were described in previous reports [26,36]. The pH of the simulated wastewater was adjusted to7.0 in both the anodic and cathodic chambers. After the feed change, both anodic chambers were purged with N_2 gas for at least 20 min to restore and maintain the anaerobic micro environment. The two MFC cathodic chambers were continuously supplied with air through an air-pump (ELITE −801, Holf C. Nagen, UK Ltd.) to maintain the oxygen and electron acceptor constant. Once the voltage dropped to 50 mV, the media were changed and the biomass was reserved to form a complete fed-batch cycle. MFCs were operated at room temperature (25°C) for 220 days (total of 28 fed-batch cycles); electrodes were connected through copper wires, isolated from the solution, using a fixed load of external resistance of 1000Ω.

2.3. Analysis

Voltage and current produced by MFCs were measured using a digital multimeter (Model 2700, Keithley, USA). Power density (PD, in mW/m^2) was calculated by the anode surface area and net liquid volume of the anodic compartment. Polarization studies were carried out by varying the external resistance in the range of 15–10000Ω. Electrochemical analysis was performed using a potentiostat (CHI 440B, CHI Instruments, USA), considering the chambers anode and cathode carbon felts as the working electrodes and/or secondary electrodes in the opposite chamber to close the circuit; an Ag/AgCl reference electrode was used by introducing it in the anodic or cathodic chambers by the available setup ports. Cyclic voltammetry (CV) was performed at a scan rate of 20 mV/s in the potential window from +0.8 to −0.8 V. The chemical oxygen demand (COD) influence and effluent concentration were measured according to standard methodology [37]. The p^H (Metrohm, model 691), conductivity (OAKTON, model 35,607–20) and dissolved oxygen (Hach, model HQ40D) were measured with portable digital meters. The coulombic efficiency (CE) was calculated by integrating the measured current over time, relative to the possible maximum current based on the observed chemical oxygen demand

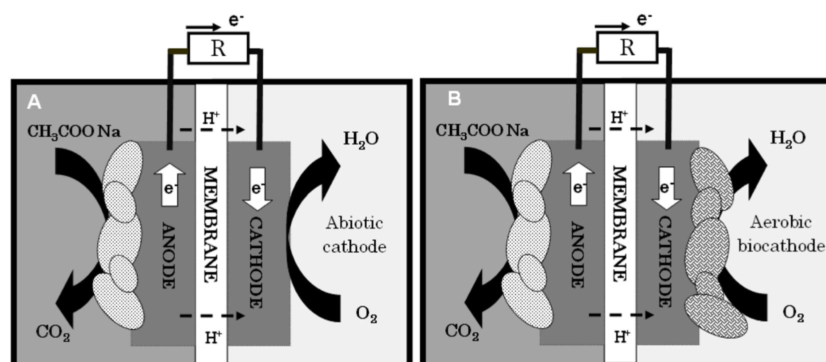


Fig 1. Schematic drawings of the two chambered microbial fuel cells, (A) MFC-1 (abiotic cathode); and (B) MFC-2 (biocathode) with different cathode conditions.

(COD) removal (**Eq. (1).**). The CE evaluated over a period of time 't' is calculated as reported elsewhere [38].

$$CE = \frac{M \int_0^{t_b} I\ dt}{Fbv_{An} \Delta COD} \quad (1)$$

Eq. (1). Coulombic efficiency determination equation

In Eq. (1), M is the molecular weight of oxygen (32 g mol^{-1}), F is Faraday's constant (96,485 C mol^{-1}), b = mol of electrons, ν_{An} is the volume of liquid in the anode compartment, and ΔCOD depicts the change in COD over a period of time (t_b).

2.4. Cloning and restriction fragment length polymorphism (RFLP)

Identification of microorganisms was done according to the protocol from Ramos et al., 2010 [35]. 16S rRNA gene sequence analysis was done to identify the taxonomic affinities of a broad range of taxa [39]. Universal primers for archaea and bacteria were chosen (based on previous literature) to amplify the partial sequence of 16S rRNA in order to comprise the largest number of microorganisms. Nucleotide sequences of 16S rRNA encoding genes were retrieved from the Ribosomal Database Project [40] and GenBank (https://www.ncbi.nlm.nih.gov/genbank/). The details of primers used are presented in **Table S1.**

Biofilm samples were obtained from the two MFCs anodes and biocathode chambers, which were operating continuously for more than 220 days using synthetic wastewater at different organic loads. Biofilm samples were collected and stored at −80°C. Total DNA was extracted using the High Pure PCR Template Preparation Kit (Roche) according to the manufacturer's instructions. PCR amplification was done using the purified DNA according to the protocol from Ramos et al., 2010 [35]. Amplified fragments were purified from agarose gels using the Jet Quick kit (Genomed) according to the manufacturer instructions. Purified fragments were then ligated into the TA region of the pCR 2.1 cloning vector and screened according to the protocol from Sambrook and Russel,2001 [41]. Plasmid DNA from the selected clones was purified using the Miniprep plasmid kit (Qiagen), and 200 ng of plasmid DNA were digested with 1 U of restriction enzyme *Hae*III for 30 min at 37°C. RFLP was carried out according the procedure described elsewhere [35], the restriction patterns obtained were stored and analyzed with the Lab Image 1D software http://www.labimage.com/1dsoftware/labimage/index.html. Plasmids containing correct size inserts were sequenced by Eurofins MWG. The sequences obtained were compared in a pair wise mode and in the BLAST searches [42]. Sequences obtained in this work and exhibiting an overall pair wise identity with sequences deposited in databases equal or lower than 98% were considered as new according to the protocol of Skirnisdottir et al., 2000 [43].

Microbial populations present in the long-term MFC reactors were evaluated using polymerase chain reaction (PCR) based restriction fragment length polymorphism (RFLP) technique. Comparisons were made between the sequences identified in the current work and sequences present in data bank to determine the structure and function of microbes. RFLP analysis of the initial 281 clones with *Hae*III allowed the identification of only 39 distinct profiles. Both reactors showed dominance of organisms involved in electricity generation in MFC. Results from the molecular characterization of the microbial populations are consistent with the literature.

3. Results and discussion

3.1. Power generation

Both anodic chambers in MFC-1 and MFC-2, and the cathodic chamber in MFC-2 were inoculated with 90 mL of aerobic activated sludge, and fed with 270 mL of the simulated wastewater. After the bacteria enrichment, a slow increment of the cell voltages were observed, with a stabilization at the 5th day (after 2 fed-batch cycles) and the 16th day (after 5 fed-batch cycles) for MFC-1 and MFC-2 respectively (**Fig. 2**). The difference in time for achieving cell voltage stabilization was already reported by other authors and interpreted as the biocathode conditions implies more time for the bacterial growth and consequent stabilization [21,22,44]. Under stabilized conditions, MFC-2 (biocathode) exhibited an open circuit voltage (OCV) of 439 mV, with an external resistor of 1000 Ω, power density (PD) of 54 mW/m^2 and current density of 122 mA/m^2. The values are comparable with those observed for MFC-1 (abiotic cathode) (**Table 1**). The voltage dropped after 150 and 165 days for MFC-2 and MFC-1, respectively (**Fig. 2**). In the first stage, the voltage dropped to 233 mV with PD of 15 mW/m^2 and current density of 65 mA/m^2 for MFC-2; for MFC-1 the voltage dropped to 330 mV with PD of 30 mW/m^2 and current density of 92 mA/m^2 (**Table 1**). In the second stage, after 200 days, the values continued decreasing for both MFCs (**Fig. 2**). This decrease might be due to the reduction of the sinergetic metabolic activity of the microorganisms in the anodic chambers, together with the biofouling of the nafion membrane, subsequently contributing to a diminuished electron transfer efficiency for longer periods of time [45,46]. From the obtained results it is possible to conclude that both the MFCs showed comparable results for the parameters under analysis.

3.2. Polarization curves in MFCs

Polarization curves (PC) provide information regarding the different type of voltage losses (activation, mass transfer and ohmic drops) in MFCs due to different aspects such as kinetics of the electrochemical reactions and the internal electrical and ionic resistances. Polarization studies were conducted at different external resistances from 15 to 10,000 Ω for both MFCs; the measurements were performed taking the slope of the plot of voltage versus resistance (**Fig. 3a**).The measured OCV for both MFCs is the maximum voltage that can be obtained within the system. PC and power curves for MFC-1 and MFC-2 were determined. The OCV for MFC-1 was 516 mV (no current, infinite resistance) and the voltage falls to 449 mV at a current density of 194 mA/m^2, and linearly with the current after that point. The highest power density obtained was 52 mW/m^2. For MFC-2 the OCV was 521 mV (same conditions) and the voltage falls to 456 mV at current density of 214 mA/m^2 and also linearly with the current after that. In MFC-2, the highest power density obtained was 54 mW/m^2 (**Fig. 3b**). The PC curves for the two MFCs revealed likely patterns, in which the cell voltage dropped rapidly at low currents and was followed by a slow and near-linear drop in the remaining region. This behavior, may be attributed to the activation and ohmic losses in both MFCs. The voltage behavior in the PC supports the premise of losses during electron transfer in fuel cells [44,45,47]. Surface reactions, either oxidizing or reducing compounds, require some activation energy and are associated to those losses [44,47]. Aerobic metabolism at the cathode contributes to lower the activation losses by enhancing the final reduction reaction. Also, ohmic drops are triggered by electrical resistance of electrodes, electrolyte and membranes [44,47]. Mass transfer losses occurs when the substrate is oxidized faster at the anode, producing more electrons than those that can be transported to the cathode. From the results, it can be infered that the biocathode in MFC facilitates the reduction resulting in faster electron capture at the anode.

3.3. Wastewater treatment efficiency

The performance of both MFCs were also evaluated for the wastewater (substrate) treatment efficiency through the reduction of chemical oxygen demand (COD%) at different time periods. COD removal efficiency were observed in both MFC operations, in the first 3 and 4 fed-batch cycles for MFC-1 and MFC-2, respectively. Both MFCs stabilized in the next 18 fed-batch cycles in which the higher COD removal efficiencies varied between 81% to 90% (**Fig. 4**). MFC-2 (biocathode) has shown slightly higher COD removal efficiency (94%) with the coulombic

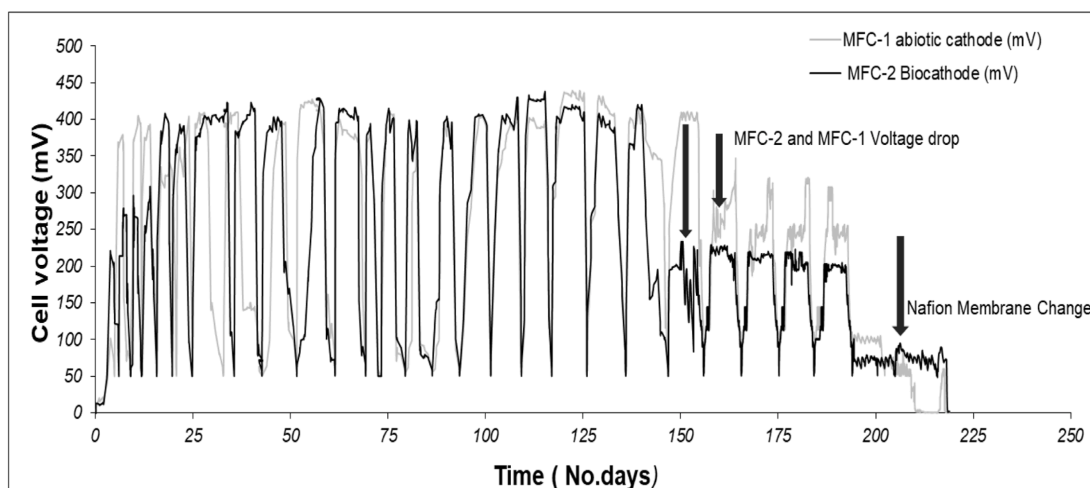
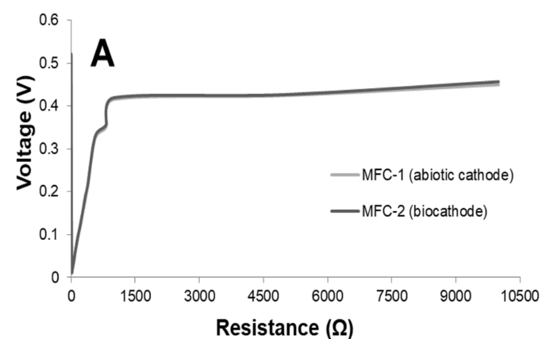


Fig 2. Cell voltage with time for the long-term operation of abiotic cathode (MFC-1) and biocathode (MFC-2).

Table 1
Consolidated data obtained during operation of both MFC-1 and MFC-2.

Characterstics	Biocathode (MFC-2)		Abiotic (MFC-1)	
	BVD	AVD	BVD	AVD
Batch mode operation time (days)	150	-	165	-
[1]OCV (mV)	439	233	435	330
Power density (mW/m^2)	54	15	51	30
[2]COD removal efficiency (%)	94	66	90	69
Coulombic efficiency (%)	33	15	31	15
Current density (mA/m^2)	122	65	121	92

[1]OCV: open circuit voltage;.
[2]COD: chemical oxygen demand; BVD: Before voltage drop; AVD: After voltage drop.



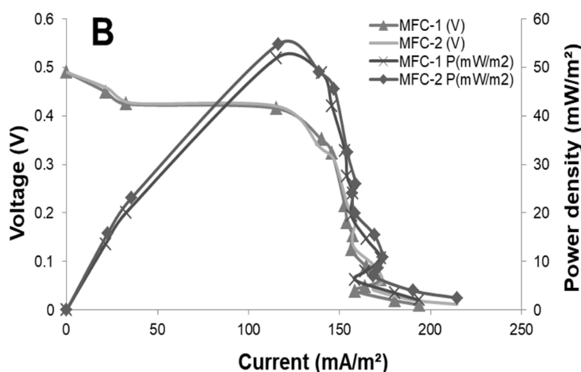


Fig 3. (A) Cell voltage as a function of resistance, and (B) Polarization curve plotted with the voltage and power density against current density.

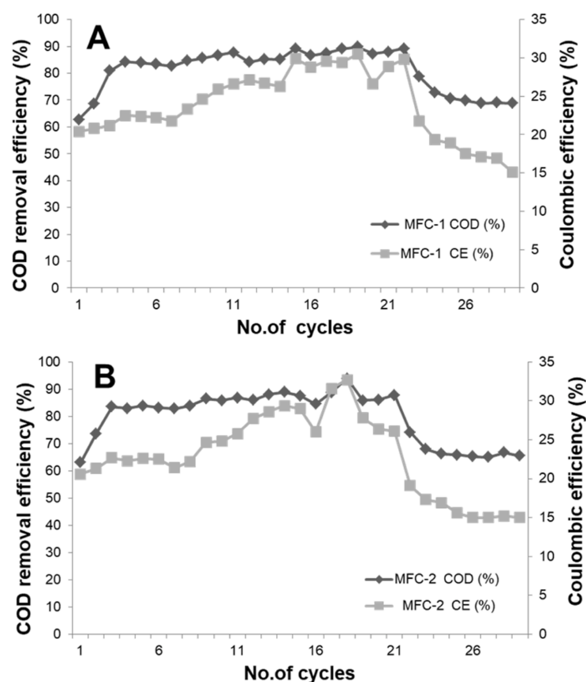


Fig 4. Differences in COD removal (%) and CE efficiency (%) for (A) MFC-1; and (B) MFC-2, at various operating cycles.

efficiency (CE) of 33%, than MFC-1 that showed 90% of COD removal efficiency and 31% of CE (Table 1). The availability of oxygen and convenient redox partners and respective compatible potential values at adjacent neutral conditions in the MFC-2 chamber supports the fast metabolic activities of the bacteria leading to the slightly higher values of COD removal efficiency. Consumption of protons and electrons in the aerobic metabolic process seem to be responsible for the maximum COD removal efficiencies obtained for longer period operations in MFCs [5, 21,23,24,45]. Over the tested period when the voltage drops in MFCs, COD and CE removal efficiencies also decrease in both MFCs (Fig. 4).

During the operation, the pH values were measured at the starting and final time of each fed-batch cycle (Fig. 5). The pH was found to be stabilized at neutral condition (7.0) in the anode and cathodic chambers for almost all cycles. In few cycles, the pH at anode chambers was found to have a slight variation between 6.9–7.3. The pH has stabilized in the range of 6.5–7.3 in cathodic chambers. The pH stabilization is due to the

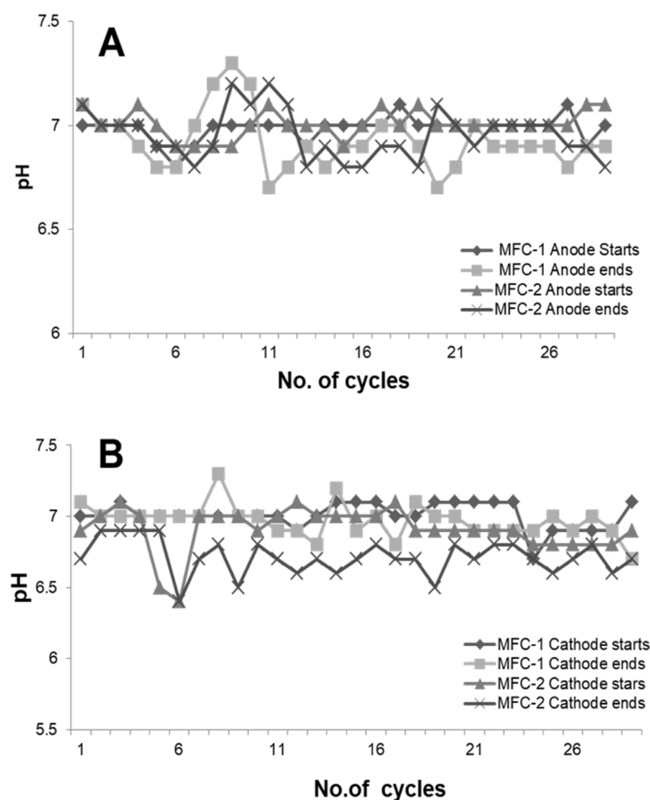


Fig 5. Variations in pH profiles in each fed-batch cycle for (A) anodic and (B) cathodic chambers of MFCs.

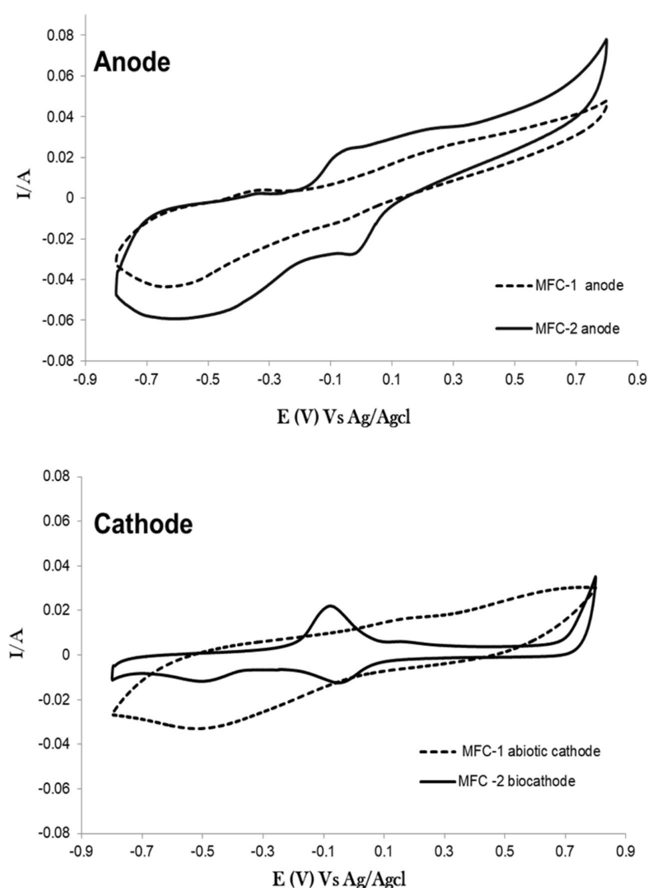


Fig 6. Typical cyclic voltammograms from anodes and cathodes of the two type of MFCs.

bicarbonate buffering mechanism, inspite of the continuous reduction reactions occurring in the cathodic chambers. The *in-situ* buffering mechanism in the cathodic chamber that prevents the pH drop is vital for the ongoing reduction reactions and for the power generation and the maximum substrate degradation [45].

3.4.Electrochemical. behavior of biotic and abiotic MFC cathodes

Cyclic voltammetry (CV) is a helpful tool to elucidate the electrochemical reactions occurring at the electrodes surface. The electrochemical behavior of the MFC chambers was evaluated by CV during operation and current generation, *in-situ* using the anode or the cathode in each chamber as working electrodes. Cyclic voltammograms obtained using the MFC anodes (anaerobic conditions) as working electrodes demonstrated distinctions between the MFC-1 and MFC-2, although the initial media and electrodes are the same in both anodic chambers (Fig. 6). Around 0 V vs Ag/AgCl a well defined redox process in MFC-2 is visible, where in MFC-1 is almost unnoticeable, and probably results from small contamination from the cathodic chamber (probably though the membrane). As expected, the electrochemical behavior of the MFC-1 and MFC-2 cathodes operating in aerobic conditions are clearly distinct (Fig. 6). Once more, a well-defined redox process, close to 0 V vs Ag/AgCl, is observed in the MFC-2 that also shows lower capacity current. An anodic process around +0.2 V and a counterpart cathodic process at −0.5 V vs Ag/AgCl are observed for both MFC-1 and MFC-2. These processes were already detected in the anodic chambers and were associated to oxygen reactions. MFC-2 (biocathode) presents an enhanced anodic current starting at +0.7 V vs Ag/AgCl, not found at MFC-1 that seems similar to the behavior observed for the anodic chambers and was assigned to microorganisms metabolism. In general, the electroactivity observed in the biotic aerobic chamber is higher than in the abiotic one. The observed redox processes are consistent with the presence of extracellular active redox components, resultant from the microorganisms metabolism [48,37], and a more extended analysis of these components is currently in progress. The electrochemical behavior observed in biocathodes indicates that the microorganisms population in the chamber influences the exchange current density enhancing the electron transfer from and towards the electrode, in agreement with other reports [49–51]. Additionally, in the biocathode the oxygen reduction wave is found around −0.5 V vs Ag/AgCl, being less pronounced and starting at more negative potential values. This may be due to a slowed down diffusion towards the electrode surface due to biofilms' presence. Yet, in spite of less extent oxygen reduction in the electrode surface, the operation of the MFC-2, as discussed before, is comparable to MFC-1, which points to the active role of microorganisms as electron acceptors, also in agreement with other reports [33,37].

3.5. Microbial diversity analysis

One of the goals of this work is to identify the microbial communities present in the MFC's abiotic and biotic cathode. In conventional methodology, several biological methodologies, such as DNA isolation, PCR amplification, cloning, and sequencing are performed. All these methods are time consuming, so, in here it was decided to perform the cloning and restriction fragment length polymorphism (RFLP) analysis of clones before analyzing their nucleotide sequences, foreseeing the minimization of the time and methodologies. This strategy was already used by Ramos et al., 2010 [35], as stated before, and is based on the combination of DNA isolation, PCR amplification, cloning, and sequencing of plasmids initially characterized by RFLP using the recurrently cutting restriction enzyme *Hae*III. Using this methodology, 281 clones were identified, and among these, 104 discrete profiles were observed.

3.5.1. Optimization of PCR conditions

After DNA isolation, optimization studies were done for the PCR amplification, to amplify the DNA isolated from the organisms present in MFC-1 and MFC-2 cathodes biofilms used in the long-term operation. Optimization was done by varying the temperatures from 50°C to 72°C by using three pairs of primers as mentioned in **Table S1**. Among various annealing temperatures, best amplification was observed at the temperature of 58°C with bacteria and archaea. Amplified PCR products were loaded on agarose gels. To amplify the bacterial domain, two sets of primers were used, both the primers showed good amplification results, but the one set of primers showed more intense bands than other. So, further amplifications were carried out using those primers. The expected size of the amplified products was 605 bp. Moreover, the best temperature to use in the amplification is 58°C for both the domains (bacteria - A) and (archaea- B).

3.5.2. RFLP and sequencing analysis

After optimization of experimental conditions, PCR amplification was carried out and expected gene sizes for bacteria and archaea were obtained and confirmed through agarose gel electrophoresis. But, expected PCR amplification results were not obtained with the biofilm collected from the cathode of MFC-1 and MFC-2. The amplified products obtained were purified using a commercial kit and then cloned into the pCR2.1 cloning vector. The resulting ligation mixture was transformed in *E.coli* DH5α competent cells, and the transformants were spread onto the surface of LB solid medium supplemented with appropriate antibiotics. Therefore, plasmid DNA from the selected clones was purified using the Miniprep plasmid protocol. The presence of plasmid DNA was confirmed trough electrophoresis gel and DNA concentration was estimated using a NanoDrop ND 1000 spectrophotometer (**SFig. 2a**). Restriction digestion was carried out with the enzyme *Hae*III. A total of 281 recombination plasmids were obtained, out of the plasmids showed distinct profile were sequenced (**SFig. 2b**).

Database searches for homologous sequences were performed using the 16S rRNA encoding genes, 104 matched sequences showed maximum identity of more than 99%, and 18 sequences showed below 99%. **Tables 2** and **3** shows the bacteria and archaea identified in MFC-1 and MFC-2. For some samples one has two entries from NCBI, because the first one is a partial sequence or corresponds to uncultured species.

3.5.3. Dominant bacteria existed in MFC −1

The bacteria Acinetobacter baylyi, *Rhodocyclaceae, Pseudoxanthobacter* sp. DDT-1, *Flavobacteriaceae* 18–10 PB, *Acidovorax* sp. CSC45, *Pseudomonas geniculata* H10, *Pseudomonas* sp.a-1–7, *Burkholderia* sp. CRRI-84 and *Aminobacter* sp. ZYYR1were observed in the MFC-1 (**Table 2**). A. baylyi belongs to the order Pseudomonadales, class γ-proteobacteria, it is a non-pathogenic soil bacterium involved in the degradation of aromatic compounds and the production of triacylglycerols and wax esters [52]. *Rhodocyclaceae* bacteria belong to the order Rhodocyclales, class β-proteobacteria. Oren, 2014 [53] reported that the family Rhodocyclaceae are anoxygenic photoheterotrophs, can degrade the waste materials; aromatic compounds and also perform propionic acid fermentation. *Pseudoxanthobacter* sp. DDT-1 belongs to the order Rhizobiales, class α-proteobacteria. Liu et al., 2014 [54] isolated *Pseudoxanthobacterliyangensis*sp DDT-3(T) from dichlorodiphenyltrichloroethane contaminated soil. *Flavobacteriaceae* 18–10 PB belongs to the order Flavobacteriales and class Flavobacteria. These bacteria were isolated from municipal wastewater by Allen et al., 200 [55]. *Acidovorax* sp. CSC45 belongs to the order Burkholderiales and class β-proteobacteria. Vayenas, 2011 [56] reported that *Acidovorax* bacteria species have been isolated from the mixed cultures in denitrification reactors. Bacteria belongs to *Pseudomonas* group, *P. geniculata* H10 and *Pseudomonas* sp. a-1–7 were identified in the reactor which belongs to the order Pseudomonadales and class γ-proteobacteria. Many reports were available about electricity generation using *Pseudomonas* group of bacteria. Qiao et al., 2017 [57] used the bacterium *P. aeruginosa* for the electricity generation. *Burkholderia* sp. CRRI-84 belongs to the order Burkholderiales and class β-proteobacteria. Hunter and Manter, 2011 [58] reported that *Burkholderiacenocepacia* released oxidizers into the media to improve the electrical output of MFC. *Aminobacter* sp. ZYYR1 belongs to the order Rhizobiales and class α-proteobacteria also important strains. McDonald, 2005 [59] isolated the bacterial strains IMB-1 and CC495 from agricultural soil which can grow on methyl chloride and methyl bromide. Presence of all these organisms showed positive influence on electricity generation and substrate degradation in MFCs.

3.5.4. Dominant bacteria existed in MFC-2

The bacteria *Propionivibriomilitaris, Geobacter* sp., *Cupriavidus* sp., *Azospira* sp., *Chryseobacteriumkoreense,Rhodanobacter*sp.,*Methyloversatilisuniversalis, Mesorhizobium, Terrimonas* sp., *Nitrosomonas* and *Acidobacteria* were observed in the MFC-2 (**Table 3**). *Propionivibriomilitaris* belongs to the order Rhodocyclales, class, β-proteobacteria, can use chemoorganotrophic substrates such as acetate and propionate as electron donors for growth [60]. *Geobacter* sp. belongs to the order Desulfuromonadales and class Δ-proteobacteria. Sun et al., 2014 [61] reported that *Geobacter* sp. SD-1 produced current of $220 \pm 4 Am^{-3}$ in a highly saline water. *Cupriavidus* sp. belongs to the order Burkholderiales and class β-proteobacteria. Friman et al., 2013 [62] reported that *C. basilensis* cells growing in the anode in a defined medium with acetate or phenol produced current of 902 and 310 mA m^{-2} respectively. *Azospira* sp. belongs to the order Rhodocyclales and class

Table 2
Bacteria and archaea (anode) identified in the MFC-1 using RFLP analysis.

Sample	Closest relative	Accession number	Similarity (%)	Order/class	Phylogenetic affiliation
Bacteria					
A-9	*Acinetobacter baylyi*	AB859675.1	99	Pseudomonadales/γ-proteobacteria	Proteobacteria
A-12	*Rhodocyclaceae bacterium*	AB723853.1	100	Rhodocyclales/β-proteobacteria	Proteobacteria
A-28	*Uncultured bacterium*	FJ416412.1	99	–	Proteobacteria
A-29	*Uncultured bacterium*	KC521781.1	98	–	Proteobacteria
A- 65	*Rhodocyclaceae bacterium*	AB723853.1	99	Rhodocyclales/β-proteobacteria	Proteobacteria
A-69	*Pseudoxanthobacter* sp. *DDT-1*	FJ587218.1	98	Rhizobiales/α-proteobacteria	Proteobacteria
A-75	*Flavobacteriaceae bacterium 18–10PB*	JX491324.1	93	Flavobacteriales/Flavobacteriia	Bacteroidetes
A-85	*Acidovorax* sp. *CSC45*	JN541154.1	99	Burkholderiales/β-proteobacteria	Proteobacteria
Archaea					
A-9	*Methanosarcinales QEBH4ZF091*	KF198803.1	99	Methanosarcinales/Methanomicrobia	Euryarchaeota
A-12	*Uncultured archaeon*	JX000057.1	100	–	Euryarchaeota
A-16	*Methanolinea* sp. *B1-A-15*	JN836394.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-20	*Methanomicrobiales 108ZC12*	KF198732.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-35	*Methanosarcinales QEBH4ZF091*	KF198803.1	99	Methanosarcinales/Methanomicrobia	Euryarchaeota
A-53	*Methanomicrobiales 108ZC12*	KF198732.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-147	*Methanosarcinales QEBH4ZF091*	KF198803.1	99	Methanosarcinales/Methanomicrobia	Euryarchaeota

Table 3
Bacteria (anode and cathode) and archaea (anode) identified in the MFC-2 using RFLP analysis.

Sample	Closest relative	Accession number	Similarity (%)	Order/class	Phylogenetic affiliation
Bacteria					
A-1	*Pseudomonas* sp. *X-b2*	JX997894.1	99	Pseudomonadales/γ-proteobacteria	Proteobacteria
A-8	*Propionivibriomilitaris MP*	EU849004.2	99	Rhodocyclales/β-proteobacteria	Proteobacteria
A-11	*Geobacter* sp. *OSK2A*	AB762695.1	97	Desulfuromonadales/Δ-proteobacteria	Proteobacteria
A-13	*Cupriavidus* sp. *B55*	KF788067.1	99	Burkholderiales/β-proteobacteria	Proteobacteria
A-21	*Pseudomonas* sp. *G1–10*	KF578422.1	99	Pseudomonadales/γ-proteobacteria	Proteobacteria
A-22	*Geobactersulfurreducens PCA*	NR_075009.1	99	Desulfuromonadales/Δ-proteobacteria	Proteobacteria
A-29	*Cupriavidus* sp. *B55*	KF788067.1	99	Burkholderiales/β-proteobacteria	Proteobacteria
A-34	*Azospira* sp. *Tagus*	KC247691.1	99	Rhodocyclales/β-proteobacteria	Proteobacteria
A-48	*Pseudomonas* sp. *X-b2*	JX997894.1	99	Pseudomonadales/γ-proteobacteria	Proteobacteria
A-62	*Chryseobacteriumkoreense*	AB681907.1	99	Flavobacteriales/ Flavobacteria	Bacteroidetes
C-7	*Uncultured bacterium*	JX883006.1	99	–	Proteobacteria
C-8	*Rhodanobacter* sp. *BJQ-6*	EU876661.1	99	Xanthomonadales/γ-proteobacteria	Proteobacteria
C-26	*Methyloversatilis universalis FAM5*	NR_043813.1	99	Rhodocyclales/β-proteobacteria	Proteobacteria
C-33	*Rhodanobacter* sp. *BJQ-6*	EU876661.1	99	Xanthomonadales/γ-proteobacteria	Proteobacteria
C-41	*Mesorhizobium* sp. *DLS-79*	FN646688.1	98	Rhizobiales/α-proteobacteria	Proteobacteria
C-45	*Acidovorax* sp. *Van62*	HQ222278.1	99	Burkholderiales/β-proteobacteria	Proteobacteria
C-57	*Terrimonas* sp. *YJ03*	JN848793.1	99	Sphingobacteriales/ Sphingobacteria	Bacteroidetes
C-67	*Terrimonas* sp. *CR94*	FJ772030.2	97	Sphingobacteriales/ Sphingobacteria	Bacteroidetes
C-74	*Nitrosomonas* sp. *HP8*	HF678378.1	98	Nitrosomonadales/β-proteobacteria	Proteobacteria
C-117	*Acidobacteria P105*	KJ461654.1	99	Acidobacteriales/ Acidobacteria	Acidobacteria
Archaea					
A-15	*Methanosarcinales archaeon S4*	GU475184.1	100	Methanosarcinales/ Methanomicrobia	Euryarchaeota
A-21	*Methanobrevibactersmithii*	NR_074235.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-31	*Methanomicrobiales*	KF198732.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-35	*Uncultured archaeon*	KF670358.1	100	–	Euryarchaeota
A-42	*Methanolineatarda NOBI-1*	NR_028163.1	98	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-50	*Methanobrevibacter AZ*	AY196663.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota
A-63	*Nitrososphaera gargensis Ga9.2*	NR_102,916.1	98	Nitrososphaerales/Nitrososphaeria	Thaumarchaeota
A-71	*Methanomicrobiales 108ZC12*	KF198732.1	99	Methanomicrobiales/Methanomicrobia	Euryarchaeota

β-proteobacteria. Yong et al., 2015 [63] reported that Azospira, *Azospirillum, Acinetobacter, Bacteroides, Geobacter, Pseudomonas,* and *Rhodopseudomonas* are active communities present in BES reactor. No reports were found about the role of *Chryseobacteriumkoreense* in MFC which belongs to the order Flavobacteriales and class Flavobacteria. *Rhodanobacter* sp. belongs to the order Xanthomonadalesand class γ-proteobacteria. Patil et al., 2009 [64] reported that *Rhodanobacterlindaniclasticus* was present in the anodic chamber of MFCs. *Methyloversatilis universalis* belongs to the order Rhodocyclales and class β-proteobacteria, and these strains were facultative methylotrophs that can grow on a variety of C1 and multi carbon compounds [65]. *Mesorhizobium* belongs to the order Rhizobiales and class α-proteobacteria. Studies found that denitrification bacteria *Mesorhizobium* was involved in the NO_2^-, NO_3^- reduction process in MFC [66]. *Terrimonas* sp. belonging to the order Sphingobacteriales and class Sphingo bacteria were also identified in MFC [67]. *Nitrosomonas* belongs to the order Nitrosomonadales and class β-proteobacteria. Khunjar et al., 2012 [68] used *Nitrosomonas europaea*, as biocatalyst to utilize ammonia as its sole energy source for growth in a reverse MFC. *Acidobacteria*belongs to the order Acidobacteriales and class Acidobacteria, these are physiologically diverse, able to utilize different substrates in MFC and produce electricity [69].

3.5.5. Dominant archaea existed in MFC-1 and MFC-2

Almost similar types of archaea were observed in both MFC-1 and MFC-2. *Methanosarcinales*, uncultured archaeon, *Methanolinea,* and *Methanomicrobiales* were observed in the MFC-1. *Methanobrevibactersmithii* and *Nitrososphaeragargensis* were observed in the MFC-2. *Methanosarcinales* QEBH4ZF091 belongs to the order Methanosarcinales and class Methanomicrobia. Olubunmi, 2016 [70] reported that Methanosarcinales related species are present in sediment and sludge inoculated reactors, and have the ability of using a variety of substrates. These archaea species are dominating and produce methane in the anaerobic digestion process. Kendall and Boone, 2006 [71] reported that these microbes catalyze the terminal step in the degradation of organic matter. *Methanomicrobiales* are strictly carbon dioxide reducing methanogens and using hydrogen or formate as the reducing agent [72].Wu et al., 2019 [73] analyzed the abundance of methanogens, *Methanomassiliicoccus, Methanoregula* and *Methanolinea* by quantifying and sequencing the *mcrA* gene. *Methanobrevibactersmithii* belongs to the order Methanomicrobiales and class Methanomicrobia. *Nitrososphaeragargensis*is ammonia and nitrite oxidizing organism belongs to the order Nitrososphaerales and class Nitrososphaeria [74].

4. Conclusions

MFC in long-term operation with a aerobic biocathode showed better electrogenesis, power production and substrate degradation than the corresponding MFC with an abiotic cathode. RFLP analysis identified the potential electricity producing organisms such as Acinetobacter, *Acidovorax, Pseudomonas, Geobacter, Cupriavidus* and *Burkholderia*. Main advantages of the RFLP method include cost diminishing by reducing the sequencing reactions, and also the easier manipulation allowing lesser skilled personnel to do it. Our findings clearly demonstrated that MFC which contains biocathode has wider potential applications than other more convencional MFCs using abiotic cathodes, in particular due to simpler conditions and the reduction of costs. But, for future routine applications, the biocathode containing MFC need to overcome some limitations, which are mainly caused due to contaminations and biofouling.

Declarations

Author's contributions

S.V. Ramanaiah, Cristina M. Cordas and Sara C. Matias: experimental procedures, results discussion, data treatment, draft revision; M. Venkateswar Reddy, J. H. Leitão and Luis P. Fonseca, results discussion and draft revision. All authors have read and agreed to the manuscript before submission to the journal.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data Availability Statement

The datasets generated for this study are available on request to the corresponding.

Ethics approval and consent to participate

Not applicable.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2021.e00693.

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