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ELECTRICAL STRESS-DIRECTED EVOLUTION OF BIOCATALYSTS COMMUNITY SAMPLED FROM A SODIC-SALINE SOIL FOR MICROBIAL FUEL CELLS

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ABSTRACT

Anode-respiring bacteria (ARB) perform an unusual form of respiration in which their electron acceptor is a solid anode. The focus of this study was to characterize the electrical stress direct evolution of biocatalysts as a way of enriching the community with ARB for microbial fuel cell. The original microbial consortium was sampled from a sodic-saline soil (Texcoco Lake). Interestingly, the most probable number of iron (III) reducing bacteria in the original consortium was 8500 ± 15 MPN/100 mL, since iron (III) is reported to be associated to anode-respiring capabilities. Cyclic voltammetry studies of electrochemical stressed biofilm-ARB were conducted at 135th day, and an irreversible electron transfer reaction of alkaliphilic cytochrome, due to the electrode fouling was found. The electrochemical impedance spectroscopy results revealed that the resistance of the biofilm-ARB decreases with the time, associated to the adaptability of electroactive biofilm on the graphite electrode surface. Confocal microscopy revealed that the biofilm-ARB attained ~40 μm thickness. Electrical stressed-ARB gave a maximum power density of 79.44 mW/m^2, which was greater than that obtained by the chemical stressed-ARB (48.48mW/m^2) in a single-chamber microbial fuel cell (SCMFC).

Key words: Anode-respiring bacteria, Cyclic Voltammetry, Extra cellular electron transfer

1. INTRODUCTION

In Microbial fuel cells (MFCs) efficient extracellular electron transfer microbes (EETM) also known as anode-respiring bacteria (ARB) can play an important role on cell performance. There are some studies about modified electrode materials showing significant improvement in MFCs [1,2]. Hunting the suitable microbes for MFCs changes the various environmental stresses, such as oxygen, low pH, low temperature, external resistance [3-7] and electrode potential [8-12]. In addition to the different redox potentials of electron acceptors, the energetic requirements of a cell vary depending upon the terminal electron acceptor, for example: i) internal electron acceptor (such as fumarate), ii) external electron acceptor such as insoluble Fe (III) and iii) solid electrode with an internal electron acceptor. The protons produced from substrate oxidation are transported out of the cytoplasm, in this way the pH remains constant within the cytoplasm. The proton transport requires an external electron acceptor such as a solid electrode [13,14]. Thus, the anode potential, not the acceptor concentration, regulates the thermodynamic energy available for ARB to grow. It is generally accepted that ARB communities should be capable of switching their respiratory mechanism in order to maximize the energy obtained for ATP production as the anode potential changes. In a mixed ARB community, several respiratory pathways could be available, and the community may be able to maximize energy efficiency by adapting to the anode potential. Torres et al., [9] used waste water-activated sludge as inoculum and found that the two electrodes at the lowest potential showed a faster biofilm growth and produced the higher current densities reaching up to 10.3 A/m^2. At low anode potentials, clone libraries showed a strong selection (92-99% of total clones) of an ARB that is 97% similar to *G.sulfurreducens*. Cyclic voltammograms performed on various electrodes suggest that the ARB grown at the lowest potentials carried out extracellular electron transport exclusively by conducting electrons through the extracellular biofilm matrix. Therefore, anodic potential regulation as incisive selective pressure on microbial community is important for enrichment of efficient and selective ARB community growth.

The focus of this study was to induce an electrical stress directed evolution of ARB biocatalysts at low anode potential of -150mV vs. SCE, starting with a saline-sodic soil inoculum. The final terminal electron acceptor was a solid electrode surface through this more efficient ARB community selected for MFCs applications. Electrochemical and confocal microscopy tools were used to characterize the ARB community developed at anode surface. Results concluded that the bio electrolysis was an efficient method for developing a rich ARB community

2. MATERIALS AND METHODS

2.1 Sample collection and enrichment

A. Texcoco Soil samples were collected in a sterile anaerobic container and preserved aseptically. Chemically enriched (Ferric citrate 20mM as terminal electron acceptor) Texcoco bacterial community was enriched in modified Soap Lake basal medium (SLBM) called SL3 medium [15]. The medium has the following components (per liter of de-ionized water): K_2HPO_4, 13.5g; NaCl, 70g; Na_2MoO_4, 4.84g; Cysteine-HCl (10%), 1.5 ml; Na_2CO_3, 40g; $CaCl_2$. $2H_2O$, 0.42g; SiO_2. $2H_2O$, 0.75g; $MgCl_2$. $2H_2O$, 0.852g; $MnCl_2$, 0.448g;NH_4Cl, 5g; Trace metal solution, 10ml. The trace metal solution consists of the following in 1 liter of de-ionized water: Nitrilotriacetic acid, 1.63 g; $MgSO_4$. $7H_2O$, 3 g; $MnCl_2$. $4H_2O$, 0.5 g; $CaCl_2$, 0.1 g; $CoCl_2$. $2H_2O$, 0.1 g; $ZnCl_2$, 0.13 g; $CuCl_2$. $2H_2O$, 0.007 g; AlK $(SO_4)_2$.$12H_2O$, 0.01 g; H_3BO_3, 0.01 g; Na_2MoO_4, 0.025 g; $NiSO_4$. $6H_2O$, 0.03g; Na_2WO_4. $2H_2O$, 0.025g and NaCl, 1g.The medium was made anaerobic by boiling and cooling under an atmosphere of N_2:CO_2 (80:20) and subsequent placement in an anaerobic glove bag. The glove bag was maintained under a constant N_2:H_2 (90:10) atmosphere. Cysteine-hydrochloride was added to the medium in the glove bag, and the pH was brought to ~10.5-11 with 10N NaOH. Both Na_2CO_3 and iron (III) citrate were added after autoclaving.

2.2 Electrochemical Characterization

2.2.1 Electrochemical set-up

The working (geometrical area- 14.05 cm^2) and counter electrodes (geometrical area- 20.5 cm^2) were Graphite rods. A saturated calomel electrode was used as a reference electrode. The modified SL3 medium with 15mM of sodium acetate was used as carbon source. Potentials were applied with a 273A Potentiostat/Galvanostat from EG&G Princeton Applied Research. Temperature was set at 30ºC.

2.2.2 Electrode Preparation

Graphite rods were submerged in 0.5 M KCl solution for 3 h, after that the graphite rods were polished with 1500b sand paper and rinsed with deionized water before use. The graphite rods were submerged in 0.5 M KCl solution overnight in order to activate them.

2.2.3 Electrochemical impedance spectroscopy studies

Impedance spectra of biofilm were obtained at the open circuit potential (E_{ocp}). The amplitude of the signal perturbation was 10 mV, the frequency range scanned was from 100 kHz to 1 mHz. Impedance experiments were performed in the potentiostat/galvanostat Volta lab model PGZ402.

2.2.4 Evaluations of vertical single-chamber MFC efficiency using Linear sweep Voltammetry

Liner sweep Voltammetry (LSV), was run at the recommended scan rate of 1mV s^{-1} starting from the measured open circuit potential [16] using 273A Potentiostat/Galvanostat from EG&G Princeton Applied Research.

2.3 Construction of the vertical single-chamber microbial fuel cell (SCMFC)

MFC consisted of a vertical cylinder built in Plexiglass 9 cm long and 5.6 cm internal diameter (Fig. 1). An assemblage of anode-proton exchange membrane-cathode was fitted at the bottom of the cell. For brevity, this 'sandwich' arrangement was coined as AMC for the Anode-proton exchange Membrane Cathode. This AMC consisted of a sandwich of stainless steel plate 1 mm thickness with a Toray flexible carbon-cloth sheet placed in one circular face and a cathode in the opposing face made of (from inside to outside): proton exchange membrane (Nafion 117), the

cathode made of flexible carbon-cloth containing 0.5mg cm^{-2} Pt catalyst (10wt%/C-ETEK) and a perforated plate of stainless steel 1 mm thickness.

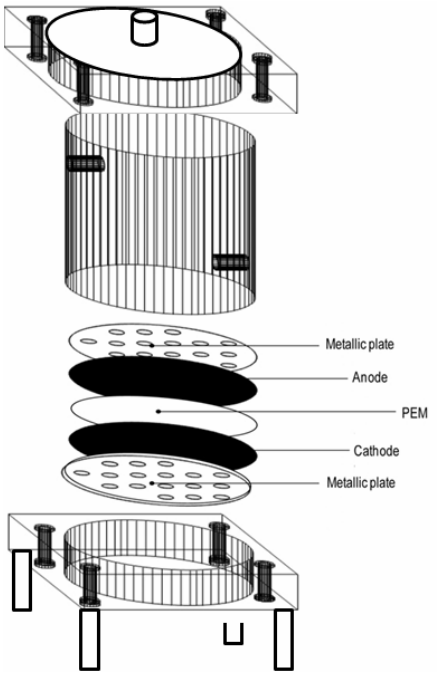

Figure 1. Schematic diagram of the vertical single-chamber MFC

2.4 Confocal Microscopy studies

The biofilm on the surface of graphite rod was aseptically scraped with the help of sterile glass rod and placed on a glass slide. The films were stained with 5mM of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and incubated for 2 hours. Morphology was analyzed by using Leica confocal microscope.

2.5 Most probable number

Initial inoculum of Texcoco soil was incubated in 10 g of soil into 90 ml modified SL3 medium with 15mM of sodium acetate used as carbon source and 20 mM iron (III) citrate as terminal electron acceptor, set to be 10^{-1} dilution and 1 g of soil into 99 ml of SL3 medium set to be 10^{-2} dilution, from that 10 ml of inoculum transferred into 90 ml of SL3 medium as 10^{-3} dilution.

Similarly continue the dilution up to 10^{-5} dilution. After the incubation period (~14 days) it was performed the iron reduction test in a plastic well using ferrozine technique [17].

3. RESULTS AND DISCUSSION

Most probable number (MPN) method adapted to quantify the number of iron (III) reducing microbial community in the original Texcoco inoculum gave a value an interesting 8500 ± 15 /100 mL (Fig. 2), since iron (III) dissimilatory reducing bacteria are reported to be associated to anode-respiring capabilities [17].

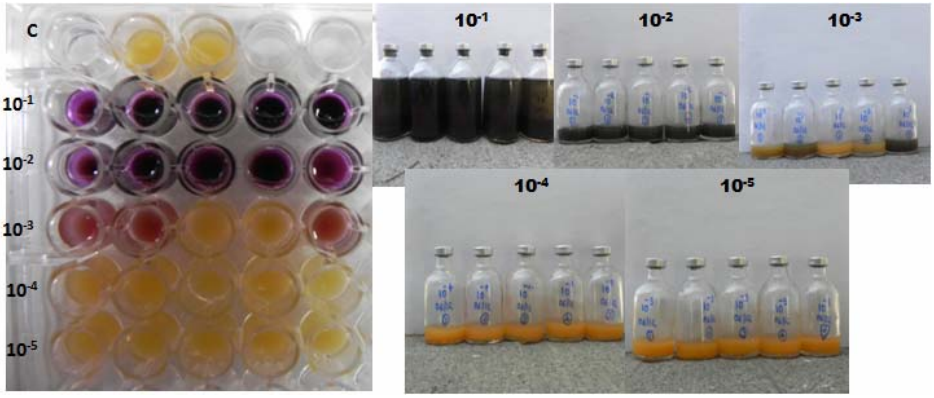

Figure 2. MPN method of Iron (III) reducing microbial community using ferrozine as indicator

In order to selectively grow the electro active biofilm of ARB, a potential step of -150 mV was applied over 150th days; during this period, the current was monitored. The shape of the curve in Figure 3 resembled the bacterial sigmoidal growth curve; the latter is in agreement with previous reported works [3, 5]. During the first 5 days, the recorded current was very small. After 5 days, the current started to increase and attained the maximum value of 1.8 mA in the 28th day. After 30 days the current decreased down to 0.9mA. Addition of carbon source at 75 th day lead to an increase in the current up to 0.8mA in the 135th day.

In the initial 4 days, the growth of microbial colonies was observed in the log phase related to low current. After 4 days, the biofilm ARB grew and attained the maximum current in the 28th day, probably due to microbial colonies in stationary phase. The depletion of nutrient lead to the microbial colonies to decline phase of growth, started in the 30th day. By adding the carbon

source at the 75th day the bacterial community started to grow again; this was reflected by the current increment and attained 0.8mA in the stationary phase.

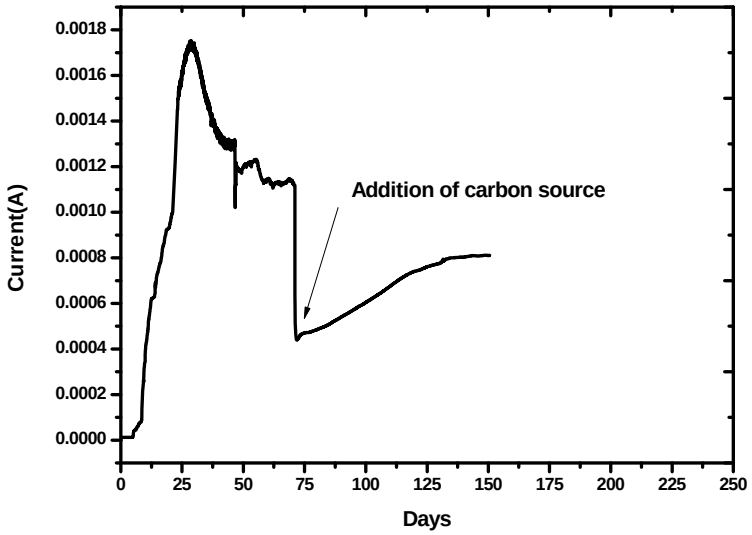

Figure 3. Variation of Current (A) during biofilm formation at -150mV vs SCE.

In order to check the activity of the biofilm, a cyclic voltammetry study was performed (Fig. 4). In the initial 4 days, the cyclic voltammograms showed no peaks, presumably due to the fact that the biofilm has not been completely formed (results not shown). At the 28th day, the voltammograms at a different scan rate up to 15mV/s presented an anodic peak. By increasing the scan rate the anodic peak shifted to more positive values. Even though there was an anodic peak, the corresponding cathodic peak did not provide the same area under the anodic curve (anodic charge). This suggests that an irreversible process is occurring. A similar pattern occurred at the 136th day (data not shown). Concomitantly, large amounts of membrane-bound cytochromes occur in alkaliphilic bacteria [18], the potential increased in parallel with the pH of the growth medium [19, 20], indicating an adaptation to environmental stress [21]. Midpoint potential deduced from the CV, was +108mV vs SHE. This value was draw closer to the alkaliphilic cytochromes potential range [22]. It could be the Soluble/membrane-bound cytochromes from alkaliphilic bacteria are characterized [18,20,23,24], a feature which could facilitate electron

transfer to the membrane- bound terminal oxidase in the presence of the large negative membrane potentials was associated with the alkaline pH growth medium [19]. One of the possible reasons for the irreversible process would be the electrode surface that apparently fouled by strong irreversible adsorption [25], due to long term growth of bio films. Membrane bound enzymes (cytochrome) of biofilm activity was independently of the electroactive ARB`s metabolism [26].

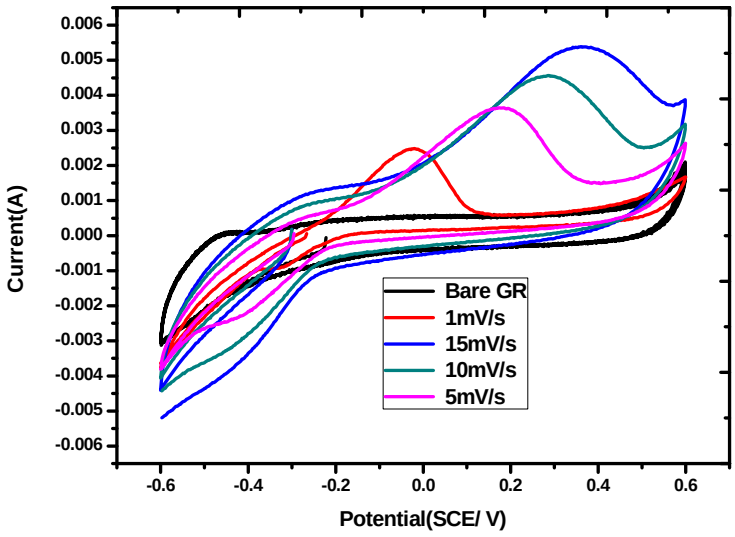

Figure 4. Cyclic Voltammetry of initial GR at 15mV/s. the cyclic Voltammetry of Biofilm in bicarbonate buffer (0.1M) with 15mM of sodium acetate as carbon source were measured at different scan rates.

Further characterization of ARB biofilm by electrochemical impedance spectroscopy (EIS) at 28th day and 136th day revealed two semicircles; one at high frequencies and another at low frequencies (Fig. 5). High frequency semicircle was associated to ARB biofilm electrical properties. The low frequency semicircle was ascribed to the processes occurring at the biofilm/solution interface. The impedance spectra at 28th day and 136th day were fitted with an appropriate equivalent circuit (EC). A simple EC used for describing the electrochemical properties of the ARB biofilm is shown inlet scheme, which has the following elements: R1 - solution resistance (ohmic resistance), R2 - ARB biofilm resistance, R3 - mass transfer/ diffusion

resistance and C – capacitance (ie, accumulation of charges at the electrode-solution interface). A constant phase element (CPE) usually substitutes the capacitance in ECs because of the inhomogeneous conditions (e.g. electrode roughness, coating, and distribution of reaction rate). [27]. One or two time constants are usually sufficient to interpret the impedance data for most cases in bio electrochemical studies [28]. Data fitting is accomplished by appropriate software, such as Z-view. The obtained values of the resistance of the film (R2) were 11.11Ω for the 28th day and 5.5 Ω for the 136th day. This may be due to the adaptability of electroactive biofilm on the surface of the graphite rod which may reduce the internal resistance.

The procedure for preparing the SEM images can alter the appearance of the biofilm. The treatment with ethanol might wash away constituents, and dehydration might affect structure and thickness [29]. Therefore, the ARB biofilm was analyzed by confocal microscopy. The biofilm formed after 150th days is shown in Figure 6. In this figure the biofilm reduced 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) into water insoluble crystalline CTC-formazan, would be visualized as red crystals inside the membrane bound enzymes [30]. This represents the presence of bacteria using electron-transport for energy generation, i.e., respiration. In the biofilm anode fed by acetate, only ARB was stained red. Most of the cells in the biofilm underneath was stained red, indicating that most of the cells were metabolically active [31] along with green color areas, which indicate the nucleic acid staining of acridine orange. With longer incubation, the biofilm became thicker, ca. ~40 µm.

Polarization curves of electro active biofilm-ARB and chemically active iron reducing biocatalysts enriched by the method are shown in Figure 7. Electro active biofilm-ARB was gave the maximum power density of 79.44 mW/m^2 at the current density of 261.65 mA/m^2 and 0.296V, while in chemically active ARB, the power density was 48.48 mW/m^2 at the current density of 161.16mA/m^2 and 0.300V.

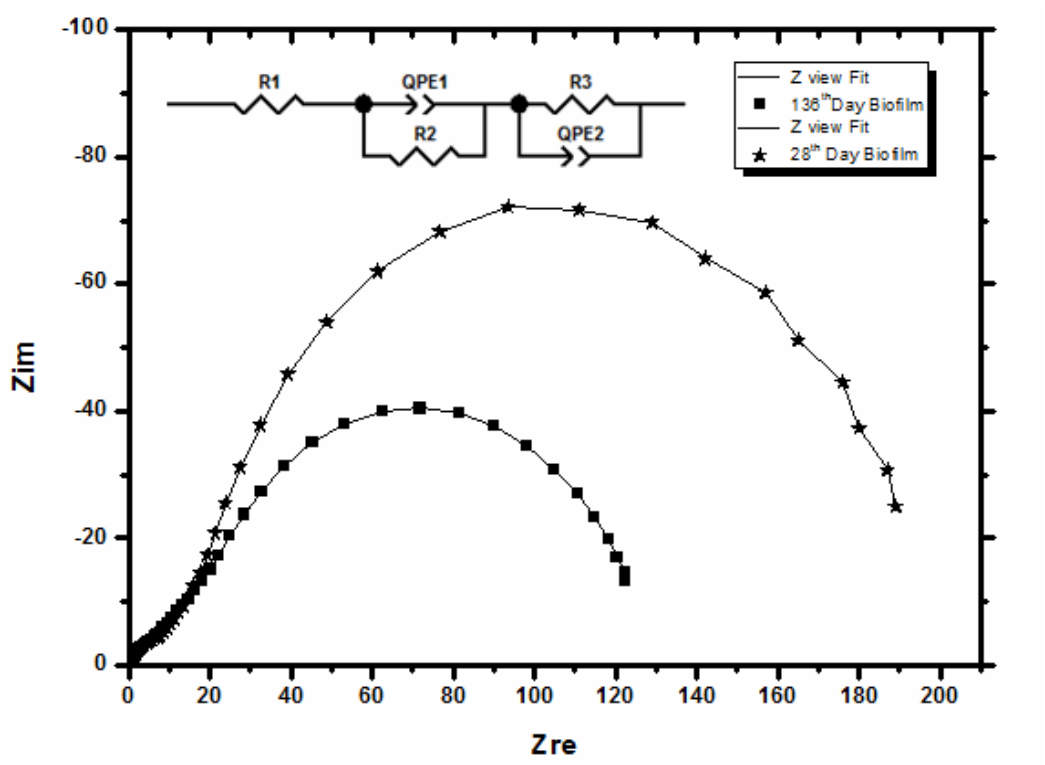

Figure 5. Electrochemical Impedance Spectroscopy of Biofilm electrode

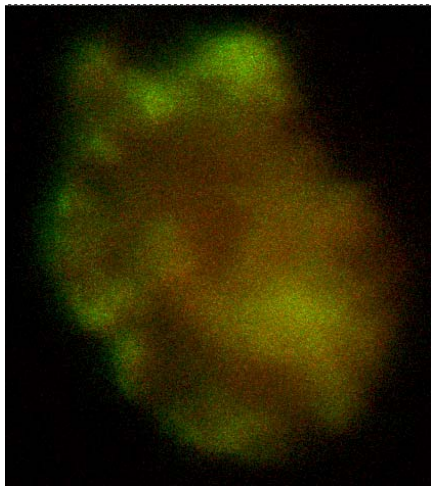

Figure 6. Image of biofilm using confocal microscopy

The power density in the present work was higher than the MFCs operated at hypersaline soda lake environment (6 X 10^{-5}Wm^{-2}) [32] and alkalophilic Corynebacterium sp. strain MFC03 gave

41.8 mWm^{-2} [33], and alkaline condition for rice mill waste water MFC (50 mWm^{-2}) [34]. However, haloalkalophilc microorganisms provide greater tolerance to fluctuations in chemical composition, which makes them useful candidates for operating in MFCs under various waste water treatment process.

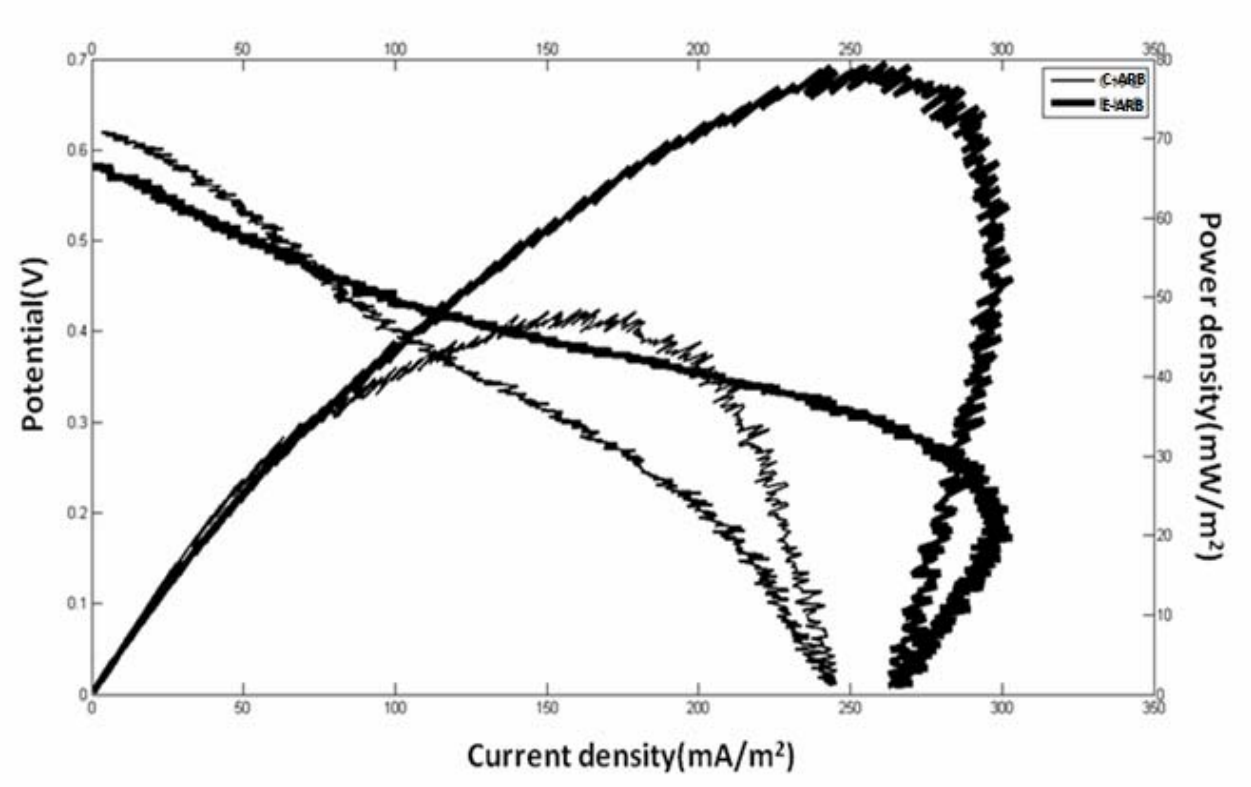

Figure 7. Polarization curve of C-ARB and E-ARB using LSCV

Figure 8 shows the EIS results of the inocula Chemical stressed-ARB (C-ARB) and Electrochemical stressed-ARB (E-ARB). The cell loaded with C-ARB inoculum gave a R_{int} of 1230Ω (R_{anode}-967 Ω, $R_{cathode}$-263 Ω and $R_{membrane}$-0.65 Ω) whereas the E-ARB inoculum showed a R_{int} of 445 Ω (R_{anode}-358 Ω, $R_{cathode}$-86 Ω and $R_{membrane}$-0.62 Ω). The R_{int} of the E-ARB was dramatically lower than that of C-ARB. This clearly suggests that the bio electrolysis was an efficient method for developing a rich ARB community [35].

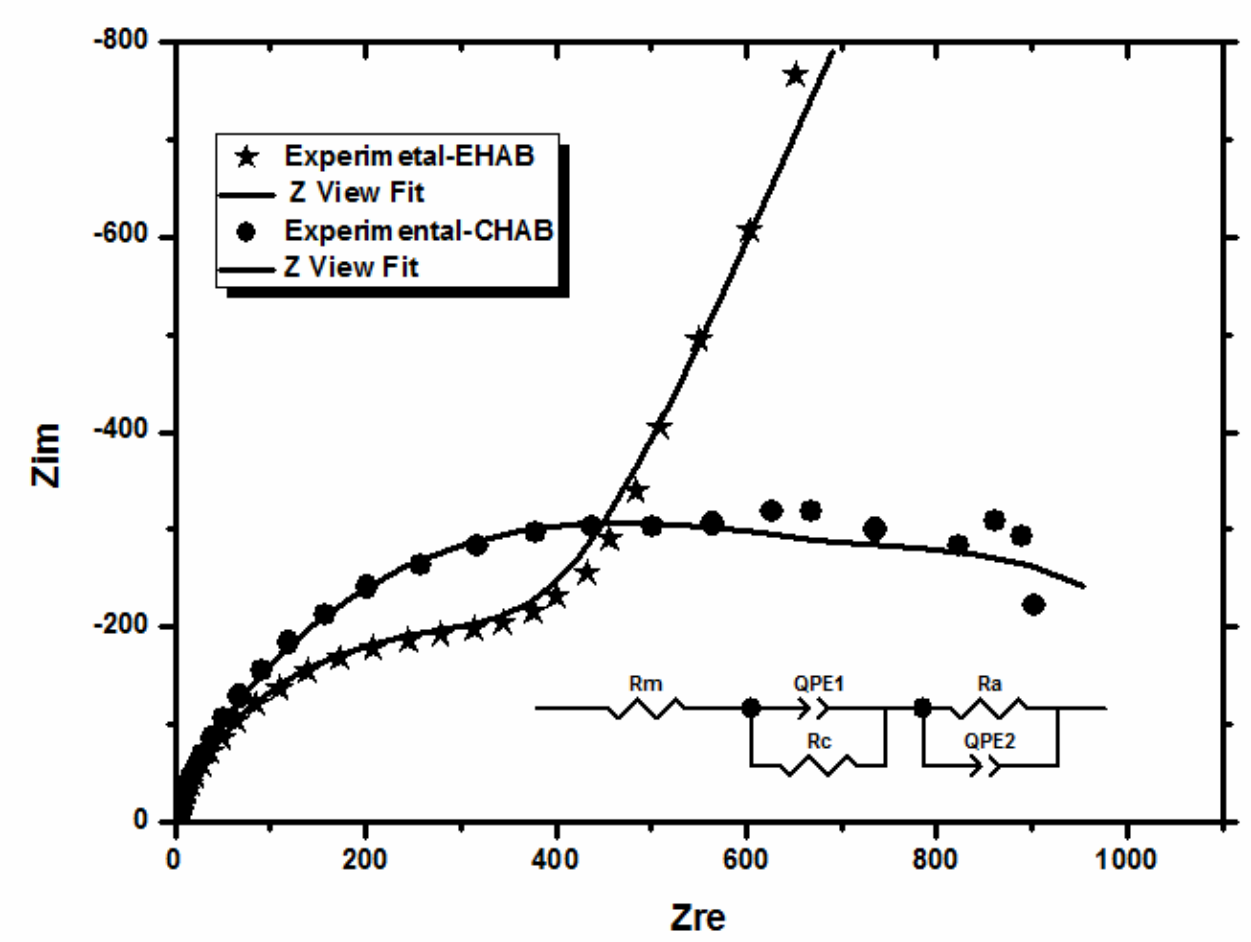

Figure 8. EIS of SCMFC wore C-ARB and E-ARB inoculum.

4. Conclusions

The electrical-stress direct evolution of biocatalysts was evaluated as a way of enriching the community with ARB. The biofilm was grown on a graphite rod using a potential step at -150mV/SCE, from Texcoco soil bacterial community. Cyclic voltammetry study showed the irreversible electron transport reaction occurs on biofilm adsorbed on the graphite rod surface. This behavior is attributed to electrode fouling. The electrochemical impedance spectroscopy revealed that the resistance of the biofilm (R2) was 11.11Ω for the 28th day and 5.5 Ω for the 136th day. The inference of the R_{int} reduction is due to the adaptability of electroactive biofilm on

the surface of the graphite rod. Confocal microscopy revealed that the thickness of biofilm is ~40 μm. Electrical stressed-ARB gave a maximum power density of 79.44mW/m^2, this value was greater than that obtained by the Chemical stressed inoculum (48.48mW/m^2) in a single-chamber microbial fuel cell (SCMFC). All these electrochemical experiments and evaluation in SCMFC studies suggest that the electrical-stress directed evolution of ARB community was associated to a more efficient extracellular electron transfer process in SCMFC.

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