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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 bottom soil (Texcoco Lake). Interestingly, iron (III) reducing bacteria consortium in the sodic-saline bottom soil was 8500 ± 15 MPN/100 mL by the most probable number method, since microbial reduction of iron (III) is reported to be associated to anode-respiring capabilities. Cyclic voltammetry studies of electrochemical stressed biofilm-ARB were conducted at 28th and 135th days, and an irreversible electron transfer reaction was found possibly related to electron transfer reaction of the cytochrome. The electrochemical impedance spectroscopy results revealed that the resistance of the biofilm-ARB decreased with time (28th day-11.11Ω and 135th day- 5.5Ω), possibly associated to the adaptability of electroactive biofilm on the graphite electrode surface. Confocal microscopy showed that the biofilms are active in nature and the biofilm-ARB attained ~40 μm thickness at the 136th day. Electrical stressed-ARB gave a maximum power density of 79.4mW/m^2, and unstressed-ARB gave a maximum power density of 41.0mW/m^2 in a single-chamber microbial fuel cell (SCMFC). All these electrochemical experiments and evaluation suggest that the electrical-stress directed evolution of ARB community was associated to a more efficient extracellular electron transfer process in SCMFC.*

Keywords: 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) and 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 involves changes by the various environmental stresses, such as external resistance, varying electrode potential, and low pH level, [3-12]. 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 anode potential, not the acceptor concentration, regulates the thermodynamic energy available for ARB to grow [9]. It is generally accepted that ARB communities should be capable of switching their respiratory mechanism in order to maximize the energy obtained for Adenosine triphosphate (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] uses activated sludge as inoculum and found that the two electrodes at the lowest potential showed a faster biofilm growth and produced the highest current densities, reaching a value up to 10.3 A/m^2. At low anode potentials, shows a strong selection of an ARB that is 97% similar

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ABBREVIATIONS

AMC	assemblage (sandwiched) anode-membrane-cathode
ARB	anode repairing bacteria
ATP	adenosine triphosphate
C	capacitance
CV	cyclic voltammetry
CPE	constant phase elements
EIS	electrochemical impedance spectroscopy
LSV	linear sweep voltammetry
MFC	microbial fuel cell
MPN	most probable number
OCP	open circuit potential
ORR	oxygen reduction reactions
R_a	anodic resistance
R_c	cathodic resistance
R_{int}	internal resistance
R_m	membrane resistance
R1	solution resistance
R2	ARB biofilm resistance
R3	mass transfer/diffusion resistance
SCMFC	single chamber microbial fuel cell

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 outer cell membrane of 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. saturated calomel electrode (SCE), starting with a saline-sodic soil inocula. 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 the anode surface. Results concluded that the bio electrolysis was an efficient method for developing a rich ARB community.

2. EXPERIMENTAL

2.1. Inoculum and medium

The sampling site is located at the former lake Texcoco (Northern Latitude 19º 30' 52" Western Longitude 98º 59' 24") in the state of Mexico, Mexico, D.F. Soil samples were collected in a sterile anaerobic container and preserved aseptically [13]. That soil sample contains the electrolytic conductivity in saturation extracts 150 dS m^{-1}. This paves the attention to use as inoculum for MFC. The medium has the following components (per liter of deionized 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 consisted of the following in 1 liter of de-ionized water: nitrilotriacetic acid, 1.63g; $MgSO_4$-$7H_2O$, 3g; $MnCl_2$-$4H_2O$, 0.5g;$CaCl_2$, 0.1g;$CoCl_2$-$2H_2O$, 0.1g;$ZnCl_2$, 0.13g; $CuCl_2$-$2H_2O$, 0.007g;AlK $(SO_4)_2$-$12H_2O$, 0.01g; H_3BO_3, 0.01g; Na_2MoO_4, 0.025g; $NiSO_4$-$6H_2O$, 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 box. cysteine-hydrochloride was added to the medium in the glove box, and the pH was brought to ~10.5-11 with 10N NaOH. Both Na_2CO_3 and iron (III) citrate (20mM) were added after autoclaving. This medium was used in enrichment of un-stressed haloalkaliphilic bacteria (ARB), preservation and subculture process. The culture was preserved at -10 ºC.

2.2. Electrochemical characterization

2.2.1. Electrochemical set-up and electrical stressed enrichment

The graphite was procured from GraphiteStore.com. Inc. US (resistivity-5.5ohm/in$\times 10^{-4}$; porosity-12%;thermal conductivity-83W/(m^2.K/m).The working (geometrical area- 14.05 cm^2) and counter electrodes (geometrical area- 20.5 cm^2) were graphite rods. A SCE was used as a reference electrode. The modified SL3 medium with 15mM sodium acetate was used as carbon source, without any final terminal acceptor and remaining chemicals were same as described in section 2.1. 1g of Texcoco soil sample was added into the modified SL3 medium. Potentials were applied with a 273A Potentiostat/Galvanostat from EG&G Princeton Applied Research. Temperature was set at 30 ºC. Graphite rods were submerged in 0.5M KCl solution for 3 h, polished with 1500b sand paper submerged in 0.5M KCl solution overnight in order to activate them and rinsed with deionized water before use. In order to selectively grow the electro active biofilm of haloalkaliphilic bacteria (E-HAB), a potential step of -150 mV was applied over 150 days. During this period, the current was monitored. Later on the biofilm was subculture using modified SL3 medium.

2.2.2. Electrochemical impedance spectroscopy, cyclic voltammetry and linear sweep voltammetry studies

Impedance spectra of the biofilm were obtained at the open circuit potential (Eocp). The amplitude of the signal perturbation was 10mV; the frequency range scanned was from 100 kHz to 1mHz. Impedance experiments were performed in a Potentiostat/Galvanostat Volta lab model PGZ402. Data fitting was accomplished by appropriate software, such as Z-view. Linear sweep voltammetry (LSV) was run at the recommended scan rate of 1mV/s starting from the measured open circuit potential [14] and using the 273A Potentiostat/Galvanostat from EG&G Princeton Applied Research. Cyclic voltammetry was performed by three electrode system using the 273A Potentiostat/Galvanostat from EG&G Princeton Applied Research.

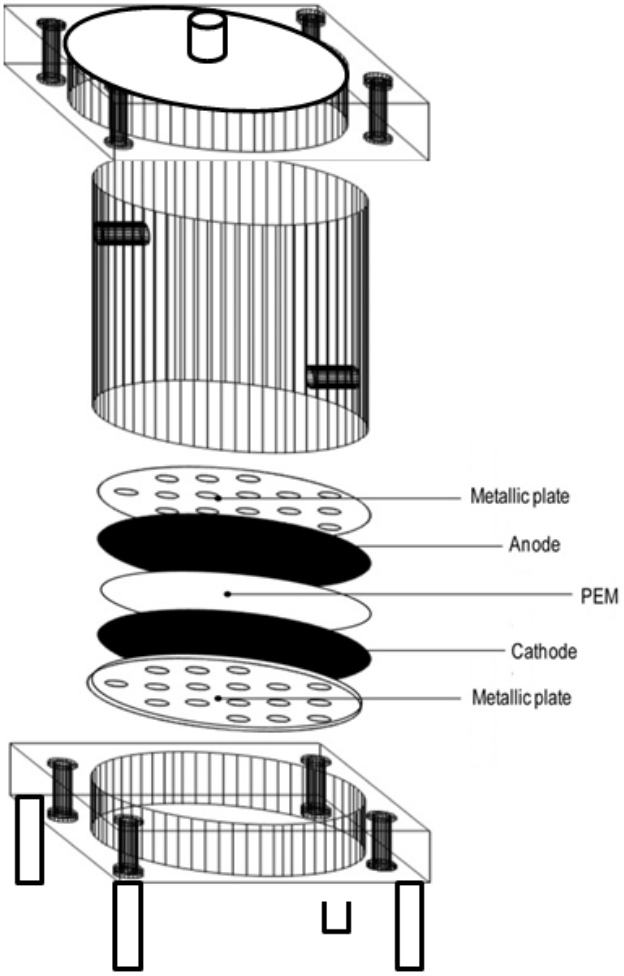

Figure 1. Schematic diagram of the vertical single-chamber microbial fuel cell

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

The MFC consisted of a vertical cylinder built in Plexiglas's, its dimensions: 9cm long and 5.6cm 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 an arrange of a stainless steel perforated plate 1mm 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): a proton exchange membrane (Nafion 117), the cathode made of flexible carbon-cloth containing 0.5mgcm^{-2} Pt catalyst (10wt%/C-ETEK) and a perforated plate of stainless steel 1mm thickness.

2.4. Confocal microscopy studies

The electrochemical stressed grown biofilm was scarped from graphite rod under aseptic conditions and analyzed by Leica TCS SP5X confocal microscopy. 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 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), acridine orange and incubated for 2 hours.

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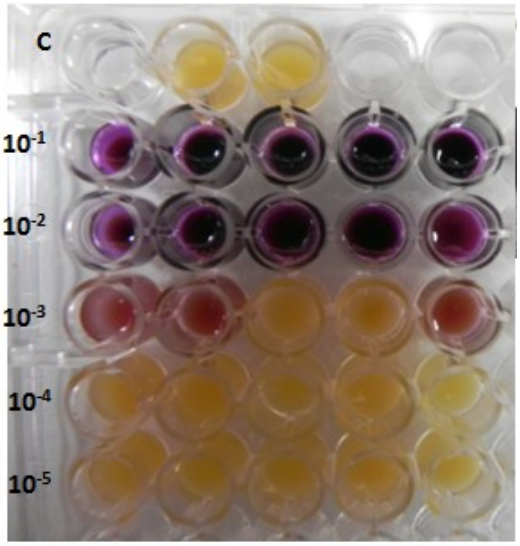

Figure 2. The results of the most probable number method of Iron (III) reducing microbial community using ferrozine as indicator

2.5. Most probable number of iron reducing bacteria

Initial inoculum of Texcoco soil was incubated in 10 g of soil into 90 ml of modified SL3 medium with 15mM of sodium acetate used as carbon source and 20mM iron (III) citrate as terminal electron acceptor, set to be 10^{-1} dilution; in 1g of soil into 99 ml of modified SL3 medium set to be 10^{-2} dilution;, from that 10 ml of inoculum transferred into 90 ml of modified SL3 medium as 10^{-3} dilution. Similarly continue the dilution up to 10^{-5} dilution. After the incubation period (~14 days) the iron reduction test was performed using the ferrozine technique [15].

3. RESULTS AND DISCUSSION

The most probable number (MPN) method adapted to quantify the number of iron (III) reducing microbial community in the original Texcoco inoculum gave an interesting value 8500 ± 15 cells/100mL, since iron (III) dissimilatory reducing bacteria are reported to be associated to anode-respiring capabilities in MFCs [15]. Fig.2 shows the reduction of iron appeared as purple colour in the well regard as MPN end point. Variation of current intensity during different time intervals leads to biofilm formation at -150mV vs SCE is shown in Fig.3a. Current production was directly associated to the formation of biofilm on the surface of the graphite rod. It 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. Subsequent to 30 days the current decreased down to 0.4mA might be associated to depletion of nutrient leads to arrest the growth of biofilm. Addition of carbon source at the 75th day

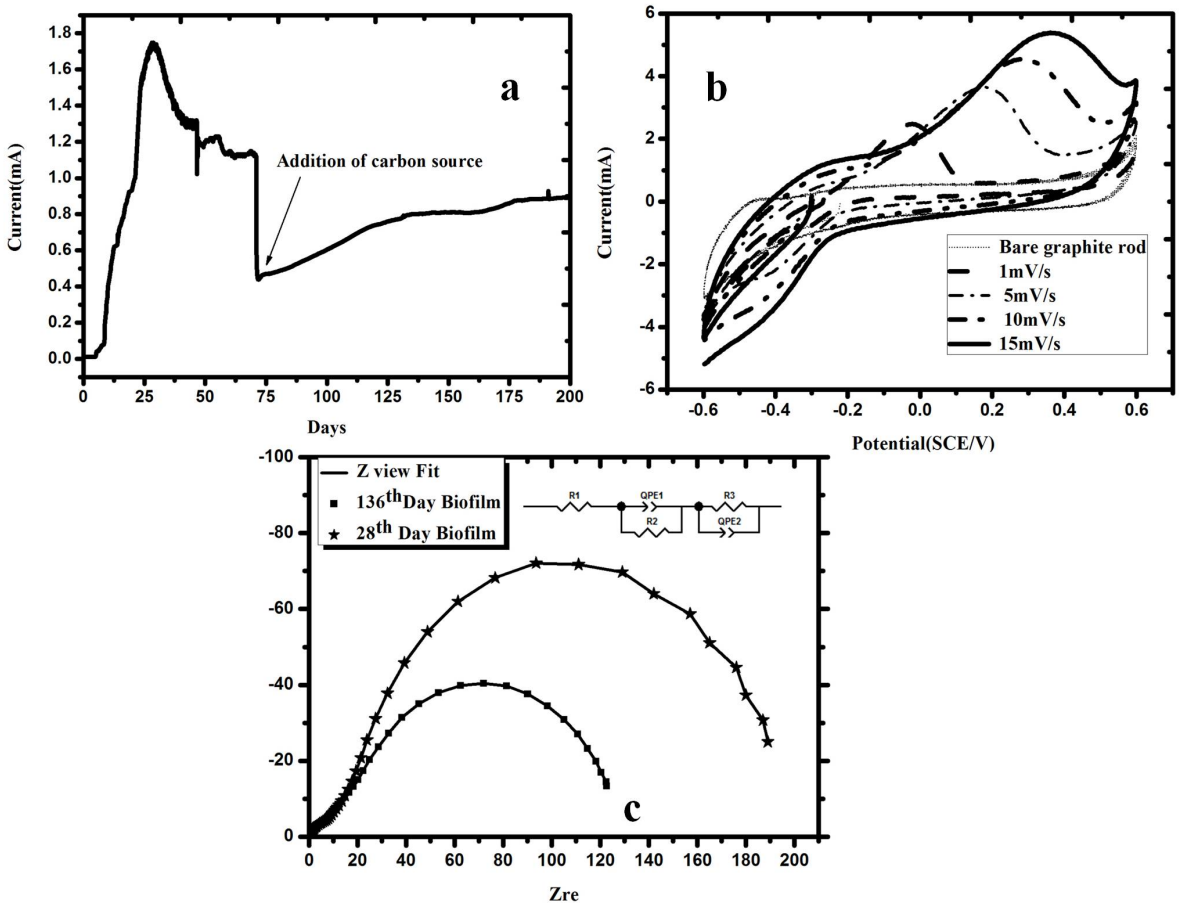

Figure 3. (a) Variation of current during biofilm formation at -150mV vs saturated calomel electrode. (b) Cyclic voltammetry of initial GR at 15mV/s the cyclic voltammetry of biofilm in bicarbonate buffer (0.1M) with 15mM of sodium acetate as carbon source were measured at different scan rates. (c) Electrochemical impedance spectroscopy of the biofilm electrode.

leads to an increase in the current up to 0.8mA at the 135th day. During the first 5 days, the growth of microbial colonies was observed in the log phase related to low current. Following 5 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 organic matter probably 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.

Cyclic voltammetry (CV) experiments (Fig. 3b) were performed for further investigate the electrochemical character of the biofilm. 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). CV was performed at the 28th day at a different scan rate up to 15mV/s. The midpoint potential deduced from the cyclic voltammetry, was +108mV vs standard hydrogen electrode (SHE). This value is close to the alkaliphilic cytochromes potential range [16]. Concomitantly, it could be the soluble/membrane-bound cytochromes of alkaliphilic bacteria [16-19]. Haloalkaliphilic bacteria require high negative membrane potentials in order to stand high alkaline pH conditions [20]. By increasing the scan rate the anodic peak shifted to more positive values (Fig. 3b). 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). One of the possible reasons for the irreversible process would be the electrode surface that could be fouled by strong irreversible adsorption [21], due to the thickness of biofilms. Membrane bound enzymes (cytochrome) of biofilm is required to be independent of the electroactive ARB`s metabolism [22].

Electrochemical impedance spectroscopy (EIS) at the 28th day and the 136th day revealed two semicircles; one at high frequencies and another at low frequencies (Fig. 3c). The high frequency semicircle could be associated to ARB biofilm electrical properties. The low frequency semicircle was probably linked to the processes occurring at the biofilm/solution interface. The impedance spectra were fitted with an appropriate equivalent circuit (EC). A simple EC used for describing the electrochemical properties of the ARB biofilm is shown in the Fig. 3c. inset, which has the following elements: R1 - solution resistance between the electrode surface and medium (ohmic resistance), R2 - ARB biofilm resistance, R3 - mass transfer/diffusion resistance and C – capacitance (ie, accumu-

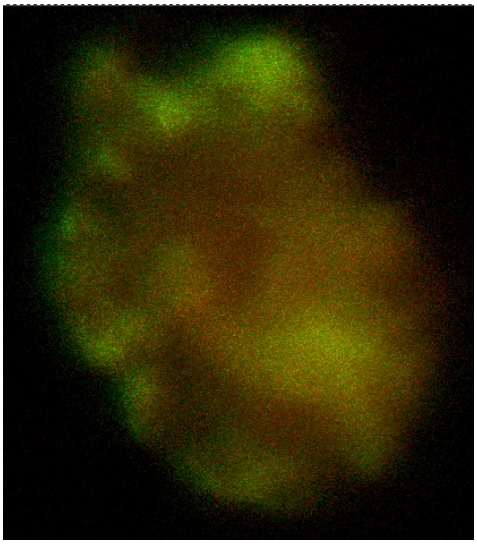

Figure 4. Image of biofilm surface using confocal microscopy at ×400 magnification

lation 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) [23]. One or two time constants are usually sufficient to interpret the impedance data for most cases in bio electrochemical studies [24,25].

The obtained values of the resistance of the bio film (R2) were 11.1Ω for the 28th day and 5.5 Ω for the 136th day, i.e., this tendency decreased with time of operation (Table 1). This may be due to the adaptability or enrichment of electroactive bacteria on the surface of the graphite rod which may reduce the internal resistance. The resistance of the mass transport/diffusion (R3) was reduced at the 136th day; due to electroactive biofilm reduces the diffusion resistance on the surface of the graphite rod. The biofilm formed after 150 days is shown in Figure 4. The biofilm reduced 5-cyano-2,3- ditolyl tetrazolium chloride (CTC) into water insoluble crystalline CTC-formazan, visualized as red crystals and accumulate inside the membrane bound enzymes [26]. 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 were stained red, indicating that most of the cells were metabolically active [27] along with green colour areas, which indicate the nucleic acid staining of acridine orange. This also further confirmed the presence of membrane bound enzymes actively involved in the

Table 1. Electrochemical impedance spectroscopy of biofilm anode

Time (Days)	R1(Ω)	R2(Ω)	R3(Ω)	R(Ω)
28	3.81±0.01	11.11±0.01	235±1.0	250±1.0
136	3.80±0.01	5.5±0.01	120.7±0.1	130±1.0

R1-Solution resistance; R2-Biofilm resistance; R3-mass transfer/diffusion resistance;R=R1+R2+R3

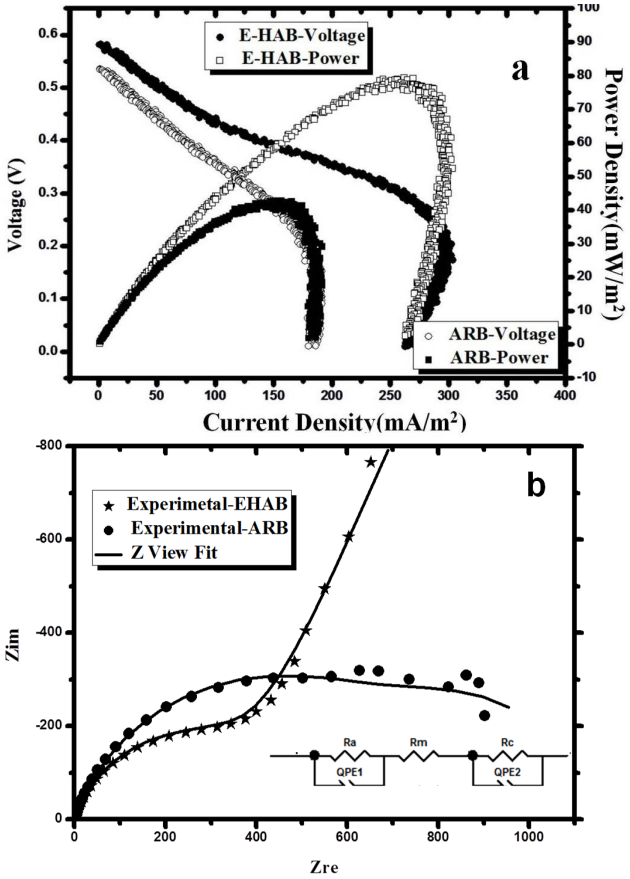

Figure 5. (a) Polarization curve of electrochemical stress (E-HAB) and unstressed (ARB) using linear sweep voltammetry (b) Electrochemical impedance spectroscopy of single chamber microbial fuel cell wore electrochemical stress-anode respiring bacteria

extracellular electron transfer. With longer incubation, the biofilm became thicker, ca. ~40 μm.

Electroactive biofilm- haloalkaliphilic bacteria (E-HAB) and anode respiring bacteria (ARB) were inoculated in SCMFC and monitor the open circuit potential (OCP), after 24 hours both cultures attain the maximum OCP (data not shown) than proceed the polarization. The polarization curves of E-HAB and ARB biocatalysts by the LSV method were shown in Figure 5(a). E-HAB gave the maximum power density of 79.4±0.81mW/m^2 at the current density of 261.7±0.44mA/m^2 and 0.296±0.01V, ARB (HAB-without any electrochemical stress) gave the maximum power density of 41.0±0.58mW/m^2 at the current density of 138.0±0.61mA/m^2 and 0.294±0.01V. The power density in the present work was higher than those of MFCs operated at hypersaline inoculum (0.06mWm^{-2}) [28] with alkalophilic Corynebacterium sp. strain MFC03 (41.8 mWm^{-2}) [29], and alkaline condition for rice mill waste water MFC (50 mWm^{-2}) [30]. These values of power densities are lower with respect to enrichment of E-HAB. However, haloalkalophilc microorganisms provide greater tolerance to fluctuations in chemical composition, which makes them useful candidates for operating in MFCs under various special waste water treatment processes.

Figure 5(b) shows the EIS results of the inocula E-HAB and haloalkaliphilic bacteria-without any stress (ARB). The cell loaded with E-HAB inoculum showed a R_{int} of 445 Ω (R_{anode}-358 Ω, $R_{cathode}$-86 Ω and $R_{membrane}$-0.62 Ω). The cell loaded with ARB inoculum showed a R_{int} of 1532Ω (R_{anode}-1044Ω, $R_{cathode}$-487Ω and $R_{membrane}$-1.3Ω). The anode resistance drastically reduced in the case of E-HAB. This clearly suggests that the bio electrolysis was an efficient method for developing a rich ARB community [31].

4. CONCLUSION

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 at -150mV/SCE potential, from Texcoco soil bacterial community. Midpoint potential deduced from the cyclic voltammetry, was +108mV vs standard hydrogen electrode (SHE). This value was close to the alkaliphilic cytochromes potential range. The electrochemical impedance spectroscopy of electrolysis cell revealed that the resistance of the biofilm (R2) decreased from 11.1Ω at the 28th day to 5.5Ω at the 136th day, i.e., this resistance decreased with time of operation. Confocal microscopy analysis allowed to appreciate the activity of the membrane bound enzymes involved in the extra cellular electron transfer process, with the thickness of biofilm around ~40μm. Electrical stressed-HAB gave a maximum power density of 79.4mW/m^2 when compare with the un-stressed HAB (ARB) (41.0mW/m^2), in a single-chamber microbial fuel cell (SCMFC). All these electrochemical experiments and evaluations suggest that the electrical-stress directed evolution of ARB community was associated to a more efficient extracellular electron transfer process in SCMFC.

5. ACKNOWLEDGMENTS

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