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RESEARCH ARTICLE - BIOLOGICAL SCIENCES

MFC—An Approach in Enhancing Electricity Generation Using Electroactive Biofilm of Dissimilatory Iron-Reducing (DIR) Bacteria

C. Yuvraj[1] · **V. Aranganathan**[1]

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Abstract A potential dissimilatory iron-reducing bacteria *Klebsiella pneumoniae* was employed in dual chamber microbial fuel cell for the formation of biofilm on the anode surface. Biofilm development on the electrode was examined as extracellular polymeric substances and phospholipids quantitatively. Significant increase in open circuit voltage and the current was observed from first cycle (0.950 V, 1.250 mA) to the last cycle (1.2 V, 1.683 mA) of microbial fuel cell operation. Increasing columbic efficiency from 8 to 62% showed the amount of electrons available from the oxidation of organic matter into electricity. Chemical oxygen demand removal efficiency increment from 44 to 85% establishes effective utilization of organic matter by *K. pneumoniae*. The scanning electron microscopic observations proved the ability to form a biofilm on an electrode surface. Results of the present study suggested that increasing power output is directly proportional to biofilm formed on the electrode surface. Biofilm development enhances the current production as a result of effective electrocatalysis by *K. pneumoniae*.

Keywords Biofilm · Microbial fuel cell · COD · Columbic efficiency · *K. pneumoniae*

1 Introduction

Fossils fuels account for nearly 80% of world energy production, among that petroleum being in limited stock has led to energy crisis [1]. Furthermore, combustion of these natural combustible sources adds to the CO_2 profiling and causes a global climate alteration. To diminish the after effect of global climatic change and energy crisis, mankind needs to develop the carbon neutral sustainable energy sources.

Microbial fuel cell (MFC) is one such sustainable and renewable technology that works on the exploitation of microorganisms as a biocatalyst for the electricity generation from organic matter [2]. Although MFC is going through the pace of significant development in recent years, the power generation is not adequate for practical applications [3]. In order to increase MFC performance, exertion has been made to enrich electrogenic bacteria [4].

In MFC system, electroactive microorganism facilitates the transfer of electrons to an electrode as a final acceptor. In a classic MFC, electrochemically active bacteria oxidize organic matter and grow as a biofilm on the anode surface. In recent years, several studies have been done to prove that electrogenic bacteria such as *Geobacter* and *Shewanella* genera are capable of forming biofilm over the anode surface [5]. Thus, the formation of bacterial biomass on anode can be critical to increase the current production in MFCs. Wei et al. [6] investigated the power output and biomass production of *G. sulfurreducens* growing on anode surface, and the results indicated the higher current generation on greater biomass production.

In the present study *K. pneumoniae*, a potential isolate from local sewage water exhibiting dissimilatory iron-reducing (DIR) activity was employed to investigate the correlation between electricity generation and biofilm formation in MFC. Quantitative characterization of biofilm was performed at the end of each batch operation. Scanning electron microscope (SEM) analysis was performed to visualize the surface morphology of the electrode. The results of this study are expected to afford a good understanding of the correlation between biomass of biofilm and enhanced electricity generation.

✉ V. Aranganathan
bioarang@yahoo.co.in

[1] Department of Biochemistry, Jain University, 18/3, 9th Main, 3rd Block, Jayanagar, Bangalore, Karnataka 560 011, India

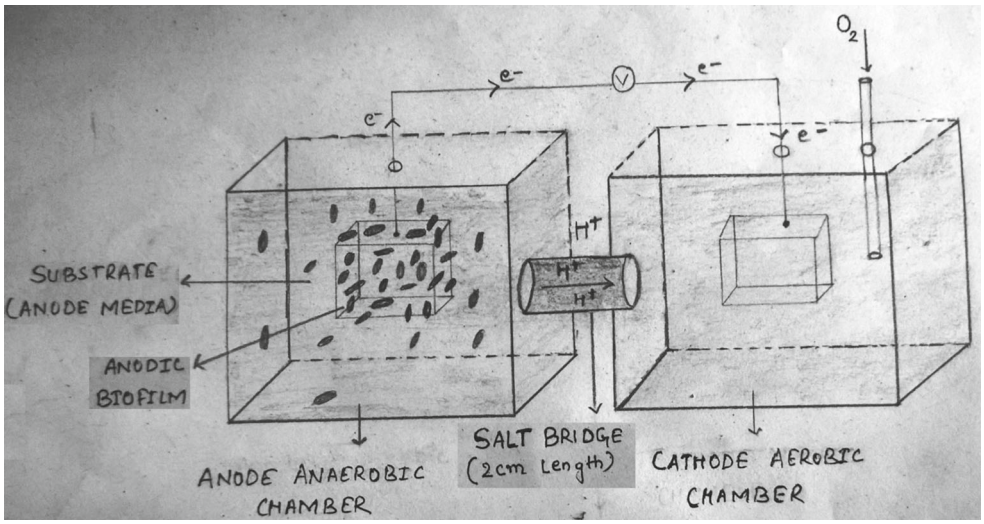

Fig. 1 Schematic diagram of dual chambered MFC setup

2 Materials and Methods

2.1 Chemicals

Glucose, yeast extract, peptone, asparagine, KH_2PO_4 (potassium di-hydrogen phosphate), K_2HPO_4 (di-potassium hydrogen phosphate), KCl (potassium chloride) , H_2O_2, vitamins and amino acid solution were purchased from Himedia, India. Potassium ferricyanide, Trizma base, EDTA, sodium potassium tartrate and FC reagent were procured from Fisher scientific, India.

2.2 Microorganism and MFC Configuration

The efficient electrogenic bacterial strain was isolated from sewage water collected from Cubbon park sewage treatment plant, Bangalore, India. Selective enrichment culture technique was used to select the DIR bacteria. The most efficient bacterial strain was selected based on reducing the capability of iron oxide and identified through 16S rRNA gene sequencing as *K. pneumoniae* [7]. The preserved stock culture was used after preculturing in glucose basal medium containing the following components (g/l): KH_2PO_4 (0.8), K_2HPO_4 (3), KCl (0.2), yeast extract (0.5), glucose (20), asparagine (5) along with 5 ml of vitamin (407 nM) and 5 ml of amino acid solution (2 mg/L).

A dual chamber MFC was constructed using polycarbonate bottles (50 ml each) connected together with the salt bridge as shown in Fig. 1. The anode chamber contains glucose basal media as anolyte and potassium ferricyanide with an electrolyte solution containing 100mM phosphate buffer solution (pH 7.0) as catholyte. The MFC configuration, operation, and optimized media composition were prepared and used as previously described by Yuvraj and Aranganathan [8]. All experiments were done in triplicates, and the average was considered for the calculation.

2.3 Biofilm Development

The 24 h activated culture of *K. pneumoniae* was inoculated in anode chamber (1×10^6 cells) and was incubated at room temperature under open circuit conditions for the development of biofilm over a period of 60 days. Prior to inoculation, the anaerobic condition was maintained in anode chamber by sparging N_2 for 10 mins at 4°C. To facilitate water formation in cathode chamber, catholyte was continuously aerated. Anode chamber was replenished in anaerobic condition with a fresh glucose basal media when the open circuit voltage (OCV) dropped less than 0.2 V considered as one cycle.

2.4 Electrochemical Measurements

2.4.1 Analytical Measurements

OCV and other parameters required for calculating power (W) and power density (mW m^{-2}) were recorded every 5 h of each cycle using auto-range digital multimeter (MECO, USA) with1 kΩ external load [8].

2.4.2 Cyclic Voltammetry (CV)

Bio-electrochemical characteristics of biofilm on electrode surface were studied under steady conditions using cyclic voltammeter (CHI instrument, USA). CV analysis was performed on the anode during mid of each cycle in the range of −1.0 to +1.0 V (vs. Ag/AgCl) at a scan rate of 0.025 V s^{-1}, followed by reverse scan to the original value of −1.0 V.

The cathode and Ag/AgCl was used as counter and reference electrode, respectively.

2.5 Quantification of Biofilm Components

2.5.1 Attachment Assay

Cells attached on anode surface were evaluated by staining the electrode with 1% (w/v) crystal violet. The adsorbed dye on the cell surface was eluted using ethanol (70%) and quantified by measuring the optical density (OD) at 580 nm, which is directly proportional to biofilm size [9].

2.5.2 Extracellular Polymeric Substances (EPS) Characterization

EPS of anodic biofilm were characterized at the end of each cycle as described by Zhang et al. [3] with minor modifications. Electrodes containing biofilm was immersed immediately after removed from MFC setup in 25 ml Tris-EDTA buffer (10 mM Trisbase, 10 mM EDTA and 2.5% NaCl; pH 7.5) followed by scrapping of biofilm and centrifuging in the same buffer at 3500 rpm for 20 mins. The supernatant containing microbial biomass was resuspended using 0.5% formaldehyde. The dissolved EPS in formaldehyde solution was extracted by centrifuging at 3500 rpm for 20 mins.

The amount of carbohydrate and protein concentration of EPS was measured from the extract using dinitro salicylic acid method [10] and Lowry's method [11], respectively. The sum of carbohydrate and protein concentration represents the total EPS content of the biofilm.

2.5.3 Biomass Estimation

Biomass can be accurately estimated by measuring lipid-bound phosphates concentration. Electrodes containing biofilm was taken from MFC at the end of each cycle and analyzed for phosphate concentration according to the protocol described by Aelterman et al. [12]. Phosphate concentration released from phospholipids was determined at 610 nm using spectrophotometer (Shimadzu, USA). Cell numbers in the total active biomass was correlated with phosphate concentration using the conversion factor of 1.0 nmol of lipid-bound phosphate $= 3.43 \times 10^7$ cells.

2.6 Chemical Oxygen Demand (COD) Analysis

COD was measured before inoculation of the first cycle (COD_i) and end of each cycle (COD_t) by using spectrophotometer (DRB200, Hach Co., USA). 2 ml of samples was taken from the anode chamber and subjected to COD analysis for the calculation of COD removal efficiency at the end of each cycle.

The amount of electrons available from the total electrons for oxidation of organic matter into electricity was calculated in terms of columbic efficiency (CE) as a function of COD according to Logan et al. [13] as:

$$\mathrm{CE} = \frac{M \int_0^t I \mathrm{d}t}{nvF\,(\mathrm{COD}_i - \mathrm{COD}_t)}$$

where M is the molecular weight of oxygen, I is the current, F is Faraday's constant, $n = 4$ (number of electrons exchanged per mole of oxygen), and v is the anolyte volume.

2.7 SEM Characterization

Surface morphology of biofilm on anode surface at the end of each cycle was evaluated by using SEM (S-3000H, Hitachi, Japan). At the end of each cycle, the electrode was removed from the anode chamber and rinsed with phosphate buffer (pH 7.0), and then fixed with 2% glutaraldehyde at 4°C for 1 hr. After fixation, the electrode was subjected to dehydration using ethanol (30, 50, 70, 80, 90, and 100%) at room temperature. Then the electrodes were washed thrice with tert-butyl ethanol, air-dried and sputter layered with a thin layer of gold [3]. Fresh graphite electrode was used as control.

3 Results and Discussion

3.1 Electricity Generation

Current and OCV production was observed over a period of time after inoculation. A maximum value of current (1.250 mA) and OCV (0.950 V) was observed during the mid of the first cycle, followed by a gradual decrease in the production of current and OCV towards the end. The inability to maintain continuous electricity generation may be due to the limitation in fuel availability. The slow pace of electricity generation at the initial stage of MFC operation can be correlated with the lag period of bacterial cell formation on the anode surface [14].

Once the media was replenished, unlike first cycle current and OCV production started immediately and reached a maximum value as well as sustained for more time when compared to the previous cycle as shown in Fig. 2. The maximum electricity output at each cycle and its corresponding parameters are shown in Table 1. The power density of 1037 mW m^{-2} produced in the present study is much higher than what reported for *K. pneumoniae* L17 (410 mw m^{-2}) according to Zhang et al. [15].

Klebsiella pneumoniae used in this study has advantages of transferring electrons directly to the anode without any

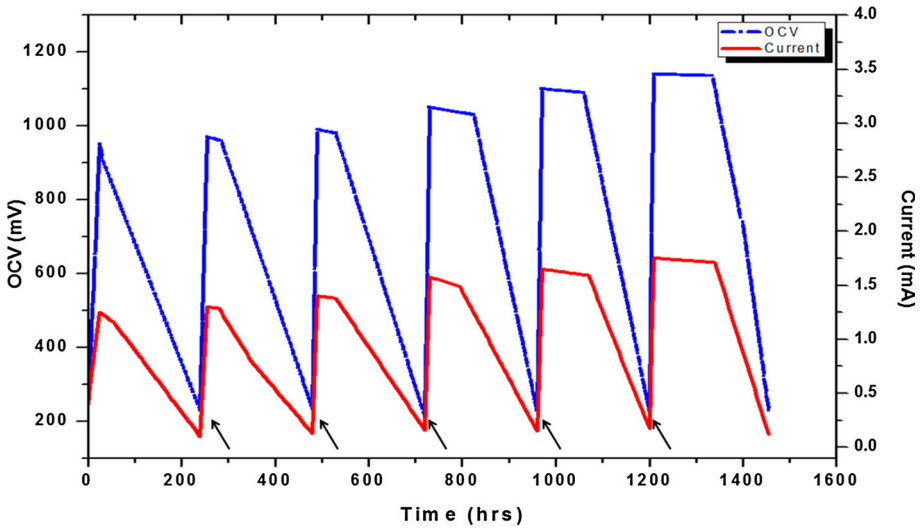

Fig. 2 Electricity generation by *K. pneumoniae* in MFC at different cycles (*arrows* indicating replenishment of media)

Table 1 Electricity profile of MFC at different cycle

Cycle	Time (h)	OCV (V)	Voltage (V)	Current (mA)	Power (mW)	Current density (mA/m^2)	Power density (mW/m^2)
1st	25	0.950	0.735	1.250	0.92	833	612.5
2nd	255	0.970	0.755	1.317	0.99	878	662.7
3rd	490	0.990	0.775	1.397	1.08	931	721.6
4th	730	1.050	0.835	1.473	1.23	982	820.0
5th	970	1.100	0.885	1.637	1.45	1091	965.7
6th	1210	1.140	0.925	1.683	1.56	1122	1037.7

external mediators, which in turn facilitates the biofilm formation. In addition it does not require any special conditions for its cultivation in laboratory scale unlike *Geobacter* spp. which need special pressure conditions for culture maintenance.

3.2 Biofilm Quantification

Figure 3 shows the significant increase in absorbance of ethanol-dye complex solution from the 1st cycle (0.33 OD) to 6th cycle (1.02 OD). About threefold increase in absorbance proves the significant growth of microbial biomass on the electrode surface. The increase in biofilm thickness was observed with age over a period of 60 days on the anode surface. Results of the increasing protein and polysaccharide concentration on the biofilm are evident with the growth of microbial biomass on electrode surface.

The total changes in the EPS concentration on biofilm is shown in Fig. 4. Protein concentration at the end of first cycle was found to be 0.314 mg protein cm^{-2}, and it increased up to 1.10 mg protein cm^{-2} at the end of last cycle. Similarly, polysaccharide concentration on electrode surface also gradually increased up to 3 cycles, followed by a little decrease

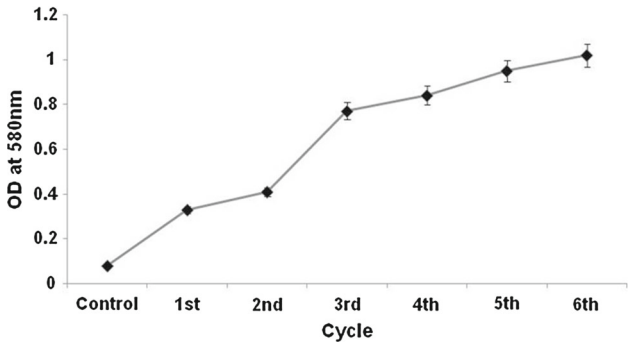

Fig. 3 Crystal violet assay of electrodes at each cycle

in subsequent cycles, which may be due to detachment of old microbial cells from anode surface.

The analysis of phospholipids has been providing relatively simple and convenient method for estimating microbial biomass [16]. On the basis of conversion factor, number of cells was increased from 8.23×10^7 to 13.17×10^7 from first to last cycle respectively (Table 2). All major classes of macromolecules, i.e., polysaccharides, proteins, nucleic acids, peptidoglycan, and lipids can be present in the biofilm. Ahimou et al. [17] clearly pointed out the relationship between the EPS concentration and biofilm development.

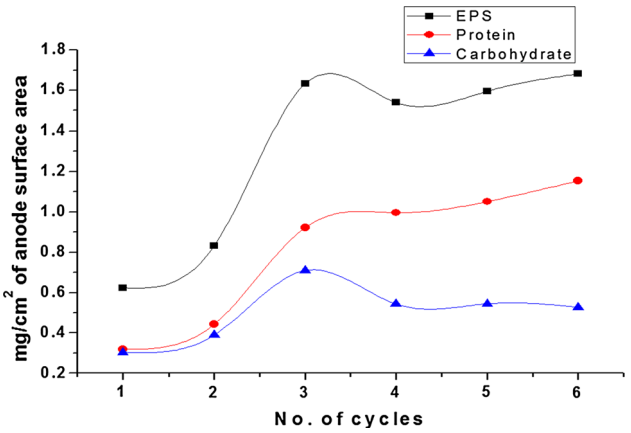

Fig. 4 Extracellular polymeric substance profile on electrode surface cm^{-2} at different cycle

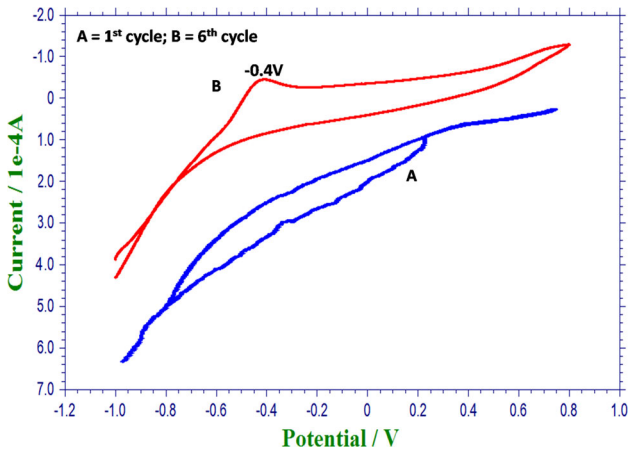

Fig. 5 CV curve of MFC at 1st and 6th cycle

Table 2 Lipid-bound phosphate conversion to microbial cell number at different cycle

Cycle	OD	µg of P_i	nmol of P_i	No. of cells (10^7)
1st	0.134	0.084	2.40	8.16
2nd	0.197	0.123	3.78	12.85
3rd	0.239	0.149	4.47	15.19
4th	0.295	0.184	5.52	18.70
5th	0.241	0.151	4.50	15.30
6th	0.206	0.129	3.84	13.05

Several investigators have also profiled the thickness of biofilm dependence on these parameters. Polysaccharide interacts with proteins when attached to the surface of the microbial cells and form polysaccharide-protein matrix, which induces both structural and functional properties on biofilm development [18]. In another study, Albelo and Domenech [19] described the increasing membrane content of *P. aeruginosa* during biofilm formation. Results of the present study show the increasing EPS profile on anode surface indicating the biofilm adhesion strength, which is well correlated with the previous reports on the influence of EPS on biofilm formation [20,21].

3.3 Electrochemical Behaviour of Biofilm

CV helps in the elucidation of potential redox reactions happening at the interface of electrode solution and electrochemical reactions happening at the electrode surface. Results of the cyclovoltammetric measurement depict the changes in electrochemical behaviour of *K. pneumoniae* due to biofilm formation on electrode surface are shown in Fig. 5.

The electrochemical activity of bacterial strain significantly increased in each cycle, which confirms the biofilm establishment on anode surface. Appearance of reduction peak at −0.4 V (6th cycle) along with increment in OCV further confirms the electrochemical activity of biofilm. Results in the present study are in accordance with that reported by Zhang et al. [15]. Accordingly, development of biofilm on anode surface is the key factor for electron transfer, which is confirmed through an increase in the reduction peak. Otherwise, along with the used media redox molecules also get removed from anode which can further decrease the electrochemical activity of *K. pneumoniae*.

3.4 COD Analysis

COD removal efficacy is used as a measure of oxygen requirement of the sample that is susceptible to oxidation by the strong chemical oxidant. Increment around twofold in COD removal efficiency from first cycle (44%) to the final cycle (85%) clearly exhibits the feasibility of MFC on removing organic carbon and its utilization by microorganism for biofilm formation. Columbic efficiency was greatly increased from 8% (1st cycle) to 62% (6th cycle), and its corresponding COD removal efficiency is shown in Fig. 6.

The COD removal efficiency (85%) reported in the present study is much higher than reported by Baranitharan et al. [22], where electricity generation and treatment of palm oil effluent was carried out simultaneously. Based on this result, it is clear that the organism *K. pneumoniae* effectively utilized the media components for its growth on the anode surface leading to development of biofilm. Similarly, Khater et al. [23] used glucose as main substrate yielding 55% CE in oxidizing glucose which is lower than that (62%) reported in the present study.

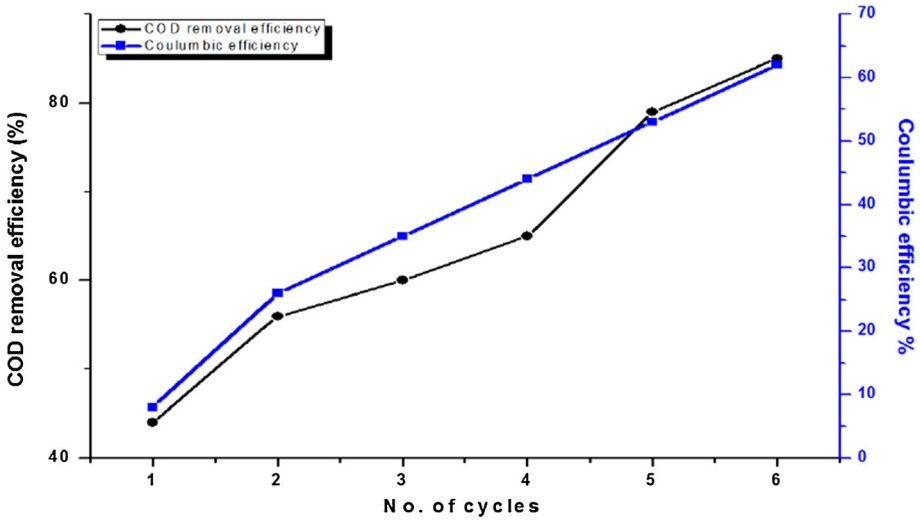

Fig. 6 COD and columbic efficiency at different cycle

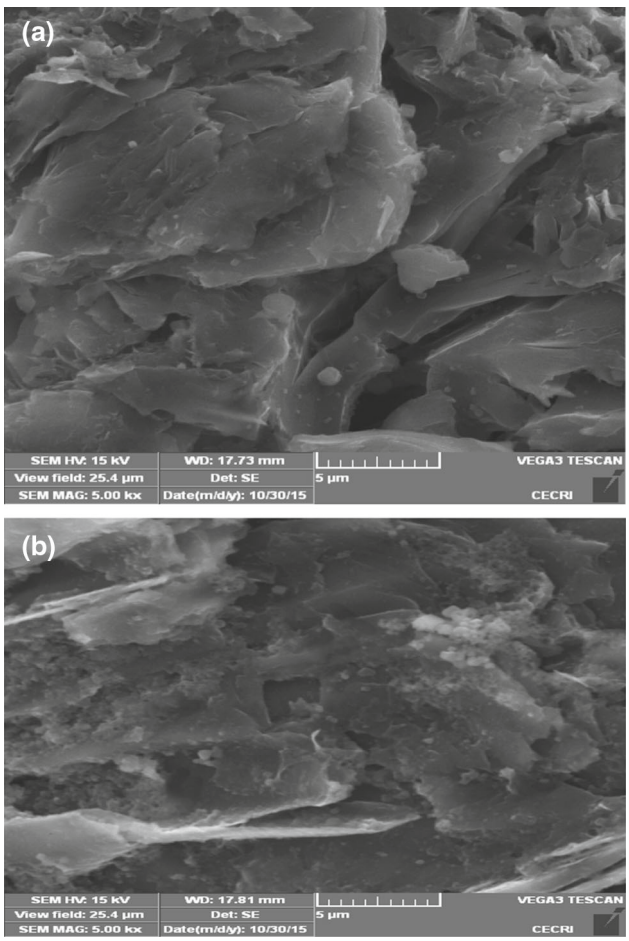

Fig. 7 SEM image of fresh and 6th cycle electrode

3.5 Biofilm Formation on Anode

The dehydration process during fixation may have altered the biofilm morphology, even though SEM result provides good visual comparison demonstrating architectural difference of biofilm at different cycles. Figure 7a, b displays the SEM microphotographs of the control and 6th cycle anode surface. The SEM results indicated that *K. pneumoniae* can form electroactive biofilm on the anode surface and facilitate electron transfer to the electrode through the biofilm network in MFC operation for electricity generation.

4 Conclusion

The present study proves that *K. pneumoniae* was able to form an electroactive biofilm on electrode surface over a period of MFC operation. Increasing biofilm density on anode surface was well correlated with enhanced electricity generation that proves the role of bacteria in MFC to transfer electron from organic media components to the anode. Findings of the present study shows electrogenic microorganisms are capable of using organic fuels for generating electricity by biofilm formation. This is potentially beneficial to increase the performance of MFCs for various applications.

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