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Enrichment of Electricigenic Biofilm for Synchronized Generation of Electric Current and Waste Water Treatment in Microbial Fuel Cells

Zargona Zafar, Kamran Ayaz, Iqra Sharafat, Shabeena Shah, Sarwat naz zafar, Naeem Ali[*]

Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan
[*]E-mail: naeemali95@gmail.com, naeemali2611@gmail.com, naeemali@qau.edu

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Enrichment of electricigens was carried out in double chamber Microbial fuel cells (MFCs) for synchronized organic waste treatment and bioenergy recovery. Activated sludge was inoculated in MFCs to measure the effect of two carbon sources (sucrose and acetate) on current output. MFCs operated with sucrose showed current generation of 5µA and 3µA with COD removal efficiency (86.04% and 77.85%) in two respective stages of enrichment. Whereas, the relative efficiency of MFCs run with acetate remained considerably low in the 1st stage though it inclined to 4µA with 81.17% removal of COD after 2nd stage of enrichment. Culture based analysis of anodic biofilms indicated the presence of different bacterial species i.e. *Pseudomonas* sp., *Proteus* sp., *Citrobacter* sp., with different biofilm forming capabilities (strong 5%, moderate 15% and weak 14.8%) in sucrose and acetate fed MFCs. 454 pyrosequencing of acetate-fed MFC indicated clear difference between established biofilm communities on anode and sludge sample. Anodic biofilm was covered with both culturable and un-culturable *Pseudomonas* species along with *Nitrosomonas europaea* and *Massilia* sp., etc. Principle component analysis also confirmed that major contributing classes were α-proteobacteria (48.51%), β-proteobacteria (31.48%) and γ-proteobacteria (16.16%). The results of current study implied that enrichment technique resulted in better selection of electricigens along with waste water treatment.

Keywords: Microbial fuel cells; Enrichment; Biofilm; Current; Pyrosequencing

1. INTRODUCTION

Worldwide, the energy demand is increasing with the expansion of human population. Apart from energy crises, the global concern regarding environmental impacts of huge consumption of fossil fuels is another challenge to the sustainability of earth. A significant portion of energy is also

consumed in waste water treatment systems, that also demands alternate cost effective and sustainable energy solutions. Currently, several alternate energy sources have been promoted at large scale, however, few innovative technologies are undergoing through experimental trials. Among different innovative solutions, microbial fuel cells (MFCs) emerged as one of the most promising technologies. MFCs works on the principle of bio-electrochemistry where energy (current) is recovered from oxidation of reduced organic compounds [1] by the catalysis of bacteria. Basically, electrons from these substrates are picked up on anode and channelized towards cathode through in an electric circuit [2]. Microbial fuel cells have a distinctive advantage of utilizing low grade biomass and even waste water to generate electricity. MFCs simultaneously treat waste water and recover bio-energy in terms of electricity generation [3-11].

Domestic waste water contains easily bio-convertible organic substrates that are used by bacteria in MFCs to generate electricity [12-18]. Some electrochemically active bacteria (EAB) known as anode-respiring bacteria or electricigens are able to donate their electrons to electron acceptors like metal oxide and electrode surfaces etc., via extracellular electron transfer mechanisms [19, 20]. EAB are reported to be present in diverse and complex environments such as municipal wastewater, seawater, soils and sediments etc. Generally mixed bacterial communities include fermenters and EAB like *Shewanella putrificians* [16], *Geobacter metallireducens* [21],*Geobacteraceae sulferreducens* [18] and *Rhodoferax ferrireducens* [22], *Pseudomonas aeruginosa* [23] and *Enterococcus Faecium* [23] are abundant in nutrient rich environment. The hidden potential of EAB was reported about 100 years ago that they produce current [24], however, their effective utilization in any MFC devise is still under investigation. The advantage of applying electrochemically active bacteria in MFC not only help in waste water treatment, it simultaneously generates electric current. Despite intensive research, MFC technology has yet to achieve a final architecture for potential application [25]. In this reference search for potentially active electricigens is considerably vital and currently under investigation.

Keeping in view the immense hidden diversity of bacteria, typically electricigens in nature, isolation of potentially active ones is essentially important to extend the scope of MFC technology in future. The present research work evaluated the succession and enrichment of bacterial communities from activated sludge during repeated operations of double chamber MFCs. Enrichment method developed by J.F. Miceli et al.[26] was applied to find efficient electrochemically active bacteria (EAB) for electric current generation along with waste water treatment. The enriched biofilm bacterial community structure was determined on the basis of culture dependent and independent techniques (pyrosequencing). Furthermore, the biofilm forming abilities of each culturable bacterial isolate was separately determined under in-vitro condition.

2. MATERIAL AND METHODS

2.1 Sludge sampling

Activated sludge sample was collected from Waste water treatment Plant I-9, Islamabad, Pakistan in a sterile polythene container. The samples were transported and stored at 4^{o}C before use.

2.2 MFC construction and operation

Dual chamber Microbial fuel cells (MFCs) were constructed using polyacrylic bottles (500ml each). Salt bridge was made between anode and cathode by a glass rod (length; 5cm). The total working volume of each chamber was approx. 300ml. The joints of the chambers were sealed with epoxy glue and silicon sealant to prevent the leakage. The anodic chamber was kept air tight throughout the incubation period. Carbon cloth (EC-CC1-060, no wet proofing) was used as electrodes (5 x 5cm) in salt bridge MFCs. Cathode surface was coated with Pt catalyst following Cheng et al., (2006) protocol.

Almost 240ml autoclaved synthetic waste water containing sucrose and potassium acetate as the only carbon source was added as a feed solution in anode [Sucrose 450mgl^{-1}/ Potassium acetate 450mgl^{-1}, $NaHCO_3$ 480mgl^{-1}, NH_4Cl 95.5 mgl^{-1}, K_2HPO_4 10.5 mgl^{-1}, KH_2PO_4 5.25 mgl^{-1}, $CaCl_2.2H_2O$ 63.1 mgl^{-1}, $MgSO_4.7H_2O$ 19.2 mgl^{-1} (Trace metals:$FeSO_4.7H_2O$ = 10 mgL^{-1}, $MnSO_4.H_2O$ = 0.526 mgl^{-1}, $NiSO_4.6H2O$ = 0.526 mgl^{-1}, H_3BO_3 = 0.106 mgl^{-1}, $ZnSO_4.7H_2O$ = 0.106 mgl^{-1}, $CoCl_2.6H_2O$ = 52.6 µgl^{-1}, $(NH_4)_6Mo7O_{24}.4H_2O$ = 52.6 µgl^{-1} and $CuSO_4.5H_2O$ = 4.5 µgl^{-1})] and 60ml of activated sludge inoculum was added into the anode chamber. Anaerobic conditions were produced by sparging (N_2) for 20 min in anode chamber. In cathode chamber, Potassium permanganate ($KMnO_4$) 0.6mM was used as a catholyte along with Phosphate buffer solution (PBS: NaH_2PO_4 4.904gl^{-1}, Na_2HPO4 9.125gl^{-1}, NH_4Cl 0.62gl^{-1}, KCl 0.26gl^{-1} in 1L distilled water, pH was adjusted to 7). Initially, COD of synthetic waste water was about 237mgL^{-1}. when the inoculum was added to the feed solution, COD exceeded to ≥1500mgL^{-1} (pH was adjusted to 7.3-7.6). The fuel cells were placed in incubator at 35±2^{o}C for 16 days. After six hours, open circuit voltage was measured with the help of precision multimeter (UT33C; UNI-T). The circuit was closed by applying 1000kΩ resistor and the voltage of microbial fuel cell was continuously monitored. After 16 days, the fuel cells were refreshed with synthetic waste water and operated with detached biofilm (2%) from anode surface and 10% sludge inoculum. Data was recorded and compared.

2.3 Power calculation

Voltage during enrichment stage 1 (*V1o, V2o, V3o)* and stage 2 (*V1E, V2E, V3E)* (mV) was recorded for 32 days for all microbial fuel cells with precision multimeter (UT33C; UNI-T). Ohm's law was used for calculation of current (I): $V = IxR$. where, I = current, V = voltage, R = Resistance.

2.4 Molecular phylogeny of anodic biofilm

Total DNA was extracted from activated sludge and anodic biofilm after enrichment in acetate fed MFCs. For DNA extraction, soil DNA isolation kit (Norgen biotek corp, product Catalog: 26500) was used. DNA samples were sent to Molecular Research (MRDNA) (http://www.mrdnalab.com) for 454 pyrosequencing analysis. 16S rRNA gene V4 variable region was targeted, 515/806 PCR primers were used in a single-step PCR 30 cycle using the HotStarTaqPlus Master Mix Kit (Qiagen, USA). Ion

Torrent PGM Sequencing was performed according to manufacturer's guidelines. The data was processed by means of proprietary pipeline analysis (MR DNA, Shallowater, TX, USA).

2.5 Heterotrophic plate count and bacterial isolation from anode surface

After completion of each stage of bacterial enrichment, a small piece of anode was cut with the help of sterile scissors and placed in phosphate buffer solution. The solution was sonicated for 5mins to remove attached cells from anode surface under anaerobic conditions. Standard serial dilution and pour plate techniques were used to isolate the bacteria. The culture plates were incubated at 35±2^{o}C in anaerobic jar for 24-48 hours. Morphologically distinct bacterial colonies were purified on nutrient and blood agar plates. Sub-culturing was carried out until individual separated colonies were obtained. Pure culture bacterial colonies were differentiated and partially identified on morphological and biochemical behavior using Bergey's Manual of Determinative Bacteriology [27]. Biofilm forming capability of each strain was checked by Microtiter dish biofilm formation assay.

2.8 Scanning electron microscopy

Biofilm formed during enrichment stage 1 and 2 with activated sludge on carbon cloth in anode chamber was cut with sterile scissors and placed in sterile phosphate buffer solution. The samples were sent to centralized resource laboratory, university of Peshawar, Khyber Pakhtunkhwa. The samples were scanned with scanning electron microscope at 20kV at different resolutions.

3. RESULTS AND DISCUSSION

3.1 Current output Vs different carbon sources in MFC

Enrichment of anode respiring bacteria from activated sludge was carried out in salt bridge double chamber microbial fuel cells with two different carbon sources i.e. sucrose and acetate. An open circuit voltage (OCV) 107.5mV was developed in sucrose fed MFCs (S-MFCs), whereas, 229mV in acetate fed MFC (A-MFCs) after one day of operation. As OCV potential develop, the circuit was closed by connecting 1000kΩ resistor across the circuit. During 1st stage of enrichment, maximum voltage (597mV) in S-MFCs and 136.2mV in A-MFCs was recorded. During enrichment stage 2, the biofilm developed in stage 1 was re-suspended into newly assembled fuel cells. Besides, the maximum voltage across 1000kΩ was found to be 345.7mV and 398mV in the two cells respectively (Figure 1A).

The low voltage generation during enrichment stage 2 in S-MFCs might be due to competitive inhibition of electrogenic bacteria during substrate consumption by other non-electrogenic bacteria [28]. Insufficient supply of electron acceptors in cathodic chamber sometimes cause accumulation of electrons in the anodic compartment thereby inhibiting the growth of EAB[28] that work in combination. Altered voltage output in MFC reactors during operation under batch mode condition

might be due to depleting nutrients in the first phase. Besides, development of altered community after succession may have varying nutritional requirements based upon their physiological status and adaptation in a close circuit electrical system. Therefore, it is always vital to timely replenish nutritional inputs as anolytes and catholytes in the system keeping in view the biofilm status and age [28, 29].

In previous studies, effect of different carbon sources (glucose [22], acetate [31], sucrose [32], synthetic waste water [33], domestic [34], recycling of paper waste water [35] and swine wastewater [36]) (in pure and mixture culture communities) on bioelectricity generation in MFCs has been investigated [30]. It has been proved from the current and previous studies that type of substrate has great influence on performance of MFCs. Primarily, the anodic and cathodic anolytes help in shaping of specific bacterial community structures integrate and adapt with MFC circuit and set-ups [37, 38] (Table 1).

3.2 Effect of External Resistance

Table 1. Current outputs with diverse substrates in microbial fuel cells (MFCs)

Substrate type	Concentration	Source of Inoculum	MFC Type (cell volume/electrode surface area)	Current density ($mAcm^{-2}$) at maximum power	References
Acetate	1 g/L	Pre-acclimated bacteria from MFC	Cube shaped single-chamber MFC with anode made of graphite fiber brush (7170 m^2m^{-3} brush volume)	0.8	[39]
Glucose	6.7 mM	Mixed bacterial culture maintained on sodium acetate for 1 year (*Rhodococcus* and *Paracoccus*)	One-chamber air-cathode MFC (12 mL) with anode (non-wet proofed carbon cloth) (2 cm^2) and wet proofed carbon cloth as cathode (7 cm^2)	0.70	[40]
Sucrose	2674 mg/L	Anaerobic sludge from septic tank	Dual-chambered mediator-less MFC with stainless steel mesh anode (213.29 cm^2) and cathode (176.45 cm^2); KMnO4 (0.2 gL^{-1}) as catholyte	0.19	[32]
Artificial wastewater with glutamate and glucose	300 mg/L	Anaerobic sludge	Membrane-less MFC with anode (465 cm^2) at bottom and cathode (89 cm^2) at top of cylinder; graphite felt as both electrode	0.02	[41]
Synthetic wastewater	510 mg/L	Anaerobic culture from a preexisting MFC	Dual chamber MFC with stainless tell as anode (170 cm^2) and graphite rods as cathode (150 cm^2)	0.008	[42]
Sucrose	450mg/L	Activated sludge	Dual chamber MFC with carbon cloth as anode and cathode (50cm^2)	0.04	Present study
Acetate	450mg/L	Activated sludge	Dual chamber MFC with carbon cloth as anode and cathode (50cm^2)	0.04	Present study

To determine the effect of external resistance on voltage and current density, external resistor was varied from 10 to 1000kΩ (Figure 1B). Maximum current density of 0.04$mAcm^{-2}$ was recorded

across 10Ω resistor in both S-MFCs and A-MFCs. As the resistance was increased to 10Ω from 1000kΩ, current density was decreased from 0.04mAcm^{-2} to 0.0001mAcm^{-2} in S-MFCs. Whereas, in A-MFCs current density decreased from 0.04 mAcm^{-2} to 0.00007mAcm^{-2}. However, voltage output increased in reverse order. With increase in resistance, increase in voltage was observed.

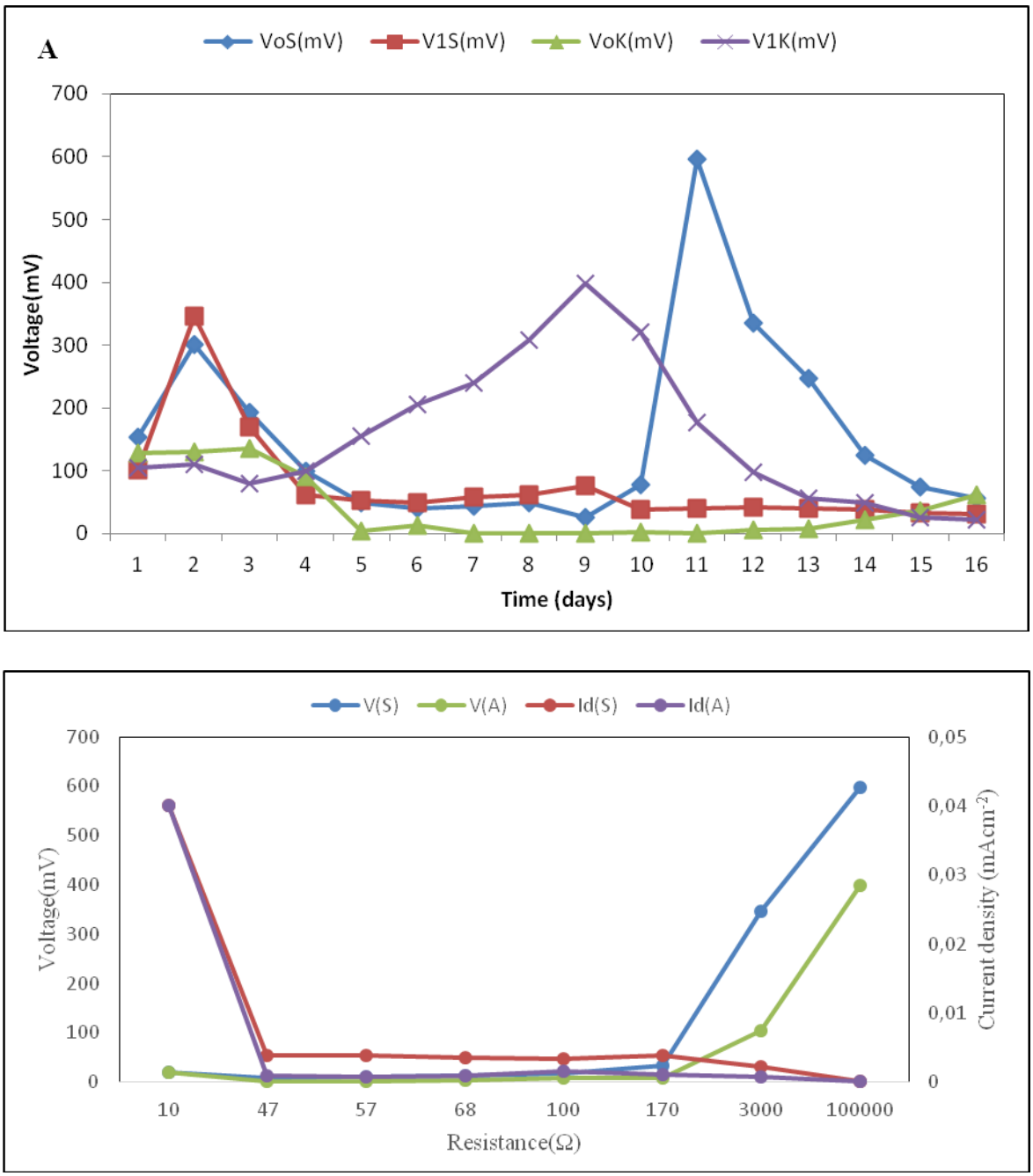

Figure 1(A). Effect of sucrose (S) and acetate (A) on voltage (mV) output during enrichment stage 1 (Vo) and enrichment stage 2 (V1) MFCs reactors (B) Effect of external resistance on current density (mAcm^{-2}) and voltage (mV)

3.3 Treatment of wastewater with current output

COD has been considered as one of basic parameter to determine the real-time status of oxidable nutrients in liquid mixture. Initially, COD of the MFC reactors was ≥1500mgl^{-1} (Figure. 2). However, after 5 days of operation during enrichment stage 1 of, COD removal efficiency recorded 37% in S-MFC and it was 6.62% lower than in A-MFC reactors. COD removal efficiency increased (37.04 - 86.04%) and (43.66 - 88.42%) in both fuel cells (S-MFCs and A-MFCs) from 5th to 16th day during operation (Figure 2). Decrease in the concentration of COD suggested the oxidative demineralization of specific electron donors by some bacteria. Catabolic products generated were then further metabolized by other bacteria that were enriched on anode surface during working of MFCs [43].

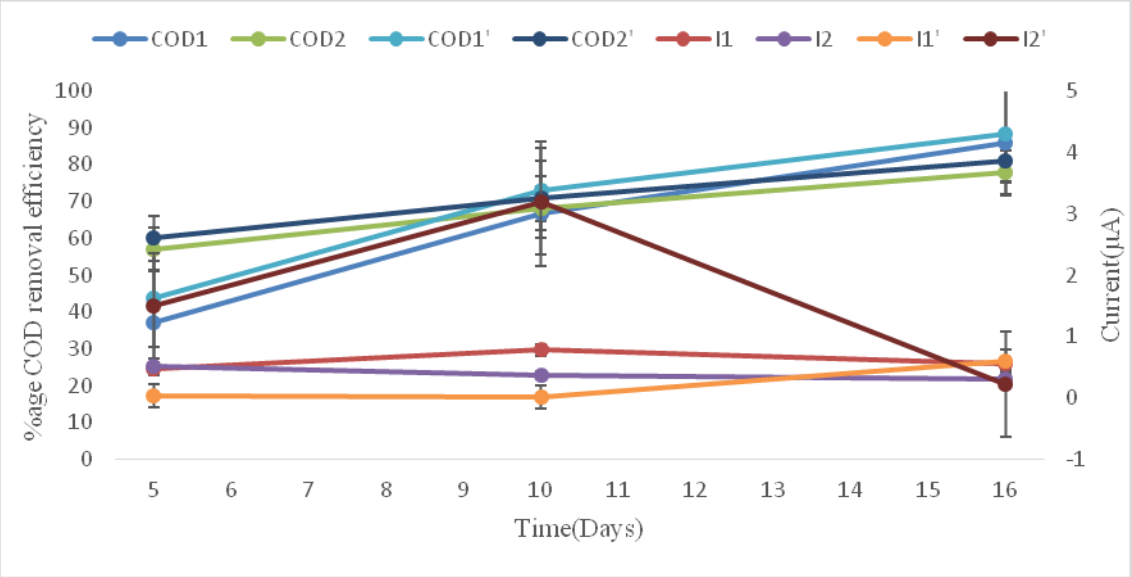

Figure 2. %age COD removal efficiency Vs current (µA) in sucrose and acetate fed MFCs. [Scale: COD1 = COD removal efficiency of S-MFC during 1st stage of enrichment, I1 = current of S-MFC during 1st stage of enrichment, whereas, COD2 and I2 for 2nd stage of S-MFC. While COD1', COD2' and I1', I2' shows COD removal for A-MFCs for two respective stages]. Bars show standard error

During enrichment stage 2, initial COD (850mgl^{-1}) in A-MFC whereas, 971mgl^{-1} in S-MFCs. Increase in COD removal versus time could be better adaptation of bacteria to actively degrade organic constituents. In comparison to the current generation capabilities of electricigens, at the fuel concentration of ≥517mgl^{-1} resulted in maximum current generation of about 5µA in S-MFCs across 1000kΩ. Whereas, maximum current (1µA) was observed with higher %age COD removal during first five days of operation of A-MFCs. During 2nd stage of enrichment COD removal remained low i.e., 57.04% as compared to 1st stage of enrichment (86.04%), resulted in lower maximum current generation 3µA. Previously, higher current output has always been linked with higher concentration of

anolyte. However, increase in substrate beyond certain limits creates toxicity to bacteria thereby decrease MFC performance by bacteria [43, 44].

3.4 Culturable Electricigens anodic biofilms

A total of 62 different bacterial isolates were isolated from anode surfaces (S-MFCs and A-MFCs). Microtiter plate assay revealed that almost 5% of the bacterial isolates were strong and 15% were moderate biofilm formers. Few non-adherent bacterial isolates (14.8%) were also examined. On morphological and biochemical basis, bacterial isolates were mostly identical as *Pseudomonas* sp., *Salmonella* sp., *Proteus* sp., *E. coli*., *Citrobacter* sp., *Vibrio* sp in S-MFCs and A-MFCs. Similar bacterial species [*Pseudomonas* sp. [45, 46], *Proteus vulgaris* [47], *Enterobacter cloacae* [48], *Klebsiella* sp. [49], *Lactococcus lactis* [50], *Clostridium butyricum* [51], *Shewanella* sp.[52], *Corynebacterium* sp.[53] etc] were reported previously with varying bioelectricity production ability.

3.5 Molecular Phylogeny of Electricigens on anodic Biofilms

454 pyrosequencing targeting the variable region V4 of 16S rRNA gene (515/806 PCR primers) showed that sludge sample contained 31 different phyla (cut-off value was set at 0.1%, the bacterial phyla and classes appearing below 0.1% were not included). After enrichment, only 14 phyla were observed in anodic biofilm in A-MFC. The relative abundance of species corresponding to phylum Proteobacteria was maximum (96.44%) in biofilm (Figure 3A). The abundance of phylum Proteobacteria increased from 50% to 96% approximately. Whereas, the percentage of other phyla decreased significantly after enrichment which indicated that only specific phylum associated with electric potential output were enriched on anode surfaces. Bacteriodates decreased from 5.6% to 0.96% in, 4.99% to 0.71% actinobacteria, 2.18% to 0.38% in chloroflexi, 12.64% to 0.24% in gemmatimonadetes, 1.25 to 0.10% in planctomycetes etc (Figure 3A). Same results have been discussed by Mei et al. (2015) with high percentages of Proteobacteria i.e. 76% revealing that enrichment technique resulted in a better selection of electricigens.

Classes belonging to phylum proteobacteria were abundant in sludge inoculum. Major classes identified were α-proteobacteria (48.51%), β-proteobacteria (31.48%), γ-proteobacteria (16.16%), sphinogobacteria (0.61%), actinobacteria (0.60%). Decrease in bacterial density of some major classes (α-proteobacteria 38.67%, β-proteobacteria 12.34% and γ-proteobacteria 4.9%) occurred in biofilm of A-MFC. Classes like bacteriodetes and armatimonadetes were completely eradicated or absent in anodic biofilms (figure 3B). The inoculum effect has been investigated previously to find out the performances of single-chamber air-cathode [54]. Our findings were comparable with previously published data that showed the effects of different environmental samples on the power density of MFCs. Initially, MFCs inoculated with activated sludge revealed higher peak voltages implying that exoelectrogenic bacteria are widely distributed in natural habitats and can be used as inoculum for MFCs.

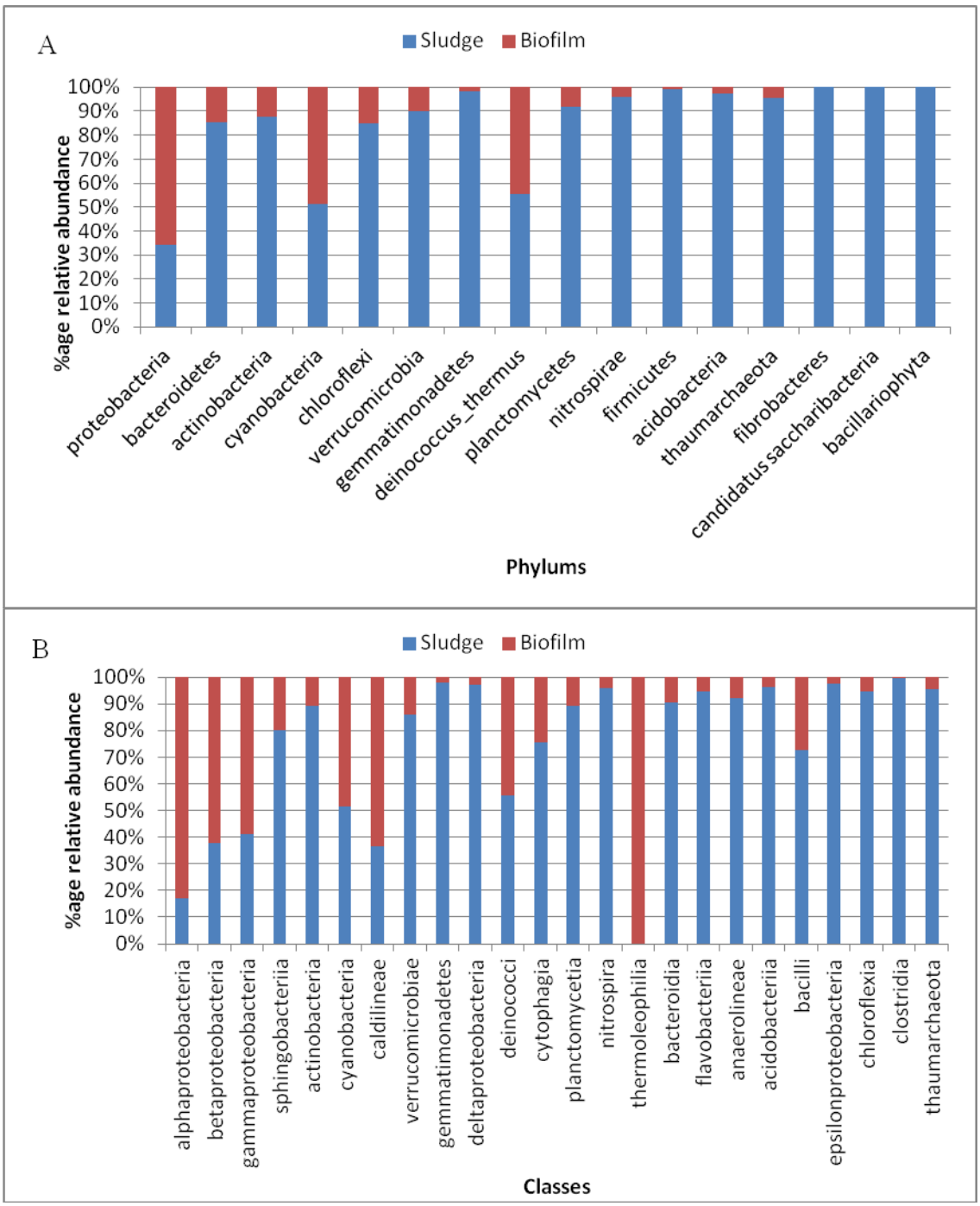

Figure 3. (A) Relative abundance (%) between biofilm and sludge communities in acetate fed double chamber microbial fuel cell at (A) phylum level and (B) Class level (cut-off value was set at 0.1%)

3.6 Principal component analysis (PCA)

Principal component analysis (PCA) illustrated a visual demonstration of bacterial diversity between raw inoculum and biofilm community on anode surface. Two components in PCA plot, PC1 and PC2 explained 92% and 8% variation of total bacterial population (Figure 4). Majority of classes were clustered together showing that they belong to closely related genera. PC2 is positively correlated

to PC1. Small angle between two variables (sludge and biofilm) represented that both variables have almost similar response to all classes. However, biofilm has strong response towards α-Proteobacteria than activated sludge community, while sludge shows response more strongly towards β-Proteobacteria, γ-Proteobacteria and δ-Proteobacteria than biofilm. Clusters formed near the principal component axis have least variation as they can be adapted to both environments of activated sludge environment and anodic biofilm.

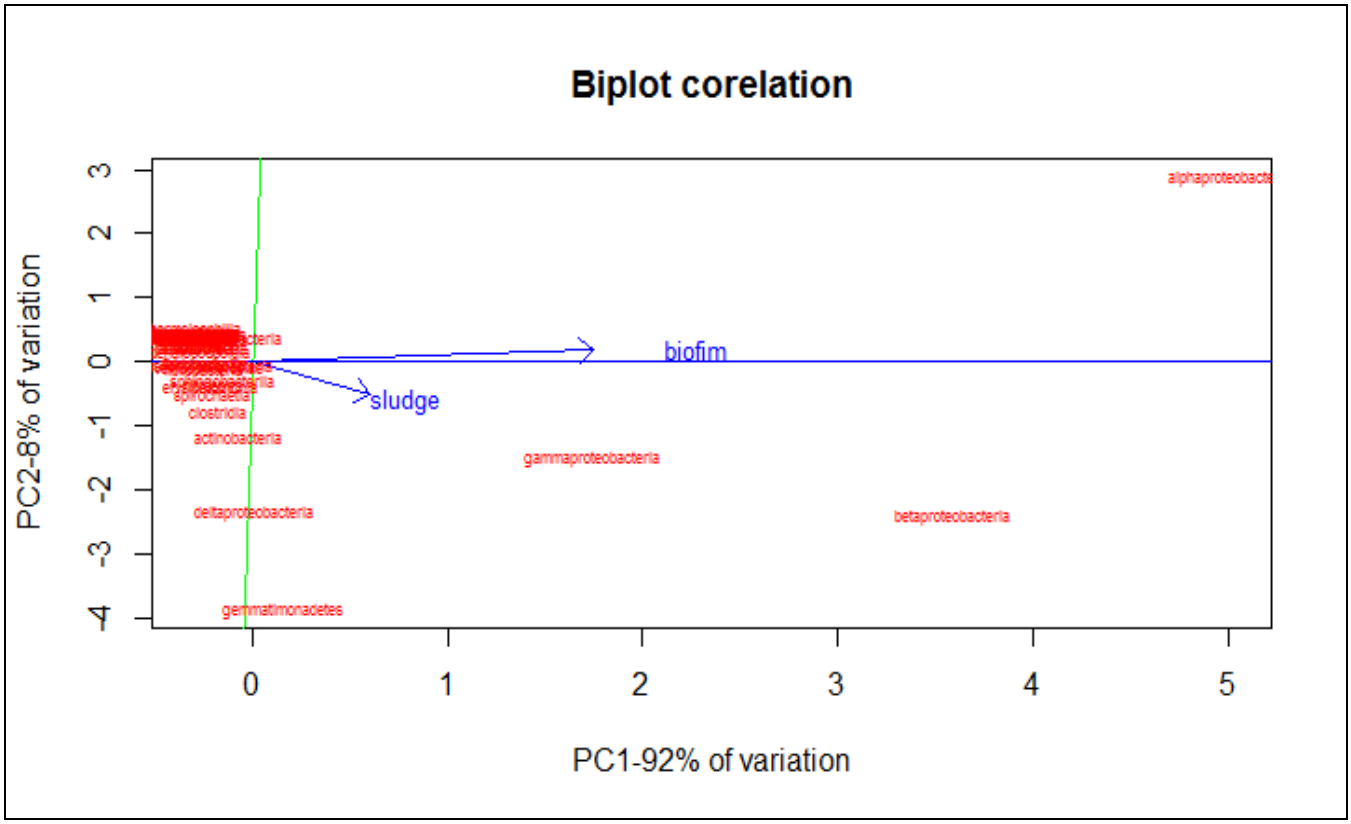

Figure 4. Principle component analysis (PCA) of dominant bacterial classes in sludge Vs biofilm on anode surface in A-MFC (cut-off value was set at 0.1%)

3.7 Phylogenetic analysis

Phylogenetic diversity of culturable and non-culturable bacteria based on partial 16S rRNA sequences in biofilm indicated presence of anode respiring bacteria in A-MFC (Figure 5). BLAST search of the sequences revealed that anode surface was mainly covered with biofilm forming culturable and un-culturable *Pseudomonas* sp., [*Pseudomonas aeruginosa* (KX692287.1), *Pseudomonas fluorescens strain AAU_PR3* (Kj161327.1), *Pseudomnas putida strain J-18* (JX122835.1)]. Sequences also showed 96% similarity with *Nitrosomonas* sp. *HPS* (HF678378.1), *Nitrosomonas europaea* strain ATCC 25978 (NR117649.1). It has been reported that *Nitrosomonas* sp. have power generating capabilities because of their power generating membranes. Schmidt and Bock [55] have confirmed that *Nitrosomonas europaea* was also able to oxidize ammonium anaerobically consuming nitrite as oxidant. It was expected that on the anode surface majority of aerobic ammonium

oxidation was performed by *Nitrosomonas europaea* consuming oxygen, and thus created an anoxic atmosphere for the minor community of *N. europaea* to carry out ammonium oxidation anaerobically to transfer electrons to the surface of anode [56]. It has been revealed that anode surface was harbored with large number of un-culturable bacteria like 96% similarity with uncultured beta-Proteobacterium clone WCB190 (AY217460.1), 98% similarity with uncultured gamma-Proteobacterium clone MI-072 (KF182916.1), 97% similarity with Uncultured *Pseudomonas* sp. clone OSU6-002A (JX467538.1) etc. Our results are similar with previously reported anode respiring bacteria in terms of electricigens found on anode surface [57].

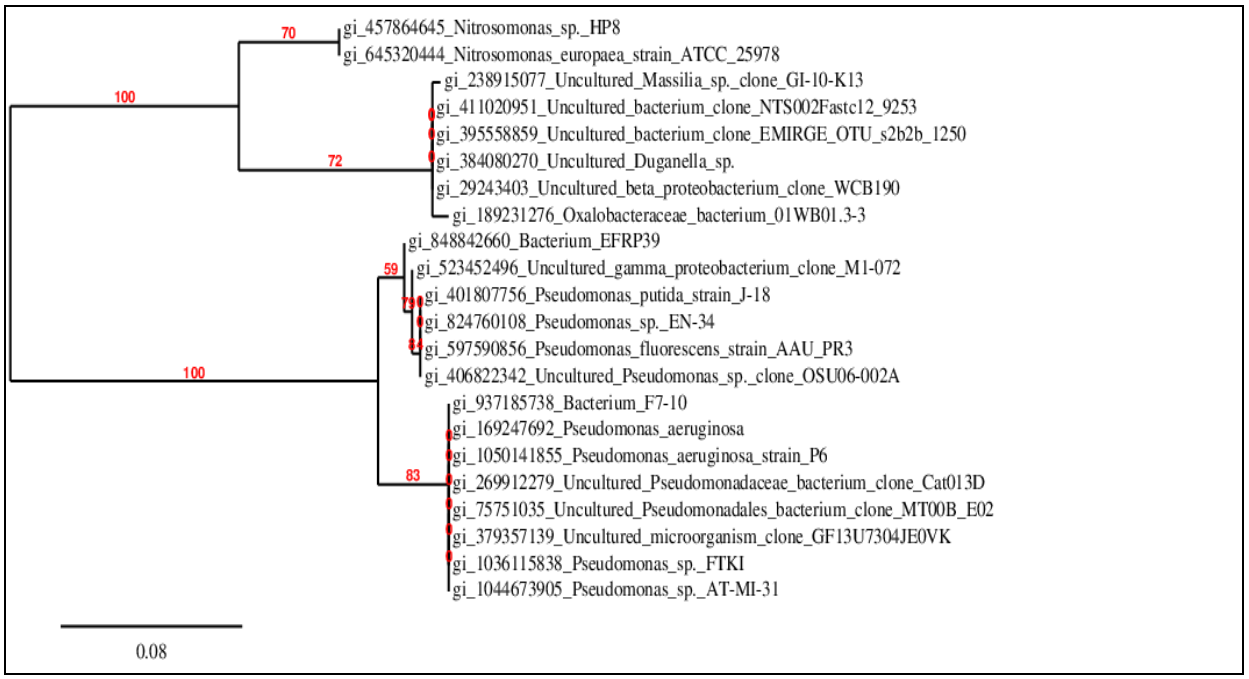

Figure 5. Phylogenetic tree of closely related species found on anode surface based on partial 16S rRNA gene sequences [Scale bar = 8% divergence]

3.8 Ultra Structure Analysis of Anodic Biofilms

Ultra-structure of biofilms on carbon cloth (anode) was examined under scanning electron microscope at different resolutions. The images demonstrated thick matrix of different bacteria on anodic surface as attached biofilm (Figure 7). Electricigens might be attached with each other through pilus like structures. Bacterial community was tightly amalgamated with thick layers of exopolysaccharide substances (EPS). The presence of highly diverse ARB communities in biofilms might have resulted from either unknown synergistic factors or functional redundancy among bacteria [26].

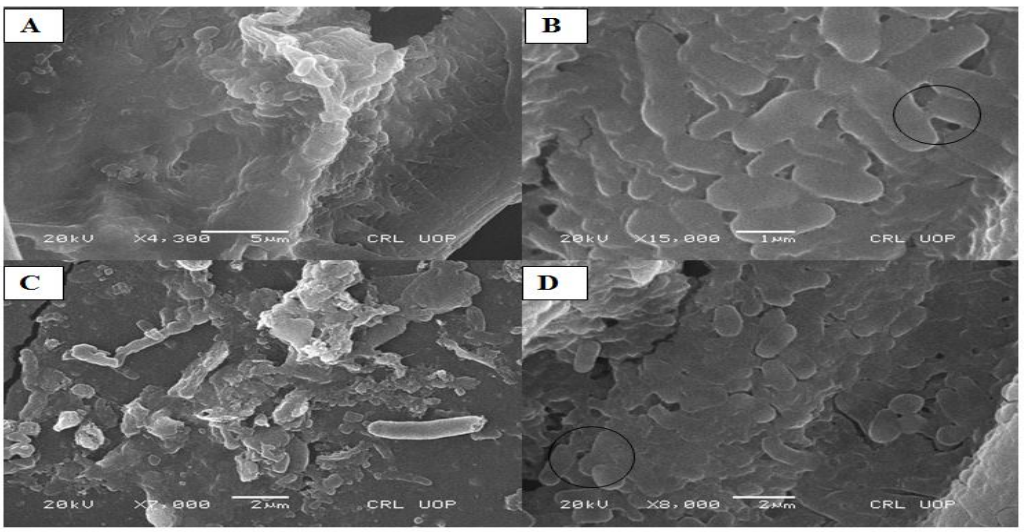

Figure 6. Scanning electron micrographs of anodic biofilms (A-B) 1st stage of enrichment in S-MFCs and A-MFCs (C-D) S and A-MFCs after 2nd stage of enrichment

4. CONCLUSIONS

From the present study, it was concluded that enrichment technique resulted in a better selection of electricigens from sludge inoculum. The simultaneous current generation with treatment of waste water was successfully proved. It was also demonstrated that almost similar electrochemical performance of MFC reactors was achieved with S-MFCs and A-MFCs with maximum current generation 0.04mAcm^{-2} along with 88% COD removal efficiency with negligible differences. It is illustrated that in addition to molecular based techniques, SEM also confirmed the results of community analysis and substantiated the findings of bacterial abundance in biofilms. Overall, MFC systems could be beneficial for utilization of this technology for simultaneous treatment of waste water along with generation of electricity.

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