

# Comparing Natural and Artificially Designed Bacterial Consortia as Biosensing Elements for Rapid Non-Specific Detection of Organic Pollutant through Microbial Fuel Cell

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The standard 5-days biochemical oxygen demand (BOD) method used for determination of biologically oxidizable organic material in wastewater considered to be laborious, time consuming and costly. Mediator-less microbial fuel cell (MFC) based biosensor offers an efficient alternative approach for real time monitoring of biodegradable organic matter in wastewater. Here we constructed an H-shaped MFC biosensor for comparing the efficiency of a complex natural (activated sludge) and artificially designed bacterial (*Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus circulans*) consortia as biological sensing elements for BOD measurements. Initially, the MFC biosensor was optimized and calibrated at pH 7 and temperature 37°C using 100 mM phosphate buffer with 100 mM NaCl solution as catholyte at 10 kΩ external resistance. Maximum power density of 14.2 mW/cm<sup>2</sup> was generated by MFC-I with sludge consortium and it was 5 folds higher than MFC-II with artificial consortium. Standard glucose and glutamic acid (GGA) solutions were used for establishing the calibration curves between different BOD concentrations (50-250 mg/L) and voltage (mV) outputs in MFC. The regression equations for MFC-I and MFC-II biosensors were recorded as  $y_1 = 0.7834x - 11.638$  and  $y_2 = 0.1667x + 0.8476$  respectively. Linear regression analysis revealed that 1 unit (mg/L) increase in organic load caused a voltage increase of 0.78 mV and 0.16 mV in the MFCs (I and II) reactors respectively. The relative performance in terms of stability (55-60 days) and reproducibility (within ±15.4%) of MFC-I BOD biosensor was almost double than MFC-II. The varying low concentrations of different electron acceptors (phosphate, nitrate and nitrite) in anodic compartments did not affect the performance of MFC biosensors.

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**Keywords:** MFC biosensor, Biochemical oxygen demand (BOD), Natural consortia, Artificial consortia, Pyrosequencing

## 1. INTRODUCTION

Biochemical oxygen demand (BOD) is commonly used as international index by environment protection agencies for assessment of organic matter load (pollutants) in wastewater. Many industries

regularly monitor their effluents to comply with the requirements of regulatory agencies [1]. Generally, the conventional BOD method used to analyze wastewater is not only time consuming (almost 5 days) but it also demands nitrification inhibitors (such as allyl-thiourea) and extensive training of the technicians to achieve reproducible results. Therefore, BOD<sub>5</sub> method is not considered suitable for dynamic intervention and real time monitoring in aquatic environments (such as ponds, lakes, rivers and ground water and wastewater). Several BOD biosensors based on bioluminescent, UV absorbance, enzymatic reactions and oxygen consumption by immobilized bacterial cells have been designed to overcome the aforementioned drawbacks. However, these methods demonstrated relatively unstable performance due to membrane fouling, volatile operation and limited utilization of substrate by reference bacteria [2]. A microbial fuel cell (MFC) based non-specific biosensor has been considered as a feasible alternative to the aforesaid methods for in-situ and ex-situ BOD monitoring of water bodies [3].

Microbial fuel cell (MFC) is an electrochemical device that converts chemical energy of wastewater into electric energy by using bacterial catalytic reaction [4]. Therefore, it is an environment friendly approach for wastewater treatment and renewable energy generation (in the form of electric current or bio-hydrogen). Recently, MFC has been viewed as a potential biosensing device for organic load measurement in aqueous environment. Redox electrochemical mediators that are phenolic toxic compounds (e.g. phenazine derivatives and flavins) have been usually employed to facilitate electrons transfer from bacterial cells to electrode [5]. Exoelectrogenic metal reducing bacteria such as *Shewanella putrefaciens*, *Rhodospirillum rubrum* and *Geobacter sulfurreducens* have also been reported in direct shuttling of electrons attached to anode [6]. Considering the operational suitability, lower electronic requisites, mechanical simplicity, good signal acquisition, sensitivity to change in organic load, high reproducibility and cost effectiveness, mediator-less MFC is considered more promising approach for developing an organic matter biosensor [7].

Previously, various MFC biosensors have been fabricated by immobilizing biological recognition element (bacterial cells) on physical transducer or in close proximity with transducer to convert biochemical changes into readable signals [8]. These biosensors have been successfully tested for monitoring COD, BOD, volatile fatty acid, anaerobic digestion and toxic components (cadmium, sodium acetate, chromium, and nitrate) in analyte. Pure culture biofilms of *Bacillus subtilis*, *Serratia marcescens* and *Photobacterium phosphoreum* have been designed for assessment of assimilable organic contamination in wastewater [9]. The use of single bacterial species in MFC biosensor has challenges of substrates selection and toxicity due to hazardous compounds that ultimately limit the electrode sensitivity. The major drawback of pure culture biosensor is inaccurate measurement of BOD concentration as only limited range of sample contaminants are metabolized [10]. Therefore, some studies have used complex bacterial community in MFC based BOD biosensors to estimate the total biodegradable organic matter of wastewater. Although the diverse nature of unknown bacterial community in sludge has been recognized as a limiting factor in terms of repeatability and stability of biosensing system [11]. Micro-aerophilic, anoxic and anaerobic bacteria produce different electric outputs utilizing organic matter through various metabolic pathways following Monod growth kinetics. Hence, it is essential to consider the effects of bacterial substrate consumption rate on MFC performance and BOD measurements of wastewater [8, 12]. The concept of MFC based biosensor has

been known for decades but uncertainty in architecture, instability, unavailability of standardized bacterial consortium and continuous maintenance limit its commercial applications [13].

Bio-electrochemical system generates a specific measurable current signal that depends on organic analyte concentration. Theoretically, a high signal output results from high BOD concentration but in MFC infrastructure various operating parameters such as fuel (substrate) type, nature of biological recognition element, substrate utilization rate, electron acceptors, co-existing ions and liquid retention time in anode greatly affect its biosensing ability [13, 14]. Various positively and negatively charged ions (such as nitrate, nitrite, ferric, phosphate and sulfate) are potential redox electron acceptors and commonly found in wastewater at concentration much higher than  $H^+$  ions. Therefore, the function of traditional cation exchange membranes in MFC significantly reduce the signal production by MFC biosensor [15]. To accurately measure the BOD in wastewater, electron acceptors must be removed or anode must be essentially supplemented with respiratory inhibitors (azide and cyanide) to reduce the interference from co-existing electron acceptors [16].

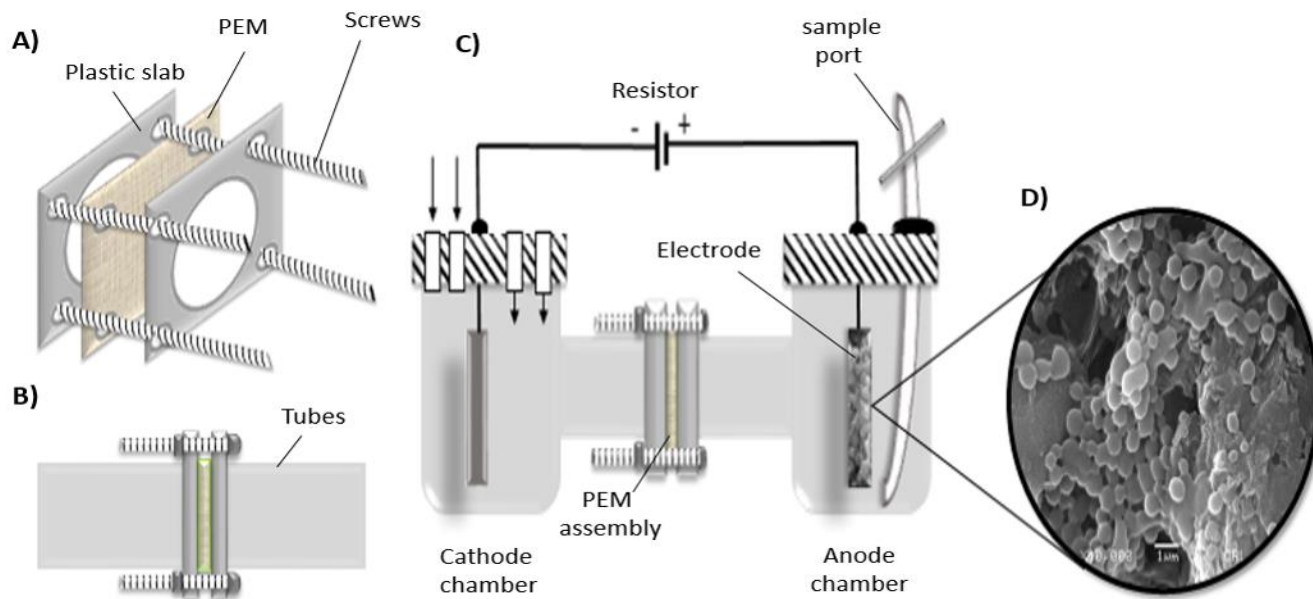
This study highlighted the comparative efficiency of an artificially designed bacterial consortium with sludge biofilm in analogous setup of MFC based biosensor to analyze the BOD concentrations of wastewater through voltage generation performance. The operational properties of MFC biosensors including start up time, inoculation, immobilization, stability, response time, repeatability and linearity were established and validated. The structure of bacterial community developed on anode from activated sludge was characterized in detail through next generation pyrosequencing sequencing using 16S rRNA gene. The relative abundance of different ions (phosphate, ammonium, nitrate and nitrite) in the anolytes affecting the signal transduction and performance of MFC were continuously monitored.

## 2. MATERIALS AND METHODS

### 2.1 Microbial fuel cell setup:

A dual chamber MFC was built with 120 mL polyacrylic plastic bottles joined together by two tubes (length 2.5 cm and diameter 25 cm<sup>2</sup>) holding a piece of proton exchange membrane (5.0 cm×5.0 cm, Nafion 115, Gas Hub Pte Ltd, Du Pont Company, USA). The proton exchange membrane was pre-treated by immersing in 0.1 M HCl for 3-4 hours at room temperature to remove any possible contamination. Holes of 26 cm<sup>2</sup> diameter were drilled in the two prosaic plastic slabs (5 cm x 5 cm) to insert the tubes. Screws (2 inches) were used to join the mouth of tubes holding membrane (Fig. 1). The internal volume of 100mL was used for anolyte and catholyte. Polished graphite rods (2 mm×22 mm) were suspended via copper wire (diameter= 0.8 mm) in both chambers to complete the external circuit. Black Ice T 401 Waterproof Sheets (9" x 11") were used to polish graphite rods in order to enhance the microbial adsorption on the surface of rod during biofilm formation. Inlet and outlet ports (4 mm diameter) with plastic stoppers were installed at the top of anode chamber for inoculation, replacement of medium and sampling through syringe. Reactors were operated in fed-batch mode at 37°C in an incubator. Air saturated sterile buffer and salt solution (100 mM phosphate buffer saline + 100 mM NaCl solution) were fed into cathode chamber of reactors. The potential difference between

anode and cathode was measured continuously via digital multimeter (model: UT33B; UNI-T) by varying the resistance from 50  $\Omega$  to 20,000  $\Omega$ . All experiments were performed in triplicate and the mean values were recorded.



**Figure 1.** Double chamber H-shaped mediator less MFC biosensor A) Proton exchange membrane (Nafion) assembly B) Plastic slabs holding a piece of PEM through slabs and screws C) Schematic diagram of MFC setup D) Biofilm on anode.

## 2.2. Enrichment of electrochemical active bacteria:

MFC-I was operated by feeding domestic wastewater (Table: 1) inoculated with activated sludge in anodic compartment with retention period of 10 days. For MFC-I, an aliquot of 4 mL activated sludge was mixed with 96 mL domestic wastewater (inoculum/analyte ratio: 1/24 [v/v]) in serum bottle and introduced in anode chamber under anaerobic conditions to initiate the experiment. After approximately 5 weeks of continuous operation under optimum conditions (temp.: 37°C and pH: 7) the cell potential reached a steady state indicating the development of mature biofilm. An artificial consortium of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus circulans* was developed on anode surface of MFC-II by inoculating the full loop colony of each bacterial species in 100 mL sterilized nutrient broth. These bacterial cultures were isolated on nutrient medium from 1% inoculum of domestic wastewater used in MFC-I by means of standard microbiological cultural techniques. Sub-culturing of *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Bacillus circulans* at 37°C for 24 hours was repeatedly done in nutrient agar for 4-5 days before using as inoculum. MFC-II was incubated at 37 °C for 4 weeks after inoculation and nutrient broth was refreshed after every 72 hours to avoid the organic matter exhaustion. Anode solution was purged with oxygen free nitrogen gas for 10-15 min after every 24 hours to maintain anoxic conditions. Both reactors were operated in fed batch mode and operational conditions were kept constant.

**Table 1.** Physico-chemical characteristics of domestic wastewater (I-9, Islamabad, Pk) used in double chamber MFC

Parameter	Concentration range	Mean value
pH	5.9-7.3	6.6±0.7
COD (mg/L)	497-780	638±141
BOD <sub>5</sub> (mg/L)	169.8-485.2	327.5±157
Conductivity (S/m)	0.01-0.13	0.07±0.06
TS (mg/L)	870-10500	960±1500

### 2.3. Operation of MFC Biosensors:

Once biofilms were matured, anode chambers were fed with fresh mineral medium (MM) containing 50 mg L<sup>-1</sup> glucose at 24 hours retention period until new steady state of cell potentials were reached (approximately 3-4 days). Activated sludge and artificially designed bacterial consortia were then used in MFCs as biological sensing elements. The operating properties of MFC biosensors were evaluated by feeding the five different concentrations (50, 100, 150, 200 and 250 mgL<sup>-1</sup>) of glucose and glutamic acid (GGA) standard solution to validate the relationship between different BOD concentrations and system respective voltage response. The basic mineral composition of GGA medium was: NaHCO<sub>3</sub> 2.5 gL<sup>-1</sup>, KCl 0.1 gL<sup>-1</sup>, CaCl<sub>2</sub> · 2H<sub>2</sub>O 0.1 gL<sup>-1</sup>, NH<sub>4</sub>Cl 1.5 gL<sup>-1</sup>, MgSO<sub>4</sub> · 7H<sub>2</sub>O 0.1 gL<sup>-1</sup>, MgCl<sub>2</sub> · 6H<sub>2</sub>O 0.1 gL<sup>-1</sup>, NaCl 0.1 gL<sup>-1</sup>, MnCl<sub>2</sub> · 4H<sub>2</sub>O 0.005 gL<sup>-1</sup>, NaH<sub>2</sub>PO<sub>4</sub> 0.6 g L<sup>-1</sup> and NaMoO<sub>4</sub> · 2H<sub>2</sub>O 0.001 gL<sup>-1</sup>. Each concentration was introduced at least three times to get the average results.

## 3. ANALYSIS AND CALCULATION

### 3.1 Physicochemical analysis

Standard methods from American Public Health Association (APHA, 20<sup>th</sup> Edition, 1995) were used to analyze the quality of domestic wastewater immediately after collection. All samples were filtered prior to analysis through 11 µm size filter paper. Soluble chemical oxygen demand (COD) and pH of wastewater were estimated using HACH 5220 COD method and bench top pH meter respectively. The COD removal of natural and artificial consortia was determined between 10 and 40 days. The COD removal efficiency (%) was calculated according to:  $COD_r = \frac{COD_{in} - COD_{out}}{COD_{in}}$  where  $COD_{in}$  is the COD (mgL<sup>-1</sup>) concentration measured in the influent of anode compartment and  $COD_{out}$  is the COD (mgL<sup>-1</sup>) concentration measured in the effluent of anode compartment. The BOD of wastewater samples were determined via Standard 5-Days respirometric Method (5210B) by using DO meter. After 5 days of incubation, BOD was calculated as:  $BOD \text{ mg/l} = (\text{Initial DO} - DO_5) \times \text{Dilution Factor}$ . Where Dilution Factor = Bottle Volume (300 ml)/Sample Volume, DO = initial DO of diluted sample and DO<sub>5</sub> = DO of diluted sample after 5 days incubation at 20°C under darkness in order to avoid algal respiration. Measurements of inorganic compounds: ammonium (Nessler reagent method), nitrate (Phenol di-sulphonic acid colorimetric method) and nitrite (Sulfanilamide colorimetric method) along with phosphate (Stannous chloride method) were

made in duplicate. The dry masses of biofilms were determined by drying the electrodes at 105 °C and heating it at high 550 °C before and after the biofilms formation. The weights of the electrodes were determined in order to calculate the volatile suspended solid per cm<sup>2</sup> of electrode surface area.

### 3.2 Electrochemical analysis

MFCs were initially operated at open circuit voltage (OCV) without any external circuit resistance for several hours. Once voltage became stable the system circuit was closed with 10kΩ resistor. The voltage between anode and cathode was measured by using a digital multimeter (model: UT33B; UNI-T). For the applied resistance methods, cell current (I) was calculated from Ohm's law:  $I=U/R$ . Where, U is the recorded potential and R is the external resistance. Polarization studies were conducted using single cycle method by varying external resistor load from 50 Ω to 20 KΩ in decreasing order and each resistance was connected for 20 min. These polarization curves were used to evaluate the internal resistance and maximum power. The current and power densities were normalized by the projected electrode surface area (1.45 cm<sup>2</sup>). The power density (mW/cm<sup>2</sup>) was calculated by using the formula  $P.D = IU/A$  and current density (mA/cm<sup>2</sup>) as  $C.D = U/.A$ ; where A (cm<sup>2</sup>) is the projected surface area of electrode.

### 3.3 Microbiological analysis

The anodes from MFC-I and MFC-II with biofilm were washed with distilled water prior to immersion in 2% formaldehyde (v/v) for sample fixation. These samples were further rinsed carefully 3 times with 100mM PBS (pH: 7) and then with deionized water. The fixed anode samples were then sent to centralized resource laboratory of Physics department, UOP, Pk for scanning electron microscopic (SEM) analysis.

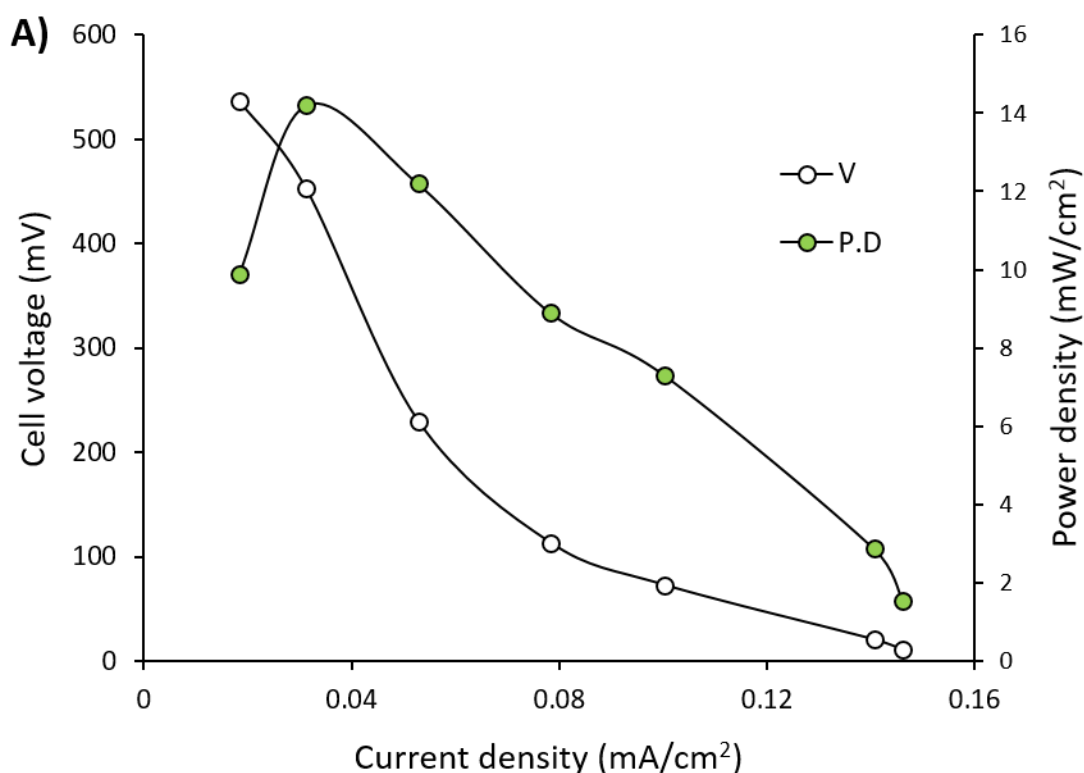
The diversity of bacterial community on the sludge inoculum and anodic biofilm in MFC-I was determined through 454 pyrosequencing. Following 5 weeks of incubation, anode was transferred to a saline phosphate buffer and sonicated for 30 sec in 2-3 intervals to dislodge the cells from the anodic biofilm in the buffer solution. 1 ml of biomass suspension was centrifuge separately at 10,000 rpm for 4-5 mins and pellets were re-suspended in DNA isolation Kit (Cat: 24700, Norgen biotek corp product). Manufacturer protocols were followed for total DNA isolation. The quantity and quality of purified genomic DNA was confirmed prior to pyrosequencing by using NanoDrop 2000 UV-Vis Spectrophotometer. Purified genomic DNA samples were sent to Molecular Research LP (MR DNA) 503 Clovis Road Shallowater Texas for 454 pyrosequencing analysis.

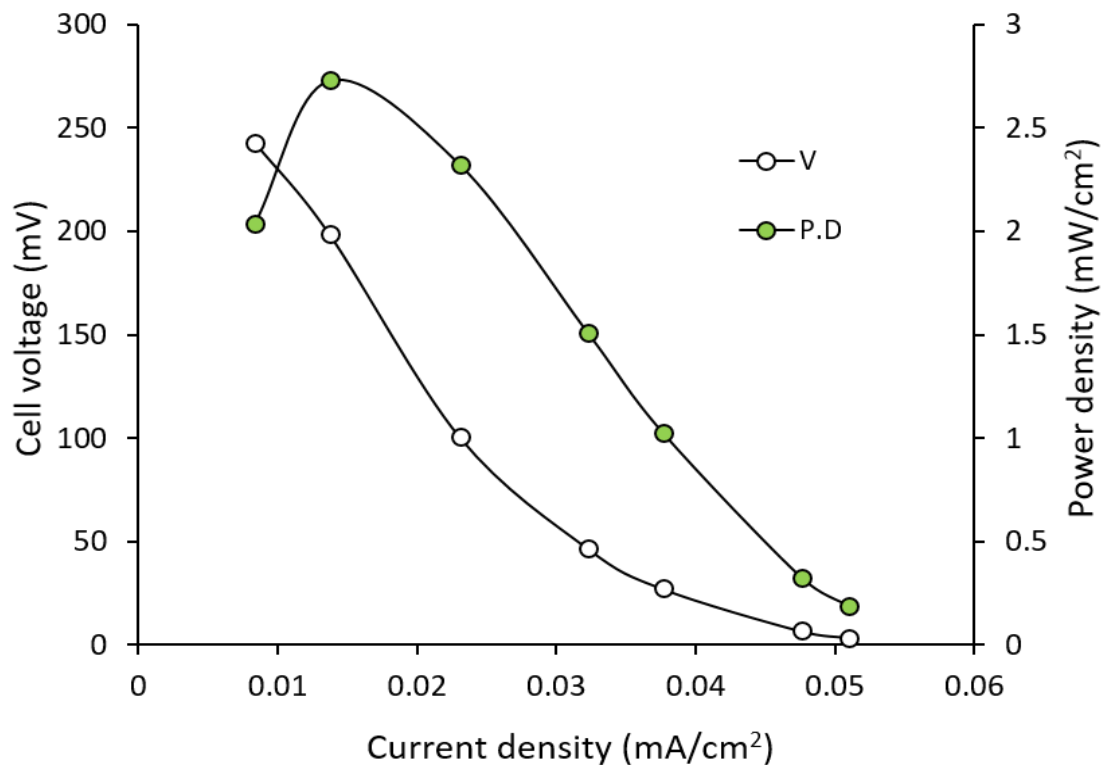
## 4. RESULTS AND DISCUSSION

### 4.1 Electrochemical characterization of MFC biosensor:

MFC reactors exhibited consistent reproducible cycles of voltage generation after two successive weeks of inoculation, indicating successful acclimation of bacteria on anodes. Mineral

medium containing  $50 \text{ mgL}^{-1}$  of glucose was fed into the reactors for 3-4 days prior conducting biosensing operation in order to ensure the stability of the reactors. A pre-developed activated sludge biofilm was used in anodic chamber of MFC-I biosensor. The corresponding open circuit voltage (OCV) of  $0.68 \pm 0.1 \text{ V}$  was recorded immediately after MFC was fed with sewage sludge and wastewater. Initially, the potential remained below  $250 \text{ mV}$  then the voltage gradually increased to  $453 \pm 5 \text{ mV}$  on 18<sup>th</sup> day. During first 35 days of operation MFC-I generated maximum power of  $14.2 \pm 1 \text{ mWcm}^2$ , afterwards, a decline in system performance was observed. Whereas, MFC-II operated with artificial consortium produced a maximum voltage of  $198.9 \pm 2 \text{ mV}$  on 13<sup>th</sup> day. A stable voltage with average current of  $0.031 \pm < 0.01 \text{ mA}$  and  $0.019 \pm < 0.005 \text{ mA}$  was recorded in MFC-I and MFC-II respectively during the last 2 weeks of operation. The maximum current density of MFC-I with sludge was 66% higher than that of MFC-II with artificial consortium (*Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus circulans*). During single cycle polarization studies voltage output was observed with each resistance loads (from  $50 \Omega$  to  $20 \text{ K}\Omega$ ) after 20 min interval. Voltage exhibited a sharp decline at higher current densities resulting in occurrence of power overshoot but then power decreased rapidly. While, current density increased continuously (Fig. 2). The COD removal was monitored during the start and stable phase of MFCI operation between days 10-40. The current density of MFC-I was around  $0.02 \pm < 0.005 \text{ mAcm}^2$  during day 10<sup>th</sup> to 15<sup>th</sup>, thereafter, it exponentially increased up to  $0.041 \pm 0.005 \text{ mA}$  on 30<sup>th</sup> day. The COD removal efficiency of MFC-I was increased from 58% to 74% as current density increased between 10<sup>th</sup> to 20<sup>th</sup> days and ultimately it reached up to 95.7% on 40<sup>th</sup> day.





**Figure 2.** Polarization and Power density curves of; A) MFC-I and B) MFC-II

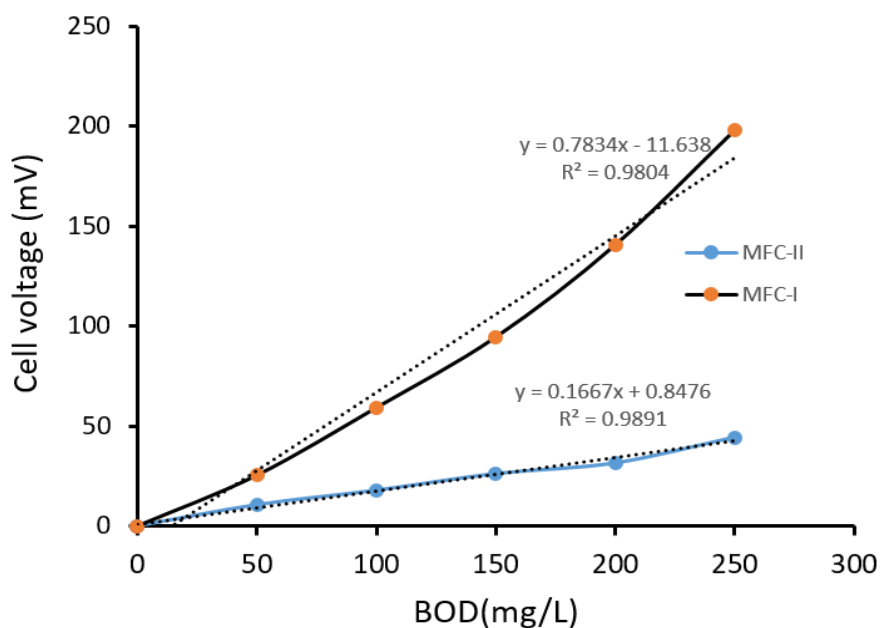
This indicated that electrochemically active bacteria turned highly metabolically active during the last two weeks. Whereas, the COD removal efficiency of MFC-II with artificial consortium remained lower and it was 72% during the final week of experiment.

#### 4.2 Calibration of MFC Biosensor

MFC-I and MFC-II were supplied with different concentrations (50-250mg/l) of standard solution of glucose and glutamic acid (GGA) under optimal working conditions to establish a relation between BOD and respective voltage outputs. The steady state response times of BOD biosensors were monitored following replacement of anodic GGA standard solution with its new successive concentration (Fig. 3). An acceptable positive correlation ( $> 90\%$ ,  $r^2 = 0.9$ ) was achieved between different concentrations of standard solutions and voltage outputs validating MFC as a BOD biosensor. The regression equations for MFC-I and MFC-II biosensor were established to be  $y_1 = 0.7834x - 11.638$  ( $r^2 = 0.9804$  or 98%) and  $y_2 = 0.1667x + 0.8476$  ( $r^2 = 0.9891$  or 98.9%) respectively. Where, 0.7834 and 0.1667 were slopes of regression lines (b), x represented the predictor variable (organic load of wastewater) and y was the response variable (voltage output of system). The Y-intercept (a) was the point where line cut the y-axis and can be intercepted as the value predicted for voltage only if  $x = 0$ . Thus, it was inferred from the regression equations that with each 1 mg/L increase in organic load the voltage responses were increased by 0.7 mV for MFC-I and 0.1 mV for MFC-II biosensor (Fig. 3). Furthermore, in extrapolating the BOD (mg/L) samples from different water bodies (stream, wetland



and domestic treatment plants) same regression equations were applied. To interpret the biodegradable organic load of sample through biosensor a variation in voltage was recorded after introducing the wastewater in anode chamber and voltage response was used for calculating the BOD (mg/L) through these equations.



**Figure 3.** Relationship between different BOD concentration and voltage outputs of MFC-I and MFC-II biosensors

#### 4.3 Performance characteristics of MFC biosensor

Different performance parameters of MFC biosensors including stability, response time and reproducibility were analyzed during operation and the detailed are as follows:

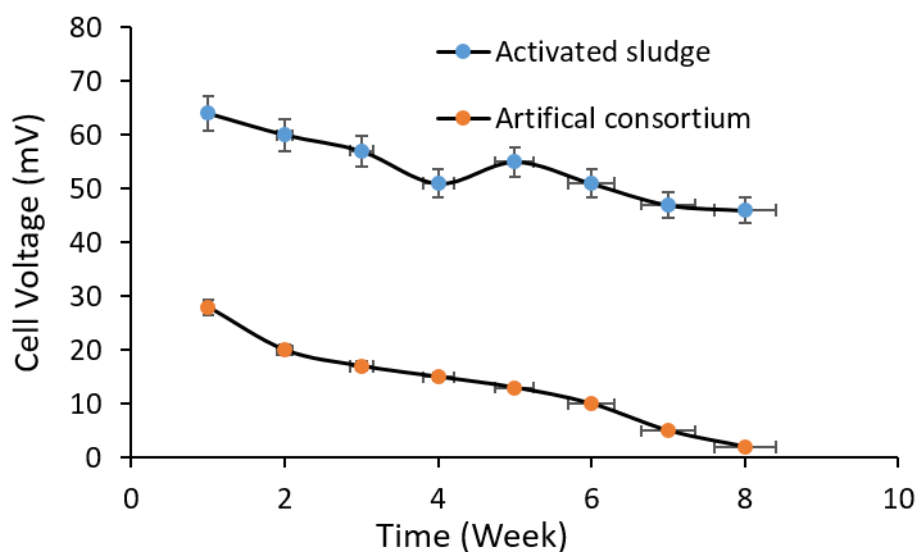
##### 4.3.1 Response time

The time period required to achieve a stable operating voltage (OV) following the addition of organic matter in anode compartment was recorded as the response time. Response time of MFC biosensor depends upon BOD concentration, type of MFC and operational conditions. Higher the sample strength longer the time required to measure the BOD in MFC biosensor. MFC-I required approximately 60 min to detect a BOD concentration of 13.6 mg/L, whereas, it took about 21 h for BOD concentration of 989.4 mg/L. Therefore, samples with high COD were diluted before subjecting to bio-sensing test (Table: 2). Dilution technique was also used to avoid extrapolation outside the range of collected data points because relationship may not be linear beyond that point. In terms of voltage output, the response of MFC-II was much quicker than MFC-I but has shown a percentage error of about 25%.

**Table 2.** BOD of different water samples (stream, wetland and domestic treatment plants) measured by MFC biosensors and standard 5-days method

No	Wastewater Type	MFC BOD Biosensor			BOD <sub>5</sub> (mg/L)
		Type	(mg/L)	Response Time (min)	
1	Stream	MFC-I	16±0.9	60±1	13.6±0.2
2	Domestic wastewater	MFC-I	980±15	1260±3	989.4±1.2
3	Stream	MFC-I	40±0.2	180±1	29.9±0.5
4	Wetland	MFC-I	30±0.5	255±0.9	42.1±0.3
5	Domestic wastewater	MFC-I	60±0.7	210±2	36.04±0.2
6	Wetland	MFC-II	100±1.4	120±2	102±1
7	Domestic wastewater	MFC-II	57±3.5	160±1	273±0.3
8	Stream	MFC-II	60±0.2	70±3	67±2
9	Wetland	MFC-II	90±0.8	100±2	88±0.9

4.3.2 Repeatability and comparison of MFC BOD biosensor with standard BOD<sub>5</sub> method



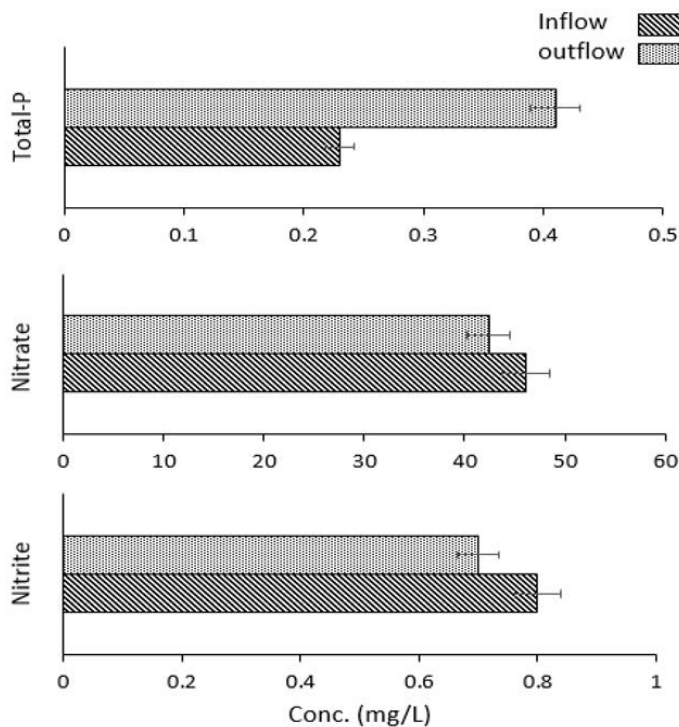
**Figure 4.** Repeatability and comparison of MFC BOD biosensor with standard BOD<sub>5</sub> method in successive 7-8 cycles using regular intake of mineral medium (COD 100 mg/L) after every 24 hours

According to the APHA BOD<sub>5</sub> method, a standard deviation of ±30.5 mg/L is acceptable that is equivalent to ±15.4% variation in precision [11]. To determine the repeatability of biosensing system anodic chamber was operated with mineral medium (COD = 100 mg/L) for 24 hours in repeated 7-8 cycles and the voltage responses were recorded. The variation in repeatability was ±5 mV in MFC-I biosensor qualifying acceptable limit. However, a considerable variation in MFC-II biosensor signals was observed after 3 weeks (Fig. 4). Furthermore, the values from BOD biosensors were comparable to BOD<sub>5</sub> standard method and measured results remained within APHA acceptable limits (Table: 2).

#### 4.3.3 Operational stability

The stability of MFC biosensors in terms of voltage outputs was monitored regularly throughout the operation. During the entire period voltage output remained considerably stable. A voltage fluctuation of about 65.8±0.3 to 45.7±1 mV was recorded in 2 months for MFC-I and 25.8±1 to 11.2±1.1 mV in 1.5 months for MFC-II when fed with BOD standard solution (100 mg/L) of GGA. Overall, the stability of bacterial consortium of activated sludge was 55% higher than artificially developed consortium.

#### 4.4 Effect of co-existing ions on MFC-I biosensor

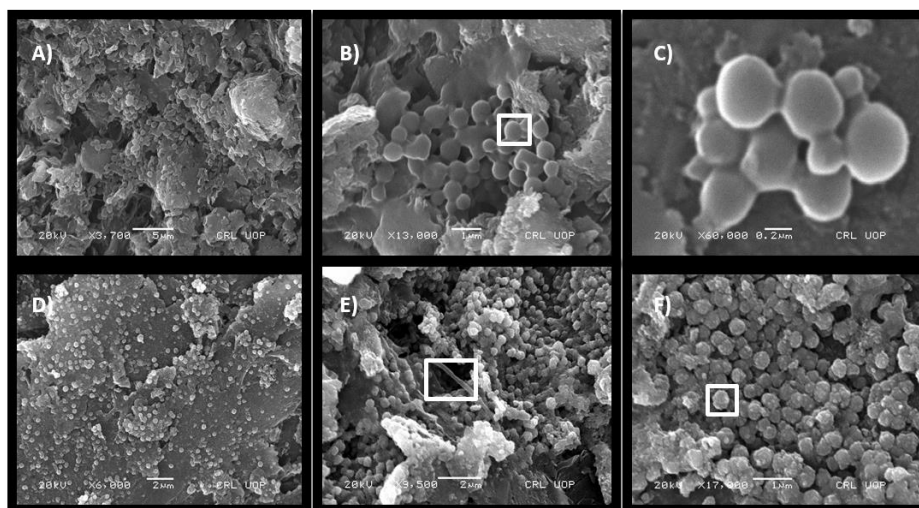


**Figure 5.** Variation in electron acceptors (conc.) during development of biofilm in MFC-I

Domestic and industrial wastewater generally contain high concentrations of oxygen gas (O<sub>2</sub>), charged ions and inorganic compounds such as phosphate (PO<sub>4</sub><sup>3-</sup>), ammonium (NH<sub>4</sub><sup>+</sup>) nitrate (NO<sub>3</sub><sup>-</sup>), nitrite (NO<sub>2</sub><sup>-</sup>) and sulfate (SO<sub>4</sub><sup>2-</sup>) that can greatly influence the signal output. Therefore, domestic

wastewater sample was purged with oxygen free nitrogen gas to create the anaerobic conditions before introducing into MFC. Phosphate ( $\text{PO}_4^{3-}$ ), ammonium ( $\text{NH}_4^+$ ) nitrate ( $\text{NO}_3^-$ ) and nitrite ( $\text{NO}_2^-$ ) concentrations were continually measured in wastewater samples during immobilization process through standard methods. The average amount of different electrons acceptors were; Total-P  $0.23 \pm 0.1$  mg/L,  $\text{NO}_3^-$   $46.1 \pm 3$  mg/L, and  $\text{NO}_2^-$   $0.8 \pm 0.1$  mg/L. Under optimum conditions ammonium was removed by 37%, whereas, nitrite ( $\text{NO}_2^-$ -N) and nitrate ( $\text{NO}_3^-$ -N) by 5-10% in 10 days. A slight increase ( $0.23 \pm 0.1$ - $0.41 \pm 0.13$  mg/L) in orthophosphate ( $\text{PO}_4^{3-}$ ) was occurred during MFC operation (Fig. 5).

#### 4.5 Ultrastructure and quantitative analysis of bacteria in anodic biofilms



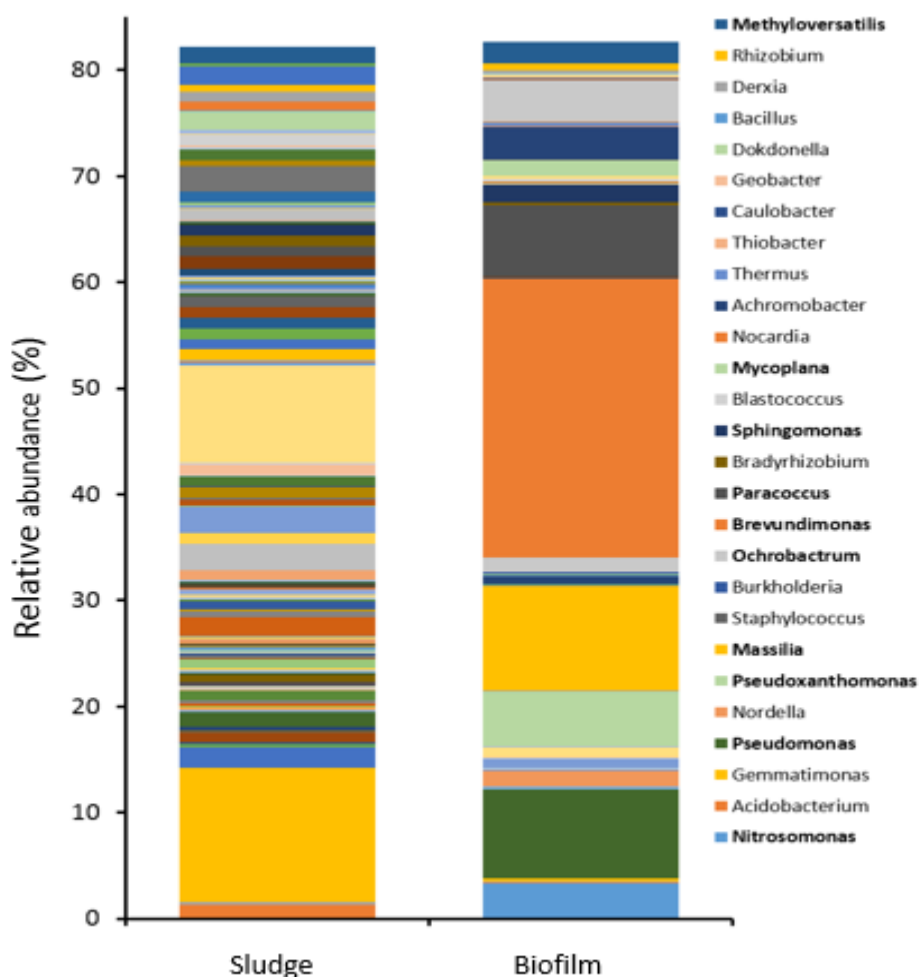
**Figure 6.** Scanning electron microscopic (SEM) A) A broad overview of the MFC-I biofilm at 3700X B) Multiple layers of cells extending from the anode of the MFC-I and white square indicate round shaped cell C) Aggregate of round cells D) A broad overview of MFC-II anode biofilm at 6000X E) Multiple layers of cell extending from anode surface and white square indicate filament shaped structure extending between cells F) Round shaped cells held by extracellular polymeric substances (EPS)

The structure and morphology of biofilms formed on anode surfaces were analyzed by scanning electron microscopy (SEM) (3400N, HITACHI instrument). A thick smooth coverage of biofilm biomass was observed on anodes of MFC-I and MFC-II (Fig. 6). Figure 6A highlights the anode surface of MFC-I with appreciable amount of biofilm attached and majority of individual bacterial cells were uniform spherical shaped cocci. Under higher magnification (60,000 X), a compact biofilm structure was evident where clumps of heterogeneous shaped cells were tightly interconnected by polymeric viscous materials (Fig. 6C). SEM of artificial consortium in MFC-II illustrated less biofilm density than MFC-I. The extracellular polymeric matrix was holding ovoid shape bacterial cells on anode with interconnecting filaments (Fig. 6D). The high density of bacterial cells on MFC-I anode surface was further confirmed by the biofilm dry mass analysis. The dry weight of mature (5 weeks) sludge biofilm was  $3.31 \text{ mg/cm}^2$ , whereas, it was  $0.81 \text{ mg/cm}^2$  for artificial

consortium. An about 75% less biofilm biomass was found on anodic surface of MFC-I compared to MFC-II.

#### 4.6 Molecular Phylogeny of Bacteria (454-Pyrosequencing)

454-Pyrosequencing was used to reveal the diversity of bacterial community present in activated sludge inoculum and biofilm enriched on MFC-I anode (Fig. 7). The resultant sequences were grouped into 24 different phyla in sludge and 12 phyla in anodic biofilm. The most active genera in sludge inoculum were *Gemmatimonas* (12.6%), *Schlegelella* (9.1%), *Syntrophus* (3.5%), *Nitrospira* (2.5%), *Beggiatoa* (2.4%), *Bulleidia* (2.4%), *Nitrospira* (2.4%), *Denitratisoma* (1.9%) and others (<1.7%). However, *Brevundimonas* (26.2%), *Massilia* (9.9%), *Pseudomonas* (8.3%), and *Paracoccus* (6.7%) were the most active genera comprising 51% of the total anode bacterial community in MFC. (Fig: 6). *Caulobacteraceae* (26.6%), *Oxalobacteraceae* (13.6%) and *Pseudomonadaceae* (8.3%) were the most dominant families in anodic biofilm.



**Figure 7.** Relative abundances of dominant bacterial genera in activated sludge inoculum and anodic biofilm

#### 4.7 Discussion

Microbial fuel cells (MFC) is a growing alternative green energy technology that also offers a wide range of other applications [17]. Previous research has been mainly focused on the use of MFC as electric energy generation device. Recently, MFC has gained much attention in environmental sector to use it as a potential biosensing element in monitoring of wastewater treatment. MFC biosensor is emerging as a promising option for BOD measurements considering its high operational stability, reproducibility and broad substrate versatility [18]. In this study, the maximum power density achieved by artificially developed consortia was  $2.7 \text{ mW/cm}^2$ . This was lower than power density achieved with natural consortia on MFC-I electrode i.e. up to  $14.2 \text{ mW/cm}^2$  at  $10\text{K}\Omega$  circuit load. Similar properties of MFC biosensor was evident in the current study, however, current density versus BOD sensing were comparatively higher with natural bacterial consortium than artificial consortium used. Enhanced performance of MFC reactors in terms of current generation has been previously linked with highly active electrogenic biofilms [19] encompassing greater diversity of anodophilic bacteria [20] with pili network of appendages [21].

An equitable analyte detection can be achieved with the microbial electrochemical system [22]. This could offer an economically attractive, environment friendly and rapid alternative for measuring the organic load in water as BOD because it minimizes the input and later exhaustion of expensive reagents that are used in standard BOD protocols [23]. A good linear relationship was observed between MFC voltage responses and organic loading rates (Figure: 3). A similar linear relationship between electrical signals and different BOD concentrations ranging from 20–100 mg/L and 1–25 g/L was reported by Chang [8] and Kumlanghan [24] respectively. Both MFC types showed good performance in terms of BOD sensing when GGA solution was used as a standard [25]. However, while sensing organic pollutant load in domestic wastewater sample (7), MFC-II showed 25% error that was far out of acceptable range of  $\pm 15 \text{ mg/L}$ . This might have happened due to limited substrate utilization spectrum of artificially developed bacterial consortium. Presence of toxic compounds and heavy metal ions in wastewater have also been associated towards creating erratic electronic response by MFC reactors [26].

Higher the organic strength of water the longer the time MFC biosensor took for measuring the BOD (Table: 2). Thus, dilution techniques were employed to analyze the high strength organic wastewater in terms of BOD [27]. Overall, the electrical response time (min) increased with greater bacterial diversity on anode. Slow electrogenic response of natural consortium than artificial consortium might be due to excessive accumulation of extra polymeric substance (EPS) on anode. Besides, development of thick biofilm on anode surface has been reported to suppress the mass transfer rate of nutrients between bacteria and the outside environment resulted a rapid decline of catabolic reactions of bacteria [9]. Both MFC configurations used in this study remained stable for 30 days following the biofilm immobilization, however, stability and biosensing response output decreased afterwards. MFC-I proved to be 57% more stable in operation as a biosensing device than MFC-II. Greater bacterial diversity and associated environmental compatibilities in sludge consortium with broad substrate specificity and resistance to adverse environmental conditions might have offered

better stability and reproducibility to MFC-I biosensor [18, 28]. Nevertheless, MFC-I with sludge bacterial consortium needed continuous maintenance after construction [29].

Despite successful operation of MFC biosensor, challenges of varying bacterial diversity and density in natural consortium always vary with time, source and environmental conditions. [30]. 454 pyrosequencing successfully revealed the molecular phylogeny of bacterial community composition in MFC-I anode developed from sludge. *Proteobacteria*, *Chlorflexi*, *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria* were the most dominant phyla in biofilms. However, chances of duplicating the same bacterial community in other MFC biosensors would always be a great challenge. Such limitations always create drawbacks in marketing the MFC reactors as biosensors. Though laboratory defined consortia have limited substrate utilization spectrum and issues in dealing with diverse wastewater samples [31], still they should be developed more comprehensively taking some vital environmental bacterial isolates with greater physiological capability. In this context, new combinations of ATCC bacterial species should be tested to develop a universal bacterial consortia for optimum and sustainable commercial use in MFC biosensor [2, 22].

Generally, increase in nitrites and nitrates are inversely related with the decreasing levels of ammonium in any organic wastewater system. However, in this case slight decrease (5-10%) in nitrogenous electron acceptors after 10-days retention time of MFC was observed indicated low density and related functionality of denitrifies in bacterial community. Therefore, signal output of the reactor remained high and stable during operation. Though, higher concentration of different inorganic compounds could be negatively effecting performance of MFC [32]. A slight increase (0.23-0.41mg/L) in orthophosphate ( $\text{PO}_4^{3-}$ ) during MFC operation might has been occurred from degradation of organic matter at low redox potential in the reactor. Overall, low concentrations of different inorganic electron acceptors did not pose any major limitation in the biosensing step as was reported previously [33].

## 5. CONCLUSION

The study validated the importance of microbial fuel cell (MFC) as a biosensor device for efficient and continuous monitoring of BOD in water. Comparatively, MFC enriched with complex bacterial community proved to be more stable and efficient than that run with simple artificial bacterial consortium in generating electric signals thereby sensing BOD loads. Molecular base phylogeny revealed sludge as a source of various bacterial species, besides; the community structure was considerably changed during enrichment in MFC. Still, development of better combination of electroactive bacteria as biosensing element in MFC biosensor is obligatory to avoid maintenance issues in future. The co-existence of different electron acceptors (phosphate, nitrate and nitrite) at low concentration in the anolyte demonstrated an insignificant effect on MFC performance.

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