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Improvement of power generation using *Shewanella putrefaciens* mediated bioanode in a single chambered microbial fuel cell: Effect of different anodic operating conditions

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HIGHLIGHTS

- Taguchi design to find out the key anodic process parameter.
- Impedance study to evaluate the influence of riboflavin addition to anolyte.
- Cyclic voltammetry to find out the effect of nano hematite modified anode.
- Microscopic study of biofilm formation using different nano hematite loaded anode.

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ABSTRACT

Three different approaches were employed to improve single chambered microbial fuel cell (sMFC) performance using *Shewanella putrefaciens* as biocatalyst. Taguchi design was used to identify the key process parameter (anolyte concentration, $CaCl_2$ and initial anolyte pH) for maximization of volumetric power. Supplementation of $CaCl_2$ was found most significant and maximum power density of 4.92 W/m^3 was achieved. In subsequent approaches, effect on power output by riboflavin supplementation to anolyte and anode surface modification using nano-hematite (Fe_2O_3) was observed. Volumetric power density was increased by 44% with addition of 100 nM riboflavin to anolyte while with 0.8 mg/cm^2 nano-Fe_2O_3 impregnated anode power density and columbic efficiency increased by 40% and 33% respectively. Cyclic voltammetry revealed improvement in electrochemical activity of *Shewanella* with nano-Fe_2O_3 loading and electrochemical impedance depicted inverse relationship between charge transfer resistance and nano-Fe_2O_3 loading. This study suggests anodic improvement strategies for maximization of power output.

1. Introduction

Microbial fuel cell (MFC) uses electrochemically active bacteria (EAB) as biocatalyst to convert biodegradable waste into electricity (Yang et al., 2012). EABs are gaining importance because of their electron donating ability to the electrodes in MFC. Microorganisms such as members of the *Geobacter* family, *Shewanella putrefaciens* and *Shewanella oneidensis*, *Rhodoferax ferrireducens*, *Pseudomonas aeruginosa*, *Clostridium butyrium* and *Aeromonas hydrophila* have been reported to oxidize organic matter at anode to complete their metabolism process (Logan and Regan, 2006). In spite of being a promising technology, MFC suffers from limitations such as low power density, high cost etc. MFCs will be a viable option for power generation if the current production of this device is improved (Kim et al., 2007). The electrochemical performance of the bio-anode is one of the major factors that affect the bioelectricity generation process therefore identification of key operational factors and their optimization is of utmost important for the maximization of the power output (Pham et al., 2009). Electrode modification is another strategy to improve the performance of an MFC (Wei et al., 2011). Thus the present study endeavored in this direction and was focused in improving power generation using *Shewanella* sp. as anodic biocatalyst in a single chambered MFC (sMFC).

Many MFCs operation were reported with *Shewanella* sp. as a model organism (Kim et al., 2002; Sharma and Kundu, 2010) which

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is a facultative anaerobic bacteria that can use a wide range of electron acceptors and are classified in the group of dissimilatory metal-reducing bacteria (DMRB) (Lovley et al., 2004). Intact cells of anaerobically grown *S. putrefaciens* are electrochemically active and can grow in a MFC using anode as its terminal electron acceptor. The anode performance in *Shewanella* inoculated MFC is influenced largely by factors such as anolyte pH; inoculum age; concentration of electron donor: Calcium chloride ($CaCl_2$) riboflavin, etc (Kim et al., 2002; Park and Zeikus, 2002). Previous studies were carried out to evaluate the individual effect of these operating parameters in the anode chamber that influenced the activity of *Shewanella*. However, the combined effects of these parameters have not yet been explored. A better understanding of interrelationship between the operational parameters is needed prior to successful MFC application. The present study addressed the individual as well as the combined effect of the above mentioned operating parameters. Taguchi method was adapted to evaluate the influence of anolyte pH, $CaCl_2$ and substrate concentration on volumetric power generation.

Initially, it was assumed that outer most cytochrome C type protein is involved in exocellular electron transfer (EET) and riboflavin plays significant role in EET by donating electron to anode (Kim et al., 2002). Recent work has shown that *Shewanella* species secrete riboflavin that facilitates electron transfer to both insoluble metals and anodes. The removal of accumulated soluble flavins decreases the rate of electron transfer by *Shewanella* biofilm to electrodes over 80%. Commercial riboflavin is much cheaper considering the requirement in anolyte of MFC (Marsili et al., 2008). Thus, in our second approach, riboflavin was supplemented to anolyte to study the improvement in power output. Electrochemical impedance spectroscopy was employed to estimate the reduction in internal resistance.

In a third approach, the influence of anode modification (impregnated) with Nano Fe_2O_3 on power output was investigated. Anode activity of a MFC can be improved by introducing electrocapacitive component into it. It is well known that a capacitive anode can perform much efficiently than its noncapacitive counterpart. So, incorporation of electrochemically charge storage materials on anode surface is desirable. Now-a-days transitional metal oxides are extensively studied as electrode materials for electrochemical capacitors. Among these oxides Fe_2O_3 has gained much attention for its low cost, non toxicity, high performance and long cycle life (Park et al., 2008). It was previously reported that *Shewanella* was attracted by hematite or Fe_2O_3 particles and it had a tendency to reduce hematite (Bose, 2009). Thus hematite can be utilized as an anode modifier. In the present study, (hematite) Fe_2O_3 nano structure was prepared, characterized and impregnated in the carbon cloth anode and its loading effect was evaluated on current output of sMFC. In addition, cyclic voltammetry was utilized to evaluate the capacitance of different nano Fe_2O_3 loadings on anode of sMFC.

2. Methods

2.1. Microbial strain, media and growth conditions

S. putrefaciens (ATCC® BAA1097™) was used as biocatalyst in the anode chamber. Colonies of *S. putrefaciens* were grown on LB agar (HiMedia Laboratories Pvt. Ltd., India) at 37 °C for 24 h. Single colonies were incubated in 50 mL LB broth (HiMedia Laboratories Pvt. Ltd., India) in 100 mL conical flasks, shaken continuously on a rotary shaker (180 RPM) for at least 24 h under aerobic conditions. 10 mL of the culture was used as inoculum for the MFCs.

For experiment purposes, lactate based medium was used as anolyte in MFCs; for 1000 mL anolyte preparation- 3 mL Na-lactate (60% v/v); 0.425 g KCl; 2.028 g anhydride Na_2SO_4; 0.19 g $CaCl_2{\cdot}2H_2O$; 0.105 g $MgSO_4{\cdot}7H_2O$ and 1.004 g yeast extract (Difco) were taken. The anolyte pH was adjusted to 7 (unless stated otherwise) (Khilari et al., 2014) with 1.0 M carbonate/bicarbonate buffer. Desirable chemical oxygen demand (COD) was maintained by altering the Na-lactate concentration in the medium. Since the unmodified lactate medium contained 0.19 g $CaCl_2$, all concentrations of $CaCl_2$ added for Taguchi optimization process were normalized to the un-modified medium.

2.2. Synthesis of nano Fe_2O_3

Fe_2O_3 nano structure was prepared by a simple solvothermal method. In a typical synthesis, 30 mL 0.05 M ethanolic solution of $FeCl_3$ was taken in a beaker and 360 mg urea was added to this solution followed by stirring for 10 min. After stirring a clear solution was formed which was transferred to a 50 mL Teflon-line stainless steel autoclave. Hydrothermal dwell temperature was set at 150 °C for 12 h. Then the autoclave was allowed to cool down naturally to room temperature and precipitate formed inside Teflon line container was collected by centrifugation. Subsequently the product was washed with distilled water followed by ethanol for several times. Finally the washed precipitate was dried in an oven at 60 °C for 6 h.

2.3. Physical characterizations of nano Fe_2O_3

The crystallographic phase and structure were evaluated from X-ray diffractogram. Powder X-ray diffraction was carried out in a Rigaku Ultima III using Cu K_α (40 kV, 40 mA) radiation over a range of 20–80°. The surface morphology and composition was studied with a Zeiss Supra scanning electron microscope at an accelerating voltage of 5 kV.

2.4. Nano Fe_2O_3 decorated anode preparation

The anode was modified with nano Fe_2O_3 containing electrode modifier ink. In a typical ink preparation, requisite amount of nano Fe_2O_3 (0.2, 0.4, 0.8 mg/cm^2) was dispersed in acetone-isopropyl alcohol (1:1) solution with 5 wt% polytetrafluoroethylene (PTFE; 60 wt%; Sigma Aldrich). The ink was subsequently sprayed on a carbon cloth by a gravity spray gun. Then the modified carbon cloth was allowed to dry at 70 °C in an oven for 6 h. Finally the dried Fe_2O_3 loaded carbon cloth was used as anode in MFCs.

2.5. MFC assembly construction

Nine identical sMFCs were used for the experiments. The MFC consisted of an anode compartment and a membrane cathode assembly (MCA) placed on opposite sides. The cuboidal anode chamber is made from transparent polyacrylic material with outer dimensions $7 \times 8 \times 3.5$ cm^3 of 110 mL capacity. The anode chamber had two ports at the top, one for electrode terminal and the other for reference electrode (Ag/AgCl, saturated KCl; +197 mV, Equiptronics, India) and sampling. The anode consisted of a carbon cloth of working surface area 12 cm^2 with a stainless steel wire welded to form the terminal. A low cost custom made KOH doped polyvinyl alcohol (PVA) -polydiallyldimethylammonium chloride (PDDA) anion exchange membrane (AEM) was used as separator in sMFCs. The thickness of anion exchange membrane was found to be 110 ± 05 μm. Membrane cathode assembly (MCA) was prepared by coating membrane with catalyst loaded cathode. In order to prepare cathode, conductive ink containing cathode catalyst [0.15 mg/cm^2 Manganese cobaltite nanorods ($MnCo_2O_4$-NRs) dust as reported in our earlier work and carbon black Vulcan XC-72 (0.35 mg/cm^2; Cabot Corp.; India)] was taken in 20 mL 1:1

acetone-isopropyl alcohol solution with 0.3 mg/cm^2 PVA (1% w/v) aqueous solution as a binder. Ultra-sonication of the PVA-$MnCo_2O_4$-NRs loaded carbon black aqueous-acetone solution was done in 30 min and used as ink to spread on the cathode. Ink containing cathode catalyst was sprayed on the preheated membrane and was kept in the oven at 60 °C (Khilari et al., 2014). The MCA was manufactured by bonding the carbon ink coated AEM [consist of KOH doped PVA–PDDA polymer electrolyte] directly onto a flexible stainless steel (SS) mesh as current collector. SS mesh (8 cm^2) was attached with all the membrane using conducting paint (Siltech corp., India) on the air facing side (Khilari et al., 2013). The SS mesh used in the present study was of SS-304 type with 50×50 number of openings per square inch (wire diameter 0.17 mm). It was connected with a concealed copper wire as cathode terminal.

Concealed copper wires were used to connect the external resistance to close the circuit. The inter-electrode distance was kept constant in all the experiments (~2.5 cm), the anodes being placed equidistant from the MCA. The additional ports were sealed with clamped tubes to ensure anaerobic environment. The sMFC were washed with 70% alcohol and put in the UV chamber for 30 min before the experiment.

2.6. MFC operation and experimental variations

Details about the operational parameters are given in Table 1. The anode chamber was kept airtight and filled completely with 100 mL lactate broth with the inoculum added to achieve anaerobic conditions in the least operation time. The anolyte for each MFC was prepared as per the chart (Table 2). The MFCs were operated in close circuit mode using a 100 Ω external resistance to determine the COD removal and Columbic efficiency.

2.7. Analytical measurements and calculations

The COD values of the anolyte were measured using a COD measurement instrument set (DRB200 & DR2800 Portable Spectrophotometer, HACH, USA). The pH values were monitored using a desktop pH meter (pH510, Cyberscan, Singapore). The operating voltage (OV) was measured using a data acquisition system (USB-6009, National Instruments, Texas, USA with NI LabVIEW–based customized software, Core Technologies, India).

Prior to polarization study, the sMFCs were kept in open circuit mode to let it reach maximum voltage. Polarization curves were obtained by varying the external resistance of the closed circuit using a variable resistance box (range 90 kΩ–20 Ω) in discrete steps and measuring the corresponding voltage drop. The average time required for obtaining a stable reading was 5–7 min. The volumetric current density and volumetric power density was calculated as given elsewhere (Khilari et al., 2014).

2.7.1. Scanning electron microscopy (SEM)

To examine the surface morphology of the anode, SEM was performed. A few fibers from carbon cloth bio-anode were removed aseptically in a laminar hood before and after the experiment. The cells, if any, on the surface of the electrode were fixed using 2% formaldehyde/0.02% picric acid in 0.1 M sodium phosphate buffer, pH 7.2 and were subsequently dehydrated in ethanol gradient of 40–100% (5 min each). Samples were then stored in desiccators. Gold sputtering was carried out in a HITACHI E-101 ion sputter unit maintained at 0.1–0.01 Torr for a uniform coating of 300–350 Å. SEM images of prepared samples were then obtained using a Zeiss EVO 60 scanning electron microscope with incident electron beam energy of 10 keV and working distance of 6 mm.

Table 1
Experimental design chart.

Shewanella putrefaciens mediated bioanode	
Experimental parameters	Range
$CaCl_2$ concentration (μM)	750, 1500, 2250
Initial anolyte pH	6, 7, 8
Lactate medium concentration (mg/L)	2000, 4000, 6000
Riboflavin addition (nM)	100, 50, 25, control
Nano hematite (Fe_2O_3 nanoplates) loading (mg/cm^2)	Control, 0.2, 0.4, 0.6, 0.8 mg/cm^2

Table 2
Taguchi orthogonal matrix of different anodic operational parameters and response.

Anolyte pH	Lactate (mg/L)	$CaCl_2$ (μM)	Power density (W/m^3)	Standard deviation (±)
6	2000	750	2.44	0.12
6	4000	1500	3.67	0.15
6	6000	2250	3.28	0.01
7	2000	1500	4.32	0.16
7	4000	2250	3.93	0.11
7	6000	750	2.69	0.09
8	2000	2250	4.64	0.16
8	4000	750	3.04	0.12
8	6000	1500	4.8	0.12

2.7.2. Fluorescence microscopic study of carbon cloth bioanode

In order to perform fluorescence microscopy study, cell fixation was performed following the method described by Das et al., 2013. The biofilm of *S. putrefaciens* grown on different modifier loaded carbon cloth anode were fixed with 4% paraformaldehyde (w/v, in phosphate buffer saline) at 4 °C overnight, subsequently washed with 1X PBS twice and finally stored in 1X PBS: ethanol (1:1) at −20 °C. 10 μL of 4,6 diamidino-2-phenylindole (DAPI) (1 μg/mL) were added in same amount of fixed sample and incubated for 15 min at room temperature in dark condition. Excess DAPI was removed by washing the sample with 1X PBS. All microscopic observations and image acquisition were performed with an Olympus model epifluorescence microscope equipped with the UV filter set (excitation wavelength 330–385 nm) to visualize bacteria stained with DAPI at 40× magnification. Different microscopic fields on each slide were analyzed to confirm the results. All image combining, processing and analysis were performed with the software package Soft Imaging Systems and CCD camera.

2.7.3. Bioelectrochemical measurements

The cyclic voltammogram (CVs) of bioanode were recorded with a CHI 660E at a scan rate of 1 mV/S. A three electrode configuration consisting of bioanode, Pt wire and Ag/AgCl as working, counter and reference electrode, respectively, was used for electrochemical measurements. Electrochemical impedance spectroscopy (EIS) of bioanode was performed with the same electrode configuration. EIS was done over a frequency range of 100 kHz to 1 Hz with a sinusoidal perturbation of 5 mV (Khilari et al., 2014).

2.8. Taguchi optimization of process parameter

The relative importance of anolyte pH, concentration of lactate medium and $CaCl_2$ for power generation was investigated by Taguchi design. Taguchi method of the design of experiments (DOE) is a factorial based approach which involves establishment of large number of experimental situations described as orthogonal array (OA) to reduce errors and to enhance the efficiency and reproducibility of the laboratory experiments. In this method, the

performance is measured by the deviation of a characteristic from its target value and a loss function [L(y)], as represented by

$$L(y) = k(y - m) \quad (1)$$

where, '*k*' denotes the proportionality constant, '*m*' represents the target value and '*y*' is the experimental value obtained for each trial. In the case of 'bigger is better' quality characteristics, the loss function can be written as

$$L(y) = k\left(\frac{1}{y^2}\right) \quad (2)$$

and the expected loss function can be represented by

$$E[L(y)] = \mathrm{kE}\left(\frac{1}{y^2}\right) \quad (3)$$

Where, $E(1/y^2)$ can be estimated from a sample of n as

$$E\left(\frac{1}{y^2}\right) = \sum_{i=1}^{n}\left[\frac{1}{y^2}\right]/n \quad (4)$$

The key objective of employing the DOE methodology was to understand the influence of selected factors individually and in combination on the performance of MFC.

In experimental design three factors i.e., anolyte pH, lactate concentration and concentration of $CaCl_2$ were considered with three levels of factor. Subsequently, matrix experiments (9 experiments with different combinations) were designed and the data analysis procedure was defined. Levels of factor variation were considered and the diversity of factors was studied by crossing the orthogonal array (OA) as tabulated in Table 2. The obtained experimental data was processed using MiniTab15 software.

3. Results and discussion

The details of results on Taguchi Optimization of process parameter, influence of riboflavin supplementation, SEM and fluorescence microscopic studies of the microbial cells are presented in the supplementary information (SI).

3.1. Nano Fe_2O_3 modified anode

3.1.1. Morphology and crystal structure of the anode modifier

Field emission scanning electron microscope (FE-SEM) was used to evaluate the surface morphology of as-prepared Fe_2O_3 nanostructure (Fig. S2a). The FE-SEM micrographs represent the pancake like morphology of as-prepared Fe_2O_3 which are assembled to form a flower like cluster. The thickness of each pancake like Fe_2O_3 nanostructure was found to be in the range of 100–260 nm. A high magnification FE-SEM image clearly revealed that the pancake like nanostructures were self-assembled particles with diameter <25 nm. X-ray diffractrogram was analyzed to study the crystal phase and structure of as-prepared nano Fe_2O_3. The XRD pattern of nano Fe_2O_3 composed of sharp diffraction features indicating well crystallinity (Fig. 1). Again the XRD pattern was found to be well matched to the standard JCPDS (File No. 087-1166) with α-Fe_2O_3 phase and rhombohedra crystal system.

3.1.2. Charge discharge experiment

Capacitive behavior of an electrode can be evaluated by employing charge discharge technique. A series of anode materials consisting of nano Fe_2O_3 modified on the carbon cloth were considered for experimental study. The capacitances were determined before the addition of inoculum onto the anodes of MFCs. Specific capacitance of anode was measured by following equation

$$C = \frac{I_{\text{charge-discharge}} \times t}{U_{\text{charge-discharge}} \times A} \quad (5)$$

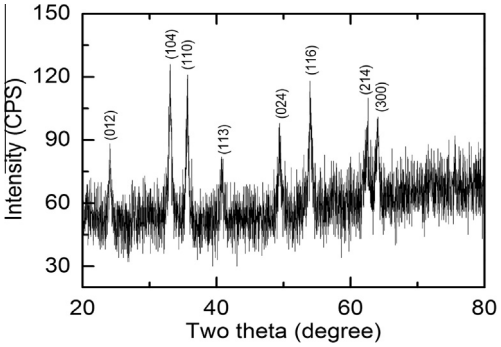

Fig. 1. X-ray diffraction patterns of nano Fe_2O_3.

where $I_{\text{charge-discharge}}$ is the charge-discharge current; t is the discharge time; $U_{\text{charge-discharge}}$ is the potential window; and '*A*' is the projected anode surface area. Capacitance of the anode improved with increase in the nano Fe_2O_3 loading to carbon cloth (Fig. 2a). It was observed that the increment of capacitance decreased after certain loading. Initially 0.2 mg/cm^2 Fe_2O_3 loading showed specific capacitance of 0.05 F/cm^2 which was higher than unmodified anode. Further doubling the initial nano Fe_2O_3 loading from 0.2 mg/cm^2 to 0.4 mg/cm^2 resulted in ~2 times improvement in specific capacitance. However, increase in capacitance was found insignificant on further addition of nano Fe_2O_3 loading possibly due to an increase in electrode surface resistance with formation of thick layer on anode surface. This thicker layer restricts the diffusion of electrolyte ions to the capacitive nano Fe_2O_3 layers thereby reducing capacitance.

3.1.3. Effect of nano Fe_2O_3 loading on Shewanella grown anode

Four sMFCs were operated using same cathodic configuration; while quantity of nano Fe_2O_3 in anode configuration was varied keeping other anodic conditions unaltered. The anolyte pH was kept at 7 while the concentration of lactate medium and $CaCl_2$ were 6 g/L and 1500 μM respectively. The performances of sMFCs with nano Fe_2O_3 catalyst based anode were investigated using four different loadings (0.2–0.8 mg/cm^2), one modifier free anode was taken as control to evaluate relative increase in power output. Stable power was generated after three to five sequential transfer of lactate medium into the anode chamber of mediator-less sMFC. However, significant variation was found in the anode half-cell potential. The corresponding polarization curves of the MFCs, as shown in Fig. 2b, are obtained by varying the external resistance from 10 Ω to 90 kΩ. It was observed that presence of nano Fe_2O_3

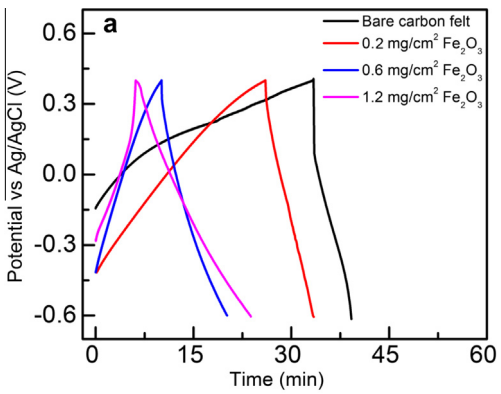

Fig. 2a. Charging discharging plot of anode at different modifier loading.

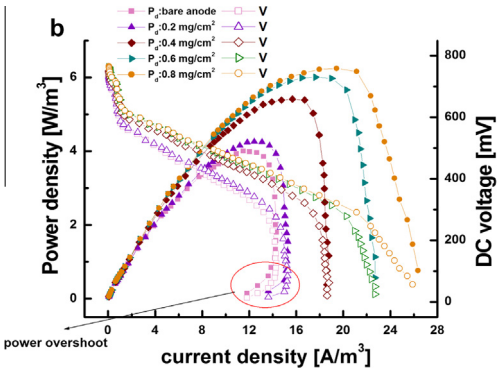

Fig. 2b. Polarization plots for sMFC (power density and D.C. voltage as a function of current density) with different nano Fe_2O_3 loaded anode. The power density and voltage data points are presented as solid and open symbols, respectively.

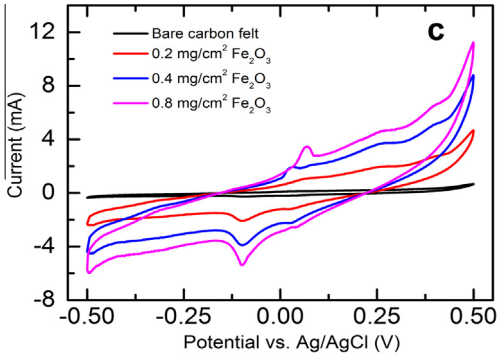

Fig. 2c. Cyclic voltammograms recorded at scan rate 1 mV/s with different nano Fe_2O_3 loaded anode.

(0.8 mg/cm^2 loading) on anode induced maximum OV of ≈0.26 V and higher power densities (6.24 W/m^3) whereas MFCs with unmodified anode had OVs of around 0.15 V and significantly lower power densities. The MFC without nano Fe_2O_3 (uncoated) anode produced a maximum power density of 3.96 W/m^3; At high electrical current regime power overshoot occurred in sMFCs having bare anode and anode containing little amount of modifier (0.2 mg/cm^2). It was observed that both cell voltage and electrical current declined at reduced external loads during polarization study. Overshoot problem was absent with the increase of anode modifier loading. Increasing the quantity of nano Fe_2O_3 from 0.2 mg/cm^2 to 0.8 mg/cm^2 substantially enhanced the maximum volumetric power density (32.2%); however increasing the nano Fe_2O_3 quantity from 0.6 to 0.8 mg/cm^2 power density improved only 4.32%. This increase in power density with nano Fe_2O_3 on anode was possibly due to large population of bacteria that consequently lead to large negative potential through electron donation by EAB at anode even at low external resistance.

The performance of MFC is usually estimated by polarization curve or linear sweep voltammetry (LSV) (Pinto et al., 2011). Power overshoot commonly makes the performance evaluation of MFCs inaccurate. Two type of power overshoot was observed; V and M type, V type was commonly associated with a sharp fall in anode potential whereas type M was due to fast scan rate of linear sweep voltammetry (LSV) and less time available for stabilization during polarization study (Hong et al., 2011). It was demonstrated that mass transport limitation is not responsible for overshoot problem (Winfield et al., 2011). Electrical and ionic depletion on the anode under low external resistances did not influence overshoot (Ieropoulos et al., 2010). Peng et al. suggested that the power overshoot was due to the lack of anodic capacitance (Peng et al., 2013; Marsili et al., 2008). Winfield et al. observed that one of the reasons for the power overshoot seen in power curves from MFCs might be due to immature biofilm on the anode electrode. Herein power overshoot problem was reduced through the modification of anode surface by blending with nano Fe_2O_3 modifier (Winfield et al., 2011). Therefore, it may be inferred that anode modifier might help in increasing capacitance and developing mature biofilm.

3.1.4. Cyclic voltammetry investigation of bioanode

Cyclic voltammetry (CV) was extensively used to study the catalytic and capacitive behavior of different bio electrodes (modified and unmodified). The specific capacitance of various anodes was measured by the following equation;

$$C = \frac{\int_{V1}^{V2} \mathrm{IdV}}{A\Delta V(dV/dt)} \quad (6)$$

where '*C*' '*A*', 'ΔV' and '(dV/dt)' indicated specific capacitance per unit area (F/cm^2), surface area of anode (cm^2), potential window (V) and scan rate (V/s) respectively. Bare bio anode showed distinct difference in the CV with its only catalyst loaded counterpart (Fig. 2c). Bare anode did not exhibit any redox peak whereas bioanode shows well defined anodic and cathodic peak at 0.018 and −0.188 V, respectively. Further increase in catalyst loading on anode improved the peak current as well as the area under the peak. The specific capacitance measured for cyclic voltammogram of different anodes was found to be maximum of 0.16 F/cm^2 for 0.8 mg/cm^2 nano Fe_2O_3 loaded anode and minimum for bare anode (0.008 F/cm^2). This indicated that the electron generated on the anodic oxidation process was efficiently stored on the pseudocapacitive bioanode as compared to noncapacitive counterpart. It was also observed that the nano Fe_2O_3 content played an important role in the specific capacitance of bio anode. Initially 0.2 mg/cm^2 nano Fe_2O_3 loaded anode showed specific capacitance of 0.07 F/cm^2 which was far higher than the unmodified anode. Further doubling the initial (0.2 mg/cm^2) nano Fe_2O_3 loading to 0.4 mg/cm^2 resulted in ~2 times improvement in specific capacitance. However no significant effect on capacitance was found on further increment in nano Fe_2O_3 modifier on carbon cloth anode. This anomaly was observed may be due to formation of thick layer on anode surface leading to increase in surface resistance. As nano Fe_2O_3 is a good pseudocapacitive electrode material, it improves the charge storage on the anode surface (Lv et al., 2014). In a similar study, the biocompatibility of Fe_2O_3 allowed *S. putrefaciens* to grow on its surface thereby improving charge storage on anode (Royer et al., 2002). Recently few research groups suggested that accumulation of charge generated during anodic oxidation through an external capacitor followed by short time decapitation can help in improved performance of MFCs (Kalathil et al., 2013). It was also reported that the incorporation of capacitive material on anode can replace external capacitor and behave like a capacitive anode which performs better as compared to the noncapacitive anode due to more charge accumulation (Lv et al., 2014). The transient charge storage in biofilm resulted in a significant increase in total capacitance for anodes, which was the possible reason for the elimination of power overshoot in sMFCs with higher amount of (0.4–0.8 mg/cm^2) Fe_2O_3 loaded anode after multiple batch cycle operation.

3.1.5. Microscopic analysis

3.1.5.1. Scanning electron microscope image of the carbon cloth anode fiber. SEM micrographs studies on the morphology of nano Fe_2O_3 loaded carbon cloth before and after biofilm formation, demonstrating good bacterial adhesion. Due to the high surface area, total biomass attached to the modified anode was relatively high and

morphology of biofilm was uniform, which facilitated electron generation and transfer. High cell density (*S. putrefaciens*) was observed on the carbon cloth anode fiber (Fig. S2b) which implies that EABs like *Shewanella* have a high binding affinity towards Fe(III) oxide. High specific area of the anode assists bacteria to attach to the electrode easily and form uniform morphology (Bose, 2009). It has been experimentally demonstrated that outer membrane c-type cytochromes (OM c-Cyts) have a high binding affinity towards Fe(III) oxide. Furthermore, Fe(III) oxide can be recognized by dissimilatory iron reducing bacteria (DIRB) and utilized as electron shuttle to reduce distant terminal acceptors (Li et al., 2011). The role of (outermost cytochrome c) OM c-Cyts for respiratory Fe(III) oxide reduction has also been suggested by some researchers (Korenevsky and Beveridge, 2007). They found that the modification of anode by Fe_3O_4 enhanced the kinetic activity by 120% compared to that of the unmodified anode in sediment MFCs. Wei et al also reported that the Fe_2O_3 electrode prepared through layer-by-layer self-assembly technique can be used as the anode which has noticeable improvement in power generation compared to the bare anode (Wei et al., 2011) The improved electrochemical performance could be ascribed to the synergetic effect of its unique structures, higher surface area, which could provide more active sites for interface electrochemical reaction and better biocompatibility (Zhou et al., 2011).

3.1.5.2. Epifluorescence microscope. The biofilm growth of *S. putrefaciens* on the surface of carbon cloth was studied using epifluorescence microscope equipped with the UV filter set. The density of cells on anode surface was visualized by DAPI staining. It was indicative of total microbial population on anode. Maximum biofilm growth was found in 0.8 mg/cm^2 loaded anode (Fig. S2c). The order of cell population which was clearly visualized from the photographs was: 0.8 mg/cm^2 nano Fe_2O_3 >0.4 mg/cm^2 nano Fe_2O_3 >0.2 mg/cm^2 nano Fe_2O_3 > bioanode without modifier. From this study it can be concluded that modification of anode with nano Fe_2O_3 facilitates reducing anodic half cell overpotential by promoting biofilm growth.

3.2. Bioelectrochemical treatment

Coulombic efficiency (C_E) describes the number of electrons that can be recovered as current versus that initially present in the biodegradable matter of the anolyte in MFC under close circuit mode at a particular external resistance. An average C_E observed in MFC having nano Fe_2O_3 loaded was (0.8 mg/cm^2 loading) 7.3% and (0.8 mg/cm^2 loading) 6.15% respectively compared to 4.98% in MFC with bare anode after 7th cycle. MFC supplemented with riboflavin showed higher value C_E of 7.5% (with 100 nM addition) because of improved electron transfer from EAB to anode compared to control MFC. More than 80% COD removal was achieved with all systems suggesting the suitability of sMFCs for wastewater treatment or BOD biosensor application purposes.

4. Conclusion

Taguchi design suggested that supplementation of $CaCl_2$ to anolyte was most influential parameter for maximization in power production followed by anode pH and substrate concentration. Impedance studies indicated reduction in charge transfer resistance with the supplementation of riboflavin to anolyte. Cyclic voltammogram indicated that sMFC having nano Fe_2O_3 loaded bioanode was kinetically more advantageous as it increased the number of active sites and improved biocompatibility and capacitance of the bioanode. The present work thus helped in determining the proximate values of these process parameters which ultimately resulted in maximum bioelectricity production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2014.05.075.

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