Annexure A
MATERIALS AND METHODS

A.1 MATERIALS

The reagents and chemicals used in the study are listed below:

Table A.1: List of all the chemicals and reagents used in the complete study

Chemicals/Reagents/ Materials	Make
COD estimation Reagents	Merck-Spectroquant kit
Basic salt & chemicals	Fischer scientific/ Merck
Culture Media Supplements	Himedia, India
Nafion-117 Proton Exchange Membrane	Nickunj Eximp Pvt. Ltd.
Graphite felt (Electrode)	Nickunj Eximp Pvt. Ltd.
Rock phosphate	Jhamarkotra Mines, Udaipur (Rajasthan)
Low-density Polythene Bags	Unique Plastic Industries
PCR reagents	Himedia, India
High range standard DNA ladder	Himedia, India
Soil DNA purification kit	Himedia, India
Gel extraction kit	Himedia, India
Primers	Xceleris, Ahmedabad
Cupric nitrate for nanoparticle synthesis	Sigma-Aldrich
$KMnO_4$	Fischer scientific/ Merck
$FeCl_3$	Sigma-Aldrich

A.2 METHODS

A.2.1 Construction of MFC reactors

The MFC reactors used in the study were fabricated at IIT Jodhpur. The details regarding MFC construction are described below:

(a) Polyacrylic- Dual Chambered MFC

Dual-chamber MFC reactors were fabricated using two equal-sized rectangular chambers made of acrylic material. The use of gaskets between both of the compartments established an airtight assembly. The working volume of both the chambers was 100 ml. Graphite felt (4 cm×4 cm×0.56 cm) was used as the anode and the cathode. A Cu wire connected both the anode and the cathode (gauge 2 mm). Each chamber was equipped with one sampling port. The photograph of the actual MFC reactor, used for the proposed system is shown in figure A1. Nafion-117(DU Point, USA) was used as a separator between the anode and cathode chamber. Both anode and cathode were kept at a distance of 2.5 cm. A fixed 1000 ohm resistor was plugged between anode and cathode. This MFC reactor was used in the study described in chapter 3.

Figure A1: MFC reactor used in the study.

(b) Pre-Treatment of Nafion membrane

The Nafion-117 membrane was treated as described previously [Ghasemi *et al.*, 2013]. The membrane was placed in distilled water at 80° C for one h following heating in a 3% hydrogen peroxide solution (H_2O_2). Next, the membrane was boiled in distilled water at 80° C, followed by treatment with 0.5 M sulphuric acid (H_2SO_4) for one h. Finally, the membrane was washed in distilled water at 80° C for two h and subsequently kept in distilled water at room temperature.

(c) Scaled-up MFC reactors

Clayware alone and clayware blended with rock phosphate served as the anodic chamber (1L). Rock phosphate was obtained from Jhamarkotra mines located at Udaipur (Rajasthan). Rock phosphate was mixed with clay (Black soil) at 5% & 10% loading. Low-density polythene (LDPE) bags (10 L) having dimensions (L*W*D) as 1 m × 0.2 m × 200 µm served as the cathodic chamber. The anodic inoculum was taken from previously operating MFCs degrading lipid extracted algae (LEA) biomass. The source of inoculum was pre-treated cow-manure. The electrode material as an anode (11cm × 11cm) and the cathode (18cm × 36cm) was graphite felt. Copper wire (gauge 2 mm) connected anode and cathode through a 1000 ohm resistor. The MFCs labeled as 5% RP-MFC, 10% RP-MFC, and CW-MFC (Clay only) were operated outdoor. Synthetic wastewater containing KH_2PO_4 4.4 g/l, K_2HPO_4 3.4 g/l, NaCl 0.5 g/l, $MgSO_4$ 0.2 g/l, $CaCl_2$ 0.014 g/l and KNO_3 1 g/l constituted the anolyte (Vijay et al., 2016). LEA biomass served as an electron donor substrate with anodic inoculums acclimatized on it previously. The anode chamber or the clayware was a closed cylinder with the detachable lid for medium exchange. The lid was closed and sealed properly to enable anaerobic conditions (Figure A2). This scaled-up reactor was utilized in the experiments stated in chapter 4. Catholyte consisted of algae cultivation medium and *Chlorella vulgaris.*

The MFC reactors operated in a fed-batch mode in triplicates. Drop-in voltage & COD after each cycle accompanied LEA addition and medium replenishment.

Figure A2: Outdoor MFC reactor used in the study.

(d) Top-bottom MFC configuration

Membrane filter holder unit (make Tarson) having a volume of 250 ml was employed as an MFC reactor. The lower chamber was converted to the anode, and the upper one served as a cathodic chamber. The ultra-filtration membrane (Diameter 47 mm) served as a separator. Graphite felt (4 cm × 4 cm × 0.56 cm) was used as the anode. The nanoparticle-based composite electrode (Diameter- 1.3 cm, height- 0.8 cm) served as cathodes. Cu wire connected anode and cathode, forming an external circuit. Catholyte consisted of a BG-11 media. Pre-treated Cow-manure was used as an inoculum source at the anode. The cathode was inoculated with microalgae *Chlorella vulgaris*. MFCs were operated in fed-batch mode. At the end of every batch cycle, all the MFC reactors were supplemented with fresh media.

A.2.2 Lipid extraction & pre-treatment of LEA biomass

Total lipid extraction from *C. vulgaris* was done using chloroform- methanol 1:2 as per the modified Bligh and Dyer extraction method [Kanaga et al., 2016]. Briefly, the algal biomass was treated with chloroform- methanol (1:2) with constant stirring, followed by the addition of distilled water. The above mixture was then subjected to centrifugation resulting in the separation of layers. The lower layer containing dissolved lipids was taken in a separate flask. The lipids were obtained after evaporation of the solvents.

LEA biomass obtained after the lipid extraction step was further treated before feeding the anode. It was placed at 82 °C in an oven overnight to remove residual chloroform and methanol traces. Alternatively, it was kept in sunlight for a period of 48 h. Both strategies were equally effective in eliminating unwanted organic leftovers. This was followed by crushing in a

pestle-mortar to convert it into a fine powder. These steps were done to remove any traces of inhibitory organic solvent and increase the surface area for the microbial attack at the anode. When the two steps mentioned above were bypassed, MFCs failed in terms of power output, COD removal, etc.

A.2.3 CHARACTERIZATION OF CLAYWARE AND ROCK PHOSPHATE AMENDED CLAYWARE

For physical Characterization on various parameters, separators (Thickness – 0.4 cm, Diameter – 6 cm) were constructed using both clay and RP blended clay.

A.2.3.1 Proton conductivity

Proton conductivity measurements involved soaking the RP blended separators in 0.1 M H_2SO_4 for 30 minutes, followed by washing with the DI water till neutral pH. The proton mass transfer coefficient and proton diffusion coefficient were calculated as described previously [Zhang et al., 2009]. The measurements were done on an MFC with the two chambers separated by clay or RP blended membrane. One of the chambers was filled with DI water of pH 6.8, while the other set at pH 10 using KOH solution. A pH probe (INFORS HT multifors) continuously read the change in pH of the alkaline chamber due to flow of protons from the low-pH chamber. Proton mass transfer coefficient (KH) was calculated using the following equation:-

$$K_H = -\frac{V}{2At}\ln[\frac{C_{1.0} + C_{2.0} - 2C_2}{C_{1.0}}]$$

Where $C_{1.0}$ is initial proton concentration in the first chamber; $C_{2.0}$ is initial proton concentration in the second chamber; C_2 is proton concentration in the second chamber at time t; V is the working volume of the reactor; A is the area of the membrane.

The proton diffusion coefficient (K_D) was calculated as follows:-

$$K_D = K_H \cdot L_D$$

Where, L_D is the average membrane thickness.

A.2.3.2 Oxygen diffusion

To measure the oxygen diffusion, one of the chambers was sealed after purging with oxygen–free nitrogen gas. The other chamber was continuously purged with air to maintain high DO. DO probe read the changes in DO in the anaerobic chamber indicative of oxygen diffusion [Zhang et al., 2009]. The oxygen mass transfer coefficient was calculated using the formula-

$$K_O = -\frac{V}{At}\ln[\frac{C_{1.0} - C_2}{C_{1.0}}]$$

Where V is the liquid volume of the first chamber where the DO probe is inserted, A is the separator's cross-sectional area, $C_{1.0}$ is the saturated DO concentration in the first chamber, and C_2 is the DO concentration in the second chamber at time t. The diffusion coefficient (D_O) was determined as follows:-

$$D_O = K_O \cdot L_D$$

L_D is the average membrane thickness.

A.2.3.3 Acetate diffusion coefficient

The acetate diffusion coefficient (D_a – cm^2/sec) was calculated according to the process described by Neethu et al. (2018). A dual chamber MFC described in the above sections was

used to determine acetate diffusion coefficient. Briefly, acetate was added in one of the chamber and the change in acetate concentration was measured in the adjoining chamber. The Da was determined using the following equation:

$$D_a = -\frac{VL_D}{2At}\ln\left[\frac{C_{1.0} - 2C_2}{C_{1.0}}\right]$$

A.2.3.4 Water holding capacity (WHC)

The separators were immersed in water for 24 hours, followed by drying in an oven at 60°C until a constant weight was achieved [Neethu et al., 2018]. Water holding capacity (%) was calculated by measuring the weight difference between the wet and dry separator.

A.2.4 SYNTHESIS OF NANO-PARTICLES

A.2.4.1 CuO nanoparticle synthesis

The cupric nitrate and citric acid were mixed into 1:1.5 ratios, respectively. The solution was heated at 300 °C in a heating mental. This was followed by dehydration, decomposition, and combustion. The combustion step was followed by the separation of nanoparticles [Christy et al., 2013].

A.2.4.2 MnO_2 Nano-particle synthesis

MnO_2 nanoparticles were synthesized according to the process described by Zhou et al., 2018 [Zhou et al., 2018]. Briefly, 2.7 ml of ethanol and 1.2 g of $KMnO_4$ were mixed in DI water. The solution was heated at 125° C for 24 h, followed by filtration, washing, and drying of the precipitate at 100° C in a vacuum oven. The precipitate was then subjected to annealing in the air for five h at 300° C.

A.2.4.3 Fe_3O_4 Nanoparticle synthesis

A 10 ml of the iron salt solution having 0.3 M Fe^{+3} was mixed with 10 ml of 0.05 M glucose solution, and this mixture was refluxed at 90° C for 12 h. The solution was mixed drop-wise with 50 ml of 1 M NH_4OH solution. The nanoparticles were then separated from sediment suspension and washed several times with deionized water [Gonzalez et al., 2011].

A.2.5 ANALYTICAL TECHNIQUES

A.2.5.1 pH Measurement

The pH meter (make- Hanna Instruments, US) was employed to determine the pH. The pH of the anodic and cathodic chamber was measured periodically.

A.2.5.2 Chemical Oxygen Demand Estimation

Chemical oxygen demand (COD) was determined according to the standard protocol [APHA, 2005]. The acidified potassium dichromate solution was used as an oxidizing agent with silver sulphate as a catalyst. The reaction mixture comprised of 2.5 ml of sample, 1.5 ml of potassium dichromate, mercury sulfate & sulfuric acid solution, and 3.5 ml of silver sulfate solution made in sulfuric acid. The reaction mixture was then subjected to a COD digester at 148°C (Merck) for in glass ampules for 2 h. The absorbance of the samples was taken at 600 nm.

A.2.5.3 Algal growth estimation

Algal growth was determined by taking either the dry weight of cells or cell numbers on every alternate day. The specific growth rate was computed during the exponential growth phase. Specific algal growth rate (μ) was calculated using the following formula:-

$$\mu = \frac{\ln N_2 - \ln N_1}{t_2 - t_1}$$

Where, N_1 and N_2 are a number of cells/dry weight at t_1 and t_2 days, respectively.

A.2.5.4 GC Analysis

Lipids were converted into fatty acid methyl ester (FAME), as described previously [Vyas & Chhabra, 2017]. The fatty acids profile of FAME was analyzed by gas chromatography with a mass spectrometer (GC-MS model- Auto system XL GC with turbo mass, make Perkin Elmer). GC-MS analysis was carried out at 250° C by introducing 1µl of the sample at a split ratio of 50:1, employing helium as a carrier gas. The column temperature was raised from 60°C to 150°C at the rate of 12°C/min. The sample was held at this temperature for 1 min. The temperature was further increased to 240°C at a rate of 50°C/min and kept at 240°C for 5 min. The FAME derived from algal lipids was analyzed for the fatty acid profile using known standards.

A.2.6 STATISTICAL ANALYSIS

All of the experiments were conducted multiple times, each time in triplicates. The reported results are the average values with a standard deviation ranging from 5 to 20%.

A.2.7 ELECTROCHEMICAL ANALYSIS

A.2.7.1 Measurement of Power Density and Voltage

Voltage was measured in every 10 seconds using Keysight 34972A data acquisition system. Polarization curves were obtained by varying external resistance from 51 ohms to 39000 ohms. Power density (W/m^3) was estimated by normalizing the power with the volume of the anodic chamber, as

$$Power\ density = \frac{V \cdot I}{Anodic\ Volume}$$

Where V is the voltage in volt (V), and I is current in ampere (A).

A.2.7.2 Columbic Efficiency

Columbic efficiency (CE) was calculated by using the following formula [Logan et al., 2006] .

$$CE = \frac{M \int_0^t I dt}{FnV\Delta COD}$$

Where M is the molecular weight of oxygen, I is current, F is Faraday constant, n is the number of electrons required for reduction of 1 mole of oxygen, V is the working volume of anodic chamber ΔCOD is the difference between initial and final COD after time t.

A.2.7.3 Cyclic Voltammetry

Cyclic voltammetry (Autolab Potentiostat PGSTAT302N, Metrohm electrochemical work station) was used to investigate the redox reactions occurring at the electrode surface. The anode was used as the working electrode, Ag/AgCl as a reference electrode and cathode as a counter electrode. All the experiments were performed within the scanning window of -0.7 V to 0.6 V at 1mV/s of scan rate. All the analysis including electrochemical analysis were done in 6th and 7th batch cycle, during which high operating voltage was achieved.

A.2.8 MICROBIAL COMMUNITY ANALYSIS BY NEXT-GENERATION SEQUENCING

Scraps of pooled anodic biofilms were subjected to community analysis. The genomic DNA was isolated using the Soil DNA purification kit, Himedia. Next-Generation Sequencing (NGS) of the V3-V4 hyper-variable region of 16s rDNA was done at Xcleris genomics facility (Ahmedabad, India). The V3-V4 hyper-variable region of bacteria & archaea was amplified using the primer pair (Prokaryote-V3 forward: 5'CCTACGGGNBGCASCAG3' and Prokaryote-V4-reverse-5' GACTACNVGGGTATCTAATCC3'). The amplicon libraries were constructed and sequenced. QIIME (Quantitative Insight into Microbial Ecology) tool helped to analyze the data. UCLUST algorithm identified an operational taxonomic unit (OTU), a taxonomic unit assigned to similar sequences (97% similarity). Green gene database helped make taxonomic assignments at a 90% threshold value. Alpha-diversity indices, for example, Chao1, Shannon index (H), and observe species were determined using QIIME. A heat map relating to OTUs data with the relative abundance was also obtained. The significant OTUs prevailing in the system (with >1200 sequences) were used for the heatmap. UPGMA hierarchical clustering method was used to cluster the log-transformed OTUs. Rarefaction plot was constructed using QIIME. Sequences were deposited at the NCBI Sequence Read Archive (SRA) database with the accession number PRJNA579083.

A.2.9 SEM AND EDX ANALYSIS

Scanning electron microscope (SEM) enables to investigate the surface morphology of any given sample. Both bacterial and algal biofilm from anode & cathode, respectively were analyzed using scanning electron microscope Carl Zeiss EVO 18 special edition. The EDX (Energy dispersive X-ray) analysis was carried out to investigate the elemental composition of fabricated composite cathodes.

A.2.10 X-RAY DIFFRACTION (XRD) ANALYSIS

XRD is generally utilized to investigate the crystal structure of unknown samples. After obtaining the XRD spectra, it is matched with standard database for the identification of given sample. X-ray diffraction (XRD) analysis was done using a Bruker AXS D8 Advance.

A.2.11 FOURIER TRANSFORMATION-INFRARED SPECTROSCOPY (FTIR)

FTIR was performed using Bruker FTIR spectrometer vertex 70V. In order to compare the key functional groups of intact algal biomass, extracted lipids and LEA biomass, FTIR analysis of all these three samples was done. The spectrum was recorded in the range of 4000-400 cm^{-1}[Phukan et al., 2011].

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List of Publications

Peer reviewed International Journals (Published)

1) **Khandelwal, A.**, Vijay, A., Dixit, A., & Chhabra, M. (2018). Microbial fuel cell powered by lipid extracted algae: a promising system for algal lipids and power generation. *Bioresource Technology*, *247*, 520-527.
2) **Khandelwal, A.**, Chhabra, M., & Yadav, P., (2020). Performance evaluation of algae assisted microbial fuel cell under outdoor conditions. *Bioresource. Technology*, 310, 123418.
3) Vijay, A., **Khandelwal, A.**, Chhabra, M. & Vincent, T. (2020). Microbial fuel cell for simultaneous removal of uranium (VI) and nitrate. *Chemical engineering Journal, 388*, 124157.

Under review or preparation

1) **Khandelwal, A.** & Chhabra, M. Superiority of activated graphite/CuO composite electrode over Platinum based electrodes as cathode in algae assisted microbial fuel cell (To be submitted).
2) **Khandelwal, A.,** & Chhabra, M. Integration of third generation biofuels with microbial fuel cell: A biorefinery approach (To be submitted).

NCBI Sequence Submission

1) **Khandelwal, A.**, & Chhabra, M., (2019). ''Microbial diversity growing in a microbial fuel cell fed by lipid extracted residual algal biomass''. **BioProject Accesion**: PRJNA579083; **BioSample:** SAMN13091731; **SRA Submission**: SRR10341464.

Peer reviewed International Journals <u>not part of the Thesis</u> (Published)

1) Chaubey, B., Narawal, P., **Khandelwal, A.** & Pal, S. (2021). ''Aqueous photo-degradation of Flupyradifurone (FPD) in presence of a natural Humic Acid (HA): A quantitative solution state NMR analysis''. *Photochemistry and Photobiology A: Chemistry*, *405, pp.112986*.

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