Materials & Methods

Chapter-3

CHAPTER 3

MATERIALS & METHODS

3.1 Dye degrading bacteria isolation, and their study for dye decolorizing capacity

3.1.1 Sample selection for isolation of bacteria

The wastewater from the primary clarifier of wastewater treatment plant Bhagwanpur, Varanasi, India, was procured and used to isolate bacterial species. Initially, the collected wastewater was supplemented with glucose as an external carbon source to enhance the bacterial community present in it. Parameters of wastewater are given in **Table 3.1**

Table 3.1 Wastewater characteristics used for MFC

S. No.	Parameters	Concentration	Units
1	pH	6.65	-
2	COD	384	mg/L
3	BOD	115	mg/L
4	TDS	2.53	g/L
5	TSS	0.15	g/L
6	CFU/ml	9.25×10^5	CFU/ml

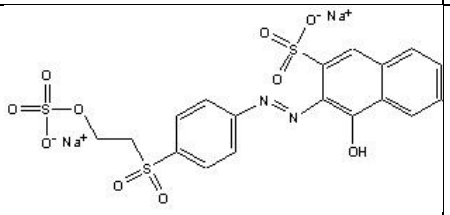
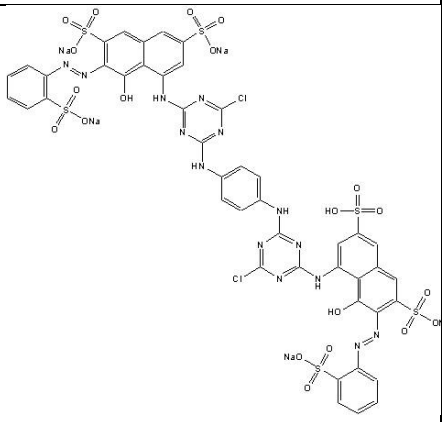
3.1.2 Dye selection

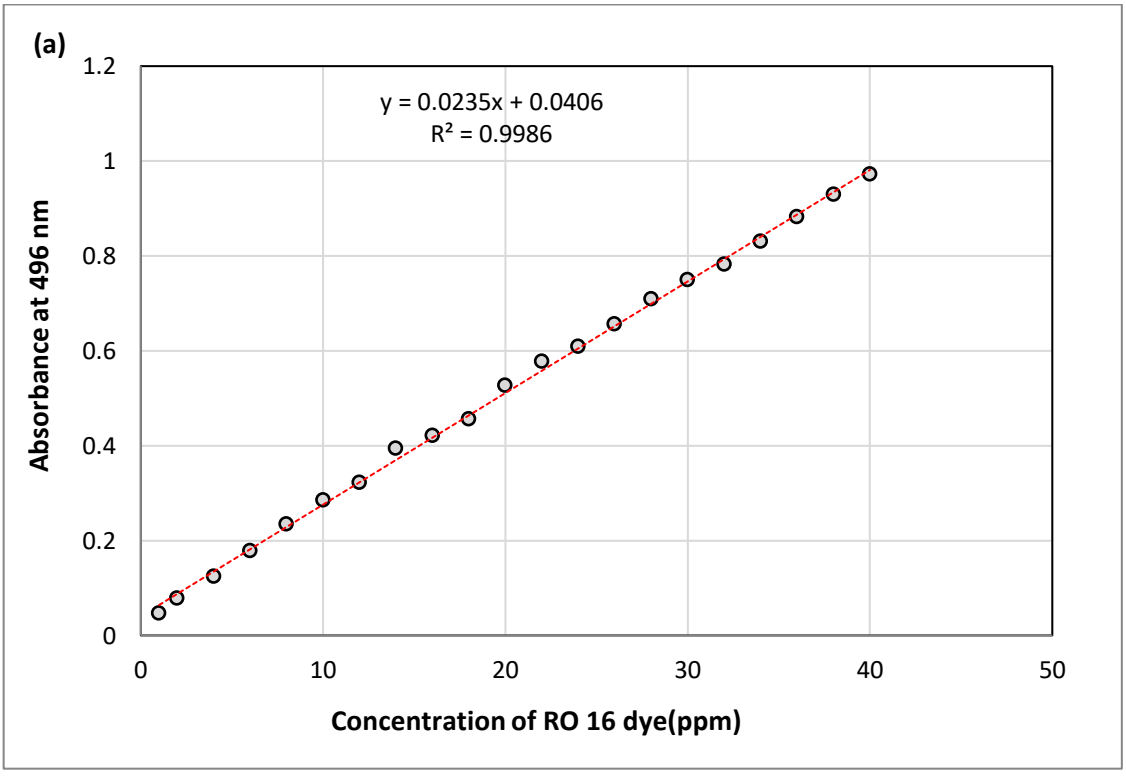
Based on the literature review, two Azo dyes are selected for the present study. These two dyes are reactive and mostly used due to their brilliant sheds. These dyes are harmful to human health as well as the environment. Some reports also classified this dye as carcinogens (Muniyasamy et al., 2020; Padmanaban et al., 2016). Very little work has been reported on Azo dyes bioremediation in MFC till date. The two model dyes, **Reactive Orange 16 (RO 16)** and **Reactive Red 120 (RR120)** dye of high purity (>98%) were procured from Sigma Aldrich. Chemical properties and other information about RO

16 and RR 120 were given in **Table 3.2** (Bharti et al., 2017; Svobodová et al., 2007). 1000 ml stock solution (1000 ppm) of dye was prepared in distilled water and further, it was diluted according to the requirement. For measuring the concentration of dye in solution UV-Visible spectrophotometer was used (SYSTRONICS 119, INDIA). The wavelength corresponding to which dye solution provides maximum absorbance was obtained by operating the UV- Visible spectrophotometer in scanning mode. The calibration curves for each dye were obtained and shown in **Figure 3.1.**

Table 3.2 Physicochemical Characteristics of Dyes

Name	**Reactive Orange 16**	**Reactive Red 120**
Chemical formula	$C_{20}H_{17}N_3O_{11}S_{3.2}Na$	$C_{44}H_{24}Cl_2N_{14}O_{20}S_6Na_6$
Synonyms	6-(Acetylamino)-4-hydroxy-3-[[4-[[2-(sulfooxy)ethyl]sulfonyl]phenyl]azo]-2-naphthalene sulfonic acid disodium salt	sodium 5-((4-chloro-6-((4-((4-chloro-6-((8-hydroxy-3,6-disulfonato-7-((E)-(2-sulfonatophenyl)diazenyl)naphthalen-1-yl)amino)-1,3,5-triazin-2-yl)amino)phenyl)amino)-1,3,5-triazin-2-yl)amino)-4-hydroxy-3-((E)-(2-sulfonatophenyl)diazenyl)naphthalene-2,7-disulfonate
Molecular weight	617.54 g/mol	1469.95

CAS Number:	12225-83-1	61951-82-4
Maximum Wavelength	496 nm	511 nm
Structure		



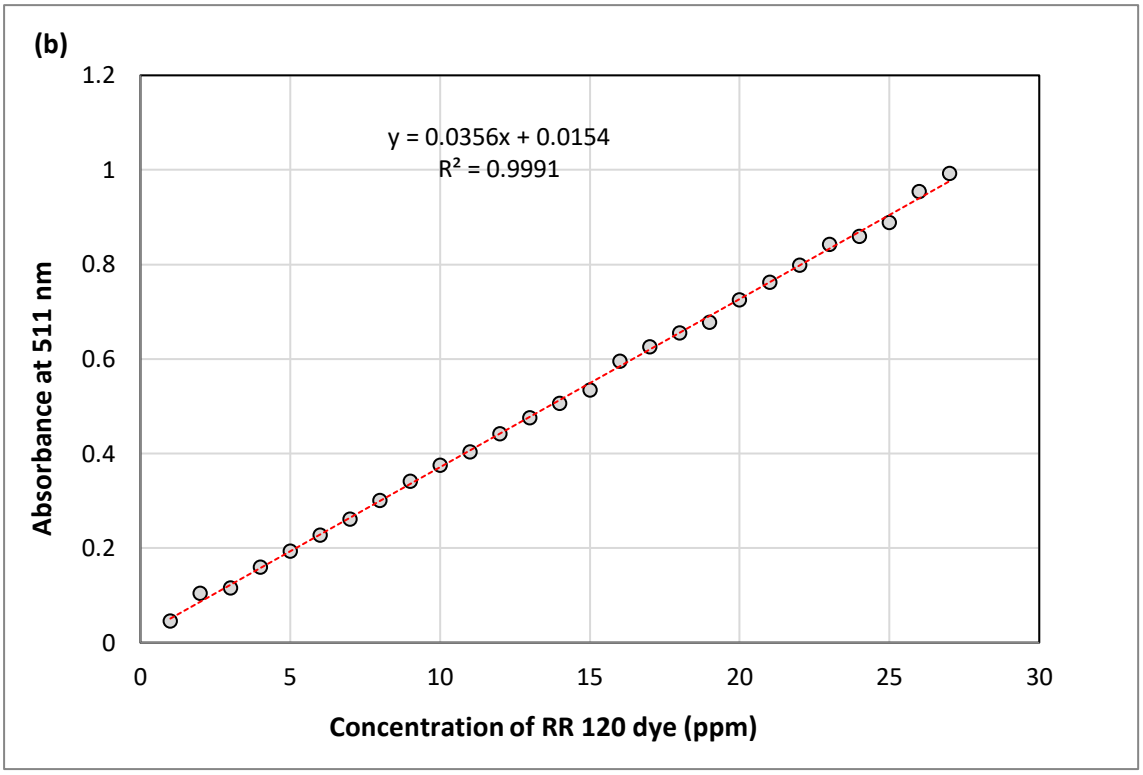


Figure 3.1Calibration plot for model dyes (a) Reactive orange 16 (b) Reactive red 120

3.1.3 Media used for the growth of Culture

Nutrient broth and Agar-Agar of analytical grade were purchased from Hi-Media Chemicals and used to isolate bacterial species for the decolonization of RO 16 and RR120. The *Geobacter sp.* medium was procured from Hi-Media and used for growth and maintenance of culture as given in **Table 3.3** (Kang et al., 2017; Lin et al., 2019).

Table 3.3 Medium specific to *Geobacter sp.* (For1 L).

Ferrous Sulphate	$FeSO_4$	0.1 g
Sodium Citrate	$Na_3C_6H_5O_7$	1 g
Sodium Hydrogen Carbonate	$NaHCO_3$	2.5 g
Ammonium Chloride	NH_4Cl	0.25 g

Di-Sodium Hydrogen Phosphate	Na_2HPO_4	9 g
Potassium Di-Hydrogen Phosphate	KH_2PO_4	1.5 g
Sodium Di-Hydrogen Phosphate	NaH_2PO_4	0.6 g
Potassium Chloride	KCl	0.1 g
Ammonium Acetate	$C_3H_2O_2NH_4$	6.8 g
Mineral Solution		10 ml
Mineral Solution composition (1 L)		
Magnesium Sulphate	$MgSO_4$	3 g
Manganese Sulphate	$MnSO_4$	0.5 g
Sodium Chloride	NaCl	1g
Ferrous Sulphate	$FeSO_4$	0.1 g
Cobalt Chloride	$CoCl_2$	0.1 g
Nitrilotriacetic acid	$N(CH_2COOH)_3$	1.5 g
Calcium Chloride	$CaCl_2$	0.1g
Zinc Sulphate	$ZnSO_4$	0.1 g
Copper Sulphate	$CuSO_4$	0.01g
Aluminium Potassium Sulphate	$AlK(SO_4)_2$	0.01 g
Boric Acid	H_3BO_3	0.01 g
Sodium Molybdate	Na_2MoO_4	0.01 g

3.1.4 Screening and identification of the isolated organism

The wastewater from the primary clarifier of wastewater treatment plant Bhagwanpur, Varanasi, India, was used to isolate bacterial species. For this, initially, MFC was operated with this wastewater along with glucose (1gm/L) as a substrate, and then glucose was replaced with a particular dye in MFC. When the voltage produced in the MFC reached maximum, then this sample is used for the isolation of potent bacteria for dye decolorization.

- **For RO 16 potent decolorizing bacterial isolation**

Serial dilutions (10^{-5}, $10^{-6,}$ and 10^{-7}) of the wastewater from MFC mixed with RO 16 (100 ppm to 500 ppm) or mixed with RR120 (100 to 300 ppm), and the nutrient solution was performed on the agar plates for isolation of bacterial species. Several specific media agar plates were prepared. Many random colonies after 10^{-5}, $10^{-6,}$ and 10^{-7} dilutions were streaked onto fresh RO 16 media agar plates using the aseptic streaking technique. The plates were incubated for 24- 48 h at 37 ºC. After incubation, the single colonies on the plates were sub-cultured onto fresh agar plates. Several subsequent subcultures were done to ensure the purity of a single strain. The bacteria showing higher zones of decolorization were isolated and used for further study.

- **Identification of model dye decolorizing microorganisms by 16S rDNA sequencing method**

  After obtaining the pure culture in the Petri plate, the cultures were transferred into an agar slant and stored at 4ºC. The morphological characteristics of all the isolated strains were recorded. These strains were sent to Bioraj Laboratories, Nagpur, India, for sequencing and identification based on 16S ribosomal RNA gene sequencing. The obtained sequences were subjected to NCBI-BLAST for sequence alignment with reported sequences. Similar sequences were subjected to multiple sequence alignment using Clustal W followed by phylogenetic analysis.

3.1.5 Study the effect of different parameters on dye decolorizing capacity

The effect of time, pH, and concentration of dye on the removal of dye was studied. The optimum value of process parameters was obtained one by one by varying one parameter and keeping all other parameters constant.

- **Time**

To evaluate the effect of time, a fixed concentration of 100 ppm of model dyes solutions was prepared in *Geobactor* media and from the prepared dye solution, 30 ml was taken into culture bottles. For maintaining anoxic condition, the bottles were sealed with a screw. Previously isolated bacterial strains (1% v/v) were inoculated into the bottles and then kept in the incubator at 37°C along with a control (without inoculation) to check the abiotic decolorization of the dye. At every 6 h, samples were taken aseptically and centrifuged at 5000 rpm for 10 minutes and the supernatant is used to measure % decolorization according to equations 3.1 and 3.2 using UV-Visible Spectrophotometer (SYSTRONICS 119, INDIA).

$$\% \text{ Decolorization} = 100 \times (1 - \text{concentration of dye}_{final} / \text{concentration of dye}_{initial}) \quad \textbf{(3.1)}$$

$$\text{Dye degraded} = (\text{concentration of dye}_{initial} - \text{concentration of dye}_{final}) \quad \textbf{(3.2)}$$

- **pH**

From the literature review, it was found that most Azo dye decolorizing bacteria, degrade the dye most efficiently around neutral pH. To optimize the pH, the % decolorization study was conducted at 5, 7, and 9 pH taking 100 ppm of a dye prepared in Geobactor media. And then the rest procedure was the same as discussed for optimization of time.

- **Concentration**

To evaluate the effect of the concentration of dyes on % decolorization, the experiments were conducted at optimum pH and time obtained for maximum decolorization at different concentrations of dyes. For RO 16 concentrations ranging from 100 to 500 ppm and for RR 120 ranging from 100 to 300 ppm were taken. Rest procedures were the same as given in pH and time optimization sections.

3.2 MFCs fabrication, calibration, and performance evaluation

Two different laboratory-scale MFCs were fabricated for the present study. Nafion and Agar salt bridge was used as proton exchange membranes whereas Carbon cloth and Aluminum mesh were used as electrodes (current collector) in the present study. Initially, to validate the fact of electricity production in MFC, glucose was taken as a substrate because glucose can be easily assimilated by bacteria. The performance of MFC-1 was evaluated with glucose as a substrate. In MFC-1 agar salt bridge and Nafion were used as PEM whereas aluminum mesh was as an electrode. Details of the MFC-1 were given in section 3.2.1. MFC-1 operated successfully with glucose as substrate and power generation from MFC-1 was confirmed. More electrical output was obtained when Nafion was used as PEM, therefore, based on the results Nafion was selected for dyes degradation studies in MFC-1. From the literature, it was found that carbon clothes have better properties than aluminum for biofilm growth and overall electricity production. Based on observations in MFC-1, another modified setup (MFC-2) was fabricated with Nafion as PEM and carbon cloth as an electrode. Various drawbacks of MFC-1 such as very high internal resistance, no provision for the addition of nutrients and substrate during the operation of MFC were removed in MFC-2. Details of MCF setups used in the present study are given in 3.2.2.

3.2.1 MFC 1

MFC was constructed with two cylindrical plastic compartments of equal dimensions with a working volume of 2 L. The two compartments were connected by a separator (2.54 cm diameter, 10 cm length) in which agar salt and Nafion were used as proton exchange membranes. Agar salt bridge was prepared with 3% agar and 1 M KCL and Nafion were procured fromVinpro Tech, Hyderabad, India. Aluminum mesh (5×5 cm^2) was used as an electrode. The compartment that functioned as the anode chamber was sealed with a parafilm to maintain anaerobic conditions. The other compartment, which

was used as a cathode chamber, was filled with 2 L of distilled water. A small aquarium air pump was used to aerate the cathode chamber to maintain aerobic conditions (**Figure 3.2)**. Before starting the experiments, Nitrogen gas was sparged into the anodic chamber to create an anaerobic condition. A variable resistance box (40 Ω to 1000 Ω) was connected in the circuit of MFCs to measure potential drop. The current generated was measured using a digital multi-meter (MASTECH ms8340B) which is connected to the computer for data recording.

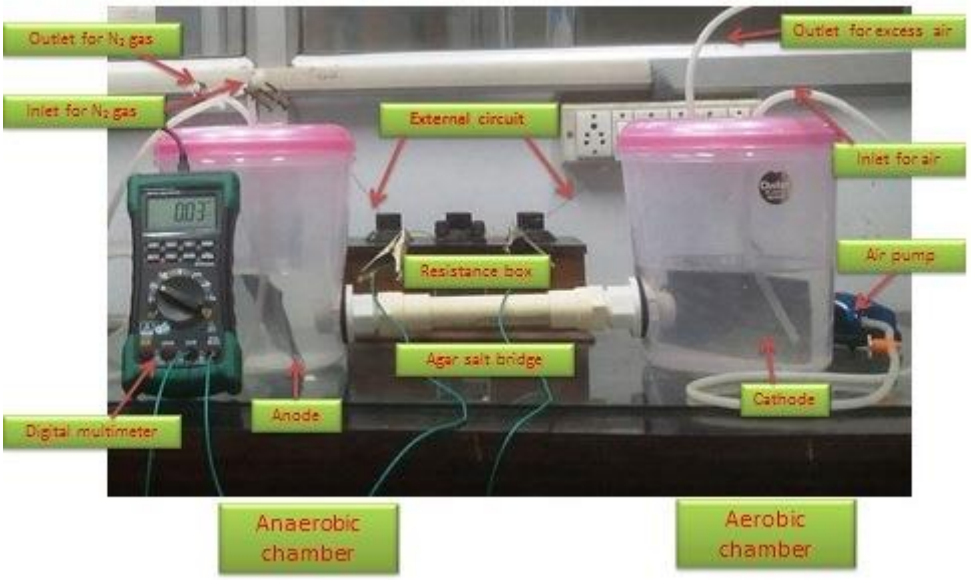


Figure 3.2 MFC1

3.2.2 MFC- 2

MFC-2 was fabricated based on experience obtained during the operation of MFC-1. Various drawbacks of MFC-1 were removed in MFC-2. MFC-2 consists of a dual-chambered design with a rectangular side (11×13 cm^2) made up of Perspex. A rectangular joint was provided for placing the proton exchange membrane at the center of MFC which divides the cell into two equal parts of 450 ml working volume. Nafion 117 was used as a proton exchange membrane and carbon cloths were used as electrodes. The electrode is

suspended from the top of each chambered in such a way that they are fully submerged in the anolyte. At the anode, dye solution at different concentrations along with nutrients and media is used as an anolyte. For closed circuit operation and monitoring voltage across MFC, multimeters along with a variable resistance box of 10 Ω to10 KΩ were connected in series. The cell is connected with two digital multi-meters (MASTECH ms8340B, USA and MECO 81-USB, INDIA) for recording voltage and current respectively, and data were transferred directly to the computer. The anolyte consists of 400 ml Geobactor media along with dye at different conditions according to the experiment. Catholyte consists of distilled water. Aeration is provided at the cathode side with help of an aquarium pump. CO_2 gas produced during the biodegradation of dye was collected in an airtight flask for measurement.

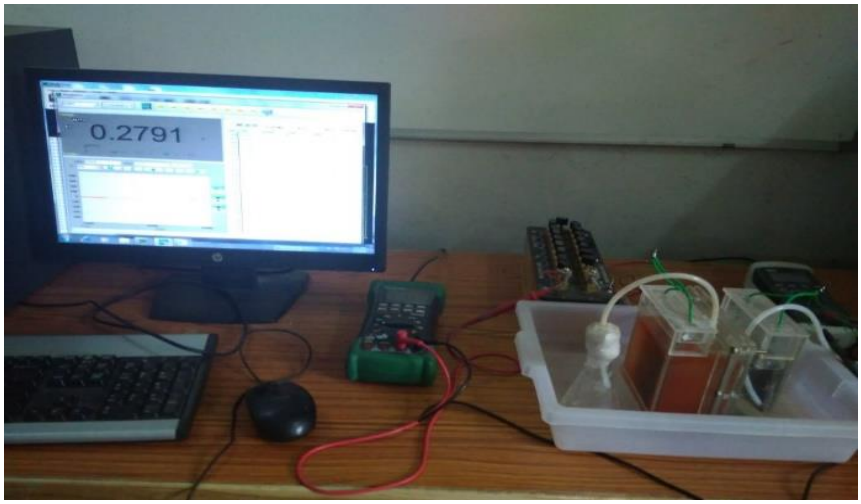


Figure 3.3 MFC2

Nafion regeneration: Nafion of area 14.5 cm^2 was procured and regenerated following the procedure provided by Li et al., 2017 (Li et al., 2017). The membrane was kept in distilled water for 30 min then dipped in 3% hydrogen peroxide for 1 hour. Now the membrane was removed and dipped in 1M sulfuric acid for 1 hour again and finally washed with distilled water and placed in MFC setup.

3.3 Analytical Procedure

3.3.1 Assessment of dye decolorization in the batch study and MFC

0.10 ml of the sample was collected from degraded dye solution obtained from the microbial fuel cells and batch culture bottles are used for plating for CFU determination, and then the remaining portion was centrifuged at 5000 rpm for 10 minutes using a centrifuge (REMI 8C PLUS, INDIA). The supernatant was collected in a 20 ml vial, and in the left pellet, fresh media were added to measure bacterial optical density at 600 nm. The supernatant was used further to test for change in COD and % decolorization.

- **Dye Degradation**

The change in absorbance was monitored at 511 nm and 495 nm using a UV-Visible spectrophotometer (SYSTRONICS 119, INDIA). The percentage of degradation (%decolorization / %removal efficiency) and the concentration of dye degraded were calculated using **Equations 3.3** and **3.4**.

$$\% \text{ Degradation} = [100\% \times (1-(\text{Final Concentration}/\text{Initial Concentration}))] \quad (3.3)$$

$$\text{Concentration of dye degraded} = (\text{Initial concentration} - \text{Final concentration}) \quad (3.4)$$

- **Bacterial densities**

Bacterial densities were also measured at 600 nm of each sample per day of the collected pellet after centrifuging and resuspending it in fresh media.

3.3.2 COD removal efficiency

The chemical oxygen demand (COD) removal efficiency was determined using a COD analyzer (UNIPHOS, INDIA) by measuring the initial and final COD of the samples from the MFC chamber and was calculated using **Equation 3.5**.

% COD removal efficiency = [100% × (1-(Final COD/Initial COD))] (3.5)

3.3.3 Dry biomass estimation

A fixed Sample volume was taken from MFC and was subjected to centrifugation as mentioned in section 3.3.1. Bacterial cell obtained after centrifugation was dried on filter paper (0.2 μ) and the weight of the filter paper was measured before and after filtering the bacterial cells. The difference in the weight of the filter paper is measured as dry biomass.

3.3.4 CO_2 measurement

CO_2 gas produced during the biodegradation of dye was collected in an airtight flask for measurement. CO_2 was determined using the Gas detector (UNIPHOS ASP 40, INDIA).

3.3.5 CFU estimations

The wastewater which was taken out from MFC was used to calculate the colony-forming unit (CFU). Two types of media were used- Nutrient Agar and *Geobacter* media containing agar. Nutrient Agar was used to determine the total bacterial load in the wastewater when mixed culture is used whereas Geobacter media was used to determine the fraction of *Geobacter*-related species in the sample when isolated strain is used in MFC. Serial dilutions of 10^{-1} to 10^{-8} were prepared and 0.1 ml from different dilutions was pipetted out and spread on both sets of the agar plate. It was spread on the plate with the help of a glass spreader using the aseptic spread plate technique. These plates were then incubated at 37°C for 24 h to 48 h. After incubation, colonies were counted and CFU was recorded.

3.3.6 Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR (Thermoscientific Nicolet, USA) analysis of dye solution before and after degradation was done to know the presence of metabolites after degradation. The identification of metabolites will help to develop the metabolic pathway of dye degradation. The analysis was carried out using the KBr pellet method in which samples are mixed with KBr (in a ratio of 1:10). % Transmittance was recorded in the range of wavenumber 400-4000 cm^{-1} (Unnikrishnan et al., 2018).

3.3.7 Identification of metabolites using Liquid chromatography and mass spectroscopy analysis (LCMS)

LCMS was used for the identification of metabolites using the liquid chromatography-electrospray ionization (LC-ESI) technique. The extracted metabolites from MFC were sent to a Sophisticated Analytical Instrumentation Facility (SAIF) laboratory, Central Drug Research Institute (CDRI), Lucknow, India. LCMS (Waters UPLC-TQD Mass spectrometer, Japan) was used to carry out the analysis in scan mass range of (m/z) 100-2000 negative mode-ESI-MS using the C-18 column for water as solvent (Ramya et al., 2010).

3.3.8 SEM analysis

Scanning electron microscopy (FEISEM QUANTA 200, USA) was done to analyze the morphology of isolated bacteria. The sample collected from the microbial fuel cell was passed through a 0.2-micron cellulose filter, dried, and sputter-coated with gold under vacuum for SEM examination (Feng et al., 2017).

3.3.9 Electrochemical monitoring

The voltage across the cell is measured using a digital multimeter (MECO 81-USB, INDIA) across 10 – 10 KΩ external resistance. Data were continuously transferred from

the multimeter to the computer. Current density and power density were calculated using **Equations 3.6 and 3.7.**

Current density (A/m^3) = (voltage /resistance) per unit volume of the MFC (3.6)

Power density (W/m^3) = current density × voltage. (3.7)

Coulombic efficiency (CE) was calculated based on a change in COD at 1000Ω resistance with equation

$$\mathbf{CE= (} Mo_2 \int_0^t I\,dt \mathbf{) / (4FV \Delta COD)} \quad (3.8)$$

where Mo_2 is the molar mass of oxygen (gm/mol), V is the volume of anode chamber (lt), F is the Faraday constant (96485 C/mole), I is the current (A), t is the change in time (sec),Δ CODis changed in COD over time t (gm/L)(Logan et al., 2006).

3.4 Kinetic Study

Monod and Haldane's models were used to study the kinetics of biodegradation in the present study. The Monod model **(Equation 3.9)** is widely used by researchers to represent bacterial growth kinetics under non-inhibitory conditions.

$$\mu = \frac{\mu_{max} S}{K_s + S} \quad (3.9)$$

$$\frac{1}{\mu} = \frac{1}{\mu_{max}} + \frac{Ks}{\mu_{max}} . \frac{1}{S} \quad (3.10)$$

Where μ is specific growth rate (1/day), μ_{max} is the maximum specific growth rate (1/day), Ks is half-saturation constant (mg/L).

Under the high loading condition, the Monod model does not fit with the experimental data and the results suggested the possibility of substrate inhibition. Therefore, the

Haldane model was tested to confirm the possibility of substrate inhibition. Haldane's inhibitory growth kinetics model is given by **Equation 3.11.**

$$\mu = \frac{\mu_{\max} S}{K_s + S + \frac{S^2}{K_i}} \tag{3.11}$$

All other parameter is same as Monod equation except K_i which is the substrate inhibition constant (mg/L).

At higher substrate concentrations, Ks<<S, the above equation reduces **Equation 3.12.**

$$\mu = \frac{\mu_{\max} S}{S + \frac{S^2}{K_i}} \tag{3.12}$$

Taking double reciprocal **Equation 3.12,** we have simplified **Equation 3.13.**

$$\frac{1}{\mu} = \frac{1}{\mu_{\max}} + \frac{S}{\mu_{\max}} . \frac{1}{K_i} \tag{3.13}$$

3.5 Response Surface Methodology (RSM) modeling for the analysis and optimization of dye decolorization and current density

Central composite design-based RSM was applied for optimization of the process parameters to achieve better degradation and current density in the MFC chamber. Design Expert (DX) 12 software (Stat-Ease Inc., Min- Neapolis, MN, USA) was used for this purpose. In this study, a face-centered central composite design with alpha value (1) was chosen to analyze the optimal level of three variables and their interaction for maximizing degradation of dye with factors A, B, C as pH, initial concentration of dye, and time respectively. The number of experimental runs was 20 including 6 center points and 14 (axial and star) points. The summary of the design

of experiments is shown in **Tables 3.4** and **3.5.** Based on the experimental data, the model was further subjected to analysis of variance (ANOVA) and multiple regression with the help of DX 12.0 software.

Table 3.4:Experimental Design for the degradation of RR120

Factor	**Name**	**Units**	**Mini**	**Max**	**Mean**	**Std. Dev.**
A	pH		5.00	9.00	7.00	1.45
B	Conc. of RR120 dye	ppm	100	300	200	72.55
C	Time	h	24	72	48	17.41
Response 1	Concentration of dye degraded	ppm	0.13	197.7	98.12	139.70
Response 2	Current density	A/m^3	0.018	1.07	0.54	0.74

Table 3.5: Experimental Design for the degradation of RO 16

Factor	**Name**	**Units**	**Mini**	**Max**	**Mean**	**Std. Dev.**
A	pH		5	9	7	1.414
B	Conc. Of dye	ppm	100	500	300	141.42
C	Time	h	24	72	48	16.97
Response Y1	Dye Degraded	ppm	13.37	223.08	118.59	69.74
Response Y2	Current Density	A/m^3	0.08	1.04	0.63	0.32

3.6 Phytotoxicity Studies

The purpose of this study is to evaluate the reduction in toxicity of dye solution after its degradation in the microbial fuel cell. By this test, we can know the possibility of using treated dye effluent for different purposes such that irrigation, domestic use, and direct discharge into the water bodies. The initial and treated dye solutions were used to germinate the seeds of wheat (*Sorghum vulgare)* and mung (*Phaseolus mungo)* at ambient

conditions. Both seeds were irrigated with 20 ml of pure RO 16 dye (500 ppm), treated dye solution, and distilled water as a control. Percentage germination for seeds irrigated with RO 16, treated dye, and distilled water was recorded after seven days (Shobana and hangam, 2012).