4
Performance Evaluation of Photosynthetic Microbial Fuel Cells using *Chlamydomonas reinhardtii* at Cathode

The most common microalgae used in PMFCs are *Chlorella*, *Scenedesmus*, *Desmodesmus*, *Microcystis, Spirulina,* etc. [Reddy *et al*., 2019; Apollon *et al*., 2021]. One of the promising and less-explored microalgae at the cathode is *C. reinhardtii*. The biomass of *C. reinhardtii* consists of lipids (16.6-25% DCW), carbohydrates (17% DCW), and protein (48% DCW) [Reddy *et al*., 2019]. *C. reinhardtii* is also reported for its high antioxidant and antibacterial properties compared to other commonly used microalgae species [Jayshree *et al*., 2016]. Previous studies report the use of *S. obliquus*, *Desmodesmus* sp. A8 (99 mW/m^2), *C. vulgaris* (35.09 mW/m^2) at the cathode chamber when acetate served as an electron donor at the anode [Wu *et al*., 2014; Yadav *et al*., 2020]. Microalgae species at the cathode impact the power output from the system, as indicated by these studies. An ideal species should quickly adapt to an electrochemical system, exhibit efficient photosynthesis and faster growth rates, and produce industrially compatible VAPs. Literature studies report using *C. reinhardtii* as anodic species along with iron-reducing *Geobacter* sp. The study indicates it a valuable source of electron donors [Nishio *et al*., 2013]. Similarly, several studies show the usefulness of this species as an electron donor at the anode [Rosenbaum *et al*., 2005; Lan *et al*., 2013; Mao and Verwoerd, 2014; Huang *et al*., 2015]. However, the application of *C. reinhardtii* at PMFC cathode was never tested.

In this study, the performance of PMFCs with *C. reinhardtii* at the MFC cathode chamber was evaluated. To support the growth of anodic bacteria in PMFC, acetate served as an electron donor in the anode chamber. The study assesses the power output, biomass production, and energy output in a lab-scale setup. The study also includes an assessment of *C. reinhardtii* biomass grown at PMFC cathode for the therapeutic properties, including the presence of pigments, total phenol, antioxidant, and antibacterial activity.

4.1 EXPERIMENTAL

The schematic of acrylic MFC reactors used in this study is shown in Figure 4.1. The Nafion membrane was used as a proton exchange membrane between the anode and cathode chambers. Before starting the experiments, graphite felt electrodes and Nafion-117 were pre-treated, as described in section 3.3.1 [Khandelwal *et al*., 2018]. Further, 10 ml of the pre-treated cow-manure culture was inoculated into the anode chamber as stated in section 3.3.2. Oxygen-free nitrogen gas was purged into an anode chamber to make it anaerobic. The anolyte consisted of sodium acetate, KH_2PO_4, K_2HPO_4, NaCl, $MgSO_4$, $CaCl_2$, and KNO_3, and trace metal solution at a concentration 2 g/l, 4.4 g/l, 3.4 g/l, 0.5 g/l, 0. 2 g/l, 0.014 g/l, 1 g/l and 1 ml/l, respectively [Vijay *et al*., 2019]. The working volume of each chamber was 100 ml while the total volume was 120 ml for both anode and cathode chambers.

The cathode chamber was inoculated with *C. reinhardtii* CC-503 from an exponential stage culture having an initial concentration of 18.00 ± 2.49×10^4 cells/ml. A fixed resistor of 1000 Ω was used to complete the circuit and enable the current flow. The experiments were conducted in a fed-batch mode. One batch cycle sustained power production for 7-8 days. The medium was replenished following a voltage drop. All the experiments were done in triplicates, with results reported as an average of three values [Khandelwal *et al*., 2018]. The open and closed-circuit

voltage along with the electrochemical performance were assessed. CV curves were obtained in a scanning window of -0.7 V to 0.7 V (vs. Ag/AgCl as reference electrode) at a scanning speed of 1 mV/s.

Microalgae growth was measured using a Hemocytometer, and the specific growth rate (μ) was calculated. Microalgae biomass was characterized for pigment concentration [Lichtenthaler and Buschmann, 2001]. For the assessment of biochemical properties, the ABME was prepared and used in subsequent assays as stated in Section 3.2.7 [Jayshree *et al.*, 2016]. The ABME was used to assess the total phenol content, total antioxidant activity, and antimicrobial activity.

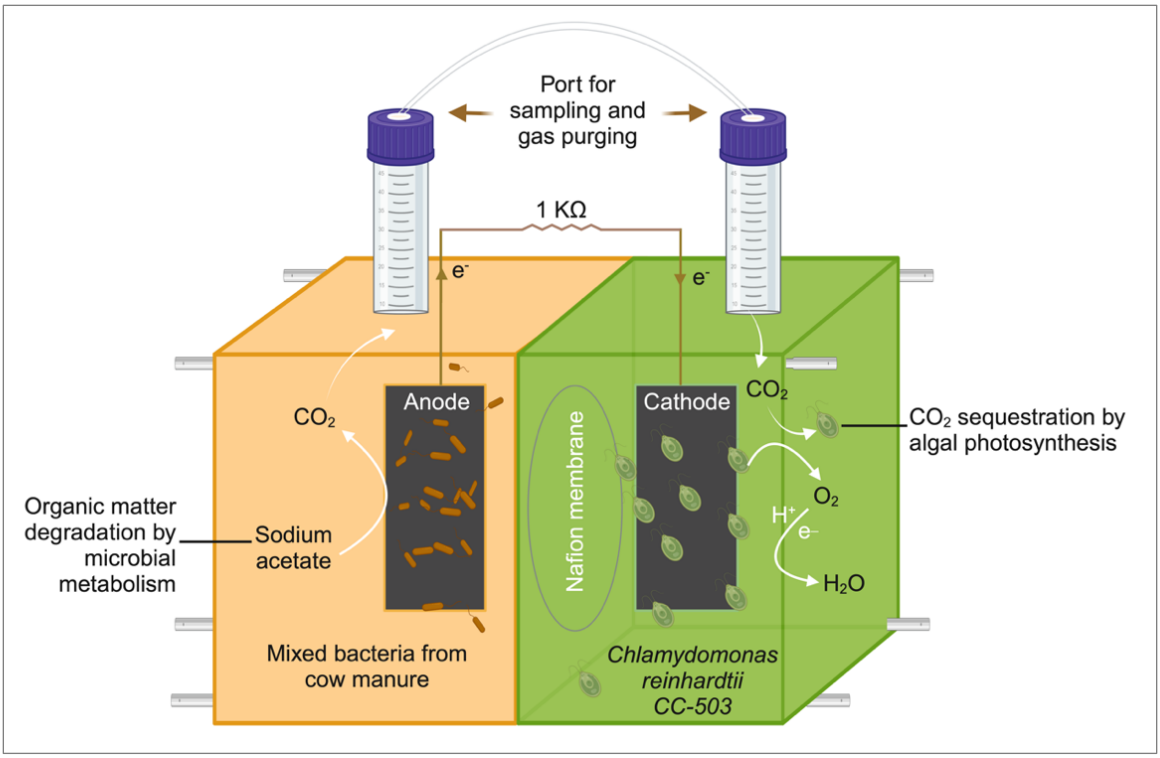

Figure 4.1 : The schematic shows the reactor design of PMFC used in this study

4.2 RESULTS AND DISCUSSION

4.2.1 Voltage Generation

The MFC reached the OCV of 134 ± 14 mV after one day. The start-up time was low due to the use of acclimatized microbial consortia and readily utilizable electron donor acetate [Liu *et al.*, 2005]. The current study noted an OCV of 0.997 V while *C. vulgaris* produced 0.53 V OCV when grown in similar reactor system. The experimental condition differed in terms of substrate provided as acetate is supplied in the present study [Khandelwal *et al.*, 2018]. Also, the PMFC performance is dependent on various factors, including reactor volume, photoperiod and light intensity, which should be accounted while drawing comparisons from literature studies. Figure 4.2 shows the MFC Closed-Circuit Voltage (CCV) with a time profile. The MFC reached an operating voltage of 563 mV on the 36th day. The results have confirmed the optimal performance of *C. reinhardtii*-based PMFCs [Kakarla and Min, 2014; Wu *et al.*, 2014; Yadav *et al.*, 2020]. Each batch cycle was characterized by a rise and drop in voltage with time, like a wave pattern. Also, the voltage drop indicates substrate depletion, and the addition of substrate helps regain the voltage. This trend is typical of a fed-batch MFC reported in the literature [Liu *et al.*, 2005; Kakarla and Min, 2014].

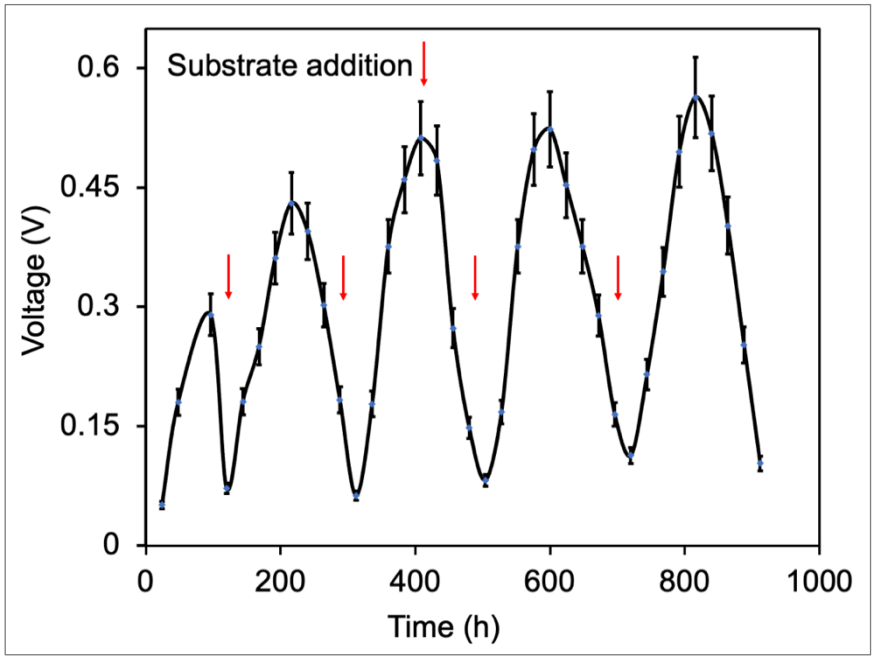

Figure 4.2 : The schematic showing the pattern of the closed-circuit voltage generation (external resistor 1000 Ω) with time. Arrows indicate the addition of sodium acetate

4.2.2 Power Output

Polarization curves help measure an electrochemical system's highest power output, optimum load, internal resistance, and highest current density. The volumetric power density realized by this acetate-fed MFC was 15.21 W/m^3 (371.34 mW/m^2), as shown in Figure 4.3. The current density stood at 39 A/m^3 at the external resistance of 100 Ω. The volumetric power density was 3.36 W/m^3 at 1000 Ω during the 5th cycle of operation. The slope of the linear region of the curve indicated internal resistance at 86 Ω. This value was considerably less than reported in similar systems [Kakarla and Min, 2014], allowing the system to capture more energy from the circuit. Lower internal resistance indicates a better MFC design that enables efficient current flow and electrode reactions.

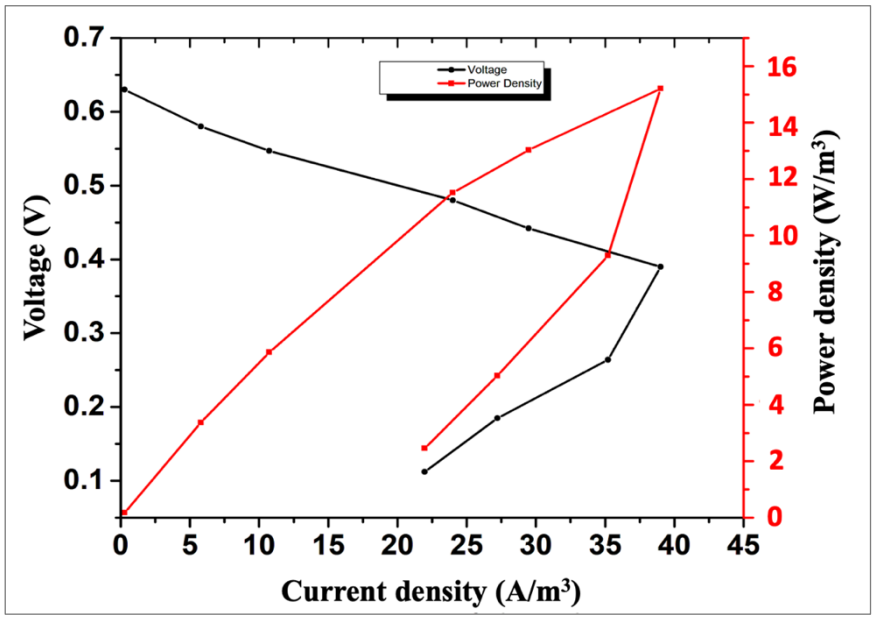

Figure 4.3 : Polarization curve for acetate-fed MFC

The H-type MFC design generally has high internal resistance and leads to low power output [Liu *et al*., 2015]. Low-power output results in such systems even when the electron acceptor has a high reduction potential, for example, ferricyanide [Ali *et al*., 2020]. Therefore, MFC design is crucial for high-efficiency energy capture, and the scope for improvement always exists. With reference to the power outputs obtained in prior arts using acetate-fed dual-chamber MFC which is up to 38 mW/m^2 [Min *et al*., 2005a], 45 mW/m^2 [Min *et al*., 2005b], 40 mW/m^2 [Kim *et al*., 2005], 43 mW/m^2 [Oh *et al*., 2004]. The power output from this study was high, indicating effective cathodic culture, MFC design, and other operating parameters. While chemical electron acceptors offer high operating voltages and power outputs [Ndayisenga *et al*., 2018], the system remains unsustainable from an environmental point of view. Microalgae-based aeration is a green technology and has several advantages over conventional systems. The slight dip in the energy output from the microalgae-based system is compensated well by the generation of microalgae biomass that serves as an energy source [Khandelwal *et al*., 2018, 2020].

The choice of microalgae species is also a critical factor that impacts MFC performance. Regarding testing different microalgae species in PMFCs, the literature is limited [Apollon *et al*., 2021]. To the best of our knowledge, this is the first report that uses *C. reinhardtii* at the cathode chamber. One of the commonly used microalgae species at PMFC cathode is *C. vulgaris*. In terms of power output, systems based on *C. vulgaris* fare well when employed in various studies. For example, Cui *et al*. supplied algae as a substrate at anode and also as biocathode which resulted in a power output of 4.98 W/m^3 [Cui *et al*., 2014]. Similarly, the power density in a clay based MFC with *C. vulgaris* as a cathodic specie and acetate as an electron donor was 35.09 mW/m^2 [Yadav *et al*., 2020]. Therefore, considering the literature studies, *C. reinhardtii*-based PMFCs are promising in terms of power production. In addition, *Chlamydomonas* is amenable to genetic manipulation. Advanced cloning and molecular biology tools are available for the species [Song *et al*., 2020]. Power output reduces with an increase in the volume or scale of the MFC system. Micro-sized reactors realize high power output owing to increased mass transfer and diffusion rates [Mink *et al*., 2014]. However, applications such as energy generation and water treatment require large-volume reactors where efficiency is achieved by optimizing microbiological and electrochemical parameters. The power output of the system was comparable to that of air-cathode MFC having carbon cloth as the anode which is reported to be 165.03 mW/m^2 [Elmekawy *et al*., 2014]. This is an important lead as two-chambered MFCs always lag air cathode ones in terms of operation cost, power output, and water treatment efficiency [Sravan *et al*., 2021]. However, if the comparable power output is obtained along with the value-added microalgae biomass, the overall energy output from the system may be considerably higher than the air-cathode MFCs.

The area under the power v/s time curve estimates the energy produced in kWh is shown in Figure 4.4. The estimated energy output was 0.029 Wh or 0.29 kWh/m^3, which is normalized with MFC reactor working volume of 100 ml. As expected, the energy output using a simple substrate such as acetate was higher than a complex substrate, such as lipid-free microalgae biomass in lipid-extracted algae-fed MFC (0.1 kWh/m^3) [Khandelwal *et al*., 2018].

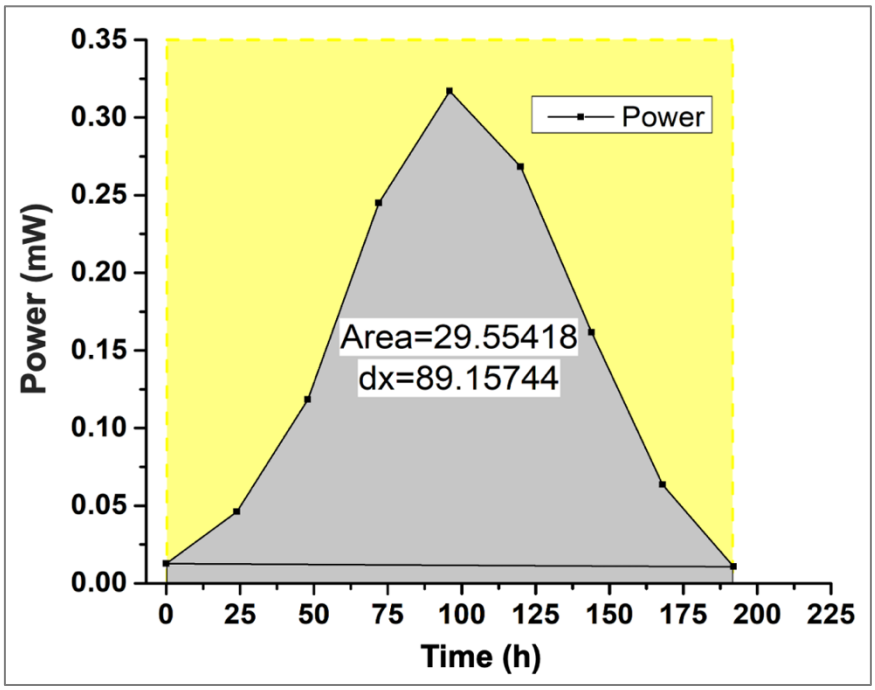

Figure 4.4 : Energy obtained in the 5th batch cycle is represented here as the area under the curve

4.2.3 Dissolved Oxygen at Cathode Chamber

The DO concentration at the cathode was monitored using a DO probe (HI 9829, Hanna multi-parameter). The DO values ranged from 8.7 mg/l in light to 3.1 mg/l in the dark. The DO level in the cathode was always above zero, sustaining continuous power production. This ensures continuous power output and high photosynthetic efficiency of *C. reinhardtii* in MFC conditions [Del Campo *et al*., 2013]. Very high DO levels are undesirable as oxygen may diffuse in the anode, causing a drop in voltage and coulombic efficiency. Similar DO profiles have been observed with *C. vulgaris*. The values ranged from 6.6 mg/l to 11.2 mg/l [Khandelwal *et al*., 2018; Yadav *et al*., 2020]. Previous studies have observed higher voltage generation during daytime than nighttime which was majorly contributed due to the active photosynthesis and continuous evolution of oxygen, an electron acceptor (Figure 4.1).

4.2.4 Electrochemical Analysis

The CV analysis helps identify the electrochemically active molecules and their location. The CVs were obtained in the 5th batch cycle when MFC displayed high operating voltage. The scanning window of the experiment was -0.7 V to 0.7 V (vs. Ag/AgCl as reference electrode) at a scanning speed of 1 mV/s, and the change in current was recorded continuously. The CV profile acquired with anode as a working electrode in Figure 4.5 showed two major redox peaks, one in reverse scan at -0.433 V and the other in forward scan at 0.362V (vs. Ag/AgCl) with oxidation current of 6.79 mA and 5.15 mA, respectively. These values likely correspond to NADH oxidation and outer membrane c-type cytochromes of *Omc*B/*Omc*Z, respectively [Christwardana and Kwon, 2017]. It has been reported in the prior arts that both the coenzyme and cytochromes are crucial for exo-electrogenesis [Okamoto *et al*., 2012]. Another oxidation peak at 0.110 V (vs. Ag/AgCl) with a 4.44 mA peak current was observed. This value relates to the phenazine mediators produced through microbial metabolism [Ali *et al*., 2020]. The findings suggest that both the mediated and direct electron transfer contributed to power generation. The results are following the previous reports using acetate as a carbon source and are similar to cathode-grown *M. aeruginosa* [Liu *et al*., 2005; Ali *et al*., 2020].

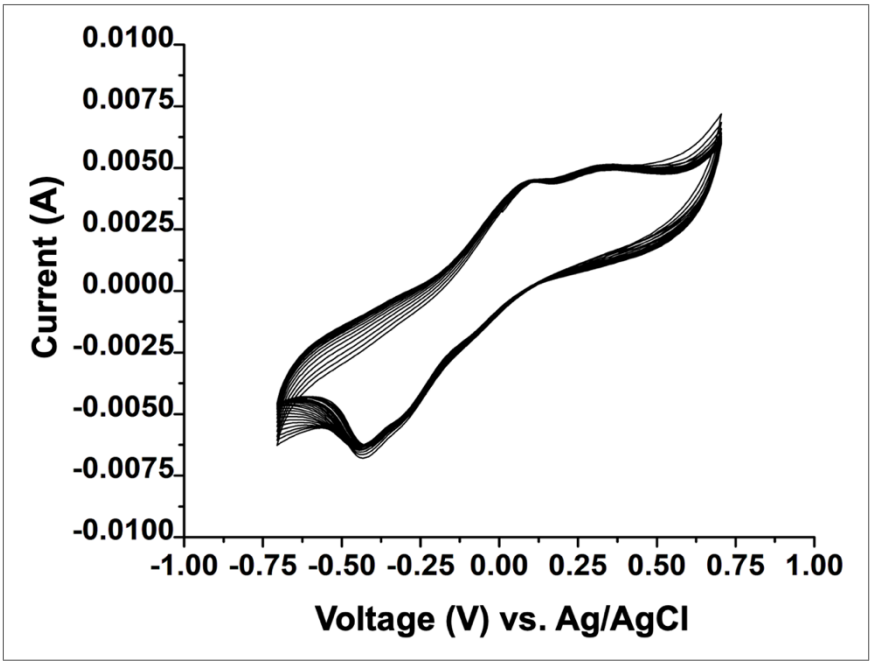

Figure 4.5 : CV curve of acetate fed MFC with biofilm in 5th batch cycle

4.2.5 COD Removal and Coulombic Efficiency

MFCs are highly efficient wastewater treatment systems. At the anode chamber, an input COD of 3108 ± 5.65 mg/l was reduced to 830 ± 3 mg/l after the 7th day of operation. This corresponds to 73.30% removal at 0.32 ± 0.0004 Kg/m^3/d. The COD removal rate and efficiencies aligned with previous reports [Cui *et al*., 2014; Ali *et al*., 2020; Yadav *et al*., 2020]. Simple substrates are assimilated faster by bacteria, resulting in high COD removal rates [Liu *et al*., 2005]. The complex substrates, on the other hand, take a long time and lead to low COD degradation rates [Khandelwal *et al*., 2018]. Previous study has identified the most common bacterial phyla in the cow manure inoculum responsible for COD removal and electrons generation, which includes, Proteobacteria, Bacteroidetes, Firmicutes, Synergistetes, Verrumicrobia, Deferribacteres, Spirochaetes, Actinobacteria, Chloroflexi, Acidobacteria, and others. However, the dominance of these bacterial groups may vary as per the growth substrate provided [Khandelwal *et al*., 2020]. Nevertheless, when discussing wastewater treatment, total COD removal and COD removal rate are critical parameters that determine the efficiency of any treatment system. As the synthetic wastewater was utilized in this study, further studies may focus on using real wastewater and provide comprehensive analysis on pollutant removal.

The CE for the present study is 9.068%, indicating significant electron losses. The CE depends on the number and kind of electron acceptors present in the system, microbial growth rate, maintenance coefficient, and formation of secondary metabolites or soluble microbial products. The change in the input COD also determines the CE. In one study, an increase in acetate concentration from 80 mg/l to 800 mg/l led to more than a 50% decline in CE [Liu *et al*., 2005]. The CE values reported in the literature vary from as low as 7% [Ali *et al*., 2020] to as high as 71.2% [Ndayisenga *et al*., 2018]. Higher CE not only helps with increased current density but also reduces the amount of sludge formed at the end of the operation as suggested previously [Kim *et al*., 2008]. The sludge needs disposal, and the lower is better [Karlikanovaite -Balıkçı and Karahan Özgün, 2019]. The pilot scale study could provide the detailed investigation on the sludge generation in these PMFC reactor systems.

4.2.6 Evaluation of Electrode Surfaces

The successful operation of an MFC depends on forming a biofilm on the electrode surface that aids in direct electron transfer. The biofilm also imparts significant physiological and ecological advantages to the mixed microbial communities prevailing in the MFC anode. The graphite felt surface was examined under a SEM to understand the biofilm morphology. These

findings were consistent with the previous studies [Liu *et al*., 2015; Khandelwal *et al*., 2018]. Dense clusters of bacterial cells are present at the anode, while microalgae cells adhere to the cathode surface, as shown in Figure 4.6.

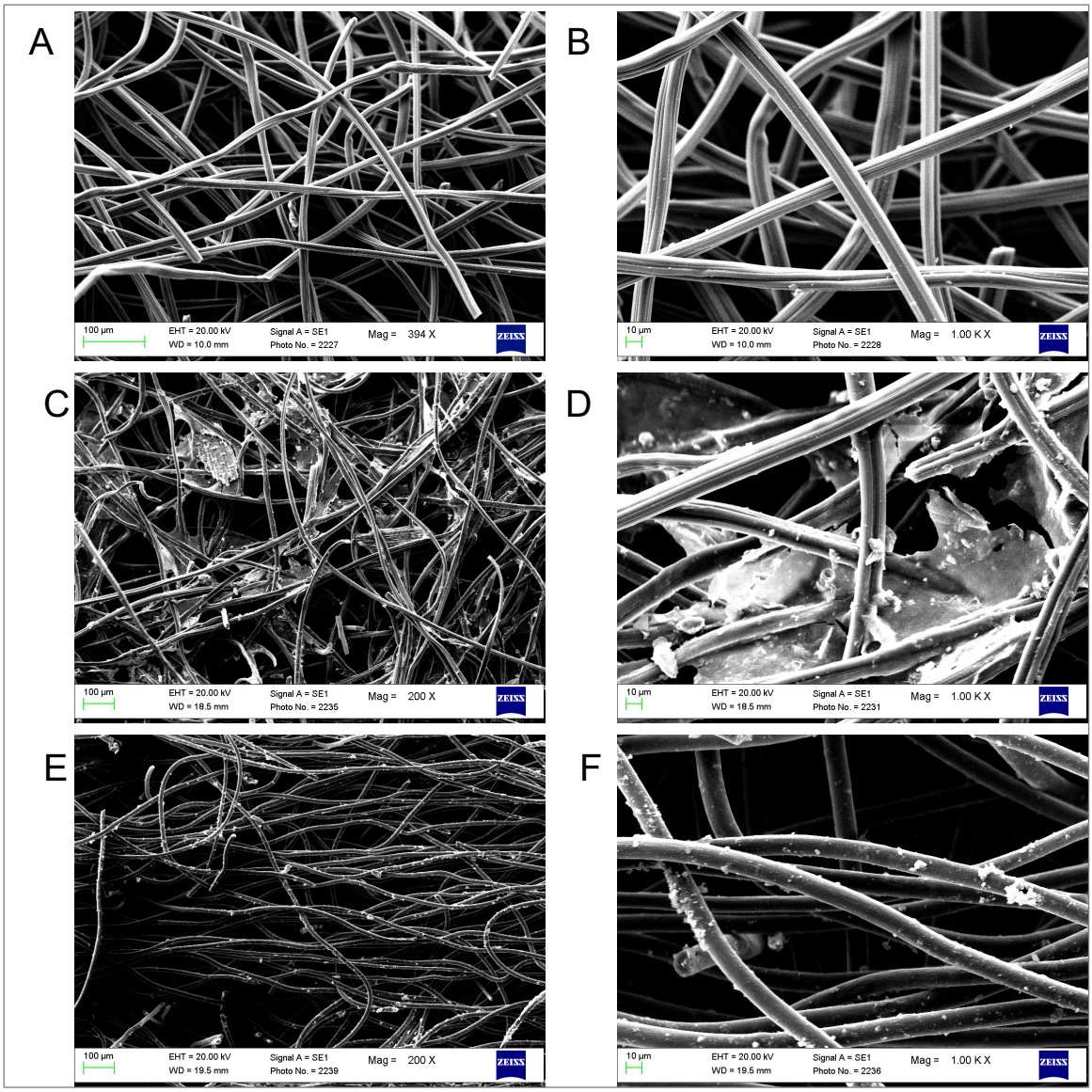

Figure 4.6 : SEM images of electrodes used in this study. A&B) treated electrode, C&D) anode electrode showing bacterial biofilm, and E&F) cathode electrode showing microalgal biofilm at different magnifications

4.2.7 pH at Anode and Cathode Chambers

Biological processes happen within a specific pH range, and therefore pH values were monitored regularly to ensure that values are not falling in the extreme ends. The anodic pH did not vary much; however, the catholyte pH increased by one unit at the end of a batch cycle. Similar observations were made earlier [Liu *et al*., 2015]. The increase can be attributed to the utilization of bicarbonate and the production of OH^- ions [Popat *et al*., 2012]. The adjustments were made to ensure process continuity and optimal microalgae growth.

4.2.8 Microalgae Growth at Cathode Chamber

The specific algal growth rate was observed as 0.284 ± 0.03 d^{-1}. Figure 4.7 depicts the growth curve of *C. reinhardtii* grown in TAP media at PMFC cathode. The microalgae growth complements the power generation from MFC as it produces oxygen for ORR at the cathode. *C. reinhardtii* naturally grows faster, making it a suitable candidate for products such as biodiesel, bioethanol, and animal feed [Yadav *et al*., 2021]. The specific growth rate in this system is slightly

less than what is obtained in a photobioreactor (0.66 d^{-1}) [Singh and Singh, 2015]. However, cultivating microalgae in a cathode chamber is beneficial in various ways. Oxygen inhibits microalgae growth at high concentrations. It is quenched through ORR at the MFC cathode, ruling out the need for a de-gasifier. The anodic off-gas contains sufficient CO_2 to support microalgae growth, eliminating the need to cool the flue gas and use that for microalgae growth. Small organic molecules that may diffuse through the porous separator help microalgae grow mixotrophically, leading to high growth rates. It also eliminates the need to supply costly organic substrates for heterotrophic microalgae growth.

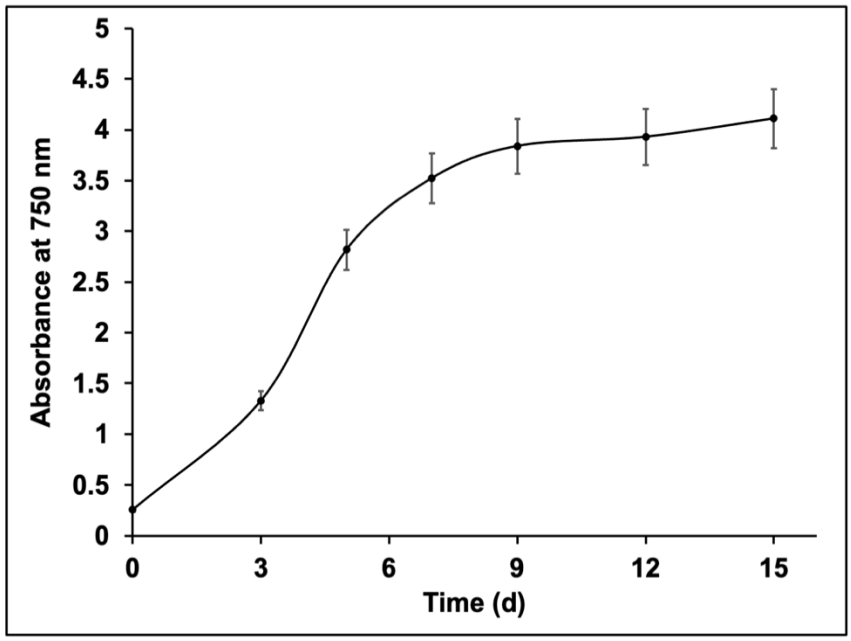

Figure 4.7 : The schematic shows the growth curve as absorbance at 750 nm vs time (d) for cathode grown algae

4.2.9 Pigment Production

The microalgae biomass harvested from the cathode chamber was processed for pigment extraction. The total chlorophyll and carotenoid content were 18.95 ± 0.0.20 mg/g and 2.40 ± 0.03 mg/g, respectively. The carotenoid content in this study is comparable to the bubble-column photobioreactor-grown microalgae [El-Mekkawi *et al*., 2019]. The high pigment content indicates good growth as the pigments are the main participants of the photosynthetic reaction [Wood *et al*., 2020]. Moreover, carotenoids have therapeutic properties that drive the process towards sustainability [Song *et al*., 2020]. Recently, the nutritional value of *C. reinhardtii* was assessed and compared with that of *Chlorella* and *Spirulina*. These two species are frequently used in PMFC applications and are considered for food and feed supplements as both fall under the GRAS category [Reddy *et al*., 2019; Fields *et al*., 2020; Apollon *et al*., 2021]. *Chlamydomonas* was found to be superior to *Chlorella* and *Spirulina* in terms of nutritional properties. Also, the pigment content of *Chlamydomonas* was significantly higher than those of these species [Darwish *et al*., 2020] making the *C. reinhardtii* a suitable candidate for PMFC application. Moreover, the increased biomass productivity of *C. reinhardtii* (820 - 2000 mg/l) was also noted, pointing to the utility of the studied microalgae species [Yadav *et al*., 2021]. As the anode and cathode chamber are separate, cross-contamination can be prevented by adequate operational controls which will help in maintain algae culture for food and feed applications. Also, the algae cultivation was done in nutrient media rather than wastewater which makes it safe for consumption. The results of the present study and the previous arts indicate the superiority of *C. reinhardtii* over conventional PMFC species such as *C. vulgaris and Spirulina*, in terms of power generation in a PMFC and microalgae biomass value.

The usefulness of *C. reinhardtii* as anodic species along with iron-reducing bacteria was studied previously [Nishio *et al*., 2013]. Also, it was reported to be used as an electron donor substrate for anodic bacteria [Mao and Verwoerd, 2014; Huang *et al*., 2015]. Moreover, the PMFC operation also generates resourceful microalgal biomass, which can be processed into biodiesel, biofuels, and other VAPs. The cultivation of photosynthetic organisms needs a continuous supply of light and CO_2. In PMFCs, CO_2-rich anodic-off gases, when channeled into the cathode chamber, help with the production of high-density microalgae biomass [Naina Mohamed *et al*., 2020]. Therefore, *Chlamydomonas*-based PMFCs can carry out power generation, waste treatment, CO_2 fixation, and microalgae biomass production simultaneously [Khandelwal *et al*., 2018, 2020].

4.2.10 Total Phenol Content and Antioxidant Activity of ABME

Phenolic compounds exhibit antibacterial, anthelmintic, anti-inflammatory, and cytotoxic properties, enhancing the therapeutic potential of microalgae biomass [Belinda *et al*., 2019]. The total phenolic activity in the methanol extract of *C. reinhardtii* biomass was found to be 157.69 ± 0.91 mg GAE/g DCW. The results are consistent with the literature, further indicating the healthy growth of *Chlamydomonas* at PMFC cathode. The microalgae extract prevented oxidation by 86.78 ± 0.25% inhibition at 1000 µg/ml concentration with an IC_{50} value of 67.07 µg/ml. It is superior to the previous study [Jayshree *et al*., 2016]. The presence of antioxidants in microalgae biomass extracts further enhances the commercial potential of such a system. However, these compounds need to be characterized well for further application.

4.2.11 Antibacterial Activity of ABME

The microalgae-based antibacterial compounds have gained attention in recent years [Falaise *et al*., 2016; Santhakumaran *et al*., 2020]. Species of *Chlorella* and *Chlamydomonas* are known to have antibacterial activity [Little *et al*., 2021]. Since this is an essential aspect from the point of view of microalgae biorefinery, it also has implications for the successful operation of PMFC for bioenergy purposes. These antibacterial compounds permit uncontaminated microalgae growth. In this study, the common bacterial pathogens, namely *P. aeruginosa*, *B. licheniformis*, and *E. coli* were inhibited by the *C. reinhardtii* microalgae extract, indicating high antibacterial activity Results showed the ABME effectively inhibited the growth of *P. aeruginosa*, the most drug-resistant bacterial species known to date. This is a breakthrough observation as none of the tested antibiotics effectively prevented its growth, as reported in Table 4.1. Similarly, the microalgae extract inhibited the growth of *E. coli* (inhibition zone of 13 ± 0.3 mm) and *B. licheniformis* (inhibition zone of 11 ± 0.1 mm). The antibacterial activity of microalgae helps to maintain the axenic cultures.

Table 4.1 : Antibacterial Assay for *C. reinhardtii* Algae Biomass Methanol Extract and Control Antibiotics

Antibacterial agent	Inhibition zone (mm)		
	P. aeruginosa	*B. licheniformis*	*E. coli*
Ampicillin	Negative	15 ± 0.5	13 ± 0.1
Tetracycline	Negative	19 ± 0.3	11 ± 0.2
Chloramphenicol	Negative	13 ± 0.15	14 ± 0.1
C. reinhardtii algae biomass methanol extract	19 ± 0.2	11 ± 0.1	13 ± 0.3

The study results indicated that *Chlamydomonas* at the cathode could be a source of antibacterial compounds while supporting its uncontaminated growth for bioenergy production. Major antibacterial activity in microalgae can be attributed to bioactive compounds such as carotenoids, chlorophyll derivatives, and unsaturated fatty acids [Falaise *et al*., 2016]. Previous study by Vishwakarma and Vavilala have reported that the sulphated polysaccharides extracted from *C. reinhardtii* also possess antibiofilm properties against known pathogenic bacteria, such as *E. coli*, *B. subtilis*, and *Streptococcus* sp. which inhibited bacterial growth and biofilm formation [Vishwakarma and Vavilala, 2019]. Therefore, the antibacterial agents in microalgae extract need

further exploration, and add another dimension to the successful use of such technology. The performance summary from the present study is shown in Table 4.2.

Table 4.2 : The Performance Summary of *Chlamydomonas*-based PMFC

Parameters	Results
Open circuit voltage (mV)	997
Voltage across 1000 Ω (mV)	563
Power density (W/m^3)	15.21
Power density (mW/m^2)	371.34
Current density (A/m^3)	39
COD removal (%)	73.30 ± 2
COD degradation rate ($Kg/m^3/d$)	0.325 ± 0.0004
Coulombic efficiency (%)	9.068
Energy output (kWh/m^3)	0.29
Specific growth rate (d^{-1})	0.284 ± 0.03
Total lipid content (%DCW)	19.5
Total chlorophyll (mg/g DCW)	18.95 ± 0.20
Total carotenoids (mg/g DCW)	2.40 ± 0.03
Total Phenolic content (mg GAE/g algae DCW)	157.69 ± 0.91
Total antioxidant activity (IC_{50}) (µg/ml)	67.07

4.3 CONCLUSIONS

The study has assessed the utilization of *C. reinhardtii* as a cathodic culture in MFC. The system turns out superior in terms of power density, current density, energy output, microalgae growth, and COD degradation. Microalgae biomass also showed potential as a therapeutic agent by its phenol content, antioxidant activity, and antibacterial properties. These aspects need detailed investigations. Further studies on using complex and readily available substrates at the anode, in particular, industrial wastewaters, compositional analysis of microalgae, system scale-up, and sustainability analysis of microalgae-based biorefinery, need to be done to prove the system's applicability at pilot scale along with the LCA. Also, the microbial diversity at anode chamber can be studied extensively to check their impact on MFC performance.