4

Removal of organic pollutants from saline waste using *Dunaliella* and halophilic bacteria in photomicrobial fuel cells

Saline wastewater contains high levels of salts and organic contaminants, making it difficult to treat as salt interferes with the activity of many heterotrophic bacteria [Siman'kova and Zavarzin, 1992]. Salt must be removed prior to organic contaminants removal, or else membrane-based processes suffer from biofouling. The food industry, textile industry, and gas and oil wells are some prominent sources of saline water. The successful treatment process must remove both salts and organic contaminants. Saline wastewater is commonly treated using physicochemical methods since traditional biological treatment methods are less effective in these cases [Guo and Liu, 2020]. Salts, particularly sodium chloride (NaCl), have a significant inhibitory effect on biological processes [Lefebvre *et al.*, 2012]. Literature shows various methods for removing organic pollutants, focusing on anoxic or oxic biological treatment methods [Lefebvre and Moletta, 2006]. The ability of halotolerant organisms to break down organic substances through anoxic digestion is widely studied. Halophiles belong to the archaea domain and can flourish in environments characterized by elevated salt concentrations. Based on the salt concentration, halophilic microbes are divided into three categories: slight, moderate, and extreme halophiles [Lederberg, 2000]. These salt-loving microbes thrive on high salinity by modifying their ion uptake process and increasing the solute concentration in their cytoplasm [Oren, 2013]. Anaerobic cellulose degrading halophiles showed increased cell count when salinity was 5 to 15%, respectively [Siman'kova and Zavarzin, 1992].

Microbial fuel cells (MFCs) have been studied worldwide because of their application in wastewater remediation, bioelectricity generation, and sensing [Logan *et al.*, 2006]. Guo *et al.* [2021] reported that wastewater with high salt concentrations may be remediated using MFC efficiently. The efficacy of the MFC depends on the biofilm formation by the electrogenic bacteria (EGB) on the anode in an anaerobic environment [Guo and Liu, 2020]. It is reported in the literature that an increase in salt concentrations results in a decrease in the internal resistance (IR) of the system because of the conductance of the ions, which ultimately leads to an enhancement in power generation [Dopson *et al.*, 2016]. Therefore, the efficiency of the MFC depends upon the salt and halophilic microbes. Anaerobic halophiles can degrade the organic carbon to support their metabolism in wastewater [Lederberg, 2000]. For such systems, halophilic algae also play a crucial role in generating electron acceptors at the cathode. The algae biomass can also serve as an electron donor substrate to sustain power [Lakaniemi *et al.*, 2012; Mishra and Chhabra, 2022]. Since the microalgae can be used as an electron donor at the anode, a study reported that the LEA harvested from *Chlorella vulgaris* reduces the dependency on chemical carbon sources by providing a sustainable substrate for the EGB and produces the power density (PD) 2.3 W/m^3 [Khandelwal *et al.*, 2018]. So, while the system can degrade organic pollutants in saline water, adding additional substrates, such as algae biomass, can enhance its power density. Some studies that discuss algae biomass as an electron donor report good power densities and the diversity of usable algae species. For example, a sediment MFC built using a multi-anode reactor produces 2965 mW/m^2 of PD with a mixture of algae biomass as the anode substrate [Taşkan *et al.*, 2021]. Lakaniemi *et al.* [2012] used pretreated biomass of *C. vulgaris* and *Dunaliella tertiolecta* in dual chamber MFC as an anodic substrate. They found that

the PD was higher for *C. vulgaris* than *for D. tertiolecta*, i.e., 15 and 5.3 mW/m^2, respectively. The anode chamber of the three-chambered PMFC was dosed with oil-free algal biomass as a co-substrate for the EGB, producing a PD of 10 W/m^3 [Das *et al.*, 2022]. A study by Rashid *et al.* [2013] with LEA as a substrate showed an open circuit voltage (OCV) of 0.242 V. A separate investigation revealed that an outdoor PMFC fueled with LEA produces a PD of 1200 ± 152 mW/m^3 [Khandelwal *et al.*, 2018]. These studies indicate the usability of algae biomass.

The usability of algae biomass in saline MFC systems is uninvestigated. Moreover, the simulation of saline effluent using algae biomass also closely mimics the complex organic substrates that may be found in actual saline waste. Previous studies that discussed saline effluent in MFC used simpler carbon sources like glucose, fructose, lactose, etc, for the EGB at the anode. A study by Vijay *et al.* [2018] demonstrated that starch, which is a complex sugar, serves as an electron donor for halophilic bacteria in an MFC for the first time and reported a PD of 207.05 mW/m^2 at1000 mM salinity. The literature lacks studies on saline waste treatment using a combination of halophilic bacteria and halophilic algae. Also, the effect of outdoor operation of these systems needs investigation. The following objectives were set for the study: to assemble and operate PMFC with modified saline wastewater containing starch and LEA and to study the effect of salt concentration on PMFC performance and bacterial diversity at the anode.

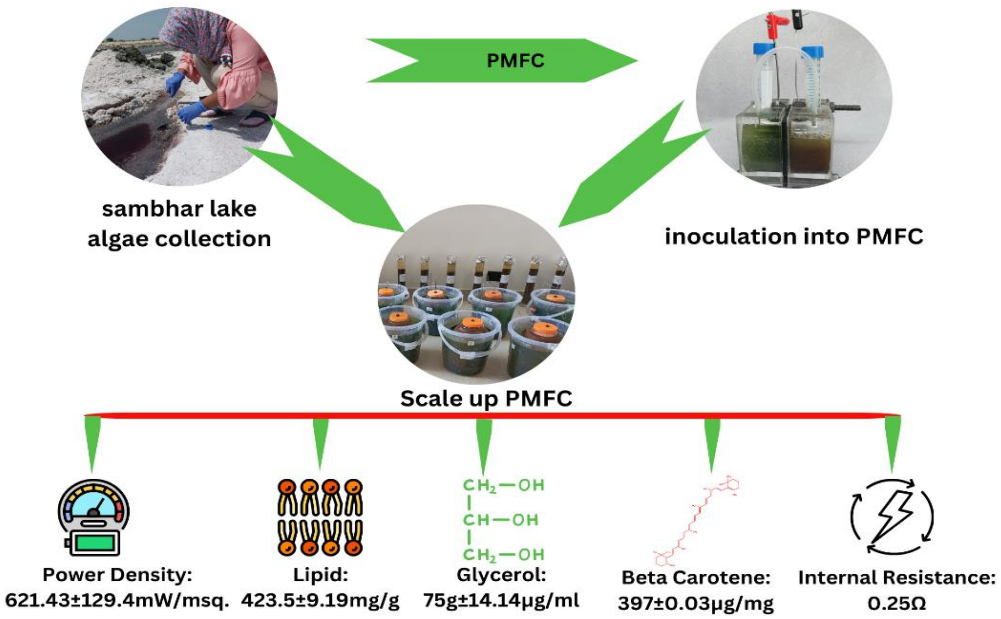


Fig. 4.1: Schematic presentation of the strategy employed in the current study.

4.1 PMFC CONSTRUCTION AND OPERATION

Dual chamber PMFCs (Fig. 4.2) were assembled by using a clay ware pot (Unnada pvt ltd, Banar, Jodhpur) as an anodic chamber (2 l) and a transparent plastic bucket as the cathodic chamber (15 l). The electrodes were graphite felt (Nickunj eximp enterprises pvt ltd, Mumbai) with dimensions of 10 × 10 × 0.56 cm and 30 × 15 × 0.56 cm, respectively. The pretreatment of the graphite felt was performed using the acid activation method, as reported previously [Venkata Mohan *et al.*, 2008]. Titanium wire (Manhar metals, Mumbai) of 2 mm gauge was used to connect electrodes. To ensure experimental consistency, all PMFCs were assembled with identical construction, ensuring uniform electrode placement and electrical connections. The assembly was securely fastened to prevent leakage or contamination between chambers. Nine different PMFC systems were operated based on salt concentrations and the substrate used in the anode (Table 4.1). The anodic and cathodic chambers were filled with modified saline wastewater (wastewater collected from the sewage treatment plant, IIT Jodhpur, and

supplemented with different salt concentrations). The anode chambers were inoculated from previously operating saline MFCs, and the cathode chambers with a pure culture of microalgae *D. salina* (57.5 ± 10.7 × 10^4 cells/ml), respectively. The anode chamber was sealed with a detachable cap for medium exchange. To maintain optimal reactor conditions, the cathodic chamber was continuously aerated to enhance oxygen availability and prevent mass transfer limitations. The anode chambers were kept in an anaerobic environment by sealing with gas-tight caps to minimize oxygen intrusion, which could interfere with microbial electron transfer. Each reactor was placed in a controlled environmental setup to ensure uniform exposure to light and temperature conditions across all replicates. All the PMFCs were operated at a photoperiod of 14:10 (D/L), light intensity of 2860 ± 876 lux measured by digital lux meter (Metravi®1300), CO_2 concentration of 650 ± 163 ppm measured by CO_2 meter (LT-Lutron, GC-2028), and a temperature of 28 ± 5˚C.

The PMFC reactors were operated in triplicates (n = 3 per condition) under a fed-batch mode, ensuring statistical reproducibility of the data. Each reactor underwent multiple operational cycles to assess performance stability over time. A single batch cycle of LEA-fed PMFC sustained 80-170 hours of power production. Adding starch/LEA and replenishing the medium (modified saline wastewater) coincided with a decrease in the drop in voltage after each cycle. Regular monitoring of voltage output was conducted to track system stability, with data acquisition performed at fixed intervals to minimize experimental variability. The electrochemical analysis was conducted during the cycle wherein the PMFCs demonstrated the highest operating voltages.

Table 4.1. Labelling of the PMFC systems used for the study based on salt concentrations and the substrate used in the anode.

Operational PMFC	Anolyte	Catholyte	Substrate at anode
0.5 S	0.5 M wastewater	0.5 M wastewater	Starch
0.5 LEA	0.5 M wastewater	0.5 M wastewater	LEA
0.5 C	0.5 M wastewater	0.5 M wastewater	Without substrate
1.0 S	1.0 M wastewater	1.0 M wastewater	Starch
1.0 LEA	1.0 M wastewater	1.0 M wastewater	LEA
1.0 C	1.0 M wastewater	1.0 M wastewater	Without substrate
1.5 S	1.5 M wastewater	1.5 M wastewater	Starch
1.5 LEA	1.5 M wastewater	1.5 M wastewater	LEA
1.5 C	1.5 M wastewater	1.5 M wastewater	Without substrate

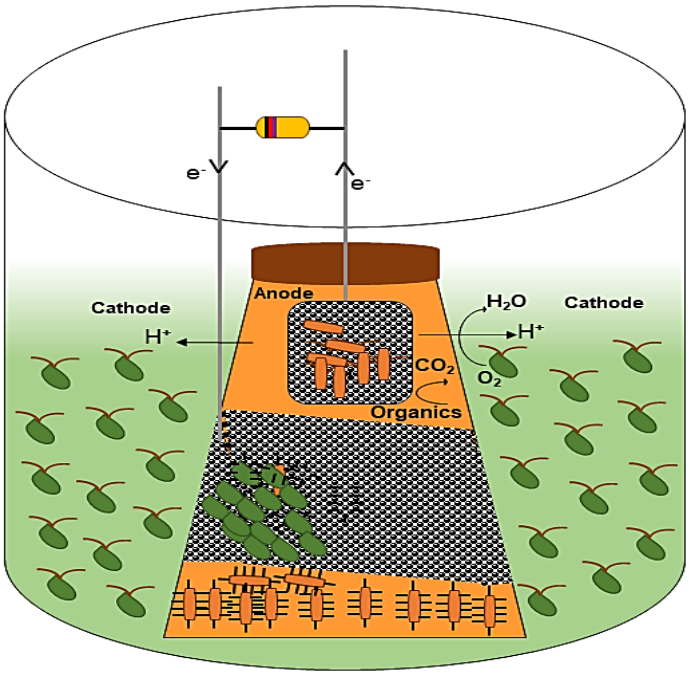


Fig. 4.2: Schematic diagram showing clay ware and plastic bucket-based low-cost PMFC.

4.2 RESULTS AND DISCUSSION

The study's primary objective is to see the efficacy of organic matter removal in saline wastewater using *Dunaliella*-assisted MFCs. LEA and starch were used as representative complex substrates found in the saline wastewater. The base wastewater was supplemented with salts and LEA/starch to assess MFC performance.

4.2.1 Fourier Transform Infrared Spectroscopy (FTIR) analysis of LEA biomass

The absence of lipid characteristic absorption bands in the LEA or de-oiled biomass sample suggests that the lipid content from the algal biomass has been effectively eliminated (Fig. 4.3). According to Stehfest *et al.* [2005], the absorption of LEA biomass was detected at a wavenumber of 1523 cm^{-1}. This absorption can be ascribed to the stretching vibration of the C=O bond and the bending vibration of the N-H bond within the peptide bond. Moreover, the LEA biomass exhibits absorption spectra spanning from 1700 cm^{-1} to 500 cm^{-1}; these spectrum ranges encompass the stretching vibrations of polysaccharides, specifically the N-H, C-C, C-O, C-O-C, and C-O-P vibrations [Phukan *et al.*, 2011]. The observations mentioned above indicate that the biomass of LEA primarily consists of carbohydrates and proteins with minimal lipid content.

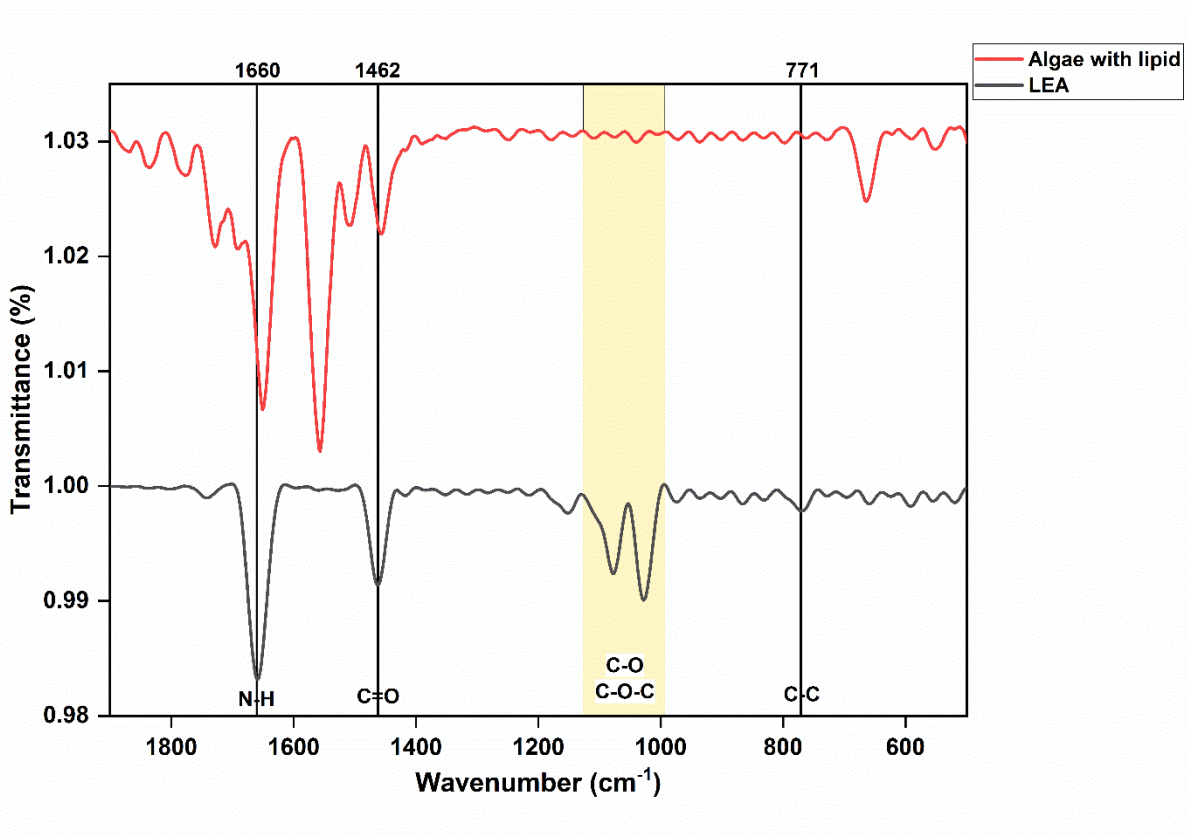


Fig. 4.3: FTIR spectrum obtained for intact algae biomass and LEA biomass.

4.2.2 Effect of salinity on voltage generation and other parameters

The Fig. 4.4 illustrates the temporal change in voltage for all working PMFCs. The LEA-fed 0.5 M PMFC exhibited a consistently good operational voltage of 615 ± 79 mV throughout 800 h. The second best was LEA-fed 1.0 M PMFC, followed by the control (base wastewater)-1.5 M PMFCs. The trends indicated LEA as a preferred substrate by anodic bacteria. LEA is a pre-treated substrate with complex carbohydrates, proteins, and other biomolecules, which would require many complex biological pathways and bacterial diversity. The successful acclimatization of anodic culture was evident here. The voltage was highest for 0.5 M, followed by 1.0 M and 1.5 M salt.

On the other hand, starch exhibited voltage values between 230-550 mV. The values were lower than LEA. Effective starch utilization requires good amylase production, and anodic bacteria produce the same (results not shown). Starch is a carbohydrate and does not contain nitrogen, phosphorus, or any other element that can support effective microbial growth. All the elements are needed to meet bacterial stoichiometric requirements for cell synthesis. LEA met the demands of elements better and therefore supported better microbial growth and power output.

Nevertheless, the voltage values were good, indicating successful acclimatization of EGB on both starch and LEA. The wastewater base provided some of the essential nutrients needed for microbial growth. The voltage values of 784.5 ± 4.5 mV were observed during the 7th batch cycle, at 0.5 M and PMFC fed with LEA, as shown in Fig. 4.4. The voltage output was consistent for all the operating PMFCs.

Every cycle was linked to a substrate replenishment, followed by a gradual increase in voltage. The present study is comparable to previous work where thermal pre-treated algal biomass produced a potential of 242 mV [Rashid *et al.*, 2013]. All operating PMFCs were operated for 800 hours to attain high operating voltages. The voltage value of 603 ± 26.1 mV was observed for starch-fed PMFC, which was low as compared with the LEA-fed PMFC at the

same salinity of 0.5M. Halophiles are anticipated to exhibit sluggish growth rates when starch is present as a substrate [Cui *et al.*, 2016]. Soluble mediators facilitate electron transport in a system. As the EGB grows, the number of produced mediators also increases, subsequently impacting the potential output [Lefebvre and Moletta, 2006]. However, the system consistently maintains a high level of effectiveness even after the final operational cycle, showcasing its optimal performance.

The fluctuation in voltage was observed due to the increase in the microalgae biomass at the cathodic chamber during the light/dark period, which resulted in the release of DO. The DO levels for the 1.0 M LEA-fed PMFC were higher than other operating PMFCs, and the values varied from night to day by 4.1 ± 2 mg/l and 6.8 ± 3 mg/l, respectively, affecting the power output.

COD removal in wastewater is indicative of its treatment. Moreover, the power generation from MFC depends on the rate of COD degradation. The PMFC fed with 1.0 M LEA exhibited the COD removal of 36.38 ± 3.5%. The COD removal was less, indicating the system's high level of inert biomass. It also reflects the limited metabolic capacity of halophiles to degrade organic pollutants. Only a fraction of COD is effectively utilized. Additional research is needed on the COD degradation capabilities of halophiles. Three significant factors contribute to the low COD removal in saline waste. Increasing salinity reduces microbial metabolic activity as energy is allocated for osmotic balance within the cell, leading to low COD removal [Lefebvre and Moletta, 2006]. The second reason for low organic removal is the decreased availability of organics to microbes caused by the salting-out effect, which reduces the solubility of organics in saline waste [Castillo-Carvajal *et al.*, 2014]. Sohn *et al.* [2004] used the biosurfactant 2-hydroxypropyl β-cyclodextrin to address this issue, enhancing organic compound solubility in saline waste. Lastly, the high salinity causes the formation of intermediate compounds, which the microbes cannot use for their metabolism [Castillo-Carvajal *et al.*, 2014].

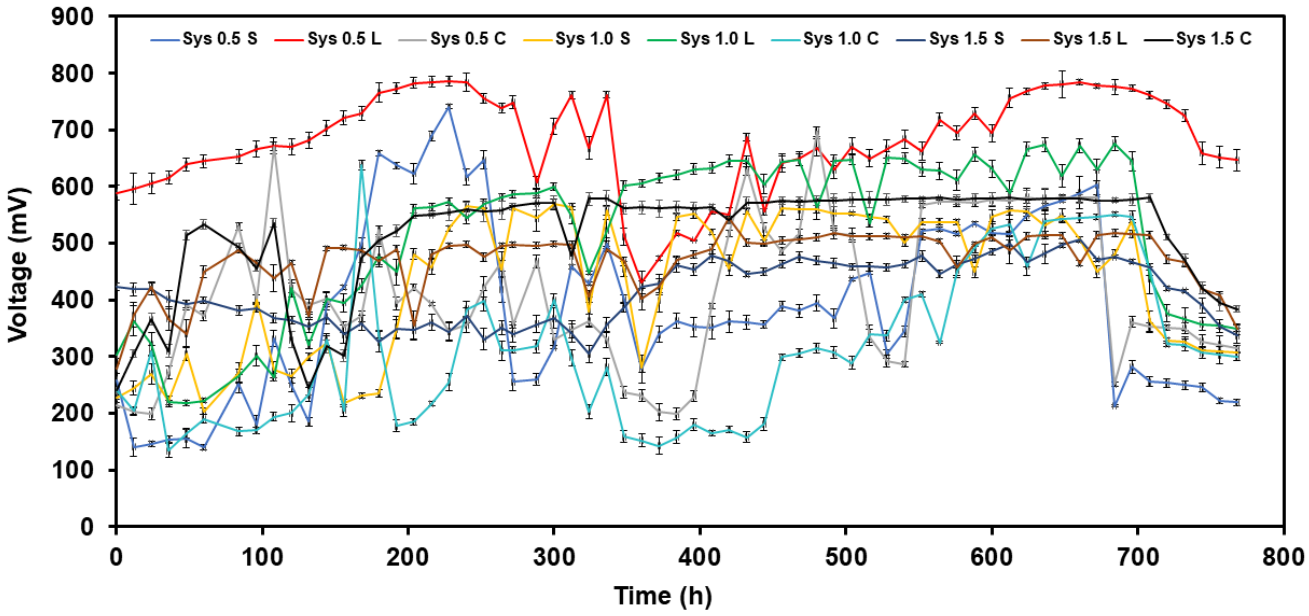


Fig. 4.4: The profile of voltage generation of all the operating PMFC across the external resistor 100 Ω with time in hours.

4.2.3 Effect of salinity on power generation of PMFCs

Polarization curves were generated by systematically altering the resistance values from 20 kΩ to 10 Ω. Fig. 4.5 displays the polarization curves illustrating the maximum power outputs achieved by various operating PMFCs. The 0.5 M LEA-fed PMFC achieved a maximum power yield of 621.43 ± 129.47 mW/m^2. The PD of the 0.5 M LEA-fed PMFC was 92.17% and 97.34% higher than the power density of the 1.0 M and 1.5 M LEA-fed PMFCs, respectively. This drastic

drop indicated that salinity values greater than 0.5 M are detrimental to COD degradation even with halophiles. The effluent treatment with higher salinities would require dilution before treatment. In addition, the power densities of 0.5 M LEA-fed PMFCs were 93.74% and 92.16% higher than those of starch-fed and control PMFCs with the same concentrations. Table 4.2 provides a summary of the PD (mW/m^2), CD (mA/m^2), maximum voltage (mV), CE, IR (Ω), energy (kWh/m^3), and COD removal (%) for all operating PMFCs. The PMFCs fed with a 0.5M concentration along with LEA as a substrate exhibited the highest current density, specifically 1665.18 ± 183.04 mA/m^2 (at 10 Ω), along with the lowest IR of 0.25 Ω, IR of the PMFCs were determined through the slope of the polarization curves. The power generated by 0.5M LEA-fed PMFCs exceeds that of starch-fed PMFCs. This demonstrates that LEA biomass can serve as an anodic substrate. The 0.5 M LEA-fed PMFC power density is comparable with the acetate-fed PMFC, i.e., 213.38 mW/m^2 [Mishra and Chhabra, 2022]. The power output of the *D. salina* LEA-fed PMFC is comparable to that of the *C. vulgaris* LEA-fed PMFC, precisely 67.07 mW/m^2 [Khandelwal *et al.*, 2018]. The power density observed in the present study is 2.01 times high as compared with the results of Vijay *et al.* [2018], where the anode was saline with halophiles as electron donors and the cathode was a chemical electron acceptor and the reactor volume of 100 ml, which resulted in the power density of 207.05 ± 5 mW/m^2. A study by Lakaniemi *et al.* [2012] showed that the biomass of the microalgae *D. tertiolecta,* when used as an anodic carbon source, results in a power density of 25.7 mW/m^2. Therefore, all the above-reported results showed the use of *Dunaliella* LEA biomass as a potential carbon source for the anodic EGB.

The 0.5 M LEA-fed PMFC CE was the highest compared to all other operating PMFCs, specifically at 12.2%. CE of the present work is comparable with the MFC, with the pre-digested biomass of *D. tertiolecta* was 7.2% [Lakaniemi *et al.*, 2012]. High CE for the PMFC has been achieved by transforming organic matter-energy into bioelectricity.

The estimated energy produced per batch cycle was calculated using the power time curve (Fig. 4.6). Integration of the curve was divided with the anodic volume observed to be around 0.496 kWh/m^3 for 0.5 M LEA-fed PMFC (Fig. 4.6). A study reports the use of LEA biomass of microalgae *C. vulgaris* as a carbon source at the PMFC anode generates the total energy of 0.1 kWh/m^3 [Khandelwal *et al.*, 2018]. *Dunaliella*'s LEA biomass generates more energy than the *Chlorella* [Lakaniemi *et al.*, 2012; Mishra and Chhabra, 2022]. The energy output is a crucial parameter for the assessment of PMFC performance.

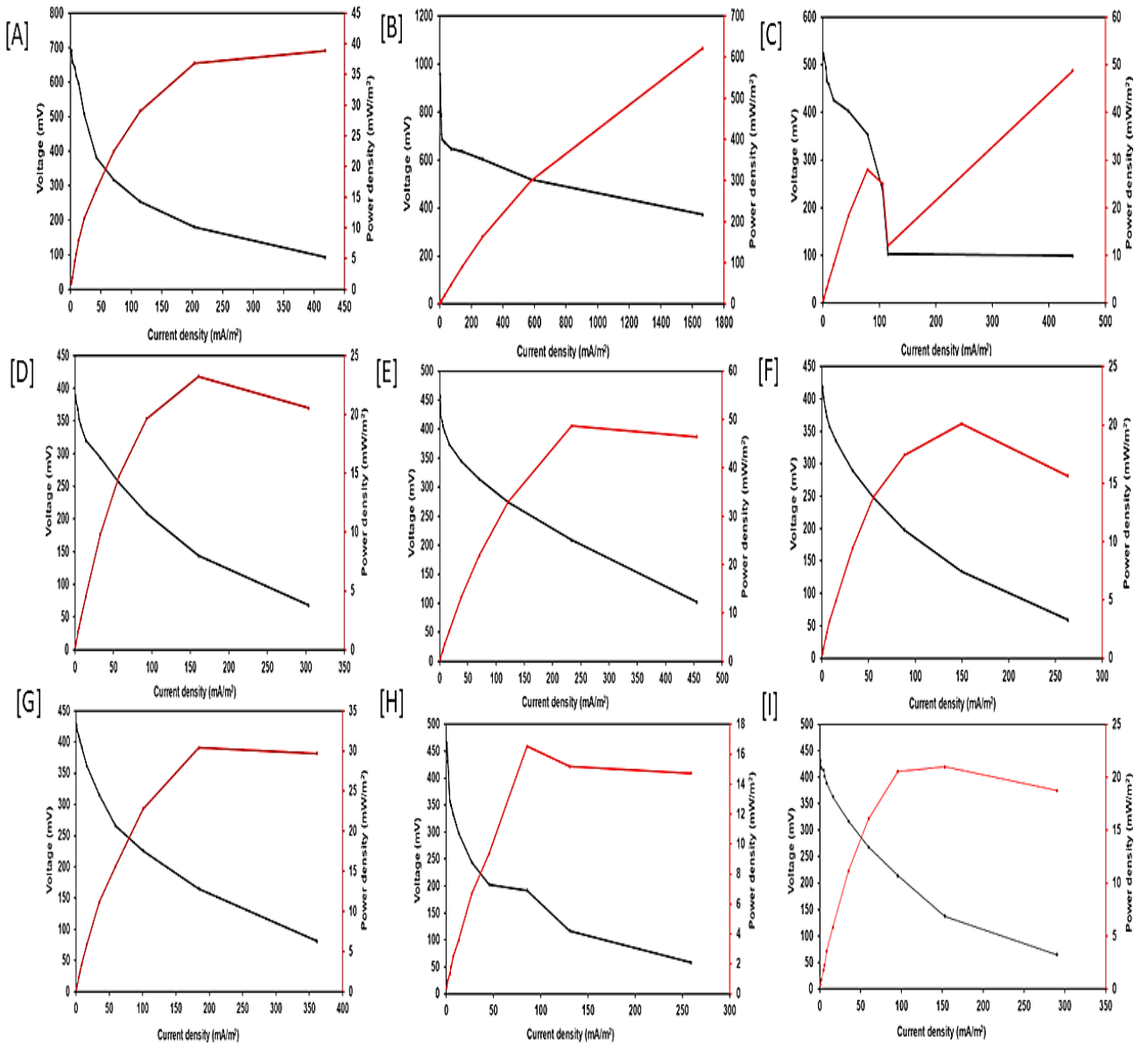


Fig. 4.5: Polarization curve for the PMFCs at different NaCl concentrations and anodic substrates: A, B, and C with 0.5 M; D, E, and F with 1.0 M; G, H, and I with 1.5 M salinity, whereas A, D, and G fed with starch; B, E, and H with LEA; C, F, I with no substrates at the anode.

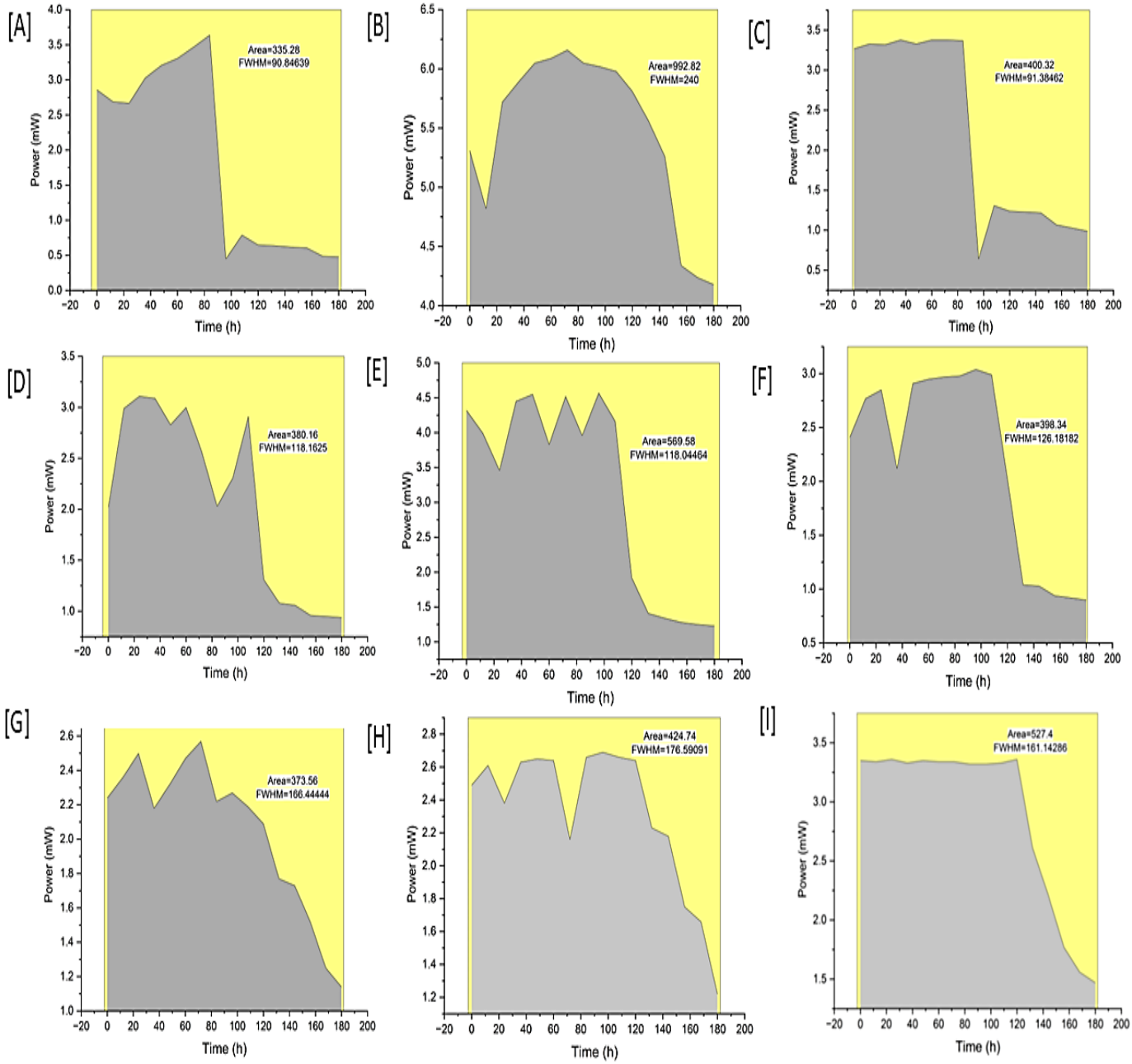


Fig. 4.6: Energy obtained in the last operating batch cycle of the PMFCs is represented as the area under the curve (shaded gray) for the PMFCs at different NaCl concentrations and anodic substrates: A, B, and C with 0.5 M; D, E, and F with 1.0 M; G, H, and I with 1.5 M salinity, whereas A, D, and G fed with starch; B, E, and H with LEA; C, F, I with no substrates at the anode.

4.2.4 CV analysis

CV is an important method that helps elucidate the redox reactions happening at the electrode [Rabaey *et al.*, 2004]. The analysis of the CV curves revealed a substantial change in the redox peaks with different salinities and substrates (Fig. 4.7). The 0.5 M LEA-fed PMFC showed two distinct oxidation and two reduction peaks. The oxidation peaks were observed at 0.396 V with a current of 225.54 mA and at 0.692 V with a current of 134.23 mA. The reduction peaks were observed at -0.302 V with a current of 41.19 mA and at -0.858 V with a current of 130.03 mA. These peaks are likely caused by the oxidation of LEA biomass through bio-electrocatalytic processes and the high oxygen reduction rate at the cathode [Khandelwal *et al.*, 2018]. The mediators produced by mixed electrogenic bacterial populations could explain the observed spikes in the anodic biofilm [Liu *et al.*, 2005]. Also, the electric current produced by these redox pairs ranged from 41.19 to 225.54 mA, indicating that specific mediators were present in reasonable quantities within the biofilm.

The present study of the CV of all the working PMFCs fed with LEA at different saline concentrations showed that they had more prominent oxidation and reduction peaks than the PMFCs that were fed with starch and the control group (see Fig. 4.7). Therefore, from the above it confirmed the presence of mediators in the biofilm. The salinity level can also impact the oxidation/reduction reaction due to its influence on biofilm formation and microbial diversity [Venkata Mohan *et al.*, 2008].

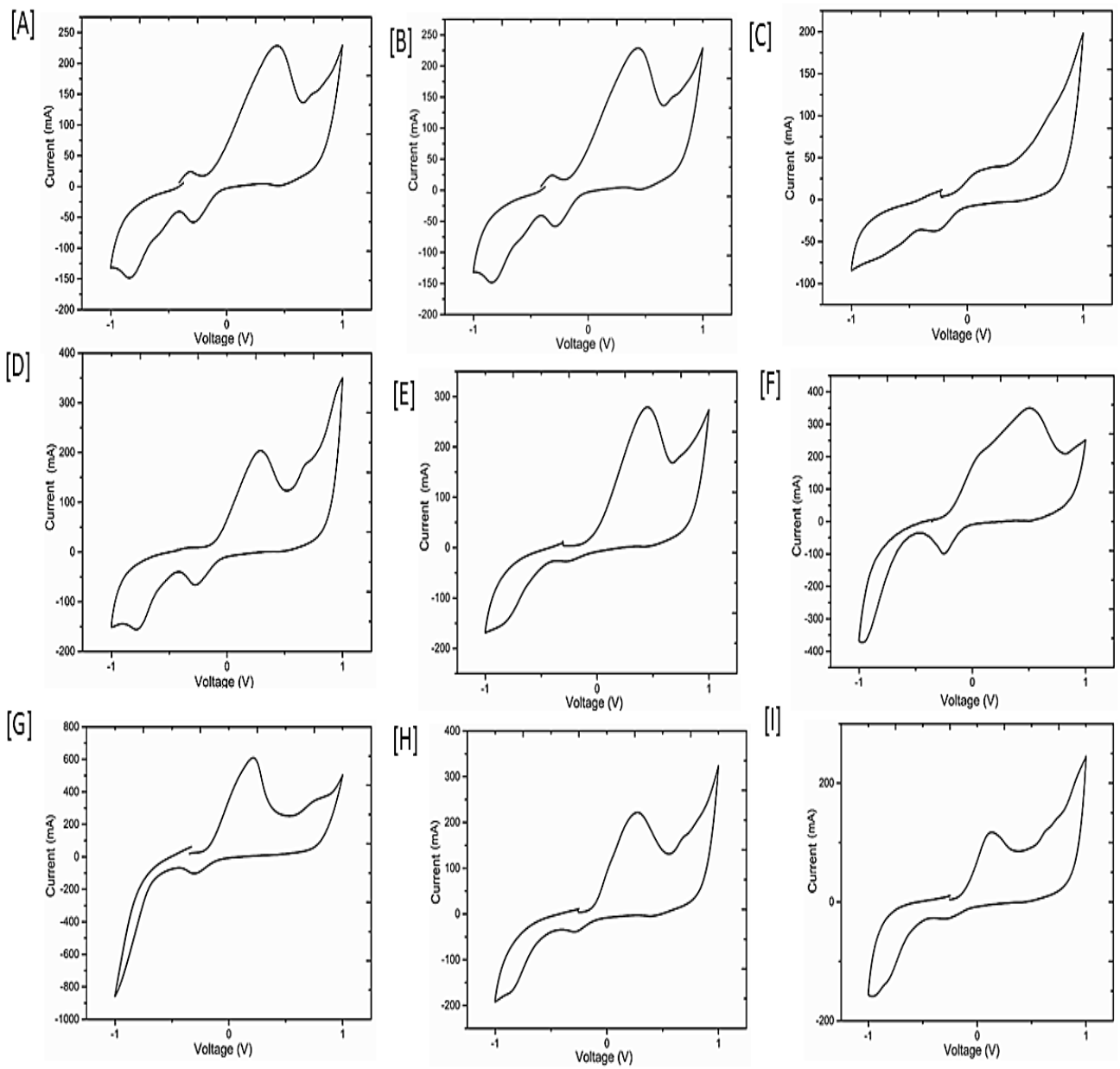


Fig. 4.7: CV curve for the PMFCs at different NaCl concentrations and anodic substrates: A, B, and C with 0.5 M; D, E, and F with 1.0 M; G, H, and I with 1.5 M salinity, whereas A, D, and G fed with starch; B, E, and H with LEA; C, F, I with no substrates at the anode.

4.2.5 Effect of salinity concentration on algae growth and other value-added product production

The growth rate of algae is a crucial parameter that impacts power generation [Khandelwal *et al.*, 2020]. The algae *D. salina* exhibited a maximum specific growth rate of 0.42 ± 0.082 day^{-1} when fed with 1.0 M LEA in the PMFC with a doubling time of 1.65 days. The next highest growth rate of 0.39 ± 0.041 day^{-1} was observed when fed with 0.5 M LEA in the PMFC with a doubling time of 1.78 days (Fig. 4.8). After the last operating cycle in PMFCs, the algae concentration reached 920.8 ± 114.84 × 10^4 cells/ml and 769.6 ± 77.21 × 10^4 cells/ml for the LEA-fed 1.0 and 0.5 M operating systems, respectively. A study by Singh *et al.* [2019] showed a

doubling time of 2.65 days when the microalgae *D. salina* grew at 0.5 M of salinity. This indicates that the MFC enhances algae growth and reduces the doubling time.

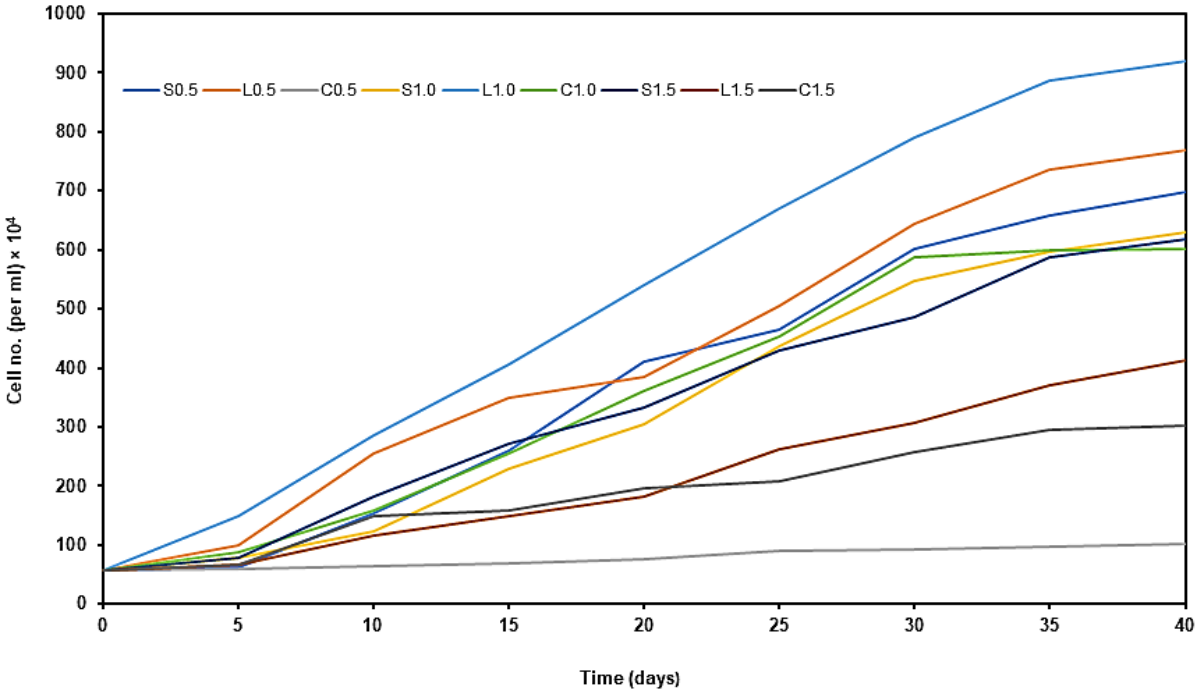


Fig. 4.8: Algae growth as observed in PMFC at the cathode at different salinities.

The algae from 1.0 M LEA exhibited the highest lipid content of 566.5 ± 9.19 mg/l. The lipid content of algae in operating PMFCs with low and high salinity was lower compared to the 1.0 M PMFCs, as shown in table 4.2. Therefore, these results represent the role of salinity in the enhancement of lipid formation in microalgae by changing the path of conversion of carbon into lipid instead of starch to protect the cell from osmotic pressure [Ahmed *et al.*, 2017; Khoo *et al.*, 2023]. The main reason behind the conversion of carbon into lipid is the overexpression of the functioning of ACCase enzymes (responsible for lipid synthesis) and suppression of the functioning of AGPase enzymes (responsible for starch synthesis) ([Khoo *et al.*, 2023].

The lipid content of the 1.5 M PMFCs declined due to elevated salinity and reduced biomass content. Lipid content in microalgae varies with the salt concentration and pH among different algal strains. A study showed a 7.93% enhancement in the lipid content when the salinity increased from zero to 0.5 M, but when the salinity reached 1.1 M, the lipid content decreased [Rai *et al.*, 2015].

Therefore, the present study has determined that PMFC displayed a high lipid concentration because of high algal biomass. The lipid content per unit of dry weight for the 1.0 M PMFC fed with LEA was precisely measured to be 56.5%. The growth of *D. salina* in outdoor ponds was effective, resulting in a lipid yield of 46 mg/l/day [Weldy and Huesemann, 2007]. *D. salina* offers the benefits of thriving in saline waste environments and exhibiting resilience to extreme temperatures. High salt concentration efficiently inhibits other microbial contaminations to a substantial extent. The *D. salina* strain showed a lipid content of 35% when subjected to a nitrogen concentration of 0.05 mM and a salinity of 5% [Yilancioglu *et al.*, 2014]. Another study found that *D. salina* grown in a solution containing 2M NaCl produced 248.33 mg/l of lipids, with a lipid content of 22.28% [Ahmed *et al.*, 2017].

The concentration of glycerol in *D. salina* cells is directly correlated with the level of salt stress because the cells regulate their osmolarity by adjusting the amount of glycerol present within the cell [Singh *et al.*, 2019]. The glycerol concentration in the 1.0 M LEA-fed PMFC was determined to be 620 ± 24.04 µg/ml, the highest among all the functioning PMFCs. The glycerol

concentration decreases as the salinity increases from 1.0 M to 1.5 M due to a decrease in cell biomass/ml. Similarly, the glycerol content is lower in the 0.5M solution compared to the 1M solution due to reduced biomass production (Table 4.2). A study by Kaçka and Dönmez [2008] showed that the microalgae *D. salina* stores a glycerol content of 452.5 µg/ml at 0.343M, less than the present study.

Like the lipid and glycerol content, the β-carotene level was also elevated in the PMFC fed with LEA and exposed to 1.0 M salinity. It was measured to be approximately 25.41 ± 0.203 µg/100 mg biomass. The concentration of β-carotene rises as salinity increases until it reaches its optimal level, but then it decreases with further increases in salinity. A study on the microalgae *D. salina* by [Hashemi *et al.*, 2020] generates a β-carotene concentration of 4.85 µg/mg, comparable with the present work. β-carotene is a high-valued secondary metabolite found in plants and algae, and *D. salina* is the strains that produce a high content of β-carotene [Aluç *et al.*, 2018]. β-carotene has a worldwide market and is utilized for food supplements, cosmetics, medications, and other applications [Aluç *et al.*, 2018].

The study results thus indicated that while 0.5M PMFCs produced high power, the value-added product generation in 1.0 M systems was highest. The MFCs are compartmentalized systems that can be integrated with multiple applications. Wastewater treatment and power generation is one aspect, while algae growth and intracellular metabolite content of algae is another. The increase in other parameters compensates for the drop in performance in one parameter. This further testifies the utility of MFCs for saline water treatment integrated with algae cathodes.

Table 4.2 Performance of the PMFC reactors operated at different salinity concentrations and substrates.

Parameters	0.5 M starch	0.5 M LEA	0.5 M control	1.0 M starch	1.0 M LEA	1.0 M control	1.5 M starch	1.5 M LEA	1.5 M control
Voltage (mV)	603 ± 26.1	784.5 ± 4.5	581 ± 8	555.5 ± 14.84	674.5 ± 12.2	547 ± 10	506.5 ± 4.1	518.5 ± 6.8	580 ± 15
PD (mW/m^2)	38.89 ± 23.79	621.43 ± 129.47	48.75 ± 1.42	23.22 ± 0.45	48. 66 ± 16.43	20.09 ± 2.43	30.41 ± 9.5	16.52 ± 6.03	20.98 ± 3.43
CD (mA/m^2)	418.17 ± 157.38	1665.18 ± 183.04	441.97 ± 10.11	303.57 ± 1.2	455.36 ± 75.89	263.39 ± 22.13	361.61 ± 107.14	258.93 ± 138.39	290.18 ± 5.61
CE	2.6	12.2	8.3	9.3	9.3	10.4	9.08	8.5	9.9
IR (Ω)	1.42	0.25	0.97	1.06	0.72	1.35	0.97	1.37	1.32
Energy (kWh/m^3)	0.167	0.496	0.2	0.192	0.28	0.19	0.186	0.22	0.26
COD removal (%)	9.14 ± 1.5	29.94 ± 5.1	33.66 ± 1.2	27.03 ± 8.08	36.38 ± 3.5	23.08 ± 3.07	26.08 ± 4.6	29.48 ± 2.9	26.87 ± 1.9
Specific algal growth rate (day^{-1})	0.39 ± 0.034	0.39 ± 0.041	0.21 ± 0.06	0.28 ± 0.04	0.42 ± 0.04	0.38 ± 0.057	0.36 ± 0.025	0.34 ± 0.011	0.31 ± 0.022
Lipid (mg/l)	385 ± 2.12	423.5 ± 9.19	345 ± 7.07	540 ± 4.29	566.5 ± 9.14	520.5 ± 13.43	518.5 ± 4.45	520 ± 5.55	439 ± 1.41
Glycerol (µg/ml)	62.5 ± 9.67	75 ± 14.14	40 ± 7.07	190 ± 7.19	620 ± 24.04	212.5 ± 1.06	222.5 ± 10.8	202.5 ± 3.537	123 ± 4.242
ß-carotene (µg/100mg)	2.92 ± 0.033	3.97 ± 0.34	1.26 ± 0.067	18.54 ± 0.14	25.41 ± 0.203	18.42 ± 0.03	18.69 ± 0.067	18.99 ± 0.03	18.54

4.2.6 Biofilm analysis of the electrodes

The biofilm is essential for the functioning of the PMFCs [Lefebvre *et al.*, 2012]. Therefore, the current work used SEM to examine the biofilm that developed on the surfaces of both cathodic and anodic electrodes, as depicted in fig. 4.9. The cell clusters are observable and adhere to the surface of graphite felt fibers. The electrode's roughness and pore size significantly impact the stability and formation of biofilm [Schneider *et al.*, 2016]. An electrode comprised of a big pore size is less prone to clogging due to cell growth [Schneider *et al.*, 2016].

The current study demonstrated that PMFCs at 0.5 M exhibited a significant amount of biofilm after the operation, in contrast to the 1.5 M PMFC. This disparity can be attributed to the elevated salinity levels, which hindered the proliferation of electrogenic bacteria. The concentration of salinity has an impact on the formation of biofilm at the electrode [Lefebvre *et al.*, 2012]. The development of biofilms is a critical stage in generating power through PMFCs.

EGB utilize a protein, which is a component of their type-IV pili, to transport electrons across significant distances [Bosch *et al.*, 2010]. FTIR spectroscopy and an *in vivo* detection technique were employed to examine the presence of type-IV pili on various anodes following the completion of the last operational cycle of the PMFCs. The FTIR scanning in the wavelength range of 1900-500 cm^{-1} showed the peaks that interpret the abundances of type-IV pili. Peaks were divided into three windows, W_1 (550-650 cm^{-1}), W_2 (1050 cm^{-1}), and W_3 (1350-1680 cm^{-1}) depicting the mix region, including protein glycosylation [Bosch *et al.*, 2010; Nath and Ghangrekar, 2020]. In the W_3 region, type-IV pili were detected and showed abundance for the 0.5 M LEA-fed PMFC (FTIR plot (Fig. 4.10)). Further analysis is required to verify the effect of salinity on the over-expression of type-IV pili with specific antibody-based western blotting experiments.

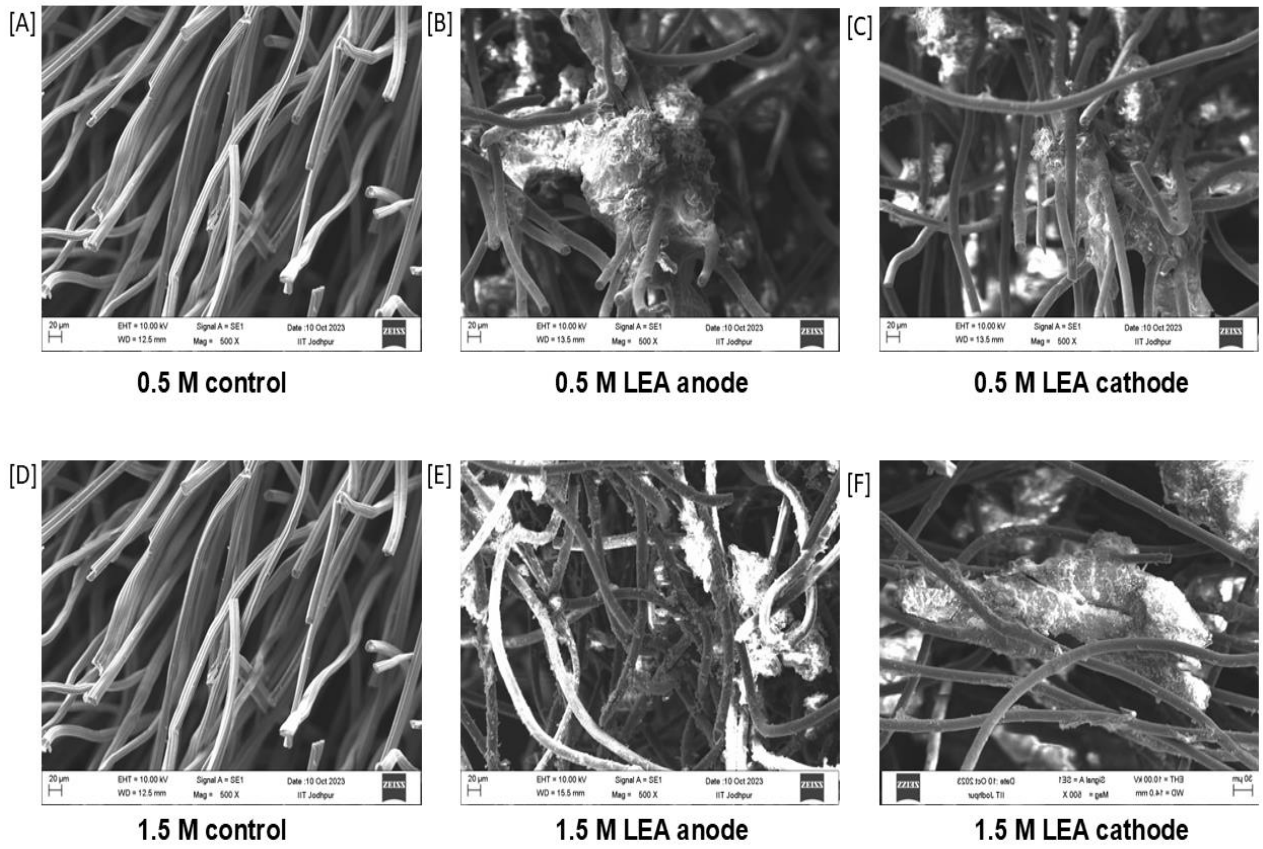


Fig. 4.9: SEM images obtained for the observation of PMFCs operated under different salinity conditions comparing 0.5 M and 1.5 M LEA-fed PMFCs, where the images A and D represent control showing pre-treated graphite felt electrodes without biofilm at 0.5 M and 1.5 M salinity respectively; B and C showing the biofilm on anodic (bacteria) and cathodic (algae) electrodes of 0.5 M system respectively; E and F showing the biofilm on anodic (bacteria) and cathodic (algae) electrodes of 1.5 M system respectively.

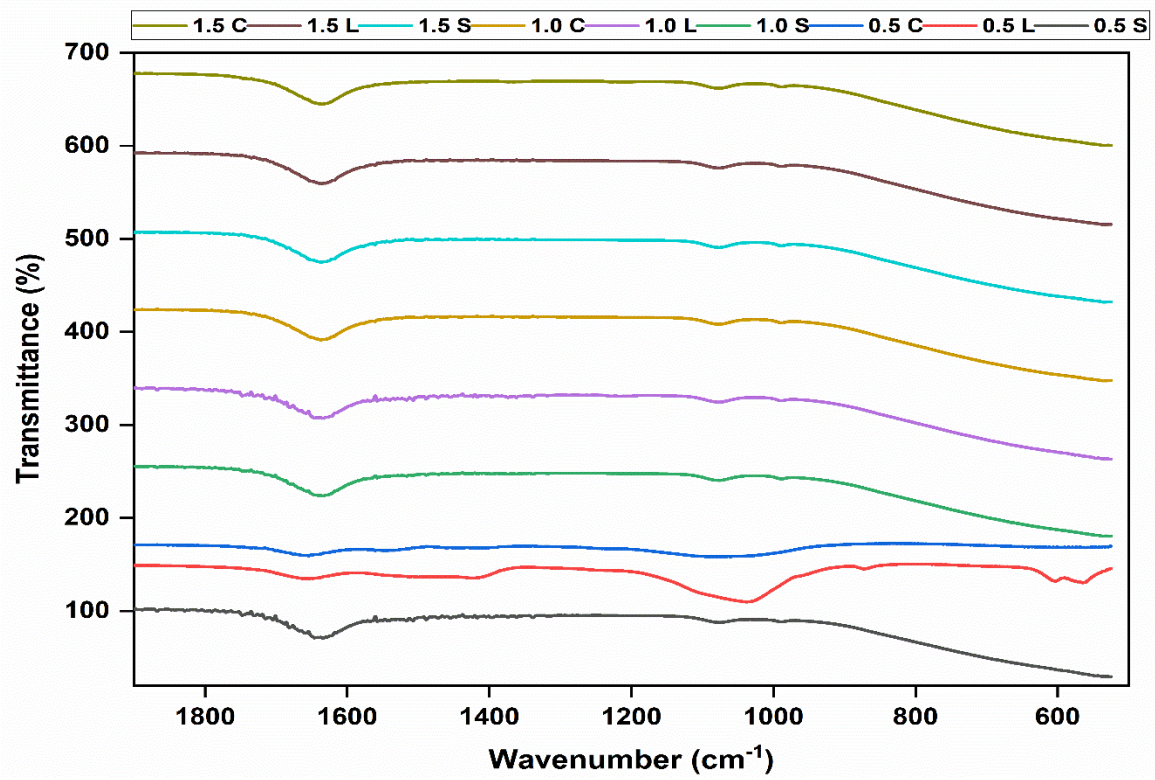


Fig. 4.10: FTIR spectrum bands (in the range of 1900–500 cm^{-1}) for type-IV pili activity in anode biofilms.

4.2.7 Metagenome analysis of anodic biofilm

LEA biomass is a complex substance made up predominantly of polypeptides and hydrates of carbon. The cell walls of algae are made up of complex carbohydrates, and their application necessitates specialized microbial communities. The anodic biofilm was analyzed using 16s rRNA sequencing. The results showed that Proteobacteria was the dominant phylum in both the 0.5 M LEA-fed PMFC and 1.5 M LEA-fed PMFC, with a relative abundance of 62.35% and 74.81%, respectively. This was followed by Bacteroidota (0.5 M-14.74%; 1.5 M-11.51%), Plactomycetota (0.5 M-11.11%; 1.5 M-0.64%), Firmicutes (0.5 M-3.59%; 1.5 M-6.35%), and Actinobacteriota (0.5 M-1.82%; 1.5 M-0.88%) (Fig. 4.11A). The analysis at the class level revealed that Alphaproteobacteria (0.5 M-33.52%; 1.5 M-26.94%) and Gammaproteobacteria (0.5 M-28.54%; 1.5 M-47.71%) were the most prevalent bacterial classes (Fig. 4.11B). The other classes that are present in large quantities are Bacterioida (0.5 M-14.79%; 1.5 M-11.61%), Plectomycetes (0.5 M-11.14%; 1.5 M-0.61%), Bacilli (0.5 M-2.87%; 1.5 M-5.69%), and Actinobacteria (0.5 M-1.42%; 1.5 M-0.88%).

In terms of family composition, the majority of microbes are found in Pseudomonadaceae (0.5 M-14.54%; 1.5 M-1.95%) and Halomonadaceae (0.5 M-1.33%; 1.5 M-31.23%) (Fig. 4.11C). In addition to these, the presence of Rhizobiaceae (0.5 M-1.42%; 1.5 M-0.88%), Pirellulaceae (0.5 M-1.42%; 1.5 M-0.88%), Caulobacteriaceae (0.5 M-1.42%; 1.5 M-0.88%), Lentimicrobiaceae (0.5 M-1.42%; 1.5 M-0.88%), Stappiaceae (0.5 M-1.42%; 1.5 M-0.88%), Marinilabiliaceae (0.5 M-1.42%; 1.5 M-0.88%), and Bacillaceae (0.5 M-1.42%; 1.5 M-0.88%) was also observed. Marinilabiaceae are chemoheterotrophic organisms that derive energy by metabolizing complex substances and can thrive on these substrates. The genera within this family have been observed in marine sediment that contains decomposing algae [Khandelwal *et al.*, 2020]. Microbes belonging to the Lentimicrobiaceae family grow chemoorganotrophically in an anaerobic environment by using carbohydrates as a carbon source [Sun *et al.*, 2016]. Microbial strains belonging to the family Caulobacteriaceae show carbohydrate metabolism under anoxic conditions [Manzoor *et al.*, 2021] and have specific enzymes that can monomerize lignin of the cell wall [Wilhelm *et al.*,

2019]. Pirellulaceae family members utilize sugars as an energy source in an anaerobic environment [Elshahed *et al.*, 2007].

The heatmap displays the quantity of operational taxonomic units (OTUs) in each sample without any modifications or conversions. The red color indicates OTUs with a higher abundance, while the blue color indicates OTUs with a lower abundance. OTUs are groups of sequences that demonstrate a certain level of taxonomic similarity, with each group usually representing a genus. The following genera was observed in the PMFC system: *Pseudomonas* (0.5 M-13.61%; 1.5 M-1.73%), *Blastopirellula* (0.5 M-13.44%; 1.5 M-0.62%), *Lentimicrobium* (0.5 M-8.67%; 1.5 M-3.92%), *Stappia* (0.5 M-8.93%; 1.5 M-0.16%), *Halomonas* (0.5 M-1.21%; 1.5 M-33.26%), *Nitratireductor* (0.5 M-1.15%; 1.5 M-12.96%), *Brevendimonas* (0.5 M-3.78%; 1.5 M-9.34%), *Idiomarina* (0.5 M-2.62%; 1.5 M-4.75%), *Marinilabilia* (0.5 M-1.83%; 1.5 M-1.04%), *Marinobactor* (0.5 M-0.78%; 1.5 M-6.64%), *Advenella* (0.5 M-5.06%; 1.5 M-0.03%), *Pelagibacterium* (0.5 M-3.56%; 1.5 M-0.56%), and *Anaerophaga* (0.5 M-0.14%; 1.5 M-6.16%) (Fig. 4.11D). *Blastopirellula* is a bacterium that thrives in moderate temperatures and relies on seawater to grow by utilizing carbohydrates as its primary carbon and energy sources [Schlesner, 2015]. *Brevendimonas* is a bacterium that breaks down cellulose and shows promise in researching new enzymes involved in cellulose degradation and enhancing the conversion of lignocellulosic biomass into biofuels [Hu *et al.*, 2014]. *Pseudomonas* is a recognized electrogenic microorganism that has been documented in numerous studies on MFCs [Vijay *et al.*, 2018].

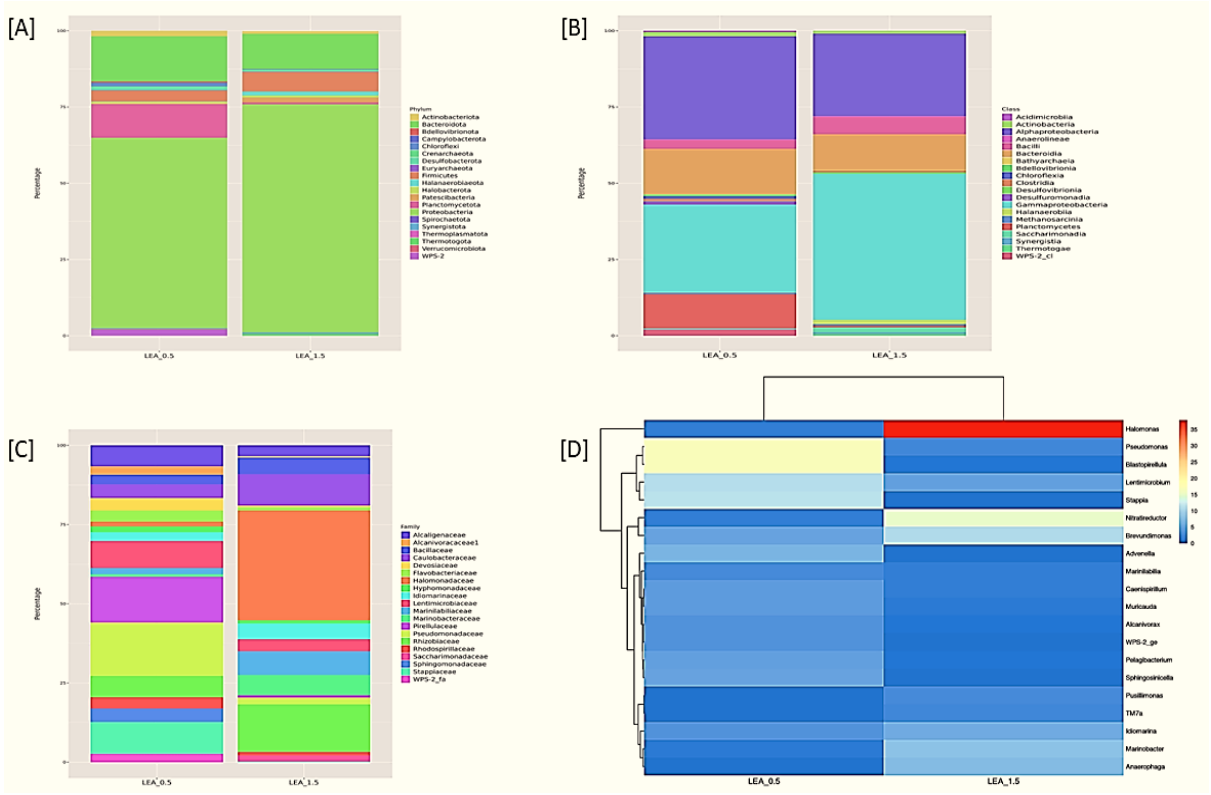


Fig. 4.11: Microbial community structure under different salinity conditions comparing 0.5 M and 1.5 M LEA-fed PMFCs at (A) Phylum, (B) Class, and (C) Family level, (D) A heat map showing the taxonomic assignment for each OUTs where, intense color (Red) represents OTUs having a relatively higher abundance, whereas light color (blue) represents OTUs having a relatively lower abundance.

4.3 CONCLUSIONS

PMFC with a *Dunaliella* at different salinities efficiently treats saline wastewater in outdoor conditions. The 0.5 M LEA-fed operating system showed the highest operating voltage, power, and current density. PMFCs fed with 1.0 M LEA exhibit the highest algal growth, lipid and glycerol concentrations, and the highest β-carotene content. The *Dunaliella*-based PMFCs have the capability to remediate waste sustainably and can be used for large-scale systems. Further investigation is necessary to understand the effect of multiple variables on the overall energy output of the system. Further modified electrodes, efficient separators, and microbial consortium can be tested that facilitate the maximum production of electrical energy.

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