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 and chemicals	Merck/SRL
Culture Media Supplements	Himedia, India
Nafion-117 Proton Exchange Membrane	Nickunj Eximp Pvt. Ltd.
Graphite felt (Electrode)	Nickunj Eximp Pvt. Ltd.
Titanium wire	Mnahar metals, Mumbai
Clayware	Unnada Pvt. Ltd., Jodhpur (Rajasthan)
Plastic buckets	Manish plastic, Jodhpur
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

A.2 METHODS

A.2.1 Sample collection

a) **For algae**

The colored water samples and soil crust with a patch of algae were collected from the fields of Hindustan Salt Ltd. in the Sambhar district of Rajasthan, India. The samples were taken in the autoclaved centrifuge tubes and kept at four degrees before subsequent use. The samples were tested for TDS, Color, resistance, conductivity, salinity, and pH using a multimeter (EUTECH instrument, PC 2700) (Table A.2).

Table A.2: The parameters of the collected water samples from the Sambhar Lake.

Parameters	**Value**
TDS content (ppt)	201.3 ± 0.6
pH	9.18 ± 0.1
Resistance (Ω)	2.47
Conductivity (mS)	209.8 ± 0.2
Salinity (ppt)	239.3

Ultrastructural features and genetic observations were carried out to ensure detailed and unambiguous algae identification. The microscopic images of algae were observed under a compound (Leica) and a fluorescence microscope (Olympus, IXX-83) (Fig. A.1).

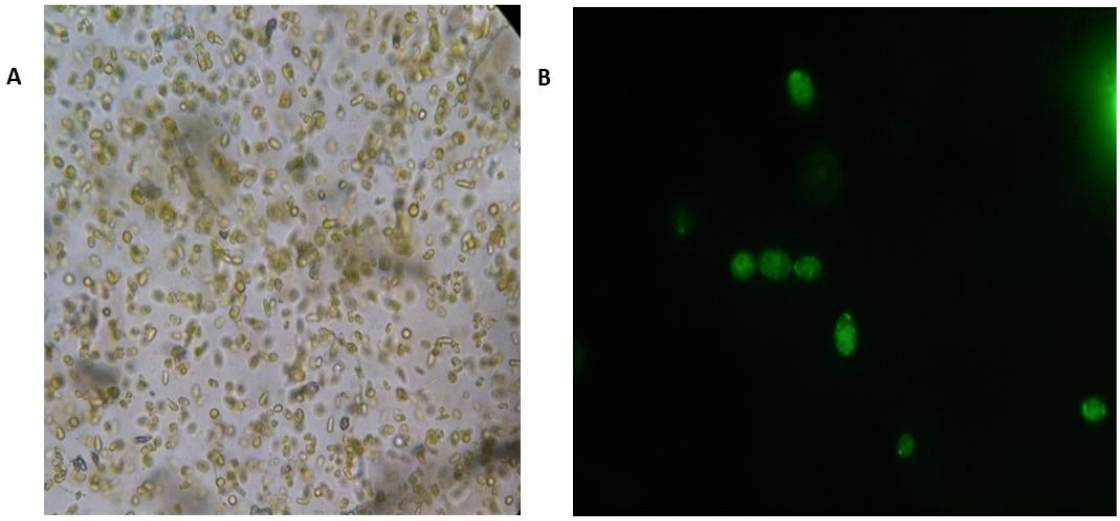


Fig. A.1: A. Compound microscopic image of Sambhar Lake algae *D. salina* showing characteristic shape and size and cup-shaped chloroplast (Left image); B. Fluorescence image of *D. salina* under the FITC filter (495 nm to 516 nm) (Right image).

b) For starch degrading bacteria

Samples were collected in sterilized tubes and bottles from Sambhar Lake, Rajasthan, India, and stored at four degrees centigrade for further use. The samples were tested for parameters like pH, conductivity, and total dissolved solids (TDS) with the help of the multiparameter (made-Eutech) (see supplementary materials). The samples collected from Sambhar Lake were added to the medium containing casein hydrolysate (8 g/l), yeast extract (4 g/l), KH_2PO_4 (0.1 g/l), K_2HPO_4(0.1 g/l), $MgSO_4$ (0.1 g/l), and 1000 mM NaCl at a pH of 7.0 ± 0.2 [Atlas and Atlas, 2004]. The anaerobic halophiles were enriched using the following procedure. Firstly, 60 g of soil sample was added to the freshly prepared anolyte, and the medium was purged with nitrogen gas for one hour to maintain an anoxic environment. The anodic EGB bacteria were adapted to the LEA by mixing two g/l of LEA in the pre-treated inoculum and subjecting it to anaerobic incubation at 30˚C for ten days.

Table A.3 The parameters of the collected water samples from the Sambhar Lake.

Parameters	Value
Color	Pale, green, reddish
TDS content (ppt)	361.1 ± 0.4
pH	9.76 ± 0.03
Resistance (Ω)	2.03 ± 0.07
Conductivity (mS)	170.6 ± 0.5
Salinity (ppm)	199.3 ± 1.8

A.2.2 Algae cultivation

The green microalgae isolated from the Salt Lake, India, was initially grown at the different salinity (0.5 M to 2 M NaCl) in Modified Johnson's media (MJM) [Singh *et al.*, 2019] consisting of macronutrients $MgCl_2.6H_2O$ (1.5 g/l), $NaHCO_3$ (0.043 g/l), $MgSO_4.7H_2O$ (0.5 g/l), $CaCl_2.2H_2O$ (0.2 g/l), KCl (0.2 g/l), KNO_3 (1.0 g/l), KH_2PO_4 (0.035 g/l), Iron solution (Sodium EDTA (94.5 mg/l), $FeCl_3.6H_2O$ (122 mg/l)), and micronutrients $CoCl_2.6H_2O$ (5.1mg/l), H_3BO_3 (61 mg/l), $MnCl_2.4H_2O$ (4.1 mg/l), $(NH_4)_6MO_7O_{24}.4H_2O$ (38 mg/l), $CuSO_4.5H_2O$ (6 mg/l), $ZnCl_2$ (4.1 mg/l), pH 7.5, and optimum conditions (temperature 28 ± 2º C, light intensity 9800 lux, photoperiod dark/light regime of 16/8). The medium was sterilized by autoclaving. The Algae sample was streaked successively on MJM-agar plates where 2% agar was mixed with MJM media to obtain the pure strain that was used for further studies.

A.2.3 Lipid extraction and pre-treatment of LEA biomass

The mass-scale cultivation of the microalgae takes place in a 5-liter capacity flask within an algae growth chamber. After 30 days of culture, algal biomass was harvested and washed multiple times to remove salt, followed by drying. Dried algal biomass was processed for lipid extraction [Bligh and Dyer, 1959]. After the lipid extraction, the left biomass was further pretreated for LEA formation. The LEA was subjected to an oven temperature of 80 ˚C for 24 hours to eliminate the organic solvent. Additionally, the resulting dried biomass was pulverized using a mortar and pestle. The LEA obtained at this step was further used as the anodic substrate for the EGB.

A.2.4 Analytical techniques

a) DO

The DO at the PMFC of the cathode was tracked with the help of a DO meter (HI-9829, Hanna Instruments, USA).

b) Carbon dioxide estimation (CO_2)

The CO_2 concentration in the room was consistently monitored using a CO_2 meter (LT-Lutron, GC-2028).

c) COD

The colorimetric dichromate oxidation method was used to measure COD [Raposo *et al.*, 2008]. Briefly, the diluted sample (2.5 ml) was taken in a COD tube (Merck) and mixed with 1.5 ml of potassium dichromate-mercuric sulfate digestion solution ($K_2Cr_2O_7$ (10.216 g/l), $HgSO_4$ (33 g/l), and H_2SO_4 (167 ml/l) followed by addition of 3.5 ml of silver sulfate (22 g $AgSO_4$ dissolved in 9-pound bottles of concentrated H_2SO_4). All the samples were refluxed at 148º C for 2 hr in a digester (Merck, Spectroquant TR-320), and optical density was measured at 605 nm after the solution cooled down to room temperature.

d) Algal growth estimation

The algae growth rate was recorded by taking the absorbance at 750 nm every alternate day, and cell count was computed using a hemocytometer (RHOM-INDIA, B.S. 748). The specific growth rate (μ) of *D. salina* was calculated after algal growth reached the end of its exponential growth phase with the help of the formula:

$$\mu = \frac{\ln n_2 - \ln n_1}{t_2 - t_1} \qquad \text{(A.1)}$$

Here, n_1 and n_2 are no. of cells on t_1 and t_2 days, respectively, and the generation time (g) of algae was computed using the given formula:

$$g = \frac{0.693}{\mu} \qquad \text{(A.2)}$$

A.2.5 Electrochemical analysis

a) Measurement of power density and voltage

Voltage and current measurements were done across the 1000 Ω and 100 Ω resisters at different time intervals with the digital multi-meter (Metravi-451) for both works, respectively. The polarization curve was made by recording the potential and current with the variation of exterior resistance from 22000 Ω to 5 Ω over the fixed time interval of one hour [Vijay *et al.*, 2018]. Power density (mW/m^2) was determined by the division of the cumulative power with the surface area of graphite felt with the help of the following formula: P = V × I/ Surface area of the electrode, where V and I are voltage in volts (V), current in ampere (A) respectively [Vijay *et al.*, 2018]. Similarly, the current per unit electrode area was calculated employing the formula: I = I/Electrode surface area [Vijay *et al.*, 2018].

b) CE

The CE of the PMFCs was calculated to quantify the electron recovery by using the equation (1);

$$CE = \frac{M \int_0^t I \, dt}{f \, n \, V \, \Delta COD} \qquad \text{(A.3)}$$

This equation illustrates the relationship between current, measured in amperes (A), and the change in time, measured in seconds (t). The symbol M denotes the molar mass of oxygen, f denotes Faraday's constant, n represents the number of electrons needed to reduce 1 mole of oxygen, V denotes the volume of the anodic chamber, and ΔCOD indicates the change in COD from the initial to the final value over a given period. The electrical energy generated by the functioning PMFC was determined by calculating the integral of the power versus time plot obtained during the last operating batch cycle [Khandelwal *et al.*, 2020].

c) CV analysis

The CV for each PMFC was determined using an electrochemical workstation (SP-300 model of Biologic) and a three-electrode setup system. The anode functioned as the working electrode, while the cathode served as the counter and Ag/AgCl as the reference electrode. The scan rate for each PMFC examination was 10 mV/s across the voltage range of -1.0 V to +1.0 V. The setups were examined during the final batch cycle when PMFC exhibited high operational potential.

A.2.6 Value-added product estimation

a) Total lipid extraction

Total lipid from the *D. salina* was harvested by the modified Bligh and Dyer method by using methanol and chloroform as the main constituents as described in the literature [Bligh and Dyer, 1959].

b) Glycerol estimation

The glycerol content was determined as per the method reported by Chen *et al.* [2009]. Briefly, the harvested algal cells (after centrifugation at 4500 rpm for 10 min at room temperature), were washed with deionized water (DI) thrice. The washed cells were centrifuged, and pellets were suspended in a solution consisting of 1.5 ml:0.2 ml DI water and chloroform. Sonication followed by centrifugation was done. Sodium periodate reagent (3 mM sodium periodate + 100 mM ammonium acetate in 6% acetic acid solution) was added to the supernatant. The aliquots were mixed thoroughly. Acetylacetone reagent (acetylacetone: isopropanol; 1:99, v/v) was then added to the mixture [Singh *et al.*, 2019]. The samples were incubated at 60º C in the water bath for 30 min. The optical density was taken at 410 nm using a spectrophotometer (SHIMADZU Corp., UV-1800).

c) Total Carotenoid extraction

Total carotenoid was measured as per Aluç *et al.* [2018] procedure. The biomass was freeze-dried, and 0.1 g of the same was suspended in a 3 ml solution of hexane-acetone-ethanol-toluene (10: 7:6:7, v/v/v/v). This was followed by shaking for 60 min. The saponification process was done by adding 2 ml of 40 % KOH (methanolic) followed by incubation in the dark for 16 hr at 25ºC. The next step involved the addition of hexane (3 ml), mixing, and one-minute settling. The aqueous and organic layers were separated after adding 10 % sodium sulfate solution, followed by shaking the solution for one minute. The upper organic layer contained the total carotenoids [Aluç *et al.*, 2018]. The organic layers were pooled, and the solvent evaporated. The dried aliquots were solvated in the mobile phase before being filtered (through 0.22 µm filter material polyether sulfone). All the experiments were performed in low light with nitrogen gas purging to arrest any isomerization of the carotenoid [Aluç *et al.*, 2018; Chen *et al.*, 1991].

A.2.7 FTIR

a) For LEA

An FTIR analysis was conducted using a Bruker-70V instrument from the United States to compare the primary functional groups in intact algal and LEA biomass. The spectrum was recorded within the wavelength range of 400-4000 cm^{-1}.

b) For anodic biofilm

As previously explained, the pili expression was conducted using FTIR-based spectral analysis [Nath and Ghangrekar, 2020]. Two methods were available for detecting pili in biofilm. An approach entails allowing the electrode to dry naturally in the air for 48 hours at room temperature, eliminating any contaminants from the surface of the electrode, and subsequently blending it with potassium bromide using a mechanized blending tool for pellet formation. Afterward, the pellet was transferred onto the ZnSe windows for scanning. The second approach involves delicately cleansing the biofilm scraped from the anodic electrode, suspending it in distilled water, and subjecting it to vertexing to ensure thorough mixing. The FTIR spectra were obtained between 700 and 2000 cm^{-1}, and 64 scans were taken for each sample.

A.2.8 SEM

Anodic and cathodic bio-film were examined after completion of the operation with the help of SEM after gold sputtering (SEM, Carl-Zeiss, EVO-18), and electrode without biofilm was also examined as a blank to acknowledge the biofilm formation.

A.2.9 HPLC

The samples were subjected to HPLC for separation, quantification, and identification (WATERS-2489), 2489 UV/Visible detector, and system software Empower. A C-18 column (4.6mm * 250 mmL, 5.0 µm) separated the molecules. Mobile phases (MP) contained solution A water: methanol**:** acetonitrile**:** (2:84:14) and solution B dichloromethane or methylene chloride (100%). The mobile phase flow rate was 0.6 ml/min [Aluç *et al*., 2018].

The gradient method was applied [Aluç *et al*., 2018] with an initial flow containing 100 % A, then 95 % A and 5 % B for 8 min, 100 % A for 9 min, 75 % A and 25 % B for 25 min, 72 % A and 28 % B for 30 min, 45 % A and 55 % B for 50 min, and back to 100 % A for 52 min. The peaks were read at 450 nm. The high-pressure limit was 1500 psi, the sampling rate was 1 point/sec, and the injection volume was 20 µl. Synthetic β-carotene (C452-5MG, Sigma) was used as standard.

A.2.10 Strain identification

The molecular identification of *D. salina* was carried out by isolating genomic DNA (gDNA) using a HIMEDIA HiPurA® marine algae DNA purification kit. The gDNA was amplified using universal primers, NS1-GTAGTCATATGCTTGTCTC; NS4-CTTCCGTCAATTCCTTTAAG (Eurofins Genomics India Pvt. Ltd), and PCR was carried out in the thermocycler (Agilent technologies, Super cycler-8800) under the given conditions, 95º C of initial denaturation (3min), then 35 cycles of denaturation at 95º C for 30sec, annealing at 55º C for 30sec, extension at 72º C for 1min30sec and, final extension at 72º C for 5 minutes. The Basic local sequence alignment tool (BLAST) tool was used for data analysis [Taşkan *et al*., 2021]. The sequence was submitted to the NCBI GeneBank, with an assigned accession number (ON753766, ON753767). The algae were named *D. salina* after the sequence's taxonomic positioning and phylogeny construct by MEGA 11 software [Vyas and Chhabra, 2017].

A.2.11 Metagenomic analysis of anodic biofilm

In the form of biofilm, the anodic bacterial consortia were manually removed from the electrodes of operating systems 0.5-LEA and 1.5-LEA. The biofilm scratch was washed twice with deionized water and stored in phosphate buffer saline (PBS) for further use. Individual biofilms were subjected to DNA extraction using a soil DNA isolation kit (Himedia) to obtain total genomic DNA. The amplification of 16S rRNA gene fragments was carried out using the primer pairs P63F (GACTCCTACGGGAGGCAGCAG) and 518R (ATTACGGCGGCTGCTGG) [Vijay *et al*., 2018]. PCR amplification was conducted using an Agilent Supercycler 8800 thermocycler. The reaction mixture consisted of 50µl, containing 200µM dNTPs (3µl) from GeNei, 1µM of each primer (3.5µl) from Bioserve, 3 units/µl Taq polymerase from GeNei, 1x reaction buffer (5µl) from GeNei, and 25ng genomic DNA (5µl). The process of thermal cycling involved an initial denaturation step at a temperature of 95°C for 5 minutes. This was followed by 40 cycles of 30 seconds at 95°C, 45 seconds at 55°C, and 30 seconds at 72°C. Finally, there was an extension period of 10 minutes at 72°C. The 500-base pair PCR product was purified using the QIAEX-11® gel extraction kit and sequencing at Lifecell international pvt. ltd., Chennai, India.

The amplicon libraries were constructed and sequenced. The reads that passed the quality control examination were imported into mothur v1.46.1 [Schloss *et al*., 2009], and the pairs were aligned to create contigs. A post-assembling procedure was conducted to eliminate contigs that exhibited ambiguous base calls. The contigs of superior quality were examined for complete sequences, and any duplicates were combined. Although the primers for the experiment were designed for 16s bacterial rRNA, there are good chances for non-specific amplification of other regions. The contigs were aligned to a SILVA v138.1 database [Quast *et*

al., 2013] dedicated to 16s rRNA to rectify this issue. Depending on the variable region being amplified, most contigs were aligned to their respective regions in the database. A post-alignment processing procedure was conducted to eliminate ambiguous contigs and align with other regions within the database. Following the previously mentioned methodology, the gaps and overhang at the ends of the contigs were removed. The cleaned contigs are then processed for chimera removal, which may have formed due to amplification errors. The contigs containing chimeric regions were identified using the UCHIME v4.2.40 algorithm [Edgar *et al.*, 2011]. The filtered contigs were analyzed and categorized into taxonomic labels using the SILVA v138.1 database. A clustering process was employed to group the contigs into operational taxonomic units (OTUs). After the classification, the OTU abundance was estimated.

A.2.12 Statistical interpretation

The standard deviation was computed using the same settings in three separate trials to confirm the reproducibility of data and minimize any potential errors.

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List of publications

Peer-reviewed International Journals (Published)

1) **Mishra, A.**, and Chhabra, M. (2022). Performance of photo-microbial fuel cell with *Dunaliella salina* at the saline cathode. *Bioresource Technology Reports*, 19, 101199.
2) **Mishra, A**., and Chhabra, M. (2023). Co-culturing *Chlorella vulgaris* and *Cystobasidium oligophagum* JRC1 in the microbial fuel cell cathode for lipid biosynthesis. *Environ Sci Pollut Res*, 1614-7499.
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4) Chalka, Vandana; **Mishra, Akanksha**; Chhabra, Meenu; Rangra, Kamaljit; Dhanekar, Saakshi Prototype for Rapid Electrochemical Detection of *Escherichia coli* in Water using Modified Screen-Printed Electrodes. ACS Appl. Bio Mater. 10. 1021. https://doi.org/10.1021/acsabm.4c00952.
5) **Mishra, A**., and Chhabra, M. (2025). Removal of organic pollutants from saline waste using *Dunaliella* and halophilic bacteria in photomicrobial fuel cells. *Biomass and Bioenergy*, 195, 107713. https://doi.org/10.1016/j.biombioe.2025.107713.

NCBI Sequence Submission

1) **Mishra, A**., and Chhabra, M., (2022). *Dunaliella salina* isolates ebl1 and ebl2 small subunit ribosomal RNA gene, partial sequence. Accession number: ON753766, ON753767.

Book chapter

1) **Mishra, A.**, and Chhabra, M. (2023). Microalgae cultivation in bio-electrochemical systems. Algal systems for resource recovery from waste and wastewater, 129. IWA publications. doi: 10.2166/9781789063547_0129.
2) **Mishra, A.**, Khandelwal, A., Chhabra, M., and Lens, P. N. (2024). Algae-powered versatile microbial fuel cells for energy and resource recovery from different waste streams. Emerging Trends and Advances in Microbial Electrochemical Technologies (pp. 627-646). Elsevier. https://doi.org/10.1016/B978-0-443-15557-4.00022-8.

Patents

1) Arti Sharma, Ajay Kumar, **Akanksha Mishra**, Amitap Khandelwal, Meenu Chhabra, Arvid Kumar, Hardik B. Kothadia, Shefali Gupta, Prasanjeet Sarkar. "Onsite CO_2 capture from industrial gaseous emissions". Patent no. 202211028952. Indian Patent. (Under examination)
2) **Akanksha Mishra**, Arti Sharma, Meenu Chhabra. "Electrochemical potentiometric device". Patent no. 202311016263. Indian Patent. (Provisional)

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