Chapter 3

Bioelectricity generation using sweet lemon peels as anolyte and cow urine as catholyte in a yeast-based microbial fuel cell

Bioelectricity generation using sweet lemon peels as anolyte and cow urine as catholyte in a yeast-based microbial fuel cell

3.1 Introduction

A MFC is a system where the metabolic activity of microorganisms converts chemical energy into electrical energy [1] – [3]. MFC is based on an extracellular electron transfer system in electrogenic microbes to transport electrons to the anode. The substrate composition in an anode chamber remarkably affects the microbial consortia of anode biofilm, coulombic efficiency and power density of MFC [349]. An extensive range of substrates has been explored for MFC. Earlier, MFC technology was operated using low molecular weight substrates like acetate, butyrate, fructose, glucose, malate, maltose, propionate, succinate, sucrose, lactate, trehalose and xylose [350]. Bai et al. (2021) used co-culture of *Saccharomyces cerevisiae* and *Shewanella oneidensis* to utilise glucose-xylose as a substrate in MFC and obtained the maximum power density of 238.7 ± 14.5 mW/m^2 [351]. Several efforts have been made in order to increase charge transfer rate from yeast cell to electrode [352], [353]. An MFC was constructed utilising yeast modified by pyrrole and compared to the features of an MFC based on non-modified yeast. The MFC with modified yeast generated maximum power density was 47.12 mW/m^2, which was significantly higher than the MFC with unmodified yeast [354]. Andriukonis et al. (2022) tested two electroconductive polymers, polypyrrole and polydopamine for yeast cell modification. Results indicated an increase in the charge transfer from the modified yeast cells to the anode [355]. Ali et al. (2017) obtained a maximum power density of 3.6 ± 1.6 mWm^2 from fructose, $8.606 \pm$ mWm^2 from sucrose and 136 ± 87 mWm^2 from glucose [356]. Complex substrates such as cellulose, chitin, dextran, starch, molasses and pectin have been utilised [350]. Cellulose showed a maximum power density of 44 mW/m^2 in a single chamber air cathode MFC [357]. Ebadinezhad et al. (2019) evaluated MFC performance utilising different substrates in a dual chamber MFC and obtained maximum

power density of 10, 315, 328, 349, 376, 388 and 513 mW m^{-2} from methanol, starch, molasses, sucrose, ethanol, lactose and acetate respectively [54]. Pure organic substrates like glucose, acetate, lactate, sucrose or different synthetic growth media are too expensive. Organic waste and different wastewater sources can serve as an inexpensive alternative substrate for MFC operation [337], [358]. Microbes in the anode chamber can efficiently utilise organic and inorganic nutrients in the wastewater as substrate and thus simultaneously contribute to the bioremediation of wastewater with bioelectricity generation. Wastewater emanating from the food processing units [80], starch manufacturing plants [359], swine farms [360] and meat production industry [361], fruit and vegetable wastes [338], food waste [362], and dairy wastewater [363] have been used as cost-effective substrates in MFC. These wastewater sources have been explored for effluent treatment and recovery of their complex carbon source through microbial biomass production [350]. Agricultural wastes such as corn stover [364], cashew apple juice [365], orange peel [23] and lemon peel [338] have also been tested as substrate after pre-treatment. Citrus fruits, grown in over 100 countries, thrive in tropical and subtropical regions. Valued in fresh and processed markets, 33% of the sweet lime harvest is used in the processing industry, with about 70% of sweet lime resulting in waste (peels, seeds, and membrane residue). In the present work, a pretreated slurry made up of *Citrus limetta* (Sweet lime) peel has been used as a microbial substrate in the anode chamber. Sweet lime is a native fruit of Asia cultivated mainly in China, Japan, India, Indonesia, Malaysia, Thailand and Vietnam. Sweet lime peel is a waste material generated from the juice processing industries. As the juice yield is less than half of the fruit weight, a large portion of peel and seed wastes are produced every year [366]. Sweet lime peels could be extremely valuable in terms of their remarkable nutritional composition [367] as a substrate in MFC. Sweet lime peel powder has approximately, 17.58% crude fibre, 5.39% protein, 3.39% ash, 1.58% fat [368].

Diluted cow urine, considered as dairy farm wastewater, has been used as a catholyte in the present work. Cow urine is widely available, inexpensive and has high electrical conductivity [369]. Cow urine has a nitrogen to phosphorus ratio of 11:1 and 2.5 parts potassium. Thus, making it a suitable medium for algae growth instead of other synthetic algae culture media [370]. Synthetic algae culture media like chu's medium No.10, bold basal medium, and BG11 are also costly for large-scale application [371]. Many studies are available on the uses of human urine as an anolyte in MFC [15], but no literature available to the best of our knowledge where diluted cow urine has been used as a catholyte and simultaneously supports microalgae growth at cathode chamber. Dairy farm effluents comprise cow urine in bulk, rich in organic molecules and minerals, causing eutrophication when discharged in excess into the freshwater sources [372]. Poor handling and disposal of dairy farm wastewater can cause water and odour pollution [373]. Containing nitrogen compounds and potential pathogens, untreated urine may runoff into water bodies, causing nutrient enrichment and harming aquatic life. Groundwater contamination is a concern when urine infiltrates the ground. In regions with concentrated cattle farming, cumulative impacts amplify the risk of water pollution. By integrating cow urine as a nutrient source in the MFC cathode for microalgae cultivation, present study creates a closed-loop system that not only produces bioenergy but also recycles and reuses valuable nutrients for sustainable agriculture and energy production. Wastewater remediation by conventional methods such as the activated sludge process is an expensive energy option concerning the generation of secondary sludge. Treatment via microalgae is more suitable as it has high treatment efficiency, and nutrients present in the wastewater are recovered in the form of valuable biomass [345]. Zalneravicius et al. (2022) designed a microalgae-based anode by immobilizing glucose oxidase and a microalgae (*Spirulina platensi*s) lysate on multiwall carbon nanotube to facilitate the electron transfer and reduce the open circuit potential drop along the electron transfer pathway [374]. Hence, the use of microalgae becomes

predominantly favourable because it acts as an in-situ oxygen producer, which facilitates enhanced oxygen reduction reaction in the cathode chamber. Further, microalgae also assist in efficiently recovering nitrogen and phosphorous from the cow urine [375]. Therefore, *Chlorella pyrenoidosa* was used as a cathode biocatalyst along with simultaneous nutrient recovery in the form of biomass from cow urine. *Chlorella* species has been widely used in wastewater remediation due to its high growth rate, robustness, huge biomass productivity and high removal efficiency of nutrients [345].

Consequently, an extensive diversity of microorganisms has been explored for power generation in MFC. Potter (1911) constructed MFC for the first time by employing glucose as a feed with baker's yeast and bacteria as inoculum. Later, tremendous efforts have been made to evaluate the performance of yeast as anode biocatalyst [1] yet none of them assessed the performance of baker's yeast with sweet lime peel waste. Yeast is a eukaryotic cell having features of an ideal biocatalyst for MFC. *Saccharomyces cerevisiae* is non-pathogenic and can grow on a vast range of organic substrates [16].

Therefore, the present chapter aims to simultaneous bioelectricity generation and wastewater treatment at both anode and cathode chambers. H-type dual chamber MFC was fabricated to generate bioelectricity using sweet lime peel slurry as substrate in the anode chamber and diluted cow urine as catholyte in the cathode chamber. Pectin and cellulose are the key components of sweet lime peel. For the better cellulose digestion co-culture of an isolated cellulolytic bacterium with *Saccharomyces cerevisiae* was used as inoculum at the anode chamber. Co-culture of *Saccharomyces cerevisiae* with cellulolytic bacterium provides better degradation of sweet lime peels than *Saccharomyces cerevisiae* alone. Anolyte and catholyte used in this study are considered equivalent to wastewater effluents from juice processing industries and dairy farms, respectively.

3.2 Material and methods

3.2.1 Raw material collection

Sweet lime peels were collected from a local fruit shop located inside the institute campus in November 2021. Cow urine was collected in a dark glass bottle from a dairy farm located in the university campus and immediately stored at 4ºC for further study. The trunk and roots of the Teak tree (located inside campus) were scraped and collected for isolation of cellulolytic bacteria.

3.2.2 Substrate preparation for the anode and its pre-treatment

Fresh Sweet lime peels were washed twice with distilled water. After that, peels were grounded into a mixer grinder in order to make a fine slurry. In the pre-treatment process, 100 ml of this fine, concentrated slurry was diluted with 900 ml of distilled water and pretreated at high temperature (121ºC) and high pressure (15 psig) for 15 min in an autoclave. The pretreated sweet lime peel slurry (HPTCL: High pressure temperature treated sweet lime peel slurry) was then used as substrate (anolyte) in the anode chamber of MFC.

3.2.3. Substrate characterisation

Elemental analysis of the substrate was done by CHNS Analyser (Euro EA 3000, Elemental analyser make, Italy). Ash [376], moisture [377], volatile content [378] and total carbon content were analysed by proximate analysis as provided in the American Society for Testing and Materials methods (ASTM) [379]. The total sugar content of HPTCL was determined by the Anthrone method, and reducing sugar content was determined by the DNSA (3,5-dinitrosalicylic acid) method. Ammonium-nitrogen, nitrate-nitrogen and phosphate-phosphorus were determined by standard methods provided by American Public Health Association (APHA) [380]. The pH of the slurry was determined by a pH meter (Eutech pH tutor, USA). The chemical oxygen demand (COD) of prepared HPTCL was estimated by the

closed reflux method. Standard methods of APHA were used to analyse the total dissolved solids (TDS) and total solids (TS) of water [380].

3.2.4. Catholyte preparation & characterisation

The catholyte for the MFC operation was prepared by diluting 10 ml of fresh cow urine in 990 ml of distilled water and then autoclaved at 121°C at 15 psig pressure for 15 mins. Hence, cow urine has been diluted 100 times. Ammonium-nitrogen, nitrate-nitrogen and phosphate-phosphorus were determined by standard methods provided in APHA guidelines [380]. The pH of the cow urine was determined by a pH meter (Eutech pH tutor make, USA). The catholyte's chemical oxygen demand (COD) was estimated by the closed reflux method [380]. Standard methods of APHA were used to analyse the total dissolved solids (TDS) and total solids (TS) of water [380].

3.2.4 Anode biocatalyst

Two kinds of anode biocatalyst were used in the present work (i) *Saccharomyces cerevisiae* (Baker's Yeast- KOTHARI'S four-season instant Yeast, India), and (ii) isolated cellulolytic bacteria (CB). The utilisation of CB as anode biocatalyst was related to the microbial digestion of complex saccharides in the sweet lemon peel.

3. 2.4.1 Isolation and identification of cellulolytic bacteria

A salt media was prepared using KH_2PO_4 2 g; $NaNO_3$ 2.5 g; NaCl 0.2 g; $CaCl_2 \cdot 6H_2O$ 0.1 g; $MgSO_4$ 0.2 g; in a litre of distilled water. Whatman filter paper no. 1 was added into the above salt media as the sole carbon source. Prepared filter paper salt media was inoculated with teak wood scrap to isolate CB in Erlenmeyer Flasks (250 ml capacity). This culture was incubated for a week in a shaking incubator at 37°C and 180 rpm. Microbial colonies capable of utilising

cellulose were isolated on cellulose agar media (pH 6.8-7.2) composed of cellulose 2.0 g; agar 15 g; $MgSO_4$ 0.25 g; KH_2PO_4 0.5 g; and one litre distilled water [381]. Later, 10 ml (0.1%) congo red was poured on replica petri-plates. After 20 minutes of congo red incubation, Petri plates were rinsed with 1 M NaCl for 10 minutes. Congo-Red was used as a rapid indicator for sensitive screening of cellulose degradation by microbes in cellulose agar media. After that, plates were observed for a clear white zone nearby the microbial colonies [382]. The clear white zone formed by microbial isolates indicated cellulase activity [381], [383]. Pure culture of the isolated CB underwent 16s rRNA sequencing.

3.2.4.2 Isolation of genomic DNA

Genomic DNA of CB was obtained using the procedure instructed in [384] and [385] [384], [385]. The CB isolate was collected and homogenised with 1 ml of extraction buffer. The homogenate was poured into a microfuge tube (2 ml). 1 ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added. Tubes were centrifuged at 14,000 rpm for 15 min at room temperature. 1 ml of supernatant was collected, and the same volume of chloroform: isoamyl alcohol (24:1) was mixed and centrifuged at 14000 rpm for 10 min. Again, the supernatant was extracted. In order to precipitate DNA from the solution, 0.1 volume of sodium acetate (3M, pH 7.0) was added with 0.7 volume of Isopropanol and incubated for 15 min at room temperature. After incubation, the tubes were centrifuged at 14,000 rpm for 15 min at 4ºC temperature. DNA pellet obtained after centrifugation was rinsed thoroughly with 70% ethanol and briefly with 100% ethanol, followed by air drying. The DNA was dissolved in Tris-EDTA (Tris-Cl 10 mM, EDTA 1 mM, pH 8.0). To get rid of RNA, 5 µl of RNAse A (10 mg/ml) was added to the DNA. RNAse A must be free of DNAse.

3.2.4.3 16S rRNA gene sequencing

For microbial identification, the 16s rRNA gene of the CB was amplified via polymerase chain reaction (PCR). 129 ng of isolated genomic DNA was used for amplification along with 10 pM of each primer. For 16s rRNA gene amplification 5'- GGATGAGCCCGCGGCCTA-3' forward primer and 5'-CGGTGTGTACAAGGCCCGG-3' reverse primer was used. The PCR product obtained after amplification was validated by agarose gel electrophoresis (70 Volt, 2 h). The amplified 16s rRNA gene was separated from agarose gel and underwent sequencing using the primers as mentioned above. The nucleotide sequence obtained was evaluated by the Basic Local Alignment Search Tool (BLAST), NCBI. The phylogenetic tree was created for the CB with the reference sequence accessible in the GenBank (NCBI). ClustarW alignment tool was used for the nucleotide sequence alignment. The phylogenetic tree was built by MEGA X software applying the Tamura-Nei model and the Maximum Likelihood method. The 16s rRNA sequence of CB was submitted to the GenBank (NCBI) to obtain an accession number.

3.2.5. Microalgae culture

Chlorella pyrenoidosa (NCIM No. 2738) was acquired on an agar slant from the National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (NCL), Pune, India. The strain was revived in a 500 ml Erlenmeyer flask with a sterilised Bold Basal medium (250 ml). Flask was set aside in a shaking incubator lightened by 20 W cool fluorescent tube lights (2500 lux, continuous photoperiod) at 120 rpm and 25°C. After revival, the strain was acclimatised to grow in 100 times diluted cow urine (sterilised) after two weeks of growth. The adapted microalgae biomass was used as cathode biocatalyst (inoculant) at the cathode. After experiments (30 days), catholyte obtained from catholyte was filtered using a pre-weighted filter paper. Algae biomass collected on filter paper was then placed in an air oven at 105 ºC

overnight (drying). The final weight of dried filter paper was taken to measure algae biomass weight.

3.2.6. MFC construction

Three H-shaped dual chamber MFCs were fabricated by using two plastic containers (700 ml) and connected via an agar salt bridge (2-5% agar in 1M KCl (w/v)) as shown in Figure 3. 1. All the three MFCs had the same anode surface area, anode orientation, anode chamber volume, cathode material, cathode surface area and cathode chamber volume. The anode was prepared using rectangular stainless-steel mesh with mesh size 13 and a total area of 60 cm^2 (grade: 304). Cylindrical graphite rods of 10 cm length and 0.5 cm radius was used as cathode. Copper wires were used for connecting the cathode and anode. An air pump sparged the air (3L/min) into the cathode chamber. An external resistance of 1000 Ω was connected between the electrodes in each MFC.

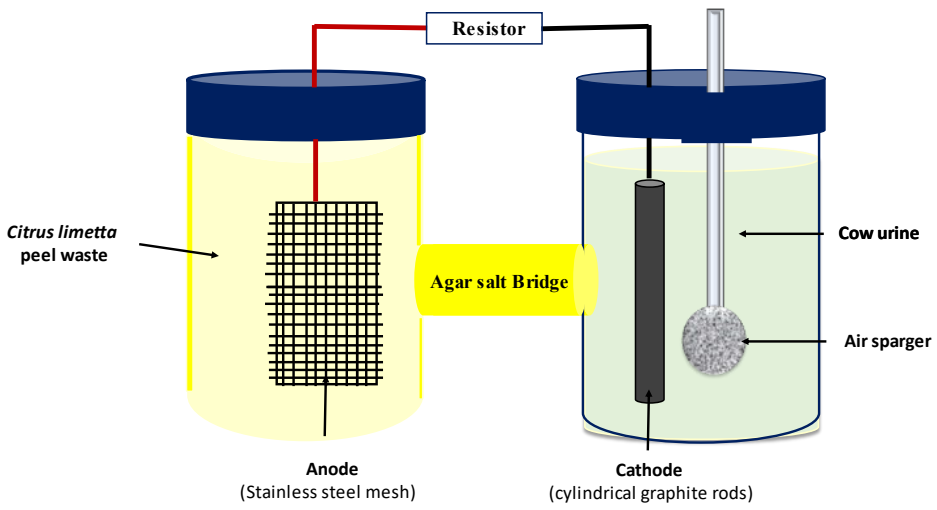

Figure 3. 1. Schematic representation of Dual chamber MFC.

3.2.7. MFC operation

Three dual-chamber H-shaped MFC (working volume 500 mL) was fabricated and operated as PS (pretreated slurry)-MFC, PSY (pretreated slurry with yeast)-MFC and PSYB (pretreated slurry with yeast-bacteria)-MFC as shown in Table 3. **1**.

Table 3. 1. Substrate and inoculum used at anode and cathode chamber.

MFC	Anolyte	Pre-treatment	Anode biocatalyst	Catholyte	Cathode biocatalyst	Aeration
PS	Pretreated slurry (100ml/L)	High temperature (121ºC) and 15 psig pressure	No	Cow Urine (100x diluted)	No	No
PSY	Pretreated slurry (100ml/L)	High temperature (121ºC) and 15 psig pressure	*S. cerevisiae* (1% v/v)	Cow Urine (100 times diluted)	No	Aeration (3L/min)
PSYB	Pretreated slurry (100ml/L)	High temperature (121ºC) and 15 psig pressure	*S. cerevisiae* (1% v/v) + *CB* (1% v/v)	Cow Urine (100x diluted)	*Chlorella pyrenoidosa* (NCIM No. 2738) (3% v/v)	Aerated (3L/min)

PS: pretreated slurry; PSY: pretreated slurry with Yeast; PSYB: pretreated slurry with yeast bacteria.

The MFC set-up was washed and rinsed with 70% ethanol to eliminate the microbial contaminants. After this, for sterilisation purposes, all MFC setups were exposed to UV light inside the laminar hood for 20 minutes. The PSY-MFC was initially acclimatised with *S. cerevisiae* (1% v/v) in a YPD medium, and PSYB-MFC with *S. cerevisiae* and CB isolate (1% v/v) into glucose as carbon source till a stable voltage generated (7 days). For acclimatisation of PS-MFC, glucose solution (20 g/L) was fed into the anode chamber without any external inoculation. During acclimatisation cathode compartment of each MFC was loaded with diluted cow urine, whereas *Chlorella pyrenoidosa* (3% v/v) was inoculated at the cathode chamber of PSYB-MFC. Light intensity was controlled using electronic regulator and measured using Lux meter. Once this initial acclimatisation (7 days) was complete and generated stable voltage, a series of batch experiments were carried out by feeding MFC with HPTCL slurry.

PS-MFC was operated with HPTCL slurry at the anode chamber without external inoculant, whereas at the cathode, sterilised cow urine (100 times diluted) was used as catholyte. Next, PSY-MFC was set up using *S. cerevisiae* (1% v/v) as biocatalyst with raw sweet lime peel slurry in the anode chamber and sterilised diluted cow urine was used with continuous aeration at a rate of 3L/min in the cathode chamber. PSYB-MFC was operated by using *S. cerevisiae* (1% v/v) and isolated bacterial culture (1% v/v) as anode biocatalyst with HPTCL slurry in the anode chamber, and *Chlorella pyrenoidosa* (3% v/v) was inoculated as cathode catalyst (under 2000 Lux; continuous light intensity) with diluted cow urine as catholyte. The cathode chamber was under continuous aeration of 3L/min. In PCLY-MFC, *S. cerevisiae* (1% v/v) and in PCLYB-MFC, co-culture of *S. cerevisiae* and isolated bacteria (1% v/v) was fed at the interval of every 7 days.

3.2.8. MFC characterisation

The voltage generated during the operation was measured by a multi-meter (Mextech Mas 8301 make, India) against external resistance of 1000 Ω. Once voltage was stabilised for several hours, the voltage was recorded for polarisation studies. MFCs were connected to resistors ranging from 10 to 15000 Ω, and a multi-meter was used to measure the voltage of MFC at each resistance. The current was computed using Ohm's law (Eq.3.1), and power was calculated from Eq. 3.2. Power (P) produced by current (I) passing through an electric potential difference (V) was measured in milliwatts (mW) according to Eq. 3.2. The internal resistance value (R_{int}) for each MFC was calculated from the slope of the I/V plots [386] from Eq. 3.3:

$$I = \frac{V}{R} \tag{3.1}$$

Where, V is voltage (mV), I is current (mA), and R is external resistance (Ω) across MFC.

$$P = I.V \tag{3.2}$$

$$V = E_{cell} - I\,R_{int} \tag{3.3}$$

Where E_{cell} is the electromotive force and R_{int} is the internal resistance of MFC. In order to obtain power and current density, power (P) and current (I) were divided by the surface area (A) of the anode (0.006 m^2). Eq. 3.4 was used to compute the power density (mW/m^2).

$$PD = \frac{I \times V}{A} \tag{3.4}$$

Where PD stands for power density (mW/m^2), A denotes the surface area of the anode (m^2), V denotes the voltage (mV), and I represents the current (mA). Eq. 3.5 was used to obtain the current density (mA/m^2) [387].

$$J = \frac{I}{A} \tag{3.5}$$

Where J stands for current density (mA/m^2), when the feed sample was withdrawn from the MFC at the end of the treatment procedure, the COD (mg/L), nitrate-nitrogen (mg/L), ammonium-nitrogen (mg/L), Phosphate-phosphorous (mg/L) were measured. The treatment efficiency (TE) represents the percentage of COD eliminated throughout the operational time. It was determined using the formula in Eq. (3.6) [387].

$$TE = 100 \times \frac{\mathrm{COD_{t=0} - COD_{t=f}}}{\mathrm{COD_{t=f}}} \quad (3.6)$$

where, $COD_{t=0}$ and $COD_{t=f}$ the initial and final COD of the wastewater. The removal percentage for phosphate-phosphorous, nitrate-nitrogen, and ammonium-nitrogen was also calculated. Columbic efficiency (CE) was determined by integrating the current (I) as a function of time (t). It was compared with the theoretical current derived from the change in chemical oxygen demand (△COD) (Eq. 3.7) [388]:

$$\mathrm{CE} = \frac{8 \int_0^t \mathrm{I\ d(t)}}{\mathrm{F}\, V_{anode}\, \Delta\mathrm{COD}} \times 100 \quad (3.7)$$

where 8 is a constant used for COD, based on MO_2= 32 gram/mole, 4 electrons exchanged per mole of oxygen, F is the Faraday's constant (96485 C/mole – electrons), V is the volume of the anode chamber, and ΔCOD is the change in the COD with time (t).

3.2.9. Statistical analysis

All experiments were performed in triplicate, and average values were reported. All results and error bars were plotted using Microsoft Excel (Version 2019). Calculation of mean values, standard deviation and standard error were calculated by a standard procedure using Microsoft Excel (Version 2019).

3.3 Results and discussion

3.3.1. Ultimate and Proximate analysis of sweet lime peel

Table 3.2 shows the % of C, H, N, S, moisture content, ash, volatile content and fixed carbon content present in the sweet lime peel powder on a dry weight basis. CHNS elemental analysis determines the percentage of carbon, hydrogen, nitrogen, and sulphur present in biomass [23], [338].

Table 3.2. Ultimate and proximate analysis of sweet lime peel powder (dry weight basis)

Element	Amount (%)
C	36.197
H	5.751
N	1.419
S	-
Moisture content	3.33 ± 0.026
Ash	0.49 ± 0.01
Volatile content	96.13 ± 0.03
Fixed carbon content	0.04 ± 0.02

Table 3. 3 reflects the values of total sugar, reducing sugar content and COD of HPTCL (substrate used at anode chamber).

Table 3. 3. Characterisation of Anolyte

Total sugar (mg/L)	24145 ± 157
Reducing sugar (mg/L)	9824.56 ± 109.5
Initial COD (mg/L)	7680 ± 105
Total Dissolved Solid (mg/L)	700

Total Solids (mg/L)	13200 ± 200
pH	6.5
Nitrate-Nitrogen (mg/L)	3.48 ± 0.15
Phosphate-Phosphorus (mg/L)	0.25 ± 0.0
Ammonium-Nitrogen (mg/L)	0.48 ± 0.0

HPTCL had COD of 7680 ± 105 mg/L. HPTCL was also analysed for total sugar and reducing sugar content in order to consider their potential in microbial growth and oxidation for the release of electrons. HPTCL had total sugar and reducing sugar content of 24145 ± 157 mg/L and 20350 ± 109 mg/L, respectively. Hence, the HPTCL substrate was found suitable for microbial growth at the anode and helpful in sustaining power generation [23]. Table 3. 4 shows catholyte characterisation.

Table 3. 4. Characterisation of diluted cow urine wastewater

Initial COD (mg/L)	2386 ± 100
Total Dissolved Solid (mg/L)	100.33 ± 0.58
Total Solids (mg/L)	520 ± 26
pH	7.2
Ammonia (mg/L)	9.8 ± 0.0
Nitrate (mg/L)	3.87 ± 0.026
Phosphate (mg/L)	3.74 ± 0.34

Diluted cow urine had ample organic and inorganic compounds with an initial COD of 2386 ± 100 mg/L. Cow urine is appropriate for microalgae growth in the cathode chamber [372].

3.3.2. 16s rRNA sequencing of isolated bacteria (CB)

On spread plate culture, only one kind of colony formed. All colonies have similar morphology, slightly transparent, raised- convex, and circular (1-2 mm diameter). All colonies on the replica plate were found to be positive (producing clear zone) on cellulose congo-red agar media, as shown in Figure 3. 2A. Gram staining of isolated bacteria revealed that it was a gram-negative rod-shaped bacterium (Figure 3. 2 C and D).

The CB isolate showed maximum similarity with *Enterobacter cloacae strain* HS-6. Gene sequence of *Enterobacter cloacae strain* IIT BHU M2V2 was submitted to the GenBank database (NCBI), and accession number OM214000 was acquired. The evolutionary analysis of *Enterobacter cloacae strain* IIT BHU M2V2 disclosed its nearest evolutionary relationship with *Enterobacter cloacae strain* HS-6. *Enterobacter cloacae strain* IIT BHU M2V2 exhibited alliance with other *Enterobacter cloacae* strains. Figure 3. 2E shows a phylogenetic tree of *Enterobacter cloacae strain* IIT BHU M2V2 generated by MEGA X software. Several studies have reported cellulase activity in different strains of *Enterobacter cloacae* [389]–[391]. Lokapirnasari et al. (2015) found cellulolytic activity in *Enterobacter cloacae* WPL 214, which was isolated from bovine rumen fluid waste [391]. Maminska et al. (2015) isolated *Enterobacter cloacae* from *Coptotermes curvignathus* (termite) gut [389].

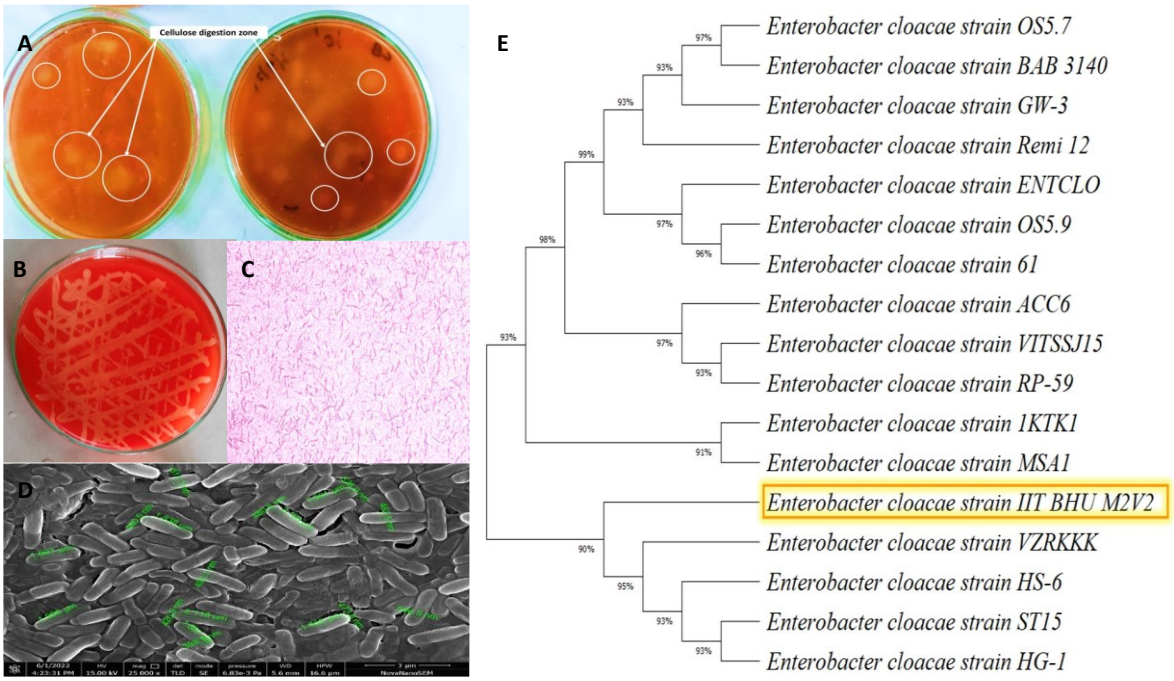

Figure 3. 2 (A) Colourless colonies on replica plate; (B) Colourless zones on streak plate; (C) Gram staining of isolated bacteria; (D) SEM image of isolated bacteria; (E) Phylogenetic tree of *Enterobacter cloacae* IIT BHU M2V2 (accession number OM214000).

3.3.3 Analysis of power generation/polarisation curve and internal resistance

HPTCL was evaluated as an anodic substrate in PCL-MFC, PCLY-MFC and PCLYB-MFC. PCL-MFC was used as control, fed with sterilised HPTCL without any external inoculation. However, PCLY-MFC and PCLYB-MFC were inoculated with yeast and yeast with CB, respectively. Bioelectricity generation began rapidly, with the maximal stable voltage achieved within five days in all cases. The voltage was stabilised at 241±1.0 mV (PCL-MFC), 282±1.7 mV (PCLY-MFC) and 284±2.0 mV (PCLYB-MFC), after 24 hours, 48 hours and 48 hours, respectively (Figure 3). The voltages of PCL-MFC and PCLY-MFC showed a fall from the 6^{th} to the 30^{th} day. However, PCLYB-MFC displayed a steady rise in voltage till day 17^{th} and a continuous fall after 18^{th} day. The profiles of voltage (at 1000Ω) obtained from PCL-MFC, PCLY-MFC and PCLYB-MFC in a duration of 30 days have been shown in Figure 3. 3.

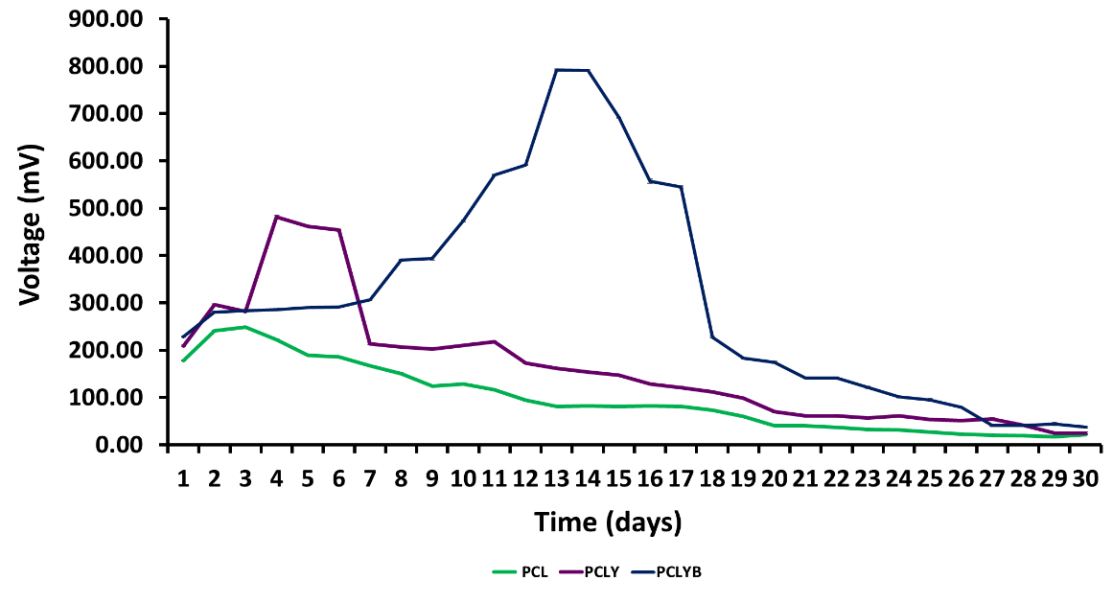

Figure 3. 3. Voltage vs Time profile for MFC

The outcomes revealed that a higher voltage was generated in PCLYB-MFC (792.33 ± 1.53 mV) as compared to PCLY-MFC (481.33± 3.51 mV) and PCL-MFC (248 ± 0.58 mV). Priya and Setty (2019) characterised a dual chamber MFC feed with clarified cashew apple juice and obtained a maximum voltage of 400 mV. Rojas-Flores et al. (2021) used blueberry waste in a two-chambered MFC by the copper anode and zinc cathodes and achieved a maximum voltage of 1.127 ± 0.096 V. Despite minor variation in the electric potential, a fall was noticed from the start of day 3 in PCL-MFC, and 21.67±1.15 mV was attained, whereas PCLY-MFC showed a decrease in voltage from day six and declined up to 25.33±2.08 mV till 30th day. In a study, researchers used potato waste as substrate in a single chamber MFC and obtained 1.12 V on 37th day of operation and noticed the voltage fall because of reduced microbial activity [392]. Yaqoob et al. (2021) remarked that the fall in voltage was due to exhausted carbohydrate content for oxidation and microbial activity [393]. PCLYB showed a consistent rise till day 14 and then waned up to 37.33±1.53 mV. The consistent voltage in PCLYB-MFC was due to co-culture of S. cerevisiae and isolated bacteria at anode whereas consistent microalgae growth from day 1 to day 14 led to enhanced oxygen reduction rate (ORR) at the cathode [394]. The

outcomes of the electric current measurements from PCL-MFC, PCLY-MFC, and PCLYB-MFC in a period of 30 days has been shown in Figure 3. 4.

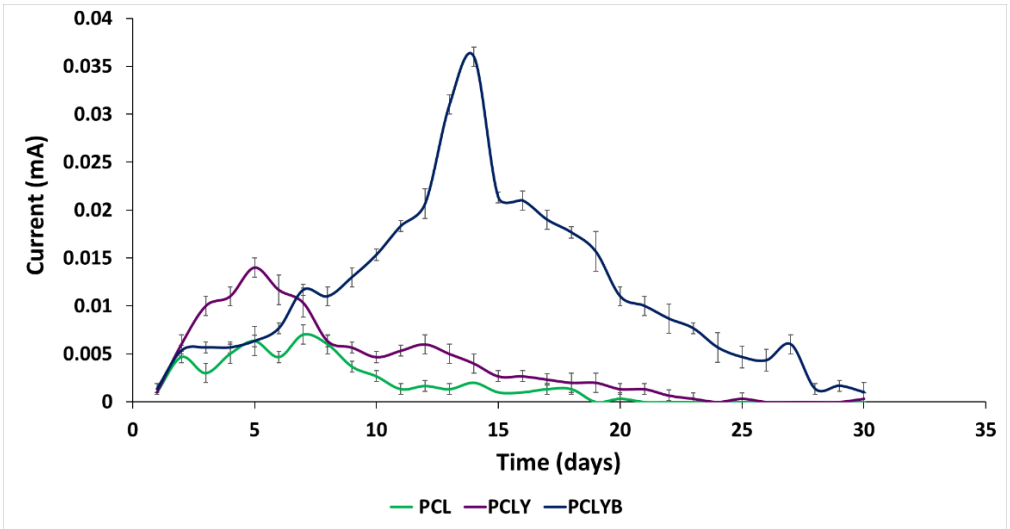

Figure 3. 4. Current vs Time profile for MFC

It became evident from Figure 3. 4 that PCLYB-MFC (0.036 ± 0.001 mA) generated current greater than PCLY-MFC (0.014 ± 0.001 mA) and PCL-MFC (0.007 ± 0.001 mA). Evaluation of optimal external resistance (R_{ext}) is a significant aspect for achieving maximum power, as R_{ext} affects anode potential and current generation in MFCs [395]. In the present work, the cumulative power and voltage generation were measured under steady conditions for different R_{ext} values (100 Ω, 470 Ω, 560Ω, 1000 Ω, 1500 Ω, 2200 Ω, 3300 Ω, 4700 Ω, 5600Ω, 8200Ω, 10000Ω, and 15000Ω). The cumulative power improved with a decline in voltage and the decrease in externally applied resistance from 15000 Ω to 10 Ω. Escalation in resistance values raised the voltage across MFCs. Polarisation curves for PCL-MFC, PCLY-MFC and PCLYB MFC have been shown in Figure 3.5.

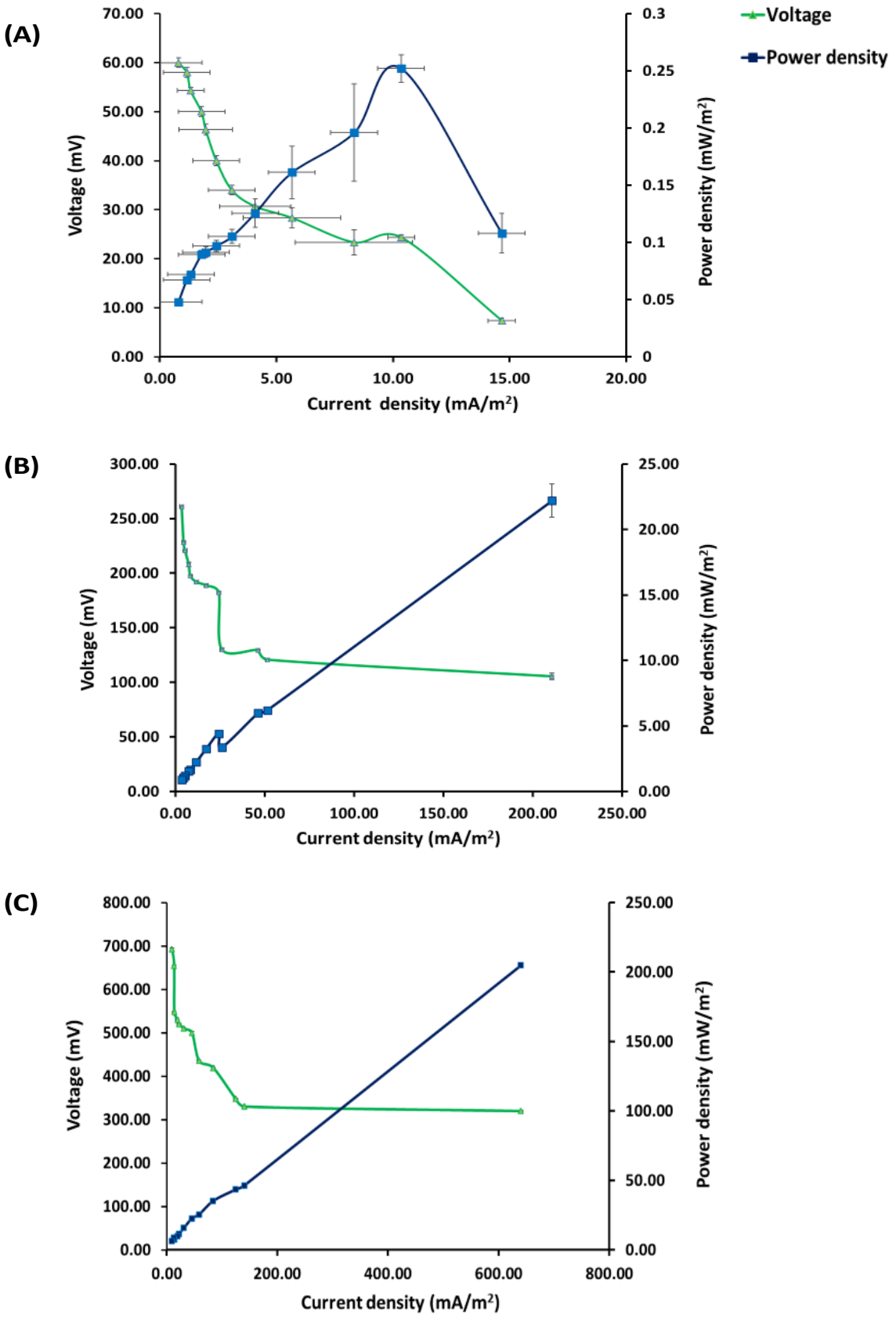

Figure 3. 5. Polarisation curve for: (A) PCL-MFC; (B) PCLY-MFC; (C) PCLYB-MFC

The maximum power density obtained by PCL-MFC was 0.252 ± 0.01 mW/m^2 with a current density of 10.35 ± 0.24 mA/m^2. In contrast, maximum power densities for PCLY-MFC and PCLYB-MFC were 22.20±1.28 mW/m^2 (at 210.66 ± 6.11 mA/m^2) and 204.80±1.28 mW/m^2 (at 640.0±2.0 mA/m^2) respectively. Polarisation curves exhibited a sharp drop in voltage at higher current densities, indicating conventional ohmic and mass transfer losses in these MFCs. The pattern of power density curve obtained in Figure 3.5B and Figure 3.5C is conceivable, similar pattern was reported by Priya et al. (2016) [396]. Still, these outcomes show significant improvement compared to other lignocellulosic biomasses used in MFCs, including composite vegetable waste and orange peel waste as substrates [23], [338], [397]. It is unrealistic to compare maximum power densities among different MFC systems having several variations in MFC variables. These variables involve various electrolytes, different anode biocatalyst, metallurgy of electrodes, and diverse separators/cation exchange membrane with the fluctuating distance between electrodes [398].

Similarly, maximum power densities of 371 mW/m^2 and 358.8 mW/m^2 were obtained using lemon peel and orange peel waste, respectively [23], [338]. The *Candida boidinii* was used with blueberry waste in a single chamber MFC, and a maximum power density of 3.155 ± 0.24 W/cm^2 was obtained [24]. Zaini Makhtar et al. (2020) constructed a sludge supplemented membrane-less MFC with graphite felt electrodes. Authors tested banana peel, corn bran and palm oil mill effluent as substrate in MFC and reported a maximum power density of 23.75 mW/m^2 by using the banana peel, whereas corn bran obtained a maximum power density of 12.65 mW/m^2 [336]. Priya and Setty (2019) utilised cashew apple juice as a substrate and reported a maximum power density of 31.58 mW/m^2 [365]. Kalagbor and Akpotayire (2020) studied the effect of the varying weight of papaya and watermelon waste in a single chamber MFC. Authors found that power density is directly associated with the amount of substrate. 1 kg of papaya waste generates 0.0108 mW/cm^2, and 10 kg of papaya waste generates 0.868

mW/cm^2 due to the increase in the amount of saccharides available to the microorganisms [399]. The internal resistance of the PCL-MFC was up to 833.33 Ω as compared to PCLY-MFC (243.90 Ω) and PCLYB-MFC (217.39 Ω). Lower internal resistance is easier than the flow of electrons from the cathode to the anode. Anode biofilms are vital in dropping the internal resistance and increasing the power generation in the MFC [400], [401]. In addition, substrate, anode biofilm, and electrode/electrolyte spacing also play a significant role in dropping the internal resistance of the MFC. The current generation is dependent on the above factors [402]. SEM images of stainless-steel mesh anode biofilm from each MFC are shown in Figure 3. 6. The stainless-steel mesh anode biofilm was characterised (after 30-days) by a scanning electron microscope (Carl Zeiss Microscopy Ltd., Germany, EVO, SEM MA15/18) to determine the possibility of yeast-anode biofilm attachment. Figure 3. 6 (A, B) shows the bare anode's SEM before performing the experiment. Figure 3. 6 (C, D) depicts the SEM images of PCL-MFC. Figure 3. 6 (E, F) and (G, H) display anode biofilm of PCLY-MFC and PCLYB-MFC, respectively. Figure 3. 6 confirmed the plentiful microbial adhesion on the stainless-steel mesh surface compared to the bare image of the stainless-steel mesh anode (Figure 3. 6 A, B). The difference in the anodophillic morphology was induced by the yeast.

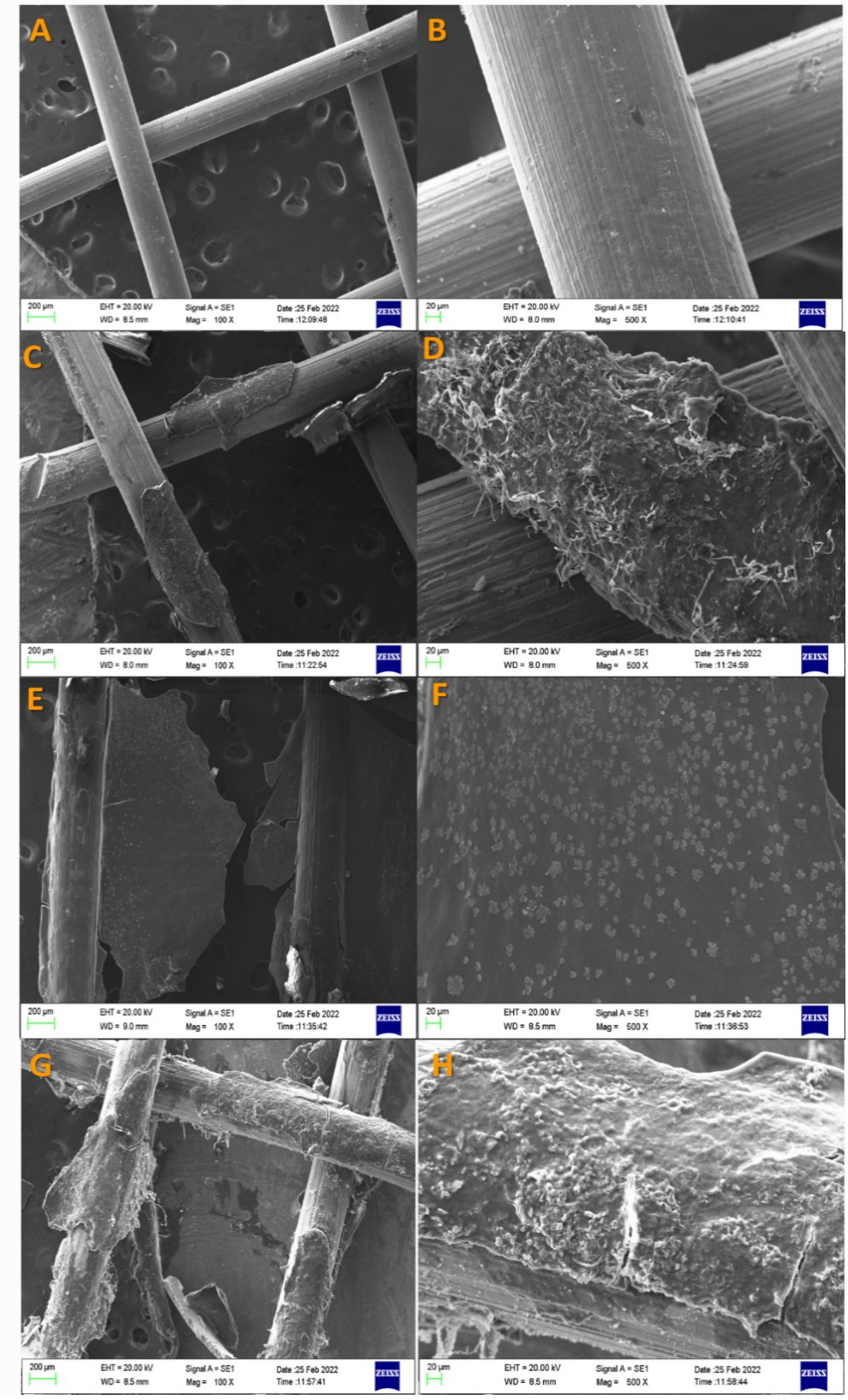

Figure 3. 6. SEM images of anode biofilm at 100x and 500x resolution (A, B) Anode prior experiment; (C, D) PCL-MFC; (E, F) PCLY-MFC; (G, H) PCLYB-MFC

Incorporating microalgae as cathode biocatalyst could be a possible reason for the power density increase of PCLYB-MFC compared to PCLY-MFC. The present study recorded a significant improvement in power densities by using sweet lime peel slurry with *S. cerevisiae* and bacteria. Mixed microbial consortia, including yeast, improved MFC performance [267].

3.3.4 Pollutant removal and organic substrate consumption

The performance characteristics of the dual chamber MFC based on pretreated sweet lime peel slurry and diluted cow urine were studied in terms of COD removal. At the anode, the maximum COD removal was 95% for HPTCL from PCLYB-MFC, which is 15% higher than PCLY-MFC (80%). Coulombic efficiency of PCLYB-MFC was utmost 41.27%; however, PCLY-MFC and PCL-MFC had coulombic efficiencies of 3.42% and 0.36%, respectively. Table 3. 5 shows the removal of COD, nitrate-nitrogen, ammonium-nitrogen, and phosphate-phosphorous from HPTCL for each MFC with the remaining total and reducing sugar.

Table 3. 5. Removal of organics and substrate consumption in HPTCL anolyte

	PCL-MFC		**PCLY-MFC**		**PCLYB-MFC**	
	Final concentration	**% Removal**	**Final concentration**	**% Removal**	**Final concentration**	**% Removal**
COD (mg/L)	6573 ± 733	14	1466 ± 266	80	360 ± 160	95
Total sugar content (mg/L)	21395 ± 95		20187 ± 125		19479 ± 130	
Reducing sugar content (mg/L)	9263.15±52.6		4596.5 ±80.4		1070.18± 109.5	
Nitrate-nitrogen concentration (mg/L)	3.05 ± 0.15	12.5	2.78 ± 0.3	20	2.52 ± 0.15	27.5
Ammonium-nitrogen	0.46 ± 0.00	3.3	0.39 ± 0.00	18.5	0.36 ± 0.00	23.9

concentration (mg/L)						
Phosphate-phosphorous concentration (mg/L)	0.23 ± 0.05	7.69	0.19 ± 0.03	23.07	0.09 ± 0.03	61.53

Table 3. **6** shows COD, nitrate-nitrogen, ammonia-nitrogen, and phosphate-phosphorous removal efficiency from diluted cow urine for each MFC.

Table 3. 6. Removal of organics in diluted cow urine at the cathode

	PCL-MFC		**PCLY-MFC**		**PCLYB-MFC**	
	Final concentration	**% Removal**	**Final concentration**	**% Removal**	**Final concentration**	**% Removal**
COD (mg/L)	2040 ± 262	14.25	906 ± 184	62	293 ± 122	87
Nitrate-nitrogen concentration (mg/L)	3.84 ± 0.06	0.67	3.72 ± 0.05	3.82	0.18 ± 0.04	95.27
Ammonium-nitrogen concentration (mg/L)	9.67 ± 0.03	1.41	9.63 ± 0.09	1.82	2.44 ± 0.05	75.10
Phosphate-phosphorous concentration (mg/L)	3.55 ± 0.59	5.26	3.35 ± 0.34	10.52	1.5 ± 0.34	57.89

Usage of *Chlorella pyrenoidosa* (microalgae biocatalyst at cathode chamber) resulted in significant nutrient recovery from diluted cow urine [394]. PCLYB-MFC achieved a maximum

COD removal of 87%, which was 25% more than PCLY-MFC (62%). In PCLYB-MFC, diluted cow urine reflected its significance for being inexpensive, conductive, and easily accessible waste effluent as catholyte with the alliance of microalgae. Kumari et al. (2021) reported removal of nitrate, phosphate and COD as 99.2%, 70.1% and 62.0% respectively during the growth of *Chlorella pyrenoidosa* in synthetic wastewater [403]. Microalgae consume organic pollutants and provide an enhanced ORR at the cathode chamber. These outcomes signify that the worth of the MFC system is limited to power generation from waste and can be used to reduce the volume of agriculture and fruit waste along with waste management.

3.3.5 Algae biomass yield

A total of 0.24 g/L of dried algae biomass was obtained from PCLYB-MFC. The obtained biomass in the present case was similar to values reported in previous literature [404] Gajda et al. (2015) designed an MFC where anaerobic biofilm at anode half-cell generated current and algae biofilm enhanced ORR at the cathode [404]. This MFC generated electricity with simultaneous algae biomass production at the cathode. The authors obtained 0.25 g/L algae biomass from a catholyte with a non-modified cathode [404].

3.4 Conclusions

The possibility of electricity generation was effectively demonstrated from the degradation of sweet lime peel waste in dual-chambered H-shaped MFC. Considerable performance was observed from each MFC setup. In PCLYB-MFC, additional inoculation of cellulolytic bacteria with yeast at the anode and *Chlorella pyrenoidosa* at the cathode generated significant improvement in power density and organic removals compared to PCLY-MFC and PCL-MFC. The maximum power density realised by PCL-MFC was 0.252 ± 0.01 mW/m^2 at a current

density of 10.35 ± 0.24 mA/m^2. The maximum power densities for PCLY-MFC and PCLYB-MFC were 22.20 ± 1.28 mW/m^2 (at 210.66 ± 6.11 mA/m^2) and 204.80 ± 1.28 mW/m^2 (at 640.0±2.0 mA/m^2) respectively. Utilising diluted cow urine as catholyte to support the growth of Chlorella pyrenoidosa at the cathode chamber was advantageous in terms of biomass production and obtaining 0.24 g/L dried microalgae biomass. Microalgae growth at the cathode might also be a reason for the remarkable difference in power densities of PCLY-MFC and PCLYB-MFC. At the anode, the maximum COD removal was 95% for HPTCL from PCLYB-MFC, which is 15% higher than PCLY-MFC, whereas at the cathode, PCLYB-MFC achieved a maximum COD removal of 87%, which was 25% more than PCLY-MFC. For diluted cow urine at cathode chamber, PCLYB-MFC reported 95.27% nitrate-nitrogen removal, 75.10% ammonium-nitrogen removal, 57.89% Phosphorus-phosphate removal by *Chlorella pyrenoidosa*.