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Sensing oxy anodic microbial fuel cell potential of halotolerant protease producer *Priestia megaterium* BorS17B13 belonging to mangrove biota

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ABSTRACT

Exploring the uncharted nooks of mangrove-rich biota is the key to unlocking the hidden world of industrial opportunities and keeping up with demanding products like microbial-based enzymes. A salt-tolerant bacteria, *Priestia megaterium* strain, BorS17B13 was isolated from the mangrove-rich biota of Borivali monari creek, Maharashtra, India. The organism was Gram-positive, aerobic, and capable of secreting protease enzymes in abundance using a crude source (wheat flour) as the sole source of carbon and nitrogen. Optimum protease activity was 739 U/mL at 72 h. Further, its potential was securitized for bioenergy production in a dual chamber of the oxy anodic microbial fuel cell (OA-MFC) with an impeller for analog configurations, like a bioreactor, to achieve homogenized medium. The maximum OCV was 614 mV and the SCC was 3.5 Am^{-3}; they were also studied with an external load/resistor (47 ohm and 100 ohm). The maximum power output was 454 mW, and the volumetric power density was 0.65 mW/m^3 (100 ohm). The novel concept of OA-MFCs will highlight the potential for combining technologies and will take aerobic microbes strands into the future by utilizing MFC techniques into "Aerobic biosensors." Our research as a whole is attractive, as mangrove-associated halotolerant microbes are rarely explored for their OA-MFC potential to date.

1. INTRODUCTION

Mangrove forests around the shore of oceans and seas can be distinguished by their capacity to survive in salty or brackish water. Carbon sequestration, coastline security, and habitat for a wide variety of flora and animals are some of the ecological benefits they provide as a cohesive ecosystem [1]. Mangrove coastal ecosystems, with several large stretches of mangrove forests, are found in Mumbai (India). These woods play a crucial role in shielding the city from flooding and other coastal hazards. However, human activities such as reclamation of soil, pollution, and exploitation pose a threat to these ecosystems [2]. Proteins can be cleaved into smaller pieces called peptides with the help of a special type of enzyme called a protease. Bacterial proteases have many applications in industries ranging from food and leather industries to detergent production [3]. Applications of the microbial fuel cell (MFC) technology were initiated in the 1970s [4] with major emphasis on the application of this technology for wastewater treatment. Improvements in terms of electricity output were recently explored and these have enhanced the hope of renewable energy for the world. Biological waste can be converted into energy via MFCs [5]. Transforming biological waste substrates to energy and applications in many other fields ignited the ideas about wider applications of MFC technology. This occurs when microbes take a switch over from natural electron acceptors to other insoluble acceptor-like electrodes in MFC [6,7].

Highly productive mangrove environments harbor a wide variety of microorganisms including bacteria, fungi, microalgae and cyanobacteria. Bacteria, found in mangroves, can survive and even grow because of their specific adaptations to the conditions [8]. Scientists globally are trying to produce protease in bulk using crude agro-industrial waste including wheat flour, whey, and soybean meal. [9]

The MFCs succeeded to attract people as a green and long-lasting way to turn waste into electricity. MFCs have been investigated for wider applications like biosensor, in which microorganisms or enzymes serve as bio recognition components for the detection of a wide range of analyses [10]. The present study describes the use of a crude source (wheat flour) as sole source of carbon and nitrogen for the production of industrially valuable protease enzyme from *Priestia megaterium* strain, BorS17B13 from mangrove-rich biota from Borivali monari creek, Maharashtra, India. Isolate was further checked for its electrogenic potential with the novel concept of aerobic

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anode MFC, also called oxy anodic MFC (OA-MFC). An emerging technology, OA-MFC, broadens the potential uses of MFCs beyond anaerobic microorganisms; however, the future of this technique may lead to exploring new horizons for "Aerobic Biosensors."

2. MATERIALS AND METHODS

2.1. Enrichment and Isolation

Samples were collected in clean containers, while the type of sampling was "Random Sampling." Soil, water, and root samples were collected from Borivali, monari creek (19.2179° North, 72.8087° East) of Maharashtra, western India. Samples were processed by enrichment method where the sample amount was 1 g (w/v) for soil sample, 1 mL (v/v) for water sample and 1 g (w/v) for root samples. Samples were incubated for 24 h under shaking conditions at 37°C and 180 rpm. Isolates were obtained by streaking on the complete medium plate (g/l; glucose, 10; yeast extract, 5; peptone, 5; Agar-agar, 3; and K_2HPO_4, 5). The medium contained varied concentrations of NaCl concentration ranging from 10% to 20% (w/v) and the pH of the medium (7.0 pH) was set using separately sterilized Na_2CO_3 (20%, w/v) [11].

2.2. Growth and Enzyme Kinetics in Submerged Fermentation (Flask)

Growth and Enzyme kinetics were performed in a 250 mL flask with 150 mL broth. The medium contained (%, w/v), wheat flour, 1; K_2HPO_4, 0.5; KH_2PO_4, 0.5 and NaCl, 0.25, and the pH of the medium was adjusted to 7.0 by separately autoclaved 20% w/v of Na_2CO_3. Submerged fermentation was carried out in flask inoculated with 9% v/v of the active culture followed by incubation at 37°C at 180 rpm on a rotary shaker [12]. Growth (600 nm) and Enzyme activity (280 nm) were monitored at 24 h, 48 h, and 72 h time intervals. The enzyme assay of protease was carried out by Anson-Hagihara's method [13] using casein as a substrate. Enzyme activity was calculated using tyrosine as the standard where one unit of enzyme activity (protease activity) was the amount of enzyme releasing 1 µg of standard (tyrosine) per minute under the standard assay conditions.

2.3. Media Optimization

The medium was optimized with one variable at a time (OVAT) methodology, where parameters such as carbon sources, nitrogen sources, cations, inoculum size, pH, salt concentration (NaCl), and crude sources were optimized. Submerged fermentation in flask was carried out for all the above parameters followed by measurement of growth (λ_{600}) and enzyme activity (λ_{280}) [11,12].

2.4. OA-MFC Setup

OA-MFC was specially designed for aerobic bacterial applications in MFCs. The MFC setup was based on the dual chamber model of MFCs [Figures 1 and 2], where an anode chamber contained bacterial broth and a cathode chamber contained electrolyte potassium ferricyanide (16.46 g/L) and di-hydrogen potassium phosphate (2.71 g/L) and water (v/v). There were two electrodes in the unit: one was anodic electrode, and another was cathodic electrode. In both cases, the electrode material was aluminum mesh. Four crocodile pins with wire on both electrodes were arranged to withdraw current and complete the close loop. Electrons drawn from wire were collected on a plane of aluminum strip (1 ohm resistance) for ease of measuring current and voltage from the unit. The OA-MFC was of dual chamber and as proton exchange membrane (PEM), ceramic material was used.

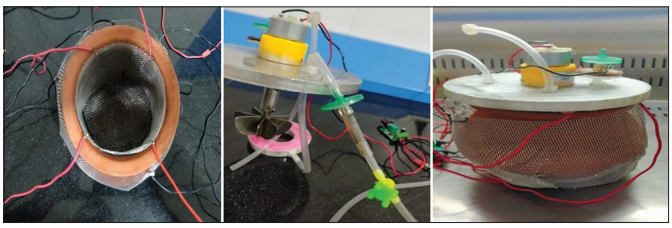

Figure 1: Oxy anodic microbial fuel cell.

Electrons in circuit flow from anode to cathode through wire and proton flow through PEM and released a water molecule along with generating current impulse. The anode chamber was supplied with oxygen (OA-MFC) as the bacteria used were aerobic in nature. Here in the setup, the cathode was also supplied with oxygen for completing the close circuit. The impeller was adjusted from the top of the setup; its main function was mixing medium broth, so that bacteria get enough motion to utilize media and simultaneously blend oxygen molecules and dissolve them in the anodic chamber for easy availability of oxygen for aerobic bacteria in the OA-MFC. Impeller speed was 100 rpm, and there was a sampling pipe for sample withdrawal and an aerated pipe with a bacterial filter to release pressure created in the unit during bacterial growth.

Open-circuit voltage (OCV) was measured when there was no external resistance being applied to the circuit and a multimeter was used to measure a direct connection. When a very low external resistor (R_{ex}), which in our case was 10 ohms, was connected in series to the circuit, short circuit current (SCC) was measured. This measurement was used to determine the circuit's maximum output in relation to the time. The open current was measured with the Load/Resistor (47 ohm and 100 ohm) in series for circuit connection, and for voltage measurement, Load/Resistor (47 ohm and 100 ohm) were arranged in parallel to complete the close circuit.

3. RESULTS AND DISCUSSION

Samples (soil, water, and root samples) were collected from mangrove rich area of Borivali, monari creek and Jhow Island, Maharashtra, western India. About 62 isolates were obtained from 30 samples when processed by enrichment method. The isolates were further screened for industrially important enzymes including protease, amylase, and cellulase. Protease enzyme, being industrially important and immensely stable in crude broth for a longer time as compared to other enzymes, was selected for further studies. Halotolerant BorS17B13, a Gram-positive, optimum protease producer was selected for further studies. It was identified as a close relative of *Priestia aryabhattai* strain based on 16s rRNA gene sequencing. Sequence of the isolate was submitted in NCBI (NCBI accession no. OM743775, *P. megaterium* B21). Further, this strain was evaluated for electricity potential through MFC technologies.

3.1. Growth and Enzyme Kinetics Submerged Flask Fermentation using Wheat Flour (Crude Source)

Wheat flour was used as the sole source for carbon and nitrogen in a minimal medium to facilitate protease production from BorS17B13. Bacterial growth and protease enzyme activity increased with respect to time [Figure 3]. Protease activity was 72 U/mL at 24 h, 664 U/mL at 48 h, and at the end of 72 h, enzyme activity was 739 U/mL with growth density 0.69 (λ_{600}). Patel *et al.* produced protease from haloalkaliphilic *Bacillus* sp. using wheat flour as sole source of carbon and nitrogen [12]. They obtained about 183 U/mL of protease activity which is significantly lesser than that of our isolate (739 U/mL). In another study, alkaliphilic *Bacillus* sp. I-312 was grown on soybean meal compiled with wheat flour for protease production [14]. Very similar to our investigations, strain

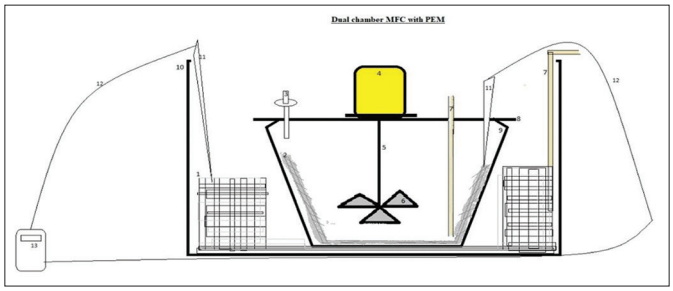

Figure 2: Oxy anodic microbial fuel cell Sketchmatic diagram.

Label 1: (Description of Figure 2)

1	Cathode electrode (alumium mesh)	8	lid
2	Anodic electrode (alumium mesh)	9	Clay pot (internal anodic chamber)
3	Air outlet and air filter	10	Cathode chamber
4	Motor	11	Crocodile pins
5	Shaft	12	Wire to withdraw current
6	Impeller	13	Multimeter
7	Oxygen supply pipe		

APP1; a *Bacillus* Sp. was studied for its protease production using wheat flour and soybean meal with other minimal media [15]. Mehta *et al.'s* [16] report enhanced production of protease from alkaliphilic *Actinomycete* sp. on wheat flour. Our data also supported the hypothesis of using wheat flour for enhanced protease production with significant reduction of overall cost on raw material (upstreaming process) at bioreactor scale. BorS17B13 protease was active optimally at pH 7 and temperature 37°C.

3.2. OA-MFC Parameters in Pot Model-Biosensor Approach

OA-MFC, a new and trending approach to magnify the horizons of MFC applications from anaerobic to aerobic bacteria. Till date, a notable number of aerobic bacteria have been exploited for numerous applications in human welfare. The concept of OA-MFCs was based on the futuristic applications of aerobic bacteria as biosensors using MFC techniques. Oxy indicates oxygen, which is crucial for aerobic bacteria. A dual-chamber of MFC was used with a PEM (a ceramic pot) [Figure 4]. The anodic chamber contained bacterial broth and the cathodic chamber contained electrolyte solution. Electrons flew from the anode to the cathode, completing the closed circuit. Protons (H+ ions) flew out of the inner anodic chamber through PEM. R_{ex}/Load was used for measuring close circuit current. Many of the major studies were carried out to study the effect of salinity on MFC parameters. The conductivity of cultural broth and the impact of nutrients in them play impacting role on MFC output and a positive correlation was observed in current output and conductivity [17]. Similar research with respect to PEM was carried out by Gajda *et al.* [18]. They used low cost ceramic-based MFC that worked on electron osmotic mechanisms and produced electrical impulses in the form of electricity. Similar to our setup, they used anodic chamber filled with culture broth, whereas cathodic chamber was filled with electrolyte solution. The proton was transferred from anode to cathode from PEM and electrons were transferred through external circuit for making close circuit conditions [18]. Our setup was novel and have not been reported earlier where we applied advancement using impeller in the unit and kept anode and cathode, both oxygenated, as our organism (BorS17B13) was aerobic in nature. OA-MFC was run for 8 h, and

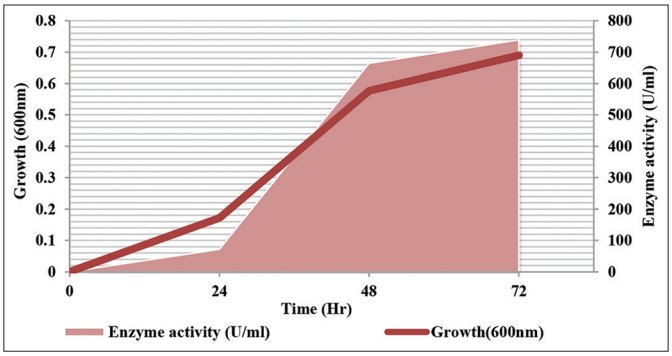

Figure 3: Growth and enzyme kinetics of BorS17B13 using wheat flour.

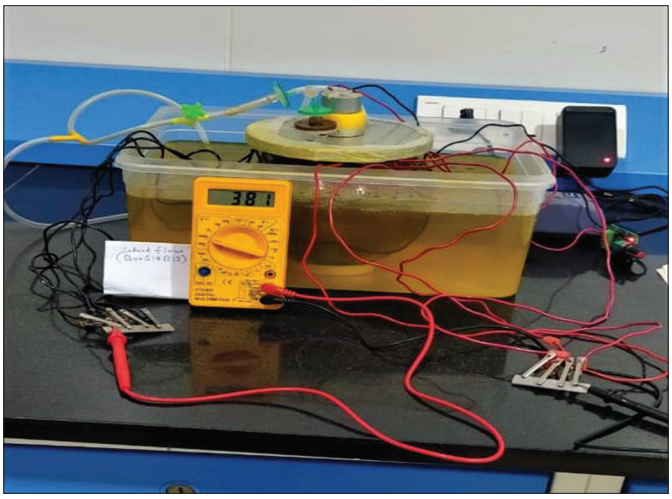

Figure 4: Working model of oxy anodic microbial fuel cell.

growth and protease activity was measured at the end of 8 h. Growth was 0.64 (λ_{600}) and protease activity was 603 U/mL. OA-MFC was studied on the base of the OVAT method where the focus was on the electrogenic property of isolate BorS17B13.

3.2.1. Correlation of OCV and SCC with respect to time

The results obtained from OA-MFC [Figure 5], with respect to OCV and SCC revealed that at growth 1.2 (λ_{600}) at 8 h, the maximum voltage was 614 mV, whereas current production was steady at about 3.5 Am^{-3}. Similar to our study, OCV and SCC were used by many researchers. Gajda *et al.* [18] used six different MFCs from two groups and obtained optimum OCV 750 mV with a group 3 MFC. These results are in contrast to ours as we obtained similar results from a single MFC unit.

3.2.2. Correlation of Voltage and Current with R_{ex} (47 ohm and 100 ohm)

The voltage and current output of the MFC increased over time [Figure 6]. An external resistance of about 47 ohm was applied in a series and was parallel for current and voltage measurement, respectively, where at the initial 1st h, the voltage output was 65 mV and the current output was 1.21 Am^{-3}. By the end of 8 h, the voltage output increased to 122 mV, and the current output increased to 2.5 Am^{-3}. Maximum voltage and current were obtained at 5 h, with 132 mV and 2.67 Am^{-3}, respectively. An external resistance of about 100 ohm was applied in a series and was parallel for current and voltage measurement [Figure 7]. While initially voltage output was 73 mV and the current output, 1.4Am^{-3}; by the end of 8 h, the voltage output increased to 102 mV, and the current

was 1.92 Am^{-3}. Maximum voltage (216 mV) and current (2.1 Am^{-3}) were obtained at 5 h. Many studies were carried out with different.

R_{ex}s, where they were used considering the internal resistance of the MFC setup to overcome the ohmic losses and to get desired output in the form of current [19-22]. To study the effect of different R_{ex} on MFC sets, a polarization study was performed [23,24]. In another report, authors used 100 ohm R_{ex} to study the half cell with the help of a linear sweep voltammeter [18].

3.2.3. Maximum power with R_{ex} (47 and 100 ohm) and volumetric power density

From Figure 8, maximum power peak was calculated by plotting power (mW) versus current (Am^{-3}) graph. Maximum power peak was observed at 5 h for circuit with 47 ohms resistor which was 352.44 mW. In addition, our MFC unit also produced protease enzyme inside the anode chamber as the (Isolate- BorS17B13) could utilize wheat flour [Figure 3] medium for protease production. Maximum power peak was calculated by plotting power (mW) versus current (Am^{-3}) graph for 100 ohm R_{ex} [Figure 9]. Maximum power peak (454 mW) was observed at 5 h. Figure 10 shows correlation between voltage and volumetric power density. Volumetric power density was defined with respect to the volume of the anode compartment (bacterial broth), which was 700 mL in our case. As the voltage increased, volumetric power density also increased, and both reached a peak at the 5th h with a maximum volumetric power density of 0.65 mW/m^3 that slowly dropped until the 8th h. In a research by Freguia *et al.,* a stable current was produced by applying noncatalyzed cathode and the measuring parameter was power density (21 W/m^3) [25]. These findings support our MFC model with ceramic PEM and also represented MFC potential in terms of measuring power in the form of volumetric power density. In addition, our MFC unit also produced protease enzymes inside the anode chamber as the isolate BorS17B13 could utilize wheat flour [Figure 3] as the sole source for the production of protease.

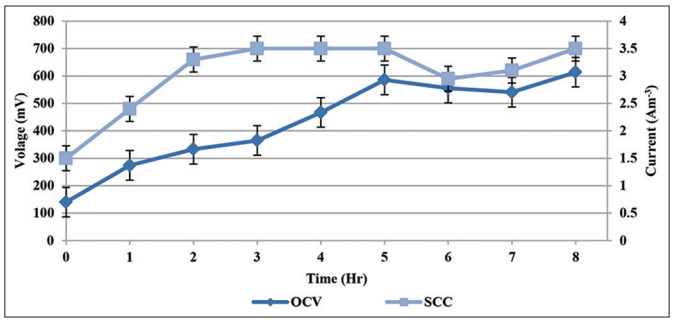

Figure 5: Open-circuit voltage versus short-circuit current.

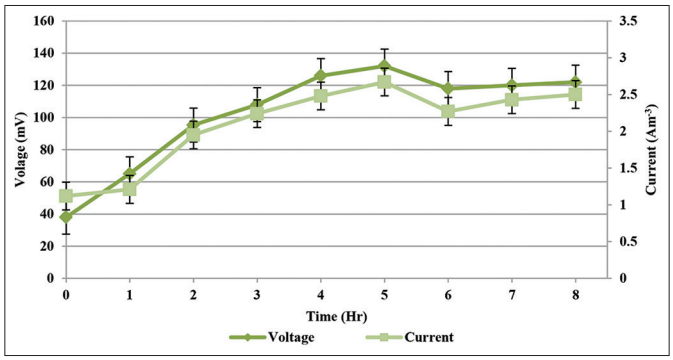

Figure 6: Voltage (mV) versus current (Am^{-3}) with R_{ex} 47 Ohm.

3.2.4. Effect of various resistors on voltage and voltage drop phenomenon

The above experimental result [Figure 11] represents the change in voltage with respect to time and external Load/Resistor. The major difference between the OCV, R_{ex} 100 ohm, and R_{ex} 47 ohm displayed a voltage drop after the applied load. Voltage drop was greater in the 47-ohm close circuit than 100-ohm close circuit, but interestingly, current (2.67 Am^{-3}) was greater with 47-ohm close circuit than 100 ohm (2.1 Am^{-3}) close circuit. A study, supporting our results, by Huangand Logan [12], focused on the influence of external resistance on the efficiency of MFC using effluent from paper recycling. After initially evaluating the MFC in open-circuit mode, they determined the OCV values 0.45 V. The authors subsequently applied different R_{ex}s ranging from a thousand to ten ohms. They discovered that the MFC's voltage output dropped along with the external resistance. The authors hypothesized that this is because MFC's internal resistance

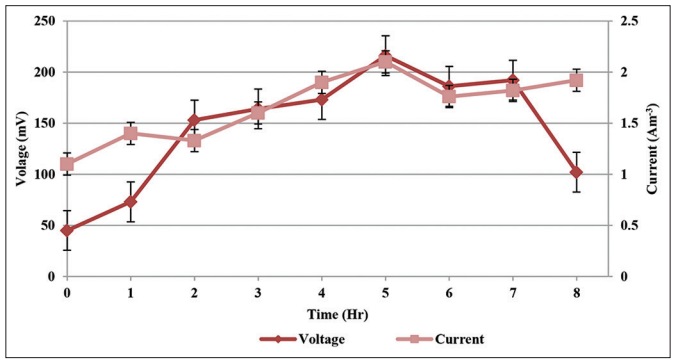

Figure 7: Voltage (mV) versus current (Am^{-3}) with external resistance 100 Ohm.

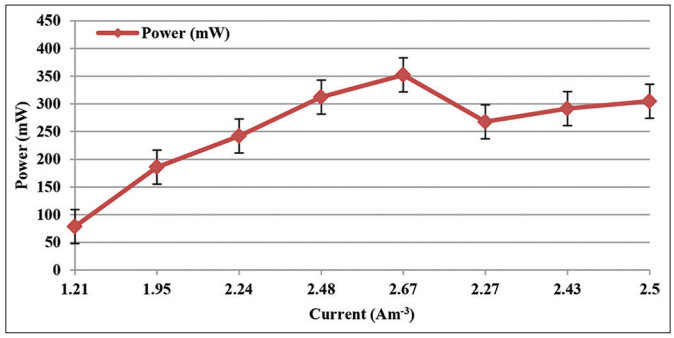

Figure 8: Power (mW) versus current (Am^{-3}) for external resistance 47 Ohm.

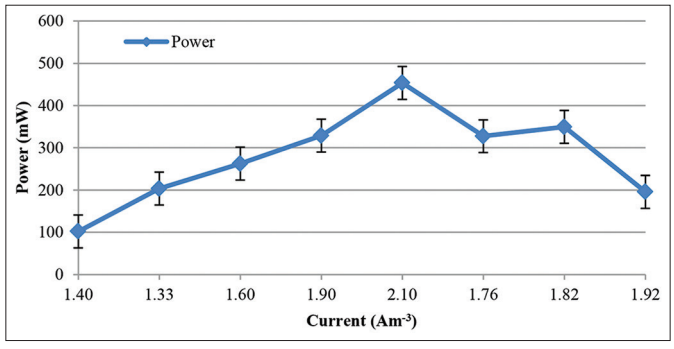

Figure 9: Power (mW) versus current (Am^{-3}) for external resistance 100 Ohm.

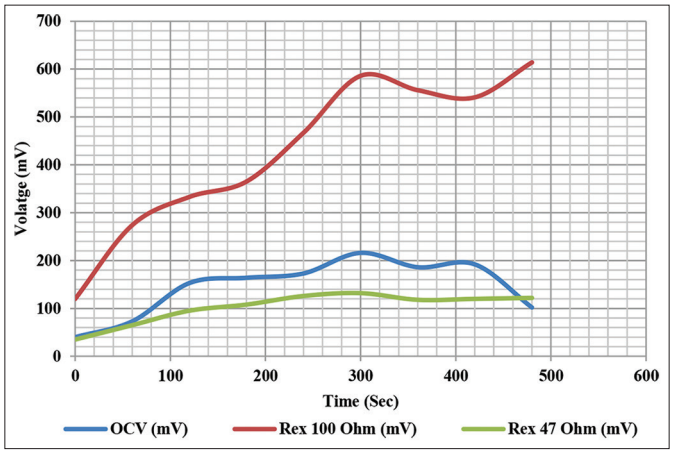

Figure 10: Voltage (mV) versus volumetric power density (mW/m³) with external resistance 100 Ohm.

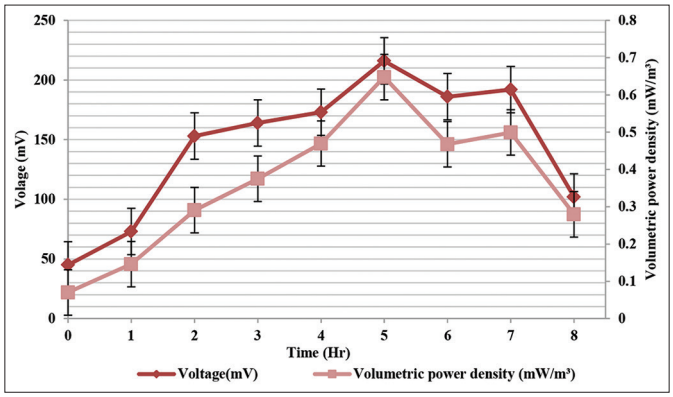

Figure 11: Voltage (mV) versus time (s).

dropped with increasing current through it. Further, this finding also supports our conclusion that there was a voltage drop when switching the MFC system from OCV to connecting a load or R_{ex}s (47 ohm and 100 ohm). In many reports, evidence of current and voltage was observed with the change in the R_{ex} [19,26]. Further, ceramic PEM has been offered as a technique to boost the efficiency of air-anode MFCs in real-world applications like renewable energy. This enhancement might be accomplished by the incorporation of ceramic PEM. These findings lead credence to our MFC concept, which is based on the use of a ceramic PEM. This research also provides a hint of the potential of MFCs for measuring power and converting that power into electrical signal impulses for use in aerobic biosensor applications.

4. CONCLUSION

Mangrove-rich biota was used to isolate halotolerant bacteria, which were widely studied for their potential to produce protease from a cheap source like wheat flour. In addition, the potential of employing an oxygenated (aerated) anodic chamber in an MFC for electricity generation (power impulse); from aerobic bacteria will drive the development of a novel concept of "Aerobic Biosensor" in the near future. Our study provides empirical evidence for the feasibility of employing distinct microbial communities in biotechnological contexts and underlines the opportunity for combining technologies to realize synergistic effects. Oxy Anodic MFC s, also known as OA-MFCs, will be the latest and most cutting-edge method for expanding the scope of MFC applications beyond anaerobic bacteria to include aerobic bacteria. Significant numbers of aerobic bacteria have already been utilized for a variety of applications in the field of human welfare. The use of aerobic bacteria, isolated from geographically rare mangrove forests, as biosensors using MFC techniques was the basis of the idea behind OA-MFCs, which looks to the future for further inspiration.

5. ACKNOWLEDGMENT

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6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

The dataset generated in this study has been deposited in the NCBI GenBank, accession number: OM743775.

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