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***Bacillus amyloliquefaciens*strain NSB4 bacteria for treating wastewater for fuel cell application**

ABSTRACT

Pollutants in water bodies come from a variety of sources, including but not limited to domestic, industrial, municipal etc. Water contamination and energy shortages are global problems that require significant attention. Therefore, it is essential to synthesize sustainable energy and transport waste-free water to the water reception points. Concerns about energy shortages and water contamination have prompted the development of microbial fuel cell technology. Microorganisms are used by electrochemical cell nature of MFCs to anaerobically digest the organic wastes and produce energy. Focusing on a single-chambered mediator-less MFCs operating in batch mode, this study assesses the efficacy of a novel bacterial strain Bacillus amyloliquefaciens NSB4, as an exoelectrogen in terms of electricity yield and waste elimination. Results from the strain's electrochemical characterisation showed a maximum current density of 0.4804A/m^2 and a power density of 41.281mW/m^2. Additionally, the columbic efficiency (72%) and COD reduction efficiency (90.46%) was also remarkably high. Growth of the anodic biofilm during the MFC process displayed the crucial performance of the exoelectrogen used. SEM images of the biofilm are also presented in the study.
Keywords: *Microbial fuel cells, Mediator-free MFC, Separator, Biofilm, Waste-water treatment*

1. INTRODUCTION

Human activities as well as rainwater runoff generate wastes in waterbodies and thus create a global challenge with respect to the water pollution. Wastewater can be typically categorised by the source of its generation such as domestic, industrial, municipal wastewater etc. [1]. In 2004 waste water, used as a feed for microbes in Microbial Fuel Cell (MFC) technology, possessed the advantage towards the treatment of sludge and energy savings from wastewater aeration [2,3]. Microbial fuel cell technology is an emerging environment friendly, eco-efficient and sustainable method to treat wastewater besides generating electricity from the organic and inorganic waste substances[4,5].

Electrochemical cell nature of MFC engages the microbes to anaerobically digest the organic wastes, thereby generates electricity. The energy transformation from chemical to electrical, by virtue of electrochemically active bacteria and the extracellular electron transfer mechanism along with simultaneous wastewater treatment largely escalates the interest towards the use of MFCs[6-8]. In the MFC system, the coherence of waste-water treatment as well as electricity production depends uponvarious factors viz. Electrochemically active microorganisms (exoelectrogens), architecture of microbial fuel cells (type of MFC, electrode materialetc.), substrate used in the MFC, pH, temperature, and inoculum size [9]. One of the most significant factors mentioned above, is the electrochemically active bacteria (EAB), which acts as a biocatalyst, by transferring the electrons, generated during the degradation of pollutants present in the wastewater, to an extracellular electron acceptor and in doing so, it produces electrical energy and thus, affects the overall MFC performance[10,11]. These bio-catalytically active EABs passes on the electrons, generated during

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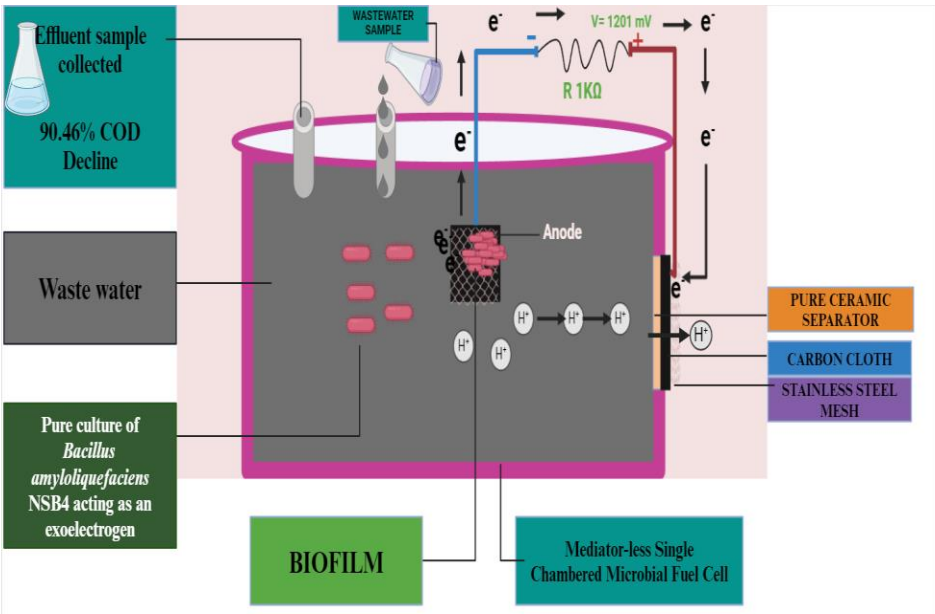
the metabolic processes, towards the anode surface and thus, makes the MFCs, mediator less. Some bacteria in MFC can boost the power generation while other plays an elemental role in eliminating the pollutants from the wastewater[12]. A huge variety of EABs have been isolated till date, from cow dung, soil, aerobically or anaerobically digested sludge, and/or anaerobically decomposed compost[13-15] , the most studied genus acting as an exoelectrogen belong to *Shewanella*, *Proteobactor*, and *Pseudomonas* bacteria[16]. The current research is focussed on the role of an electrochemically active pure culture *Bacillus amyloliquefaciens* NSB4 in electricity generation and removal of pollutants from domestic wastewater using batch MFC system. The robust treatment was evaluated through power production in mW/m^2, decrement in COD value and achievement of high columbic efficiency. Although, several researchers have reported the involvement of mixed microbial cultures in MFCs, for the treatment of wastewater, the employment of a pure culture strain as an exoelectrogen has not been reported till date and hence, **this is the first report to explore the capability of using pure strain of *Bacillus amyloliquefaciens* NSB4 in MFCs** for producing electricity as well as removing the pollutants from the wastewater.



2. MATERIALS AND METHODS

Sampling of wastewater and its preparation as anolyte for MFC

Wastewater sample was collected in a pre-sterilized bottle from wastewater treatment plant (WWTP), Sharda University, Greater Noida, India. Sample was kept undisturbed for 24-48 h under sterilized conditions in order to ensure the settling down of solid particles. After 48 h of time interval, the liquid supernatant collected, was further sieved through a muslin cloth to remove the unwanted fine granules of sand, wood etc. and was stored at 4^0 C before usage. The Chemical Oxygen Demand (COD), Total Suspended Solids (TSS) and pH of the black-brown coloured, foul-smelling waste-water was recorded to be 1071.50 mg/L, 2540 mg/L, and 7.8 respectively.

MFC construction, inoculation and operation

A 300 ml, 10 cm × 6 cm plastic container (purchased from local market) was used to design an air-cathode MFC. Four identical chambers were designed after partitioning the container. The experiments were run in triplicates, in three chambers, while the fourth chamber was kept as control. A piece of carbon cloth (25cm^2) attached to a stainless-steel mesh was placed in the centre of each MFC chamber in order to be used as an anode. Further, a cut of 25cm^2 was made on one of the walls of MFC chamber and a ceramic separator of same dimension was fixed over this cut. The carbon cloth was placed on the outer layer of each separator using a conductive graphite adhesive. Finally, a stainless-steel mesh was attached as current collector. MnO_2 was used as a catalyst on cathode assembly. To ensure the prevention of water crossover, a conductive electric paint (Aerol silicon conformal coating, Grade 9114) was used on the inner side of the separator. Connections for electricity production and measurement were established using the stainless-steel wire end, extended from the mesh of both the electrodes. The wires were further connected to a digital multi-meter via an insulated copper wire, to record the voltage and current output[17-19]. External resistance was fixed at 10kΩ. Each reactor was surface sterilized using ethanol (70%) and UV rays before placing the anolyte in it. The autoclaved anolyte (prepared as mentioned in section 2.1) was transferred in each chamber of the reactor under aseptic conditions (using a biosafety cabinet). The reactor box was tightly sealed with the top-lid attached with a gasket to avoid any oxygen diffusion. Two separate capped openings (one for inoculation and reference electrode as well as the other for anode connection wire) were maintained. Each anodic chamber (except one, set as control) was inoculated with an overnight grown pure culture of *Bacillus amyloliquefaciens* NSB4 (40 ml each). The MFCs were allowed to run in a batch mode for 15 days at room temperature (25^0C). For maintaining an anaerobic environment in the anodic chamber, purging of nitrogen gas was carried out to remove the dissolved oxygen in the anolyte. Digital multi-meter was used to record the voltage results in millivolts. On the drop of voltage (below 300 mV), half of the pre-fed anolyte was replaced with the fresh feed. The experiment was monitored keeping the optimum parameters for all reactors uniform and their average results were recorded.

Electrical measurements

Electrical measurements like voltage, current and power density of the reactor were conducted according to the methods mentioned in previous literature and the voltage was assessed by a digital multimeter (HTC ^{TM}) only when the reactor was balanced[20]. After the attainment of balanced voltage by the MFCs, different external resistances were applied through a resistor box ranging from 10 Ω, to 1000k Ω to obtain the polarization results. The current was measured in milli-ampere (mA), next day after the start of the experiment using a fixed external resistance (1000 Ω). Similarly, the voltage was recorded in millivolts (mV). Both these values were calculated using Ohms' law using the following formula.

$$I = V/R$$

Where, V =voltage and R = external resistance applied.

For calculating power, the following formula was used

$$P = IV$$

Where I is the current generated by MFC and V is the voltage generated by MFC

Power density was calculated using the formula

$$Pd = IV/A,$$

Where, A is the surface area of operational electrode which is 25 cm^2 in current study.

Columbic efficiency (CE) and COD measurement

The efficiency of substrate utilization by the bacteria, *Bacillus amyloliquefaciens* NSB4 was studied by analysing the columbic efficiency, which is actually the ratio of total coulombs shifted from the substrate towards the anodic surface, to the highest attainable coulombs, if whole organic load is removed to generate the current[21,22]. The COD concentration of the anolyte wasrecorded according to the standard procedure [23]. Conside-ring the reduction in the COD concentration, the measurement of columbic efficiency was calculated using the equation given below [24].

$$CE(\%) = \frac{Ms.\int_0^{tb} I.dt}{F.b.Van.\Delta COD} \times 100$$

WhereMs is the molecular weight of O_2 (32 g/mol), I is the current density (mA cm^{-2}), tb is the operation time (days), F is the Faraday's constant (96,487 C/mol), b= 4 (number of electrons exchanged per mole of Oxygen)

Van is the volume of the anode (L) and ΔCOD is the change in COD (g/l) over time tb

Redox reaction study and internal resistance effect

To transfer the electrons to the anode surface, bacteria may either use mobile redox shuttles also called mediators or directly, without involving any mediator via their membrane associated cytochrome compounds[25-27]. Cyclic voltammetry study using electrochemical workstation (Bio-Logic, SP-150, Sharda University, Greater Noida, India) was conducted. The three-electrode system: carbon cloth (working electrode), platinum (refere-nce electrode) and saturated calomel electrode (counter electrode) were used to analyse either of the above mechanism. Oxidation reduction process (redox reaction) was analysed during the electron shifting from the bacteria to the anodic surface and finally towards the cathode. Cyclic voltammograms were recorded between -0.6 to + 0.6 V potential range and 5mV/s scan rate to define the cyclic voltammetry curves. For the examination of the interference caused by the internal resistance created during the MFC run, electrochemical impedance spectroscopy (EIS) was conducted with the frequency range and an amplitude of 100 kHz to 1 Hz and 5.0 mV, respectively[28-30].

SEM analysis of Biofilm

For determining the relation of an anolyte usage, power production and biofilm growth on the electrode surface, scanning electron microscopy

(SEM) was conducted. This study allows us to analyse the attachment of metabolites secreted extracellularly by the exoelectrogens[31]. In this study, several pieces of size 1cm^3 were cut aseptically from the anode surface (before and after the power production), keeping the biofilm intact and were prepared for SEM analysis. The anode sample pieces were fixed in glutaraldehyde (2.5% v/v) for 48 h, followed by serial dehydration in ascending alcohol concentration (10%, 30%, 60% and 100%)[32-34] , and finally, the samples were desiccated for 48 h. Samples were sent to Sophisticated Analytical Instrumentation Facility (SAIF), Punjab University for SEM imaging analysis.

3. RESULTS AND DISCUSSION

Electricity performance of the strain

Once the settling of the MFC was done, the voltage was monitored regularly up to 15 days across an external resistance of 1kΩ. The operating voltage during the 15 days MFC run was recorded using a digital multi meter and the peak was noticed at 1201 mV on 15th day (Fig. 1) The other electricity associated components like, current, current density and power density were also recorded as shown in Table 1. The results achieved during this study were comparable with the earlier reports[35,36], and witness the high efficiency of *Bacillus amyloliquefaciens* NSB4 for power production from the wastewater.

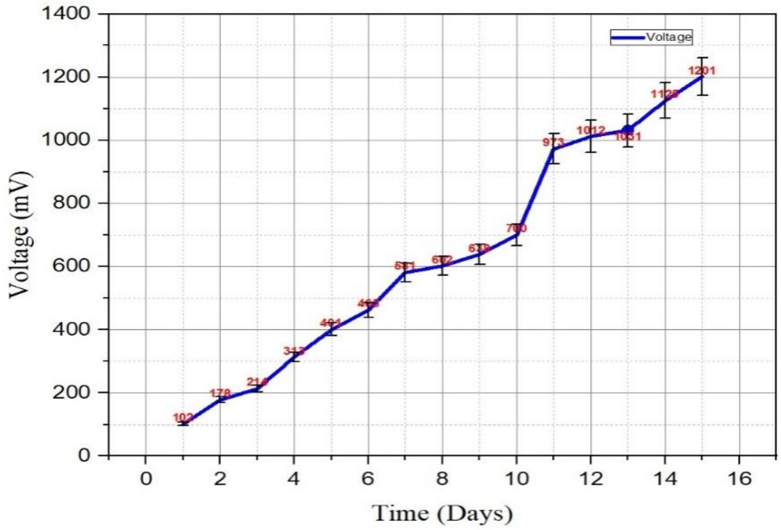


Figure 1. Graph represents the voltage (mV) generation by Bacillus amyloliquefaciensNSB4

Table 1. Electrical measurements and COD of the MFC recorded during the experiment

Time (Days)	Voltage (mV)	Current (mA)	Current Density (mA/m^2)	Power (mW)	Power Density (W/m^2)	COD (mg/L)
1	102	0.102	40.8	0.010404	0.04	1071.50
2	178	0.178	71.2	0.031684	0.12	-
3	214	0.214	85.6	0.045796	0.18	-
4	313	0.313	125.2	0.097967	0.39	-
5	401	0.401	160.4	0.160801	0.64	-
6	463	0.463	185.2	0.214369	0.85	-
7	581	0.581	232.4	0.337561	1.35	-
8	602	0.602	240.8	0.362404	1.44	-
9	638	0.638	255.2	0.407044	1.62	-
10	700	0.7	280	0.49	1.96	-
11	973	0.973	389.2	0.946729	3.78	-
12	1012	1.012	404.8	1.024144	4.09	-
13	1031	1.031	412.4	1.062961	4.25	-
14	1125	1.125	450	1.265625	5.06	-
15	1201	1.201	480.4	1.442401	5.77	102.134

Columbic Efficiency (CE) and COD reduction

The current produced by the strain using wastewater as substrate transferred the coulombs to the anode which was measured by columbic efficiency. The columbic efficiency of the strain was found to be 72%, which is higher as compared to previous studies[37-39] and therefore, claimed that our bacteria, *Bacillus amyloliquefaciens* NSB4, is a reliable exoelectrogen to be used in MFC technology. The effluent collected at the end of the experiment was examined to determine the reduction in the organic load while generating volts also. The COD values of the wastewater was found to be decreased from 1071.50 mg/L to 102.134 mg/L, after 15 days of the MFC run process which accounts for 90.46% decline in the COD. Significant decrement in the COD values of wastewater (Table 1) witnesses as one of the vital parameters for determining the efficiency of the bacteria involved in the mediator-free single-chambered MFC. The results are in agreement with the previous findings (Table 2)[39-59]. Few workers have reported the current density of 366 mA/m^2[40] using *Shewanella oneidensis* MR-1 and 369.4mA/m^2 using *S. oneidensis* and *S. cerevisiae* microbial strains[48]. Similarly, power density ranging from 2.15 mW/m^2 to 2720 mW/m^2 has also been reported in literature[40-59]. These findings suggest that the current density of 480.4 mA/m^{2}and the power density of 41.281 mW/m^2 reported in the current study, are in close agreement with the result reported in literature till date.

Table 2. Comparative study of different bacteria studied for wastewater treatment and power production

Bacteria studied	Type of culture used	MFC used	highest current density	highest Power Density	COD removal Efficiency	Columbic Efficiency	Ref.
Gluconobacteroxydan	Pure	Dual chambered MFC	Not available	81 mW/m^2	32%	40%	[39]
Shewanellaoneidensis M R-1	Pure	Double chambered	366 mA/m^3	14466 mW/m^3	65%	5.70%	[40]
Pseudomonas aeruginosa PBH03	Pure	Not available	9.01 µA/cm^2	Not available	Not available	Not available	[41]
Castellaniella sp. A5, Castellaniella sp. B3, andCastellaniella sp.A3	Mixed	single chambered	3.19 A/m^2	320 mW/m^2	91.15 ± 0.05%	54.81 ± 4.18%	[43]
G. sulfurreducens, E.coli	Mixed	Not available	NR	918 mW/m^2	Not available	Not available	[44]
P. aeruginosa, E. aerogenes	Mixed	Not available	212 µA/cm^2	NR	Not available	Not available	[45]
P. aeruginosa, K.variicola	Mixed	Not available	NR	12.88 W/m^3	Not available	Not available	[46]
G. sulfurreducens, C. cellulolyticum	Mixed	Not available	NR	143 mW/m^2	Not available	Not available	[47]
S. oneidensis, S. cerevisiae	Mixed	Not available	369.4 mA/m^2	123.4 mW/m^2	Not available	Not available	[48]
K. pneumonia, L. stakeyi	Mixed	Not available	NR	12.87 W/m^3	Not available	Not available	[49]
S. oneidensis, K.pneumonia	Mixed	Not available	10 mA/m^2	2.15 mW/m^2	Not available	Not available	[50]
S. oneidensis, E.coli	Mixed	Not available	3.0 µA/cm^2	NR	Not available	Not available	[51]
Rhodospirillumrubrum	Pure	Double chambered	Not available	1.25 W/m^2	Not available	Not available	[52]
R. sphaeroides	Pure	single chambered	Not available	790 mW/m^2	Not available	Not available	[53]
R. palustris	Pure	Not available	Not available	2720 mW/m^2	Not available	Not available	[54]
Ochrobactrumanthropi	Pure	Not available	Not available	89 mW/m	Not available	Not available	[55]
Acidiphiliumcryptum	Pure	Not available	Not available	12.7 mW/m^2	Not available	Not available	[56]
Shewanellaoneidensis M R-2	Pure	Dual cham-berredMFC	31 mA/m^2	12.9 mW/m	Not available	81%	[57]
Shewanellabaltica 21	engineered strain	Dual chambered MFC	Not available	1304 mW/m^2	Not available	Not available	[58]
Pseudomonas aeruginosa PBH04	Pure	Not available	125 mA/m^2	26 mW/m^2	Not available	Not available	[59]
*Bacillus amyloliquefaciens*NSB4	Pure	Single Chambered	0.4804A/m^2	41.281 mW/m^2	90.46%	72%	This study

Polarization study and Cyclic Voltammetry

The curve recorded during the polarization study, displays the effect of external resistances (10 Ω, 100 Ω, 1000 Ω, 10kΩ, 100kΩ and 1000kΩ) on the power generation by the MFC, under particular operating conditions.

Fig. 2 displays the current as well as power. In current study, highest power density recorded was 5.77W/m^2. These results are also comparable to the previous reports[35] and therefore, highlight the typical role of *Bacillus amyloliquefaciens* NSB4 in power generation. To further confirm the oxidation reduction status of MFC during the substrate utilization and electron transport by the bacteria, cyclic voltammetry (CV) was performed using the Potentiostat (BioLogic SP-150, Department of Life Sciences, Sharda University, Greater Noida, India).

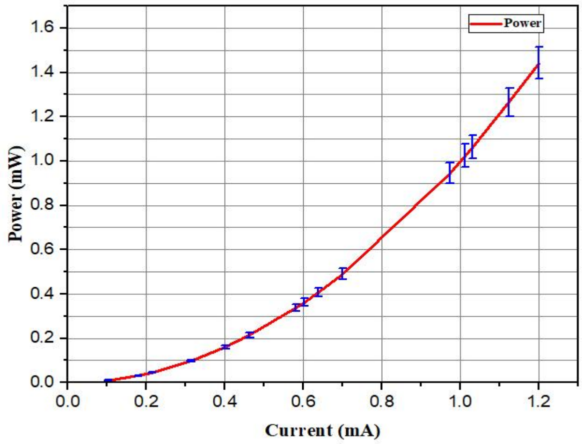
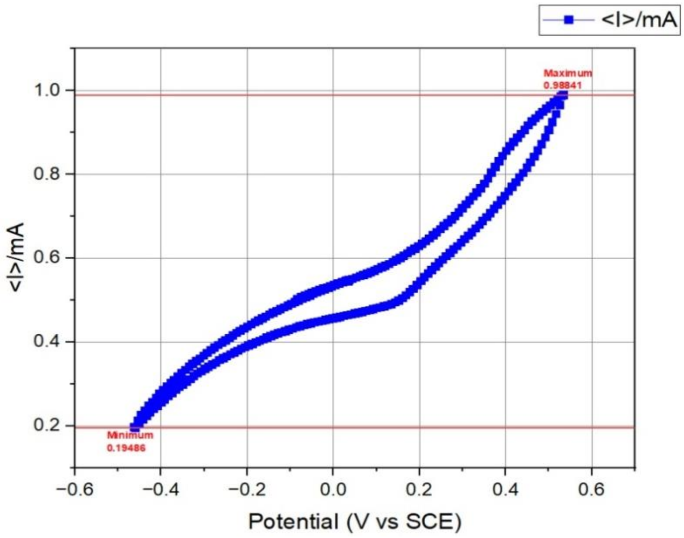
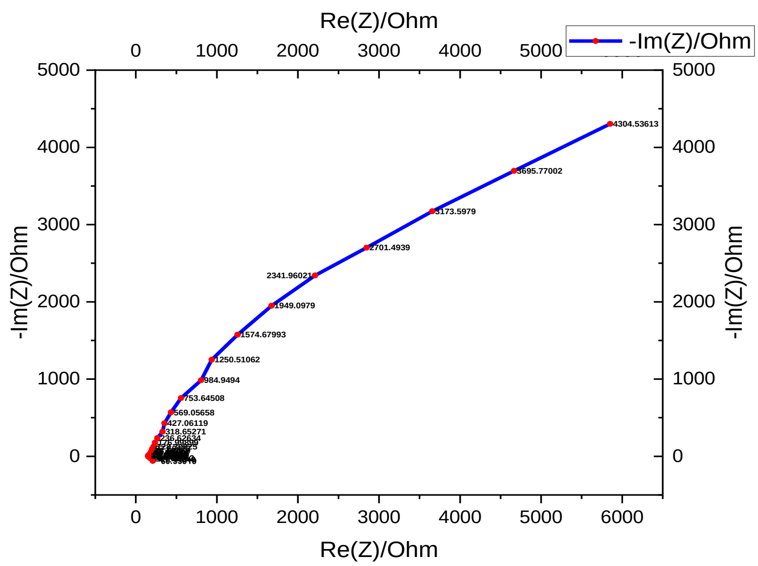


Figure 2. Graph represents the current (mA) and power (mW) produced during the treatment of wastewater by Bacillus amyloliquefaciensNSB4



a)



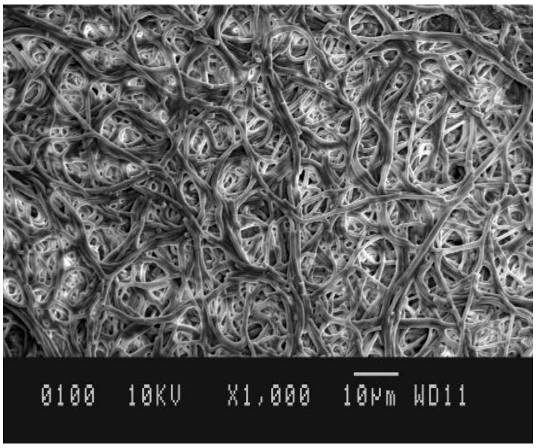
b)

Figure 3.Graph shows (3a) the voltammogram recorded during the oxidation reduction status of the MFC and the graph and (3b)the relation of internal resistance and external resistance of the MFC studied

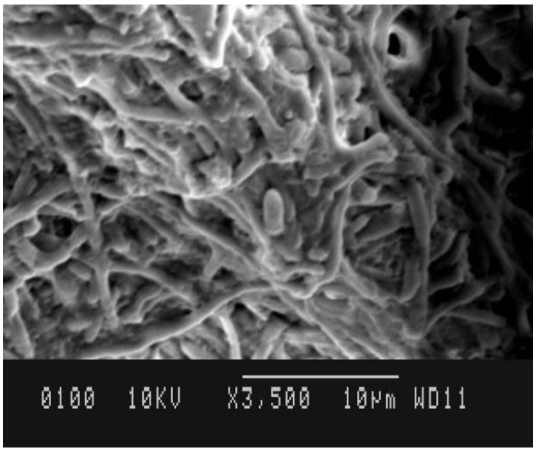
The CV observations revealed the presence of oxidation reduction peaks, associated with the electrode.The anode of the MFC displays oxidation (0.988 mA at 0.533 V) and reduction peak (-0.194mA at -0.460 V) (Fig. 3a). The position of the redox peak displays the redox capability of the components involved in Extracellular Electron Transfer (EET). Comparatively, the results are in satisfaction with the work already reported[60]. The Electrochemical Impedance Spectroscopy (EIS) was also conducted in this study (Fig. 3b).

SEM observation of biofilm

For analysing the biofilm growth, the anode pieces of both the stages, before and after the power production from the wastewater as well as COD removal, were examined under SEM. No growth was observed before the start of MFC whereas, growth of rod-shaped bacteria was visible on the anode surface post 15 days of operation (Fig. 4a and 4b). This finding clearly displays the exoelectrogen-anode association as well as the transfer of electrons without the aid of any mediator.



a)



b)

Figure 4. Scanning Electron Micrograph shows (4a) no growth on anode surfaceand (4b)Biofilm attached on the anode surface

4. CONCLUSION

Microbial fuel cell technology exhibits a great applicability in the wastewater treatment and power production. The design as well as the constructing material of the reactor significantly enhances the efficiency of the MFC operation. The pure ceramic material-built separator used in the current study effectively boosted the ion exchange performance of the process. Besides the design of the reactor, the involvement of a pure bacterial strain, *Bacillus amyloliquefaciens* NSB4 as a biocatalyst, also plays a vital role in this technology by acting as an extracellular electron transfer system and generated the electric power. Additionally, the COD reduction efficiency recorded was also quite high, as near about 90.46% reduction in COD of the wastewater was noted before and after the MFC process. Therefore, it can be concluded that the strain *Bacillus amyloliquefaciens* NSB4 could be best utilized as a low-cost and highly-efficient exoelectrogen in a single-chambered mediator-free MFC technology.

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IZVOD

*BACILLUS AMILOLIKUEFACIENS*SOJ BAKTERIJA NSB4 ZA PREČIŠĆAVANJE OTPADNIH VODA ZA PRIMENU GORIVIH ĆELIJA

Zagađivači u vodnim telima dolaze iz različitih izvora, uključujući, ali ne ograničavajući se na domaće, industrijske, komunalne itd. Zagađenje vode i nedostatak energije su globalni problemi koji zahtevaju značajnu pažnju. Zbog toga je neophodno sintetizovati održivu energiju i transportovati vodu bez otpada do prihvatnih mesta. Zabrinutost zbog nestašice energije i kontaminacije vode podstakla je razvoj tehnologije mikrobnih gorivih ćelija. Mikroorganizmi se koriste od strane elektrohemijske ćelijske prirode MFC-a za anaerobno varenje organskog otpada i proizvodnju energije. Fokusirajući se na jednokomorne MFC-ove bez medijatora koji rade u serijskom režimu, ova studija procenjuje efikasnost novog bakterijskog soja Bacillus amilolikuefaciens NSB4, kao egzoelektrogena u smislu prinosa električne energije i eliminacije otpada. Rezultati elektrohemijske karakterizacije soja pokazali su maksimalnu gustinu struje od 0,4804 A/m2 i gustinu snage od 41,281 mV/m2. Pored toga, Kolumbijska efikasnost (72%) i efikasnost smanjenja COD-a (90,46%) su takođe bile izuzetno visoke. Rast anodnog biofilma tokom MFC procesa pokazao je ključne performanse korišćenog egzoelektrogena. SEM slike biofilma su takođe predstavljene u studiji.
Ključne reči: *mikrobne gorivne ćelije, MFC bez posrednika, separator, biofilm, tretman otpadnih voda.*

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