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Simultaneous cellulose hydrolysis and bio-electricity generation in a mediatorless Microbial Fuel Cell using a *Bacillus flexus* strain isolated from wastewater

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The ability of electrochemically active bacteria to degrade natural wastes suggests a wide array of applications for waste management as well as electricity generation. This study reports the results of an experimentation where five bacteria were isolated from waste water and the electrochemical activity of one of them was confirmed by their use in a Microbial Fuel Cell. The bacterium was identified to be *Bacillus flexus* with a 99% similarity to the IFO 15715 strain using partial 16S rDNA sequencing. Phylogenetic analysis based on the BLAST results indicated close proximity to *Bacillus megaterium*, a known exoelectrogen. The cellulolytic ability of the bacteria was established in an MFC containing 1% Carboxy-Methyl-Cellulose as the sole carbon source which yielded a peak current density of 267.81±1 mA.m^{-2} of the anode, and a peak voltage of 0.421 V. The results clearly indicate that the bacterium is an exoelectrogen and can hydrolyse cellulose. This is the first report of the electrochemical ability of *Bacillus flexus*. The fact that *Bacillus flexus* can utilise cellulose for electricity generation gives way for future research on electricity production from natural cellulosic wastes.

Keywords: *Bacillus flexus,* MFC, Cellulose, mediator less, cmc.

The world's need for a sustainable and clean energy source is fast becoming a major issue for all the countries. Rising sea levels, increasing pollution and changing weather patterns globally has forced man to search for new alternatives to conventional sources of energy which need to be replaced by non-polluting resource which is available in abundance. Another major problem being faced by developing countries such as India is the problem of waste management. India generates approximately 350 million tonnes of agricultural wastes every year (Pappu et al. 2007). This is a staggering amount of waste that is mostly dumped into landfills and causes the growth of unwanted microbes. A small fraction of this colossal number is used for the generation of energy from biomass using biogas plants in the rural areas (Elangoa et al. 2007). An even smaller fraction is used as compost to obtain manure for agriculture. However, the amount recycled is so little compared to the amount generated that it can almost be neglected.

An ideal solution to these problems would be to convert the waste into energy [Liu et al. 2004; Moon et al. 2006; Logan 2008; Mohan et al. 2008; Zain et al. 2011). This can be achieved by a variety of conventional means involving physical and

chemical methods. Popular among these treatment methods to generate energy include gasification, steam explosion, incineration and pyrolysis. These methods, while being expensive and tedious, damage the environment quite badly by the liberation of toxic gases. Also, the energy yield by these processes is very low. This warrants the use of biological methods of control because, firstly the pollution levels are almost negligible in these methods and secondly, the manpower and the costs involved are very low. Since the major component of these wastes is cellulose, the production of cellulosic ethanol by saccharification followed by fermentation is one method of biological conversion (Gomathi et al. 2012).The ethanol can then be used for other purposes such as electricity generation or as a fuel for automobiles. However, the use of cellulose for direct production of electricity scores over the ethanol production method, due to the lesser number of steps involved, and also due to the savings in the costs of processing and purifying the ethanol. This direct conversion of cellulose into electricity is possible by use of a microbial fuel cell (MFC).

An MFC works on the same basic principle as any other fuel cell, i.e. the usage of a fuel source for the generation of electricity. The fuel source here is bacteria which colonize the anode. The bacteria breakdown the organic matter by their metabolism and their respiration generates electrons, which flow through the external circuit, to be finally accepted by a terminal acceptor at the cathode. The essence of the working of the MFC lies in the interruption of the usual electron transport chain of the bacteria by the electrode. The electrode acts as the terminal electron acceptor instead of the traditional sulphates or nitrates. The protons generated in the bacterial respiration process pass through a Cation exchange membrane into the cathode where they reduce the dissolved oxygen to water (Logan 2008).

Ren et al. (2008) made use of crystalline cellulose for its direct conversion into electricity by microbial action in amediatorless microbial fuel cell. They used a mixed culture of two bacteria, *Clostridium cellulolyticum* and *Geobacter sulfurreducens* for cellulose hydrolysis and electricity production respectively. A year later, Rezaei et al. (2009) used a single bacterium, *Enterobacter cloacae,* to perform both the tasks, which was the first of its kind.

This encouraged researchers across the world to look for other, more efficient electrochemically active bacteria that could utilise cellulose and produce electricity. This is because cellulose is the most abundant bio-polymer on earth (Rezaei et al. 2009). Since it forms a component of most plant and algal cell walls, the issue of availability of the substrate gets ruled out and so it forms an ideal solution to the world's energy crisis.

In this study, we extend the previous research by demonstrating the feasibility of using cellulose for electricity production by a novel exoelectrogen isolated from waste water. This manuscript details the isolation, characterization and testing of the electrochemical ability and cellulose hydrolysis potentials of this bacteria.

Materials and Methods:

MFC Architecture:

The MFC was fabricated out of acrylic plastic with suitable attachments in the middle for inserting and removing the membrane. The anode and cathode volumes were one litre each and contained valves for adding and removing the anode and cathode components to the MFC. Graphite plates were obtained from Graphite India Ltd and pre-treated by heating in a chamber containing ammonia gas as described by Wang et al. (2009).The area of the plates was78.6 cm^2 each and they were connected to wires by means of epoxy material (which also served the purpose of insulating the region of contact between the wires and the electrode with the electrolytes)and used as electrodes (Gregory et al. 2004). The membrane used was a Cation Exchange Membrane (CMI-

7000, Membranes International Inc.). Membrane pre-treatment was done by immersing the membrane in a solution of 1 M NaCl for a period of 2 hours before its use, as indicated by the manufacturers. The membrane was cleaned between uses with 2 M NaOH solution. Water was used as the catholyte and was frequently sparged with air using an air pump to maintain sufficient dissolved oxygen (Kim et al. 2008). A 200 Ω resistor(R) was connected across the two electrodes to facilitate the measurement of voltage (V). All measurements were taken using a multimeter. The current (I)and the power (P) were computed using I=V/R and P = VI respectively.

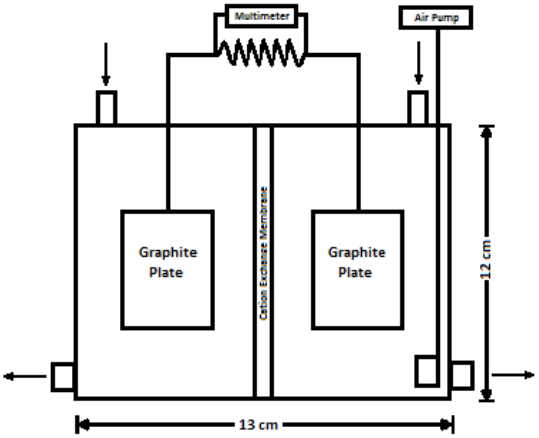

Fig 1 Schematic Diagram of MFC

Isolation of Electrochemically active bacteria

Waste water was collected from Vrishabhawathi River, at the spot behind RV College of Engineering, Bangalore and filtered using Whatman No.1 filter paper before its use in the MFC. The MFC was allowed to function for a period of 10 days with periodic enrichment of wastewater after every 2 days to allow for selective growth of the electrochemically active bacteria (Chang et al. 2006). The anode from this MFC was placed in a solution of sterile water and shaken gently to liberate cells adhering to the electrode. The solution was then serially diluted and plated into Luria Bertani (LB) agar plates and incubated at 30°C for 24 hours.

Test for electrochemical activity

Five bacteria were selected based on varying morphology and were sub cultured in LB broth. These five bacteria were then tested separately in the MFC for electrochemical activity by aseptically inoculating 20 ml of the respective liquid broth into 1 litre of sterile LB broth in the anode of the MFC. Electrochemical activity was established by observing for development of voltage over a period of 1 week.

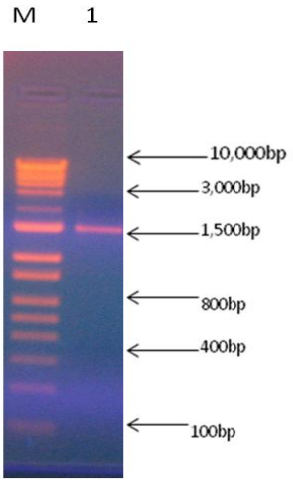

Fig 2: 1500 bp amplicon of 16s r DNA from *Bacillus flexus* strain

DNA Isolation and PCR

The genomic DNA was isolated from the organism displaying electrochemical activity in the MFC using genomic DNA extraction Kit (BHAT BIOTECH, Bangalore, India) in accordance with the instructions provided by the maker. The DNA was run on a 1 % agarose gel to confirm its integrity and Polymerase Chain Reaction (PCR), was performed to amplify the 16S rDNA gene using the forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and the reverse primer 5'-ACG GCT ACC TTG TTA CGA CTT-3'. The amplification was carried out in a Master cycler® Thermocycler (Eppendorf, Germany) using the following conditions - Initial denaturation at 94°C for 2 minutes followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55°C for 1 min and extension at 72°C for

1.5 minutes. Final extension was carried out at 72°C for 10 min. The PCR product was purified to remove unincorporated dNTPs and primers before sequencing using a PCR purification kit (Norgen Biotech, Canada). The PCR product integrity was then verified by using a 1.2% agarose gel (figure 2).

DNA Sequencing and Analysis

Both strands of the DNA amplified by PCR were sequenced in an automated DNA sequencer (3037xl DNA analyser, Applied Biosystems) by using BigDye® Terminator v3.1 cycle sequencing Kit (Applied Biosystems). Sequence data was then aligned and dendrograms were generated using Sequence analysis software version 5.2 (Applied Biosystems). The sequences obtained for plus and minus strands were aligned using DNA baser software before performing the bioinformatics analysis.

Sequences were compared to the non-redundant NCBI GenBank database using BLASTN, with the default settings. On the basis of this, the most similar sequences were identified and sorted by the E score. A representative sequence of 10 most similar neighbours was then aligned using CLUSTAL W2 alignment server for multiple alignment with the default settings. The multiple-alignment file was then used to create the phylogram using MEGA 5 (Molecular Evolutionary Genetics Analysis) software.

Test for Cellulolytic Ability in MFC

The media used in the anolyte contained 1% Carboxy Methyl Cellulose-Sodium Salt (CMC-Na) as the sole carbon source. Other media components include yeast extract (0.5%), KH_2PO_4 (0.15%), $(NH_4)_2SO_4$ (0.15%), NaCl (0.3%), Peptone (1.5%), LiCl (0.05%), $CaCl_2$ (0.1%), and $MgSO_4$ (0.1%). A single colony of the bacteria from LB agar plates was aseptically inoculated into 200 ml of this media and allowed to incubate on a rotary shaker (LABTOP Instruments, India) at 100 rpm, 30 °C overnight. 20 ml of this overnight grown culture was used as the inoculum for the MFC. The pH of the medium was maintained at 7.2 by the use of phosphate buffer (Jadhav and Ghangrekar 2009)

Comparison of Electrochemical Ability

The culture of *Shewanella putrefaciens,* a known exoelectrogen (Rabaey and Verstraete 2005) was obtained from the Microbial Type Culture Collection (Accession Number 8104), Chandigarh, India. 1 litre of LB broth was prepared and autoclaved and *Shewanella* was aseptically inoculated into the media and used in the MFC anode to compare its electrochemical activity with that of *Bacillus flexus.*

Results

Isolation and testing of Electrochemically Active Bacteria

Five distinct colonies were chosen based on the morphological differences in their growth on the LB agar plate. One particular bacterium (Later identified by 16s rDNA sequencing as *Bacillus flexus)* generated a voltage when used in the MFC. A peak voltage of 0.542 V was generated on the second day of the MFC run as compared to 0.631 V generated by *Shewanella*. This equated to a current value of 2.71 mA for the isolated bacteria and 3.16 mA for *Shewanella.* The power density values on day 2 were at 186.8 $mW.m^{-2}$ and 253.28 $mW.m^{-2}$ of the electrodes respectively. These values experienced a steady decline after this point with the voltage finally dropping to 0.092 V on day 7 for the isolated bacteria and 0.079V for *Shewanella putrefaciens* as can be seen from figure 3.

Identification and Phylogenetic Analysis

The results of the BLAST search conducted for the partial 16S rDNA sequence obtained after sequencing of the PCR product indicated a 99% homology with the *Bacillus flexus* strain IFO15715, with an e value of 0.

The evolutionary relationship was inferred using the Neighbour-Joining method. The optimal tree with the sum of branch lengths equal to 0.15316644 is shown in figure 3. The percentages of replicate trees in which the associated taxa

are clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved the use of 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. A total of 1417 positions in the final dataset were obtained (Figure 4)

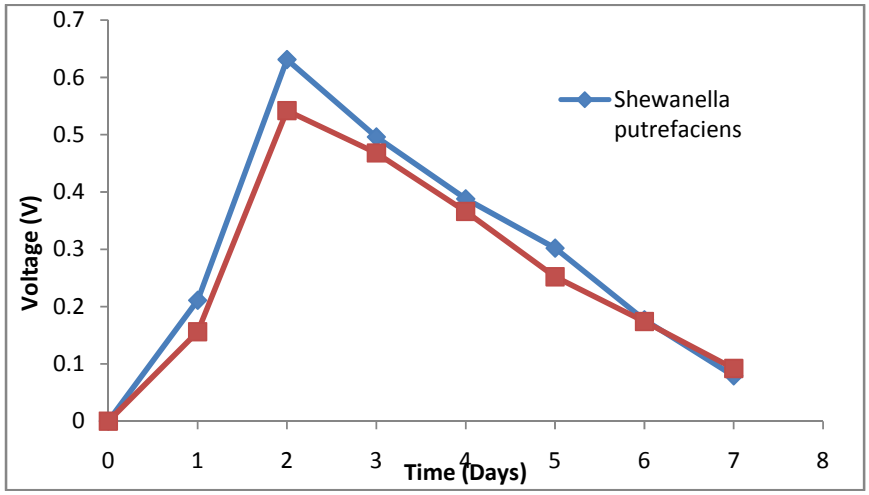

Fig. 3. Comparison of Voltage generated over time between *Shewanella putrefaciens* and *Bacillus flexus*

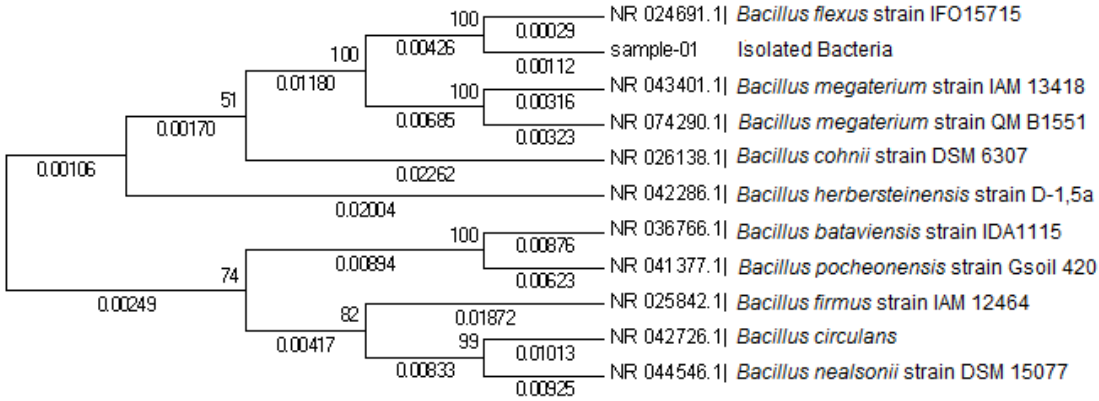

Fig. 4 Phylogenetic tree showing evolutionary distance between the isolated bacteria and closely related species based on 16 S rDNA sequencing results. The tree was constructed using the Neighbour joining method and the bootstrap values at the nodes were calculated based on 500 replicates

Cellulolytic Ability

Voltage and current readings experienced a sharp climb on the second day of the MFC run with a peak voltage of 0.421 mV (fig 5) which corresponded to a current value of 2.11 mA. The voltage readings then declined steadily with values falling daily until day 7. The peak current density obtained was 267.81 ± 1 $mA.m^{-2}$ on day 2, and current density values showed a similar trend as the voltage with an initial sharp climb on the second day of operation and a gradual decline leading to zero ultimately.

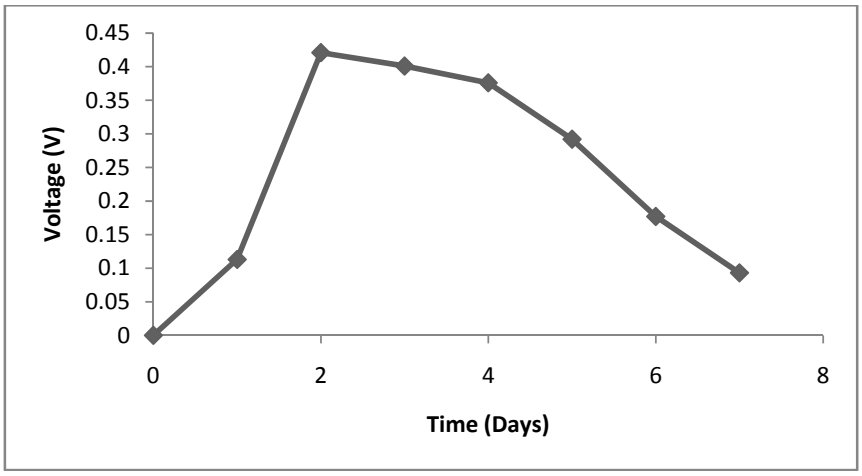

Fig. 5. Voltage generated over time with CMC-Na as the sole carbon source

Discussion

The electrochemical activity of *Bacillus flexus* has been reported for the first time in this manuscript. This is advancement towards making the MFC a reality for large scale power generation. Although the electrochemical ability of *Bacillus flexus* might be lesser than that of *Shewanella putrefaciens,* it has the advantage of possessing the cellulase gene, which puts it above *Shewanella* when it comes to the overall costs of using MFCs for large scale power generation.

The reason for the voltage readings making a sharp climb on the second day of the MFC run can be attributed to the bacteria being in their most active state (late log phase) at the time. However, as the growth curve progressed and the culture entered the stationary and ultimately the death phase, the voltage readings showed an appropriate fall. Also, this hypothesis of the nutrient limitation being the prime reason for fall in the MFC performance after the second day is backed by the fact that all other parameters such as dissolved oxygen, electrode spacing, etc were maintained at constant levels.

While the work of Ren et al. (2008) was novel at the time, the use of two electrochemically active bacteria meant the substrate provided in the media was competed for by two bacteria. Thus, the output of the MFC was not at its optimum best since half the population of the MFC anode could not produce electricity. This drawback is overcome by the use of *Bacillus flexus.* The ability of the bacteria to utilise cellulose, has also been reported by Trivedi et al. (2013) and this makes it even more economical for large scale use due to reasons mentioned earlier.

Another unique ability of *Bacillus flexus*is its tolerance to high levels of salts due to its marine habitat, which puts it among a number of unique bacteria (Borah et al. 2013) which can be used for electricity generation in the deeper regions of the oceans. The output of the MFC functioning on *Bacillus flexus* can be further increased by increasing the pH of the anode to 10, at which the bacterial cellulase is at its most active (Trivedi et al. 2013). However, at this pH, the growth of the bacterium is extremely slow. Thus a balance needs to be struck between the optimum pH for growth and that for best cellulase activity.

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