Chapter 6

Microbiology of Electrogenic Activity

6.1 Introduction

Substrate oxidation resulting in conversion to electricity requires catalysis by biofilms (Zhou et al. 2013). New breakthroughs are required in the field of MFC for practical application in wastewater treatment. One of these is a highly efficient biofilm.

In anaerobic digestion, a “food-chain” type consortia catalyses biogas formation wherein the role of methanogens is vital (Pham et al. 2006). On the other hand, in MFCs inoculated with mixed consortia, not all bacteria are electrogenic. However, they provide organic nutrients to the electrogenic bacteria and play crucial roles in biofilm growth (Zhou et al. 2013). Considering the above two aspects, it was interesting to explore and elucidate the presence of electrogenic bacteria from the consortia responsible for biogas formation. Also, the formation of biofilms and the mechanism of electron transfer are key parts of the study in this chapter.
6.2 Materials and Methods

6.2.1 General

The materials used and the methods adopted to elucidate the bacteria responsible for the activity as well as the mechanism of electron transfer is presented in the following sections.

6.2.2 Bacteriological Media and Chemicals

Bacteriological media such as nutrient agar, anaerobic agar, and tomato juice agar, as well as Anaerogas pack all were purchased from HiMedia (India). All chemicals used were of ExcelaR grade obtained from Qualigens (Fisher Scientific, India).

6.2.3 Analysis of pH, Dissolved Oxygen and Viable Count of ADDW

The pH of ADDW in the anodic chamber was analysed at 0, 30 and 50 h using a pH meter (model 1500, Eutech Instruments, India). Similarly, dissolved oxygen was measured using a DO probe at the same intervals. ADDW at 50 h was serially diluted and viable count was determined using the spread plate technique on nutrient agar and anaerobic agar for aerobes and anaerobes, respectively. Nutrient agar plates were aerobically incubated at 37°C for 24 h; anaerobic agar plates were incubated at 37°C for 48 h under anaerobic conditions, created by Anaerogas pack in an anaerobic jar.
6.2.4 Electricity Generation from Acetic Acid as Carbon Source

6.2.4.1 Monitoring of Acetic Acid, Butyric Acid and Propionic Acid by Gas Chromatography

Samples were taken every 10 h. Quantitative analyses of metabolic acids were performed using external standard method. Standards of acetic acid (0.084%), butyric acid (0.0505%), and propionic acid (0.0652%) were used. The analyses were conducted on a Shimadzu Gas Chromatograph (Model 2010, Shimadzu, Japan) at Clariant Chemicals Ltd. (Thane, India). A 30 m × 0.25 mm × 0.25 μm DB-5ms capillary column (Agilent J &W) was used with helium carrier gas at 0.70 mL/min. Column temperature was 85°C for 4 min, increased to 250°C at 10°C/min, held for 5 min. Injector temperature was 250°C, as was the FID detector. Split injection (50:1) was achieved using an AOC-5000 autosampler (Shimadzu, Japan) which injected 1 μL doses through a glass wool liner to keep non-volatiles from entering the column. The run time for each analysis was approximately 25 min.

6.2.4.2 MFC Experiment Confirming Role of Acetic Acid

MFC experiments were performed to determine the role of acetic acid. In each, two MFCs were held at optimum conditions, and at 55 h, the anodic chamber, containing ADDW, was spiked with 0.2% acetic acid in one of them. The other was used as a control. The MFCs were held for a total run of 100 h and qualitative changes in voltage generation were analysed. All experiments were carried out in triplicates.

6.2.5 Analysis of Biofilm by Scanning Electron Microscopy

Anodes removed at 0, 30 and 50 h from the optimized MFC were subjected to scanning electron microscopy (SEM). The analysis was performed at Sophisticated Analytical Instrument Facility, IIT Bombay using ESEM (model FEI Quanta 200).
6.2.6 Isolation and Identification of Electroactive Bacteria from Biofilm

Anode from the MFC was immersed in sterile saline and sonicated for few seconds to suspend microorganisms present in the biofilm, which was then used as a microbial source for isolation. Organisms were isolated under aseptic conditions in Laminar airflow (Esco, India) on anaerobic agar, tomato juice agar special and nutrient agar. All media plates were incubated in an incubator (Equitron, Medica Instruments Mfg. Co., India) at 37°C for 72 h under anaerobic conditions created by Anaerogas Pack in an anaerobic jar. Single colonies were removed from the plates and sub-cultured on new plates, then repeated to obtain pure cultures. The outline of morphological and physiological characteristics of pure cultures were determined following the methods of Cappuccino and Sherman (2005). All required media for biochemical tests were prepared in test tubes and petri dishes in accordance with the test. Reagents required for biochemical tests were prepared freshly. 10 µL of the overnight grown cultures were inoculated in the biochemical test media and incubated at 37°C for 48 h. The results were compared to different representative genera in Bergey’s manual of determinative bacteriology. All isolates were then sent for identification to BioAxis DNA Research Centre, Hyderabad.

Following the manufacturer's instructions, BioPure DNA Isolation and Purification Kits, developed by BioAxis DNA Research Centre, were used to extract DNA. Extracted DNA was amplified using universal primers 8F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R: (5’-ACGGCTACCTTGTTACGACTT-3’). The PCR was carried out using 11 µL reaction mixture containing 1 µL Taq polymerase, 1 µL primer 8F, 1 µL primer 1492R, 2 µL DNA, 2.5 µL Taq buffer, 2 µL dNTP mix, and 1.5 µL MgCl₂. The system was programmed with 5 min at 94°C, 35 cycles for 30 sec at 94°C, 30 sec at 56°C, 30 sec at 72°C and final extension at 72°C. The results were analysed using 1.2% agarose gel electrophoresis. The sequencing of the product was carried out by ABI 3100 using Sanger’s method.
6.2.7 Denaturing Gradient Gel Electrophoresis

6.2.7.1 DNA Extraction and Conditions

DGGE analyses were performed by BioAxis DNA Research Centre, Hyderabad. In brief, total DNA from the 50 h anodic biofilm suspension was extracted using FastDNA SPIN Kit for Soil (MP Biomedical, India). The DNA was amplified using PCR using the universal primers F984 (5′-CCCCGCGGCGGCGGCGGCGGCGGCGGGGCACGGCGAAACCTTAC-3′) and R1378 (5′-CGGTGTGTACAAGGCCCGGGAACG-3′) (Costa et al. 2006). The DGGE analysis was performed in DGGE apparatus (Bio-rad, India). PCR product was loaded onto 2% (w/v) polyacrylamide gels in 10x TAE (15 mM Tris-HCl, 50 mM acetate, 0.5 mM EDTA, pH 7.4). To separate the amplified DNA fragments, the polyacrylamide gels were made with denaturing gradients ranging from 0% to 80%. The electrophoresis was run for 16 h at 60°C and 40-50 V. Bands were visualized using a UV trans-illuminator at 256 nm after staining the gel for 15 min with 0.5 µg/mL ethidiumbromide and then destaining with gel in water for 5 min.

6.2.7.2 Sequencing of DGGE Bands

Selected bands were carefully cut from the gel and spun to separate the acrylamide. After adding 10-15 µL of sterile distilled water, the acrylamide slice was broken with a pipet tip. The DNA was allowed to elute for 10-20 minutes at room temperature, then 1-2 µL were checked for purity and used as templates in the PCR reaction using the conditions described in section 6.2.6, followed by sequencing using an ABI 3100 sequencer (Sanger method).

6.2.8 Evaluation of Mechanism of Electron Transfer

6.2.8.1 Modified U-tube MFC

Two chambered MFCs constructed earlier were modified appropriately for this experiment. As shown in Figure 6.1, the anodic chamber was divided into two components separated by a sterile 0.2 µm membrane. One component was filled with 200 mL sterile distilled water, while the other was filled with 200 mL modified ADDW. The anode was placed in the water-
containing component, which was joined to the cathodic chamber using a salt bridge. Both electrodes were connected to copper wires, which in turn were connected to 1000 Ω external resistance. The cathodic chamber was filled with 200 mL of 100 mM potassium ferricyanide prepared in 100 mM phosphate buffer at pH 7. All experiments were carried out at room temperature (27 ± 3°C) in triplicates.

![Image](image-url)

**Figure 6.1** Modified U-tube MFC Experiment

### 6.2.8.2 Cyclic Voltammetry

An Ag/AgCl electrode was calibrated with the help of standard reversible hydrogen electrodes in order to estimate its voltage in a 25 mL electrochemical cell. The pre-standardized electrode was used as reference in cyclic voltammetry experiments carried out using conventional three-electrode systems in which the anode was used as a working electrode while the cathode was used as counter electrode. The cyclic voltammograms were obtained using a potentiostat (model VSP-300; Biologic Instrument, Germany) at a scan rate of 5 mV/s over a range of 0.5 to -0.6 V. Cyclic voltammetry experiments were performed to analyse the anodes after 0, 30 and 50 h for direct electrode reaction of bacterial cells on the anode surfaces. All experiments were repeated at least five times.
6.3 Results and Discussion

6.3.1 Analysis of pH, Dissolved Oxygen and Viable Count of ADDW

Generally, the anaerobic digestion process and production of methane is divided into different stages. Often, three stages are defined to illustrate the sequence of microbial events that occur during the digestion process and the production of methane (Figure 6.2). These stages are hydrolysis, acidogenesis and methanogenesis.

![Figure 6.2 Stages of Anaerobic Digestion](image)

When the anaerobic digestion process is efficient, the degradation rates of all three stages are equal. If the first stage is inhibited, then the substrates for the second and third stages will be limited, decreasing methane production. If the third stage is inhibited, the acids produced in the second stage will accumulate. Inhibition in the third stage occurs because of an increase in acids, signified by a decrease in pH. The most common upsets of anaerobic digesters occur because of inhibition of methane-forming bacteria in the third stage. The anaerobic digestion process involves different groups of bacteria. These groups work in sequence, and the products of one group serve as substrates for another group. Therefore, each group is linked to
the other groups in a chain-like fashion, with the weakest links at the acetate and methane production stages (Gerardi 2003). Because ADDW was used in this study, all stages of anaerobic digestion were expected to occur.

Table 6.1 shows that ADDW became acidic over a period of 50 h under optimized MFC conditions. This confirmed the role of fermentative bacteria responsible for the generation of acids. Bacteria can generate electricity from dextrose (Rabaey et al. 2003), in which bacteria start generating electricity within 2 h, but in present study, the longer lag phase (30 h) indicated that electricity is generated from a carbon source other than dextrose. The acids produced during anaerobic digestion could be the substrate from which bacteria are deriving electrons. Similar findings were observed by Lee et al. (2008) where *Geobacter sulfurreducens* was used as the major bacterial species in the MFC, and were fed glucose as well as acetate. They observed that the time required was longer when glucose was used rather than acetate because *G. sulfurreducens* is unable to ferment glucose and hence, depends on other fermentative bacteria to produce acids.

Anaerobic digestion is a biochemical process in which organic matter is converted to biogas in the absence of oxygen (Botheju and Bakke 2011). Since the wastewater used in this study is from an anaerobic digester, little or no dissolved oxygen was expected. As seen in Table 6.1, the dissolved oxygen (DO) was less than 1 mg/L. Anaerobic conditions exist when DO is less than 1.0 mg/L (Castle and Nemeth-Harn 2007). The low DO at 0 h was reduced further as time progressed, which indicated the growth of bacteria. Also, bacterial growth was predicted with the increase in acidity (Table 6.1) as well as foam formation (Chapter 4).

Viable counts of aerobes as well as anaerobic bacteria over a period of 50 h were monitored to determine a means of elucidating the activity and the bacteria responsible for it. It was observed that aerobic bacteria increased from 0 h to 30 h, but remained constant thereafter, or until 50 h. In contrast, the anaerobes increased continuously from 0 h to 50 h (Table 6.1). It may be concluded that aerobes are utilizing the dissolved oxygen for growth and are responsible for acid accumulation. Formation of acid could also be caused by growth of anaerobes. Considering the very low DO in ADDW and the possibility of obligate anaerobes, facultative anaerobes and aerotolerants cannot be ruled out. Also, as known that two processes occur during anaerobic digestion: first, acid is formed by obligate anaerobes, facultative anaerobes, and aerotolerant bacteria utilizing organic matter and producing gases such as CO₂.
and H₂S, and second, methane is formed by methanogenic bacteria (Gerardi 2003). The presence of H₂S was detected by using filter paper dipped in lead acetate near the sidearm of the anodic chamber, which turned black upon reacting with H₂S. There was increase in electricity generation from 30 h to 50 h in optimized MFCs and until that time, only anaerobic bacterial growth was observed. Because the microbial growth curve corresponds to the MFC curve; the role of anaerobes in electricity generation was confirmed.

Table 6.1 Analysis of pH, Dissolved Oxygen and Viable Count

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>pH of ADDW (mg/L)</th>
<th>Dissolved Oxygen (mg/L)</th>
<th>Viable Count (CFU/mL)</th>
<th>Aerobic</th>
<th>Anaerobic</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.96 ± 0.1</td>
<td>0.54 ± 0.01</td>
<td>3 ± 1.67 × 10⁵</td>
<td>2 ± 1.2 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>6.69 ± 0.2</td>
<td>0.19 ± 0.01</td>
<td>4 ± 2.3 × 10⁷</td>
<td>2 ± 0.3 × 10⁵</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>4.96 ± 0.3</td>
<td>0.05 ± 0.01</td>
<td>2 ± 0.96 × 10⁷</td>
<td>4 ± 0.07 × 10⁶</td>
<td></td>
</tr>
</tbody>
</table>

(Results expressed as Mean ± SD; n = 3)

6.3.2 Electricity Generation from Acetic Acid as Carbon Source

It is important to confirm the presence of acid produced by fermentative bacteria. Acetic acid, butyric acid, or propionic acid are expected because they are major by-products of fermentation metabolism (Gerardi 2003). Rabaey et al. (2005c) confirmed the fermentation of glucose into acetic acid using ion chromatography. Acetate is produced via several fermentative pathways. A large diversity of bacteria, collectively known as acetogenic or acetate-forming bacteria, produce nongaseous acetate. These organisms include bacteria in the genera Acetobacterium, Clostridium and Sporomusa. Along with acetate, butyrate is a fermentative product of many bacteria. Strict anaerobes such as Clostridium sp. and Butyrivibrio sp. ferment a variety of sugars to produce butyrate. Anaerobic Propionibacteria sp. or propionate-forming bacteria are known to ferment glucose and lactate with the help of Bacteriodes sp., Clostridium sp., Peptostreptococcus sp., Ruminococcus sp., Selenomonas sp., Succinovibrio sp. and Veillonella sp. (Gerardi 2003).

Because most carbohydrates are first fermented in mixed anodic communities, electricity generation from carbohydrates is almost entirely a syntrophic process. However, it is not a process that creates electricity in MFCs because many electrons remain in the fermentation product and are unable to react with the anode. Effective anaerobic oxidation must combine
the fermentation products to complete electron transfer to the anode (Damiano 2009). Acetate might be the organic compound that is bacterially converted into electricity the fastest (Freguia 2010a). Others have reported on propionate (Bond and Lovely 2005) and butyrate (Liu et al. 2005a). In the present research, gas chromatographic analysis of ADDW was performed for acetic acid, butyric acid and propionic acid using method outlined in section 6.2.4.1. Figure 6.3 shows that acetic acid accumulated until 30 h, then suddenly decreased. Figure 6.4 clearly shows that electricity is simultaneously generated as acetate decreases in concentration.

![Graph of Concentrations of Acetic Acid, Butyric Acid and Propionic Acid over a Period of 50 h](image1)

**Figure 6.3** Concentrations of Acetic Acid, Butyric Acid and Propionic Acid over a Period of 50 h

![Graph of Comparing Utilization of Acetic Acid with Electricity Generation in an Optimized MFC](image2)

**Figure 6.4** Comparing Utilization of Acetic Acid with Electricity Generation in an Optimized MFC
This postulate was confirmed when the ADDW was spiked with 0.2% acetic acid (devoid of dextrose) at 55 h, a decrease in voltage was observed (Figure 6.5). As soon as the ADDW was spiked with acetate, a boost in electricity generation was observed, and this was not seen in the control. Acetate is a well-known substrate for electricity production (Das and Mangwani 2010; Lee et al. 2008, 2003; Liu et al. 2005a; Mathuriya and Sharma 2010; Nevin et al. 2008; Rabaey et al. 2003; Ren et al. 2011; Yates et al. 2012). Thus, the increase in generated electricity was immediate because the biofilm had already been formed on the surface of the anode, allowing immediate oxidation of the acetic acid.

![Figure 6.5 Confiming the Role of Acetate as a Substrate in Electricity Generation when Compared with MFC without Acetate. Arrow Represents the Time at which 0.2% Acetate was Spiked in the Anodic Chamber (Mean; n = 3; %CV < 10)](image)

6.3.3 Analysis of Biofilm Development

It was important to analyse the development of the biofilm on the anodic surface over a period of 50 h in an optimized MFC. All electrodes taken for SEM analysis were from the same MFC chamber to minimize the error. Figure 6.6 shows no bacterial cells on the anode surface at 0 h but as time progressed, the round-shaped bacteria along with deposition of exopolysaccharide on the anode surface were observed. Figure 6.6 showed oval-shaped bacteria covering the anode surface at 50 h, when electricity generation was at a maximum. Bacteria seen at 50 h were different from those observed at 30 h. Bacterial growth was found
to be uniform throughout the anode surface at 50 h, hence, this anode was considered as the best from which to isolate the bacteria responsible for electricity generation.

**Figure 6.6** Development of Biofilm at (a) 0 h, (b) 30 h and (c) 50 h on Anode Surface under 4000x Magnification
6.3.4 Isolation and Identification of Bacteria from Anode Biofilm

From the anodic biofilm, bacteria were inoculated on nutrient agar, tomato juice agar and anaerobic agar to isolate the wide variety of anaerobes. Though nutrient agar is a generalized media for aerobes, nutrient agar used was incubated under anaerobic conditions because the ADDW was anaerobic (DO < 1 mg/L). Tomato juice agar was used for growing lactic acid bacteria because ADDW became acidic over a period of time and the electricity was generated at an acidic pH. Anaerobic agar is specialized for cultivation of anaerobes, especially *Clostridia sp.* because *Clostridium sp.* is involved in all stages of anaerobic digestion except methanogenesis. However, only the anaerobic agar plates showed growth of bacteria derived from the anodic biofilm (Figure 6.7). These anaerobes were further purified to obtain three types of bacterially pure cultures (An1, An2 and An3) (Figure 6.7). An3 was visible only when observed against the light.

![Figure 6.7 Bacterial Growth on Anaerobic Agar Plate (Master Plate) from Anode Biofilm and Further Isolation of An1, An2 and An3](image-url)
Because wastewater was used, different types of microorganisms were expected to be responsible for the activity. The Gibbs free energy, available for the microorganisms during substrate oxidation, is proportional to the number of electrons transferred to the electrode and hence, scientists operate MFCs for extended times to establish highly active anodic biofilms (Cheng et al. 2008; Logan et al. 2006). One study also showed that MFCs could be used to enrich a bacterial consortium that oxidizes acetate with concomitant electron transfer to the electrode because the bacterial populations in the MFCs were different from the activated sludge that was used to inoculate them (Lee et al. 2003). That also means that anodes in MFCs selectively attract electrogenic bacteria to their surfaces. This could be the reason for obtaining very few culturable bacteria from the anode's surface. The morphological and biochemical characteristics of isolates are shown in Table 6.2 and Table 6.3. Closely related organisms from Bergey’s Manual showing similar characteristics were Clostridium beijerinckii, Clostridium butyricum, Clostridium clostridioforme, Clostridium coccoides, Clostridium ramosum, Clostridium tertium, Clostridium thermohydrosulfuricum, and Clostridium thermosaccharolyticum (Cato et al. 2009).

### Table 6.2 Morphological Characteristics of Isolates

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Organism 1 (An1)</th>
<th>Organism 2 (An2)</th>
<th>Organism 3 (An3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiration</td>
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<td>Anaerobic</td>
<td>Anaerobic</td>
</tr>
<tr>
<td>Shape</td>
<td>Circular</td>
<td>Circular</td>
<td>Circular</td>
</tr>
<tr>
<td>Size</td>
<td>5 mm</td>
<td>2 mm</td>
<td>Pinpoint</td>
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<tr>
<td>Colour</td>
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<td>Off-white</td>
<td>Colourless</td>
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<tr>
<td>Margin</td>
<td>Entire</td>
<td>Entire</td>
<td>Entire</td>
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<tr>
<td>Opacity</td>
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<td>Translucent</td>
<td>Translucent</td>
</tr>
<tr>
<td>Elevation</td>
<td>Concave</td>
<td>Concave</td>
<td>Concave</td>
</tr>
<tr>
<td>Consistency</td>
<td>Sticky</td>
<td>Sticky</td>
<td>Sticky</td>
</tr>
<tr>
<td>Gram Nature</td>
<td>Gram positive</td>
<td>Gram positive</td>
<td>Gram positive</td>
</tr>
</tbody>
</table>
Table 6.3 Biochemical Characteristics of Isolates

<table>
<thead>
<tr>
<th>Biochemical Test</th>
<th>Organism 1 (An1)</th>
<th>Organism 2 (An2)</th>
<th>Organism 3 (An3)</th>
</tr>
</thead>
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<tr>
<td>Endospore Staining</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lipase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Galactose Fermentation</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>Lactose Fermentation</td>
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<td>Raffinose Fermentation</td>
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<td>Arabinose Fermentation</td>
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<td>Sorbitol Fermentation</td>
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<td>Starch Fermentation</td>
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<td>Sucrose Fermentation</td>
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<td>+</td>
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<td>Xylose Fermentation</td>
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<td>Indole</td>
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<td>Nitrate reduction</td>
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</tr>
<tr>
<td>Lipase</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Key: + Positive Test ; - Negative Test

The three bacterial isolates were sent to BioAxis DNA Research Centre, Hyderabad for 16s rRNA identification. The PCR product of all three isolates as provided by them is shown in Figure 6.8.

The sequences of the products, when carried out using an ABI 3100 and the Sanger method are shown in Figure 6.9, Figure 6.10 and Figure 6.11. All isolated bacteria belonged to *Clostridium sp.*, shown in Table 6.4. The phylogenetic analysis for all isolates is shown in Figure 6.12, Figure 6.13 and Figure 6.14.
Figure 6.8 PCR Product of the Three Isolates

Legend: Lane 1 (M) – 1 Kb DNA Ladder (Sigma Aldrich), Lane 2 (An1) – Isolate 1, Lane 3 (An2) – Isolate 2, Lane 3 (An3) – Isolate 3

Figure 6.9 16s rRNA Sequence of Organism An1

Figure 6.10 16s rRNA Sequence of Organism An2
Table 6.4 Identification of the Three Isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Biochemical identification</th>
<th>Identification by 16s rRNA</th>
<th>Identities</th>
<th>% homology</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>An1</td>
<td>Clostridium sp.</td>
<td>Clostridium sp. 16 rRNA</td>
<td>1426/1437</td>
<td>99%</td>
<td>L23477.1</td>
</tr>
<tr>
<td>An2</td>
<td>Clostridium sp.</td>
<td>Clostridium sp. CFU-1</td>
<td>1334/1372</td>
<td>99%</td>
<td>AB673455.1</td>
</tr>
<tr>
<td>An3</td>
<td>Clostridium sp.</td>
<td>Clostridium sp. JCC</td>
<td>1200/1209</td>
<td>99%</td>
<td>HG726039.1</td>
</tr>
</tbody>
</table>

Figure 6.11 16s rRNA Sequence of Organism An3

Figure 6.12 Neighbour-Joining Tree of 16S rRNA Gene Sequences of An1. Phylogenetic Relationship of Organism An1 with many other Strains of Thermoanaerobacter sp. is Covered under Dendrogram
Figure 6.13 Neighbour-Joining Tree of 16S rRNA Gene Sequences of An2. Phylogenetic Relationship of Organism An2 with many other Strains of Clostridium sp. is Covered under Dendrogram

Figure 6.14 Neighbour-Joining Tree of 16S rRNA Gene Sequences of An3. Phylogenetic Relationship of Organism An3 with many other Strains of Uncultured Bacterial Clones is Covered under Dendrogram

6.3.5 DGGE Analysis

Using conventional microbiological techniques, identification of just three types of bacterial species was possible. However, the shortcomings of the conventional methods, such as incomplete knowledge about microbial physiological requirements and the complex syntrophic and symbiotic relationships, which were expected in this study, make it impossible to obtain pure cultures of most microorganisms in natural environments. Moreover, most culture media tend to favour the growth of certain groups of microorganisms, whereas others
that are important in the original sample do not proliferate (Sanz and Köchling 2007). Hence, use of a molecular technique, such as DGGE was found to be important to increase the possibility of identifying different microorganisms present in the mixed consortia on the anodic biofilm in their native habitat, without the need to isolate them (Sanz and Köchling 2007). Because the anodes removed after 50 h were uniformly covered by bacteria (as observed in SEM), these were used in DGGE analysis by BioAxis DNA Research Centre, Hyderabad. Figure 6.15 shows the DGGE pattern provided by them, from which five prominent bands were selected for further identification (Figure 6.15). The three bands that coincided with the molecular weights of the identified *Clostridium sp.* were excluded.

**Figure 6.15** DGGE Analysis of 50 h Biofilm showing Five Bright Bands (Arrows) Selected for Further Purification and Identification. From Top to Bottom, Bands were Labelled 1 through 5

Each band was further sequenced and identified in a way similar to the identification of individual isolates. The sequences of bands 1 through 5 are shown in Figure 6.16 through Figure 6.20. Table 6.5 represents the identification of all sequences and their phylogenetic relationships are shown in Figure 6.21 through Figure 6.25.

**Figure 6.16** 16s rRNA Sequence of Band 1
Figure 6.17 16s rRNA Sequence of Band 2

ATGCTTAGAATCGCTGCTGATGAGGGAACAGTGTTCAAGAAAGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT
CTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTGGTTGATGAGGGAACAGTGTTCAAGAAAGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT

Figure 6.18 16s rRNA Sequence of Band 3

CCGCACTTAAAGGGGCGCCATTAAATGTGCACTGCGACGGGATGGATTAAAGAGCTTGGCTCTATTAGTGAAGTAGGCGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT
CTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTGGTTGATGAGGGAACAGTGTTCAAGAAAGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT

Figure 6.19 16s rRNA Sequence of Band 4

AATGTGTCAGAATCGCTGCTGATGAGGGAACAGTGTTCAAGAAAGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT
CTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTTGGCTCTAATCAGTGACTGACCTGGTAGATGACGGTGGTGAATGCTGCGATAGCGATAGCTGACGGGGAGAGTTATATGACGATGGATTAT
GCCTGGTTGATGAGGGAACAGTGTTCAAGAAAGACAGGCGTACACGCATTACACCGCTGAGAATATATGACGATGGATTAT
Table 6.5 Identification of DNA Bands Analysed by DGGE with Scoring, Homology, and Accession Numbers (obtained from NCBI)

<table>
<thead>
<tr>
<th>Band number</th>
<th>Identification by 16s rRNA</th>
<th>Identities</th>
<th>% homology</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus licheniformis strain AK02</td>
<td>1057/1167</td>
<td>91</td>
<td>JX286652.1</td>
</tr>
<tr>
<td></td>
<td>Pseudomonas fluorescens strain CB32</td>
<td>1135/1142</td>
<td>99</td>
<td>FJ422406.1</td>
</tr>
<tr>
<td>3</td>
<td>Pseudomonas sp. PcFRB039</td>
<td>1042/1063</td>
<td>98</td>
<td>AB569921.1</td>
</tr>
<tr>
<td>4</td>
<td>Bacillus sp. JSG1</td>
<td>1413/1413</td>
<td>100</td>
<td>KF908820.1</td>
</tr>
<tr>
<td>5</td>
<td>Corynebacterium freiburgense strain 1045</td>
<td>1346/1356</td>
<td>99</td>
<td>NR044584.1</td>
</tr>
</tbody>
</table>

Figure 6.20 16s rRNA Sequence of Band 5

Figure 6.21 Phylogenetic Tree of 16s rRNA Gene Sequences of Band 1 Shows Close Relationship with many other Strains of Bacillus sp. Covered under Dendrogram
Figure 6.22 Phylogenetic Tree of 16s rRNA Gene Sequences of Band 2 Shows Close Relationship with many other Strains of Pseudomonas sp. Covered under Dendrogram

Figure 6.23 Phylogenetic Tree of 16s rRNA Gene Sequences of Band 3 Shows Close Relationship with many other Strains of Pseudomonas sp. Covered under Dendrogram

Figure 6.24 Phylogenetic Tree of 16s rRNA Gene Sequences of Band 4 Shows Close Relationship with many other Strains of Bacillus Cereus and Bacillus Subtilis Covered under Dendrogram
From Table 6.4 and Table 6.5, it is seen that the identified bacteria most commonly belonged to Firmicutes and \( \gamma \)-proteobacteria. In a biogas digester, Liu et al. (2009) found 47% and 0.5% of Firmicutes and Proteobacteria respectively, while Chouari et al. (2005) observed 22% and 14%-21% of Firmicutes and Proteobacteria respectively. Firmicutes and Proteobacteria are the predominant varieties found on the anode when analysed by DGGE (Aelterman et al. 2006b; Logan and Regan 2006; Parthasarathy and Chellaram 2014). Zhang et al. (2011b) analysed the change in the bacterial community in response to the change in the substrate in the anodic chamber. In an acetate-enriched MFC, *Clostridium* sp. and *Bacilli* sp. of phylum Firmicutes were observed, but when acetate was replaced with glucose, Firmicutes completely disappeared. The same was observed in this study as well, and electricity generation was found to occur through utilization of acetate by Firmicutes and/or Proteobacteria.

In one of the study, the dominant fermentative bacteria, *Bacillus licheniformis*, was isolated from an anaerobic digester at 35°C mesophilic temperature. It had the ability to consume glucose along with xylose and xylan at the optimum growth temperature of 37°C (Pantamas et al. 2003). Using a mixed acid fermentation pathway, *B. licheniformis* is known to ferment glucose under anaerobic conditions, producing acetate (Shariati et al. 1995). It was also found to produce electricity at 50°C (Choi et al. 2004). Based on the literature, *B. licheniformis* seems to be most important in creating an acidic environment. As mentioned in Chapter 3, *Bacillus* sp. is a natural inhabitant of distillery wastewater and is also known to be part of a consortia in anodic biofilms, detected by a DGGE technique similar to that described in Chapter 2 (Ren et al. 2011). *Bacillus* sp. is also found to be a dominant community member.

**Figure 6.25** Phylogenetic Tree of 16s rRNA Gene Sequences of Band 5 Shows Close Relationship with many other Strains of *Corynebacterium* sp. Covered under Dendrogram
when electricity is generated from propionate and butyrate (Chae et al. 2009; Freguia et al. 2010).

*Pseudomonas fluorescens* and *Pseudomonas aeruginosa* were biocatalysts in electron transfer in a single chambered MFC (Cournet et al. 2010). To decolorize molasses wastewater that was formed under conditions similar to that of ADDW, *P. fluorescens* was used by Dahiya et al. (2001). *Pseudomonas* genus is known to be highly aerobic, but work by Schreiber et al. (2006) showed a pyruvate fermentation pathway by *P. aeruginosa* under anaerobic conditions. This pathway was found to be essential for the survival of *P. aeruginosa* in deeper layers of biofilm. But apart from the above, the role of *Pseudomonas sp.* in anaerobic digestion remains unknown. Similarly, apart from the one study that showed electrogenic activity of *Corynebacterium humireducens* (Wu et al. 2011), the role of *Corynebacterium* in anaerobic digestion and electricity generation in MFC is not known. Much remains to be discovered about the physiology of these bacteria as individuals and community members as well as their roles in extracellular electron transfer.

Considering the fact that any bacteria could not be isolated that were identified by DGGE under strictly anaerobic conditions and that growth of anaerobes on anaerobic agar increased with the increase in electricity, *Clostridium sp.* must have been responsible for the electrogenic activity by oxidizing acetate. *Clostridium sp.* is known to oxidize acetate into CO$_2$ and H$_2$ (Karakašhev et al. 2006). The closely related *Clostridium* species *C. acetobutylicum*, *C. thermohydrosulfuricum* (Mathuriya and Sharma 2009), *C. acetobutylicum*, *C. cellulolyticum* (Finch et al. 2011), *C. butyricum* (Rabaey and Verstraete 2005), and *C. beijerinckii* (Scott and Murano 2007) are also reported to be responsible for electricity generation, further supporting the role of *Clostridium sp.* in electricity generation.

**6.3.6 Mechanism of Electron Transfer**

*Clostridium sp.* is known to transfer electrons directly through either surface cytochrome receptors (Rabaey and Verstraete 2005) or by reducing iron (Min et al. 2005). The mechanism of electron transfer, direct or mediated, was determined in this study using two techniques: a modified U-tube MFC followed by a confirmatory cyclic voltammetry technique.
6.3.6.1 Modified U-tube MFC Experiment

The preliminary technique used to elucidate mechanism of electron transfer involved an experiment similar to the U-tube experiment performed by Davis in 1959. The MFC design was modified to make it suitable to carry out a U-tube experiment (Figure 6.1). The anode was separated from the bacteria using 0.2 µm membrane. If the mechanism of electron transfer is by direct contact between the bacteria and anode, the MFC will not generate electricity. Only if electron transfer is through mediators that can pass through the 0.2 µm membrane, electricity will be produced by MFC.

Figure 6.26 shows the increased voltage until 20 h in control, which was devoid of endogenous consortia from ADDW as well as in MFC with bacterial growth (test). The pattern of initial increase in voltage could be from slow diffusion of ADDW and water through the 0.2 µm membrane. The continuous increase in electricity generation after 20 h indicated the presence of mediators in electron transfer to the anode. However, the pattern of electricity increase did not coincide with the previous MFC curves. Hence, the mediated electron transfer mechanism could not be confirmed using the modified U-tube MFC. The MFC curve found in this experiment was different because of a slow diffusion of electrons to the anode from the start, which took place because there was no biofilm on anode. Alternatively, it could have been caused by a change in MFC assembly. In the control, because ADDW was devoid of any endogenous microbial source, it lacked electron transfer after 20 h.

Figure 6.26 Modified U-tube MFC Experiment with Inoculum (Test) and without Inoculum (Control) (Mean; n = 3; %CV < 10)
6.3.6.2 Cyclic Voltammetry

Mediators are important chemical entities that facilitate electron transfer from bacteria to the anode. They are generally redox compounds that play roles as oxidizing and reducing agents. Because of their dual natures, cyclic voltammetry can be an important tool to detect them.

**Calibration of Reference Electrode**

The cyclic voltammetry set-up involves three-electrodes where one is a reference. The pH of ADDW was initially 8, and over a period of 50 h, it changed to 5. Hence, the silver reference electrode (Ag/AgCl; Sensor Technologies, India) was preferred because it was stable over a wide range of pH (0–13.5). The Ag/AgCl electrode was calibrated first using a reversible hydrogen electrode. The assembly for calibration is shown in Figure 6.27.

![Figure 6.27 Ag/AgCl Electrode Calibration Assembly](image)

Calibration of the Ag/AgCl electrode involved monitoring the current for steadiness. Figure 6.28a shows a constant current where the open circuit voltage for the Ag/AgCl electrode stabilized between -0.637 and -0.639 V, as shown in Figure 6.28b. Hence, the potential of the Ag/AgCl reference electrode was +0.638 V.
**Cyclic Voltammetry**

Initially, cyclic voltammetry experiments were carried out using optimized parameters; but use of a 55.26 cm$^2$ surface area anode led to a very high current response, beyond the detectable limit. Hence, an anode (working electrode) and cathode (counter electrode) with surface areas of 6.14 cm$^2$ were used. One special MFC system was constructed with two anodes suspended in ADDW. One was evaluated for the presence of mediators at 0 and 30 h, and the other at 50 h. The voltage scan rate selected was 5 mV/sec; higher scan rates (50 mV/sec) did not show oxidation or reduction peaks (Figure 6.29 and Figure 6.30). A scan rate of less than 10 mV/sec is recommended for the detection of mediators in MFCs (Logan 2008).

Figure 6.30 shows oxidation and reduction peaks at 0 h, similar to those observed for reversible redox species. Though the response of the current was very low, it confirmed the
presence of mediators in ADDW. Distillery wastewater is known to contain heavy metals such as Fe, Mn, Zn, and Cu (Jain and Srivastava 2012), which have the ability to donate and accept electrons. The ADDW used in study was checked for the presence of metals using Atomic Absorption Spectrophotometer (model 902, GBC, Australia) and it was found to contain 26.7 mg/L Fe, 0.61 mg/L Mn, 0.58 mg/L Zn, 2.14 mg/L Pb and 0.65 mg/L Ni. These could very well act as mediators.

Figure 6.29 Cyclic Voltammogram of MFC at 0 h at 50 mV/sec Scan Rate

Figure 6.30 Cyclic Voltammogram of MFC at 0 h at 5 mV/sec Scan Rate

Over a period of 30 h, when the bacteria start depositing on the anode, the oxidation and reduction peaks (Figure 6.30) were similar to those observed in at 0 h (Figure 6.31), confirming the absence of electricity generation.
The cyclic voltammogram after 50 h is shown in Figure 6.32. Two reduction peaks and one oxidation peak are observed. The oxidation peak and first reduction peak were similar to those observed at 0 and 30 h. However, the second reduction peak at -0.38 V showed a very high current response, observed only at 50 h. This high response was seen at maximum electricity generation. In other words, when bacteria transfer their electrons to the anode (working electrode), the response is seen as a second reduction peak in the cyclic voltammogram. Figure 6.32 also shows the importance of the biofilm in electron transfer because it was absent at 0 h, but at 30 h, the SEM also showed the presence of few scattered bacteria on the anode surface.

Figure 6.31 Cyclic Voltammogram of MFC at 30 h with 5 mV/sec Scan Rate

Figure 6.32 Cyclic Voltammogram of MFC at 50 h with 5 mV/sec Scan Rate
To confirm the role of biofilm in electron transfer, another fresh electrode devoid of biofilm was dipped in ADDW and used as the working electrode. The cyclic voltammogram, shown in Figure 6.33, does not have a second reduction peak. Though mediators, responsible for the mediated transfer of electrons, were present, this shows that the presence of biofilm was indispensable for the generation of electricity.

Figure 6.33 Cyclic Voltammogram of MFC using Working Electrode Devoid of Biofilm at 50 h with 5 mV/sec Scan Rate

The Modified U-tube MFC experiment showed that mediators could be present as part of the mechanism of electron transfer, but cyclic voltammetry experiments confirmed that mediators were playing role and also that the biofilm in the direct transfer of electrons by Clostridium sp. was indispensable.