Chapter 7

Summary, Conclusion and Future Scope

7.1 Summary

In MFCs, anaerobic conditions are important because lack of oxygen as terminal electron acceptor forces bacteria to transfer their electrons outside the cell to anode. Hence, in single chambered MFC, because both anode and cathode are in the same chamber, there is a possibility of diffusion of oxygen near anode. This makes the configuration less efficient as oxygen acts as the terminal electron acceptor and hence efficiency of the system decreases (Das and Mangwani 2010). Keeping this in mind, two chambered MFC was constructed first by using plastic reservoirs connected by filter paper dipped in 1.3 M KCl as salt bridge. One chamber had anode known as anodic chamber while other chamber had cathode known as cathodic chamber. This configuration worked well only for maximum 15 h as the problem of frequent drying of filter paper was observed due to exposure to atmosphere. Hence, another
design was constructed using H-shaped PVC pipes. Two cylindrical anodic and cathodic chambered were connected by salt bridge (1.3 M KCl and 2.5% agar). MFC with PVC pipes worked very well, however, due to opacity of PVC pipes, similar construction was made using glass.

Two chambered MFCs were constructed appropriate for the batch operation. The anodic and cathodic chambers of MFC of 500 mL each were joined by a glass rod containing salt bridge. Graphite material was used as electrodes. Anodes and cathodes were connected to the copper wires, which in turn were connected to 1000 Ω external resistance. The anodic chamber was filled with 200 mL of wastewater as feed, while cathodic chamber was filled with 200 mL of 100 mM potassium ferricyanide prepared in 100 mM phosphate buffer of pH 7. Different microbial cultures were inoculated in the feed depending on the experiments. All the experiments were carried out at room temperature (27 ± 3°C).

Two types of graphite electrodes were used, one were cylindrical rod removed from exhausted Eveready AA batteries while other were graphite plate (Schunk, Germany). The conductivity of cylindrical graphite rod was found to be 23.67±0.14 S/m while conductivity of graphite plate was 8.14 ± 0.01 S/m. Since the conductivity of graphite rods removed were 4 times higher, they were used as anode as well as cathode in further study.

Next, the volume of salt bridge was varied from 5 mL to 25 mL. It was found that 15 mL was the minimum volume required to inhibit mixing of anodic and cathodic solution. Also, it is known from the literature that the salt bridge increases an internal resistance which eventually decreases the efficiency of the system (Min et al. 2005). Hence, it was not advisable to go beyond 15 mL volume of salt bridge, thus 15 mL volume of salt bridge was used in further experiments.

Commercial voltage data logger Picolog 1216 was purchased from Picotech, UK. Picolog 1216 and was used throughout the experiments. The voltage was recorded every 5 minutes. The current was calculated using Ohm’s law V = I × R, where V is the voltage across resistance, I is the current generated and R is the external resistance. The power generated by the system was calculated as P = V × I where, V is the voltage and I is the current generated by the system. During calculations, the baseline voltage was considered as 0 V and any rise in the voltage was calculated in the cumulative manner to find area under the curve which gave
the total electricity generated by the system. In pharmacokinetics, area under the curve is considered very important as it represents the total drug exposure over time (Mross et al. 1996). Similarly in MFC, total power generated over time is thought to be important because it will represent the total power generated by the system and it may evaluate the system differently as generally highest voltage is considered for evaluation of MFC performance.

Validation of constructed MFC design was important before starting the experiments using real wastewater. The design was validated using artificial wastewater as feed inoculated with laboratory cultures of *Bacillus cereus* (MTCC 7409) and *Bacillus megaterium* (MTCC 8371). The composition of the artificial wastewater optimized in order to prevent precipitation was as follows (in litre): \( \text{NH}_4\text{Cl} 0.02 \text{ g}, \text{CaCl}_2.2\text{H}_2\text{O} 0.015 \text{ g}, \text{KCl} 0.033 \text{ g}, \text{NaCl} 0.003 \text{ g}, \text{MgSO}_4.7\text{H}_2\text{O} 0.015 \text{ g}, \text{CuSO}_4.5\text{H}_2\text{O} 0.001 \text{ g}, \text{ZnSO}_4.7\text{H}_2\text{O} 0.001 \text{ g}, \text{MnSO}_4 0.001 \text{ g}, 0.1 \text{ M phosphate buffer of pH 7, Dextrose 5 g keeping in view COD of distillery wastewater. The selection of Bacillus cultures was based on the literature as it was found that Bacillus sp. was predominant in distillery wastewater (Jain et al. 2002; Krzywonos 2014). Also, evidences are available that these two cultures are used in MFC for electricity generation (Jain et al. 2002). Out of the two cultures, *B. cereus* did not show electricity generation while *B. megaterium* showed rise in the voltage from 0.397 V to 0.491 V, when compared with control. Irrespective of the activity of *B. megaterium* and absence of electrogenic activity of *B. cereus*, the two chambered design of MFC was proved to be functional and hence was used for further studies using ADDW.

ADDW was procured from distillery producing alcohol using sugarcane molasses as raw material. The characterization of ADDW was carried out as per standard methods (APHA, 1995). It was found that the dark brown coloured ADDW had high COD of 18560 ± 640 mg/L, while TOC was found out to be 6519 ± 89 mg/L. Since, the wastewater was generated after anaerobic digestion; the biodegradability was expected to be low which was confirmed by the ratio of BOD/COD being 0.13. pH of the ADDW was found to be 7.96 ± 0.1. Also, ADDW was high in total suspended and total dissolved solids.

ADDW was used as a source of microorganisms for isolation. Nutrient broth and alternative thioglycollate medium were used for enrichment for aerobic and anaerobic microorganisms respectively. Anaerobic organisms were isolated on anaerobic agar under anaerobic
conditions created by Anaerogas Pack in anaerobic jar. Total four different types of colonies were found, two each aerobic and anaerobic.

MFC experiments were performed using isolated cultures under aseptic conditions. However, none of the isolates could generate electricity. Even MFC with mixed consortia of all four isolates did not produce electricity. Since, the isolated organisms did not produce electricity and same was the case with mixed consortia, modification in ADDW was necessary. Firstly the ADDW was diluted four times to increase the chance of electrogenic activity of bacteria if they were in low concentrations. It did not show electricity generation. Next, with the addition of sugar and mineral solution, rise in the voltage was observed which was expected as this wastewater was already exhausted with nutrients during biogas formation. Also added nutrients provided favourable conditions for the growth of bacteria. During the growth of bacteria, there was lot of foam formation. To suppress the foaming around anode, antifoam agent Sag Tex PHD was found to be effective. The amended ADDW was prepared as follows: 250 mL of wastewater was diluted to 1 L with distilled water. To it, 2% dextrose along with 1 mL each of 0.1 M phosphate buffer of pH 7.2, 0.1 M MgSO$_4$.7H$_2$O, 0.2 M CaCl$_2$.2H$_2$O and 0.01 M FeCl$_3$.6H$_2$O and 1.5 mL of Sag Tex PHD antifoam were added. This amended wastewater was used as feed throughout further experiments.

Various controls were maintained in order to prove that the electricity generation was through bacterial activity. When 10 fold concentrated bacterial culture was inoculated in the anodic chamber, reduction in time required to attain highest voltage was observed. Because of high bacterial load, the rate of degradation of organic matter was high and hence it could achieve highest voltage much earlier than that of normal MFC. When bacteria culture was metabolically poisoned by addition of disinfectant in anodic chamber, no electricity generation was observed. Similarly, no culture control was kept as the feed in the anodic chamber was autoclaved before use. Due to lack of bacterial culture, no electricity generation was observed. This result confirms the role of bacteria in electricity generation. Next, when anodic chamber was continuously aerated with the help of an aerator, no power generation was observed which showed the importance of anaerobic culture in electricity generation.

The amended ADDW was used for optimizing different MFC parameters. There are two kinds of parameters in operation of MFCs. The first are physical parameters like surface areas of electrodes, presence of aeration in cathodic chamber. Another is growth affecting
parameters like concentration of antifoam agent, pH of wastewater and external resistance. Physical parameters were optimized using one parameter at a time approach while growth affecting parameters were optimized using Box-Behnken Design.

The surface areas of electrodes were varied from 6.14 cm$^2$ to 55.26 cm$^2$. It was found that power generation increased with increase in the surface area of anode, with total power generated being 0.65 ± 0.35 mW for 55.26 cm$^2$. In case of surface area of cathode, the power generated reached plateau at 18.42 cm$^2$ (0.095 ± 0.01) and any further addition of the cathodic surface area remained ineffective. Surface area of anode was found to be the critical and limiting factor than surface area of cathode. Next, an aeration pump of 3 L/min flow rate was used as an aerator in the cathodic chamber and its effect was compared to MFC without aeration. Aeration at cathodic chamber did not increase the power generation. Also aeration requires input of energy and hence MFC without aeration was found to be advantageous.

Next, growth affecting parameters were optimized using BBD for total power generation response (Y). In order to know the nature of the response surface and to find the optimal concentrations of pH, antifoam and resistance, a BBD was used by Design Expert 8.0 software. Advantage of this design is that it requires fewer treatment combinations than a central composite design in cases involving three or four factors. Selection of low, centre and high values in BBD for antifoam, pH and external resistance were required and hence to find these values, again effect of antifoam, pH and resistance was analysed individually (one parameter at a time) for maximum current generation. The effect of pH on power output was monitored over a range from 5 to 10, whereas external resistance was varied over a range of 100 Ω to 22000 Ω. Similarly, concentration of antifoam was varied from 0 mL/L to 7 mL/L. Considering the results of above study high and low values for pH of feed, concentration of antifoam (mL/L) and resistance (Ω) were selected as 7 and 9, 0 and 5 and 100 and 1000 respectively. Total 13 experiments were performed in triplicate according to the design of experiments to obtain results. Next, analysis of variance of the quadratic equation for the total power generation in MFC was performed. The statistical significance of the second-order model equation was evaluated by $F$-test. The Model $F$-value of 12.00 implies the model was significant. The model’s goodness of fit was checked by determination coefficient ($R^2$). $R^2$ of 0.8000 indicated the fitness of the model. The best solution given by the Design Expert 8.0 software for the optimum values obtained for antifoam (mL/L), pH and resistance (Ω) were 0,
8.34 and 1000, corresponding to 6.6930 mW of total power production per 200 mL. The volumetric power density of the optimized MFC was found to be 33.46 W/m$^3$.

In order to enhance the power produced by the system, MFCs were connected in series. Increase in power production was observed with increase in number of MFCs in series. Power output was found to increase in the following order: 4MFCs > 3MFCs > 2MFCs > 1MFC. More than 4 MFCs could not be used due to sensitivity of voltage data logger as it could not detect the voltage beyond 2.5 V. When 4 MFCs were connected in series, the total voltage output was found to be 2.1 V. As an application, when three MFCs were connected in series, enough energy was generated to glow LED bulb. Since, the wastewater used as feed had already undergone the process of anaerobic digestion, the purpose of optimization was to extract maximum energy rather than maximum TOC reduction. Hence, during optimization by BBD, TOC reduction was not considered as response (Y) along with cumulative power production. TOC of the optimized MFC was monitored for reduction of organic content in wastewater. It was observed that after 50 h, when maximum electricity had been generated, % TOC reduction was 60.78 ± 0.95, which corresponded to 33.47 W/m$^3$ of cumulative volumetric power density.

Since, ADDW was used in the study; all stages occurring at anaerobic digestion were expected to occur. It was very interesting and challenging to investigate the electrogenic activity by bacteria. The optimized MFC was monitored for pH, dissolved oxygen and viable count of the ADDW. Over a course of 50 h, the pH of ADDW decreased from 8 to 5. This explains the acidogenic stage of anaerobic digestion. The amended ADDW had dextrose, which was fermented by fermentative bacteria to form acid, increasing the acidity of the wastewater. The dissolved oxygen of ADDW was found to be 0.54 ± 0.1 mg/L. After 50 h of experiment, it further reduced to 0.05 ± 0.1 mg/L. Dissolved oxygen of a system below 1 mg/L is considered as anaerobic and DO of ADDW itself was less than 1 mg/L because of the anaerobic digestion treatment of distillery wastewater. Viable count of ADDW at 0, 30 and 50 h was performed on nutrient agar and anaerobic agar for the growth of aerobes and anaerobes respectively. Aerobic microbes increased from 0 to 30 h but after that, the growth remained constant and no increase in the growth was observed while anaerobic organisms continually increased over a course of 50 h. From optimization study, it was evident that the electricity generation started after 30 h and reached its peak at 50 h. It is well known fact that increase in electricity generation is directly proportional to the growth of electrogenic bacteria (Peter
Aelterman et al. 2008b; Davila et al. 2008; Reguera et al. 2006). When there was rise in electricity, growth of only anaerobic bacteria was observed. This also confirms the role of anaerobic bacteria in electricity generation.

To further analyse the microbiology behind the activity and understand the system further, GC analysis of volatile fatty acids was performed. Three different acids namely acetic acid, propionic acid and butyric acid were detected as these are the three major acids formed during fermentation in anaerobic digestion. For quantitative analysis, the external standard method was used. Standards of acetic acid (0.084%), butyric acid (0.0505%) and propionic acid (0.0652%) were used in the study. It was observed that the concentration of acetic acid increased from 10 h and reached its peak till 30 h. After 30 h, the concentration of acetic acid started declining. The drastic decrease in acetic acid after 30 h coincided with the increase in electricity generation. This indicated that the electricity generation was dependent on the acetate utilization by anaerobic consortia present in the anodic biofilm. It was confirmed by spiking 0.2% acetate at 55 h resulting in the boost of electricity generation.

Scanning electron microscopy of optimized MFC was carried for the monitoring the growth of biofilm on anode. The development of biofilm formation was observed on the surface of anode. Obviously at 0 h, not a single bacterium was observed, while few oval shaped bacteria were observed on 30 h old anode, when electricity generation was absent. After 30 h, voltage was increased and reached its maximum at around 50 h. At that time, rod shaped bacteria were observed uniformly on the surface of anode. The growth of bacteria was found to be uniform throughout the surface of anode.

Anodic biofilm was considered as the best source to isolate the bacteria responsible for electricity generation. 50 h old anode was immersed in sterile saline, which was then used as microbial source for isolation. Organisms were isolated on anaerobic agar, tomato juice agar special and nutrient agar. Different media were considered to have better chance of vast variety of bacteria on anode biofilm to grow. Since, anaerobes were responsible for generation of electricity; all plates were incubated under anaerobic conditions created by anaerogas Pack in anaerobic jar. All agar plates were incubated at 37°C for 72 h. Out of all media plates, only anaerobic agar plates showed growth of bacteria. A total of only three different types of colonies were isolated, which were further purified to obtain pure cultures. Since, the growth was observed on anaerobic agar, Clostridium sp. was expected, which was confirmed by
biochemical tests. All three isolates were sent for identification to BioAxis DNA Research Centre, Hyderabad which was found out to be *Clostridium sp* 16S ribosomal RNA, *Clostridium sp.* CfU-1 and *Clostridium sp.* JCC.

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). DGGE showed presence of different bands, however, only five prominent bands were selected for further purification and identification, which showed presence of *Bacillus licheniformis* strain AK02, *Pseudomonas fluorescens* strain CB32, *Pseudomonas sp.* PcFRB039, *Bacillus sp.* JSG1 and *Corynebacterium freiburgense* strain 1045. It is seen that the identified bacteria most commonly belonged to Firmicutes and γ-proteobacteria. Based on the literature, *B. licheniformis* seems to be most important in creating an acidic environment. The role of *Pseudomonas sp.* in anaerobic digestion remains unknown, apart from few studies on electricity generation by them. 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. Because *Clostridium sp.* is involved in all stages of anaerobic digestion except methanogenesis, and identified by culturing, they must have been responsible for the electrogenic activity by oxidizing acetate. *Clostridium sp.* is known to oxidize acetate into CO$_2$ and H$_2$ (Karakashev 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
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. Similar to U-tube by Bernard Davis, modifications were made in the construction, in which two MFC reservoirs were separated by a 0.2 µm membrane. In one reservoir, bacteria were allowed to grow while in another reservoir, anode was placed in sterile distilled water. If electron transfer from bacteria to anode is through direct contact, there won’t be increase in the voltage but if the transfer of electrons is through mediators, then electricity will still be generated even though anode is not in direct contact with the bacteria. The output was compared with control in which did not have bacterial growth and hence lack of electricity generation. The modified U-tube experiment showed rise in voltage as verses no culture control. 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.

7.2 Conclusion

The major conclusions of the present study are as follows:

1. Two chambered MFCs were constructed using glass reservoirs, separated by salt bridge. The anodic chamber consists of anode, feed and bacterial source; while the cathodic chamber contains cathode and 100 mM potassium ferricyanide.

2. The constructed MFCs were validated using standardized artificial wastewater as feed and laboratory Bacillus cultures as inoculum.

3. The ADDW was procured from distillery producing alcohol using sugarcane molasses as raw material. The characterization of ADDW showed presence of high COD and TOC, however, comparatively low BOD. BOD/COD ratio was 0.13, indicating the presence of recalcitrant or non-biodegradable substances present in ADDW.
4. After many failed attempts to use un-treated ADDW as feed in MFCs, ADDW was diluted 4 times and amended 2% dextrose along with minerals for good bacterial growth.

5. The amended ADDW was used as feed for further optimization of MFCs. Parameters such as surface areas of electrodes, aeration in cathodic chamber were optimized by one parameter at a time methodology while concentration of antifoam agent, pH of ADDW and external resistance were optimized using Box-Behnken Design. The optimized parameters are as follows: surface area of anode – 55.26 cm², surface area of cathode – 18.42 cm², aeration in cathodic chamber – no, antifoam – 0 mL/L, pH – 8.3 and resistance - 1000 Ω. The optimized total power output was 6.69 mW per 200 mL of ADDW that is, 33.46 W/m³.

6. The analysis of pH of feed in optimized MFC indicated occurrence of fermentation due to increase in the acidity up to 5. The dissolved oxygen of 0.54 ± 0.01 mg/L showed anaerobicity of ADDW while viable count showed continual increase in anaerobic count over a period of 50 h, proving the role of anaerobes in electricity generation.

7. GC analysis indicated the role of acetate in the electricity production which was later confirmed by MFC experiments by spiking acetate in ADDW.

8. Scanning electron microscopy revealed the development of biofilm on anodic surface where the rod-shaped bacteria were deposited uniformly at 50 h, when the electricity was at peak.

9. The isolation and identification bacteria from anode biofilm showed the presence of *Clostridium sp 16S ribosomal RNA*, *Clostridium sp. CfU-1* and *Clostridium sp. JCC*, while DGGE analysis revealed presence of *Bacillus licheniformis strain AK02*, *Pseudomonas fluorescens strain CB32*, *Pseudomonas sp. PcFRB039*, *Bacillus sp. JSG1* and *Corynebacterium freiburgense strain 1045* in the biofilm on anode.

10. The mechanism of transfer of electrons was analysed by modified U-tube MFC and cyclic voltammetry. The modified U-tube MFC experiment indicated presence of
mediators. Cyclic voltammetry experiments confirmed that mediators. Cyclic voltammograms also revealed the indispensable role of biofilm in the direct transfer of electrons by *Clostridium sp.*

### 7.3 Future Scope

The wastewater generated after AD will continue to be available in abundance. Thus the approach used in this study is very useful and will be helpful in the future. MFC will be a complementary technique to AD. Following studies are suggested before achieving the goal of setting the MFC plant next to anaerobic digesters.

1. First of all, to activate bacterial growth, amendment with dextrose was essential. The addition of dextrose increases the COD of wastewater which is undesirable. For this, advanced oxidation processes (AOPs) are recommended when wastewater components have a low biodegradability. Combined AOPs as pre-treatments, followed by biological processes, are both cost efficient and extremely viable from an economic perspective (Cañizares et al. 2009). As one of the treatment, ADDW was treated with Fenton’s reagent. pH (4.5), concentration of 30% H$_2$O$_2$ (15 mL/L) and FeSO$_4$ (4 g/L) were optimized to increase the BOD to COD ratio that is biodegradability from 0.13 to 0.21. A BOD/COD ratio of more than 0.4 is required for biological treatment (Forgie 2008). Hence, full scale study should be done on stronger AOPs such as Fenton’s reaction, or photo Fenton’s reaction followed by ozonation treatment, which will help increase the biodegradability of ADDW and then MFC can extract more energy with the need to add external source of sugar.

2. The next study should be undertaken to make MFC functional in a fed-batch mode followed by continuous mode of operation. The different parameters such as flow rate and hydraulic retention time can be optimized to enhance electricity generation.

3. Finally, the continuously fed MFC should be scaled up from 1 L to 10 L and then to industrial scale.